Influence of Masticatory Functional Loss on the Remodeling of Alveolar Bone and Morphology of the Tooth Root in Rats

（ラット歯槽骨のリモデリングと歯根形態における咀嚼機能低下の影響）

日本大学大学院　松戸歯学研究科　歯学専攻

山田　尚迪

（指導：清水　武彦　教授）

Abstract

There is plenty of literature on masticatory function and its impact on maxillofacial development. However, the influence of masticatory hypofunction on bone turnover in the alveolar bone and on morphology of the tooth root has hardly been studied. This study aimed to clarify the influence of tooth loss and soft diet on the alveolar bone turnover and on morphology of the tooth root during the growth period. Three-week-old Wistar rats were randomly divided into the following three groups: Hard diet group (rats raised on solid standard diet), Powder diet group (rats raised on powdered standard feed diet), and Extraction group (rats raised on powdered standard diet with maxillary molars extraction).

The bone volume (BV), bone mineral content (BMC), and bone mineral density (BMD) in the cancellous bone of mandibular first molars (M1) were measured using micro-CT analysis. To analyze the histological bone turnover, we prepared non-decalcified thin sections of alveolar cancellous bone when rats were 20 weeks old. On three-dimensional constructed images, the experimental groups (the Powder diet and Extraction groups) showed expansion of the medullary cavity of the interradicular septum of the first molar compared to controls (the Hard diet group). BV, BMC, and BMD were significantly lower in the experimental groups, with the difference from controls being greater in the Extraction group. On histomorphometric analysis, the bone mass parameters, bone formation parameters, and bone mineralization parameters were significantly lower in the experimental groups compared to controls. The bone resorption parameters were significantly higher in the experimental groups. From this study, we found that soft diet and tooth loss might worsen the bone microstructure, reduce osteogenesis, and promote bone resorption in alveolar bone.

Length, width, cross-sectional area, and volume of the root of the M1 and second molars (M2) were measured using micro-CT analysis. The root lengths of all roots in the Extraction group were significantly longer than the corresponding root lengths in the Hard diet and Powder diet groups. The root width and cross-sectional area at the apical side 1/4 of all roots in the Extraction group were significantly smaller than those in the Hard diet and Powder diet groups. The root volume of the M1 mesial root in the Extraction group was significantly smaller than that in the Hard diet and Powder diet groups. This study clarified that when masticatory stimulus in the immature teeth is reduced by the extraction of opposing teeth and a powder diet, the root length increases due to the promotion of cellular cementum addition at the apex, and the root width and cross-sectional area decrease due to the suppression of cellular cementum addition at the apical side 1/4 of the roots.

These results suggest that loss masticatory stimulation by soft diet and tooth loss have reduced bone mass in the interradicular septum because of the significant resorption uncoupling in the osteoblast-osteoclast equilibrium. Furthermore, the root length increased due to the promotion of cellular cementum addition at the apex, and the root width and cross-sectional area decreased due to the suppression of cellular cementum addition at the apical side 1/4 of the roots.

1. Introduction

Amid the growing popularity of processed food and soft food diets, children increasingly prefer softer foods that require fewer mastication iterations and less time for mastication. This preference for softer foods may affect the child's maxillofacial development, narrowing the jaw and tooth rows and resulting in malocclusion. Another pediatric dental problem is masticatory hypofunction, which may be related to congenital hypodentia or premature tooth loss caused by dental trauma or dental caries. When chewing food, children with masticatory hypofunction apply less mechanical force to their jaw and facial muscles than otherwise, which significantly affects physical growth. Thus, the consistency of food ingested and the condition of the oral cavity are critical factors in ensuring appropriate masticatory stimulation to the maxillofacial and masticatory muscles.

There is plenty of literature on masticatory function and its impact on maxillofacial development. Moss *et al*. [1] argued that biological functions are related to their corresponding skeletal components. In humans, they claimed that components of the mandible powerfully shape the performance of masticatory muscles. Specifically, the temporalis shapes the coronoid process, the masseter muscle shapes the mandibular condyle, and the medial and lateral pterygoid muscles shape the gonial angle. There are many experiments on reduced chewing stimulation with a soft diet. These experiments showed a reduction in maxillary width, mandibular height and mandibular condyle thickness, and expansion of the size of the gonial angle [2-6].

Similarly, our previous research showed masticatory hypofunction in rats, due to extraction of maxillary molars and a diet of powdered feed, was associated with reduced mandibular volume, bone density, mandibular height, and mandibular condyle thickness [7, 8]. Additionally, we indicated that reduced masticatory function led to deformation and cellular disorders in the condylar cartilage, and diminished ossification and heightened bone resorption in the secondary cancellous bone of the condylar head, resulting in slower ossification, less bone volume, and reduced trabecular thickness [9]. There are also reports on the effects of occlusal stimuli on the alveolar bone: In rats, a soft diet, tooth extraction or the insertion of a bite-opening appliance were associated with a thinner [10-12] and higher alveolar process [13,14], and sparser mandibular alveolar architecture [15].

Bone remodeling is a process in which osteoclasts break down bone tissue and osteoblasts build new bone tissue. Dental practice will benefit from a better understanding of how reduced masticatory stimuli and masticatory hypofunction affect bone turnover in the alveolar bone. To our knowledge, bone mass and the bone density of alveolar bone have been reported; however, the bone turnover of alveolar bone has been hardly studied.

Additionally, disordered periodontal ligament fibers and fibroblast arrangement [16], narrowed periodontal ligament width [17], and changes in microvasculature of the periodontal ligament [18] were reported in rats whose occlusal function was artificially reduced by opposing teeth extraction, cusp removal, or bite-raising. Furthermore, Saeki *et al*. [19] reported that cementoblast proliferation and cementum addition were observed in the apex of rats whose opposing teeth were extracted. Cementum covers the

surface of the root and determines the morphology of the root. The formation of cementum continues throughout the lifetime, and its thickness increases with age; however, its rate of formation is slow [20]. Cementum is classified into acellular and cellular cementum according to the presence or absence of cells. Acellular cementum is distributed on the cervical side 1/2-2/3, and cellular cementum is distributed on the apical side 1/2-1/3. Several researchers have reported an association between reduced masticatory function and high cementum formation [21, 22]. However, there has been no quantitative study of the effects of changes in food properties and loss of opposing teeth on root morphology.

Accordingly, the purpose of this study was to clarify the influence of tooth loss and soft diet on the alveolar bone turnover and on the morphology of the tooth root during the growth period. To assess bone remodeling, we used the alveolar bone volume, mineral content, alveolar density, which we measured using micro-CT analysis. To analyze bone turnover at a histological level, we prepared non-decalcified thin sections of alveolar cancellous bone for histomorphometric analysis. Furthermore, to assess morphology of the tooth root, the root length, root width, root cross-sectional area, and root volume of the M1 and M2 were measured and examined using micro CT in growing rats raised on powder feed and extracted opposing teeth. Furthermore, we observed the roots of the mandibular M1 histologically.

2. Materials and methods

2.1. Experimental Animals

We purchased three-week-old Wistar rats (21 male rats) from Sankyo Lab Service Co., Ltd., and randomly divided them into the following three groups (seven rats each): (a) Hard diet group: These rats were raised on solid standard feed (MF, Oriental Yeast Co., Ltd, Tokyo, Japan). (b) Powder diet group: These rats were raised on powdered standard feed. (c) Extraction group: When the rats were 4 weeks old, we intraperitoneally administered thiamylal sodium (15 mg/kg; Kyorin Pharmaceutical Co., Ltd., Tokyo, Japan) and used a spoon excavator under general anesthesia to dislocate and extract all the maxillary molars on both sides; we fed the rats on powdered standard feed.

The animals were raised in the rat facility at the Laboratory Animal Research Center of our university with a room temperature of 24 ± 1 ºC; a constant humidity level of 65 ± 5 %; and a 12-hour light/dark cycle. All rats were given distilled water to drink and had free access to feed and water.

Tetracycline (20 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) and calcein (10 mg/kg; Sigma Aldrich) were subcutaneously injected seven days and one day before euthanasia, respectively, in order to double-label the bones. At the age of 20 weeks, the rats were euthanized with carbon dioxide gas, and the mandibles on both sides were removed. The experimental schedule and procedures are summarized in Figure 1.

This study was approved by the Ethics Committee of the Nihon University School of Dentistry at Matsudo (animal experiment approval number: AP13MD020). The study design adhered to ARRIVE guidelines and was approved by the appropriate ethics review board.

2.2. Measurement of Body Weight

The rats' body weights were measured weekly from the age of 3 weeks until the age of 20 weeks.

2.3. Micro-CT Analysis

We imaged the extracted right mandibular body using micro-CT (R_mCT2, Rigaku, Tokyo, Japan). The imaging conditions were as follows: tube voltage, 90 kV; tube current, 160 μA; magnification, 2x; measurement time, 3 minutes; Field of View, 3 mm. Slice images were constructed in three dimensions using 3-D construction analysis software (TRI/3D-BON, Ratoc System Engineering Co, Tokyo, Japan).

The BV, BMC, and BMD were measured with a range of 600 μm × 600 μm × 600 μm in the cancellous bone of interradicular septum of the first molar (Figure 2).

On the cross-section passing through the buccolingual center of the tooth, the tooth root was parallel to the line connecting the mesiodistal CEJ, and was below the line passing through the root furcation (Figure 3 (a)). Using 3-D construction analysis software (TRI/3D-BON, Ratoc System Engineering Co, Tokyo, Japan), slice images of the tooth root were constructed in 3-D, and the root length, root width, root cross-sectional area, and root volume were measured for M1 (mesial root and distal root) and M2 (mesiobuccal root, mesiolingual root, and distal root) (Figure 3 (b)). Each measurement was performed three times, and the mean of these measurements was used. The measurement results were compared among the three groups.

1) Measurement of root length

   The line connecting the apex from the center of the upper margin of the root was defined as the root length (Figure 3 (c)), and this length was measured in 3-D images.

2) Measurement of root width

   The mesiodistal root width was measured in lingual view of 3-D images. The buccolingual root width was measured in distal view of 3-D images. These root width was measured at three places, as follows: the upper 1/4th part of the tooth root on the cervical side (cervical side 1/4), the lower 1/2th part of the tooth root on the apical side (central part) and the lower 1/4th part of the tooth root on the apical side (apical side 1/4) (Figure 3 (d)).

3) Measurement of root cross-sectional area

   The cross-sectional area of the root was measured at three places in 3-D images as follows: the cervical side 1/4, central part, and apical side 1/4 (Figure 3 (d)).

4) Measurement of root volume

   The root volume was measured in 3-D images.

2.4. Histological Preparation

We extracted the left mandibular body of each rat and fixed it in 70 % ethanol. We discarded the soft tissue and then immersed the bone for 4 days in Villanueva bone stain without decalcification. After dehydrating with ethanol, we wrapped the mandibular body with a methyl methacrylate resin. We cut the sagittal

sections of the alveolar part and the M1 part into sections of 5 μm thickness using a microtome and prepared non-decalcified thin-slice specimens for histological observation.

2.5. Bone Histomorphometry of Cancellous Bone

Bone histomorphometric measurements were performed using a Histometry RT digitizer (System Supply, Nagano, Japan) and specialized software (CSS-840 cancellous bone morphometry version; System Supply). The cancellous bone was measured at the interradicular septum of the lower first molar: a region of 1,217 to 1,643 mm^2 was selected. The following parameters were measured: Interradicular septum volume (I.S.V), interradicular septal width (I.S.Wi), osteoid thickness (O.Th), osteoid surface/bone surface (OS/BS), eroded surface/bone surface (ES/BS), quiescent surface/bone surface (QS/BS), osteoblast number/bone surface (N.Ob/BS), osteoclast number/bone surface (N.Oc/BS), mineral apposition rate (MAR), mineralizing surface/bone surface (MS/BS) mineralization lag time (Mlt), bone formation rate/bone surface (BFR/BS) and bone formation rate/interradicular septum volume (BFR/I.S.V). For the N.Oc, mononuclear osteoclasts (N.Mo.Oc) and multinuclear osteoclasts (N.Mu.Oc) were measured separately.

2.6. Statistical Analysis

All statistical analyses were performed using SPSS (IBM Corp., Armonk, NY). All data are expressed as the mean ± SD. ANOVA was used to determine differences between multiple groups, while differences between individual groups were determined by Tukey's test. In alveolar bone analysis, a p value equal to or less than 0.05 and in tooth root analysis, a p-value equal to or less than 0.01 was considered to be statistically significant.

3. Results

3.1. Body Weight

The mean body weight increased over time for all groups, and no significant differences were found among the groups at any point in time (Figure 4).

3.2. Micro-CT Analysis

1) CT-Based Assessment of the Alveolar Bone

In the three-dimensional constructed images, both the Extraction group and the Powder diet group showed expansion of the bone medullary cavity of the interradicular septum of the first molar compared to the Hard diet group. The tendency was recognized conspicuously in the Extraction group (Figure 5).

BV in the cancellous bone of interradicular septum of the first molar was 0.18 ± 0.02 mm^3 for the Hard diet group, 0. 13 ± 0.01 mm^3 for the Powder diet group, and 0.07 ± 0.01 mm^3 for the Extraction group. BMC in the cancellous bone of interradicular septum of the first molar was 0.126 ± 0.031 mg for the Hard diet group, 0.063 ± 0.011 mg for the Powder diet group, and 0.027 ± 0.008 mg for the

Extraction group. BMD in the cancellous bone of interradicular septum of the first molar was 674.21 ± 108.79 mg/cm^3 for the Hard diet group, 471.25 ± 51.21 mg/cm^3 for the Powder diet group, and 369.07 ± 64.02 mg/cm^3 for the Extraction group. The Extraction group and the Powder diet group showed significantly lesser values than the Hard diet group for all three parameters. The BV and BMC in the Extraction group were significantly lesser than those in the Powder diet group (Figure 6).

2) Three-dimensional reconstructed images of the tooth root

The roots of the rats in the Hard diet group and Powder diet group grew laterally around the apical side 1/4, and the apex was rounded. The morphology of the teeth in both groups was similar. On the other hand, the roots of the Extraction group did not show lateral growth, and the apex was sharp. The root length in the Extraction group was longer than that in the Hard diet group and Powder diet group (Figure 7).

3) Root length

Figure 8 shows the results of the measurement of root length. Among the three groups, the Extraction group showed a significantly longer root length than the Hard diet and Powder diet groups. No significant differences were found between the Hard diet group and Powder diet group.

4) Root width

Figure 9 shows the results of the measurement of the root width.

(1) M1 mesial root

No significant differences were found among the groups in the mesiodistal and buccolingual root widths at the cervical side 1/4. The Extraction group showed significantly thinner mesiodistal and buccolingual root widths at the central part and apical side 1/4, than the Hard diet and Powder diet groups. The Powder diet group showed a significantly thinner mesiodistal root width at the apical side 1/4 than the Hard diet group.

(2) M1 distal root

With regards to the mesiodistal and buccolingual root widths at the cervical side 1/4, no significant differences were found among the groups. The Extraction group showed a significantly thinner mesiodistal root width at the central part than the Hard diet group. The Extraction group also showed a significantly thinner mesiodistal root width at the apical side 1/4 than the Hard diet and the Powder diet groups. There were no significant differences among the groups in the buccolingual root widths at the central part and apical side 1/4.

(3) M2 mesiobuccal root

With regards to the mesiodistal and buccolingual root widths at the cervical side 1/4 and central part, no significant differences were found among the groups. However, the Extraction group

showed significantly thinner mesiodistal and buccolingual root widths at the apical side 1/4 than the Hard diet and Powder diet groups.

(4) M2 mesiolingual root

With regards to the mesiodistal and buccolingual root widths at the cervical side 1/4 and central part, no significant differences were found among the groups. The Extraction group showed significantly thinner mesiodistal and buccolingual root widths at the apical side 1/4 than the Hard diet and Powder diet groups.

(5) M2 distal root

With regards to the mesiodistal and buccolingual root widths at the cervical side 1/4 and central part, no significant differences were found among the groups. The Extraction group showed a significantly thinner buccolingual root width at the apical side 1/4 than the Hard diet and Powder diet groups.

5) Root cross-sectional area

Figure 10 shows the results of the measurement of the root cross-sectional area.

(1) M1 mesial root

At the cervical side 1/4, no significant differences were found among the groups. At the central part and apical side 1/4, the cross-sectional area of the Extraction group was significantly smaller than that of the Hard diet and Powder diet groups. At the apical side 1/4, the Powder diet group showed a significantly smaller cross-sectional area than the Hard diet group.

(2) M1 distal root

At the cervical side 1/4, no significant differences were found among the groups. At the central part and apical side 1/4, the Extraction group showed a significantly smaller cross-sectional area than the Hard diet group. At the apical side 1/4, the Powder diet group showed a significantly smaller cross-sectional area than the Hard diet group.

(3) M2 mesiobuccal root

At the cervical side 1/4, no significant differences were found among the groups. At the central part and the apical side 1/4, the Extraction group showed a significantly smaller cross-sectional area than the Hard diet and Powder diet groups. At the apical side 1/4, the Powder diet group showed a significantly smaller cross-sectional area than the Hard diet group.

(4) M2 mesiolingual root

At the cervical side 1/4 and central part, no significant differences were found among the groups. At the apical side 1/4, the Extraction group showed a significantly smaller cross-sectional area than the Hard diet and Powder diet groups.

(5) M2 distal root

At the cervical side 1/4, no significant differences were found among the groups. At the central

part, the Extraction group showed a significantly smaller cross-sectional area than the Hard diet group. At the apical side 1/4, the Extraction group showed a significantly smaller cross-sectional area than the Hard diet and Powder diet groups.

6) Root volume

Figure 11 shows the results of the measurement of root volume. In the M1 mesial root, the Extraction group showed a significantly smaller root volume than did the Hard diet. In the other roots, no significant differences were found among the groups.

3.3. Histological Observation

1) Assessment of the Alveolar Bone

Regarding the inner architecture of the interradicular septa, the specimens from the Hard diet group had narrow medullary cavities with extensive trabecular distribution, those from the Powder diet group had wide medullary cavities with isolated trabecular clusters, and those from the Extraction group had markedly wide medullary cavities with several thin trabecular beams. Among the specimens from the Hard diet group, the upper section of the septal outline (immediately below the crown) was thick and continuous (without any breaks), and the overall outline was proportionate to that of the root branches on the other side of Sharpey fibers. In contrast, in the specimens from the two experimental groups, the upper section of the outline was thick, but the sections further down the branches of the root were thin, with breaks in some places. This result was particularly conspicuous in the specimens from the Extraction group (Figure 12).

The specimens from the experimental groups had more polynuclear osteoclasts than those from the control group, suggesting the presence of deeper resorption cavities (Figure 13 (a)). The osteoblasts in the extraction specimens were different, based on the classification system used by Villanueva *et al*. [23] from those in the Hard diet and Powder diet specimens. The osteoblasts in the Hard diet specimens were identified as type II (cuboidal) osteoblasts based on the presence of cubic cytoplasm. The cytoplasm was arranged like brickwork, leaving no gaps. Furthermore, the height of osteoblasts [24] was increased and consistent. The Powder diet specimens also showed type II osteoblasts, but in lesser numbers. Additionally, the cell heights were shorter compared to those in the Hard diet specimens. In the extraction specimens, type IV osteoblasts were identified based on their flat cytoplasm and nuclei; the osteoblasts were lesser in number. In the control specimens, the osteoids were thick and even, while those in the experimental specimens were thinner by comparison (Figure 13 (b)). Regarding the double bone labels, compared to controls, the intervals were shorter in the experimental groups (Figure 13 (c)). Regarding the length of the double-labeled surfaces, in the control group, many surfaces were uninterrupted, but in the experimental groups, they were shorter by comparison (Figure 13 (d)). ISV

was greater in the control specimens than in the experimental specimens. In the control specimens, the interradicular septa had broad, sheet-like trabeculae. The trabeculae in the powder specimens were fragmentary, and those in the extraction specimens were more finely shredded (Figure 13 (e)).

2) Assessment of the roots of the mandibular M1

In all groups, the cervical root surface 1/2 was covered with a layer of acellular cementum. On the other hand, the apical root surface 1/2 was covered with cellular cementum. Thickening of the cementum was noted from the cervical 1/2 to the apex. In particular, in the Hard diet group, significant lateral thickening of the cementum in the roots was noted. In the Extraction group, the cementum thickness on the lateral surface of the apical side 1/4 was thinner, while the cementum thickness at the apex was thicker than in the Hard diet group. The cementum thickness in the Powder diet group was intermediate to that in the Hard diet group and the Extraction group. In the morphology of root dentin, no differences were found among the groups. Compared to the Hard diet group, the periodontal ligament was narrower in the Powder diet group, and was the narrowest in the Extraction group (Figure 14).

3.4. Bone Histomorphometry of Cancellous Bone in the First Molar

We performed bone histomorphometry of the interradicular septum of the cancellous bone in the first molar (Figure 15). I.S.V, a BV parameter, was 0.78 ± 0.04 mm², 0.68 ± 0.11 mm², and 0.53 ± 0.08 mm² in the Hard diet group, Powder diet group, and the Extraction group, respectively. The I.S.V in the Extraction group was significantly lower than that in the Hard diet group. I.S.Wi was 140.07 ± 15.43 μm, 120.69 ± 23.05 μm, and 97.19 ± 21.51 μm in the Hard diet group, Powder diet group, and the Extraction group, respectively. Moreover, I.S.Wi in the Extraction group was also significantly lower than that in the Hard diet group. O.Th, a bone formation marker, was 5.38 ± 0.08 %, 3.46 ± 0.82 %, and 2.99 ± 0.2 % in the Hard diet group, Powder diet group, and Extraction group, respectively. O.Th in the Powder diet and Extraction groups were significantly lower than that in the Hard diet group. OS/BS was 34.51 ± 4.52 %, 24.42 ± 5.01%, and 23.34 ± 3.44% in the Hard diet group, Powder diet group, and Extraction group, respectively. OS/BS in the Powder diet and Extraction groups were significantly lower than that in the Hard diet group. ES/BS was 9.27 ± 3.42 %, 19.77 ± 4.34 %, and 29.49 ± 6.52 % in the Hard diet group, Powder diet group, and Extraction group, respectively. ES/BS in the Powder diet and Extraction groups were significantly higher than that in the Hard diet group. QS/BS was 56.20 ± 5.30 %, 55.79 ± 4.38 %, and 47.16 ± 4.52 % in the Hard diet group, Powder diet group, and Extraction group, respectively. QS/BS in the Extraction group was significantly lower than that in the Powder diet and Hard diet groups. N.Ob/BS was 24.57 ± 3.65 N/mm in the Hard diet group, 16.99 ± 3.55 N/mm in the Powder diet group, and 10.56 ± 1.51 N/mm in the Extraction group. N.Ob/BS in the Powder diet and Extraction groups were significantly lesser than that in the Hard diet group. N.Mu.Oc/BS, a bone resorption parameter, was 0.94 ± 0.29 N/mm, 2.02 ± 0.45 N/mm, and 2.81

± 0.63 N/mm in the Hard diet group, Powder diet group, and Extraction group, respectively. N.Mu.Oc/BS in the Powder diet and Extraction groups were significantly higher than that in the Hard diet group. N.Mo.Oc/BS was 0.69 ± 0.32 N/mm, 1.04 ± 0.29 N/mm, and 1.52 ± 0.27 N/mm in the Hard diet group, Powder diet group, and Extraction group, respectively. N.Mo.Oc/BS in the Extraction group was significantly higher than that in the Powder diet and the Hard diet groups. MAR, a kinetic parameter, was 2.54 ± 0.11 μm/day, 1.83 ± 0.23 μm/day, and 2.09 ± 0.11 μm/day in the Hard diet group, Powder diet group, and Extraction group, respectively. MAR in the Powder diet and Extraction groups were significantly lesser than that in the Hard diet group. MS/BS was 39.88 ± 4.21 %, 32.23 ± 4.35 %, and 32.1 ± 3.55 % in the Hard diet group, Powder diet group, and Extraction group, respectively. MS/BS in the Extraction group was significantly lower than that in the Powder diet and Hard diet groups. Mlt was 1.86 ± 0.18 day, 1.43 ± 0.38 day, and 1.05 ± 0.13 day in the Hard diet group, Powder diet group, and Extraction group, respectively. Mlt in the Powder diet and Extraction groups were significantly lesser than that in the Hard diet group. BFR/BS was 0.36 ± 0.03 mm^3/mm^2/year, 0.21 ± 0.03 mm^3/mm^2/year, and 0.24 ± 0.02 mm^3/mm^2/year in the Hard diet group, Powder diet group, and Extraction group, respectively. BFR/BS in the Extraction group was significantly lesser than that in the Hard diet and Powder diet groups. BFR/I.S.V was 535.18 ± 87.32 %/year, 375.29 ± 126.18 %/year, and 523.57 ± 119.28 %/year in the Hard diet group, Powder diet group, and Extraction group, respectively. BFR/ ISV in the Powder diet group was significantly lesser than that in the Extraction and Hard diet groups. In summary, compared to controls, the experimental groups had lower values of bone mass parameters (I.S.V, I.S.Wi), bone formation parameters (O.Th, OS/BS, N.Ob/BS), and bone mineralization parameters (MAR, MS/BS, Mlt, BFR/BS), and higher values of bone resorption parameters (ES/BS, N.Oc/BS), and the difference from the control group were greater in the Extraction group than in the Powder diet group.

4. Discussion

Many studies have suggested the influence of masticatory functional loss on morphology, bone mass and bone density of alveolar bone; however, remodeling of alveolar bone has not been defined. Furthermore, several researchers have reported an association between reduced masticatory function and high cementum formation; however, there has been no quantitative study of the effects of changes in food properties and loss of opposing teeth on root morphology. We aimed to examine how soft diet and tooth loss in growing rats affected alveolar bone turnover and root morphology. In this study, we have shown that soft diet and tooth loss may cause a worsening of bone microstructure, reduced osteogenesis, and promotion of bone resorption in alveolar bone. Additionally, soft diet and tooth loss may cause the root length increases due to the promotion of cellular cementum addition at the apex, and the root width and cross-sectional area decrease due to the suppression of cellular cementum addition on the lateral surface of the apical side 1/4.

We used rats as test subjects because they mature quickly, allowing us to administer feed to many

subjects under controlled conditions. Another reason was that many studies have used rats to examine the craniofacial and periodontal tissues effects of a soft diet. According to one such study, the occlusal force was approximately 400 g during biting [25]. Another study indicated that active tetanic tension in the masseter muscle was significantly lower in rats fed with a soft diet compared to in those fed with a hard diet [26]. Similarly, at higher activity levels, the duty time of the superficial masseter muscle in the soft diet group was significantly lower than that in the Hard diet group [27].

In light of these findings, we prepared a powdered feed, which could be ingested without chewing or crushing, and fed it to rats in the experimental groups (Powder diet group and Extraction group) in order to minimize masticatory stimulation to the alveolar bone and to reduce activity in the masticatory muscles. In the Extraction group, we reduced masticatory stimulation to the mandibular alveolar bone even further by extraction of molars (in addition to feeding a powder diet), simulating a case of congenital hypodontia or premature tooth loss caused by dental trauma or dental caries.

Extraction was performed at 4 weeks of age, which corresponds to eruption of the third molars. We evaluated all animals at the age of 20 weeks, when the development of mandibular bone has been reported to be complete [7]. In many studies [28-30], rats with extracted molars were fed hard diet. However, we fed a soft diet in this study because we wanted to reduce the load on the mandibular molar.

We observed no inter-group differences in body weight of the rats at any point. Body weight increased over time in all three groups. This finding denoted that powdered diet and tooth loss had no physiological effect on rats. Physiological condition can therefore be excluded as a reason for the mandibular and root morphology changes observed in the experimental groups.

4.1. Influence of Masticatory Functional Loss on the Remodeling of Alveolar Bone in Rats

Previous studies showed that in contrast to that in the mandible, the values of BV/TV, trabecular thickness, trabecular number, trabecular width, and trabecular star volume in the maxillary alveolar bone were not significantly changed in rats fed a soft diet when compared with those fed a hard diet [31]. Therefore, in this study, we extracted the maxillary molars and observed the mandibular alveolar bone. In addition, the interradicular alveolar bone in the M1 was chosen because this area is exposed to concentrated occlusal stimuli and is often used for alveolar bone histomorphometry [32].

On the three-dimensional constructed images, both the Extraction group and the Powder diet group showed expansion of the medullary cavity of the interradicular septum in the first molar compared to the Hard diet group. The medullary cavities were particularly large in the Extraction group, as revealed by the size of the area in the transmission image. Our data were broadly consistent with findings of studies in rats fed a soft diet or with extracted opposing tooth [28, 31]. Literature indicates that a soft diet or extraction of the opposing tooth can cause higher alveolar bone [10, 11, 13, 14]. In our study, we used CT analysis to determine the alveolar type based on size, as we aimed to evaluate the cancellous bone without influence of the size in the interradicular septa of the first mandibular molars. The analysis revealed that BV, BMC,

and BMD were significantly lower in the experimental groups than in the control group, with the difference from control group being greater in the Extraction group. Regarding the BV in rats fed with a soft diet, similar results were reported [33]. Similarly, in the alveolar bone that extracted opposing tooth, the decrease in BV was reported [14, 28, 30]. Some studies have suggested that the mineral density in rats fed with a soft diet was significantly increased compared to in those fed with a hard diet [34, 35]. They measured the cortical portion of the alveolar bone, at an early stage. As we measured the cancellous bone of the interradicular septum in the first molar after 17 weeks in this study, we considered that a different finding was obtained. A lower cancellous BMD in the mandibular first molar was reported in rats, which caused masticatory hypofunction, by the insertion of a bite-opening appliance and soft diet [36]. Our findings are consistent with the results of these studies. We hypothesized that the experimental groups in our study had a lower BV, BMC, and BMD because masticatory stimulation to the mandibular alveolar bone was inhibited by the soft diet, and inhibited further by tooth loss in the Extraction group, leading to expansion of the bone medullary cavity, sparser trabecular density, and delay in bone deposition. We used a histological analysis to further clarify this causal relationship.

1) Bone Mass

On histo-morphometric analysis, the values of the bone mass markers (I.S.V, I.S.Wi) were lower in the experimental groups compared to controls. Regarding I.S.V, based on tissue imaging, the control specimens had broad, sheet-like trabeculae, powder specimens were fragmented trabeculae, and extraction specimens had more finely shredded trabeculae. Similar findings were observed for I.S.Wi. Another finding on tissue imaging was that the interradicular septal outline was thin and broken in several places in the experimental groups, while it was thick and unbroken in the control group. In their study on rats, Bresin *et al*. [15] found that a soft diet was associated with reduced cortical thickness below and lateral to the first molar, which is consistent with our observation in the septal outline. The septal outline was thinner and trabeculae were fragmentary in the experimental groups because masticatory stimulation was reduced by a soft diet and, in the Extraction group, tooth loss.

2) Bone Formation

The values of the bone formation markers (OS/BS, O.Th, N.Ob/BS) were significantly lower in the experimental groups. Our results are consistent with those of previous study. Ejiri *et al.* [14] have reported that bone formation was significantly suppressed in the alveolar bone by opposing tooth extraction. Levy *et al*. [37] reported that 15 days after opposing tooth extraction, the periodontal ligament was obviously narrowed, its structure was disorganized, and woven bone formation was noted at the top of the interradicular septa, beneath the sockets along their region of modeling. This is thought to be related to tooth extrusion due to opposing tooth extraction. Johnson *et al*. [29] also reported that after 1 to 5 weeks of opposing tooth extraction, new bone formation was observed in the

alveolar bone between the first and second molar in the Extraction group than the control group. The new bone mass likely resulted from supraeruption of the second molar, which displaced the mucoperiosteum and increased the strain delivered to crestal alveolar bone by alveolar crest [38]. In this study, extrusion of the second molar was observed in some individuals; however, an increase in bone formation markers was not detected because of a prolonged duration after opposing tooth extraction. We identified type II (cuboidal) osteoblasts, based on their cubic cytoplasm, in the Hard diet and Powder diet groups. In the Extraction group, we identified type IV osteoblasts based on their flat cytoplasm and nuclei. We also found that the height of the osteoblasts was maximum in the Hard diet group. Cell height was small in the Powder diet group, and even smaller in the Extraction group as compared with that in the Hard diet group. Type II refers to "active" osteoblasts, and type IV refers to "inactive" osteoblasts. Morphologically active osteoblasts synthesize the bone matrix more rapidly than morphologically inactive osteoblasts [23]. It is suggested that the reduced masticatory stimulation resulted in a slower formation of the bone matrix as well as a decrease in the numbers of osteoblasts and osteoids.

3) Resorption

The values of the bone resorption markers (ES/BS, N.Oc/BS) were significantly higher in the experimental groups. The Extraction group had a particularly high count of large, polynuclear osteoclasts, suggesting deep resorption cavities. Although mononuclear osteoclasts can resorb bone, large, polynuclear osteoclasts account for most osteoclasts actively engaged in bone resorption. Therefore, bone resorption would have been rampant in the Extraction group. Similarly, Honda *et al*. [28] reported increased N.Oc in the alveolar bone of extracted opposing tooth. In another study, Enokida *et al*. [32] fed rats a soft diet and used a metal device to prevent occlusion. They reported that these conditions led to increased N.Oc in the interradicular alveolar bone in the mandibular M1. Thus, reduced masticatory stimulation facilitated resorption as well as reduced the number of osteoclasts.

4) Bone Mineralization

Among the bone mineralization markers (MAR, MS/BS, Mlt, BFR/BS, BFR/I.S.V), MAR was lower in the experimental groups. This may have occurred because of the increase in osteoclastic resorption, which uncoupled the osteoblast-osteoclast equilibrium, resulting in lesser osteoblastic mineralization. MAR was higher in the Extraction group than in the Powder diet group. Additionally, ES/BS and N.Oc/BS showed similar results. In the Extraction group, this may have occurred because of an increase in osteoclastic resorption, which coupled the osteoblast-osteoclast equilibrium, resulting in higher mineral apposition rate. MS/BS was significantly lower in the experimental groups. Tissue imaging revealed an inter-group difference in length of the double-labeled surfaces: in the control group, many surfaces continued uninterrupted, but in the experimental groups, they were shorter in

comparison. We used two measures for bone formation rate: the bone formation rate/bone surface (BFR/BS) and the bone formation rate/interradicular septum (BFR/I.S.V). Both measures were lower in the Powder diet group than in the Hard diet group. This result is attributable to the extremely low MS/BS and MAR in the Powder diet group. On the other hand, in the Extraction group, BFR/I.S.V was similar to that in the Hard diet group. This result is attributable to the fact that the Extraction group had low MS/BS and MAR and markedly low I.S.V. The Mlt indicated a time lag between matrix production and mineralization; the values were significantly lower in the experimental groups. Therefore, histological analysis revealed that reduced masticatory stimulation was associated with a decline in the four bone mineralization markers (MAR, MS/BS, Mlt, BFR/BS).

These differences might be explained by the Wolff's Law. Julius Wolff, after discovering that trabecular architecture reflects the mechanical environment, postulated that a bone's architecture adjusts to the mechanical load under which the bone is placed, such that the bone shape and mass are commensurate with the load [39]. Thus, because the mechanical load on the alveolar bone differed between the three groups, each group developed a different trabecular architecture, adapted to withstand the particular load for that group.

Offering further insights into the results, Harold Frost, as part of his mechanostat theory, postulated that mechanical stress shapes bone structure [40-42]. Frost argued that moderate loading facilitates bone formation, while reduced loading facilitates bone resorption. Mechanical stress on the alveolar bone was low in the two experimental groups due to the powdered feed, and particularly low in the Extraction group due to the tooth loss. Hence, bone resorption was greater in the experimental groups than in the control group. This understanding is corroborated by the CT results, which indicate lower BV, BMC, and BMD values in the experimental groups.

For bone remodeling, osteoclastic resorption precedes osteoblastic formation, osteoclasts are attached to the bone matrix and initiate resorption, and the osteoblasts initiate bone formation and mineralization. Repeated iterations of resorption followed by formation determine the bone mass in the interradicular septum. Taken together, the results of our study imply that a loss in masticatory stimulation increases the number of osteoclasts in the alveolar bone, such that at each iteration of the bone remodeling cycle, the osteoblasts (proceeding the osteoclasts in said cycle) operate to a lesser extent than the osteoclasts. This significant resorption uncoupling in the osteoblast-osteoclast equilibrium results in reduced interradicular septal bone mass. Thus, masticatory mechanical stress to the alveolar bone plays a significant role in sustaining interradicular septal bone mass. Individuals who prefer a soft diet (as is increasingly the case today) or with congenital hypodontia or premature tooth loss, are more likely to have reduced bone mass in their interradicular septum, as a result of the significant resorption uncoupling in the osteoblast-osteoclast equilibrium. Therefore, prevention of dental trauma and dental caries, and early detection and treatment of dental caries are important to prevent premature tooth loss. In the case of congenital hypodontia or premature tooth loss, pediatric

denture should be attached to recover the occlusal function. In addition, a well-balanced diet without frequent eating of soft diet should be recommended. Appropriate masticatory stimulation plays an important role in the regulation of the osteoblast-osteoclast equilibrium and the maintenance of functional alveolar structure.

4.2. Loss of Masticatory Function Affects Morphology of the Tooth Root in Rats

Regarding normal root dentin length and cementum, Hoffman *et al*. [43] found that in the mandibular first molars of Wistar rats, the thickness of the acellular cementum increases in a direction that is almost parallel to the dentin-cementum boundary; however, this increase slows over time. On the other hand, cellular cementum addition begins on the 35th day after birth, and increases as the length of the root dentin grows. However, after the 12th week after birth, instead of the formation of root dentin, cellular cementum is added at the apex. This addition gradually increases over time. Therefore, in this study, to examine the effects prior to the start of cellular cementum addition, powder feed was given to the Powder diet group and the Extraction group at three weeks of age, and tooth extraction was performed at four weeks of age.

Root morphology can be observed by extracting the tooth, but at 20 weeks old, the root is longer and the alveolar bone is harder than at four weeks old, so the root is likely to break during tooth extraction. In this study, we used micro CT to separate the root and alveolar bone and construct a three-dimensional image of the root. As such, we were able to evaluate the three-dimensional morphology of the root without tooth extraction. Furthermore, it was easy to measure the length, cross-sectional area, volume, etc., and we think that micro CT was effective.

To quantitatively evaluate the morphology of the tooth root, root width and root cross-sectional area were measured at the cervical side 1/4, central part, and apical side 1/4. Additionally, regarding the root width, mesiodistal and buccolingual width were measured. To reduce measurement error, these measurements were made three times, and the mean of measurements was used. Since the root morphology of M3 differed individually, root morphology observation and measurement were performed with M1 and M2. In this study, on the cross section passing through the buccolingual center of the tooth, the tooth root was parallel to the line connecting the mesiodistal CEJ and was below the line passing through the root furcation. The root length was defined as the distance from the upper margin of the center to the apex, to avoid the influence of root dilaceration or inclination.

Based on 3-D reconstructed images, the roots in the Hard diet group and Powder diet group grew laterally near the apical side 1/4, and the apex was rounded. On the other hand, the Extraction group did not show lateral growth, and the apex was sharp and long. These findings suggested that the extraction of opposing teeth prevented the lateral growth near the apical side 1/4 and caused the apex to become pointed and longer. Furthermore, the tooth root morphology in the Hard diet group and Powder diet group were similar, suggesting that a powdered diet does not affect root morphology.

In all roots, root length in the Extraction group was significantly longer than that in the Hard diet and

Powder diet groups. The length of root dentin could not be measured in this study because separating dentin and cementum was difficult using micro CT. In the tissue images, the amount of cellular cementum at the apex in the Extraction group was significantly more than that in the Hard diet and the Powder diet groups. Johnson [29] and Gerard [37] *et al*. reported that when opposing teeth were extracted, bone formation was noted at the root furcation and between adjacent teeth at an early stage, and that the teeth were extruded. In this study, tooth extraction was performed at four weeks old when the roots were still immature and growing longitudinally. We believe tooth extrusion during this period reduced masticatory pressure in the apex, and reduced the suppression of physiologically applied longitudinal growth. The results suggested that the formation of cellular cementum at the apex in the Extraction group was actively prevalent, and that the root length increased significantly. It has been reported that in human decalcified sections, impacted teeth uninvolved in occlusion show a high degree of cementum thickening, and that thick cementum formation also occurs at the furcation [44]. It has been reported that cementoblast proliferation and cellular cementum addition was noted in rats [19], and we obtained the same results in this study. Furthermore, tooth extrusion was identified as a cause of hypercementosis, which is one of the progressive conditions involving cementum [45]. We believe that the results of our study support this finding. With reference to root length, no significant differences were found between the Hard diet and Powder diet groups. To date, several reports have stated that the alveolar bone mass and bone density decrease when rats are raised on a soft feed [15, 33], and we believe that the Powder diet group experienced a lower masticatory pressure at the apex than did the Hard diet group. However, cementum is a tissue that does not undergo bone-like remodeling, though it changes as dynamically as do bones. The results of this study suggest that the change in masticatory pressure as a result of a powder diet does not affect the formation of cementum at the apex.

With regards to the root width and cross-sectional area at the cervical side 1/4, no significant differences were found among the groups. In the M1 mesial and distal roots at the central part, root width in the Extraction group was significantly smaller than that in the Hard diet group. In all roots at the central part except the M2 mesiolingual root, the cross-sectional area in the Extraction group was significantly smaller than that in the Hard diet group. In all roots at the apical side 1/4, root width and cross-sectional area in the Extraction group were significantly smaller than those in the Hard diet and Powder groups. The root width of the M1 mesial root and the cross-sectional area of the M1 mesial and distal roots at the apical side 1/4 were significantly smaller in the Powder group than in the Hard diet group. In tissue images, no differences in the morphology of root dentin were found among the groups. Furthermore, in contrast to the root thickening caused by cellular cementum addition near the apical side 1/4 in the Hard diet group, cellular cementum addition was smaller in the Extraction group. The extent of root thickening cause by cellular cementum addition in the Powder diet group was intermediate to that in the Hard diet group and the Extraction group. We believe that this caused the difference in root width and cross-sectional area at the central and apical side 1/4 of the root among the three groups. It has been reported that cementum formation is not related to mechanical stimulation, as a high degree of cementum thickening has been observed in

unerupted teeth not involved in mastication [44–46]. On the other hand, cellular cementum has also been reported to develop on acellular cementum in response to functional requirements [47]. In this study, compared to the Hard diet group which was given solid feed, the masticatory stimulus was weaker in the experimental groups which were feed a powdered diet or extracted the opposite teeth. Based on histological images, the periodontal ligament width in the lateral side of the root was smaller in the Extraction group than in the Hard diet group. The histological features of a hypofunctional periodium include a progressive decrease in the number and density of the fibers and their disorientation, and a narrowing of the periodontal ligament [48, 49]. Based on these reports, it can be inferred that the masticatory stimulus on the lateral side of the root is weaker in the Extraction group than in the Hard diet group. Furthermore, it has been reported that the strength of masticatory muscles and the bone density of the alveolar bone decreased in rats which were fed soft food and in those which extracted the teeth [25]. We believe that these may influence a masticatory stimulus on the root. The above mentioned findings suggest that cellular cementum was added on the central and apical side 1/4 of the root in proportion to the degree of the masticatory stimulus acting on the root. Moreover, significant differences in the mesiodistal width and buccolingual width were noted between the Hard diet group and the Extraction group. Based on this finding, we believe that the cementum addition, which was a reaction to masticatory stimuli, occurred in the same way in the mesiodistal and buccolingual parts. Compared to the Hard diet group, root width or cross-sectional area of only M1 were smaller in the Powder diet group. The results of this study suggest that the change in masticatory pressure as a result of a powder diet does not affect the formation of cementum at the apical side 1/4 too much. On the other hand, on the cervical side 1/4, the results suggest that the decrease in masticatory stimulation due to a powder diet and the extraction of opposing teeth did not affect the morphology of the root.

It is well known that root resorption occurs when excessive pressure is applied to the root as a result of occlusal trauma or orthodontic treatment. It has been reported that occlusal trauma occurred when a micro-plus-screw pin was inserted into the opposing teeth in mice; additionally, multinuclear giant cells and resorption cavities were also observed on the cementum surface [50]. It has also been reported that excessive orthodontic force induces RANKL-mediated odontoclast differentiation and apoptosis in cementum, resulting in root resorption [51]. From the results of this study, the addition of cellular cementum on the lateral surface of the apical side 1/4 occurs actively under appropriate masticatory pressure. Moreover, our results clarified that when the masticatory pressure decreased, the addition of cellular cementum on the lateral surface of the apical side 1/4 was suppressed. This suggests that cementum is formed according to the mechanical environment, and is adjusted to a shape and mass suitable for maintaining its strength.

In the M1 mesial root, the root volume in the Extraction group was significantly smaller than that in the Hard diet and Powder groups. Considering the root length, root width, and cross-sectional area, the root length was longer in the Extraction group than in the Hard diet group. However, we believe that the cementum thickening on the lateral surface of the apical side 1/4 of the Hard diet group was more remarkable. Furthermore, the root length of the Powder diet group was almost the same as that of the Hard

diet group, and the root width on the lateral surface of the apical side 1/4 did not decrease as much as it did in the Extraction group. The significant effect on M1 mesial root may be related to the difference in occlusal load depending on the chewing cycle of the rat [52, 53]. On the other hand, no significant differences in the root volume of the other roots were found among the groups. In the Extraction group, the root width on the lateral surface of the apical side 1/4 was smaller than that in the Hard diet group. However, it was supposed that this decrement and increment of the tooth root length were almost equal results. In the Powder diet group, we believe that both the root length and the root width on the lateral surface of the apical side 1/4 were almost the same as in the Hard diet group.

The above findings clarified that when masticatory stimulus in the immature teeth decreases as a result of a powder diet or due to opposing teeth extraction, the root length increases due to the promotion of cellular cementum addition at the apex, and the root width and cross-sectional area decrease due to the suppression of cellular cementum addition on the lateral surface of the apical side 1/4. These results suggest that the same phenomenon may occur in cases where masticatory stimulation decreases due to early loss of teeth as a result of trauma, dental caries, or congenital deficiency encountered in pediatric dental clinics.

5. Conclusion

To summarize, the experimental groups exhibited lower bone mass in the interradicular septum below the first molar, with more osteoclasts and greater osteoclastic resorption. Compared to Hard diet group, the experimental groups also showed lower values of the osteoid surface, osteoid thickness, number of osteoblasts, mineralization surface, and MAR. Furthermore, the Extraction group exhibited longer tooth root and addition of cellular cementum at the apex. Compared to the Hard diet group, the Extraction group showed also smaller tooth width and cross-sectional area.

Individuals who either prefer a soft diet, have congenital hypodontia, or premature tooth loss are more likely to have reduced bone mass in the interradicular septum because of the significant resorption uncoupling in the osteoblast-osteoclast equilibrium. Furthermore, it is clarified that the root length increases due to the promotion of cellular cementum addition at the apex, and the root width and cross-sectional area decrease due to the suppression of cellular cementum addition at the apical side 1/4 of the roots.

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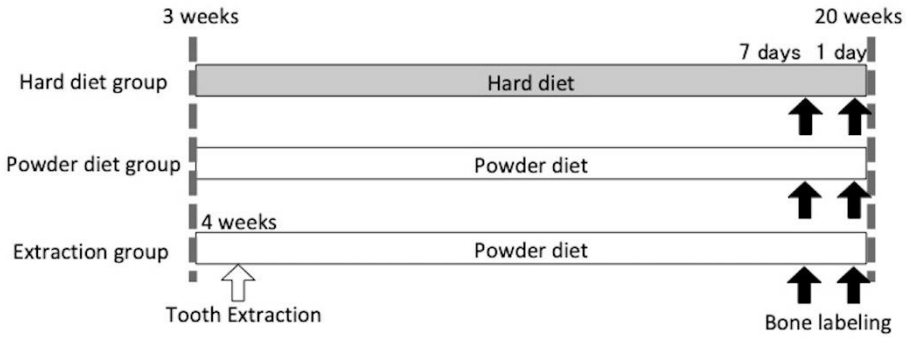


Figure 1. Time-schedule of the experiment. When the rats were 4 weeks old, we extracted all the maxillary molars on both sides (white arrow). Tetracycline and calcein were subcutaneously injected seven days and one day before euthanasia, respectively, in order to double-label the bones (black arrows).

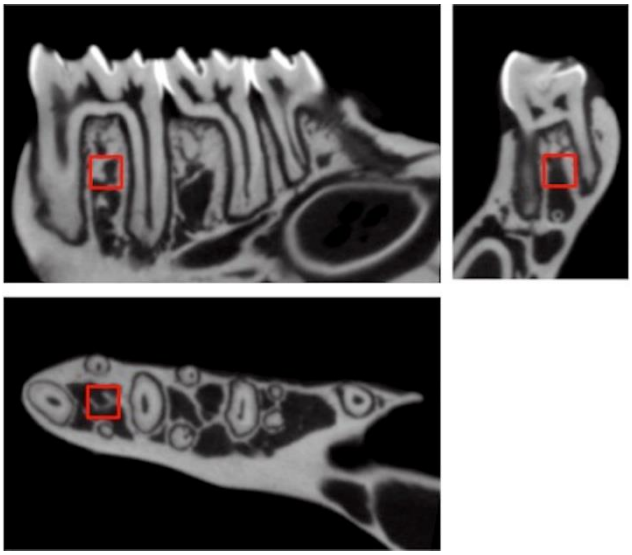


Figure 2. BV, BMC, and BMD were measured at the range of 600 μm *600 μm *600 μm in the cancellous bone of the interradicular septum in the first molar. The square indicates the measurement range.

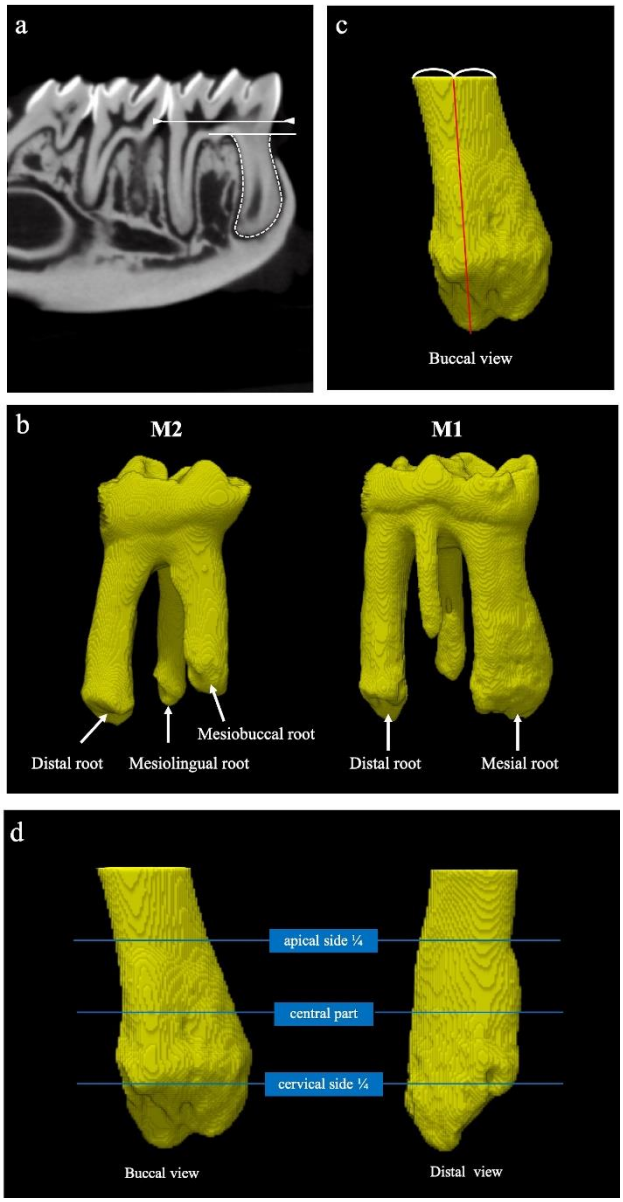


Figure 3. (a) On the cross-section passing through the buccolingual center of the tooth, the tooth root was parallel to the line connecting the mesiodistal CEJ and was below the line passing through the root furcation. **(b)** The 3-D reconstructed images of M1 (mesial root and distal root) and M2 (mesiobuccal root, mesiolingual root, and distal root). **(c)** The line connecting the apex from the center of the upper margin of the root was defined as the root length. **(d)** The mesiodistal root width was measured in buccal view of 3-D images. The buccolingual root width was measured in distal view of 3-D images. The root width and cross-sectional area were measured at three places as follows: the cervical side 1/4, central part, and apical side 1/4.

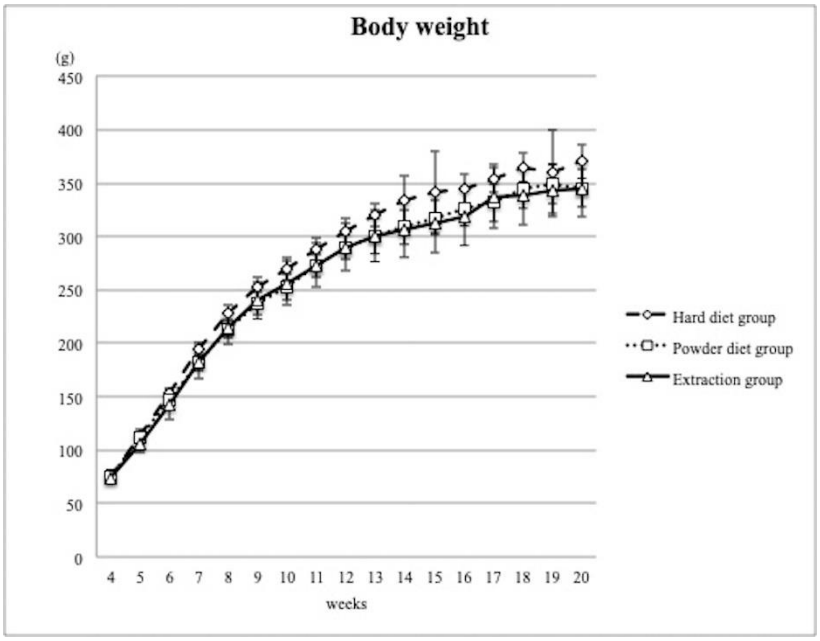


Figure 4. Body weight of the rats, by weeks. The body weight increased with age, and no significant difference was found between the groups at any point in time.

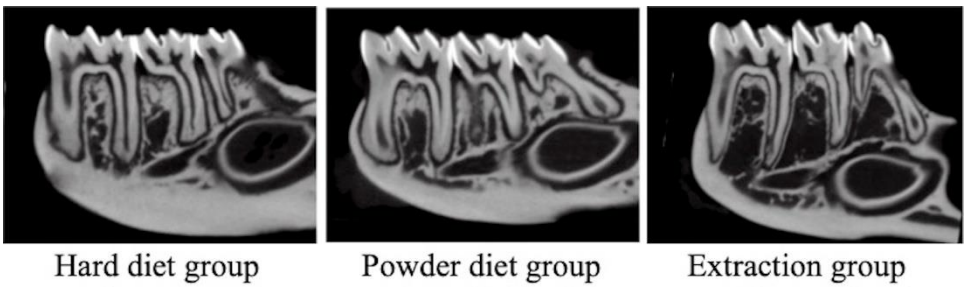


Figure 5. Micro-CT images. Both the Powder diet group and the Extraction group showed expansion of the bone medullary cavity of the interradicular septum compared to the Hard diet group. The tendency was recognized conspicuously in the Extraction group.

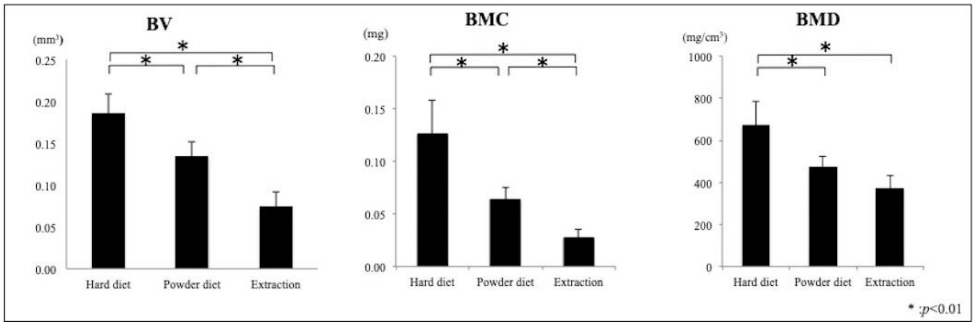


Figure 6. BV, BMC, and BMD in the micro-CT analysis. Data are presented as mean ± standard deviation (*$p < 0.01$). BV: bone volume. BMC: bone mineral content. BMD: bone mineral density.

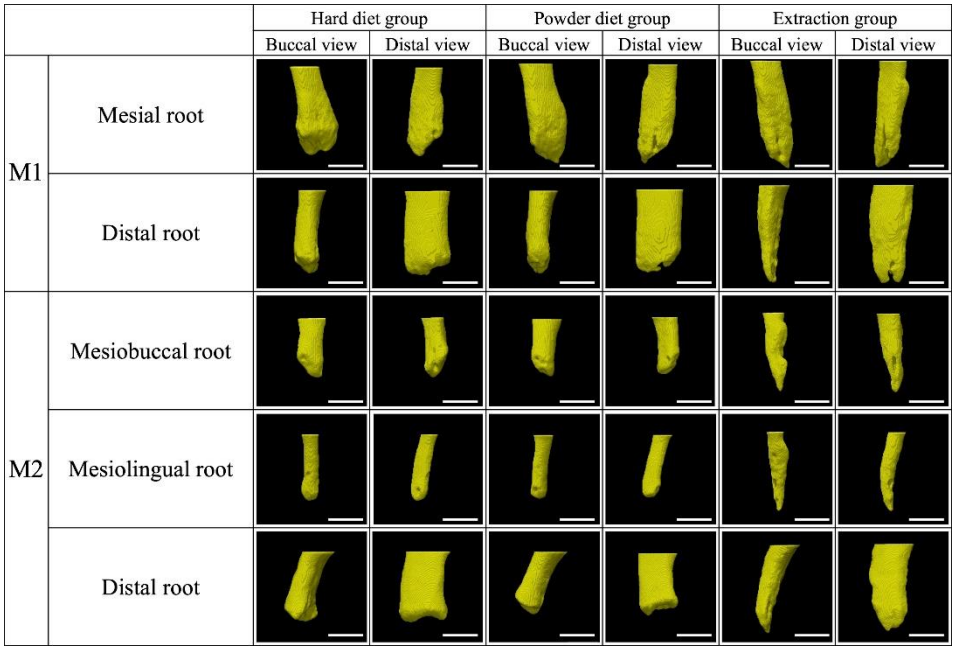


Figure 7. The three-dimensional reconstructed images of the tooth root in buccal and distal views. Bar: 1000 μm.

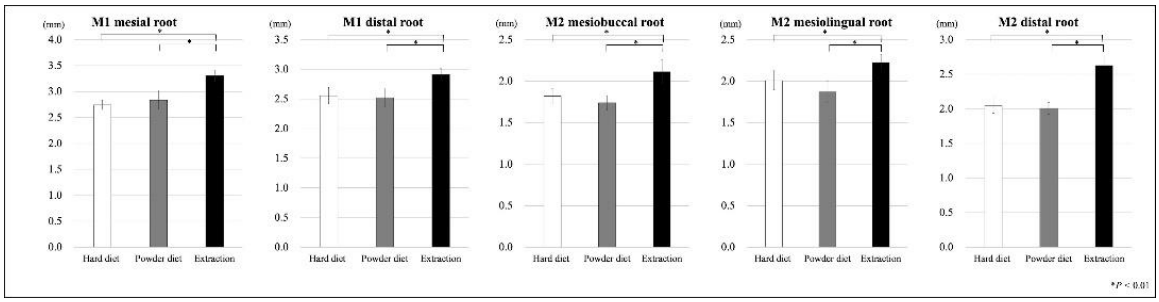


Figure 8. Root length in the micro-CT analysis. Data are presented as mean ± standard deviation (*$p < 0.01$).

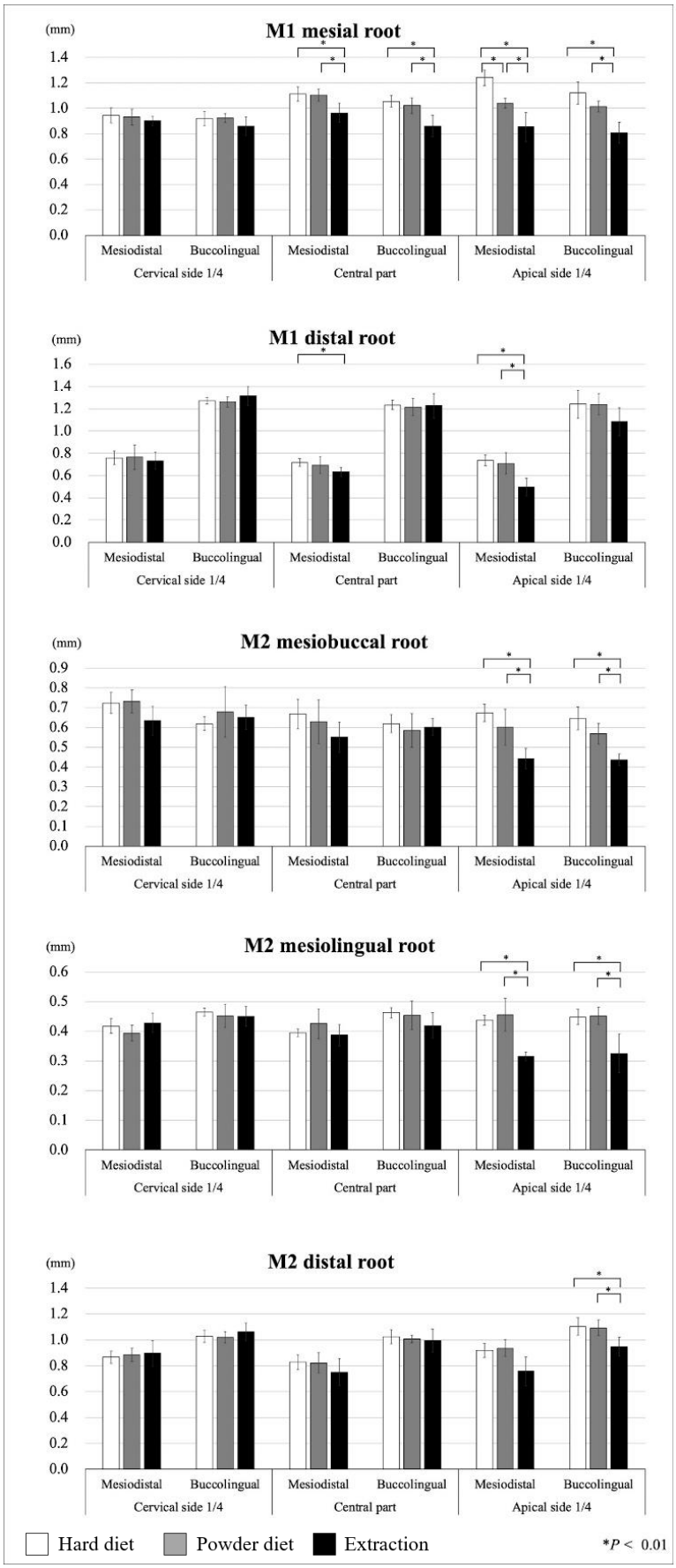


Figure 9. Root width in the micro-CT analysis. Data are presented as mean ± standard deviation (*$p < 0.01$).

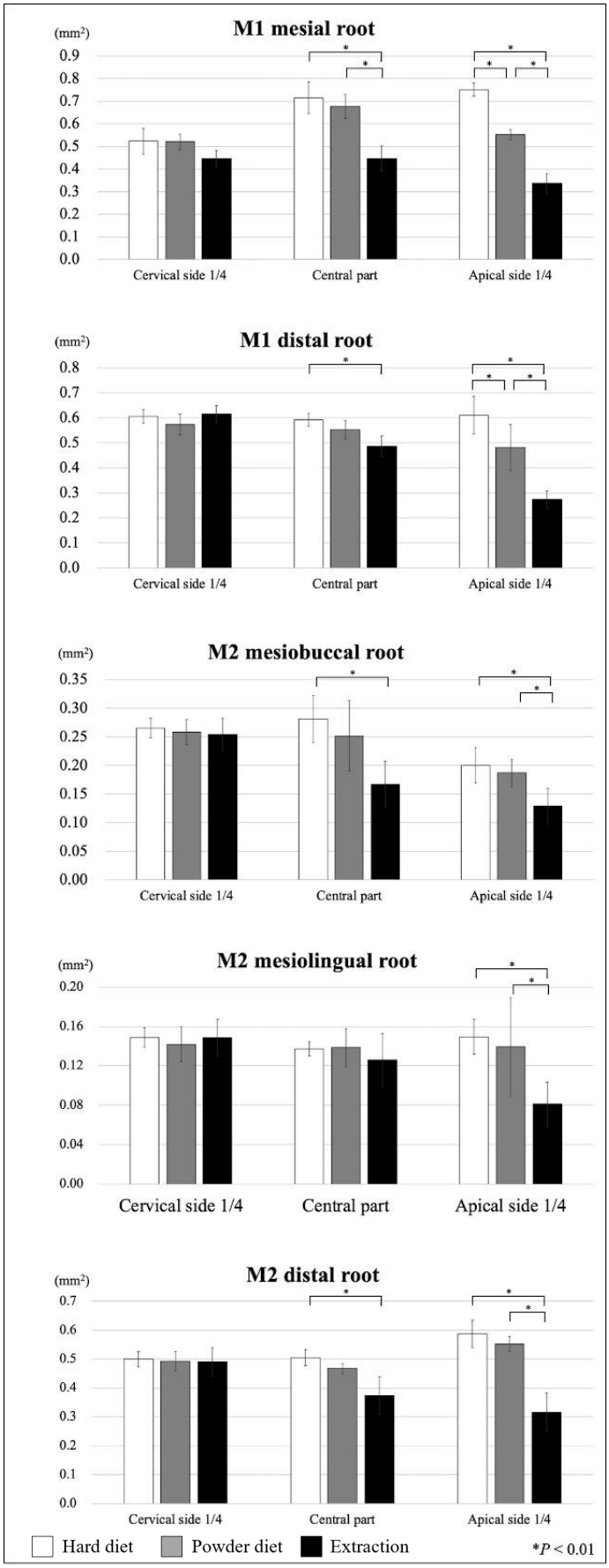


Figure 10. Root cross-sectional area in the micro-CT analysis. Data are presented as mean ± standard deviation (*$p < 0.01$).

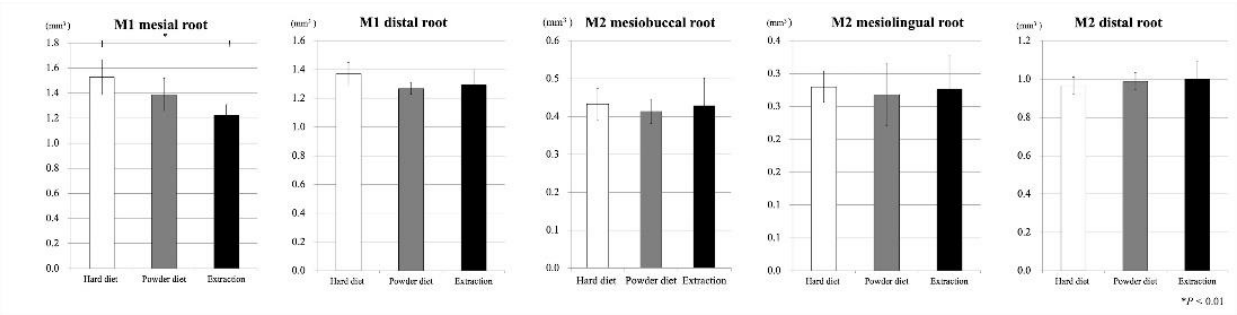


Figure 11. Root volume in the micro-CT analysis. Data are presented as mean ± standard deviation (*$p <$ 0.01).

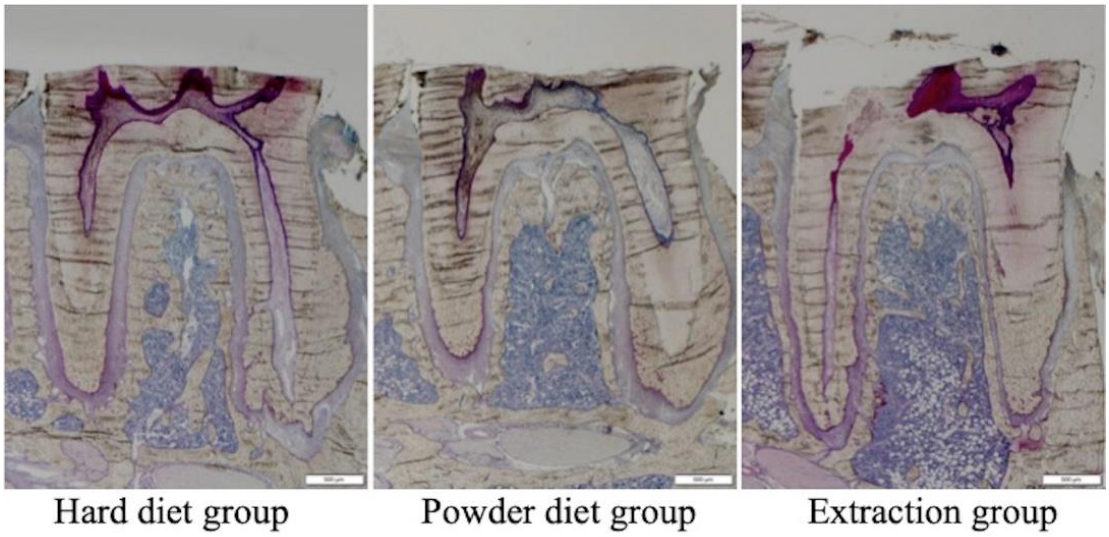


Figure 12. Tissue images of sagittal sections of the interradicular septum in the first mandibular molar. Bar: 500 μm.

Hard diet group: The septal outline is proportionate to that of the root branches on the other side of the Sharpey's fibers. The internal architecture features narrow medullary cavities with extensive trabecular distribution.

Powder diet group: The center of the septal apex is bifurcated. The upper section of the outline (directly underneath the crown) is thick, but the lines are thinner in the lower sections along the root. The internal architecture features wide medullary cavities with isolated trabecular clusters.

Extraction group: The center of the septal apex is trifurcated. The upper section of the outline (directly underneath the crown) is thick, but the lines running down the root are conspicuously thin and broken in several places. The internal architecture features markedly wide medullary cavities with several thin trabecular beams.

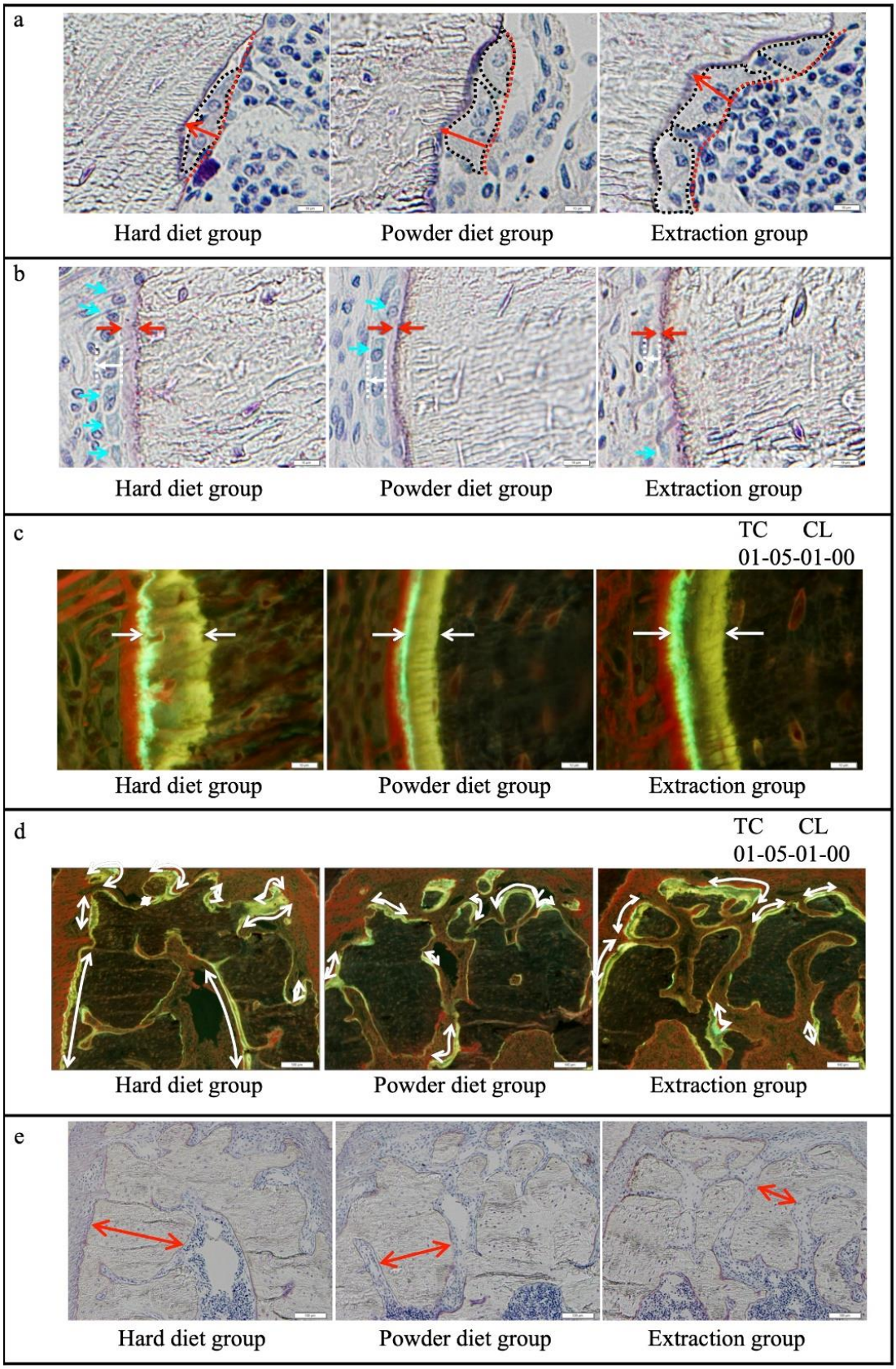
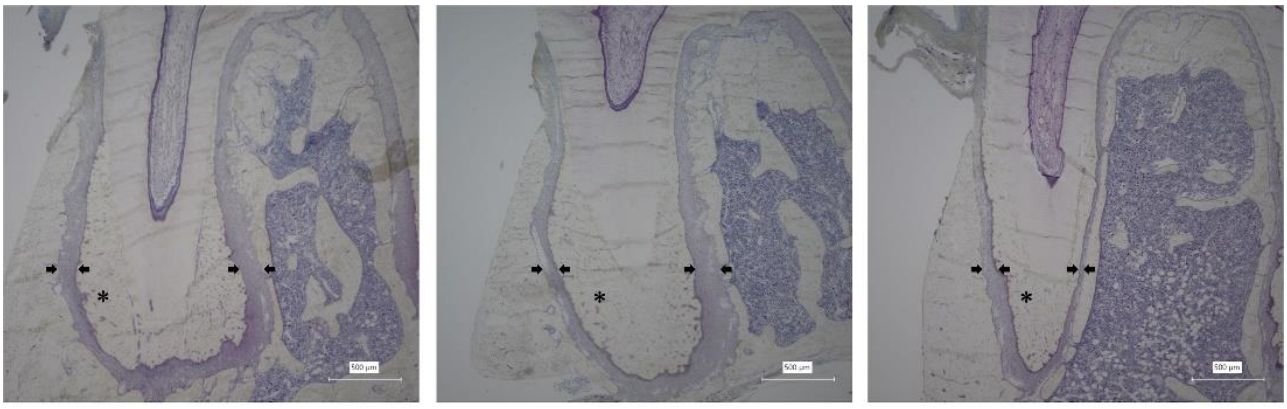

a
Hard diet group
Powder diet group
Extraction group
b
Hard diet group
Powder diet group
Extraction group
c
TC CL
01-05-01-00
Hard diet group
Powder diet group
Extraction group
d
TC CL
01-05-01-00
Hard diet group
Powder diet group
Extraction group
e
Hard diet group
Powder diet group
Extraction group

Figure 13. Tissue images of sagittal sections of the interradicular septum in the first mandibular molar; **(a)** The osteoclasts are shown within the dotted lines. Bone lining cells are seen at the apex of the osteoclasts. The red dotted line indicates the boundary between the clusters of bone marrow cells and the corresponding bone cells. The red arrows indicate the erosion depth, which is the distance to the deepest recess of the resorption cavity. Bar: 10 μm. Compared to controls, the experimental groups had greater erosion depths. **(b)** The osteoblast and osteoid thickness are shown. Blue arrows indicate osteoblasts. White arrows indicate osteoblast cell heights. Red arrows indicate osteoid thickness. Bar: 10 μm. Compared to controls, the experimental groups had few in number, short in height of the osteoblasts and thin osteoid. **(c)** Fluorescence microscopic images of the interradicular septum of the first mandibular molar. The emitted yellow and green light are tetracycline (injected seven days before imaging) and calcein (injected one day before imaging), respectively. Arrows indicates the double labeled width. Bar: 10 μm. Regarding the double bone labels, compared to controls, the intervals are shorter in the experimental groups. **(d)** Fluorescence microscopic images of the interradicular septum of the first mandibular molar. Arrows indicates the length of the double labeled surfaces. Bar: 100 μm. Regarding the length of the double labeled surfaces, in the control group, many surfaces continue uninterrupted, but in the experimental groups, they are shorter in comparison. **(e)** High magnification of the interradicular septum of the first mandibular molar. Arrows indicate the interradicular septum width. Bar: 100 μm. The Hard diet group had broad, sheet-like trabeculae, the Powder group had fragmented trabeculae, and the Extraction group had more finely shredded trabeculae.



Hard diet group Powder diet group Extraction group

Figure 14. Tissue images of the sagittal sections of the M1 mesial root. Arrows indicate the periodontal membrane width. The asterisk indicates cellular cementum.

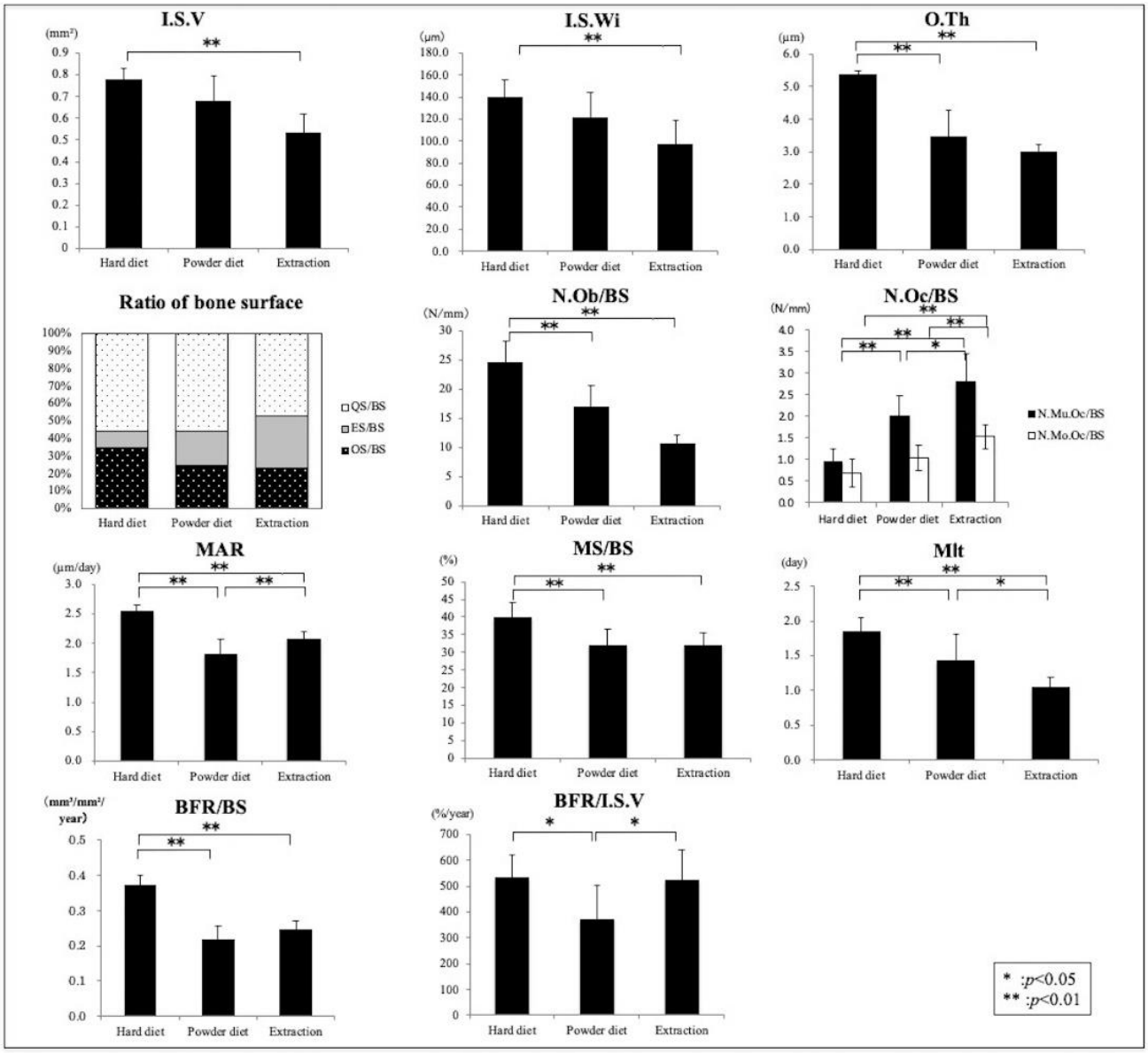


Figure 15. Histomorphometric analysis of secondary cancellous bones in the interradicular septum of the mandibular first molar. Histomorphometrical parameters (I.S.V; Interradicular septum volume, I.S.Wi; Interradicular septum width, O.Th; Osteoid thickness, ES/BS; eroded surface/bone surface, OS/BS; osteoid surface/bone surface, QS/BS; quiescent surface/bone surface, N.Ob/BS; osteoblast number/bone surface, N.Oc/BS; osteoclast number/bone surface, N.Mo.Oc/BS; mononuclear osteoclast number/bone surface, N.Mu.Oc/BS; multinuclear osteoclast number/bone surface, MAR; mineral apposition rate, MS/BS; mineralizing surface/ bone surface, Mlt; mineralization lag time, BFR/BS; bone formation rate/bone surface and BFR/I.S.V; bone formation rate/ Interradicular septum volume) of cancellous bone in the interradicular septum of the mandibular first molar. Data are presented as mean ± standard deviation. (*$p < 0.05$ and **$p < 0.01$)