Original article

Morphological characteristics of interalveolar septum and mandible in BMAL1 gene knockout mice

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Abstract

Purpose: Circadian rhythm is associated with the pathogenesis of systemic disease and bone mineral metabolism. This study aimed to radiographically evaluate morphological characteristics of the interalveolar septum in circadian rhythm deficient animals.

Methods: Heads of 10 brain and muscle arnt-like protein-1 (BMAL1)-knockout (KO) mice and 10 wild-type mice sacrificed at 36 weeks were imaged using micro-computed tomography. The mean depth from the cemento-enamel junction to the alveolar ridge (virtual bone sounding: VBS) of the interalveolar septum between the first and second molars, and the bone mineral density (BMD) of the interalveolar septum and the mandibular inferior cortex region were calculated. Tooth diameter was also measured.

Results: The VBS of the interalveolar septum in the BMAL1-KO mice was significantly deeper than that in wild-type mice. The BMD in the BMAL1-KO mice was significantly lower than in the wild-type mice in both regions. No significant difference was observed in tooth diameter between BMAL1-KO and wild-type mice.

Conclusion: These results suggest that low BMD in the interalveolar septum accelerates bone resorption in the interalveolar septum in BMAL1-KO mice.

Keywords: BMAL1, bone mineral density, circadian rhythm, periodontitis

Introduction

Periodontal disease is the one of the most common inflammatory diseases in the oral cavity, affecting 20-50% of the global population [1]. The pathology of advanced periodontal disease and chronic periodontitis is characterized by destruction of the alveolar bone and periodontal ligament by immune and inflammatory responses attributable to oral microbiota, including anaerobic bacteria such as *Porphyromonas*, *Aggregatibacter*, and *Prevotella* [2]. It has been recognized that in addition to bacterial factors, various host and environmental factors contribute to the pathogenesis and development of periodontal disease, such as age, sex, dry mouth, systemic disease, stress, obesity, pregnancy, smoking, medication, education, and socioeconomic status [3].

Circadian rhythms in mammals are physiological and behavioral cycles with a recurring periodicity that coordinates internal time with the external environment in a daily cycle of approximately 24 h [4-6]. Circadian rhythms play an important role in biological homeostasis and adaptation to

fluctuating environments such as the light-dark cycle, temperature, arousal stimuli, and feeding [6]. Several studies have shown that disruption of circadian rhythms induces the development and progression of various conditions or diseases such as aging, obesity, diabetes mellitus, cardiovascular disease, malignancies, psychiatric diseases, and sleep disorders [6-8].

Circadian rhythms are regulated by coordinated action between the central circadian clock system located in the suprachiasmatic nucleus and the peripheral clock system located in almost all cells of the body [9]. The essence of molecular interaction in the mammalian circadian rhythm is the transcription-translation feedback loop of brain and muscle arnt-like protein-1 (BMAL1) / circadian locomotor output cycles kaput (CLOCK)-period (PER) / cryptochrome (CRY) [6,7,9]. In BMAL1-knockout (KO) mice, CLOCK mutant mice, and PER- and CRY-deficient mice, significant changes have been detected in the circadian behavioral rhythms [9]. Qiu et al. [10] reported high nocturnal locomotion and reduced sleep in BMAL1-KO macaque monkeys. A previous study by Haque et al. [11] showed that BMAL1-KO mice were subject to reduced lifespan, sarcopenia, and premature aging.

It has been recognized that the circadian rhythm in mammals is also closely related to bone metabolism [12]. Reduced bone volume in BMAL1-KO and CLOCK-KO mice, and increased bone volume in PER- and CRY-deficient mice have been reported [12]. However, no research showing a relationship between alveolar bone and circadian rhythm has been published. Thus, this study was conducted to radiographically evaluate morphological characteristics of the interalveolar septum in circadian rhythm deficient BMAL1-KO mice.

Materials and Methods

The procedure for the experimental animals was approved by the Animal Experimentation Committee of Nihon University School of Pharmacy (AP15P024).

Experimental animals

Ten female C57/BL strain mice (5 BMAL1-KO mice and 5 wild-type mice as controls), which were bred in Nihon University School of Pharmacy, were included in the study. The mice were housed with five animals per cage in a facility maintained at 23 ± 1°C with 50% ± 10% relative humidity on a 12-h light / 12-h dark cycle with normal pellet feeding. The mice were sacrificed at 36 weeks at Nihon University School of Pharmacy by carbon dioxide. Dissected heads of the mice fixed in 10% formalin neutral buffer solution were transferred to the Department of Oral and Maxillofacial Radiology Nihon University School of Dentistry. There was no information on body weight.

Micro-computed tomography (micro-CT)

Micro-CT examination was performed using the R_mCT system (Rigaku, Tokyo, Japan) for all subjects. Imaging conditions were as follows: tube voltage 90 kV, tube current 200 μA, exposure time 2 m, voxel size 20 × 20 × 20 μm³, matrix size 480 × 480 × 480. The center of x, y and z axis of the FOV was set at the center of the mandible at molar region,

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Fig. 1 Location of the volume of interest (VOI) in the interalveolar septum between the first and second molars. A: Cross-sectional slice image. B: Parasagittal slice image. The VOI was set in accordance with the axis of the interalveolar ridge between the first and second molars on the sagittal image parallel to the dental arch (A). The buccolingual center of the VOI was adjusted to the buccolingual center of the first molar on the cross-sectional image vertical to the dental arch. The superior edge of the VOI was also set at the lowest point of the cemento-enamel junction at the distal interproximal surface of the first molar.

Fig. 3 Location of the volume of interest (VOI) in the inferior mandibular cortex. A: Cross-sectional slice image. B: Parasagittal slice image. The VOI was set at the inferior border of the mandible corresponding to the interalveolar septum between the first and second mandibular molars. The lower and medial edges were at the lower and medial margins of the mandible, respectively.

occlusal plane, and anterior border of mandibular first molar, respectively. The imaging datasets were stored in an image server before image evaluation.

Image processing and measurements

The cross-sectional and transverse images to the mandibular molars were reconstructed with i-view software (J. Morita MFG, Kyoto, Japan). This study employed original CT images analyzer software (3by4viewer Ver. 2, Kitasenju Radist Shika, Tokyo, Japan) and virtual bone sounding analyzer software (Depth analyzer Ver. 1, Nihon University School of Dentistry, Tokyo, Japan) to measure the four items of both sides of the mandible described below.

Virtual bone sounding (VBS) value

A cuboid volume of interest (VOI), which was $400 \times 400 \times 40 \mu\text{m}$ (20 pixels in both buccolingual and craniocaudal dimensions, and 2 voxels in the mesiodistal dimension), was set in the interalveolar septum between the first and second mandibular molars on the cross-sectional image of the mandible (Fig. 1). The buccolingual center of the VOI was positioned against the buccolingual center of the mandibular first molar. The superior edge of the VOI was also set at the lowest point of the cemento-enamel junction (CEJ) on the distal interproximal surface of the first molar. A calibration phantom (Ratoc phantom hydroxyapatite, Ratoc System Engineering, Tokyo, Japan) was employed to measure an accurate mineral-equivalent value in milligrams per cubic centimeter of calcium

Fig. 2 Extraction of interalveolar septum in the volume of interest (VOI). A: The VOI set on the image. B: The red area in the VOI indicates the area except for bone in the interalveolar septum ($<300 \text{ mg HA/cm}^3$).

Fig. 4 Measurements of tooth diameter. Maximum mesiodistal (solid double-headed arrow) and buccolingual (dotted double-headed arrow) diameters of the crowns of the mandibular second molars were measured on horizontal slice images.

hydroxyapatite as 200, 300, 400, 500, 600, 700 and 800 mg HA/cm^3 . The threshold of bone was set at over 300 mg HA/cm^3 . In Fig. 2, the red area shows the area of defect in the interalveolar septum, which was the volume from the CEJ to boundary line in the interalveolar septum. The VBS value was defined as the mean depth from the CEJ to the alveolar ridge in the VOI and was calculated using the following formula: $\text{VBS} = \text{volume from the CEJ to boundary line in the interalveolar septum} / (\text{buccolingual size of the VOI} \times \text{mesiodistal size of the VOI})$

Bone mineral density (BMD) of the interalveolar septum

The VOI was set in the interalveolar septum as well as measuring VBS. Mean BMD was automatically calculated using a bone density curve which was initially made using a calibration phantom to measure an accurate mineral-equivalent value. BMD was defined as bone mineral density in the bone structure ($>300 \text{ mg HA/cm}^3$) in the VOI.

BMD of the inferior cortex of the mandible

The VOI was set at the inferior border of the mandible corresponding to the location of the interalveolar septum between the first and second mandibular molars. The lower and medial edges were at the lower and medial margins of the mandible, respectively (Fig. 3). Other conditions were similar to those used to measure the BMD of the interalveolar septum.

Tooth diameter of the mandibular second molars

The maximum mesiodistal and buccolingual diameters of the crowns of the mandibular second molars were measured on horizontal slice images that were parallel to the occlusal plane of the mandibular molars (Fig. 4).

All measurements were performed by one periodontist. The collected measurements were used to compare BMAL1-KO and wild-type mice.

Table 1 Comparison of virtual bone sounding value and bone mineral density between BMAL1-KO and wild-type mice

	BMAL1-KO mice			Wild-type mice			<i>P</i> value
	Mean	SD	95% CI	Mean	SD	95% CI	
Virtual bone sounding value	316.2 (μm)	64.9	269.8-362.6	253.0 (μm)	65.8	205.9-300.0	<0.05
Bone mineral density (interalveolar septum)	520.4 (mg HA/cm ³)	45.5	487.9-552.9	665.1 (mg HA/cm ³)	68.3	616.2-714.0	<0.01
Bone mineral density (mandibular inferior cortex)	716.4 (mg HA/cm ³)	27.7	696.6-736.2	825.8 (mg HA/cm ³)	18.2	812.8-838.8	<0.01

BMAL1-KO, brain and muscle arnt-like protein-1 knock out; SD, standard deviation; CI, confidence interval; HA, hydroxyapatite

Fig. 5 Representative cross-sectional images in calculating virtual bone sounding value. Brain and muscle arnt-like protein-1 knockout (BMAL1-KO) mice showed more severe bone loss in the interalveolar septum than in wild-type mice. Volume of interest (VOI) was set in the alveolar septum of the BMAL1-KO (A) and wild-type (B) mice. Red area indicates volume from the CEJ to boundary line of the interalveolar septum in the VOI in the BMAL1-KO (C) and wild-type (D) mice.

Statistical analysis

Shapiro-Wilk and Levene's tests were performed to confirm the normality and homoscedasticity of the measurements, respectively. In measurements with normal distribution and homoscedasticity, Student's *t*-test was used for the comparison between BMAL1-KO and wild-type mice. Mann-Whitney's *U*-test was employed for analysis of abnormally distributed data. All statistical analyses were performed using SPSS Statistics for Windows version 25.0 (IBM Corp., Armonk, NY, USA). The statistical significance level was set at a *P* value of <0.05.

Results

VBS value

The mean VBS value in the BMAL1-KO mice (mean 316.2 μm) was significantly higher than that in the wild-type mice (mean 253.0 μm ; *P* < 0.05, Table 1 and Fig. 5).

BMD of the interalveolar septum

The mean BMD of the interalveolar septum in the BMAL1-KO mice

(mean 520.4 mg HA/cm³) was significantly lower than that in the wild-type mice (mean 665.1 mg HA/cm³; *P* < 0.01, Table 1).

BMD of the inferior cortical margin of the mandible

The mean BMD of the inferior cortical margin of the mandible in the BMAL1-KO mice (mean 716.4 mg HA/cm³) was significantly lower than that in the wild-type mice (mean 825.8 mg HA/cm³; *P* < 0.01, Table 1).

Tooth diameter of the mandibular second molars

Means of the mesiodistal diameter of the mandibular second molars in BMAL1-KO and wild-type mice were 1553.0 μm and 1540.0 μm , respectively.

Medians of the mesiodistal diameter of the mandibular second molars in BMAL1-KO and wild-type mice were 935.0 μm and 920.0 μm , respectively.

No significant difference was detected in either the mesiodistal or buccolingual diameter of the second molars in the BMAL1-KO mice compared with wild-type mice (*P* > 0.05, Table 2).

Discussion

In this study, BMD in the interalveolar septum and inferior cortex of the mandible were lower in BMAL1-KO mice than in wild-type mice. Similar results were identified in the BMD of the tibia and femur in BMAL1-KO animals [12]. Additionally, BMAL1-KO animals have been shown to have low bone mass and short bone formation in long bones and the mandibular condyle [12,13]. A deficiency of BMAL1 promotes osteoclast differentiation and suppresses differentiation of osteoblasts and chondrocytes [14,15], finally resulting in reduced bone mass and BMD. Thus, the decrease in BMD in the interalveolar septum and the inferior cortex of the mandible in BMAL1-KO mice may be one phenotype of BMAL1 deficiency. Other clock gene KO animals have also exhibited bone metabolism abnormalities such as increased or decreased bone mass [16]. Therefore, circadian function has attracted considerable attention in the field of bone research. The present study used 36-week-old mice. In general, mice as old as 48 weeks are considered to be mature adults equivalent to 42-year-old humans (Life span as a biomarker [https://www.jax.org/research-and-faculty/research-labs/the-harrison-lab/gerontology/life-span-as-a-biomarker] accessed on 30 June 2020). Thus, 36-week-old mice can be considered to be in early middle-age and do not normally show abnormal decreases in BMD. In the BMAL1-KO mice, normal growth is observed until about 11 weeks, and then pathological changes in some biological functions, such as activity levels and body weight, gradually develop [17]. Zhou et al. [13] evaluated the morphology and bone volume of the mandibular condyle in BMAL1-KO mice and showed that no significant hypoplasia or low bone volume was evident until at least 8 weeks. Thus, low BMD in the BMAL1-KO mice in the present study might not be a pathological condition, but may be a developmental or aging change in the interalveolar septum.

This study demonstrated that bone resorption in the interalveolar septum was more severe in BMAL1-KO mice than in wild-type mice, possibly because of low BMD in the interalveolar septum. It has been reported that decreased BMD is correlated with the severity of periodontal disease in postmenopausal women [18,19]. Night-shift working and rotating-shift working have been identified as risk factors for osteoporosis and osteoporotic fracture as a result of reduced BMD [20,21]. Another study revealed a relationship between shift work and periodontitis in middle-aged adults [22]. Thus, systemic decreases in BMD are associated with the severity of periodontal bone resorption as shown in the results of this study.

Table 2 Comparison of the tooth diameters of mandibular second molars between BMAL1-KO and wild-type mice

	Mesiodistal diameter			Buccolingual diameter	
	Mean (μm)	SD	95% CI	Median (μm)	IQR
BMAL1-KO	1553.0	45.0	1520.8-1585.2	935.0	52.5
Wild	1540.0	56.1	1470.3-1609.7	920.0	27.5

BMAL1-KO, brain and muscle arnt-like protein-1 knockout; SD, standard deviation; CI, confidence interval; IQR, interquartile range. No significant difference was detected in either the mesiodistal or buccolingual diameter of the second molars in the BMAL1-KO mice compared with wild-type mice. Buccolingual diameter showed abnormal distribution and was analyzed with non-parametric test.

Reduced salivary flow rate and salivary contents increase the risk of periodontal disease [23,24]. Circadian rhythm is also associated with salivary function [25]. Some researchers have established that IgA secretion in saliva is associated with sleep and circadian rhythm [26,27]. Thus, deficiencies in the circadian rhythm may be a risk factor for periodontal disease and other oral diseases.

Aging is significantly associated with the increased morbidity of periodontal disease [28]. BMAL1-KO mice have been shown to experience early aging as seen in the increase of reactive oxygen species (ROS) in whole body tissue [29]. ROS are also strongly implicated in the pathogenesis of periodontal inflammation and alveolar bone resorption in periodontitis [30,31]. In the present study, the reason for severe alveolar bone resorption in BMAL1-KO mice was unclear, but may have been due to multiple factors related to the abnormality of the bone phenotype, the low BMD regulated by circadian rhythm disturbance, salivary dysfunction, earlier aging and ROS.

There was no significant difference in tooth diameter between the two groups in the study. BMAL1 deficiency does not regulate enamel morphology because enamel formation is completed just after birth. However, the relationship between amelogenesis and circadian rhythm has been demonstrated in *in vitro* studies [32,33]. Simmer et al. [32] showed that circadian oscillations are involved in the formation of cross-striations and the striae of Retzius in enamel by regulating the secretion of enamel proteins. Therefore, the degree of mineralization may be decreased in BMAL1-KO mice. However, no studies have revealed tooth size modulation in BMAL1-KO animals or circadian rhythm deficient models.

This study has limitations. First, it was a cross-sectional study and did not evaluate the time course of bone formation, resorption, growth, or aging. Second, the study employed only radiographic evaluation. Therefore, it was not possible to clarify the mechanism involved in the acceleration of bone loss in the interalveolar septum by each effect of low BMD, salivary dysfunction, earlier aging, and ROS regulated by circadian rhythm disturbance. Future research will include time-independent pathological and genetic evaluation of alveolar bone changes.

In conclusion, this study revealed that 36-week-old BMAL1-KO mice exhibited lower BMD in the interalveolar septum and the inferior cortical margin of the mandible, and more significant bone loss in the interalveolar septum, when compared with wild-type mice. These results suggest that low BMD in the interalveolar septum accelerates bone resorption in the interalveolar septum in BMAL1-KO mice.

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Conflict of interest

The authors have no conflict of interest to declare in association with this study.

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