Research Article

Melatonin is more effective on bone metabolism when given at early night than during the day in ovariectomized rats

Yukari Miki^[1], Azusa Seki^[2], Hiroyuki Mishima[3,4], Yusuke Maruyama[5], Kazuki Watanabe[6], Jingjing Kobayashi-Sun[6,7], Isao Kobayashi[7], Kohei Kuroda[8], Shion Oshima[8], Takeru Okamoto[9,10], Hajime Matsubara[11], Ajai K. Srivastav[12], Yoshiaki Tabuchi[13], Jun Hirayama[6,9], Atsuhiko Hattori[5], and Nobuo Suzuki[8]*

[1]Shikoku Chuken, Inc., Ayauta-gun, Kagawa 761-2101, Japan
[2]HAMRI Co. Ltd., Koga, Ibaraki 306-0101, Japan
[3]Department of Dental Engineering, School of Dental Medicine, Tsurumi University, Yokohama, Kanagawa 230-8501, Japan
[4]Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan
[5]Department of Sport and Wellness, College of Sport and Wellness, Rikkyo University, Niiza Saitama 352-8558, Japan
[6]Department of Clinical Engineering, Faculty of Health Sciences, Komatsu University, Komatsu, Ishikawa 923-0961, Japan
[7]Faculty of Biological Science and Technology, Institute of Science and Engineering, Kanazawa University, Kanazawa, Ishikawa 920-1192, Japan
[8]Noto Marine Laboratory, Institute of Nature, and Environmental Technology, Kanazawa University, Ogi, Noto-cho, Ishikawa 927-0553, Japan
[9]Division of Health Sciences, Graduate School of Sustainable Systems Science, Komatsu University, Komatsu, Ishikawa 923-0961, Japan
[10]Division of Clinical Engineering, Japanese Red Cross Kanazawa Hospital, Kanazawa, Ishikawa 921-8162, Japan
[11]Noto Center for Fisheries Science and Technology, Kanazawa University, Osaka, Noto-cho, Ishikawa 927-0552, Japan
[12]Department of Zoology, D.D.U. Gorakhpur University, Gorakhpur 273-009, India
[13]Division of Molecular Genetics Research, Life Science Research Center, University of Toyama, Toyama 930-0194, Japan
^ These authors contribute equally
* Correspondence: nobuos@staff.kanazawa-u.ac.jp, Tel: 81-768-74-1151

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ABSTRACT

Melatonin has diverse effects, and has been reported to promote bone formation in addition to regulating the sleep–wake cycle. In the present study, we investigated the effects of melatonin on bone metabolism using ovariectomized (OVX) rats; a model of postmenopausal osteoporosis. Here, we focused on the differences in bone formation when melatonin was subcutaneous injected at day

or early night. The OVX rats were injected with melatonin once daily (0.8 or 8 mg/head) between 11:00 to 14:00 or 18:00 to 19:30 for the day or early night, respectively, for six weeks. After completion of the injection, the femur and tibia in the OVX rats were dissected under general anesthesia and examined by quantitative computed tomography (pQCT) and histological analysis, respectively. Interestingly, the trabecular bone mineral density in the femur metaphysis of the OVX rats receiving 8 mg/head melatonin at early night was higher than those receiving melatonin during the day and they recovered to a similar level as the rats with sham treatment. In the diaphysis, the pQCT analysis results indicated that there was no significant difference in bone mineral density between the day and early night melatonin-injected OVX rats. Histological analysis of the secondary trabecular bone in the tibia of the OVX rats, revealed that the bone matrix area of the group receiving 8 mg/head melatonin at early night was higher compared with that of the day group and had a significant difference compared with OVX treatment rats. Taken together, the subcutaneous melatonin injection in OVX rats at early night was found to promote trabecular bone formation better than melatonin injection during the day. The timing of melatonin injection is a crucial factor when examining the influence of bone metabolism.

Key words: Melatonin, ovariectomy, rats, quantitative computed tomography, bone metabolism, circadian rhythm, histochemistry

1. INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine) is an indolamine that is synthesized from tryptophan through four enzymatic reactions and is mainly secreted by the pineal gland at night (1). Sleep is induced by the elevated melatonin secretion at night. Therefore, melatonin is a therapeutic agent used for the treatment of insomnia (2, 3). In addition to regulate the sleep–wake cycle, melatonin has diverse effects (4). In recent years, attention has been focused on the bone-regulating function of melatonin (5-11). A characteristic feature of melatonin's action is its ability to inhibit osteoclast activity, and this inhibitory effect of melatonin on osteoclasts has been reported in both in vitro and in vivo experiments (4–11). Therefore, we hypothesize that melatonin is a potential curative molecule for the inhibition of bone resorption in human bone diseases such as osteoporosis.

To investigate the effects of long-term administration of melatonin on bone metabolism, it has been previously reported that melatonin (100 μg/mL) added to the drinking water and orally administered to mice from 4 to 20 months of age (12). As a consequence of this long-term melatonin treatment, melatonin was effective to maintain the bone strength of the femoral diaphysis and metaphysis without side effects (12). Bisphosphonates have been utilized as therapeutic drugs to cure postmenopausal osteoporosis (13, 14); however, bisphosphonates have been known to cause serious side effects, such as osteonecrosis of the jaw (15, 16). The use of melatonin as a medicine against an anti-osteoporosis would be beneficial as it has a high safety profile.

Based on the evidence mentioned above, the following hypothesis is proposed. We speculate that melatonin given at night would be more effective for bone metabolism than given during the day due to the fact that melatonin secretion mainly occurs at night by the pineal gland. Especially, the effects of melatonin are likely to be seen if melatonin is administered early night when melatonin begins to rise (17, 18). However, few studies have compared the effects of melatonin

injection at early night on bone metabolism with those of melatonin injection during the day. In the present study, the effects of melatonin on bone metabolism using ovariectomized (OVX) rats, a postmenopausal osteoporosis model (19) was investigated. Additionally, the differences in bone formation were examined when melatonin was subcutaneous injection at early night or during the day by quantitative computed tomography (pQCT) and histological analysis.

2. MATERIALS AND METHODS

2.1. Animals.

Female Sprague-Dawley rats (5-weeks-old, n = 43) were purchased from Charles River Japan (Kanagawa, Japan). They were fed a standard pellet chow diet containing 1.25% calcium and 0.9% phosphorus (CRF-1: Oriental Yeast, Co., Ltd., Tokyo, Japan). The animals were housed under local vivarium conditions (temperature 24 ± 3°C, humidity 50 ± 20%, and a 12 h on/off light cycle), with free access to food and water. The rats were allowed seven days to acclimatize to the new environment before the study.

These rats were maintained according to the National Institutes of Health Guide for Care and Use of Laboratory Animals, and the protocols were approved by the Laboratory Animal Care Committee of HAMRI Co. Ltd. (Approval No. 07-408).

2.2. Ovariectomy procedures.

Under anesthesia by intramuscular reagent (Somnopentyl, Schering-Plough Corporation, UJ, USA), the abdominal area of rats was shorn with electric clippers and disinfected with 70% ethanol and 10% isodine field solution (Meiji Seika Co., Ltd., Tokyo, Japan). After incision of the abdominal skin, the abdominal muscle was dissected, the ovaries were pulled out and removed. After removal of both ovaries, the peritoneum, abdominal muscle, and skin were sutured in this order. Rats in the sham-operated group underwent the same procedure as the OVX rats, in which the skin and abdominal muscle were incised and the ovaries were exposed outside the abdominal cavity. The ovaries were then returned to the abdominal cavity without removal, and the peritoneum, abdominal muscle, and skin were sutured in this order. Penicillin (200,000 units, Meiji Seika Co., Ltd.) was administered as an antibiotic, and the skin was disinfected with 10% isodine field solution (Meiji Seika Co. Ltd.).

2.3. Treatment paradigm.

OVX rats were used as a model of osteoporosis in the present study (19). The dose of melatonin was determined based on the previous report (7). The blood melatonin levels in Sprague-Dawley rats begin to rise at around 18:00 and peak at around 24:00 (18). Therefore, melatonin was administered at the time when blood melatonin concentration began to rise at 18:00 to 19:30.

The initial body weight of rats was range from 143.7g to 179.1g. Melatonin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was dissolved in dimethyl sulfoxide (DMSO) and injected subcutaneously daily. The rats were randomized by the stratified weight method into six groups: Sham + vehicle (n = 5), OVX + vehicle (n = 10), OVX + melatonin (0.8 mg/head; daytime) (n = 7), OVX + melatonin (8 mg/head; daytime) (n = 7), OVX + melatonin (0.8 mg/head; early nighttime) (n = 7), and OVX + melatonin (8 mg/head; early nighttime) (n = 7). In particular, the

present study evaluated the differences in bone formation when melatonin was subcutaneous injected at early night or during the day. The OVX rats were given melatonin once daily (0.8 or 8 mg/head) between 11:00 to 14:00 or 18:00 to 19:30 for the day or early night, respectively, for six weeks. The melatonin was administered for a period of 6 weeks because blood estrogen levels in the OVX rats were significantly lower 30 days after ovary removal than in the SHAM rats (20). In both the sham and control OVX rats, only DMSO was subcutaneous injected. After six week treatment, the femur and tibia were dissected under general anesthesia (21). Final body weight of rats was range from 268 g to 327 g. After removing the surrounding skin and muscles, each femur was fixed with 4% paraformaldehyde (FUJIFILM Wako Pure Chemical Corporation) in 0.1 M phosphate buffer consisting of disodium hydrogenphosphate 12-water and sodium dihydrogenphosphate dihydrate (pH 7.4) for 2 nights at 4°C. The fixed femoral bones were transferred to 70% ethanol, and the trabecular bone mineral density was measured by pQCT. Tibial bones were utilized for histological analyses. pQCT is a method of structurally analyzing the bone by using X-rays to nondestructively measure and analyze the properties of trabecular and cortical bone. The measurements were performed at 3 mm and 13 mm away areas from the growth plate of the distal metaphysis and middle portion of the diaphysis, respectively (Figure 1).

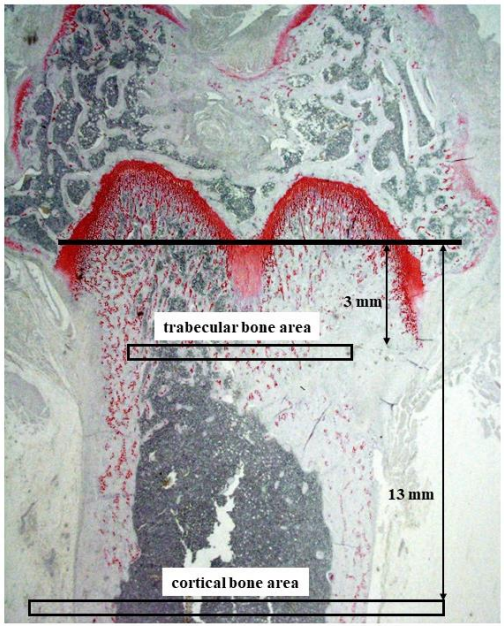

Fig. 1. Measurement area of the femur diaphysis by pQCT.

The measurements were performed at 3 mm and 13 mm away areas from the growth plate of the distal metaphysis and middle portion of the diaphysis, respectively.

2.4. Histological analysis.

After fixation of the tibia from the sham (n = 5), OVX (n = 9), OVX + melatonin (8 mg/head) daytime (n = 7), and OVX + melatonin (8 mg/head) early nighttime (n = 7), the samples were decalcified using 10% EDTA (Dojindo, Kumamoto, Japan) (pH 7.4), and embedded in paraffin. Sections of 4–5 μm thickness were prepared using a microtome (REM-710, Yamato Koki Industrial Co. Ltd., Saitama, Japan) and stained with Meyer's hematoxylin and eosin (HE). Three areas of 500 μm × 500 μm (250000 μm^2) in the secondary trabecular area 500 μm from the growth

plate were measured. The area and number in the trabecular bones were also evaluated. The average of the three areas represents the value for each rat. Measurements were performed under a light microscope (ECLIPSE 80i, Nikon Co. Ltd., Tokyo, Japan) with image analysis software (WinROOF, MITANI Corporation, Tokyo, Japan).

2.5. Statistical analysis.

All results are indicated as the mean ± standard error of the mean. Statistical analysis was assessed by one-way analysis of variance followed by the Bonferroni method. In all cases, the significance level was $p < 0.05$.

3. RESULTS

3.1. pQCT analysis of the femoral bone in OVX rats with or without melatonin administration at day or early night.

pQCT analysis results indicated that 8 mg/head melatonin given at early night appeared to promote bone formation in the trabecular bone (red areas in the image of pQCT in Figure 2) in OVX rats compared to the OVX rats without melatonin (Figure 2). Interestingly, trabecular bone mineral density in the femur metaphysis of the OVX rats receiving 8 mg/head melatonin at early night was higher than that of the OVX rats receiving 8 mg/head melatonin during the day and the density was recovered to a level similar to that of sham rats (Figure 2).

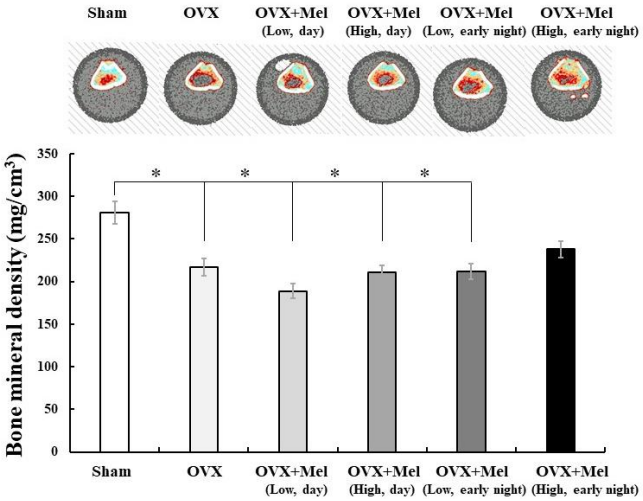

Fig. 2. Trabecular bone mineral density and image data from pQCT on femur metaphysis in ovariectomized (OVX) rats with or without melatonin (Mel) at day or early night.

*Sham rats (n = 5), OVX rats (n = 10), OVX rats with Mel (0.8 mg/head) during the day (n = 7), OVX rats with Mel (8 mg/head) during the day (n = 7), OVX rats with Mel (0.8 mg/head) at early night (n = 7), and OVX rats with Mel (8 mg/head) at early night (n = 7). *: P < 0.05.*

In the diaphysis, the results of pQCT analysis indicated that there were no significant differences in bone mineral density between the day and early night melatonin-treated rats (Figure 3).

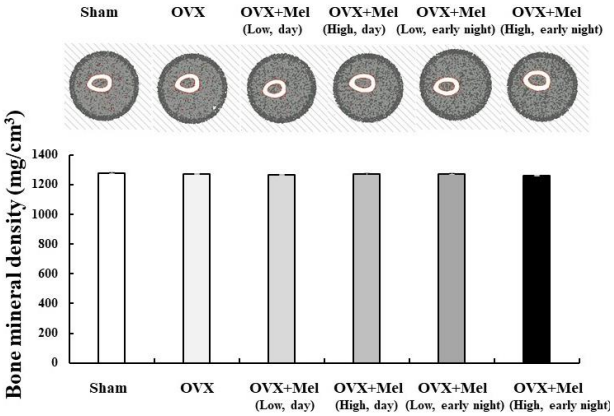

Fig. 3. Bone mineral density and image data from pQCT on the femur diaphysis in ovariectomized (OVX) rats with/out melatonin (Mel) injection at day or early night.

Sham rats (n = 5), OVX rats (n = 10), OVX rats with Mel (0.8 mg/head) during the day (n = 7), OVX rats with Mel (8 mg/head) during the day (n = 7), OVX rats with Mel (0.8 mg/head) at early night (n = 7), and OVX rats with Mel (8 mg/head) at early night (n = 7).

3.2. Histological analysis of the secondary trabecula bone in the tibia.

HE-stained sections of the tibia from rats of 4 groups (1: Sham, 2:OVX, 3:OVX + melatonin, 8 mg/head, daytime injection; 4:OVX + melatonin, 8 mg/head, early night injection) are indicated in Figure 4. The area of the bone matrix in the secondary trabecula bone of the OVX rats (Figure 4B) tended to decrease compared with that of the sham rats (Figure 4A). The area of the bone matrix (secondary trabecular bone) increased with melatonin treatments (Figure 4C, D). Furthermore, the bone matrix area receiving melatonin at early night (Figure 4D) was higher compared with those receiving melatonin during the day (Figure 4C).

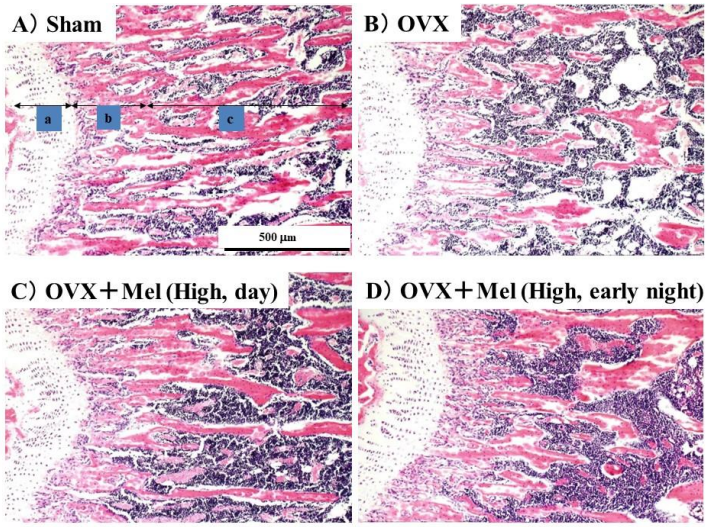

Fig. 4. Hematoxylin and eosin-stained sections of the tibial metaphysis in ovariectomized (OVX) rats with/out melatonin (Mel) injection at day or early night.

A: Sham rats; B: OVX rats; C: OVX rats with Mel (8 mg/head) injection during the day; D: OVX rats with Mel (8 mg/head) injection at early night. a: epiphyseal cartilage area; b: primary trabecular bone area; c: secondary trabecular bone area.

To examine the detailed analysis of the bone matrix area, the area (μm^2) occupied by the bone matrix per unit area (250,000 μm^2) was calculated using the HE-stained sections. There was a significant difference between the sham and OVX rats, OVX rats + melatonin-injected rats at early night (Figure 5). However, there was no significant difference in the number of trabecula bones among the sham, OVX, and OVX + melatonin-treated rats (Figure 6). Melatonin injected at early night functioned to increase the area, not the number, of trabecular bones.

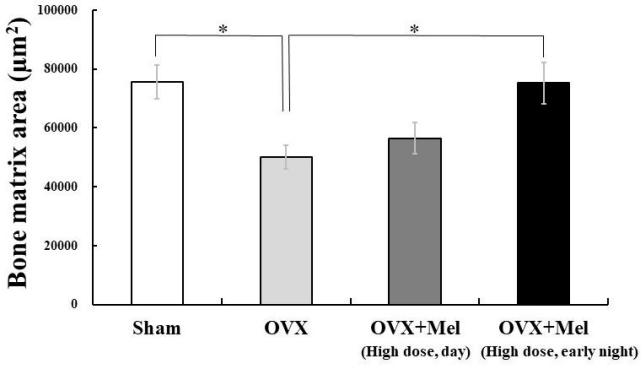

Fig. 5. Analysis of the area of the bone matrix in the tibial metaphysis of ovariectomized (OVX) rats with/out melatonin (Mel)(8 mg/head) injection at day or early night.

*Sham rats (n = 5), OVX rats (n = 9), OVX rats with Mel (8 mg/head) injection during the day (n = 7), and OVX rats with Mel (8 mg/head) injection at early night (n = 7) were used in the present study. *: P < 0.05.*

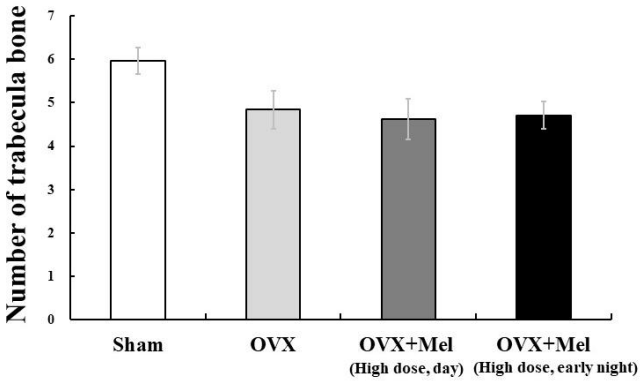

Fig. 6. Number of trabecular bones in the tibial metaphysis of ovariectomized (OVX) rats with/out melatonin (Mel)(8 mg/head) injection at day or early night.

Sham rats (n = 5), OVX rats (n = 9), OVX rats with Mel (8 mg/head) injection during the day (n = 7), and OVX rats with Mel (8 mg/head) injection at early night (n = 7) were used in the present study. There were no significant differences between the number of trabecular bones in the tibial metaphysis of the four groups.

4. DISCUSSION

Disruptions in circadian rhythms due to shift work, aging, and nighttime light exposure are known to suppress nocturnal melatonin levels and are associated with bone loss and fracture risk (22-24). It is important to restore peak nighttime melatonin to normal levels by taking melatonin.

Additionally, the serum night-time melatonin levels in the OVX rats decreased significantly and negatively correlated with serum osteoclastic markers (19). Therefore, the timing of melatonin administration is of critical importance. The present study demonstrated that melatonin subcutaneous injection at early night (18:00 to 19:30) promoted bone formation in OVX rats better than that of melatonin given during the day (11:00 to 14:00). The effect of melatonin mainly occurred in the trabecula bone of the femur and tibia, but not in the diaphysis without trabecular bone. The melatonin receptor expressed in osteoblasts is MT2 (8, 24-28). Melatonin binds to the MT2 of osteoblasts and promotes their activities and growth (6, 25, 27, 29). It has been reported that rounded osteoblasts, an active type of osteoblasts, were located in the secondary trabecula bone (12). Thus, melatonin acted on the osteoblasts and induced the promotion of bone formation in the secondary trabecula bone of OVX rats. Since the effect of melatonin on cortical bone thickness in OVX rats after 8 weeks of treatment (10 or 30 mg/kg/day) was observed (30), the effects of long-term melatonin administration with photoperiodic influence will be examined in the future studies.

It has been reported that melatonin is provided in the drinking water for a blind mouse model (MMTV-Neu transgenic mice) during the hours of darkness (representing nighttime) under 12:12-hr L/D cycle for 1 y (31). This melatonin treatment significantly increased the tibia bone density and osteoblastic marker expression. This study did not compare the effects of nighttime to daytime melatonin administration on the bone characteristics. However, couple of recent studies have compared the effects of melatonin on bone metabolism of OVX rats during the day and at night (32, 33). But, these studies examined the effects of melatonin administered at middle night (21:00 and 22:00) not in the early night (18:00 to 19:30). Their reports indicated a significant increase in bone strength and bone formation when melatonin was administered into OVX rats during the day as compared to when melatonin was administered at night (32, 33). We believe that the best time to given melatonin is at early night. A more detailed analysis of the timing of melatonin administration will be performed in the near future. In addition, as the expression of melatonin receptors including MT2 was increased at night in some tissues (34, 35), the expression of MT2 in trabecula bone may be increased at night. In the next study, the changes in the MT2 expression of trabecula bone will be examined under light and dark cycles to elucidate the efficiency of the different melatonin administration schedules.

In the case of osteoclasts, both direct, and indirect effects of melatonin on osteoclasts have been reported. Recently, in the femoral bone of mice, MT2 was detected in both osteoblasts and osteoclasts (12), indicating that melatonin directly functions on osteoclasts in addition to osteoblasts. One of the indirect effects of melatonin on osteoclasts is the involvement of the receptor activators of the nuclear factor-κB (RANK)/receptor activators of the nuclear factor-κB ligand (RANKL) system regarding the interaction between osteoclasts and osteoblasts (6, 27, 28). RANK is expressed in the osteoclasts, and osteoblasts produce its ligand, RANKL; both possess critical roles in bone metabolism (36, 37). Because RANKL triggers osteoclast activation, RANKL is a key factor in osteoclastic bone resorption (26, 36, 37). In addition, the anti-RANKL antibody was effective against the loss of bone mass (38). Both osteoblasts and multinucleated osteoclasts, an active type of osteoclast, were located in the secondary trabecula bone (12). Therefore, melatonin possibly inhibits bone resorption via the RANK/RANKL system. Recently, a novel pathway has been identified for the inhibitory effect of melatonin on bone resorption (10, 39). Melatonin suppresses bone resorption by increasing the expression of calcitonin, an osteoclast suppressive hormone, produced by osteoblasts in fish (10) and chickens (39). Additionally, it has been recently reported that 45 days of melatonin injection coupled with MEK1/2 and 5 inhibitors

only affected trabecular bone (40). In order to investigate the detailed mechanism of the inhibitory effect of melatonin on bone resorption, RNA-sequencing analysis will be examined in the next experiments.

As mentioned in the Introduction section, melatonin has been found to increase the bone strength of the femoral diaphysis and metaphysis in mice after 1.5 y with no side effects (12). There have also been studies in which melatonin has administered to humans with no side effects. Also, melatonin has a promotive action of bone regeneration (41). Specifically, bone mineral density in the femoral neck of postmenopausal osteopenic women was upregulated in response to melatonin in a dose-dependent manner compared to the placebo group (42). The present study demonstrated that melatonin injected in OVX rats at early night promoted trabecular bone formation better than melatonin injected during the day as analyzed by pQCT and histochemistry. The timing of melatonin injection should be considered because melatonin has potential as a curative drug for human bone diseases such as osteoporosis.

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AUTHORSHIP

YM, AS, HM, AH, and N.S.: conception of the idea, design of the study, and drafted the manuscript; YM, KW, JKS, IK, KK, SO, TO, HM, AKS, YT, and J.H.: analysis and data interpretation. All authors examined/evaluated the data and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors have no competing interests to declare.

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