Research Article

Oral administration of melatonin increases plasma calcium and magnesium and improves bone metabolism in aged male mice

Junko Igarashi-Migitaka[1], Yusuke Maruyama[2], Azusa Seki[3], Jun Hirayama[4], Atsuko Kamijo-Ikemori[1], Kazuaki Hirata[1], Ryoya Kawamura[5], Hajime Matsubara[6], Ajai K. Srivastav[7], Yoshiaki Tabuchi[8], Hiroyuki Mishima[9], Atsuhiko Hattori[2], and Nobuo Suzuki[5*]

[1]Department of Anatomy, St. Marianna University School of Medicine, Miyamae-ku, Kanagawa 216-8511, Japan
[2]Department of Biology, College of Liberal Arts and Sciences, Tokyo Medical and Dental University, Ichikawa, Chiba 272-0827, Japan
[3]HAMRI Co. Ltd., Koga, Ibaragi 306-0101, Japan
[4]Department of Clinical Engineering, Faculty of Health Sciences, Komatsu University, Komatsu, Ishikawa 923-0961, Japan
[5]Noto Marine Laboratory, Institute of Nature and Environmental Technology, Division of Marine Environmental Studies, Kanazawa University, Ogi, Noto-cho, Ishikawa 927-0553, Japan
[6]Noto Center for Fisheries Science and Technology, Kanazawa University, Ossaka, Noto-cho, Ishikawa 927-0552, Japan
[7]Department of Zoology, D.D.U. Gorakhpur University, Gorakhpur 273-009, India
[8]Division of Molecular Genetics Research, Life Science Research Center, University of Toyama, Sugitani, Toyama 930-0194, Japan
[9]Department of Dental Engineering, Tsurumi University School of Dental Medicine, Yokohama, Kanagawa 230-8501, Japan
*Correspondence: nobuos@staff.kanazawa-u.ac.jp, Tel: 81-768-74-1151, Fax 81-768-74-1644

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ABSTRACT

We previously reported that the oral administration of melatonin from 4 to 20 months to male mice improved femoral bone strength and bone density during the aging. Additionally, melatonin receptor, MT2, was immunologically detected in both osteoblasts and osteoclasts of the mouse femoral bone. Thus, melatonin can act on both osteoblasts and osteoclasts to maintain bone strength during the aging process. Here, we analyzed plasma calcium (Ca^{2+}), magnesium (Mg^{2+}), and inorganic phosphorus ($[PO_4]^{3-}$) in 20-month-old male mice with or without administration melatonin (15-20 mg/kg/day) in drinking water. We found that plasma Ca^{2+} and Mg^{2+} levels in melatonin-treated mice increased significantly as compared with control mice. In $[PO_4]^{3-}$, melatonin administration tended to increase its plasma level, but did not reach statistical significance. The potential association between these divalent ions and metabolism markers of femoral bone was also examined. In the femoral diaphysis, the plasma Ca^{2+} and Mg^{2+} concentrations were positively correlated with periosteal and endosteal circumference which were significantly associated with the Strength Strain Index. Therefore, melatonin treatment enlarged femoral diaphysis and enhanced bone strength by increasing

mineral depositions. In addition, the plasma melatonin levels were significantly positive correlation with total bone density and critical thickness in the femoral diaphysis. Since we had not observed the primary trabecular bone and osteoclasts in 20-month-old mice previously, it is suggested that plasma Ca^{2+} and Mg^{2+} are not elevated due to bone resorption. The increased plasma Ca^{2+} and Mg^{2+} by melatonin may originate from the intestinal absorption of these ions since melatonin binds to the vitamin D_3 receptor, its activation is known to promote the intestinal absorption of Ca^{2+}.

Key words: melatonin, calcium, magnesium, inorganic phosphorus, bone metabolism, femoral diaphysis, aging.

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1. INTRODUCTION

Melatonin is a pleiotropic molecule synthesized from tryptophan by four enzymatic reactions, and it is mainly secreted by the pineal gland at night (1). As this molecule controls the sleep–wake cycle, it has been utilized for the treatment of insomnia (2, 3). Recently, the bone regulatory function of melatonin has been attracting attention of researchers because the inhibitory effect of melatonin on osteoclasts has been reported in the in vitro and in vivo studies (4–10). Therefore, we speculated that melatonin has potential as a drug for inhibiting bone resorption in bone diseases.

In aged population, osteoporosis has become a major social problem. Accordingly, anti-resorptive medicines such as bisphosphonates are widely used for curing postmenopausal osteoporosis (11, 12). However, these drugs have been reported to induce serious side effects, such as osteonecrosis of the jaw (13, 14). We emphasize the need for low-risk drugs to prevent bone resorption. For this reason, melatonin has been selected for consideration as an anti-osteoporosis due to its very high safety profile.

We previously developed an age-related bone disease model with naturally-aged male mice (15). Using this model, we added melatonin (100 μg/ml) into drinking water to treat them for a long term (from 4 to 20 months of age) (15). We found that melatonin effectively maintained the bone strength of the femoral diaphysis and metaphysis during the aging process of these male mice (15). Thus, in the present study, we will measure plasma calcium (Ca^{2+}), magnesium (Mg^{2+}), and inorganic phosphorus ($[PO_4]^{3-}$) levels in 20-month-old male mice treated with/without melatonin for 16 months. The total bone density and critical thickness in the femoral diaphysis will also be examined to find the potential association between melatonin treatment and bone metabolism in the aged mice.

2. MATERIALS AND METHODS

2.1. Animals.

To exclude the potential interference of endogenous melatonin on the current study, male BALB/c mice (n = 14) were selected since these mice were reported being unable to synthesize melatonin due to the deficient mutations in the respective genes for the two critical enzymes (aralkylamine N-acetyltransferase and acetylserotonin O-methyltransferase) in the synthetic pathway of melatonin (16, 17). The mice were purchased from a commercial supplier (Japan SLC, Inc. Shizuoka, Japan) at the age of 8 weeks. The purchased mice had been bred at St. Marianna University School of Medicine Animal Experiment Facility. Animal experiments were performed according to the St. Marianna University School of Medicine Institutional Guide for Animal Experiments (Nos. 0808011 and 0910002).

2.2. Long-term oral administration of melatonin in mice.

In the experiment, mice at 4-month-old were divided into two groups (control and melatonin-administered group, 7 mice for each group) and the treatment continued to that the mice have reached 20 months of age. In the melatonin-administered group, melatonin was provided in drinking water. Melatonin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was first dissolved in ethanol to make a stock solution. Then, the stock solution was diluted with distilled water with the final melatonin concentration of 100 µg/ml and the ethanol concentration of 0.5%. Based on the body weight and the water volume drunken by the mice, the calculated melatonin dose for mice was 15-20 mg/kg/day (15). The control group received only the solvent ethanol (final ethanol concentration: 0.5%). Drinking water was changed every 3–4 days. All animals were housed in the room with the temperature 22-23 °C and the light/dark cycle of 12 /12 hrs. The standard mouse diet including calcium (1.15 g/100 g), magnesium (0.32 g/100 g), and phosphorus (1.08 g/100 g) was fed to these mice. When mice reached 20 months of age, the blood was collected from their inferior vena cava under anesthesia with Nembutal. After centrifugation, the plasma samples were kept at -80 °C until analyzed.

2.3. Measurement of plasma Ca^{2+}, Mg^{2+}, and $[PO_4]^{3-}$ concentrations.

Plasma samples of the mice with/without melatonin treatment were sent to a commercial analysis vendor (Oriental Yeast Co., Ltd., Tokyo, Japan) for analysis of their mineral concentrations. The plasma Ca^{2+}, Mg^{2+}, and $[PO_4]^{3-}$ levels (mg/dL) were determined using assay kits (Ca^{2+}: Ca II, Shino-Test Corporation, Tokyo, Japan; Mg^{2+}: Mg・N, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan; $[PO_4]^{3-}$: IP-II, Kyowa Medex Co., Ltd., Tokyo, Japan) followed the manufacture's instruction.

The values of these divalent ions were compared with markers of bone metabolism in each individual mouse as reported by Igarashi-Migitaka *et al*. (2020).

2.4. Analyses of bone metabolism markers in the femoral bone of naturally aged mice.

The left hindlimbs of the mice (20 months of old) were dissected under anesthesia with Nembutal (Dainippon Sumitomo Pharma, Osaka, Japan). After removing the surrounding skin and muscles, each femur was fixed with 4% paraformaldehyde (PFA)(FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) in 0.1 M phosphate buffer (pH 7.4) for 2 days at 4 °C. The fixed bones were transferred to 70% ethanol, and markers of bone metabolism such as bone density and bone strength were measured by peripheral quantitative computed tomography (pQCT) as described in Igarashi-Migitaka *et al*. (2020). The measurements by pQCT were carried out in areas 1.2 mm from the growth plate of the distal metaphysis and in the middle portion of the diaphysis.

2.5. Measurement of plasma melatonin concentrations.

The plasma melatonin was measured by HPLC. Briefly, plasma (50 µl) was adjusted to 1 ml by adding 950 µl of distilled water. Then, 4 ml of chloroform (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) were added to the plasma samples and mixed. The chloroform phase was evaporated to dryness using N_2 gas. The extracts were redissolved in 300 µl of HPLC mobile phase solution, consisting of 50 mM of ammonium acetate (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and 30% methanol (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) (vol/vol), adjusted to pH 4.8 with acetic acid. After centrifugation at 500 × g for 1 min at room temperature, the supernatant filtrated through a

Millex® LH 0.45 mm filter unit (Merck Millipore, Darmstadt, Germany) was subjected to HPLC for melatonin measurement.

2.6. Statistical analysis.

All data were expressed as the mean ± S.E.M. Statistical analyses between two groups were carried out using the Student's t-test. Spearman's or Pearson's correlation coefficient was used to examine the correlation between plasma minerals and bone metabolism markers or among bone metabolism markers in mice. In all cases, the significance level was $p < 0.05$.

3. RESULTS

3.1. Plasma Ca^{2+}, Mg^{2+}, and $[PO_4]^{3-}$ concentrations in 20-month-old mice with/without melatonin treatment.

The plasma Ca^{2+} levels in melatonin-treated mice increased significantly as compared with those in control mice (Figure 1A). Plasma Mg^{2+} concentrations also increased significantly with melatonin treatment (Figure 1B). Melatonin treatment also increased plasma $[PO_4]^{3-}$ level, but it failed to reach statistical significance (Figure 1C).

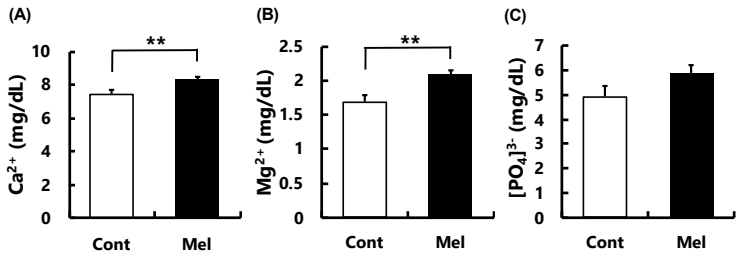

Fig. 1. Plasma Ca^{2+} (A), Mg^{2+} (B), and $[PO_4]^{3-}$ (C) levels in 20-month-old mice with/without melatonin treatment.

*Control group (Cont): n = 7; Melatonin-treated group (Mel): n = 7. **: p < 0.01.*

3.2. Co-relationship between divalent ions (Ca^{2+}, Mg^{2+}, and $[PO_4]^{3-}$) and bone metabolism markers in 20-month-old mice with/without melatonin treatment.

In the femoral metaphysis, we compared plasma Ca^{2+}, Mg^{2+}, and $[PO_4]^{3-}$ to the total bone density, trabecular bone density, X-strength strain index (SSI), Y-SSI, and Polar-SSI. However, there was no significant difference between the levels of plasma divalent ions (Ca^{2+}, Mg^{2+}, and $[PO_4]^{3-}$) and these bone metabolic markers (Table 1).

Table 1 Relationship between divalent ions and bone markers in the femoral metaphysis.

	Ca^{2+}	Mg^{2+}	$[PO_4]^{3-}$
Total bone density	0.313	0.204	0.216
Trabecular bone density	0.137	0.164	0.361
X-SSI	0.322	0.275	-0.216
Y-SSI	0.335	0.319	0.011
Polar-SSI	0.405	0.295	-0.051

Data indicate each r_s value. SSI: Strength strain index.

In femoral diaphysis of the mice, we observed that several bone metabolic markers correlated with plasma Ca^{2+} and Mg^{2+} concentrations. The plasma Ca^{2+} concentrations were positively correlated with periosteal circumference (r_s = 0.806), endosteal circumference (r_s = 0.716), cortical area (r_s = 0.645), X-SSI (r_s = 0.655), and Polar-SSI (r_s = 0.590) (The results are summarized in Table 2 and Figure 2).

Table 2 Relationship between divalent ions and bone markers in the femoral diaphysis.

	Ca^{2+}	Mg^{2+}	$[PO_4]^{3-}$
Total bone density	-0.176	-0.299	0.033
Cortical bone density	0.216	0.195	0.145
Cortical area	0.645*	0.487	0.158
Cortical thickness	0.188	0.121	0.197
Periosteal circumference	0.806**	0.722**	0.141
Endosteal circumference	0.716**	0.735**	0.200
X-SSI	0.655*	0.559*	0.251
Y-SSI	0.511	0.556*	0.183
Polar-SSI	0.590*	0.620*	0.218

Data indicate each r_s value. SSI: Strength strain index.
**: $p < 0.05$; **:$p < 0.01$*

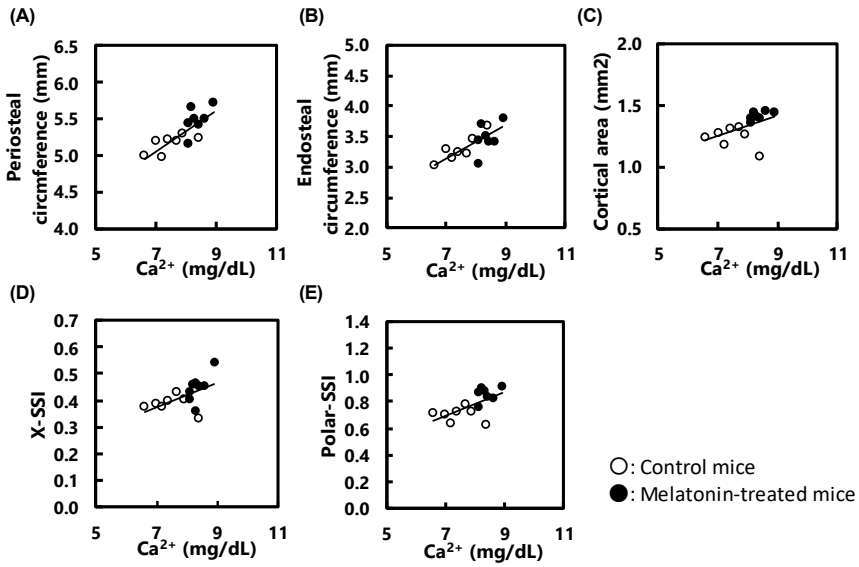

Fig. 2. Co-relation between plasma Ca^{2+} and bone metabolic markers.

(A). periosteal circumference, (B). endosteal circumference, (C). cortical area, (D). X-SSI, and (E). Polar-SSI in the femoral diaphysis of 20-month-old mice.

Plasma Ca^{2+} concentrations were positively correlated with periosteal circumference (r_s = 0.806, $p < 0.01$), endosteal circumference (r_s = 0.716, $p < 0.01$), cortical area (r_s = 0.645, $p < 0.05$), X-SSI (r_s = 0.655, $p < 0.05$), and Polar-SSI (r_s = 0.590, $p < 0.05$). ○: Control mice (n = 7); ●: Melatonin-treated mice (n = 7).

Furthermore, there was a significant co-relation between plasma Mg^{2+} concentrations and bone markers (periosteal circumference, r_s = 0.722; endosteal circumference, r_s = 0.735; X-SSI, r_s = 0.559; Y-SSI, r_s = 0.556; Polar-SSI, r_s = 0.620) in diaphysis (Figure 3). However, there was no significant difference between plasma $[PO_4]^{3-}$ concentrations and the above-mentioned bone markers.

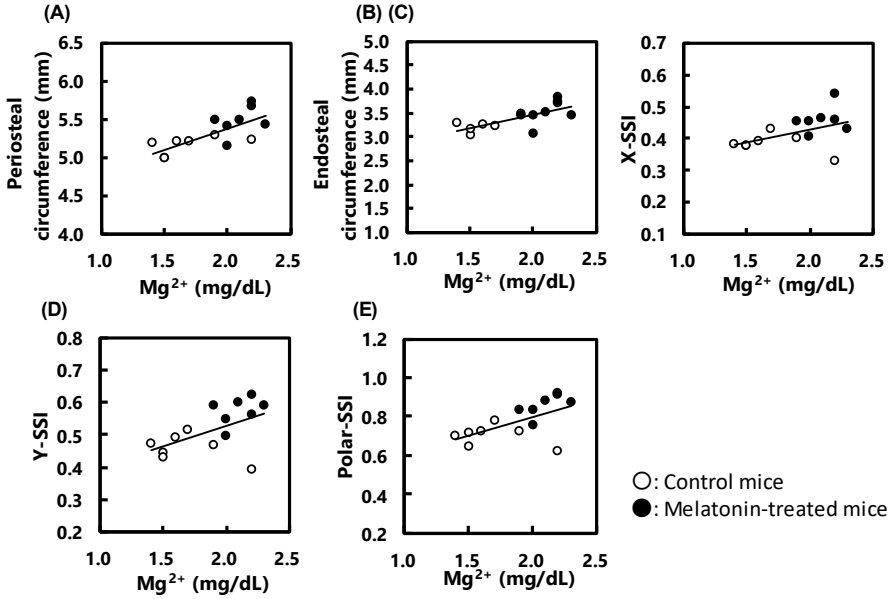

Fig. 3. Co-relation between plasma Mg^{2+} and bone metabolic markers.

(A). periosteal circumference, (B). endosteal circumference, (C). X-SSI, (D). Y-SSI, and (E). Polar-SSI in the femoral diaphysis of 20-month-old mice.

Plasma Mg^{2+} concentrations were significantly positively correlated with periosteal circumference (r_s = 0.722, $p < 0.01$), endosteal circumference (r_s = 0.735, $p < 0.01$), X-SSI (r_s = 0.559, $p < 0.05$),Y-SSI (r_s = 0.556, $p < 0.05$), and Polar-SSI (r_s = 0.620, $p < 0.05$). ○: Control mice (n = 7); ●: Melatonin-treated mice (n = 7).

As both plasma Ca^{2+} and Mg^{2+} concentrations were positively correlated with periosteal circumference and endosteal circumference with significance, we examined the co-relationship between the periosteal and endosteal circumferences and bone strength (X-SSI, Y-SSI, and Polar-SSI). The results are presented in Table 3. In the control mice, there was no significant difference between circumferences and bone strength, while in the melatonin-treated mice, a significantly positive co-relationships were observed between periosteal circumference with Polar-SSI (r_s = 0.786). Additionally, the endosteal circumference was positively correlated with X-SSI (r_s = 0.821) and Polar-SSI (r_s = 0.964) in the melatonin-treated mice.

Table 3 Relationship between strength strain index and periosteal or endosteal circumference in the femoral diaphysis.

	Periosteal circumference	Endosteal circumference
Control		
X-SSI	0.306	-0.018
Y-SSI	0.107	-0.143
Polar-SSI	0.214	-0.250
Melatonin		
X-SSI	0.750	0.821*
Y-SSI	0.536	0.643
Polar-SSI	0.786*	0.964**

*Data indicate each r_s value. SSI: Strength strain index. *: $p < 0.05$; **:$p < 0.01$*

3.3. Co-relationship between plasma melatonin levels and bone metabolic markers in 20-month-old mice with/without melatonin treatment.

In the control mice, the plasma melatonin was not detectable due to their genetic mutation on melatonin synthetic enzymes in this mice strain. Therefore, plasma melatonin levels in the melatonin-treated mice could be used to compare the correlation with bone metabolism markers. It was found that in the femoral diaphysis of the melatonin-treated mice, some bone metabolic markers positively correlated with plasma melatonin concentrations. These included total bone density (r = 0.807) and cortical thickness (r = 0.748) (Table 4 and Figure 4).

Table 4 Relationship between plasma melatonin concentration and bone markers in the femoral metaphysis and diaphysis.

	Plasma melatonin conc.
Femoral metaphysis	
Total bone density	-0.133
Trabecular bone density	0.122
Femoral diaphysis	
Total bone density	0.807*
Cortical bone density	0.175
Cortical area	-0.384
Cortical thickness	0.748*

Data indicate each r value. SSI: Strength strain index.
*: $p < 0.05$; **:$p < 0.01$

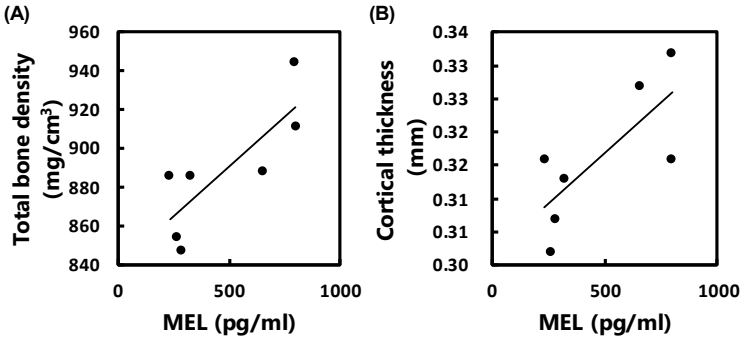

Fig. 4. Co-relationship between plasma melatonin and bone metabolic markers in the femoral diaphysis of 20-month-old mice.

(A). total bone density and (B). cortical thickness. Plasma melatonin concentrations in the melatonin-treated mice (n = 7) were positively correlated with total bone density (r = 0.807, p < 0.05) and cortical thickness (r = 0.748, p < 0.05).

4. DISCUSSION

In the present study, we reported that the plasma Ca^{2+} and Mg^{2+} levels in melatonin-treated mice were significantly higher than those in control mice (Figure 1). Melatonin treatment also increased plasma $[PO_4]^{3-}$ levels but failed to reach the statistical significance (Figure 1). These findings show that melatonin functions in mineral metabolism in the aged mice.

In human adults, Ca makes up 1–2% of the body's weight (18). Most Ca (99%) in the human body is in hard tissues—bones and teeth (18–20). Ca and phosphorus form the hydroxyapatite which is a main component of bone (19–21). Recently, Mg has been reported

to be an essential component of bone (21). Mg influences osteoblastic and osteoclastic activity and regulates bone metabolism (22, 23). Thus, it is noteworthy that our results showed a significant increase in both plasma Mg^{2+} and Ca^{2+} in the melatonin-treated mice. We surmise that the plasma Ca^{2+} and Mg^{2+} elevated by melatonin administration are correlated with bone strength. Thus, we compared plasma minerals with bone metabolic markers. The results showed that in the femoral diaphysis, both plasma Ca^{2+} and Mg^{2+} levels had significantly positive relationships with the periosteal circumference and endosteal circumference (Figures 2, 3 and Table 2). Then, the correlation between these circumferences and the markers of bone strength were also examined. We found that the periosteal and the endosteal circumferences were significantly associated with X-SSI and Polar-SSI (Table 3). Therefore, melatonin induced enlargement of femoral diaphysis and enhanced bone strength by the deposition of divalent ions in the aged mice. The fact that both plasma Ca^{2+} and Mg^{2+} concentrations were positively correlated with bone strength markers such as X-SSI, Y-SSI and Polar-SSI (Figures 2 and 3) supported the possible improvements of bone strength with melatonin supplementation. We also found that plasma melatonin levels are positively correlated with total bone density and cortical thickness (Table 4 and Figure 4). We previously demonstrated that a low concentration of melatonin (100 μg/ml) supplemented in drinking water to male mice from 4 to 20 months improved both bone strength and trabecular bone density in the femoral bone (15). Additionally, the melatonin receptor (MT2) in both osteoblasts and osteoclasts of the femoral bone of male mice was detected (15). Considering all data together, it seemed that melatonin acted on both osteoblasts and osteoclasts to improve bone strength and bone density by mineral depositions during the aging process.

Previously study showed that the primary trabecular bone was not detectable in 20-month-old mice, and also identification of osteoclasts was difficult in these aged mice (15). Therefore, it was suggested that increased plasma Ca^{2+} and Mg^{2+} levels were not attributed to bone resorption but might be from the increased intestinal absorption of Ca^{2+} and Mg^{2+} with melatonin treatment. Vitamin D_3 is well-known as a hormone that promotes the intestinal absorption of Ca^{2+} (19, 24, 25). Recently, it has been reported that melatonin binds to the receptor of vitamin D_3 (26). We thus believe that the long-term administration of melatonin acts on vitamin D_3 receptor of the intestines to promote intestinal resorption of Ca^{2+} and Mg^{2+} (Figure 5). The elevated plasma Ca^{2+} and Mg^{2+} improve total bone density and critical thickness in the femoral diaphysis in the aged mice (Figure 5). In consistent with our findings, it has been reported that melatonin administration enhances bone growth in aged women (7, 27). Thus, the results of current study provide further evidence to support use of melatonin in treatment of osteoporosis in aged population.

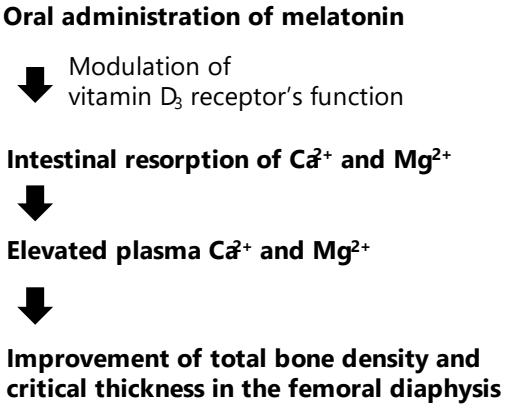

Fig. 5. A model depicting the mechanism by which oral administration of melatonin improves bone metabolism in naturally-aged male mice.

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AUTHORSHIP

JIM, AH, HM, and NS: conception of the idea, design of the study and drafted the manuscript; YM, AS, JH, AKI, KH, RK, AKS, HM and YT: 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 interest to declare.

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