Melatonin is a potential drug for the prevention of bone loss during space flight

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Abstract

Astronauts experience osteoporosis-like loss of bone mass because of microgravity conditions during space flight. To prevent bone loss, they need a riskless and antiresorptive drug. Melatonin is reported to suppress osteoclast function. However, no studies have examined the effects of melatonin on bone metabolism under microgravity conditions. We used goldfish scales as a bone model of coexisting osteoclasts and osteoblasts and demonstrated that mRNA expression level of acetylserotonin O-methyltransferase, an enzyme essential for melatonin synthesis, decreased significantly under microgravity. During space flight, microgravity stimulated osteoclastic activity and significantly increased gene expression for osteoclast differentiation and activation. Melatonin treatment significantly stimulated Calcitonin (an osteoclast-inhibiting hormone) mRNA expression and decreased the mRNA expression of receptor activator of nuclear factor κB ligand (a promoter of osteoclastogenesis), which coincided with suppressed gene expression levels for osteoclast functions. This is the first study to report the inhibitory effect of melatonin on osteoclastic activation by microgravity. We also observed a novel action pathway of melatonin on osteoclasts via an increase in CALCITONIN secretion. Melatonin could be the source of a potential novel drug to prevent bone loss during space flight.

KEYWORDS
calcitonin, fish scales, microgravity, osteoblasts, osteoclasts, RANKL

1 | INTRODUCTION

Exposure to microgravity during space flight leads to rapid bone loss in humans and animals. The rapid and vigorous bone loss is one of the key challenges that remain to be solved to enable humans to pursue healthy lives in outer space. Bone mass often reflects a balance between bone formation by osteoblasts and bone resorption by osteoclasts. An increasing number of studies have demonstrated the effects of microgravity on bone metabolism and osteoblast activity, and
increased bone resorption under microgravity has been widely reported. However, the cellular and molecular mechanisms underlying the phenomena remain unclear. Therefore, further investigations on the mechanisms that could facilitate the discovery of medical drug that effectively addresses bone loss in space are required.

In bone tissue, differentiation and activation of osteoclasts are generally influenced by interactions with osteoblastic lineage cells. Osteoclasts express the receptor activator for nuclear factor κB (RANK), which is a receptor for the RANK ligand (RANKL). RANKL is expressed in some stromal cells such as osteoblasts and bone marrow cells, and is required for osteoclastogenesis and osteoclast activation. Therefore, interactions between osteoclasts and stromal cells are essential for evaluating the mechanisms underlying rapid bone loss during space flight. Although coculture experiments of isolated osteoblasts and osteoclasts are feasible, they do not accurately reflect natural cellular microenvironments. Therefore, the lack of appropriate experimental models for interactions between osteoclasts and stromal cells implies that no published data currently demonstrate the precise mechanisms underlying bone loss during space flight.

Teleost scales are calcified tissues that exhibit multiple similarities with mammalian membrane bone tissues. Morphological features of goldfish scales are illustrated in Figure S1. Fish scales can regenerate following scale removal. The regenerating scales contain multinucleated osteoclasts with actin rings, well-developed ruffled borders, and clear zones. Active cuboidal osteoblasts are observed at the periphery and on the dermis side of the scale. They represent a highly developed rough endoplasmic reticulum and a Golgi apparatus. In addition, biological responses to mechanical stimuli are maintained in regenerating scales after storage at 4°C for 1 week. Therefore, regenerating scales are the most suitable materials for a space experiment examining interactions between osteoblasts and osteoclasts.

Antiresorptive drugs, such as bisphosphonates, are expected to prevent bone loss during space flight. However, some antiresorptive drugs cause unfavorable side effects in some cases such as osteonecrosis of the jaw. Therefore, riskless drugs are required to prevent bone loss during space flight. Melatonin is the principal secretory product of the pineal gland and is synthesized almost exclusively in the dark. Therefore, its blood levels exhibit a discernible daily rhythm. In 2002, we reported, for the first time, that melatonin suppresses osteoclast activity using a goldfish scale culture system. The report predicted the potential application of melatonin as a therapeutic drug for bone loss. Furthermore, following surgical ablation of the pineal gland, spinal malformations have been observed in Atlantic salmon, chicks, and rats. In humans, bone mineral density in the femoral neck of postmenopausal osteopenic women increased in response to melatonin in a dose-dependent manner when compared with a placebo. In addition, in melatonin-treated perimenopausal women, the ratio of bone resorption marker:formation marker exhibited a decreasing trend. The results indicate that treatment with melatonin is a superior method for suppressing osteoclast activity under microgravity conditions, in addition to on the ground. It was recently reported that melatonin is produced in the brain, retina, intestine, ovary, skin, and crystalline lens, which implies that melatonin could have widespread physiological functions in numerous tissues.

Investigating whether melatonin increases an osteoclast-inhibiting hormone, CALCITONIN, in goldfish scales and whether the increased CALCITONIN regulates osteoclast function via paracrine system could offer insights on the role of melatonin. The present study performed space experiments to analyze bone metabolism in goldfish scale during space flight to determine the melatonin-dependent regulatory mechanisms of osteoclasts.

## METHODS

### 2.1 Goldfish

Goldfish (*Carassius auratus*) specimens were purchased from Higashikawa Fish Farm (Yamatokoriyama, Japan) and were artificially fertilized at Tokyo University of Marine Science and Technology. Experiments with growing fish (10-12 cm) were performed according to the recommendations in the ethical guidelines of Kanazawa University and Tokyo Medical and Dental University.

### 2.2 Comparison of melatonin production between ontogenic and regenerating goldfish scales

Regenerating scales on day 14 were obtained from goldfish. The samples from the ontogenic and the regenerating scales were subjected to liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) as illustrated in Figure S2.

### 2.3 Space flight experiment

The regenerating scales were packed into culture chambers (60 scales in each chamber) and 96-well plates (1 scale in each well) with and without melatonin (1 μM; Sigma-Aldrich Co. LLC) as illustrated in Figure S3 and flown on the space shuttle Atlantis flight STS-132 (NASA, USA) as illustrated in Figure S4. After arrival at Japan’s space laboratory (KIBO) at the International Space Station (ISS), one 96-well plate was frozen at −95°C as a launch control for enzyme activity.
analysis. Before incubation, the other 96-well plates and the culture chambers were kept at 4°C and then incubated for 86 hours at 22°C in in-flight microgravity (F-μg) or in-flight artificial 1 gravity (F-1g) at the Cell Biology Experiment Facility (Videos S1 and S2).

Subsequently, the scales in 96-well plates were frozen at −95°C for tartrate-resistant acid phosphatase (TRAP) activity analysis. The culture medium was replaced with RNA later (Sigma-Aldrich) for gene expression analysis or with 4% paraformaldehyde (PFA) phosphate buffer for morphological analysis (Videos S1 and S2). Specimens were stored at −95°C for gene expression analysis or at 4°C for morphological analysis until the return of STS-132 to the Kennedy Space Center in Florida.

Some scales were incubated with melatonin (1 μM) in F-μg conditions and processed for gene expression analysis.

### 2.4 Histological analyses

The scales fixed with 4% PFA were incubated in 0.1% Triton X-100 for 10 minutes. They were then used for actin ring formation and TRAP activity analyses (Figure S1).

For the immunohistochemical analysis, the fixed scales were used as whole-mount samples or cryosections. They were incubated with a blocking solution and then with primary antibodies: anti-acetylserotonin O-methyltransferase (ASMT) (ab180511; Abcam; ×100), anti-CALCITONIN (×50 000),22 or anti-melanatonin receptor (anti-Mel-R) (MTNR1B/MT2 antibody; LS-A934; LifeSpan BioSciences; ×1000). For the secondary antibody, Alexa Fluor® 488-labeled anti-rabbit IgG (A11034, LifeSpan BioSciences; ×1000). For the secondary anti-ABCPos, Alexa Fluor® 594-labeled anti-mouse IgG (A11033, LifeSpan BioSciences; ×1000) was used. For the negative controls, immunostaining was also performed using anti-CALCITONIN or anti-Mel-R that had been pre-absorbed with 5 μg/mL of salmon CALCITONIN or 2 μg/mL of peptide for Mel-R (LS-E29610; LifeSpan BioSciences), respectively.

For in situ hybridization, digoxigenin (DIG)-labeled sense and antisense single-stranded RNA probes for goldfish Asmt were prepared using a cDNA fragment containing 324-1244 bases in GU205783. The signals of specific transcripts were detected using a kit (Boehringer Mannheim). The sections were counterstained with methyl green.

### 2.5 Histomorphometry

Regenerating scales had enlarged foci with complex mesh-like networks of grooves (Figure S1B). In the histomorphometric analysis, the areas observed were restricted to regions with enlarged foci that were not covered with epithelia. For the space flight experiment, six (for F-μg) or eight (for F-1g) scales were randomly selected and histomorphometric analysis was performed on six 0.31 mm² observation areas. Mean measurements from the six areas were regarded as representative values for each scale.

The numbers of the osteoclasts per mm² were recorded, and the mean number of nuclei per multinucleated osteoclast and the percentage of osteoclasts with actin rings were calculated. The width of the grooves at the middle of each groove, actin ring size, and the percentage of groove lengths covered with actin rings were measured using NIH Image J (https://imagej.nih.gov/ij/).

### 2.6 Quantitative real-time PCR

Total RNA isolation and cDNA synthesis were performed using a kit (Qiagen GmbH).23 Primers used for quantitative real-time PCR (qPCR) are listed in Table S1. A TaqMan probe was used for Calcitonin (Figure S5). Elongation factor 1 alpha (EFlα) was used for the standardization procedure.24

### 2.7 Reanalysis of data in an open public repository

To compare arylalkylamine N-acetyltransferase (Aanat) and acetylserotonin O-methyltransferase (Asmt) expression levels between space flight and ground control conditions in medaka brain samples, raw fastq files of RNA-sequencing (RNA-seq) data were downloaded from the DNA Data Bank of Japan database (accession no. DRA003542).25 De novo assembly using Trinity, mapping using Bowtie2, annotation using BLASTN, and normalization using the fragments per kilobase of exon per million mapped reads method were performed. Among the Aanat and Asmt candidates, the longest transcripts that exhibited the highest homologies (Aanat: 4506 bp/85.25%; Asmt: 1333 bp/99.86%) were predicted to be the promising transcripts in this study.

### 2.8 Effects of melatonin on CALCITONIN production in cultured regenerating goldfish scales and rat calvariae on the ground

The regenerating scales were incubated in a culture medium containing 10 nM to 1 μM of melatonin for 1, 2, and 4 days. Some scales were incubated with Mel-R antagonist luzindole (10 μM; Tokyo Chemical Industry, Co., Ltd.), and melatonin (1 μM) for 4 days. In addition, the CALCITONIN concentrations in the media were measured using ELISA for salmon CALCITONIN antibody22 and for rat CALCITONIN antibody (Peninsula Laboratories International Inc).

To examine the effects of melatonin in mammalian bones, 10 calvariae excised from 2-day-old Wistar rats were cut in half, and two of the calvaria halves were incubated in a well with BGJb medium (Gibco BRL, Life Technologies, Inc; 10% FCS, antibiotics) containing melatonin (1 μM) or vehicle at 37°C for 1 day. The CALCITONIN concentrations...
in the culture media (n = 5 for each group) were measured as described above. Experiments with rats were performed according to the recommendations in the ethical guidelines of Okayama University.

2.9 | Statistical analyses

Student’s t test or paired t test was performed to analyze significant differences between the two groups. In addition, one-way repeated-measures analysis of variance (ANOVA) or one-way ANOVA followed by Tukey’s post hoc test was used to examine significant differences in the values among three or four groups.

3 | RESULTS

3.1 | Comparison of melatonin levels between ontogenic and regenerating scales

The extracts from both ontogenic and regenerating scales were subjected to LC-MS/MS (Figure S2). The melatonin contents in the regenerating scales were significantly higher than those in the ontogenic scales (Figure 1A). Both the Aanat and Asmt mRNA levels in the regenerating scales were also significantly higher than those in the ontogenic scales (Figure 1B,C). Asmt mRNA (Figure 1Da,Db) and the protein (Figure 1Dc) were also detected in osteoblasts on the fibrillary plate.

3.2 | Comparison of melatonin-related enzyme expression in regenerating scales cultured on the ground with those cultured in space

The Asmt mRNA expression levels in F-μg scales decreased significantly when compared with those on the ground (Figure 2A). The Aanat expression levels, which were measured in goldfish scales on the ground (Figure 1B), were not detected in both F-μg and F-1g scales in the present stage. We aligned the raw sequence data with the reference sequences in medaka brain25 based on de novo assembly to quantify medaka Aanat and Asmt expression levels and found that they decreased during space flight (Figure 2B,C).

3.3 | Osteoclast multinucleation and resorption activity in regenerating scales during space flight

TRAP activity was mainly observed along the edges of grooves in the scales subjected to F-μg and F-1g (Figure 3A,B). The groove widths were significantly greater in F-μg scales than in F-1g scales (Figure 3C-E). Furthermore, TRAP activity significantly increased in F-μg scales when compared with activity in F-1g scales (Figure 3F).

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in F-μg scales than in F-1g scales (Figure 3G,H,K). However, the total nuclear number of mono- and multinucleated osteoclasts exhibited no significant difference between the F-μg and F-1g groups (Figure S6).

The percentage of actin ring-positive multinucleated osteoclasts and the actin ring size were significantly greater in the F-μg scales (Figure 3I,J,L,M) than in the F-1g scales. In addition, since actin rings were mostly observed along scale grooves, the percentage of lengths of grooves associated with the actin rings was higher in F-μg scales than in the F-1g scales. (Figure 3N).

3.4 | Effects of microgravity and melatonin on bone-related genes’ expression in regenerating scales

Among the factors stimulating matrix resorption, expression level of matrix metalloproteinase‐9 (Mmp‐9) significantly increased in F-μg condition (Figure 4A), while the levels of cathepsin K (Ctsk; Figure 4B) and Trap (Figure 4C) exhibited an upward trend in F-μg condition, compared with those in F-1g condition. Microravity significantly stimulated the expression of Rankl (Figure 4E) but significantly suppressed the expression of Opg (Figure 4F), which led to a remarkable increase in the ratio of Rankl:Opg (Figure 4G). In addition, the expression of cyclooxygenase-2a (Cox2a) significantly increased under F-μg condition (Figure 4H). In contrast, the expression of Calcitonin, whose translated product has inhibitory effects on osteoclast activity,26,27 reduced significantly in F-μg condition compared with that in F-1g condition (Figure 4I).

Notably, melatonin treatment changed the expression levels of the genes analyzed to levels comparable with those in F-1g. Melatonin significantly suppressed the increase in the expression of genes for the osteoclastogenic factors in F-μg (Figure 4A,B,E,G,H). Conversely, it increased the expression levels of osteoclast inhibitory factors: Opg and Calcitonin (Figure 4F,I). In addition, melatonin significantly reduced the expression level of the osteoclast marker Rank (Figure 4D).

The expression levels of osteoblastic factors—runt-related transcription factor 2b (Runx2b), osterix (Osx), type 1 collagen 1a (Col1a), and osteocalcin (Ocn)—were lower in F-μg scales than in F-1g scales (Figure 4J-M). Melatonin treatment maintained the expression levels of Runx2b, Osx, and Col1a at F-1g levels (Figure 4J-L).

3.5 | Effects of melatonin on CALCITONIN production in cultured regenerating scales and rat calvariae on the ground

CALCITONIN and Mel-R were immunolocalized in mononuclear cells forming the scale fibrillary plate (Figure 5A-D). The cells exhibited cuboidal form and cover the surface of the fibrillary plate (Figure 5E). Melatonin treatment significantly increased Calcitonin gene expression by days 1 and 2 (Figure 5F-H). It is well established that melatonin shows its effects by binding to Mel-R in the plasma membrane to activate intracellular signaling pathways. To test whether melatonin-induced Calcitonin expression is mediated by Mel-R, we used the Mel-R antagonist, luzindole, and found that luzindole treatment inhibited melatonin-induced Calcitonin expression (Figure 5I). Validations of housekeeping genes are a crucial component in qPCR analysis, because variation of reference gene expression may cause misinterpretation of the target gene expression level. Accordingly, expression levels of several housekeeping genes, β-Actin, Gapdh (glyceraldehyde 3-phosphate dehydrogenase), and Efla, in regenerating scales cultured in dish for 1 day, 2 days, and 4 days were analyzed by qPCR. We then presented
raw Ct values for housekeeping genes analyzed (Table S2), as well as expression levels of each housekeeping gene (Figure S7). In the goldfish scales, expression level of *Efla* was higher than those of *β‐Actin* and *Gapdh* (Table S2). It was also stable over days in the cultured goldfish scales providing evidence that variation of *Efla* expression level does not cause misinterpretation of the target genes’ expression in our qPCR analyses.

In addition, CALCITONIN levels detected in the culture medium of the scales increased significantly by day 4 (Figure 5J). CALCITONIN production also increased significantly in rat calvariae treated with melatonin for 1 day (Figure 5K).

**4 | DISCUSSION**

In the experiments based both on the ISS and on the ground, to the best of our knowledge, we are the first to demonstrate a novel melatonin action pathway on bone metabolism using goldfish scales (Figure 6), a bone model with osteoclasts and osteoblasts coexisting on a calcified matrix (Figure S1).

We chose goldfish scales as experimental materials suitable for the analysis of bone metabolism in outer space. Although fish scales and mammalian mineralized skeleton are both derived from dermal skeleton, there is a long evolutionary gap between them. Fish scales originate from the postcranial dermal skeleton, which has been largely lost in mammals. In humans, the dermal skeleton is only found as the calvaria and clavicle, which are formed by intramembranous ossification in the same manner as fish scales, and most of the endoskeleton is formed by endochondral ossification. Nevertheless, fish scales and mammalian mineralized skeleton share many similarities regarding matrix components, cellular morphology, and responses to hormones and mechanical stress. In addition, fish scales can be easily obtained in large numbers and are easy to handle as experimental materials. Therefore, it is considered...
advantageous to use fish scales as a bone model in experiments and to be able to obtain useful information about the phenomenon occurring in bone tissue under microgravity. We detected melatonin and melatonin-synthesizing enzymes in the scale (Figure 1). We also observed that in regenerating scales, melatonin induced \textit{Calcitonin} mRNA expression and \textit{CALCITONIN} release into media. In addition, Mel-R was detected in the scale osteoblasts (Figure 5C,D). The results suggest that melatonin increases \textit{CALCITONIN} production from the osteoblasts via paracrine or endocrine mechanisms to inhibit osteoclast activity in the scales. In our preliminary experiment, melatonin levels in female scales exhibited significant annual variations, with higher levels in April (late reproductive season). In the reproductive period, the plasma calcium levels in female fish increase remarkably to facilitate production of vitellogenin, which is a major egg protein and a calcium-binding protein. We think that
melatonin protects scales from excess calcium degradation. Such regulation mechanisms were first reported in goldfish scales. Furthermore, preliminary results showed that melatonin is detected in the culture media of human primary osteoblasts 4 days postincubation Tabuchi Y, Hattori A, and Suzuki N (unpublished data). Human primary osteoblasts also expressed CALCITONIN and Mel‐R. These findings, along with our results of culturing rat calvariae (Figure 5K), indicated that melatonin can regulate CALCITONIN production in mammalian bone as well as in goldfish scales.

In the space experiments, we could evaluate resorptive activities using increased groove widths as indicators, and activated osteoclasts in the regenerating scales (Figure 3). Our results are consistent with findings of previous studies analyzing the impact of microgravity on different aspects of osteoclasts. In medaka bone during space flight for 56 days, osteoclast multinucleation also increased, although the total number of nuclei exhibited no significant difference between the ground and flight groups. The findings support our results, showing that increased resorptive activity in space flight depends on accelerated multinucleation and osteoclast activity but not on increased osteoclast proliferation. A potential explanation for osteoclastic activation in medaka could be the decline in melatonin since Aanat and Asmt expression levels decreased in the medaka brain including the pineal gland in space (Figure 2B,C).

Microgravity increased the expression ratio of Rankl:Opg (Figure 4G), a key indicator of bone resorption. In a space experiment using mice, OPG administration could prevent space flight‐associated increases in bone resorption. Therefore, the upregulated gene expression ratio of Rankl:Opg could be due to the activation of osteoclast and bone resorption. Increased expression level of Cox2a could facilitate the upregulation of Rankl, since COX2 is an isozyme of cyclooxygenase, which is required for the synthesis of prostaglandin E2, a stimulator of bone resorption.
Under microgravity conditions, melatonin maintained the gene expression levels detected at F-1g for Mmp-9, Ctsk, Rankl, and Cox2a, and the Rankl:Opg ratio at F-1g (Figure 4A,B,E,G,H). Such conditions also reduced the expression of the osteoclast marker Rank (Figure 4D). The results suggest that melatonin suppresses osteoclast activity under microgravity conditions, potentially via the regulation of the expression of Rank in osteoclastic cells and Rankl in osteoblastic cells, as above reported in mammalian cells. The suppression of Rankl or RANKL by melatonin has been widely reported. Melatonin also tended to increase Opg expression (Figure 4H), which is specifically detected in osteoblasts, indicating the inhibition of synthesis of prostaglandin E2, a stimulator of RANKL expression. Furthermore, microgravity-dependent induction of osteoblastic markers, Osx and Col1a, was maintained by melatonin (Figure 4K,L), implying that melatonin could also target specific genes in osteoblasts under microgravity, probably via Mel-R signaling in scale osteoblasts.

To comprehensively identify the genes whose expression levels were altered in the scales kept in F-μg condition, we performed next-generation sequencing analysis (Figure S8). Our analysis found that the F-μg condition induced the altered expression patterns of osteoclast (Pth1r and Irs1) and osteoblast-related genes (Cebpb, Fos, Twist1, and Osr2). These identified genes constituted the networks associated with bone resorption and its formation (Figure S8A,B). Notably, melatonin treatment canceled the alteration of genes’ expression (Figure S8C,D). Taken together, these findings provided evidence that melatonin regulates bone resorption and its formation by competing with the
microgravity effect on them, consistent with results of the qPCR analysis in Figure 4. Whether microgravity-induced changes in mRNA expression of analyzed genes translate to an altered expression of the corresponding proteins will be tested in the future.

In the present study, we observed a loss of the melatonin effect on Calcitonin expression on day 4 in ground basis experiment (Figure 5H). One possible explanation for this might be a reduction in Mel-R, as reported by Witt-Enderby et al. and our previous study. In fish scales and rat calvariae, on the other hand, we found melatonin-induced increases in CALCITONIN (Figure 5I,K). This indicates that increase in Calcitonin mRNA expression translates to the induced expression of CALCITONIN in fish scales on day 4. Notably, Calcitonin expression in the regenerating scales significantly decreased during space flight (Figure 4I). This is the first report of the suppression of Calcitonin expression in bone tissues under microgravity conditions. Consistent with our findings, the serum CALCITONIN in monkeys exhibited modest decreases during space flight, while in humans, the blood CALCITONIN levels decreased during 120 days of head-down tilt bed rest. Therefore, the suppression of CALCITONIN could play a key role in enhancing bone resorption during space flight. Remarkably, the suppression of Calcitonin expression in bone tissues was counteracted by treatment with melatonin (Figure 4I). Although our results strongly indicate that astronauts would benefit from taking melatonin to effectively prevent bone loss, further effort will be required to increase utility of melatonin in the prevention of bone loss. For example, the chemical modification of melatonin would increase its efficacy as a drug preventing bone loss in astronauts.

To further assess the morphological changes taking place in osteoclasts and the mechanism by which melatonin could suppress bone loss during space flight, we cultured the regenerating scales under simulated microgravity conditions created using a 3-dimensional clinostat (Ground-µg; Figure S9). After 4 days, the number of nuclei per multinucleated osteoclast (Figure S9G) and the percentage of osteoclasts expressing actin rings (Figure S9H) were higher in scales cultured in Ground-µg condition than in Ground-1g condition. The presence of melatonin considerably reduced the impact of simulated microgravity treatment on the ground on both parameters (Figure S9G,H).

Circadian clocks regulate various biochemical, cellular, and physiological processes with a periodicity of approximately 24 hours in order to maximize an organism's physiological efficiency. Studies have reported molecular links between circadian clocks and bone metabolism in mammals, including humans. For example, in humans, levels of the C-terminal cross-linked telopeptide of type I collagen (a bone resorption marker) and fibroblast growth factor 23 (a regulator of mineral metabolism) display a circadian rhythm. On the basis of these findings, one important issue we raised was whether goldfish scales have circadian clock machinery. In our preliminary experiments, we observed a circadian expression of clock genes in goldfish scales kept under constant light conditions—evidence that goldfish scales have an intrinsic circadian clock. Fish scales have a distinctive utility as experimental systems. They are small and are present on the body surface, facilitating the extraction and analysis of bone components. Therefore, they are an attractive vertebrate model for examining molecular links between circadian clocks and bone metabolism.

Overall, the present study provides evidence that melatonin suppresses the bone-resorbing activity of osteoclasts in bone tissues under microgravity conditions via the upregulation of Calcitonin and the downregulation of Rankl in osteoblasts (Figure 6). In humans, melatonin treatment has been reported to increase bone mineral density and decreases the bone resorption marker:formation marker ratio in perimenopausal women. These studies indicated that melatonin can work as an anti-osteoclastic drug in humans, consistent with our findings. Altogether, we propose melatonin as a potentially promising drug for the prevention of bone loss during space flights.

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AUTHOR CONTRIBUTIONS
MI, MJT, AH, KK, YT, TY, TS, IT, YF, YM, AK, RA, MN, YS, TN, MN, HI, SW, HA, KM, MS, HM, HF, MM, HU, SY, RM, TI, AH, and NS planned the space experiment. ME and TT prepared materials. TT, HN, NS, MN, TK, TH, KH, IT, TY, VSC, KI, TS, SE, YMT, AK, AS, YW, AT, AH, and NS analyzed the data. MI, YF, YT, JH, AH, and NS wrote the manuscript.

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REFERENCES

1. Arfat Y, Xiao W-Z, Iftikhar S, et al. Physiological effects of microgravity on bone cells. *Calcif Tissue Int*. 2014;94(6):569-579.

2. Caillot-Augusseau A, Vico L, Heer M, et al. Space flight is associated with rapid decreases of undercarboxylated osteocalcin and increases of markers of bone resorption without changes in their circadian variation: observations in two cosmonauts. *Clin Chem*. 2000;46(8 Pt 1):1136-1143.

3. Collet P, Uebelhart D, Vico L, et al. Effects of 1- and 6-month spaceflight on bone mass and biochemistry in two humans. *Bone*. 1997;20(6):547-551.

4. Pardo SJ, Patel MJ, Sykes MC, et al. Simulated microgravity using the random positioning machine inhibits differentiation and alters gene expression profiles of 2T3 preosteoblasts. *Am J Physiol Cell Physiol*. 2005;288(6):C1211-1221.

5. Zayzafoon M, Gathings WE, McDonald JM. Modeled microgravity inhibits osteogenic differentiation of human mesenchymal stem cells and increases adipogenesis. *Endocrinology*. 2004;145(5):2421-2432.

6. Kears AE, Khosa S, Kostenik PJ. Receptor activator of nuclear factor kappaB ligand and osteoprotegerin regulation of bone remodeling in health and disease. *Endo Rev*. 2008;29(2):155-192.

7. Kondo Y, Irie K, Ikegame M, Hanada K, Ozawa H. Role of stromal cells in osteoclast differentiation in bone marrow. *J Bone Miner Metab*. 2001;19(6):352-358.

8. de Vrieze E, Moren M, Metz JR, Flik G, Lie KK. Arachidonic acid enhances turnover of the dermal skeleton: studies on zebrafish scales. *PLoS ONE*. 2014;9(2):e89347.

9. Suzuki N, Somel M, Seki A, Reiter RJ, Hattori A. Novel bromomelatonin derivatives as potentially effective drugs to treat bone diseases. *J Pineal Res*. 2008;45(3):229-234.

10. Suzuki N, Tabata MJ, Omori K, et al. Fish scale study for spaceflight on bone mass and biochemistry in two humans. *Bone*. 2005;76(23):2699-2709.

11. Yano S, Masuda D, Kasahara H, et al. Excellent thermal control ability of cell biology experiment facility (CBEF) for ground-based experiments and experiments onboard the KIBO Japanese experiment module of International Space Station. *Biol Sci Space*. 2012;26:12-20.

12. Cole AA, Walters LM. Tartrate-resistant acid phosphatase in bone and cartilage following decalcification and cold-embedding in plastic. *J Histochem Cytochem*. 1987;35(2):203-206.

13. Suzuki N, Yamamoto K, Sasayama Y, et al. Possible direct induction by estrogen of calcitonin secretion from ultimobranchial cells in the goldfish. *Gen Comp Endocrinol*. 2004;138(2):121-127.

14. Ishizu H, Sekiguchi T, Ikari T, et al. alpha-Melanocyte-stimulating hormone promotes bone resorption resulting from increased osteoblastic and osteoclastic activities in goldfish. *Gen Comp Endocrinol*. 2018;262:99-105.

15. St Hilaire MA, Rahman SA, Gooley JJ, Witt-Enderby PA, Paulo S, Abrantes AM, Laranjo M, et al. Bisphosphonate-related osteonecrosis of the jaw: specificities. *J Pineal Res*. 2015;59(2):221‐229.

16. Kotlarczyk MP, Lassila HC, O’Neil CK, et al. Melatonin osteoporosis prevention study (MOPS): a randomized, double-blind, placebo-controlled study examining the effects of melatonin on bone health and quality of life in perimenopausal women. *J Pineal Res*. 2012;52(4):414-426.

17. Acuña-Castroviejo D, Escames G, Venegas C, et al. Extraperinuclear melatonin: sources, regulation, and potential functions. *Cell Mol Life Sci*. 2014;71(16):2997-3025.

18. Suzuki N, Kitamura K-I, Omori K, et al. Response of osteoblasts and osteoclasts in regenerating scales to gravity loading. *Biol Sci Space*. 2009;23:211-217.

19. Yoshikubo H, Suzuki N, Takemura K, et al. Osteoblastic activity and estrogenic response in the regenerating scale of goldfish, a good model of osteogenesis. *Life Sci*. 2005;76(23):2699-2709.

20. Murata Y, Yasuda T, Watanabe-Asaka T, et al. Histological and transcriptomic analysis of adult Japanese medaka sampled onboard the international space station. *PLoS ONE*. 2015;10(10):e0138799.

21. Keller J, Catala-Lehnen P, Huebner AK, et al. Calcitonin controls bone formation by inhibiting the release of sphingosine 1-phosphate from osteoclasts. *Nat Commun*. 2014;5:5215.

22. Suzuki N, Suzuki T, Kurokawa T. Suppression of osteoclastic activities by calcitonin in the scales of goldfish (freshwater teleost) and nibbler fish (seawater teleost). *Peptides*. 2000;21(1):115-124.

23. Sato M, Yachiguchi K, Motohashi K, et al. Sodium fluoride influences calcium metabolism resulting from the suppression of osteoclasts in the scales of nibbler fish, *Girella punctata*. *Fish Sci*. 2017;83(4):543-550.

24. Verasper JY, Gooley JJ, Witt-Enderby PA, Paulo S, Abrantes AM, Laranjo M, et al. Bisphosphonate-related osteonecrosis of bone formation by inhibiting the release of sphingosine 1-phosphate from osteoclasts. *Nat Commun*. 2014;5:5215.

25. Suzuki N, Suzuki T, Kurokawa T. Suppression of osteoclast activities by calcitonin in the scales of goldfish (freshwater teleost) and nibbler fish (seawater teleost). *Peptides*. 2000;21(1):115-124.

26. Sire JY, Huyseaine A. Formation of dermal skeletal and dental tissues in fish: a comparative and evolutionary approach. *Biol Rev Camb Philos Soc*. 2003;78(2):219-249.

27. Sire JY, Akimenko MA. Scale development in fish: a review, with description of sonic hedgehog (shh) expression in the zebrafish (Danio rerio). *Int J Dev Biol*. 2004;48(2–3):233-247.

28. Iimura K, Tohse H, Ura K, Takagi Y. Expression patterns of runx2, osteopontin, and osteocalcin and cartilage following decalcification and cold-embedding in plastic. *J Histochem Cytochem*. 1987;35(2):203-206.

29. Suzuki N, Yashima S, Iwamuro S, Hattori A. Physiological role of melatonin derivatives as potentially effective drugs to treat bone diseases. *Endocrinology*. 2006;23(11):1021-1029.

30. Nakamura Y, Sato M, Yachiguchi K, et al. Sodium fluoride influences calcium metabolism resulting from the suppression of osteoclasts in the scales of nibbler fish, *Girella punctata*. *Fish Sci*. 2017;83(4):543-550.

31. Murata Y, Yasuda T, Watanabe-Asaka T, et al. Histological and transcriptomic analysis of adult Japanese medaka sampled onboard the international space station. *PLoS ONE*. 2015;10(10):e0138799.

32. Keller J, Catala-Lehnen P, Huebner AK, et al. Calcitonin controls bone formation by inhibiting the release of sphingosine 1-phosphate from osteoclasts. *Nat Commun*. 2014;5:5215.

33. Suzuki N, Suzuki T, Kurokawa T. Suppression of osteoclastic activities by calcitonin in the scales of goldfish (freshwater teleost) and nibbler fish (seawater teleost). *Peptides*. 2000;21(1):115-124.

34. Sire JY, Huyseaine A. Formation of dermal skeletal and dental tissues in fish: a comparative and evolutionary approach. *Biol Rev Camb Philos Soc*. 2003;78(2):219-249.

35. Sire JY, Akimenko MA. Scale development in fish: a review, with description of sonic hedgehog (shh) expression in the zebrafish (Danio rerio). *Int J Dev Biol*. 2004;48(2–3):233-247.

36. Iimura K, Tohse H, Ura K, Takagi Y. Expression patterns of runx2, osteopontin, and osteocalcin and cartilage following decalcification and cold-embedding in plastic. *J Histochem Cytochem*. 1987;35(2):203-206.

37. Suzuki N, Yashima S, Iwamuro S, Hattori A. Physiological role of melatonin derivatives as potentially effective drugs to treat bone diseases. *Endocrinology*. 2006;23(11):1021-1029.
35. Azuma K, Kobayashi M, Nakamura M, et al. Two osteoclastic markers expressed in multinucleate osteoclasts of goldfish scales. *Biochem Biophys Res Commun*. 2007;362(3):594-600.

36. Drissi H, Hott M, Marie PJ, Lasmoles F. Expression of the CT/CGRP gene and its regulation by dibutyryl cyclic adenosine monophosphate in human osteoblastic cells. *J Bone Miner Res*. 1997;12(11):1805-1814.

37. Man G-W, Wang W-J, Yeung B-Y, et al. Abnormal proliferation and differentiation of osteoblasts from girls with adolescent idiopathic scoliosis to melatonin. *J Pineal Res*. 2010;49(1):69-77.

38. Chatani M, Mantoku A, Takeyama K, et al. Microgravity promotes osteoclast activity in medaka fish reared at the international space station. *Sci Rep*. 2015;5:14172.

39. Lloyd SA, Morony SE, Ferguson VL, et al. Osteoprotegerin is an effective countermeasure for spaceflight-induced bone loss in mice. *Bone*. 2015;81:562-572.

40. Blackwell KA, Raisz LG, Pilbeam CC. Prostaglandins in bone: bad cop, good cop? *Trends Endocrinol Metab*. 2010;21(5):294-301.

41. Koyama H, Nakade O, Takada Y, Kaku T, Lau KH. Melatonin at pharmacologic doses increases bone mass by suppressing resorption through down-regulation of the RANKL-mediated osteoclast formation and activation. *J Bone Miner Res*. 2002;17(7):1219-1229.

42. Maria S, Samsonraj RM, Munmun F, et al. Biological effects of melatonin on osteoblast/osteoclast cocultures, bone, and quality of life: implications of a role for MT2 melatonin receptors, MEK1/2, and MEK5 in melatonin-mediated osteoblastogenesis. *J Pineal Res*. 2018;64(3):e12465.

43. Wang J, Xiao X, Zhang Y, et al. Simultaneous modulation of COX-2, p300, Akt, and Apaf-1 signaling by melatonin to inhibit proliferation and induce apoptosis in breast cancer cells. *J Pineal Res*. 2012;53(1):77-90.

44. Saini V, Marengi DA, Barry KJ, et al. Parathyroid hormone (PTH)/PTH-related peptide type 1 receptor (PPR) signaling in osteocytes regulates anabolic and catabolic skeletal responses to PTH. *J Biol Chem*. 2013;288(28):20122-20134.

45. Ogata N, Chikazu D, Kubota N, et al. Insulin receptor substrate-1 in osteoblast is indispensable for maintaining bone turnover. *J Clin Invest*. 2000;105(7):935-943.

46. Ichida F, Nishimura R, Hata K, et al. Reciprocal roles of MSX2 in regulation of osteoblast and adipocyte differentiation. *J Biol Chem*. 2004;279(32):34015-34022.

47. Johnson RS, Spiegelman BM, Papaioannou V. Pleiotropic effects of a null mutation in the c-fos proto-oncogene. *Cell*. 1992;71(4):577-586.

48. Huang Y, Meng T, Wang S, et al. Twist1- and Twist2-haploinsufficiency results in reduced bone formation. *PLoS ONE*. 2014;9(6):e99331.

49. Kawai S, Yamauchi M, Wakisaka S, Ooshima T, Amano A. Zinc-finger transcription factor odd-skipped related 2 is one of the regulators in osteoblast proliferation and bone formation. *J Bone Miner Res*. 2007;22(9):1362-1372.

50. Witt-Endersby PA, Bennett J, Jarzynka MJ, Firestone S, Melan MA. Melatonin receptors and their regulation: biochemical and structural mechanisms. *Life Sci*. 2003;72(20):2183-2198.

51. Arnaud SB, Navidi M, Defos L, et al. The calcium endocrine system of adolescent rhesus monkeys and controls before and after space-flight. *Am J Physiol Endocrinol Metab*. 2002;282(3):E514-521.

52. Korol’kov VI, Dotsenko MA, Larina IM, Shakhmatova EI, Natochin I. The indices of water-salt metabolism and of the endocrine status in monkeys after flights on the Kosmos biological satellites. *Aviakosm Ekolog Med*. 1996;30(3):32-35.

53. Morukov BV, Orlov OI, Grigoriev AI. Calcium homeostasis in prolonged hypokinesia. *Physiologist*. 1989;32(1 Suppl):S37-40.

54. Takahashi JS. Transcriptional architecture of the mammalian circadian clock. *Nat Rev Genet*. 2017;18(3):164-179.

55. Song C, Wang J, Kim B, et al. Insights into the role of circadian rhythms in bone metabolism: a promising intervention target? *Biomed Res Int*. 2018;2018:9156478.

56. Kawai M, Kinoshita S, Shimba S, Ozono K, Michigami T. Sympathetic activation induces skeletal Fgf23 expression in a circadian rhythm-dependent manner. *J Biol Chem*. 2014;289(3):1457-1466.

57. Swanson C, Shea SA, Wolfe P, et al. 24-hour profile of serum sclerostin and its association with bone biomarkers in men. *Osteoporos Int*. 2017;28(11):3205-3213.

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Additional supporting information may be found online in the Supporting Information section at the end of the article.

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