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Effects of acute- and long-term aerobic exercises at different intensities on bone in mice

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Abstract

Introduction

Exercise intensity determines the benefits of aerobic exercise. Our objectives were, in aerobic exercise at different intensities, to determine (1) the changes in bone metabolism-related genes after acute exercise and (2) the changes in bone mass, strength, remodeling, and bone formation-related proteins after long-term exercise.

Methods

Total 36 male C57BL/6J mice were divided into a control group and exercise groups at 3 different intensities: low, moderate, or high group. Each exercise group was assigned to acute- or long-term exercise groups. Tibias after acute exercise were evaluated by real-time PCR analysis. Furthermore, hindlimbs of long-term exercise were assessed by micro-CT, biomechanical, histological, and immunohistochemical analyses.

Results

Acute moderate-intensity exercise decreased RANKL level as bone resorption marker, whereas low- and high-intensity exercise did not alter it. Additionally, only long-term exercise at moderate intensity increased bone mass and strength. Moderate-intensity exercise promoted osteoblast activity and suppressed osteoclast activity. After low- and high-intensity exercise, osteoblast and osteoclast activity were unchanged. An increase in the number of β-catenin-positive cells and a decrease in sclerostin-positive cells were observed in the only moderate group.

Conclusion

These results showed that moderate-intensity exercise can inhibit bone resorption earlier, and long-term exercise can increase bone mass and strength through promoted bone formation via the Wnt/β-catenin activation. High-intensity exercise, traditionally considered better for bone, may fail to stimulate bone remodeling, leading to no change in bone mass and strength. Our

findings suggest that moderate-intensity exercise, neither too low nor high, can maintain bone health.

1. Introduction

Physical exercise promotes bone formation and maintains skeletal and bone health. Among various modes of exercise, aerobic exercise has benefits, such as improving bone mineral density (BMD) [1–3]. Increased bone mass and strength due to exercise can reduce fracture risk in advanced age [4, 5], and establishing optimal conditions for aerobic exercise is required. Exercise intensity determines the benefits of aerobic exercise [6]. For example, low-intensity aerobic exercises, such as swimming and cycling [7], do not affect BMD. Previous systematic reviews and meta-analyses have shown that high-intensity aerobic exercise effectively increases BMD [1, 8]. However, exhaustive aerobic exercise promotes bone resorption and causes fatigue fractures in elite athletes [9, 10]. In earlier studies, the optimal exercise conditions for the bone remained unclear because of the lack of detailed investigation of the bone response to exercise intensity.

Acute aerobic exercise changes the dynamic balance between bone formation and resorption, leading to positive adaptation. The assessment of bone metabolism after acute exercise provides a basis for earlier benefits. However, clinical studies suggest that acute exercise promotes catabolic response [11–13]. Similarly, in animal studies, no beneficial effects, including an increase in catabolic gene expression, were observed in the bones of mice subjected to acute exercise [14]. Unfortunately, these studies examined the acute response of bone metabolism at a single exercise intensity. Investigations into bone metabolism-related factors after acute exercise at various intensities may identify the optimal intensity; however, to the best of our knowledge, there are no such studies.

Unlike acute exercise, long-term aerobic exercise can increase bone mass and strength in response to the intensity. Animal studies have shown that high-intensity exercise effectively increases bone mass and strength [15–17]. However, several reports show that treadmill exercise at 85%VO2 max rather than 95%VO2 max increases bone mass and

strength [18, 19]; therefore, the appropriate intensity remains controversial. On the other hand, little is known about local bone remodeling by osteoblasts and osteoclasts. According to serum markers, long-term exercise promotes bone formation [18, 20] and inhibits bone resorption [21]. However, evaluating bone turnover using serum markers is indirect [13], which may not accurately explain local changes in bone structure and mechanical properties after exercise. Thus, the direct evaluation of bone remodeling should be emphasized. It has also been postulated that mechanical stress plays a crucial role in exercise-induced bone formation [6, 22]. Mechanical stress enhances osteoblast activity through Wnt/β-catenin signaling in an intensity-dependent manner [23–25]. Depending on the exercise intensity, such mechanisms may also be involved in osteoblastic activation; however, this is not yet understood.

We adapted treadmill exercise at different intensities as aerobic exercise for mice, once or for 4 weeks. Using aerobic exercise at different intensities, the goal of this study was to determine (1) changes in bone metabolism-related genes after acute exercise and (2) changes in bone mass, strength, remodeling, and proteins of bone formation-related pathways after long-term exercise.

2. Materials and Methods

Experimental design

All experimental procedures were examined by the Institutional Animal Care and Use Committee, approved by the president of Kobe University, and performed according to the Kobe University Animal Experimentation Regulations (approval number: Pl90814). Total 54 male C57BL/6J mice (7 weeks old, Japan SLC Inc., Shizuoka, Japan) were used in this study. To avoid sex-related confounding effects on bone, only male mice were used in this study. All mice were housed in standard cages under a 12 h dark/light cycle at a constant temperature of

22 ± 1°C. Mice were randomly divided into a control group and exercise groups at the following 3 different intensities: low, moderate, or high group. Exercise was assigned only once in acute exercise groups (low, moderate, or high group, n = 4 per intensity) or for 4 weeks in long-term exercise groups (low, moderate, or high group, n = 5 per intensity). The control group was kept in a cage without exercise for the periods corresponding to acute exercise groups (n = 4 per group) or long-term exercise groups (n = 5 per group). In some experiments, mice in the low and moderate groups (low and moderate, n = 6 per intensity) ran daily for 8 weeks to examine prolonged exercise duration. The control group (n = 6 per group) was housed without exercise for 8 weeks.

Treadmill exercise protocol

A treadmill device (MK-680, Muromachi kikai Co, Ltd., Tokyo, Japan) was used for aerobic exercise intervention. In all exercise groups, mice ran at an intensity of 8 m/min for 5 days as acclimation exercise. During this acclimation period, exercise duration was increased by 15 min per day. After rest for 2 days, mice were subjected to treadmill exercise at intensities of 8 m/min (low group), 16 m/min (moderate group), and 24 m/min (high group) at 0˚ slope for 60 minutes. These exercise intensities correspond to 65, 80, and 90%/VO_2max, respectively [26, 27]. Exercise protocols at different intensities were adapted to mice only once (acute exercise group) or 7 days/week for 4 weeks (long-term exercise group) after acclimatization (Table.1). Low and moderate groups were also subjected to daily exercise for 8 weeks to examine prolonged exercise duration.

Tissue preparation

At the end of experimental period, mice were euthanized by exsanguination under anesthesia and sacrificed. One of tibias from acute exercise group was used for real-time polymerase

chain reaction (PCR) analysis. Hindlimbs in the long-term exercise group were randomized and, one for micro-computed tomography (μCT) analysis and biomechanical test, and the other for histological and immunohistochemical analyses. For frozen section preparation, tibias were embedded in gel according to Kawamoto`s method [28]. Frozen blocks were sectioned at a thickness of 50 μm for real-time PCR analysis or 5 μm for histological and immunohistochemical analyses.

Real-time PCR analysis

We assumed that metaphyseal bone metabolism plays a critical role due to its susceptibility to exercise. Sclerostin can inhibit suppress osteoblastic activity [23, 24]. Runt-related transcription factor 2 (Runx2) is a transcription factor essential for osteoblast differentiation[29]. Receptor activator of nuclear factor-κB ligand (RANKL) plays a crucial role in promoting osteoclast differentiation, and osteoprotegerin (OPG) inhibits its function as a decoy receptor [30]. Total RNA of the tibia was extracted with a modification of previous methods [31, 32]. Total RNA of bone tissue was isolated from 5 sections 50 μm in thickness using tweezers under an actual microscope. Bone tissue (trabecular bone, cortical bone, and bone marrow) consisted of the region starting distal to the growth plate and extending 2 mm. We have before detected the osteocyte-specific gene (Sost; sclerostin) and the osteoblast-specific gene (Bglap3; osteocalcin), suggesting that total RNA was mainly extracted from bone-derived cells in this method. The preliminary study showed that our target genes were much less or undetectable in total RNA from bone marrow (Table S2); thus, its effect was minimized. Obtained tissue was thoroughly homogenized with the common homogenizer (BioMasher, Nippi, Tokyo, Japan) in QIAzol Lysis Reagent (Qiagen, Hilden, Germany) until the lysate was uniform. Subsequently, according to the manufacturer's protocols, total RNA was extracted using RNeasy Plus Universal Mini kit (Qiagen, Hilden, Germany). The purity

and concentration of total RNA were checked by BioPhotometer D30 (Eppendorf, Hamburg, Germany). The mean concentration of RNA samples was 65.6 ± 13.0 μg/μL (Table S1), which is sufficient for the following analysis. Total RNA and the TaqMan™ Fast Virus 1-Step Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA) were used for reverse transcription. RNA was quantitatively analyzed with the StepOne real-time PCR system (Thermo Fisher Scientific Inc., Waltham, MA, USA) with TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA) for Sclerostin (Sost: Mm00470479_m1), Runx2 (Mm00501580_m1), RANKL (Tnfsf11; Mm00441906_m1), OPG (Tnfrsf11b: Mm00435454_m1) and ribosomal proteins S18 rRNA (Mm03928990_g1). Ribosomal protein S18 rRNA as an internal control was used for calculating the relative expression of each gene by using the $2^{-\Delta\Delta Ct}$ method.

μCT analysis

After 4 and 8 weeks of exercise, the bone structure was evaluated using micro 3D X-ray CT system (R_mCT2; Rigaku, Tokyo, Japan). Each tibia was scanned separately with an isotropic vowel resolution of 10 μm (X-ray tube potential = 90 kV, current = 160 μA, and a scan time of 3 min per sample). 3D images of bone were reconstructed and analyzed with TRI/3D-BON software (Ratoc, Tokyo, Japan). For evaluation of trabecular bone microstructure, proximal tibial metaphyseal regions 1.7 mm from immediately below the growth plate were selected [33]. The cortical bone region was 1.4 mm thick and located 5 mm away from the growth plate [34]. Trabecular bone microstructure was determined by trabecular bone structure, trabecular bone volume/tissue volume (BV/TV), trabecular bone thickness (Tb.Th), trabecular bone number (Tb.N), trabecular bone separation (Tb.Sp), and bone mineral content/tissue volume (BMC/TV). Cortical bone structure was characterized by cortical thickness (Ct.Th), cross-sectional area (CSA) and cortical volume to all tissue

126 volume (Cv/Av). In accordance with previous studies [35], the thresholds for trabecular and
127 cortical bone were set at 500 and 700 mg HA/cm3, respectively. Bone microstructure was
128 analyzed by an examiner blinded to the grouping of each 3D-image.
129
130 *Mechanical testing*
131 The mechanical properties of the proximal tibial metaphysis were evaluated by mechanical
132 testing device (AUTOGRAPH, Shimadzu, Kyoto, Japan), referring to previous studies [36,
133 37]. The tibia was stabilized and fixed on the acrylic plate. The roller stamp was positioned
134 1.5 mm below the tibial growth plate and dropped at a speed of 70 mm/min until bone
135 fracture. The maximum load, energy to failure, and stiffness were determined from the load-
136 deflection curve. Mechanical properties were unblinded to measure in all bone samples.
137
138 *Histological analysis*
139 Frozen sections in long-term exercise group were stained with tartrate-resistant acid
140 phosphatase (TRAP)/ alkaline phosphatase (ALP) stain kit (Wako Pure Chemical, Osaka,
141 Japan) in an accordance with the manufacturer's instructions. TRAP and ALP staining were
142 counter stained with Nuclear Staining Reagents. They were captured with a light microscope
143 (BX-53, Olympus, Tokyo, Japan) at a magnification of 20X. TRAP- and ALP-positive
144 surface within trabecular bone surface was measured using Image J 1.50 (National Institutes
145 of Health, Bethesda, MD, USA), and expressed as the TRAP- and ALP-positive surface/bone
146 surface by averaging measurements from different 3 regions. For all quantification, an
147 examiner was blinded to grouping of each image.
148
149 *Immunohistochemical analysis*
150 We evaluated proteins of sclerostin and β-catenin, which are associated with the Wnt/β-

catenin pathway [6]. Following the protocols established in our laboratory [38], sections were immunostained using primary antibody of sclerostin (diluted 1:300; AF1589, R&D Systems, Minneapolis, MN, USA) and β-catenin (diluted 1:200; ab32572, Abcam, Tokyo, Japan). Briefly, sections of the tibia were fixed in 100% ethanol for 2 min in 4% paraformaldehyde/0.01M phosphate-buffered saline (PBS, pH 7.4). The sections were then decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for 1 hour and incubated overnight with each primary antibody. Immunoreactivity was amplified with Elite ABC kit (diluted 1:50; PK-6100; Vector Lab., Burlingame, CA, USA) and visualized with diaminobenzidine tetrahydrochloride reagent (ImmPACT™ DAB peroxidase substrate kit, SK-410, Vector Lab). Finally, sections were counterstained with Mayer's hematoxylin. For sclerostin, the ratio of number of sclerostin-positive cell per total osteocytes was manually counted and calculated. For β-catenin, the number of positive cells around the trabecular surface was counted and expressed as the number of positive cells/bone perimeter (mm). All images from immunochemical staining were blinded for unbiased quantification.

Statistical analysis

All results were analyzed statistically using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). First, all values were checked for normality with the Shapiro-Wilk test, where $P > 0.05$ indicates a normal distribution. As a result, normality was observed in all values; thus, the results were compared with one-way analysis of variance (ANOVA) and Tukey's HSD test as post-hoc test. All data are presented as the mean and standard deviation (SD). In these analyses, $P < 0.05$ was defined as statistically significant.

3. Results

Bone metabolism-related gene expressions in acute exercise group

The expression of sclerostin mRNA was significantly lower in the low, moderate, and high dose groups than in the control group ($P < 0.05$) (Fig. 1a). No exercise group showed affected Runx2 mRNA ($P > 0.05$) (Fig. 1b). Moderate-intensity exercise decreased the mRNA level of RANKL compared to the control group ($P < 0.05$) (Fig. 1c). No significant changes in OPG mRNA expression were observed in any of the exercise groups ($P > 0.05$) (Fig. 1d). The calculated RANKL/OPG ratio did not differ significantly between the control and exercise mice ($P > 0.05$) (Fig. 1e).

Bone Microarchitecture in long-term exercise group

After 4 weeks of long-term exercise, an increase in trabecular bone mass in the moderate exercise group was observed using 3D images (Fig. 2). In trabecular bone parameters (Table. 2), BV/TV, BMC/TV, and Tb.N were significantly higher in the moderate group than in the control group ($P < 0.05$). In all exercise groups, Tb.Th was longer and Tb.Sp was shorter than the control group ($P < 0.05$). Cortical bone parameters revealed no significant changes in Ct.Th, CSA, and Cv/Av among all the groups ($P > 0.05$).

After 8 weeks, low- and moderate-intensity exercise significantly increased BV/TV and BMC/TV compared to the control ($P < 0.05$) (Fig. S1a and b). In addition, BV/TV and BMC/TV in the moderate group were greater than those in the low and control groups ($P < 0.05$).

Mechanical testing in long-term exercise group

The maximal load in the moderate-intensity group tended to be higher than that in the control group ($P = 0.054$) (Table. 3). There were no significant differences in the energy to failure or stiffness between the control and exercise groups ($P > 0.05$).

ALP and TRAP activity in long-term exercise group

ALP and TRAP staining revealed the presence of osteoblasts and osteoclasts on the trabecular bone surface, respectively (Fig. 3a and b). The quantitative results of ALP staining showed that the osteoblast surface area was significantly higher in the moderate group than in the control, low, and high groups ($P < 0.05$) (Fig. 3c). TRAP staining revealed a decrease in the percentage of osteoclast surfaces in the moderate group ($P < 0.05$). In addition, a smaller TRAP-positive osteoclast surface was observed in the low and moderate groups than in the high group ($P < 0.05$) (Fig. 3d).

Immunohistochemical analysis of sclerostin and β-catenin in long-term exercise group

Immunohistochemical staining revealed sclerostin proteins in osteocytes (Fig. 4a). Moderate- and high-intensity exercise significantly decreased the percentage of sclerostin-positive cells in osteocytes compared to the control ($P < 0.05$) (Fig. 4c). β-catenin protein was localized to the surface of the trabecular bone (Fig. 4b). The number of β-catenin positive cells was greater in the moderate group than in the control group ($P < 0.05$) (Fig. 4d).

4. Discussion

Our objectives were to determine (1) changes in bone metabolism after acute exercise and (2) changes in bone mass, strength, remodeling, and proteins of bone formation-related pathways after long-term exercise at different intensities. Acute moderate-intensity exercise may suppress bone resorption by decreasing RANKL mRNA levels. Long-term moderate-intensity exercise increased trabecular bone mass and strength. Moderate-intensity exercise also promoted osteoblast activity and suppressed osteoclast activity in the trabecular bone. Furthermore, Wnt/β-catenin activation via suppressing sclerostin was implicated in osteoblast

activation by moderate-intensity exercise.

Aerobic exercise alters bone metabolism-related factors during the early phase [14, 39]. The mRNA expression of sclerostin, an inhibitor of osteoblast activation [23, 24], decreased at all exercise intensities. This result indicates an increase in the expression of osteoblast factors. However, Runx2 mRNA, which promotes osteoblast differentiation [29], did not change in any exercise group. Treadmill exercise for 8 weeks increased Runx2 mRNA levels in the bone tissues of rats [40, 41], but the differences in these results may be due to exercise duration. Indeed, acute exercise for 4 days did not change ALP and Col1a1 mRNA levels, which are bone formation markers [14]. The results from these and our studies suggest that aerobic exercise in the early phase, regardless of intensity, may not alter bone formation. The RANKL/RANK/OPG system is essential for bone remodeling [42]. RANKL plays a crucial role in promoting osteoclast differentiation [30]. As a decoy receptor, OPG can inhibit RANKL function by preventing RANKL-RANK binding; therefore, the RANKL/OPG ratio indicates bone resorption [30]. In our study, only moderate-intensity exercise decreased RANKL mRNA levels. The response of RANKL to multiple intensities remains elusive; however, our results in the moderate exercise group are consistent with an animal study that evaluated the tibia after exercise at a single intensity of 15 m/min [39]. Similar to previous studies [39, 43], we found no alteration in the RANKL/OPG ratio after any exercise intensity. This result may be attributed to the lack of changes in the OPG in all exercise groups, as previously described [44]. Moreover, a decrease in RANKL levels during moderate-intensity exercise may not affect the RANKL/OPG ratio owing to constant OPG levels. Given the critical role of RANKL in the regulation of osteoclasts [30, 42], decreased RANKL levels in the moderate group may have contributed to suppressing osteoclast activity. After 4 weeks, only moderate-intensity exercise resulted in reduced osteoclast activity in this study. Additionally, treadmill exercise recovered glucocorticoid-induced

RANKL expression and bone loss in osteoporosis rats [45], which shows that exercise suppresses bone resorption by inhibiting RANKL. Conversely, exercise may not cause osteoclastic suppression when RANKL levels are unaltered during low- and high-intensity exercises. Therefore, our study indicates that only moderate-intensity exercise can decrease RANKL levels, which may effectively suppress early-phase bone resorption.

Many studies have shown that long-term aerobic exercise improves trabecular microarchitecture [15, 18, 21, 39, 46, 47]. μCT analysis revealed that only moderate-intensity exercise at 16 m/min for 4 weeks increased BV/TV and BMC/TV, although low- and high-intensity aerobic exercise did not change them. This finding is supported by several reports investigating the effects of multiple intensities on the bone [18, 19], indicating the benefits of moderate-intensity exercise. Prolonged exercise duration can potentially enhance bone anabolism [18, 47, 48]. Bone morphology was analyzed after 8 weeks of low- and moderate-intensity exercise to assess the effects of duration. The results showed that BV/TV and BMC/TV increased after 8 weeks of low-intensity exercise, with no change after 4 weeks of exercise. Therefore, in line with Chen's study [18], low-intensity exercise may promote bone anabolism by prolonging exercise duration. In contrast, BV/TV and BMC/TV in the moderate group were higher than those in the low and control groups after 8 weeks. Chen's study using senescence-accelerated mice found no difference in bone mass between low-(8 m/min) and medium-intensity (18 m/min) mice [18], which is inconsistent with our results using young mice. However, because bone anabolism is greater in non-aging mice [39], moderate-intensity exercise may remain effective in increasing bone mass, at least in adulthood, even with prolonged exercise duration. Consistent with several studies [21, 46, 49], the present study also showed no cortical microarchitecture changes after any exercise intensity. Consequently, we presumed that the morphological changes in the trabecular bone contributed to the increased bone strength. The standardized three-point bending test often

assesses the diaphyseal cortical bone, which is unaffected by exercise [19, 21]. As an alternative assessment method to evaluate mechanical properties of metaphysis, the bending and breaking test was applied to the proximal diaphyseal tibia [36, 37]. The mechanical testing revealed a tendency to increase the maximal load in the moderate group. With a few exceptions, such as bone quality [50], the mechanical behavior of bone is largely determined by its mass [51]. Hence, an increase in the metaphyseal mechanical properties following moderate-intensity exercise may be caused by an improvement in the trabecular bone mass. Long-term exercise increases bone mass by suppressing bone resorption or promoting bone formation [11, 13, 17]. The quantitative results of ALP and TRAP staining showed that only moderate-intensity aerobic exercise promoted osteoblast activity and suppressed osteoclast activity in the trabecular bone. In contrast, osteoblast and osteoclast activities remained unchanged after low- and high-intensity exercise. Whether or not bone remodeling is altered depends on the threshold range of bone strain [1, 52]. Low-intensity exercise has often been reported not to affect bone remodeling because of its lower load on the bone [6] , which is consistent with our results. By contrast, high-intensity exercises with loads above the threshold promote bone formation [1, 50, 53]. Although the causes have not been examined, some studies have shown that higher-intensity exercise (28 m/min) than our intensity (24 m/min) could not affect bone formation and resorption [18, 19]. In support of these findings, our study revealed that high-intensity exercise resulted in unchanged bone remodeling, including that of osteoblasts and osteoclasts. In summary, our results suggest that increased trabecular bone mass following moderate-intensity exercise may be caused by promoted osteoblast activity and suppressed osteoclast activity.

Sclerostin proteins expressed in osteocytes suppress osteoblastic activity by inhibiting Wnt/β-catenin signaling [25]. After 4 weeks of moderate- and high-intensity aerobic exercise, we found a decreased number of sclerostin-positive osteocytes. As in vivo

studies demonstrated [54, 55], mechanical stress can suppress sclerostin expression depending on its intensity. Aerobic exercise can load the bone through floor reaction forces and muscle contractions [34]. Therefore, mechanical stress higher than moderate intensity may be required to suppress sclerostin proteins. β-catenin, the final transducer for Wnt/β-catenin signaling, promotes osteoblastic activity and differentiation [56]. Only moderate-intensity exercise increased the number of β-catenin-positive cells around the trabecular bone. In an earlier study [57] , downhill running increased the β-catenin protein, but swimming did not result in a change, suggesting that higher intensity is required to stimulate β-catenin. Therefore, it is reasonable that moderate-intensity exercise increased the number of β-catenin-positive cells compared with low-intensity exercise. However, high-intensity exercise maintained the number of β-catenin-positive cells despite suppressing sclerostin proteins. High-intensity exercise at 24 m/min can induce inflammatory cytokines [58], potentially preventing an increase in β-catenin [59]. Such factors have not been investigated, but high-intensity exercise may not influence osteoblastic activity if β-catenin remained consistent. Therefore, our findings suggested that Wnt/β-catenin activation via sclerostin suppression through mechanical stress contributes to osteoblastic activation of moderate-intensity exercise.

This study has several limitations. First, we analyzed bones from healthy young mice. Young bones can adapt to exercise for a short period, although whether they can be applied to other bone conditions (e.g., aging or osteoporosis) should be confirmed in further studies [39]. Second, we did not examine oxidative stress or inflammatory cytokines [59, 60]. because our study focused on bone adaptation at different exercise intensities. Further investigations are required to clarify the harmful effects of high-intensity exercise. Third, the mechanical stress on the bone was not directly evaluated. Fiber-optic sensors can measure bone strain [61]. Alternatively, measuring temporal or cumulative bone strain may explain

changes in sclerostin as a mechanosensitive protein. Fourth, total RNA was extracted from bone tissue including bone marrow. Therefore, we must be mindful that the contaminated bone marrow cell might have affected real-time PCR analysis.

In conclusion, our study showed that only moderate-intensity exercise can inhibit bone resorption at the early phase, and long-term exercise can increase bone mass and strength through promoted bone formation via activation of the Wnt/β-catenin pathway. We also found that high-intensity exercise, traditionally considered better for the bone, failed to alter bone remodeling, mass, and strength. Our findings suggest that moderate-intensity aerobic exercise, neither too low nor high, can maintain bone health. A systematic review suggested that aerobic exercise at an intensity of 40– 60% of maximal oxygen intake was effective in increasing BMD in middle-aged and older men [1]. However, many studies have not standardized age, sex, exercise type, or methods of intensity setting; thus, the optimal intensity in humans is not fully understood. Future studies should limit the characteristics of the participants and standardize the exercise conditions to determine whether our results apply to humans.

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Author contributions

JH and HM conceived and designed research. SI, HJ, CL, DT, and HK collected and analyzed

data. HK contributed analytical tools. JH wrote the manuscript. JH, HM, and HK reviewed the manuscript. All authors read and approved the manuscript.

Conflict of Interest

All authors have no conflicts of interest.

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Figure Legends

Fig. 1 The graphs show the expression of (a) Sost, (b) Runx2, (c) RANKL, (d) OPG, and (e) RANKL/OPG ratio in each acute exercise group. Data are shown as the mean ± SD (n = 4 per group). *P < 0.05 vs. Control group.

Fig. 2 Representative 3D images of proximal tibia obtained by μCT analysis in long-term exercise groups after 4 weeks. Scale bar = 1.0 mm.

Fig. 3 Representative histological images in the tibial trabecular bone stained with (a) ALP and (b) TRAP after long-term exercise. Scale bars = 50 μm. (c) Quantification of the ALP staining by osteoblast surface per trabecular bone surface. (d) Quantification of the TRAP staining by osteoclast surface per trabecular bone surface. *P < 0.05 vs. Control group; †P < 0.05 vs. Low group; ‡P < 0.05 vs. High group.

Fig. 4 Representative immunohistochemical images in the tibial trabecular bone stained with (a) sclerostin and (b) β-catenin after long-term exercise. The graphs show (c) the ratio of sclerostin-positive cells in osteocytes and (d) β-catenin-positive cells the per trabecular bone surface. Scale bars = 50 μm. *P < 0.05 vs Control group.

Fig. S1 Representative 3D images of proximal tibia obtained by μCT analysis in long-term exercise groups after 8 weeks. Scale bar = 1.0 mm. The right graphs show (a) BV/TV and (b) BMC/TV in each group. Data are shown as the mean ± SD (n = 6 per group). *P < 0.05 vs. Control group; †P < 0.05 vs. Low group.

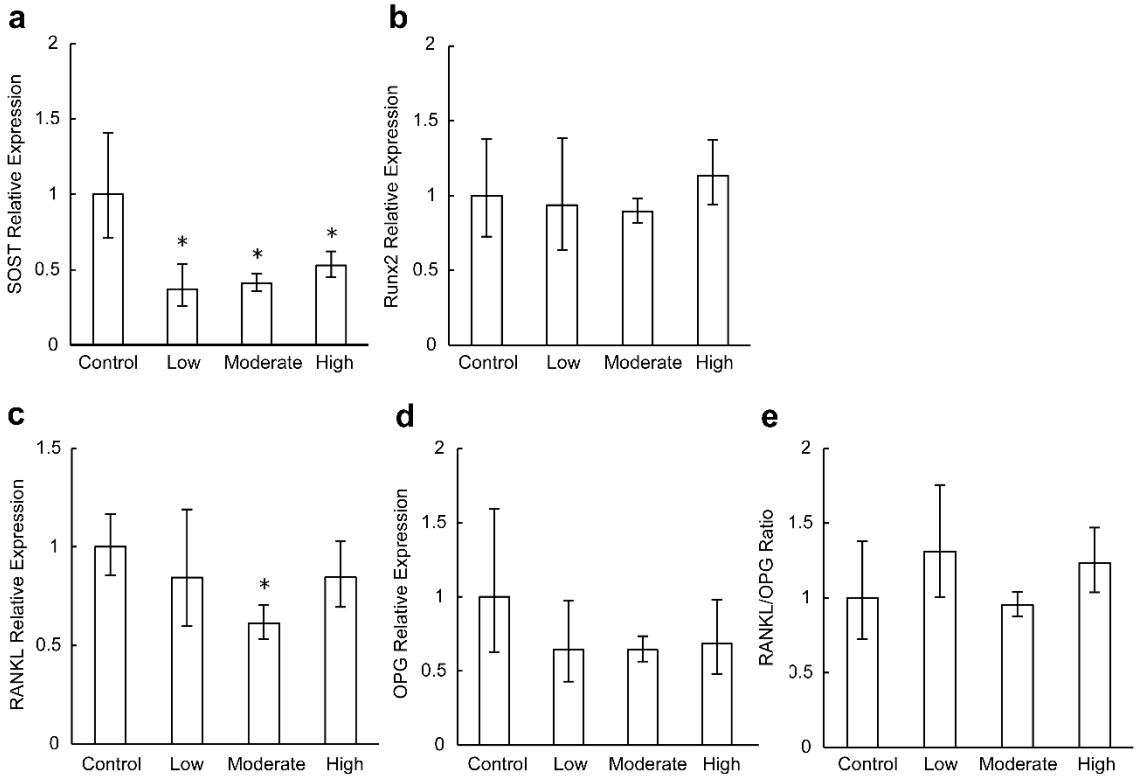
a
SOST Relative Expression
2
1.5
1
0.5
0
*
*
*
Control
Low
Moderate
High
b
Runx2 Relative Expression
2
1.5
1
0.5
0
Control
Low
Moderate
High
c
RANKL Relative Expression
1.5
1
0.5
0
*
Control
Low
Moderate
High
d
OPG Relative Expression
2
1.5
1
0.5
0
Control
Low
Moderate
High
e
RANKL/OPG Ratio
2
1.5
1
0.5
0
Control
Low
Moderate
High

Figure 1

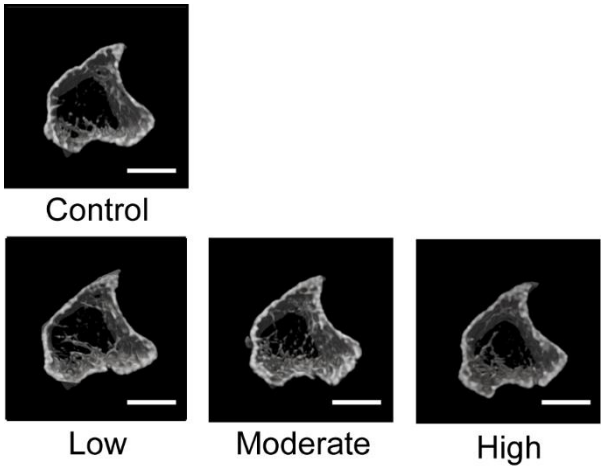

Figure 2

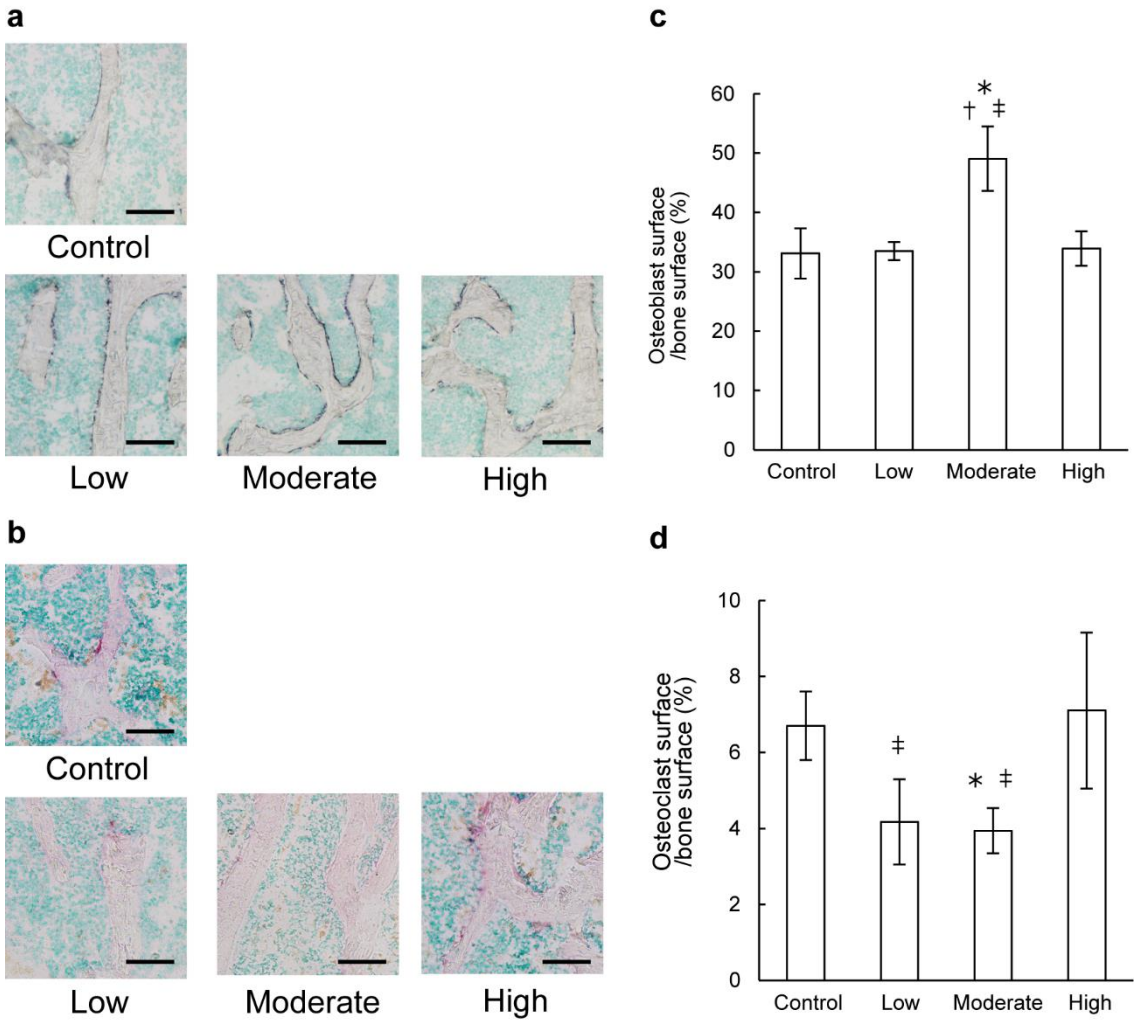
a
Control
Low
Moderate
High
b
Control
Low
Moderate
High
c
Osteoblast surface /bone surface (%)
60
50
40
30
20
10
0
Control
Low
Moderate
High
d
Osteoclast surface /bone surface (%)
10
8
6
4
2
0
Control
Low
Moderate
High

Figure 3

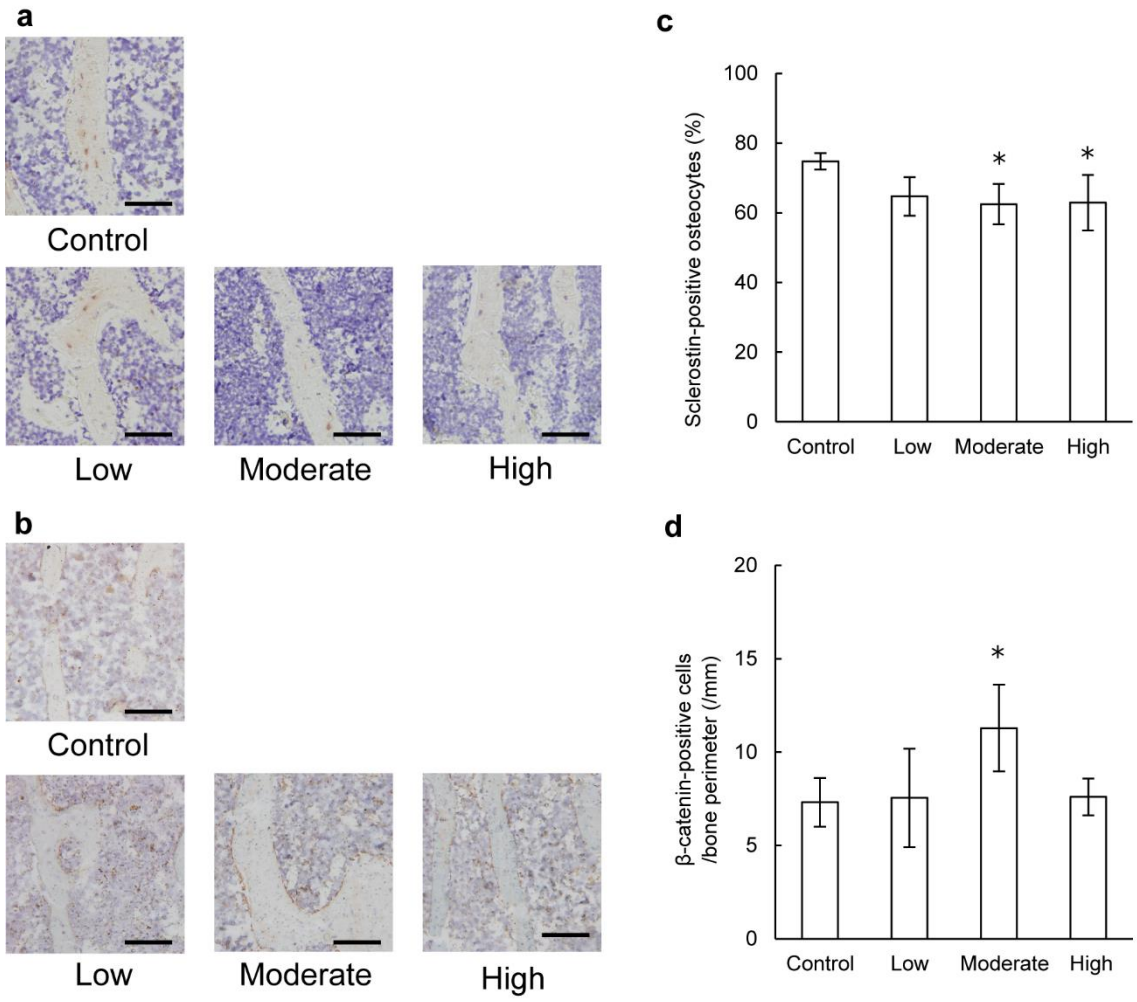
a
Control
Low
Moderate
High
b
Control
Low
Moderate
High
c
Sclerostin-positive osteocytes (%)
100
80
60
40
20
0
*
*
Control
Low
Moderate
High
d
β-catenin-positive cells /bone perimeter (/mm)
20
15
10
5
0
*
Control
Low
Moderate
High

Figure 4

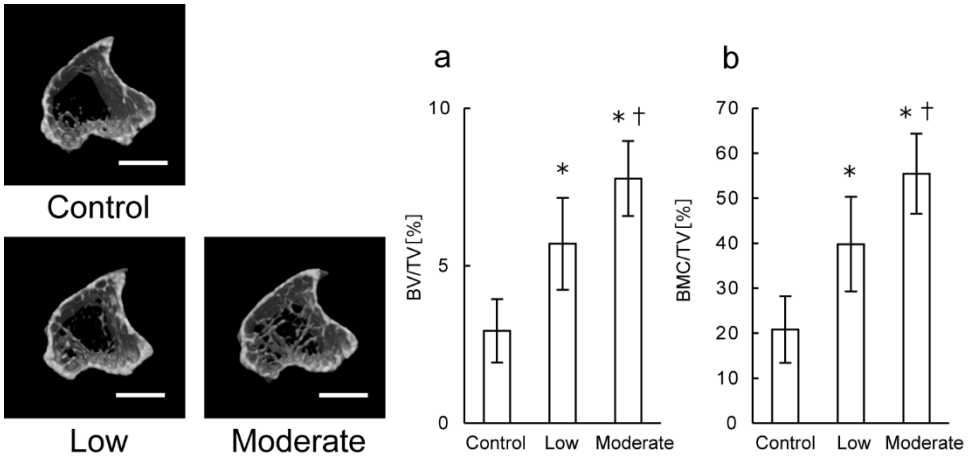
Control
Low
Moderate
a
b
BV/TV[%]
BMC/TV[%]
10
5
0
70
60
50
40
30
20
10
0
*
* †
Control
Low
Moderate

Figure S1

Table 1. Treadmill exercise protocols

	Group	Intensity	Time	Frequency	Duration
Acute exercise	Low (n =4)	8 m/min	60 min	—	Once
	Moderate (n =4)	16 m/min			
	High (n =4)	24 m/min			
	Control (n =4)	-			
Long-term exercise (4 weeks)	Low (n =5)	8 m/min	60 min	7 days/week	4 weeks
	Moderate (n =5)	16 m/min			
	High (n =5)	24 m/min			
	Control (n =5)	-			
Long-term exercise (8 weeks)	Low (n = 6)	8 m/min	60 min	7 days/week	8 weeks
	Moderate (n = 6)	16 m/min			
	Control (n = 6)	-			

Table 2. Bone microarchitecture of tibial trabecular and cortical bone by μCT

	Control	Low	Moderate	High
BV/TV (%)	3.5 ± 0.8	5.4 ± 1.5	8.4 ± 2.0*	6.0 ± 1.6
Tb.Th (μm)	26.4 ± 1.2	30.8 ± 1.6*	33.2 ± 3.0*	31.9 ± 1.3*
Tb.N (1/μm)	1.3 ± 0.3	1.7 ± 0.4	2.5 ± 0.4*	1.9 ± 0.4
Tb.Sp (μm)	766.2 ± 148.9	531.7 ± 98.4*	379.6 ± 68.0*	516.9 ± 118.9*
BMC/TV (mg/cm^3)	23.9 ± 5.4	38.3 ± 11.2	62.6 ± 18.1*	43.2 ± 11.9
Ct.Th (μm)	109.3 ± 4.2	113.7 ± 10.1	112.2 ± 9.6	109.2 ± 7.0
CSA (mm^2)	0.19 ± 0.01	0.20 ± 0.02	0.20 ± 0.03	0.20 ± 0.02
Cv/Av (%)	64.8 ± 1.3	65.9 ± 2.8	63.9 ± 1.4	63.1 ± 1.4

*$P < 0.05$ vs. control group

BV/TV trabecular bone volume/tissue volume, Tb.Th trabecular bone thickness, Tb.N trabecular bone number, Tb.Sp trabecular bone separation, BMC/TV bone mineral content/tissue volume, Ct.Th cortical thickness, CSA, cross-sectional area, Cv/Av, cortical volume/all tissue volume

Table 3. Mechanical testing for proximal diaphyseal tibia

	Control	Low	Moderate	High
Maximal load (N)	15.3 ± 3.1	17.9 ± 1.9	21.1 ± 3.6*	18.1 ± 2.6
Energy to failure (mJ)	5.6 ± 1.0	6.4 ± 2.2	8.2 ± 1.8	6.3 ± 1.7
Stiffness (N/s)	26.4 ± 5.9	30.6 ± 7.5	31.3 ± 9.1	34.2 ± 7.5

*$P < 0.1$ vs. control group

Supplemental Table 1. RNA concentration in tibia

Group	No.	RNA concentration [ng/µL]
Control	1	47.25
Control	2	67.60
Control	3	41.05
Control	4	74.12
Low	5	78.56
Low	6	65.93
Low	7	63.53
Low	8	54.73
Moderate	9	74.82
Moderate	10	80.53
Moderate	11	79.21
Moderate	12	43.93
High	13	63.56
High	14	61.06
High	15	75.55
High	16	61.71

Supplemental Table 2. Real-time PCR in total RNA from bone marrow (n = 2)

Target gene	Ct value (bone marrow)	Ct value (bone tissue)
Bglap3	Undetectable	32.3
Sost	39.7 (Undetectable)	26.5
Rankl	Undetectable	31.8
Opg	Undetectable	28.4
Runx2	37.5 ± 1.4	27.1