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Short bouts and long-term exercise reduce sedentary-induced bone loss and microstructural changes by modulating bone formation and resorption in healthy young male rats

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Although the toxic effect of Sedentary behavior (SED) on bone health has been demonstrated in the previous study, the underlying mechanisms of SED, or break SED to bone health remain unclear. In this study, we aim to investigate the effects of sedentary behavior (SED) on bone health, as well as the potential favor effects of moderate to vigorous physical activity (MVPA) and periodic interruptions of SED. To simulate SED, we used small Plexiglas cages (20.0 × 9.0 × 10.0 cm) to restrict animal movement. Short bursts of exercise to break SED and continuous long-term exercise were also designed. After an 8-weeks period of SED, we observed decreased bone mass and bone microstructure. Specifically, there was a notable decrease in the bone mineral density (BMD), bone surface (BS) and cortical thickness (Ct.Th) significantly reduced in cortical bone. In the trabecular bone, parameters such as trabecular separation (Tb.Sp), trabecular number (Tb.N), BS, connectivity density (Conn.D), BS/BV, bone volume/tissue volume (BV/TV), degree of anisotropy (DA), and structural model index (SMI) were also significantly reduced. In addition, we detected an increase in serum tartrate-resistant acid phosphatase (TRAP) levels in SED rats at both 4 and 8 weeks. At 8 weeks, the osteoclast number and surface with TRAP-staining were significantly increased, however, the OPG mRNA and proteins level were significantly decreased. After daily short bouts exercise and long-term exercise, we observed improvements in bone mass and microstructure. These improvements included increasing BMD and BV/TV of cortical bone, and improving Conn.D, BV/TV, DA and SMI of trabecular. Meanwhile, we found that, at 4 and 8 weeks, there was an increase in serum ALP. At 8 weeks, the mineralized nodules surface with Alizarin Red S-staining, and OPG mRNA and proteins level in bone tissue were significantly increased. Our findings suggest that SED leads to alterations in the bone mass and microstructure, which are associated with the changes in the OPG protein and bone remodeling. Exercise, whether in short daily bouts or continuous long-term sessions, can ameliorate the harmful effects of SED. Similarly, the changes in bone mass and microstructure from exercise are also associated with the changes in the OPG protein and bone remodeling by upregulated osteoblast activity to bone formation. Overall, our findings indicate the importance of physical activity in maintaining bone health and preventing the negative impacts of prolonged SED.

Keywords Sedentary behavior, Physical activity, Bone health, OPG, TRAP

Abbreviations

SED	sedentary behavior
MPA	Moderate Physical Activity
MVPA	moderate to vigorous physical activity
VPA	Vigorous Physical Activity

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VILPA	vigorous intermittent lifestyle physical activity
BMD	bone mineral density
BS	bone surface
Ct.Th	cortical thickness
Tb.Sp	trabecular separation
Tb.N	trabecular number
Conn.D	trabecular connectivity density
BS/BV	bone surface per total bone volume
BV/TV	bone volume per total volume
DA	degree of anisotropy
SMI	structure model index
ALP	alkaline phosphatase
TRAP	tartrate-resistant acid phosphatase
Oc.S	osteoclast surface
Oc.N	osteoclast number
No.S	mineralized nodules surface

Adolescence is an important period for bone health, characterized by since it is associated with rapid physical growth and substantial bone development. In this period, bones undergo continuous remodeling, contributing to the enough of bone mass for bone development[1,2]. Bone remodeling includes three key processes, including osteoblast-mediated bone formation, osteoclast-mediated bone resorption, and osteoblast-derived lining cells on the bone surface[3]. Adverse living habits during adolescence can disrupt the balance between bone resorption and formation, potentially leading to bone disorders such as bone loss and osteoporosis[4]. Of note, sedentary lifestyle or lack of physical activity are associated with negative outcomes of bone health[5,6]. Previous studies found that esports players, who often spend prolonged periods sitting, exhibited less bone mineral content than non-esports players[7]. Pelegrini et al. confirmed that normal weight adolescents who spend more time on the computer have lower BMD[8].Sedentary behavior (SED), characterized by activities during waking hours that involve low energy expenditure (≤ 1.5 METs/Metabolic Equivalents), typically performed in a sitting or reclining posture[9]. The 2020 WHO guidelines on physical activity and sedentary behavior for children and adolescents aged 5–17 years recommend that SED should be limited in daily life[10]. The relationship between SED and bone health outcomes has been extensively investigated. Gracia-Marco et al.[11] found a negative association between whole-body bone mineral content (BMC) and total sedentary time in adolescents aged 12.5–17.5 years. Similarly, Vaitkeviciute et al.[12] observed a negative correlation between whole-body BMC and daily objectively sedentary time in adolescents aged 11–12 years. However, the negative relation disappeared after adjusting for the moderate to vigorous physical activity (MVPA).

These results verify the existence of the negative relation between sedentary and bone health, and the affection can be verified by MVPA. Besides, Chastin et al.[13] emphasized the importance of breaking up sedentary time. The pattern of intermittence between SED periods and activity appears to play a role in BMC with clustered short bouts of activity interspaced with long periods of SED appearing to be more beneficial than activities more evenly spread in time.

Detrimental effect of SED on bone health has been demonstrated in the previous studies. However, several critical questions remain unresolved. For instance, the underlying mechanisms driving this phenomenon are not fully understood, and it is unclear whether short bouts of exercise confer greater benefits compared to prolonged daily exercise. Therefore, we will attempt to provide experimental answers to such problems in this research.

Result

SED causes bone loss and changes bone microstructure in rats.

SED has been linked to adverse bone health outcomes in adolescents, as evidenced by numerous observational studies. However, the underlying mechanisms of this association remain unclear.

As expected, Micro-CT analysis revealed a significant reduction in bone size (S group) compared to controls (Fig. 1a). Additionally, cortical bone parameters such as bone mineral density (BMD), bone surface (BS) together with cortical thickness (Ct.Th) was significantly reduced in cortical bone (Fig. 1b). Most importantly, the trabecular separation (Tb.Sp), trabecular number(Tb.N), BS, trabecular connectivity density (Conn.D), BS per total bone volume (BS/BV), bone volume per total volume (BV/TV), degree of anisotropy (DA) and structure model index (SMI) were also significantly reduced in trabecular bone (Fig. 1c, d). To investigate whether the reduced bone mass in SED rats was due to altered bone formation or resorption, we conducted bone histomorphometric analysis. Light microscope revealed that serious bone loss was observed in SED group, with the notably thinner trabecular bone compared to the control group, accompanied with increased bone marrow area, which was subjected to fracture (Fig. 1e). Tartrate-resistant acid phosphatase (TRAP) staining indicated an increase in osteoclasts, with a significant augmentation in surface (Oc.S) and osteoclast number (N.Oc) in SED rats at 8 weeks (Fig. 2a, b). Conversely, the difference of Alizarin Red S staining of the coronal section was not observed both at 4 and 8 weeks (Fig. 2a, b). Similarly, the serum concentration of alkaline phosphatase (ALP), an osteoblast-related marker, was not altered in SED rats in 4 weeks at 8 weeks. However, TRAP levels, indicative of osteoclast activity, were significantly elevated in the SED group at both 4 weeks and 8 weeks (Fig. 1f). These results indicated that SED dramatically changed bone remodeling especially in osteoclast activity and causes bone loss and microstructure changes.

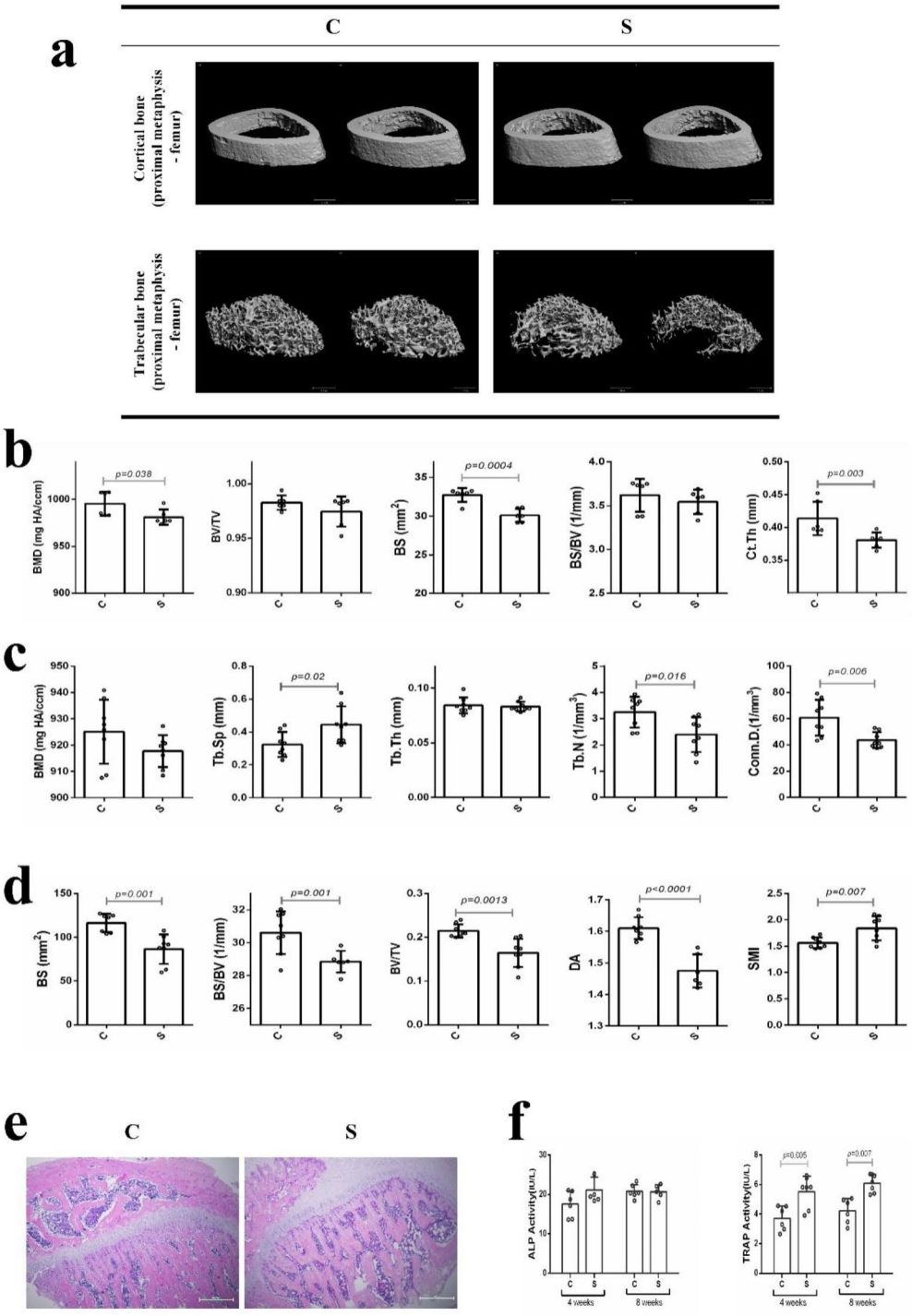

Fig. 1. Bone loss and microstructure in SED rats. Representative Micro-CT images from each group showing the three-dimensional (3D) architecture of cortical bone and trabecular bone within the distal metaphyseal femur region **(a)**. BMD, BV/TV, BS, BS/BV and Ct.Th of the cortical bone are shown **(b)**. BMD, Tb.Sp, Tb.Th, Tb.N, Conn.D, BS, BS/BV, BV/TV, DA and SMI of the trabecular bone are shown **(c, d)**. Representative images at 8 weeks of left femurs sections stained with H&E **(e)** (The H&E images at 4 weeks were available in ***Supplement file***: Fig. 1). Scale bar = 500 μm. Enzyme-linked immunosorbent assays were performed to ALP and TRAP in the serum at 4 or 8 weeks **(f)**. Data are shown as the mean ± SD.

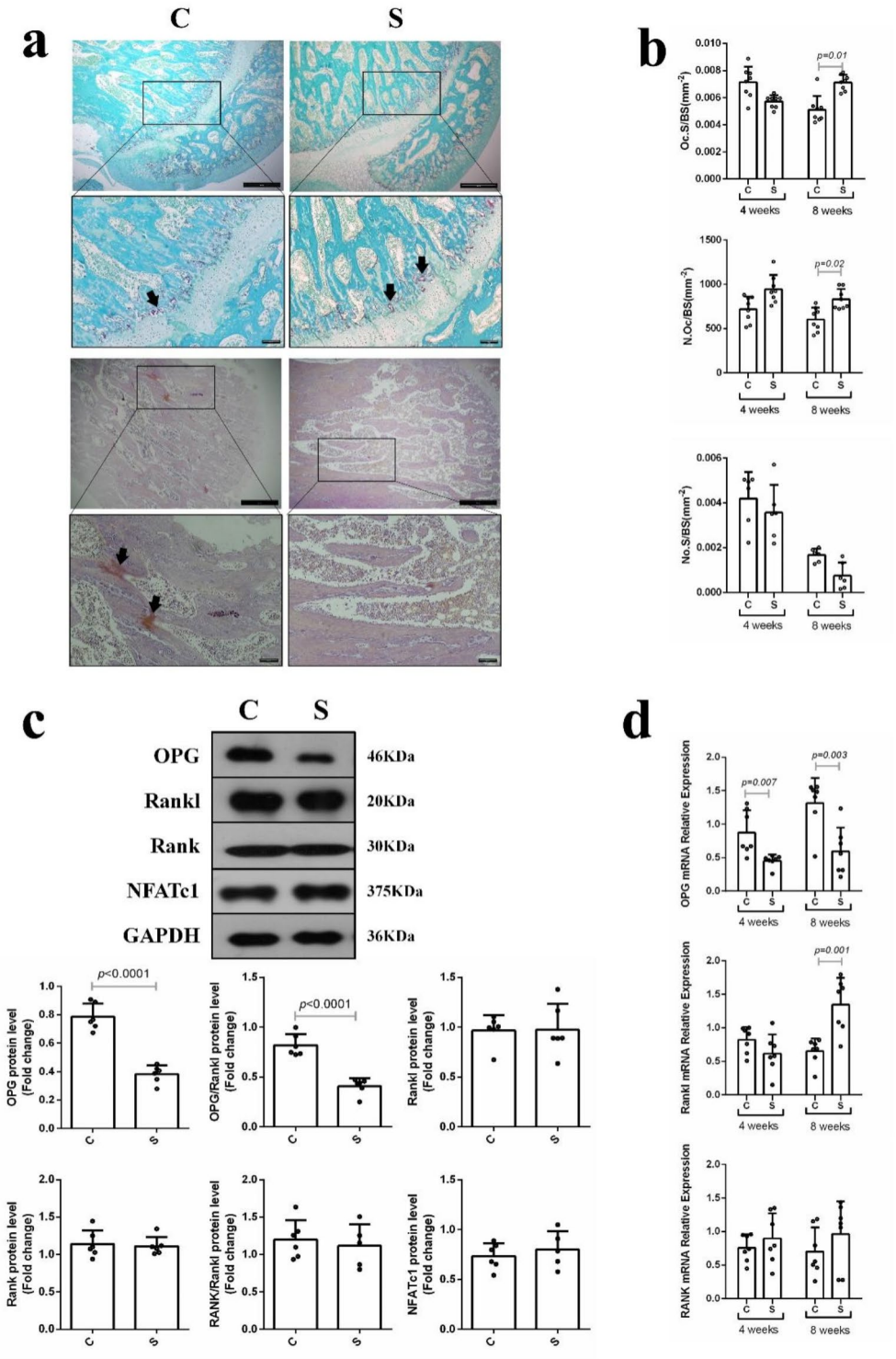

Fig. 2. Bone remodeling changes in SED rats. Representative TRAP staining and Alizarin Red S staining images of coronal sections of left femur at 8 weeks **(a)** (The TRAP staining and Alizarin Red S staining images at 4 weeks were available in ***Supplement file***: Fig. 1). Scale bar = 500 μm–100 μm. Osteoclast surface (Oc.S/BS), and osteoclast number (N.Oc/BS) and mineralized nodules surface (No.S) are presented **(b).** Protein level of OPG, Rankl, RANK and OPG/Rankl, RANK/Rankl in bone tissue of rats at 8weeks **(c)**. mRNA expression of OPG, Rankl and RANK in bone tissue of rats at 4 or 8weeks. Data are shown as the mean ± SD.

SED alters bone remodeling in rats via modulation of OPG protein expression

The OPG/Rankl/RANK signaling pathway, known for its critical role in maintaining bone remodeling, is highly responsive to mechanical stimulation and regulates bone metabolism. Our study revealed that, compared to the control group, SED significantly reduced mRNA and protein levels of OPG ($p < 0.05$). However, no significant changes were observed in the protein levels of Rankl, RANK and NFATc1 protein level (Fig. 2c). Similarly, the mRNA expression levels of Rankl and RANK mRNA expression level remained unchanged, except at 8 weeks of Rankl mRNA expression (Fig. 2d). Taken together, these findings indicate that OPG is a key mediator in the SED-induced alterations of bone remodeling.

Exercise can modulate bone formation and resorption in ameliorating SED-induced bone loss and microstructure changes

To determine whether exercise can ameliorate SED-induced bone loss and microstructure changes, and distinguish the health effects of the two types of exercise at 4 or 8 weeks. We analyzed the changes in bone remodeling, bone mass, and microstructure resulting from short bouts of exercise (S-B group) and long-term exercise (S-S group) in daily life. Micro-CT analysis showed that while bone size was increased in exercise rats (S-B and S-S group) as expected (Fig. 3a), BMD and BV/TV was significantly increased in cortical bone (Fig. 3b). Moreover, the Ct.Th was significantly increased in S-B group than S group. Most importantly, the Conn.D, BV/TV, DA and SMI of trabecular in S-B and S-S was improved than S. Besides, we observed an increase in BMD and Tb.Th in S-S, but not in S-B (Fig. 3c, d). Similarly, we found S-B have better Tb.Sp than S in trabecular, but not in S-S. Histomorphometric analysis further indicated changes in both exercise groups. The S-B and S-S group exhibited greater bone mass and thicker trabecular bone compared to S group, accompanied by decreased bone marrow area (Fig. 3e). Alizarin Red S staining of the left femur demonstrated mineralized nodules surface (No.S) were increased in S-S group at both 4 and 8 weeks. Similarly, the same change was also observed in S-B group, except in 4 weeks. On the contrary, the osteoclasts, visualized by TRAP staining, were slightly decreased, with a significant reduction in osteoclast surface (Oc.S) in both the S-B and S-S groups at 8 weeks (Fig. 4a, b). Interestingly, we found a totally different result with SED rats in ELISA test, the serum concentration of ALP, were augmented significantly in S-B and S-S at 4 weeks and 8 weeks, while TRAP levels remained unchanged (Fig. 3f). Collectively, these data suggest that both short bouts of exercise and long-term exercise can alter the bone remodeling, particularly by enhancing osteoblast activity, and improve bone mass and microstructure. However, no significant differences were found between the effects of short bouts of exercise and long-term exercise in daily life.

Exercise ameliorates SED-induced bone remodeling alterations also via OPG/Rankl/RANK pathway

To verify whether exercise could attenuate SED-induced bone remodeling changes by OPG/Rankl/RANK pathway, we assessed the expression levels of OPG, Rankl, RANK and NFATc1 in bone tissues. At 8 weeks, the mRNA and protein expression levels of OPG were significantly higher in the S-B and S-S groups compared to the S group, whereas no significant changes in OPG mRNA expression were detected at 4 weeks. Additionally, NFATc1 protein expression was markedly elevated in the S-S group comparing with the S group at 8 weeks. Conversely, we observed no changes in Rankl and RANK mRNA or proteins, except for a difference in RANK mRNA expression between the S-B and S groups at 8 weeks (Fig. 4c, d). Overall, these data suggest that OPG as a crucial role participated in exercise ameliorates SED-induced bone remodeling changes.

Discussion

Infancy and adolescence are crucial periods for bone health. A large number of previous studies have demonstrated that SED is an independent risk factor for deficient bone deposition in youth[14]. However, most researchers have only conducted observational studies to examine the association between SED and bone health outcomes, with limited experimental investigations demonstrating a cause-and-effect relationship, and there is an absence of an appropriate animal model for previous studies.

Recently, researchers have endeavored to design innovative enclosures to simulate sedentary behavior (SED) in laboratory settings. In the study by Alabi's et al.[15], a steel compartment of 20 cm length, 9 cm breadth and 10 cm height was developed to induce SED. A key advantage of this cage is that it can be used to house rats within and restrict their movement as much as possible without causing them any pain. In which, Yoshihara T et al.[16] found that the serum corticosterone level and adrenal weight, which are markers of stressors, did not differ between the inactivity group and control groups. Therefore, compared to the immersion model, bed rest, limb immobilization, and other models, the "small cages" can provide better adequate free-living environments without unnecessary restraint stress[16,17].

This model effectively replicates the mechanistic onset of risk factors associated with sedentariness, mirroring the typical sedentary behavior observed in adolescents today. Similarly, Tyganov et al.[17] utilized a small Plexiglas cage ($17.0 \times 9.6 \times 13.0$ cm) to limit rat activity, and they observed the variation of mechanical properties in fast and slow skeletal muscle. Besides, Marmonti et al.[18] kept rats 7, 14, and 28 consecutive days in a confined space ($12 \times 12 \times 8$ cm) to study disuse-induced changes in muscle and bone, observed during prolonged bed rest in humans. Similarly, Siripoksup et al.[19] developed a mouse model of sedentariness using a small mouse cage, which, unlike other traditional models of disuse in mice, accurately recapitulated metabolic responses that occur in humans. This model was developed to explore the mechanism by which physical inactivity promotes metabolic inflexibility in skeletal muscle. Overall, these "small cage" was utilized to simulate sedentariness and identify the precise relationship between SED and health. In the current study, we used small Plexiglas cages ($20.0 \times 9.0 \times 10.0$ cm) restricting animal movement to simulate the SED of animals (Fig. 5a, b). We investigated the effects of 4 and 8 weeks of SED on the bone remodeling, bone mass and bone microstructure. Results revealed

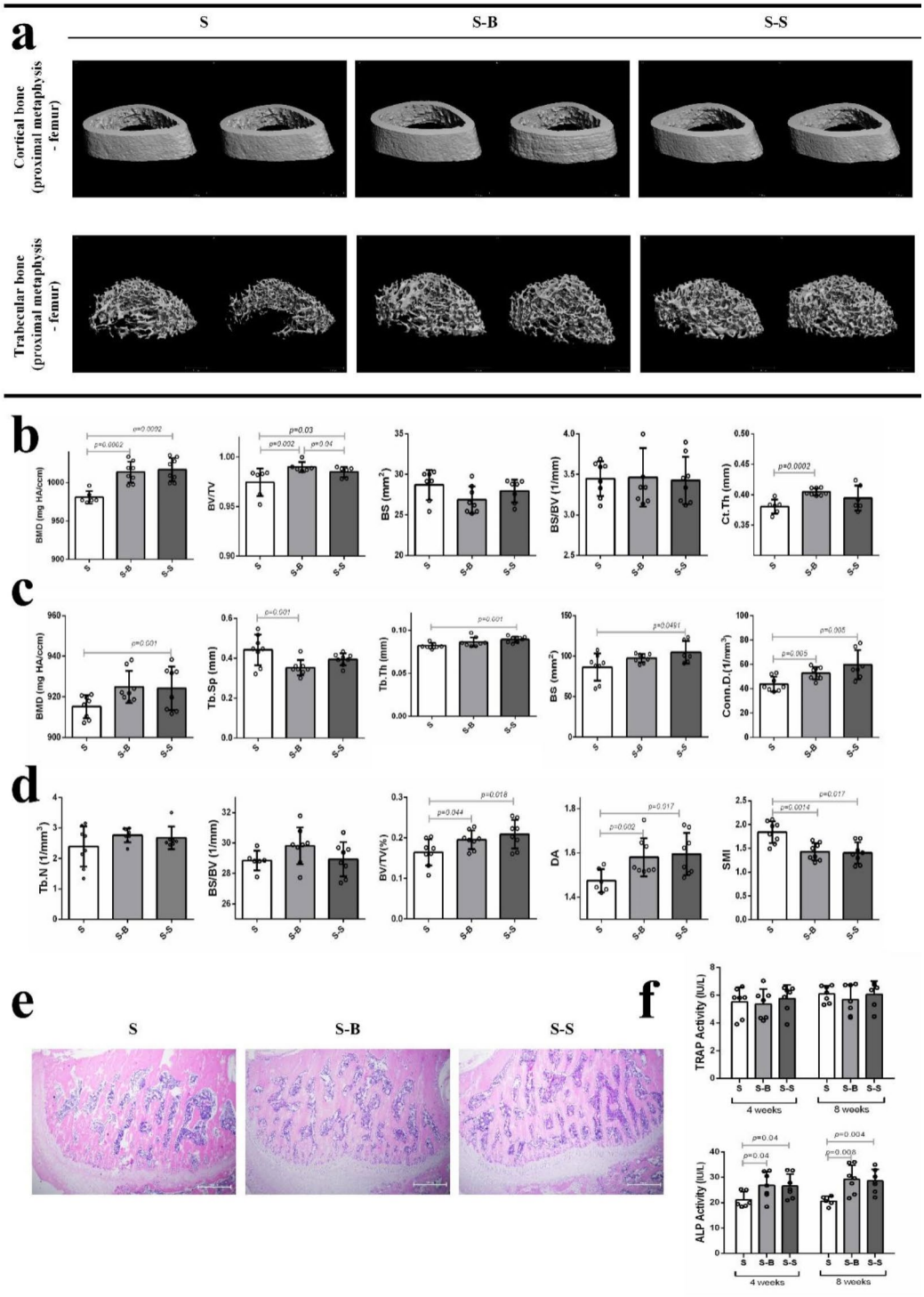

Fig. 3. Exercise modulate bone formation and resorption in ameliorates SED-induced bone loss and microstructure changes. **(a)** Representative Micro-CT images from each group showing that three-dimensional (3D) architecture of cortical bone and trabecular bone within the distal metaphyseal femur region. BMD, BV/TV, BS, BS/BV and Ct.Th of the cortical bone are shown **(b)**. BMD, Tb.Sp, Tb.Th, Tb.N, Conn.D, BS, BS/BV, BV/TV, DA and SMI of the trabecular bone are shown **(c, d)**. Representative images at 8 weeks of left femurs sections stained with H&E **(e)** (The H&E images at 4 weeks were available in ***Supplement file***: Fig. 1). Scale bar = 500 µm. Enzyme-linked immunosorbent assays were performed to ALP and TRAP in the serum at 4 or 8 weeks **(f)**. Data are shown as the mean ± SD.

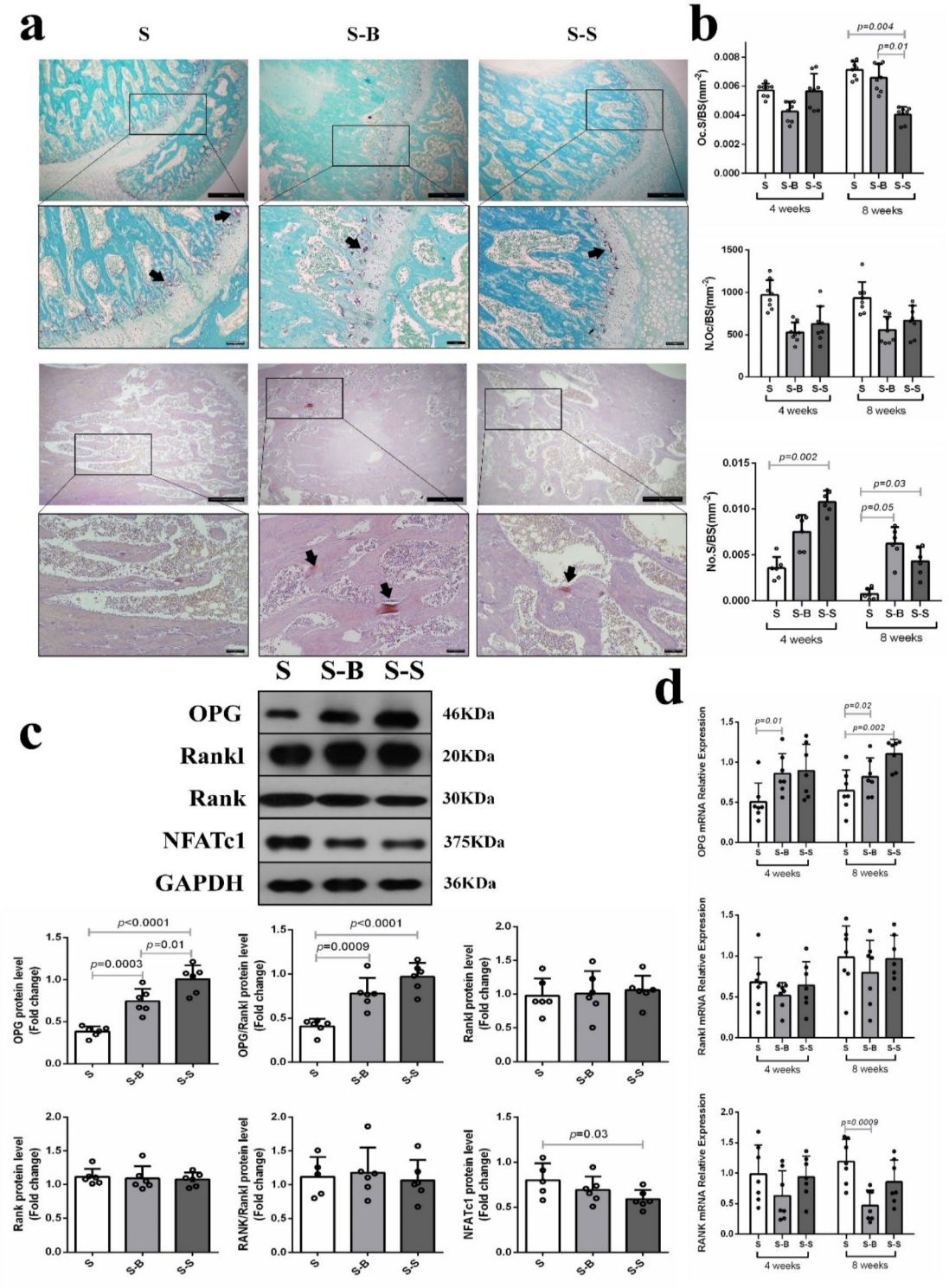

Fig. 4. Bone remodeling in Exercise rats. Representative TRAP staining and Alizarin Red S staining images of coronal sections of left femur at 8 weeks **(a)** (The TRAP staining and Alizarin Red S staining images at 4 weeks were available in ***Supplement file***: Fig. 1). Scale bar = 500 μm–100 μm. Osteoclast surface (Oc.S/BS), and osteoclast number (N.Oc/BS) and mineralized nodules surface (No.S) are presented **(b).** Protein level of OPG, Rankl, RANK and OPG/Rankl, RANK/Rankl in bone tissue of exercise rats at 8weeks **(c)**. mRNA expression of OPG, Rankl and RANK in bone tissue of exercise rats at 4 or 8weeks. Data are shown as the mean ± SD.

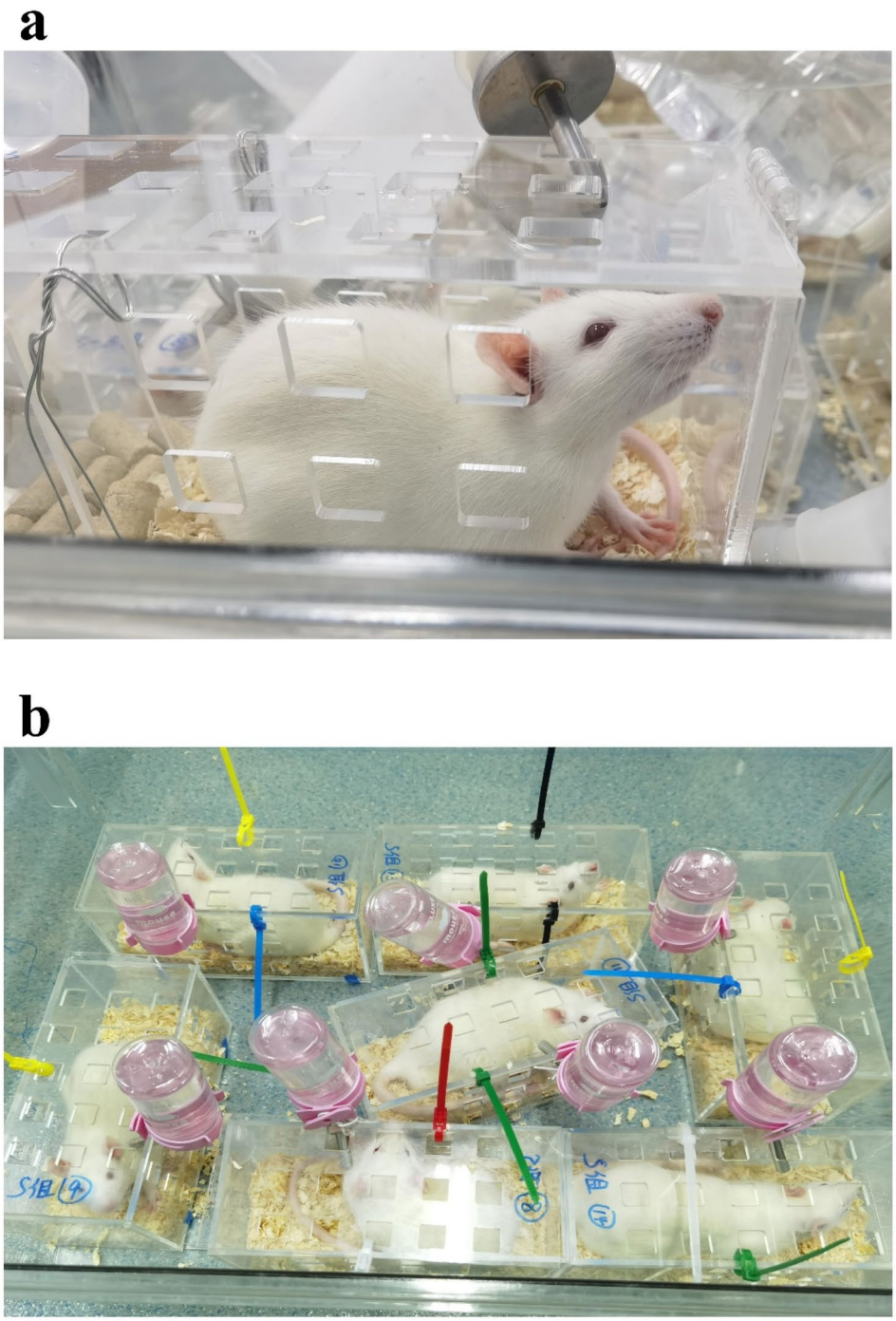

Fig. 5. Design of the plexiglass sedentary behavior cages. View from the side **(a)** and from the top **(b)**.

that 4- and 8-week SED had bad effects on bone health and a big decrease in bone mass. The bone microstructure significantly altered at 8 weeks of SED, along with a decrease in BMD, BS and Ct.Th in femur cortical bone, and a bad variation in Tb.Sp, BS, Conn.D, Tb.N, BS/BV, BV/TV, DA with SMI. The skeleton is a metabolically active organ that constantly remodels during life[4]. Bone remodeling, a cellular process performed by bone multicellular units (BMUs), comprising osteoclast and osteoblast, maintains the volume, microstructure, and material composition of bone[20]. At the same time, the TRAP activity, a histochemical marker of osteoclasts, also

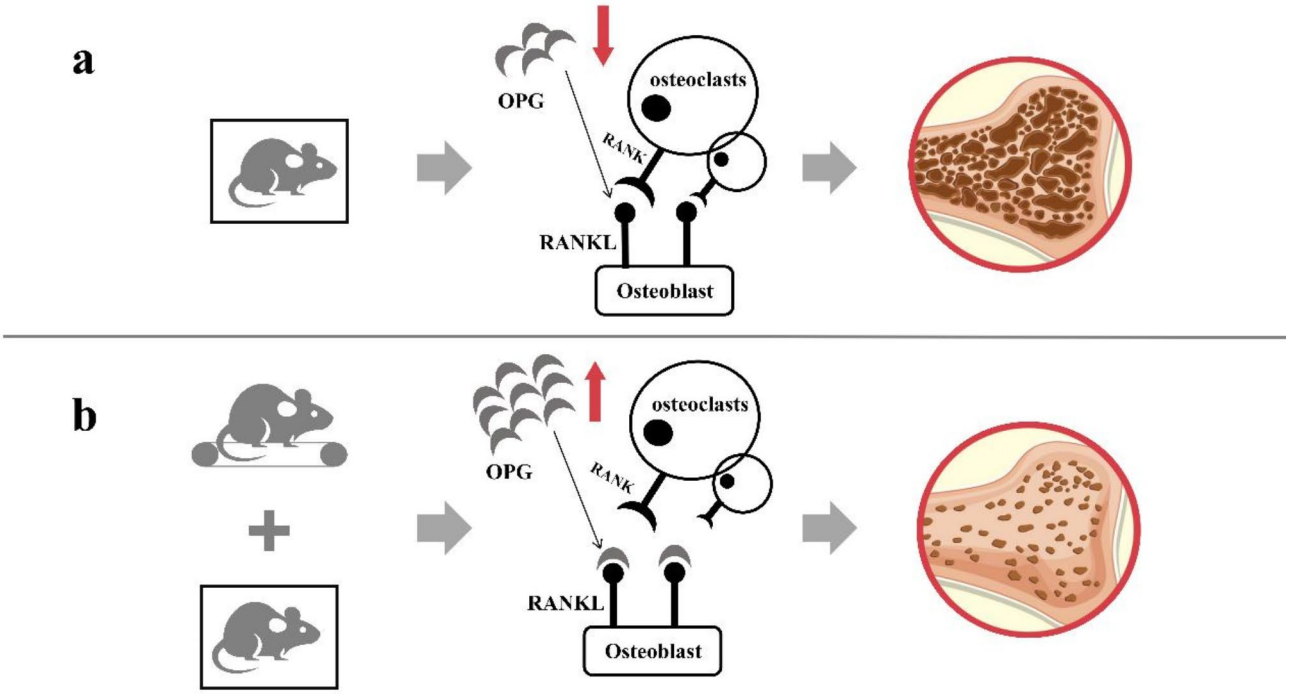

Fig. 6. An illustration of the mechanism of sedentary-induced bone loss and microstructural changes relating to OPG expression **(a)**. In addition, both short and long-term exercise reduced sedentary-induced bone loss and microstructural changes also relating to OPG expression **(b)**.

increased in serum after 8 weeks of SED. Regarding the OPG/Rankl/RANK pathway, as key mediators of bone remodeling, we observed a significant decrease in OPG protein content[21].

The health outcomes of bone following SED have not been studied thoroughly. Several studies utilizing Dual-energy X-ray Absorptiometry (DXA) or quantitative ultrasound have suggested that objectively measured SED is negatively associated with lower extremity bone outcomes, such as femoral neck bone mineral density in human[22,23]. In the research conducted by Marmonti et al.[18], Immobilization animals showed a significant decrease in bone mineral density, consistent with our findings. Bone is a dynamic structure maintained by a balance between bone formation by osteoblasts and resorption by osteoclasts. These opposite processes allow the bones to adapt to dynamic mechanical loads[24–26]. SED typically involves prolonged siting, reclining and bed rest posture, which decrease the mechanical load to weight-bearing bone. Besides, several studies have identified a link between bone cells and global metabolism, highlighting hormonal interactions between the skeleton and other tissues[27]. Overall, insufficient mechanical load on bones and elevated global metabolism may be the crucial factors contributing to bone loss and microstructure changes associated with SED. In order to track changes in bone remodeling, we measure serum ALP and TRAP activity in addition to TRAP staining and Alizarin Red S, which indicate osteoblast and osteoclast activity, respectively. We observed that, compared to osteoblast, osteoclast activity appears to be more susceptible to SED. Moreover, we noted significant variations in OPG protein and mRNA expression in SED rats. OPG is a circulating decoy receptor for RANKL, a multifunctional cytokine essential for the osteoclast's differentiation, which can induce apoptosis of mature osteoclasts and inhibit the activity of mature osteoclasts, thereby exerting its role in protecting bone tissue[28,29]. OPG's functions limits its functions within the tissue where it was produced[30]. Therefore, decreased OPG levels in bone tissue fail to inhibit osteoclast activity, leading to bone loss and altered microstructure in SED rats (Fig. 6).

Recent updates to physical activity guidelines[31,32] suggest 150–300 min of moderate-intensity or 75–150 min of vigorous-intensity (≥6 metabolic equivalents) per week. A renewed focus is on "all activity counts," which include all life domains and bout duration. This statement is in opposition to earlier guidelines[33] which undervalued the importance of short bouts (<10 min) of physical activity for health [34]. Vigorous intermittent lifestyle physical activity (VILPA)[35] refers to short, sporadic bursts of high-intensity physical activity integrated into everyday life, including stair climbing and rapid walking during commuting[36]. Researchers have begun to focus on VILPA. Because VILPA requires no special equipment, preparation, or facility access, and only requires a modest time investment, it may be more practical for most individuals than structured exercise[37]. Studies on bone tissue indicate that the quantity of intense physical activity bouts each day appear to be more significant for optimal bone strength than the total cumulative duration[38,39]. Besides, break/interrupting SED seems more important than amount of exercise[9,40,41]. Therefore, we investigated whether short bouts of exercise are more effective for improving bone health in SED rats than long-term exercise in daily life. We found that exercise can modulated the bone mass loss and bone microstructure changed from SED. The benefits of short bouts exercise vs. long-term exercise, however, did not differ significantly in terms of bone health.

Both short bouts exercise and long-term exercise can increase the BMD of femur, alter bone microstructure, and influence bone remodeling by modulating serum TRAP and ALP activity. Gemma`s research indicated a greater benefit of Vigorous Physical Activity (VPA) over Moderate Physical Activity (MPA) / MVPA[42]. Some researches indicated that the detrimental effect from SED to bone health can by modified if counting the VPA in daily life[12,13,43]. Additionally, some researchers proposed that not all SED is detrimental to bone health, particularly for highly active adolescents, as SED can provide a recovery period between loading bouts, facilitating optimal biomechanical adaptation and restoration of mechano-sensitivity of bone cells[44]. In contrast with the VPA, we used an MPA exercise to interfere with the sedentary activity rats; perhaps this is why we are unable to distinguish between the short bouts exercise and long-term exercise. Therefore, in order to better explore the optimal exercise scheme for ameliorating SED-induced bone remodeling changes, we will consider different ways of exercise intensity in the future studies.

Interestingly, our findings highlight that the OPG still plays an important role in the process of exercise ameliorates SED-induced bone loss. The OPG protein and mRNA expression level was significant increase in both short bouts exercise and long-term exercise. Following the addition of mineralized nodules surface and stimulation of osteoblast activity, serum ALP activity increased at 8 weeks. Osteoclast area decreased at 8 weeks, suppressing osteoclast at the same time. These processes collectively contribute to increased bone mass and bone growth.

Materials and methods

Experimental design

64 Sprague-Dawley (SD) male rats were obtained from the Guangxi Medical University Laboratory Animal Center (Nanning, Guangxi Province, China). This study was approved by the the Animal Care & Welfare Committee of Guangxi Medical University (202408005). All methods were carried out in accordance with relevant guidelines and regulations. All methods are also reported in accordance with ARRIVE guidelines.

Six-week-old male rats were divided into four groups ($n = 16$ per group), including baseline control group (C group), SED group (S group), daily short bouts exercise to break SED group (S-B group) and daily long-term exercise and keep SED group (S-S group). Animals in the C group were kept in typical cages ($31.0 \times 23.0 \times 36.0$ cm), and activities were not restricted. The SED rats were kept in small Plexiglas cages ($20.0 \times 9.0 \times 10.0$ cm) to restrict rat's activities in 16 h a day (Fig. 5a, b and Supplement file). The animals were kept in a temperature-controlled and light-controlled room on a 12:12-hlight-dark cycle. All rats had access to a standard diet with food pellets and water adlibtum. All the exercise group (S-B and S-S group) rats were adapted to 8 m/min, 15 min treadmill training each day for 1 week to reduce exercise-induced stress. The velocity of treadmill was weekly increased until rats running for 30 m/min, which was reached, on the 6 weeks of training. Exercise sessions were performed during the light cycle and consisted of 7 days per week for 4 or8 weeks. S-B group daily exercise consisted of 6 short bouts of exercise, 8 min per bout, to break 16 h of daily SED. The daily exercise regimen for the S-S group included a 48 min exercise that did not interrupt the 16 h of SED.

At 4-week, 8 SD rats for each group were sacrificed for bone histomorphometry, ELISA analysis and Rt-PCR analysis. And at 8-week, 8 SD rats for each group were sacrificed for bone histomorphometry, ELISA, Micro-CT, Western Blotting and Rt-PCR analysis. Prior to dissection, the animals were anesthetized with an intraperitoneal injection of tribromoethanol (400 mg/kg), and sacrificed by collecting big volume blood by the heart punctures.

Micro-CT analysis

Each group rat's right femur in 8 weeks was removed in order to use Micro-CT to assess the cortical bone and trabecular bone microarchitecture of the femoral metaphysis (SCANCO viva CT 80, Scanco Medical, Bruttisellen, Switzerland) with the following parameters: (isotropic nominal resolution: 9 μm scan slice thickness; 70kVp, 144 μA, 450ms integration time; 500 projections per 180°; 31.0 mm field of view (FOV); 29 μm^3 resolution, scanning mode was continuous ROT; scan duration 450ms; BH calibration was 1200mgHA/cc).

The femur was scanned from the proximal growth plate in the distal direction (1 mm/slice). The cortical bone volume of interest (VOI) was set as a region 2 mm away from the growth plate at the proximal end of the femur to 70 slices. The trabecular bone VOI was set as a region 1 mm away from the growth plate at the proximal end of the femur to 70 slices. The 3D images were obtained for visualization and display.

Bone histomorphometry

The slices of femurs for each group rats in 4 and 8 weeks were subjected to hematoxylin and eosin (H&E) staining, TRAP staining and Alizarin Red S staining. After soft tissue removal, the left femurs were fixed in 4% paraformaldehyde for 24 h at 4℃, decalcified with 10%EDTA PH = 7.2(G1105, Servicebio, China) for 3 weeks, during which the decalcifying solution was refreshed every 3 days, and then dehydrated. Subsequently, the specimen was embedded in wax, cut into 4 mm paraffin slices, and baked at 60°C. The slices were deparaffinated before being subjected to H&E staining (GP1031, Servicebio, China) and TRAP staining PH = 5.0 (G1050-50T, Servicebio, China). After Micro-CT analysis, the right femurs were fabricated as frozen sections. After thawing with room temperature, the frozen sections were dipped into Alizarin Red S solution (G1038, Servicebio, China) for 10 min.

After staining, the slices were sealed in neutral gum and analysis was performed with NIKON Eclipse Ci microscope equipped with a digital camera. TRAP-positive cells, which were stained to amaranth were identified as osteoclasts, and the osteoclast surface/bone surface and osteoclast number/bone surface were counted in three randomly selected pictures taken from each sample by Image-pro plus 6.0. Alizarin Red S-positive were verified the formation of mineralized nodules, the mineralized nodules surface/bone surface was also counted in three randomly selected pictures taken from each sample calculated by Image-pro plus 6.0.

Enzyme-linked immunosorbent assay (ELISA)

The blood for each group rats in 4 and 8 weeks were subjected to ELISA analysis. After anesthetized, the heart puncture method was used for blood collection. Each blood sample was centrifuged at 3000 g for 15 min and the serum supernatant was removed and placed in a fresh centrifuge tube. Quantitative determination of ALP and TRAP was performed using a Rat/Mouse ALP ELISA Kit and TRAP ELISA Kit (Jianglai, Shanghai, China), respectively. according to the manufacturer's instructions. Absorbance at 450 nm was measured using a microplate reader (Spark, Tecan, Germany, Switzerland).

Western blotting

The right tibias for each group of rats in 8 weeks were subjected to Western Blotting analysis. To isolate the total protein fraction, after removing soft tissue, the tibias were lysed by the RIPA reagent kit (R0020, Solarbio, China). The samples were diluted in a 4X sample electrophoresis buffer (5.4 mM Tris-HCl (pH 6.8), 4%-Ds-Na, 20%-glycerin, 10%−2-mercaptoethanol, and 0.02%-bromophenol blue). Trophoresis was performed in 10% separating PAGE. The primary antibodies were used as follows: GAPDH (Feiyuebio, FY-AB37472, 1:1000 Wuhan, China), OPG (Feiyuebio, FY-AB34533, 1:1000 Wuhan, China), Rankl (Feiyuebio, FY-AB36074, 1:1000 Wuhan, China), RANK (Feiyuebio, FY-AB37704, 1:1000 Wuhan, China) and NFATc1 (Feiyuebio, FY-AB33777, 1:1000 Wuhan, China). As secondary antibodies, goat anti-rabbit antibodies conjugated with horseradish peroxidase (SantaCruz, Dallas, TX, USA) were used at a dilution of 1:50,000. The blots were revealed by using the Clarity Western ECL Substrate (Biosharp, Guangzhou, China). Protein bands were analyzed by using a C-DiGit Blot Scanner (Lincoln, NE, USA). Images from Western blots were processed with Image Studio Digits Ver4.0. (Lincoln, NE, USA).

RT-qPCR

The left tibias for each group rats in 4 and 8 weeks were subjected to RT-qPCR analysis. Minced bones were lysed using TRIzol reagent to purify RNA, and 1 μg of total RNA was subjected to reverse transcription using the Verso cDNA Kit (BL1018A, Biosharp, Guangzhou, China). Relative amounts of gene transcripts were determined by real-time qPCR using qPCR SYBR Green Master Mix (11198ES, Yeasen, Shanghai, China). ***Supplementary file Table 1*** lists the sequence information for the primers used. All primer sets are for rat's genes.

Statistical analysis

Data are presented as mean ± SD from three independent experiments. All data were analyzed by Prism (GraphPad Software, San Diego, CA, USA). Statistical differences between the two groups were determined using a two-tailed unpaired Student's t-test. One-way analysis of variance (ANOVA) and Tukey's post hoc test was used for comparing multiple groups. P values < 0.05 were considered statistically significant.

Conclusions

This study demonstrates that SED leads to profound specific changes in the mass and microstructure of bone tissue. The changes in bone parameters are associated with the changes in the OPG protein and bone remodelling. Notably, exercise can ameliorate the detrimental effect from SED, with no difference between daily short bouts exercise and daily long-term exercise. The beneficial effects of exercise on bone mass and microstructure are associated with increased OPG levels, promoting bone formation. To the best of our knowledge, this is the first research that explore the specific effect from SED to bone health outcomes. In this study, we made an initial attempt to investigate the specific effect between SED and bone health from experiment. But there still have some limitations of this study as following: 1) Research on the relationship between SED, exercise, and bone health remains limited due to a lack of reliable data collection. For Micro-CT analyses, the spatial resolution (29 μm^3) is insufficient to adequately investigate cancellous bone, given the average size of a trabecula in rats[45]. 2) It is unfortunate that the samples only conducted with rapidly growing rats (6 weeks of age), which may limit the applicability of the data to adult models or older models. 3)Regarding the effects of estrogen on bone, only male rats were included in the study, which may limit our understanding of the effect of estrogen, and this would be a fruitful area for future study. 4) This is despite the fact that we uniformly controlled for diet and water use in all rats, and we recorded weekly body mass changes(in *Supplementfile* Fig. 4), but there is no consideration was given to the effect of obesity on bones in our study, which may have limited our understanding of body fat influence[46,47]. In the future, several unresolved questions still require attention, such as "Is that that adipose tissue mediates the adverse effect of SED on bone?" and "Whether there is a gender different effect from SED to bone health?" or pay attention to VPA and continue discuss the difference between daily short bouts exercise and daily long-term exercise to determine the optimal strategy for mitigating SED effects.

Data availability

Data of the analysis are available upon reasonable request to L.Y Wang (lin-yuanw@sr.gxmu.edu.cn).

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Author contributions

LY. Wang collected and did data analysis, prepared all figures, and wrote the main manuscript text. LM. Liang, XX. Zhang and H. Chi collected and did data analysis. FL. Peng designed the experiments and fund acquisition. All authors reviewed the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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