Original Article
The effect of tail suspension and treadmill exercise on LRP6 expression, bone mass and biomechanical properties of hindlimb bones in SD rats

Haobo Jia[1,2*], Aixian Tian[3*], Xiaoyu Zhang[4*], Xinlong Ma[1], Jianxiong Ma[3], Jie Wang[1], Lei Sun[3], Bin Lu[3]

[1]Orthopaedics Institute, Tianjin Hospital, Tianjin University, Jiefang Nan Road 406, Hexi District, Tianjin 300211, P. R. China; [2]Tianjin Medical University, Qixiangtai Road 22, Heping District, Tianjin 300070, P. R. China; [3]Orthopedic Research Institute, Tianjin Hospital Heping Branch, Tianjin University, Munan Street 122, Heping District, Tianjin 300050, P. R. China; [4]Orthopaedics Institute, First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, Chang Ling Road 88, Xiqing District, Tianjin 300381, P. R. China. *Equal contributors.

Abstract: *Purpose:* To investigate whether mechanical load regulates LRP6 expression and whether different intensities of treadmill exercise have different effects on LRP6 expression and the biomechanical properties of hindlimb bones in Sprague-Dawley (SD) rats. *Methods:* Fifty-six three-month-old virgin female SD rats were randomly divided into seven groups (n=8). Each group was subjected to tail suspension, free physiological activity or different intensities of treadmill exercise according to the experimental design for four or eight weeks. Rats were sacrificed after the intervention based on experimental design, and fresh femurs, tibias and fibulas were harvested for molecular biological analysis, biomechanical testing and micro-CT analysis. *Results:* LRP6 expression and the Wnt/β-catenin pathway activity decreased, and bone mass and biomechanical properties decreased after loss of mechanical stimulation. For disuse osteoporosis, even physiological activity could improve LRP6 expression, Wnt/β-catenin pathway activity, bone mass and biomechanical properties. Compared with physiological activity, treadmill exercise had better and faster effects on bone recovery. Compared with the Low intensity Exercise Group (LE group), the Medium intensity Exercise Group (ME group) and High intensity Exercise Group (HE group) had higher LRP6 expression, bone mass and biomechanical properties, while there were no significant difference between the ME group and HE group. *Conclusions:* Mechanical load appears to be a regulator of LRP6 expression, and it further affects the Wnt/β-catenin pathway activity and bone mass. The LRP6 expression, bone mass and biomechanical properties gradually improve as treadmill exercise intensity increases, while there is no significant difference between the ME group and HE group.

Keywords: Mechanical load, disuse osteoporosis, LRP6, Wnt/β-catenin pathway, treadmill exercise, biomechanical property

Introduction

Disuse osteoporosis is a secondary osteoporosis due to loss of mechanical load, such as long-term weightlessness in space flight, long-term bed rest due to fracture or motor paralysis caused by spinal cord injury, which leads to local or whole body bone loss [1-3]. Under conditions of reduced stress stimulation, osteoclast resorption is enhanced, and osteoblast-mediated bone formation is inhibited; thus, bone remodeling activity is unbalanced, eventually leading to bone loss and fragility fractures [4, 5].

The Wnt/β-catenin signaling pathway plays an important role in bone metabolism, as it is involved in osteoblast differentiation, proliferation, maturation and functional expression [6]. Furthermore, the Wnt/β-catenin pathway has been determined to play a role in osteocyte sensing and transducing mechanical stimuli to other bone cells [7]. LRP6, LRP5 and Frizzled protein together form a ternary complex that acts as a receptor for the Wnt/β-catenin pathway. To investigate the role of LRP5 in bone responses to loading, Leanne Saxon et al. analyzed changes in tibias subjected to loading or disuse in male mice with the LRP5 loss-of-

function mutation (LRP5^{-/-}) with a heterozygous LRP5 G171V High Bone Mass (HBM) mutation (LRP5[HBM+]) [8]. The male WT([+/+]) controls showed significant strain-response curves for cortical area and trabecular thickness, but the LRP5([-/-]) mice showed no detectable strain-response in those same outcomes, and the LRP5([-/-]) mice had greater loss in cancellous bone than the WT([+/+]) controls. In contrast, the tibias of male (LRP5[HBM+]) mice showed greater osteogenic responsiveness to loading and less bone loss associated with disuse than their WT controls. These results indicated that LRP5 plays an important role in bone responses to mechanical load. While LRP6 is also the component of the Wnt pathway coreceptor, its role in the regulation of the Wnt pathway by mechanical stimulation still remains unclear.

There are many ways to induce disuse osteoporosis animal models, such as local plaster fixation, spinal cord semi-transection, sciatic nerve cutting and tail suspension. The tail suspension method has obvious effects and causes no harm to animals, which makes it an ideal method for long-term research. Lecoq et al. compared the effect of bone loss in female rats induced by hindlimb unloading and ovariectomy, and they found that hindlimb unloading induced a greater degree of bone loss [9]. YC Lau confirmed that hindlimb unloading for 14 days may result in significant bone loss in the rat tibia [10].

Knowledge of microgravity- or disuse-induced bone loss and the development of anti-catabolic strategies that inhibit bone loss are important [11]. Physical activity and mechanical load can increase bone formation and inhibit bone resorption through complex mechanisms involving mechanotransduction, without the side effects of drugs [12, 13]. Many studies have explored the impact of running on bone, but the conclusion is still inconsistent. Many studies suggest that moderate-intensity treadmill exercise can increase bone mass [12, 14]. Bourrin et al. found that high-intensity treadmill exercise is not conducive to bone growth and development. These authors found that strenuous training in young rats reduces longitudinal bone growth and induces bone loss [15, 16]. Other treadmill exercise studies have yielded other conflicting or inconclusive results [17-20]. In the present study, we used the tail suspension method to induce hindlimb disuse osteo-

porosis models. Disuse osteoporosis rats were trained in different intensities of treadmill exercise or just free physiological activity, and we observed the effect of mechanical stimuli on LRP6 expression and bone mass recovery. This study preliminarily revealed the role of LRP6 in mechanical loading in promoting osteogenesis.

Materials and methods

Animals and treatments

Healthy three-month-old female Sprague Dawley rats were purchased from the Animal Center of the Chinese Academy of Science and were randomly divided into seven groups (8 rats/group). The animals were housed under a 12-h light/dark cycle and given ad libitum access to food and water. The experimental design is shown in **Figure 1A**. The first group of rats was allowed to move freely in their cage, and they were sacrificed four weeks after the start of the experiment as the Control Four-week Group (C4W group). The rats in the second group were tail-suspended for 4 weeks and then sacrificed as the Suspension Four-week Group (S4W group). The rats in the third group were allowed to move freely in their cage for 8 weeks, as the Normal activity Four week + Normal activity Four week Group (NN group). The rats in the fourth group were tail-suspended for 4 weeks and then allowed to move freely in their cages for 4 weeks, as the Suspension Four-week + Normal activity Four week Group (SN group). The rats in the remaining three groups were all tail-suspended for 4 weeks and then trained with relatively low, medium and high intensity treadmill exercise, as the Low intensity Exercise Group (LE group), Medium intensity Exercise Group (ME group) and High intensity Exercise Group (HE group), respectively. The treadmill exercise was performed with a motor-driven leveled treadmill that was equipped with electrical stimulation to provide a stimulation voltage 0.05~4 mA (Treadmill Exerciser ZH-PT, Zhenghua Biologic Apparatus Facilities, Huaibei, Anhui, China). The velocity was 15 m/s, 25 m/s and 35 m/s for the LE group, ME group and HE group, respectively [17-20]. For all groups, the declination was 15°. Training was divided into two 30-min sessions per day, 5 days/week for 4 weeks. The rats were sacrificed after the intervention, and fresh femurs, tibias and fibulas were harvested for molecular
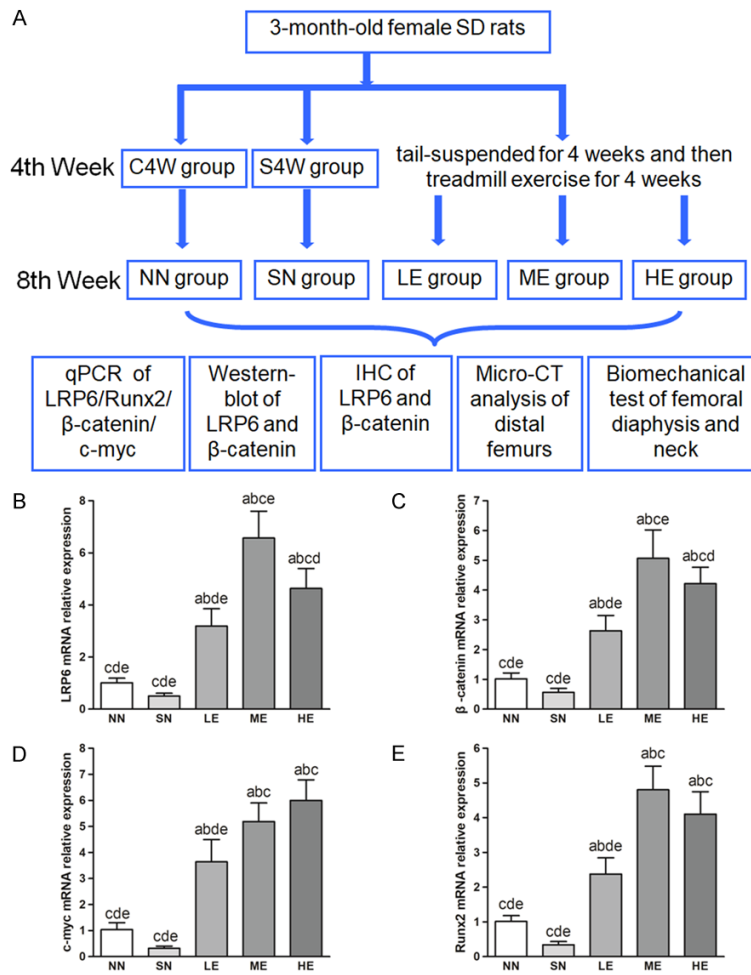
Figure 1. The experimental design (A) and LRP6 (B), β-catenin (C), c-myc (D) and Runx2 (E) mRNA expression in each group relative to the NN group calculated using the $2^{-\Delta\Delta Ct}$ method. a: P<0.05 compared with the NN group. b: P<0.05 compared with the SN group. c: P<0.05 compared with the LE group. d: P<0.05 compared with the ME group. e: P<0.05 compared with the HE group.

biological analysis, biomechanical tests and micro-CT analysis. The rats in the C4W group and the S4W group were only subjected to the biomechanical test and micro-CT analysis to confirm the effect of tail suspension, and their results were used as numerical references for the other groups. All treatment procedures were approved by the Ethics Committee of Tianjin hospital.

Quantitative real-time PCR

After the marrow was flushed with DEPC-treated water, the left tibias and fibulas were immediately immersed in RNA storage reagent and frozen with liquid nitrogen until use in mRNA analysis. Total RNA was extracted from tibias and fibulas using TriPure Isolation Rea-

gent (Roche Diagnostics, Indianapolis, IN, USA), and then RNA (2 μg) was reverse-transcribed using a First-Strand cDNA Synthesis kit (Gene-Copoeia, Guangzhou, China). The transcripts of interest and GAPDH were amplified from first-strand cDNA by real-time PCR using a qPCR mix and primers (LRP6, Product ID: RQP067324; β-catenin: Sequence (5'-3'), Forward-GA-GTGCTGAAGGTGCTGTCT, Reverse-ATCCCTTCCTGCTTAGTC-GC; Runx2, Product ID: RQP-084949; c-myc, Product ID: RQP048938; GAPDH, Product ID: RQP049537; Gene-Copoeia, Guangzhou, China). Amplification and detection were performed with the iQ5 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) as follows: denaturation at 95°C for 10 min, and 40 cycles of amplification including denaturation at 95°C for 10 s, annealing at 60°C for 20 s and extension at 72°C for 15 s. The results were analyzed by the comparative Ct method of quantification ($2^{-\Delta\Delta Ct}$ method) [22].

Western-blot analysis

After the marrow was flushed with normal saline, right tibias and fibulas were crushed using a mortar and pestle in liquid nitrogen. For total protein extraction (LRP6), the lysates were collected in ice-cold RIPA buffer (containing 1 μM pepstatin A, 1 mM Iodo and 0.4 mM phenylmethylsulfonyl fluoride). For nuclear protein extraction (β-catenin), nuclear proteins were isolated from the lysates using a Nuclear Extract Kit (Active Motif, Carlsbad, CA). Total protein was quantified by a BCA assay, separated on a 12% sodium dodecyl sulfate gel and transferred to an Immobilon-PVDF membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% nonfat dry milk in PBST for 2 h at room temperature and then incubated with goat polyclonal anti-rat LRP6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or β-catenin

antibody (Abcam, Cambridge, United Kingdom) (1:200 in 5% nonfat milk) for 2 h at room temperature. After rinsing, the membranes were incubated with rabbit anti-goat-horseradish peroxidase secondary antibody (1:10000 in 5% milk) for 1 h and were then developed with an enhanced chemiluminescence reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Immunohistochemical staining

The right femurs were fixed in neutral buffered formalin, decalcified with 10% EDTA and embedded in paraffin. The sections were deparaffinized, and endogenous peroxidase activity was inhibited by 3% H_2O_2 treatment for 10 min. After pretreatment with 5% BAS at 37°C for 30 min, the slides were incubated with 1:100 diluted goat polyclonal anti-rat LRP6 antibody or 1:200 diluted goat polyclonal anti-rat β-catenin antibody at 4°C overnight. After washing, the sections were incubated at 37°C for 30 min with biotin-coupled secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and streptavidin-biotin complex diluted to 1:150. The color reaction was developed with a 3, 3'-diaminobenzidine-tetrachloride kit (DAB kit, Boster, Wuhan, China). The sections were washed and counterstained with hematoxylin and observed with a Nikon Ni-E Digital Image Analyzer.

Micro-CT analysis

The left femurs were harvested and frozen in normal saline for micro-CT analysis and biomechanical tests. Micro-CT scanning was performed using a SIEMENS Inveon PET.SPECT.CT (SIEMENS, Berlin, Germany) with energy settings of 500 mA and 80 kV. The region of interest (ROI) was defined as the region 100-250 slices away from the distal femoral growth plate. The thickness of each slice was 17 μm. The bone volume to tissue volume (BV/TV), bone surface to bone volume (BS/BV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and trabecular number (Tb.N) in the ROI were calculated with the associated software with 3D images reconstructed for visualization.

Biomechanical test

After micro-CT analysis, the femurs (femoral diaphysis and neck) were subjected to biomechanical tests with the Electro-Force 3230 Material Testing System (BOSE, Minnetonka,

MN, USA). The femur shaft was fixed between two supporting points with a distance of 20 mm. A load was vertically applied to the femoral midshaft in the middle of the supporting points with a displacement speed of 0.01 mm/s at 100 Hz until the diaphysis fractured. Then, the femurs were cut at the midshaft level for the femoral neck biomechanical test. The femoral diaphysis region was fixed using polymethylmethacrylate and was maintained in a vertical posture. Then, the femoral head was loaded with a vertical compression that was parallel to the femur long axis with a speed of 0.01 mm/s until the femoral neck fractured. The load-deformation curves were recorded automatically to calculate the maximum load, stiffness, and energy absorption.

Statistical analysis

All data are presented as the mean ± SD. Significant differences were determined by one-way ANOVA followed by Dunnett's test for post hoc comparisons with GraphPad PrismV5.01. $P<0.05$ was considered significant.

Results

Effects of tail suspension and treadmill exercise on LRP6, β-catenin, c-myc and Runx2 mRNA expression

As shown in **Figure 1B**, the rats in the SN group, which were tail-suspended for 4 weeks and then allowed to move freely in their cages for 4 weeks, exhibited decreased LRP6 mRNA expression compared with the NN group (0.49-fold vs 1.01-fold), though the difference was not statistically significant. The rats in the LE group, ME group and HE group, which were all tail-suspended for 4 weeks and then trained with different intensities of treadmill exercise for 4 weeks, had dramatically increased LRP6 mRNA expression (LE group 3.10-fold, ME group 6.57-fold and HE group 4.63-fold) compared with the NN group ($P<0.05$). The different intensities of treadmill exercise had different effects on LRP6 mRNA expression, and the differences among the three groups were significant ($P<0.05$).

As shown in **Figure 1C**, for the rats in the SN group, the β-catenin mRNA expression decreased compared with the NN group (0.56-fold vs 1.0-fold), though the difference was not statistically significant ($P>0.05$). For the rats in the LE group, ME group and HE group, β-catenin expression dramatically increased (LE group
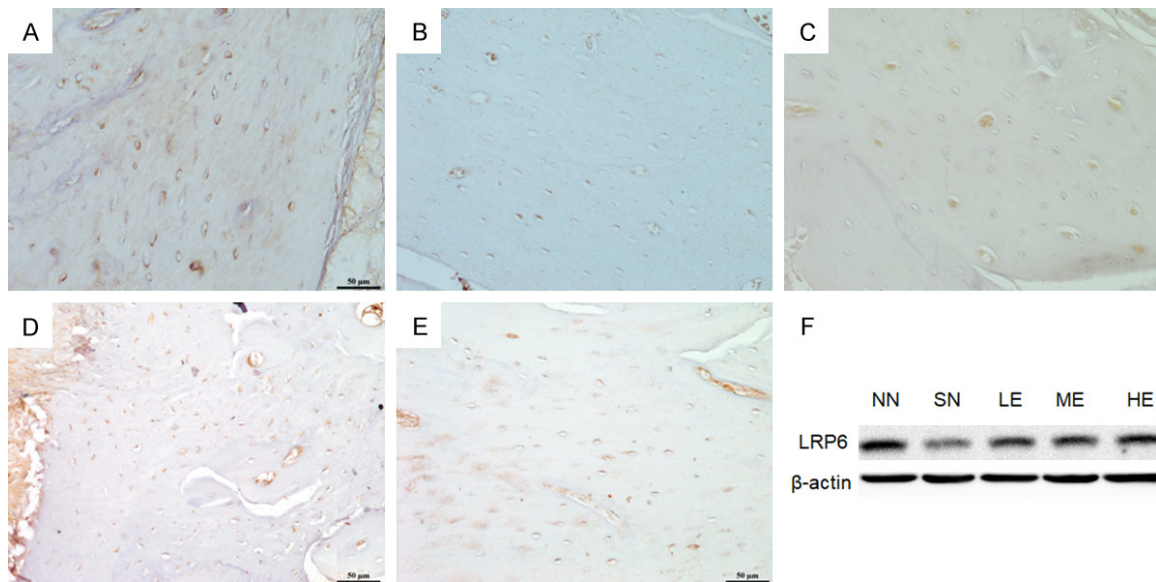
Figure 2. LRP6-positive osteocyte distribution according to immunohistochemical staining in each group (A-E) and Western blot analysis of LRP6 protein (F). (A) The NN group, (B) The SN group, (C) The LE group, (D) The ME group, (E) The HE group. Original magnification ×200.

2.63-fold, ME group 5.06-fold and HE group 4.21-fold) compared with the NN group (*P*< 0.05). The results showed that different intensities of treadmill exercise had different effects on β-catenin mRNA expression, and the differences among the three groups were statistically significant (*P*<0.05).

As shown in **Figure 1D** and **1E**, for the rats in the SN group, c-myc and Runx2 mRNA expression decreased compared to those in the NN group (0.32-fold vs 1.0-fold, 0.34-fold vs 1.0-fold, respectively), though the difference was not statistically significant (*P*>0.05). For the rats in the three treadmill exercise groups, the c-myc mRNA expression (LE group 3.64-fold, ME group 5.19-fold and HE group 5.99-fold) and Runx2 mRNA expression (LE group 2.38-fold, ME group 4.80-fold and HE group 4.11-fold) dramatically increased compared to those in the NN group (*P*<0.05). The c-myc and Runx2 mRNA expression in the ME group and the HE group were higher than those in the LE group (*P*<0.05), while the difference between the ME group and the HE group was not statistically significant (*P*>0.05).

Effects of tail suspension and treadmill exercise on LRP6 protein expression

As shown in **Figure 2A**, the rats in the NN group that were allowed to move freely in their cages for 8 weeks showed significant positive ex-

pression of LRP6, as detected by immunohistochemical staining. For rats in the SN group which had decreased LRP6 mRNA expression, LRP6 protein expression was also significantly reduced, as shown in **Figure 2B**. The increase in LRP6 mRNA expression caused by treadmill exercise resulted in an increase in LRP6-positive osteocytes in the three treadmill exercise groups, and LRP6-positive osteocytes in the ME group and HE group were more abundant than in the LE group (**Figure 2C-E**). Consistent with the results of qPCR and IHC, the LRP6 protein concentration in the SN group was the lowest among the five groups, and treadmill exercise improved the LRP6 protein concentration (**Figure 2F**).

Effects of tail suspension and treadmill exercise on the Wnt/β-catenin signaling pathway

LRP6 is a component of the Wnt/β-catenin pathway receptor; when LRP6 expression varies, the Wnt/β-catenin pathway may be accordingly affected. The LRP6 expression decreased due to hindlimb immobilization in the SN group, and the number of β-catenin-positive osteocytes also decreased in the SN group compared with the NN group (**Figure 3A** and **3B**). Furthermore, the percentage of β-catenin-positive osteocytes increased after treadmill exercise, and β-catenin-positive osteocytes in the ME group and HE group were more abundant
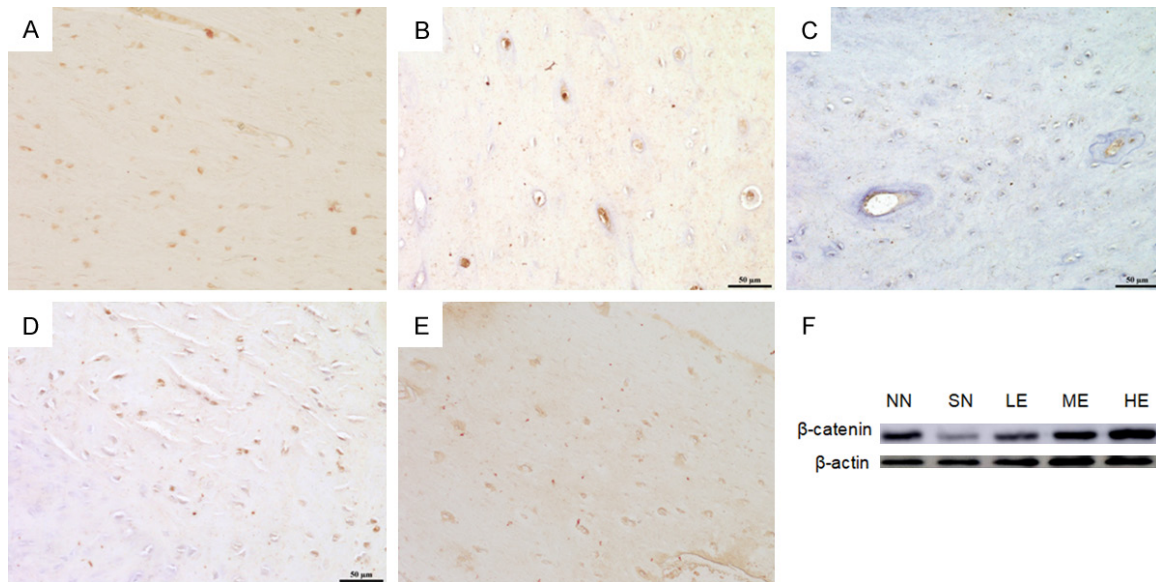
Figure 3. β-catenin-positive osteocyte distribution by immunohistochemical staining in each group (A-E) and Western blot analysis of β-catenin (F). (A) The NN group, (B) The SN group, (C) The LE group, (D) The ME group, (E) The HE group. Original magnification ×200.

than that in the LE group (**Figure 3C-E**). Consistent with the IHC and qPCR results, the nuclear β-catenin protein concentration in the SN group was the lowest among the five groups, and treadmill exercise significantly improved the nuclear β-catenin protein concentration (**Figure 3F**).

Effects of tail suspension and treadmill exercise on trabecular bone mass and structure of distal femurs

Micro-CT analysis showed that after four weeks of tail-suspension, the rats in the S4W group had lower BV/TV, Tb.Th and Tb.N levels and higher BS/BV and Tb.Sp levels than the age-matched rats in the C4W group (**Figure 4A-E**, $P<0.05$). The results confirmed the effect of the tail suspension modeling method and can be used as numerical references for other experimental groups. Due to continued growth over four weeks, the rats in the NN group had higher BV/TV and Tb.N levels and lower Tb.Sp levels than the rats in the C4W group (**Figure 4A**, **4D** and **4E**, $P<0.05$). Compared with free physiological activity (SN group), different intensities of treadmill exercise significantly improved the BV/TV, Tb.Th and Tb.N levels and decreased the BS/BV and Tb.Sp levels (**Figure 4A-E**, $P<0.05$). After treadmill exercise, the rats in the LE group still has lower BV/TV and Tb.N levels

and higher Tb.Sp levels than rats in the NN group (**Figure 4A**, **4D** and **4E**, $P<0.05$). The rats in the ME group and HE group had recovered parameters relative to the NN group (**Figure 4A-E**, $P>0.05$). The rats in the ME group and HE group had higher Tb.N and lower Tb.Sp levels than the rats in the LE group (**Figure 4D** and **4E**, $P<0.05$), and the differences between the ME group and HE group were not significant ($P>0.05$). **Figure 5** shows the representative three-dimensional reconstructed images from each group.

Effects of tail suspension and treadmill exercise on biomechanical properties of femoral diaphysis and neck

Four weeks of tail-suspension in the S4W group resulted in significant reductions in maximum load (-20.9%), stiffness (-18.4%), and energy to maximum load (-29.9%) of the femoral diaphysis and significant reductions in the maximum load (-28.9%), stiffness (-26.3%), and energy to maximum load (-37.6%) of the femoral neck compared with the C4W group (**Figure 6A-F**, $P<0.05$). Compared with the SN group, low intensity treadmill exercise increased the stiffness (+11.9%) of the femoral diaphysis, maximum load (+14.8%), stiffness (+13.4%), and energy to maximum load (+23.3%) of the femoral neck (**Figure 6B**, **6D-F**, $P<0.05$), although
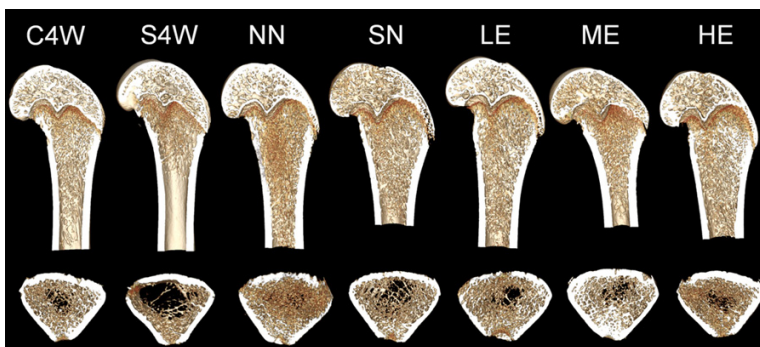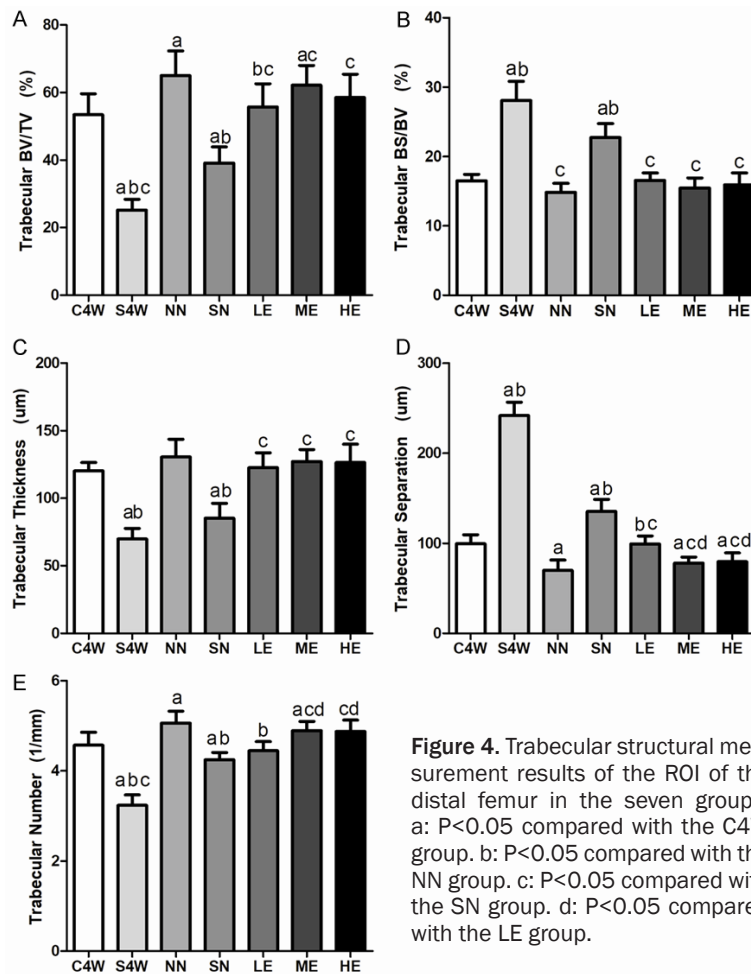
Figure 4. Trabecular structural measurement results of the ROI of the distal femur in the seven groups. a: P<0.05 compared with the C4W group. b: P<0.05 compared with the NN group. c: P<0.05 compared with the SN group. d: P<0.05 compared with the LE group.

Figure 5. Representative 3D reconstruction images of the distal femur and distal femoral growth plate.

Discussion

In the present study, the tail suspension method was used to induce hindlimb disuse osteoporosis. A free physiology activity group and three different intensity treadmill exercise groups were established to evaluate the changes in LRP6 and Wnt/β-catenin pathway expression, bone mass and biomechanical properties under different intensity mechanical stimulations. The results revealed that LRP6 expression, Wnt/β-catenin pathway activity, bone mass and biomechanical properties decreased after losing mechanical stimulation. For disuse osteoporosis, even physiological activity could improve LRP6 expression and bone mass. Compared with physiological activity, treadmill exercise had better and faster effects on bone recovery. Compared with the LE group, the ME group and HE group had higher LRP6 expression, bone mass and biomechanical properties.

The traditional view that bone acts as a passive organ in response to hormones and diet has changed over the past half-century, and it is now considered a dynamic adaptive organ that actively responds to mechanical needs [23, 24]. Mechanical load causes increased strain-related stimulation, induces elevations in early markers of bone formation, and reduces osteoblastic-expressed regulators of osteoclasts [25, 26]. The low-density lipoprotein receptor-related protein (LRP) was identified by Herz in 1988 and contains the same repeat sequence as the LDL receptor [27]. LRP6, LRP5 and Frizzled protein together form a ternary complex that is a coreceptor for the canonical Wnt pathway. Once the LRP6/LRP5/Frizzled
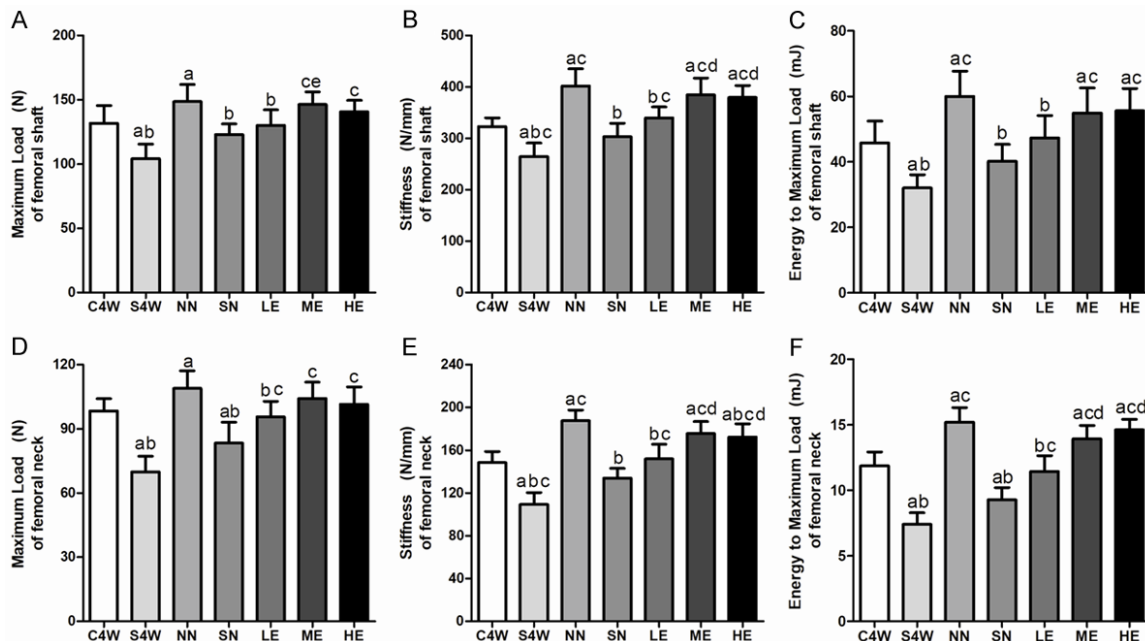
the values did not reach the level of those in the NN group (P>0.05). Among the three treadmill exercise groups, the ME group and HE group had higher biomechanical properties than the LE group in some parameters (**Figure 6B**, **6E** and **6F**, P<0.05), and the differences between the ME group and the HE group were not significant (P>0.05).

Figure 6. Biomechanical parameters of the femoral diaphysis and neck. (A-C) Maximum load (A), Stiffness (B) and Energy to maximum load (C) of the femoral shaft; (D-F) Maximum load (D), Stiffness (E) and Energy to maximum load (F) of the femoral neck. a: P<0.05 compared with the C4W group. b: P<0.05 compared with the NN group. c: P<0.05 compared with the SN group. d: P<0.05 compared with the LE group. e: P<0.05 compared with the MM group.

coreceptor is activated by mechanical load, it then activates a downstream cascade reaction to allow β-catenin to enter the nucleus and activate downstream target genes. There have been many studies on LRP5, and it has been proven to play an important role in bone metabolism and mechanotransduction. Though LRP6 is similar in sequence and structure to LRP5, there are also some differences between them. However, there have been few studies on LRP6, and the specific role and effect of LRP6 in mechanical regulation of bone metabolism remain unclear. Takuo Kubota et al. studied bone phenotypes in mice harboring the LRP6 hypomorphic mutation ringelschwanz (rs). LRP6[rs/rs] mice exhibited reduced trabecular BMD, low bone volume, decreased trabecular number, and increased urinary deoxypyridinoline excretion. RANKL expression was increased in LRP6[rs/rs] osteoblasts both in vivo and in vitro, and osteoclastogenesis and bone-resorbing activity in vitro were accelerated in LRP6[rs/rs] cells. These results indicated that LRP6-mediated signaling controls postnatal bone mass [28]. Changjun Li deleted LRP6 in mature osteoblasts in mice (LRP6 KO) and found that compared with wild-type littermates, LRP6 KO mice had a significant reduction in bone mass and osteoblasts, with higher percentage of apoptotic osteo-

blasts at the bone surface, indicating that LRP6 in osteoblasts is essential for osteoblastic differentiation during bone remodeling [29]. Travis Burgers investigated the process of mid-diaphyseal femur fracture healing in LRP6^{+/-} mice using micro-CT scans, biomechanical testing, and histological analysis. LRP6^{+/-} mice had significantly decreased stiffness and strength 28 days post-fracture and significantly decreased BV/TV and immature bone density [30]. In the present study, with tail suspension to induce decreased mechanical load on the hindlimbs, LRP6 expression was decreased, as was the Wnt/β-catenin pathway activity. Treadmill exercise dramatically increased LRP6 mRNA and protein expression and also increased activity of the Wnt/β-catenin pathway. As a preliminary animal experiment, our results suggest a role of LRP6 in mechanotransduction and the regulatory effect of mechanical load on LRP6. Further research is needed to identify the role of LRP6 in mechanotransduction and to explore the specific mechanisms involved.

Wang et al. analyzed osteocyte strain amplification under static and cyclic loading with a finite element study, and they found that the strain amplification factor of the osteocyte-lacunar-canalicular system increased with incre-

ased loading frequency and loading strain [31]. Following other scholars' treadmill exercise design, we used the following parameters in the present study: velocity of 15 m/s, 25 m/s and 35 m/s for the LE group, ME group and HE group, respectively [12, 15, 21], which have different mechanical loading frequencies. It is generally believed that the mechanical stimulus has a threshold for bone; within the threshold, exercise may increase bone mass and biomechanical properties. Beyond this threshold, exercise even does harm to bone. Many studies suggest that moderate-intensity treadmill exercise can increase bone mass [12, 14]. Bourrin et al. found that high-intensity treadmill exercise it is not conducive to bone growth and development. In Bourrin's research, 5-week-old male Wistar rats were trained on a treadmill for 5 days per week for 11 weeks with 105 min/day at a rate of 30 m/min with a 10° incline. These results showed that compared with controls, the bone volume and trabecular thickness for primary and secondary spongiosa of the tibia in trained rats significantly decreased [15, 16]. In Starnes's research, seven-month-old female SD rats were trained on a treadmill 5 days/week for 5 weeks at a rate of 30 m/min for 60 min at a 6° grade, and femurs were harvested for BMD and biomechanical testing. These results showed that there was no difference between the trained rats and the controls [20]. Our results showed that compared with the LE group, the ME group and HE group had higher LRP6 and Wnt/β-catenin pathway expression and greater bone mass and biomechanical properties. However, the difference between the ME group and HE group was not statistically significant. Other treadmill exercise studies have yielded other conflicting or inconclusive results; these inconsistencies may be due to the interactions between bone remodeling and exercise depending upon the exercise mode, intensity, duration, animal species and skeletal age [17-19].

Our study has some limitations. The present study preliminarily explored the effect of hindlimb immobilization and different intensities of treadmill exercise on LRP6 and Wnt/β-catenin pathway expression. Only one time point was chosen in the present study; a dynamic analysis of LRP6 expression caused by hindlimb immobilization and treadmill exercise will help us to better understand the regulatory effects of mechanical load on LRP6 expression. In addi-

tion to biomechanical testing and micro-CT analysis, dynamic histomorphometry should be conducted in our future studies to fully understand the action of hindlimb immobilization and treadmill exercise. Finally, the sample size in each group was not very large, thus the p value between different groups may become larger in statistical analysis. A larger sample size would be more effective at verifying the conclusions.

In conclusion, mechanical load appears to be a regulator of LRP6 expression, and it also affects Wnt/β-catenin pathway activity and bone mass. LRP6 expression, bone mass and biomechanical properties gradually improve as treadmill exercise intensity increases, while there is no significant difference between the ME group and HE group.

Acknowledgements

Disclosure of conflict of interest

None.

Address correspondence to: Xinlong Ma, Orthopaedics Institute, Tianjin Hospital, Tianjin University, Jiefang Nan Road 406, Hexi District, Tianjin 300211, P. R. China. Tel: 0086 15002276378; Fax: 0086 15002276378; E-mail: maxinlong2007@163.com; Jianxiong Ma, Orthopedic Research Institute, Tianjin Hospital Heping Branch, Tianjin University, Munan Street 122, Heping District, Tianjin 300050, P. R. China. Tel: 0086 15002276378; Fax: 0086 1500-2276378; E-mail: majianxiong0007@163.com

References

[1] Wang H, Wan Y, Tam KF, Ling S, Bai Y, Deng Y, Liu Y, Zhang H, Cheung WH, Qin L, Cheng JC, Leung KS and Li Y. Resistive vibration exercise retards bone loss in weight-bearing skeletons during 60 days bed rest. Osteoporos Int 2012; 23: 2169-2178.

[2] Garland DE, Adkins RH, Stewart CA, Ashford R and Vigil D. Regional osteoporosis in women who have a complete spinal cord injury. J Bone Joint Surg Am 2001; 83: 1195-1200.

[3] Cappellesso R, Nicole L, Guido A and Pizzol D. Spaceflight osteoporosis: current state and fu-

ture perspective. Endocr Regul 2015; 49: 231-239.

[4] Xiao W, Wang Y, Pacios S, Li S and Graves DT. Cellular and molecular aspects of bone remodeling. Front Oral Biol 2016; 18: 9-16.

[5] Yuan Y, Chen X, Zhang L, Wu J, Guo J, Zou D, Chen B, Sun Z, Shen C and Zou J. The roles of exercise in bone remodeling and in prevention and treatment of osteoporosis. Prog Biophys Mol Biol 2016; 122: 122-130.

[6] Kobayashi Y, Uehara S, Udagawa N and Takahashi N. Regulation of bone metabolism by Wnt signals. J Biochem 2016; 159: 387-392.

[7] Duan P and Bonewald LF. The role of the wnt/beta-catenin signaling pathway in formation and maintenance of bone and teeth. Int J Biochem Cell Biol 2016; 77: 23-29.

[8] Saxon LK, Jackson BF, Sugiyama T, Lanyon LE and Price JS. Analysis of multiple bone responses to graded strains above functional levels, and to disuse, in mice in vivo show that the human Lrp5 G171V high bone mass mutation increases the osteogenic response to loading but that lack of Lrp5 activity reduces it. Bone 2011; 49: 184-193.

[9] Lecoq B, Potrel-Burgot C, Granier P, Sabatier JP and Marcelli C. Comparison of bone loss induced in female rats by hindlimb unloading, ovariectomy, or both. Joint Bone Spine 2006; 73: 189-195.

[10] Lau YC, Qian X, Po KT, Li LM and Guo X. Electrical stimulation at the dorsal root ganglion preserves trabecular bone mass and microarchitecture of the tibia in hindlimb-unloaded rats. Osteoporos Int 2015; 26: 481-488.

[11] Xin M, Yang Y, Zhang D, Wang J, Chen S and Zhou D. Attenuation of hind-limb suspension-induced bone loss by curcumin is associated with reduced oxidative stress and increased vitamin D receptor expression. Osteoporos Int 2015; 26: 2665-2676.

[12] Murai IH, Roschel H, Pabis LV, Takayama L, de Oliveira RB, Dos Santos Pereira RT, Dantas WS, Pereira RM, Jorgetti V, Ballester RY, Gualano B. Exercise training, creatine supplementation, and bone health in ovariectomized rats. Osteoporos Int 2015; 26: 1395-1404.

[13] Compston J. Pathophysiology of atypical femoral fractures and osteonecrosis of the jaw. Osteoporos Int 2011; 22: 2951-2961.

[14] Fang J, Gao J, Gong H, Zhang T, Zhang R and Zhan B. Multiscale experimental study on the effects of different weight-bearing levels during moderate treadmill exercise on bone quality in growing female rats. Biomed Eng Online 2019; 18: 33.

[15] Bourrin S, Genty C, Palle S, Gharib C and Alexandre C. Adverse effects of strenuous exercise:

[16] Bourrin S, Ghaemmaghami F, Vico L, Chappard D, Gharib C and Alexandre C. Effect of a five-week swimming program on rat bone: a histomorphometric study. Calcif Tissue Int 1992; 51: 137-142.

[17] Gao J, Fang J, Gong H and Gao B. Morphological and microstructural alterations of the articular cartilage and bones during treadmill exercises with different additional weight-bearing levels. J Healthc Eng 2017; 2017: 8696921.

[18] Wheeler DL, Graves JE, Miller GJ, Vander GR, Wronski TJ, Powers SK and Park HM. Effects of running on the torsional strength, morphometry, and bone mass of the rat skeleton. Med Sci Sports Exerc 1995; 27: 520-529.

[19] Rico H, Crespo E, Hernandez ER, Seco C and Crespo R. Influence of boron supplementation on vertebral and femoral bone mass in rats on strenuous treadmill exercise. A morphometric, densitometric, and histomorphometric study. J Clin Densitom 2002; 5: 187-192.

[20] Starnes JW, Neidre DB, Nyman JS, Roy A, Nelson MJ, Gutierrez G and Wang X. Synergistic effect of exercise and statins on femoral strength in rats. Exp Gerontol 2013; 48: 751-755.

[21] Xu SY, He YB, Deng SY, Liu SY, Xu L and Ni GX. Intensity-dependent effect of treadmill running on rat Achilles tendon. Exp Ther Med 2018; 15: 5377-5383.

[22] Jia HB, Ma JX, Ma XL, Yu JT, Feng R, Xu LY, Wang J, Xing D, Zhu SW and Wang Y. Estrogen alone or in combination with parathyroid hormone can decrease vertebral MEF2 and sclerostin expression and increase vertebral bone mass in ovariectomized rats. Osteoporos Int 2014; 25: 2743-2754.

[23] Grabowski P. Physiology of bone. Endocr Dev 2015; 28: 33-55.

[24] Wang Y, Jia L, Zheng Y and Li W. Bone remodeling induced by mechanical forces is regulated by miRNAs. Biosci Rep 2018; 38.

[25] Saunders MM, Taylor AF, Du C, Zhou Z, Pellegrini VJ and Donahue HJ. Mechanical stimulation effects on functional end effectors in osteoblastic MG-63 cells. J Biomech 2006; 39: 1419-1427.

[26] Skerry TM. The response of bone to mechanical loading and disuse: fundamental principles and influences on osteoblast/osteocyte homeostasis. Arch Biochem Biophys 2008; 473: 117-123.

[27] Herz J, Hamann U, Rogne S, Myklebost O, Gausepohl H and Stanley KK. Surface location and high affinity for calcium of a 500-kd liver

membrane protein closely related to the LDL-receptor suggest a physiological role as lipoprotein receptor. EMBO J 1988; 7: 4119-4127.

[28] Kubota T, Michigami T, Sakaguchi N, Kokubu C, Suzuki A, Namba N, Sakai N, Nakajima S, Imai K and Ozono K. Lrp6 hypomorphic mutation affects bone mass through bone resorption in mice and impairs interaction with Mesd. J Bone Miner Res 2008; 23: 1661-1671.

[29] Li C, Xing Q, Yu B, Xie H, Wang W, Shi C, Crane JL, Cao X and Wan M. Disruption of LRP6 in osteoblasts blunts the bone anabolic activity of PTH. J Bone Miner Res 2013; 28: 2094-2108.

[30] Burgers TA, Vivanco JF, Zahatnansky J, Moren AJ, Mason JJ and Williams BO. Mice with a heterozygous Lrp6 deletion have impaired fracture healing. Bone Res 2016; 4: 16025.

[31] Wang L, Dong J and Xian CJ. Strain amplification analysis of an osteocyte under static and cyclic loading: a finite element study. Biomed Res Int 2015; 2015: 376474.