

The role of body fluid shifts on hindlimb bone loss in tail suspended rats using a novel body fluid alteration device

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ABSTRACT

In space, the mechanical loading of the weight-bearing bone decreases and the body fluid shifts cephalad. However, the correlative researches are rarely reported on how the body fluid shift affects the bone, especially how it affects the weight-bearing bone in space or in bed rest on the ground. In our study, a novel model was designed to investigate the effect of body fluid shifts on the bone loss of the hindlimbs in the tail-suspended rats. The model can keep the rat hindlimb unloaded, and change the distribution of the body fluid by altering rats' body position. The study was conducted for 21 days. Thirty-six female Sprague-Dawley rats were randomly divided into four groups ($n = 9$, each): control group (CON), head-down tail-suspension group (HDT), head-horizontal tail-suspension group (HHT), head-up tail-suspension group (HUT). The results indicated that body fluid shifting towards the tail end may inhibit the bone loss of the rat hindlimbs, that means the absence of cephalad body fluid shifting resulted in the positive effect on the bone microstructure parameters and the mechanical properties. The results suggested that the backflow of the body fluid in rat hindlimbs was able to alleviate the disuse osteoporosis in a certain extent.

1. Introduction

Serious bone loss which occurs in astronauts can lead to health problems and increase fracture risk. As we all known, the reduction of mechanical load on weight bearing bone in space is one of the major causes of bone loss [1–5]. Although the efficacy of some physical exercises against osteoporosis were good on earth, it was not ideal when they were adopted in space. It is pity the mechanisms are still unknown.

In space, the body fluid shifts from lower body to cephalad. Some studies have shown such body fluid shifting could increase the bone mass of skull [6,7]. Bergula's research indicated that the bone mass of the hindlimb unloading rats increased by femoral vein ligation [8]. Hargens et al. inhibited the fluid shift cephalad by lower body negative pressure (LBNP) and the bone loss of lower body was ameliorated [9]. These studies indicated body fluid/blood alternation should play an important role in the mechanism of microgravity-induced bone loss

though the mechanism was unclear.

Colleran's study had demonstrated that the amount of femoral and tibial blood perfusion reduced in tail-suspended rats [10]. Prisby had observed that the femoral artery of hindlimb blood flow decreased during suspension [11]. What's more, Dillaman and Li found that interstitial fluid flow (IFF) decreased in tibia of the tail-suspended rats [12,13], while increasing the IFF can inhibit bone loss in tail-suspended rats [14]. Either blood flow/blood perfusion or interstitial fluid flow of bone has a close relationship with body fluid. Therefore, investigating the impact and mechanism of body fluid alterations on bone metabolism under microgravity will help us understand the microgravity-induced bone loss and improve the countermeasures.

The rat tail-suspended model has been widely accepted to simulate microgravity and induce osteoporosis of hindlimbs [15], in which the rat lower body are elevated to produce a 30° head-down tilt body position, leading to both hindlimbs unloading and body fluid shifts

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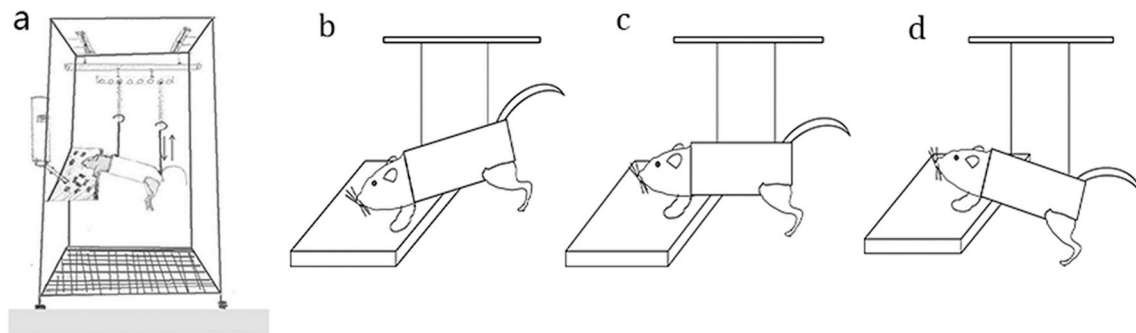


Fig. 1. The sketch picture of the device. (a) The rats can move on the sheaf while free access to food and water, and the height of the hindlimb can be adjusted by the ropes in the posterior of the sleeve. (b) Head-down tail-suspension rat (HDT). The body fluid is distributed to the head. (c) Head-horizontal tail-suspension rat (HHT). The body fluid is distributed average. (d) Head-up tail-suspension rat (HUT). The body fluid is distributed to the hindlimb.

cephalad. However, the effect of mechanical load and body fluid on bone loss could not be investigated individually in this model. To solve this problem, a new custom-made device based on the rat tail-suspended model was developed by our group to change the body fluid by altering the rat's body position meanwhile keeping hindlimb unloading. Using this new device, the effect of body fluids on bone mass of tail-suspended rat was investigated to make sure the role of body fluid in the mechanism of microgravity-induced bone loss.

2. Materials and methods

2.1. Establishment of a new model device

This new device kept the rat hindlimb unloaded while the distribution of the body fluid was changed by altering rats' body position in the cage. The device was composed of a tail box, a sheaf for providing food and water and offering activity space to rats, a sleeve for support of the rats and a sliding rail (Fig. 1). After placing the sleeve with a rat, the height of rat hindlimb was changed by adjusting the latter rope of the sleeve. Briefly, the rat was put in the sleeve and the tail was fixed to the end of the sleeve. The sleeve was hung on the sliding rail, the front of the sleeve was in the same height with the sheaf, the height of the end was adjusted by the rope in order to manipulate distribution of the body fluid.

2.2. Experimental design and animal model

8-week-old female Sprague-Dawley rats weighing 190 ± 10 g were obtained from the Experimental Animal Center of Peking University. The animal care and experimental procedures were approved by the Beihang University, China. The housing unit of the animal facility of our Department in Beihang University was maintained at $23 \pm 2^\circ\text{C}$ with a reversed 12/12 h light-dark cycle. Each rat was fed separately in

a tail box. The rats were given regular chow and water ad libitum.

Thirty-six female Sprague-Dawley rats were randomly divided into four groups ($n = 9$): control group (CON), head-down tail-suspension group (HDT), head-horizontal tail-suspension group (HHT), head-up tail-suspension group (HUT). The study was conducted in 21 days.

2.3. Dual-energy X-ray absorptiometry (DXA) analysis

Bone Mineral content (BMC) and Bone Mineral Density (aBMD) of tibia were measured on a DXA scanner (Discovery A, Hologic, USA). The region of interest was shown by Fig. 2a.

2.4. Microcomputed tomography (Micro-CT) analysis

At the end of experiment (day 21), rats were anaesthetized with 1% pentobarbital sodium (4 mL/kg), and then scanned by uCT (Skyscan 1076, Belgium) in vivo. The proximal tibia of rats was scanned as previously reported [16]. Briefly, all scans were performed at the following settings: 70 kV X-ray voltage, 140 μA current, 1 mm aluminum filter, 18 μm pixel size and a rotation step of 0.6° . All scans were reconstructed with the same parameters. The measured region started at the position of 1.898 mm to the growth plate level and extended to the diaphysis to analysis cancellous bone, covering a total length of 1.898 mm. The measured region of cortical bone started at the position of 0.949 mm to the bottom of the cancellous bone measured region and extended to the diaphysis, covering a total length of 0.949 mm. The region of interest was delineated by freehand drawing from the same investigator (Fig. 2b), then vBMD of cortical bone and cancellous bone, the trabecular microstructural parameters of proximal tibia were calculated, including: BV/TV (Percent bone volume), Tb.Th (Trabecular thickness), Tb.N (Trabecular number), Tb.Sp (Trabecular separation).

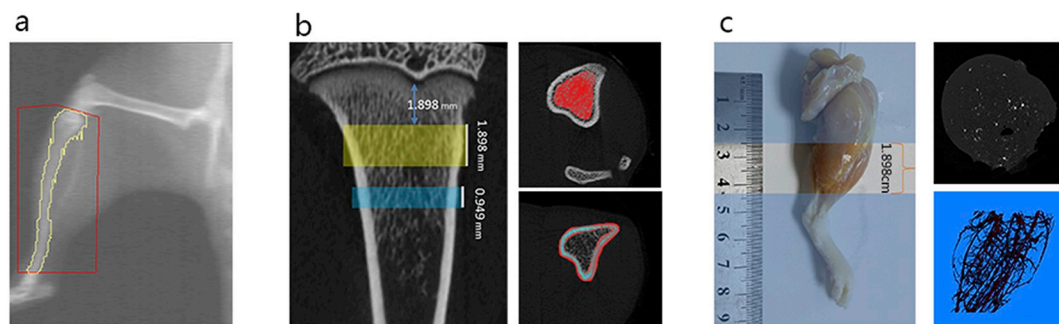


Fig. 2. The region of interest with DXA scan and uCT. (a) The region of interest with DXA scan, the yellow line enclosed area were calculated. (b) The scan region by uCT and corresponding cross sections. (c) The scan region of vessel by uCT, reconstructed cross section image of vessel and reconstructed 3D image of vessel. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
The BMC and aBMD of tibia.

	CON	HDT	HHT	HUT
BMC of Tibia	0.2561 ± 0.0218	0.2028 ± 0.0097 ^a	0.2060 ± 0.0151 ^a	0.2164 ± 0.0131 ^{a,b}
aBMD of Tibia	0.2000 ± 0.0080	0.1761 ± 0.0056 ^a	0.1797 ± 0.0068 ^a	0.1821 ± 0.0064 ^{a,b}

Values are expressed as mean ± SD. (n = 9).

^a Significant difference from the CON group with P < 0.05.

^b Significant difference from the HDT group with P < 0.05.

2.5. Biomechanical test and measurement of bone dimensions

After euthanasia, the tibiae were removed from the lower limbs and wrapped in wet gauze which was saline soaked in a centrifuge tube, and stored at −20 °C. Before measurement, the right tibiae were restored to room temperature. The length and minimum diameter of the bone are measured with a Vernier caliper and the weight is measured with a balance.

The bone strength was measured with a three-point bending test (Instron E10000, USA), 250 N mechanical sensor. Refer to the previous described methods [17,18], bones were put on two supports spaced 20 mm apart, and load was applied to the middle of the bone at a deformation rate of 2.5 mm/min until fracture occurred. The central loading point was displaced and then the force and displacement data were recorded. The parameters of structural properties included maximum load, yield load, yield displacement, stiffness, breaking load and energy to failure were calculated. After bone fracture, the external and internal diameters of the cross-section at the fractured point were measured by a Vernier caliper. The parameters of bulk material properties included Young's modulus, maximum stress and breaking stress were calculated.

2.6. Blood vessel perfusion

Vascular perfusion was performed at the 21st day. Rats were sacrificed by anesthesia overdose with 1% pentobarbital sodium (4 mL/kg), and abdominal aorta and venae cava inferior vessels were exposed after skin incision, to perform the process of vascular perfusion. Heparinized saline (100 U/mL, 12 mL/kg) was then injected into the abdominal aorta and kept for 5 min until the blood of rats turned heparinized. Abdominal aorta and venae cava inferior vessels were clamped at the upper position of the injection spot by the haemostatic forceps. Afterwards, the abdominal aorta vessel was injected with normal saline and venae cava inferior vessel was opened in order to substitute normal saline for blood in the Lower extremity circulatory system. Then, injected barium sulfate solution (30 g/100 mL, approximately diameter 0.7 µm) until barium sulfate began to flow out of the venae cava inferior vessel. After the perfusion of blood vessels, the upper position of the thigh was tied with a rope tied to reduce barium sulfate leakage, the bilateral hind limbs were dissected, fixed in 4% paraformaldehyde solution and placed in a 4 °C refrigerator overnight. The next day, placed them into 8% hydrochloric acid solution for decalcification about 30 h [19]. At last, they were scanned by uCT and reconstructed. (Fig. 2c). Calculated parameters included: Vessel volume (VV), vessel surface (VS), Vessel volume ratio (VV/TV), and vessel surface density (VS/TV).

2.7. Statistical analysis

The statistical analysis results were reported as mean ± SD. Statistical analyses were performed with SPSS16.0. One-way analysis of variance (ANOVA) was used for evaluating the existence of differences among the four groups. When comparing any two groups, the LSD test will be applied if the variance is homogeneous, otherwise Tamhane's T2 test will be used. P values < 0.05 were considered statistically significant.

3. Results

3.1. BMC and aBMD by DXA

As shown in Table 1, after 21 days' experiment, BMC and aBMD of tibia in the HDT group, HHT group and HUT group were significantly lower than the CON group (p < 0.05). BMC and aBMD of tibia of the HUT group was significantly higher than the HDT group (p < 0.05, increased by 6.7% and 3.4% respectively).

3.2. vBMD and microstructure by Micro-CT

Representative uCT images for trabecular bone microarchitecture of the four experimental groups are shown in Fig. 3a–c. The vBMD of cortical bone and vBMD of cancellous bone in the HDT, HHT and HUT groups were significantly lower than those in the CON group (p < 0.05), except vBMD of cortical bone in the HUT group, which is higher significantly than the HDT group (Fig. 3d and e). As shown in Fig. 3f–i, The trabecular BV/TV and Tb. N were significant decrease in the HDT, HHT and HUT groups than those in the CON group (p < 0.05), there was no significant difference between trabecular Tb.Th in the four groups. Moreover, the trabecular Tb.Sp was significantly increased in the HDT and HHT groups compared to the CON group (p < 0.05), except HUT group.

3.3. Biomechanical testing

The results of tibial structural properties by three-point bending test are shown in Fig. 4. In the mechanical parameters of the rat tibia, maximum load, yield load, stiffness, breaking load, maximum stress and breaking stress in the HDT group were significantly lower than those in the CON group (p < 0.05), however, maximum load, stiffness, breaking load and breaking stress were no significant difference in the HHT and HUT groups than those in the CON group. The yield load showed no significant difference in the HHT group to CON group. Young's modulus showed there were no significant difference among the four groups. Moreover, breaking load in the HHT group were significantly higher than the HDT group (p < 0.05). Energy absorption in the HDT, HHT and HUT groups were significantly lower than the CON group.

3.4. Bone dimensions of tibia

As shown in Table 2, after 21 days' experiment, The weight of tibia in the HDT group, HHT group and HUT group were significantly lower than the CON group. However, length and diaphyseal width showed no obvious difference.

3.5. 3D vascular reconstruction and analysis

Typical 3D vascular reconstruction images of the rat shank from micro-CT imaging are shown in Fig. 5a. The vessel volume (VV), vessel surface (VS), Vessel volume ratio (VV/TV), and vessel surface density (VS/TV) in the HDT group were lower than those in the CON group, and those in the HHT and HUT groups were higher than the HDT group, however, there is no significant difference in four groups (Fig. 5b–e).

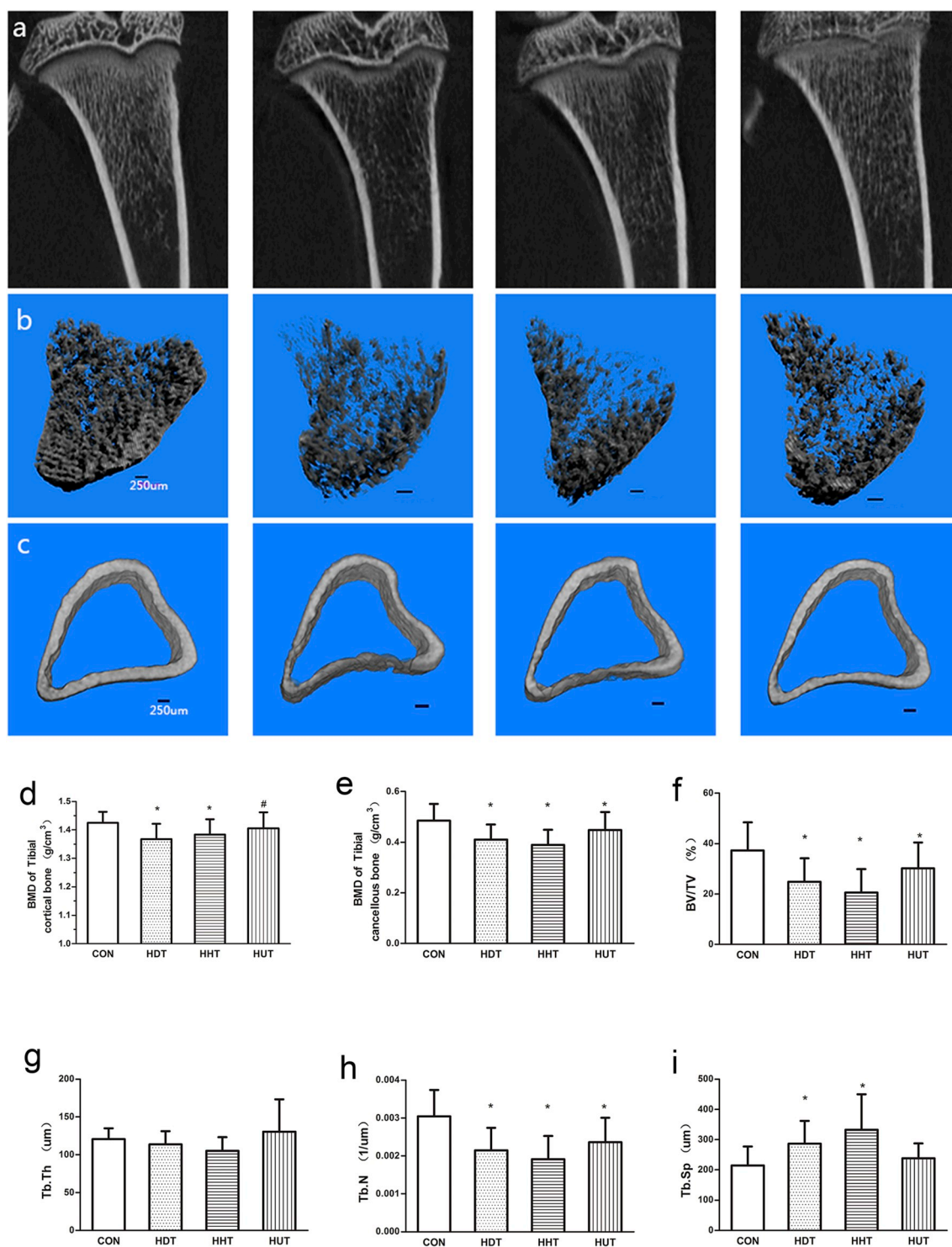


Fig. 3. Micro-CT images for tibia bone microarchitecture in the four experimental groups and comparisons for trabecular bone microarchitecture. (a) 2D uCT images of trabecular bone microarchitecture from the axial plane observation in the distal tibia. (b) 3D uCT images of trabecular bone microarchitecture. (c) 3D uCT images of cortical bone. (d) vBMD of cortical bone. (e) vBMD of cancellous bone. Statistical comparisons of indices of trabecular bone microarchitecture included (f) BV/TV, (g) Tb.Th, (h) Tb.N, (i) Tb.Sp. Values are all expressed as mean $\pm$ SD (n = 9). BMC = bone mineral content; vBMD = bone mineral density; BV/TV = bone volume per tissue volume; Tb.Th = trabecular thickness; Tb.N = trabecular number; Tb.Sp = trabecular separation. *Significant difference from the CON group with $P < 0.05$. #Significant difference from the HDT group with $P < 0.05$.

4. Discussion

In this study, we developed a novel model to keep the rat hindlimb unloading and alter rats' body position to change the distribution of the body fluid. The model allows for the examination of body fluid shifts in

different angles, making it a valuable tool to investigate the effect of body fluid shifts on musculoskeletal system and cardiovascular system in the rat.

Our data showed that the increase of body fluid had inhibitory effects on bone loss in tibia of rats to some extent. This is consistent with

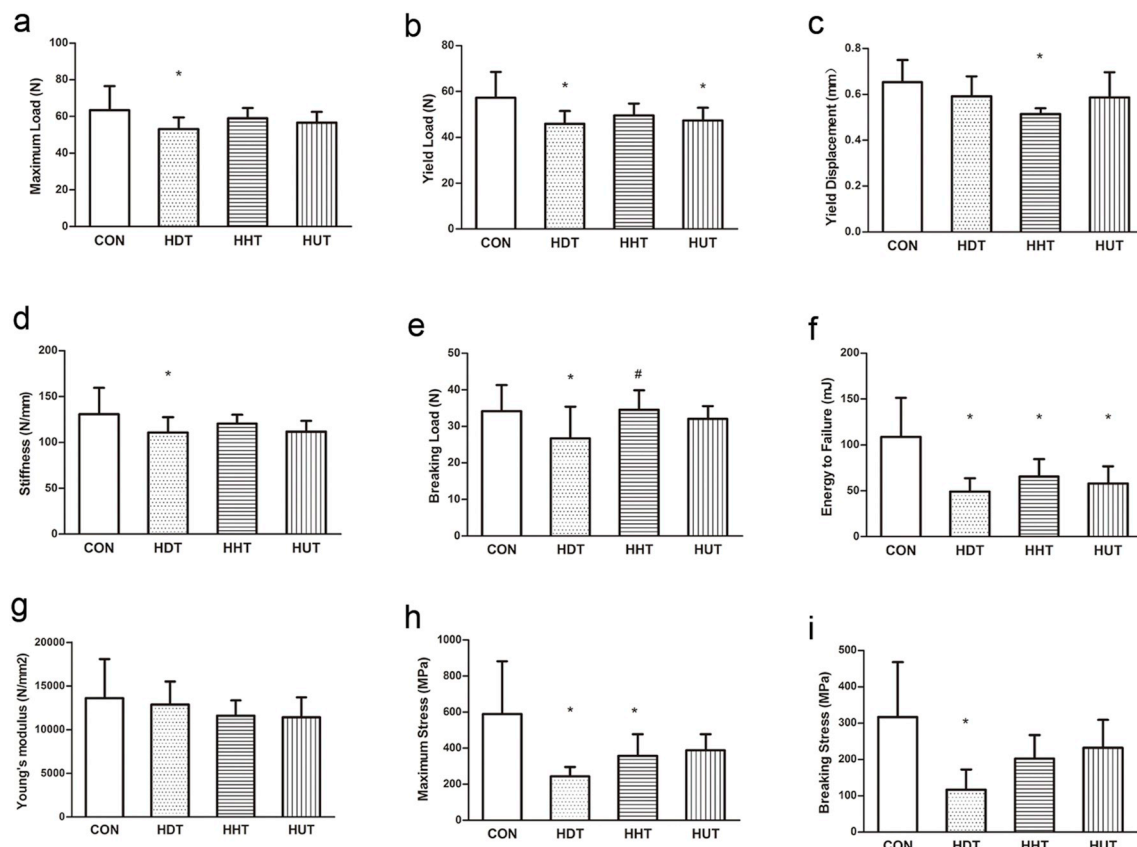


Fig. 4. Tibial biomechanical properties in rats were measured via three-point bending test, including: (a) maximum load, (b) yield load, (c) yield displacement, (d) stiffness, (e) breaking load, (f) energy absorption, (g) Young's modulus, (h) maximum stress, (i) breaking stress. Values are all expressed as mean $\pm$ SD (n = 5). *Significant difference from the CON group with $P < 0.05$. #Significant difference from the HDT group with $P < 0.05$.

the previous findings [8]. In addition to BMD, bone strength has been considered an important factor contributing to bone quality [20]. Bone strength can reflect fracture risk [21]. In this study, the structural properties including maximal load, yield load and fracture load of the tibia in HHT and HUT group were higher than those in the HDT group, which indicated that the increase of body fluid improved the mechanical properties. The bulk material parameters including maximum stress, breaking stress in HHT and HUT group were higher than those in the HDT group, which revealed potential improvement of bone's intrinsic properties. Meanwhile, the present results showed that the effect of body fluids on the cortical bone was greater than that on the cancellous bone.

Blood is the main component of body fluids. Blood supply plays an important role in bone development, bone repair and bone remodeling [22,23]. Disuse bone loss induced by hindlimb unloading [11], bed-rest [24], neurectomy and spinal cord injury [25], is mostly association with changed indexes of bone blood perfusion. The way to characterize vasculature and blood supply is vascular perfusion. Through vessels perfusion, the vessel-imaging is attained from micro-CT scanning [26,27]. Our results showed that compared with the TS group, the hindlimb blood vessels of the HDT group decreased, while those of the

HHT and HUT groups increased, but the difference was not significant. Hindlimb suspension could lead to vascular compliance increase [28], we inferred that the vascular changes was not obvious, because the hindlimb vascular became thicker in hindlimb unloading rats after vascular perfusion.

Besides, change of blood pressure may affect the bone loss due to hindlimb unloading. In vitro, it has been demonstrated that pressure can promote bone formation and inhibit resorption by regulating osteoblasts [29,30], osteocytes [31], and marrow cells [32,33]. Some studies have shown that blood pressure has little or no effect on bone mass [34], while intramedullary pressure was associated with bone formation [14]. In the present study, compared with the HDT group, the hindlimb venous pressure increased in the HHT and HUT groups because of the different body position, which can induce increase of intramedullary pressure. Suggesting that the effect of body fluids on the bone mass and biological properties of the rats hindlimbs may be related to increase of intramedullary pressure. Moreover, blood flow can affect rat bone tissue reconstruction [35], changes of body position may alter blood flow and microvascular of rats hindlimbs, which may affect bone remodeling.

As a main countermeasure against bone loss, exercise have been

Table 2

Comparisons for tibial bone dimensions.

	CON	HDT	HHT	HUT
Length (mm)	37.192 $\pm$ 2.253	37.104 $\pm$ 1.318	36.936 $\pm$ 0.813	36.712 $\pm$ 1.632
Weight(g)	0.758 $\pm$ 0.110	0.600 $\pm$ 0.085 ^a	0.628 $\pm$ 0.066 ^a	0.632 $\pm$ 0.076 ^a
Diaphyseal Width (mm)	1.988 $\pm$ 0.091	2.020 $\pm$ 0.047	2.204 $\pm$ 0.156	1.908 $\pm$ 0.477

Values are expressed as mean $\pm$ SD. (n = 9).

^a Significant difference from the CON group with $P < 0.05$.

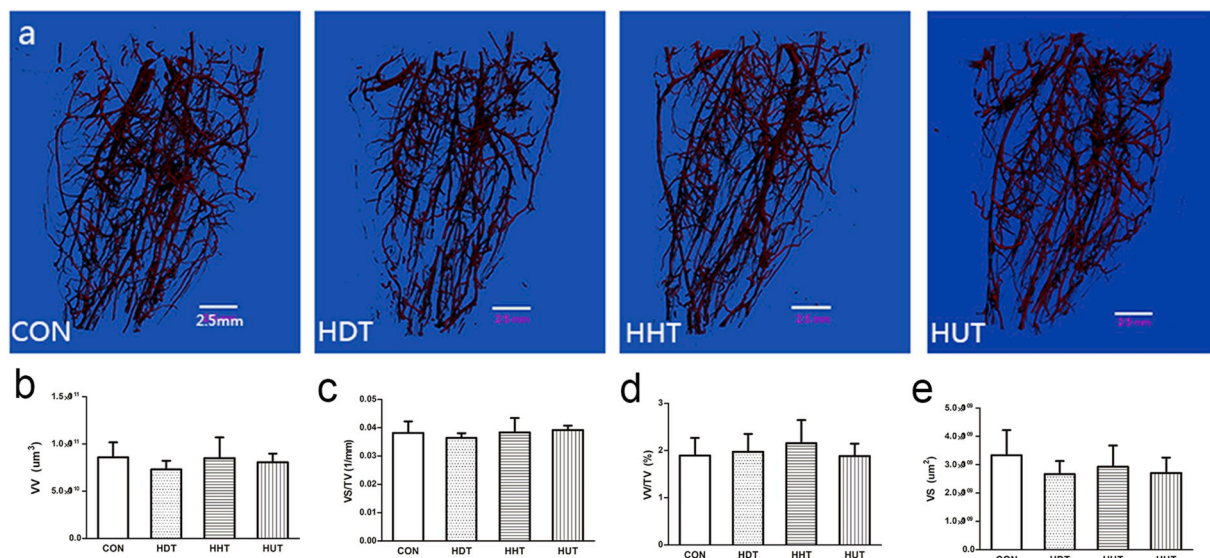


Fig. 5. Typical 3D vascular reconstruction of the rat shank with micro-CT imaging in four groups. (a) Comparisons for vascular parameters included (b) VV, (c) VS/TV, (d) VV/TV, (e) VS. Values are all expressed as mean $\pm$ SD ($n = 4$). Vessel volume (VV), vessel surface (VS), Vessel volume ratio (VV/TV), and vessel surface density (VS/TV).

studied for the past four decades in spaceflights [4,36]. Hyper-gravity resistance exercise is considered as a countermeasure to microgravity, it has proven it has a positive effect on the musculoskeletal system [37]. Localized vibration of 35 Hz to hindlimb could be used to counteract microgravity-induced bone loss in tail-suspended rats [38,39], and a specially developed stepper-device was able to keep the bone and muscle structure [40]. Combination of exercise and anti-resorptive drug may be more useful to protect bone health than exercise during long duration spaceflight and bed rest [37,41]. We think that exercise combined with increased body fluid may be more effective to combat lower body bone loss induced by microgravity. Therefore, we need to confirm the effect of the combination of body fluids and exercise in the follow-up study. Besides, it has been found that increased bone vessel function was related to the increase of trabecular bone after training in rats [42]. Although the adaptation of bone to mechanical loads involves changes of bone blood supply and vascular function [43,44], the link between the physical and molecular mechanisms is not fully understood. So, further research is necessary to examine the relationship between bone microvascular and bone remodeling in the hindlimb unloading rats.

In conclusion, we developed a novel model to examine the effect of body fluid shifts on bone loss in tail suspended rats, and our study demonstrated that increase of body fluid inhibited bone mass loss. The increase of body fluid had some positive effects on the bone mass, trabecular parameters and bone mechanical properties of hindlimb. The study suggested that mechanism of effect of fluid shift on bone remodeling in hindlimb unloaded rats need further investigations.

Disclosures

All authors state that they have no conflicts of interest.

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