



Musculoskeletal Alterations of Hindlimb During Simulated Microgravity and Reloading in Rats: Compared by Gender

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Abstract

Although several researches have reported potential gender differences in adaptations during microgravity, the musculoskeletal alterations of different genders are still under debate. Besides, there were few researches which involved recovery phase after reloading. In the present study, we investigated the musculoskeletal alterations of rats hindlimb via tail-suspension in both genders, and analyzed the serum sex hormone at the same time. Thirty-two Sprague Dawley rats were equally divided by gender into four groups randomly: control of male (CONM), tail-suspension of male (TSM), control of female (CONF) and tail-suspension of female (TSF). The experiment consisted two phases, which were 21 days of tail suspension phase and 21 days' reloading phase, and parameters of bone, muscle and serum were tested at the end point of each phase. The results indicated that during tail suspension phase, male rats showed a greater and earlier bone loss and microstructure deterioration compared with females, but they could maintain muscle strength much better. In addition, during reloading phase, male rats showed a slower bone recovery and some parameters could not recover until the end, while loss in muscle mass and strength in both genders could recover to control level.

Keywords Gender · Simulated Microgravity · Musculoskeletal Alterations · Reloading

Introduction

A series of detrimental adaptations caused by unloading in humans have been found during and after spaceflight, while the musculoskeletal disorders are particularly serious (Grimm

et al. 2016; Hughes-Fulford and Lewis 1996; Okoro et al. 2017). Physical exercise as a countermeasures have been shown a positive effect on the treatment of osteoporosis, such as treadmill, resistance exercise and vibration training (Karin et al. 2013; Castrogiovanni et al. 2016; Musumeci 2017). However, exercise can not completely alleviate weightlessness-induced bone loss. The mechanism of bone loss by microgravity-induced is still unknown.

With the rise in the number of female astronauts, potential gender differences in adaptations in space have received more attention. However, on the contrast to a number of single-sex studies, there are only a few investigations which involve both genders. Allen et al. (2001) compared the bone response to 28-d hindlimb unloading (HU) in male and female rats and their data suggested that there were gender-specific responses to HU in adult rats at sites composed of both cortical and cancellous bone, neither female rats not exhibiting the decrements in Bone Mineral Density (BMD) nor bone area observed in males. Park et al. (2015) also found a more serious bone loss in male groups than the females, but the females were also found to suffer a significant bone loss induced by unloading. Nonetheless, Harm et al. (2001) believed that gender differences had nothing to do with weightlessness bone loss

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because their data showed the similar results compared the women with the men during 17 weeks of bed rest ($n=6$) and during 4-6 months of spaceflight ($n=2$).

Moreover, although studies on weightlessness countermeasures have attracted many scientists, there are a few published data for the recovery phase after reloading. Based on the data of the previous researches, astronauts would suffer a fast bone loss in space and recover at a very low space after coming back to earth. For instance, one astronaut was reported to loss 12% of bone mass after 4.5 months' spaceflight but recovered only 6% after 1-year return to the ground (Clément 2005). Also there was another research reported that 28 days' reloading still could not restore the bone mineral content loss induced by head-down suspension to control levels (Vico et al. 1995). In addition, some data demonstrate the differential recovery rate of bone and muscle adaptations caused by unloading. Allen et al. (2006) reported that the rat BMD remains suppressed for a long time after reloading, while muscle strength recovers within a period equal to one-half of the unloading duration, and they questioned if this mismatch would heighten risk of bone injury. To improve our understanding on weightlessness osteoporosis and countermeasures, it is important to determine if there are gender differences in recovery phase during reloading.

Tail-suspended rat model is a commonly used model in simulating microgravity (Morey-Holton and Globus 2002). Tail-suspended rats can keep their hind limbs unloaded to provide a simulated microgravity effect. The effects of exercise on musculoskeletal system can be observed and studied through intervention measures such as exercise or vibration (Huang et al. 2017; Sun et al. 2013; Luan et al. 2018).

Thus, the aim of our study was (1) to present comparative analysis between males and females in the alterations of musculoskeletal system under simulated microgravity provided by tail-suspension and, (2) to compare those alterations of the recovery phase during reloading. That will further extend our knowledge among the musculoskeletal alterations in space and its recovery after returning to earth.

Materials and Methods

Experimental Animals and Animal Care

Sixteen eight-week-old female Sprague Dawley rats and sixteen eight-week-old male Sprague Dawley rats were purchased from Vital River Laboratory Animal Technology Co. (Beijing, China) and were housed under the same nursery conditions with 12 h dark-light cycles, food and water ad libitum for 21 days in the animal facility of Biological Science and Medical Engineering Department at Beihang University, China. All the treatments were carried according to Regulations for the Administration of Affairs Concerning

Experimental Animals promulgated by Decree No.2 of the State Science and Technology Commission of China and the Guiding Principles for the Care and Use of Animals set by Beijing Government. All protocols were approved by the Animal Care Committee of Beihang University.

After three days of adaptation, thirty-two rats were equally divided by gender into four groups respectively: control group of male rats (CONM), tail-suspension group of male rats (TSM), control group of female rats (CONF) and tail-suspension group of female rats (TSF). In the TS groups (TSM and TSF), rats were subjected to tail suspension for 21 days, in order to simulate weightlessness as previously reported (Morey-Holton and Globus 2002). In the roughly 30-degree head down tilt, their hind paws could not touch the floor but their fore paws could bear load and hold the position. on another hand, in CON groups (CONM and CONF), rats were housed normally. After the tail suspension phase, half rats of each group was sacrificed by hyper anesthesia and the others remained alive for further experiments. In the next 21 days, the two TS groups were released from suspension devices and treated under the same conditions as the control groups. After reloading phase, the remained rats were also sacrificed by hyper anesthesia. On the 21st day and the 42st day, the blood was collected from abdomens aorta after anesthesia and serum was separated by centrifugation for the following analyzed before the rats were sacrificed. Then the rats were sacrificed, the left tibias were excised all the surrounding tissues, wrapped by saline-soaked gauze bandage and were preserved at -20°C. Meanwhile, the left soleus muscles were separated for muscular tension measures (Fig. 1).

Bone Mineral Density (vBMD) and Microstructure Measured with Micro-CT

On the day 0, 7, 14, 21, 28, 35, 42 of the experiment, the rats were subjected to an in-vivo scan by micro-CT (SkyScan 1076, Belgium). According to previous study report (Luan et al. 2014), all scans used 70 kV X-ray voltage, 140 μ A current, a 1 mm aluminum filter, 18 μ m pixels, 360° tomographic rotation and a rotation step of 0.6°. Left tibias of the rats were analyzed and the region of interest commenced at the position of 1.898 mm to the growth plate level and extended to the diaphysis 3.796 mm (Fig. 2a). The region of interest was delineated automatically by CT Recon and then vBMD and trabecular microarchitecture parameters were calculated. Morphometric variables of trabecular included BV/TV (percent bone volume), Tb.N (trabecular number), Tb.Th (trabecular thickness) and Tb.Sp (trabecular separation).

Biomechanics of Tibia Measured with Instron E10000

The tibias were slowly thawed to room temperature and still wrapped by humid gauzes during the following

Fig. 1 Study design. This study was divided into two phases and each phase contained 21 days



measurements. Three-point bending of rat tibia was carried out by Instron testing machine (Instron E10000, USA). The span was about 20 mm. Before the actual testing, 5 cycles' stabilizing preload (5 N) were applied to the medial surface at rate of 0.1 mm/s and then the bending load was applied at rate of 0.2 mm/s until fracture of the specimen. After three-point bending experiments, both the external and internal diameters of the cross-section at the site of fracture were measured and then the maximum load, breaking load, stiffness and Young's modulus were analyzed (Sun et al. 2009).

Muscle Analysis with Multi-Channel Physiological Signal Tester

Muscular tension of left soleus was measured by the RM6240 multi-channel physiological signal tester (Chengdu, China) under single-twitching stimulus. The voltage of the square wave was 4.0 volts and the waviness width was 900 μ s (Close 1964). Then, the soleus was weighted by analytical balance.

Serum Bone Turnover Markers and Sex Hormones Measured by ELISA

Serum bone turnover markers, which included PINP (type I procollagen N-terminal propeptide) and β -CTX (c-terminal telopeptide region of collagen type I), sex hormones which included E2 (estradiol) and T (testosterone). All were measured by the corresponding double antibody radioimmunoassay kit (Baiaobolai Science and Technology Ltd., Beijing China) respectively. All the treatments were carried out as

per manufacturer's recommendations and duplicate determinations were made for every serum sample.

Statistical Analyses

All data were reported as means $\pm$ SD (standard deviation). Change rate of each parameter was calculated as $\Delta X = (X_{DX} - X_{D0})/X_{D0}$, where X representing the value of parameter and DX showing the date when the parameter measured. Two-way ANOVA was used for the comparison of multiple independent groups. The group differences were determined by T-test, with statistical significance defined as $P < 0.05$. All analyses were performed with SPSS 20.0.

Results

Cortical and Trabecular vBMD of Tibia

The tibia cortical vBMD and trabecular vBMD measured by micro-CT are shown in Fig. 3. Compared with CONM group, cortical vBMD of TSM group showed significant decrease at day 14 and could not recover until the end of our experiment. However, there appeared no remarkable difference between CONF and TSF group. According to data of the 21st day, the extent of decrease of cortical vBMD between male groups tended to be 10 times as large as female groups. As for trabecular vBMD, both gender showed significant difference between CON and TS groups, which was 122.5% in male groups and 41.1% in female groups, when under tail suspension. And similar to data from DXA, the significant difference

Fig. 2 Viewing position of tibia with micro-CT measurements.

a The 2D view of tibia and the analyzed region of interest. **b** 3D view of tail suspension after 21 days. **c** 3D view of reloading after 21 days

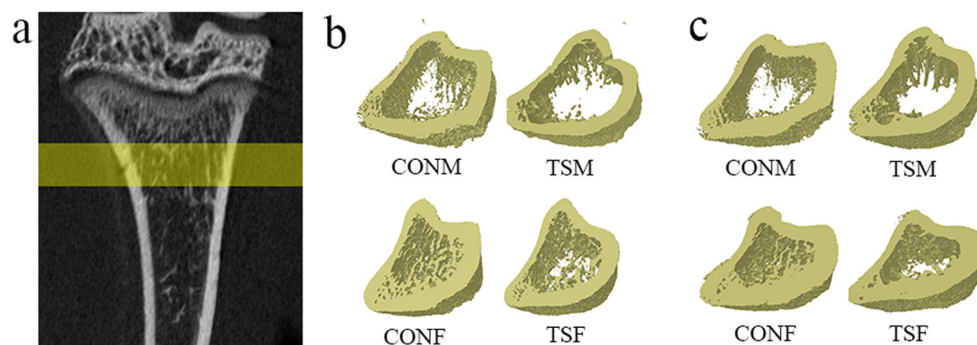
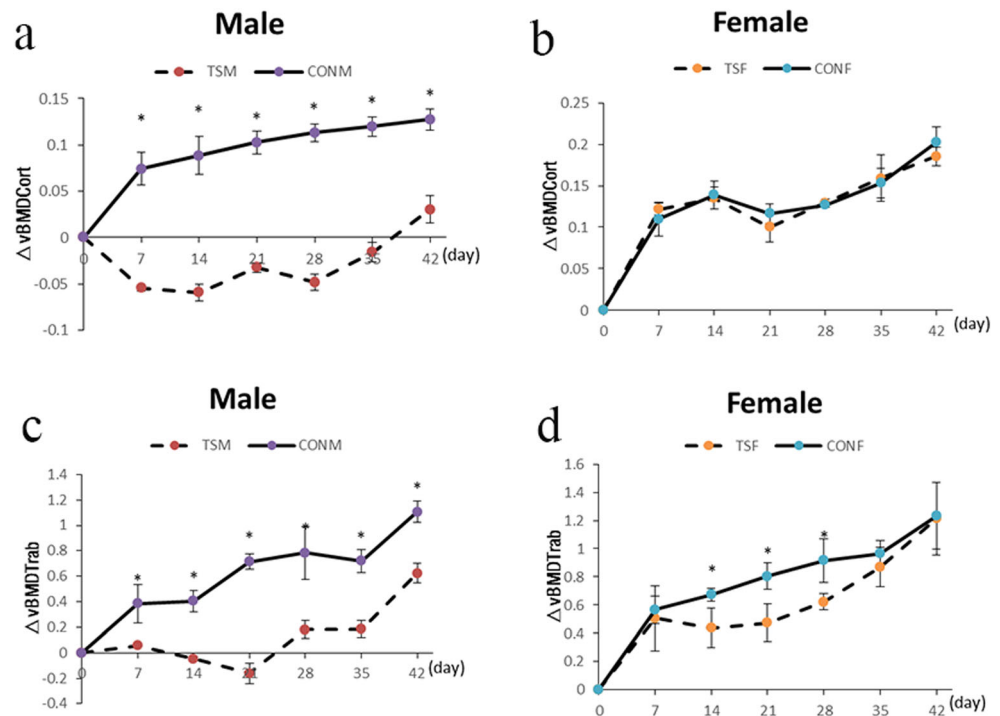


Fig. 3 Change rate of cortical and trabecular vBMD of left tibias measured by micro-CT according to time. Δ vBMD_{cort} and Δ vBMD_{trab} of tibia were measured every 7 days during the tail-suspension phase and reloading phase and T-test was used to determine the group differences between TS groups and CON groups of same gender (* $P < 0.05$). **a** Δ vBMD_{cort} of TSM and CONM group. **b** Δ vBMD_{cort} of TSF and CONF group. **c** Δ vBMD_{trab} of TSM and CONM group. **d** Δ vBMD_{trab} of TSF and CONF group



between CONM and TSM group started at day 7 and was not recovered until day 42 and the significant difference between CONF and TSF group started at day 14 and recovered at day 35. Micro-CT results also showed that male rats suffered earlier and more severe impairment in bone mineral density when under tail suspension than females. There was a remarkable phenomenon in our results that male rats suffered a serious deterioration in cortical bone due to tail suspension, while females showed almost no significant change.

Trabecular Microarchitecture

The tibia trabecular microarchitecture measured by micro-CT is shown in Fig. 4. In TS groups, trabecular microarchitecture in tibia of both genders tended to deteriorate among tail-suspension phase, as BV/TV, Tb.N and Tb.Th decreased and Tb.Sp increased significantly compared with CON groups. Tb.Sp of TSM group showed significant increase compared with CONM group while the two female groups presented no significant difference. In addition, the change extent of all parameters between the two male groups tended to be higher than the two female groups. Among the recovery phase, the trabecular microarchitecture of TSF group could recover to CONF level but BV/TV, Tb.N and Tb.Th of TSM group were still significantly below CONM group on day 42.

According to the parameters we showed above, TS groups in both genders showed remarkably worse bone microarchitecture compared with CON groups when exposed to tail suspension, but male rats' trabecular microarchitecture seemed more sensitive to simulated weightlessness than females.

Bone Mechanical Properties

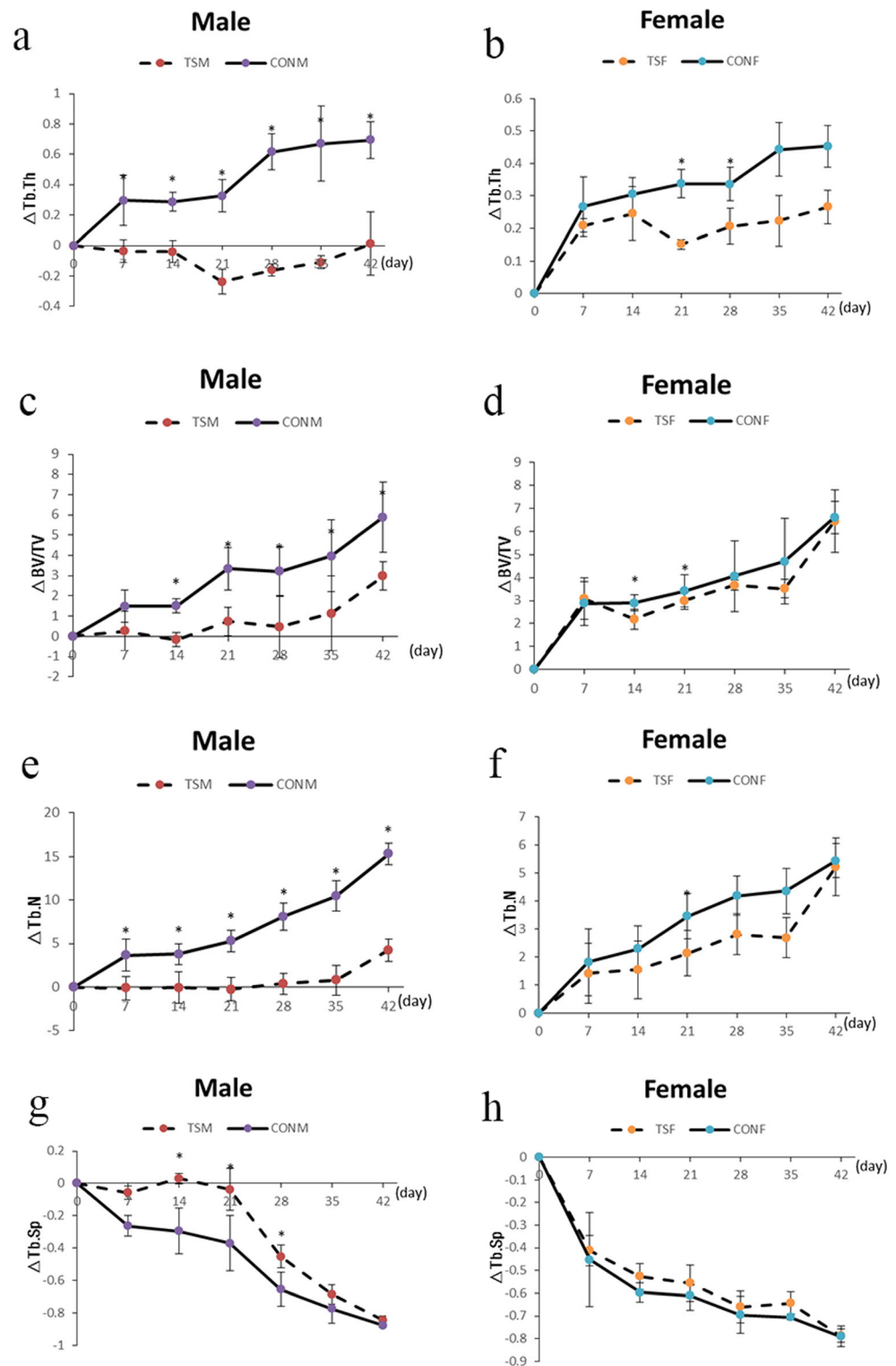
The tibia bone mechanical properties measured by three-point bending test are shown in Table 1. On day 21, in TS groups, both genders' maximum load, breaking load, stiffness and Young's modulus of tibia decreased significantly compared with corresponding CON groups and those changes could be recovered after 21 days' reloading. Other data of BMD, the extents of variations from three-point bending test between TS groups and CON groups were similar between male groups and females.

Wet Weight and Muscle Tension

The soleus wet weight and tension measured by multi-channel physiological signal tester are shown in Table 2. After 21 days' tail suspension, wet weight of soleus decreased in TS groups significantly compared with CON groups in both male and female groups and the muscular tension of single twitching contraction in soleus also showed the similar results. While, after 21 days' reloading, not only the wet weight but also the muscular tension in TS groups recovered to the CON level. According to data of day 21, although the wet weight change between TSM and CONM group tended to be very close compared with the two female groups, the change rate of muscle strength showed the difference in results, where male rats seemed to be much in maintaining of muscle contraction strength.

Fig. 4 Change rate of trabecular BV/TV, Tb.N, Tb.Th and Tb.Sp of left tibias measured by micro-CT

according to time. Δ BV/TV, Δ Tb.N, Δ Tb.Th and Δ Tb.Sp of tibia were measured every 7 days among the tail-suspension phase and reloading phase and T-test was used to determine the group differences between TS groups and CON groups of same gender (* $P < 0.05$). **a** Δ BV/TV of TSM and CONM group. **b** Δ BV/TV of TSF and CONF group. **c** Δ Tb.N of TSM and CONM group. **d** Δ Tb.N of TSF and CONF group. **e** Δ Tb.Th of TSM and CONM group. **f** Δ Tb.Th of TSF and CONF group. **g** Δ Tb.Sp of TSM and CONM group. **h** Δ Tb.Sp of TSF and CONF group



Serum Bone Turnover Markers and Serum Sex Hormones

The serum bone turnover markers and serum sex hormones measured by ELISA are shown in Table 3. After 21 days' tail suspension, the concentration of serum PINP in TS groups

showed no significant difference compared with CON groups and the concentration of serum β -CTX also tended to be no remarkable difference between the two male groups. Only TSF group showed significant increase in the concentration of serum β -CTX compared with CONF group. Then after 21 days' reloading, both genders showed no significant

Table 1 Tibia bone mechanical properties measured by three-point bending of tibia

		Maximum load (N)	Breaking load (N)	Stiffness (N/mm)	Young's modulus (N/mm ²)
After tail suspension	CONM	90.82 ± 6.81*	78.67±12.29*	173.89±18.07*	14801.32±2515.30*
	TSM	64.41 ± 5.71	52.79±12.04	145.70±9.76	10743.84±871.15
	CONF	76.04±7.26*	69.32±12.01*	176.35±27.71*	17497.45±3395.37*
	TSF	59.89±5.81	50.38±9.82	135.58±13.53	11606.06±1799.88
After reloading	CONM	103.54±15.76	97.92±20.74	229.21±30.93	15210.95±1824.54
	TSM	89.68±5.52	82.16±5.89	206.75±26.07	14811.73±1355.47
	CONF	77.04±10.50	70.59±13.40	174.58±59.76	17728.48±2220.41
	TSF	68.43±1.57	61.87±2.72	148.66±29.85	16022.80±740.53

Values are showed as mean ±SD and T-test was used to determine the group differences between TS groups and CON groups of same gender (*P<0.05)

difference between TS groups and CON groups. After 21 days' tail suspension, serum E2 of TSF group showed a remarkable decrease compared with CONF group and this adaptation recovered during reloading period. However, serum T remained similar between the two male groups all through the whole experiment.

Results from Two-Way ANOVA of Δ BMD and Soleus Parameter

Results from Table 4 showed that after tail suspension, gender and tail suspension had significant effects on the Δ BMD of cortical bone and trabecular bone, and there was interaction between them. After the recovery, the Δ BMD of trabecular bone was not affected by tail suspension and gender. After tail suspension, gender and tail suspension had significant effects on wet weight and muscle tension of soleus, but there was no interaction between them. After recovery, soleus muscle wet weight and muscle tension were not affected by tail suspension.

Discussion

In this study, we investigated the variation of musculoskeletal system caused by simulated microgravity induced in tail-

suspension rats and its recovery after reloading in both males and females. Our results showed that both male and female rats exhibited degenerative changes in the musculoskeletal system under the influence of tail-suspended simulated microgravity effect, including decreased tibia mass, deterioration of bone microarchitecture, decreased bone mechanics, and soleus muscular atrophy. Overall, males deteriorated earlier than females. The improvement of male musculoskeletal system was later than that of females during recovery, but most of the changes could attain the level of the control group on day 21 of recovery.

According to our results in bone variation, we found that male rats suffered more severe impairment induced by simulated weightlessness than female rats. Not only a greater degree of bone loss and microstructure deterioration, but also a significant earlier degradation phenomenon and a slower recovery during reloading phase were appeared in male groups compared with female groups. Some studies also showed the similar results (Park et al. 2015; Allen et al. 2006), where bone of male seems to be more sensitive to weightlessness, but their studies did not involve the reloading phase. Also, there is no clear reason why such differences happened. On one hand, the reduction of mechanical loading occurred during tail suspension induced lower stresses and strains in bone tissue, and consequently bone mass would be lower to maintain the structural integrity of the skeleton. In general, female rats are

Table 2 Soleus wet weight and muscle tension

		Wet weight of soleus (g)	Tension of soleus (g)
After tail suspension	CONM	0.17 ± 0.02*	0.90±0.21*
	TSM	0.10 ± 0.02	0.63±0.08
	CONF	0.13±0.02*	1.67±0.27*
	TSF	0.08±0.01	0.80±0.16
After reloading	CONM	0.20±0.03	1.93±0.60
	TSM	0.17±0.01	1.87±0.32
	CONF	0.14±0.02	1.67±0.60
	TSF	0.13±0.02	1.58±0.35

Values are showed as mean ±SD and T-test was used to determine the group differences between TS groups and CON groups of same gender (*P<0.05)

Table 3 Serum bone turnover markers and Serum sex hormones measured by ELISA

		PINP (ng/mL)	β -CTx (ng/mL)	Testosterone (ng/mL)	Estradiol (ng/mL)
After tail suspension	CONM	15.15 $\pm$ 1.05	14.86 $\pm$ 0.72	2.75 $\pm$ 0.03	-
	TSM	14.37 $\pm$ 1.78	17.12 $\pm$ 1.27	2.44 $\pm$ 0.22	-
	CONF	15.30 $\pm$ 2.15	14.62 $\pm$ 0.73*	-	22.76 $\pm$ 2.02*
	TSF	13.76 $\pm$ 3.04	17.38 $\pm$ 1.48	-	18.73 $\pm$ 0.80
After reloading	CONM	18.86 $\pm$ 3.11	13.92 $\pm$ 1.90	2.18 $\pm$ 0.18	
	TSM	20.76 $\pm$ 5.40	13.41 $\pm$ 2.39	2.14 $\pm$ 0.07	
	CONF	18.20 $\pm$ 1.37	6.21 $\pm$ 1.31	-	33.01 $\pm$ 6.26
	TSF	18.19 $\pm$ 2.04	6.25 $\pm$ 1.15	-	38.08 $\pm$ 3.15

Values are showed as mean $\pm$ SD and T-test was used to determine the group differences between TS groups and CON groups of same gender (*P<0.05)

lighter than males in the same age. From the perspective of adaptation, because of the adaptation to the changed mechanical environment induced by weightlessness, males may suffer more serious bone loss than females. Meanwhile, due to a greater extent of variation, males may need longer time to recovery from those changes. On the other hand, gender differences in tibia length, bone area, bone microarchitecture, and bone formation could also be the influential factors. Compared with female rats, male rats have longer tibia, greater cortical bone area, and greater trabecular separation. Conversely, female rats have higher cortical and cancellous bone formation rates, more cancellous bone, and greater trabecular number (Hefferan et al. 2003). Considering the above gender differences, it may be rational that male rats suffered serious deterioration in cortical bone due to tail suspension, while females showed almost no change. In addition, estrogen, as a menstrual hormone for female, has generally been considered to be the major endocrine regulator of bone metabolism, resulting in increased bone mass in adolescence (Manolagas et al. 2002). Hence, estrogen may play an important role in female's bone maintaining as well.

Obviously, there tended to be significant reduction in mechanical properties of bone between TS groups and CON groups in both genders. However, other than the bone mass and bone microarchitecture results, the effects of tail suspension seemed to be nondiscriminatory in this regard. In view of three-point bending test only reflecting the effect of

weightlessness on the whole bone, which depends on both material and architecture of the bone, the mechanical measurements at the material level should also be tested to better explain these phenomena in our following researches.

Trying to find possible mechanisms on weightlessness bone loss, we also analyzed the concentration of serum bone turnover markers. On the basis of our results (data from both genders) and some other studies (Smith et al. 2005), the bone turnover biochemical markers tests did not detect a change in bone formation during spaceflight or simulated weightlessness. Meanwhile, data of calcium kinetics from other researchers also suggested that bone formation is unchanged (Smith et al. 2005). In this sense, bone formation seemed not be an important factor in weightlessness-induced bone loss. However, we found significant increased bone resorption after 21 days of tail suspension in female rats but not in males. In consideration of estrogen's main function in suppressing bone resorption (Jagger et al. 1996), there was evidence that lack of estrogen could lead to activation of osteoclasts, increased reabsorption of cartilage and increase in bone turnover (Manolagas et al. 2002), these bone resorption marked differences appeared between TSF and CONF group may partly attributed to the decreased of estrogen during tail suspension, which were also observed in our study.

In the results of the muscle parameters, we found that male rats' soleus suffered more severe muscle mass loss but could better maintain muscle strength during tail suspension

Table 4 Results from two-way ANOVA

	Measurement	Δ BMDcort	Δ BMDtrab	Wet weight of soleus	Tension of soleus
After tail suspension	CON/TS	0.003*	0.019*	0.001*	0.032*
	Sex	0.011*	0.011*	0.004*	0.010*
	Interaction effects	0.042*	0.031*	0.423	0.421
After reloading	CON/TS	0.003*	0.730	0.075	0.850
	Sex	0.001*	0.446	0.002*	0.473
	Interaction effects	0.008*	0.567	0.168	0.965

*P<0.05

compared with corresponding female groups. In general, along with decreased protein synthesis and increase protein degradation caused by weightlessness, the amount of myofibril and cross-bridge of unit cross-sectional area would reduce consequently. And then, the lighter muscle wet weight and less cross-sectional area of muscle fiber would also lead to the reduction in muscle contractility. However, there were some researchers reported that changes in muscle size or mass did not explain the exacerbated strength losses noted in women following unloading (Yasuda et al. 2005). In addition, estrogen administration in females was considered to help maintain soleus mean fiber cross-sectional area (McClung et al. 2006) and muscle mass (Yasuda et al. 2005). Therefore, we have reasons to believe that this effect of estrogen may help female rats better maintenance of their muscle mass. Certainly, there were several differences between female and male skeletal muscles, including differences in energy metabolism, contractile speed (Glenmark et al. 2004) and different types of muscles, such as fast-twitch and slow-twitch muscles, may have various responses to weightlessness, but estrogen administration may play an important role as well.

Except for some of males' bone parameters, most parameters of females have recovered to CON level after 21 days' reloading. Considering the factors that we mentioned above, it seems acceptable that males in TS group also have slower recovery after reloading compared to females. Moreover, different from female groups which time equal to tail suspension was sufficient to return to CON level, male rats may need more time to recovery from weightlessness osteoporosis. In addition, there are some shortcomings in this study, such as some morphological measurements of bone and muscle have not been analyzed, this is a preliminary study and further studies are needed to consolidate these results. As the increase of the astronauts and the time that they stay in space, these results can be used as a reference.

In conclusion, this study analyzed the changes of musculoskeletal system under simulated microgravity provided by tail-suspension and the recovery phase in both male and female rats. The performance of bones and muscles in both male and female rats under simulated microgravity was inconsistent, male rats appeared earlier and greater deterioration of musculoskeletal system during unloading phase and slower recovery during reloading phase compared with the corresponding females.

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Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical approval All animal treatments were approved by the Animal Care Committee of Beihang University in accordance with the Regulation of Administration of Affairs Concerning Experimental Animals of State Science and Technology Commission of China.

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