

Would Interstitial Fluid Flow be Responsible for Skeletal Maintenance in Tail-Suspended Rats?

Wen-Ting Li¹ · Yun-Fei Huang¹ · Lian-Wen Sun^{1,2} · Hui-Qin Luan¹ · Bao-Zhang Zhu¹ · Yu-Bo Fan^{1,3}

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Abstract Despite the fast development of manned space flight, the mechanism and countermeasures of weightlessness osteoporosis in astronauts are still within research. It is accepted that unloading has been considered as primary factor, but the precise mechanism is still unclear. Since bone's interstitial fluid flow (IFF) is believed to be significant to nutrient supply and waste metabolism of bone tissue, it may influence bone quality as well. We investigated IFF's variation in different parts of body (included parietal bone, ulna, lumbar, tibia and tailbone) of rats using a tail-suspended (TS) system. Ten female Sprague-Dawley (SD) rats were divided into two groups: control (CON) and tail-suspension (TS) group. And after 21 days' experiment, the rats were

injected reactive red to observe lacuna's condition under a confocal laser scanning microscope. The variations of IFF were analyzed by the number and area of lacuna. Volumetric bone mineral density (vBMD) and microarchitecture of bones were evaluated by micro-CT. The correlation coefficients between lacuna's number/area and vBMD were also analyzed. According to our experimental results, a 21 days' tail-suspension could cause a decrease of IFF in lumbar, tibia and tailbone and an increase of IFF in ulna. But in parietal bone, it showed no significant change. The vBMD and microarchitecture parameters also decreased in lumbar and tibia and increased in ulna. But in parietal bone and tailbone, it showed no significant change. And correlation analysis showed significant correlation between vBMD and lacuna's number in lumbar, tibia and ulna. Therefore, IFF decrease may be partly contribute to bone loss in tail-suspended rats, and it should be further investigated.

✉ Lian-Wen Sun
sunlw@buaa.edu.cn

✉ Yu-Bo Fan
yubofan@buaa.edu.cn

Wen-Ting Li
lwt028@163.com

Yun-Fei Huang
282785222@qq.com

¹ Key Laboratory for Biomechanics and Mechanobiology of Ministry of Education, School of Biological Science and Medical Engineering, Beihang University, No. 37 Xueyuan Road, Haidian district, 100191, Beijing, China

² International Joint Research Center of Aerospace Biotechnology and Medical Engineering, Ministry of Science and Technology of China, Beijing, China

³ National Research Center for Rehabilitation Technical Aids, Beijing, China

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Introduction

During spaceflight, astronauts have been known to suffer a series of adaptive physiological changes, such as cardiovascular disorder, osteoporosis, calcium-phosphorus metabolism imbalance, muscular atrophy, etc. (Xianyun 2013) and decrements of bone mineral density (BMD) in weight-bearing bones could be 1.0 %-2.0 % per month (LeBlanc et al. 2000). Therefore, the problem above has become one of the main restrictions in manned space development.

Studies have showed that astronauts suffered different degrees of bone loss in different parts of the body when

in space (Ma et al. 2003) and different loading change in each part was regarded as the main reason. However, body fluids, which on the basis of Dillaman's fluid flow theory, should also be considered. Dillaman believed that the source power of bone stress adaptability was the IFF, which also undertook the metabolism in bone (Dillaman et al. 1991). Moreover, IFF is believed to influence osteocytes' cellular environment and communications (Dong et al. 2014) as well as bone tissue's nutrient supply and waste metabolism (Knothe Tate et al. 2000). Therefore, we believed that the investigation on IFF in tail-suspended rats will lead to a better understanding of bone loss caused by unloading.

However, there were few studies focusing on the whole body distribution change of IFF in tail-suspended rats. The aim of the study was (1) to describe the variation of IFF in different parts of body in tail-suspended rats simultaneously and, (2) to study the possible relationship between IFF and bone loss in microgravity.

Materials and Methods

Experimental Animals and Animal Care

Ten eight-week-old female Sprague Dawley rats were purchased from Experimental Animal Center of Beijing University and were housed under the same nursery conditions with 12h 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' adaptation, ten animals were equally divided into two groups: control (CON) and tail-suspension

(TS). In the TS group, rats were subjected to tail suspension for 21 days, in order to simulate weightlessness as previously reported (Morey et al. 1979). In the roughly 30-degree head down tilt (Morey-Holton and Globus 2002), their hind paws could not touch the floor but their fore paws could bear load and hold the position. On day 22, the rats were released from tail suspension devices and were injected reactive red (Sigma, USA) (1ml/100g body weight) after anesthesia (1 % pentobarbital sodium: 6mg/100g body weight). After three hours postinjection, the rats were sacrificed.

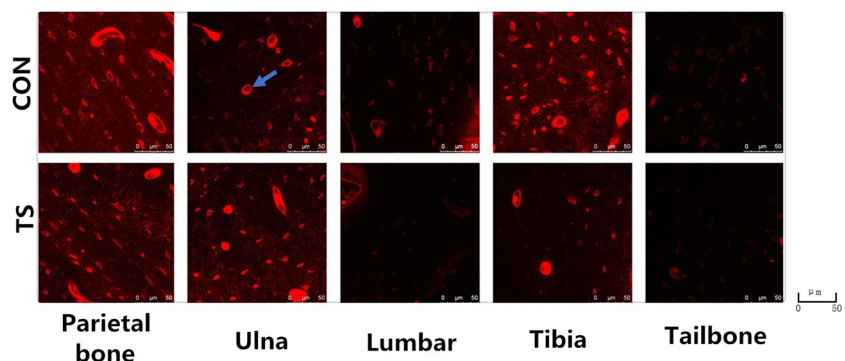
Bone Mineral Density (vBMD) and Microstructure by Micro-CT

The parietal bones (n=10), left ulnae (n=10), lumbar (n=10), left tibiae (n=10) and tailbones (n=10) of the rats (n=10) were dissected, cleaned of soft tissue, and scanned by micro-CT (SkyScan1076, Belgium). According to study reported previously (Luan et al. 2014), all scans used 70Kv X-ray voltage, 143 μ A current, a 1mm aluminium filter, 18 μ m pixels, 360° tomographic rotation and a rotation step of 0.6°. In parietal bone, ulna, lumbar, tibia and tailbone, the region of interest commenced at the position of 0.142mm, 0.855mm, 1.709mm, 1.898, and 1.424mm to the growth plate level, respectively and all extended to the diaphysis 2.373mm. All scans were reconstructed with the same parameters. 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, mm⁻¹), Tb.Th (trabecular thickness, mm) and Tb.Sp (trabecular separation, mm).

IFF by Confocal Laser Scanning Microscope (CLSM)

After the micro-CT scan, all bone specimens were longitudinally sectioned at 100 μ m using a heavy-duty sliding microtome (EXAKT CT300, Germany) and 5 sections of

Fig. 1 The image of lacuna after 21 days' experiment. An example of lacuna is indicated by the arrow



each bone specimen were obtained (250 sections in total). Images of each section were obtained by CLSM (Leica TCS SPE CTP6500, Germany) and then the number and area of lacuna were calculated by ImageJ.

Statistical analyses

All data were reported as means \pm SD (standard deviation). Group differences were considered significant at

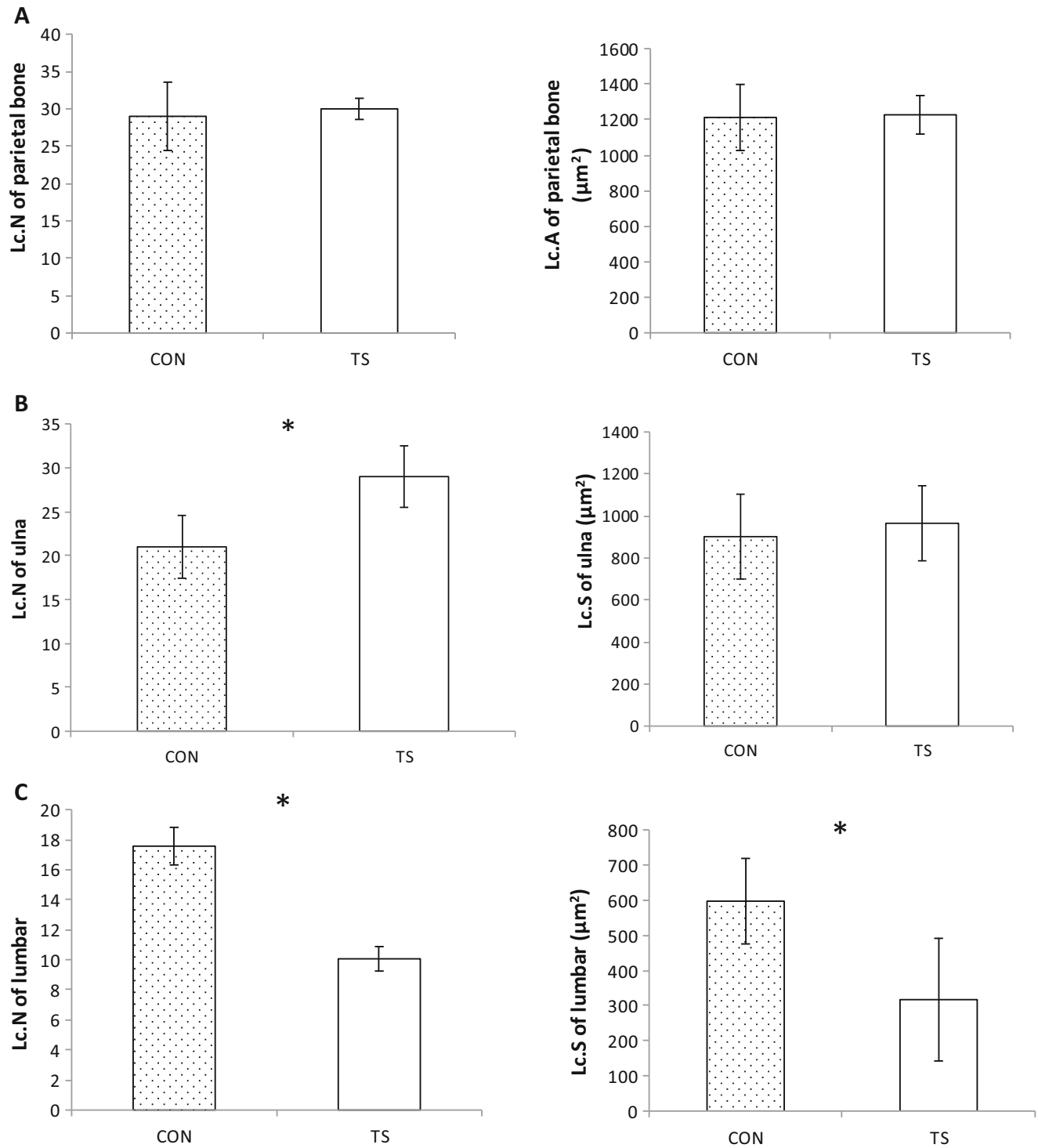


Fig. 2 The number and area of lacuna after 21 days' experiment. **A** parietal bone; **B** ulna; **C** lumbar; **D** tibia; **E** tailbone. * $p < 0.05$

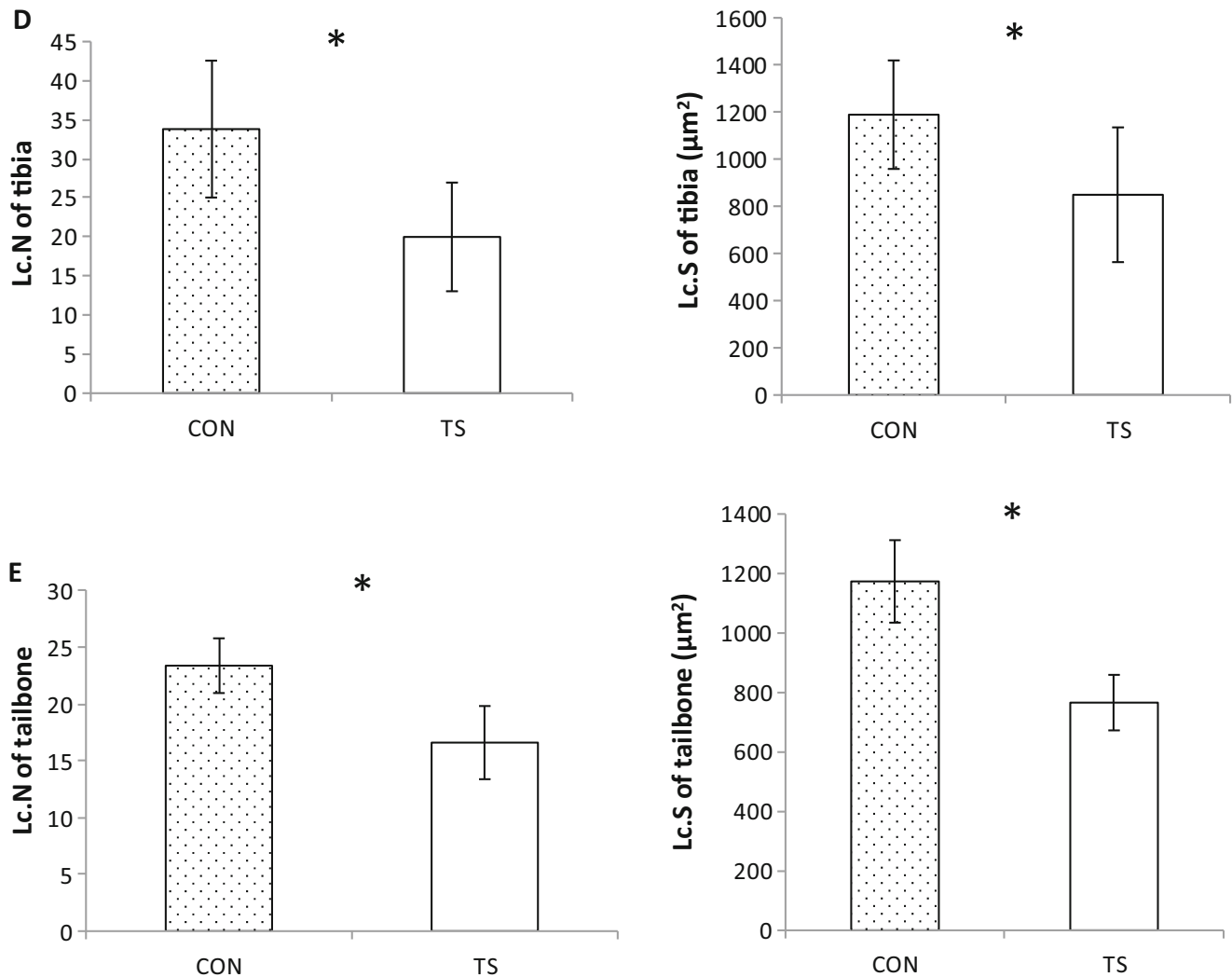


Fig. 2 (continued)

$p \leq 0.05$. Pearson correlation analyses were used to assess the relationships between lacuna's number/area and vBMD. All analyses were performed with SPSS 20.0 using T-test analysis.

Results

Variations of IFF by CLSM

After 21 days' experiment, IFF of TS group showed significant changes compared with CON group (Fig. 1). In lumbar, tibia and tailbone, both the number and area of lacuna in TS group significantly decreased compared with the CON group, which were 43 %, 41 %, 29 % in number and 47 %, 29 %, 35 % in area respectively (Fig. 2C/D/E). In ulna, compared with CON group, the number of lacuna increased 38 % significantly in TS group, but appeared no

influence in the area (Fig. 2B). In parietal bone, the two groups expressed no significant difference in number or area (Fig. 2A).

Bone Mineral Density (vBMD) by Micro-CT

After 21 days' experiment, compared with CON group, in TS group trabecular vBMD of lumbar and tibia decreased significantly by 18 % and 19 % respectively (Fig. 3C/D), while the ulna trabecular vBMD increased significantly by 9 % (Fig. 3B). As for parietal bone and tailbone, there appeared no remarkable difference (Fig. 3A/E).

Trabecular Bone Microarchitecture by Micro-CT

As was seen in vBMD, in the TS group, trabecular microarchitecture at lumbar and tibia tended to deteriorate, such that at tibia BV/TV, Tb.N and Tb.Th decreased and Tb.Sp

increased significantly compared with CON group, while at lumbar BV/TV, Tb.N and Tb.Th also diminished and Tb.Sp increased significantly. However, there appeared a little appreciable impact at ulna and tailbone between two groups (Table 1). The parameters of parietal bone were not

collected due to the lack of trabecular at parietal bone we selected (Table 2).

Rats exposed to tail suspension showed remarkably worse bone microarchitecture compared to CON group, according to the parameters showed above.

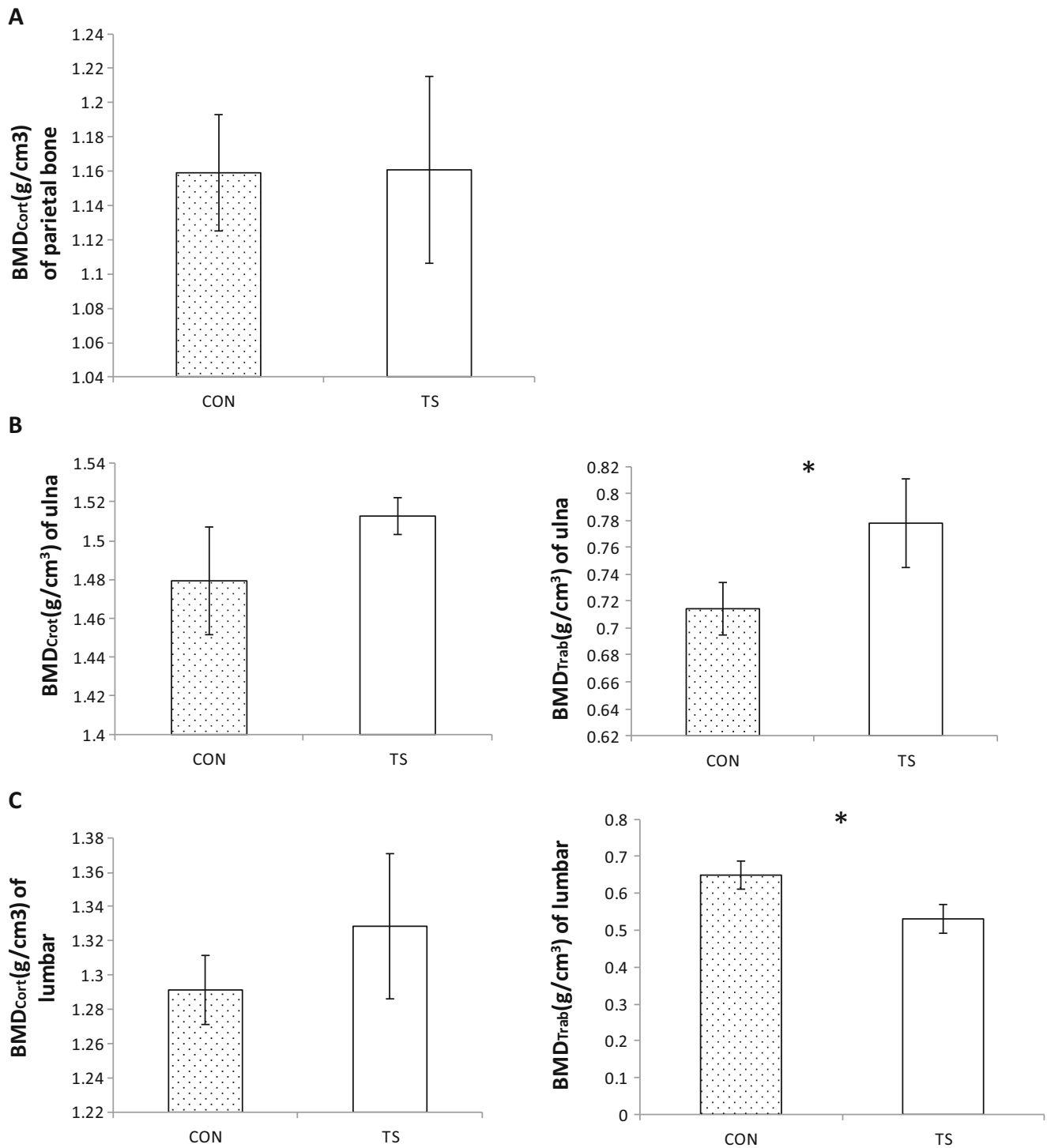


Fig. 3 Cortical and trabecular vBMD after 21 days' experiment by micro-CT. **A** parietal bone; **B** ulna; **C** lumbar; **D** tibia; **E** tailbone. *p<0.05

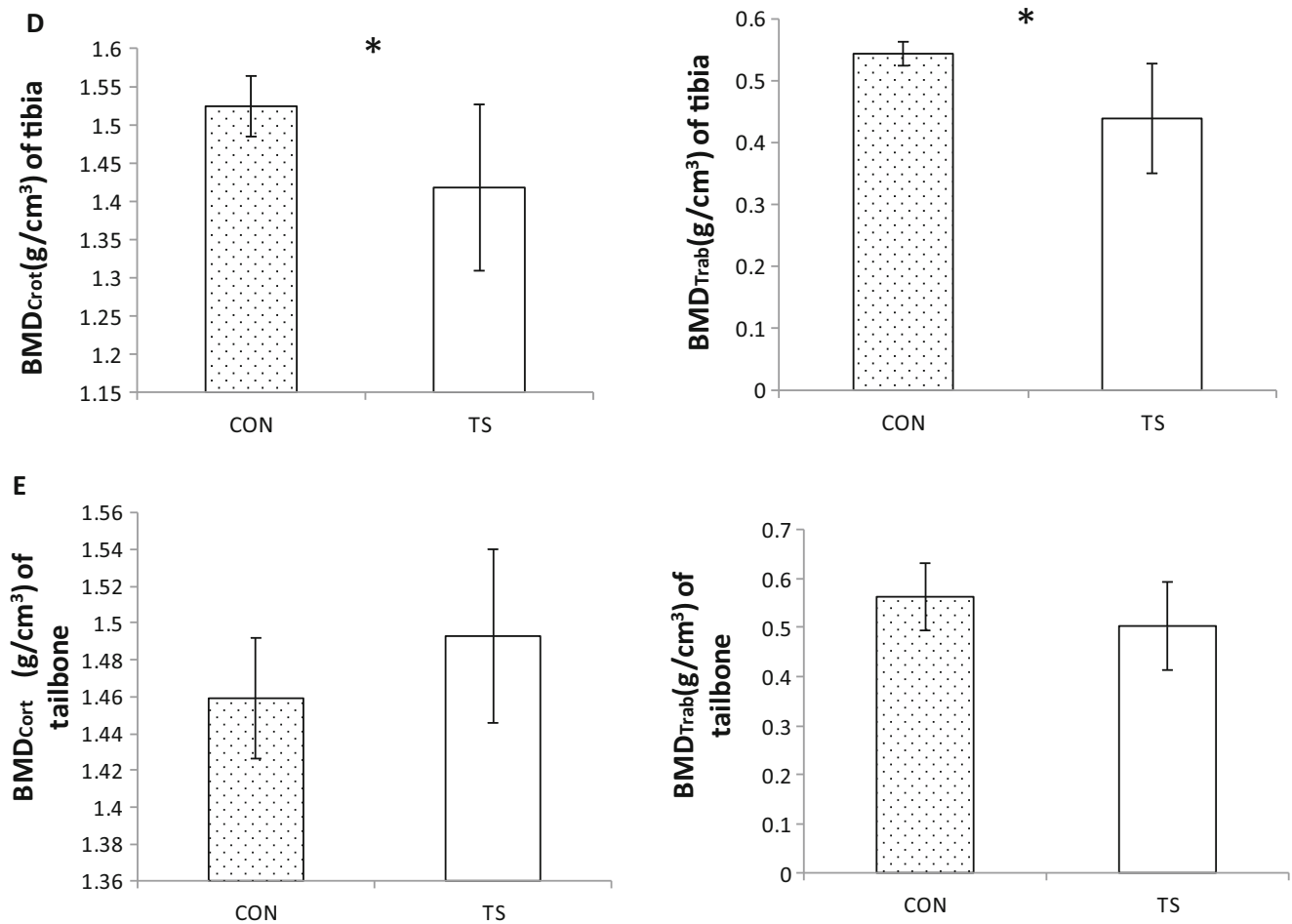


Fig. 3 (continued)

Correlation Between Parameters of IFF and Bone Mineral Density

In lumbar, tibia and ulna, the number of lacuna and vBMD showed significant correlations, but parietal bone and tailbone showed no significant correlations. In all five parts of bones, the area of lacuna showed no significant correlations with vBMD.

Discussion and Conclusion

In this study, we investigated the variation of bone quality and IFF distribution (described by lacuna number and area) in different parts of tail-suspended rats simultaneously and analyzed the possible factors contribute to bone loss caused by unloading (Overall change tendency of IFF, bone mineral density (vBMD) and general weight-bearing caused by tail-suspension is shown in the table below: - = no significant change; ↑= increase significantly; ↓= decrease significantly).

	IFF	vBMD	Weigh-bearing
parietal bone	—	—	—
ulna	↑	↑	↑
lumbar	↓	↓	↓
tibia	↓	↓	↓
tailbone	↓	—	↑

In this study, we used lacuna number and area to describe IFF distribution. To confirm that all the red dots we collected are lacunae, we use diameter as a criterion since lacuna’s diameter is approximately between 5 and 15 according to other researches (Wang et al. 2004; Ciani et al. 2005). Also, we contrasted photos in consecutive sections collected by confocal laser scanning microscope to reconfirm the results.

After 21 days’ experiment, in lumbar and tibia, IFF in TS group reduced significantly compared with CON group while in ulna, IFF in TS group increased significantly compared with CON group. On the one hand, tail-suspension induced cephalic fluid shift (Hargens et al. 1984; Maurel et al. 1996; Provost and Tucker 1992) could diminished

blood flow to hindquarter arteries through an increase in vascular resistance (Colleran et al. 2000). This means the blood flow in lumbar and tibia decreased but in ulna it increased. On the other hand, during tail-suspension, weight-bearing in tibia and lumbar decreased but in ulna increased compared with the CON group (Sun et al. 2009). Since IFF arises from the vascular pressure gradient between the medullary cavity and the lymphatic drainage at the periosteal surface and is enhanced by mechanical loading events (Stevens et al. 2006), change in blood flow and weight-bearing caused by tail-suspension could be the possible explanation for the IFF change. In lumbar and tibia, the vBMD and microarchitecture were decreased significantly compared to CON group, as a result that IFF (Fig. 2), blood flow (Hargens et al. 1984) and weight-bearing (Sun et al. 2009) were diminished stimulated by tail suspension. And in ulna, where both IFF and weight-bearing slightly increased (Sun et al. 2009) in TS group, an increase in vBMD was observed. As in parietal bone, IFF and bone quality showed no remarkable change between TS group and CON group, but an increase in BMD was found at the skull of astronauts in space flights (Alexandre and Vico 2011). This discrepancy maybe because that when under tail-suspension, the rat's head almost remains in state of nature and there seemed little change in distance between head and heart, that was neither weight-bearing or blood flow was remarkable changed in tail-suspended rats' parietal bone. And the unchanged carotid blood volume between tail suspension and control group according to Chew and Segal (Chew and Segal 1997) also supported this point. This may indicate a difference between tail-suspension model and astronauts in space flights in general.

The results indicated that the change of IFF caused by tail-suspension in lumbar, tibia and parietal bone showed the same tendency with bone quality change and correlation analysis also supported this point.

However, an unexpected result came to tailbone that its vBMD and microarchitecture showed no remarkable difference between the two groups, but its IFF reduced significantly in TS group compared with CON group. Owing to a cephalic fluid shift and an elimination of the head-to-foot hydrostatic pressure gradient (Thornton et al. 1987; Watenpugh and Hargens 1996), the vascular blood pressure induced (Alexandre and Vico 2011) IFF decreased significantly in tailbone. Although IFF decreased in tailbone, tail tension increased during tail-suspension (Hargens et al. 1984). Therefore, we speculated that increased force might offset the effect on bone loss caused by IFF and blood flow to some extent and these results also indicated that IFF change may partly contribute to bone loss caused by unloading.

In conclusion, this study used a tail suspension model to describe the variation of bone quality and IFF distribution

(described by lacuna number and area) in different skeletal bones of tail-suspended rats simultaneously. According to our experimental findings, IFF may be one of the notable factors for skeletal maintenance in tail-suspended rats and more specifically, a decreased IFF could partly contribute to the reduction in bone density. Previous studies also indicate that a decrease in total osteocyte lacunar density may contribute to failure or delayed bone repair in aging bone and decreased osteocyte lacunar density may cause deteriorations in the canalicular fluid flow and reduce the detection of microdamage (Busse et al. 2010). According to this point of view, the weightlessness-induced reduction in number of lacunae showed in our research may partly be a reflection of a deteriorated bone remodeling. However, the specific relationship between IFF and bone remodeling, and the regulation of IFF, which has been implicated as the mediator of load-induced bone remodeling (Hillsley and Frangos 1994; Reich et al. 1990) in bone loss, may be very complicated, and further study should be carried out to understand its mechanism.

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Disclosures We declare that for all authors listed, no competing financial interests exist.

Appendix

Table 1 Trabecular parameters of ulna, lumbar, tibia and tailbone after 21 days' experiment by micro-CT

	BV/TV (%)	Tb.N (1/mm)	Tb.Th (mm)	Tb.Sp (mm)
ulna				
CON	76.83± 2.10	4.54± 0.21	0.16± 0.01	0.11± 0.01
TS	79.99± 2.66	4.75± 0.21 ^a	0.16± 0.01	0.11± 0.02
lumbar				
CON	61.46± 6.73	3.88± 0.09	0.16± 0.01	0.17± 0.03
TS	50.4± 4.05 ^a	3.24± 0.17 ^a	0.15± 0.01 ^a	0.21± 0.03 ^a
tibia				
CON	59.09± 2.75	3.90± 0.21	0.15± 0.01	0.14± 0.01
TS	50.4± 6.26 ^a	3.21± 0.66 ^a	0.14± 0.01 ^a	0.19± 0.05 ^a
tailbone				
CON	39.12± 5.41	2.92± 0.17	0.13± 0.01	0.26± 0.03
TS	30.6± 6.66 ^a	2.56± 0.41	0.13± 0.01	0.31± 0.06

Values are mean± SD.

CON = control; TS = tail-suspended;

BV/TV = percent bone volume; Tb.N = trabecular number; Tb.Th = trabecular thickness; Tb.Sp = trabecular separation

^aDifferent from CON by T-test (p < 0.05)

Table 2 Correlation coefficients between parameters of IFF and bone mineral density

	r between vBMD and Ln.N	r between vBMD and Ln.S
parietal bone	0.64	0.71
ulna	0.894 ^a	0.399
lumbar	0.72 ^a	0.581
tibia	0.657 ^a	0.519
tailbone	0.51	0.56

Ln.N = lacuna's number; Ln.S = lacuna's area.

^ap<0.05

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