Original Article

Moderate tibia axial loading promotes discordant response of bone composition parameters and mechanical properties in a hindlimb unloading rat model

Peng-Fei Yang[1,2], Ling-Wei Huang[1], Xiao-Tong Nie[1,2], Yue Yang[1], Zhe Wang[1,2], Li Ren[1,2], Hui-Yun Xu[1,2], Peng Shang[1,2]

[1]Key Laboratory for Space Bioscience and Biotechnology, Institute of Special Environmental Biophysics, School of Life Sciences, Northwestern Polytechnical University, Xi'an, China; [2]Research & Development Institute in Shenzhen, Northwestern Polytechnical University, Xi'an, China

Abstract

Objectives: The purpose of the present study was to characterize the dynamic alterations of bone composition parameters and mechanical properties to disuse and mechanical intervention. **Methods:** A tail suspension hindlimb unloading model and an *in vivo* axial tibia loading model in rats were used. A moderate mechanical loading that was capable of engendering 800 με tibia strain was applied to the right tibia of rats in both control and hindlimb unloading group across 28 days of the experimental period. The contralateral tibia served as control. **Results:** Hindlimb unloading led to bone loss in tibia from day 14. Bone mineral density, mineral content and mechanical properties responded differently with microstructure to disuse in timing course. Mechanical loading of 800 με tibia strain failed to alter the bone of the control group, but minimized the detrimental effects of unloading by completely prohibiting the decrease of bone mineral content and main mechanical properties after 28 days. Less obvious influence of mechanical loading on bone microstructure was found. **Conclusions:** The moderate mechanical loading is not able to stimulate the mechanical response of healthy tibia, but indeed lead to discordant recovery of bone composition parameters and mechanical properties.

Keywords: Mechanical Loading, Bone, Disuse, Mechanical Properties, Composition Parameters

Introduction

The capability of bone to withstand and adapt to habitual mechanical loading has been well documented. Dramatic amount of mineral loss at the load-bearing bones occurs during long-term space flight[1-3] and bed rest[4,5]. On the other hand, sufficient amount of mechanical loading can prohibit bone loss during disuse[6] or enhance bone recovery during the re-loading period[7].

Bone is designed to resist body weight and mechanical load from the adjacent tissue or external substrate. Bone quality is determined by its mass and structure, as well as its mechanical properties. As the matter of fact, evidence from experiments and modelling computations suggested that the indicators of bone integrity, e.g. bone mineral density, bone mineral content, structure and mechanical properties, respond differently to mechanical environment[2,8]. Discordant dynamic adaptation of bone parameters has been observed at specific sites of bone[8]. Bone mineral content was recovered prior to bone mineral density in astronauts during one year of recovery phase after space flight. Moreover, calculated bone strength indicators were recovered slower than the other bone parameters[2]. The mis-match between the bone strength indicators and the composition parameters of bone increased the risk of mis-interpreting data from many studies.

To date, extensive studies have shown the anabolic effect of mechanical loading on bone[9,10]. Mechanical stimulation has also been taken as one of the countermeasures against bone loss[6,11]. However, the exact dynamic alterations of bone to mechanical intervention across the disuse period have not been detailed investigated. Previous study with hindlimb unloading model has suggested a discordant response of

bone parameters in rats during the recovery phase after disuse[8]. Nevertheless, it remains unclear that how the mechanical stimulation influences the discordant response of bone to abnormal mechanical environment. Whether the mechanical properties and mineral phase of bone respond equally to mechanical stimulation remains unknown. Further the current understanding of the discordant response of bone parameters to mechanical loading during disuse was one of the novelties of the present study.

The purpose of the present study was to outline the time course of bone response to disuse and mechanical stimulation. To achieve this purpose, the modulation effects of a moderate mechanical loading on bone compositional parameters and mechanical properties during disuse were investigated. A tail-suspension disuse model and an *in vivo* tibia axial loading model in rats were used. The moderate mechanical loading that engender 800 με tibia strain was determined with an *in vivo* strain gauge approach weekly and applied to the tibia of rats with the *in vivo* tibia axial loading model. Bone mineral density, bone mineral content, bone microstructure, weekly mechanical load to engender 800 με tibia strain and mechanical properties of tibia were assessed.

Materials and methods

Animals and experimental design

Seventy-five skeletal mature male Sprague-Dawley rats were obtained (The Lab Animal Center of the Fourth Military Medical University, Xi'an, Shaanxi, China) at 4-month of age and allowed to acclimate for 1 month prior to the study. All animals were given standard rodent chow and water *ad libitium* with housing in a room with temperature maintained at 22°C and 12 h light/dark cycle. The experiment and animal care were approved by the animal ethics and welfare committee of Northwestern Polytechnical University.

The animals (20 weeks old) were randomly assigned to three groups: baseline control group (BC, n=7), age-matched group (AC, n=28) and hindlimb unloading group (HU, n=40). All animals were housed individually in a same custom-made cage. The only difference between the AC and HU groups was the hindlimb loading situation of the animals. The rats in BC group were sacrificed on day 0 to harvest bone samples for further measurements. The entire experiment lasted for 28 days. During the 28-day experimental period, the left tibia of the rats in both AC and HU groups remained untreated, which was referred to AC Untreated (ACU) and HU Untreated (HUU) bone, respectively. The right tibia of the rats in both AC and HU groups was loaded 5 days/week with *in vivo* axial mechanical loading from the first day of experiment until the day of sacrifice. By doing this, the impact of mechanical loading on both healthy and disused bone can be assessed during 28-day experimental period. The right tibia of the animals from AC and HU groups was referred to AC Loaded (ACL) and HU Loaded (HUL) bone. On the experimental day 7, 14, 21 and 28, the animals (n=7 for AC group and n=10 for HU group at each time point) were sacrificed for bone sample
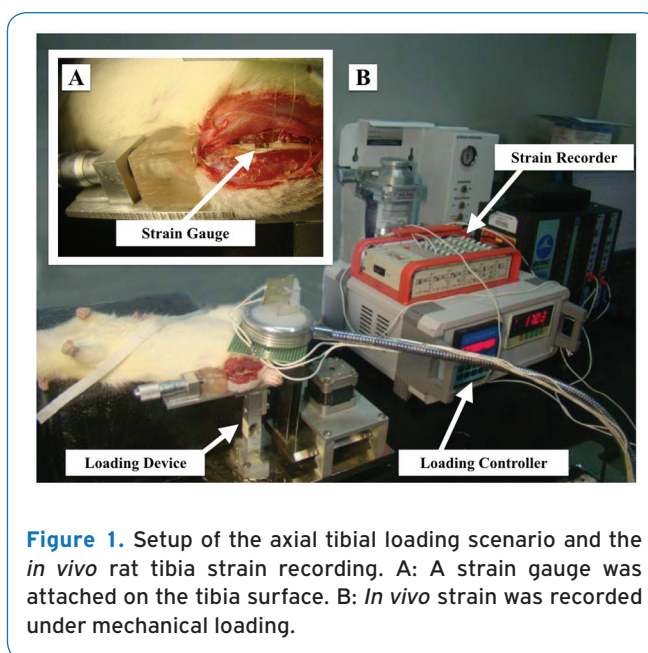

Figure 1. Setup of the axial tibial loading scenario and the *in vivo* rat tibia strain recording. A: A strain gauge was attached on the tibia surface. B: *In vivo* strain was recorded under mechanical loading.

collection of bilateral tibia and femur. The bones were cleaned to remove the adjacent soft tissue for further microCT scans and mechanical tests. Body mass was recorded weekly throughout the experiment.

Tail-suspension animal model

As previously described in our publications[7,12], a well-established tail-suspension model with 30° head down titling in rats was used to induce hindlimb disuse of the rats. The animals had free access to food and water with their forelimbs during hindlimb unloading. All HU animals were monitored twice per day for health evaluation.

Mechanical loading devices and loading protocol

A non-invasive axial tibia loading model was custom-built for the present study (Figure 1)[13]. A force sensor (L6D21, Zhonghang Electronic Measuring Instruments Co., Ltd., Hanzhong, China) was used to provide feedback to maintain the desired loading amplitude.

With anesthesia, the right tibia of the animals in AC and HU groups was preloaded with 1 N to avoid loosening during the loading procedure. One of the main aims of the present study was to assess the dynamic adaptation process of bone to mechanical loading. The present study emphasized on the modulation effects of mechanical loading on the discordant response between bone mechanical properties and structural parameters. Therefore, a moderate level mechanical loading protocol was chosen in the present study. Sinusoidal mechanical loading was applied to the right tibia of the rats at 1 Hz with force maximally engendering 800 με bone strain, 600 cycles/day, 5 days/week for 1, 2, 3, and 4 weeks, respectively. Although the adopted loading amplitude

and frequency were not very large, 800 µε bone strain is generally conceived to be within the range of physiological strain level of bone. Moreover, low loading frequency has also been used to assess the bone formation capabilities previously[14].

Strain gauge approach

On the experimental day 0, 7, 14, 21 and 28, three animals were taken from each of the AC and HU group to conduct bone strain assessments. At each chosen time point of the experiment, the loading amplitude that can engender 800 µε bone strain at the medial mid-diaphysis of the right tibia in these three animals was characterized using strain gauge approach. Briefly, with anesthesia (2% isoflurane, 1.0 L/min O_2), an approximately 1-cm incision was made over the anterior surface of the tibia. The antero-medial tibia surface was exposed and prepared by gently scraping the periosteum with a scalpel, following with the cleaning and degreasing procedure using ethanol. Wired single element strain gauge (ZF350-1AA-W-X, Zhonghang Electronic Measuring Instruments Co., Ltd., Hanzhong, China) was attached in longitudinal alignment onto the tibia surface using ethyl cyanoacrylate (Figure 1A). Strain data was amplified with an amplifier and monitored. The maximum loading amplitude was adjusted to engender 800 µε of tibia strain. The loading curve was recorded simultaneously. The peaks of the loading and strain amplitude were detected with a custom-written Matlab routine. Bone samples of the animals were collected after the strain assessments for further bone measurements.

Bone mass and structure assessments

Dual-energy x-ray absorptiometry (DEXA) (Lunar Prodigy, GE Medical Systems, Madison, WI, USA) in the small-animal mode was used to quantify bone mineral density (BMD) and bone mineral content (BMC) of hindlimb bones. On day 0, 7, 14, 21, 28, the animals to be sacrificed at different time points of the experiment (n=7 for the control group, n=10 for the hindlimb unloading group) were anesthetized using intraperitoneal injection of 3% pentobarbital sodium (1.5 ml/kg) and placed in the prone position with the hindlimb naturally extended. BMD and BMC of bilateral femur and tibia in rats were analyzed.

Left tibia from the BC and AC group and bilateral tibias from HU group (n=3) were fixed with paraformaldehydeand and stored until the microCT scans. The mineral content and microstructure of the tibia were assessed using a quantitative microCT system (Inveon Micro CT, Siemens, Germany). Diaphyseal scan of the proximal tibia was taken with 10.56 µm isotropic voxel resolution, with the X-ray tube operated at 50 kV and 200 mA, 1600 ms exposure time with a 0.5 mm aluminum filter and a focal spot size of 5 mm. The bone section with the thickness of 1 mm, located 1.5 mm below the growth plate, was selected as the region of interest for 3D reconstruction and further analyses. Bone volume fraction (BV/TV), trabecular space (Tb. Sp), trabecular thickness (Tb. Th), bone surface to bone volume ratio (BSV/BV), cortical wall thickness (CWT) and trabecular number (Tr. N) were calculated to evaluate bone structure alterations.

Mechanical testing of hindlimb bone

The mechanical properties of bilateral tibia and femur from all groups (n=3) were assessed using the conventional 3-point bending test in a universal testing machine (AGS - 10 kNG, Shimadzu, Japan). The support span was set at 20 mm. The orientation of the bone samples on the support roller was adjusted as identical as possible. Loading speed was set at 2 mm/min until bone fracture. A load - deformation curve was obtained eventually. The dimension of the bone fracture cross-section was measured using a stereo microscope to calculate the cross-section moment of inertia about the neutral axis. The Young's modulus of bone was therefore determined. Stiffness, maximum load, maximum stress and toughness of the bones were derived from the load - deformation curve or calculated accordingly.

Statistics

Data are presented as means and limits of 95% confidence interval of the mean. Statistical analyses were conducted with the GraphPad Prism software (GraphPad software, Inc., La Jolla, CA, USA). The relationship between loading amplitude and tibia strain was analyzed using linear regression. The differences on the forces to engender 800 µε of tibia strain between the loaded and contralateral tibia were assessed using unpaired student's t-test. Difference on BMD and BMC across groups and time was analyzed using two-way repeated ANOVA. Structure and mechanical properties of bone were analyzed using two-way ANOVA to identify the effects of time and group. Group difference on body weight across different time points was identified using one-way ANOVA. Statistical significance was accepted while the p value was 0.05 or less.

Results

Body mass

The body mass of the rats from the AC and HU group remained nearly constant across the experimental time period (p=0.69 for the AC group, p=0.06 for the HU group, Figure 2). There was no significant difference between the AC and HU group at each time point (p>0.99 at day 7, p=0.21 at day 14, p>0.99 at day 21, p>0.99 at day 28, Figure 2).

Force to engender 800 µε tibia strain

The mechanical load to engender 800 µε of tibia strain did not change over the experimental time period for ACL tibia (p=0.35). By contrast, dynamical changes of the mechanical load for HUL tibia were found (p=0.03). In particular, at day 14, the force amplitude to engender 800 µε of tibia strain between the ACL and HUL was statistically different (p=0.0013, Figure 3). Moreover, the required force to induce
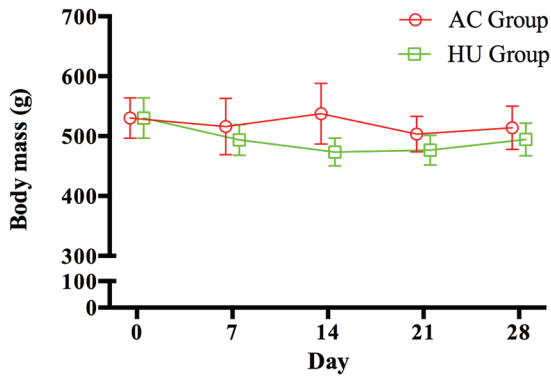
Figure 2. Body mass alteration of the animals in age-matched control (AC, n=7 at each time point) and hindlimb unloading (HU, n=10 at each time point) groups during the intervention time period. No significant change between groups was found at different time points.
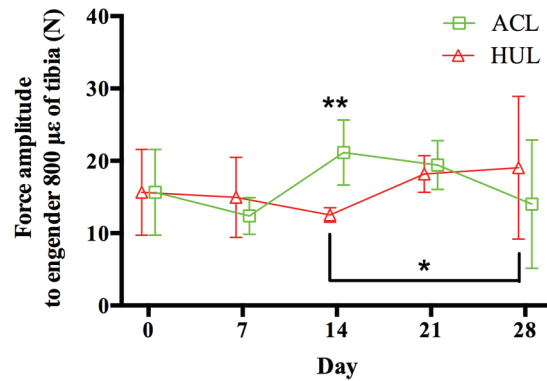

Figure 3. Force amplitude to engender 800 με of tibia strain in both control and hindlimb unloading group. ACL: tibia with mechanical loading from the age-matched control group (n=3 at each time point). HUL: tibia with mechanical loading from the hindlimb unloading group (n=3 at each time point). *: $p<0.05$, **: $p<0.01$.

the anticipate tibia strain was the larger at day 28 than day 14 ($p=0.039$).

Bone mineral density of tibia

Significant difference on BMD and BMC of tibia among groups was only found after day 7 (Figure 4). Statistical analysis yielded main and interaction effects of the group and time on BMD of tibia ($p=0.045$ for group, $p=0.0010$ for time, $p=0.0086$ for the interaction of time and group). At day 14, BMD of both HUU and HUL tibia was lower than ACU ($p=0.0023$ and $p=0.039$, respectively). Likewise, BMD of HUU tibia was lower than ACL tibia ($p=0.043$). Similar trend remained until day 21. At day 21, BMD of ACU tibia was still higher than the HUU ($p=0.048$) and HUL bone ($p=0.0081$). At day 28, BMD of HUL tibia was significantly lower than ACU ($p=0.0015$) and ACL bone ($p=0.024$). Across 28 days, no significant difference on BMD between HUU and HUL tibia was found ($p=0.93$, Figure 4A). Significant effects of time on BMD was only found in HUU tibia ($p=0.025$ for Day 0 v.s. Day 7, $p=0.013$ for Day 7 v.s. Day 14). There was no significant main and interaction effects of group and time on BMC ($p=0.052$ for group, $p=0.16$ for time, $p=0.056$ for the interaction of time and group). BMC of HUU tibia was the lowest in all groups at day 14 ($p=0.0001$ v.s. ACU, $p=0.0001$ v.s. ACL and $p=0.020$ v.s. HUL). At day 21, BMC of both HUU and HUL tibia were lower than ACU bone ($p=0.041$). No difference on BMC among groups was found at day 28 (Figure 4B).

Bone structure alteration: microCT analyses

Considering that there is no significant difference on BMD and BMC between ACU and ACL across the entire experiment, the ACL tibia was excluded from the microstructure analyses. The microstructure of the ACU, HUU and HUL tibia was further analyzed. Statistical analysis
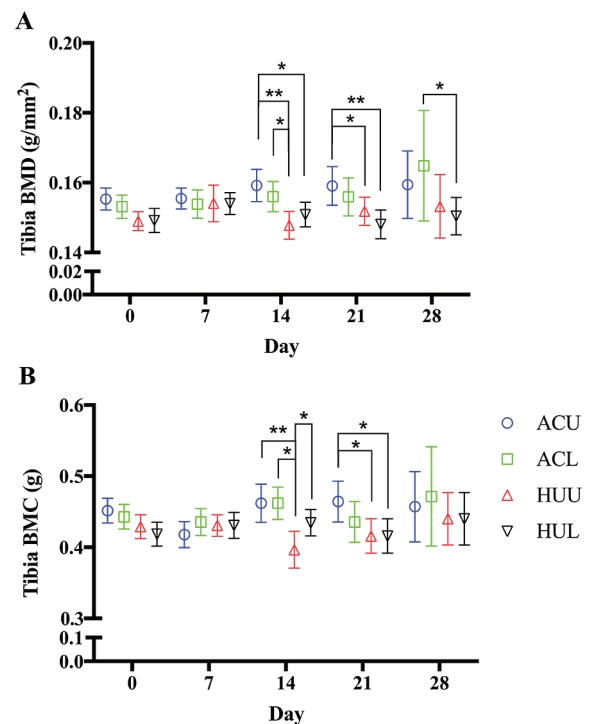

Figure 4. Bone mineral density (A) and bone mineral content (B) changes of tibia during the intervention. ACU: untreated tibia from the age-matched control group (n=7 at each time point). HUU: untreated tibia from the hind limb unloading group (n=10 at each time point). ACL: tibia with mechanical loading from the age-matched control group (n=7 at each time point). HUL: tibia with mechanical loading from the hindlimb unloading group (n=10 at each time point). *: $p<0.05$, **: $p<0.01$, ***: $p<0.01$.
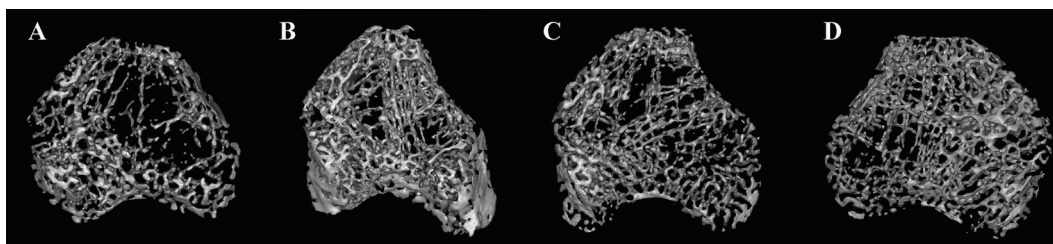
Figure 5. 3D microstructure of trabecular bone in the proximal tibia at day 14. A: Baseline, B: untreated tibia from the age-matched control group (ACU), C: untreated tibia from the hindlimb unloading group (HUU), D: tibia with mechanical loading from the hindlimb unloading group (HUL).

Table 1. Microstructure alteration of the proximal tibia during the intervention time period (n=3).

Variable	Day/Group	ACU	HUU	HUL
BV/TV (%)	Baseline	24 (22, 27)		
	Day 7	32 (23, 42)	33 (23, 43)	32 (22, 43)
	Day 14	34 (14, 54)	25 (17, 33)*	24 (16, 32)*
	Day 21	27 (13, 41)	27 (15, 39)	25 (14, 36)
	Day 28	29 (17, 41)	25 (12, 38)	24 (14, 34)
Tb. Sp (mm)	Baseline	0.20 (0.17, 0.23)		
	Day 7	0.14 (0.11, 0.18)	0.14 (0.07, 0.22)	0.14 (0.08, 0.20)
	Day 14	0.14 (0.06, 0.22)	0.19 (0.13, 0.24)*	0.20 (0.13, 0.26)*
	Day 21	0.18 (0.09, 0.26)	0.18 (0.08, 0.27)	0.19 (0.08, 0.29)
	Day 28	0.17 (0.10, 0.24)	0.18 (0.11, 0.26)	0.19 (0.11, 0.26)
BSA/BV (%)	Baseline	31 (29, 32)		
	Day 7	30 (24, 36)	29 (28, 30)	30 (27, 32)
	Day 14	28 (17, 39)	32 (29, 35)	32 (28, 37)
	Day 21	32 (24, 39)	31 (26, 37)	33 (31, 36)
	Day 28	29 (24, 35)	33 (24, 43)*	34 (28, 40)*
Tb. Th (mm)	Baseline	0.065 (0.062, 0.068)		
	Day 7	0.067 (0.054, 0.081)	0.069 (0.066, 0.072)	0.068 (0.061, 0.074)
	Day 14	0.072 (0.046, 0.098)	0.062 (0.057, 0.068)*	0.062 (0.053, 0.070)*
	Day 21	0.064 (0.048, 0.080)	0.064 (0.054, 0.075)	0.060 (0.056, 0.064)
	Day 28	0.069 (0.056, 0.082)	0.060 (0.043, 0.078)	0.059 (0.049, 0.07)
CWT (mm)	Baseline	0.55 (0.52, 0.58)		
	Day 7	0.50 (0.44, 0.57)	0.57 (0.51, 0.63)	0.55 (0.43, 0.67)
	Day 14	0.53 (0.44, 0.63)	0.52 (0.38, 0.66)	0.51 (0.43, 0.59)
	Day 21	0.54 (0.41, 0.67)	0.53 (0.41, 0.66)	0.52 (0.38, 0.66)
	Day 28	0.56 (0.54, 0.58)	0.54 (0.49, 0.60)	0.55 (0.45, 0.65)
Tr. N	Baseline	3.8 (3.3, 4.3)		
	Day 7	4.6 (4.0, 5.2)	4.8 (3.1, 6.5)	4.8 (3.6, 5.9)
	Day 14	4.7 (3.5, 5.8)	4.1 (3.2, 4.9)	3.9 (3.0, 4.8)
	Day 21	4.2 (2.9, 5.5)	4.2 (2.5, 5.8)	4.1 (2.5, 5.7)
	Day 28	4.2 (3.0, 5.3)	4.1 (3.1, 5.2)	4.1 (3.0, 5.2)

*ACU: untreated tibia from the age-matched control group. HUU untreated tibia from the hindlimb unloading group. HUL: tibia with mechanical loading from the hindlimb unloading group. BV/TV: Bone volume fraction, BSA/BV: Bone surface area to bone volume ratio, Tb. Sp: Trabecular Space, Tb. Th: Trabecular thickness, CWT: Cortical Wall Thickness, Tr. N: Trabular Number. *: comparison with the ACU bone at the same time point. *: p < 0.05, ***: p < 0.001.*

Table 2. Macro-mechanical properties of tibia during the intervention time period (n=3).

Variable	Day	ACU	ACL	HUU	HUL
E (MPa)	Baseline	3635(2173, 5097)			
	Day 7	4325 (2969, 5681)	4038 (3815, 4260)	4661 (4028, 5293)	5272 (3362, 7217)
	Day 14	4889 (3115, 6664)	4673 (2300, 7045)	3800 (863, 6737)*##	3605 (2391, 4819)##
	Day 21	5482 (4392, 6572)	4215 (2338, 6093)	3981 (3469, 4493)*	4216 (2111, 6320)
	Day 28	4132 (3674, 4590)	4732 (2572, 6892)	3754 (2638, 4870)#	4562 (1194, 7930)
Stiffness (N/mm)	Baseline	85 (36, 134)			
	Day 7	96 (67, 126)	85 (58, 112)	78 (71, 85)	90 (52, 129)
	Day 14	119 (96, 143)	109 (84, 133)	62 (36, 88)***###	128 (69, 186)+++
	Day 21	117 (93, 141)	103 (87, 119)	101 (96, 107)*	110 (67, 154)
	Day 28	110 (91, 130)	120 (60, 180)	89 (81, 98)*	104 (98, 110)++
M. Load (N)	Baseline	62 (54, 70)			
	Day 7	60 (59, 62)	60 (43, 77)	69 (46, 92)	73 (45, 101)
	Day 14	68 (53, 83)	65 (53, 78)	55 (44, 66)	53 (35, 70)
	Day 21	67 (55, 78)	64 (58, 69)	62 (51, 73)	76 (49, 104)
	Day 28	74 (69, 80)	82 (61, 103)	59 (49, 70)*#	55 (51, 58)*##
M. Stress (MPa)	Baseline	70 (64, 76)			
	Day 7	66 (55, 77)	70 (54, 87)	73 (54, 91)	69 (40, 99)
	Day 14	68 (56, 81)	65 (61, 70)	61 (47, 74)	57 (42, 72)
	Day 21	65 (54, 76)	62 (53, 72)	68 (55, 81)	73 (46, 100)
	Day 28	72 (50, 94)	67 (45, 89)	72 (35, 110)	89 (38, 139)
Toughness (J)	Baseline	0.068 (0.059, 0.077)			
	Day 7	0.081 (0.072, 0.090)	0.073 (0.043, 0.100)	0.093 (0.063, 0.120)	0.083 (0.056, 0.110)
	Day 14	0.087 (0.078, 0.097)	0.078 (0.066, 0.090)	0.072 (0.061, 0.084)*	0.058 (0.024, 0.092)*#
	Day 21	0.083 (0.065, 0.100)	0.081 (0.062, 0.099)	0.076 (0.053, 0.100)	0.069 (0.039, 0.099)
	Day 28	0.094 (0.062, 0.130)	0.091 (0.061, 0.120)	0.100 (0.048, 0.150)	0.070 (0.037, 0.100)+

ACU: untreated tibia from the age-matched control group. HUU untreated tibia from the hindlimb unloading group. ACL: tibia with mechanical loading from the age-matched control group. HUL: tibia with mechanical loading from the hindlimb unloading group. E: Elastic Modulus. M. Load: maximum load. M. Stress: maximum stress. *: comparison to ACU bone at the same time point. *: p<0.05, **: p<0.01, ***: p<0.001. #: compare to ACL bone at the same time point. +: comparison to HUU bone at the same time point.

identified main effects of time on BV/TV ($p=0.0053$), Tb. Sp ($p=0.0029$) and Tb. N ($p=0.0029$). Main effects of group on BSV/BV ($p=0.042$) and Tb. Th ($p=0.031$) were found as well. No interaction effects of time and group on any structural parameter were yielded. At day 14, BV/TV of HUU and HUL tibia was lower than ACU ($p=0.047$ and $p=0.019$). By contrast, Tb. Sp of HUU and HUL tibia was larger than ACU ($p=0.017$ and $p=0.045$). Tb. Th of HUU and HUL tibia was less than ACU tibia ($p=0.037$ and $p=0.028$). BSV/BV of HUL tibia was larger than ACU tibia at day 28 ($p=0.040$). No significant difference on CWT and Tr. N was found among groups at the chosen time points (Figure 5, Table 1).

Mechanical properties of tibia

The main effects of time on elastic modulus ($p=0.016$), stiffness ($p<0.0001$) and toughness ($p=0.017$) of tibia were identified. Main effects of group on stiffness ($p<0.0001$) and toughness ($p=0.032$) of tibia were also found. Elastic modulus of tibia decreased by 22.3% ($p=0.043$) and 27.3% ($p=0.043$) after 14 and 21 days of disuse (HUU) than the

control (ACU), respectively. No significant difference on elastic modulus between HUL and ACU tibia were found at day 14 ($p=0.13$), although with up to 26.2% decrease in HUL tibia. At day 14, the elastic modulus of HUU and HUL tibia were lower than ACL by 18.7% ($p=0.0070$) and 22.9% ($p=0.0052$). At day 28, the elastic modulus of HUU tibia was lower than ACU tibia ($p=0.029$). Disuse lead the reduction of tibia stiffness by 47.9% than ACU ($p<0.0001$) at day 14, by 13.7% ($p=0.025$) at day 21 and by 19.1% ($p=0.023$) at day 28. At day 14 and 28, HUL tibia stiffness was 51.6% ($p<0.0001$) and 14.4% ($p=0.031$) larger than HUU. At day 28, maximum load of HUU and HUL tibia was 20.3% and 25.7% lower than ACU ($p=0.019$ and $p=0.045$) and ACL ($p=0.013$ and $p=0.0023$). No significant difference on maximum stress was found among the groups. Disuse decreased the tibia toughness by 17.2% than ACU ($p=0.019$) at day 14. At day 14, HUL tibia toughness was 33.3% ($p=0.037$) lower than ACU. HUL tibia toughness was 25.6% ($p=0.025$) lower than ACL as well. At day 28, HUL tibia toughness was 30% lower ($p=0.034$) than HUU tibia (Table 2).

Discussion

In this paper, we found that the moderate mechanical stimulation of 800 με bone strain failed to affect the tibia in the control group over 28 days, but completely prohibited the decrease of bone mineral content and mechanical weakness of the tibia after 28 days. Interestingly, bone mineral phase, microstructure and mechanical properties responded differently to mechanical loading in timing course during the experimental period,. Discordant recovery of bone composition parameters and mechanical properties was induced by the moderate mechanical loading.

Disuse induced bone mineral loss has been well documented in both animal and human models[15,16]. As expected, the present study found that tail suspension leads to bone loss in tibia from as early as day 14 and last until day 21 (Figure 4). Similar trend of femur responses to disuse was also indicated (Supplementary Materials). Surprisingly, the effects of tail suspension on the mineral composition of tibia disappeared on day 28. Bone mineral alterations are generally accompanied with structural changes. However, obvious microstructure changes of the proximal tibia were only found on day 14 in the present study (Table 1, Supplementary Figure 2). Recent studies have suggested that the mechanical properties of bone respond differently with mineral composition to abnormal mechanical environment[8]. Similar discordant response between bone structure and the estimated bone strength has also been observed in the femur of astronauts[2]. Results from the present study suggested that the main mechanical properties of tibia respond similarly with bone mineral phase and structure to tail suspension disuse. The difference was that tibia microstructure seems to be only affected at day 14 and is barely affected by either tail suspension or mechanical loading at the other chosen time points (Table 1), which seems to be inconsistent with previous studies[13,17]. This might due to limited sample size. Larger sample size for microstructure assessments in the future study might be necessary to clarify the present observations.

The moderate axial mechanical loading adopted in this study was capable of maintaining bone mineral compositions and mechanical integrity to certain extent (Figure 4). The decrease of BMC and BMD induced by disuse was completely prevented by mechanical stimulation in both tibias of tail suspension mice after 28 days (Figure 4B). However, no significant impact of mechanical loading on the microstructure of bone was found. By contrast, mechanical weakness of tibia was fully prevented by mechanical loading across the entire experimental period (Table 2), which was shown by the results of strain gauge measurements as well. The amplitude of mechanical load to generate 800 με of tibia strain was lower at day 14 (Figure 3). This trend was reversed by weekly mechanical stimulation on tibia after day 14. These results clearly showed an incoherent response of bone composition parameters, microstructure and mechanical properties to mechanical loading. Previous study showed that the mechanical properties of mice tibia respond differently with the geometrical changes to two weeks of mechanical loading[18]. However, to date, little was known regarding whether and how the mechanical competence of bone responds differently with composition parameters during disuse and mechanical stimulation. One of the novel findings in the present study was that mechanical loading seems to rescue mechanical properties of tibia ahead of bone mineral compositions and structure during disuse. Although bone mineral content or bone mineral density is the commonly used indicator of bone quality, the ultimate goal of maintaining or increasing BMC or BMD induced by disuse is to decrease the fracture risk, or in other words, improving mechanical competence of bone. The present results therefore implied that mechanical loading during disuse may contribute to restore bone integrity much more than what is commonly thought.

It has been well accepted that bone mineral composition can not fully represent bone quality or bone mechanical competence[19], which was further supported with the present evidence. In detail, the elastic modulus and stiffness of the loaded tibia in the group of tail suspension were larger than the non-loaded tibia across most of the experimental time. It may well indicate the discordant recovery response between bone mineral phase and its mechanical properties. It is not surprising because the mineral phase is only one of the main constituents in bone matrix. Traditionally, bone mineral density or bone mass are used as the standard measure for assessing bone quality. However, it lacks specificity to do that by using mineral quantity alone. Type I collagen, non-collagenous proteins and water play significant roles in determining the mechanical competence of bone tissue[20]. In particular, collagen has been shown to be vital in determining mechanical properties of bone[21,22], as the soft collagen assigns bone its ability of dissipating energy under deformation. The toughness of bone correlates tightly with the collagen network in bone[23,24]. These non-mineralized constituents can not be identified with the X-ray based CT scanning techniques. The present results indicated that the toughness of tibia was impaired by disuse. Mechanical loading failed to rescue the attenuation of toughness in tibia induced by disuse (Table 2). Moreover, the alterations of toughness and bone mineral content or bone mineral density remained closely in timing. It may well indicated that the collagen network has been altered with the losing of mineral crystals of hydroxyapatite deposited on the collagen. Moreover, the quality of cortical bone, e.g. bone porosity, is also crucial for determining the mechanical properties of bone. Further analysis on cortical bone properties would be necessary in the future studies. More detailed studies are expected to draw a firm conclusion on the response of collagen network to disuse and how it may relate to the macro-scale tissue behavior of bone. It would be of interest to understand the mechanisms underlying the present observations with future investigation.

Comparatively, the main mechanical properties of femur were slightly improved by the mechanical loading after 28-day (Supplementary Materials, Suppl.Table 1). Given that the axial mechanical loading primarily acts on tibia rather

than femur, the distinct response of the femur and tibia was reasonable and may partially due to the site-specific changes of bone to mechanical unloading, which was consistent with the previous findings[25]. Another interesting observation of the present study was that mechanical loading did not change the structure and mechanical properties of the loaded tibia of the control group. It was inconsistent with the previous findings in mice with greater tibia compressive loading[17,26]. The relatively low level of mechanical loading adopted in the present study might be one of the explanations for such response. In the present study, the contralateral bone, e.g. the non-loaded tibia in tail suspension rats, seems to be influenced by mechanical loading as well (Figure 4). Previous research showed that the mechanical adaptation process is confined to the loaded bones[27]. By contrast, previous studies concluded that remote anabolic response to mechanical loading also exists in the contralateral control bone, while a single bone is loaded[28]. It is still controversial regarding whether the interplay between the unilateral bone and contralateral bone occurs during the mechanical adaptation process[13,28,29], although using the contralateral bone as internal control to investigate the mechanical adaptation of bone has become a common practice in many studies[30]. For clarifying this issue, an additional control group that only experienced tail-suspension hindlimb unloading will be needed in the future studies.

In the present study, bone strain with the amplitude of 800 με remained in the range of physiological strain. The present findings suggested that this moderate mechanical loading failed to increase the capability of tibia to resist external loading for the control bone. The limited amplitude of bone strain might be one of the causes for such consequences. However, besides strain amplitude, loading cycles, frequency and loading type were also conceived to be anabolic and be able to maintain bone integrity[14,31]. Bone strain amplitude was not the only parameter to determine the mechano-response of bone[32]. Furthermore, the main focus of the present research was to provide insights on the dynamic response of bone to mechanical loading, rather than assessing the potential anabolic effects of mechanical loading itself. From this point of view, the initial intent of the present research has been achieved. As discussed above, one of the shortcoming of the present study might be the relatively small sample size. Although the measurement error remained at a low level (Supplementary Materials, Suppl. Table 2, 3 and Suppl. Figure 2), larger sample size for the assessments of bone parameters and the effects of mechanical loading on bone adaptation might greatly enhance the statistical power and help to draw a firm conclusion in the future studies.

In summary, the present results suggested that tail-suspension induced disuse impaired tibia integrity from several different aspects, which was congruent with previous findings. Weekly mechanical stimulation of 800 με tibia strain did not alter the mechanical response of tibia to loading for the control animals, but was capable of prohibiting most of the detrimental effects of unloading on bone. The response of the bone mineral composition, structure and mechanical properties show certain discordant in timing. Mechanical weakness of tibia induced by disuse was prevented ahead of bone mineral composition. Therefore, we conclude that moderate mechanical loading is not able to stimulate the mechanical response of healthy tibia, but indeed promote discordant recovery of different components indicating bone integrity from disuse.

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Supplementary Materials

*Bone mineral density and bone mineral content of femur*

Significant difference on BMD and BMC of femur among groups was only found at the chosen time points after day 14. The alteration trend of femur BMD and BMC remained similar across the intervention time period. Statistical analysis yielded main effects of time and interaction effects of time and group on BMD of femur ($p<0.0001$ for time, $p=0.79$ for group, $p=0.026$ for the interaction of time and group). BMD of HUU ($p=0.035$) and HUL ($p=0.023$) femur were lower than ACU at day 21. Significant effects of time on BMD was found in ACU femur ($p=0.0092$ for Day 0 v.s. Day 21, $p=0.043$ for Day 0 v.s. Day 28) and HUL femur ($p=0.039$ for Day 7 v.s. Day 21, $p=0.0016$ for Day 7 v.s. Day 28, $p=0.039$ for Day 14 v.s. Day 28). No significant main effects and interaction effects of time and group on BMC of femur were observed ($p=0.099$ for time, $p=0.36$ for group, $p=0.60$ for the interaction of time and group). BMC of ACU femur was larger than HUU ($p=0.023$) and HUL ($p=0.0039$) femur at day 21 and HUL femur at day 28 ($p=0.025$). BMC of ACU femur increased after 21 ($p=0.042$) and 28 days ($p=0.042$).

Mechanical properties of femur

Main effects of time and group on fracture load ($p=0.0006$ for time and $p=0.021$ for group) of femur were observed. Main effects of group on maximum load ($p=0.030$) were also identified. Disuse failed to reduce the elastic modulus of femur across the intervention time period. The elastic modulus of ACL femur was 40.4% larger than ACU femur ($p=0.048$) and 35.6% higher than HUL ($p=0.047$) at day 28. At day 28, the elastic modulus of HUL femur was higher than ACU bone ($p=0.023$). By contrast, disuse lead significant decrease of femur stiffness by 16.7% at day 14 (ACU v.s. HUU, $p=0.028$). HUL femur stiffness decreased by 13.5% and 7.3% than ACU at day 7 ($p=0.74$) and day 14 ($p=0.96$), respectively. Disuse significantly decreased the maximum load of HUU femur at day 7 ($p=0.037$) and 14 ($p=0.027$). The maximum load of the HUL femur was lower than ACU femur at day 14 ($p<0.0001$) and 28 ($p=0.037$). No significant difference on maximum stress of femur among groups was found. Significant reduction on the fracture load were found in HUU femur at day 7 ($p=0.015$), 14 ($p=0.041$) and 28 ($p=0.037$). At day 7, mechanical loading increased the fracture load of HUL femur by 31.5% ($p=0.014$) than HUU femur. No significant difference on femur fracture stress and toughness was found among the groups.
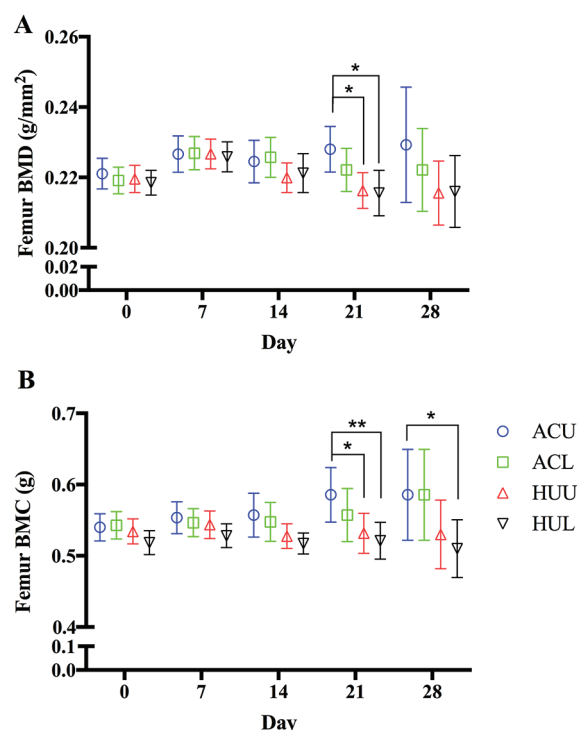
Suppl. Figure 1. Bone mineral density (A) and bone mineral content (B) changes of femur during the intervention. ACU: untreated femur from the age-matched control group. HUU untreated femur from the hind limb unloading group. ACL: femur with mechanical stimulation from the age-matched control group. HUL: femur with mechanical stimulation from the hind limb unloading group. *: $p<0.05$, **: $p<0.01$.
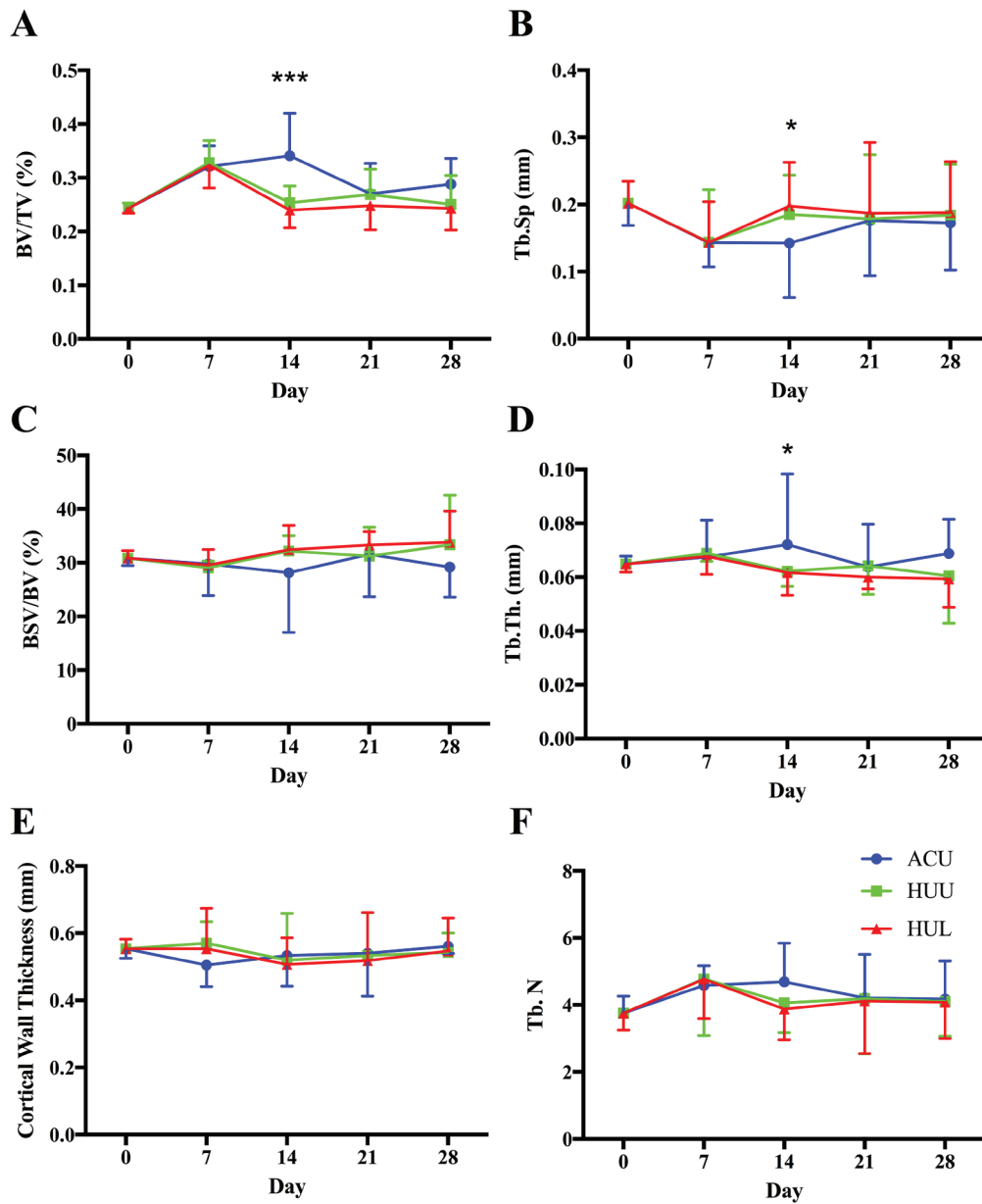
Suppl. Figure 2. Microstructure alteration of the proximal tibia over the time course. ACU: untreated femur from the age-matched control group. HUU untreated femur from the hind limb unloading group. HUL: femur with mechanical stimulation from the hind limb unloading group. BV/TV: Bone volume fraction, Tb. Sp: Trabecular Space, Tb. Th: Trabecular thickness, CWT: Cortical Wall Thickness, Tr. N: Trabecular Number. *: $p<0.05$, ***: $p<0.001$.

Suppl. Table 1. Mechanical property alterations of femur during the intervention time period.

Variable	Day	ACU	ACL	HUU	HUL
E (MPa)	Baseline	2115 (-602, 4832)			
	Day 7	1803 (463, 3143)	1792 (813, 2771)	1987 (691, 3283)	2265 (727, 3803)
	Day 14	1858 (466, 3249)	2336 (501, 4171)	1598 (1161, 2034)	1862 (430, 3293)
	Day 21	2141 (1340, 2941)	2358 (1580, 3137)	2099 (999, 3198)	1885 (559, 3211)
	Day 28	1881 (1627, 2135)	2641 (1767, 3515)*	1845 (867, 2823)	1948 (432, 3464)*#
Stiffness (N/mm)	Baseline	242 (71, 412)			
	Day 7	289 (227, 350)	298 (227, 370)	253 (184, 322)	250 (229, 270)
	Day 14	262 (188, 336)	288 (140, 436)	218 (206, 229)**	243 (135, 350)
	Day 21	280 (163, 398)	271 (182, 360)	274 (254, 294)	244 (188, 301)
	Day 28	274 (153, 396)	324 (146, 502)	284 (179, 389)	251 (100, 403)
M. Load (N)	Baseline	143 (53, 234)			
	Day 7	164 (140, 188)	165 (119, 211)	145 (134, 156)*	150 (143, 157)
	Day 14	157 (154, 160)	149 (114, 184)	127 (104, 150)*	130 (102, 158)***
	Day 21	163 (119, 208)	157 (118, 197)	150 (121, 180)	139 (129, 148)
	Day 28	163 (100, 226)	160 (113, 207)	142 (126, 159)##	136 (126, 146)*###
M. Stress (MPa)	Baseline	74 (35, 114)			
	Day 7	74 (40, 108)	68 (52, 84)	75 (52, 98)	74 (50, 99)
	Day 14	74 (67, 81)	70 (59, 80)	64 (35, 92)	68 (37, 100)
	Day 21	86 (62, 110)	81 (57, 105)	76 (50, 101)	69 (63, 75)
	Day 28	69 (46, 92)	75 (42, 109)	77 (61, 92)	64 (35, 93)
F. Load (N)	Baseline	100 (71, 128)			
	Day 7	131 (96, 167)	130 (118. 142)	98 (93, 103)*###	143 (127, 158)+
	Day 14	112 (63, 161)	99 (31, 168)	74 (47, 100)*	102 (67, 137)
	Day 21	118 (54, 181)	123 (80, 167)	122 (55, 190)	107 (59, 156)
	Day 28	106 (82, 131)	135 (68, 203)	98 (42, 155)*	114 (95, 133)*++
F. Stress (MPa)	Baseline	52 (26, 79)			
	Day 7	59 (48, 69)	54 (41, 67)	51 (35, 67)	70 (56, 85)
	Day 14	53 (32, 74)	46 (17, 75)	37 (12, 63)	53 (35, 71)
	Day 21	62 (28, 96)	64 (32, 96)	62 (19, 109)	54 (24, 84)
	Day 28	46 (15, 77)	64 (31, 96)	45 (41, 49)	54 (21, 87)
Toughness (J)	Baseline	0.15 (-0.15, 0.46)			
	Day 7	0.20 (0.11, 0.28)	0.21 (-0.047, 0.47)	0.26 (0.19, 0.34)	0.17 (0.047, 0.30)
	Day 14	0.20 (0.13, 0.27)	0.21 (0.17, 0.25)	0.13 (0.011, 0.25)	0.13 (0.04, 0.23)
	Day 21	0.22 (-0.024, 0.46)	0.19 (0.14, 0.24)	0.21 (0.016, 0.40)	0.19 (0.089, 0.29)
	Day 28	0.20 (0.043, 0.35)	0.14 (0.089, 0.20)	0.27 (-0.068, 0.61)	0.14 (0.073, 0.21)

ACU: untreated femur from the age-matched control group. HUU: untreated femur from the hind limb unloading group. ACL: femur with mechanical stimulation from the age-matched control group. HUL: femur with mechanical stimulation from the hind limb unloading group. E: Elastic Modulus. M. Load: maximum load. M. Stress: maximum stress. F. Load: fracture load. F. Stress: fracture stress. *: comparison to ACU bone at the same time point. *: p<0.05, **: p<0.01, ***: p<0.001. #: compare to ACL bone at the same time point. +: comparison to HUU bone at the same time point.

Suppl. Table 2. Standard Error of Mean (SEM) of the bone mineral density (BMD) and bone mineral content (BMC) of tibia during the experiment.

Variable	Day	ACU	ACL	HUU	HUL
BMD (g/mm²)	Baseline	0.004	0.003	0.003	0.002
	Day 7	0.003	0.003	0.003	0.002
	Day 14	0.003	0.002	0.004	0.003
	Day 21	0.003	0.003	0.003	0.002
	Day 28	0.004	0.006	0.004	0.002
BMC (g)	Baseline	0.009	0.018	0.013	0.020
	Day 7	0.009	0.018	0.013	0.021
	Day 14	0.013	0.018	0.031	0.016
	Day 21	0.013	0.020	0.013	0.013
	Day 28	0.020	0.029	0.016	0.016

ACU: untreated femur from the age-matched control group. HUU: untreated femur from the hind limb unloading group. ACL: femur with mechanical stimulation from the age-matched control group. HUL: femur with mechanical stimulation from the hind limb unloading group.

Suppl. Table 3. Standard Error of Mean (SEM) of the mechanical properties of tibia during the experiment.

Variable	Day	ACU	ACL	HUU	HUL
E (MPa)	Baseline	340			
	Day 7	437	52	147	303
	Day 14	412	551	683	282
	Day 21	253	436	119	489
	Day 28	144	679	259	783
Stiffness (N/mm)	Baseline	40			
	Day 7	14	17	26	4.7
	Day 14	17	34	2.7	25
	Day 21	27	21	4.6	13
	Day 28	28	41	24	35
M. Load (N)	Baseline	3.2			
	Day 7	0.46	5.2	7.3	10
	Day 14	4.7	4.0	3.4	4.0
	Day 21	3.6	1.8	3.6	9.9
	Day 28	1.8	6.5	2.4	1.2
M. Stress (MPa)	Baseline	2.5			
	Day 7	3.4	5.3	5.9	9.3
	Day 14	3.9	1.4	4.3	3.5
	Day 21	3.5	3.1	4.1	9.8
	Day 28	6.9	5.1	2.9	16
Toughness (J)	Baseline	0.070			
	Day 7	0.020	0.060	0.018	0.029
	Day 14	0.017	0.0099	0.028	0.022
	Day 21	0.056	0.012	0.044	0.023
	Day 28	0.035	0.013	0.079	0.016

ACU: untreated tibia from the age-matched control group. HUU untreated tibia from the hindlimb unloading group. ACL: tibia with mechanical loading from the age-matched control group. HUL: tibia with mechanical loading from the hindlimb unloading group. E: Elastic Modulus. M. Load: maximum load. M. Stress: maximum stress.