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Original Article

The hypobaric hypoxia environment impairs bone strength and quality in rats

Wei Wang[1,2*], Zhe Yun[1,2*], Hua-Zhi Peng[1*], Shi-Ju Yan[1], Hong-Tao Zhang[1], Xiu-Chun Qiu[1], Yan-Hua Wen[1], Hua Long[1], Bao-An Ma[1]

[1]Department of Orthopedic Surgery, Tangdu Hospital, Fourth Military Medical University, Xi'an, Shaanxi, People's Republic of China; [2]Department of Orthopedic Surgery, The Fourth Hospital of PLA, Xining, Qinghai, People's Republic of China. [*]Equal contributors.

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Abstract: *Introduction:* Several studies report that bone development and regeneration at high altitude are different from those at low altitude. However, the conclusions are inconsistent. This study was designed to elucidate the effects of a hypobaric hypoxia environment on bone strength and quality. *Methods:* A total of 8 twelve-week-old male Sprague-Dawley rats were housed in different environments: hypobaric hypoxic (HX, n=4) and normobaric normoxic (NX, n=4). All rats were euthanized for examination at 21 d of the experimental period. The body weights were registered; the haematological values were counted; and the femurs were dissected for micro-CT, histological observation, and biomechanical testing. *Results:* After the 21-d treatment, the variation of body weights and haematological values confirmed that the simulative hypobaric hypoxia environment was effective, and the rats in the HX group had physiologically adapted to it. The micro-CT realistic 3D images and the histological observation showed that the HX group exhibited less trabecular bone in the distal metaphysis of their femurs. A quantitative analysis of the micro-CT data further validated this observation; rats in the HX group had lower trabecular bone mineral density (BMD), bone volume (BV/TV), trabecular thickness (Tb. Th), and trabecular number (Tb. N) and higher bone surface density (BS/TV) and trabecular separation (Tb. Sp) in the distal metaphysis of their femurs. The biomechanical properties (maximum load, stiffness and Young's modules) of the femoral diaphysis were impaired by the hypoxic environment. *Conclusion:* These findings reveal that the hypobaric hypoxia environment negatively influenced bone strength and quality in rats.

Keywords: High altitude, hypobaric hypoxia, bone strength, bone quality

Introduction

High altitude environments exist in the three largest high altitude regions (Qinghai-Tibetan Plateau, Andean Altiplano, and Semien Plateau of Ethiopia) and mountains around the globe. This environment is characterized by severe cold, dry air, high wind, ionizing radiation, and primarily, hypobaric hypoxia.

Although high altitude environment is a minor region on Earth, more than 140 million people live above 2500 m, and millions more visit regions of high altitude every year [1]. Some high altitude populations (live permanently) have lived for hundreds of generations in this unique environment, and they generally have different physiological mechanisms than low altitude populations. In addition, altitude acclimatization has been an important subspecialty of high altitude medicine. As a consequence of the globalization process, human migration has accelerated. More people enter high altitude for political, economic, military, or other reasons. Sudden ascents to high altitude without the benefits of acclimatization may induce significant variations in the respiratory, cardiovascular, and haematology system primarily to improve O_2 transport and utilization, and these are the focus of traditional high altitude medicine.

The influence of high altitude is extensive and profound for an incomer from low altitude. There have recently been some reports indicating that bone development and regeneration at

high altitude are different than low altitude. However, the conclusions have been inconsistent. The current prevailing viewpoint is that the negative effects on the bone mass, the structural properties of bone tissue, the biomechanical behaviour, and bone repair are consistent with decreased body weight in hypobaric hypoxia environments [2-5]. However, a converse view is that hypoxia has a positive influence on the bone tissue and can accelerate bone healing [6-8]. Notably, to date, the evidence is insufficient to draw a definite conclusion whether bone is impaired by hypobaric hypoxia and should result in medical treatment. This study was designed to clarify the variations of bone induced by the hypobaric hypoxia environment.

Materials and methods

Animals and experimental design

All procedures were approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University. Twelve-week-old male Sprague-Dawley rats (320-383 g, provided by Experimental Animal Center of The Fourth Military Medical University) were used as the experimental subjects. A total of 8 rats were randomly divided from random numbers tables into two groups: hypobaric hypoxic (HX, n=4) and normobaric normoxic (NX, n=4). 4 rats in each group were housed in a stainless steel cages (485×350×200 mm) and allowed free access to water and a standard pelleted chow diet. Hypobaric hypoxia was induced by a controlled atmosphere animal chamber (9000×3200×3200 mm, Guizhou Fenglei Oxygen Cabin Co, Guizhou, China) in which the air pressure was maintained at 48.5 kPa and pressurized to a level corresponding to an altitude of 6000 m. The cage in which the 4 rats of HX group were housed was moved into the animal chamber. Hypobaric hypoxia was interrupted for 40 minutes every day to renew the food and water and clean the animal cages. The air pressure of the chamber altered moderately in the 6 m/s when the lower pressure in the chamber was interrupted. The cage in which 4 rats of the NX group were housed was placed in the animal room with normal atmosphere (96.4 kPa and an altitude of 400 m). Both of the groups had the same controlled temperature (22-24°C) and a 12:12 h on/off light cycle. All rats were euthanized after 21 d of the experimental period.

Body weight and haematological values

The body weight was registered when the rats were taken out from the chamber/animal room. Then the rats were anesthetized with intraperitoneal injection of 2% pentobarbital sodium (50 mg/Kg) immediately. Whole blood (2 ml) was collected in vacuum blood collection tube (purple top) by cardiac puncture, and haematological values, included erythrocyte (RBC), haemoglobin (HGB), haematocrit (HCT), platelet (PLT), and plateletcrit (PCT), were assessed with an automated haematology analyser (XE-2100D; Sysmex, Kobe, Japan).

Micro-CT analyses

After blood was obtained, all rats were euthanized by cervical dislocation, both femurs of each rat were dissected and cleaned of the surrounding soft tissue in the operation room. The right femurs (n=4/group) were collected and then fixed in 10% formalin until they were ready (within 1 week) for further micro-CT imaging and analysis (Y. Cheetah; YXLON, Hamburg, Germany). The distal metaphysis of each femur was scanned and reconstructed into a 3D-structure (VGStudio MAX 2.1 V; Volume Graphics, Heidelberg, Germany) with a voxel size of 15.28 μm (80 kV, 50 μA, 600 ms exposure time). The X-ray projections were obtained at 0.5° intervals with a scanning angular rotation of 360°, and six frames were averaged for each rotation. For the quantitative analysis, the volume of interest (VOI) was extracted with a 2000-μm-long region of trabecular bone in the distal metaphysis starting 1 mm proximal to the growth plate. The following parameters of the VOI were measured and calculated: bone volume (BV/TV), bone surface density (BS/TV), trabecular number (Tb. N), trabecular thickness (Tb. Th), trabecular separation (Tb. Sp) and trabecular bone mineral density (BMD).

Histological observation

After the micro-CT analysis, the distal femurs were dissected and decalcified in 15% EDTA at room temperature for 10 days. The specimens were then embedded in paraffin, sagittally sectioned (5 μm), and stained with haematoxylin-eosin (HE) staining for histological evaluation.

Biomechanical testing

Left femurs (n=4/group) were subjected to the three-point bending test for mechanical proper-

Table 1. Comparison of body weight and haematological values between the analyzed groups. If normal distribution was accepted, the results were presented as mean and standard deviation. If not, the results were presented as 5% percentile, median, 75% percentile

Variables	NX	HX	*p*-Value
Weight beginning (g)	340.3±7.5	351.0±11.3	0.4591[a]
Weight end (g)	447.5±12.3	368.8±10.5	0.0028[a]
Weight increased (g)	107.3±5.3	17.8±3.9	<0.0001[a]
RBC ($\times10^{12}$/L)	7.888-8.205-8.433	9.135-10.150-10.220	0.0286[b]
HGB (g/L)	153.30±2.29	227.00±5.26	<0.0001[a]
HCT (%)	43.98±0.59	67.48±0.97	<0.0001[a]
PLT ($\times10^9$/L)	781.00±46.88	404.50±33.89	0.0006[a]
PCT ($\times10^9$/L)	0.5925±0.0459	0.3000±0.0227	0.0012[a]

RBC, erythrocyte; HGB, haemoglobin; HCT, haematocrit; PLT, platelet; PCT, plateletcrit. [a]Significant difference between groups (normal distribution was accepted and Student's t-test for unpaired data was applied). [b]Significant difference between groups (normal distribution was not accepted and Mann-Whitney was applied).

ties. Femurs were individually excised, stored in saline-soaked gauze, and frozen at -80°C. Before testing, they were thawed and soaked with saline during the entire testing. Before the three-point bending test, we scanned the shaft of each femur by micro-CT (Y. Cheetah; YXLON, Hamburg, Germany) and obtained images of the transverse cross-section of the midpoint of the distance from the proximal surface of the trochanter major to the distal surface of both femoral condyles. The scanning parameters were set as described above. Then, the measurements were performed using a bone strength tester (Model CMT4000; SANS, Shenzhen, China) and analysed using a strength inspection system (PowerTest_D00C; SANS, Shenzhen, China). The femurs were placed on their posterior surface on top of two metal supports located 20 mm apart in the tester, making sure the midpoint of the metal supports copied the midpoint of the distance from the proximal surface of the trochanter major to the distal surface of both femoral condyles. So, the posterior point of the transverse cross-section obtained from micro-CT was the intended site of load application. Then a bending force was applied midway at the rate of 5 mm/min until a fracture occurred. The bending force and response to loading was automatically obtained and showed in the form of a load-deformation curve by the strength inspection system. Stiffness (N/mm) was calculated as the slope of the curve in the pre-yield region. To determine the intrinsic mechanical properties, the load-deformation curve was converted to a stress-strain curve as follows: images of midiaphysis captured from micro-CT were analysed with AUTOCAD 2014 (Autodesk, Inc., San Rafael, CA, USA) to measure geometric parameters, including the femoral diaphysis cross-sectional moment of inertia ($I_{femoral}$, mm^4) and the distance from the load application point to the centroid of the bone (d, mm). Then, the strain (ε) and stress (σ) of each femur were calculated according to Eqs. 1 and 2, respectively, as detailed elsewhere [9] (Y, deformation in mm; L, distance between the lower supports in mm; F, load applied to the specimen in Newtons):

$$\varepsilon=(12\times d\times Y)/L^2 \quad (1)$$

$$\sigma=(F\times L\times d)/(4\times I_{femoral}) \quad (2)$$

The intrinsic mechanical properties were then determined from the generated stress-strain curves: maximum stress (megapascals, MPa) and Young's modulus (calculated as the slope of the stress-strain curve within the elastic region) (MPa).

Statistical analysis

The results were summarized as mean, standard deviation, 25% percentile, median and 75% percentile. To compare the two groups, initially, we tested the normality of data distribution (Shapiro-Wilk test) and equality of variances (Fisher's exact test). If the normal distribution was accepted and the variances were equal, Student's t-test for unpaired data was applied. If not, the non-parametric test, Mann-Whitney, was applied. The statistical software used for analysis was GraphPad Prism 7.00 (GraphPad Software, San Diego, CA, USA). All differences were considered significant at $P<0.05$.

Results

Body weight and haematological values

There was no difference in the pre-experimental body weights between the groups. The body weights were measured again before the rats

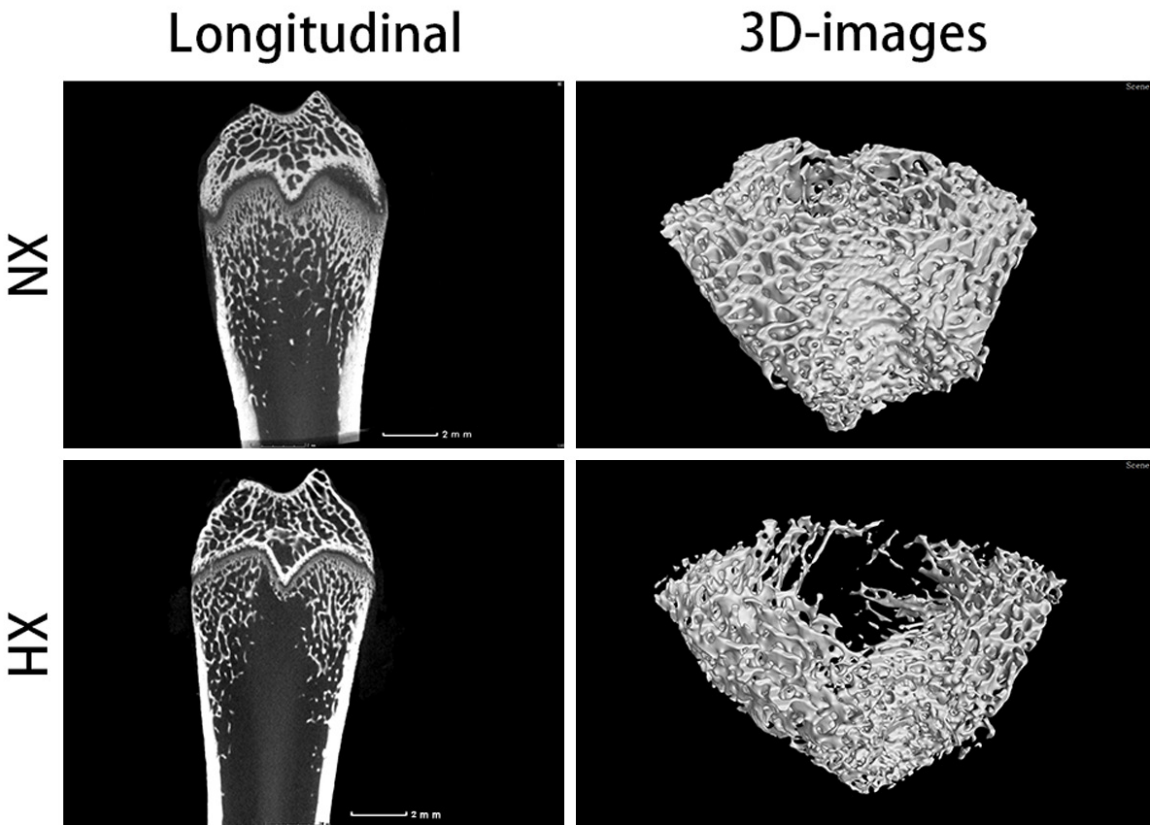

Figure 1. Rats treated by hypobaric hypoxia exhibited less trabecular bone in the distal metaphysis of the femurs. Left, longitudinal Images of the distal femur by micro-CT (bar =2 mm). Right, transverse 3D images of trabecular bone in the distal metaphysis (volume of interest (VOI)).

Table 2. Comparison of micro-CT analyses between the analyzed groups. The results were presented as mean and standard deviation

Variables	NX	HX	*p*-Value
BV/TV (%)	0.3465±0.0177	0.2209±0.0182	0.0026[a]
BS/TV (mm^{-1})	25.78±1.23	30.34±1.99	0.0992[a]
Tb. N (mm^{-1})	4.438±0.107	3.302±0.116	0.0004[a]
Tb. Sp (mm)	0.1476±0.0061	0.2372±0.0126	0.0007[a]
Tb. Th (mm)	0.07811±0.00371	0.06677±0.00435	0.0947[a]
BMD (mg/cm^3)	341.9±4.5	321.9±6.0	0.0373[a]

BV/TV, bone volume; BS/TV, bone surface density; Tb. N, trabecular number; Tb. Th, trabecular thickness; Tb. Sp, trabecular separation; BMD, trabecular bone mineral density. [a]Significant difference between groups (normal distribution was accepted and Student's t-test for unpaired data was applied).

were euthanized, and the increased weights during the experimental period were calculated. As shown in **Table 1**, rats in the NX group showed larger increases in body weight (107.3± 5.3 g (n=4)) than the HX group (17.8±3.9 g (n=4)) (*P*<0.001).

The haematological values between the groups were evaluated. As shown in **Table 1**, RBC (*P*=0.0286), HGB (*P*<0.0001), and HCT (*P*<0.0001) were higher in the HX group, while the NX group had more PLT (*P*=0.0006) and PCT (*P*=0.0012) than the HX group.

HX group had deteriorated trabecular bone microarchitecture and lower bone density

The HX group exhibited less trabecular bone in the distal metaphysis of the femurs (**Figure 1**). The quantitative analysis of the micro-CT data further validates this observation. As shown in **Table 2**, rats in the HX group had lower trabecular BMD (*P*=0.0373), BV/TV (*P*= 0.0026), Tb. N (*P*=0.0004), higher Tb. Sp (*P*=0.0007) in the distal metaphysis of the femurs.

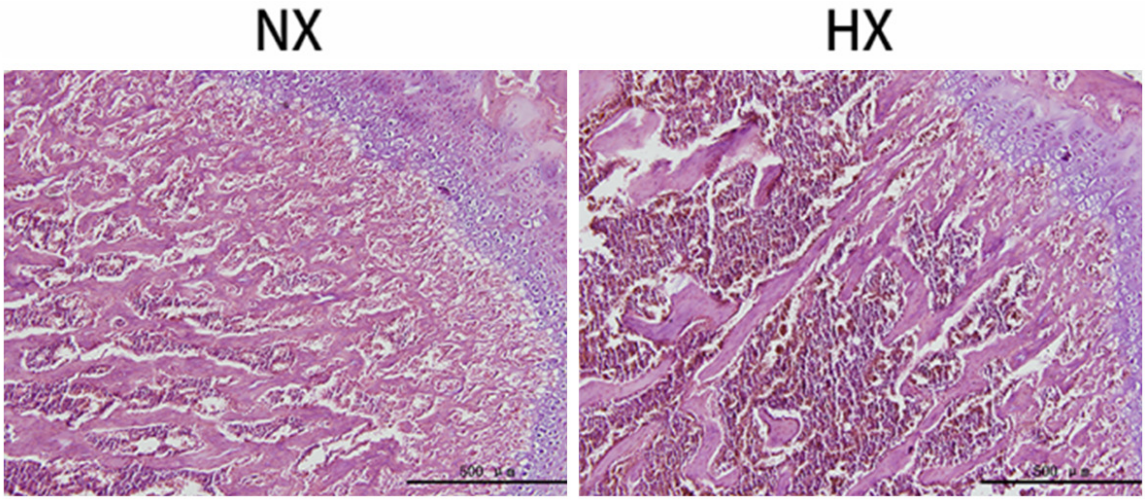

Figure 2. Effects of haematoxylin-eosin (HE) staining for histological observation in the distal metaphysis of the femurs (close to the growth plate) in the two groups (bar =500 μm).

Table 3. Comparison of biomechanical properties between the analyzed groups. If normal distribution was accepted, the results were presented as mean and standard deviation. If not, the results were presented as 5% percentile, median, 75% percentile

Variables	NX	HX	*p*-Value
Maximum load (N)	149.8±14.8	100.5±5.3	0.0201[a]
Stiffness (N/mm)	211.7-228.9-374.1	117.4-129.1-164.8	0.0286[b]
Maximum stress (MPa)	178.7±22.0	142.7±8.9	0.1798[a]
Young's modulus (N/mm^2)	5368-5527-7928	3780-3846-4731	0.0286[b]

MPa, megapascals. [a]Significant difference between groups (normal distribution was accepted and Student's t-test for unpaired data was applied). [b]Significant difference between groups (normal distribution was not accepted and Mann-Whitney was applied).

Fewer trabeculae was shown in HX group by histological observation

A histological observation of the distal metaphysis of femurs confirmed the fewer trabeculae in the HX group and is consistent with the micro-CT analysis. As shown in **Figure 2**, the trabecular bone structure of hypoxia rats was much looser than that of the normoxic rats.

Lower bone mechanical properties was revealed in HX group

The mechanical bone strength of the femoral diaphysis (n=4/group) was investigated by the three-point bending test and is summarized in **Table 3**. The HX group showed lower structural properties as averaged maximum loads (149.8±14.8 N for the NX and 100.5±5.3 N for the HX; *P*=0.0201). The NX group had a higher stiffness than the HX (*P*=0.0286). For intrinsic mechanical properties, a significantly smaller Young's modulus was observed in the HX group than in the NX group (*P*=0.0286). The maximum stress was lower in the HX group, but the difference was not significant (*P*=0.1798).

Discussion

The results of our study indicated that the rats in the chamber gained body weight during the experimental period; however, the increase in body weight for the HX group was less than that of the NX group. In addition, the rats had higher RBC, HGB, and HCT and lower PLT and PCT. These findings were in perfect accordance with other studies of high altitude medicine. The micro-CT and histological observation showed less trabecular bone in the distal metaphysis of the femurs in the hypoxia rats, and the quantitative analysis confirmed the observation. Less structural properties and intrinsic mechanical properties in the HX group demonstrated impaired bone strength.

The high altitude environment is a complex system involving many factors. It is now recognized that hypobaric hypoxia is the most important characteristic for high altitude, and it is reason-

able to use hypobaric hypoxia as a simulant of high altitude. The hypobaric hypoxia chamber used in this study was airtight and could accurately decrease the air pressure to 48.5 kPa using vacuum pumps pressurized to a level corresponding to an altitude of 6000 m. We elevated or reduced the pressure at a moderate speed (corresponding to 6 m/s) when altering the pressure in the chamber to avoid adverse effects, such as decompression sickness, which is mainly observed in aviation and space activities.

The high altitude environment has multiple effects on the overall organism. Initially, high altitude research was focused on the adjustments of the respiratory and circulatory systems to hypoxia [10]. After decades of research, the scope of this research has broadened considerably. It is now known that more mechanisms are involved in the adaptation of an incomer to constant hypoxia at high altitude. As a consequence of hypobaric hypoxemia induced by the decreased partial pressure of oxygen (PO_2), the respiratory rate, heart rate, and systemic blood pressure increase to improve O_2 transport and utilization [11-14]. After a few days, polycythaemia develops due to erythropoietin secretion by the kidney cells. If exposure to high altitude is maintained for a sufficient period of time, body weight would decrease due to anorexia induced by hypoxia [15-17]. Mechanisms for setting the target body weight may be responsible for body weight retardation, and it was suggested that a reduced body weight may be adaptive to high altitude by reducing oxygen needs [18]. Our study and others showed that hypobaric hypoxia had a great influence on body weight [3]. Interestingly, one report observed no significant change of body weight in a normobaric hypoxia environment, which was induced by infusing more compressed nitrogen [2]. This might suggest that air pressure plays an important role in the regulation of body weight.

Additionally, haematological values revealed that treatment with hypoxia increased the RBC, HGB, and HCT levels and reduced the levels of PLT and PCT. The reduced PLT in our study agreed with previous observations that PLT are activated at high altitude, which leads to PLT aggregation and consumption [19, 20]. Increased HGB, RBC, and HCT at altitude are also very well documented [21, 22]. Our data indicate that the simulative hypobaric hypoxia environment in the chamber was effective, and the rats in the HX group had physiologically adapted to it.

In our study, micro-CT, HE stain, and a three-point bending test were performed to investigate the bone properties influenced by hypoxia. Micro-CT can provide a detailed quantitative analysis of 3D microscopic bone architecture and has been widely used in osteoporosis studies [23, 24]. Our results of micro-CT and histological observation demonstrated that the HX group exhibited less trabecular bone in the distal metaphysis of their femurs (**Figures 1** and **2**). Quantitative analysis of the micro-CT data further validated this observation. The rats in the HX group had lower trabecular BMD, BV/TV, Tb. N, and higher Tb. Sp in the distal metaphysis of the femurs (**Table 2**).

Biomechanical analysis of bone can provide useful information regarding the integrity of bone function [25]. Mechanical properties of bones are the most important predictors of fracture risk, and bone strength is determined by bone mass and the intrinsic properties of the bone material [26]. The key indicators of biomechanical properties are based on the relationship between applied load and the resulting deformation in bone. Structural properties, which are related to the geometric properties of femurs, could be calculated from the load-deformation curves, and the intrinsic properties describing bone tissue material properties could be calculated from the stress-strain curves.

In our results, the hypobaric hypoxia environment induced lower maximum loads and stiffness of the femurs, and both of these indicators belong to the structural properties. For the intrinsic properties, a significantly smaller Young's modulus was observed in the HX group than in the NX group (**Table 3**). Ultimate stress was also lower in the HX group, but the difference was not significant. The lower structural properties might have a relationship with the lower body weight because of the corresponding reduced femur geometry. The impaired intrinsic properties indicated that the hypobaric hypoxia environment negatively influenced bone mass and bone material properties. Our findings have many similarities with other studies. The biomechanical behaviour of the diaphy-

sis that was negatively affected in the animals exposed to high altitude has been reported by other authors [3, 4]. In addition, some of our observations differ from previous studies, which showed that hypoxia had no effect on Young's modulus [3]. This discrepancy may be partially related to the different methods of converting the load-deformation curve to a stress-strain curve. In our studies, before the three-point bending test, we obtained the image of the transverse cross-section of the midpoint of the diaphysis by micro-CT, and the bending force was performed on the right point of the images. Then, the images were analysed by AUTOCAD to calculate the cross-sectional moment of the inertia ($I_{femoral}$) and the distance from the load application point to the centroid of the bones. The two geometry parameters were helpful in accurately obtaining the strain and stress. Therefore, the intrinsic mechanical properties based on the stress-strain curve should be errorless and the conclusion credible.

The above observations confirmed the impairment of the hypobaric hypoxia environment on bone strength and quality in rats. Multiple mechanisms might be responsible for the negative effects. First and most important, the bone micro-environment has lower oxygen tension, and this affects the activity of multiple skeletogenic cells, which are important for bone development. Local oxygen tension of the bone micro-environment corresponds with breathing environment oxygen levels [2]. The PO_2 of inspired air decreases with the lower barometric pressure at high altitude; the estimated PO_2 is reduced to 68 mmHg in the 6000 m altitude, whereas it is 149 mmHg at sea level [27]. The local PO_2 of the bone micro-environment was 45.5±15.3 mmHg at sea level [28], and it would be dramatically lower at the 6000 m altitude [29] as in our studies. Other studies have demonstrated that when tissue PO_2 falls below 40 mmHg, angiogenesis, extracellular matrix formation, and resistance to infection are all impaired [30].

Many *in vitro* experiments have demonstrated that oxygen tension could affect many skeletogenic cells, including osteoblasts [31, 32], chondrocytes [33-35], osteoclasts [36, 37], and mesenchymal stem cells (MSCs) [38], and many genes are involved in those biological processes [39-43]. However, the conditions performed *in vitro* studies are quite different from those *in vivo*. In most *in vitro* studies, the results in 2-5% oxygen were compared with 20-21% oxygen and the influence of air pressure were not considered. Furthermore, the *in vivo* environment is much more complex, and more factors related to hypoxia may be responsible for the impaired bone strength and quality, such as an imbalanced acid-base level in the micro-environment, loss of appetite, a lower basal metabolic rate, and reduced activity. Thus, the results of the *in vitro* studies have a limited contribution to the perspective of the bone influenced by the hypobaric hypoxia environment *in vivo*, and more meticulous research should be performed to determine the exact mechanism.

Our study has observed several important variations in bone induced by hypobaric hypoxia environment; however, it also has some limitations. First, for the purpose of finding more positive results in a shorter period, we set an ultimate altitude for 6000 m in the chamber, and the environment is rare in reality. Second, hypobaric hypoxia is just one, although the most important, characteristic at high altitude. Thus, the observation in the bone in this study cannot be recognised as the actual effects at high altitude, although the reduced body weight and variations of haematological values are corresponding with them in the animals and human at high altitude [3, 19-22]. Third, we ignored the influence of anorexia and decreased physical activity induced by hypobaric hypoxia, which play a pathogenic role in bone metabolism. Additionally, 21-d, the period of treatment in this study, is enough to cause physiological adaptation as shown in our results, the long-time effect have to be observed in further studies yet.

In conclusion, we successfully simulated the high altitude environment using a hypobaric hypoxia chamber and observed that hypobaric hypoxia had negative effects on bone strength and quality in rats. To clarify the mechanisms in the intricate alteration, we have performed RNA-seq to compare the gene expression differences in the femoral diaphysis in the two groups, and the results will be reported later. The outcomes of future studies at different time points, multiple altitudes and more influences at high altitude and a better understanding of the pathogenesis will add to our knowl-

edge with the potential to find a new strategy to prevent bone dysfunctions for people who enter high altitudes.

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Disclosure of conflict of interest

None.

Address correspondence to: Bao-An Ma and Hua Long, Department of Orthopedic Surgery, Tangdu Hospital, Fourth Military Medical University, Xi'an, Shaanxi, People's Republic of China. E-mail: mbaoan@163.com (BAM); 1903426246@qq.com (HL)

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