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Spatiotemporal characterization of microdamage accumulation in rat ulnae in response to uniaxial compressive fatigue loading

Xuhui Zhang [a,b,1], Xiyu Liu [a,1], Zedong Yan [a,1], Jing Cai [a], Fei Kang [c], Shuai Shan [a,d], Pan Wang [a], Mingming Zhai [a], X. Edward Guo [e], Erping Luo [a,*], Da Jing [a,*]

[a] Department of Biomedical Engineering, Fourth Military Medical University, Xi'an, China
[b] Department of Medical Engineering, 456th Hospital of Chinese People's Liberation Army, Jinan, China
[c] Department of Nuclear Medicine, Xijing Hospital, Fourth Military Medical University, Xi'an, China
[d] Department of Medical Engineering, 150th Hospital of Chinese People's Liberation Army, Luoyang, China
[e] Bone Bioengineering Laboratory, Department of Biomedical Engineering, Columbia University, New York, USA

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ABSTRACT

Repetitive fatigue loading can induce microdamage accumulation in bone matrix, which results in impaired mechanical properties and increased fracture susceptibility. However, the spatial distribution and time-variant process of microdamage accumulation in fatigue-loaded skeleton, especially for linear microcracks which are known to initiate bone remodeling, remain not fully understood. In this study, the time-varying process of the morphology and distribution of microcracks in rat ulnae subjected to uniaxial compressive fatigue loading was investigated. Right forelimbs of thirty four-month-old male Sprague-Dawley rats were subjected to one bout of cyclic ramp loading with 0.67 Hz at a normalized peak force of 0.055 N/g body weight for 6000 cycles, and the contralateral left ulnae were not loaded as the control samples. Ten rats were randomly euthanized on Days 3, 5, and 7 post fatigue loading. Our findings *via* two-dimensional histomorphometric measurements based on basic fuchsin staining and three-dimensional quantifications using contrast-enhanced micro-computed tomography (MicroCT) with precipitated $BaSO_4$ staining demonstrated that the accumulation of linear microcracks (increase in the amount of linear microcracks) on Day 5 was significantly higher than that on Day 3 and Day 7 post fatigue loading. Our histological and histomorphometric results revealed that linear microcrack density (Cr.Dn) in the tensile cortex at Days 3, 5 and 7 post fatigue loading was significantly higher than that in the compressive side, whereas linear microcrack length (Cr.Le) in the tensile cortex at Day 3 was significantly lower than that in the compressive cortex. Our findings revealed that microcrack accumulation exhibited a non-linear time-varying process at 3, 5 and 7 days post axial compressive fatigue loading (with observable peak Cr.Dn at Day 5). Our findings also revealed distinct distribution of microcrack density and morphology in rat ulnae with tensile and compressive strains, as characterized by more microcracks accumulated in tensile cortices, and longer cracks shown in compressive cortices.

1. Introduction

Normal bone is capable of bearing external mechanical loading in daily life and resisting fracture like most engineering materials, whereas microdamage may accumulate in both cancellous and cortical bone in response to repetitive fatigue loading [1–3]. In the 1960s, Frost for the first time reported the observation of *in vivo* bone microcracks with 30–100 μm in length at the microscopic level using the basic fuchsin bulk staining technique to distinguish pre-existing microdamage induced by daily loading from artificial damage caused during specimen preparation [4]. It has been substantiated that microdamage accumulation in bone matrix may contribute to the degradation of skeletal mechanical properties (*e.g.*, elastic modulus and stiffness), and accordingly result in the increase of bone fragility [5–7]. However, unlike engineering materials, bone tissues have an inherent ability to repair microdamage on the osseous surface and maintain the fracture resistance *via* targeted bone remodeling process. Coupled bone resorption and bone formation are two major steps involved in the process of targeted bone remodeling, referring to the removal of "dead" bone *via* osteoclast recruitment and subsequent replacement with new bone involving osteoblasts [8, 9]. However, if the production of microdamage exceeds the damage-related remodeling process, overwhelmed self-repair mechanisms tend to result in the accumulation of microdamage in

* Corresponding authors at: Department of Biomedical Engineering, Fourth Military Medical University, 17 West Changle Road, Xi'an 710032, China.
E-mail addresses: luoerping@fmmu.edu.cn (E. Luo), jingdaasq@163.com (D. Jing).
[1] These authors contributed equally to this work.

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bone matrix and subsequent occurrence of stress fractures, which are commonly seen in the populations of soldiers, athletes, joggers and dancers [10–14]. Thus, characterizing the morphology and distribution of microcracks in fatigue-loaded skeleton and clarifying damage-related repair process are beneficial for providing further insight into the etiology of stress fractures and age-related fragility fractures.

Basically, microscopic fatigue microdamage can be categorized into two major types according to their different morphological features, including linear microdamage and diffuse microdamage. It has been documented that the linear microcracks rather than diffuse microcracks have the capacity of initiating targeted bone remodeling *via* osteoclast recruitment activated by osteocyte apoptosis [15–17]. The linear microcracks play an essential role in triggering remodeling-mediated damage repair process. Thus, understanding the time-variant process of linear microcrack accumulation associated with targeted bone remodeling is of great importance for deciphering remodeling-mediated damage repair mechanism. Silva et al. focused on time-dependent responses to fatigue accumulation associated with bone remodeling during the first 30 days following fatigue loading using ^{18}F-fluoride with positron emission tomography (PET) [18]. However, this PET-based study did not investigate the time-variant process of the amount and morphology of the linear microcracks as well as their distributions in the three-dimensional bone tissues after fatigue loading. However, to our knowledge, the potential time-dependent changes of linear microcracks in bone subjected to fatigue loading have never been fully characterized by previous investigators.

In addition to the time-variant process of microdamage accumulation, understanding the spatial distribution of microdamage also holds great significance for advancing our basic knowledge about the microdamage-related characteristics and mechanisms. Several investigators have endeavored to predict the potential features of microdamage distribution within trabecular bone [19, 20], whereas few studies have systematically identified the spatial distribution characteristics of microdamage in cortical bone under fatigue loading. Boyce et al. performed four-point bending tests on machined beam specimens of *ex vivo* human tibial cortical bone, and found that linear microcracks tended to accumulate in compressive region, while diffuse microdamage was more common in tensile area [21]. However, the *ex vivo* segmented diaphysial beam specimens *via* four-point bending tests could not provide sufficient information concerning the spatial distribution of microdamage within cortical bone in response to cyclic fatigue loading. It has been reported that the rodent ulnae under axial compressive loading exhibited maximal strains in the mid-diaphysial region, and the medial and lateral cortex of the ulnar mid-diaphysis are subjected to compressive and tensile strain, respectively [22, 23]. However, it remains unclear whether the accumulation of linear microcracks exhibit different characteristics in areas with different strain patterns in response to whole-bone compressive fatigue loading.

Therefore, the spatial distribution and time-variant process of the microcrack density and morphology in rat ulnae subjected to one single bout of uniaxial compressive fatigue loading were systematically investigated in the current study. We hypothesized that microcrack accumulation in rat ulnar bone cortices would exhibit a non-linear time-varying process at 3, 5 and 7 days post the stimulation of a bout of compressive fatigue loading. Moreover, we also hypothesized that most of linear microcracks would accumulate in the tensile cortices of rat ulnae in response to compressive fatigue loading, but relatively longer linear microcracks would be shown in the compressive cortices post uniaxial compressive fatigue loading.

2. Materials and methods

2.1. Animals

Thirty male adult Sprague-Dawley rats (4 months, 554 ± 99 g) purchased from the Animal Center of the Fourth Military Medical University were used in the current study. All rats were housed under controlled temperature (23 ± 1 °C) and relative humidity (50%–60%) with a regular 12 h light-dark cycle. Animals were allowed *ad libitum* access to standard rodent chow and clean tap water until they were sacrificed. The experimental protocol of our study was approved by the Institutional Animal Studies Committee of The Fourth Military Medical University, and all animal procedures were strictly performed in accordance with the approved guidelines.

2.2. Mechanical loading system and fatigue loading protocol

A custom-designed mechanical loading system was employed in the current study to generate uniaxial compressive loading *in vivo* to the ulnae of rats (Fig. 1A). Both ends of the ulna were fixed using two differently shaped holders. The olecranon of the ulna was fixed by an immovable holder, and the flexed carpus was fixed by a movable holder (Fig. 1B). The sample holders were made of aluminium alloy, and the surface of the holders was covered with nylon plastic to help maximize the friction between the ends of ulna and holders. The motion of the movable sample holder was motivated by a linear actuator (M-235.2DG, Physik Instrumente, Germany) with 0.1 μm minimum incremental motion and 120 N maximum push/pull force. The force magnitude was measured using a 200-N load cell (LH-S05, Liheng Instrument Co., Ltd., Shanghai, China). A linear guide was mounted along the loading axis to constrain the lateral motion and avoid the lateral movement of the sample holders during mechanical loading. Displacements applied on the ulna were precisely determined using a laser displacement transducer (ACR-LDS131, SHDallas Instrument Co., Ltd., Shanghai, China). The force and displacement data after being instrumentally amplified were captured with a 16-bit data acquisition (DAQ) card NI USB-6210 (National Instruments, TX, USA). Real-time data were automatically acquired in conjunction with a custom-written LabView computer program. The applied force was controlled *via* a proportional-integral-differential (PID) controller, which was able to ensure that the waveform did not overshoot or undershoot the desired load and minimize the deviation between the output applied force and the force set point. The load cell, displacement transducer and linear actuator were all precisely calibrated, and the mechanical loading system was also well validated using the UHMWPE rods by comparing the measurement accuracy of elastic modulus with the value measured by a Bose mechanical testing system (ElectroForce3220, Eden Prairie, MN).

Fatigue loading generated by the load-controlled mechanical loading system was applied on the right forelimbs of the rats as described previously [24–26], and the contralateral ulnae were not subjected to mechanical loading and used as the control samples. Before applying *in vivo* fatigue loading, all rats were weighed and then intraperitoneally anesthetized with 3% pentobarbital sodium (30 mg/kg). A preload of 1 N was applied to immobilize the ulna prior to fatigue loading. Prior to fatigue loading, preconditioning of cyclic compressive loading with 50 cycles was applied on the rat forelimb to minimize the influence of soft tissue compliance as reported by several previous studies [23, 27] to ensure that the displacement measurements from the laser displacement transducer mainly reflected the deformation of bone during fatigue loading. Then, cyclic uniaxial compressive loading was applied as ramp loading at 0.67 Hz with 0.7 s ramp up, 0.7 s ramp down and 0.1 s ramp wait (Fig. 1C). Most of previous studies investigating fatigue damage also employed the loading frequency with the range of 0.5–2 Hz [25, 26, 28–32], which was a typical physiological-related frequency range. Right forelimbs of the rats were loaded at a normalized peak force of 0.055 N/g body weight (with averaged ~30.5 N peak force, Fig. 1C). Substantial previous studies have also employed this normalized peak force of 0.055 N/g body weight as the magnitude of fatigue loading on rat forelimbs [25, 26, 28, 29], and the objective was to counteract the difference in bone size related to the different body weight of rats and the consequent difference in the strains in ulnae induced by external loading. Cyclic fatigue loading was ceased until 6000 cycles were reached.