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Knee Loading Dynamically Alters Intramedullary Pressure in Mouse Femora

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Abstract

Dynamic mechanical loads have been known to stimulate bone formation. Many biophysical factors such as number of daily loading cycles, bone strain, strain-induced interstitial fluid flow, molecular transport, and modulation of intramedullary pressure have been considered as potential mediators in mechanotransduction of bone. Using a knee loading modality that enhances anabolic responses in mouse hindlimb, we addressed a question: Do oscillatory loads applied to the knee induce dynamic alteration of intramedullary pressure in the femoral medullary cavity? To answer this question, mechanical loads were applied to the knee with a custom-made piezoelectric loader and intramedullary pressure in the femoral medullary cavity was measured with a fiber optic pressure sensor. We observed that in response to sinusoidal forces of 0.5 Hz and 10 Hz, pressure amplitude increased up to 4-N loads and reached a plateau at 130 Pa. This amplitude significantly decreased with a loading frequency above 20 Hz. To confirm alteration of intramedullary pressure, real-time motion of microparticles in a glass tube inserted to the femoral medullary cavity *ex vivo* was visualized. Taken together, these data reveal that knee loading dynamically alters intramedullary pressure as a function of loading intensities and frequencies.

Keywords

Mechanical stimulation; knee joint; medullary cavity; pressure; microparticles

Introduction

A long bone such as a femur or a tibia constitutes a mechanosensitive system [1,2], and dynamic loading through walking and jogging are well-known to enhance its remodeling [3–5]. To devise effective mechanical intervention for promoting bone formation [6], treating osteoporosis [7], or accelerating fracture healing [8], elucidation of the mechanism underlying

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load-driven remodeling is indispensable [9]. Biophysical mediators for mechanotransduction in bone have been searched for, and conceivable candidates include strain magnitude or rate [10,11], number of daily loading cycles [12], interstitial fluid flow [13,14], transport of nutrient and/or waste molecules [15,16,17], and intramedullary pressure [18,19]. Among them, intramedullary pressure has been shown to enhance bone formation, where a turkey ulna *ex vivo* was linked to an external pressure source for oscillatory modulation of the ulnar intramedullary pressure [20,21]. Although load-driven alteration in intramedullary pressure is considered as a potential cause for anabolic responses, it is unclear whether pressure alteration in the turkey study is actually inducible in a medullary cavity *in vivo* with any existing loading modality.

Thus, the present study addressed the following questions: Is intramedullary pressure in a medullary cavity *in vivo* altered synchronous to dynamic loading? And if so, is the amplitude of modulation affected by loading intensities and frequencies? In order to examine those questions, a relatively novel loading method, “knee loading,” was employed using a mouse femur as a model system [22]. Knee loading belongs to a joint loading modality, in which lateral loads are applied to the epiphysis for enhancing bone formation throughout a long bone. In our previous mouse studies, knee loading was shown to increase formation of cortical bone in tibiae [22,23]. Furthermore, elbow loading, the other form of the joint loading modality, was shown to elevate bone formation in mouse ulnae [24].

Joint loading apparently stimulates anabolic responses in cortical bone with *in situ* strain < 100 μ strain at the site of bone formation. One hypothesis for explaining the mechanism of anabolic effects is that dynamic loads to the joint induce modulation of intramedullary pressure throughout a long bone. In this study, we examined this hypothesis by detecting minute alteration of intramedullary pressure in the femoral medullary cavity with knee loading. Our hypotheses were (a) intramedullary pressure is altered synchronous to dynamic loading, and (b) the amplitude of pressure oscillation depends on loading intensities and loading frequencies.

Materials and Methods

Animal use

Femora of C57BL/6 mice (female, ~ 16 weeks old) with a body weight of ~ 20 g were used in the experiments. The procedures for use of mice were approved by Institutional Animal Care and Use Committee at Indiana University School of Medicine. The mice were anesthetized with 2% isoflurane and the periosteal surface of femora was exposed. For *in vivo* pressure calibration, a pair of holes (~ 0.3 mm in diameter) was generated with a blunt drill bit at the site ~ 2 mm and ~ 8 mm to the distal femoral end. The former was used for connecting to a water column and the latter for inserting a cannula linked to a pressure sensor.

For *in vivo* measurements of intramedullary pressure, a single hole at ~ 8 mm to the distal end was created. For *ex vivo* monitoring of microparticles in the femur, the hole at the same location was generated and a glass tube was inserted. The femur for the *ex vivo* experiment was immediately harvested and maintained in the α MEM culture medium supplemented with 10% fetal calf serum.

Calibration of a pressure sensing system

Prior to measuring intramedullary pressure in response to knee loading, the pressure sensing system was calibrated using the glass tube filled with a saline solution as well as the femur *in vivo* (Fig. 1). The sensing system consisted of a fiber optic pressure sensor (FOP-MIV, FISO Technologies Inc., Ontario, Canada), a fiber optic cable, and a pressure monitor (PM-250, FISO Technologies Inc., Ontario, Canada). The sensor was designed to measure the relative pressure

with thermal sensitivity of $\leq \pm 1\%/^{\circ}\text{C}$. The sensor tip was 0.95 mm in diameter, and it is connected to a fiber optic cable. We confined the sensor tip and a part of the cable into a 3-ml syringe filled with a saline solution containing 10 units/ml of heparin, and inserted a 23GTW cannula (Becton Dickinson & Co., Franklin Lakes, NJ) into the femoral medullary cavity. In order to minimize pressure alterations due to insertion of the cannula, we made a shallow indentation on the cortical surface and inserted the cannula into the cavity without releasing the medullary pressure outside the cannula. The height of the water column was adjustable in the range up to 2000 Pa (20 cmH₂O). The optical signal from the pressure sensor was relayed through a fiber optic cable and converted into a voltage signal with the optic pressure monitor. The voltage signal was transmitted to a PC through the BNC-2110 interface.

The baseline intramedullary pressure was measured as difference between an ambient atmospheric pressure and the pressure inside the medullary cavity. The cannula was inserted into the femoral medullary cavity and the sensor system was equilibrated for 30 min. The pressure in the cannula was abruptly released to the ambient pressure by opening a dilator switch (Pinnacle 15–725, Terumo Medical Co., MD), which was connected to the syringe. We employed three mice and conducted three measurements for each of the three mice.

In vivo pressure measurements with knee loading

The custom-made piezoelectric mechanical loader was used to apply lateral loads to the knee (Fig. 2). Intramedullary pressure in the femoral medullary cavity was measured using 3 animals. The same animals were used for knee loading and sham loading. The loading signal was sinusoidal, and we employed the actuator voltage from 10 to 100 V (peak-to-peak). Since the applied loads were calibrated to 0.05 N per actuator voltage, the actual force applied to the knee was estimated from 0.5 N (10 V) to 5 N (100 V). For sham loading, the piezoelectric loader was activated without contacting the knee to the loading rod. To evaluate the frequency responses, the knee was loaded at varying frequencies ranging from 0.05 Hz to 50 Hz. Note that the pressure sensor was zeroed prior to measurements, and therefore the observed signal represented the pressure alteration from the unloaded state.

Ex vivo monitoring of microparticle motions with knee loading

The real-time motion of microparticles (3 μm in diameter, Polysciences Inc., Warrington, PA) was monitored using the femur *ex vivo*. The bone sample was immobilized in the sample holder of the *ex vivo* piezoelectric loader using super glue (Henkel Loctite Co.), and the loading rod was placed at the distal epiphysis. The loader was mounted on a NIKON microscope (E600 eclipse, Kanagawa, Japan), and a cylindrical glass pipette (340 μm in internal diameter, and 6 mm in length; Drummond Scientific Co., Broomall, PA) was inserted into the femoral bone cavity. This tube was filled with a saline solution containing 10 units/ml of heparin and 0.01 g/ml of microparticles. Note that this *ex vivo* loader was previously used to monitor molecular transport with a “fluorescence recovery after photobleaching technique (FRAP)” [16]. The rod was slid by the piezoelectric actuator (LPD12060X, Megacera Inc.), which was regulated with a voltage amplifier (PZD700 M/S, TREK) through a BNC-2110 interface (National Instruments). We employed 4 N force with the loading frequency at 0.5 Hz. Using a 10X objective, the real-time motion of microparticles inside the glass tube was monitored. The time-lapse images with 250 msec intervals were collected by a CCD camera (RET/CCD-1300-Y/HS Princeton Instruments, Inc., Trenton, NJ). Using MetaMorph software (version 3.6, Universal Imaging Corp., West Chester, PA), the microparticle motion was traced and analyzed. The experiments were conducted using three samples and the images were analyzed three times for each sample.

Statistical analysis

All measurements were conducted three times for each of three samples, and the mean and the standard deviation were calculated using Microsoft Excel (version 2003). A spectral analysis was conducted to identify a peak frequency component using MatLab software (version 7.0, The MathWorks, Inc.).

Results

Calibration of the pressure sensing system

Using a water column with known heights from 2 cm H₂O (200 Pa) to 20 cm H₂O (2000 Pa), a pressure calibration reference was established using an increase in water pressure and the voltage signal from the pressure sensor (Fig. 1A). A best-fit linear regression analysis determined a calibration slope of 17.5 Pa/mV (pressure in the glass tube; $r^2 = 0.99$) and 20.4 Pa/mV (pressure in the *in vivo* femur; $r^2 = 0.99$), indicating that a voltage signal of 1 mV corresponded to pressure modulation of 17.5 Pa (glass tube) and 20.4 Pa (femur *in vivo*) (Fig. 1B). The difference between these two slopes is probably induced by viscoelasticity of the femoral bone as well as marrow fluid inside the bone cavity. Hereafter, we used the slope of 20.4 Pa/mV to evaluate pressure alteration driven by knee loading.

Integration of the *in vivo* piezoelectric loader with the pressure sensor system

The piezoelectric loader was previously used for studying bone formation, and the same piezoelectric actuator was used for pressure measurements with a minor modification of the shape of the loading rod (Fig. 2A & 2B). Prior to loading, the baseline intramedullary pressure was measured as 1290 ± 150 Pa (mean $\pm$ SD; equivalent to 9.5 ± 1.1 mmHg) (Fig. 2C). Using sham loading, we first evaluated any spurious signal inherent to activation of the piezoelectric actuator or ambient vibration of the sample table and the optical cable. The signal level for sham loading has a root-square-mean noise of 0.99 mV with an actuator voltage of 60 V (peak-to-peak) and a loading frequency at 0.5 Hz (Fig. 3A & 3B). This noise level was not dependent on loading frequencies (data not shown).

Using the above loading condition, intramedullary pressure in the femoral bone cavity was measured (Fig. 3C). The alteration of pressure signal (peak-to-peak) was estimated as 6.0 ± 1.0 mV with knee loading (mean $\pm$ SD). We conducted a spectral analysis to identify a peak frequency component in the observed pressure signal. The results clearly showed that the peak frequency coincided with the loading frequency at 0.5 Hz (Fig. 3D). No frequency peaks were detected in the signal for sham loading (data not shown).

Effects of loading intensities and loading frequencies

Increasing loads to the knee elevated pressure alterations with the actuator voltage ranging from 10 V (0.5 N) to 80 V (4 N). The pressure elevation then reached a plateau and no significant increase was observed from 80 to 100 V (Fig. 4A). This two-phase trend was common with the loading frequency at both 0.5 Hz and 10 Hz. At 0.5 Hz, for instance, the pressure alteration (peak-to-peak) was observed as 0.34 ± 0.24 mV (mean $\pm$ SD) at 20 V, 2.8 ± 0.40 mV at 40 V, and 7.0 ± 0.72 mV at 80 V.

Alteration in intramedullary pressure was dependent on the loading frequency. In response to a 40-V actuator voltage with the loading frequency ranging from 0.5 Hz to 50 Hz, the pressure alterations were nearly constant up to ~ 10 Hz and significantly decreased at > 20 Hz (Fig. 4B). For instance, the measured pressure value was 2.8 ± 0.36 mV (mean $\pm$ SD) at 0.5 Hz, 2.4 ± 0.32 mV at 5 Hz, and 0.76 ± 0.013 mV at 50 Hz.

Real-time monitoring of the microparticles

Real-time microparticle motion was observed in response to 4-N force (peak-to-peak) at 0.5 Hz (Fig. 5A & 5B). First, sham loading presented a constant linear motion outwards to the open end of the tube, since the pressure inside the femoral cavity was partially released but still higher than the atmospheric pressure. Second, in response to knee loading, the microparticles exhibited a combined motion of linear translation and sinusoidal oscillation. Based on a trace of 4 microparticles near the radial center, their motion along the length of the tube was modeled as $\alpha \sin(2\pi ft + \theta_0) - \beta t$ with $\alpha = 10.6 \mu\text{m}$ (amplitude of the oscillatory motion), $\beta = 16.2 \mu\text{m/s}$ (linear translational speed) at $f = 0.5 \text{ Hz}$, where “ t ” = time, and θ_0 = phase angle (Fig. 5C & 5D). Note that stationary particles immobilized on the glass tube indicated no obvious vibration of the tube in response to knee loading.

Discussion

The current study demonstrates dynamic alterations of intramedullary pressure in the mouse femur in response to knee loading. The observed pressure amplitude (half of peak-to-peak) in the femoral bone cavity ranged from approximately 3 to 130 Pa depending on the loading conditions (0.5 to 4 N at 0.5 to 50 Hz). Note that 1 cm H₂O is equivalent to 100 Pa, and therefore the observed maximum pressure alteration of 130 Pa corresponds to 1.3 cm H₂O. Using sham loading, the background signal level (rms error) was estimated as 20 Pa. The pressure modulation synchronous to dynamic knee loading was consistent with the oscillatory motion of the microparticles in the glass tube connected to the femoral cavity. These results clearly reveal that intramedullary pressure in the femur is sensitive to dynamic lateral loads applied to the femoral epiphysis. Since knee loading was shown to enhance bone formation in the hindlimb [22,23], the results here suggest a potential linkage between modulation of intramedullary pressure and load-driven bone formation throughout the length of long bones.

The data further reveal nonlinear dependence of pressure alterations on loading conditions. First, increasing loads elevated the amplitude of modulation monotonously from 1 N to 4 N at the rate of $\sim 20 \text{ Pa per N}$, but no significant increase was observed above 4 N. Second, the loading effect was significantly reduced at a loading frequency above 20 Hz. The viscoelastic nature of tissues likely determines their ability to respond to loading and exhibits the lower response to higher frequencies. It is possible that at 4 N the bone structure reaches its elastic limit and further deformation is restrained. In the current *in vivo* studies, the cannula was filled with the saline solution and we occasionally observed that this saline solution was mixed with a small amount ($< 0.05 \text{ ml}$) of fluid from the bone cavity. Therefore, the femur *ex vivo* might not faithfully represent the undisturbed condition for nominal knee loading. Nevertheless, our observations suggest intensity and frequency dependence of the pressure modulation and indicate an advantage of loading frequencies below 20 Hz to effectively alter intramedullary pressure. The observation is consistent with frequency dependence of anabolic effects in previous studies [22,23], although dependence on loading frequencies is expected to be altered by loading modalities and target bones.

The observed pressure alteration can be induced by load-driven volume alteration in the femoral bone cavity. Since a long bone like a femur is less rigid in the lateral direction than the axial direction and the epiphysis is more deformable than the diaphysis in response to the same magnitude of loads, knee loading is considered to be an efficient method to dynamically alter the volume of the femoral cavity and thus intramedullary pressure. It is possible that the rapid change in intramedullary pressure creates swift alterations in a pressure gradient which in turn drive the interstitial flow in the lacunocanicular network. Our measurement of the baseline intramedullary pressure of approximately 10 mmHg is in good agreement to the data published by Stevens et al. [25]. Although the observed pressure alteration with knee loading is 0.2 ~ 10% of the baseline intramedullary pressure, it is a dynamic change rather than static.

Dynamic pressure oscillations in a tube have been shown to enhance solute dispersion even at a low-level fluctuation [26,27].

The current pressure data are suggestive of the role of intramedullary pressure in load-driven bone formation, but they do not establish a causal relationship between the observed pressure alteration and anabolic effects with knee loading. Many biophysical factors have been considered as a potential determinant in load-driven bone remodeling. Besides alterations in intramedullary pressure, threshold strain [10,11], daily loading cycles [12], interstitial fluid flow [13], and enhancement of molecular transport [16,17] are considered to be influential. In whole body vibration, for instance, a nonlinear relationship of strain and loading cycles is employed to define the loading conditions required for anabolic responses [28]. It is unclear, however, how the relationship is linked to molecular transport or interstitial fluid flow. With knee loading, we have previously shown using FRAP that molecular transport is significantly elevated in response to sinusoidal loads to the femur *ex vivo* [16]. It is possible that load-driven intramedullary pressure in the femoral bone cavity is a cause of molecular transport. Although knee loading is capable of elevating bone formation in the hindlimb, our preliminary studies indicate that effective loading frequencies differ. Furthermore, the periosteal surface in general exhibits stronger anabolic responses than the endosteal surface. These observations suggest that the anabolic responses in response to knee loading are affected by not only biophysical alterations but also anatomical and physiological microenvironments in the long bone. Comparison of the frequency response of molecular transport to that of pressure modulation might facilitate linking these biophysical phenomena to bone formation.

In conclusion, we have demonstrated that intramedullary pressure in the femoral bone cavity was altered in synchrony with mechanical loads applied with knee loading. The load-driven anabolic effect is a complex interplay among viscoelastic tissues, pressure in the medullary cavity, interstitial fluid in porous bone matrix, and other biochemical factors. Knee loading was reported to enhance formation of diaphyseal cortical bone with *in situ* strain < 100 μ strain at the site of bone formation, and therefore it is conceivable that intramedullary pressure plays an important role in connecting the loaded epiphysis to the unloaded diaphysis. The current pressure study is limited in its scope, since the measurement was directed in the intramedullary cavity and not in the porous matrix of cortical bone. Thus, further analysis will be required to more fully evaluate the role of pressure modulation and fluid flow in load-driven bone formation.

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Figure 1.

Pressure calibration. (A) Schematic illustration of an experimental setup for pressure calibration using a femur *in vivo*. Two surgical holes were generated for connecting a pressure sensor and a water column to a femoral cavity. Pressure calibration was conducted using the femur *in vivo* and a glass tube filled with a saline solution. (B) Relationship between the applied pressure in Pa and the sensor signal in mV. The best fit regression line is $y = 0.057x + 2.236$ ($r^2 = 0.99$; water column), and $y = 0.049x - 0.488$ ($r^2 = 99$; *in vivo* femur).

Figure 2.

Experimental setup for pressure measurements with the femur *in vivo* and baseline intramedullary pressure. (A) Syringe consisting of a pressure sensor and an optic fiber. (B) Schematic illustration of an experimental setup. (C) Baseline intramedullary pressure. At $t = 30$ s, the pressure in the syringe was released to an ambient atmospheric pressure.

Figure 3. Pressure measurements in response to 0.5 Hz dynamic loading. (A) Input voltage to the piezoelectric loader at 0.5 Hz. (B) Pressure signal for sham loading (control). (C) Pressure signal with knee loading. (D) Fourier spectrum corresponding to the data in C.

Figure 4.

Effects of loading intensities and frequencies on intramedullary pressure. (A) Alteration in pressure amplitudes as a function of actuator voltages at 10 to 100 V (0.5 to 4 N). (B) Alteration in pressure amplitudes as a function of loading frequencies at 0.5 to 50 Hz.

Figure 5.

Real-time monitoring of the microparticles with knee loading. (A) Piezoelectric loader with a femur *ex vivo* mounted on a NIKON microscope. (B) Schematic illustration of the microparticle experiment. (C) Time elapse images at 250-msec intervals in response to the loading at 2 Hz. The left panel shows the raw images, and the right panel presents a trace of 4 particles. (D) Temporal alterations in particle positions. Based on 4 particles the sinusoidal motion model, depicted in the dotted line, was built as $\alpha \sin(2\pi ft + \theta_0) - \beta t$