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Knee Loading Accelerates Bone Healing in Mice

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Abstract

Knee loading is an anabolic loading modality that applies lateral loads to the knee. The present study demonstrates that loads applied to the proximal tibial epiphysis stimulate healing of surgically generated wounds in the tibial diaphysis.

Introduction—Wound healing is sensitive to mechanical stimulation such as various forms of stress and different magnitudes of strain. Knee loading has been shown to induce anabolic responses to murine tibiae and femora when a strain of 10 – 20 μ strain is applied at the site of new bone formation. The object of this study was to address a question: Does knee loading accelerate closure of open wounds in the tibia?

Material and Methods—Fifty-three C57/BL/6 female mice were used. A surgical wound (0.5 mm in diameter) was generated in the left tibia (loaded) as well as the right tibia (sham-loaded control). From the fourth postoperative day, knee loading was performed to the left knee with a custom-made piezoelectric loader for 3 min/day for 3 consecutive days. The peak-to-peak force was 0.5 N. Animals were sacrificed 1, 2, or 3 weeks after the surgery, and the healing process was evaluated with microcomputed tomography (μ CT), peripheral quantitative computed tomography (pQCT), and bone histomorphometry with calcein labeling.

Results—The measured strain was < 20 μ strain with 0.5-N force regardless of the presence or absence of surgical wounds. Compared to sham-loaded control, the results showed load-driven acceleration of wound healing. First, μ CT data revealed that knee loading reduced the size of surgical wounds by 13% ($p < 0.01$; 1 week), 25% ($p < 0.001$; 2 weeks), and 15% ($p < 0.01$; 3 weeks). Second, pQCT data indicated that total BMD and BMC as well as cortical BMD and BMC were significantly increased in the third postoperative week. Lastly, bone histomorphometry revealed that bone formation was stimulated from the site proximal (close to the knee) to the wound.

Conclusions—The reparative and remodeling phases of wound healing were enhanced by loads applied to the knee without inducing significant *in situ* strain at the site of wounds. Noninvasive knee loading might therefore be useful clinically to stimulate bone healing in the entire tibia along its length (including cast immobilized wounds).

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Keywords

knee loading; wound healing; mouse; tibia; bone formation

INTRODUCTION

The effects of mechanical loading on fracture repair depend on loading conditions such as stress types (compressive, tensile, and shear) and strain magnitudes.(1–7) The results of various studies support the notion that relatively low stress or strain promotes callus formation and increases bone strength.(8–14) However, application of higher loads appears to result in a deleterious outcome. (8) The appropriate loading type and its magnitude are related to the type of fractures.(1,15,16) Furthermore, loading modalities such as tensile, axial loading and bending are often ineffective when fractures are immobilized by a cast. (17) Thus, although mechanical loading can noninvasively stimulate fracture healing, a seemingly biphasic outcome with high sensitivity to a loading intensity makes clinical applications somehow restricted.(18,19) We sought a mechanical loading modality with strong anabolic potential that does not require direct contact with the fracture site, and induces only small mechanical strain.

In this study we investigated the effects of a knee loading modality on healing of surgical wounds in the murine tibia. Like elbow and ankle loading,(20,21) knee loading applies lateral loads to the synovial joint with relatively small force (0.5 N, peak-to-peak) for several minutes per day. Knee loading causes enhanced bone formation throughout the tibia and femur(22,23) when the measured strain is $\sim 10 \mu\text{strain}$ and intramedullary pressure was modulated synchronously with a loading frequency. (24) Knee loading therefore seems to satisfy the above requirements of an indirect contact and induction of small mechanical strain. In this study, we determined its efficacy for accelerating bone healing.

This study addressed two questions: 1) Does knee loading accelerate a closure of surgical wounds in the tibial diaphysis? 2) Does the closure result from uniform or site-specific formation of callus and/or remodeling towards lamellar bone? We hypothesized that by knee loading a growth of callus would take place from the site proximal to surgical holes (close to the loading site at the knee) and remodeling would be stimulated. Those hypotheses were tested using strain measurements, structural analyses with micro-computed tomography, measurements of bone mineral density with peripheral quantitative tomography, and bone histomorphometry. We chose to employ surgical wounds in the tibia, since this wound model is well-established for studies on bone defect repair in normal and transgenic mice. (25,26) The model allowed induction of reproducible bone lesions in a readily identifiable location adjacent to the distal end of the tibial crest with a defined size of 0.5 mm in diameter.

MATERIALS AND METHODS

Animal preparation

Fifty-three C57/BL/6 female mice (14 weeks of age) were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Four to five mice were housed together in a cage, and mice chow and water were given *ad libitum*. All procedures performed in this study were in accordance with the Indiana University Animal Care and Use Committee guidelines and were in compliance with the Guiding Principles in the Care and Use of Animals endorsed by the American Physiological Society. Mice were allowed to acclimate for 2 weeks before the experiment. Note that we used 9 mice for strain measurements, 34 animals for μCT and pQCT analyses, and 10 mice for bone histomorphometry.

Surgical procedure

A round surgical wound (0.5 mm in diameter), penetrating the medial and lateral surfaces, was generated in the left and right tibiae of 44 mice at a site ~ 4 mm distal (25% along the length of the tibia) from the proximal tibial end (Fig. 1). The animal was anesthetized with 2% isoflurane and the hindlimb was shaved and sterilized with 10% providoneiodine solution. A 5-mm longitudinal skin incision was made over the antero-medial side of the hindlimb. Using a new drill bit a hole of 0.5 mm in diameter was generated on the medial cortex and the lateral cortex.(25,26) A plunge router attachment was used to maintain stability and consistency of the operation. Subsequently, the muscles and skin were sutured and closed. The same surgical procedure was used to the left and right tibiae. Antibiotic prophylaxis (Enrofloxacin, 5 µg/g body mass) and analgesia (Morphine, 5 µg/g body mass) were administered for the first two postoperative days. Following the operation mice were allowed full unrestricted cage activity.

Measurement of mechanical strain

Axial strain, induced by knee loading, was performed on the procedure described previously (22,23) (Fig. 1). We employed 9 mice in total with surgical holes (n = 5) and without them (n = 4). The mouse was sacrificed, and the medial periosteal surface of a left tibia was exposed and cleared. A uniaxial strain gauge (0.7 mm × 2.8 mm; Model EA-06-015DJ-120, Measurements Group Inc.) was glued on the medial periosteal surface at a site corresponding to the surgical hole, which was located ~ 4 mm (25%) distant from the proximal end of the tibia. Sinusoidal loads were applied to the knee at 5 Hz with 0.5, 1 or 2 N force (peak-to-peak) in the lateral-medial direction. Voltage signals from the strain gauge were sent to the computer via a signal conditioning amplifier (2210, Measurement Group Inc.), and Fourier analysis was applied to remove the noise from the signal. The measurement was repeated five times, and the peak-to-peak voltage was recorded and converted to the strain value.

Mechanical loading

On days 4, 5 and 6 after the surgery, knee loading was conducted to the left knee. (22,23) In brief, the mouse was anesthetized in an anesthetic induction chamber and then mask-anesthetized using 2% isoflurane. Using the custom-made piezoelectric loader, loads were applied for 3 minutes/day for 3 consecutive days at 5 Hz with a peak-to-peak force of 0.5 N (Fig. 1). The right hindlimb was used as sham-loaded control, while the right knee was placed under the loading rod in the same procedure used for the left knee without applying a voltage signal to the loader. On days 8 and 14 after the surgery, the mice were given an intraperitoneal injection of calcein (Sigma, St. Louis, MO) at 30 µg/g body mass.

Sample harvest

Thirty-four animals were sacrificed for µCT and pQCT (n = 5, 7, 6, and 16 on the operative day, 1 week, 2 weeks, and 3 weeks after surgery, respectively). For bone histomorphometry, 10 mice were sacrificed 3 weeks after surgery. Soft tissues were removed from the isolated tibiae, and the distal and proximal ends were cleaved to allow infiltration of the fixatives containing 10% neutral buffered formalin. After 48 h in the fixatives bones were transferred to 70% ethanol for storage.

Micro-computed tomography

Micro CT was performed using a desktop µCT-20 (Scanco Medical AG, Auenring, Switzerland) (Fig. 2). The harvested tibia was placed in a plastic tube filled with 70% ethanol and centered in the gantry of the imaging device. A series of cross-sectional images

were captured at 30- μ m resolution. The transverse and the axial sizes were estimated from 3D reconstructed images, and the size of the hole was defined as a mean of those sizes.(27)

Peripheral quantitative computed tomography

Eight bone samples were used for pQCT (XCT Research SA Plus, software 5.40; Norland-Stratec Medizintechnik GmbH, Birkenfel, Germany).(28,29) The surgical wounds were scanned for five consecutive cross-sections with a sectional distance of 0.4 mm, where each section was 260 μ m in thickness with a voxel size of 7 μ m.(25) For each slice, total volumetric bone mineral density (vBMD_t; mg/cm³) and cortical bone mineral density (vBMD_c; mg/cm³) were computed together with total bone area (A_t; mm²) and cortical area (A_c; mm²). From those data, we derived total bone content (vBMC_t = vBMD_t $\times$ A_t; mg/mm), and cortical bone content (BMC_c = BMD_c $\times$ A_c; mg/mm). An outer threshold of 400 mg/cm³ and an inner threshold of 710 mg/cm³ were used to distinguish the cortical shell from soft tissues and cortical bone from the endocortical surface, respectively.(30)

Bone histomorphometry

Bone histomorphometry was conducted for three cross sections (proximal, middle, and distal sections to the wound). Those sections were located ~ 3 mm (proximal - outside of the wound), ~ 4 mm (in the middle of the wound), and ~ 5 mm (distal - outside of the wound) distant from the tibial end at the knee, respectively. We determined labeled surface, mineralizing surface (MS/BS), mineral apposition rate (MAR), bone formation rate (BFR/BS), cross-sectional cortical area, and cortical thickness as described previously.(22,23) The relative parameters such as surface labeling, rMS/BS, rMAR, and rBFR/BS were calculated as $([L - S]/S \times 100 \text{ in } \%)$, where L = “loaded” and S = “sham loaded control.”

Statistical analysis

The data were expressed as mean $\pm$ SEM. Statistical significance among groups was examined using one-way ANOVA. For pair-wise comparisons a post-hoc test was conducted using Fisher’s protected least significant difference tests. A paired *t*-test was employed to evaluate statistical significance between the loaded samples and sham-loaded control. All comparisons were two-tailed and statistical significance was assumed for $p < 0.05$. The single, double and triple asterisks in figures indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

RESULTS

No infections were detected at the surgical site during the 3-week course of experiments. We did not observe any abnormal behavior, weight loss, or diminished food intake.

Strain at the site of wound with and without knee loading

In response to loads (0.5, 1, and 2 N, peak-to-peak), strain on the periosteal surface of the tibia was determined with and without the surgical hole (Fig. 1). In the tibia with the surgical hole, the strain value over the surgical hole was $10 \pm 1 \mu\text{strain}$ (0.5 N), $23 \pm 2 \mu\text{strain}$ (1 N), and $48 \pm 3 \mu\text{strain}$ (2 N). The tibiae without a surgical hole did not show a statistically significant difference ($p = 0.07 \sim 0.20$), and the strain values were $12 \pm 2 \mu\text{strain}$ (0.5 N), $26 \pm 2 \mu\text{strain}$ (1 N), and $44 \pm 3 \mu\text{strain}$ (2 N). Hereafter, we employed 0.5-N loads for knee loading.

Accelerated closure of surgical holes with knee loading

Micro CT images displayed accelerated closure of the surgical hole in the tibia with knee loading (Fig. 2). One week after the surgery, the size of the wounds on the medial cortex

was decreased from 0.48 ± 0.02 mm (control) to 0.40 ± 0.01 mm (loaded; $p < 0.01$). The wounds on the lateral cortex were also reduced from 0.44 ± 0.03 mm (control) to 0.40 ± 0.01 mm (loaded; $p = 0.18$), although the reduction was not statistically significant. In the second and the third weeks, the wound size was further decreased and the decrease was statistically significant both on the medial and the lateral surfaces. The overall improvement of bone wound closure by knee loading was 13% (1 week, $p < 0.01$), 25% (2 weeks, $p < 0.001$), and 15% (3 weeks, $p < 0.01$).

Interestingly, the wounds did not heal uniformly in the axial and lateral directions (Fig. 3). Three weeks after the surgery, for instance, the size of cortical wound in the axial direction was 0.35 ± 0.01 mm (control) and 0.31 ± 0.01 mm (loaded, $p < 0.001$), and 0.18 ± 0.02 mm (control) to 0.14 ± 0.01 mm (loaded, $p < 0.05$) in the transverse direction.

Increased vBMD and BMC with knee loading

Evaluation of bone quality with pQCT revealed an increase in vBMD and BMC with knee loading (Fig. 4). Total vBMD was elevated 13% from 588 ± 14 mg/cm³ (control) to 666 ± 12 mg/cm³ (loaded, $p < 0.01$), while cortical vBMD was increased 6% from 997 ± 14 mg/cm³ (control) to 1058 ± 7 mg/cm³ (loaded, $p < 0.01$). Furthermore, total BMC was 13% higher from 3.19 ± 0.09 mg/mm (control) to 3.46 ± 0.09 mg/mm (loaded, $p < 0.05$) with 20% increase of cortical BMC from 2.16 ± 0.06 mg/mm (control) to 2.6 ± 0.08 mg/mm (loaded, $p < 0.01$).

Increase in bone formation with knee loading

Bone morphometry was conducted using the samples harvested 3 weeks after the surgery (Fig. 5). At the proximal site, the calcein-labeled surface was increased by loading from $42 \pm 4\%$ to $56 \pm 4\%$ on the periosteal surface ($p < 0.001$), and from $40 \pm 6\%$ to $64 \pm 4\%$ on the endosteal surface ($p < 0.01$). The loading-induced increase was also observed at the middle site: from $48 \pm 3\%$ to $62 \pm 3\%$ on the periosteal surface ($p < 0.01$), and from $39 \pm 4\%$ to $63 \pm 3\%$ on the endosteal surface ($p < 0.001$). Interestingly, no significant changes were observed at the distal site most distant from the knee ($p = 0.13 \sim 0.27$). Compared to the distal site on the periosteal surface, higher calcein labeling was observed at the proximal site ($p < 0.01$) and the middle site ($p < 0.05$). On the endosteal surface there was no difference among the three sites, although the increase in the proximal and middle sites approached statistical significance (both $p = 0.06$).

Load-driven increase in bone morphometric parameters is summarized in Table 1. The maximum bone formation rate was detected at the proximal site on the periosteal surface (1.8 X; $p < 0.01$) and the endosteal surface (2.8 X; $p < 0.01$). An increase in rMS/BS, rMAR, and rBFR/BS was determined and the percent change in three parameters was calculated (Fig. 6). On the periosteal surface, the proximal site resulted in a significantly greater value of rMS/BS than the distal site ($p < 0.05$). The proximal and middle sites exhibited a larger value of rMAR than the distal site (both $p < 0.01$). A highest value of rBFR/BS was detected at the proximal site ($p < 0.001$ to distal; and $p < 0.05$ to middle) and the second was the middle site ($p < 0.05$ to distal). The endosteal surface presented a larger rMS/BS at the proximal site and the middle site than the distal site (both $p < 0.05$). A higher rMAR was determined at the proximal site ($p < 0.001$) and the middle site ($p < 0.05$) than the distal site, and the change in rBFR/BS was significantly larger at the proximal site ($p < 0.001$) and middle site ($p < 0.01$) than that at the distal site.

Load-driven alteration in skeletal geometry

Enhancement of the cortical area and cortical thickness was observed with knee loading (Fig. 7). Compared to the sham-loaded controls, the cross-sectional cortical area was

increased 18% at the proximal site from $0.79 \pm 0.02 \text{ mm}^2$ to $0.94 \pm 0.02 \text{ mm}^2$ ($p < 0.001$). The cortical area was elevated by 9% at the middle site from $0.78 \pm 0.02 \text{ mm}^2$ to $0.85 \pm 0.02 \text{ mm}^2$ ($p < 0.05$). Similarly, the cortical thickness was increased from $0.152 \pm 0.004 \text{ mm}$ to $0.180 \pm 0.005 \text{ mm}$ at the proximal site ($p < 0.001$), and from $0.161 \pm 0.004 \text{ mm}$ to $0.175 \pm 0.005 \text{ mm}$ at the middle site ($p < 0.05$). There was no statistical difference in the cortical area or cortical thickness at the distal site (both $p = 0.07$). Among the three sections, the proximal site presented the highest increase in cortical area ($p < 0.001$). In addition, the proximal site presented the maximum elevation in cortical thickness followed by the middle site.

DISCUSSION

The present study demonstrates that knee loading (900 daily cycles for 3 consecutive days) accelerates a closure of surgical holes in the tibial diaphysis and remodeling of callus towards lamellar bone. Knee loading provides some advantages for potential clinical application. For instance, there was no direct contact to the fracture site. Forces were applied at the knee, which was 4 mm away from surgical wounds in the tibia. Loads of 0.5 N (peak-to-peak) applied at 5 Hz induced strain of only about 10 μ strain at the fracture site. With 0.5 N force, the wound closure was improved significantly by 13%, 25%, and 15% in the 1st, 2nd, and 3rd week, respectively. Measurements of by pQCT in the 3rd week demonstrated elevated total vBMD (13% increase compared to sham loading) as well as BMC (9% increase). The results with bone histomorphometry show that the growth of callus is most active proximal to the wound site close to the knee.

Previous studies demonstrated that microstrain induced by axial loading shortened the healing time,(2,13,31) and mechanical compression was shown to accelerate the growth of cartilaginous tissue.(32) However, those loading models directly applied loads to a fracture site through axial force, bending moment or vibratory oscillation. Deformation and induced strain at the site of fracture are heavily affected therefore by geometry, fixation, and healing phases of individual fractures.(1,33) Compared to these loading modalities, knee loading has the advantage or relative non-sensitivity of induced strain to fracture conditions.(1,34) The strain gauge data indicate that the presence of surgical wounds did not affect the measured strain values. Another advantage of knee loading includes a short loading duration (3 min/day for 3 days) as well as its applicability to fractures immobilized by casting. Since fractures in long bones are in many cases immobilized by casting, knee loading seems to have a wider application of mechanical stimulations.

The mechanism underlying fracture repair with knee loading is yet to be understood and it is likely to be different from the mechanism for anabolic responses in intact tibiae. Knee loading causes cyclic alteration of intramedullary pressure (24,27) which may affect interstitial molecular transport driven by pressure gradients.(35,36) In the current study, however, surgical holes apparently reduce pressure sealing in the medullary cavity yet knee loading remained effective for improving healing. We speculate that knee loading would stimulate molecular and cellular transport in the medullary cavity, and this transport would be stronger at the proximal site than the distal site. Furthermore, knee loading is reported to generate an oscillatory motion of micro particles in the glass tube connected to the surgical hole.(24) Further biophysical and biochemical examinations are necessary to understand the interplay between the loaded epiphysis and the fractured diaphysis.

The current study is limited in its scope to healing of surgical holes in the tibial diaphysis and a specific loading condition and timeline. We chose a surgical hole because of its well-documented procedure and reproducibility to evaluate a proof of principle with knee loading. Although the current study did not evaluate angiogenesis, previous studies have indicated an important role of load-driven vascularization.(37,38)

In summary, we demonstrate that knee loading can be a useful means to accelerate wound healing in the tibial diaphysis with very small mechanical strains at the wound site. The results extend our knowledge of load-driven fracture repair and the interplay between the epiphysis and the diaphysis. Based on previous studies on bone formation, the described knee loading modality would indicate a novel strategy for stimulating bone repair in the entire tibia and the femur throughout its length in a relatively short loading period. The current proof-of-principle results with knee loading should be further examined using larger experimental animals where biomechanical conditions can be closer to those in humans.

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Figure 1.

(A) Time line of the study. μ CT = micro computer tomography; and pQCT = peripheral quantitative computed tomography. (B) Left tibia with a surgical hole. Bar = 2 mm. (C) Left knee with a surgical hole on a loading table for knee loading on the fourth postoperative day. Bar = 4 mm. (D) Strain gauge attached to the tibia for the strain measurement with knee loading. Bar = 4 mm. (E) Force - strain relationship with and without a surgical hole. The results are expressed as mean $\pm$ SEM.

Figure 2. Micro CT images of the surgical holes. Bar = 1 mm. (A) Control tibiae 1, 2, and 3 weeks after the surgery. The upper and lower images are outside and inside of the tibia lesion, respectively. (B) Loaded tibiae 1, 2, and 3 weeks after surgery.

Figure 3. Alteration in wound size with and without knee loading. (A) Wound size on the medial surface (mm). (B) Wound size on the lateral surface (mm). (C) Wound size along the axial direction (mm). (D) Wound size along the transverse direction (mm).

Figure 4. Alteration in bone mineral density and bone mineral content in the third postoperative week. (A) Total bone mineral density and cortical bone mineral density (mg/cm³). (B) Total bone mineral content and cortical bone mineral content (mg/mm).

Figure 5.

Calcein labeling with and without knee loading. (A) Micro CT image of the control tibia in the third postoperative weeks. Note that “p-p” = proximal section; “m-m” = middle section; and “d-d” = distal section for bone histomorphometry. (B) Control cross-sections. The labels are medial (med), lateral (lat), and posterior (post). Bar = 200 μ m. (C) Loaded cross-sections. Bar = 200 μ m. (D) Percentage of the calcein-labeled surface on the periosteum and the endosteum for the three cross-sections. (E) Increase in labeling (% of control) on the periosteum and the endosteum.

Figure 6. Alterations in the morphometric parameters. (A) Increase in relative MS/BS (% of control). (B) Increase in relative MAR (% of control). (C) Increase in relative BFR/BS (% of control).

Figure 7. Alteration in the cross-sectional area and cortical thickness. (A) Cross-sectional area (mm²). (B) Increase in the cross-sectional area (% of control). (C) Cortical thickness (mm). (D) Increase in the cortical thickness (% of control).

Table
Histomorphometric parameters in the periosteum and endosteum near the surgical hole

		MS/BS (%)	fold change <i>p</i> value	MAR (μm/day)	fold change <i>p</i> value	BFR/BS (μm ³ /μm ² /year)	fold change <i>p</i> value
proximal							
periosteum	control	37.96±4.97	1.4 X < 0.05	0.35±0.02	1.3 X < 0.05	49.42±7.02	1.8 X < 0.01
	loading	54.28±4.68		0.44±0.03		89.24±10.44	
endosteum	control	39.48±6.32	1.8 X < 0.01	0.33±0.02	1.5 X < 0.01	49.06±8.79	2.8 X < 0.01
	loading	71.68±9.05		0.49±0.05		138.93±27.45	
middle							
periosteum	control	46.55±5.16	1.2 X NS	0.38±0.02	1.2 X < 0.01	65.15±7.76	1.6 X < 0.05
	loading	57.71±4.21		0.47±0.02		101.77±10.83	
endosteum	control	39.24±8.03	1.6 X < 0.05	0.30±0.01	1.3 X < 0.01	42.71±7.84	2.1 X < 0.01
	loading	61.52±6.10		0.40±0.02		90.77±11.37	
distal							
periosteum	control	44.91±4.74	1.2 X NS	0.34±0.01	1.1 X NS	55.70±5.76	1.3 X NS
	loading	54.09±3.57		0.36±0.01		71.04±4.95	
endosteum	control	40.57±3.95	1.2 X NS	0.31±0.01	1.1 X NS	45.89±4.78	1.2 X NS
	loading	47.05±4.04		0.33±0.01		56.16±5.02	

NS indicates that the *p* value exceeds 0.05.