Pause Insertions During Cyclic In Vivo Loading Affect Bone Healing

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Abstract Fracture repair is influenced by the mechanical environment, particularly when cyclic loads are applied across the fracture site. However, the specific mechanical loading parameters that accelerate fracture healing are unknown. Intact bone adaptation studies show enhanced bone formation with pauses inserted between loading cycles. We hypothesized pause-inserted noninvasive external loading to mouse tibial fractures would lead to accelerated healing. Eighty mice underwent tibial osteotomies with intramedullary stabilization and were divided into four loading protocol groups: (1) repetitive loading (100 cycles, 1 Hz); (2) pause/time-equivalent (10 cycles, 0.1 Hz); (3) pause/cycle-equivalent (100 cycles, 0.1 Hz); and (4) no load control. Loading was applied daily for

2 weeks. Healing was assessed using histology, biomechanical bending tests, and microcomputed tomography. The pause-inserted, cycle-equivalent group had a greater percentage of osteoid present in the callus cross-sectional area compared with no-load controls, indicating more advanced early healing. The pause-inserted, cycle-equivalent group had a failure moment and stiffness that were 37% and 31% higher than the controls, respectively. All three loaded groups had smaller overall mineralized callus volumes than the control group, also indicating more advanced healing. At an early stage of fracture healing, pause-inserted loading led to more histologically advanced healing.

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Introduction

Numerous characteristics of the mechanical environment directly affect the subsequent course of fracture healing. The magnitude of the applied load across the fracture site, the size of the fracture gap, the relative fracture motion and resulting strain, and the timing of mechanical intervention all impact healing [1, 7, 11, 12, 18]. Cyclic compression applied at an appropriate time, rate, and magnitude across the fracture site potentiates healing, leading to earlier and more advanced callus formation [6, 7, 11, 12, 18, 20, 22, 30]. Early dynamization of external fixation also leads to accelerated healing compared with rigid fixation [6–8, 20]. Conversely, the beneficial healing effects of intermittent loading across a fracture are negated when superimposed on static compression [2, 3, 13, 24, 35].

Innovative loading parameter variations cause dramatic increases in bone formation in intact bone [26, 28, 31]. Cyclic compressive loads applied with a pause between each cycle are more osteogenic than repetitive, continuous cyclic loads [23, 26, 31]. An increased duration of

repetitive cyclic loading does not lead to proportional increases in bone mass, presumably because cellular sensitivity to the mechanical stimulus diminishes quickly once an initial threshold for a response has been exceeded [10, 26, 29, 33]. For example, in a rat jumping model, the femoral compressive force in rats that jumped five times a day led to considerable bone hypertrophy compared with controls, but when jumps were increased to 100 per day, no additional increase occurred [33]. The cellular sensitivity to load can be recovered, however, as seen in studies in which loading sessions were separated into discrete temporal bouts [5, 16].

Accelerating fracture repair through mechanical intervention is a potentially noninvasive treatment supplement. Although used clinically in one trial [22], this approach has not become a standard treatment method, partly because the ideal loading parameters have not been fully defined. Previously, we showed that repetitive cyclic noninvasive loading without pause insertions in the loading regimens led to improved fracture healing after 2 weeks in a mouse tibial fracture model [18].

We hypothesized insertion of pauses in the loading waveform would lead to improvements in fracture healing compared with healing of nonloaded and repetitively loaded fractures, specifically in the early phases of healing.

Materials and Methods

To address our hypothesis, we divided 80 C57Bl/6 12-week-old male mice (Jackson Laboratories, Bar Harbor, ME; body weight 29 ± 2 g) into four groups ($n = 20$ per group). After tibial osteotomy creation, stabilization, and loading, the animals were euthanized by carbon dioxide inhalation. The experimental hind limbs were disarticulated at the hip, the tibiae were dissected free of soft tissue, and the intramedullary pin was removed. All specimens except those designated for histologic sectioning were frozen in moist gauze at -20°C in preparation for mechanical analysis and microcomputed tomographic imaging.

All mice underwent surgical osteotomy and intramedullary nailing of the left tibia as described previously [18]. In three of the groups, cyclic compression was applied to the ends of the tibia with a noninvasive external loading device (Fig. 1) [17]. Daily loading was begun after a 4-day fracture consolidation period to allow for stabilization of the hematoma and early angiogenesis [19, 34] and continued for 5 days per week for 2 weeks. One group had 100 load cycles applied daily at a rate of 1 Hz. A second group had a 9-second pause inserted between each load such that each load-pause cycle was 10 seconds. This loading continued for 10 cycles and was the time-equivalent (100 seconds) of the first group but with one-tenth the

Fig. 1 The external loading device consists of titanium plates that cup the calcaneus and femoral condyles. The actuator is driven by a function generator, an amplifier, and an electromagnetic coil to apply loads across the stabilized fracture.

number of cycles. A third group also had a 9-second pause inserted between loads, but continued for 100 cycles at 0.1 Hz (1000 seconds), which was the cycle equivalent of the repetitive loading group (Fig. 2). All loading was applied with the mice under general anesthesia with a triangle waveform with a peak load amplitude of 1 N and a preload of 0.5 N. In a fourth (control) group, mice underwent surgical osteotomy and intramedullary stabilization and were placed in the loading device daily but without any load application. A pre-hoc power analysis determined that 10 mice per group, plus an additional five specimens for histologic analysis, would provide greater than 80% power to detect a difference in biomechanical and microcomputed tomography parameters of 1.2 times the pooled standard deviation using a one-factor analysis of variance design (α , 0.05). Our protocol had prior approval of our Institutional Animal Care and Use Committee.

With the animal under general anesthesia, an incision was made over the dorsal aspect of the knee, and a 25-gauge needle was used to bore a hole in the proximal tibia. An oscillating saw was used to create a transverse osteotomy 5 mm distal to the patellar tendon insertion. The fragments were held in alignment, and a 27-gauge needle was inserted across the osteotomy into the distal fragment and cut to the appropriate length. All fibulas were verified as intact postoperatively using radiography. Buprenorphine was administered ad libitum for pain relief; no nonsteroidal antiinflammatory medications were used to avoid pharmacologic interference with fracture healing. All mice were allowed full, unrestricted cage activity after surgery. The animals did not favor the nonoperated limb starting on the first postoperative day.

The formation of mineralized tissue at the fracture site was assessed by quantitative microcomputed tomography (micro-CT) (MS-8 Small Specimen Scanner; GE

Fig. 2 A plot of the waveforms for the loaded groups is shown. The two pause-inserted loading groups had a 9-second pause between each load application with cyclic loading continuing for either 100 or 1000 seconds.

Healthcare, formerly EVS Corp, Ontario, Canada). The tibiae were placed in a saline-filled scan tube and scanned at 80 V and 80 μ A. Each scan included a phantom containing air, saline, and an SB-3 bone analog (1.18 g/cc) for calibration of image Hounsfield units to tissue mineral density in milligrams per centimeter. Reconstruction of the individual projections to CT volume data was accomplished with a modified Parker algorithm [15, 25] with an isotropic voxel resolution of 24 μ m.

To distinguish mineralized tissue from marrow, water, and unmineralized callus, microCT images were thresholded using 25% of the mineral attenuation value of the cortical bone determined for each specimen. Regional analyses of the thresholded scans were performed using the system software (MicroView; GE Healthcare Technologies, Waukesha, WI). To characterize calluses globally and locally, two volumes of interest were selected. First, a best-fit volume around the entire callus was used to determine the total mineralized callus volume, a slightly different method than has been used previously [18]. An elliptic cylinder, 1 mm in height, was centered at the osteotomy site and used to determine callus mineral density and content. Care was taken to ensure this section did not contain a cortical overlap that would affect density measurements.

Bone tissue from five specimens from each group underwent histologic analysis. Immediately after harvesting and dissection, the bones were preserved in 10% phosphate-buffered formalin for 2 days at 4° C. After formalin fixation, samples were washed overnight in running water, dehydrated through alcohol at 70%, cleared in xylene, and embedded in methyl methacrylate [14]. Calcified tissues were then sectioned longitudinally at 5- to 7- μ m thickness along the extramedullary portion of the callus. Sectioning was performed with a tungsten carbide blade on a microtome (Reichert-Jung Ultracut E; Leica Microsystems, Wetzlar, Germany). Nine sections were subsequently stained with von Kossa to evaluate the mineralized tissue and with Goldner's trichrome to evaluate the osteoid

deposition. Measurements were taken at x40 magnification. The percentage of mineralized tissue and osteoid at the osteotomy site was quantified by a blinded investigator (BFR) using a BioQuant morphometric analysis system (Bioquant, Nashville, TN). Because of the early time studied, no attempt was made to quantify trabecular morphology or other indicators of advanced bone formation. Rather, we considered advanced histologic healing to be the presence of more osteoid and total mineralized tissue.

All remaining specimens from each group ($n = 7-9$) were tested to failure using four-point bending on an electromagnetic-based load frame (EnduraTEC ELF 3200; Bose Corp, Minnetonka, MN). Tibiae were placed with the flat anteromedial surface on the lower supports, which were set 8.4 mm apart; the upper load points were set 3.5 mm apart. Displacement was applied at 0.033 mm per second until failure occurred. The bending moment to failure (Nmm) and stiffness (Nmm²) were calculated from the load-deflection curves and the dimensions between the loading points. Contralateral intact bones were not tested.

We calculated the mean and standard deviation of the percent osteoid, percent mineralized tissue, bone mineral density, bone mineral content, bone volume, callus volume, failure moment, and stiffness for each group. One-way analysis of variance tests, followed by Bonferroni post hoc tests, were used to compare differences among the means of each variable tested for each group using a p value < 0.05 (PASS 6.0; NCSS, Kaysville, UT). The number of mice per group was based on a power analysis performed before the experiment.

Results

Histologically, the cycle-equivalent pause-inserted group (Fig. 3A) had a greater ($p = 0.015$) percentage of osteoid present in the callus compared with controls (Fig. 3B), indicating a more advanced early healing process. The

Fig. 3A–B (A) Examples of a histologic specimen after trichrome staining and microcomputed tomography scans from the cycle-equivalent pause-inserted group are shown. The area staining positive for osteoid in the lower portion of the callus is highlighted in red (Magnification, $\times 12.5$). (B) Examples of a histologic specimen after

trichrome staining and microcomputed tomography scans from the control group are shown. The area staining positive for osteoid in the lower portion of the callus is highlighted in red (Magnification, $\times 12.5$). The histology showed more osteoid in the pause-inserted group, and calluses appeared radiographically more advanced.

percentage of mineralized callus tissue was similar between the groups (Fig. 4).

All three loaded groups had smaller mineralized callus volumes than the control group (Fig. 5). Additional measurements of callus characteristics such as bone mineral density, bone mineral content, and total bone volume were similar between the groups.

Bending strength and stiffness were similar across the treatment groups. (Fig. 6; Table 1).

Fig. 4 The percentage of osteoid relative to total callus tissue area in the pause-inserted cycle-equivalent group was greater ($p = 0.015$) than the control group. No considerable differences were found in callus mineralization among groups. Means and standard deviations are shown.

Discussion

Directed mechanical stimulation of fracture healing may be a potent treatment supplement in difficult fractures. To become potentially clinically useful, however, the specific loading parameters which optimize osteogenesis must be further defined. The insertion of pauses into mechanical loading regimens is particularly osteogenic in intact bone, and the purpose of this study was to determine the effect of pause-inserted loading on fracture healing. After 2 weeks of healing, we found histologic evidence that pause-

Fig. 5 All three loaded groups had smaller callus volumes than the control group as measured by microcomputed tomography. Means and standard deviations are shown.

Fig. 6 The biomechanical parameters tested, failure bending moment and stiffness, were similar between the groups. Means and standard deviations are shown.

inserted loading accelerates the fracture healing process compared with controls.

This study had limitations. Most notably, we studied only one healing time. Although this time period was

chosen for the specific purpose of analyzing early healing differences resulting from loading, this choice was nonetheless somewhat arbitrary, and different results may occur at later stages of healing. Our microCT and mechanical testing data showed larger variability than observed previously [18], contributing to the lack of considerable effects despite an appropriate power calculation as part of the planning for this experiment. Reasons for this are unclear, but may have been partly attributable to technical error with testing, and the biomechanical results should be interpreted with caution. Another limitation was the exact strains at the osteotomy site were unknown. The curvature of the mouse tibia induces bending moments that produce compressive and tensile strains at the fracture site. Strains induced at the middiaphysis with loading have been characterized for intact tibia of mice using this loading device [9, 17], but the strains that occur with loading across a stabilized osteotomy in this model are unknown.

The specimens with 100 cycles of daily pause-inserted loading had histologic evidence of accelerated fracture healing compared with controls. This group, together with

Table 1. Groupwise comparisons of bone healing

Variable	Group			
	Control	Repetitive loading	Pause-inserted, time-equivalent	Pause-inserted, cycle-equivalent
Percent osteoid	13.0	19.4	17.4	24.8*
SD	5.0	5.8	5.3	1.9
Number	5	5	5	5
Percent mineralized tissue	25.0	26.8	32.5	31.8
SD	1.7	7.0	9.1	7.8
Number	5	5	5	5
BMD (mg/cm ³)	473.80	491.84	488.26	519.24
SD	41.87	64.59	55.96	75.74
Number	16	14	15	16
BMC (mg)	2.38	2.07	2.29	2.23
SD	0.37	0.51	0.73	0.53
Number	16	14	15	16
Bone volume (cm ³)	4.40	3.72	4.16	3.87
SD	0.81	0.93	1.42	0.86
Number	16	14	15	16
Callus volume (cm ³)	0.035	0.024*	0.026*	0.026*
SD	0.010	0.007	0.010	0.008
Number	16	14	15	16
Failure moment (N·mm)	16.7	18.4	20.4	22.8
SD	6.9	5.2	7.4	9.5
Number	8	7	9	9
Stiffness (N·mm ²)	741.4	719.4	776.1	974.7
SD	312.6	223.8	311.3	463.5
Number	8	7	9	9

* $p < 0.05$ versus control; SD = standard deviation; BMD = bone mineral density; BMC = bone mineral content.

the other loaded groups, also had a smaller mineralized callus volume. In general, fracture callus increases in size before complete calcification and remodeling, which then leads to a decrease in size. Thus, the smaller size coupled with increased osteoid in our study may support the advanced stage of healing afforded by loading.

Substantial data suggest specific loading regimens in intact bone induce increased bone formation [23, 26–28, 31]. Although fracture repair mechanisms are clearly more complex than intact bone adaptation, the mechanisms involved in periosteal osteoblast proliferation in intact bone are important in fracture healing as well, and activation of a mechanically induced response may similarly result in increased bone formation. In a noninvasive mouse tibia cantilever bending model, a low-magnitude force applied for 100 cycles daily was minimally osteogenic, whereas the same load applied for 10 cycles daily with 10-second pauses between cycles was a potent stimulant of bone formation [31]. In a similar study loading the rat ulna, a 14-second pause between individual load cycles led to a greater response than insertion of shorter pauses [26].

The overall effect from pause-inserted loading during fracture healing was modest, but present nonetheless. We found no mechanical differences among the pause-inserted and repetitively loaded groups despite observing considerably greater strength using the same repetitive loading protocol previously [18]. Perhaps the beneficial effects of pause insertions over repetitive loading results from saturation of the continuous cyclic protocol after a set number of cycles [31, 32]. Based on our data, the 100-cycle/1-Hz repetitive loading protocol may not saturate the mechanosensitivity of the healing tissue. Superimposing externally applied load on routine weightbearing, as was the case in this study, also may lessen the effect of load augmentation [36].

We chose the 2-week loading period to determine the differences in the early healing phase. Concordantly, healing appeared at an accelerated stage in the cycle-equivalent pause-inserted group as indicated by increased osteoid deposition. This time in the healing process may have been before substantial mineralization [4, 21], accounting for the lack of differences in von Kossa staining or microCT-based bone mineral content and density. Considerably smaller mineralized callus size with applied loading was measured by microCT compared with controls. The smaller size of calluses in the loaded groups may reflect early organization of the healing callus tissue.

We found axial loading of the fractured mouse tibia with pauses inserted may lead to acceleration of bone healing compared with nonloaded controls in the early phases. We suspect the increased osteoid deposition after pause-inserted loading would likely lead to increased mineral deposition and earlier fracture consolidation in the later

phases. Additional studies will be required to evaluate the effects of loading and pause insertions at later times.

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