Published in final edited form as:

J Orthop Res. 2007 December ; 25(12): 1552–1558. doi:10.1002/jor.20435.

The Effects of Delayed Stabilization on Fracture Healing

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Abstract

Previous studies have revealed that delayed internal fixation can stimulate fracture callus formation and decrease the rate of non-union. However, the effect of delayed stabilization on stem cell differentiation is unknown. To address this, we created fractures in mouse tibiae and applied external fixation immediately, at 24, 48, 72, or 96 hours after injury. Fracture healing was analyzed at 10 days by histological methods for callus, bone, and cartilage formation, and the mechanical properties of the calluses were assessed at 14 days post-injury by tension testing. The results demonstrate that delaying stabilization for 24 to 96 hours does not significantly affect the volume of the callus tissue (TV) and the new bone (BV) that formed by 10 days, or the mechanical properties of the calluses at 14 days, compared to immediate stabilization. However, delaying stabilization for 24 to 96 hours induces 10–40 times more cartilage in the fracture calluses compared with fractures stabilized immediately. These findings suggest that delaying stabilization during the early phase of fracture healing may not significantly stimulate bone repair, but may alter the mode of bone repair by directing formation of more cartilage. Fractures that are not rigidly stabilized form significantly larger amount of callus tissues and cartilage by 10 days post-injury than fractures stabilized at 24 to 96 hours, indicating that mechanical instability influences chondrocytes beyond the first 96 hours of fracture healing.

Keywords

fracture; delayed stabilization; delayed fixation; chondrocyte

Introduction

Over 6,300,000 people in the United States sustain fractures each year¹. The current trend in treating adult diaphyseal long bone fractures is to surgically stabilize them early after injury, in order to more rapidly mobilize patients. However, an increased “healing rate” has been observed in patients treated with delayed fixation, suggesting that there may be benefits in waiting to stabilize fractures^{2–9}. These biological benefits include the stimulation of callus formation, the improvement of mechanical properties of the callus, and a decreased rate of non-union^{2–9}. Although it has been proposed that delaying fixation may boost inflammatory response and provide extra stimulation to cells that are responsible for fracture healing^{4, 9, 10}, the exact mechanisms underlying fracture repair stimulation remain largely unknown.

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Whether delayed stabilization can affect stem cell differentiation and alter the mode of fracture healing has not been well determined.

Epigenetic factors, such as mechanical forces, are critical regulators of chondrocyte and osteoblast differentiation during fracture healing. Previous data from our group and others ^{11, 12} have shown that non-stabilized fractures heal through endochondral ossification, during which a cartilage template forms and is subsequently replaced by bone. In contrast, rigidly stabilized fractures heal through the process of intramembranous ossification where bone forms directly without a cartilage intermediate. These data indicate that mechanical stability can influence stem cells or progenitor cells to differentiate into chondrocytes or osteoblasts. However, the precise time period during which mechanical stimuli induce the commitment of cells to chondrogenic or osteogenic fates is unknown. There is evidence suggesting the early period of healing is crucial for cells to become cartilage or bone. In mice, chondrocyte (e.g. *collagen type II*) and osteoblast-specific (e.g. *osteocalcin*) transcripts are detected in the fracture callus as early as three days post-fracture ^{11, 13, 14}. Therefore, we hypothesize that delaying stabilization for various amounts of time during the early period of fracture healing affects cell fate decisions, and thereby influences the mode of fracture healing. To test this hypothesis, tibial fractures were created in mice, and then the bone segments were rigidly stabilized using external fixators immediately, or at 24, 48, 72, or 96 hours after surgery. Fracture healing and the callus tissues were assessed at 10 and 14 days post-injury by histological and mechanical methods.

Materials and methods

Surgical procedures and external fixation

All surgical procedures were approved by UCSF Institutional Animal Care and Use Committees. Male 129J/B6 mice (3-month old, weighing 25–30g) were used in this study. Mice were anesthetized by intraperitoneal injection of 2 % Avertin (0.015 ml/g). To avoid excessive displacement of the fracture ends, which itself causes cartilage formation and makes it difficult to analyze the effects of delayed stabilization on chondrogenesis ¹¹, a 0.25mm intramedullary pin was placed prior to the creation of fracture. The external fixator was then applied as previously described ¹¹. Briefly, the proximal and distal metaphyses of the tibia were transfixed using four 0.25 mm pins, which were oriented perpendicular to the long axis of the tibia, 45° to the sagittal plane, and 90° to each other (Fig. 1A). Two rings were positioned above the proximal and distal pins and secured to the pins using hexagonal nuts (Fig. 1B). Closed transverse mid-diaphyseal fractures of the tibia were created with a three-point bending apparatus (Fig. 1C). Radiographs were taken immediately after injury to confirm the extent of fracture. After recovery, animals were allowed to ambulate ad libitum and analgesics were provided for the first 48 hours (Buprenorphine, ZT Sigma, St. Louis, MO).

Following surgery, the tibiae were stabilized immediately, at 24, 48, 72, or 96 hours by connecting the rings using three longitudinal threaded rods (Fig 1D). Another group of fractures were left with the rings un-connected until sacrifice as non-stabilized controls. In the interim period, the mice were allowed to ambulate as tolerated, which typically happened within 24 hours of the surgery. The intramedullary pin maintained axial alignment while allowing for longitudinal and rotational motion of the segments during the interim period. Since mice normally exhibit the largest amount of cartilage in the soft callus stage of fracture healing¹¹ and exhibit calluses suitable for mechanical testing during the hard callus phase of repair¹⁵, animals in this study were sacrificed at 10 days post-fracture for histological analysis and 14 days post-fracture for mechanical testing. Mice with loose fixators or comminuted fractures were excluded from further analyses.

Tissue preparation for histological analysis

Ten days after fracture, animals were euthanized and the fractured tibiae were collected and fixed at 4°C in 4% PFA overnight. Tissues were decalcified in 19% EDTA for 10–14 days (4°C), and then dehydrated in a graded ethanol series and embedded in paraffin¹⁶. Sections of 10 µm were prepared through the whole callus, and 3 sections were mounted on each slide. Forty to 70 slides were collected for each sample depending on the size of callus.

Histological and histomorphometric analyses

Safranin O/Fast Green staining (SO/FG) was performed on every 10th slide to visualize cartilage (red). Trichrome staining (TC) was performed on the slides adjacent to that used for SO/FG staining to visualize new bone formation (blue) in the fracture callus.

Histomorphometric analysis of fracture healing was performed as described previously¹⁴. Briefly, sections stained by SO/FG and TC were viewed under the microscope and images were exported to Adobe Photoshop. The area of the whole callus, cartilage, or bone was selected by either a lasso tool, or using color range command. The total volume of the callus (TV), the total volume of cartilage (CV), and the total volume of new bone (BV) were then calculated.

Biomechanical testing

A second group of mice with fractures stabilized immediately, at 48 hours, at 96 hours, or left non-stabilized were sacrificed at 14 days after fracture and the fractured tibiae were collected for tension testing. Tissues were kept in PBS at –20°C until the day before testing and were thawed at 4°C overnight. The intramedullary pins were carefully removed prior to the testing. Both the proximal and distal pins were kept in situ to provide an extra anchor for potting. The proximal end of the fractured tibia was mounted in a pot using PMMA, secured onto a custom-designed mechanical testing apparatus, and then the distal end was mounted into another pot. Tension testing was performed at a linear rate of 0.10mm/s. Two parameters were derived from tension displacement curves: failure load, which represents the maximum tension required for failure of the callus, and slope of the load displacement curve, which represents the overall stiffness of the callus.

Statistics analysis

The data were analyzed in SAS. A step-down boot-strap method with 10,000 re-samples of multiple t-tests was used to assess which group had the maximum amount of cartilage and bone, or the best mechanical property.

Results

Mouse surgery

Mice with fractured tibiae began ambulating immediately after recovery from anesthesia. Infections or foot necroses were not observed during the post-operative period. Eleven mice were excluded from this study due to post-operative death, loose fixators, or comminuted fractures. The number of mice analyzed at each time point for each group is shown in Table 1.

Delaying stabilization during the first 96 hours after fracture does not significantly affect fracture healing

At 10 days post-surgery, callus tissue formed around the fractured bone of all mice regardless of the time of stabilization (Fig. 2A–E). A small quantity of new bone was present in the periosteum and the marrow cavity adjacent to the fractured bone ends (data not shown), and a thin layer of new bone was occasionally observed around the intramedullary pins.

Histomorphometric analyses revealed that there were no significant differences in the callus volume (TV, Fig. 3A) or bone volume (Fig. 3B) among the fractures stabilized at each time studied.

In fractures that were stabilized immediately a trace amount of cartilage was observed in 4 of 7 animals ($CV = 0.01 \pm 0.02 \text{ mm}^3$, Fig. 3C and 2A). In contrast, when fractures were stabilized 24 (8/8, $CV = 0.41 \pm 1.00 \text{ mm}^3$, Fig. 3C and 2B) and 48 (6/6, $CV = 0.34 \pm 0.50 \text{ mm}^3$, Fig. 3C and 2C) hours after injury, cartilage was observed in all animals. Delaying stabilization for 72 (5/7, $CV = 0.10 \pm 0.14 \text{ mm}^3$, Fig. 3C and 2D) and 96 (5/7, $CV = 0.11 \pm 0.11 \text{ mm}^3$, Fig. 3C and 2E) hours also resulted in the formation of a relatively large amount of cartilage in the majority of animals. In general, delaying stabilization increased the amount of cartilage present in the fracture callus 10–40 fold compared to immediate stabilization (Fig. 3C). However, statistical analyses indicated that the difference in cartilage formation was not significant among fractures stabilized at the different time points. This may result from the large variance in the amount of cartilage present in the animals.

Control fractures with sustained instability developed a big callus comprised of a large amount of cartilage at 10 days post-surgery (Fig. 2F). Histomorphometric analyses confirmed that both the volume of the callus (TV, Fig. 3A, $p < 0.05$) and the volume of the cartilage (CV, Fig. 3C, $p < 0.05$) of non-stabilized fractures were significantly larger than any other group of stabilized fractures. The amount of new bone that formed in the non-stabilized control fractures was not significantly different from that of the stabilized fractures (BV, Fig. 3B).

Mechanical analysis

To determine whether delaying stabilization affects the mechanical property of the fracture calluses, mechanical testing by load to failure was performed on 14-day old calluses of non-stabilized control fractures and calluses that were stabilized immediately, at 48, or 96 hours after fracture. As shown in Table 2, failure loads were similar among animals that were stabilized immediately, at 48 hours, at 96 hours, or left non-stabilized. The stiffness of fractures that were stabilized at 48 hours was greater, but not statistically significantly so, to that of other groups.

Discussion

Delayed stabilization during early phase of fracture healing affects cartilage formation

The results of this study demonstrate that delayed stabilization during the early stages of fracture repair influences cartilage formation in the callus. Compared to fractures stabilized immediately after injury, a trend towards more cartilage formation was observed in fractures stabilized at 24 to 96 hours (Fig. 2 and 3C). Growing evidence indicates that the mechanical environment plays a crucial role in cell differentiation during fracture repair. In a theoretical model, compression induces chondrocyte differentiation while tension leads to the formation of fibrous tissue¹⁷. *In vitro* studies have also demonstrated that cyclic, hydrostatic or static compression enhances formation of the cartilaginous matrix produced by bone marrow-derived mesenchymal cells¹⁸. However, the length of time required for a mechanical stimulus to influence cell fate decisions in a fracture environment is not known. In mice, tibial fractures heal quickly and molecular markers of chondrocyte (*collagen type II*) and osteoblast (*osteocalcin*) differentiation are detectable within 3 to 5 days after injury^{11, 13, 14}. The results of this study indicate that mechanical instability for as little as 24 hours may be sufficient to influence chondrocyte differentiation.

In this study, the non-stabilized control fractures formed a large amount of cartilage in the calluses at 10 days post-injury, indicating that the intramedullary pins provided only partial

stabilization of the fracture ends, with endochondral ossification being the principle mode of fracture healing. The non-stabilized control fractures exhibited significantly more callus and cartilage, compared to the fractures stabilized at 24 to 96 hours. This finding indicates that the period between 96 hours to 10 days after fracture is also crucial for cartilage formation. Chondrocyte differentiation and proliferation might be dynamically regulated by mechanical stimuli. Stabilization might suppress chondrocyte differentiation and/or proliferation¹⁹, while continuous instability may induce more chondrocyte differentiation and/or enhance chondrocyte proliferation²⁰. Furthermore, mechanical stability may also influence the rate of chondrocyte apoptosis²¹.

Timing of delayed stabilization affects the outcome of fracture healing

Data from this study demonstrates that delayed stabilization employed during the first 96 hours of fracture healing leads to a trend of increased chondrogenesis without significantly enhancing fracture repair in mice. Histomorphometric analyses of the amount of callus tissue and bone that formed at 10 days post-fracture, and biomechanical analyses at 14 days post-fracture, reveal that there are no differences among fractures stabilized immediately and after 24 to 96 hours of instability. A period of 24 to 96 hours of delayed stabilization could have been too short to have an enhancing effect on fracture healing. A previous study in rabbits found that delaying fixation for 10 days enhances fracture healing, but this effect is not observed if the fractures are fixed at 5 or 17 days post-injury⁶. These findings suggest that the timing of delayed fixation may affect the fracture healing outcome.

In addition, fractures in this study were stabilized by closed external fixation and only minimal injury was introduced to the callus tissues at the time of stabilization. Compared to delayed internal fixation^{4, 9, 10}, the magnitude of the “second injury” response may have been much lower in our model. Therefore, the effects we observed could be mainly due to delayed stabilization itself and not to a second injury.

Acknowledgments

We would like to thank Zheng Xu for technical help and Stuart Gansky and Sara Shain for statistical analysis. This work was supported by NIH-NIAMS (K08-AR002164 and R01-AR053645-01 to T.M.).

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Fig. 1. Procedures of creating and stabilizing tibia fracture

(A) One 0.25mm pin (arrow) was placed into the marrow cavity. Two 0.25 mm pins (arrowheads) were placed 90° to each other in the proximal and distal segments of tibia. (B) A circular ring oriented perpendicular to the long axis of the tibia was then fixed to the pins in each segment. (C) A closed fracture (arrow) in the tibial diaphysis was created by three-point bending. (D) The tibiae were stabilized immediately, at 24, 48, 72, or 96 hours by connecting the rings with three longitudinal threaded rods (arrows). (This figure is modified from Fig. 2 in “A model for intramembranous ossification during fracture healing. Thompson et al. *J Orthop Res.* 2002;20(5):1091–8” with permission from John Wiley & Sons, Inc.)

Fig. 2. Comparison of cartilage formation at 10 days post-fracture

(A) A representative histograph of a fracture stabilized immediately after injury, (B) at 24 hours, (C) at 48hours, (D) at 72 hours, (E) at 96 hours, or (F) left non-stabilized (Ctrl). Cartilage was stained red by Safranin O/Fast Green (SO/FG) staining. Scale bar: A–E = 200μm, F = 1mm.bm = bone marrow.

Fig. 3. Histomorphometric analyses of fracture healing at 10 days post-injury
 (A) TV (total volume of callus). (B) BV (total volume of new bone). (C) CV (total volume of cartilage). * The non-stabilized control fractures exhibit significantly more callus tissue (TV) and cartilage (CV) compared to the animals with fractures stabilized at 0, 24, 48, 72, or 96 hours (p < 0.05).

Table 1

Number of animals analyzed for each group.

	Stabilized immediately	Stabilized at 24 hours post-surgery	Stabilized at 48 hours post-surgery	Stabilized at 72 hours post-surgery	Stabilized at 96 hours post-surgery	Non-stabilized control
Day 10 (histology)	7	8	6	7	7	8
Day 14 (mechanical testing)	6		4		6	6

Table 2

Tension test at 14 days post-fracture.

	Failure Load (N)	Stiffness (N/mm)
Non-stabilized control (n=6)	5.09±1.33	4.51±1.81
Stabilized immediately (n=6)	3.94±1.10	1.94±0.65
Stabilized at 48 hours post-surgery (n=6)	3.98±1.45	9.59±9.41
Stabilized at 96 hours post-surgery (n=4)	4.53±0.54	2.19±0.63