Development of an Atrophic Nonunion Model and Comparison to a Closed Healing Fracture in Rat Femur

Takeshi Kokubu, David J. Hak, Scott J. Hazelwood, A. Hani Reddi

Department of Orthopedic Surgery and Center for Tissue Regeneration and Repair, University of California Davis

Although most fractures heal, some fail to heal and become nonunions. Many animal models have been developed to study problems of fracture healing. The majority of nonunion models have involved segmental bone defects, but this may not adequately represent the biologic condition in which nonunions clinically develop. The objective of the present study is to develop a nonunion model that better simulates the clinical situation in which there is soft tissue damage including periosteal disruption and to compare this model to a standard closed fracture model utilizing identical fracture stabilization, providing a similar mechanical environment. A total of 96 three month old Long Evans rats were utilized. A 1.25 mm diameter K-wire was inserted into the femur in a retrograde fashion, and a mid-diaphyseal closed transverse fracture was created using a standard three-point bending device. To create a non-union, 48 of the rats received additional surgery to the fractured femur. The fracture site was exposed and 2mm of the periosteum was cauterized on each side of the fracture. Fracture healing was evaluated with serial radiographs every two weeks. Animals were maintained for intervals of two, four, six or eight weeks after surgery. Specimens from each time interval were subjected to biomechanical and histological evaluation. None of the cauterized fractures healed throughout the eight weeks experimental duration. The radio-graphical appearance of nonunion models was atrophic. This investigation showed pronounced differences between the experimental nonunions and standard closed fractures both histologically and biomechanically. In conclusion, we have developed a reproducible atrophic nonunion model in the rat femur that simulates the clinical condition in which there is periosteal disruption but no bone defect.

Introduction
Fracture healing is a complex process involving the coordination and regulation of multiple cells, regulatory cytokines and morphogenetic proteins [2,4-6,11,21, 25,26]. Although most fractures heal, some fail to heal and become established nonunions. The known causes of fracture nonunions are multifactorial and include instability, infection, soft tissue interposition, distraction of fracture fragments, loss of vascularity, and soft tissue damage [1,15,16]. Most previous nonunion models have utilized a large segmental defect at the fracture site [8,14,19,27]. These models may not adequately represent the clinical situation in which most nonunions develop. They do not heal without the implantation of graft material because of the defect size, not because of altered biology at the fracture site [7].

Clinical and experimental evidence of healed fractures has highlighted the importance of preserving the periosteum at the fracture site and avoiding periosteal disruption [9,18,21-23,28]. During an initial stage of the fracture healing process, the periosteum cells first differentiate into osteoblasts, followed by intramembranous ossification [21]. If the periosteum is damaged, fractures may not heal. Fracture healing is also influenced by the mechanical environment, such as the stiffness and fit of the intramedullary fixation devices [24]. Hietaniemi et al. have reported a rat femoral nonunion model produced by an open osteotomy with loose K-wire fixation and cauterization of the periosteum [16]. In attempting to recreate the Hietaniemi model we found a high rate of wire migration and fatigue failure when 0.7 mm diameter K-wires were used. In addition, an open osteotomy may not accurately model the amount of energy imparted to the bone or the surrounding zone of soft tissue injury that occurs as the result of a fracture.

We therefore developed specific modifications to the nonunion model developed by Hietaniemi. Rather than performing an open osteotomy, the three-point bending impact model of Bonarens and Einhorn was utilized to create a transverse diaphyseal fracture [3]. The fractures were stabilized with 1.25 mm diameter K-wires providing more stable and rigid fixation, avoiding any subsequent fatigue wire failure. We further compared this model to a standard closed fracture model utilizing identical fracture stabilization that would produce a similar mechanical environment.
Materials and Methods

Experimental Nonunion Model

Ninety-six Long Evans rats were used in this study. The mean age of the rats was approximately 14 weeks (range 13-17 weeks), and their mean body weight was approximately 330 g (range 300-426 g). All surgical procedures were performed under anesthesia and normal sterile conditions. Anesthesia was performed with 4% Halothane in-halation, followed by Ketamine hydrochloride (80 mg/kg) and Xylazine hydrochloride (8 mg/kg) administered intraperitoneally.

A lateral parapatellar knee incision on the right limb was made to expose the distal femoral condyle. A 1.25 mm diameter K-wire was inserted from the trochlear groove into the femoral canal in a retro-grade fashion with use of a motor-driven drill. The wire was advanced through the greater trochanter and out of the skin until its distal end was positioned deep to the articular surface of the knee. A 5 mm incision in the skin was made around the K-wire and the wire was then cut close to the proximal femur. After irrigation the wounds were closed with 5-0 nylon suture. A closed transverse femoral shaft fracture was then created in the right femur of each rat using a three-point bending apparatus with a drop weight (R. Zarb. State University New York, Brooklyn, NY) following the method of Bonnarens and Einhorn [3]. Following this procedure, half (48) of the rats received additional surgery to create a nonunion in the fractured shaft. In order to produce the nonunion, the fractured site was minimally exposed through a lateral approach. The periosteum was then cauterized (Loop tip surgical cautery, Ahco Dealers Inc, Nashville, TN) circumferentially for a distance of 2 mm on each side of the fracture. The muscle was protected to preserve all soft tissue except the periosteum around the fracture site. We were careful to cauterize the periosteum only once to prevent excess thermal necrosis of bone. The wound was then irrigated with 10 cc of sterile saline and the muscle and skin were closed in layers with 5-0 nylon sutures. Post-operative pain was managed by administration of subcutaneous injection of buprenorphine hydrochloride after surgery. The rats were fed a standard maintenance diet and provided water ad libitum. Unprotected weight bearing was allowed immediately post-operatively. The left
nonfractured femur served as a control.

Twelve animals were assigned to each group and were maintained for intervals of two, four, six or eight weeks (Table 1). Eight specimens from each time point were randomly selected for biomechanical testing as described below. The four remaining specimens from each group were processed for histological study. If the fracture produced was not a stable transverse fracture or if evidence of deep infection developed then the animal was excluded from the study and replaced with an other animal. Thus, ten rats with comminuted fractures and four rats with infectious findings in radiographs were replaced during the experiment. This research protocol was approved by the Institutional Animal Care and Use Committee, following all appropriate guidelines.

Radiological evaluation

Radiographs of all rats were obtained following creation of the fracture and at two week intervals until the time of sacrifice. This was done under anesthesia with the animal prone and with both limbs fully abducted. Fracture union was determined by the presence of bridging callus on two cortices. Radiographs of each animal were assessed by three blinded independent observers to judge whether the fractures united or not.

Histological evaluation

At the end of the maintenance intervals, the 32 rats utilized for histological evaluation were euthanized with an excess of carbon di-oxide gas. Right femurs were harvested and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 24 h at 4° C, defatted in ethanol, decalcified with 10% formic acid in citrate for 4 days at 4° C and embedded in paraffin. Paraffin sections 4μm thick were cut and stained with toluidine blue for histological observation. Histology was evaluated to confirm that the standard closed fracture model produced normal stages of fracture healing and that the nonunion model produced an established nonunion.

Biomechanical evaluation
Sixty-four animals were used for biomechanical evaluation. Following euthanasia the fractured femurs and the contralateral nonfractured intact femurs were dissected free of surrounding muscle. After the intramedullary K-wires were removed, the ends of both femurs were imbedded in Wood's metal (Alfa Aesar, Ward Hill, MA). Specimen length was statistically similar between nonunions (17.5 ± 1.9 mm) and standard closed fractures (17.2 ± 1.7 mm) (p > 0.05). The specimens were mounted in a Frankel-Burstein axial torsion-testing machine [10] that was modified to operate under computer control. The standard swinging-pendulum mechanism was replaced with a stepper motor (model 083062-1-8-031-010, Parker Compumotor, Rohnert Park, CA). Rotational displacement was measured with a precision potentiometer (model 793341-14092, Gould, Inc, Cleveland, OH), and torque was measured with a torque cell (model 2105, Eaton Corporation, Tray, MI). Specimens were tested in torsion at a rate of 500/mmn until failure or through an arc of 45°. Rotational displacement and torque data were collected at 60 Hz using a digital data acquisition system (model K500A, Keithley Instruments, Cleveland, OH). Maximum torque to failure was measured directly from the data and torsional stiffness was calculated from a regression of the linear portion of the torque versus angular displacement curve. The energy absorption to maximum torque was also calculated as the area under the curve to the maximum value. The nonfractured contralateral intact femur was also tested to calculate normalized values of maximum torque to failure (percent maximum torque), stiffness (percent stiffness) and energy absorption to maximum torque (percent energy) in order to reduce the influence of individual animal differences.

**Statistical analysis**

A two factor analysis of variance (ANOVA) was performed on the values of the biomechanical properties. The factors were the time point and the fracture model (nonunion or standard closed fracture). Significance was defined as p values less than 0.05. When appropriate, a Bonferroni-Dunn post-hoc test was performed to determine differences between the models at the various time points. For the post-hoc test, the level of significance was adjusted to 0.0018 to account for multiple comparisons.
Results

Radiographs

Radiographs taken just after surgery showed transverse mid-diaphyseal femoral fractures in both the standard closed fracture model and the nonunion model (Fig. IA and E). The numbers of radiographic fracture unions during each period are shown in Table 2. Seventy-eight percent of the closed fractures healed at four weeks after fracture, and they all healed at six weeks after fracture. In contrast, bridging callus was not seen in the cauterized fractures, even at eight weeks after surgery, with the vast majority of them classified as atrophic nonunions based on their radiographical appearance (Fig. IH). The radiographic findings were inconclusive in three of the cauterized cases, but their biomechanical properties confirmed the development of a nonunion.

The standard closed fractures generated by three-point bending formed abundant callus around the fracture sites at two weeks (Fig. 1B). At four weeks after fracture most of the callus bridged between both ends of the fractured bones and the cortical gaps disappeared (Fig. 1C). After forming a bridging callus, bone remodeling began (Fig. 1D). On the other hand, the nonunions did not have much callus formation around the fracture sites. Some callus did form along the periosteum away from the fracture site, but this never extended to bridge the fracture site (Fig. IF and G). The ends of the fractured bone became round and were resorbed, appearing to be established nonunions at eight weeks after fracture (Fig. 1 H).

Histology

At two weeks after fracture, the standard closed fracture model rats displayed intramembranous ossification in the periosteal tissue and endochondral ossification at the fracture site (Fig. 2A). They formed a thick callus consisting of chondrocytes and newly formed trabecular bone, and the two calluses on each side of the fracture nearly united. The gap between the endochondral ossification areas was filled with mesenchymal cells. At two weeks, the nonunions also exhibited chondrocytes and endochondral ossification, similar to the standard closed fractures (Fig. 2D), but there was no bone formation on the
site of periosteal cauterization. In addition, in the nonunion group the gap between the calluses was greater. At four weeks after fracture, the callus in the standard closed fracture had united and the chondrogenic areas almost had disappeared (Fig. 2B). The fractured bone was covered with newly formed trabecular bone and achieved bony union. In contrast, a large gap persisted between the surfaces of woven bone in the nonunions at four weeks (Fig. 2E). At eight weeks, the united bone in the standard closed fracture had remodeled with progressive decrease in the thickness of the woven bone (Fig. 2C). The fracture gap at the interface of the original cortical bone and the border between the cortical bone and the newly formed woven bone was indistinguishable. In comparison, at eight weeks in the nonunion model, the fibrous tissue was surrounding the fracture site and we began to see resorption of the ends of the cortical bone (Fig. 2F). This is consistent with the histological finding of atrophic nonunions.

_Biomechanical evaluation_

The means and standard deviations of the maximum torque to failure, the stiffness and the energy absorption to maximum torque are shown in Table 3. The maximum torque, stiffness and energy absorption values were normalized by the respective values from the intact femurs (percent maximum torque, percent stiffness and percent energy) and average values were calculated for each group. Significant differences were found between nonunions and standard closed fractures at each time point in most mechanical properties. Significant reductions in the mechanical properties were seen in the nonunion model compared to the standard closed fractures at all time points (Fig. 3).

For the standard closed fracture model, percent maximum torque and percent energy were less than 50% of the intact values at two weeks after surgery (Fig. 3A and C). Over time, all the mechanical properties of the standard closed fracture gradually increased, with significant differences observed in percent maximum torque, percent stiffness, and percent energy of the standard closed fractures between two weeks and eight weeks (Fig. 3). At eight weeks the mean values of percent maximum torque and percent stiffness of the standard closed fractures exceeded 100% (Fig. 3A and B).
The normalized mechanical properties of the non-unions increased slightly with time, but there were no significant changes in percent maximum torque, stiffness and energy absorption between two weeks and eight weeks (Fig. 3). All the mechanical properties of the nonunions were less than 50% of their corresponding intact values at each time point (Fig. 3).

**Discussion**

Development of an appropriate animal nonunion model for the investigation of fracture healing requires consideration of the propensity for small animals to rapidly heal their fractures. Previous animal studies evaluating long bone nonunion formation have utilized a segmental defect model [8,14,19,27] but this may not adequately simulate the biological and mechanical environment that clinically leads to nonunion development. While large bone defects may occur due to extensive loss of bone as a result of trauma or musculoskeletal tumor resection, many nonunions develop in the absence of significant bone loss. Clinical and experimental evidence indicates that periosteal disruption at the fracture site [9,21] or gross instability of the fracture fragments [13,15] may impair fracture healing.

Hietaniemi et al. have described a reproducible rat femoral nonunion model using an open osteotomy technique [16]. Rather than create a segmental defect, they destroyed 2 mm of periosteum on each side of the fracture by electrocautery. They apparently described "loose" fixation with a 0.7 mm intramedullary K-wire. In our pilot study to recreate the Hietaniemi model we found a high rate of fatigue failure and backing-out of K-wires when 0.7 mm K-wires were used. Therefore we utilized a 1.25 mm K-wire which was strong enough to avoid fatigue failure throughout the experimental period. In addition, more rigid fixation was achieved by the use of the thicker diameter K-wire. Park et al. have reported that open osteotomy causes more severe periosteal damage and delays biologic healing and restoration of biomechanical properties [23]. In order to standardize fracture production, and to permit comparison to a standard closed fracture model, we utilized the closed technique of Bonnarens and Einhorn [3]. To simulate the periosteal disruption that may occur following fracture or internal
fixation, the periosteum was cauterized after the method of Hietaniemi [16]. By using the same closed fracture technique and an identical diameter K-wire in both the nonunion model and the standard closed fracture model we attempted to eliminate the effects of differences in the mechanical environment on fracture healing.

Hausman et al. recently described that an inhibitor of angiogenesis prevents fracture healing in rats [12]. Fracture callus is not observed radiographically and histologically at three weeks after fracture by subcutaneous injection of angiogenesis inhibitor, TNP-470. Kowalski et al. reported the periosteum supplies blood for up to one-third of the cortical bone [17]. These findings imply that vascularization around the fracture site is essential for normal healing, and that periosteal disruption will interfere blood supply at the fracture site. In our study, we circumferentially disrupted 2 mm of periosteum on each side of the fracture site. While we did not perform any studies about vascularization, we propose that this resulted in loss of periosteal vascularity at the fracture site and interfered with normal fracture healing.

The use of the cautery clearly impaired the process of normal bone healing. Possible limitations of this technique to create a nonunion include additional thermal necrosis of the cortical bone near the fracture site and variations in the degree of surrounding soft tissue dissection during the open surgical procedure. We examined the effect of periosteal cauterization on the underlying cortical bone in three animals without producing any associated fractures. Histological evaluation at one and three weeks showed no evidence of deep cortical necrosis with normal appearing marrow. We also examined the effect of surgical exposure of the fracture in two additional animals. After production of the closed fracture the fracture site was exposed but no cauterization was performed. The radiographic pattern of healing in these procedures was similar to that seen in the standard closed fracture model, with evidence of callus formation present by three weeks. This suggests that the open surgical exposure of the fracture site did not dramatically impair fracture healing. While we attempted to standardize the duration and region of cautery and to perform a similar soft tissue dissection in each animal, these variables may potentially affect the healing
response in the nonunion model.

A criticism of this study is that the closed fracture model we used as a comparison does not represent a valid sham control. It was not our intent to evaluate the independent role of periosteal cauterization on fracture healing. This would require an additional sham procedure in which the fracture was produced and surgically exposed but not cauterized. The purpose of our investigation was to develop a nonunion model that simulates the clinical situation in which there is soft tissue damage including periosteal disruption. We studied the closed fracture model to obtain comparison biomechanical data of normal fracture healing at each time point. Future studies using this nonunion model in which additional intervention is performed, such as application of bone morphogenetic proteins or vascularized endothelial growth factor, can then be compared to normal fracture healing.

Additional criticism of this study includes the use of extensive biomechanical testing at the early stages of fracture healing. However, since one goal of future fracture intervention is the acceleration of fracture healing biomechanical parameters at early time points will provide useful comparison data in evaluating these interventions.

In the absence of a universal definition of nonunions, it has been suggested that nonunion should not be defined in arbitrary terms of duration, but rather as the cessation of the intramembranous healing response [20]. At eight weeks, our experimental nonunion model radiographically appeared atrophic and histologically there was no evidence of intramembranous ossification in the cauterized region throughout the experiment, indicating that the fracture had become an established nonunion.

Our experimental model showed pronounced differences between nonunions and standard closed fractures in radiographs, histology and biomechanical properties. Serial radiographs indicated that 78% of the standard closed fractures united radiographically at four weeks and at six weeks all displayed bridging callus indicating a united fracture. In contrast, there was no evidence that the healing process was progressing in the cauterized nonunions throughout the experimental time course. The radiographic
findings were confirmed by histological analysis. The standard closed fracture model followed a normal process toward fracture healing by four weeks after surgery, with a newly formed bridging callus at the fracture site. Numerous proliferating osteoblasts and chondrocytes were seen in the woven bone. On the other hand, the cauterized nonunion model did not heal by eight weeks after fracture. Intramembranous ossification was not observed in the area of the cauterized periosteum throughout the experimental time course. At eight weeks the fibrous tissue was surrounding the fracture site and there were no new chondrocytes or osteoblasts present. In normal fracture healing, early intramembranous ossification is followed by endochondral ossification. Chondrocytes are normally observed throughout the phases of endochondral ossification. In the nonunion specimens, the lack of chondrocytes after two weeks implies that normal endochondral ossification did not occur. Absence of reparative chondrocytes also suggests that the fracture healing process was quiescent and the nonunion was established.

Biomechanical torsional testing revealed that percent maximum torque was six times larger, percent stiffness was 12 times larger, and percent energy was three times larger for the healed standard closed fracture model compared to the experimental nonunions at eight weeks. Each of these mechanical properties had significant differences between the standard closed fracture and nonunion groups. The nonunions did not have the re-modeling callus around the fracture site and the two fractured fragments were connected with only fibrous tissue, making the nonunion weaker than the standard closed fracture model throughout the study.

We have developed a reproducible atrophic nonunion model in the rat femur with atrophic radiographic appearance and significantly decreased biomechanical properties compared to the standard closed healing fracture model. Using a standard fracture production method and identical K-wire stabilization permits comparison of the nonunion model with the standard closed fracture model, setting the stage for further investigations to study the influence of different interventions on bone repair and to systematically study the cellular and molecular mechanisms underlying nonunions.
Acknowledgements

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References

Table 1
Experimental plan and the number of animals in each experimental group

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<th>6 weeks</th>
<th>8 weeks</th>
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<td>Biomechanical evaluation</td>
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<td><strong>Standard closed fracture</strong></td>
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<td>Biomechanical evaluation</td>
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Table 2
Serial radiographic evaluation of fracture union

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<th>Period after surgery</th>
<th>Experimental group</th>
<th>Standard closed fracture group</th>
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<tr>
<td>2 weeks</td>
<td>0/48 (0%)</td>
<td>6/48 (13%)</td>
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<td>4 weeks</td>
<td>0/36 (0%)</td>
<td>28/36 (78%)</td>
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<td>6 weeks</td>
<td>0/24 (0%)</td>
<td>24/24 (100%)</td>
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<td>8 weeks</td>
<td>0/12 (0%)</td>
<td>12/12 (100%)</td>
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Table 3
Biomechanical evaluation in nonunions and standard closed fractures

<table>
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<tr>
<th>Period after surgery</th>
<th>Nonunion*</th>
<th>Standard closed fracture</th>
<th>p-value*</th>
<th>Intact*</th>
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<td><strong>Maximum torque to failure (Nm)</strong></td>
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<td>2 weeks</td>
<td>0.024 ± 0.021</td>
<td>0.110 ± 0.031</td>
<td>0.0019</td>
<td>0.290 ± 0.050</td>
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<td>4 weeks</td>
<td>0.052 ± 0.024</td>
<td>0.367 ± 0.085</td>
<td>&lt;0.0001</td>
<td>0.367 ± 0.048</td>
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<td>6 weeks</td>
<td>0.099 ± 0.049</td>
<td>0.348 ± 0.058</td>
<td>&lt;0.0001</td>
<td>0.451 ± 0.077</td>
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<td>8 weeks</td>
<td>0.088 ± 0.067</td>
<td>0.493 ± 0.046</td>
<td>&lt;0.0001</td>
<td>0.490 ± 0.096</td>
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<td><strong>Stiffness (Nmmrad)</strong></td>
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<td>2 weeks</td>
<td>0.052 ± 0.045</td>
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<td>&lt;0.0001</td>
<td>1.276 ± 0.429</td>
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<td>4 weeks</td>
<td>0.308 ± 0.191</td>
<td>1.284 ± 0.248</td>
<td>&lt;0.0001</td>
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<td>6 weeks</td>
<td>0.232 ± 0.130</td>
<td>1.930 ± 0.385</td>
<td>&lt;0.0001</td>
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<td>8 weeks</td>
<td>0.309 ± 0.209</td>
<td>2.641 ± 0.398</td>
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<td>2.094 ± 0.432</td>
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<td><strong>Energy absorption to maximum torque (Nmmrad)</strong></td>
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<tr>
<td>2 weeks</td>
<td>0.0065 ± 0.0062</td>
<td>0.0115 ± 0.0044</td>
<td>0.5756</td>
<td>0.0542 ± 0.0203</td>
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<td>4 weeks</td>
<td>0.0184 ± 0.0105</td>
<td>0.0573 ± 0.0221</td>
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<td>6 weeks</td>
<td>0.0260 ± 0.0193</td>
<td>0.0510 ± 0.0220</td>
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<td>8 weeks</td>
<td>0.0301 ± 0.0225</td>
<td>0.0634 ± 0.0219</td>
<td>0.0004</td>
<td>0.0842 ± 0.0456</td>
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*The values are given as the mean and standard deviation.

b The p-values are derived from comparison of nonunions and standard closed fractures.
Fig. 1. Radiographs of both standard closed fracture models (A–D) and nonunion models (E–H) obtained immediately after surgery (A and E), at two weeks after surgery (B and F), four weeks after surgery (C and G), and eight weeks after surgery (D and H). Note the union in standard closed fracture group and the gap in the nonunion group.
Fig. 2. Histological appearances of standard closed fracture models (A–C) and nonunion models (D–F). Sections were stained with toluidine blue. Both models at two weeks after surgery had endochondral ossification with chondrocytes, but there was larger gap in the nonunion (A and D). The standard closed fracture united at four weeks after surgery (B), however the nonunion still had a large gap (E). At eight weeks, the united bone in the standard closed fracture had remodeled (C). In the nonunion group no healing was observed and the cortical bone was resorbed (arrowheads, F). en: endochondral ossification, co: cortical bone. Bar, 1 mm.
Fig. 3. Biomechanical properties of the closed standard fracture and nonunion group normalized by the respective values of the intact femurs: (A) percent maximum torque (maximum torque to failure of fractured femur/nonfractured intact femur × 100); (B) percent stiffness (stiffness of fractured femur/nonfractured intact femur × 100); (C) percent energy (energy absorption to maximum torque/nonfractured intact femur × 100). Significant differences were found between nonunions and standard closed fractures in percent maximum torque, percent stiffness, and percent energy measurements (except percent energy at two weeks). Significant differences were also found between two weeks and eight weeks. Note: *p < 0.0018, †p < 0.001, **p < 0.0001.