

A Fracture Pain Model in the Rat

Adaptation of a Closed Femur Fracture Model to Study Skeletal Pain

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Background: Because of the relative lack of understanding of the mechanisms that drive skeletal pain, the purpose of this study was to adapt a previously validated closed femur fracture model to quantitatively evaluate skeletal pain in female and male rats.

Methods: Three-month-old female and male Sprague-Dawley rats were anesthetized, and a stainless steel pin was inserted into the intramedullary space of the left femur. Three weeks later, the rats were reanesthetized, and left femoral diaphyses were fractured using a standardized impactor device. At 1–21 days after fracture, skeletal pain was measured by quantitatively assessing spontaneous guarding, spontaneous flinching, and weight bearing of the fractured hind limb.

Results: Females and males showed highly robust pain behaviors that were maximal at day 1 after fracture and returned gradually to normal nonfractured levels at days 14–21 after fracture. The magnitude of fracture pain was not significantly

different at most time points between female and male rats. In both females and males, the pain-related behaviors were attenuated by subcutaneous morphine in a dose-dependent manner.

Conclusions: This model may help in developing a mechanism-based understanding of the factors that generate and maintain fracture pain in both females and males and in translating these findings into new therapies for treating fracture pain.

FRACTURE pain is a common form of pain in the young and, even more so, in the old.^{1–3} In young individuals, (aged <30 yr), the majority of fractures are due to sports, motor vehicle-related accidents,⁴ and combat-related injuries.⁵ Although young males have historically had a higher incidence of fractures than young females,⁶ with the increasing number of women participating in sports⁷ and military roles,⁸ this sex difference in fracture incidence in the young will likely decline.

As humans age, bone fractures not only become more frequent but have a significant impact on quality of life, morbidity, and mortality.^{9–11} In humans, peak bone mass is reached at 25–30 yr of age, after which bone loss exceeds bone formation.^{12,13} As humans age (>30 yr), there is an increase in bone loss (osteopenia) that, if it becomes severe enough, is termed *osteoporosis*.^{12,13} Osteopenia and osteoporosis are characterized by low bone mineral density and compromised bone strength, which predisposes individuals to an increased risk of fractures.^{12,14} Whereas women and men (aged >50 yr) are equally likely to have osteopenia, women are three times more likely to have osteoporosis.¹⁵ In the United States, approximately 8 million women have osteoporosis, 22 million have low bone mineral density of the hip,¹⁶ and more than 1.5 million osteoporotic-related fractures occur each year.¹⁷

Osteoporotic fractures can be highly disabling (because they heal slower and therefore remain painful for a longer period of time)^{18,19} and are associated with a decreased quality of life and significantly contribute to morbidity and mortality in this population.^{9–11} This is especially true of hip fractures (90% of “hip” fractures are actually a fracture of the proximal head of the femur²⁰) because these almost invariably result in loss of function, loss of mobility, and hospitalization.^{21,22} Because bone healing is slow^{18,19} and it is painful to walk on the fractured bone (resulting in loss of bone and muscle mass), rehabilitation is often incomplete so that only 60% of patients with osteoporosis-related hip fractures will regain their prefracture mobility at 6 months

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after fracture.²³ Furthermore, many of these patients now find walking painful, which contributes to loss of mobility, independent living, and social interactions so that approximately 20% of patients die within a year after osteoporotic-related fracture of the hip.^{24,25}

A major problem in treating chronic fracture pain is that the number of available analgesic therapies is limited. Nonsteroidal antiinflammatory drugs (NSAIDs) are effective in attenuating many musculoskeletal pain states.^{26–28} However, NSAIDs have been shown to inhibit bone healing after fracture in rodents.^{29–32} In addition, in older patients where bone loss occurs^{12,13} and in many young patients from the military with explosion-induced fracture⁵ who have also experienced traumatic brain injury, opiate-induced side effects such as sedation, cognitive impairment, clouding of mental status, and depression tend to be more severe.^{33,34} For these reasons, there is a significant need to develop novel mechanism-based analgesics to treat chronic fracture pain without the unwanted side effects of currently available analgesics.

We recently characterized fracture-induced pain behaviors in a murine closed femur fracture model^{35,36} that has been previously used to study bone regeneration after fracture.³⁷ Although the mouse model has many advantages, the rat model has advantages of its own. In addition to being a commonly used animal species for studying pain³⁸ and bone healing,^{29,39} advantages of the rat model may include a more accessible central nervous system for the study of electrophysiologic properties of pain-transmitting neurons and the ability to implant indwelling catheters for the delivery of potentially therapeutic compounds.⁴⁰ Because this model can be used to simultaneously assess bone pain and bone healing, it may aid in developing new mechanism-based therapies for treating acute and chronic fracture pain that lack the side effects of currently available analgesics.

Materials and Methods

Animals

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Minnesota (Minneapolis, Minnesota) and were in accordance with the National Institutes of Health guidelines for care and use of laboratory animals. Experiments were performed in 30 adult male (330–370 g) and 30 female (220–250 g) Sprague-Dawley rats (Harlan, Indianapolis, IN). The rats were housed in conventional facilities with a 12-h light–dark cycle and were given food and water *ad libitum*.

Surgical and Fracture Procedure

Before femoral pin placement, rats received an intraperitoneal injection of 100 mg/kg ketamine and 10

mg/kg xylazine to provide anesthesia. An incision of approximately 6 mm was made in the skin, and the proximal patellar ligament of the left femur was severed, revealing the synovial space of the knee joint as previously described.^{37,41} A-20 gauge needle was used to core between the condyles and into the medullary canal of the left femur. Rats were immediately radiographed to ensure proper coring; any rat with a needle protruding outside of the medullary canal was killed. A precut 0.8-mm-diameter (length: 25 mm for females and 27 mm for males) stainless steel wire (pin) (Small Parts Inc., Miami Lakes, FL) was inserted into the medullary space for fracture stabilization. Dental amalgam was used to secure the pin and close the hole. Wound clips (MikRon Precision Inc., Gardena, CA) were used to close the incision and were removed 7 days after pin placement.

A closed mid-diaphyseal fracture of the left femur was produced 21 days after pin placement in rats during anesthesia (100 mg/kg ketamine and 10 mg/kg xylazine, intraperitoneal) as originally described by Bonnarens and Einhorn.⁴² The three-point impactor device (BBC Specialty Automotive Center, Linden, NJ) used to fracture was based on the original design of Bonnarens and Einhorn (illustrated in article)⁴² and subsequently adapted by Simon *et al.*³² The left femur of the anesthetized rat was secured between two lower supports and an upper impactor head. A guillotine-like effect was created by dropping a rod-guided 411-g weight from a height of 20 cm onto the spring-loaded upper impactor head, creating a femoral fracture. Immediately after fracture, rats were radiographed to ensure localization of a mid-diaphyseal fracture. Exclusion criteria were adapted from Gerstenfeld *et al.*⁴³ and included fractures located too far from the mid-diaphyseal region of the femur, dislodged pins, and nonvisible fracture after impact. Only one rat met the exclusion criteria and was immediately killed and was not used for the current study. After recovery from anesthesia after fracture, rats were allowed unrestricted movement and hind limb weight bearing.

Pain-related Behaviors

Female and male rats were behaviorally analyzed before fracture (day 0) and at days 1, 2, 4, 7, 10, 14, 18, and 21 after fracture to assess ongoing (spontaneous) fracture pain-related behaviors (guarding and flinching) as previously described.^{35,36} Briefly, the number of hind limb flinches and time spent guarding over a 2-min observation period were recorded as measures of ongoing pain, because these endpoints are similar to observations in patients who protect their fractured limb.⁴⁴

Fracture-induced pain was also assessed by differences in the distribution of weight in the left (fractured hind limb) *versus* the right hind limb (intact hind limb) using an incapacitance meter as previously described.⁴⁵ Weight bearing was used as an endpoint in this study because it has been widely used in humans to evaluate bone heal-

ing after fracture.^{46,47} Briefly, the mean force applied during 3 s by each hind limb was measured in five trials. Weight bearing on the left hind limb (fractured or pinned femur) was calculated as percentage of total weight bearing on fractured hind limb by the following equation:

$$\left[\frac{\text{Weight on fractured hind limb}}{(\text{weight on fractured hind limb} + \text{weight on intact hind limb})} \right] \times 100$$

Experimental Groups

To determine possible sex-related differences in pain-related behaviors after fracture, our experimental protocol consisted of three different groups for female and male rats: naive ($n = 4$), pin ($n = 4$), and pin + fracture ($n = 16$ for female and $n = 15$ for male). Rats were behaviorally analyzed (guarding, flinching, weight bearing distribution) at the time points previously described. At days 7 and 14 after fracture, female ($n = 5$ for each time point) and male ($n = 4$ for day 7 and $n = 5$ for day 14) rats were killed and processed to evaluate the callus histology (see Radiographic, Histologic, and Micro-Computed Tomography Analyses in the Materials and Methods section) of the fractured bones. To monitor the general health of the rats, body weights were recorded throughout the experiment.

To evaluate possible sex-related differences in the response to morphine, female and male fracture rats ($n = 10$ for female and $n = 9$ for male) at day 7 after fracture received cumulative doses of morphine sulfate (0.3, 1.0, 3.0, and 10.0 mg/kg, subcutaneous) 20 min before behavioral testing. Rats first received sterile saline (vehicle) followed by four cumulative doses of morphine having a 30-min interval between each dose of morphine.⁴⁸ Behavioral analysis was completed within 30 min after injection to ensure that the animals were tested within the known therapeutic window of drug action in rats after subcutaneous injection.⁴⁹

Morphine-induced side effects were determined by measuring the number of total spontaneous vertical stands and locomotor activity in an open field. Vertical stands were defined as the number of times the animal stood on both hind limbs, supporting their entire body weight, in a 2-min period.^{50,51} Locomotor activity was measured by counting the number of floor units the animal crossed during a 2-min period. The animal was placed in the center of a circular container (108 cm diameter \times 38 cm height equally divided into 29 floor units) at the beginning of the 2-min period.⁵²

Radiographic, Histologic, and Micro-Computed Tomography Analyses

Radiographic images (Specimen Radiography System Model MX-20, Faxitron X-ray Corporation, Wheeling, IL; Kodak film Min-R 2000, Rochester, NY) of fractured femurs were obtained immediately after fracture and at all behavior time points.

Female and male rats with fracture were killed at days 7 and 14 after fracture and processed for histologic analysis as previously described.⁵³ Briefly, rats were perfused intracardially with 200 ml phosphate-buffered saline (PBS), 0.1 M, followed by 200 ml 4% formaldehyde-12.5% picric acid solution in 0.1 M PBS. The femurs were removed, postfixed for 4 h in the perfusion fixative, and placed in a PBS solution. Micro-computed tomography (μ CT) images of fractured femurs of female and male rats were obtained with an eXplore Locus SP μ CT (GE Healthcare, London, Ontario, Canada). The cone beam μ CT scanner used a $2,300 \times 2,300$ charge-coupled device detector with current and voltage set at 80 μ A and 80 KVp, respectively. A 360° scan was performed with a 3,000-ms integration time with images reconstructed at $29 \mu\text{m}^3$ resolution. Three-dimensional images were created using MicroView analysis software (version 2.2; GE Healthcare).

After μ CT analysis was performed, femurs of fracture rats were decalcified in 10% EDTA at 4°C for no more than 3 weeks. After complete bone demineralization, determined radiographically, bones were embedded in paraffin and serially sectioned on the longitudinal axis using a Leica Microsystems RM2135 microtome (Wetzlar, Germany) at a thickness of 7 μm . Five sections at least 150 μm apart spanning at least 0.75 mm from the center of the fracture callus of each animal were stained with hematoxylin and eosin.⁴¹ Images of sections were digitally captured at $10\times$ using a SPOT II digital camera with SPOT image capture software (Diagnostic Instruments, Sterling Heights, MI) attached to an Olympus BX41 microscope (Olympus America Inc., Melville, NY).

Euthanasia and Processing of Tissue for Periosteum Immunohistochemistry

Naive female ($n = 6$) and male ($n = 6$) rats were killed and perfused as described above. Periosteum from the diaphyseal shaft was removed as a whole mount and processed for immunohistochemistry as previously described.⁵³ Briefly, whole mount preparations were washed in PBS 3×10 min and incubated for 60 min at room temperature in a blocking solution of 3% normal donkey serum in PBS with 0.3% Triton-X 100 and then incubated overnight at room temperature with primary antibodies. Unmyelinated primary afferent sensory nerve fibers were labeled with polyclonal rabbit anti-rat calcitonin gene-related protein (CGRP, 1:15,000 dilution; Sigma Chemical Co., St. Louis, MO). Myelinated primary afferent sensory nerve fibers were immunostained for 200-kd neurofilament H (NF200, polyclonal chicken anti-mouse NF200, 1:2,000, Chemicon, Temecula, CA). Preparations were then washed in PBS and incubated for 3 h at room temperature with secondary antibodies conjugated to fluorescent markers (Cy3 1:600; Jackson ImmunoResearch, West Grove, PA). Finally, tissue was washed in PBS and dehy-

drated through an alcohol gradient (70, 90, and 100%), cleared in xylene, mounted (attached muscle layer in contact with the slide) on gelatin-coated slides, and coverslipped with di-n-butylphthalate-polystyrene-xylene. To confirm the specificity of the primary antibodies, controls included preabsorption with the corresponding synthetic peptide and omission of the primary antibody. Images of periosteal whole mount preparations were captured using an Olympus Fluoview FV1000 laser scanning confocal imaging system (software version 5.0; Olympus America Inc.).

Quantification of CGRP⁺ and NF200⁺ fibers in periosteal whole mounts preparations from rats was performed as previously described.⁵⁴ Briefly, digital confocal images for each periosteal layer (400× magnification; two random sections per rat) were acquired as described above. Images were viewed on a high-resolution monitor, and the number of intersections between nerve fibers and the vertical grids (7.35 μm spacing, Adobe Photoshop software version 7.0; San Jose, CA) was quantified. Results were expressed as number of intersections per mm².

Statistical Analysis

The percent of antinociception was calculated according to the following equation⁵⁵:

Percent of antinociception

$$= \left[\frac{(\text{vehicle behavior} - \text{postcompound behavior})}{\text{vehicle behavior}} \right] \times 100$$

SPSS version 15 statistics package (SPSS, Chicago, IL) was used to perform statistical analyses. Frequency distributions of the behavioral dependent variables guarding, flinching, and incapacitance appeared markedly nonnormal, each failing the Lilliefors test for normality ($P < 0.05$). Therefore, response measures of guarding, flinching, and incapacitance for pin and fracture groups were compared separately for each sex on each outcome measure at each postintervention time point using Mann-Whitney nonparametric t tests, with significance

levels Bonferroni adjusted for multiple comparisons. With eight postintervention time points for each outcome measure, the Bonferroni-adjusted significance level for a single-comparison P value was therefore set at $P < 0.006$ ($0.05/8 = 0.006$).

Percent analgesic effect under differing morphine dosages was compared between sexes using a two-way repeated-measures analysis of variance, with sex as a between-group factor and dose as the repeated factor. A significant sex × dose interaction effect was observed ($P = 0.018$, Greenhouse-Geisser corrected). While *post hoc* comparisons of analgesic response at each dose level revealed significant sex differences at 1.0, 3.0, and 10.0 dose levels for both guarding and flinching responses (all $P < 0.05$, unadjusted), only sex comparisons of guarding response at 3.0 and 10.0 remained statistically significant after the more stringent Bonferroni adjustment for multiple comparisons.

Results

Effect of Pin Placement on Bone

Radiographic evaluation indicated no significant bone remodeling after intramedullary pin placement (fig. 1). Age-matched naive and pin rats were radiographically similar in appearance at all time points examined. In addition, body weight (data not shown; $P > 0.05$, Bonferroni adjusted), and pain related behaviors (fig. 2) were not significantly different between naive and pin rats during the length of the experiment.

Fracture Production in Sprague-Dawley Rats

The three-point fracture protocol resulted in reproducible transverse or slightly oblique mid-diaphyseal femoral fractures (figs. 1E and F). There were no sex-related differences in the success rate of usable fractures. After the surgical procedure, 0 rats, both female and male, were excluded because of protruding pins. In the current study, of the 16 female rats fractured, 0 were ex-

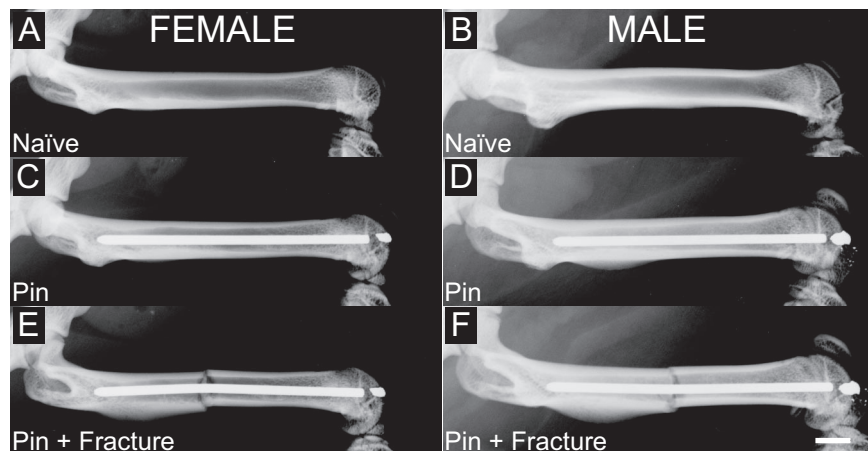


Fig. 1. Representative radiographs showing a naive, pin, and pin + fracture femur in the female and male adult Sprague-Dawley rat. A stainless steel pin was implanted into the intramedullary space of the femur 21 days before fracture to provide mechanical stability to allow bone healing. Closed mid-diaphyseal fractures of the left femur were produced in female and male rats using a three-point impactor device. Radiographic images of femurs from naive (A and B), pin (C and D), and pin + fracture 2 days after fracture (E and F). Scale bar = 3.0 mm.

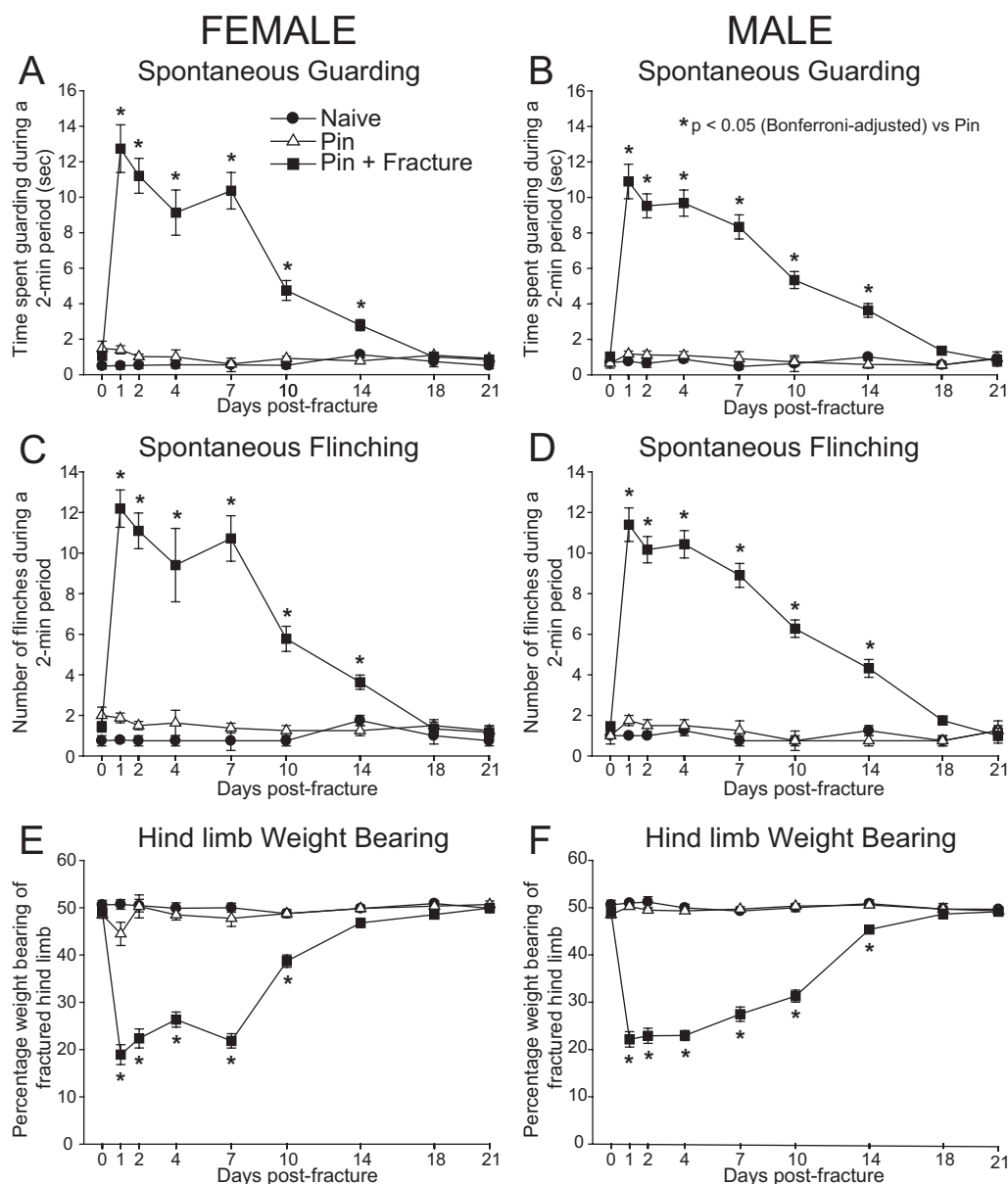


Fig. 2. Pain-related behaviors after a closed fracture of the femur in female and male rats. Female and male pin + fracture rats (closed squares) exhibited a greater time spent spontaneously guarding (A and B), a greater number of spontaneous flinches (C and D), and reduced weight bearing of the fractured limb (E and F) as compared with pin rats (open triangles) or age-matched naive rats (closed circles). There were no differences in pain-related behaviors between female and male rats at nearly all time points. Data are presented as mean \pm SEM (* $P > 0.05$, Bonferroni adjusted, *vs.* pin).

cluded, and of the 16 male rats fractured, 1 was excluded using previously described exclusion criteria.⁴³

Femoral Fracture Produces Pain-related Behaviors in Female and Male Rats

Spontaneous guarding, spontaneous flinching, and weight bearing in the left hind limb were analyzed in naive, pin, and pin + fracture rats. Pin + fracture rats exhibited a greater time spent guarding, increased number of flinches, and marked reduction in weight bearing as compared with naive and pin rats (fig. 2) from day 1 through day 14 after fracture for both female and male rats. In both female and male rats, spontaneous pain-

related behaviors peaked at day 1 after fracture, decreased gradually, and continued through day 18 after fracture (figs. 2A–D). In regard to weight bearing, there was a marked reduction after fracture. The greatest reduction was observed at day 1 after fracture and remained present until day 10 for female rats and day 14 for male rats (figs. 2E and F). At day 18 after fracture, all pain-related behaviors in fracture rats were not significantly different from those in pin rats. There were no significant differences in the magnitude of the pain-related behaviors between female and male rats at nearly all time points evaluated (data not shown, $P > 0.05$, Bonferroni adjusted). Rats with an intramedullary pin (pin, figs. 2A–D) showed mini-

mal number of flinches, time spent guarding, and hind limb weight bearing, which was not significantly different from that observed in naive rats (baseline values).

Morphine Treatment Reduces Fracture-induced Pain

Acute subcutaneous administration of morphine administered at day 7 after fracture significantly reduced ongoing guarding and flinching behaviors in a dose-

dependent manner (fig. 3). In female rats, administration of morphine at 3.0 and 10.0 mg/kg significantly reduced the fracture-induced guarding, and only 10.0 mg/kg significantly reduced flinching behaviors (figs. 3A and C; $P < 0.05$, Bonferroni adjusted, *vs.* vehicle-treated rats). In male rats, a significant reduction in these pain-related behaviors was observed after 3.0 and 10.0 mg/kg morphine (figs. 3B and D; $P < 0.05$, Bonferroni adjusted, *vs.* vehicle-treated rats).

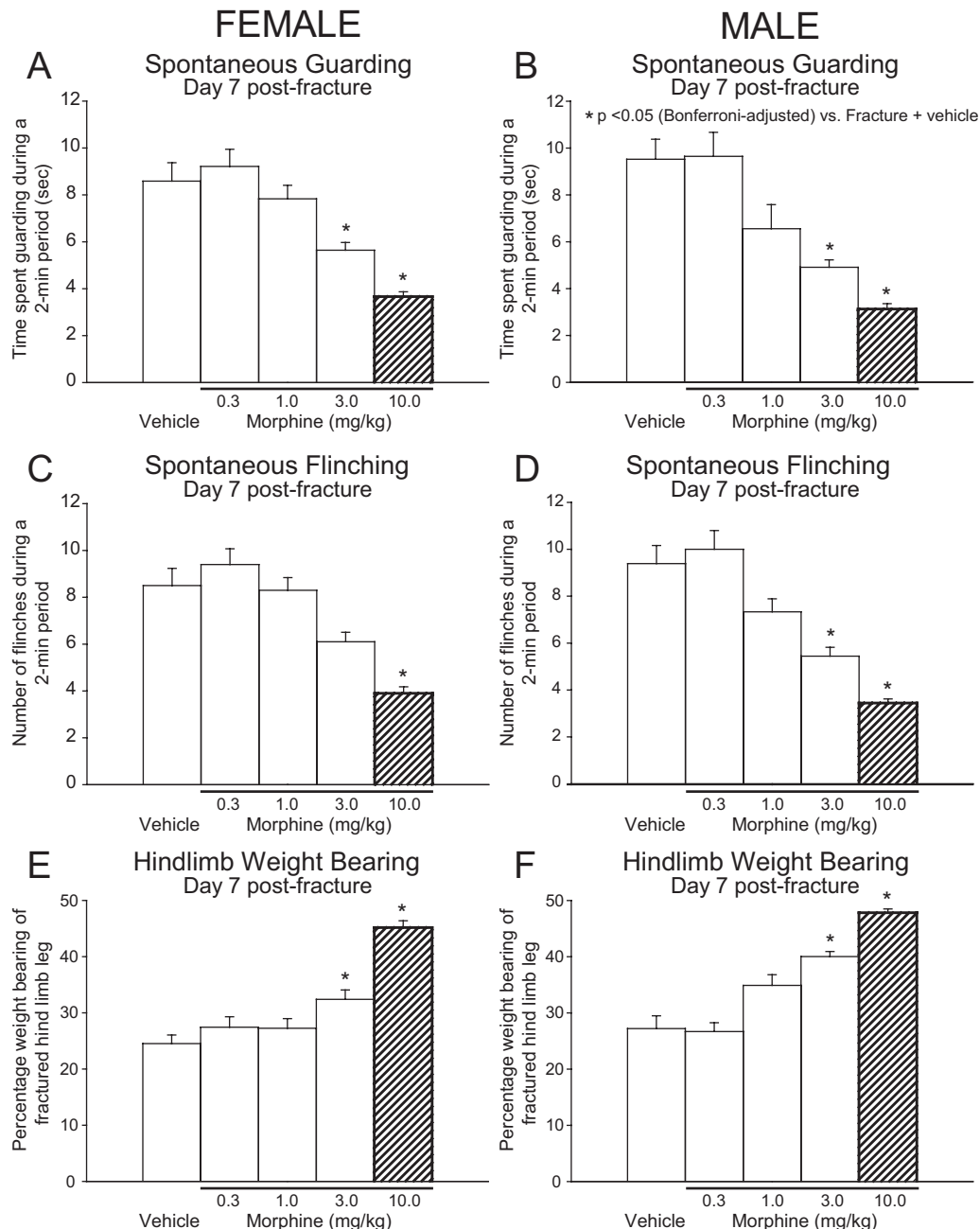


Fig. 3. Morphine reverses pain-related behaviors after fracture in female and male rats. Cumulative doses of morphine sulfate (0.3, 1.0, 3.0, and 10.0 mg/kg, subcutaneous) were administered 20 min before behavioral evaluation and 30 min between doses. Subcutaneous administration of morphine reduced fracture-induced guarding behavior (A and B), reduced number of flinches (C and D), and improved hind limb weight bearing (E and F) day 7 after fracture in a dose-dependent manner. Cumulative dose of 10 mg/kg resulted in side effects (hatched bars) as determined by reduction vertical stands and open field activity. Data are presented as mean \pm SEM (* $P > 0.05$, Bonferroni adjusted, *vs.* vehicle-treated rats).

For guarding behavior, the percentage of analgesia induced by morphine in female rats was smaller than that in male rats after 1.0 (8.8% for female and 31.1% for male), 3.0 (34.3% for female and 48.2% for male), and 10 mg/kg (57.3% for female and 67.1% for male). For flinching behavior, the percentage of analgesia induced by morphine in female rats was smaller than that in male rats after 1.0 (2.3% for female and 21.1% for male), 3.0 (28.2% for female and 42.0% for male), and 10 mg/kg (54.1% for female and 63.3% for male). While *post hoc* comparisons of analgesic response at each dose level revealed significant sex differences at 1.0, 3.0, and 10.0 mg/kg for both guarding and flinching responses (all $P < 0.05$, unadjusted), only sex comparisons of guarding response at 3.0 and 10.0 mg/kg remained statistically significant after the more stringent Bonferroni adjustment for multiple comparisons. Morphine at 10 mg/kg resulted in side effects including decreased locomotor activity (likely caused by lethargy) and decreased vertical stands on hind limbs (likely caused by sedation), which made it difficult to interpret the antinociceptive effect of morphine at this dose (additional information regarding this is available on the ANESTHESIOLOGY Web site at <http://www.anesthesiology.org>).

In addition, morphine treatment reversed the reduction of hind limb weight bearing in a dose-dependent manner in female and male rats (figs. 3E and F). Administration of 3.0 and 10.0 mg/kg morphine for female rats and 3.0 and 10.0 mg/kg for male rats significantly reversed the reduction in hind limb weight bearing ($P < 0.05$, Bonferroni adjusted, *vs.* vehicle-treated rats). For hind limb weight-bearing analysis, the percentage of analgesia induced by morphine in female rats was not significantly different as compared with male rats after 1.0 (11.9% for female and 28.2% for male), 3.0 (32.1% for female and 47.1% for male), and 10 mg/kg (80.7% for female and 75.7% for male) ($P > 0.05$, Bonferroni adjusted).

Soft Callus Formation and Mineralization after Fracture

Soft callus formation around fracture site in rats can be visualized at early time points after fracture by histologic analysis but not by x-ray and μ CT (fig. 4). Femoral fracture resulted in formation of mineralized callus (radiopaque area around the fractured cortical walls), which was minimal at day 7 after fracture (figs. 4A and B). Three-dimensional μ CT images of the same bone also show the relative absence of calcified callus at day 7 after fracture (figs. 4C and D). However, histologic analysis (hematoxylin and eosin) revealed the presence of a soft, cartilaginous callus around the fracture line as well as endochondral calcification at this time point in both groups (figs. 4E and F). At day 14 after fracture, mineralized callus was more visible as determined by radiographic and three-dimensional μ CT analysis (figs. 4G–J).

Histologic analysis shows a greater cartilaginous callus around the fracture line (figs. 4K and L).

Density of Sensory Nerve Fibers in Diaphyseal Periosteum of Female and Male Naïve Rats

The periosteum is a fibrous and cellular sheath that covers the outer surface of nearly all the bones of the body.⁵⁶ To elucidate what sensory fibers could be involved in the detection of fracture-induced pain, we determined the density of CGRP⁺ and NF200⁺ nerve fibers in the femoral periosteum of female and male rats.

Confocal micrographs of whole mount mid-diaphyseal periosteum preparations show that CGRP⁺ and NF200⁺ nerve fibers have a linear and bifurcating pattern of fibers. These sensory fibers form a mesh-like network that envelopes the naïve, unfractured bone (figs. 5A–D). Sensory fibers in the periosteum can be found as single nerve fibers or nerve bundles. The density of CGRP⁺ fibers in the periosteum of naïve female rats was not significantly different when compared with naïve male rats ($2,045 \pm 132$ and $1,928 \pm 209$ CGRP⁺ fiber intersections per mm² in female and male rats, respectively). Likewise, there were no significant differences in the density of NF200⁺ fibers in the periosteum between naïve female and male rats ($2,224 \pm 403$ and $2,093 \pm 201$ NF200⁺ fiber intersections per mm² in female and male rats, respectively).

Discussion

The Rat Model of Fracture-induced Pain

Jimenez-Andrade *et al.*³⁶ and Koewler *et al.*³⁵ have previously described models of bone fracture pain in C3H/HeJ and C57BL/6J mice, respectively. In the current study, we modified this model for use in the rat because this species has been widely used in pain³⁸ and bone healing research.^{32,43} We directly measured flinching, guarding, and weight bearing of the fractured hind limb. This latter behavioral endpoint may have significant utility in assessing the effects of novel analgesics have on use and rehabilitation of the fracture limb because the ability of the patient to voluntarily bear weight on the affected extremity is frequently used as one measure of successful bone healing during and after rehabilitation.^{46,47}

Previous reports examining other rodent preclinical models of acute and chronic pain have reported significant differences in the time course of spontaneous pain-related behaviors (guarding, lifting/licking) in the mouse *versus* the rat.^{57–60} In comparing the current results in the rat model with our previous results in the mouse model,^{35,36} it is remarkable how similar the guarding and flinching pain behaviors are in terms of the pain scores over time and the reduction in the pain scores that occurs with callus induced stabiliza-

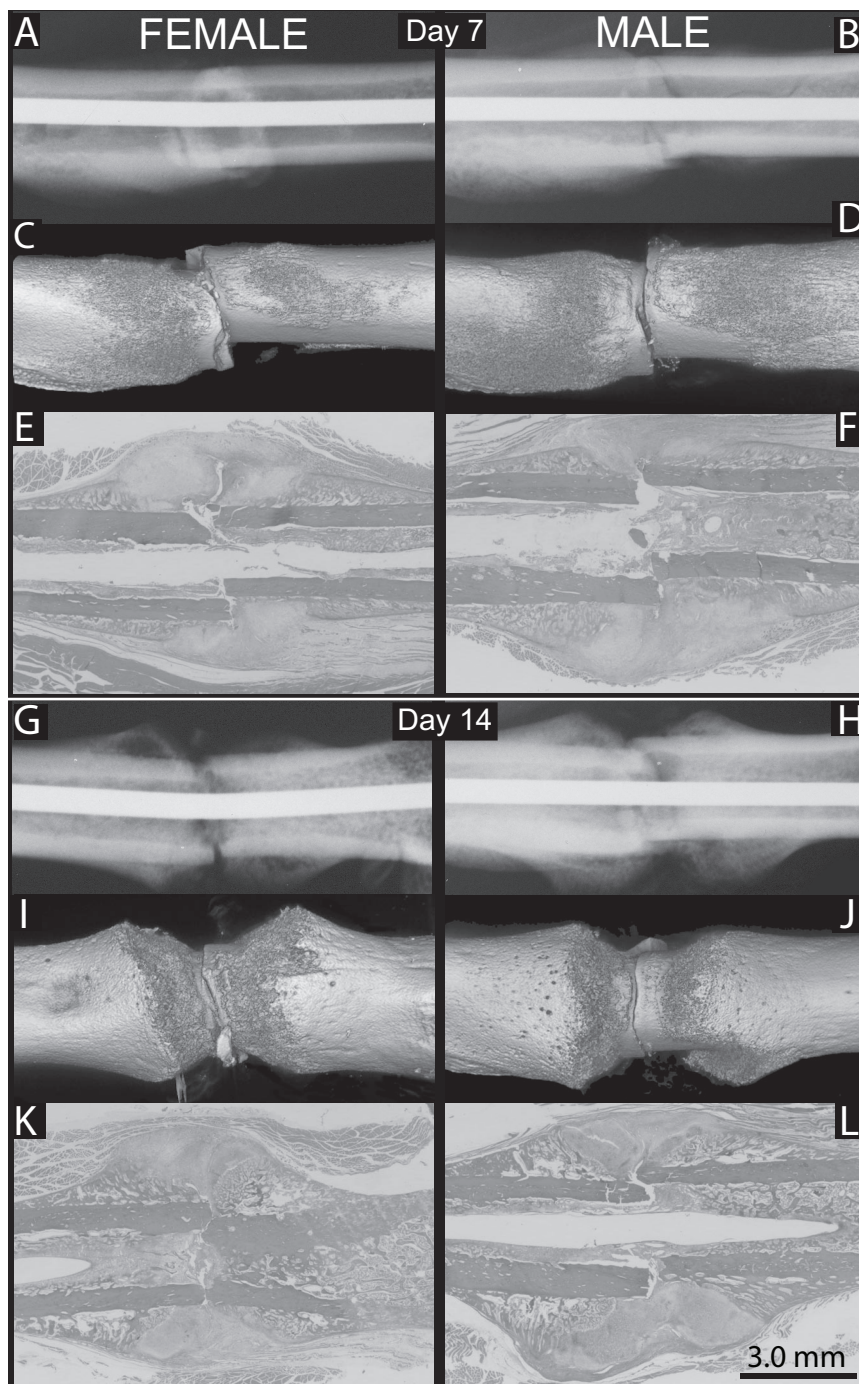


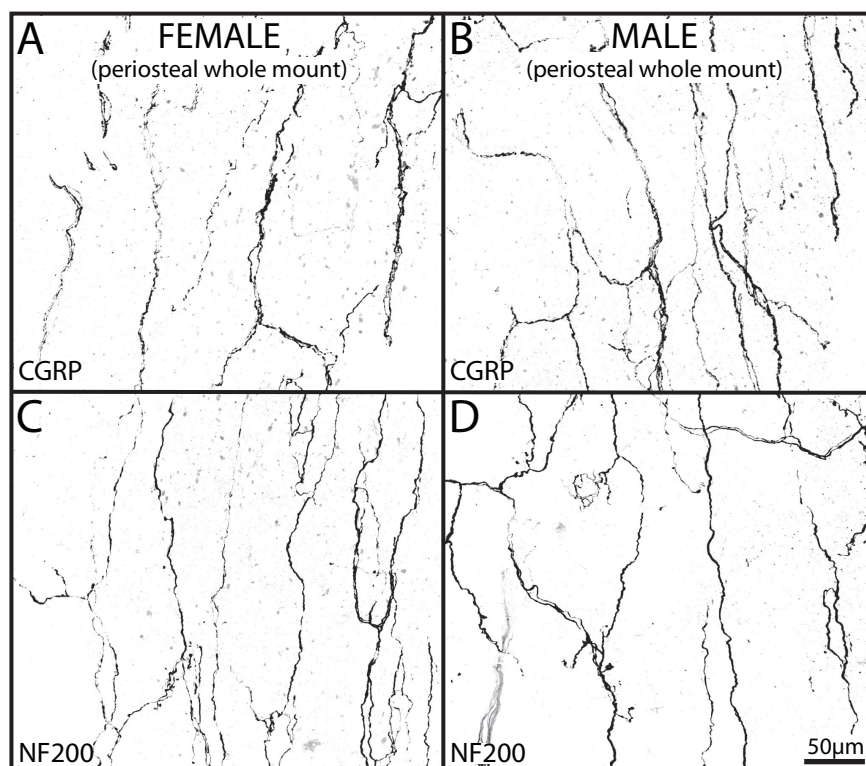
Fig. 4. Soft callus formation, which results in stabilization of the fracture site, is evident at days 7 and 14 after fracture by histologic but not radiographic or micro-computed tomography analysis. At day 7 after fracture, although there is little mineralized callus around the fracture site in female and male rats as shown by radiographs (A and B) and three-dimensional micro-computed tomography images of the mid-diaphysis (C and D), there is extensive soft callus formation around the fracture site as shown by longitudinal cross-section (7 μ m thick) of the femur stained with hematoxylin and eosin (E and F). At day 14 after fracture, calcification of the callus around the fracture site has begun in female and male rats as shown in the radiographs (G and H) and three-dimensional micro-computed tomography images (I and J) of the mid-diaphysis. Additional soft callus formation has occurred at day 14 after fracture (hematoxylin and eosin) (K and L). This soft callus provides mechanical stabilization of the fractured bone and may in part be responsible for attenuation of acute fracture pain. Scale bar = 3.0 mm.

tion of the fractured bone. Guarding and flinching behaviors are spontaneous, nonevoked pain behavior because animals withdraw their paw (flinching) and then guard their paw (guarding) to minimize the use of the fractured hind limb.³⁶ In contrast, weight bearing measured by incapacitance meter is a measure of the load the animal is willing to place on the fractured hind limb as compared with the nonfractured hind limb.⁴⁵ This latter measure seems to be analogous to the amount of weight a human would be willing to place on a fractured bone without pain.^{46,47}

Pain after Femoral Fracture in Male and Female Rats

The influence of sex on pain sensitivity is of great interest to pain research.⁶¹⁻⁶³ In the current study, we found that there was no significant difference between females and males when comparing fracture-induced pain behaviors, which included flinching, guarding, and weight bearing. In humans, recent data regarding the effects of sex on musculoskeletal pain are mixed. Therefore, whereas it was shown that women have greater postoperative pain than men after arthroscopic anterior

Fig. 5. The femoral periosteum of female and male rats receives a significant innervation by calcitonin gene-related peptide (CGRP)⁺ and 200-kd neurofilament H (NF-200)⁺ sensory fibers. Whole mount preparations of periosteum isolated from femurs of naive female (A and C) and male (B and D) rats were immunohistochemically labeled with antibodies against CGRP, a neuropeptide found in predominantly unmyelinated and thinly myelinated sensory fibers and NF200, which labels myelinated primary afferent sensory nerve fibers. The periosteum of naive female and male rats was densely innervated by CGRP⁺ and NF200⁺ sensory fibers (A–D), which formed a net-like meshwork that may be involved in detecting mechanical distortion of underlying mineralized bone. Confocal images (30- μ m z-series) were projected from 120 optical sections acquired at 0.25- μ m intervals. Scale bar A–D = 50 μ m.



cruciate ligament reconstruction⁶⁴ and tooth extraction,⁶⁵ no sex differences were noted after intracranial surgery.⁶⁶ In contrast to the fracture-induced pain related behaviors, small but significant sex differences were noted in the analgesic response of guarding behavior at 3.0 and 10 mg/kg morphine. The current results are consistent with sex differences in the antinociceptive response to morphine in the warm-water tail withdrawal assay,⁶⁷ the hot plate assay,⁶⁷ and the abdominal constriction test, which measures visceral pain.⁶⁷ It should be noted that we did not examine whether this effect was complicated by the estrous cycle of the female because the literature on this is not consistent.⁶⁸ Thus, there does seem to be a small male-*versus*-female difference in the ability of morphine to relieve fracture pain over and above any changes due to the estrous cycle.

Potential Mechanisms That Drive Fracture Pain

Worldwide, musculoskeletal pain is the main complaint of 30% of all medical consultations.⁶⁹ Musculoskeletal pain is responsible for 40% of all chronic pain states and 54% of all long-term disability and work absenteeism.⁷⁰ Despite these facts, it is remarkable how little is known about the specific mechanisms that drive skeletal pain. While bone is frequently thought of as a rather static organ, in fact bone is remarkably malleable and one of the most dynamic organs of the body in that it is constantly being remodeled in response to general use, loading of the bone, injury, and aging.^{71,72} Our lack of knowledge of what drives skeletal pain is in large part due to the dearth of animal models that closely mirror common painful conditions such as fracture

or osteoarthritis that are usually accompanied by significant skeletal pain.^{2,26,73}

In the current article, we have characterized a rat model of skeletal pain and, using this model, along with the human clinical literature on fracture pain, suggest that there are several distinct but overlapping mechanisms that drive this pain. The initial pain that follows acute fracture of the human femur is most frequently described as sharp, stabbing, aching, burning, and very intense.⁷⁴ For example, patients often refer to the pain that follows fracture of the femur as the worst pain that they have ever felt in their life.^{44,74} Based on the nature of the pain after fracture, we believe that this initial pain is due to mechanical activation of mechanosensitive nociceptors (C and A- δ nerve fibers) that innervate the periosteum, mineralized bone, and marrow.^{2,26} Therefore, stabilization of the fracture site by internal or external fixation in humans^{44,75–77} results in a significant attenuation of fracture pain. Second, within minutes to hours of the initial fracture, there is a marked influx of hematologic and inflammatory cells into the fracture site, which results in activation of nociceptors that express receptors for cytokines, chemokines, and inflammatory factors such as bradykinin, nerve growth factor, or prostaglandins that are frequently released upon tissue injury.^{78–80} Therefore, blockade of prostaglandin production by NSAIDs²⁶ or sequestration of nerve growth factor by anti-nerve growth factor antibodies^{35,36} results in a significant attenuation in fracture pain. Third, these factors may directly excite as well as sensitize and induce sprouting of mechanosensitive nociceptors in the

bone and induce a central sensitization characterized by neurochemical and cellular changes in the dorsal horn of the spinal cord and brain that facilitate the transmission and perception of pain in the central nervous system.⁸¹ Last, in cases where significant fracture-induced nerve injury occurs after fracture, the peripheral and central sensitization may be maintained and accompanied by inappropriate sprouting. These changes may contribute to a component of the chronic pain observed in individuals with complex regional pain syndrome. In fact, in approximately 45% of complex regional pain syndrome patients, fracture seems to be the precipitating event.^{82,83}

Current Analgesics Used to Treat Fracture Pain

A major reason fracture pain remains a significant health problem is the limited repertoire of analgesics available to treat this pain without negatively influencing fracture healing and/or the ability of the patient to participate in effective rehabilitation. For example, NSAIDs, which are effective in reducing a variety of musculoskeletal pains,^{27,28} have been shown to inhibit fracture healing in both mice³¹ and rats,³² although these results are less clear in humans.^{84–86} These data, together with recent reports that show selective prostaglandin agonists of the prostaglandin receptor E2 accelerate bone healing after fracture,^{87,88} indeed suggest that the use of NSAIDs and cyclooxygenase-2 inhibitors may delay fracture-induced bone healing.

Opiates are currently the mainstay for treating moderate to severe chronic pain.² However, opioids do have a variety of nonskeletal side effects that could inhibit bone healing. Opioid side effects include sedation, cognitive impairment, clouding of mental status, and depression, which can reduce mobility, resulting in loss of bone and muscle mass.⁸⁹ In young individuals with severe fractures, long-term opiate use can result in dependence and a reduced ability to promptly and fully participate in effective musculoskeletal rehabilitation that is necessary for early and effective bone healing.^{90,91} In elderly patients and patients with traumatic brain injury, opioid side effects tend to be more pronounced.^{33,34} After osteoporotic fractures in the elderly, minimum bed rest is desired to minimize inactivity-induced loss of bone and muscle mass.²⁰ Yet administration of strong opiates will, in general, reduce the ability of these patients to effectively engage in the exercise and rehabilitation necessary for more rapid bone healing. Together, these data highlight the need for the development of novel, mechanism-based therapies to treat skeletal pain that have negligible or a positive effect on bone healing. The current model seems to offer an attractive platform for the preclinical screening of novel therapies to treat fracture pain.

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