Development of a novel murine femur fracture and fixation model

Joey P. Johnson^{a,*}, Christopher T. Born^b, Nathan Thomas^d, Jeremy Truntzer^c,
P. Kaveh Mansuripur^b, Justin Kleiner^d, Scott T. McAlister^b, Dioscaris Garcia^b, Sarath Koruprolu^b

^a Department of Orthopedic Surgery, Loma Linda University, 11406 Loma Linda Drive, Suite 128, Loma Linda, CA, 92354, USA

^b Department of Orthopaedic Surgery, Warren Alpert Medical School, Brown University, Providence, RI, 593 Eddy Street, Providence, RI, 02903, USA

^c Department of Orthopedic Surgery, Stanford University, 291 University Drive, Stanford, CA, 94305, USA

^d Warren Alpert Medical School of Brown University, 222 Richmond Street, Providence, RI, 02912, USA

ARTICLE INFO

Keywords:

Basic science
Fracture model
Femur fracture

ABSTRACT

Background: Animal models have been used for decades to simulate human fractures in the laboratory setting. Fracture models in mice are attractive because they offer a high volume, relatively low-cost method of investigating fracture healing characteristics. We report on the development of a novel murine femur fracture model that is rapid, reproducible and inexpensive.

Methods: As part of a pilot study to investigate the effects of smoking on fracture healing, fifteen 35–43 g twelve-week old female CD-1 mice underwent a novel surgical protocol using direct visualization of femur fracture creation and fixation. Following surgery, mice were sacrificed at 14 days, 28 days and 42 days. After sacrifice, the femora were analyzed using MicroCT and histology to evaluate progression of healing.

Results: Of the 14 mice that survived the surgical procedure (one succumbed to a complication of anesthesia), two lost reduction and did not heal. Histology demonstrated at 14 days 44.1% (SD $\pm$ 2.9%) of callus composed of cartilage. At 28 days there was 19.0% (SD $\pm$ 3.4%) of callus composed of cartilage. At 42 days there was 8.4% (SD $\pm$ 2.6%) callus composed of cartilage ($p < 0.005$). MicroCT demonstrated that from 14 to 42 days the average callus volume decreased from 101.6 mm³ to 68.2 mm³ while the relative bone volume of callus increased from 14 to 42 days (15%–31%) ($p = 0.068$).

Conclusions: Our novel fracture and fixation model is an effective, rapid, reproducible and inexpensive method to simulate a fracture in a laboratory setting. Additionally, our model reliably creates a reproducible progression of radiographic and histological bone healing.

1. Introduction

Fracture systems created in laboratory animals have been used for decades for a variety of applications. Recently, mouse and rat fracture models have become more popular because of the ability to produce high volume genetically similar groups. Fracture models in mice often use ribs, tibiae or femora.¹ For surgical purposes, ribs do not provide an adequate model, and while tibiae provide an adequate long bone model for healing studies, intramedullary instrumentation of mice tibiae is technically difficult and there is limited soft tissue coverage.²

Manigrosso and O'Connor described mouse femur intramedullary fixation placed over a tungsten monofilament guidewire utilizing a drop-weight method for fracture creation.^{3,4} This technique provided a moderately reproducible closed fracture model. Twenty-two of 246 fractures had to be discarded.³ They were able to describe a stepwise histologic evolution of fracture healing similar to that seen in humans.³

Through biomechanical testing they were able to demonstrate that these fractures reached peak strength between 6 and 12 weeks.³

This fracture model was subsequently modified by Holstein et al. to provide a rotationally stable fracture model.⁵ Although not reported by the authors, this flattening of one end might theoretically make these intramedullary devices more challenging to pass through the medullary canal.

Both of these techniques require closed fracture creation by a 3-point drop-weight device as described by Bonnarens in rats.⁴ The Holstein technique requires violation of the knee joint and is technically demanding in the smaller medullary anatomy of mice. Given the diminutive size of the anatomy and implants involved in a mouse model, these closed fracture models require costly specialized instrumentation and were extremely difficult to reproduce in our laboratory. The drop-weight can bend implants and guidewires, leading to malunion, and/or a loss of intramedullary fixation. It also results in differing amounts of

* Corresponding author. Department of Orthopedic Surgery, Loma Linda University, 11406 Loma Linda Drive, Suite 128, Loma Linda, CA, 92354, USA.

E-mail address: joejohnson@llu.edu (J.P. Johnson).

<https://doi.org/10.1016/j.jor.2019.06.015>

Received 27 April 2019; Accepted 9 June 2019

Available online 15 June 2019

0972-978X/ © 2019 Professor P K Surendran Memorial Education Foundation. Published by Elsevier B.V. All rights reserved.

comminution, making stabilization more difficult and significant variability of fracture healing. Additionally, fracture creation is difficult to target with a drop-weight because of the short length of the mouse femur. We describe a femur fracture and fixation model that can be fully executed in minute by generating a highly reproducible transverse, noncomminuted mid-shaft fracture. The entire procedure is carried out under direct vision mitigating the need for fluoroscopy and the healing characteristics are highly replicated across specimens as determined by histopathology and microCT. This study was performed in an Association for Assessment and Accreditation of Laboratory Animal Care International (AALAC) approved laboratory. Prior to initiation of our study, all parts of our protocol were reviewed and approved by our Institutional Animal Care and Use Committee (IACUC).

2. Materials and methods

Fifteen 34–43 g CD-1 strain, three month old female mice were obtained.⁶ All anesthetic and surgical events were observed and monitored with assistance from our institution's veterinary staff. One mouse died unexpectedly on the day of surgery, from an adverse anesthetic event. Four mice were allowed to live for 14 day, five mice for 28 days and five mice for 42 days until sacrifice.

2.1. Fracture creation

Prior to surgery, mice were anesthetized with inhaled isoflurane. Clippers were used to remove hair from the entire lateral femur and the skin was sterilized with alcohol and povidone-iodine. A lateral incision to the femur was used. The underlying femur was palpated and the plane between the anterior and lateral musculature was bluntly spread longitudinally with straight iris scissors to expose the bone. This helps

to minimize soft tissue damage and bleeding (Fig. 1a). Once this was done, the femur was grasped with curved ophthalmologic forceps (Fig. 1b). The midshaft of the femur was identified and a transverse fracture was created using the iris scissors. Scissors were held with the tips completely perpendicular to the shaft of the femur to ensure a transverse cut was made. No fractures were excluded for fracture quality as this provided a uniform, transverse midshaft femur osteotomy with limited comminution or crush.

2.2. Intramedullary fixation system

Prior to fracture creation, a 24 gauge blunt tip needle was cut to a length of 10 mm; though this length could be varied as needed based on mouse femur morphology. Under direct vision, the proximal fracture fragment was cannulated in a retrograde manner with the 24 gauge needle (Fig. 1c). The distal fragment was then reduced onto the intramedullary fixation device by grasping it with curved forceps and placing the medullary shaft around the intramedullary device through the osteotomy (Fig. 1d) (See Video, Supplemental Digital Content 1).

Supplementary video related to this article can be found at <https://doi.org/10.1016/j.jor.2019.06.015>.

Following stable fixation, subcutaneous tissue was closed with simple buried 6-0 monocryl, and skin was closed with small veterinary skin staples. These staples were removed 10 days post operatively. This procedure is somewhat analogous to the open nailing popularized by Küntscher, but there is no need to breach either the proximal or distal ends of the femoral canal.⁷ The reduction of the fracture is accomplished by placing the soft tissues under light stretch to allow the distal medullary opening to be reduced onto the nail. The natural tension of the surrounding musculature then creates impaction and compression at the osteotomy site. This surgical procedure took on average 5 min to

Fig. 1. Surgical technique. A-lateral skin incision made over the femur. B- femur grasped with curved ophthalmologic forceps. C- post fracture creation with 24 gauge needle in the proximal fragment. D-distal fragment.

complete (range: 4–7 min). Under the guidance of our veterinary staff, it was recommended given the short operative time and skin sterilization that antibiotics not be used, and no post-operative infections were encountered. Post operatively, the mice were placed in recovery cages with soft food and water *ad libitum* and on heating pads to maintain core body temperature. Mice were checked every twelve hours for three days, and given weight based subcutaneous buprenorphine for post-operative analgesia. They were allowed immediate weight bearing to tolerance.

2.3. Callus evaluation

At time points of 14 days, 28 days and 42 days our mice were euthanized via carbon monoxide exposure. The femurs were disarticulated and stripped of tissues. Osteotomy at the femoral neck allowed for easy removal of the intramedullary device without disruption to fracture callus.

All specimens were stored in 10% buffered formalin. MicroCT (μ CT 40, Scanco Medical, Brüttisellen, Switzerland), as described by Zheng et al., was used to determine callus volume and density of the callus to assess radiographic adequacy of healing.⁸ This included calculations of total volume of callus (Bone Volume/BV) and the volume of callus that had become calcified (CV). Relative bone volume (RBV) was determined by dividing CV by BV. All samples were processed by two technicians who had been trained extensively in MicroCT analysis and had used the system with other projects (JPJ, SK).

Following MicroCT analysis specimens were decalcified, prepared in axial sections of 5 μ m thickness and stained with hematoxylin and eosin for light microscopic examination. The samples were analyzed for cartilaginous content and ImageJ (National Institutes of Health, Bethesda, MD) imaging software was used to determine the percentage area of callus composed of cartilage. ImageJ is an open access image processing program provided by the National Institutes of Health (NIH) that allows for precise color-metric analysis of digital images. Two blinded reviewers trained in the use of this software calculated the area of callus composed of cartilage for each sample, inter-observer error was within 3% on all measurements (JPJ, SK). Additional sections were stained with Safranin O and Mason's trichrome stains to qualitatively evaluate fracture healing.

2.4. Statistics

Standard descriptive statistics were used to compare groups. Means and standard deviations were used to compare groups between time points for MicroCT and histologic data. ANOVA analyses were used to compare differences in RBV and cartilage content between the three euthanasia time points. All statistical analyses were performed using Microsoft Excel Stats Package (Microsoft Office, 2007). Significance was set to $p < 0.05$ *a priori*.

3. Results

3.1. Radiographic evaluation

All mice that survived surgery survived to the time of sacrifice and none suffered infectious or wound healing sequelae. Of the fourteen mice that survived the surgical procedure, two lost reduction (14%) and did not heal (Fig. 2), one was euthanized at two weeks and one at 4 weeks. All twelve mice that did not lose reduction had bridging callus evident at time of sacrifice, with the mice at the post op day 42 time point showing evidence of fracture remodeling. In total, three mouse femora were available for analysis at the sacrifice point of 14 days, four at 28 days and five at 42 days.

Fig. 2. Two mice lost reduction and went on to nonunion.

3.2. MicroCT

Following microCT scanning, three dimensional reconstructions were created. In the 14 day sacrifice group, bridging soft callus was evident in all fractures. In the 28 day sacrifice group, bridging hard callus was evident in all fractures. In the 42 day sacrifice group, remodeling had begun (Fig. 3).

In the 14 day sacrifice group average BV of callus was 101.6 mm³ (range: 81.8–112.5 mm³), while the CV of callus was 15.0 mm³ (range: 14.4–16.1 mm³) which gave a RBV of 15% (range: 13%–20%; SD 4.5%). In the 28 day sacrifice group the average BV of callus was 84.7 mm³ (range: 62.4–117.9 mm³), while the CV of callus was 20.5 mm³ (range: 12.2–30.7 mm³) which gave a RBV of 25% (range: 15–34%; SD 9.1%). In the 42 day group the average BV of callus was 68.2 mm³ (50.8–86.22 mm³), while the average CV of callus was 20.0 mm³ (range: 19.1–21.1 mm³) giving a RBV of 31% (range: 24–41%; SD 7.4%) (Table 1). There was a trend toward significance with regards to RBV with later euthanasia date associated with higher RBV ($p = 0.068$).

3.3. Histologic evaluation

Our histologic findings largely mirrored our MicroCT analysis. At 14 days, hematoxylin and eosin staining demonstrate that fracture callus is comprised of islands of cartilage interwoven with trabecular bone and deposits of fibrinous tissue. At 28 days there is marked remodeling of the callus with conversion of nearly all islands of cartilage to woven bone and progressive lysis of the cortices characterized by increased porosity and thinning. At 42 days there is continued remodeling of the callus with conversion of nearly all islands of cartilage to woven bone and reduction in callus size (Fig. 4).

At 14 days, the average percent area of callus composed of cartilage was 44.1% (SD 2.9%), while at 28 days the average percent area of callus composed of cartilage was 19.0% (SD 3.4%) and at 42 days the average percent area of callus composed of cartilage was 8.4% (SD 2.6%) (Table 2) ($p < 0.005$).

4. Discussion

This study demonstrates a novel murine fracture and stabilization system that is reliable, reproducible and does not require specialized instrumentation to perform. The fracture model described by Bonnarens et al., was carried out in rats, which have larger anatomy allowing for a greater ease of fracture creation and instrumentation.⁴ Subsequent studies have been performed validating this in a mouse model reporting a high rate of failure due to nail migration, as the method for instrumentation relied on unlocked intramedullary implants being introduced retrograde through the knee joint.^{3,5} Subsequently, a “locked” nailing technique was developed, although this method still had a 10% failure rate, and required insertion through the mouse knee joint.^{3,5}

While developing our model, we found the retrograde intramedullary instrumentation described by previous authors difficult to reproduce, especially in the smaller sized mouse. During trialing of

Fig. 3. Fractures with bridging callus, from left to right. 6a) POD14 6b) POD28 6c) POD42 with remodeling already evident.

Table 1

MicroCT results of healing mouse fractures.

Mouse Group	Bone Volume (BV)	Cortical Volume (CV)	Relative Bone Volume (CV/BV)
2 weeks			
10	112.48	14.43	0.13
13	81.75	16.11	0.2
11	110.58	14.57	0.13
	101.6	15.04	0.15
4 weeks			
2	62.41	21.16	0.34
15	66.35	12.23	0.18
1	117.93	17.8	0.15
3	92.19	30.66	0.33
	84.72	20.46	0.25
6 weeks			
8	74.64	19.28	0.26
7	76.9	19.61	0.26
5	50.76	20.77	0.41
12	86.23	21.12	0.24
14	52.52	19.09	0.36
	68.21	19.97	0.31

these procedures, we found that fracture creation by drop-weight would often kink the tungsten monofilament guidewire making passage of the pre-flattened 24-gauge needle difficult, if not impossible. Additionally, we found that because of the small femur size of the mice, placement and standardization of fracture creation was very difficult and time consuming. Given the direct visualization used in our approach, none of our fractures were excluded for fracture quality because the fractures were very reproducible. In contrast to the technique described by Manigrasso et al., no fluoroscopy or immediate post fixation radiographs were needed to verify fracture creation and stabilization given the direct visualization afforded by our technique.³ The average time needed for our surgical procedures was five minutes (range: 4–7 min). Also, given that we did not breach the distal cortex of the femur, a

“locking” mechanism was not necessary. Our rate of loss of reduction (2/14) is similar to that reported previously in the literature.^{3,5} Anecdotally, these failures occurred in two specimens that utilized a shorter intramedullary device, and we no longer use precut needles that are shorter than 10 mm for this particular mouse size and population.

Radiographically, abundant callus formation was visible at 14 days, bridging callus was evident at 28 days and remodeling was evident at 42 days. These results are supported by our MicroCT data which show large callus volumes at 14 days, that steadily decrease to 42 days as the percentage of calcified bone increased. As would be anticipated, from days 14–42, BV steadily decreased as callus matured. During this time, CV simultaneously increased. Increasing CV and decreasing BV lead to an increase in RBV (CV/BV). These differences showed a trend towards significance, with later euthanasia date associated with higher RBV ($p = 0.068$). This shows that over time, callus is remodeling and being replaced by bone, an observation that corresponded with our histological results. Our histological analysis showed decreased cartilage content with later euthanasia date as well ($p < 0.005$). The rate and sequence of bone healing is similar to endochondral bone healing in humans as demonstrated by previous groups.^{3–5} Additionally, as this was a proof of concept pilot for a larger study, the authors subsequently performed this procedure on 78 mice. 76 survived to sacrifice and only 4 lost reduction and had to be excluded, further demonstrating the reliability and reproducibility of this method.⁹

Previously, open fracture models have been described in the literature.^{10–12} Klein et al., describe an open fracture and fixation system that requires a prefabricated intramedullary screw made specifically for mice, and that utilizes a separate incision for instrumentation.¹⁰ In contrast, our model does not require a specialized and costly prefabricated implant, and does not require the use of a second incision that violates the knee joint. The relative bone volume as measured by MicroCT found in their study at 2 weeks is similar to what was found in our study at 2 weeks (20%–15.1%). Wehrle et al., utilize a similar approach for fracture creation that we describe, but utilize an external fixator for fracture stabilization.¹¹ Their sacrifice time point of 21 days

Fig. 4. Histologic progression of enchondral healing at Day 14, Day 28 and Day 42. Top row demonstrates hematoxylin and eosin staining, middle row shows safranin and O staining and bottom row demonstrates Mason's trichrome staining.

Table 2
Histologic evaluation, percentage of callus composed of cartilage per mouse femur.

Mouse Number	Cartilage composition
2 Weeks	
10	41.02%
13	44.57%
11	46.80%
Average	44.13%
4 weeks	
2	24.09%
15	17.45%
1	17.82%
3	16.61%
Average	18.99%
6 weeks	
8	10.10%
7	9.80%
5	5.79%
12	5.16%
14	10.86%
Average	8.34%

showed similar results to our 28 day group with regards to MicroCT, however, this comparison is not perfect given the differing time points and an external fixation technique vs intramedullary fixation. Kawada et al., utilized a similar approach for fracture creation, but again, require a separate, or extensile, incision to cannulate the femur through the knee joint.¹² Our method negates the need to operate through the knee joint, allowing for more rapid stabilization, without an extensile, or secondary incision.

Our study does have limitations. Specifically, we were unable to provide biomechanical data regarding the strength of callus at these time points. However, we could not biomechanically load these femurs to failure and obtain histologic data. The reproducibility of the progression to healing by both MicroCT and histology might obviate the requirement for formal biomechanical testing validation for this model. Also, our imaging data closely resembles previous published models and closely approximates our histologic data strengthening our analysis

of interval healing from 14 to 42 days.^{3–5,11,12} Our histology does demonstrate an increased amount of cartilage on histology as compared to other groups, however, this may be due to differences in slide preparation. Furthermore, we analyzed the entire femur distal to the proximal extent of callus, including the articular surface. While the articular surface was not involved in the fracture callus, it did allow for standardization of our measurements. Additionally, this reports on a relatively small number of animals, however our data appears reliable between animals and follows a logical progression of endochondral healing. Our fracture model does involve opening the skin at the site of fracture and necessitates some soft tissue stripping of the femur, which could alter the healing environment when compared to a closed fracture model. Eighty percent of our pilot study animals were available for analysis for healing. These animals demonstrated similar radiographic and histologic healing progression to other published models. There were no infectious sequelae of our technique in this study. We do not have a control population of the previously described percutaneous technique for comparison to our novel open technique. However, our results do stand up to historical controls, and more recently described open methodologies^{3–5,11,12}.

5. Conclusions

The method we describe is reliable and reproducible and allows for high volume throughput. Additionally, it does not require the use of any specialized equipment, implants or intraoperative fluoroscopy. This novel method also does not require violation of the knee joint, allowing for maintained knee function, and allowing for normal ambulation. It produces a uniform fracture and a uniform healing process as shown both by histology and MicroCT. It is also technically less demanding than previously described procedures, extra-ordinarily cost effective and utilizes materials available in most laboratory settings. We hope that this model can provide a useful technique for future investigators who require a rapid, reliable and inexpensive animal model with which to examine fracture healing.

Declarations of interest

Dr. Born is a paid consultant for Stryker Trauma, and has stock or stock options in Illuminoss and BioIntraface. No other authors have any conflicts to report.

Previous publication declaration

This manuscript, including the related data, figures and tables has not been published previously, nor is it under consideration elsewhere.

Conflicts of interest

Dr. Born is a paid consultant for Stryker Trauma, and has stock or stock options in Illuminoss and BioIntraface. No other authors have any conflicts to report.

Author contributions

Dr. Joseph Johnson carried out the research plan, assisted in writing the manuscript and approved the final version. Dr. Christopher Born formulated the research plan, assisted in carrying out the research plan, edited the manuscript and approved the final version. Nathan Thomas assisted in carrying out the research plan, edited the manuscript and approved the final version. Dr. Jeremy Truntzer assisted in formulating the research plan, edited the manuscript and approved the final version. Dr. P. Kaveh Mansuripur assisted in formulating the research plan, assisted in carrying out the research plan, edited the manuscript and approved the final version. Justin Kleiner assisted in formulating the research plan, assisted in carrying out the research plan, edited the manuscript and approved the final version. Scott McAlister assisted in formulating the research plan, assisted in carrying out the research plan, edited the manuscript and approved the final version. Dioscaris Garcia assisted in formulating the research plan, assisted in carrying out the research plan, edited the manuscript and approved the final version. Sarath Koruprolu assisted in carrying out the research plan, edited the manuscript and approved the final version.

Acknowledgements

This study was supported in part by a grant from the National

Institutes of Health; (2P20 GM104937) (COBRE Bioengineering Core).

This study was supported in part by OTA Resident Grant 529037.

Neither of these entities played any role in study design or collection, analysis or interpretation of data. Neither funding source assisted in writing this manuscript or played any role in the decision to submit this for publication.

The authors would like to acknowledge Dr. Matthew Deren and Douglas Moore for their assistance with this project.

The authors would also like to acknowledge the assistance of the Brown University Department of Veterinary Medicine, specifically Dr. Lara Helwig, Pamela Norberg and Veronica Bouvier.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jor.2019.06.015>.

References

- Cheung JP-Y, Samartzis D, Cheung KM-C. A novel approach to gradual correction of severe spinal deformity in a pediatric patient using the magnetically-controlled growing rod. *Spine J. Off. J. North Am. Spine Soc.* 2014;14:e7–13.
- Hiltunen A, Vuorio E, Aro HT. A standardized experimental fracture in the mouse tibia. *J. Orthop. Res.* 1993;11:305–312.
- Manigrasso MB, O'Connor JP. Characterization of a closed femur fracture model in mice. *J. Orthop. Trauma.* 2004;18:687–695.
- Bonnarens F, Einhorn TA. Production of a standard closed fracture in laboratory animal bone. *J. Orthop. Res.* 1984;2:97–101.
- Holstein JH, Menger MD, Culemann U, Meier C, Pohlemann T. Development of a locking femur nail for mice. *J. Biomech.* 2007;40:215–219.
- Born C. *IACUC Approval RE: What Is the Optimal Timing for Cessation of Smoking to Reduce the Impact on Bone Healing?* 2016; 2016 (#1504000131 Amendment #002).
- Kuntscher GB. The Kuntscher method of intramedullary fixation. *J. Bone Joint Surg. Am.* 1958;40-A:17–26.
- Zheng LW, Ma L, Cheung LK. Changes in blood perfusion and bone healing induced by nicotine during distraction osteogenesis. *Bone.* 2008;43:355–361.
- Johnson, J. et al The Impact of Smoking Cessation Timing on Bone Healing- Manuscript in Progress.
- Klein M, et al. Comparison of healing process in open osteotomy model and open fracture model: delayed healing of osteotomies after intramedullary screw fixation. *J. Orthop. Res.* 2015;33:971–978.
- Wehrle E, et al. Distinct frequency dependent effects of whole-body vibration on non-fractured bone and fracture healing in mice. *J. Orthop. Res.* 2014;32:1006–1013.
- Kawada S, Wada E, Matsuda R, Ishii N. Hyperbaric hyperoxia accelerates fracture healing in mice. *PLoS One.* 2013;8:e72603.