

Dear Editors, *Journal of Visualized Experiments*,

We thank the reviewers for their comments regarding our manuscript “A Simple Critical-Sized Femoral Defect Model in Mice.” by Clough *et al.* We have endeavored to address each of the concerns with existing data and observations but were unable to perform additional experiments within the timeframe set by the JoVE editors. We appreciate the reviewers’ understanding in this matter.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This paper proposes a novel method for mouse long bone defect model. The small, thin and fragile nature of the femoral bone of mice impedes the establishment of easy, reproducible bone defect model, despite its wide application for gene knock-out/knock-in models. By using self-made prosthesis consisted of only common supplies, the author provided us with useful and relatively easy way to make delayed or non union model of femoral bone.

Major Concerns:

There is no major concerns in this paper.

Minor Concerns:

1. Bone marrow cavity size and shape may vary between individual variability. Polishing metal shaft and reaming femoral cavity to adjust the very same size seems to demand highly sophisticated techniques and this might limit the reproducibility and universality of the methods.

Comparison of this method to intramedullary nails or external fixations might be better to show its superiority, in terms of learning curve, morbidity or mortality rate, if possible.

This is a good question and a valid concern. In fact, have found that the key to reproducibility lies in careful matching of the age, gender and strain of mice rather than the prototyping of the pin, although this should be done carefully.

Regarding comparisons with other methods, we propose that the ability to easily manufacture a pin in-house to the exact specifications of the mice to be studies represents a distinct advantage over established fixation systems.

In response to this concern, we have added the following to the revised manuscript:

INTRODUCTION, page 3, line 119: “With careful selection of inbred age, gender and strain-matched mice, the the result is a highly reproducible *hypertrophic non-union defect*²² which can be easily evaluated radiologically.”

PROTOCOL: page 3, line 133: “Since strains of mice vary slightly in terms of anatomy and growth rate, we advise that the fabrication of pins is optimized to the strain, gender

and age of the recipients prior to implantation into live subjects. **If the strains are carefully matched, the interference fit between the pin and marrow cavity is highly reproducible.”**

DISCUSSION, page 10, line 447: “While some torsional motion may occur during the early stages of healing, this is minimized by careful attention to the pin diameter and adequate reaming of the medullary canal so as to attain a firm interference fit between the implant and endosteum. **With careful selection of inbred strain and matching of age and gender, the fit becomes reproducibly robust within a few days. Nevertheless, with the advent of 3D printing techniques, it is expected that torsional motion can be further reduced** by more sophisticated versions of the pin that incorporate roughened surfaces and/or barbed attachment sites.”

And line 453: “The ease of pin fabrication, the availability of a wide variety of hypodermic tubing sizes also permit the optimization of the technique for virtually any adult inbred mouse, irrespective of natural or experimental bone phenotype.”

2. No adhesives or glues were applied to the interface between the bone cavity and metal implant with smooth surface. Is the friction resistance strong enough to prevent the metal shaft to come out from the bone cavity?

Please refer to our modifications for point 1.

3. Figure 2: The function of gel foam applied to the collar is not described and uncertain in this paper. Is it for cell transplantation?

Sorry for the omission, we have provided clarification:

METHODS, page 4, line 171: “We have found that the positioning of a gelatin or collagen scaffold at the site of the defect improves cell retention and induces longitudinal growth along the axis of the bone. Use a 4 mm diameter punch-biopsy...”

FIGURE 2 LEGEND, page 9, line 403: “...Gelfoam cylinder is trimmed, positioned over the steel collar (*middle*), then autoclaved, resulting in a sterile pin coated with dried Gelfoam. **This improves cell attachment at the site of injury and maintains the direction of healing (*below*).**”

Reviewer #2:

Manuscript Summary:

The manuscript describes techniques for generating a segmental defect in the femur of a mouse using a femoral medullary pin for stability. The technique is described in a comprehensive manner that makes it comprehensible to all scientists irrespective of whether they have veterinary training. As one who has performed similar work, I can attest to how difficult it is to generate reproducible bone defects in mice. Therefore, I suspect the article will be very well received and should open the door for a variety of new experimental approaches, such as testing new therapeutics and conducting basic studies using transgenic and knockout mice.

Major Concerns:

There are no major concerns with the manuscript.

Minor Concerns:

The manuscript would be further improved if the authors described several examples of how the technique could be used to test therapeutics, e.g. growth factor, cell-based, or materials-based treatments. Providing a simple example of how one might develop such an experiment, e.g. route and timing of delivery of test agent, and whether differences in bone production would yield significant differences between treatment groups based on the methods of analysis described in the text would be informative.

We also agree that this would benefit the readers of the article. However, experiments involving novel cells, growth factors and scaffolds could be highly variable and subject to very specific requirements. Nevertheless, we have recently utilized the approach to demonstrate efficacy of novel stem cells and stem cell derived scaffolds for femoral healing and the findings have been approved for publication in *Journal of Bone and Mineral Research*. We believe this article would showcase the technique and address many of the concerns raised here. In response to this comment, we have added the citation and the following text.

DISCUSSION, page 11, line 476: “For example, there is a variety of immune-compromised strains that permit testing of human cells and proteins without fear of immunological rejection, and their small size reduces the need for excessive amounts of valuable experimental materials, cells or compounds. **This is exemplified by our recent study demonstrating the efficacy of adult human stem cells and their extracellular proteins for osteoregeneration.**”

Reviewer #3:

The objective of this study is to demonstrate a new simple method to generate a critical-sized pin stabilized defect of murine femur in the standard laboratory. The manuscript claims that the defect 21 days after surgery is a hypertrophic non-union defect. It would be better to follow up the healing process for a longer time period, for instance, 12 weeks. In the previous study, a non-union fracture is defined as failure of healing even after the time period three times longer than that of the normal healing. In this study, the control group is missing. More quantitative data should be added to make the authors' conclusion. Major revision should be made for paper resubmission.

[Editorial comment: We do not require in depth or novel results for publication in JoVE, only representative results that demonstrate the efficacy of the protocol. However, please ensure that all claims made throughout the manuscript are supported by either results or references to published works.]

1, Lines 113-114: The authors state that the superior point to Garsia's is a simple method to generate the defect of the murine femur. Other superior points should be discussed. There are no data to compare with other methods. Comparing with other methods, the present method should be evaluated.

We concede that the primary advantage of our system over others is the simplicity of pin manufacture and implantation (see Introduction, page 3, line 116-124). Another major advantage is that the unique collar permits definition of the defect margins and thus, the region of interest for microCT scanning (Introduction, page 3, line 122-124 and Discussion, page 10, line 453-467). While we believe it is beyond the scope of the manuscript to compare every mouse fixation method in the literature, we appreciate that the method of Garcia *et al.* is the most comparable to ours. As such, we have made the following additions to the text in support of the proposed system and included the citation.

Introduction, page 3, line 112: “For example, Garcia *et al.* [new ref 23] devised an elegant interlocking pin system for use in mice, but the procedure involves incisions at two separate sites and extensive modification of the femur to accommodate the pins. These procedures were performed under a dissecting microscope.”

2, Lines 162-167, Lines 437-444: The reason to fix the pin into the femoral medullary cavity should be described clearly. The torsional motion may occur during the early stages of healing, and the pin became immovable. The statement is difficult to understand. It should be revised. For the osseointegration taking place in the space between the pin and endosteum, the authors should demonstrate that phenomenon scientifically.

Confidential comment to Editor: We are unclear exactly what reviewer 3 is referring to here, but we have attempted to clarify the wording a little more.

Protocol, page 4, line 167: “Make sure that the ends of the pin penetrate into the trabecular bone of the diaphysis so as to maximize fixation and improve the interference fit (Fig2E).”

Results, page 8, line 355: “In rare instances (<5% of cases), pins can become dislocated at early stages of healing and animal use protocols should provide for revision surgery if this occurs. While it is technically challenging to quantify torsional motion on murine femurs, manual palpation of specimens confirmed that torsional and longitudinal motion is marginal after 7 days as connective tissue accumulates around the pin.”

3, Lines 296-299: The follow-up time of healing is too short. Generally mice have a great potential for bone repair and the unstabilised fracture can be healed without any delay. If the authors want to claim that this is a model of permanent bone failure for bone healing, the longer-term follow-up, for instance, 12 weeks, should be performed.

A 12-week experiment is beyond our deadline and also unlikely to be approved by our IACUC given that much of this work has already been done but your point is well taken. We propose the following response to your concern: our assay is designed to assess healing during the anabolic phase of bone which is 3-4 weeks in rodents, thereafter slow bone remodeling takes place which is unlikely to be associated with bridging of the defect. Nevertheless, our defect size (3 mm) conforms to the criteria of Key *et al.* and is much smaller than the 1.8 mm defect used in Garcia's work. Therefore, we predict that the 3 mm defect is critical sized and will remain for up to 12 weeks.

Discussion, page 11, line 468: “In the experiments described here, the permitted healing time was relatively short at 3 weeks, which corresponds to the rapid, anabolic phase of bone healing. Thereafter, bone remodeling is a very slow process²⁸. Generally, if bridging is not observed after 4 weeks, healing is unlikely to occur and in agreement, we observe very little additional bone growth after 4 weeks in this system. Furthermore, a 3 mm gap meets the criteria of Key *et al.*¹⁶ for a critical sized defect and Garcia *et al.* demonstrated that a gap as narrow as 1.8 mm does not sufficiently heal after 10 weeks and this could be delayed to 15 weeks with stripped perichondrium²³.”

4, Lines 378-383: In this study, the pins were removed after decalcification. I am afraid that the removal may lead to the destruction of the surrounding tissue. To demonstrate the immobility of pins, the surrounding tissue should not be destroyed. This point should be discussed.

In response to this concern, we have added the following text and data.

Results, page 9, line 386: “While some damage will inevitably occur during dissection, the histological structure of bone and connective tissue remains clear if the pin is removed carefully. Alternatively, methyl methacrylate embedding and sectioning of non-demineralized sections may be performed with the pin in place (**Fig6F**).”

We have added data in Fig 6 showing an MMA embedded, non-decalcified section with the pin in place.

5, The strain and age of mice to use must affect the healing process of bone fracture. This point should be discussed referring some related research papers.

6, The present procedure is also applicable to the disease models of mice, such as diabetic and ovary-extracted mice. This point should be discussed.

In response to these concerns we have added the following text and references:

Discussion, page 11, line 481: “The relatively short lifespan of mice also present the opportunity for research into aging³⁰ and the wide variety of inbred strains permit the study of global genotype on healing³¹. There are also a number of disease models that are easily established in mice such as diabetes and osteoporosis^{32,33}.”