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TITLE:

A Reliable and Reproducible Critical-Sized Segmental Femoral Defect Model in Rats Stabilized with a Custom External Fixator

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SUMMARY:

In vivo mammalian models of critical-sized bone defects are essential for researchers studying healing mechanisms and orthopedic therapies. Here, we introduce a protocol for the creation of reproducible, segmental, femoral defects in rats stabilized using external fixation.

ABSTRACT:

Orthopedic research relies heavily on animal models to study mechanisms of bone healing *in vivo* as well as investigate the new treatment techniques. Critical-sized segmental defects are challenging to treat clinically, and research efforts could benefit from a reliable, ambulatory small animal model of a segmental femoral defect. In this study, we present an optimized surgical protocol for the consistent and reproducible creation of a 5 mm critical diaphyseal defect in a rat femur stabilized with an external fixator. The diaphyseal osteotomy was performed using a custom jig to place 4 Kirschner wires bicortically, which were stabilized with an adapted external fixator device. An oscillating bone saw was used to create the defect. Either a collagen sponge

alone or a collagen sponge soaked in rhBMP-2 was implanted into the defect, and the bone healing was monitored over 12 weeks using radiographs. After 12 weeks, rats were sacrificed, and histological analysis was performed on the excised control and treated femurs. Bone defects containing only collagen sponge resulted in non-union, while rhBMP-2 treatment yielded the formation of a periosteal callous and new bone remodeling. Animals recovered well after implantation, and external fixation proved successful in stabilizing the femoral defects over 12 weeks. This streamlined surgical model could be readily applied to study bone healing and test new orthopedic biomaterials and regenerative therapies *in vivo*.

INTRODUCTION:

Orthopedic trauma surgery focuses on treating a wide range of complex fractures. Critical diaphyseal segmental bone defects have proven difficult to treat clinically due to the decreased regenerative ability of the surrounding muscle and periosteum as well as the failure of localized angiogenesis¹. Modern treatment techniques include operative fixation with bone grafting, delayed bone (Masquelet) grafting, bone transport, fusion, or amputation²⁻⁴. In most patients who have ambulatory function preserved after their trauma, with well-functioning distal limbs, limb salvage is clearly a better treatment option⁵. These salvage treatments often require staged surgical interventions over a long treatment course. Some authors have suggested that external fixation is superior as compared to the internal fixation for these applications due to the decreased tissue damage during implantation, decreased implanted surface area, and increased postoperative adjustability of the fixator⁶. However, a prospective randomized controlled trial is currently underway to help clarify this controversy of internal versus external fixation in severe open fractures of the tibia⁷. Unfortunately, with either treatment selected, significant complication and failure rates persist^{8,9}. With either treatment method, with respect to the segmental bone loss, the surgeon must contend with segmental diaphyseal defects that present significant challenges. Corrections of segmental defects must maximize bone stabilization and simultaneously enhance the osteogenic process^{10,11}.

Due to the clinical importance, yet the lower volume, of critical-sized diaphyseal segmental defects, an effective, reproducible animal model is necessary to enable research teams to advance treatment techniques and ultimately improve clinical outcomes. Researchers need to study *in vivo* physiologic healing mechanisms in a mammalian animal model. While such models of external fixation already exist¹²⁻¹⁵, we hope to provide a more reliable method for non-unions in the untreated animals, decrease costs through the choice of affordable fixator materials, and outline a straightforward surgical protocol for the easy application to future studies. The primary goal of this protocol is to establish a reliable and reproducible model of a critical diaphyseal defect in rats. The procedure was evaluated by assessing the stabilization and bone healing in rat femurs over 12 weeks. The secondary goals included: making an affordable model as a cost effective as possible, simplifying the surgical approach and stabilization, and ensuring ethical care of the animals. The authors and research team conducted preliminary experiments with a range of different biomaterials and potential regenerative therapies to improve healing in this segmental defect.

PROTOCOL:

The rats used in this study received daily care in accordance with the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition¹⁶. The Institutional Animal Care and Use Committee at the University of Wisconsin-Madison evaluated and approved this experimental protocol before the project began.

1. Animals

1.1. Use the outbred Sprague-Dawley male rats weighing approximately 350 g.

2. Preparation of Bone Morphogenetic Protein-2 (rhBMP-2) soaked sponge scaffolds

NOTE: Scaffold preparation should occur just before implantation in the femur (see Step 6.14).

2.1. Follow manufacturer instructions for the use of an established rhBMP-2 bone graft kit containing a collagen sponge, lyophilized rhBMP-2, and sterile water for reconstitution¹⁷. Maintaining sterility, reconstitute the rhBMP-2 with the sterile water to a concentration of 1.5 mg/mL.

2.2. Using sterile scissors and a sterile ruler, trim the rhBMP-2 soaked collagen sponge to reshape to fit a 5 mm x 3 mm x 3 mm defect.

2.3. Using a syringe, distribute the rhBMP-2 solution evenly over the collagen sponge so that it is absorbed.

3. Preparation of custom external fixation device

NOTE: See **Figure 1A** for the more complete listing of dimensions.

3.1. Cut aluminum sheet stock (type 6061, 0.088" thickness) to two pieces (1.4" x 6") using a jigsaw or other appropriate tool.

3.2. Mount one piece in the milling machine and, using a 1/8" 90°-point carbide drill mill, cut four 'V' grooves (0.035" deep) lengthwise. Leave the other piece free of cuts.

3.3. Cut individual plates of 0.3" width from the two pieces (**Figure 1B**). Measure and drill screw holes for 4-40 thread. Tap plate with 'V' grooves with the 4-40 thread. Drill the plate without grooves for a #4 screw body drill.

3.4. Sand both pieces to round corners and reduce weight (**Figure 1C**).

3.5. Screw pieces together with 4-40, 18-8 stainless steel button head cap screws (0.25") so that grooves are flush against the plain plate (**Figure 1D**)

4. Anesthetic procedure and analgesia

133
134 4.1. Induce anesthesia by placing the rat in induction chamber delivering 4 L O₂/min with 4%
135 isoflurane.

136
137 CAUTION: Research personnel must avoid inhalation of anesthetic gas and maintain proper hood
138 and ventilation in the laboratory.

139
140 4.2. Remove the rat from the chamber after the rat loses righting reflex, attach a nose cone and
141 place at the maintenance dose of anesthesia through the nose (O₂ delivery rate to 2-3 L/min and
142 0.8% isoflurane).

143
144 4.3. Place the rat on the heating pad or under the warming light to prevent hypothermia.

145
146 4.4. Confirm the adequate depth of anesthesia by pinching the toe or testing the palpebral reflex.

147
148 4.5. Apply lubrication to eyes to prevent drying out of the cornea.

149
150 4.6. Deliver a subcutaneous injection of extended-release buprenorphine (1 mg/kg) on the
151 trunk/dorsum of the rat, far from the surgical site, to provide analgesia for up to 3 days following
152 surgery.

153 154 **5. Aseptic preparation and antibiotic preventatives**

155
156 5.1. Shave area around hindleg using the 13th rib, the foot, the dorsal midline, and the ventral
157 midline as margins.

158
159 5.2. Scrub shaved area using sterile 2 x 2 gauze soaked with 10% povidone-iodine followed by
160 70% EtOH (4 times each, alternating).

161
162 5.3. Administer an intramuscular injection of cefazolin (20 mg/kg) into the operative quadriceps.

163
164 5.4. Administer enrofloxacin (0.25 mg/ml) in drinking water for 7 days postoperatively for
165 continued antibiotic protection.

166
167 5.5. Place rats on medicated feed (e.g., Uniprim) for the duration of the study to prevent pin tract
168 infections.

169
170 5.6. Apply double antibiotic ointment to the skin-pin interface once daily for 3 days
171 postoperatively.

172
173 NOTE: Avoid any external fixation pin or clamp loosening which can contribute to the
174 development of an infection.

175 176 **6. Surgical procedure**

NOTE: Make a concerted effort to maintain a sterile field and workspace and follow sterile technique throughout the entirety of the case.

6.1. Extend shaved leg through fenestrated, clear sticky drape and cover surgical bench in sterile towels to create a sterile field.

6.2. Palpate the femur and use a #15 blade to create an anterolateral incision through the skin extending from the patella to the greater trochanter at the proximal femur.

6.3. Carefully incise the lateral leg fascia along the intermuscular septum to separate the vastus lateralis muscle of the quadriceps anteriorly from the hamstrings posteriorly until the lateral femur is exposed. Preserve the abductor gluteal tendon insertion on the greater trochanter.

6.4. Perform a careful, atraumatic circumferential soft tissue dissection and expose the femur at its mid-diaphysis starting on the lateral surface. To do this, use a #15 blade to gently cut the muscle away from the underlying bone by keeping the blade parallel against the contour of the bone surface. Use a periosteal elevator to lift the muscle away from the exposed bone as it is dissected and proceed around the femoral shaft until 7-10 mm of central diaphysis has been cleared of soft tissue on all sides to prepare for osteotomy.

NOTE: Avoid injury to the medial femoral neurovascular bundle.

6.5. Insert four 1.0 mm Kirschner (k) wires: 2 proximal and 2 distal in the femur perpendicular to the lateral femur, directed straight lateral to medial. Ensure all pins engage both cortices (bicortical) for adequate stability (**Figure 2A**).

6.6. Start with the distal-most pin first, just at the level of the lateral epicondyle. Place jig flush to the lateral distal femur and insert a 1.0 threaded tip k-wire.

6.7. Maintaining the position of the jig on the bone, identify where the most proximal pin will enter the bone based on the jig holes. Once the position is determined, carefully incise parallel to the fibers of the gluteal tendon as needed to create a small gap in the tissue for the proximal pin to pass through, thus minimizing iatrogenic damage to the tendon. Drill a 1.0 mm non-threaded k-wire in this gap, again ensuring the pin engages both cortices (**Figure 2B**).

6.8. Maintain the jig's position in contact with the bone and drill two 1.0 mm threaded k-wires, one on either side of the future defect site. Ensure pins engage both cortices (**Figure 2C**).

6.9. Place the external fixator bar level 1 cm above skin and screw tightly, locking the bar in place. Clip the excess pin lengths (**Figure 2D**).

6.10. Prepare for the osteotomy (defect creation) by placing a small, curved retractor around the anterior and posterior femur to protect the surrounding soft tissue, muscle, and neurovascular

bundle. Utilizing a ~5 mm sagittal oscillating saw blade, very cautiously create a 5 mm segmental defect through the mid-diaphysis. Apply a light, even pressure with the saw to avoid unnecessary fracture (**Figure 2E**).

6.11. Apply small amounts of irrigation (room temperature 0.9% sterile normal saline (NS)) as needed while creating defect to avoid thermal necrosis of the bone.

6.12. Flush the wound using 10 mL of NS after creating the defect.

6.13. Administer 0.1 mL of a 0.25% bupivacaine with epinephrine (1:200,000) to the wound as an analgesic and vasoconstrictor.

6.14. Insert the scaffold (5 mm x 3 mm x 3 mm) of collagen sponge or rhBMP-2 soaked sponge (from **Step 2**) into the defect. Each scaffold should be sized appropriately to span the length and volume of the defect, helping the sponge stay in position.

NOTE: At this point, mRNA complexes may be prepared and injected as outlined in Steps 7.1-7.3 below if performing bioluminescence imaging.

6.15. Close the muscle plane using the simple interrupted pattern with 4-0 absorbable suture. Close the skin layer using a running subcuticular pattern with 4-0 absorbable suture and skin glue to close gaps around the protruding pins.

6.16. Remove the rat from the nose cone, remaining on the heating pad, and monitor continuously until the rat is able to consistently maintain an upright posture. At this point, place in a clean cage to recover.

7. Preparation of complexed mRNA and bioluminescence imaging

NOTE: Transfection with mRNA complexes should be performed during surgery 1 day before luminescence imaging. Use sterile techniques when handling mRNA.

7.1. Mix 10 μ L of mRNA encoding for Gaussia luciferase (stock concentration of 1 μ g/ μ L) with 30 μ L of the lipidic transfecting agent.

7.2. Allow for the mRNA-lipid complexes to form by incubating for at least 5 min at room temperature. The lipidic transfecting agent will condense the mRNA molecules, stabilizing them and enhancing transfection efficiency.

Note: If the complexes are not used immediately, store them in ice for a maximum of 1 h.

7.3. Using a 20 μ L pipette equipped with filtered tips, inject half of the volume of mRNA complexes to the distal and proximal ends of the defect, respectively.

7.4. The following day, 3 min before imaging, anesthetize the rat using inhaled isoflurane as previously described in Step 4.1.

7.5. Position the rat in an *in vivo* imaging chamber equipped with a nose cone delivering maintenance isoflurane (0.8% isoflurane, O₂ delivery rate of 2-3 L/min).

7.6. Inject coelenterazine resuspended in saline at a dose of 4 mg/kg body weight in the proximity of the defect.

7.7. Acquire bioluminescence images with the *in vivo* imaging system (IVIS) according to the manufacturer's instructions¹⁸.

8. Imaging Protocol

8.1. After calibrating the plain radiographic machine, an X-ray system¹⁹, anesthetize rat using inhaled isoflurane as previously described (see **Step 4.1**) and position the rat in a nose cone with inhaled isoflurane (0.8% isoflurane, O₂ delivery rate of 2-3 L/min) for an anteroposterior (AP) femur radiograph.

8.1.1. While the rat is in sternal recumbency, advance the surgical hindlimb forward, flexing at the hip and stifle joint. Flex the stifle joint to approximately 90°. Tape the paw plantar side down, close to the body wall. Position the tibia forward from the femur to eliminate the possibility of superimposing the bones. To provide slight abduction of the hip, place a translucent sponge (approximately 15 mm thick) in the groin region. Then obtain an anterior-posterior (cranial-caudal) image of the femur.

8.2. Repeat this AP femur radiographic view immediately following surgery, 4 weeks, and 12 weeks. Use tape and gauze to appropriately position the animal's extremity for quality and consistent imaging.

8.3. Remove the rat from the nose cone and monitor continuously until the rat is able to consistently maintain an upright posture. Then, place back into the cage.

9. Histological Procedure

9.1. Euthanize rats in a chamber with inhaled CO₂ according to AVMA ethical standards¹⁶.

9.2. Following euthanasia, shave the hindlimb, remove the skin from the operative extremity and disarticulate femur at the hip. Carefully remove all soft tissue from the operative femur (including all muscles, tendons, and ligaments). Leave only a thin layer of muscle surrounding the defect site to protect the healing region from inadvertent damage during dissection.

9.3. Place the femur in 10% Neutral Buffered Formalin at room temperature for 3-4 days to allow for fixation. Keep a 15:1 formalin to tissue volume ratio. Change the solution once halfway through the fixation process.

9.4. Decalcify the femur in a 15% Ethylenediaminetetraacetic acid (EDTA) pH 6.5 solution for 3-4 weeks. Collect serial radiographs to determine decalcification endpoint.

9.5. Bisect the femur longitudinally with a cut from anterior to posterior in the mid-sagittal plane. Submit tissue for standard paraffin embedding and hematoxylin and eosin (H&E) staining.

9.6. Send H&E slides to a pathologist for histological assessment.

REPRESENTATIVE RESULTS:

Surgeries were performed in approximately one hour by one surgeon with the help of one assistant. After surgical optimization, intra- and postoperative complications were greatly minimized and use of the jig apparatus ensured consistent size (5 x 3 x 3 mm) and localization of femoral defects. Rats were ambulatory immediately following recovery from anesthesia and did not appear to have any altered behavioral patterns; their gait was not antalgic, and they did not appear to be disturbed by the external fixator.

Non-threaded k-wires were chosen for the most proximal pin (**Figure 2B**), as the proximal pin had the highest risk of breaking when threaded wires were used. In some instances, particularly in control animals without rhBMP-2 or scaffolds whose defects showed no evidence of healing/bone formation, one or more k-wire tips broke after about 8 weeks as seen in the sponge only control radiograph of the excised femur (**Figure 3**).

Radiographs and histology (H&E stain) were analyzed to assess levels of bone healing. Negative control defects containing only a collagen sponge showed no evidence of bridging osteogenesis between the proximal and distal bone edges (**Figure 3, Figure 4**). A small amount of new bone remodeling can be seen directly adjacent to the cut femur edge; the defect itself shows a lack of bony material, the presence of cartilage, and some residual hematoma (**Figure 4**). Defects containing rhBMP-2 soaked sponge demonstrated significant bone healing as early as 4 weeks after surgery, as shown by the radiopaque callous bridging across the defect in **Figure 3**. By 12 weeks, significant new mineral deposition (**Figure 4**, NB: new bone, PC: periosteal callous) has formed throughout the defect. Significant new periosteal bone can be seen in the callous extending from the cut femur edge, and spicules of woven and lamellar bone have developed throughout the defect. Cartilage deposition is not seen (**Figure 4**).

Histology (H&E stain) was also performed for both an uninfected control and an example of an infected femur (**Figure 5**). The infected femur is significantly enlarged, showing signs of an endosteal reaction infiltrating the bone cortex. Arrows indicate areas of osteoclast-mediated pathologic bone resorption. The uninfected femur cortex remains compact and with a clearly delineated lamellar cortex. Antibiotic dosing was optimized to include maximal coverage postoperatively. While infection around the defect site can occur, continued administration of

antibiotics topically around pin sites and in water and diet proved successful in minimizing postoperative infection.

Further imaging using *In Vivo* Imaging System (IVIS) illustrates the ability of bioluminescent cells to be visualized within the defect after implantation of the external fixator (**Figure 6**). The external plate can be easily removed for imaging and replaced upon completion. Cells in the medullary cavity luminesce after transfection with complexed mRNA encoding for *Gaussia luciferase*. The highest level of luminescence is focused at the site of the femoral defect and the signal is not obstructed by the fixation device pins. This is promising for future studies relying on bioluminescence or fluorescence to measure biological changes such as a gene or protein expression during the healing process.

FIGURE LEGENDS:

Figure 1: External fixator fabrication. **A:** CAD schematic of the assembled external fixator with annotated dimensions for proper fabrication. Each fixator is composed of two aluminum plates held together by two screws. **B:** Plates are cut from 1.4" x 6" aluminum sheets with 'V' grooves cut into the bottom sheet. **C:** Screw holes are drilled into the plates (threaded in the plate with 'V' grooves) and all edges and corners are sanded to round and reduce weight. **D:** Assembled external fixator is tightened with screws (4-40 x 0.25", 18-8 stainless steel button head cap) once pins are in place in the 'V' grooves on the insides of the aluminum plates. The left pin is non-threaded and is most proximal on the femur.

Figure 2: Schematic of pin placement, fixator placement, and defect creation. **A:** The distal pin (1.0 mm threaded k-wire) is placed at the epicondyle metaphyseal region using the jig (blue rectangle) to guide proper pin insertion. The jig is placed onto the anterolateral femoral surface. **B:** The proximal pin (1.0 mm non-threaded k-wire) is placed using the jig after making a small incision in the gluteal tendon. **C:** The middle pins (1.0 mm threaded k-wire) are inserted using the jig. **D:** The jig is removed and the 2 plates are attached to the pins using the 2 screws to secure the plates. The plates are tightened 1 cm above skin level to avoid pressure on the skin. **E:** A sagittal oscillating saw is used to create a 5 mm defect between the two middle pins.

Figure 3: Representative high-resolution radiographs show bone healing with rhBMP-2 treatment. Images for the negative control collagen sponge and the rhBMP-2 soaked sponge groups are shown at 0, 4, and 12 weeks postoperatively. The rhBMP-2 treatment group exhibits significant healing after 4 weeks with callous spanning the defect. The negative control femur ends do not heal with bridging bone and the defect remains a non-union.

Figure 4: Significant new bone formation is seen with rhBMP-2 treatment. Representative 4x magnified H&E histological images for the negative control collagen sponge and the rhBMP-2 soaked sponge groups both at the cut femur edge and within the defect. New bone formed around the control femur edge, but significant extensions of both new trabecular bone as well as the periosteal callous project from the treated femur. No bony material is seen within the control defect, while significant bone formation can be observed throughout the rhBMP-2 treated

defect. NB: new bone, F: femur, C: cartilage, H: hemorrhage, PC: periosteal callous. Scale bar: 200 μ m.

Figure 5: Infected femur exhibits hypertrophy and inflammatory cell markers. H&E histological images of an uninfected femur compared to an infected femur, in full view and at 4x magnification of boxed locations. The uninfected femoral cortex remains organized and delineated, with little sign of inflammation. The infected femur enlarges greatly, as seen in full view, and the cortex is broken up by areas of resorption and necrosis (purple cell clusters indicated by black arrows). F: femur. Scale bar: 200 μ m.

Figure 6: Gaussia luciferase signal detected in the defect. Luminescence of cells transfected with Gaussia luciferase mRNA is imaged with IVIS after external plate removal. Red indicates the highest luminescence intensity at the site of the femoral defect.

DISCUSSION:

Small animal models of orthopedic injuries such as complete bone fractures enable research that explores the mechanisms of osteogenesis and assessing the therapeutic potential of biomaterials²⁰. This study introduces a rat segmental defect model stabilized by a custom external fixator that a lab and biomedical engineering team can readily reproduce for further studies of load-bearing osteosynthetic bone repair.

Previous studies using critical-sized defects in rat models commonly rely on internal fixation plates^{21–24}. Although either fixation method is clinically acceptable, external fixation has the distinct advantages of causing less soft tissue disruption and blood loss, decreasing the total implanted surface area to minimize opportunities for bacterial colonization, and allowing for postoperative adjustability and staged surgical interventions⁶. Previous external fixation animal fracture models have used different plate materials, pin securement, and/or bone cutting methods^{12–14}. Because the external fixator in this protocol was made in the lab's workshop with low-cost aluminum and can be easily refurbished, expenses were minimized. This provides an economical external fixator allows researchers to perform experiments with multiple animal groups without being limited financially. As compared to an internal fixation model, we believe that this system is technically simpler and more reproducible in a small animal model. In our experience with these models, internal fixation is significantly more demanding technically and can require custom-machined implants. To the authors' knowledge, this overall protocol is unique in its use of a custom-designed jig combined with an adapted metal external fixator design¹⁵, as well as its use of an oscillating bone saw to better represent the common clinical scenario of periosteal stripping performed to prepare fracture sites for fixation. A final note on the use of an oscillating saw in this model is that the heat generated and the periosteal disruption that occurs provides a final element of control in forming a non-union model. Our experience has been that with other methods, in these animal models, investigators run the risk of the controls healing.

To ensure surgical success, care should be taken at several critical steps: when incising and performing the circumferential dissection to expose the femur from surrounding muscle, avoid

disturbing the sciatic nerve caudally, the femoral vessels medially, and the gluteal tendon proximally. Take care to position the jig apparatus parallel and flush against the flat, anterolateral face of the femur so that all pins are precisely perpendicular to the bone. This will confirm the proper alignment of the external fixator and reduce the likelihood of pin breakage. The order of pin placement found to be most straightforward was distal first, followed by proximal and then both middle pins. This allowed for less disruption of the gluteal tendon by the proximal pin. Finally, it is important that each pin is placed bicortically, penetrating both cortices so that it does not back out of the bone or shift into the medullary cavity.

An x-ray machine capable of high-resolution radiography was used to monitor bone-healing status as qualitative changes can be visualized easily and non-invasively over time. However, consistent leg positioning is crucial for accurate radiographic interpretation as differences in positioning may be misleading. Results from this study are in agreement with previous work demonstrating that a 5 mm femoral defect prevents spontaneous bone healing in normal rats²⁵. Therefore, any healing that is noted with added therapies such as the rhBMP-2 soaked sponge can be definitively attributed to the respective treatment (**Figure 3**).

Possible concerns for this technique include pin breakage, loosening, and infection. Problems with proximal pin breakage in preliminary testing prompted a switch from threaded to non-threaded k-wire. Non-threaded pins are mechanically stronger but also pose more risk of backing out of the cortex. Particularly in empty bone defects, k-wires may break or be displaced around 8 weeks due to the prolonged cyclic pin loading and lack of healing (similarly to cycling/bending a paperclip). A strict antibiotic regimen was utilized to include an immediate cefazolin injection, daily application of antibiotic ointment at the incision site, 7 days of enrofloxacin added to drinking water, and a medicated feed. This antibiotic protocol, along with the proper surgical techniques outlined above, minimized infection (**Figure 5**).

One of the additional advantages of using external fixation in an animal model is easy removal for an unobstructed view of the defect and replacement after imaging. This enables more effective *in vivo* imaging techniques relying on fluorescence or luminescence to assess changes such as the gene or protein expression. For instance, we have shown that cells in the medullary cavity transfected with complexed mRNA encoding for Gaussia luciferase could be visualized with IVIS. **Figure 6** illustrates that luminescence signal detection capability is not obstructed by this external fixation approach as it may be with internal plates, screws, or intramedullary nails^{21–24}. This cost effective and reproducible surgical protocol allows for consistent creation and stabilization of a critical-sized femoral defect that mimics the initial clinical management of these complex fractures. The establishment of a reliable animal model is critical for any experimental treatments intended for eventual clinical use. Our model has demonstrated predictable results and minimal behavioral changes or discomfort in our animals. This model may be used with a variety of biomaterial-based scaffolds in conjunction with the imaging techniques utilized in this paper for future translational testing purposes. It is our hope that in working with this model, researchers will be able to devise novel ways to treat critical bony defects in trauma patients. This could help avoid the morbidity and cost in lengthy treatments currently employed and possibly decrease the number of amputations.

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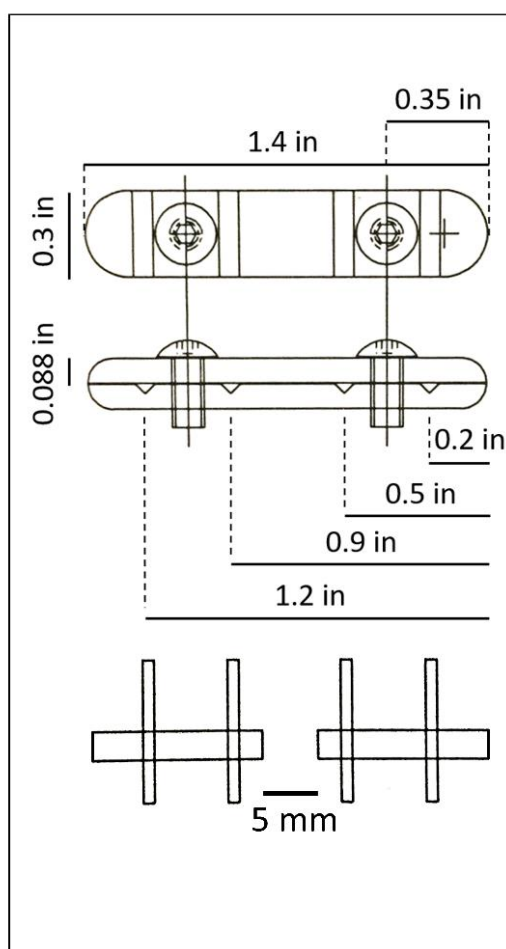
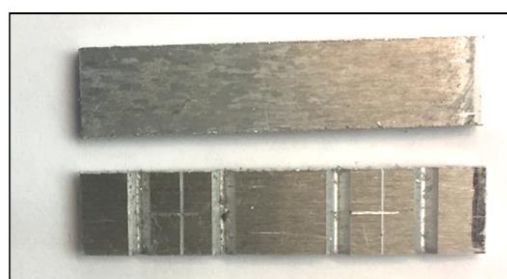
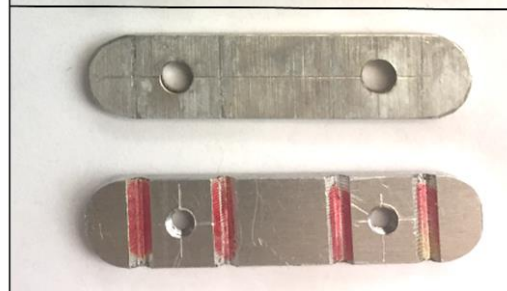
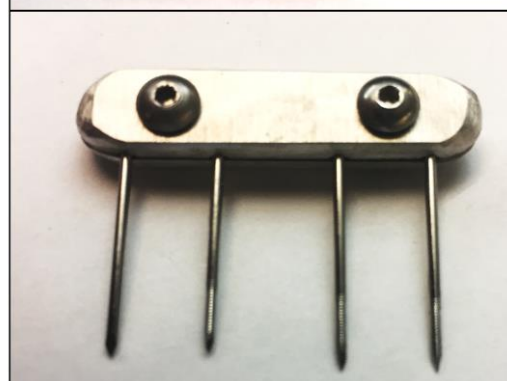
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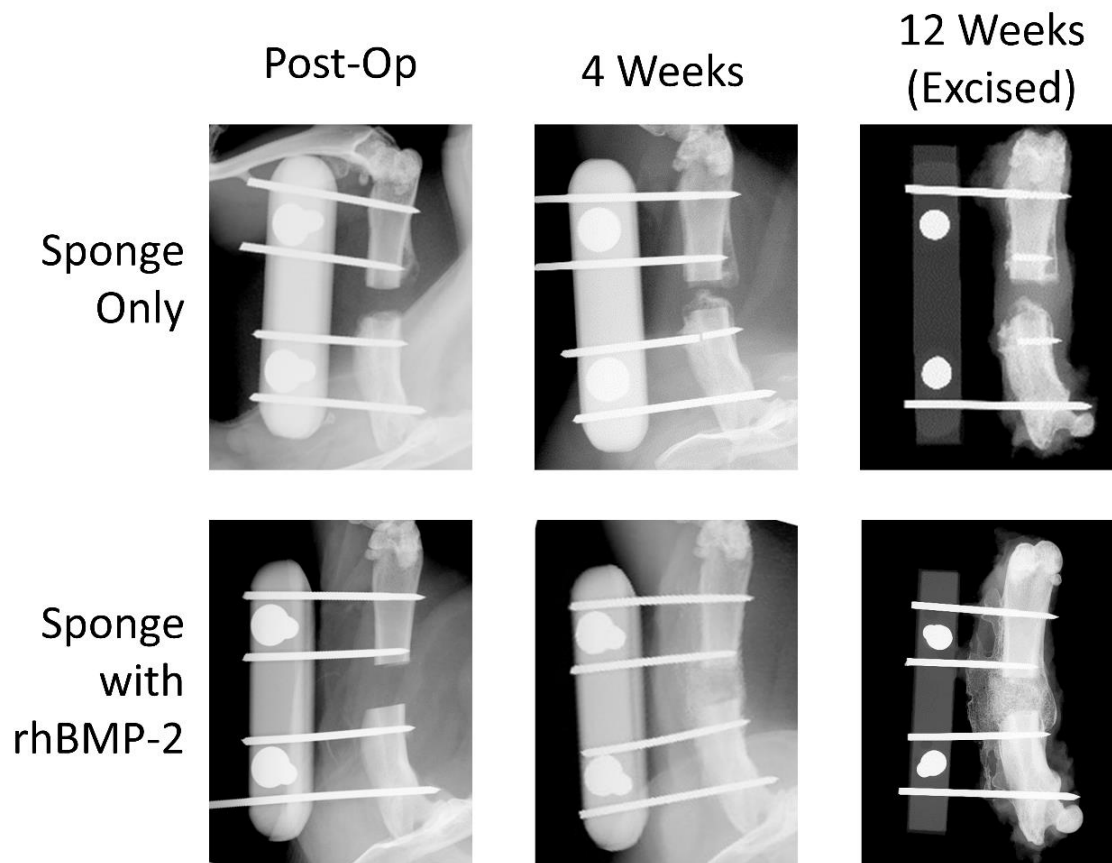
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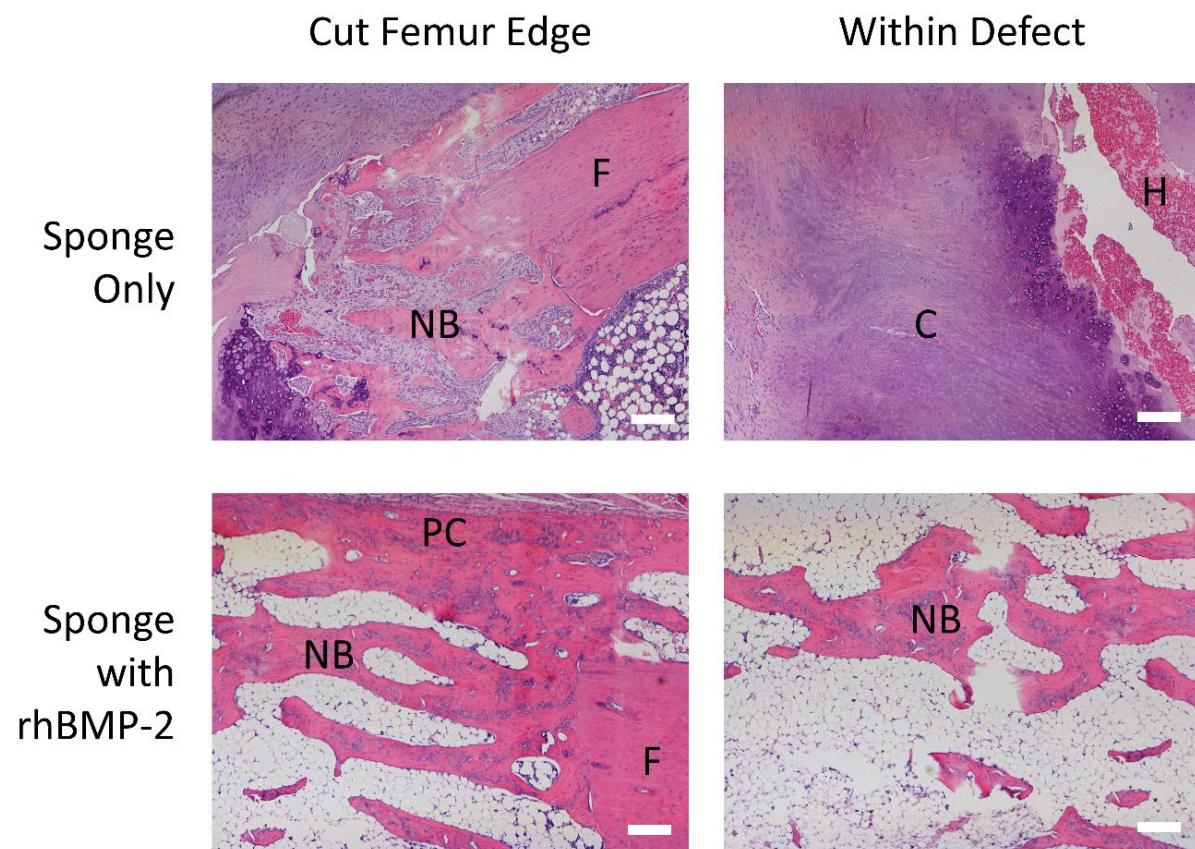
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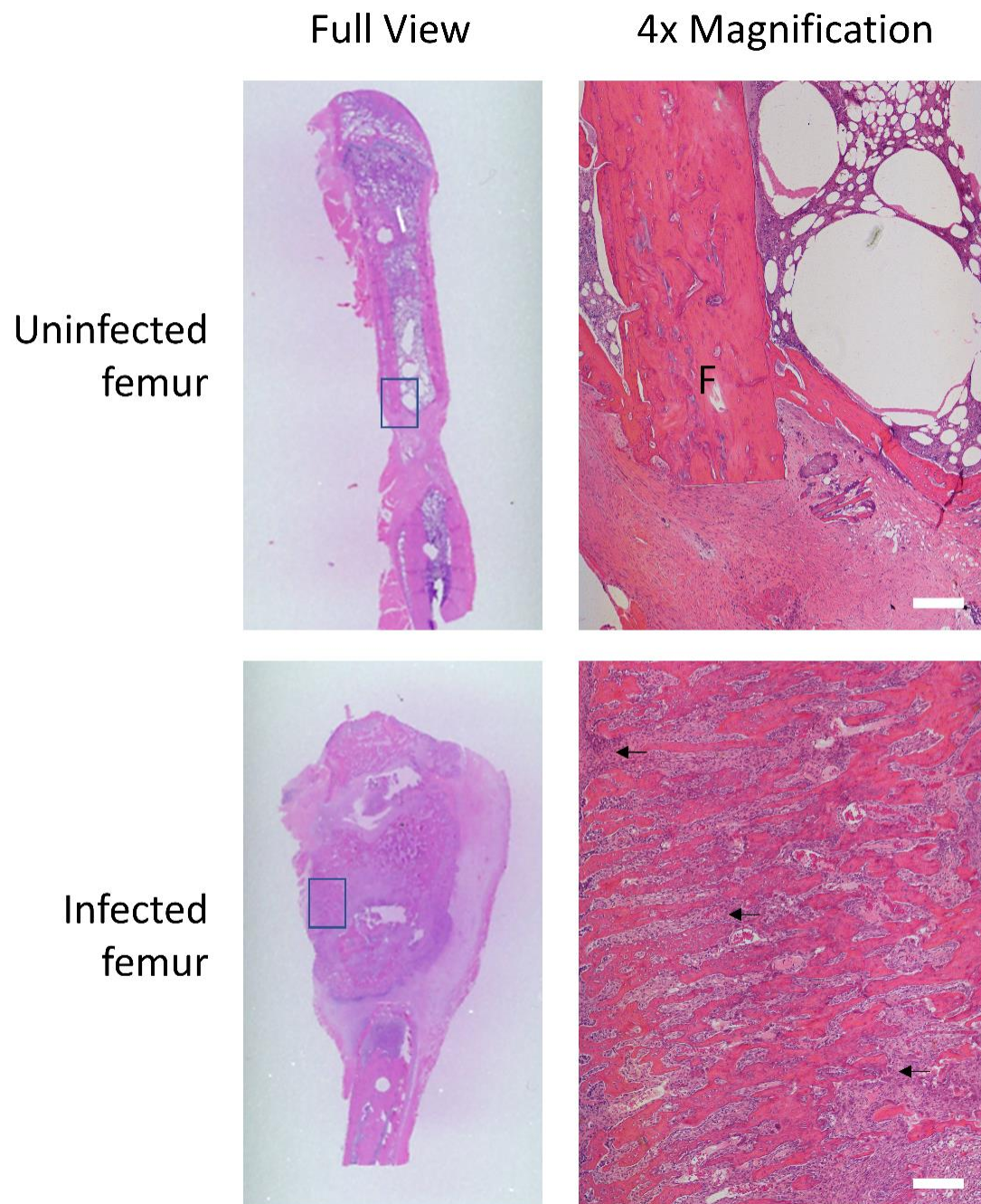
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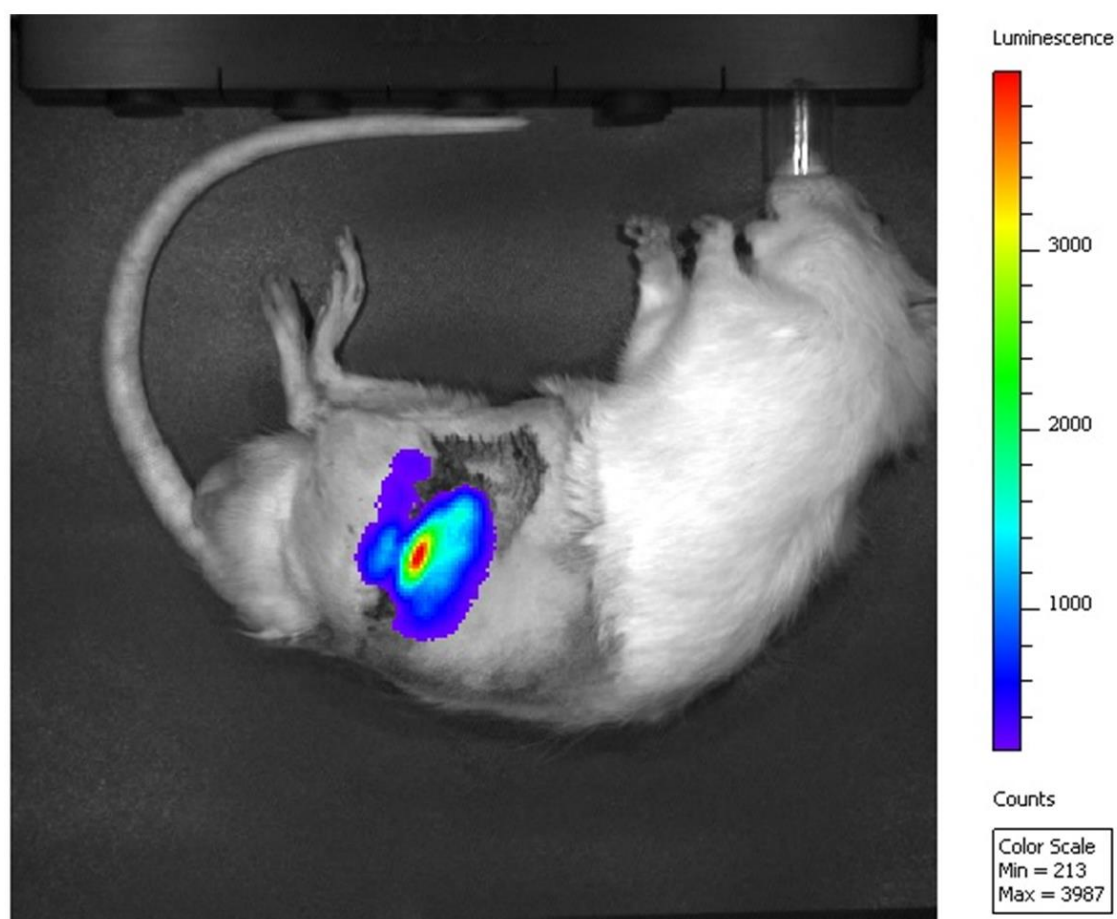
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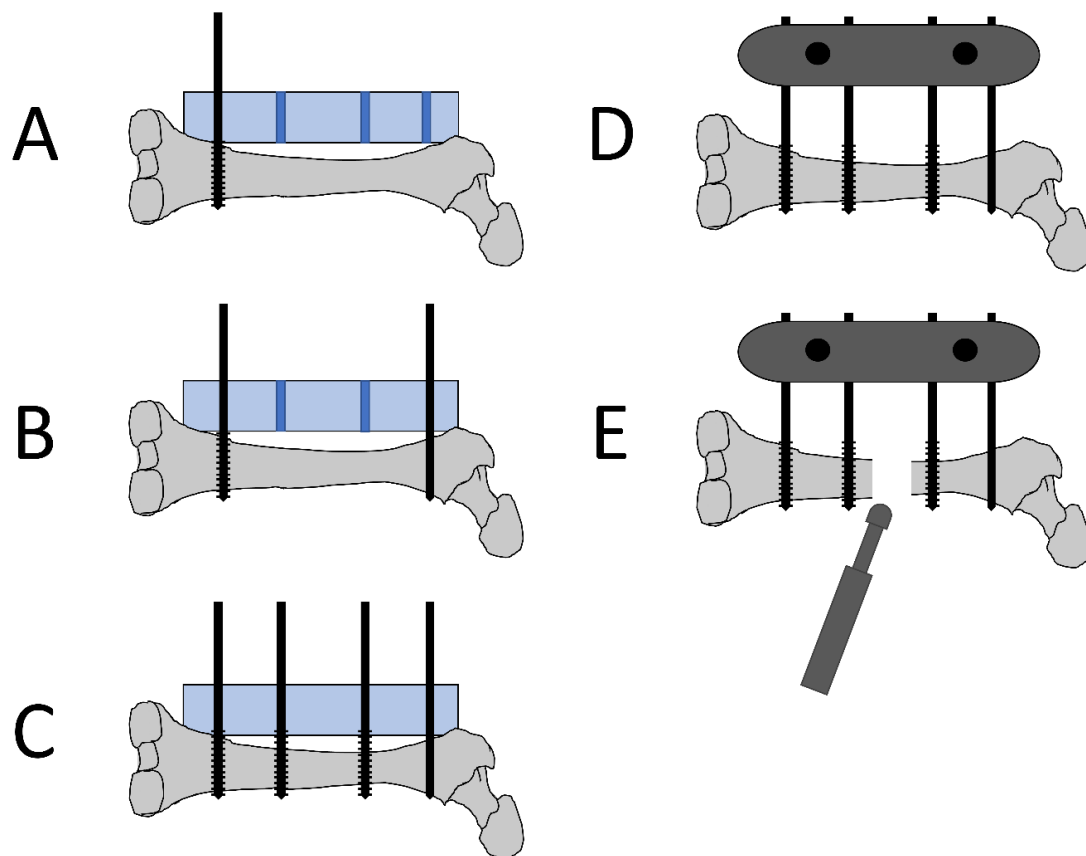
A**B****C****D**











Name of Reagent/ Equipment	Company
0.9% Sterile Saline	Baxter
10% Iodine/Povidone	Carefusion
10% Neutral Buffered Formalin	VWR
1mm non-threaded kirschner wire	DePuy Synthes
1mm threaded kirschner wire	DePuy Synthes
2x2 gauze	Covidien
4-0 Vicryl Suture	Ethicon
4-40 x 0.25", 18-8 stainless steel button head cap screws	Generic
4200 Cordless Driver	Stryker
4x4 gauze	Covidien
70 % Ethanol	
Baytril	Bayer Healthcare LLC, Animal health division
Cefazolin	Hikma Pharmaceuticals
CleanCap Gaussia Luciferase mRNA (5moU)	TriLink Biotechnologies
Coelenterazine native	NanoLight Technology
Double antibiotic ointment	Johnson & Johnson consumer Inc
Dual Cut Microblade	Stryker
Ethylenediamine Tetraacetic Acid (EDTA)	Fisher
Extended Release Buprenorphine	ZooPharm
Fenestrated drapes	3M
Handpiece cord for TPS	Stryker
Heating pad	K&H Pet Products
Hexagonal head screwdriver	Wiha
Induction chamber	Generic
Infuse collagen sponge with recombinant human Bone Morphogenic Protein-2	Medtronic
Isoflurane	Clipper
IVIS	Perkin Elmer
Jig	Custom
Lipofectamine MessengerMAX	Fisher Scientific
Sensorcaine-MPF (Bupivacaine (0.25%) and Epinephrine (1:200,000))	APP Pharmaceuticals, LLC
Sterile water	Hospira
Titanium external fixator plates	Custom
Total Performance System (TPS) Console	Stryker

TPS MicroSaggital Saw	Stryker
Ultrafocus Faxitron with DXA	Faxitron
Uniprim rat diet	Envigo
Universal Handswitch for TPS	Stryker
Vetbond Tissue Adhesive	3M

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OR-S-5100-1

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TD.06596
OR-S-5100-9
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Comments/Description
Used for irrigating wound and rehydration
Used to prep skin
Used as fixative
Sterilized, used for the most proximal pin
Sterilized, used for the 3 most distal pin slots
Sterilized, used to prep skin and absorb blood
Used to close muscle and skin layers
External fixator assembly
Used to drill kirschner wires
Sterilized, used to absorb blood
Used to prep skin
Added to water as an antibiotic
Pre-op antibiotic
Modified mRNA encoding for Gaussia Luciferase, keep on ice during use
Substrate for Gaussia Luciferase, used to assess luciferase activity <i>in vivo</i>
Applied to pin sites post-op as wound care
Used to create 5mm defect in femur
Used to decalcify bone to prep for histology
Used as 3 day pain relief
Used to establish sterile field
Used to create 5mm defect in femur
Rat body temperature maintenance
External fixator tightening
Anesthesia for rats
Clinically relevant treatment used as positive control
Anesthesia for rats
Bioluminescence imaging modality
Used to place bicortical pins
mRNA complexing agent that enables mRNA delivery
Applied to surgical site for pain relief and vasoconstriction
Used as solvent for cefazolin powder
Prepared in house with scrap titanium and milling machine
Used to create 5mm defect in femur

Used to create 5mm defect in femur
High resolution radiographic imaging modality
Medicated rat diet
Used to create 5mm defect in femur
Skin closure



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Author(s): F. Kerzner, H. L. Martin, M. Weiser, G. Fontana, N. Russell, W. L. Murphy, E. A. Lund, C. J. Doro

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December 28, 2018

Dear Dr. Bajaj,

Thank you for the additional review of our manuscript. We have addressed your constructive comments and have made revisions according to your suggestions. Please find below each comment addressed individually, as well as our attached updated manuscript with tracked changes. We hope that you find our responses satisfactory, and we look forward to hearing from you.

Thank you,

Christopher J. Doro, MD

Department of Orthopedics and Rehabilitation

University of Wisconsin-Madison

Editorial comments:

- 1. The editor has formatted the manuscript to match the journal's style. Please retain the same.**

We have retained the formatting style.

- 2. Please address all specific comments in the manuscript.**

Please see Manuscript Comments below for our point-by-point responses.

- 3. Once done please ensure that the highlighted text is no more than 2.75 pages including headings and spacings.**

We have ensured that the highlighted text is less than 2.75 pages. It is slightly over 2 pages.

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All of the figures used in this manuscript are original and have not been used in previous publications.

Manuscript comments:

- 1. What kind of complexed mRNA? What is the significance with respect to the present study? (Line 244)**

The Gaussia luciferase mRNA is complexed with a lipidic transfecting agent (Lipofectamine MessengerMAX), which serves to stabilize the mRNA molecules and enhance transfection efficiency. We have added the following clarification to Step 7.2.

"Allow for the mRNA-lipid complexes to form by incubating for at least 5 min at room temperature. The lipidic transfecting agent will condense the mRNA molecules, stabilizing them and enhancing transfection efficiency."

Injection of these reporter mRNA complexes causes successfully transfected cells to express luciferase protein, which can then be visualized with bioluminescence imaging. Please see Comment 7 below for a more detailed explanation of the significance of these results.

2. How? (Line 276, regarding positioning for radiographs)

We have clarified Step 8.1 regarding the positioning of the animal during radiographic imaging. We have also removed “coronal” from Step 8.1. We have added the following substep below to describe AP imaging.

“8.1.1. While the rat is in sternal recumbency, advance the surgical hindlimb forward, flexing at the hip and stifle joint. Flex the stifle joint to approximately 90 degrees. Tape the paw plantar side down, close to body wall. Position the tibia forward from the femur to eliminate possibility of superimposing the bones. To provide slight abduction of the hip, place a translucent sponge (approximately 15 mm thick) in the groin region. Then obtain an anterior-posterior (cranial-caudal) image of the femur.”

3. Please include post anesthesia recovery steps as well. (Line 280)

We have added a step to address post-anesthesia recovery in the surgical section and imaging section to ensure proper monitoring.

“6.16. Remove the rat from the nose cone, remaining on the heating pad, and monitor continuously until the rat is able to consistently maintain an upright posture. At this point, place in a clean cage to recover.”

“8.3. Remove the rat from the nose cone and monitor continuously until the rat is able to consistently maintain an upright posture. Then, place back into cage.”

4. Need some result for this part. (Line 322)

We apologize for being unclear. The callous shown at the 4-week timepoint in Figure 3 is our result demonstrating the significant bone healing. We have combined the sentences below to avoid confusion.

“Defects containing rhBMP-2 soaked sponge demonstrated significant bone healing as early as 4 weeks after surgery, as shown by the radiopaque callous bridging across the defect in **Figure 3.**”

5. Please mark this in the figure as well. (Line 324, regarding mineral deposition)

The new bone growth and periosteal callous are labeled in Figure 4 as NB and PC, respectively.

We have added the following reference to the figure:

“By 12 weeks, significant new mineral deposition (**Figure 4**, NB: new bone, PC: periosteal callous) has formed throughout the defect.”

6. We cannot have data not shown. Please either remove this sentence or include data for the same. (Line 333)

We have removed the sentence.

7. Please bring out clarity on why this result is important. (Lines 339-340, regarding bioluminescence imaging)

For the purposes of this manuscript, bioluminescence imaging of tissues expressing the luciferase enzyme demonstrates just one potential experimental application where our surgical procedure may be advantageous. Due to the easy removal and replacement of the external fixation plate, longitudinal *in vivo* imaging of the site is possible. By demonstrating successful transfection of cells *in vivo*, we also show that the cells at the defect site are amenable to gene delivery. This is an important precedent for future studies that may use therapeutic genes to elicit a healing response in our defect model. Bioluminescence and fluorescence are also commonly used by many research teams as biomarkers, so our results of successful localized bioluminescence are promising for future studies using *in vivo* imaging to monitor biological changes.

We have added the following sentence to the end of the Results section to show clarity.

“This is promising for future studies relying on bioluminescence or fluorescence to measure biological changes such as gene or protein expression during the healing process.”