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Preparation of a femoral defect model in rats for bone healing studies

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Abstract
Any bone graft or tissue engineered constructs has to be tested in animal models before it is put to clinical studies. Though properties like toxicity or cell integration could be evaluated *in vitro*, assessment of osteointegration, osteoconduction etc has to be tested *in vivo* using animal models. A lot number of models have been suggested and is used for bone healing studies. The choice of model depends on the research question as well as on personal and institutional capabilities, experiences and preferences. This study is intended to provide researchers characteristics, advantages, and limitations of this model, which might be very useful to investigate bone graft substitutes in a standardized and well-defined fashion.

Keywords: Critical size defect, bone healing, rat model, biomaterials

Introduction
Healing of bones is limited or will never occur in scenarios like tumor resection, management of non-union or delayed union or comminution after a high energy trauma which result in large defect in the diaphysis (Sen and Miclau, 2007) [1]. In such cases, bone grafting is still considered as the gold standard. Limited availability, donor site morbidity, prolonged surgery time and therefore increased risk of infection are the main risks associated with allogenic bone grafts. To tackle these issues researchers are developing synthetic bone graft substitutes (Goulet *et al.*, 1997) [2]. Recently, osteoconductive bone substitutes have been combined with osteogenic cells and/or bioactive (osteoinductive) factors. Although acute toxicity, biological activity, cytocompatibility and fundamental biological mechanisms can be assessed *in vitro*, such systems cannot provide a reproducible approximation of the real life *in vivo* settings. Biocompatibility, degradation properties of implant materials, survival of transplanted cells, tissue response and mechanical function can only be investigated *in vivo*. For such investigations well-defined and standardized animal models are needed.

One experimental approach for the *in vivo* assessment of tissue engineered constructs is a "critical size defect" (CSD) models. In the ASTM Standard Guide for Preclinical *in vivo* evaluation in critical sized segmental bone defects (F2721-09) [3], CSD model is defined as "a defect that will not heal without intervention". It is also defined as a defect does not heal spontaneously within the lifetime of the animal or experiment (Gosain *et al.*, 2000) [4]. Rat is a well-established animal model for bone healing studies. Mills and Simpson (2012) [5] recommended that male rat osteotomy models could effectively be used for evaluating healing of segmental defects of long bones. Relatively low expenses, availability and hardy nature of rats made them the best model for preliminary evaluation of all bone grafts (Hollinger and Kleinschmidt, 1990) [6].

Even though a number of different fixation systems for CSD in rats have been published, reproducible mechanical fixation resulting in consistent loading conditions has been difficult to achieve with these systems. The goal of this study was to develop and validate *in vivo* a standardized CSD model in the rat suitable for screening new bone substitutes/ tissue engineered constructs in bones.

The study was conducted in accordance with the guide lines laid by the CPCSEA as per the principles of guide for care and management of experimental animals. The study was approved by the Institutional Animal Ethics Committee of College of Veterinary & Animal Sciences, Pookode of Kerala Veterinary and Animal Sciences University. (IAEC/COVAS/PKD/22/2/2024)

Materials and Methods

1. Selection of experimental animals

Thirty adult male wistar rats aged 16 weeks with an average body weight of 300g were selected for the study. They were housed and maintained under identical conditions with natural day and night light cycles and fed with standard mash for rats' *ad-libitum*. Clean drinking water was provided throughout the period of observation.

2. Preparation of the animal

The animals were selected randomly. Feed was withdrawn one hour prior to induction of anaesthesia and water was provided till they were anaesthetised.

Right lateral thigh of all the animals were prepared aseptically as for routine surgery. The thigh region was shaved off hair and cleaned with soap and water, the area was de-greased with 70% alcohol and disinfected using povidone Iodine solution.

3. Anaesthesia and control

The rats were premedicated with 0.05 mg/kg body weight of buprenorphine hydrochloride, administered subcutaneously 20 minutes before induction of anaesthesia. Induction of anaesthesia was done by intraperitoneal injections of xylazine hydrochloride and ketamine hydrochloride at a dose rate of 3 mg/kg and 30 mg/kg body weight respectively (IACUC Guidelines: Rodent Anesthesia and Analgesia Formulary, 2017) [7]. A mixture of 0.9 ml xylazine hydrochloride (100 mg/ml) and 18 ml ketamine hydrochloride (50 mg/ml) was diluted to 20 ml with distilled water and this stock solution was used at a rate of 0.07 ml per 100 g body weight. Anaesthesia was maintained with 1 to 2 percent isoflurane in 100 percent oxygen and the animals were placed in left lateral recumbency on heating pad for maintaining normal body temperature.

4. Approach to the femur and defect surgery

The shaft of the right femur was approached as per Costa *et al.* (2011) [8]. A linear skin incision was made on the lateral aspect of the thigh extending from the greater trochanter to the femoro-patellar joint. The junction between the superficial gluteus and the biceps femoris muscle was identified as a white line (Figure 1). The two muscles were separated by blunt dissection to identify the femoral diaphysis. The muscular attachments of the shaft were dissected all around. The shaft was isolated and lifted using Backhaus' forceps. A six-millimeter defect was measured and marked using a vernier calipers and the bone segment in between the markings were removed using a dental bur (Figure 2).

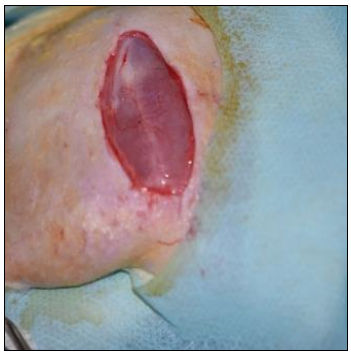

Fig 1: The junction between the superficial gluteus and the biceps femoris muscle was identified as a white line

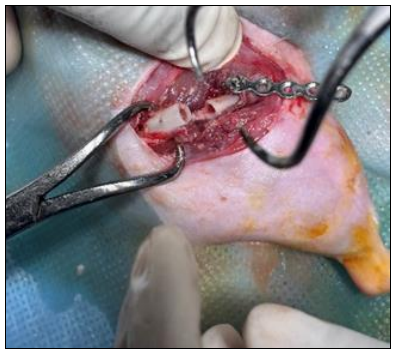

Fig 2: The markings were removed using a dental bur

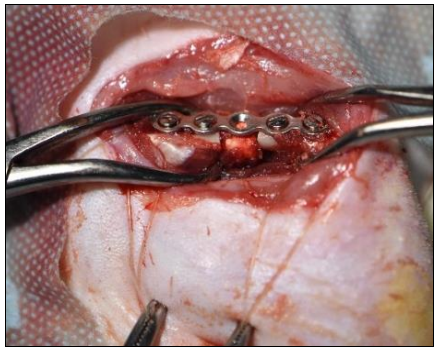

Fig 3: The plates were fixed on the anterior aspect of the femur

The defect was bridged with pre-sized biomaterial and held in position using 1.5 mm five-hole micro plates and 1.5 mm x 6 mm cortical screws. The plates were fixed on the anterior aspect of the femur (Figure 3). The dissected muscle bellies were apposed in simple interrupted pattern using mono-filamentous fine nylon. Skin incision was apposed in same pattern using the same material.

5. Post operative management

Postoperatively the animals were administered with ceftriaxone sodium at a dose rate of 40 mg/kg body weight intramuscularly twice a day for seven days. Analgesia was achieved by giving buprenorphine injection at a dose rate of 0.05 mg/kg subcutaneously once a day for three days and meloxicam injection at a dose rate of 2.0 mg/kg subcutaneously for five days postoperatively as per IACUC Guidelines. The skin wound was dressed with povidone iodine ointment twice daily. The skin sutures were removed on 10th postoperative day.

6. Assessment of healing

6.1 Radiography

Immediate postoperative radiographs were taken to assess the extent of reduction of the fracture and position of graft. Healing of the defect was assessed radiographically under light anaesthesia. The parameters used for radiography were 45 kV and 9.5mAS at 100 cm FFD.

6.2 Histologic evaluation

Three rats each were sacrificed at regular intervals during weeks 2, 4, 8 and 12th postoperatively to evaluate healing through histological examination. The implantation site, along with normal bone was harvested and fixed in 10% neutral buffered formalin. Bone pieces were decalcified, sectioned and stained using routine haematoxylin and eosin stain.

Representative sections from each sample were subjected to Masson's Trichrome staining to demonstrate new bone formation.

Results and Discussion

A lot number of models have been suggested and is used for bone healing studies. The choice of model depends on the research question as well as on personal and institutional capabilities, experiences, and preferences. Other than these, ethics and economics are also to be considered. This study is intended to provide researchers characteristics, advantages, and limitations of this model, which might be very useful to investigate bone graft substitutes in a standardized and well-defined fashion.

In this model approach to the bone, implant and graft placement is easy and reliable. No implant associated complications were experienced in these animals, indicating that the fixation system is appropriate and safe. In this study, a 6 mm defect in the rat femur was of critical size, since it did not heal without intervention. Even though 6 mm is the typical size for a diaphyseal CSD in the rat (ASTM Standard F2721) [3], it has to be emphasized that the size of a CSD depends on the rat strain, weight, age, sex, metabolism status, and the fixation system used. If one of these factors is changed, the size of the defect might need to be changed to remain of critical size.

A disadvantage with this model is the use of stainless-steel plates which are radio-opaque. This will hinder radiographic evaluation of the fracture healing during the observation period. Radiopaque materials such as stainless steel or titanium do not allow for a proper visualization of the defect. This can be countered by using plates or other fixtures made of radiolucent materials like Polyether ether ketone (PEEK). (Poser *et al.* 2014) [9].

Conclusion

The study developed a reliable rat critical size defect (CSD) model for evaluating bone graft substitutes. A six-millimetre femoral defect was confirmed as critical, requiring intervention for healing. The fixation system was effective with no complications, though stainless-steel plates hindered radiographic monitoring, suggesting radiolucent materials like PEEK as an alternative. In short, this standardized model provides a valuable tool for preclinical bone healing research.

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