

## A CRITICAL SIZE DEFECT MODEL IN THE RADIUS OF RABBITS

### MODELO DE FALHA ÓSSEA SEGMENTAR EM RÁDIO DE COELHOS

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#### SUMMARY

This study aimed to characterize a preclinical model of critical size defect (CSD) in the radius of rabbits. Twenty adults (> 7 months), female, New Zealand Rabbits, weighing between 3,5 to 5 Kg were used. They underwent a 1.5 cm long osteotomy of the diaphysis of the radius, starting 2 cm from the carpus joint. Radiographic analyses were performed at 15, 30, 60, and 90 days postoperatively, in order to evaluate bone callus formation, periosteal reaction, and bone bridge formation. The methodology allowed to precisely create bone defects of 1.5cm. Over time, no bone deposition was found at 15 days, but mild bone callus formation was observed in three animals after 60 days postoperative. At 90 days postoperatively only one rabbit presented bone consolidation radiographically, the others presented non-union. The critical bone defect proposed in this study was satisfactory, feasible with very low risk of complications.

**KEY-WORDS:** Bone regeneration. Critical bone defect. Radius defect. Segmental defect.

#### RESUMO

O objetivo do presente estudo é caracterizar um modelo pré-clínico de falha óssea crítica em rádio de coelhos. Vinte coelhos adultos (> 7 meses), fêmeas, da raça New Zealand, pesando entre 3,5 e 5 kg foram utilizados. Os animais foram submetidos à osteotomia segmentar de 1,5 cm na diáfise do rádio, iniciando 2 cm acima da articulação carpo radial. As análises radiográficas foram feitas aos 15, 30, 60 e 90 dias pós-operatório, para avaliação de formação de calo ósseo, reação periosteal e formação de ponte óssea. Na metodologia permitiu a criação precisa de defeitos ósseo segmentar de 1,5 cm. Durante o experimento, não se observou deposição óssea na primeira avaliação radiográfica, porém após 60 dias foi possível visibilizar formação de calo ósseo em 3 animais. Aos 90 dias apenas um animal apresentou consolidação óssea radiograficamente; o restante apresentou não-união óssea. A falha óssea foi eficaz para estabelecer um defeito ósseo crítico além de apresentar fácil aplicação, apresentando pequeno índice de complicação.

**PALAVRAS-CHAVE:** Reparação óssea. Defeito ósseo crítico. Defeito em rádio. Defeito segmentar.

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## INTRODUCTION

Tissue engineering has been deeply involved into the purpose of improving the repair of bone defects, especially those ones created from high energy trauma, neoplasm removal or avascular non-union (NAUTH et al., 2011). Most of non-critical bone defects can heal spontaneously in a healthy patient, even without mechanical stabilization. However, in large tissue loss, microenvironment lacking of osteogenic cells, low metabolism and poor vascularization, non-union is likely to occur, characterizing the critical bone defect (CSD) (CLEMENTS; CARPENTER; POURCIAU, 2008). Many authors describe a critical sized bone defect as one that will not consolidate without clinical intervention (SANDERS et al., 2014). Clinically, multiple factors can affect bone defects, such as age, weight, circumferential losses, anatomical location, soft tissue environment, and other systemic comorbidities (SCHEMITSCH, 2017).

Animals have been historically used for testing orthopaedic technologies and they are indispensable for the improvement of researches in this field. Selecting the ideal animal model that will mimic the target specie's bone biology is critically important (MUSCHLER et al., 2010). Many different species are potentially suitable and have been used for studying bone repair (LI et al., 2015). However, the *Oryctolagus cuniculus* (rabbit) presents similar microstructure, bone density and resistance of the diaphysis compared to the human bone. Currently, the rabbit has been widely used for this purpose and they are considered the most potential bone model after primates and dogs. Additionally, they are easy to acquire and manage (LI et al., 2015; WANG; MABREY; AGRAWAL, 1998).

Although, different bones and sites have been described, the radius has been considered one of the best,

once it is possible to use the critical size without additional fixation (LI et al., 2015). Several studies have shown that using a radial osteotomy without cutting the ulna allows early and full limb function in rabbits, which is remarkably desirable (GEIGER et al., 2007; NIEMEYER et al., 2010; ZHAO et al., 2016). The bone region may vary between diaphyseal or metaphyseal, being the first one most commonly used to perform critical size defects in rabbits (CSD) (MA et al., 2018; WANG et al., 2019; XIE et al., 2017; ZHANG et al., 2019).

Standardizing a CSD is essential in tissue engineering, especially when investigating biomaterials and bone healing (MUSCHLER et al., 2010). Establishing a CSD model in the New Zealand White rabbit radius is an indispensable step for *in vivo* investigation of mechanical and biological bone repair and the compatibility of bone substitute materials. The purpose of this study was to validate a preclinical model of CSD in the radius of rabbits.

## MATERIAL AND METHODS

This study was approved by the ethics committee on the use of animals of the faculty of agrarian and veterinary sciences, UNESP/Jaboticabal, Brazil under protocol number 9417/2015. To elect the location and size of the defect, an anatomical study of the forearm of five adult female New Zealand rabbits was carried out. From these anatomical pieces, it was determined the length of the defect based on the radius diameter in the region to be removed. The diameter was measured in three points: at the site of the proximal cut, the distal cut and between the two cuts (Figure 1).



**Figure 1** - Forearm of a 7 months old, female, New Zealand Rabbit used for radius diameter measuring. A) Osteotomy cut 1.5cm from the first cut, 2 cm above from radiocarpal joint. B) Measurement of the radius diameter between the proximal and distal osteotomy cuts.

The mean value of the largest measure between the bones was obtained (0.56cm) and multiplied by 2.5 resulting in 1.5 cm (GUGALA; LINDSEY; GOGOLEWSKI, 2007; LINDSEY et al., 2007). The most distal incision of the osteotomy was stipulated at 2 cm from the radiocarpal joint. The radius was chosen in view of that rabbits present a broader ulna than radius. The osteotomy location was based on the anatomical conformation of the radius. In this area, the bone is thinner and less curved, facilitating the cut.

Twenty adults (> 7 months) female New Zealand Rabbits, weighing between 3.5 to 5 Kg were undergone pre-anaesthetic medication consisted of ketamine hydrochloride (20mg/kg), midazolam maleate (2mg/kg) and morphine sulphate (2mg/kg) intramuscular. Induction and anaesthetic maintenance were achieved with isoflurane using masks in open inhalation system vaporized in oxygen 100%. Afterward, brachial plexus block was performed in all animals using lidocaine (6mg/kg) 2% without vasoconstrictor.

In dorsal recumbency, a longitudinal skin incision of approximately 3 cm on the dorsomedial face of the right antebrachium was performed. In sequence, subcutaneous tissue and musculature were retracted to expose the diaphysis of the radius and facilitate the removal of the periosteum with blunt dissection. Then, the osteotomy was performed 2 cm above the carpus joint, removing a 1.5 cm of a segmental defect with the aid of an oscillating saw with irrigation (Figure 2). The cut was carefully performed to avoid any lesion on the ulna. Furthermore, the interosseous ligament was incised, which allowed removing the fragment. The bone defect was left empty and subcutaneous tissue and skin were sutured routinely. Postoperative medication consisted of dipyron 25mg/kg subcutaneously (SC/BID), tramadol hydrochloride 4mg/kg (SC/BID), meloxicam 0.1mg/kg (SC/SID), all for three days and enrofloxacin 5mg/kg (SC/BID), for five days.



**Figure 2** - Surgical procedure. A) After radius exposure, two osteotomies were performed with oscillatory saw. The distal cut was made 2 cm above the radiocarpal joint. B) Removal of the radius segment of 1.5cm, creating the critical bone defect.

For radiographic evaluation, craniocaudal and mediolateral views (mA 100, Kv 70) , using digital radiographical equipment (Digital Radiography Equipment; Siemens RG150/100gl, Munique – Alemanha, Germany), were taken in the immediate postoperative period and after 15, 30, 60 and 90 days

postoperatively. Radiographs were blinded analysed by three evaluators in relation to the groups. The images were assessed for periosteal reaction (Table 1), bone callus formation (Table 2) and bone bridge quality (Table 3), receiving scores from 1 to 4.

**Table 1** - Scores classifying the volume of bone callus used in the postoperative radiographic evaluation of rabbits submitted to osteotomy of the right radius.

SCORE	BONE CALLUS VOLUME
1	0-25% of bone failure is filled by bone callus
2	25%-50% of bone failure is filled by bone callus
3	50-75% of bone failure is filled by bone callus
4	75-100% of bone failure is filled by bone callus

**Table 2** - Scores classifying the periosteal reaction used in the postoperative radiographic evaluation of rabbits submitted to ostectomy of the right radius.

SCORE	PERIOSTEAL REACTION
1	Absence periosteal reaction
2	Discrete periosteal reaction
3	Moderate periosteal reaction
4	Intense periosteal reaction

**Table 3** - Scores classifying the bone bridge formation used in the postoperative radiographic evaluation of rabbits submitted to ostectomy of the right radius.

SCORE	Bone bridge formation
1	Absence of bone bridge between the fragments of radius
2	Discrete bone bridge formation in the ventrolateral region of the radius
3	Formation of a bone bridge of a thickness less than the diameter of the radius
4	Formation of bone bridge of thickness equal or greater than the diameter of the radius restoring the bone column

For categorical variables, the Kruskal Wallis test was used, and for p values equal or less than 0.05 ( $p \leq 0.05$ ) the differences between the medians were considered statistically significant and Dunn test was used to identify which groups presented that difference. Statistical analyses were performed with the software BioEstat (version 5.0).

## RESULTS AND DISCUSSION

The defect was properly created in all animals, but one that an injury in the distal ulna cortex of the ulna was observed. Moreover, on the 7th postoperative day, this animal presented complete transverse ulna fracture and it was removed from the study. Except for this case, no other complication was observed. Shortly after anaesthetic recovery, the animals were able to support the operated limb, although a mild degree of

claudication was observed in some animals. Moreover, no assumed signs of infection were noted.

A defect non-union was observed in all radius at the end of experimental period, excepted in one animal that showed bone consolidation at 90 days (Figure. 3). Radiographic evaluation immediately after surgery confirmed the size of defects (1,5cm precisely). At 15 days postoperatively no bone deposition was found, however, one animal presented intense periosteal reaction. Furthermore, it was possible to visualize mild bone callus formation in three animals after 60 days postoperatively. Using the Kruskal Wallis test, it was found difference between times ( $p > 0,05$ ). In sequence, Dunn test was applied to define which group was different. Was founded that group 1 (15 days) was different of all other groups.



**Figure 3** - Mediolateral and craniocaudal radiographs of the right thoracic limb of a New Zealand rabbit. A and B) 90-day postoperative radiographic examination of a rabbit submitted to a 1.5cm ostectomy in the radius diaphysis resulting in a non-union.

Non-union is a common challenge in the treatment of long bone fractures. Many factors can contribute to this, such as comorbidities, infection, bone devitalization, mechanical environment, and poor vascularity in extensive soft tissue damage (MILLS et al., 2016). Defect non-union represents the most dramatic clinical scenario, once the surgeon has to deal with a critical sized bone gap, and just few options are available for modify the devastated biological and mechanical environments (GIANNOUDIS; EINHORN; MARSH, 2007). Numerous biomaterials have been investigated in the field of bone tissue engineering and the critical sized bone defect has been the most common and effective experimental model for testing then (CASTAÑEDA et al., 2006; NIELSEN; KARRING; GOGOLEWSKI, 1992; NIEMEYER et al., 2010; WANG et al., 2019; ZHANG et al., 2019).

Animal models are essentials for bone engineering, especially, when in vitro alternatives cannot reproduce a clinical scenario for the target species. Many different animals can be suitable for this purpose, depending on the study's question (NAFF; CRAIG, 2012). However, no one can exhibit human-like or small animal-like responses perfectly, making the decision regarding the experimental model challenging. The most appropriate animal model is that one with high level of physiological similarities to the target species and accompanied by easy acquisition, low maintenance costs, and feasible ethical issues (EGERMANN; GOLDHAHN; SCHNEIDER, 2005; LIEBSCHNER, 2004). For humans primates would be best comparative model, however the handling, expensive cost to maintaining, zoonotic potential, and possible aggressive behaviour make its your potential limited (WANG; MABREY; AGRAWAL, 1998). On the other hand, **rabbits**, present similar bone microstructure, density and resistance of the diaphysis compared to the human or canine bone. Currently, the rabbit has been widely used for this purpose and they are considered the most potential bone model after primates and dogs. Additionally, they are easy to acquire and manage (LI et al., 2015; WANG; MABREY; AGRAWAL, 1998).

The total time of the experimental phase to confirm a defect non-union is dependent on several factors, however it is short when using rabbits (AZI et al., 2012; DEN BOER et al., 1999), once they have fast bone turnover, allowing the study to be performed in a shorter period when compared to other species (CASTAÑEDA et al., 2006). Additionally, young adults were selected, facilitating and standardizing the time of achieving CSD. A longer evaluation time was not established in our study, however, based on the results of other studies, which was observed bone consolidation in CSD filled with biomaterials and cells, it may be suggested that the period of assessment was adequate (GEIGER et al., 2007; KASTEN et al., 2008; PEARCE et al., 2007)

CSD models have been performed in femur, rib, tibia, mandible and radius with great efficacy (CARLISLE et al., 2019; CLOUGH; MCCARLEY; GREGORY, 2015; LIU et al., 2016; MANASSERO et al., 2013; ZHAO et al., 2016). In rabbits a complete failure in diaphysis of radius can be performed and the

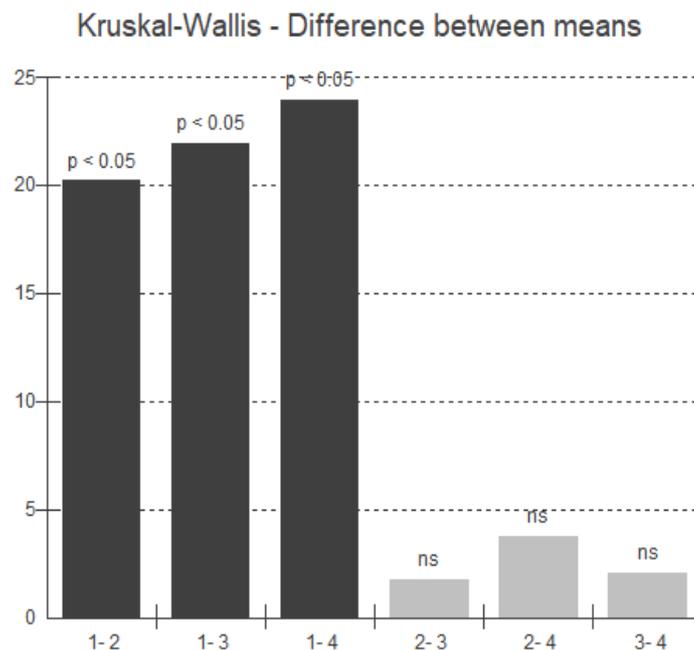
ulna work as a bridge, stabilizing the fracture and avoiding the use of implants. They have an ulna larger than radius, which allows them to support all the weight when walking. Without the need for implants the surgical time become shorter and the cost of researches decreases (ZHAO et al., 2016). In this study only one rabbit had a fracture of ulna, but was because an iatrogenic lesion during a surgery, confirming the efficacy of the ulna in bearing the weight. In addition, the radius has lesser coverage of soft tissue, principally in your distal portion. Fractures of distal radius in toy dogs are challenger due the poor vascularization of the region, leading to high rates of non-union even with surgical intervention (WELCH et al., 1997). In humans, regions of lesser tissue coverage have high incidence of complications and adjuvants technics to improve the bone regeneration should be used. Therefore, that model can mimic challenger situations in orthopaedics surgery (MILLS et al., 2016).

Different methodologies have been proposed for achieving an ideal CSD. Some of them consider the total size of the gap, and others prefer to use a proportion of the total length or width of the bone (BOLANDER; BALIAN, 1986; GUGALA; LINDSEY; GOGOLEWSKI, 2007; ZHAO et al., 2016). Spontaneously healing was detected in defects with 1,0 cm and 1,2 cm in radius of rabbits, however other study proved that no spontaneously bone healing can occur when the defect size has 1 cm and 1,2 cm, and above 1,4 cm the bone growth was significant lesser (ZHAO et al., 2016). Moreover, 1,5 cm defect with periosteum resection is the most used in bone tissue engineering and the results showed no bone union at 16 weeks after procedure (GEIGER et al., 2007; KASTEN et al., 2008; NIEMEYER et al., 2010; ZHAO et al., 2016), which made this model the standard for CSD in radius of rabbits. Thus, bone healing is difficult in larger defects associated with less red bone marrow and less muscle cover and non-union can occur without clinical intervention (JOHNSON et al., 1989). Therefore, new studies as this need be carried out to determine the more feasible CSD for each bone. In this study, the defect size was anatomically based on proposed in the literature. To achieve the gap of 1.5 cm, the length of the failure should exceed at least 2.5 times the diameter of the bone (BOLANDER; BALIAN, 1986; GUGALA; LINDSEY; GOGOLEWSKI, 2007), in addition the periosteum and the interosseous ligament were removed to potentialize the defect.

The segmental defect of 1.5cm proved to be effective, as CSD, considering the statistical analyses (Figure 4). Although the test showed significant difference, this was between group 1 (15 days) and all the others, probably because with 15 days almost no bone formation was found in radiographic evaluation, except in one animal, which presented consolidation on end of the 90<sup>th</sup> day. In addition, some animals expressed periosteal reaction e bone formation without consolidation starting on the 30<sup>th</sup> day. Our result corroborate with other study, which showed no consolidation in 1.4 cm or greater (ZHAO et al., 2016), although the one case of union. That may occur due several factors, how the persistence of periosteum or the membrane intraosseous, a great

vascularization individual, the weight and size of rabbits, making the defect lesser proportionally. Moreover, considering the results at 15 days, in which there was difference between the other groups, it is suggested that radiographic exams of less than 30 days may not be

necessary when the objective of the study is the visualization of bone activity. Other studies should evaluate the bone activity before the 30 day when grafts and scaffolds are tested, because in these situations bone repair will be enhanced.



**Figure 4** - Graphic showing the difference between the means of each time by Kruskal-Wallis and Dunn's method. The horizontal axis shows the times 1 (15 days), 2 (30 days), 3 (60 days) and 4 (90 days). Already, on the vertical axis are the differences of the means between the times. The time 1 had significant difference to all times.

## CONCLUSION

Our results demonstrate that 1.5 cm segmental bone defects in the rabbit radius can be used as a model of CSD. Moreover, the critical bone defect proposed in this study was satisfactory, feasible with very low risk of complications and radiographic evaluations lesser than 30 days may not be necessary if no adjuvant of osteogenesis is tested.

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