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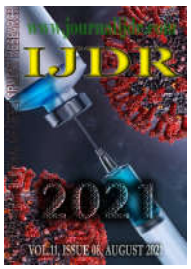

RESEARCH ARTICLE OPEN ACCESS

A NEW CRITICAL SEGMENTAR RADIAL BONE DEFECT MODEL IN RABBITS

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ABSTRACT

The loss of large bone segments remains one of the most challenging problems in orthopedic surgery and considerable research continues with different results being reported. We proposed an experimental model of critical bone segmental defect. Thirteen New Zealand rabbits were used. The animals were divided into 2 groups composed of 06 animals each and a 7 mm ostectomy was performed in the radial bone of each animal. In Group A the bone defect was kept without any filling. In Group B the bone defect was filled by allogenous cortical bone graft. The remaining animal was used for grafts` acquisition. Stabilization with plates and screws was done in animals of Group B. Clinical and radiographic evaluations were performed in the postoperative period at five specific moments. All animals in Group B showed bone union through radiographic studies, while all animals in Group A showed bone neoformation within the bone gap, but none of them showed bone union at 120 days after surgery. The segmental bone defects were easy to make, with quick surgical time, without complications, and due to the non-union observed during the bone healing process in Group A, we can state that the ostectomy length used in this study can be considered as a critical bone segmental defect and be used as experimental model for this specie.

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INTRODUCTION

The loss of large bone segments remains one of the most challenging problems in orthopedic surgery (Clements *et al*., 2008; Azi *et al*., 2012; Pobloth *et al*., 2017; Ruan *et al*., 2018; Huang *et al*., 2020) due to their weak capacity to self-regenerate (Huang *et al*., 2020). In some cases, the treatment of bone defects can lead to serious complications, such as nonunion, bone atrophy, and bone deformity (Leng *et al*., 2020). Prior to the advent of complex reconstructive procedures this kind of problems culminated in limb amputation or permanent functional deficits (Gugala *et al*., 2007; Reichert *et al*., 2009). Segmental bone defects may result in limb length inequity and may complicate a patient's postoperative course (Clements *et al*., 2008). Although there are a number of ways to treat segmental bone loss, few methods allow treatment with primary fusion and maintenance of limb length using a 1-stage procedure (Clements *et al*., 2008). Traditionally, bone grafts, either autogenous or allogenous, have been used in attempt to restore or maintain segmental length (Clements *et al*., 2008; Cipitria *et al*., 2013, Pobloth *et al*., 2017). Bone allografts are bone tissues harvested from another animal of the same specie after processing it to reduce antigenicity. However, this treatment results in a decrease of incorporation capacity of the graft with host tissue and includes the risk of immune rejection and pathogen transmission to recipient (Yassine *et al*., 2017).

The vast majority of bone defects can heal spontaneously under suitable physiological environmental conditions due to the regeneration ability of bone (Reichert *et al*., 2009), unless the segmental size defect is extensive (Zhao *et al*., 2016). However, the healing process of bone defect is time consuming, and new bone generation takes place slowly because of decreased blood supply to the fracture site and insufficiency of calcium and phosphorus to strengthen and harden new bone. In addition, large defects, also known as critical bone defects, may not heal spontaneously and lead to nonunion prognosis due to the size of defects or unstable biomechanical properties, unfavourable wound environment, suboptimal surgical technique, metabolic factors, hormones, nutrition, and applied stress (Li *et al*., 2015). For this reasons, segmental bone defect repair remains a clinical and experimental challenge in tissue engineering (Cao *et al*., 2012), and considerable research continues toward developing novel devices to restore and accelerate bone integrity (Wancket, 2015). Recently, with the advances made in the realm of bone tissue engineering, the development and use of artificial bone has been successfully utilized to fill limited and focal bone defects (Cao *et al*., 2012). Synthetic bone graft substitutes are expected to be of major importance in the treatment of these large bone defects. However, the osteoregenerative potential of these bone substitutes still needs to be improved to obtain materials equivalent to autologous bone (Bodde *et al*., 2008). Guidelines are provided for the dimensions of implants for in vivo studies, based on the size of animal and bone chosen and on the implant design, in order to avoid

pathological fracture of the test site. For example, cylindrical implants placed into rabbit tibial and femoral diaphyseal bone should be no larger than 2 mm in diameter and 6 mm in length (International Standard ISO 10993-6, 1994; Pearce *et al*., 2007). Experimental models are useful for assessing the efficacy of new treatments, and should be reproducible, well controlled and afford the application of standardized methods of analysis (Azi *et al*., 2012). An adequate animal model involves the creation of a standardized bone defect that resembles bone loss encountered in clinical situations. To eliminate the influence of instability, adequate fixation of the bone defect is mandatory (Boer *et al*., 1999). Also, must permit angiogenesis, provide all types of cells needed for bone repair in situ, and minimize the suffering of the animal (Horner *et al*., 2010). For the testing of tissue destined for long bones, the use of nonhealing segmental bone defect models is well established and includes fracture and osteotomy models, based upon those Bonnarens and Einhorn *et al*. described in 1984, it means, there are two main methods of creating a critical defect in the long bone, using either an osteotomy approach or a traumatic approach. Osteotomy utilizes a drill or saw to surgically remove the required length of bone from a predetermined site and producing a consistent defect in all subjects. The edges of the defect are usually cut straight (not jagged) with less trauma. To reflect the conditions after traumatic injury, the defect can be created via trauma, which will produce a jagged cut edge and traumatize both the bone and surrounding soft tissue (Horner *et al*., 2010). Rabbit is one of the most commonly used animal models, and it ranks first among all the animals used for musculoskeletal research (Castañeda *et al*., 2006; Li *et al*., 2015), being used in approximately 35% of studies (Pearce *et al*., 2007). They offer advantages over large animal models by reaching skeletal maturity at a relatively early age (approximately 6 months) and advantages over other rodents by undergoing more secondary osteonal remodeling (Pearce *et al*., 2007; Wancket, 2015). They also show significant intracortical remodelling, have faster bone turnover than other rodents (Li *et al*., 2015), even than primates (Castañeda *et al*., 2006). It was reported that there were similarities in bone mineral density and the fracture toughness of mid diaphyseal bone between rabbits and humans (Li *et al*., 2015). Other advantages are that they are easily available, and easy to house and handle (Pearce *et al*., 2007). These characteristics make rabbits the first choice when researchers develop animal model for the in vivo test of a new bone substitute biomaterials (Li *et al*., 2015). In the evaluation of artificial bone regenerative materials, the so-called ‘‘critical-size defect’’ (CSD) is an essential model. A CSD is defined as the smallest size of a defect, that does not heal spontaneously when left untreated for a certain time period or that shows less than 10% bone regeneration during the lifetime of an animal (Bodde *et al*., 2008; Reichert *et al*., 2009; Li *et al*., 2015; Zhao *et al*., 2016).

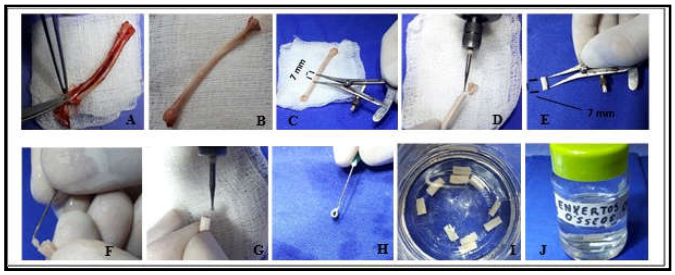
Nevertheless, a critical defect in long bone cannot simply be defined by its size but may also be dependent on the species phylogenetic scale, anatomic defect location, associated soft tissue and biomechanical conditions in the affected limb as well as age, metabolic and systemic conditions, and related morbidities affecting defect healing (Reichert *et al*., 2009). Disregarding study dissimilarities, CSD varies between 10 and 20 mm in general (Nielsen *et al*., 1992; Bodde *et al*., 2008; Xia *et al*., 2019; Huang *et al*., 2020). Earlier studies investigated scaffolds for degradation and bone formation in 15 mm segmental radial rabbit defects (Walsh *et al*., 2008; Bodde *et al*., 2008; Zhou *et al*., 2010; Ruan *et al*., 2018; Leng *et al*., 2020). Empty defects did not confirm a critical size of 15 mm for a time period of 12 weeks, because some of the defects had closed (Bodde *et al*., 2008; Cao *et al*., 2012). Gauthier *et al*. (2005) used a cylindrical, 7 e 10 mm critical-size bone defect rabbit model to investigate the efficiency of an injectable calcium phosphate bone substitute for bone regeneration. Yassine *et al*., 2017 has created a segmental defect of 5 mm in the mid-shaft of radius as a critical size defect bone model and observed for 90 days (Yassine *et al*., 2017). However, a review of the literature shows that the exact critical size of radial defects in rabbits is not clear (Bodde *et al*., 2007; Zhao *et al*., 2016). For practical purposes, if there is no mineralised area of 30% after 52 weeks, there would never be complete bony regeneration. Although the minimum size that renders a defect “critical” is not well understood, it has been defined as a segmental bone deficiency of a length exceeding 2 to 2.5 times the diameter of the affected bone (Oryan, 2014; Li *et al*., 2015; Wancket *et al*., 2015). One of the fundamental factors for studying a nonhealing bone defect model is whether the size of the defect created remains critical. A problem with comparing different models is that there is often a lack of clear evidence that the model used was actually a critical defect. In 1934, Key hypothesized that to create a critical size defect in canine ulna, the defect must be over 1.5 times the diameter of the bone. It has also been repeatedly shown that the periosteum is very important for bone regeneration and that unless this is removed the defect will often spontaneously heal (Horner *et al*., 2010). The method of fixation is another important variable to be studied in bone fracture defect models. Fixation is more important for defects within weight-bearing bones than the nonweight-bearing bones (Horner *et al*., 2010). At present, most long bone defect models use either bone plates or intramedullary rods to fix the defect, thus reflecting the situation within the clinic (Horner *et al*., 2010). A good site for studying the regeneration of segmental long bone defects is the mid-diaphyseal radius. It is claimed that this model does not need internal fixation or external splinting in small animals as the adjacent intact ulna provides stability to the created radial defect. (Bodde *et al*., 2008; Zhao *et al*., 2016). The time scale of an in vivo study will vary depending on the choice of model and species as well as the parameters to be monitored. Studies using rabbits, for example, normally takes 8 to 20 weeks observation (Horner *et al*., 2010).

MATERIALS AND METHODS

This study was previously submitted to the Bioethics Committee of the Federal University of Campina Grande (CEUA - UFCG) to assess the use of animals and was approved under protocol number CEP 046/2018. Allogeneous cortical bone graft collection and storage procedures, as well as surgical and postoperative procedures were performed at Animal Care Barueri Clínica Veterinária, in Barueri, São Paulo. For this experiment 13 New Zealand rabbits, male and female, weighing between 3 and 4 kg were used. The animals were subdivided into 2 groups consisting of 06 animals each. Group A was considered the group in which the radial diaphysis ostectomy was performed and it was kept without any filling of the bone defect by a graft. Group B was considered the control group of the study, in which radial diaphysis ostectomy was performed and cortical bone allograft was used to fill the bone gap. The other spare animal was used for the aseptic collection of the two bones of the radius for manufacturing of all the allogenous bone cortical grafts that were part of the bone bank, and which were later implanted in the radial bone of the animals of Group B.

Grafts` acquisition: After obtaining the bone fragments for grafting, they were cleaned and washed through irrigation with 0.9% saline solution, and any tissue adhering to the bone cortex, including the periosteum, was removed. The medullary canal was also cleaned with the aid of a hypodermic needle, and any structures within this same canal were removed, such as blood vessels and bone marrow. Afterwards, the grafts already completely free of any non-bone tissue were again abundantly washed with 0.9% saline solution before being stored in a bottle containing 98% glycerin solution (Padilha Filho *et al*., 2008a and 2008b), product chosen to serve as a preservative for the grafts (Figure 1). In this bottle, the bone allografts remained preserved for a minimum period of 30 days before being used.

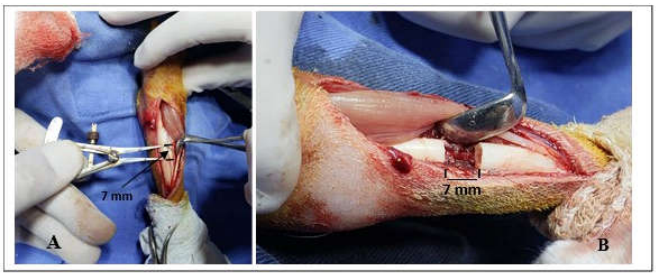
Anesthetic protocol: As pre-anesthetic protocol, Acepromazine was used at a dose of 1 mg/kg via the intramuscular route, associated with Ketamine at a dose of 40 mg/kg, also via the intramuscular route. After a 15-minute interval, the animals were catheterized in the venous blood vessel of the ear and maintained on fluid therapy with Ringer Lactate. Then, isoflurane was used for anesthetic induction by vaporization in the mask. For anesthetic maintenance, the same isoflurane was maintained in an open anesthetic circuit with the use of the mask mentioned above. The animals were monitored by electrocardiogram, heart rate, respiratory rate, blood pressure, oxygen saturation and temperature.



Removal of soft tissue adhered to the bone after dismemberment (A). Radius appearance after all soft tissue cleaning and ulna removal (B). Measurement ofgraftlength (7 mm) withCastroviejo compass (C). Cutting the grafts with high-speed burr and dental drill (D). Graft cut to desired size and measured for exactcheckingof 7 mm length (E). Removal of all tissue inside the medullary canal (F). Trimming of any irregularities of the graft`s border (G). Image after graft irrigation and medullary canal cleanning (H). Grafts are placed into 98% glycerin for conservation (I). Pot identified with the grafts to be used after 30 days of rest in 98% glycerin (J).

Figure 1. Photographic images demonstrating the procedures for collecting, manufacturing and storing the allogenous bone cortical grafts

Surgical protocol: Prior to surgery, the hairs on the left pelvic limb of each animal were shaved and then antisepsis was performed by cleaning the skin with alcoholic chlorhexidine. A longitudinal cranial incision of approximately 6 centimeters was made in the skin over the topography of the left radius of each animal and the tissues adjacent to the diaphysis of the bone were dissected. In the middle third of the radial shaft, an ostectomy was performed with the aim of removing a bone fragment of approximately 7 mm in length. The measurement was demarcated over the bone with the aid of a Castroviejo compass and electrocautery (Figure 2A). Then, the ostectomy procedure was performed with the aid of a mini electric high-speed drill and a dental burr (Figure 2B). Small holes were made transversely in the bone until it was possible to fracture and completely remove the bone fragment.



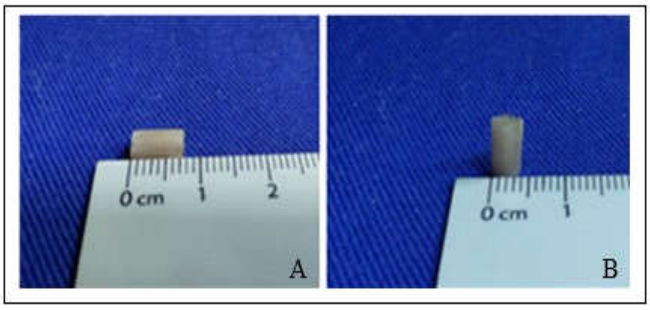
Demarcation of the osteotomy site in the middle third of the diaphysis of the left radius with the aid of a Castroviejocompass (7 mm in length) (A). Image after ostectomy, showing a 7 mm bone segmental defect in the left radius (B).

Figure 2. Photographic images demonstrating the demarcation site at the mid shaft of the radius bone and ostectomy gap created as a segmental critical bone defect

Trimmings were performed on the remaining bone through burr wear, in order to make the bone defect as homogeneous as possible, with the osteotomies well parallel. Therefore, irrigation of the ostectomized sites was performed at the time of cuts in order to prevent the occurrence of thermal necrosis. For the animals in Group A, the ostectomy site was left without any type of filling material and the anatomical planes were sutured as described below. For Group B animals, cortical bone allografts removed from the bone bank were positioned in the bone defect created by the ostectomy (Figure 3). The grafts were inserted through pressure at the ostectomy site so that their proximal and distal ends were in intimate contact and compressed with those of the animal's bone. At the moment that allogenous cortical bone graft were supposed to be used, they were removed from the 98% glycerin pot and were subjected to an abundant prior washing with 0.9% saline solution for a period of 10 minutes before being placed in bone defect, in order to remove the glycerin and rehydrate the fragment to be used. For osteosynthesis, stabilization of the operated limb, and protection and support of the graft at the ostectomy site, six holes 1.5 mm titanium locking plates and 1.5 mm titanium locking screws were used. Both the plates and screws used in this research were donated. Osteosynthesis was performed by bridging the grafted defect, that is, although the plate had 6 holes along its length, only 4 screws were used for locking, 2 of them being placed in the two most proximal holes of the plate, and another 2 placed in the two most distal holes, leaving the central part of the plate free of screws. After performing the ostectomy and osteosynthesis procedures, suturing of the muscle planes, subcutaneous tissue and skin with nylon 3.0 using simple and separate pattern were performed in a conventional manner. Dressings and care of the surgical wound stitches throughout the postoperative period were performed twice a day. The stitches were removed after 2 weeks post-operatively. For infection control, Enrofloxacin was administered at a dose of 5 mg/kg, subcutaneously, once a day, for 7 days. To control pain and inflammation, morphine was used at a dose of 2.5 mg/kg, subcutaneously in the immediate postoperative period, followed by Tramadol Hydrochloride at a dose of 5 mg/kg, subcutaneously, twice a day, for 7 days, in addition to Meloxicam, at a dose of 0.2 mg/kg, subcutaneously, once a day, for 7 days. The animals were clinically and radiographically evaluated postoperatively (PO) for a period of 120 days. The evaluated moments were considered: M0 (immediate postoperative period); M1 (30 days of PO); M2 (60 days of PO); M3 (90 days of PO); M4 (120 days of PO).

RESULTS

Clinically, all animals in Group A and Group B evolved satisfactorily. Even after the procedures, they rested their paws on the ground and walked without limping. The alignment of the operated limb remained adequate throughout the entire period. They presented mild discomfort on palpation of the site, but there were no complications in the operated region, such as infection, drainage of purulent content, limb edema, or opening of the surgical wound. Two animals tore off some of the stitches, but without any complications. Radiographically, for both Group A and Group B, it could be observed that the osteotomies were performed properly and with good positioning in the middle third of the diaphysis of each radius (Figures 4A1 and 4A2).

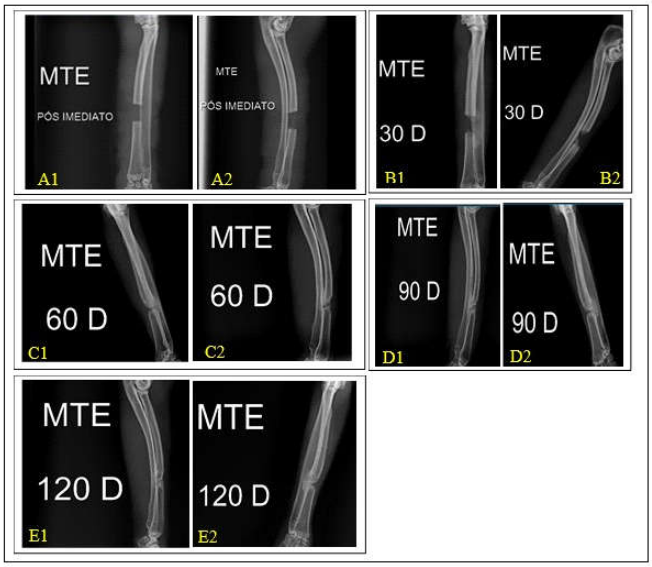


Allogenous bone cortical graft: length = 7 mm (A). Diameter = 3 mm (B).

Figure 3. Photographic images showing the shape and measurements of the allogenous bone cortical graft

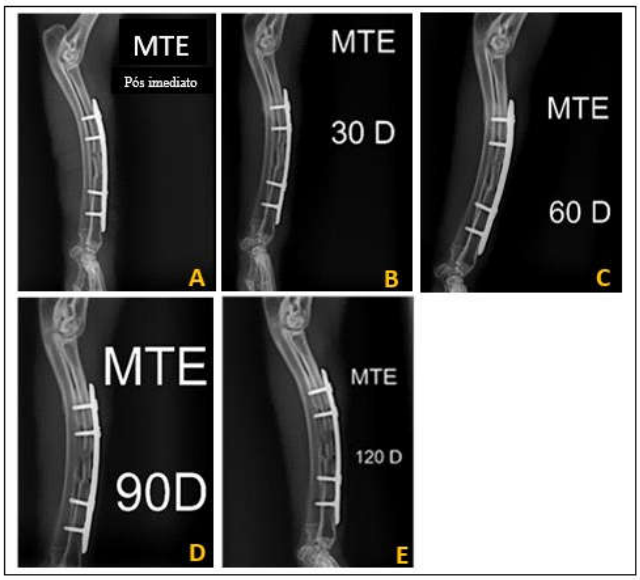
The animals in Group A showed an onset of bone mineralization on the radiographs of M1 (Figures 4B1 and 4B2), but in a slower process compared to other moments. In M2 (Figures 4C1 and 4C2), the bone healing process was more advanced, and a good part of the bone gap was already filled with new bone. However, in M3 and M4, the healing process seemed to stop, since in the images of 90 and 120 days PO were very similar in terms of bone growth within the defect, not reaching the proximal and distal ends of the ostectomized bone (Figures 4D1 and 4D2, and 4E1 and 4E2, respectively). In Group B, it was possible to notice the good positioning of the graft within the bone gap in all animals (Figures 5A to 5E). In all six animals there was bone integration of the graft and the host bone. In M1 (Figure 5B) a radiolucent line between the graft and the bone could still be

observed, but in M2 (Figure 5C) the beginning of the bone consolidation process that extended to M3 and M4 (Figures 5D and 5E, respectively).



MTE = left thoracic limb; Pós imediato = Imediate post operatory moment (M0); 30D = 30 days after surgery (M1); 60D = 60 days after surgery (M2); 90D = 90 days after surgery (M3); 120D = 120 days after surgery (M4).

Figure 4. Postoperative radiographic images from moments M0 to M4 showing the segmental bone defect performed in the middle third of the left radius of one of the animals in Group A and the organism's attempt to carry out the bone healing process over time



MTE = left thoracic limb; Pós imediato = Imediate post operatory moment (M0); 30D = 30 days after surgery (M1); 60D = 60 days after surgery (M2); 90D = 90 days after surgery (M3); 120D = 120 days after surgery (M4).

Figure 5. Radiographic images of one of the animals in Group B from moment M0 to M4 after filling the bone defect with the allogenous bone cortical graft.

DISCUSSION

In order to choose a critical bone segmental defect model, several factors must be taken into account, such as species used, animal age, bone, observation time for bone growth, speed of bone turnover and similarity of bone mineral density of the species in question (Castañeda *et al*., 2012; Li *et al*., 2015). Despite countless works already carried out with rabbits, we chose to use this species for several reasons, such as high availability, small size, easy handling, reduced shelter space (Pearce *et al*., 2007), and mainly because its turnover is faster than from other rodents and species, and have significant intracortical remodeling, as mentioned by Li *et al*., 2015, being this species the first choice in cases of in vivo experimental models for bone substitutes. Although several authors use other bones to produce the bone defect in rabbits, radius can be considered an adequate bone for simulating critical bone defects (Yassine *et al*., 2017). In our study, we used this same bone and it proved to be adequate for making the bone defect, being easy to perform, without complications in relation to the surgical and postoperative procedures, and the surgical time was considered reduced to perform the procedure. Many authors choose this bone as an experimental model because they report that it is unnecessary to stabilize the bone failure or even protect the implant or graft used in the failure, due to the integrity of the ulna that serves as support and alignment of the limb (Bodde *et al*., 2008; Horner *et al*., 2010).

However, we chose to stabilize the radius as indicated by Boer *et al*., 1999 in Group B (use of the graft), as this would mimic exactly what would happen in a clinical/surgical situation. We allow cranial support to the bone graft, reducing mechanical stress on it, which would be adequate if porous implants were to be used. On the other hand, we chose not to stabilize the animals in Group A (failure without filling), as this would reproduce extensive bone loss without treatment, simply letting the bone try to spontaneously consolidate without any help (Reichert *et al*., 2009) whether this occurred or not (Huang *et al*., 2020). The big question that arises for this type of experimental model, and there are still divergences between the studies, is in relation to the length of the critical bone defect. In the literature used as reference, the size ranged from 5 mm to 2 cm of bone defect produced in the radius of rabbits (Nielsen *et al*., 1992; Gauthier *et al*., 2005; Bodde *et al*., 2008; Walsh, 2008; Yassine *et al*., 2017; Ruan *et al*., 2018; Xia *et al*., 2019; Huang *et al*., 2020; Leng *et al*., 2020). Despite the controversy, we chose to perform the radial ostectomy length based on the definition cited by Oryan, 2014; Li *et al*, 2015 and Wancket *et al*., 2015 in which the critical bone defect is 2 to 2.5 times the diameter of the bone in question. As the radius of the animals used in this experiment was 3 mm, which was even the diameter of the allogenous grafts used, we chose to perform the critical bone defect with a length of 7 mm. Despite being a shorter length than most defects created by various authors, it is longer than the defects created by Yassine *et al*., 2017 and equal to that performed by Gauthier *et al*., 2015.

The critical defect used in this experiment did not take into account the guidelines of the International Standard ISO 10993-6, 1994 regarding the size of implants, which advocate the use of cylindrical implants in the tibia and femur of rabbits in experimental models of segmental bone defect with maximum diameter and length of, respectively, based on the possibility that larger sizes could generate greater local stress and occurrence of pathological fracture in the test area. In our study, the allogeneic bone cortical graft was 3 mm in diameter and 7 mm in length, and contrary to what was previously described, there was no fracture at the site, either in the graft or even in the host bone. Regarding the time of study, Horner et a., 2010 reports temporal observation between 8 and 20 weeks. Our study used a time limit of 4 months (16 weeks), which is longer than many studies, but a few weeks less than the longest time reported above. What we were able to assess during this period was the occurrence of graft osseointegration (Group B), which could already be observed from 60 days after the operation, and the beginning of bone growth in the critical failure of animals in Group A (without filling of failure). In Group A, this process occurred slowly in the first 30 days, but then, between 30 and 60 days, bone growth in the gap was fast and with good filling, but it did not reach the bone union until 120 days. And in fact, from the radiographs we can see that the filling in M4 is practically the same as in M2, which may mean that from M2 onwards, with no union of the bone ends, the consolidation process generated by the bone may have stopped from 60 days after surgery. We cannot say for sure, if we could observe any longer, whether there would still be enough bone activity to culminate in bone union. This

could even determine whether the size of the segmental bone defect created is really critical or not. Other studies using more observational time and different lengths of bone gap could be carried out to resolve this question.

CONCLUSION

The segmental bone defects performed in this study were easy to make, with a quick surgical time, and without complications. Although there was bone growth through both bone ends of the defects, none of the defects showed complete bone union at the end of 4 months, unlike the graft group, showing that the length of the bone defect performed in the radius of rabbits during this period can be considered as a critical defect and can be considered as an experimental model for this specie.

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