



RESEARCH ARTICLE

Use of Mesenchymal Stem Cells from Canine Bone Marrow Associated with the Use of Metallic Implants in the Repair of Distal Radius and Ulna Fractures in Toy Breed Dogs

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ABSTRACT

Cell therapy has been an effective tool for the treatment of several animal diseases in experimental and clinical studies, including osteogenic stimulation for the treatment of non-union and repair fractures of distal radius and ulna in dogs. There are many advantages of using stem cells since this differentiated cell type has high proliferation capacity, self-renewal, production of different cell lines and tissue regeneration. These cells are present in embryos and can be collected from umbilical cord and bone marrow. The aim of this study was to perform a clinical and radiographic evaluation of dogs with fractures of distal radius and ulna after surgery and treatment of fractures with stem cells from canine fetuses' bone marrow. Radiographic evaluations showed bone regeneration from 45 days post-surgery in both the groups, only with conventional surgery and implant and also in the conventional surgery group with implants associated with stem cells. Thus, cell therapy can be a favorable tool to assist in bone healing of distal fractures of radius and ulna, however, details on the quantity of applied cells and routes of administration need to be studied in more detail.

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INTRODUCTION

Fractures of the radius and ulna represent 8.5-18% of fractures in dogs and cats, with most authors reporting an average incidence of 17%, being the third most frequent type in dogs (Muir, 1997). In young dogs of medium to large breeds, prognosis progress to healing regardless of the stabilization method, while in small breeds there is a high risk for the development of delayed union and nonunion and growth deformities, stiffness joint and hyperextension of the carpus, thereby reflecting the complexity in treating these fractures in smaller animals (Fayaz *et al.*, 2011).

Non-unions are common complications in fractures located in bones with sparse muscle tissue coverage, reduced medullary canal diameter or poor vascularization. In these bones, other complications also occur more frequently and both surgical reductions and conservative treatments represent greater difficulty for some authors. In small breeds, the complication rate is even higher due to inefficient vascularization, reaching 60% of cases with nonunion in some studies. Fractures of radius and ulna are

significant examples of this type of complication in these breeds. Therefore, alternatives are being studied to enhance the approach in these cases, such as proteins that stimulate chemotaxis of undifferentiated mesenchymal cells, making these cells differentiate into osteoblasts and form callus (Ferrigno *et al.*, 2007).

The formation and bone repair depend on the presence of pluripotent mesenchymal stem cells, capable of differentiating into osteoblasts; growth and differentiation factors to direct these cells to the lesion focus; a matrix for migration and attachment of these cells; and angiogenic factors which allow the formation of a vascular network for the newly formed bone. Pluripotent cells with osteoblastic potential have been isolated from several species including canine (Nauth and Schemitsch, 2012). The repair of fractures depends on a sequence of chondrogenesis, osteogenesis and remodeling, which ultimately restores the integrity of the bone and its biomechanical characteristics (Chen *et al.*, 2003).

Despite the development of new technologies and advances in clinical orthopedics regarding fractures, there is still a subset of fractures which remain deficient in bone

repair, culminating with a bone nonunion. Currently, new strategies for the treatment of these fractures includes the application of stem cells due to their regenerative potential (Heino, 2008; Tseng *et al.*, 2008; Djouad *et al.*, 2011).

Cell therapy has currently been a useful tool in regenerative medicine with high therapeutic potential due to the capacity for self-renewal and differentiation (Heino and Hentunen, 2008; Djouad *et al.*, 2011). In recent years orthopedics has searched new methods to obtain a more effective bone repair. Therefore, many studies involving biological and molecular devices, aiming to improve bone healing, are being performed (Puleo, 2003; Crovace *et al.*, 2008). In order to seek new alternatives for effective repair in nonunion bone fractures, the aim of this study was to evaluate the therapeutic potential of mesenchymal stem cells from canine bone marrow in dogs with fractures of distal radio and ulna.

MATERIALS AND METHODS

Cultivation of cells from bone marrow in dogs' fetuses:

Culture was performed using bone marrow cells of dogs' fetuses from the cell bank of our Stem Cell Lab from the Sector of Anatomy, Faculty of Veterinary Medicine and Animal Science, University of São Paulo (FMVZ/USP). They were thawed and expanded in DMEM (LGCBio), supplemented with 10% fetal bovine serum (LGCBio), 1% streptomycin-penicillin solution and 1% nonessential amino acids, for subsequent application after surgery in the treated animals (described as follows).

These cells were previously characterized, being adherent to plastic, were of fibroblast type, expressed the canine stem cell CD90 and CD44 and it is differentiated into adipocytes, chondrocytes and osteocytes, characteristics of mesenchymal stem cells.

Selection of animals and preoperative procedures: We used 10 animals with diaphyseal fractures of radius and ulna (Tables 1 and 2), from private veterinary clinics in Guarulhos, Sao Paulo, Brazil. Selected animals had non-weight bearing lameness, with time of fracture from 1 to 7 days, 1-5 years-old and fracture in the distal third portion.

All conventional preoperative tests (CBC, ALT, AST, BUN, creatinine) indicating normal values and radiography (cranial-caudal and medial-lateral projections) for accurate determination of fracture sites were performed. Owners signed a consent form, where they were informed about all procedures to be adopted and risks associated with surgery and cell application. This study was approved by the Ethics Committee of the University of São Paulo, Faculty of Veterinary Medicine and Animal Science (Animal Bioethics Protocol 2578/2012).

Animal groups: For this study, 10 animals were divided into two groups (with five each). Group A: surgical correction with plate and bolt locked. Group B: surgical correction with plate and screw locked + stem cells from canine fetal bone marrow placement into the fracture, with the aid of fluoroscopy unit (arch surgery) every 15 days (starting the first application on the day of surgery and the other 3 every 15 days), with a total of four applications.

Table 1: Group A (Control): Animals with fracture of distal radius and ulna that have undergone orthopedic surgery and B, animals with fracture of distal radius and ulna that have undergone orthopedic surgery and application of stem cells

Animal	Age (years)	Breed	Weight (kg)
A1	4	Mongrel	6.5
A2	1	Pinscher	2.1
A3	1	Pinscher	2.9
A4	2	Pinscher	3.1
A5	2	Mongrel	2.7
B1	2	Mongrel	9.5
B2	1	Poodle	6.8
B3	1	Mongrel	11
B4	1	Pinscher	2.7
B5	1	Pinscher	2.6

Table 2: Comparative analysis between control and treated groups for the absence of fracture line and functionality of the limb

Animal	Absence of fracture line in the radius through radiography (in days) ^a	Functionality of the limb (Level III; in days) ^b
A1	45	90
A2	45	30
A3	90	30
A4	90	30
A5	75	30
B2	45	30
B3	90	60
B4	75	60
Group A average	69	42
Group B average	70	50

^aP = 0.2388; ^bP = 0.5237; *Owners of animals B1 and B5 did not return to continue the treatment.



Fig. 1: Radiographic images of the surgical procedure performed in an animal from Group A (Control). In A and B: Preoperative images; lateral and craniocaudal views, respectively. In C and D: Postoperative after 45 days, without visualization of the fracture line; lateral and craniocaudal views, respectively.

Surgery and cell transplantation: For anesthesia, animals received acepromazine (0.05mg/kg; 0.2% Acepran, Vetnil) and morphine (0.5mg/kg; 10mg/ml Dimorf, Cristália). Induction was performed using propofol (3mg/kg; Propovan, Cristália), ketamine (1mg/kg; 10% Ketamine) and maintained with isoflurane (Isoflorano, Biochimico Laboratory). 48 hours prior to



Fig. 2: Radiographic images of the surgical procedure performed in an animal treated with stem cells. In A and B: Preoperative images; lateral and craniocaudal views, respectively. In C and D: Postoperative after 45 days, without visualization of the fracture line or bone callus; lateral and craniocaudal views, respectively.

surgery, animals received cephalexin (30mg/kg/BID), tramal (2mg/kg/TID) and ketoprofen (1mg/kg/SID), being immobilized with a Robert Jones bandage. Skin incision in the cranial-medial face of the distal radius and ulna was performed, followed by location, isolation and remoteness of tendons of the muscles: flexor carpi ulnaris, extensor digitorum communis, extensor digitorum longus, extensor digitorum longus and flexor deep digitorum. Then, reduction procedures and alignment of the fracture fragments were performed, after which the plate was minimally framed manually. With a specific "reduction device", the plate was fixed to the distal fragment, and then the proximal fragment. The guide to a 1.5mm drill was placed in the distal portion of the bone and drilled with the aid of a 1.5 mm drill to a 2.0 mm plate (Ind. Engiplan Implants). The hole depth was measured and a 2.0 mm threaded screw was inserted with key until it attached to the plate. After fixing all screws, the "reduction device" was removed. The surgical suture was

performed according to established surgical standards [muscle, subcutaneous tissue and skin by simple interrupted sutures, using monofilament nylon (Bioline Ltd. Surgical Wire)]. At the end of procedures, forelimbs were immobilized with Robert Jones bandage. For the treatment group, the first application of stem cells (1×10^6 cells per application) was performed after the surgery in the fracture, into the bone, with the aid of surgical arch. Three more applications of 1×10^6 cells were performed at fracture after surgery every 15 days. On the application day, the animals were radiographed again, clinical examination was performed, gait assessment and blood tests were done. The dogs were evaluated for up to 120 days.

Statistical analysis: Unpaired T test between groups A and B (Viana *et al.*, 2012), described in Table 2 was performed, where $P=0.2388$ to bone consolidation and $P=0.5237$ to the functionality of the member, considered no significant results. Significant P value <0.05 .

RESULTS

After surgical procedures, animals from both groups were observed and clinically evaluated every 15 days for 120 days and radiographic examination, evaluation of the limb functionality and blood tests were conducted to evaluate possible changes related to stem cell therapy. The absence of fracture line was adopted as a standard measure for evaluating the effectiveness of treatment.

Based on the data obtained, no significant differences were observed in the time of bone healing (absence of fracture line) between the control and the treated group, with the consolidation being observed from 45 days of treatment (Fig. 1 and 2). There was also no significant difference in the degree of functionality of the limb between both groups (Table 2). Neither inflammatory reactions in involved and adjacent tissues nor significant change in blood tests was observed in the post injection period.

DISCUSSION

Several types of radius and ulna fractures are observed routinely in veterinary orthopedics for small animals, these being considered common injuries (Egger, 1993; Della Nina *et al.*, 2007; Ferrigno *et al.*, 2007; Hayashi *et al.*, 2008; Aronsohn and Burk, 2009; Kaya *et al.*, 2011; Piórek *et al.*, 2012; Sancak *et al.*, 2014). However, fractures of the distal radius and ulna, especially in toy breeds, have high rate of non-union, mainly due to low local vascularization (Giglio *et al.*, 2007) and cats (McCartney *et al.*, 2006).

Our results corroborate those of Zamprogno (2007), who affirm that stem cells from bone marrow are potential tools for cell therapy, through the observation of fracture healing in dogs for up to three months after cell injection, with satisfactory results for stimulation osteogenesis in dogs with a history of non-unions in previous unsuccessful surgeries.

Oliveira *et al.* (2010) using autologous mononuclear stem cells in collagen sponge, performed experimental tibial fractures in dogs and observed 100% consolidation with 45 days of treatment. Brasil (2010) performed

osteosynthesis in distal fractures of the radius and ulna in 15 small dogs using semitubular titanium plates with claws and observed bone consolidation at 30, 60 and 90 days in different dogs treated similarly. In our results, we observed consolidation with 45 days, without line of fracture in the radius.

Yaneselli *et al.* (2013) used $10 \cdot 10^6$ mesenchymal stem cells from allogenic adipose tissue in case of non-radio-ulnar union in dogs, and the bone healing was observed by radiographic evidences after 16 weeks; and the animal began to support the forelimb on the floor in the eighth week. Arinzeh *et al.* (2003) applied $37 \cdot 10^6$ bone marrow stem cells in femurs lesions of 20 adult dogs, while Semiglia *et al.* (2011) obtained success using a single dose of $1 \cdot 10^6$ autologous bone marrow cells in cases of non-natural-bone union. Based on these data, we can say that our four applications of $1 \cdot 10^6$ bone marrow cells from canine fetus, in each animal, are within the standards in research in this area. And as in the previous studies, showed satisfactory results.

Satisfactory consolidation has been observed in our study with the application of stem cells. We observed an improvement in the clinical picture of the group of animals that received cells. The treated animals did not show any adverse reaction to the application of cells and were followed for 90 days. These results are consistent with other authors who also did not notice reactions, but an improvement in the clinical condition of the animal (Arinzeh, 2003)

Conclusions: Stem cells from bone marrow, when associated with the use of metallic implants, can assist in bone healing. No adverse reactions were observed in animals that received the stem cells.

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Author's contribution: All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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