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PRELIMINARY CLINICAL EVALUATION OF MESENCHYMAL STROMAL CELL TREATMENTS FOR UNUNITED ANCONEAL PROCESS AND LEGG-CALVE'-PERTHES DISEASE IN DOGS

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Abstract

Mesenchymal stromal cells are a population of adult stem cells with a vast potential for tissue engineering as well as regenerative medicine. Ununited Anconeal Process (UAP) and Legg-Calvé-Perthes (LCP) disease are two common growth pathologies in young dogs. Even if these diseases present different manifestations and etiopathogenesis, the attention of modern research has focused on the "restoration" of the same cells that were not developed correctly in these bone defects. The administration of Bone Marrow Mesenchymal Stromal Cells and Bone Marrow Mononuclear Cells containing MSCs in these two types of pathologies could be considered as an innovative but also conservative therapy, allowing the formation of new bone tissue in a minimally invasive way. In this preliminary study we evaluated the improvement of the clinical and also the radiographic condition in 2 dogs with UAP and in 3 dogs with LCP, treated with a single administration of autologous Bone Marrow Mesenchymal Stromal Cells and Bone Marrow Mononuclear Cells.

Key words*: Ununited Anconeal Process, Legg-Calvè-Perthes disease, dog, Mesenchymal Stromal Cells, Bone marrow Mononuclear cells.*

INTRODUCTION

Recent studies have demonstrated the exciting potential of tissue regeneration *via* tissue engineering approaches.

Bone marrow-derived mesenchymal stromal cells (BM-MSCs) were first isolated in the 1970s and were the progenitors of many mesenchymal mature cells, including osteocytes, chondrocytes, and adipocytes.

BM-MSCs are an important source of osteogenic cells for bone tissue engineering, and many studies have shown evidence that they contribute to bone regeneration (Liu et al., 2014).

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposes three standard requirements to better identify MSCs (Dominici et al., 2006):

1. cells must be plastic-adherent when maintained in standard culture conditions.
2. > 95% of the MSCs population must express specific surface antigens (CD105, CD73 and CD90) analyzed by flow cytometry
3. cells must display multipotential differentiation capacity, being able to differentiate into osteoblasts, chondroblasts and adipocytes.

A number of favorable biological characteristics, including their poor immunogenicity and the ability to induce immune-tolerance, make BM-MSCs ideal therapeutic agents.

Hernigou and Beaujean first used bone marrow grafting to treat osteonecrosis and the results were encouraging (Hernigou et al., 2002).

BM-MSCs may be used after isolation and culture expansion or in the form of a concentrate of BM mononuclear cells (BM-MNCs) that includes the fraction of BM-MSCs.

In this study we treated two orthopedic diseases:

- Ununited Anconeal Process (UAP), an uncommon condition of the canine elbow joint causing pain and lameness in young medium-large breeds, thought to be due to physeal osteochondrosis or relative overgrowth of the radius.
- Legg-Calvé-Perthes (LCP) disease, a non-inflammatory aseptic necrosis of the femoral head, thought to develop secondarily to ischaemia and resulting in vascular compromise.

The aim of this study was to evaluate the therapeutic effects of culture expanded BM-MSC and BM-MNCs in the treatment of UAP and of LCP in the dog.

MATERIALS AND METHODS

Owned dogs referred at veterinary teaching The Veterinary Teaching Hospital of Perugia with a radiographic diagnosis of UAP and LCP disease were included in the study. The treatment has been authorized in all the dogs through an informed consent signed by the owners. All the dogs underwent a careful clinical examination and complete blood analysis.

Five dogs were included: 2 cases with UAP and 3 cases with LCP disease, respectively. All the dogs were less than one year of age. For the UAP cases the dogs were 2 Corso breed (8 months old), one male and one female, with mono-lateral UAP, both on the left elbow. The three LCP cases were small breed dogs [1 mixed-breed Chihuahua (6 months old), 1 Miniature Poodle (7 months old), 1 Russian Toy (7 months old)], two males and one female, all with mono-lateral disease.

- **UAP group**

The dogs were presented for a forelimb lameness arisen 2 months earlier. At the orthopedic evaluation the following clinical signs were noted: grade of lameness (Quinn et al., 2007), joint ectasia and pain at the passive manipulation of the elbow (Tables 1-3). The Range of Motion was reduced especially during flexion even in deep sedation. The diagnostic workup included: radiographic examination, dynamic fluoroscopy imaging, CT study and arthroscopy of the affected elbow joint. The aim of these diagnostic exams was to assess the grade of UAP (Table 4) and to exclude any other joint disease (eg. Radio-ulnar incongruence, fragmented coronoid process). Dogs with grade III UAP were not included. All the dogs have undergone to surgery to perform a Bi-Oblique Dynamic Proximal Ulnar Osteotomy (BODPUO) (Caron et al., 2016) in addition of the BM-MSCs treatment in order to reduce the load on the ulna compartment. BM sample was taken, at the same time of the ulnar osteotomy surgery, from the iliac crest with a 16 G Jamshidi needle. Bone marrow harvested was diluted 1:1 in phosphate-buffered saline (PBS) containing 2000 U/ml heparin. It was then centrifuged on a density gradient solution (Histopaque 1077, Sigma Aldrich) to collect the mononuclear cell fraction (BMMNCs) that was plated in collagen coated flasks. Cells were cultured in Low Glucose Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS) and 1% antibiotics. MSCs in culture adhered to the tissue culture substrate and displayed a fibroblast-like morphology. Primary cultures were maintained until passage 3 to deplete non-adherent haematopoietic cell fraction and expanded to obtain about $30x10^6$ cells. At the time of the first radiographic follow up, the cells were ready to be grafted in the ununited anconeal gap. They were detached with trypsin EDTA, washed in FBS free medium and re-suspended in Platelet Rich Plasma (PRP). PRP was activated with 10% Calcium Gluconate immediately prior to administration.

Under general anaesthesia the dogs were positioned in lateral recumbency with the limb to be treated downwards, to allow the medial access to the elbow joint: an arthroscopic visualization of the non-united portion of the Anconeal Process was performed in order to correctly insert a 18G needle into the non-vital portion of the bone in the proximal part of the process. Successively, the joint was drained from the saline solution and the stem cells compound (PRP solution and Calcium Gluconate) was inserted by means of the needle. At the end of this procedure, needle and optic were removed and the skin was closed in a routine fashion.

The follow up were made at 15, 30, 60 days, 3 and 6 months post treatment; they included a clinical and orthopedic evaluation and a radiographic examination.

• **LCP group**

The dogs with LCP disease were presented with severe hindlimb lameness, reluctance to move, severe pain at the palpation of the hip, and muscular hypotrophy. The diagnostic workup consisted in a standard radiograph projection of both hips to assess the severity and grading of LCP disease (Ljunggren, 1967; Mickelson et al., 1981; Crovace, 2013) (Table 5). Dogs with more than grade III LCP disease were not included. The owners of the dogs with LCP disease declined the standard surgical treatment (total hip replacement or head/neck femur ostectomy) and preferred cell therapy. The bone marrow sample was taken from the iliac crest with a 16G Jamshidi needle the same day of the implantation. In order to process the cells quickly, the following protocol was used: bone marrow harvested from iliac crest was diluted 1:1 in phosphate-buffered saline (PBS) containing 2000 U/ml heparin and centrifuged on a density gradient solution (Histopaque 1077, Sigma Aldrich). The BM mononuclear cells (BM-MNCs) at the interphase were transferred to a new conical tube and washed twice adding each time 30 ml of buffer before centrifugation. The cell pellet was finally re-suspended in an appropriate amount of freshly prepared PRP for surgical application. When the compound was ready to implant, dogs were placed again under general anesthesia and in dorsal recumbency, in order to perform a ventral approach to the head of the femur. Under ultrasound (US) guidance a 22G spinal needle was inserted directed to the bone lysis area. A bone tunnel was prepared with a Kirschner wire (0,8 mm in diameter) allowing the following entry of the needle, through the wire, into the bone. After wire retrieval, the injection of the BM-MNCs and PRP was monitored by US guide assuring the correct implantation. The clinical and orthopedic follow up were made at 10 days post treatment while the radiographic follow up were made at 30, 60 days and 6 months post treatment. In all the dogs were evaluated the following: grade of lameness (Table 1) (Quinn et al., 2007), reaction at manipulating the affected joint (Table 2), joint ectasia, joint hyperthermia (Table 3), radiographic score for osteoarthritis signs, radiographic score of amount of radiopacity of the bony defects.

Table 1. Lameness score

Description	Grade
Normal	0
Normal in gait, mild lameness at walk	1
Lame mild to moderate in all the gaits	2
Severe lame in all the gaits	3
Not weight bearing lameness	4

Table 2. Response to manipulation

Response to manipulation	Grade
Normal	0
Mild (turn the head towards the affected limb)	1
Moderate (limb retraction to manipulation)	2
Severe (yelp or aggression)	3
Not possible to manipulate	4

Table 3. Sspecific evaluation of the affected Joint

Clinical signs	Absent	Mild	Moderate	Severe
Ectasia	0	1	2	3
Hyperthermia	0	1	2	3
Skin swelling	0	1	2	3

Table 4. UAP grade

UAP grade	Radiographic description
I	Anconeal process is not united with the proximal metaphysis of the ulna, but still connected by fibrocartilaginous tissue, a mild radiolucent area is evident.
II	Anconeal process is separated by an evident radiolucent line; the gap is complete but the process is still in place
III	Anconeal process is completely detached from the ulna, with a large radiolucent area, erosion of the margins and sclerosis.

Table 5. LCP disease grade by Ljunggren, 1967

LCP grade	Radiographic description
I	Femoral head and neck have a normal profile; there is an increased joint space, the acetabulum is normal and there are few radiolucent areas
II	Flattening of the femoral head, the radiolucent areas are larger and more numerous, the femoral neck is involved with osteophytes
III	Greater alteration of the femoral head profile flattening and irregularity of the articular surface), irregular radiodensity and osteophyte production
IV	loss of the femoral head profile, large radiolucent areas alternated with small areas with normal density.
V	extensive fragmentation and deformation of the femoral head, signs of discontinuity of the articular surface

RESULT AND DISCUSSIONS

• **UAP group**

The clinical orthopedic evaluation showed a clinical improvement of the grade of lameness of 2 points in all the dogs at the time of final follow up (6 months post treatment), great reduction of pain during the manipulation of the affected joint, and complete owner satisfaction about the general clinical condition of their dogs (Table 6). The radiographic evaluations showed a partial reduction of the radiolucent defect on the anconeal process. The bone callus, as a result of the ulnar osteotomy, was reshaped at the radiographic follow up at 6 months post-treatment.

Radio-ulnar incongruence was reduced thanks to the dynamic osteotomy, and subsequently subchondral bone sclerosis was mild weaker at the level of the ulnar semilunar incision.

A variety of treatments have been proposed for UAP including removal of the anconeal process (Grondalen et al., 1980; Guthrie et al., 1989), proximal ulnar osteotomy (with or without intramedullary fixation) and lag screw (LS) fixation of the anconeus process with or without proximal ulnar osteotomy (PUO) (Pettitt et al., 2009). PUO has been considered satisfactory in radiographic union of the anconeus in some, but not all, cases (Sjostrom et al., 1995). A later report combining PUO with LS fixation of the anconeus reported the radiographic union in 4 of 4 cases (Krotscheck et al., 2000) but the small size of this study was a limitation. PUO alone has been reported as a treatment for UAP by putatively relieving the "excessive contact pressure" on the anconeal process (Pettitt et al., 2009). Clinical results compared favorably with excision, although fusion of the anconeus to the ulna in these studies varied from 12 to 95 per cent. Sjostrom et al. (1995) evaluated the outcome with a combination of radiographic and subjective lameness evaluations and reported 21 of 22 elbows had radiographic evidence of healing of the anconeus, where healing was considered complete if only "a narrow radiolucent line was evident" (Pettitt et al., 2009). The BODPUO is an upgraded ulnar osteotomy made-up by Fitzpatrick et al. (2014) for the treatment of elbow dysplasia. The combination of BODPUO and stem cell therapy could be a rational therapeutic strategy to treat UAP: it combines the mechanical dynamism given by the osteotomy to avoid the compression force upon the anconeus process, and the osteogenesis power of BM-MSCs at the level of non-union.

Table 6. UAP group; FU: follow up

FU	Description	1 Uriel	2 Margot
0	Grade of lameness	3	3
	Grade of Manipulation	2	2
	Joint Ectasia	3	3
	Joint Hyperthermia	2	1
	Joint Swelling	2	2
10 days	Grade of lameness	3	
30 days	Grade of lameness	2	
6 months	Grade of lameness	0	
	Grade of Manipulation	0	
	Joint Ectasia	0	
	Joint Hyperthermia	0	
	Joint Swelling	0	

• **LCP disease group**

A reduction on lameness was seen in all the dogs and the 6 months follow up, the improvement was of 2 points referred to the Quinns' scale. The improvement of the clinical condition was gradual and distributed over a period of 1-5 months.

The clinical improvement was seen since the 30 days follow up, with a reduction of 1 point of lameness in all treated cases and a pain reduction at manipulation of the joint. No sign of severe side effects was seen, except for a transient increase of the lameness' degree in one dog (n.1) during the first week post treatment (Table 7). Post treatment radiographic evaluations showed a partial filling of the radiolucent defects on the femoral head with an increase of subchondral sclerosis and partial remodeling of the shape of femoral head and neck. However, the persistence of the femoral head collapse and the incongruity of the coxo-femoral joint was still present.

Experimental research and clinical studies on human osteonecrosis of the femoral head have shown that necrotic bone tissue can be replaced by active bone tissue, but the osteogenic potential for repair is low in the case of osteonecrosis. For this reason we have excluded from this study dogs with grade IV and V according to Ljunggren's scale (Ljunggren, 1967). Many authors have considered that the grounds for insufficient bone remodeling can be linked to the small number of progenitor cells present in the femoral head (Hernigou et al., 1999; Hernigou et al., 2016). A possible explanation for the therapeutic effect of the bone marrow implant is that BM-MSCs are responsible for the production of angiogenic cytokines, which stimulate the processes of neoangiogenesis with consequent improvement of the osteogenesis. Angiogenesis allows the formation of new vessels by sprouting or dividing pre-existing vascular structures following metabolic stresses in hypoxic tissues and improving tissue capillarization. It involves the interaction of endothelial progenitor cells, pericytes, growth factors and components of the cellular matrix. Blood vessels are essential for the repair of the necrotic femoral head because they bring nutrients and allow the removal of waste substances (Wang et al., 2013). Hernigou et al. (1999) demonstrated that BM-MNCs are able to induce the formation of new blood vessels from endothelial cell progenitors or from hemangioblasts present in this cellular fraction.

To support this hypothesis, Yan Z. et al in 2009 conducted a study about the survival and differentiation of MSC transplanted in a dog in which the osteonecrosis of the femoral head was reproduced surgically. Patients were treated with autologous BM-MSCs implantation and physiological saline for the control group. The implanted cells were labeled with a green fluorescent protein, in order to monitor the transplant. The immunohistochemistry of the excised femoral heads demonstrated the presence of labeled MSCs at the necrosis site 12 weeks after implantation. Furthermore, they showed an increase in trabecular bone volume compared to the control group. Their studies showed that MSCs from the transplanted bone marrow could survive, proliferate and differentiate directly into osteoblasts, and that they also contributed to the acceleration of the repair process (Yan Z. et al., 2009). Management of avascular necrosis of femoral head can be grouped into three main categories: non-surgical management, head and neck ostectomy and prosthetic hip replacement. The non-surgical management (rest period with NSAD's treatment) is often ineffective. However, in human medicine, has been demonstrated that head preserving procedures which aims at decompression and revascularization of the femoral head have given encouraging results in pre-collapse stages (Mohanty et al., 2017). In light of these considerations, the authors preferred not to include a grade higher than III according to Ljunggren's scale (Ljunggren, 1967) in this study. The core decompression is the most popular revascularization procedure which reduces the intra-osseous pressure caused by interstitial edema, improves vascularity, enhances bone healing and therefore relieves pain. It can be achieved by drilling a large single tract into the femoral head or by drilling multiple drill holes (Mohanty et al., 2017). The mechanical activity obtained by drilling the non-vital bone could be involved in the revascularization process. Moreover, the results of the study by Dahners

& Hillsgrove (1989) suggest that a drill hole in an avascular bone, provides a path for rapid vascular invasion and quickly result in new bone formation. The combination of drilling the non-vital bone and cell therapy could be a rational therapeutic strategy to treat LCP: it combines the mechanical revascularization given by the single drilling and the osteogenesis power of BM-MNCs at the level of the head.

Table 7. LCP disease group; FU: follow up

FU	Description	1 Lucy	2 Mirò	3 Leo
Time 0	Grade of lameness	4	2	3
	Grade of Manipulation	4	3	3
	Joint Ectasia	1	1	1
	Joint Hypertermia	1	1	1
	Joint Swelling	1	1	1
10 days	Grade of lameness	3	2	3
30 days	Grade of lameness	0	1	1
6 months	Grade of lameness	0	0	0
	Grade of Manipulation	0	0	0
	Joint Ectasia	0	0	0
	Joint Hyperthermia	0	0	0
	Joint Swelling	0	0	0

CONCLUSIONS

In this preliminary study, the application of autologous cell therapy in dogs is well tolerated and requires a minimally invasive approach providing a full clinical improvement and a partially radiographical amelioration in dogs affected by Ununited Anconeal Process and Legg-Calvé-Perthes disease; for the UAP group, in addition to cell therapy, proximal ulnar osteotomy was performed. We cannot exclude that the biomechanical changes obtained by dynamic ulnar osteotomy could contribute to the clinical outcome.
In the LCP group, the injection of BM-MNCs was performed through a shaped drill hole: we performed only a single mini-tunnel drilling into the femoral head. As discussed previously, drilling a non-vital bone could induce an active revascularization and new bone formation. In our preliminary study, even if a single drilling micro-hole was made, is not possible to exclude that this minimal mechanical action has created the conditions for the improvement of the healing process.
The results obtained, allow the authors to consider this technique as a valid alternative therapy, especially when the owner refuses standard treatments (as for the LCP disease group). The technique allows a good remission of clinical signs and, moreover, represents a minimally invasive treatment compared to conventional ones. The use of Ultrasonography guidance in the LCP group could represent a non-invasive and safe way of administration avoiding the use of x-ray, and allowing a good visualization of the pathologic bone area. Further prospective studies are required to confirm whether the proposed techniques are associated with a durable clinical improvement.

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