Xenogeneic Mesenchymal Stem Cells in the Formation of Hyaline Cartilage in Osteochondral Goat Failure


ABSTRACT

Background: Osteochondral knee failures are among the most common causes of disability among the elderly human population and animal athletes. The xenogeneic transplantation of mesenchymal stem cells is a questionable therapeutic alternative that, despite the low expression of Major Histocompatibility Complex type II by these cells, still has relevant uncertainties about the safety and clinical efficacy. The main objective of the present study was to investigate whether the xenogeneic transplantation of mesenchymal stem cells induces hyaline cartilage formation, without histopathological evidence of rejection, in osteochondral failures of goats.

Materials, Methods & Results: Five female goats were used, submitted to three surgical osteocondral failures in the right knee, treated with xenogenic mesenchymal stem cells of dental pulp, xenogenic platelet-rich plasma and hemostatic sponge of hydrolyzed collagen, respectively. The lesions were evaluated after 60 days of treatment, aiming to identify the presence of hyaline cartilage or fibrocartilage and the subchondral bone pattern (regenerated or disorganized). Transplantation of xenogenic mesenchymal stem cells induced predominant formation of hyaline cartilage ($P < 0.05$), with no histopathological evidence of inflammation when compared to the other treatments. Therapies with xenogeneic platelet-rich plasma and hemostatic sponge of hydrolyzed collagen induced greater formation of fibrocartilaginous cartilage, with no significant difference between them ($P > 0.05$). Macroscopically, the lesions of the stem cell treated group showed formation of firm repair tissue, opaque staining, integrated with adjacent cartilage and with the failure filling almost completely. The groups treated with PRP and hemostatic sponge of hydrolyzed collagen presented, on average, partial filling of the lesion, with irregular shape and darkened coloration.

Discussion: The absence of macroscopic and histopathological evidences of an inflammatory process on the surface and in the internal portion of the osteochondral lesions treated with xenogeneic stem cells, probably due to the low expression of Major Histocompatibility Complex type II by these cells, which would theoretically induce low rejection response. Such observations are of great importance, since graft-versus-host disease syndrome is a serious condition, responsible for the low therapeutic efficacy with transplantation of cells or grafts in humans. The formation of fibrocartilage, although without macro and microscopic evidence of degeneration or necrosis, in the osteochondral failures treated with PRP and hemostatic collagen sponge suggest that paracrine factors of the local microenvironment of the osteochondral failure are possibly responsible for the formation of fibrocartilaginous tissue or by inhibition of normal cartilage formation. The fibrocartilage formed in the Plasma and Control groups, contributed to the commitment in the filling of the lesion, contrasting with the almost complete fill of the lesions treated with stem cells. The xenotransplantation of mesenchymal stem cells induced formation of hyaline cartilage and did not promote histopathological evidence of rejection in osteochondral lesions of goat knees. The treatments with PRP and hemostatic sponge of hydrolyzed collagen induced greater formation of fibrocartilaginous cartilaginous surface in the osteochondral failures.

Keywords: mesenchymal stem cells, hyaline cartilage, goat.
INTRODUCTION

Along the last decade, the use of mesenchymal stem cells for the purpose of bone and joint regeneration was intensively investigated [24,31]. In addition to the bone marrow, adipose tissue and dental pulp have been used as source of collection [14,26], and high in vitro plasticity of these cells have been demonstrated in mesodermal and non-mesodermal tissues [20,27].

Xenogeneic stem cell transplantation for treatment of osteochondral lesions is a therapeutic alternative that still has relevant uncertainties about clinical safety and efficacy [26]. However, a recent study has demonstrated the effective contribution of this alternative in murine model [18]. Biodegradable materials such as hemostatic collagen sponges have been used as scaffolds for repair of bone failures, especially in human dentistry [16], but its effectiveness as a carrier for mesenchymal stem cells is unknown.

In addition, platelet-rich plasma is a potent osteoinducer, rich in growth factors capable to increase mitotic activity, activate fibroblasts, inducing angiogenesis, and other features [3,19,21].

The objective of this study was to investigate the effect of xenogenic transplantation of mesenchymal stem cells on the formation of hyaline cartilage, compared to treatments with xenogenic platelet-rich plasma and hemostatic collagen sponge, in osteochondral failures of goats.

MATERIALS AND METHODS

Animals

Two animals were exclusively used as donors of stem cells and blood plasma and five animals to compose the experimental groups of the preclinical study. One male agouti (Dasyprocta primnolopha) was used as stem cells donor and one adult equine, with hematimetric values within the standard of normality for the species [10] and a total platelet count of 200 x 10³/mm³ or greater was used as a donor of blood plasma.

For the preclinical study, five native female goats (Capra hircus), clinically healthy, non-breed, pluriparous, non-pregnant, three years old and average weight of 35.5 ± 1.5 kg were used. The goats were fed with commercial food³ for the species, in addition to voluminous at will with supply of forage brachiaria grass and leguminous leucena, mineral salt and water ad libitum.

Obtaining of mesenchymal stem cells

Mesenchymal stem cells (MSC) were previously isolated from the dental pulp of an agouti [8]. The dental pulp of a lower incisor tooth was washed in sterile saline phosphate buffer solution (PBS²) 0.01 M pH 7.4, supplemented with 3% penicillin-streptomycin³ and mechanically dissociated into the Dulbecco’s Modified Eagle’s (F12 DMEM⁴) culture medium, supplemented with 20% fetal bovine serum³, 1% penicillin-streptomycin³, L-glutamine³ and non-essential amino acids³. The material was incubated at 5% CO₂ and 37ºC and the cells cultured and expanded until the fourth passage. Cells were characterized by analyzing the expression of the CD34, CD14, CD45, CD73, CD79, CD90 and CD105 markers. Subsequently, mesenchymal stem cells were cryopreserved in a medium consisting of 45% F12 DMEM⁴ supplemented with 45% fetal bovine serum³ and 10% dimethyl sulfoxide⁵. Cells remained cryopreserved for two years until thawed in 37ºC water bath, viability was assessed by the trypan blue method and expanded until they reached 80% confluency.

Interaction assay between stem cells and hemostatic sponge

Three aliquots of 1 x 10⁴ cells/mL were cultured with a 3 x 1 mm sample of a sterile hemostatic sponge of hydrolyzed collagen⁶ until they reached 80% confluency. The culture wells were evaluated and photographed to evaluate the interaction between cells and hemostatic sponge. Subsequently, the sponge fragment was removed and the mesenchymal stem cells were trypsinized and evaluated for cell viability [8]. The fragments of the hemostatic sponge were fixed in 5% formaldehyde⁷ alone for 24 h, dehydrated in increasing concentrations of alcohol (30%, 55%, 70%, 88%, 96%), diaphanized in xylol⁸, included in histological paraffin⁹ and sectioned with a rotary microtome¹⁰, adjusted to 4 μm thick. Slices were fixed in glass slide, stained by Hematoxylin & Eosin and analyzed by binocular optical microscope¹¹ for evaluation of cell adhesion.

Obtaining of platelet-rich plasma

The blood was immediately processed according to the methodology described [3]. The concentra-
tion of whole blood platelets was measured, followed by centrifugation of the sample at 600 g for five min. For each 25 μL of platelet-rich plasma obtained, 25 μL of 10% calcium gluconate was added, kept in a water bath at 37°C until the plasma was gelled.

Induction of osteochondral lesions in goats

All animals were submitted to antibiotic therapy with enrofloxacin (2.5 mg/kg) by intramuscular route and 2 h of water fasting and feeding of 6 h. The animals were sedated with intramuscular 2% xylazine (0.1 mg/kg), induced with intravenous propofol (5 mg/kg) and spinal anesthesia were performed with morphine (0.1 mg/kg) associated to 0.5% isobaric bupivacaine in the volume of 0.2 mL, followed by intubation and anesthetic maintenance with Isoflurane diluted in 100% oxygen. The surgical field was delimited on the right femur-tibial-patellar joint. An arthroscopy of the medial parapatellar region was performed, the subcutaneous tissue was incised to access the patella, which was laterally displaced by manual compression to expose the femoral trochlea. With the aid of an orthopedic drill equipped with a 4 mm steel drill, three osteochondral lesions 4 mm in diameter and 6 mm deep were produced, two in the distal part of the femoral trochlea and one in the proximal part. After immediate treatment of osteochondral failure, the joint capsule were closed with single interrupted suture with 2-0 nylon monofilament yarn. Surgical plans were approximated with Sultan interrupted suture plane and skin with single interrupted suture, both using 2-0 nylon monofilament yarn.

After the surgery, the animals were medicated with tramadol hydrochloride (2 mg/kg) by intramuscular route twice daily for five days, enrofloxacin (2.5 mg/kg) by subcutaneous route once daily for 10 days and meloxicam (0.2 mg/kg) by intramuscular route once daily for 5 days. Surgical wounds were cleaned daily with sterile saline solution and topical application of allantoin and chlorhexidine ointment.

Treatments

Each lesion represents an experimental group of treatment, coexisting all groups in each animal, totaling 15 lesions and 5 replicates for each treatment. The animals were treated once in the trans operative, according to the experimental groups: Control group: distal left lesion of the trochlea, treated with filling of the osteochondral failure with 3 mm of sterile hemostatic sponge of hydrolyzed collagen soaked in sterile physiological solution. MSC group: proximal inferior lesion of the trochlea, treated with 10^6 MSC/mL infused into 3 mm of sterile hemostatic sponge of hydrolyzed collagen. Platelet Rich Plasma (PRP) group: distal right lesion of the trochlea, treated with platelet rich plasma at a concentration of 1.3 x 10^4/mm^3 infused in 3 mm of hemostatic sponge.

Histological procedure

After 60 days of treatment, the animals were identified, weighed and submitted to euthanasia by sedation with ketamine (30 mg/kg) given intravenously and after confirmation of unconsciousness, administration of potassium chloride by intravenous route. The lesions were identified, serrated and fixed individually in 10% formaldehyde buffered for 48 h. The samples were decalcified in 20% hydrochloric acid solution for ten days and 5% for five days. The decalcified fragments were processed histologically in a conventional manner [24]. The samples were included in histological paraffin, sectioned with a rotary microtome, adjusted to 5 μm thick. Slices were fixed in glass slide, stained with Hematoxylin & Eosin and analyzed in binocular optical microscope.

Microscopic evaluation

For each osteochondral lesion, the scores were assigned: 0-absence of tissue covering the lesion, 1-formation of fibrocartilage and 2-formation of hyaline cartilage.

Statistical analysis

Statistical analysis was performed with Bioestat® software v. 5.9, applying the Kruskal-Wallis non-parametric analysis of independent samples, followed by the Dunn station median comparison test, adopting a level of rejection of the null hypothesis of 5% (P ≤ 0.05).

RESULTS

Goats remained clinically healthy after induction of osteochondral lesions, without clinical evidence of anorexia, fever and/or apathy. It was identified evident claudication in the affected joint, of moderate intensity, without significant alteration of the cardiorespiratory frequency, according to the physiological patterns for the species [6,11].

A homogeneous stem cell culture was obtained from the 10th day of culture, with fusiform morphology, forming a monolayer on the 22nd day of culture. Flow
Cytometry identified that mesenchymal stem cells were positive for CD34, CD73, CD90 and CD105 markers, and were negative for CD14, CD45 and CD79 markers (Figure 1). After the thawing of mesenchymal stem cells, after two years, 80% of viability was identified.

The culture medium of mesenchymal stem cells with a sterile hydrolyzed collagen sponge fragment identified that the cells adhered to the sponge matrix after 10 days of incubation (Figure 2A and B) and the evaluation of the mean cell viability identified after removal of the fragment was 95%.

The histological evaluation of the same fragment of sponge identified the presence of few mononuclear cells adhered to the substrate of the same, with intact nucleus and loosely condensed chromatin. The cells were sparsely distributed between the pores of the sponge, isolated from each other.

An equine plasma of high platelet concentration and smaller in volume than whole blood was obtained. As the sample was sequentially centrifuged and the haematological and superficial fractions of the plasma were withdrawn, a product exclusively composed of concentrated platelets of $1.3 \times 10^6 / \text{mm}^3$, representing 82% of the initial platelet volume concentration, was obtained but in the volume of 0.4 mL, 10 times lower than the initial one.

After 60 days of treatment, it was observed that the lesions of the stem cells group presented formation of firm repair tissue, opaque staining, integrated with the adjacent cartilage and filling the failure almost in its entirety. Among the failures treated with PRP, on average, there was partial integration of the failure with the adjacent cartilage, with partial and symmetrical filling of the lesion and irregular and depressed lesion surface, with darkened, well circumscribed staining, surrounded by macroscopically healthy hyaline cartilage. The lesions treated with hemostatic sponge of hydrolyzed collagen presented, on average, partial filling of the lesion, uneven articular surface and reddish coloration (Figure 3A and B).

Histopathological differences ($P < 0.05$) were observed between the lesions treated with xenogenic mesenchymal stem cells and the other treatments. In the MSC group there was an average predominance of the presence of hyaline cartilage and a smooth cartilaginous surface in the lesions evaluated. In the lesions of the Platelet Rich Plasma and Control groups, the average prevalence of fibrocartilage was observed in relation to the hyaline cartilage, with an irregular and depressed articular surface (Figure 3C and D). No differences ($P < 0.05$) were observed between the lesions treated with xenogenic platelet rich plasma and hemostatic sponge of hydrolyzed collagen.

The osteochondral lesions of all treatment groups showed regenerated, subchondral bone characterized by the presence of mature lamellar bone, composed of osteocytes and the organization of trabecular bone tissue without the presence of osteoblasts.

It was not identified histopathological characteristics of inflammatory activity and necrosis in none of the treated osteochondral failures.

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**Figure 1.** Flow cytometry of mesenchymal stem cells in the fourth passage, derived from the dental pulp of agoutis (*Dasyprocta primmolo*pho). A- Cells of fibroblastoid morphology and basophilic cytoplasm [Giemsa, Barra= 100 μm]. B, C, D- Negative expression for hematopoietic markers. E, F, G, H- Positive expression for markers commonly used for mesenchymal stem cell cultures.

**DISCUSSION**

The choice of goat as an animal model for the present study is due to the anatomical dimensions of the knee, similar to the dimensions of the human knee [5], allowing adequate exposure of the articular surface, favoring surgical manipulation.

The animals resumed spontaneous walking and showed appetite soon after anesthetic recovery. Similar results were described [7], using an equivalent anesthetic protocol, reiterating the effectiveness of the therapeutic pain management adopted in this study. The association between spinal local anesthesia associated...
with morphine, probably contributed to a postoperative period without clinical complications, associated with analgesic, antibiotic and anti-inflammatory use, as previously proposed [34].

The diameter chosen for the osteochondral lesions in this experiment was 4 mm in diameter, as recommended [15,34], according to them, lesions smaller than 3 mm may predispose to spontaneous repair by fibrocartilaginous tissue.

The use of hemostatic sponges such as scaffold for studies with repair of bone failures has already been described in previous research, both in humans and in animals [4,12], corroborating to justify the choice of this substrate for the model introduced. In addition, it is widely used in humans, possessing authorizations from governmental agencies of surveillance for commercialization [32], and is then adopted as the gold standard in this study.

The in vitro biocompatibility assay between mesenchymal stem cells and a fragment of the hemostatic sponge of hydrolyzed collagen demonstrated that there was no apparent cytotoxic effect on the cells, since the cultivation cells adhered to the fragment were identified, with average viability of 95% identified by the trypan blue exclusion technique (Figure 2A and B). This technique is used to determine the number of viable cells in a cell suspension. It is based on the principle that viable cells (with intact cell membrane) are able to exclude trypan blue, which crosses the membrane, from its cytosol. While unviable cells (with compromised cell membrane), such as in advanced apoptotic state, are unable to exclude it [9].

Cells adhered to the substrate of the fragment were identified after histological processing, denoting that the substrate matrix provided an environment favorable to cell adhesion. Corroborates this observation, a contemporary study that inferred that cell-matrix interaction influences cell viability, which may induce apoptosis or not, depending on the chemical nature of the material used [37].

However, it was not observed a high concentration of cells adhered to the hydrolyzed collagen sponge, fact that remains incomprehensible by the authors of the present study. It is believed that the pore size of the sponge may have favored the low retention of cells in the substrate, since the cell viability observed in the biocompatibility assay was 95%. Previous studies contribute to this hypothesis by indicating that the pore size and pore interconnectivity of a scaffold is a determinant factor for cell fixation, and should be compatible with the dimensions of the cell population used [23,29]. However, it was not performed evaluation of the hemostatic sponge of hydrolyzed collagen by scanning electron microscopy, and it was not possible to infer precisely the interference of the physical structure of the scaffold on the cellular retention. Complementary studies are needed to conduct this analysis, in addition to the reduction test of the bromide test 3-[4,5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium (MTT) to blue of formazan, as proposed in contemporary studies [1,13].

In the in vitro biocompatibility study, it was evident the alteration of the cellular morphology of MSC. This effect was expected, since MSCs are adherent mononuclear cells that assume fibroblastoid or fusiform morphology only when on a regular surface in the culture medium like in controlled incubation conditions [3]. Since the porous surface of the irregular hemostatic sponge, MSC was visualized in mononuclear oval form.

After 60 days of observation, they were not evident macro or microscopic traces of the hemostatic sponge inside the osteochondral failures evaluated, corroborating with previous reports of biocompatibility and resorption in vivo [30].

It was not observed macroscopic and histopathological evidence of inflammatory process on the surface and in the internal portion of the osteochondral lesions analyzed in this study. Such observations are of great importance, since graft-versus-host disease syndrome is an acute or hyperacute condition, responsible for the low therapeutic efficacy with transplantation of cells or grafts in humans [35]. According to these authors, approximately 40% to 50% of human patients undergoing bone marrow transplantation develop such a clinical condition [17].

It was expected absence of response macro and histopathological of rejection in osteochondral failures treated with hydrolyzed collagen sponge of porcine origin, since it is already commercially used in humans, as observed in contemporary studies [32,33].

However, the inexistence of tissue evidence of rejection in the osteochondral failures treated with PRP
and MSC is considered a positive preliminary datum, although it can not be inferred that this result is reproduced in other animals, under other methodological conditions of study.

In the present study, osteochondral failures treated with PRP and hydrolyzed collagen sponge induced formation of fibrocartilaginous cartilage, without macro and microscopic evidence of degeneration or necrosis. A similar result was obtained [7], using PRP in osteochondral lesion of sheep knee, in which there was predominance of fibrocartilage on the articular surface. Paracrine factors of the local microenvironment of the osteochondral failure are possibly responsible for the formation of fibrocartilaginous tissue or inhibition of normal cartilage formation [34]. The greater occurrence of the fibrocartilaginous surface in the Platelet Rich Plasma and Control groups contributed to the commitment in the filling of the lesion, observed in the macroscopic evaluation, where it was verified integration and partial filling of the failure, in an irregular and depressed form, with coloration varying darkened to reddish, respectively.

The PRP has been used for about 20 years with the objective of accelerating the healing and regeneration of bone resulting from surgical procedures in humans. The plasma has a high concentration of platelets in a reduced plasma volume, besides growth factors that behave like molecules of cellular adhesion in the processes of epithelial migration, osteoinduction and in the formation of bone matrix in the connective tissue. Due to the high concentration of growth factors that stimulate cell migration and tissue remodeling, it is believed that the xenogenic use of PRP may be responsible for the greater formation of fibrocartilage, as suggested in previous studies [28,38]. However, as there was no histopathological inflammatory response, new studies using immuno-histochemical techniques to elucidate the influence of xenogenic PRP on osteocartilaginous tissue are suggested.

The effect of MSC on bone tissue repair had been described in contemporary studies [2,25,33]. According to these authors, MSCs are multipotent somatic progenitor cells, capable of originating mesodermal and not mesodermal tissues. The differentiation of these cells in vitro into osteoblasts has been induced in several previous studies [3,8]. The xenogenic MSCs that remained adhered to the hemostatic sponge induced greater formation of hyaline cartilage (P < 0.05) when compared to the other treatments. In addition, the osteochondral failures treated with these cells did not present macro and histopathological evidence of degeneration, inflammation and/or tissue necrosis.

The MSCs do not express significant levels of the class II histocompatibility complex and do not have costimulatory molecules, such as B7-1 and B7-2, theoretically exhibiting a low rejection response [36]. However, MSCs express MHC class I and some lineages express binding CD40 and CD40 molecules, which may in some circumstances be identified by Toll Like Receptors which in turn could induce immunogenic activation [22]. Given such controversies, preclinical studies, as presented here, are of great relevance in assessing the clinical and histopathological response of MSCs in osteochondral failures.

It was considered the maintenance of joint surface integrity and neoformation of hyaline cartilage in the knee lesions of the animals treated in this study very satisfactory, since in the repair of osteochondral lesions it is expected that there is no significant recruitment of osteocartilaginous tissue, so as not to inducing fibrocartilage formation, which could compromise joint function [38].

In this study, a presence of mature subchondral bone was evidenced in all the treatments performed, demonstrating that there was a satisfactory repair of the deep part of the osteochondral lesion against the treatments used, without evidence of deleterious effect in the cartilaginous surface.

CONCLUSIONS

Mesenchymal stem cell xenotransplant induced hyaline cartilage formation and did not promote histopathological evidence of rejection in osteochondral lesions of goat knees.

The sterile hemostatic sponge of hydrolyzed collagen presented biocompatibility with the xenogenic MSCs, preserving its viability.

The treatments with PRP and hemostatic sponge of hydrolyzed collagen induced greater formation of fibrocartilaginous cartilaginous surface in the osteochondral failures. Macroscopically, it was evident filling of the inferior lesion for other treatments and discrepant coloring of normal cartilage.
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