Histological and immunohistochemical evaluation of two cell therapy protocols in equine suspensory ligament repair

[ Avaliação histológica e imunoistoquímica do reparo do ligamento suspensório equino tratado com dois protocolos de terapia celular ]

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ABSTRACT

This study aimed to histologically evaluate the quality of tissue repair in equine suspensory ligament treated with two cell therapy protocols. All four limbs of six animals were operated simultaneously to remove a fragment in each ligament using a skin biopsy punch. Two days later, intralesional injections were performed using bone marrow mononuclear fraction (BM group), cultivated cells derived from adipose tissue (AT group), saline (positive control group), or no treatment (negative control group), in such way that each horse received all treatments. After sixty days biopsies were performed for histological analysis (H & E, Masson’s trichrome and picrosirius red) and immunohistochemistry analysis (collagen type III). Histological findings (H & E and Masson’s trichrome), birefringence intensity (through picrosirius) and collagen type III expression (through immunohistochemistry) were analyzed. Samples from treated groups had better birefringence intensity (P= 0.007) and fiber alignment scores were superior compared to controls, though not statistically significant (P= 0.08). Presence of inflammatory cells and intense staining for collagen type III occurred in all groups demonstrating an active healing process. In conclusion, both protocols resulted in improvement of tissue repair indicating their potential to be used as an adjuvant treatment of equine suspensory ligament disorders.

Keywords: stem cell therapy, desmitis, horse

RESUMO

Este estudo teve como objetivo a avaliação histológica e imunoistoquímica do reparo do ligamento suspensório equino tratado com dois protocolos de terapia celular. Os quatro membros dos seis animais do experimento foram submetidos a procedimento cirúrgico em que um fragmento de cada ligamento foi retirado, utilizando-se punção de biópsia. Dois dias após o procedimento, aplicações intraleisionais foram realizadas, por meio de aspirado de medula óssea (bone marrow-BM), células mesenquimais derivadas de tecido adiposo (adipose tissue-AT), solução salina (positive control group-PC) ou controle (negative control-NC). Após 60 dias, biópsias foram retiradas da região do reparo dos ligamentos e foram submetidas à análise histológica (HE, tricrômio de Masson, picrosírius red) e imunoistoquímica (colágeno tipo III). Diferentes variáveis histológicas (HE e tricrômio de Masson), a intensidade de birrefringência das fibras colágenas (picrosírias red) e a expressão de colágeno tipo III foram avaliadas. Os grupos tratados apresentaram maior birrefringência (P=0,007) e alinhamento de fibras (P=0,08) comparados ao controle, para o qual o resultado não se mostrou estatisticamente significativo. Achados histológicos e imunoistoquímicos demonstraram um processo ativo de reparo tecidual em todos os grupos. Concluiu-se que os dois protocolos de terapia celular apresentaram melhoria no reparo tecidual, demonstrando potencial terapêutico adjuvante no tratamento de afecções do ligamento suspensório equino.

Palavras-chave: terapia celular, desmite, equino
INTRODUCTION

Suspensory ligament desmitis affects horses from different breeds, ages, and equestrian activities, causing prolonged illness and reducing the performance of athlete horses (Dyson, 1994; Dowling et al., 2000; McClure et al., 2004). Several therapies have been used for desmitis, ranging from the use of anti-inflammatory medication to extracorporeal shockwave therapy. Despite the new techniques for early diagnosis and treatment, the risk of recurrence of ligament lesion can reach up to 93% (Dahlgren, 2007). Such high risk of recurrence leads to an increase in interest on ligament physiology and disease etiology (Dahlgren, 2007) leading to the development of new disease-modifying treatments, which would result in a more physiological repair tissue (Nixon et al., 2008).

Cell therapy aims to repair, replace, and increase the biological functions of injured tissue through the use of autologous or allogeneic stem cells. Stem cells can potentially differentiate in tissue-specific cells and produce a functional extracellular matrix (Sutter, 2007) and modulate inflammation and cell apoptosis (Nixon et al., 2008).

The types of cells that are most commonly used to treat musculoskeletal disorders in horses are mesenchymal stem cells derived from the bone marrow or from adipose tissue (Gutierrez-Nibeyro, 2011). Despite the widespread use of stem cell therapy, its clinical use requires further studies aiming to understand the biological properties of these cells and their interaction with the microenvironment (Chen et al., 2011). A large number of studies have demonstrated the benefits of the use of cells derived from bone marrow or adipose tissue in the treatment of experimental equine tendinitis, but studies with their use in the treatment of experimental suspensory desmitis are limited (Alves et al., 2011; Carvalho et al., 2011; Crovace et al., 2010; Oliveira et al., 2011; Schnabel et al., 2009).

The aim of this study was to compare, through histological and immunohistochemistry analysis, the quality of the repaired tissue in injured equine suspensory ligaments that were treated using two cell therapy protocols: 1) the intraslesional injection of the mononuclear cell fraction of autologous bone marrow or, 2) the injection of laboratory cultivated autologous adipose tissue-derived cells.

MATERIAL AND METHODS

This study used archival suspensory ligament samples that were obtained from an experiment conducted in 2007 (Soares, 2008). This study was approved by the Animal Ethics Committee of Universidade Federal de Minas Gerais (CETEA- UFMG, #19/07).

Six healthy horses were selected based on the results of clinical exams, laboratory test, and ultrasonographic exams. One male and five female horses were used, aged 10.5±3.5 years, and weighing 375±75kg (mean ± SD).

Horses were submitted to a surgical procedure for the induction of lesion as previously described (Soares et al., 2010). Lesions were simultaneously induced in all four suspensory ligaments of each horse. Full-thickness circular lesions were concomitantly produced in the suspensory ligaments of each horse using a skin biopsy punch (Soares et al., 2010). Briefly, horses were submitted to general anesthesia and suspensory ligaments on both the fore and hind limbs were surgically exposed. An axial circular lesion was made 3cm proximal to each bifurcation, using a 0.6-centimeter diameter punch (Punch drill, Richter LTDA, Brazil) that was rotated until it reached the third metacarpal/metatarsal bone. After the lesions were induced, the skin was sutured, surgical wounds were treated with topical rifamycin and the limbs were covered with bandages. The postoperative therapy consisted of antibiotics and anti-inflammatories as described (Chen et al., 2011).

Mesenchymal cells derived from adipose tissue were collected, processed, and cultivated as previously described (Carvalho et al., 2011). Briefly, eight days before the induction of the lesions, the horses were sedated and local anesthesia was administered. Using aseptic techniques, a 10cm long skin incision was made abaxial to the base of the tail. Then, a fragment (3 x 0.5 x 0.5cm) of adipose tissue was removed from the subcutaneous tissue and immediately placed in a tube containing Roswell Park Memorial Institute (RPMI) medium. These fragments were cut into pieces in a laminar flow hood, digested with collagenase and incubated in

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culture medium at 37°C with 5% CO₂. Cells were isolated and placed in culture flasks, and after the cultures reached 80% confluence, secondary cultures were prepared, which were observed daily until use.

The bone-marow derived mononuclear cells were collected and processed as described (Oliveira et al., 2011). Briefly, two days after lesion induction, the horses were restrained in standing stocks and sedated as described. The appropriate skin region of each horse was shaved and aseptically prepared and local anesthesia with 2% lidocaine was done. Bone marrow was aspirated with an appropriate cannula (Jamshidi cannula, Euromed Catheters, Brazil) that was inserted into the 4th or 5th sternebra using ultrasound guidance. Bone marrow was collected into a 20mL syringe containing 1mL of heparin (5.000IU/mL). The sample was subjected to centrifugation at 1500rpm for 10 minutes to separate the mononuclear cell fraction. This fraction was collected for immediate application to the injury site.

Two days after lesions were surgically induced, intrallesional applications of saline or treatments were performed. This procedure was done with the horse standing, under sedation, after a high palmar/plantar nerve block using 2% lidocaine. With ultrasound guidance, 0.8mL of the bone marrow cell suspension was aseptically injected into the lesion site (BM group). An identical volume of a solution containing 1 x 10⁶ cells from adipose tissue (AT group) or a sterile 0.9% NaCl solution (PC group) was injected. A limb was left with no treatment as a negative control (NC group). All animals were subjected to all treatments and four experimental groups of six limbs each were established. The treatments were applied such that the negative controls were always in the left hind limb and the other treatments were distributed equally among the other limbs.

Sixty days after treatment the horses were subjected to another surgical procedure (under general anesthesia as described above) to obtain biopsies. After exposing the suspensory ligaments, a full-thickness fragment of approximately 2cm² that included the scar tissue was removed from each limb of each horse. Samples were immediately immersed in 10% buffered formalin for 48 hours and routinely processed for paraffin embedment.

Five-micrometer thick sections were cut from the paraffin blocks, mounted on slides and stained using the routine hematoxylin-eosin (H & E), Masson’s trichrome, and Picrosirius Red staining techniques. All of the slides were analyzed using light microscopy by the same researcher, who did not have prior knowledge of the groups to which they belonged.

The morphology of the nuclei, the presence of inflammatory infiltrates, the extent of vascularization and the presence and arrangement of collagen fibers were analyzed in sections stained with H & E and with Masson’s trichrome. For this last variable, a semi-quantitative descriptive analysis was performed, to establish scores ranging from 0 to 5, where grade 0 indicated the absence of parallelism and grade 5 indicated maximal parallelism. The number of blood vessels, fibroblasts and inflammatory cells per field were determined for each sample using an image analyzer Software (Image-Pro Plus, Media Cybernetics, United States). Twenty fields of each slide were evaluated, and the images were captured in 10 x and 40 x objectives of an Olympus BX43 microscope.

To assess the organization level of the collagen fibrils and consequent tissue repair, the intensity of birefringence under polarized light for samples stained with picrosirius red was evaluated. The birefringence level was graded from 0 to 3, where grade 0 was an absence of tissue birefringence and grade 3 represented maximum tissue birefringence.

For immunohistochemistry analysis, the sections were mounted on gelatin-coated slides. The tissue sections were subjected to deparaffinization using successive xylene washes, a graduated series of absolute alcohol solutions of decreasing concentration and running water. After hydration, the sections were incubated at 37°C in a 0.1% pepsin solution (from porcine gastric mucosa 0.7 FIP-U/mg, Merck Millipore, Germany) pH 1.7, for 30 minutes. The endogenous peroxidase was blocked with methanol (Methanol P.A., Cromoline fine chemicals, Brazil) and 9:1 dilution of peroxide solution (Hydrogen Peroxide
A.p, Synth, Brazil). Endogenous biotin was blocked with normal equine serum (Normal Horse Serum, Vectastain® Elite ABC Kit-VECTOR Laboratories, Canada) and phosphate buffer saline (PBS) solution (1 drop: 3nL).

The sections were incubated with a mouse monoclonal anti-human collagen type III [Mouse Anti Human Collagen Type III (HWD 1.1) IgG, Bio-Genex, Canada] primary antibody (1:50) in a humid chamber at room temperature for 1 hour, and then incubated with biotinylated horse anti-mouse (Horse biotinylated Anti-IgG Mouse, Vectastain® Elite® -ABC-Kit VECTOR Laboratories, Canada) secondary antibody—(1: 100) at room temperature for 1 hour.

The antibodies were diluted in a commercial diluent (Antibody Diluent with background reducing component-DAKO, Brazil) to reduce the background staining. For negative controls, the samples were incubated with the antibody diluent in place of the primary antibody. Finally, the slides were processed using an ABC kit (Vectastain® Elite® ABC – VECTOR Laboratories, Canada) that employs dianinobenzidine (DAB) as the substrate chromogen.

For immunostaining analysis 5 fields were selected across the length of the slide, with the fifth field at the center. The fields were photographed using 10x and 40x objectives of an Olympus BX43 microscope. The percentage of the immunostained area of each of the five fields was determined using the image analyzer Software Image-Pro Plus (Media Cybernetics, United States).

A repeated measure of ANOVA and ANOVA on ranks were used to analyze the quantitative and semi-quantitative data, respectively. The Student-Newman-Keuls test was used for comparisons among the groups, and the Spearman correlation test was used to verify the correlation between the scores for tissue birefringence and for the alignment of collagen fibers. The significance level of P< 0.05 was set for all of the tests.

RESULTS AND DISCUSSION

The healing area in most of the samples was characterized by high cellularity from the presence of fibroblasts with nuclei of various shapes (predominantly ovoid and spindle-shaped), little or no fiber parallelism (score of 0 to 2), the presence of inflammatory infiltrates and neovascularization. Although a few acute phase characteristics were verified in some specimens (i.e.hemorrhage, vascular congestion, and necrosis) all of the samples exhibited the predominant characteristics of a subacute inflammatory process and ongoing tissue remodeling. Similar findings were observed in a study using a collagenase-induced tendinitis model at 48 days after lesion induction (Barreira et al., 2008). Similar results were not observed at 60 days (Schnabel et al., 2009) or 120 days (Oliveira et al., 2011).

Mononuclear cells were the predominant cell type in the inflammatory infiltrate in all groups. Similar results have been reported in a collagenase-induced tendinitis model treated with bone marrow-derived mononuclear cells (Barreira et al., 2008). In the present study, no statistical differences were observed in the number of inflammatory cells among the groups. Similar results were observed in studies using the tendinitis model, where the studies failed to find any beneficial effect of cell therapy in reducing the number of inflammatory cells in the scar tissue (Schnabel et al., 2009, Carvalho et al., 2011). In addition, intense fibroplasia and neovascularization were observed in all of the groups, with no difference in the number of fibroblasts or blood vessels. Increase in tissue cellularity and angiogenesis was also observed in tendons treated with cell therapy in the collagenase-induced tendinitis model but the lower level of neovascularization was reported in the groups treated with adipose tissue-derived mesenchymal cells (Carvalho et al., 2011) and with bone-marrow aspirates (Oliveira et al., 2011) compared to untreated groups. In this experiment, although inflammatory infiltrates were observed, all the samples demonstrated the predominant characteristics of the proliferative phase and the remodeling phase of inflammation.

The biopsies from the treated groups displayed better fiber alignment than those of the control groups, although the differences between the grades were not significant (P= 0.08) (Figure 1). Similar improvement was obtained in other studies treating injured equine tendons with bone-marrow derived mononuclear cells (Crovace et al., 2010; Oliveira et al., 2011;
Schnabel et al., 2009) or adipose tissue-derived mesenchymal cells (Nixon et al., 2008). The authors believe that such results suggest improvement in ligament healing with the use of cell therapy, and that future studies with a larger number of horses may be able to demonstrate a statistical difference between treated and control groups.

Masson’s trichrome dye was used to detect collagen deposits in all of the samples, which were characterized by blue staining. Muscle tissue was easily observable because of its red staining in this technique, facilitating the interpretation of the extent of parallel fiber alignment, once differentiation collagen from muscle fibers is more difficult in H & E stained samples. Masson’s trichrome staining revealed variable amounts of muscle tissue in the samples from the different groups, which was expected once the amount of muscle tissue in the suspensory ligament varies according to the age, breed, genetics and physical activity of the horse (Halper et al., 2006; Soares, 2008).

The lesioned area was clearly identified in most of the sections stained using the picrosirius red technique. Lesions were characterized by the low birefringence and green staining observed using polarized light. The green staining is believed to be associated mainly with collagen type III fibers (which are prevalent in the sites of injury), while red/pinkish or yellow staining accompanied by high levels of birefringence is observed in the regions with predominantly type I collagen (Montes, 1996; Ortega et al., 2003). It should be noted that changes in birefringence occur in areas with high cellularity and areas where the pH has been changed by the cells present in inflammatory infiltrates (Alves et al.; 2001). In this study there was one occurrence of mononuclear cells infiltrated at the lesion site, the presence of the cells might have influenced the birefringence presented.

The ligaments surrounding the scar tissue were stained predominantly in yellow and exhibited thick bands of intense birefringence, suggesting a high level of tissue organization and the alleged predominance of type I collagen. Birefringence was not observed in the areas of the samples that consisted of muscle tissue. The grades of birefringence intensity did not differ between the treatment groups (BM and AT) but were significantly increased (P= 0.007) in both treatment groups compared with the control groups (Figure 1).

The birefringence intensity (higher values reflecting better fiber organization and the possible predominance of type I collagen) was consistently found to be directly correlated (r= 0.66, P= 0.0017) with the alignment of collagen fibers observed in the H & E stained sections (Figure 1B), which are the features associated with better fiber organization and the evolution of the ligament repair process. These results are consistent with those of other studies, which reported that the birefringence intensity is directly related to the level of fiber organization, once only organized molecules exhibit high birefringence (Rodriguez et al., 2003). Our findings corroborate the results obtained in a tendinitis model, in which the tendons treated with adipose-derived mesenchymal cells displayed better birefringence under polarized light (Nixon et al., 2008).

Intense and diffuse immunostaining for collagen type III was observed in most of the samples, which was observed in induced (Oliveira et al., 2011) and naturally occurring tendinitis (Satomi et al., 2008). No significant difference in the collagen type III immunostaining was observed among the groups. Similar results were observed in a collagenase-induced tendinitis model 60 days after treatment with adipose tissue-derived cells (Carvalho et al., 2011) and 120 days after treatment with bone marrow-derived cells (Oliveira et al., 2011). A significant decrease in collagen type III immunostaining in treated groups compared to controls was only observed at 150 days after treatment with adipose tissue-derived cells in the tendinitis model (Carvalho et al., 2011; Nixon et al., 2008).

The strong expression of collagen type III exhibited in the present study indicated that an active remodeling process was ongoing even in samples subjected to cell therapy. Other studies using a tendinitis model showed the increased expression of collagen I in the treated tendons compared with the untreated ones (Oliveira et al., 2011). In the present study we could not establish an effective technique for collagen I immunohistochemistry using our paraffin-embedded samples.
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Figure 1. Histological analysis of equine suspensory ligaments for pricosirius red staining. Suspensory ligaments were injured surgically and treated after 48 hours with either mononuclear cell fraction of bone-marrow aspirate (BM), adipose tissue-derived cells (AT), saline (positive control-PC), or no treatment (negative control). A) Intensity of birefringence analyzed under polarized light for samples stained with pricosirius red. A significant difference for birefringence was observed between the treated groups (AT and BM) compared to the controls (NC and PC). The data presented are the means and standard errors. The data in bars indicated by identical letters do not differ statistically. B) Correlation between the grades for the alignment of collagen fibers (H&E) and the intensity of birefringence observed using polarized light. The birefringence intensity was found to be directly correlated (r=0.66, P= 0.0017) with the alignment of collagen fibers observed in the H & E stained sections.

In this model of suspensory ligament injury we avoided the use of collagenase to produce four controlled homogenous lesions by concomitantly operating the four limbs of each animal. This model was adopted because of the difficulty in obtaining a large number of animals for this type of experiment. As previously observed (Soares et al., 2010), this model was well tolerated by horses, and the animals did not present significant signs of pain or lameness. In addition, analysis of the suspensory ligament is considered particularly difficult because of the presence of muscle tissue (McClure et al., 2004), and consequently, the direct comparison of the contralateral limbs in the same horse as performed in the present experiment effectively simplified the data interpretation.

There is some concern about producing more than one lesion per animal when studying cell therapy due to the existence of the homing phenomenon, in which the implanted cells migrate to other inflammation sites (Chen et al., 2011). In this particular situation, the cells derived from the bone marrow or adipose tissue could migrate from a treated limb to a control limb. However, the differences in the level of tissue repair among the treated and control groups in this experiment indicated that the homing phenomenon did not interfere with our model.

Despite the short period allowed for healing (62 days), it was possible to observe the improvement in tissue organization in samples that were treated with cells derived from bone marrow or adipose tissue compared to controls, as previously observed (Carvalho et al., 2011; Schnabel et al., 2009). These findings were comparable to those described by Nixon et al. (2008) and Alves et al. (2011), who noted that the beneficial effect of cell therapy might be more related to maintaining or stimulating tissue organization, than to tissue regeneration.

Despite the positive results, some limitations in this study should be noted. We analyzed archival paraffin-embedded samples from a study performed in 2007. In the original study it was not possible to characterize the adipose tissue-derived cells as mesenchymal stem cells. Additionally, the concentrations of the mononuclear cells in the bone marrow aspirates were not determined and were not standardized. Although it is not clear if the cells used in this experiment were indeed stem cells, our findings demonstrated that both tested protocols resulted in improved tissue repair indicating their potential use in clinical cases. This study provides additional information on the potential beneficial effect of cell therapy to improve healing of musculoskeletal lesions in horses.
CONCLUSIONS

Although an active remodeling process was still occurring in all samples of the study (treated and controls), both cell treatment protocols resulted in improvement of tissue repair. This study provides additional information on the beneficial use of cell therapies as an adjuvant on the treatment of equine suspensory ligament disorders. Further studies evaluating the effects of cell therapy in suspensory ligaments in a long-term (i.e. 6 months to 1 year after treatment) are warranted to evaluate the effects of this therapy in tissue remodeling and function.

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