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Original article

Comparison of autologous bone marrow and adipose tissue derived mesenchymal stem cells, and platelet rich plasma, for treating surgically induced lesions of the equine superficial digital flexor tendon

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ABSTRACT

Several therapies have been investigated for equine tendinopathies, but satisfactory long term results have not been achieved consistently and a better understanding of the healing mechanism elicited by regenerative therapies is needed. The aim of this study was to assess the separate effects of autologous bone marrow (BM) and adipose tissue (AT) derived mesenchymal stem cells (MSCs), and platelet rich plasma (PRP), for treating lesions induced in the superficial digital flexor tendon (SDFT) of horses. Lesions were created surgically in both SDFTs of the forelimbs of 12 horses and were treated with BM-MSCs (six tendons), AT-MSCs (six tendons) or PRP (six tendons). The remaining six tendons received lactated Ringer's solution as control. Serial ultrasound assessment was performed prior to treatment and at 2, 6, 10, 20 and 45 weeks post-treatment. At 45 weeks, histopathology and gene expression analyses were performed. At week 6, the ultrasound echogenicity score in tendons treated with BM-MSCs suggested earlier improvement, whilst all treatment groups reached the same level at week 10, which was superior to the control group. Collagen orientation scores on histological examination suggested a better outcome in treated tendons. Gene expression was indicative of better tissue regeneration after all treatments, especially for BM-MSCs, as suggested by upregulation of collagen type I, decorin, tenascin and matrix metalloproteinase III mRNA. Considering all findings, a clear beneficial effect was elicited by all treatments compared with the control group. Although differences between treatments were relatively small, BM-MSCs resulted in a better outcome than PRP and AT-MSCs.

Introduction

The superficial digital flexor tendon (SDFT) is subjected to large forces during athletic activity in horses (Dowling et al., 2000). Its limited regeneration potential makes tendon repair a slow process, resulting in the formation of scar tissue, which has inferior biomechanical properties and is prone to re-injury (Dahlgren, 2009; Dakin et al., 2014; Gulati et al., 2015). Several treatments for equine tendonitis have been investigated, but injured animals are rarely able to return to the same level of performance (Genovese et al., 1990; Nixon, 1990; Dehghan et al., 2007).

Regenerative medicine, including the intralesional use of mesenchymal stem cells (MSCs) and platelet rich plasma (PRP) is a promising approach for treating tendon injuries in horses (Bosch et al., 2010). Numerous studies have suggested potential therapeutic benefits of MSCs for the functional regeneration of tendons and ligaments (Godwin et al., 2012; Carvalho et al., 2013; Smith et al., 2013; Conze et al., 2014; Gulati et al., 2015). MSCs from different sources, such as bone marrow (BM) (Smith et al., 2013), adipose tissue (AT) (Conze et al., 2014) or umbilical cord blood (Van Loon et al., 2014), have to a certain extent shown efficacy in terms of reduction of the re-injury rate and improving outcome in both naturally occurring and experimentally induced lesions (Godwin et al., 2012; Martinello et al., 2013; Conze et al., 2014).

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However, several questions about the effectiveness of MSCs and PRP remain unanswered. Previous reports on the use of regenerative products showing superior healing of equine tendinopathies have combined different products (Pacini et al., 2007; Del Bue et al., 2008; Carvalho et al., 2013; Smith et al., 2013), making it difficult to elucidate if MSCs are more or less effective than PRP or if their effects are additive (Koch et al., 2009; Schnabel et al., 2013). The aim of this study was to separately assess the effectiveness of autologous BM-MSCs, AT-MSCs and PRP for treating induced injuries of the equine SDFT.

Material and methods

Animals

Twelve cross-breed geldings (H1–H12) aged 5–8 years were determined to be healthy and free of tendon injury, as shown by their history, clinical assessment and ultrasonographic exam. The project was approved by the Ethical Committee for Animal Experiments from the University of Zaragoza (project license PI36/07; date of approval 15 February 2008). The care and use of animals were performed in accordance with the Spanish Policy RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for scientific purposes.

Study design

In 24 tendons (both forelimbs of 12 horses), lesions were induced as described below and randomly divided into four batches. Each batch of six tendons was assigned to one treatment (BM-MSCS, AT-MSCs or PRP) or control (lactated Ringer's solution, LRS). H1–H6 received BM-MSCs in one tendon and H7–H12 received AT-MSCs in one tendon. In the other tendon, H1–H3 and H7–H9 received PRP, whereas H4–H6 and H10–H12 received LRS as a control treatment. The different treatments were administered 1 week after the lesion induction. Clinical and ultrasonographic parameters were recorded throughout the study in weeks 1 (pre-treatment), 2, 6, 10, 20 and 45, after which animals were euthanased. Subsequently, histological and gene expression analyses of tendons were performed (See Appendix: Supplementary material 1).

Autologous BM-MSCs, AT-MSCs and PRP

Procedures for preparation of MSCs were carried out as described by Ranera et al. (2011). BM was aseptically aspirated from the sternum of horses H1–H6, layered over Lymphoprep (Atom) and centrifuged at 300*g* for 20 min. Nucleated cells were harvested and suspended in basal medium, consisting of low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 1% glutamine and 1% streptomycin/penicillin (Sigma-Aldrich). Adipose tissue was aseptically collected from the supra-gluteal subcutaneous area of horses H7–H12. The stromal vascular fraction was isolated by digestion with 0.01% collagenase type I (Sigma-Aldrich) for 30 min at 37 °C with continuous shaking. Cells were suspended in the basal medium described above. BM and AT derived cells were expanded at 37 °C in 5% CO_2 until the third passage and then characterised as MSCs by their immunophenotype and their trilineage differentiation potential using methodology and markers described previously (Ranera et al., 2011). Subsequently, cells were cryopreserved and thawed for expansion 7 days before their in vivo use.

Autologous PRP was obtained by using the double centrifugation tube method (Arguelles et al., 2006). Peripheral blood was collected in citrated tubes from horses H1–H3 and H7–H9, centrifuged at 120*g* for 5 min and the 50% fraction closest to the buffy coat was collected and centrifuged again at 240 *g* for 5 min. Subsequently, the lower 25% fraction was collected and used for treatment. Platelet and white blood cell (WBC) counts were determined using a flow cytometry haematology system (LaserCyte Dx, IDEXX Laboratories).

Surgically induced injury

Horses were sedated with 0.04 mg/kg IV romifidine (Sedivet, Boehringer-Ingelheim) and 0.02 mg/kg IV butorphanol (Torbugesic, Pfizer). Anaesthesia was induced with 2.2 mg/kg IV ketamine (Imalgene, Merial) and 0.05 mg/kg IV diazepam (Valium, Roche), and maintained using a triple-drip, consisting of 15 mg romifidine, 500 mg ketamine and 25 g guaifenesin (Glicefar, DragPharma) mixed in 500 mL of 5% glucose solution at 2 mL/kg/h. Lesions of approximately 5 cm length were mechanically induced in the SDFT of both forelimbs of each animal, approximately at 18 cm distal to the accessory bone, using a controlled motor rotor and a 4 mm drill through a small longitudinal incision into the core of the SDFT (Cadby et al., 2013). The lesion was created in the central tendon area (maximal injury zone, MIZ) (Fig. 1). The incisions in the paratenon and the skin were closed in a routine fashion. Twice daily, 22 mg/kg IM procaine benzylpenicillin (Depocillin, Merck-Sharp) and once daily 6.6 mg/kg IV gentamicin (Gentavex, SP Veterinaria) were administered for 3 days. A two-layer bandage was applied and changed daily for 7 days. Pre-operative and post-operative analgesia were provided with oral phenylbutazone (EqZona, Calier) at 2.2 mg/kg twice daily for 3 days. Animals still presenting signs of pain received butorphanol at 0.05 mg/kg IV every 4 h. After surgery, horses were box-rested for 2 weeks and then daily hand-walked for 10 min until week 6. Subsequently, they were placed in small paddocks for restricted exercise and 10

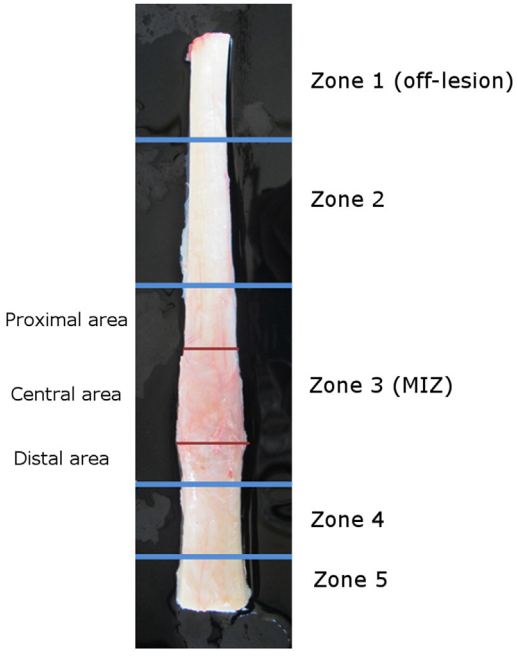

Fig. 1. Assignment of zones in the superficial digital flexor tendon (SDFT) in this study. Zone 1 was used as an off-lesion control for gene expression analysis. Zone 3 is the maximal injury zone (MIZ) and corresponds to the area of the lesion. The MIZ is subdivided into proximal, central and distal areas. Samples were collected from these areas for histopathology and gene expression analysis. Zones 2 and 4 are transition zones, while zone 5 is an off-lesion area; these zones were not used in the study.