IMMUNOHISTOCHEMISTRY OF FACTOR VIII, HISTOLOGY AND MORPHOMETRY IN EQUINE TENDON TREATED WITH PLATELET-RICH PLASMA*

Bruna Mota Zandim[1], Maria Verônica de Souza[2+], Luiza Neme Frassy[3], Marlene Isabel Vargas Vilória[4], Leandro Maia[5], Cláudio César Fonseca[6], Fabrício Luciani Valente[7], José do Carmo Lopes Moreira[8] e Pablo Costa Magalhães[3]

ABSTRACT. Zandim B.M., de Souza M.V., Frassy L.M., Vilória M.V., Maia L., Fonseca C.C., Valente F.L., Moreira J. do C.L. & Magalhães P.C. **Immunohistochemistry of factor VIII, histology and morphometry in equine tendon treated with platelet--rich plasma**. [Imunoistoquímica do fator VIII, histologia e morfometria em tendão de equino tratado com plasma rico em plaquetas]. *Revista Brasileira de Medicina Veterinária*, *35(2):169-184, 2013*. Departamento de Veterinária, Universidade Federal de Viçosa, Av. PH Rolfs, s/n, Viçosa, MG 36570-000, Brasil. Email: msouza@ufv.br

The aim of this research was to verify the therapeutic effects of platelet-rich plasma (PRP) in the treatment of tendinopathy of the superficial digital flexor tendon as assessed by histological and morphometric evaluation, as well as to evaluate early changes in the expression of factor VIII by immunohistochemical analysis during the inflammatory and proliferative phases of healing process of PRP treated tendons compared to placebo. Five days after induction of tendinopathy by collagenase, the forelimbs of six horses were treated with intralesional PRP or 0.9% NaCl. Tendon biopsy was performed at 3 or 16 days after treatments. In both phases and with both treatments, the lesion area was filled with granulation tissue containing scattered collagen fibers with loss of parallelism, fibroblasts with varied morphology, neovascularization and hypertrophied endotenon. These characteristics were more evident during the proliferative phase and no differences were observed between treatments. A negative correlation between collagen fibers and moderately plump fibroblasts was observed. Immunohistochemistry staining was positive for factor VIII, without any differences between the treatments and tendon healing phases.

KEY WORDS. Horses, Platelet-rich components, Growth factors, Tendinous structures.

RESUMO. O objetivo desse estudo foi verificar o efeito terapêutico do plasma rico em plaquetas (PRP) no tratamento de tendinopatia do tendão do músculo flexor digital superficial mediante realização de estudos histológico e morfométrico, bem como avaliar alterações iniciais na expressão do fator VIII por imunoistoquímica durante as fases inflamatória e proliferativa do processo de repara-

Rev. Bras. Med. Vet., 35(2):169-184, abr/jun 2013

169

ção do tendão tratado com PRP e com placebo. Para isso, cinco dias após indução de tendinopatia por colagenase, os membros torácicos de seis equinos foram tratados por via intralesional com PRP ou NaCl a 0,9%. Biópsia tendínea foi realizada 3 ou 16 dias após os tratamentos. Em ambas as fases e ambos os tratamentos a área da lesão estava preenchida por tecido de granulação contendo fibras colágenas esparsas, com perda do paralelismo e fibroblastos com morfologia variada, neovascularização e hipertrofia do endotendão. Essas características foram mais evidentes durante a fase proliferativa, não havendo diferença entre os tratamentos. Correlação negativa foi observada entre a presença de fibras colágenas e fibroblastos moderadamente arredondados. Ocorreu marcação positiva para o fator VIII, sem diferença entre os tratamentos e as fases da reparação tendínea.

PALAVRAS-CHAVE. Cavalos, Componentes ricos em plaquetas, fatores de crescimento, estruturas tendíneas.

INTRODUCTION

Tendinopathy is frequently observed in the superficial digital flexor tendon (SDFT) of horses, which is the most important structure for the storage of energy during locomotion in this species (Batson et al. 2003). An epidemiological study performed by Singer et al. (2008) of horses that were used in either one-day event competitions or cours complete internationale competitions revealed that 21% (25/83) of the animals were not able to participate in the complete event due to lesions. Among the lesions, those occurring in the tendons and ligaments were the most common (43.4%) and the SDFT was affected in 33.3% of the cases. Due to the frequent lesions and their dimension, as well as the superficial position, the SDFT in horses is considered to be an excellent model for studying tendinopathies and serves as a contribution for studying the injury in human athletes (Crass et al. 1992, van Schie et al. 2000).

Platelet-rich plasma (PRP) is an alternative therapy for the treatment of tendinopathies. PRP is an autogenous and economical source of growth factors that are contained in the platelet granules and are considered to be responsible for the effects of this therapy in both modulating and accelerating the healing process (de Mos et al. 2008, Foster et al. 2009). These factors are essential regulators of the cellular events that are involved in healing because they significantly influence the deposition of the extracellular matrix and promote chemotaxis to the location of the lesion by recruiting platelets and leukocytes. Additionally, these factors also promote neovascularization, cellular proliferation and differentiation and possess other functions, such as the control of cell death. Among the main substances present in platelets, transforming growth factor beta (TGF-ß) is of particular interest (Sutter et al. 2004) and has been shown to have important effects on the healing process (O'Kane & Ferguson 1997) due the increase in collagen production and cellular viability (Molloy et al. 2003). Another important factor is vascular endothelial growth factor (VEGF), which is responsible for the proliferation and migration of endothelial cells, resulting in capillary sprouting and angiogenesis (Bir et al. 2009).

In horses, some studies have shown that PRP has beneficial effects when used as an intralesional treatment of tendons or ligament structures both *in vivo* (Argüelles et al. 2008, Waselau et al. 2008, Bosch et al. 2010) and *in vitro* (Smith et al. 2006, Schnabel et al. 2007, McCarrel & Fortier 2009). However, other studies have found no difference between the effects of PRP and the saline solution (Maia et al. 2009b, Monteiro et al. 2009). Additionally, a study conducted in humans demonstrated that PRP has no effect on the treatment of chronic Achilles tendinopathy (de Vos et al. 2010), which is a tendon structure that is functionally similar to the SDFT in horses (Crass et al. 1992). Thus, the aim of the present study was to verify the therapeutic effect of PRP on the treatment of SDFT tendinopathy by histological and morphometric evaluation, as well as to evaluate early changes in the expression of factor VIII by immunohistochemical analysis performed during the inflammatory and proliferative phases of healing process of PRP treated tendons compared to placebo. We tested the hypothesis that PRP may be an effective therapy for the treatment of tendinous lesions, as it promotes a more uniform and organized healing process, and increases vascularization in the tendinous tissue.

MATERIALS AND METHODS

This work was approved by the Ethics Committee of the Veterinary Department of the Universidade Federal de Viçosa (Process no. 34/2009). The assays are in accordance with the Veterinary Professional Ethics Code, the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation and current Brazilian legislation.

Selection and management of the animals: Six healthy crossbred geldings aged 5 to 16 years (13±4.05 years) were used. The selection of the animals was based on an evaluation of the clinical variables (heart and respiratory rates, capillary refill time, body temperature, mucous membrane color, hydration and intestinal mobility), a specific examination of the locomotion system and an ultrasonographic evaluation of the palmar metacarpal region of the forelimbs.

Thirty days before the beginning of the experimental phase, the animals were moved to the individual box stalls in which they would be housed during the study. Two weeks before the induction of tendinopathy, the animals were weighed, bathed with insecticide (Ultimate®, Pffizer Saúde Animal, Belo Horizonte, Minas Gerais, Brazil) and orally dewormed with moxidectin paste (Equest®, Fort Dodge, Mogi Mirim, São Paulo, Brazil) to eliminate ectoparasites and endoparasites, respectively. At the same time, hematological variables (packed cell volume, hemoglobin concentration and erythrocyte, leukocyte and platelet counts) and biochemical variables (total plasma protein and fibrinogen concentration) were measured.

Induction of the tendinopathy: SDFT tendinopathy was experimentally induced in both forelimbs, as described by Nixon et al. (2008) with modifications, by administration of 2,090 collagen digestion units (Collagenase type 1®: C-0130, Sigma-Aldrich Brasil Ltda, São Paulo, São Paulo, Brazil) diluted in 0.3 mL of Milli-Q water.

The collagenase was injected in the SDFT palmar metacarpal region, at the midpoint (midmetacarpal region) between the distal face of the accessory carpal bone and the apical extremity of the proximal sesamoid bones to reach the center of the tendon. However, when the lesion induction region coincided with the localization of the communicating branch of the palmar nerves, the collagenase administration was performed 1 cm distal to the nerve so that the injured site varied from 13 cm to 15 cm distal to the accessory carpal bone. To perform the procedure, the forelimbs were clipped and aseptically prepared with 1% iodine-povidone and iodized alcohol. The animals were injected intravenously with 1 mg/kg of 10% xylazine (Sedazine®, Fort Dodge, Mogi Mirim, São Paulo, Brazil) and the lateral and medial palmar nerves were blocked in the proximal metacarpal region with 3 mL of 2% lidocaine chloridrate (Anestésico Bravet®, Laboratório Bravet, Engenho Novo, Rio de Janeiro, Rio de Janeiro, Brazil) without a vasoconstrictor. Then, the collagenase was injected with ultrasound guidance (7.5-MHz linear array probe, Medison, model Sonovet 600, São Paulo, SP, Brazil) through hypodermic 21-gauged needles.

After the induction of tendinopathy, a demarcation with a surgical skin marker pen and a suture point under the single interrupted pattern was established on the skin of the dorsal region of each forelimb to identify the height of the lesion located in the palmar surface and to facilitate the recognition of the site for the application of the treatments and the collection of the tendon sample for histological and immunohistochemical evaluation. After administration of collagenase, a bandage was applied to the metacarpal region for 24 h. During the period between the induction of tendinopathy and treatment, the animals rested in their stalls.

Laboratory protocol for obtaining PRP: The PRP was obtained according to the method described by Argüelles et al. (2006). For this purpose, 81-mL blood samples were collected from each animal by puncturing the external jugular vein. The samples were collected in 18 vacutainer tubes with 3.8% of sodium citrate (0.199 mol/L) (tube capacity 5 mL: 0.5 mL of anticoagulant and 4.5 mL of whole blood). Additionally, another 4.5 mL of whole blood was obtained in a tube containing EDTA for the determination of the platelet concentration, which was automatically performed (HumaCount plus, Human do Brasil, Recife, Pernambuco, Brazil). The blood samples obtained with sodium citrate were centrifuged at 120 x g for 5 min. Then 50% of the supernatant columns were discarded so that plasma with a greater platelet concentration could be obtained in a second centrifugation step. From the plasma remaining in those 18 tubes, 1.1 mL was collected just near the leukocytic layer for a total of 20 mL of plasma, which was then placed in two 10-mL sterile tubes without anticoagulant. The tubes were then centrifuged at 240 x g for 5 min. After this second centrifugation, the plasma was divided into two fractions: the supernatant (platelet-poor plasma) and the remaining fraction, the so-called PRP. Finally, an approximate volume of 75% platelet-poor plasma obtained from each tube was discarded and the remainder was conditioned in a new sterile tube without anticoagulant for a total of 5 mL of PRP. The determination of leukocyte and platelet concentrations in PRP was manually performed

Rev. Bras. Med. Vet., 35(2):169-184, abr/jun 2013

171

(Rees-Ecker method). Automatic counting would have been prejudiced or even unfeasible because of the high platelet concentration in PRP and the consequent increase in viscosity.

Treatments: five days after the induction of tendinopathy, all animals were treated by intralesional injection with platelet-rich plasma or 0.9% NaCl (Solução fisiológica sanobiol®, Laboratório Sanobiol Ltda, Pouso Alegre, Minas Gerais, Brazil). For the procedure, the horses were clipped, sedated, blocked and aseptically prepared. A total of 1.8 mL of PRP was injected into a randomly chosen limb. This total volume was divided into 0.6 mL aliquots that were applied at the site of the lesion, at a site 0.5 cm proximal to the site of the collagenase administration and at a site 0.5 cm distal to the site of the collagenase administration. In the contralateral limb, three aliquots of 0.6 mL of saline solution were administered in the same pattern as described for PRP. The treatment with PRP was performed immediately after obtaining them.

Once the treatments were performed, a bandage was applied to the metacarpal region for 24 h. During the period between treatment and biopsy (3 or 16 days after treatment), the animals rested in their stalls. Because the duration of this period was short, the animals were not subjected to progressive physical activity. In addition, the animals were not treated with anti-inflammatory drugs after the induction of tendinopathy or after administration of PRP or saline solution because these drugs could confound the evaluation of the PRP treatment and interfere with the physical and ultrasonographic examinations.

Laboratory, physical and ultrasonographic evaluations: the hematological and biochemical variables previously mentioned were monitored weekly for up to three weeks after treatment. A general physical exam was also carried out, as well as a specific examination of the locomotor system, before (T0) and after 24 (T1) and 48 (T2) hours of induction of tendinopathy. These examinations also occurred before (T3) and 24 (T4) and 48 (T5) hours after the administration of PRP or saline. Finally, the animals were evaluated weekly (T6 and T7). The physical examination consisted of monitoring the presence of swelling, any increase in the local temperature (Speirs 1999) and pain by palpation (Redding et al. 1999), which was classified as absent, discrete, moderate or intense (Redding et al. 1999, Speirs 1999) with scores ranging from 0 to 3,

respectively. The degree of lameness was also determined and classified on a scale of 0 to 5 (Kester 1991).

The ultrasonographic examinations were used only to confirm and monitor the tendinopathy and to monitor the response to treatment before the biopsies. Both the loss of collagen fiber alignment and echogenicity (Genovese et al. 1986) were evaluated.

Biopsy: To evaluate the tendon healing during both the inflammatory and proliferative phases of the healing process, the tendons of three randomly chosen animals were subjected to biopsy at three days after administration of either PRP or saline solution. In the other animals, the biopsy was performed at 16 days after treatment. For the surgical procedure, the animals were clipped and submitted to water and food fasting for 12 h. After intravenous premedication with 1 mg/kg of 10% xylazine, the horses were anesthetized with a combination of 1 g of sodium thiopental (Tiopentax®, Cristália Produtos Químicos e Farmacêuticos Ltda, Itapira, São Paulo, Brazil) and 10% guaiacol glycerine ether (EGG®, Vetec Química Fina Ltda, Duque de Caxias, Rio de Janeiro, Brazil). The anesthesia was maintained with isoflurane (Isoforine®, Cristália Produtos Químicos e Farmacêuticos Ltda, Itapira, São Paulo, Brazil).

Biopsies were performed at the sites of the PRP and 0.9% NaCl injections. The limbs were clipped and aseptically prepared. After skin incision, blunt dissection of the subcutaneous tissue, opening of the paratenon and exposure of the SDFT, a macroscopic evaluation of the local anatomical structures was performed to assess the occurrence of adherence, which was classified as absent, discrete, moderate or intense (Foland et al., 1992). When the injured area was located, a fragment of tissue with a volume of approximately 1 cm^3 was obtained using a scalpel (Embramac, Empresa Brasileira de Materiais Cirúrgicos, Itajaí, Santa Catarina, Brazil). The samples involved the entire tendon thickness in the injured area.

After the biopsy, tenorrhaphy was performed using polyglactin 910 (2-0 Vicryl®, Eletro-Light Ltda, Manaus, Amazonas, Brazil) in a locking loop pattern to avoid a possible tendon rupture during the postoperative period (Maia et al. 2009b). Paratenon synthesis was performed using the same thread in a continuous, simple pattern. The skin synthesis was performed with a separate single stitch using 00 nylon (Mononylon®, Brasuture Indústria Comér-

cio Importação e Exportação Ltda, São Sebastião da Gama, São Paulo, Brazil) stitches. After the surgical procedure, a bandage was applied to the metacarpal region for 24 h. During the postoperative period, the animals were maintained in their stalls and their wounds were cleaned until the removal of sutures on the 14th day. Meloxicam (Maxicam gel®, Ourofino Saúde Animal Ltda, Cravinhos, São Paulo, Brazil) was orally administered at a dose of 0.6 mg/kg every 24 h for seven days.

Histology: The samples collected for histology were fixed in 10% neutral buffered formalin for 48 h, dehydrated, cleared in xylene and embedded in paraffin wax using routine methods. The sections were cut at a thickness of 4 μm (transversal and longitudinal planes), dewaxed, cleared in xylene, rehydrated, and stained using the following methods: hematoxylin and eosin (HE), Picrosirius Red and Masson's trichrome (Erviegas Instrumental Cirúrgico Ltda, São Paulo, São Paulo, Brazil). The histological analyses were conducted by three observers who were blinded to the treatments using a light microscope (Olympus CX31, Olympus Optical do Brasil Ltda, São Paulo, São Paulo, Brazil) that was coupled with a photographic camera (Olympus U-CMAD3 SC20, Olympus Optical do Brasil Ltda, São Paulo, São Paulo, Brazil) and a polarized light microscope (Olympus BX 50, Olympus Optical do Brasil Ltda, São Paulo, São Paulo, Brazil) that was

also coupled with a photographic camera (Olympus Q-Color 3, Olympus Optical do Brasil Ltda, São Paulo, São Paulo, Brazil).

The cellular characterization and organization of the tendon matrix were determined in the fragments stained with HE. For this assessment, the following variables were analyzed after a general evaluation of the whole tissue fragment: the shape of the fibroblasts, the type of inflammatory infiltrate, tissue organization (linearity and crimping of the collagen fibers) and the occurrence of hemorrhage. These variables were measured using the semiquantitative criteria (Table 1) described by Nixon et al. (2008), with some modifications. For each variable, a descriptive analysis was conducted and average values were established using a scale (from 1 to 4 for each histologic variable) obtained by the three observers. In addition, morphometric analysis was used to quantitatively evaluate the density of the inflammatory infiltrate, blood vessels, fibroblasts and collagen fibers. For this purpose, six photomicrographs were randomly obtained for each sample with a 10x objective lens and placed under a point-counting grid (Aherne & Dunnill, 1982) with 352 intersections made by the PowerPoint program. The average values for each variable were obtained from the counts in the six images.

Masson's trichrome staining was used to aid in the evaluation of both the vascularization and or-

Table 1. Scoring system used for histological variables to classify the tendon healing[a].

Variables		Score and criteria
Fibroblast shape (longitudinal sections)	1	Linear (normal)
	2	Slightly oval
	3	Moderately round
	4	Predominantly round
Hemorrhage	1	None
	2	Sparse or patchy
	3	Multiple areas in each low-power field
	4	Predominant hemorrhage
Type of inflammatory infiltrate	1	Lymphocytes
	2	Neutrophils
	3	Macrophages
	4	Eosinophils
Linearity of collagen fibers	1	Linear
	2	> 50% linear
	3	20% to 50% linear
	4	No linear areas
Crimping of collagen fibers	1	Coarse, even crimp
	2	Predominantly fine, even crimp
	3	< 50% with crimp formation
	4	No crimping formation; complete disarray

[a]Adapted from Nixon et al. (2008).

ganization of the connective tissue, whereas Picrosirius Red was used to complement the analysis of the parallelism and organization of the collagen fibers and to supply information regarding the types of collagen (I and III) present.

Immunohistochemistry: The blocks that were previously prepared for histology were cut at a thickness of 4 μm (transversal and longitudinal planes) to analyse the presence of blood vessels by staining the endothelial cells with factor VIII (von Willebrand factor) using the indirect immunoperoxidase technique. The sections were dewaxed, cleared and rehydrated.

Antigen recovery was performed using pepsin (Dinâmica Química Contemporânea Ltda, Diadema, São Paulo, Brazil). Endogenous peroxidase blockage was performed using 3% hydrogen peroxide in a methanolic solution (Dinâmica Química Contemporânea Ltda, Diadema, São Paulo, Brazil) for 20 min. After blocking the peroxidase, the sections were incubated in 3% milk powder solution for one hour at 27°C and washed with Tris-buffered saline (TBS, pH 7.4). Thereafter, the sections were treated with antihuman factor VIII (Polyclonal rabbit antihuman von Willebrand Factor, 1:2000, Dako North America, California, USA) primary antibody for 18 h at 4°C. After washing with TBS, the antibodies were detected by incubation in Advance reagent system (Dako, Glostrup, Denmark). The antigen retrieval was carried out by incubation with 3,3-diaminobenzidine (Dako Cytomation, Denmark) and hydrogen peroxide for 5 min. Finally, the sections were counterstained with Harris hematoxylin (Merck, São Paulo, Brazil), dehydrated, cleared, and evaluated by three observers under a light microscope (Leica Microsystems, Wetzlar, Germany). The images were captured using the Leica Qwin program (Leica Microsystems, Wetzlar, Germany).

The staining for factor VIII along the vessels was evaluated and classified on a 5-grade semiquantitative scale as described by Bosch et al. (2011) as 0: no detectable staining, 1: mild staining of individual cells without a recognizable vascular structure, 2: staining of some small blood vessels, 3: the presence of several small vessels or some larger vessels and 4: several larger vessels. For the negative control, the primary antibody was replaced by rabbit immunoglobulin (Dako Cytomation, Denmark).

Statistical analysis: The experiment was conducted using a split-plot design with six animals (plot) to evaluate the effects of PRP in the inflam-

matory (three days after treatment) and proliferative (sixteen days after treatment) phases of tendon healing. An entirely randomized design was used with three replicates for each phase. For each animal, the choice of the treatment (PRP or 0.9% NaCl) of the forelimb was randomly performed.

After analysis of distribution normality and variance homogeneity, the data obtained were statistically submitted to the F test for the analysis of variance (ANOVA) at 5% probability to verify the effect of the treatment (PRP or 0.9% NaCl) and of the healing phases (inflammatory or proliferative), as well as the interaction between them. Additionally, a correlation analysis between the variables evaluated in morphometry was performed with a significance level of 5%. The analyses were conducted using Minitab version 16, 2010 (http://www.minitab.com/products/minitab, State College, PA).

The descriptive analyses were conducted for the data related to the hematological (packed cell volume, hemoglobin concentration, and erythrocyte, leukocyte and platelet counts), biochemical (total plasma protein and fibrinogen concentration), physical (swelling, increased local temperature and pain by palpation) and ultrasonographic (echogenicity and alignment of the collagen fibers) variables.

RESULTS

Platelet-rich plasma: The concentration of platelets in the whole blood used to prepare PRP varied from 149,000 to 177,000 (average 164,500±9,813) platelets/μL. In PRP, the concentration varied from 320,000 to 430,000 (average 368,333±39,707) platelets/μL.

Laboratory, physical and ultrasonographic evaluations: The values of the hematological and biochemical variables evaluated before and during the experiment were as follows: packed cell volume, 29.7-34.6%; hemoglobin concentration, 10.4-12.2 g/dL; erythrocyte count, 6.9-7.8 x 10^6 cells/μL; leukocyte count, 5,600-8,300 cells/μL; platelet count, 129,667-183,667 cells/μL; protein concentration, 7.6-8.0 g/dL and fibrinogen concentration, 400-600 mg/dL. The lowest values of the packed cell volume, hemoglobin concentration and erythrocyte and leukocyte counts were obtained from the last collection, three weeks after the treatment. In PRP, the leukocyte count varied from 100 to 600 cells/μL.

At 48 h after the administration of collagenase in both the right and left SDFT, the animals developed acute inflammation characterized by the presence of

intense swelling (score 3), pain by palpation (absent to intense) and an increase in the local temperature (discrete to intense). Lameness (1 to 2 degrees) was observed in only two animals and was completely absent before the treatment. At 48 h after injection of PRP or 0.9% NaCl, the following clinical signs were observed: swelling (discrete to intense), pain by palpation (absent to intense), increased local temperature (absent to discrete) and lameness (degree 1) present in only one animal.

The physical evaluation that was performed weekly in the three animals that were biopsied in the proliferative phase showed swelling (discrete to intense) until the day of surgery, which was carried out 16 days after the administration of the treatment. The pain by palpation (initially intense, reduced to discrete) remained in two animals until one week before the surgical procedure and lameness was absent in all of the horses.

Few changes were observed in the ultrasonographic evaluations performed 24 h after collagenase injection and were characterized by the partial loss of the collagen fiber alignment, which was observed in eight limbs (66.67%) and a decrease of 25% (83.33% of limbs) or 50% (16.67% of limbs) in the isoechoic pattern. In the exam performed at 48 h after the induction of tendinopathy, the following findings were observed: a partial loss of the collagen fiber alignment (partial alignment) in all tendons; a decrease in echogenicity between 25% and 75% and peritendinous edema.

At 24 h after the administration of either PRP or saline, the ultrasonographic images revealed that the SDFT of all limbs presented partial loss of collagen fiber alignment. A decrease of 25% (83.33%) or 50% (16.67%) in the isoechoic pattern, and peritendinous edema in five (41.67%) limbs was observed. In the images obtained 48 h after treatment, partial loss of collagen fiber alignment could be observed in all limbs and a decrease in echogenicity of 75%, 50% or 25% in one (8.33%), eight (66.67%) and three limbs (25%), respectively, was observed.

Biopsy: In the macroscopic evaluation performed during the surgery, no limbs presented signs of infection. The presence of adherences between the SDFT and peritenon or deep digital flexor tendon (DDFT) was observed in all limbs, independent of the treatment. The intensity varied from discrete to moderate during the inflammatory phase and from moderate to intense during the proliferative phase.

At 48 h after the biopsy, swelling, pain by palpation, and increased local temperature of intense gravity, in addition to lameness (3 to 4 degrees) were observed. In subsequent evaluations, a gradual reduction in the severity of these clinical variables was observed. Three weeks after the surgical procedure, the animals were grazing freely and no lameness, pain or locally increased temperature was observed.

Histology: In the general histological observation of the HE and Masson's trichrome staining, the injured area in both forelimb tendons could be distinguished. These areas differed in their size and characteristics, depending on the inflammatory or proliferative phase. Non-cellular areas were not found.

In the tendons of the animals submitted to biopsy 3 days after treatment (inflammatory phase), the injured area was filled with a dense connective tissue with organized collagen fibers and an increased number of plump fibroblasts surrounding the hypertrophic endotenon and dispersed into the tendon matrix (Figure 1A, B). Loss of fibroblast organization and parallelism and differential staining affinity (more intensely stained by hematoxylin, indicating a higher presence of nuclei) were observed, as well as a discrete thickness and increase in the endotenon vascularization. This injured area was larger in the 0.9% NaCl-treated limb of one (33.33%) animal and was similar in both limbs in the other horses (66.67%). Except for two tendons treated with PRP that presented an intense eosinophilic infiltrate, all of the other tendons presented a discrete inflammatory infiltrate of mixed type (66.67%). No hemorrhagic foci were observed.

Biopsy conducted 16 days after the treatments (proliferative phase) showed that the injured area was filled with dense connective tissue containing sparce collagen fibers, intense neovascularization and a large number of plump fibroblasts, especially around the hypertrophic endotenon (Figure 1C, D). Despite the absent parallelism of the collagen fibers, there was a clear tendency for this tissue to organize; tissue differentiation foci were larger in two samples, including one in a PRP-treated SFDT and another in a tendon that had received 0.9% NaCl. In contrast to the saline solution-treated tendons, cell rows of plump fibroblasts were observed in the PRP-treated tendons. Similar to the inflammatory phase, a mixed-type infiltrate was evident (83.33%), and only one PRP-treated limb presented an eosinophilic infiltrate (16.67%).

The average values established from the scores obtained by the three observers during the se-
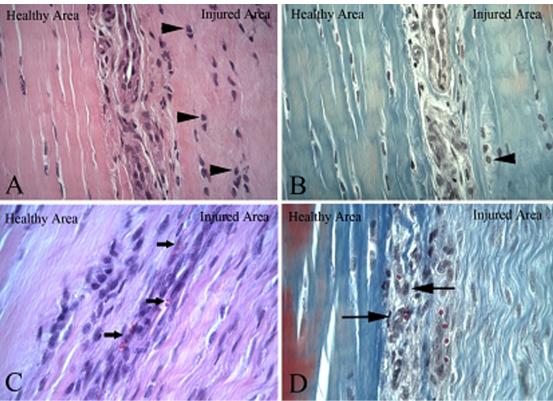
Figure 1. Photomicrography of SDFT submitted to biopsy at 3 and 16 days after PRP administration. It is noted that during the inflammatory phase (A, B) the injured area has a less organized collagen fibers and connective cells. The fibroblasts present a more plump shape (arrowheads) in contrast to the spindle-shaped observed in the normal dense fibrous connective tissue. In the proliferative phase (C, D), the injured area is filled with collagen fibers and an increase in vascularization characterized by the presence of tumescent endothelial cells (thin arrows) and extravased erythrocytes (thick arrows) (best observed by Masson's trichrome staining) is observed. Longitudinal sections in HE stain (A, C) and Masson's trichrome (B, D) stain. Magnification of 412X (A), 436X (B), 600X (C) and 588X (D).

miquantitative analysis of tendon healing are presented in Table 2. No differences (p>0.05) were detected between the averages of the treatments or between the phases of the healing process. Additionally, no interaction occurred between the tendon healing phases and the treatments performed.

Morphometric analysis revealed an ample variation among the histological sections from individual samples in the proportion of healthy and injured tendon tissue. There were no differences (p>0.1) between PRP and 0.9% NaCl treatments, or between the inflammatory and proliferative phases regarding the quantification of the fibroblasts,

blood vessels, inflammatory infiltrate and collagen fibers in the images obtained at both longitudinal and transversal planes. The average values obtained from the count of these variables in six photomicrographs are shown in Table 3. Only the longitudinal sections revealed that the collagen fibers negatively correlated with moderately plump fibroblasts (r = -0.95, p=0.04).

In both the tendons treated with PRP and those treated with 0.9% NaCl, the general descriptive evaluation of the tissue stained by the Picrosirius Red allowed the identification of the injured region, which was characterized by less organized areas and

Table 2. Quantitative histological evaluation (average values) of tendon, obtained using the scoring system, during the inflammatory and proliferative phases of the healing process.

Phases	Fibroblast shape		Hemorrhage		Type of inflammatory infiltrate		Linearity of collagen fibers		Crimping of collagen fibers	
	PRP[a]	NaCl[b]	PRP	NaCl	PRP	NaCl	PRP	NaCl	PRP	NaCl
Inflammatory	1.6	1.6	1.0	1.0	2.2	1.5	1.6	1.8	2.8	2.5
Proliferative	1.6	1.1	1.1	1.1	2.3	1.8	2.1	1.2	2.3	2.6

[a]PRP - platelet-rich plasma; [b]NaCl - saline solution.

Table 3. Quantitative histological evaluation (average values and standard deviation) of the tendon determined by morphometric analysis during the inflammatory and proliferative phases of the healing process.

Variables	Inflammatory Phase		Proliferative Phase	
	PRP[a]	0.9% NaCl[b]	PRP	0.9% NaCl
	Longitudinal Sections			
Fibroblast linear	160.3±65.2	114.6±65.0	122.3±34.2	129.0±63.6
Fibroblast slightly oval	58.0±8.8	49.6±19.2	58.6±23.1	90.0±63.0
Fibroblast moderately round	21.6±3.7	14.6±11.0	23.6±16.8	29.3±25.7
Fibroblast predominantly round	2.3±4.0	0.6±1.1	0	1.6±2.8
Blood vessels (endotenon)	36.3±31.6	34.3±20.0	42.3±11.5	41.6±22.1
Collagen fibers	1757.3±48.9	1818.6±52.7	1775.0±33.1	1717.6±154.0
	Transversal Sections			
Fibroblast	179.3±73.7	154.3±70.3	141.3±28.5	177.3±64.5
Blood vessels (endotenon)	71.0±15.8	82.0±14.4	79.3±32.5	68.3±11.0
Inflammatory infiltrate	11.3±19.6	0	0	0
Collagen fibers	1672.6±83.3	1664.6±51.6	1710.3±146.7	1737.3±56.0

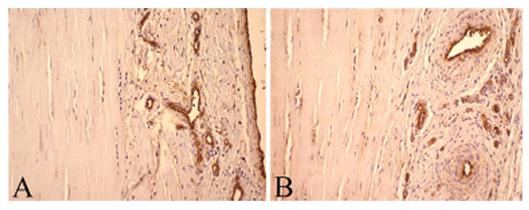[a]PRP - platelet-rich plasma; [b]NaCl - saline solution.

Figure 2. Photomicrography of factor VIII staining on the SDFT at 16 days after PRP treatment (A) or 0.9% NaCl (B) (longitudinal sections, factor VIII immunostain). There are several small vessels regularly arranged in the endotenon of the healing area (on the right of the figures), and rare large-caliber vessels can also be observed. A similar degree of vascularity was found between the treatments. Magnification of 200x.

high amounts of type III collagen fibers intermixed with type I fibers. These areas were more extensive, more disorganized and had a higher amount of type III collagen during the proliferative phase. In contrast, areas undergoing a reorganization process with high amounts of type I collagen were especially observed in the PRP-treated tendons, which also presented fiber bundles with better orientation.

Immunohistochemistry: Immunohistochemical staining for factor VIII showed a uniform distribution of vascularization, with several small vessels regularly arranged in the endotenon and, more rarely, larger-caliber vessels. Considering the semi-quantitative classification adopted for the evaluation, the staining varied between 2 and 4, with an average of 2.3 (PRP and saline) for the inflammatory phase and of 3 (PRP and saline) for the proliferative phase (Figure 2). No difference was found between the treatments or the phases of the tendon healing process.

DISCUSSION

The values for the hematological and biochemical variables evaluated during the experiment were within the reference limits for the equine species (Kaneko et al. 2008, Grondin & Dewitt 2010). The concentration of platelets in PRP (320,000 to 430,000 platelets/μL) was considered to be appropriate because it was higher than the minimum (300,000 platelets/μL) recommended by Anitua et al. (2004) and higher than that obtained in other studies performed in horses (Carmona et al. 2007, Schnabel et al. 2007, Argüelles et al. 2008). The platelet concentration in PRP was also similar to that found by Maia et al. (2009b) in healthy crossbred geldings.

An *in vitro* study showed that PRP preparations exert a dose-specific effect on oral fibroblasts and osteoblasts proliferation. Optimal effects were observed at a platelet concentration of 2.5-fold increase over the count on whole blood. On the other hand, higher concentrations (5.5-fold) resulted in a

reduction in proliferation and a suboptimal effect on osteoblasts function (Graziani et al., 2006). Therefore, different PRP concentrations may have an impact on the results that can be obtained *in vivo*. According to the authors, moderate increase of the normal platelet count provide the most optimal environment for wound healing through a more ideal balance between cell proliferation and differentiation. Considering the results of those authors, the concentration of platelets in the PRP obtained in the present study would be sufficient to achieve the effects attributed to therapy. Additionally, the number of leukocyte suggests that we used a leukocyte--poor PRP (LP-PRP), which causes the small acute inflammatory response with reduced cellularity and vascularity in rabbit tendons five days after treatment compared with leukocyte-rich PRP (LR-PRP) (Dragoo et al., 2012). In fact, because a separation system is required to exclude leukocytes, the majority of the protocols used in the current literature yield LR-PRP (Wasterlain et al., 2012).

The changes in the physical (swelling, increased local temperature, and pain by palpation) and ultrasonographic variables (echogenicity and alignment of the collagen fibers) after the induction of tendinopathy by the administration of collagenase were similar to those reported by other authors (Williams et al. 1984, Spurlock et al. 1989, Fernandes et al. 2003, Marxen et al. 2003, Dahlgren et al. 2005, Nixon et al. 2008, Maia et al. 2009a,b). Therefore, the injected collagenase (2,090 collagen digestion units) was sufficient to induce tendinopathy and mimic the naturally occurring SDFT injury.

Collagenase is a bacterial enzyme that acts at multiple sites along the collagen triple helix, releasing tropocollagen (Dahlgren et al. 2005) and promoting the destruction of cells and noncollagenous matrix with consequent rupture of the fibers (Spurlock et al. 1989, Foland et al. 1992). According to Marr et al. (1993), the reduction in the echogenicity primarily results from intratendinous hemorrhage, separation of the fibers and edema, which result from the action of collagenase. In the present study, the short time period between the administration of treatment and the biopsies for histological and immunohistochemical analysis was not long enough to monitor the evolution of the ultrasonographic variables.

The clinical signs were observed in both limbs after treatment (PRP or 0.9% NaCl). Similar results have been reported by Bosch et al. (2010) in surgically injured SDFTs that were treated by intratendinous application of PRP or saline. Those findings indicated the formation of a new focus of inflammation, possibly due to the mechanical destruction of the immature collagen fibers, caused by local administration of fluid (PRP or saline) or previous damage caused by the collagenase that was administered for induction of the tendinopathy (Maia et al. 2009b). The reduction of changes observed in the variables after two weeks of treatments was due to lower local inflammatory process. Recently Renzi et al. (2013) observed that the use of mesenchymal stromal cells resuspended in PPR did not induce any deleterious effect on the treated tendonitis and desmitis. Sporadically swelling or inflammatory responses were observed after cell implantation, which gradually resolved within 24 h. Furthemore, nor lameness neither other adverse events were clinically detected during follow up.

SDFT adhesion to peritenon, which was observed during the surgical procedure, has been reported in ponies on the 60th day after the induction of SDFT tendinopathy with collagenase (Williams et al. 1984). This observation has also been reported in horses by Maia et al. (2009b), Foland et al. (1992) and Marxen et al. (2003) at 48, 84 and 150 days, respectively, after induction of the lesion. The adherence between the SDFT and DDFT, which was present in all limbs independently of the treatment, was reported by Foland et al. (1992) and Maia et al. (2009b) in horses treated with sodium hyaluronate and PRP respectively, after collagenase-induced tendon lesion. In these studies, no differences were observed between the treated tendons and the control. According to Foland et al. (1992), this adherence can result from the disorderly and excessive growth of the granulation tissue. In addition, the adherence could be due to the stimulation of proliferation and extrinsic migration of fibroblasts of the peritenon, which contributes to the synthesis of the new tendon tissue and to the adhesion of the tendon with the adjacent tissues (Marxen et al. 2003).

These adherences may not have occurred if the animals had been submitted to controlled physical activity. According to Fortier (2005), adherence can be prevented in horses after the surgical procedure by hand-walking within 1-2 days after surgery. In contrast, in spite of the inclusion of controlled physical activity, Maia et al. (2009b) reported the occurrence of adherence between the SDFT and the peritenon in limbs infiltrated with PRP (16.67%) or

178

Rev. Bras. Med. Vet., 35(2):169-184, abr/jun 2013

0.9% NaCl (33.33%) and between the SDFT and the DDFT in one (8.33%) limb infiltrated with saline solution. In fact, the formation of adhesions is an undesirable result of the repair process itself, which can be aggravated or avoided depending on the type, frequency and intensity of the physical activity performed.

The stains used for histological verification of the tendon repair process allowed for adequate assessment of the variables. The histological sections stained with HE were essential for the cellular characterization and evaluation of the connective tissue organization. Furthermore, the sections stained with Picrosirius Red complemented the qualitative analysis, particularly regarding the evaluation of type I and III collagen fibers. Finally, Masson's trichrome staining allowed for the analysis of the organization of the collagen fibers and the verification of neovascularization.

The histological and morphometric evaluations of the inflammatory and proliferative phases revealed a disorderly regeneration that was independent of the treatment (PRP or saline). This regeneration included neovascularization in the endotenon, fibroblast hypercellularity, and the presence of few inflammatory cells, which are typical characteristics of tissue during the healing process.

Although only some histological variables were investigated in the present study, the histological characteristics observed in the biopsies obtained at three days after treatment with PRP or 0.9% NaCl revealed that the regeneration process could be classified by the formation of 'early granulation tendon tissue' described by van Schie et al. (2003), who studied histologically and by computerized ultrasound two damaged and two structurally normal SDFTs. According to the same authors, this tissue type is observed in horses with acute lesions and is composed of large numbers of swollen fibroblasts, mitotic cells, mononuclear inflammatory cells, extensive vascular proliferation, scattered small areas with ischemic changes, a loose connective tissue matrix without any regular distribution of cells or longitudinal arrangement of fibrillar components, the complete absence of primary and secondary tendon bundles, and no endotenon. As treatments were performed five days after the induction of tendinopathy, and the first biopsy was carried out three days after the treatments, the repair process was already on the eighth day, therefore the inflammatory phase had been finishing, while the proliferative phase

already started. In fact, there is controversy over the duration of the inflammatory phase of tendon repair. According to Abate et al. (2009), this phase can last from three to seven days after injury, although Wang (2006) mentions that the initial phase may last only 24 h.

The tendon tissue obtained at 16 days after the treatments was similar to the 'late granulation tendon tissue' reported by van Schie et al. (2003) in the SDFT of horses with subacute lesions characterized by the presence of large numbers of swollen fibroblasts, fibrillar components in an irregular waving pattern, loose fibrillar components of varying thickness with incomplete organization into tendon bundles, primary and secondary tendon bundles surrounded by a partially incomplete endotenon and increase in the vascularity of the endotenon.

During both phases and in both treatments, the observation of plump fibroblasts next to the hypertrophied endotenon and the intense neovascularization indicates an extrinsic migration of fibroblasts, whereas the presence of dispersed cells in the tendon matrix or the formation of rows of cells suggest that intrinsic proliferation is responsible for the increase in cellularity. According to Sharma & Maffulli (2006), intrinsic tendon repair occurs by proliferation of epitenon and endotenon tenocytes. The increase of the fibroblastic density was previously reported in the case of PRP-treated tendinopathy (Bosch et al. 2010, Maia et al. 2009b), but also with sodium hyaluronate (Foland et al. 1992), therapeutic ultrasound (Fernandes et al. 2003), laser therapy (Machado et al. 2000) and shock wave therapy (Chen et al. 2004). However, the same increase occured in tendons infiltrated with saline solution (Foland et al. 1992, Maia et al. 2009b), which corroborates the findings obtained in the morphometric analysis performed in the present study, which revealed no differences between the treatments. In contrast, Bosch et al. (2010) observed that PRP-treated tendons present higher metabolic activity relative to the control, which is due to the increase in cellularity and vascularization. According to those authors, the main effect of PRP on the healing process after a tendon injury is a "lasting" effect on cell proliferation and migration and hence, on overall metabolic activity.

The lack of any difference in the SDFT tissue organization between animals treated with PRP and those treated with saline solution has also been reported by Maia et al. (2009b) in biopsies conducted

48 days after tendinopathy induced by collagenase followed by treatment with 2.5 mL PRP at 12 days after injection of the enzyme. This histological similarity does not necessarily mean that the tissues receiving PRP and 0.9% NaCl are equivalent. Although no histological differences were found between the treatments (PRP vs. saline) regarding the general pattern of the tissue, Bosch et al. (2010) observed a higher level of organization of the collagen network in the PRP-treated SDFT. Additionally, those authors reported a better response in the biochemical variables (collagen, sulfated glycosaminoglycans and DNA content) and biomechanical variables (strength at failure and elastic modulus) in the limbs treated with the platelet-rich component. This variation in the results has been observed in few recent controlled studies with PRP in the equine tendon. This demonstrates the need for *in vivo* experiments, especially in horses of different ages submitted to different levels and types of physical activities and training, to provide more specific information about potential therapeutic approaches.

Smith et al. (2006) observed an anabolic effect of PRP on equine suspensory ligament fibroblasts *in vitro*. In addition, some studies have demonstrated that PRP influences tendon healing in dogs (Murray et al. 2006), swine (Murray et al. 2007) and rats (Kajikawa et al. 2008). In humans, the administration of PRP for the treatment of chronic elbow tendinosis (Mishra & Palveko 2006) and as an aid in the surgical repair of the Achilles tendon (Sánchez et al. 2007) has resulted in a reduction in pain and a faster return to the training activities, respectively. However, a study conducted by de Vos et al. (2010) revealed that the administration of PRP did not result in a reduction of pain and an improvement in the activity in patients with chronic midportion Achilles tendinopathy compared to the placebo group, which received a saline injection.

These controversial results emphasize that the tendon healing process represents an interesting paradigm for veterinary and human medical science. Although most tendons have a capacity to spontaneously heal after a lesion, the resulting tissue is almost always mechanically weaker than the original tissue and, therefore, less able to perform the functions of a normal tendon, predisposing the injured horse re-injury (Molloy et al. 2003, Dyson 2004, Smith et al. 2006). Thus, there is a continuous search for a treatment that promotes the regeneration or *de novo* synthesis, as well as the increase or mo-

dulation of the normal process of tendon healing, in such a way as to avoid fibrous scar tissue formation (Smith et al. 2006). Different histological results from those obtained in the present study could be possible if the animals are submitted to progressive physical activity, as observed by Bosch et al. (2010) in horses exercising under controlled conditions starting the fourth week after treatment of the SDFT with PRP.

The neovascularization observed in the endotenon under both treatments was more intense in the areas of the lesion. This is an important finding because it indicates stimulation of local angiogenesis, which favors tendon healing. In well-vascularized tendons, the tissue can quickly recover, but in hypovascular or avascular tissue, the recovery process takes longer, and there is a higher chance of re-injury if a damaging force is reapplied before this recovery is complete (Pufe et al. 2005). The neovascularization pattern observed in the present study corroborates the findings reported by Maia et al. (2009b) who investigated PRP-treated SDFTs at 12 days after induction of tendinopathy by administration of collagenase. Although no significant differences between PRP and 0.9% NaCl have been found concerning vascularization, the advantages of the angiogenic effect of PRP was previously reported in the patellar tendon of rabbits (Lyras et al. 2009). The probable mechanisms of activation of local angiogenesis in the tendon of the current study may be due to the action of growth factors naturally present in the injured tissue (independently of the PRP administration), the angiogenine or other extracellular cytokines that stimulate the proliferation, differentiation and migration of endothelial cells. Among the growth factors, VEGF stands out, whose synthesis is also stimulated by TGF-β1.

The findings obtained in the study performed by Kajikawa et al. (2008), who investigated the right patellar tendon of rats, suggest that locally injected PRP is useful as an activator of circulation-derived cells. According to the authors, PRP stimulates the mobilization of those cells between 3 and 7 days after injury. These results suggest a rapid action of PRP in the first days after injection and reinforce the need for studies that include the different phases of the regeneration process for corroboration of the efficacy of the therapy.

In the histological fragments obtained from the biopsies performed at the proliferative phase of tendon healing, the pattern of the collagen fiber distri-

180

Rev. Bras. Med. Vet., 35(2):169-184, abr/jun 2013

bution (type III collagen fibers intermixed with a type I) is an indicator that *de novo* synthesis of the collagen was occurring, which has also been observed by Maia et al. (2009b) in SDFTs biopsied at 36 days after intratendinous administration of PRP or 0.9% NaCl, and by Fenwick et al. (2001) in naturally occurring chronic Achilles tendinopathy in humans. The expression levels of type I and III collagen increase one week following injury (Dahlgren et al. 2005), as observed in the present study. With the progression of the healing process, the type III collagen fibrils, which have a smaller diameter and are more fragile and less organized, are substituted by type I fibrils, which are thicker and have greater tensile strength (Dahlgren 2007). Tendons with large amounts of type III collagen are less elastic and, therefore, more susceptible to re-injury (Sharifi et al. 2009).

The eosinophilic infiltrate present in the PRP-treated samples may be the result of the chemotactic effect of the platelet-derived histamine. This effect has been described by Chandra et al. (2007) in rabbit skin wounds treated with autologous platelet gel. According to the authors, the activated platelets can also release products of arachadonic acid metabolism, such as leukotrienes, which are chemotactic attractors of eosinophils.

Neovascularization observed by immunohistochemical staining of the vessels was present in the samples obtained 3 and 16 days after PRP administration. However, these results were also noted in tendons treated with the saline solution. These findings reinforce the results obtained in the histological and morphometric analyses, where no differences were observed between the treatments. On the other hand, our findings differ from the results obtained by Bosch et al. (2010), who also used an immunohistochemical staining of factor VIII to verify vascularization in the SDFT treated with a single dose of PRP one week after surgically induced tendinopathy. The authors observed that PRP significantly induced more neovascularization than the placebo treatment 23 weeks after administration of the platelet-rich component, when the animals were submitted to euthanasia. However, color Doppler ultrasonography carried out before and after (2, 3, 5, 8, 18 and 24 weeks) the surgical procedure revealed increased blood flow in the tendons submitted to both treatments, and differences were only found 5 weeks after surgery. According to the authors, the results obtained suggest that PRP has a long-lasting effect after a single intratendinous treatment.

Kurita et al. (2011) evaluated the tissue blood flow in the limbs of rats treated with PRP, platelet-poor plasma (PPP), or a combination of PRP and gelatin hydrogel using a laser Doppler perfusion imaging system. The study revealed that blood perfusion increased progressively from the first week after the treatment in all groups, with differences observed 3 weeks after the treatments being the perfusion significantly higher in the group treated with PRP+gelatin hydrogel. However, the comparison of the animals treated with PPP to those treated with PRP revealed improved blood perfusion in the second group only 4 weeks after treatment. Thus, taking into consideration the results obtained by Bosch et al. (2011) and Kurita et al. (2011), the period considered in this study (3 and 16 days after treatment was performed) was not sufficient to observe any differences in neovascularization based on immunohistochemistry between the groups. The absence of a difference between the treatments at this phase of the tendon healing process may be due to complex events related to neovascularization, which involve aspects such as arteriogenesis and vasculogenesis in addition to angiogenesis stimulated by growth factors, angiogegine and other extracellular cytokines.

This was a randomized controlled clinical study conducted over a period of 16 days after treatment of SDFT tendinopathy with PRP in crossbreed geldings not subjected to controlled physical activity, with an average age of 13 years. The period of time for assessment of tendon, and age of animals are some of the limitations of the study. The results did not confirm our hypothesis that PRP may be an effective therapy for the treatment of tendinous lesions. Therefore, as recently mentioned by Baksh et al. (2013), the benefits of PRP use in the clinical setting remain unclear.

CONCLUSIONS

Based on the histological, morphometric and immunohistochemical results obtained at 3 and 16 days after the induction of tendinopathy, we concluded that a single application of platelet-rich plasma 5 days after injury does not influence the tendon healing process or the presence of factor VIII. Thus, to investigate the efficacy of this low-cost and easily applied therapy, additional controlled studies are needed to evaluate the tendon tissue at different time intervals between PRP injection and biopsy. Additionally, quantitative studies are required to

Rev. Bras. Med. Vet., 35(2):169-184, abr/jun 2013

181

determine the concentration of the growth factors not only in PRP but also in the tissue receiving the therapy.

REFERENCES

Abate M., Gravare-Silbernagel K., Siljeholm C., Di Lori A., De Amicis D., Salini V., Werner S. & Paganelli R. Pathogenesis of tendinopathies: inflammation or degeneration? Review. *Arthritis Res. Ther.*, 11:1-15, 2009.

Aherne W.A. & Dunnill M.S. Point counting and the estimation of volume fraction, p.33-45. In: Aherne W.A. & Dunnill M.S. (Eds), Morphometry. Edward Arnold Ltd, London, 1982.

Anitua E., Andia I., Ardanza B., Nurden P. & Nurden A.T. Autologous platelet as a source of proteins for healing and tissue regeneration. *Thromb. Haemost.*, 91:4-15, 2004.

Argüelles D., Carmona J.U., Climent F., Muñoz E. & Prades M. Autologous platelet concentrates as a treatment for musculoskeletal lesions in 5 horses. *Vet. Rec.*, 162:208-211, 2008.

Argüelles D., Carmona J.U., Pastor J., Iborra A., Viñals L., Martínez P., Bach E. & Prades M. Evaluation of single and double centrifugation tube methods for concentrating equine platelets. *Res. Vet. Sci.*, 81:237-245, 2006.

Baksh N., Hannon C.P., Murawski C.D., Smyth N.A. & Kennedy J.G. Platelet-rich plasma in tendon models: a systematic review of basic science literature. *Arthroscopy*, 29:596-607, 2013.

Batson E.L., Paramour R.J., Smith T.J., Birch H.L., Patterson-Kane J.C. & Goodship A.E. Are the material properties and matrix composition of equine flexor and extensor tendons determined by their functions? *Equine Vet. J.*, 35:314-318, 2003.

Bir S.C., Esaki J., Maruj A., Yamahara K., Tsubota H., Ikeda T. & Sakata R. Angiogenic properties of sustained release platelet-rich plasma: characterization *in-vitro* and in the ischemic hind limb of the mouse. *J. Vasc. Surg.*, 50:870-879, 2009.

Bosch G., Moleman M., Barneveld A., van Weeren P.R. & van Schie H.T.M. The effect of platelet-rich plasma on the neovascularization of surgically created equine superficial digital flexor tendon lesions. *Scand. J. Med. Sci. Sports*, 21:554-561, 2011.

Bosch G., van Schie H.T.M., Groot M.W., Cadby J.A., van de Lest C.H.A., Barneveld A. & van Weeren P.R. Effects of platelet-rich plasma on the quality of repair of mechanically induced core lesions in equine superficial digital flexor tendons: a placebo-controlled experimental study. *J. Orthop. Res.*, 28:211-217, 2010.

Carmona J.U., Argüelles D., Climent F. & Prades M. Autologous platelet concentrates as a treatment of horses with osteoarthritis: a preliminary pilot clinical study. *J. Equine Vet. Sci.*, 27:167-170, 2007.

Chandra R.K., Handorf C., West M., Kruger E.A. & Jackson S. Histologic effects of autologous platelet gel in skin flap healing. *Arch. Facial Plast. Surg.*, 9:260-263, 2007.

Chen Y., Wang C., Yang K.D., Kuo Y., Huang H., Huang Y., Sun Y. & Wan F. Extracorporeal shock waves promote healing of collagenase-induced Achilles tendinitis and increase TGF-beta 1 and IGF-I expression. *J. Orthop. Res.*, 22:854-861, 2004.

Crass J.R., Genovese R.L., Render J.A. & Bellon E.M. Magnetic resonance, ultrasound and histopathologic correlation of acute and healing equine tendon injuries. *Vet. Radiol. Ultrasound.*, 33:206-216, 1992.

Dahlgren L.A. Pathobiology of tendon and ligament injuries. *Clin. Tech. Equine Pract.*, 6:168-173, 2007.

Dahlgren L.A., Mohammed H.O. & Nixon A.J. Temporal expression of growth factors and matrix molecules in healing tendon lessions. *J. Orthop. Res.*, 23:84-92, 2005.

de Mos M., van der Windt A.E., Jahr H., van Schie H.T.M., Weinans H., Verhaar J.A.N. & van Osch G.J.V.M. Can platelet-rich plasma enhance tendon repair? A cell culture study. *Am. J. Sports Med.*, 36:1171-1178, 2008.

de Vos R.J., Weir A., van Schie H.T.M., Bierma-Zeinstra S.M.A., Verhaar J.A.N., Weinans H. & Tol J.L. Platelet-rich plasma injection for chronic Achilles tendinopathy: a randomized controlled trial. *J. Am. Med. Assoc.*, 303:144-149, 2010.

Dragoo J.L., Braun H.J., Durham J.L., Ridley B.A., Odegaard J.I., Luong R. & Arnoczky S.P. Comparison of the acute inflammatory response of two commercial platelet-rich plasma systems in healthy rabbit tendons. *Am. J. Sports Med.*, 40:1274-1280, 2012.

Dyson S.J. Medical management of superficial digital flexor tendonitis: a comparative study in 219 horses (1992-2000). *Equine Vet. J.*, 36:415-419, 2004.

Fernandes M.A.L., Alves G.E.S. & Souza J.C.A. Efeito do ultra-som terapêutico em tendinite experimental de equinos: estudo clínico, ultra-sonográfico e histopatológico de dois protocolos. *Arq. Bra. Med. Vet. Zootec.*, 55:1-11, 2003.

Foland J.W., Trotter G.W., Powers B.E., Wringley R.H. & Smith F.W. Effect of sodium hyaluronate in collagenase-induced superficial digital flexor tendinitis in horses. *Am. J. Vet. Res.*, 53:2371-2376, 1992.

Fortier L.A. Indications and techniques for tenoscopic surgery of the digital flexor tendon sheath. *Equine Vet. Educ.*, 17:218-224, 2005.

Foster T.E., Puskas B.L., Mandelbaum B.R., Gerhardt M.B. & Rodeo S.A. Platelet-rich plasma: from basic science to clinical applications. *Am. J. Sports Med.*, 37:2259-2272, 2009.

Genovese R.L., Rantanen N.W., Hauser M.L. & Simpson B.R. Diagnostic ultrasonography of equine limbs. *Vet. Clin. North Am.: Equine Pract.*, 2:145-226, 1986.

182

Rev. Bras. Med. Vet., 35(2):169-184, abr/jun 2013

Graziani F., Ivanovski S., Cei S., Ducci F., Tonetti M. & Gabriele M. The *in vitro* effect of different PRP concentrations on osteoblasts and fibroblasts. *Clin. Oral Implants Res.*, 17:212-219, 2006.

Grondin T.M. & Dewitt S.F. Normal hematology of the horse and donkey, p.821-828. In: Weiss D.J. & Wardrop K.J. (Eds), *Schalm's veterinary hematology*. 6th ed. Blackwell Publishing, Ames, 2010.

Kajikawa Y., Morihara T., Sakamoto H., Matsuda K., Oshima Y., Yoshida A., Nagae M., Arai Y., Kawata M. & Toshikazu K. Platelet-rich plasma enhances the initial mobilization of circulation-derived cells for tendon healing. *J. Cell. Physiol.*, 215:837-845, 2008.

Kaneko J.J., Harvey J.W. & Bruss M.L. Appendix ISI Units, p.916-917. In: Kaneko J.J., Harvey J.W. & Bruss M.L. (Eds), *Clinical biochemistry of domestic animals*. 6th ed. Academic Press, Amsterdam, 2008.

Kester W.O. Definition and classification of lameness, p.19. In: Kester W.O. (Ed.), *Guide for veterinary services and judging of equestrian events*. 4th ed. American Association of Equine Practitioners, Lexigton, 1991.

Kurita J., Miyamoto M., Ishii Y., Aoyama J., Takagi G., Naito Z., Tabata Y., Ochi M. & Shimizu K. Enhanced vascularization by controlled release of platelet-rich plasma impregnated in biodegradable gelatin hydrogel. *Ann. Thorac. Surg.*, 92:837-844, 2011.

Lyras D., Kazakos K., Verretas D., Polychronidis A., Simopoulos C., Botaitis S., Agrogiannis G., Kokka A. & Patsouris E. Immunohistochemical study of angiogenesis after local administration of platelet-rich plasma in a patellar tendon defect. *Int. Orthop.*, 34:143-148, 2009.

Machado M.V.M., Vulcano L.C., Hussni C.A. & Alves A.L.G. Efeito da laserterapia em tendinite experimental no tendão flexor digital superficial em eqüinos: estudo hitológico e ultra-sonográfico. *Arch. Vet. Sci.*, 5:111-115, 2000.

Maia L., Souza M.V., Alves G.E.S., Ribeiro Júnior J.I., Oliveira A.C., Zandim B.M. & Silva Y.F.R.S. Plasma rico em plaquetas no tratamento de tendinite induzida em equinos: avaliação ultra-sonográfica. *Pesq. Vet. Bras.*, 29:241-245, 2009a.

Maia L., Souza M.V., Ribeiro Júnior J.I., Oliveira A.C., Alves G.E.S., Benjamin L.A., Silva Y.F.R.S., Zandim B.M. & Moreira J.C.L. Platelet-rich plasma in the treatment of induced tendinopathy in horses: histologic evaluation. *J. Equine Vet. Sci.*, 29:618-623, 2009b.

Marr C.M., McMillan I., Boyd J.S., Wright N.G. & Murray M. Ultrasonographic and histopathological findings in equine superficial digital flexor tendon injury. *Equine Vet. J.*, 25:23-29, 1993.

Marxen S., Neto J.C.L., Moraes J.R.E., Ribeiro G. & Neto A.Q. Efficacy of polysulphated glycosaminoglycan in the intratendinous treatment of experimental equine tendinitis. *Bras. J. Morphol. Sci.*, 20:43-46, 2003.

McCarrel T. & Fortier L. Temporal growth factor release from platelet-rich plasma, trehalose lyophilized platelets, and bone marrow aspirate and their effect on tendon and ligament gene expression. *J. Orthop. Res.*, 27:1033-1042, 2009.

Mishra A. & Pavelko T. Treatment of chronic elbow tendinosis with buffered platelet-rich plasma. *Am. J. Sports Med.*, 34:1774-1778, 2006.

Molloy T., Wang Y. & Murrell G.A.C. The roles of growth factors in tendon and ligament healing. *Sports Med.*, 33:381-394, 2003.

Monteiro S.O., Lepage O.M. & Theoret C.L. Effects of platelet-rich plasma on the repair of wounds on the distal aspect of the forelimb in horses. *Am. J. Vet. Res.*, 70:277-282, 2009.

Murray M.M., Spindler K.P., Abreu E., Muller J.A., Nedder A., Kelly M., Frino J., Zurakowski D., Valenza M., Snyder B.D. & Connolly S.A. Collagen-platelet rich plasma hydrogel enhances primary repair of the porcine anterior cruciate ligament. *J. Orthop. Res.*, 25:81-91, 2007.

Murray M.M., Spindler K.P., Devin C., Snyder B.S., Muller J., Takahashi M., Ballard P., Nanney L.B. & Zurakowski D. Use of collagen-platelet rich plasma scaffold to stimulate healing of a central defect in the canine ACL. *J. Orthop. Res.,* 24:820-830, 2006.

Nixon A.J., Dahlgren L.A., Haupt J.L., Yeager A.E. & Ward D.L. Effect of adipose-derived nucleated cell fractions on tendon repair in horses with collagenase-induced tendinitis. *Am. J. Vet. Res.*, 69:928-937, 2008.

O'Kane S. & Ferguson M.W. Transforming growth factor-*ßs* and wound healing. *Int. J. Biochem. Cell Biol.*, 29:63-78, 1997.

Pufe T., Petersen W.J., Mentlein R. & Tillmann B.N. The role of vasculature and angiogenesis of degenerative tendons disease. *Scand. J. Med. Sci. Sports*, 15:211-222, 2005.

Redding W.R., Booth L.C. & Pool R.R. The effects of polysulphated glicosaminoglican on the healing of collagenase induced tendinitis. *Vet. Comp. Orthop. Traumatol.*, 12:48-55, 1999.

Rees H.M. & Ecker E.E. An improved method for counting blood platelets. *J. Am. Med. Assoc.*, 80:621-622, 1923.

Renzi S., Riccò S., Dotti S., Sesso L., Grolli S., Cornalli M., Carlin S., Patruno M., Cinotti S. & Ferrari M. Autologous bone marrow mesenchymal stromal celss for regeneration of injured equine ligaments and tendons. A clinical report. *Res. Vet. Sci.*, 95:272-277, 2013.

Sánchez M., Anitua E., Azofra J., Azofra J., Andía I., Padilla S. & Mujika I. Comparison of surgically repaired Achilles tendon tears using platelet-rich fibrin matrices. *Am. J. Sports Med.*, 35:245-251, 2007.

Schnabel L.V., Mohammed H.O., Miller B.J., McDermott W.G., Jacobson M.S., Santangelo K.S. & Fortier L.A. Platelet Rich Plasma (PRP) enhances anabolic gene expression patterns in flexor digitorum superficialis tendons. *J. Orthop. Res.,* 25:230-240, 2007.

Sharifi D., Kazemi D. & Latifi H. Evaluation of tensile strength of the superficial digital flexor tendon in horses subjected to transcutaneous electrical neural stimulation therapeutic regimen. *Am. J. Appl. Sci.*, 6:816-819, 2009.

Sharma P. & Maffulli N. Biology of tendon injury: healing, modeling and remodeling. *J. Musculoskelet. Neuronal Interact.*, 6:181-190, 2006.

Singer E.R., Barnes J., Saxby F. & Murray J.K. Injuries in the event horse: training versus competition. *Vet. J.*, 175:76-81, 2008.

Smith J.J., Ross M.W. & Smith R.K.W. Anabolic effects of acellular bone marrow, platelet rich plasma, and serum on equine suspensory ligament fibroblasts *in vitro*. *Vet. Comp. Orthop. Traumatol.*, 19:43-47, 2006.

Rev. Bras. Med. Vet., 35(2):169-184, abr/jun 2013

183

Speirs V.C. Sistema musculoesquelético, p.109-181. In: Speirs V.C. (Ed.), *Exame clínico de equinos*. Artmed, Porto Alegre, 1999.

Spurlock G.H., Spurlock S.L. & Parker G.A. Ultrasonographic, gross, and histologic evaluation of a tendonitis disease model in the horse. *Vet. Radiol.*, 30:184-188, 1989.

Sutter W.W., Kaneps A.J. & Bertone A.L. Comparison of hematologic values and transforming growth factor-beta and insulin-like growth factor concentrations in platelet concentrates obtained by use of buffy coat and apheresis methods from equine blood. *Am. J. Vet. Res.*, 65:924-930, 2004.

van Schie H.T.M., Bakker E.M., Jonker A.M. & van Weeren P.R. Ultrasonographic tissue characterization of equine superficial digital flexor tendons by means of gray level statistics. *Am. J. Vet. Res.*, 61:210-219, 2000.

van Schie H.T.M., Bakker E.M., Jonker A.M. & van Weeren P.R. Computerized ultrasonographic tissue characteriza-tion of equine superficial digital flexor tendons by means of stability quantification of echo patterns in contiguous transverse ultrasonographic images. *Am. J. Vet. Res.,* 64: 366-375, 2003.

Waselau M., Sutter W.W., Genovese R.L. & Bertone A.L. Intralesional injection of platelet-rich plasma followed by controlled exercise for treatment of midbody suspensory ligament desmitis in Standardbred racehorses. *J. Am. Vet. Med. Assoc.*, 232:1515-1520, 2008.

Wang J.H-C. Mechanobiology of tendon. Review. *J. Biomech*, 39:1563-1582, 2006.

Wasterlain A.S., Braun H.J. & Dragoo J.L. Contents and formulations of platelet-rich plasma. *Op. Tech. Orthopaedics*, 22:33-42, 2012.

Williams I.F., McCullagh K.G., Godship A.E. & Silver I.A. Studies on the pathogenesis of equine tendonitis following collagenase injury. *Res. Vet. Sci.*, 36:326-338, 1984.

184

Rev. Bras. Med. Vet., 35(2):169-184, abr/jun 2013