

Anabolic effects of acellular bone marrow, platelet rich plasma, and serum on equine suspensory ligament fibroblasts *in vitro*

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Summary

The purpose of this study was to investigate the response of suspensory ligament fibroblasts (SLF) to *in vitro* stimulation using acellular bone marrow (ABM), platelet rich plasma (in vitro PRP), and serum as potential treatment modalities for suspensory desmitis. Blood, bone marrow, and suspensory ligaments were collected from five horses. SLF were harvested, grown until confluent, and stimulated with various concentrations of ABM, PRP, equine serum, foetal bovine serum, and medium (control). The responses to the treatments were assessed using a combination of radiolabeling for total protein synthesis and an ELISA for quantification of Cartilage Oligomeric Matrix Protein (COMP) production. Addition of all of the samples resulted in significant increases in COMP and total protein synthesis over controls ($P < 0.001$). ABM caused the greatest increase in both COMP and total protein synthesis by the SLF. Equine ABM, PRP, and serum contain anabolic factors that promote matrix synthesis by SLF *in vitro*, with ABM having the greatest effect. Application of bone marrow to injured ligaments may enhance healing by providing anabolic factors, other than or in addition to mesenchymal stem cells, which stimulate matrix production.

Keywords

Bone marrow aspirate, platelet rich plasma, serum, growth factor, suspensory desmitis.

Vet Comp Orthop Traumatol 2006; 19: 43–7

Introduction

Suspensory ligament desmitis is a common injury in athletic horses. Normal healing requires a prolonged convalescent period, ranging from three to nine months (10). The repair process results in altered fibre alignment and a mechanically inferior structure making the damaged regions prone to re-injury. In horses with severe lesions, the injuries can become career limiting.

Previous and current treatments have had limited success in returning equine athletes to sustained competition. Traditional treatments have included an initial period of rest followed by a controlled, gradually increasing exercise routine, anti-inflammatory medications, bandaging, counter-irritants, intra-lesional injections of polysulphated glycosaminoglycans, hyaluronan, or beta-aminopropionitrile fumarate, ligament splitting, and, recently, extracorporeal shock wave therapy (10). Many treatments have been aimed at enhancing or modulating the normal repair processes, but fibrous accumulation within the ligament is inadequate in restoring normal function (24). Therefore, more recently, there has been an emerging trend to develop treatments that promote regeneration (*de novo* synthesis) rather than repair (fibrous scar tissue formation). These novel treatments include intra-lesional injections using autologous bone marrow (13), growth factors (8), purified mesenchymal stem cells (25), and gene therapy (27).

Herthel (13) has previously reported the enhanced healing of suspensory ligament injuries treated with intra-lesional injection of autologous bone marrow, and proposed

that the beneficial effects of the treatment were due to the provision of pluripotential mesenchymal stem cells and/or growth factors. However, this treatment has remained controversial due to our lack of true understanding of its mechanism of action, the small number of mesenchymal stem cells (MSC) obtained in a typical bone marrow aspirate (1 MSC/100,000 nucleated cells) (18), and the lack of scientific investigations to elucidate whether the MSC or the growth factors within the bone marrow (acellular bone marrow) have the capacity to improve the healing response. Furthermore, 'growth factors' have not yet been readily identified in equine bone marrow.

'Platelet rich plasma' is considered a rich source of multiple growth factors and has been used to accelerate existing wound healing pathways (18). Processing of platelet rich plasma involves sequestration and concentration of platelets in order to amplify the delivery of the growth factors they contain to injured tissues. Unlike individual recombinant growth factors, platelet rich plasma can modulate and 'up-regulate' one growth factor's function in the presence of additional growth factors.

Experimental studies using recombinant growth factors to study soft tissue healing have yielded promising but also conflicting results (1, 4, 7–9, 14, 16, 17, 20, 26). The variation in tissue response may be due to complex interactions between growth factors and environmental factors, and/or differential growth factor receptor expression at various anatomical sites (20, 26). In addition, comparison across studies remains difficult due to the lack of an established model by which the effects of various treatments can be evaluated (6).

Approaches to studying the efficacy of treatments on soft tissue healing have employed our knowledge of the structural properties and composition of normal tendons and ligaments, and have included measurements of cellular division or proliferation, as well as extracellular matrix synthesis (proteoglycan, collagen, total protein, Cartilage Oligomeric Matrix Protein [COMP]) and organization (22). COMP is a non-collagenous glycoprotein that is found predominantly in tissues that resist load, such as tendon, ligament, cartilage, meniscus, and intervertebral disc. High levels have been found in equine tendon, whereas low levels have been found in fibrous scar tissue (21). This protein may play a substantial role in tendon development and maturation and, because it is largely absent from fibrous scar tissue, it may serve as a marker of appropriate tendon matrix (re)modeling (22). Tendon fibroblasts, in culture, were shown to synthesize COMP slowly in the absence of growth factors, but a dramatic rise in COMP synthesis was observed following the addition of various growth factors, especially transforming growth factor beta one (TGF- β 1) (22).

The purpose of this study was to investigate the metabolic response of equine suspensory ligament fibroblasts *in vitro* to stimulation with biological fluids containing mixtures of growth factors. These biological fluids consisted of acellular bone marrow, platelet rich plasma, equine serum, foetal bovine serum, and serum free medium as a control. Our hypothesis was that acellular bone marrow would contain factors that would stimulate protein and, in particular, COMP synthesis to a greater extent than that provided by platelet rich plasma, equine serum, foetal bovine serum, or without a supplement (control).

Materials and methods

Sample collection

Blood and tissue samples were harvested aseptically from five mixed age (range seven to 18 years), breed, and gender horses at an abattoir immediately post-mortem.

Serum and sodium citrate vacutainer tubes were used to collect blood intended for use as equine serum and platelet rich plasma, respectively. Bone marrow aspirates were collected aseptically from the sternum of the horses using an 11 gauge bone marrow aspirate needle^a and sterile syringe and then placed in sodium citrate vacutainer tubes. Distal forelimbs were obtained from horses without gross signs of suspensory desmitis. All samples were kept on ice until transported to the lab for further processing.

Sample processing

Samples from each horse were separated into equine serum (ES), platelet rich plasma (PRP) (15), and acellular bone marrow (ABM) by density gradient centrifugation. All of the samples were centrifuged for 15 min at 150 g at 4°C (5). The ES, PRP, and ABM were prepared by aspirating the supernatant from the centrifuged samples into separate sterile containers and discarding the concentrated cellular components. Platelet counts were performed on plasma samples before and after centrifugation using an automated counter^b. PRP was defined as plasma containing platelet counts four times greater than initial plasma platelet counts. Microscope slides of the bone marrow aspirates were also prepared for cytological evaluation to ensure marrow components had been obtained. The concentrated cellular component of the centrifuged bone marrow aspirates was discarded and the remaining supernatant was retained as ABM. The samples were initially frozen at -20°C for 24 h, followed by storage at -80°C until time of use.

Sections of suspensory ligament, mid-body and branch, from each horse were harvested aseptically. The peri-ligamentous tissues were removed and ligaments were cut into 2 mm thick strips. The sections of ligament were washed in cell culture grade phosphate buffered saline (PBS)^c contain-

ing 400 IU/ml penicillin (P) and 400 μ g/ml streptomycin (S), cut into 2 mm cubes, and placed in growth medium (DMEM)^d, and 10% foetal bovine serum (FBS)^e, containing 0.1% (w/v) bacterial collagenase^f to a final volume of 10 ml per gram of tissue. In order to release the cells, the samples were incubated at 37°C (95% humidity, 5% CO₂), with constant agitation, until the medium was cloudy. Tissue debris was removed by filtration through a 40 μ m mesh filter^g and the cells released by digestion were pelleted by centrifugation at 250 g for 10 min. The supernatant was discarded, and the cells were re-suspended and washed in DMEM-PS + 10% FBS. The cell suspensions were then transferred into a plastic tissue culture flask and incubated at 37°C (95% humidity, 5% CO₂). They were left to attach to the plastic for 48 h, at which time the medium was replaced with fresh DMEM-PS + 10% FBS. The medium was changed every 3 days until the cells had grown to a confluent monolayer within the flask. Once confluent, the cells were harvested from the flasks by trypsin and ethylenediaminetetraacetic acid (EDTA)^h digestion and centrifuged again at 250 g for 10 min. The supernatant was discarded and the pelleted cells were resuspended in DMEM-PS + 10% FBS. 500 μ l of cell suspension were placed into each well of a 48-well, flat bottomed plastic culture plateⁱ (0.95 cm²) and incubated at 37°C (95% humidity, 5% CO₂). The cells were then left to attach to the plastic for 48 h, when the medium was replaced with fresh DMEM-PS + 10% FBS. The medium was changed every three days until the cells had grown to a confluent monolayer in all the wells.

Cell stimulation

The medium was discarded from each well and the wells were washed once with PBS-

^d Dulbecco's Modified Eagle's Medium, Sigma-Aldrich, Poole, UK.

^e Foetal Bovine Serum, Sigma-Aldrich, Poole, UK.

^f Collagenase Type VII, Sigma-Aldrich, Poole, UK.

^g Falcon, BD Biosciences, San Jose, CA, USA.

^h Trypsin-EDTA solution, Sigma-Aldrich, Poole, UK.

ⁱ Costar® 48 Well TC Treated Microplates, Corning Inc., Corning, NY, USA.

PS. 500 μ l of DMEM-PS was added to each well. After maintaining the cells in serum-free medium for 24 h, the medium was discarded and replaced with a solution containing DMEM-PS, ascorbate (20 μ g/ml), 3 H-Leucine^j (20 μ Ci/ml), and 3.8% sodium citrate^k. The cells were stimulated by adding the following samples obtained from each of the five horses to the wells in triplicate: 10% ABM, 5% ABM, 10% PRP, 5% PRP, 10% ES, 10% FBS. Wells containing only the medium solution were used as controls. The plates were incubated at 37°C (95% humidity, 5% CO₂) for 48 h. Analysis was performed on both the cell-free supernatant and the cell-associated layer. The supernatant was aspirated from each well and an aliquot was precipitated twice in 95% ethanol and 50 mM sodium acetate. The cell associated layer was extracted using 4M guanidine hydrochloride containing protease inhibitors (10 mM EDTA, 100 mM E-amino-n-caproic acid, 5 mM benzamidine, 50 mM sodium acetate, and 5 mM N-ethylmaleimide) and an aliquot was also precipitated twice in 95% ethanol and 50mM sodium acetate.

The metabolic response of the cells was assessed by measuring COMP synthesis and 3 H-Leucine incorporation. COMP production was measured using a competitive inhibition, enzyme linked immunosorbent assay (ELISA) previously described by Smith (21). 3 H-Leucine incorporation (19) was determined by placing 100 μ l of the precipitated samples into scintillation vials containing 4 ml of scintillant. The amount of radiolabeled amino acid incorporated into proteins neo-synthesized by the stimulated cells was measured using a liquid scintillation counter (11).

Statistical analysis

The results were analyzed with a statistical software programme^l using analysis of variance (ANOVA) in a mixed model approach. When appropriate, a post-hoc Tukey's test

was used for 'pairwise' comparisons. Differences between the means were considered significant when $P < 0.05$.

Results

Suspensory ligament cells appeared spindle shaped and became aligned in parallel aggregate bundles resembling fibroblasts. Platelet counts performed on the platelet rich plasma were four times greater than initial plasma platelet counts with a mean platelet count of 522,000 platelets/ μ l (range: 415,000 – 650,000 platelets/ μ l). Microscope slides of the bone marrow aspirates contained fat, marrow spicules, and immature nucleated cells consistent with normal equine bone marrow.

The mean COMP production 'per well' by the suspensory ligament fibroblasts in response to the various treatments is shown in Fig. 1. There was an increase in mean COMP production by all treatments in the following order of decreasing efficacy: 10% ABM > 10% PRP > 10% ES > 10% FBS > 5% ABM > 5% PRP > medium (control). Mean COMP synthesis for cells stimulated with 10% ABM was significantly greater

than all treatments ($P < 0.0001$) other than 10% PRP ($P = 0.3956$).

The mean 3 H-Leucine incorporation per well, indicative of total protein synthesis, in response to the various treatments is shown in Fig. 2. There was a similar increase in the mean total protein synthesis by all of the treatments in the following order of decreasing efficacy: 10% ABM > 10% ES > 10% FBS > 10% PRP > 5% ABM > 5% PRP > medium (control). Mean 3 H-Leucine incorporation by cells stimulated with 10% ABM was significantly greater than all other treatments ($P < 0.0001$), except 10% ES ($P = 0.3325$) and 10% FBS ($P = 0.1257$).

Discussion

The results of this study support our hypothesis and the concept that bone marrow aspirates contain factors that may enhance ligament healing as has been previously described by Herthel (13). Due to the small number of mesenchymal stem cells obtained in a typical bone marrow aspirate (18), the beneficial effects of the treatment may be due to factors other than, or in addition to, MSC's contained within the bone marrow.

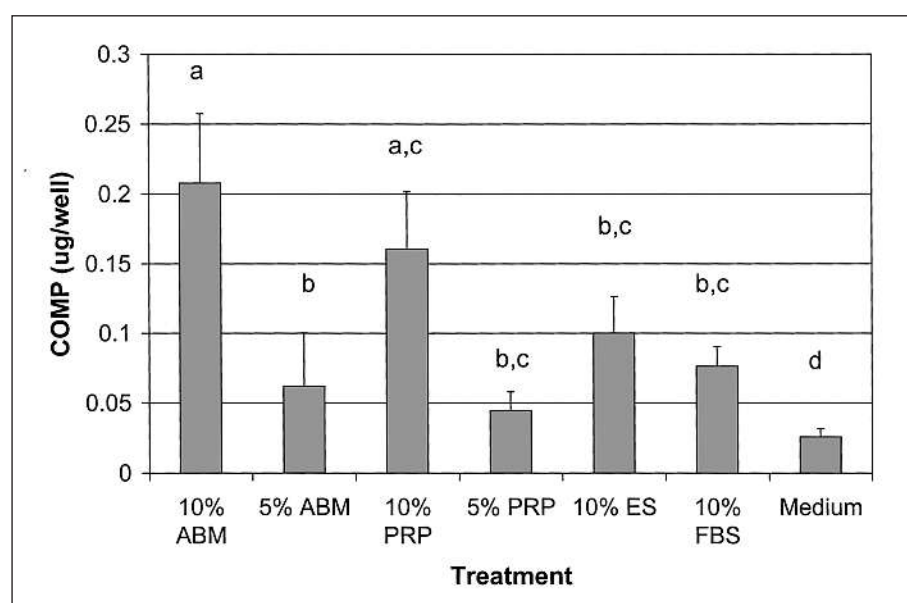


Fig. 1 Mean \pm SD values for cartilage oligomeric matrix protein (COMP) production measured in μ g per well by the suspensory ligament fibroblasts in response to the various treatments listed on the x-axis. Bars with different letters differ significantly ($P < 0.05$). Acellular bone marrow (ABM), platelet rich plasma (PRP), equine serum (ES), foetal bovine serum (FBS), control (medium).

^j 3 H-leucine, Amersham Biosciences, Little Chalfont, UK.

^k Sodium citrate, JT Baker Inc., Phillipsburg, NJ, USA.

^l Proc GLM, SAS Institute, Inc., Cary, NC, USA.

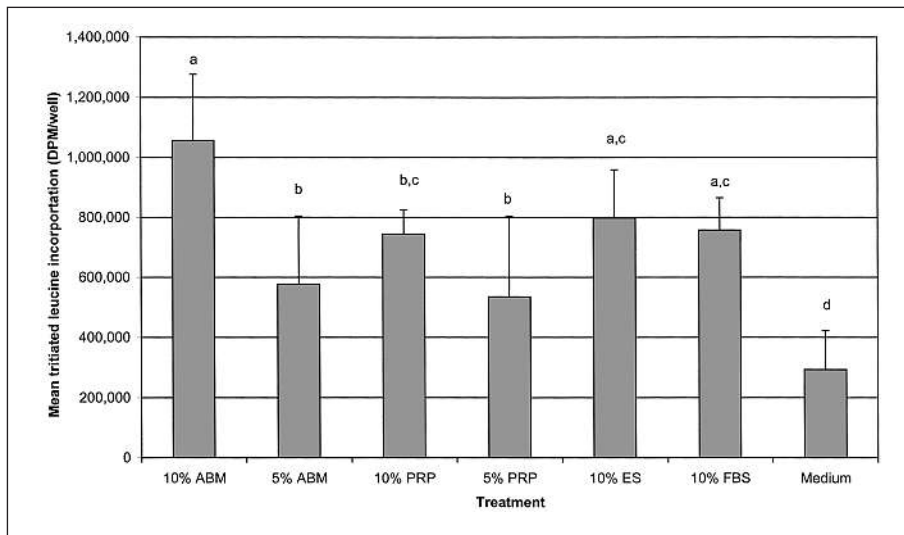


Fig. 2 Mean \pm SD values for ^3H -Leucine production measured in disintegrations per minute (DPM) per well by the suspensory ligament fibroblasts in response to the various treatments listed on the x-axis. Bars with different letters differ significantly ($P < 0.05$). Acellular bone marrow (ABM), platelet rich plasma (PRP), equine serum (ES), foetal bovine serum (FBS), control (medium).

The regulatory signals involved in tendon and ligament repair processes have not been fully elucidated. Growth factors are known to be involved in the control of cell proliferation, migration, differentiation, and synthetic activity (17, 20). However, growth factor involvement in tendon and ligament repair is not well understood (26). Recent investigations revealed various growth factors, such as TGF- β 1, insulin-like growth factor I, platelet derived growth factor, fibroblast growth factor, and epidermal growth factor (2, 12, 17, 27), are expressed in normal and healing tendons and ligaments. It is generally assumed that specific growth factors, released after injury, coordinate the repair process (12, 20). The various growth factors may act synergistically, or antagonistically, and be influenced by many environmental factors (2). Bone marrow aspirates, platelet rich plasma, and serum may contain appropriate mixtures of growth factors to enhance protein production by fibroblasts *in vitro*.

Quantitative analysis of individual equine growth factors is complicated by the lack of equine specific commercial assays, as well as the presence of active and latent forms of growth factors and growth factor inhibitors. Hence, we took an alternative approach using an activity assay to measure

the effects of the various samples on the suspensory ligament fibroblasts. A beneficial mix of growth factors for ligament repair must be able to induce appropriate protein synthesis and ligament matrix formation. The effects of ABM, PRP, ES, and FBS on the suspensory ligament fibroblasts were therefore assessed by measuring overall protein synthesis and COMP production *in vitro* by the suspensory ligament fibroblasts.

COMP was chosen because of previous work suggesting this protein may play an important role in tendon development and maturation (21). Although COMP levels have not previously been measured in equine suspensory ligaments, tendons and ligaments are similar connective tissues containing fibroblastic cells that have similar healing characteristics. While its precise role remains unclear at present, there is some evidence to suggest that COMP levels during tendon maturation correlate with tendon strength (23). COMP may be a good marker of connective tissue matrix formation since it is present in low levels in tendinous scar tissue.

Acquisition of acellular bone marrow is a minimally invasive procedure that requires minimal processing of the samples. In this study, ABM caused a significant dose-de-

pendent response by the suspensory ligament fibroblasts. The 10% ABM resulted in the highest COMP and total protein synthesis overall, with a nearly eight-fold increase in mean COMP production and a four-fold increase in mean total protein synthesis by the fibroblasts when compared to controls. Perhaps ABM contains higher concentrations of growth factors or has a more appropriate physiologic distribution of growth factors than the other biological fluids used in this study.

Platelet rich plasma and equine serum are considered to be rich sources of various growth factors (18), are less invasive to obtain than a bone marrow aspirate, and require processing that is similar to acellular bone marrow. In this study, PRP and ES also demonstrated significant anabolic effects on suspensory ligament fibroblasts. However, neither PRP nor ES were as effective as ABM. Serum, thrombin, and other platelet activators were not added to the PRP samples to encourage clot formation in our study. Whilst activation of platelets triggers the release of the growth factors and other contents of the alpha granules within the platelets, spontaneous clot formation was observed within the wells following addition of the PRP samples, and therefore further activation was deemed unnecessary. Additionally, higher levels of TGF- β 1 were quantified within PRP, as compared to platelet poor plasma, without prior activation of the samples with thrombin in a previous study (3). It was also suggested that thrombin influences wound healing (3). Therefore, the addition of thrombin to the PRP samples might have interfered with our ability to accurately assess the response of the suspensory fibroblasts to PRP alone.

Foetal bovine serum is available commercially and was shown to be the most potent stimulator of cell proliferation and protein synthesis in previous studies using rabbit flexor tendons when compared to individual growth factors (14). However, in the study presented here, FBS demonstrated minimal anabolic effects on suspensory ligament fibroblasts *in vitro*, indicating that even adult, species specific serum is more active than foetal serum from another species.

In summary, an increase in COMP and total protein synthesis by the suspensory

ligament fibroblasts was observed following stimulation with the various biological fluids. ABM, PRP, ES, and FBS all resulted in significant increases in COMP synthesis over controls while 10% ABM resulted in the greatest synthesis of COMP and total protein compared to other treatments. This suggests that ABM may contain appropriate combinations of growth factors that act synergistically to enhance healing, and which may be beneficial for the successful management of suspensory desmitis. Future work should be directed at evaluating the *in vivo* response of suspensory ligaments to various biological fluids, determining the optimal dosages and timing of administration of the fluids, and potentially using these biological fluids as vehicles for growth factor and/or mesenchymal stem cell delivery to injured soft tissues.

Acknowledgements

The authors would like to thank Dr. Joe Bird, Dr. Charlotte Scott, and Sylvia Reedy at the Royal Veterinary College for their technical assistance, Dr. Louise Southwood at the University of Pennsylvania's New Bolton Center for assistance with statistical analysis, and the Raymond Firestone Trust Research Grant for funding this study.

An abstract of this paper was presented at the ACVS Convention in Washington, DC, on October 11, 2003.

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