Journal of
Stem Cell Research & Therapy

Dhar et al., J Stem Cell Res Ther 2015, 5:4
DOI: 10.4172/2157-7633.1000278

Research Article Open Access

Platelet-Rich Plasma Enhances the Cellular Function of Equine Bone Marrow-Derived Mesenchymal Stem Cells

Madhu Dhar[1]*, Lisa Amelse[1], Nancy Neilsen[2], Pelagie Favi[3] and Jessica Carter-Arnold[1]

[1]*Department of Large Animal Clinical Sciences, University of Tennessee, Knoxville, Tennessee, USA*
[2]*Biological and Diagnostic Sciences, College of Veterinary Medicine, University of Tennessee, Knoxville, Tennessee, USA*
[3]*Material Science and Engineering, University of Tennessee, Knoxville, Tennessee, USA*

Abstract

Rationale: Equine bone marrow-derived mesenchymal stem cells (eBMMSCs) and platelet-rich plasma (PRP) are cell-based therapies being used clinically to repair damaged tissue of horses. Numerous reports including data from our laboratory show that there are variations in the biological properties of MSCs and platelet – rich plasma, which can impact their biological functions. A single study describes the use of the eBMMSCs and PRP in tendon healing, with minimal success. The exact mechanism of action using the combination of the two therapies is unknown. This study was performed to evaluate and understand the effects of PRP, if any on the cellular performance of eBMMSCs in culture.

Objective: To assess the effects of PRP *in vitro* on the rate of proliferation, expression of protein markers and osteogenic and chondrogenic differentiation of the primary cultures of eBMMSCs.

Methods and Results: A commercially available stall side, portable kit was used to isolate PRP. To investigate the effect of PRP on eBMMSCs, the rate of proliferation of eBMMSCs was measured using the MTS assay, and subsequently the viability and the stemness of eBMMSCs was assessed using fluorescent staining and the expression of CD90. Finally, the osteogenic and chondrogenic differentiation of eBMMSCs was assessed by lineage-specific staining and the expressions of lineage – specific mRNAs. All assays were performed at a concentration of 50 million platelets/mL. Significant increase in proliferation and the differentiation profiles were observed in presence of PRP. Most importantly, the stem cell characteristics of inferior eBMMSCs showed a marked improvement in presence of PRP.

Conclusions: The addition of PRP improves the *in vitro* function of eBMMSCs by enhancing the proliferation and osteo - and chondro - genesis. The presence of an optimal dose of platelets may enhance the *in vivo* performance of eBMMSCs and may be indicated when using autologous eBMMSCs therapy in the clinic.

Keywords: Equine; Mesenchymal cells; Platelet rich plasma; Platelets; Osteogenesis; Chondrogenesis

Introduction

The field of regenerative medicine is becoming an exciting area in both human and veterinary medicine that may greatly impact our ability to treat patients. Two main tools extensively being investigated are platelet-rich plasma (PRP) and mesenchymal stem cells (MSCs) and how they may aid in the treatment of orthopedic diseases. The PRP is plasma with a greater concentration of platelets than that found in whole blood, and rationale for its use arises from the release of growth factors, including platelet-derived growth factor (PDGF), transforming growth factor beta (TGFβ), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and insulin-like growth factors 1 and 2 (IGF), and other cytokines, including interleukin 8. These molecules are released from the alpha granules when platelets rupture or lyse [1-5]. This process is also referred to as activation of PRP and it is these growth factors and cytokines that locally induce endogenous bone and soft tissue regeneration. Platelet-rich-plasma also offers a significant advantage over other potential cell-based therapies in that it is autologous (i.e. the donor and the patient are the same), and hence, avoiding graft vs. host complications. Additionally, when PRP is activated it forms a platelet gel which can serve as an autologous scaffold which can serve as a delivery vehicle for stem cells or any other drug. This can have significant effect on some complex clinical cases for instance, wound healing. Hence, PRP is an attractive cell-based modality that can be beneficial in the treatment of various equine injuries or diseases.

The idea of providing a means for tissue to regenerate instead of repair with fibrous scar tissue is the basis for the use of MSC therapy. Therefore, the rational of combining MSCs and growth factors from PRP to synergistically enhance the regenerative processes of both therapies is reasonable. Synergistic effects of PRP and MSCs in cell-based therapies have been documented both *in vitro* and *in vivo* in human and rat models. *In vitro* investigations have confirmed that PRP enhances proliferation of a variety of human and rodent cell types including MSCs [6]. *In vitro* studies suggest that the combination of PRP and MSCs shows promise by the ability of PRP to increase the proliferation and differentiation of undifferentiated human MSCs [6-11]. Specifically, when human MSCs are grown in the presence of PRP *in vitro*, PRP not only enhances the MSC proliferation but also causes an increase in their osteogenic and chondrogenic differentiations [10,12]. Although these studies generally yield positive results some negative outcomes in animal studies have also been reported. For example, Ogino reported that PRP enhanced the proliferation of rat

***Corresponding author:** Dr. Madhu S Dhar, Research Associate Professor, Department of Large Animal Clinical Sciences, 2407 River Drive, University of Tennessee College of Veterinary Medicine, Knoxville, TN 37996, USA, Tel: 865-974-5703; Fax: 865-974-5773; E-mail: mdhar@utk.edu

Received April 15, 2015; **Accepted** April 29, 2015; **Published** April 30, 2015

Citation: Dhar M, Amelse L, Neilsen N, Favi P, Carter-Arnold J (2015) Platelet-Rich Plasma Enhances the Cellular Function of Equine Bone Marrow-Derived Mesenchymal Stem Cells. J Stem Cell Res Ther 5: 278. doi:10.4172/2157-7633.1000278

Copyright: © 2015 Dhar M, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

bone marrow cells but suppressed their differentiation [13]. Martinello et al. reported no synergistic or additive effect of PRP and peripheral blood derived MSCs on experimentally injured deep digital flexor tendons of sheep [10].

To the best of our knowledge, there is a single study in horses where this combination therapy has been used *in vivo.* Torricelli et al., showed that a combination of PRP and bone marrow derived mononuclear cells resulted in marked improvement in horses with tendon damage and returned 85% of those treated with this therapy to competition. Two horses that did not return to competition had relatively lower levels of growth factor and platelet counts, suggesting, that the inefficiency of PRP may be attributed to the biological status of the animals themselves rather than to an ineffective therapy [14]. Besides, musculoskeletal defects, PRP have also been used in the treatment of equine acute and chronic full thickness cutaneous wounds, again with mixed and controversial results [15-17].

Similar to as described above for PRP, *in vitro* and *in vivo* variations in the proliferation and differentiation of MSCs alone have also been demonstrated in mice, dog, human and equine donors [18-21]. These variations can be due to the source of MSCs, age-dependent or independent donor-to-donor variation, and are further complicated when cells are expanded by passaging in culture. We recently observed significant variation in the biological properties of equine bone marrow-derived MSCs (eBMMSCs) among age and gender matched horses [19]. Using molecular and cellular assays laid out by the International Society for Cellular Therapy [22], we reported that the eBMMSC population of cells generated from a 12 year old mare exhibited a higher rate of proliferation, enhanced ability for cell passaging, and relatively more robust patterns of *in vitro* differentiation. Comparatively, eBMMSCs from 2 other age and gender – matched donors demonstrated a lower rate of proliferation and lack of osteogenic and chondrogenic differentiation [19]. The clinical significance of these findings is unknown but suggests that they could potentially affect the treatment outcome. Thus, it is important to note that not all MSCs and PRP are equivalent and the quality of these must be documented and compared to improve the treatment outcome.

Due to the demand for autologous MSCs therapy, we wanted to investigate ways of enhancing the performance of the eBMMSCs specifically on those cells that demonstrate poor proliferation and differentiation, as previously reported by our group. In view of the published literature, we hypothesized that the response of eBMMSCS would differ when cultured with an optimal concentration of PRP. This response will be assessed in the rate of proliferation and osteo - and chondrogenic differentiation. Our main objective was to evaluate the effects of PRP, if any, on the cellular function of eBMMSCs. Therefore in this current study we exploited the properties of PRP to enhance the proliferation and differentiation of eBMMSCs. We performed this in focus of both the clinical and basic research in MSC therapies. To maintain consistency in the PRP preparations, for PRP isolation we selected the ProTec system from PulseVet (PulseVet, Alpharetta, GA) based on cost, portability, and ease of use. We evaluated the platelet concentration, white blood cell concentration, pH, and the effect of PRP on eBMMSCs cultured from five age and gender-matched horses. We selected equine donors from the group used previously [19]. Donor 1 is the donor from which the MSCs demonstrated superior *in vitro* properties whereas, eBMMSCs generated from donor 5 demonstrated relatively a lower rate of proliferation and reduced osteogenic and chondrogenic potentials.

Materials and Methods

Animals

All experiments were carried out using protocols approved by the Institutional Animal Care and Use Committee. All horses were age- and gender–matched (females aged 10 years to 13 years; mean $\pm$ SD, 11.4 $\pm$ 1.1). All horses were sound with no current or history of health problems and housed under the same conditions and exercise regimes. Two donors were American Quarter Horses (one 12-year old, and one 11-years-old), two were mixed breed American Quarter Horses (10-years-old) and one was a Tennessee walking horse (13-years-old).

Bone marrow derived adult mesenchymal stem cells

Previously expanded, characterized and cryporeserved eBMMSCs from donors 1- 5, described earlier were used in all experiments [19]. All cell culture experiments were carried out in triplicate and each assay was repeated with the eBMMSCs from two independent bone-marrow harvests. Each horse was sampled twice between 3-6 months. Passage 2 cells were used in all proliferation assays and passage 3 cells were used in all differentiation and protein and mRNA expression experiments.

Platelet-rich plasma, growth factors

Platelet-rich plasma was prepared from whole blood using the single step ProTec kit (PulseVet, Alpharetta, GA) according to the manufacturer's instructions. Briefly, 9 mL of whole blood was centrifuged at an optimum speed (in a centrifuge provided by the manufacturer) for 6 minutes and roughly 5 mL (50% of the whole blood volume) of PRP was obtained. Platelets and leukocytes in each PRP preparation and in whole blood were counted using the Leukochek system (Biomedical Polymers Inc. Gardner, MA).

Commercially available human ELISA kits (R&D Systems, Minneapolis, MN), previously validated for quantitation of equine growth factors [5,23-25], and were used to determine the concentrations of TGFβ1 and PDGF-BB in each sample. Two independent PRP preparations were sampled and each assay was performed in triplicate. All assays were carried out using eBMMSCs in presence of autologous PRP.

Platelet poor plasma

Platelet-poor plasma (PPP) was prepared from the plasma by centrifugation at room temperature for 15 mins at a high speed of 4600 rpm. Sample was assessed for the absence of any platelets and leukocytes. Platelet-poor plasma was used as a growth factor-free control for all cell culture assays as well as for the dilution of PRP.

Cell proliferation

A dose-response experiment was first carried out to assess the effect of varying amounts of platelets on the rate of proliferation of eBMMSCs from 2 donors (donors 1 and 5), with contrasting MSC properties. The rate of cellular proliferation of eBMMSCs from the 2 horses was assessed in the presence of PRP, PPP, and complete growth media (without any PRP or PPP) at 2 and 6 days using the CellTiter 96® Aqueous Non-Radioactive (MTS) assay according to the manufacturer's instructions (Promega Inc., Madison, WI). The PRP groups included a baseline group of 10 million platelets/mL, and groups containing 15, 30, 50, and 75 million platelets/mL, which correspond to 1.5, 3.0, 5.0 and 7.5 fold increase over the baseline. Equine BMMSCs in complete growth media served as control. The PPP groups were created to match the milliter volume of the PRP groups to confirm that the effect was a result of the

platelets.

All experiments were carried out in triplicate in 96-well plates with 3×10^3 cells seeded in 200 µL as the seeding volume. For the assay, 40 µL of the MTS reagent was added to each well and incubated for 3 h at 37°C/5% CO_2. The optical density of the complex was measured on a microplate fluorescence reader at 490 nm. Each medium combination without cells was used as the corresponding blank. Each sample absorbance was corrected by the reference blank reading. Graph of absorbance at 490 nm versus days of proliferation was generated, and data were analyzed.

Following the results of the dose response experiment the proliferation assay was repeated with the optimal deduced platelet concentrations for eBMMSCs cultured from the 3 remaining donors (donors 2-4) as described above.

Cell adhesion, viability, and immunofluorescence

Equine bone marrow-derived MSCs were seeded with 50 million platelets/mL (5 fold above baseline), that was deduced as the optimal concentration in PRP. Cell adhesion and viability was assessed at day 6 using calcein-AM (Invitrogen, Eugene, OR) and propidium iodide (PI) (Invitrogen, Carlsbad, CA). Cells were stained according to the manufacturer's protocols and subsequently visualized using an electronic camera (Nikon DS-Fi2, Japan) connected to a Zeiss microscope and evaluated with the NIS Elements imaging software (Nikon).

The expression of CD90, a reliable protein marker for MSCs [26,27] was assessed using immunofluorescence. Equine bone marrow-derived MSCs were seeded in growth medium containing 50 million platelets/mL, and were incubated at 37°C and 5% CO_2, for 6 days. At day 6, MSCs were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 (Sigma®) for 10 min, at room temperature, and blocked with 5% normal serum for 30 min, at room temperature. Cells were washed and incubated overnight with 5 µg/sample of primary antibody against FITC-conjugated anti-rat CD90 (BD Pharmingen™) at 4°C. After washing with HBSS buffer, cells were mounted with Slowfade® Gold antifade with DAPI reagent (Molecular Probes™) and images were obtained using an electronic camera (Nikon DS-Fi2, Japan) connected to a Zeiss microscope and evaluated with the NIS Elements imaging software (Nikon).

Activation of PRP and bi-lineage differentiation

For all differentiation assays, tissue culture dishes were coated with a uniform layer of activated PRP. To form a PRP gel, an appropriate volume of PRP containing an optimal concentration of platelets deduced from the above experiment, was activated by roughly 2.5% $CaCl_2$. One hundred microliter of the mixture (which contained an optimal platelet concentration of 50 million platelets/mL) was then used to coat each well of a 24 well plate using a micropipette. Plates were incubated at 37°C for 15 min and 1.0×10^4 eBMMSCs were seeded in each well for differentiation. Using PRP-coated tissue culture dishes eliminated the need of large volumes of PRP at each media change and ensured that the eBMMSCs were exposed to PRP throughout their growth and differentiation phase.

Osteogenesis and chondrogenesis were performed using standard procedures [19] with P3 eBMMSCs in presence and absence of PRP. Equine BMMSCs were seeded in presence of the complete growth medium. Seventy to eighty percent confluent cells were induced to differentiate into each of the 2 lineages. Equal numbers of cells without any differentiation media were used as controls.

Osteogenic differentiation was induced in complete growth medium supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate, and 0.25 mM ascorbic acid. The medium was changed every 3 days, and differentiation was monitored using alizarin red staining after 21 days.

Chondrogenic differentiation was induced in complete growth medium supplemented with 100 nM dexamethasone, 0.25 mM ascorbic acid, and 5 ng/mL TGFβ1. The medium was changed every 3 days, and differentiation was monitored using alcian blue staining after 14 days.

All differentiation profiles were assessed via cell-specific staining and by assessing the expression of lineage-specific mRNAs. Images were acquired with an electronic camera (Nikon DS-Fi2, Japan) connected to a Zeiss microscope and evaluated with the NIS Elements imaging software (Nikon).

RNA isolation and real time polymerase chain reaction (qPCR)

RNA isolation and qPCR from P3 eBMMSCs in presence of PRP were performed. The total RNAs from undifferentiated cells in presence of PRP (control) and differentiated cells in presence of PRP were isolated. For RNA isolation, cells were harvested using 5 mL of Accutase (Cell detachment solution in PBS) and incubated at 37°C/5% CO_2 for 30 min. Total RNA was extracted using an RNeasy Mini RNA kit according to the manufacturer's instructions. One microgram of total RNA was reversing transcribed in a 10 µL reaction volume for cDNA synthesis (Biorad, Hercules, CA).

Alkaline phosphatase (ALP) was used as an osteogenic marker and, cartilage oligometic matrix protein (COMP) was used as markers for chondrogenic differentiation. Messenger RNA levels for all the markers were quantified using mRNA expression normalized with that of equine GAPDH mRNA as the internal control. Real time PCR was carried out using the absolute SYBR Green ROX quantitative PCR mix on the Mx3005P and data were analyzed using MxPro software (Agilent Technologies, Santa Clara, CA). C_t values representing the level of each mRNA expression were calculated. Using a cDNA blank, samples with an undetectable gene expression level (i.e., a Ct value of ≥ 30) were given an arbitrary expression level of zero. The relative expression of each target gene was calculated as ΔC_T. Delta C_t values for each mRNA under a specific condition for each donor were compared. The expressions were compared between undifferentiated and differentiated samples in presence of PRP.

Statistical Analysis

All data are expressed as the mean ± standard deviation. All analyses were done using a combination of STATA 12 and SPSS 22. Normality of the data was first confirmed using the Shapiro-Wilke test and then analyses were performed using the paired, Student t-test. $P<0.05$ was considered statistically significant for all analyses.

Results

Characterization of PRP

Roughly 5 mL of PRP was obtained in one preparation from each of the five equine donors using the ProTec kit. Each horse was sampled in duplicate. The platelet counts in the whole blood ranged from 1.7 to 2.3×10^8/mL with a mean value of 1.9×10^8/mL. Comparatively, platelet

counts in PRP ranged from 2.7 to 4.3 × 10^8/mL with a mean value of 3.5 × 10^8/mL. Therefore, each preparation resulted in 1.5-1.9 fold enrichment in the platelets compared to the whole blood. Each PRP preparation contained negligible amount (<1%) of white blood cells. The pH of each PRP sample was 7.2-7.3. For all experiments the culture media was standardized to10 million platelets/mL. This is referred to as the baseline group. The TGF-b1 and PDGF-BB concentrations in the PRP were 3309.63 ± 1036.27 pg/mL and 2989.23 ± 561 pg/mL, respectively.

PRP enhances the rate of proliferation of eBMMSCs

To determine an optimal platelet concentration a dose-response experiment was carried out to assess the effect of varying amounts of platelets on the rate of proliferation of eBMMSCs from two horses with contrasting MSC properties (donors 1 and 5 from the earlier study). Cell proliferation was evaluated in presence and absence of PRP using MTS assays after days 2 and 6 (Figures 1 and 2). Cells grown in the absence of PRP served as the control. At each time point, there was a general increase in cell proliferation with increasing concentration of platelets above the baseline. The 5-fold concentration of platelets had the largest and the most consistent significant effect in both the donors and at both time points, and hence, was chosen as the optimal dose. This observation was further confirmed in eBMMSCs isolated from the remaining three equine donors 2-4 (Figure 3). Collectively, a 3.5 to 3.7 fold increase in cell proliferation on day 2 and a 2.2-3.0 fold increase in cell proliferation on day 6 were observed. PPP treatment groups equivalent to that of PRP were assayed for each donor and data showed that the effect on cell proliferation is solely due to platelets in the PRP (data not shown).

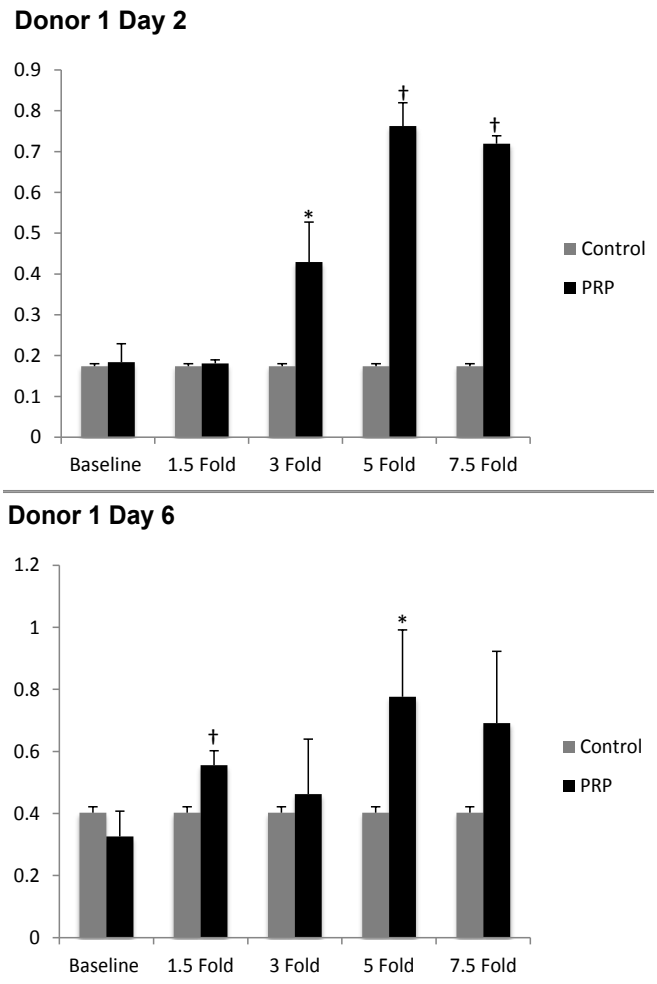

Figure 1: Measurement of rate of proliferation of eBMMSCs. The cell proliferation assay was used to assess the rate of proliferation of eBMMSCs from donor 1 over a period of 6 days, as a function of platelet concentration in PRP. The rates were obtained in presence of 10 million platelets/mL (baseline), 1.5, 3, 5 and 7.5 fold above the baseline and were compared to the rates of proliferation of cells grown in the absence of PRP (control). The single dagger symbol and the asterisk demonstrate a significant value with at least p<0.005.

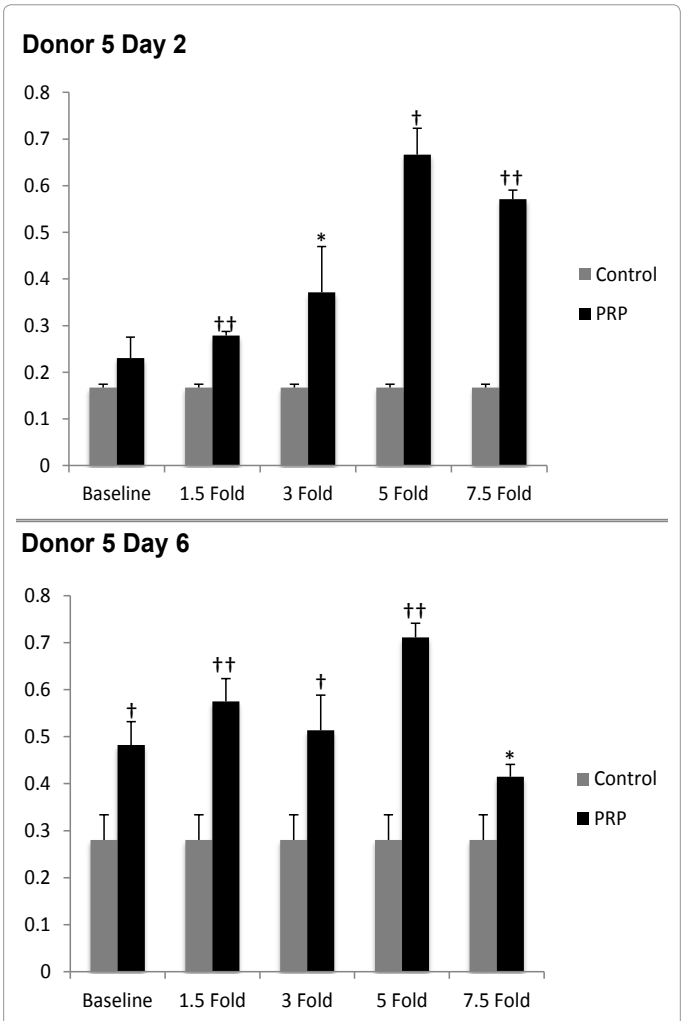

Figure 2: Measurement of rate of proliferation of eBMMSCs. The cell proliferation assay was used to assess the rate of proliferation of eBMMSCs from donor 5 over a period of 6 days, as a function of platelet concentration in PRP. The rates were obtained in presence of 10 million platelets/mL (baseline), 1.5, 3, 5 and 7.5 fold above the baseline and were compared to the rates of proliferation of cells grown in the absence of PRP (control). The dagger symbols and the asterisk demonstrate a significant value with at least p<0.005.

The cell viability and stemness is maintained in presence of PRP

Based on proliferation experiments we cultured eBMMSCs in presence of 5 fold PRP for all further experiments. The tissue culture dishes were coated with a volume which contained 5 fold platelet dose. Cell adherence and viability was tested using calcein-am/PI live-dead

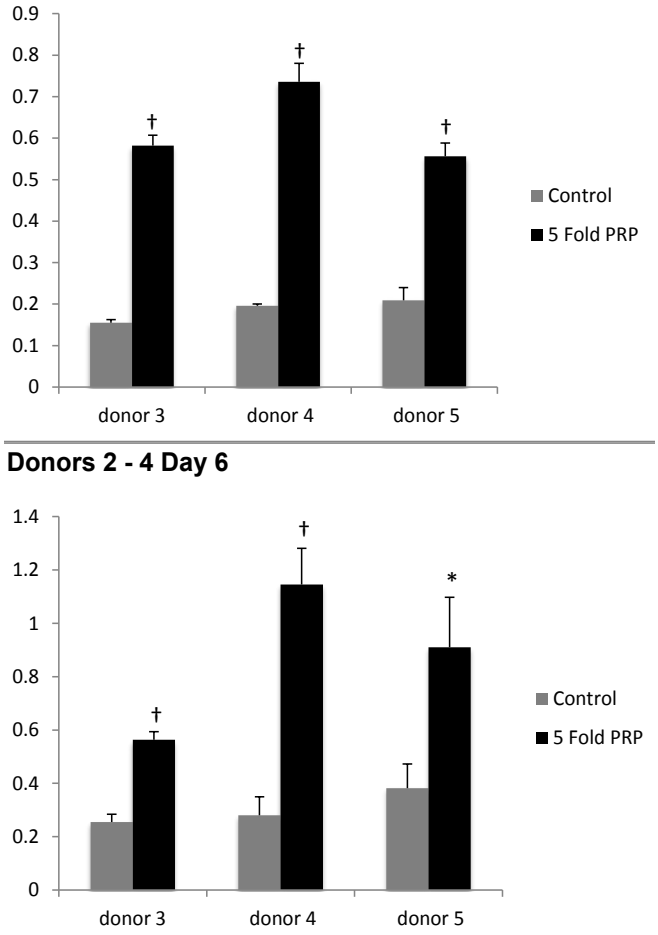

Figure 3: Measurement of rate of proliferation of eBMMSCs. The cell proliferation assay was used to assess the rate of proliferation of eBMMSCs from donors 2 - 4 over a period of 6 days, in presence of 50 million platelets/mL (5 fold above baseline) in PRP. The rates were compared to the rates of proliferation of cells grown in the absence of PRP (control). The single dagger symbol and the asterisk demonstrate a significant value with at least $p<0.005$.

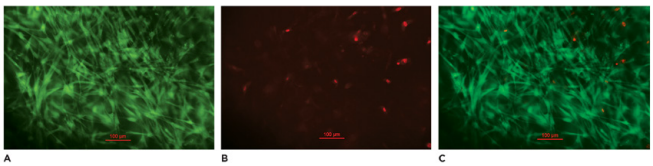

Figure 4: Cell viability of eBMMSCs after 6 days in culture. Cells were stained with calcein-AM which exhibits green fluorescence and demonstrates live cells **(A)** and propidium iodide which displays red fluorescence and demonstrates dead cells **(B)** (shown with red arrows). Panel C represents the merged image of A and B. Fluorescent micrographs showed that in presence of PRP, eBMMSCs adhered to, were viable and proliferated in culture. Scale bar=100 µm.

staining after 6 days. Positive staining of eBMMSCs with calcein-am (green) and lack of PI staining (red) of eBMMSCs demonstrate that cells adhere and are viable in presence of PRP. A representative image is shown (Figure 4). Additionally, the expression of CD90 was confirmed when the eBMMSCs were cultured in presence of 5 fold PRP (Figure 5).

Figure 5: Immunofluorescence of eBMMSCs after 6 days in culture in presence of PRP. Cells were stained with anti-rat CD90 primary antibody **(A)** and nuclei were stained with DAPI to demonstrate the expression of CD90, a protein marker for MSCs. Panel C represents the merged image of A and B. Scale bar=100 µm.

PRP promotes osteo- and chondrogenesis *in vitro*

Since the MTS assay and calcein-am/PI staining demonstrated a positive effect of PRP on the viability and rate of proliferation of eBMMSCs from the 5 donors tested, we next investigated the effect of PRP on the osteogenic and chondrogenic potentials. *In vitro* differentiation patterns were evaluated in eBMMSCs in presence of 5 fold PRP. As expected greater than 90% cells were positive for CD90.

Osteogenic differentiation of eBMMSCs was investigated after PRP stimulation at day 7. Osteogenesis was assessed qualitatively by alizarin red staining (Figure 6). Alizarin red staining increased significantly in differentiated cells compared to the control (undifferentiated) group in presence of PRP, suggesting an increase in the osteogenic capacity of eBMMSCs in presence of PRP.

Chondrogenesis of eBMMSCs in presence of PRP was assessed at day 7 by alcian blue staining. Results similar to those described above for osteogenesis were seen including an increase in cell stain uptake in the differentiated cells cultured with PRP when compared to the control (undifferentiated) group (Figure 6).

Interestingly, the eBMMSCs from donors which failed to show any osteo - or chondro - differentiation in the absence of PRP (described in our earlier study by Carter-Arnold et al. [19]) demonstrated a robust pattern of both lineages in presence of PRP. Differentiated cell-specific staining was supported by a significant increase in the expression of lineage - specific mRNAs (Figure 7). The relative expressions of ALP, Sox 9 and COMP mRNAs increased significantly in the differentiated cells compared to the control (undifferentiated) group in presence of PRP, confirming the increased differentiation potentials of eBMMSCs in presence of PRP.

Discussion

In a previous study [19] from our laboratory, we established cultures of eBMMSCs from 6 middle-aged mares. The results of this study demonstrated that cultures of eBMMSC from these donors proliferated at different rates and were able to differentiate in varying degrees into osteocytes and chondrocytes. Two out of six eBMMSCs cultures showed minimal to no osteo- and chondrogenic differentiation, and had significantly lower rates of proliferation. Since, eBMMSCs from all donors satisfied the criteria to be classified as MSCs [22,26], i.e. the cells adhered to the polystyrene surface, expressed the CD44 and CD90 proteins and were isolated and expanded using standard cell culture methods, we were confident that these are indeed MSCs with relatively inferior biological properties and hence if used in therapy, these cells may not prove to be efficient in repairing bone and cartilage tissues. The eBMMSCs from one donor, on the other hand might prove to be very useful and might result in significant improvement if they are used in a bone or cartilage injury. This same idea of intrinsic variables affecting

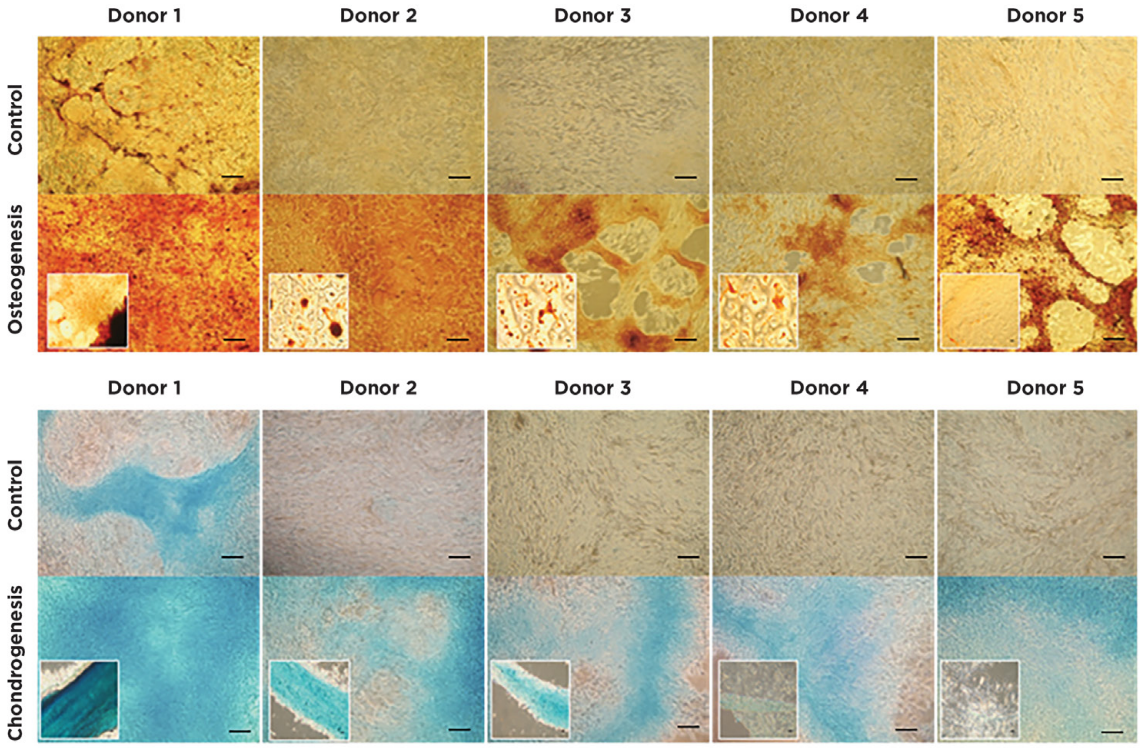

Figure 6: Osteogenesis and chondrogenesis differentiation assays. *In vitro* osteocyte differentiation (top panel) of eBMMSCs from donors 1–5. Images depict osteocyte differentiation in the presence of PRP. Mineralized nodules were visible as cells differentiated into osteocytes. Cells were fixed and stained red with Alizarin red on day 7 post differentiation. The lower panel depicts undifferentiated (control) cells in presence of PRP. Note the lack of mineralized nodules in the control cells. The insets shown in this figure demonstrate the differentiation in the absence of PRP. These data are adapted from the report published earlier [19]. Scale bar=100 µm.
In vitro chondrocyte differentiation (bottom panel) of eBMMSCs from donors 1–5. Images depict chondrocyte differentiation in the presence of PRP. Cell aggregates were visible as cells differentiated into chondrocytes. Cells were fixed and stained red with Alcian blue on day 7 post differentiation. The lower panel depicts undifferentiated (control) cells in presence of PRP. Note the lack of cell aggregates in the control cells. The insets shown in this figure demonstrate the differentiation in the absence of PRP. These data is adapted from the report published earlier [19]. Scale bar=100 µm.
Note the significant improvement in the osteocyte and chondrocyte differentiation patterns in presence of PRP.

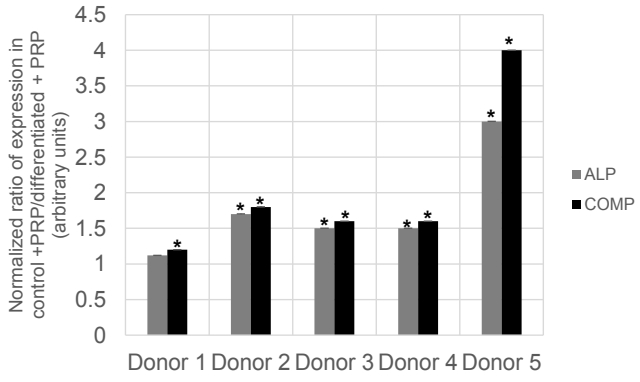

Figure 7: mRNA expression assays. Quantitative PCR was performed to compare the expression of ALP and COMP in undifferentiated (control) and differentiated eBMMSCs from donors 1–5 in presence of PRP. Relative expression of ALP and COMP mRNAs is shown. The Ct values for each target is normalized with GAPDH mRNA. Fold change in differentiated cells was calculated in comparison to the undifferentiated cells for each target gene. Asterisk demonstrates significant data with $p<0.05$. Each qPCR was repeated with at least two independent biological samples with n=3-6 for each RNA sample/ target mRNA. Note the significant increase in the expression of each of these targets after differentiation.

eBMMSCs quality and function was also hypothesized for PRP content profiles and was recently investigated by Giraldo et al. [28]. In this study the authors demonstrated that breed, age, and gender all influenced the growth factor concentrations in pure PRP and PRP gel. Hence, a detailed analysis of eBMMSCs and PRP is imperative. With this in view, in the present study, we evaluated the PRP cellular and growth factor profiles achieved by a commercial kit and determined if the addition of exogenously added growth factors in the form of PRP would have an effect on eBMMSCs in culture. We hypothesized that since PRP is an enriched reservoir of growth factors, it would rescue some of the biological properties (*in vitro* proliferation and differentiation) of eBMMSCs from all donors with a specific interest in those previously showing a lack of differentiation.

A stall-side, portable, single use commercial PRP kit was used to obtain consistent preparations of PRP and PPP samples from all donors. All samples demonstrated a 1.5-1.9 enrichment in platelets with <1% leukocytes, constituting this commercial kit as a leukocyte reduced source of PRP. Additionally, growth factor concentrations between all donors were within the ranges that have been previously reported with no statistical difference noted between any donors. A significant dose of 50 million platelets/mL ($P<0.05$) did result in increased proliferation of eBMMSCs from all donors tested (Figures 1A-C). Cell viability assays

also confirm that the presence of platelets in the culture medium is not deleterious to cell proliferation. Most importantly this data shows that there is a marked enhancement of proliferation of eBMMSCs which could not grow in the absence of PRP, suggesting that the platelets can improve the proliferation ability of eBMMSCs. Additionally, the positive expression of a reliable MSC marker protein, CD90 in all eBMMSCs generated in this study confirmed that eBMMSCs retained their stemness in presence of PRP.

In this study equine PRP exerted a positive osteogenic and chondrogenic stimulatory effect on eBMMSCs *in vitro* in all donors based on gene expression and cell specific staining (Figures 6 and 7). This was strongly supported by lineage-specific staining in addition to the changes in osteocyte and chondrocyte specific mRNA expression patterns. Significant increases ($P<0.05$) in ALP, Sox9 and COMP mRNAs in the presence of PRP confirm that eBMMSCs from all donors have the potential to differentiate, some, however due to variations in their biological properties, might require a cell-based factor i.e. platelets to help them undergo differentiation. Noteworthy, is the observation that eBMMSCs from donor 5 did not undergo an efficient osteo- and chondro-genesis in the absence of PRP, whereas they demonstrated marked differentiation-specific staining and increase in lineage-specific differentiation patterns in presence of PRP.

Similar to other published studies we also observe that this effect on cell proliferation and differentiation is dependent on the platelet concentration. Lennon and group directly related proliferation and differentiation of rat MSCs to platelet concentration and it began at a 4-5 fold increase above baseline [29]. Choi et al. showed that the proliferation of alveolar bone cells was suppressed at high and were stimulated at low PRP concentrations [30]. As a result of our dose response curve in all our experiments a 50 million platelet/mL concentration was used. Our dose was selected based on the MTS day 6 data indicating 75 million/mL concentration to have a decrease in cell proliferation when compared to the 50 million/mL concentration. Whether this is the optimal dose *in vivo* needs to be established.

The effect of PRP on the eBMMSCs in this study may be attributed to the cytokines and growth factors of platelets, however, it is still unknown whether the stimulatory effects of PRP is related to the growth factors or to other microparticles from the cytoplasm or cell membrane that are released from activated platelets [31]. Platelets release several growth factors such as TGF beta 1 and PDGF type BB, which can exert anti-inflammatory, anabolic, and angiogenic effects, and future experiments are required to assess the exact mechanism and identify the protein targets involved in this process.

The results of the current study confirm that PRP has a stimulatory effect on eBMMSCs proliferation and differentiation *in vitro* in horses. The presence of PRP does not affect the viability and the expression of cell surface marker proteins in eBMMSCs. It is most important that we were able to restore the function of eBMMSCs previously classified as poor quality demonstrated by improved osteo and chondrogenesis. Utilizing PRP with eBMMSCs that demonstrate poor proliferation and differentiation may be indicated for both *in vitro* culturing conditions and at local injection. This positive effect will hopefully improve the *in vivo* function of eBMMSCs and can be used to enhance the application of PRP and MSC – based therapies.

Conclusions

In conclusion, the *in vitro* results of this study demonstrate that the proliferation of equine BMMSCs can be improved by the presence of PRP, which can subsequently enhance their potential bone and cartilage formation abilities. The *in vivo* application of these data however, needs to be confirmed. These findings are important for further studies towards technological advances in equine regenerative medicine.

Acknowledgements

This work was supported by grants from the University of Tennessee (UT) Center of Excellence in Livestock Diseases & Human Health (MD). The authors also acknowledge the generous donation of Adrian Lock, Pulsevet, ProTec for providing us with the PRP kits.

References

1. Molloy T, Wang Y, Murrell G (2003) The roles of growth factors in tendon and ligament healing. Sports Med 33: 381-394. [PubMed]
2. Fréchette JP, Martineau I, Gagnon G (2005) Platelet-rich plasmas: growth factor content and roles in wound healing. J Dent Res 84: 434-439. [PubMed]
3. Anitua E, Sánchez M, Prado R, Orive G (2011) Plasma rich in growth factors: the pioneering autologous technology for tissue regeneration. J Biomed Mater Res A 97: 536. [PubMed]
4. Pietrzak WS, Eppley BL (2005) Platelet rich plasma: biology and new technology. J Craniofac Surg 16: 1043-1054. [PubMed]
5. Schnabel LV, Mohammed HO, Miller BJ, McDermott WG, Jacobson MS, et al. (2007) Platelet rich plasma (PRP) enhances anabolic gene expression patterns in flexor digitorum superficialis tendons. J Orthop Res 25: 230-240. [PubMed]
6. Mishra A, Tummala P, King A, Lee B, Kraus M, et al. (2009) Buffered platelet-rich plasma enhances mesenchymal stem cell proliferation and chondrogenic differentiation. Tissue Eng Part C Methods 15: 431-435. [PubMed]
7. Huang Q, Wang YD, Wu T, Jiang S, Hu YL, et al. (2009) Preliminary separation of the growth factors in platelet-rich plasma: effects on the proliferation of human marrow-derived mesenchymal stem cells. Chin Med J (Engl) 122: 83-87. [PubMed]
8. Kocaoemer A, Kern S, Klüter H, Bieback K (2007) Human AB serum and thrombin-activated platelet-rich plasma are suitable alternatives to fetal calf serum for the expansion of mesenchymal stem cells from adipose tissue. Stem Cells 25: 1270-1278. [PubMed]
9. Lucarelli E, Fini M, Beccheroni A, Giavaresi G, Di Bella C, et al. (2005) Stromal stem cells and platelet-rich plasma improve bone allograft integration. Clin Orthop Relat Res 2005(435): 62-68. [PubMed]
10. Martinello T, Bronzini I, Perazzi A, Testoni S, De Benedictis GM, et al. (2013) Effects of in vivo applications of peripheral blood-derived mesenchymal stromal cells (PB-MSCs) and platlet-rich plasma (PRP) on experimentally injured deep digital flexor tendons of sheep. J Orthop Res 31: 306-314. [PubMed]
11. Vogel JP, Szalay K, Geiger F, Kramer M, Richter W, et al. (2006) Platelet-rich plasma improves expansion of human mesenchymal stem cells and retains differentiation capacity and in vivo bone formation in calcium phosphate ceramics. Platelets 17: 462-469. [PubMed]
12. Gentile P, Orlandi A, Scioli MG, Di Pasquali C, Bocchini I, et al. (2012) Concise review: adipose-derived stromal vascular fraction cells and platelet-rich plasma: basic and clinical implications for tissue engineering therapies in regenerative surgery. Stem Cells Transl Med 1: 230-236. [PubMed]
13. Ogino Y, Ayukawa Y, Kukita T, Atsuta I, Koyano K (2009) Platelet-rich plasma suppresses osteoclastogenesis by promoting the secretion of osteoprotegerin. J Periodontal Res 44: 217-224. [PubMed]
14. Torricelli P, Fini M, Filardo G, Tschon M, Pischedda M, et al. (2011) Regenerative medicine for the treatment of musculoskeletal overuse injuries in competition horses. Int Orthop 35: 1569-1576. [PubMed]
15. Carter CA, Jolly DG, Worden CE Sr, Hendren DG, Kane CJ (2003) Platelet-rich plasma gel promotes differentiation and regeneration during equine wound healing. Exp Mol Pathol 74: 244-255. [PubMed]
16. DeRossi R, Coelho AC, Mello GS, Frazílio FO, Leal CR, et al. (2009) Effects of platelet-rich plasma gel on skin healing in surgical wound in horses. Acta Cir Bras 24: 276-281. [PubMed]
17. Theoret C (2009) Tissue Engineering in Wound Repair: The three "R"s-Repair, Replace, Regenerate. Veterinary Surgery 38: 905-913. [PubMed]

18. Alves EG, Serakides R, Boeloni JN, Rosado IR, Ocarino NM, et al. (2014) Comparison of the osteogenic potential of mesenchymal stem cells from the bone marrow and adipose tissue of young dogs. BMC Vet Res 10: 190. [PubMed]

19. Carter-Arnold JL, Neilsen NL, Amelse LL, Odoi A, Dhar MS (2013) In vitro analysis of equine, bone marrow-derived mesenchymal stem cells demonstrates differences within age- and gender-matched horses. Equine Vet J 46: 589-595. [PubMed]

20. Katsara O, Mahaira LG, Iliopoulou EG, Moustaki A, Antsaklis A, et al. (2011) Effects of donor age, gender, and in vitro cellular aging on the phenotypic, functional, and molecular characteristics of mouse bone marrow-derived mesenchymal stem cells. Stem Cells Dev 20: 1549-1561. [PubMed]

21. Phinney DG, Sensebé L (2013) Mesenchymal stromal cells: misconceptions and evolving concepts. Cytotherapy 15: 140-145. [PubMed]

22. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, et al. (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8: 315-317. [PubMed]

23. Schnabel LV, Sonea HO, Jacobson MS, Fortier LA (2008) Effects of platelet rich plasma and acellular bone marrow on gene expression patterns and DNA content of equine suspensory ligament explant cultures. Equine Vet J 40: 260-265. [PubMed]

24. Stewart AA, Byron CR, Pondenis HC, Stewart MC (2008) Effect of dexamethasone supplementation on chondrogenesis of equine mesenchymal stem cells. Am J Vet Res 69: 1013-1021. [PubMed]

25. Worster AA, Brower-Toland BD, Fortier LA, Bent SJ, Williams J, et al. (2001) Chondrocytic differentiation of mesenchymal stem cells sequentially exposed to transforming growth factor-beta1 in monolayer and insulin-like growth factor-I in a three-dimensional matrix. J Orthop Res 19: 738-749. [PubMed]

26. De Schauwer C, Meyer E, Van de Walle GR, Van Soom A (2011) Markers of stemness in equine mesenchymal stem cells: a plea for uniformity. Theriogenology 75: 1431-1443. [PubMed]

27. Dhar M, Neilsen N, Beatty K, Eaker S, Adair H, et al. (2012) Equine peripheral blood-derived mesenchymal stem cells: isolation, identification, trilineage differentiation and effect of hyperbaric oxygen treatment. Equine Vet J 44: 600-605. [PubMed]

28. Giraldo CE, López C, Álvarez ME, Samudio IJ, Prades M, et al. (2013) Effects of the breed, sex and age on cellular content and growth factor release from equine pure-platelet rich plasma and pure-platelet rich gel. BMC Vet Res 9: 29. [PubMed]

29. Lennon DP, Haynesworth SE, Young RG, Dennis JE, Caplan AI (1995) A chemically defined medium supports in vitro proliferation and maintains the osteochondral potential of rat marrow-derived mesenchymal stem cells. Exp Cell Res 219: 211-222. [PubMed]

30. Choi BH, Zhu SJ, Kim BY, Huh JY, Lee SH, et al. (2005) Effect of platelet-rich plasma (PRP) concentration on the viability and proliferation of alveolar bone cells: an in vitro study. Int J Oral Maxillofac Surg 34: 420-424. [PubMed]

31. van den Dolder J, Mooren R, Vloon AP, Stoelinga PJ, Jansen JA (2006) Platelet-rich plasma: quantification of growth factor levels and the effect on growth and differentiation of rat bone marrow cells. Tissue Eng 12: 3067-3073. [PubMed]