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## Research Article

# Effect of Autologous and Allogeneic Platelet Rich Plasma on Viability, Proliferation and Chondrogenesis of Equine Bone Marrow Derived Mesenchymal Stromal Cells

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### Abstract

Biological therapeutics are used in practice to treat musculoskeletal injuries of the equine athlete and optimization of these treatments for enhancement of the healing process is needed. Platelet Rich Plasma (PRP) and Bone Marrow Derived Mesenchymal Stromal Cells (BMSCs) are often used in combination, even though the source of these biological products may be from different donors. Bone marrow derived mesenchymal stem cells were obtained from 6 healthy adult horses, culture expanded, and characterized through tri-lineage differentiation. BMSCs were cultured for 48-72 hours in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with either 25% or 50% (v/v) autologous or allogeneic PRP. Cellular viability was measured at 24 and 72 hours. Cellular proliferation was assessed at 48 hours. Chondrogenesis was quantified after 28 days in culture. Autologous and allogeneic PRP supplementation of the culture medium caused a dose dependent decrease in BMSC viability after 24 to 72 hours of culture. Both autologous and allogeneic PRP significantly decreased BMSC proliferation after 48 hours regardless of percent supplementation. BMSC chondrogenesis was significantly enhanced with exposure to PRP compared to control chondrogenic medium, regardless of whether the PRP was autologous or allogeneic. Exposure to PRP may significantly alter BMSC viability and proliferation, but appears to enhance BMSC chondrogenesis.

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**Keywords:** Chondrogenesis; Equine bone marrow; Mesenchymal stromal cell; Platelet rich plasma

### Abbreviations

OA: Osteoarthritis

MSC: Mesenchymal Stromal Cell

MHC: Major Histocompatibility Complex

BMSC: Bone Marrow Derived Stromal Cell

PRP: Platelet Rich Plasma

GAG: Glycosaminoglycan

MDPI: Multidisciplinary Digital Publishing Institute

DOA: Directory of Open Access Journals

TLA: Three Letter Acronym

LD: Linear Dichroism

### Introduction

Joint pain due to Osteoarthritis (OA) is one of the leading causes of reduced quality of life and loss of performance in people and animals [1-3]. Spontaneous and post-traumatic OA in horses has been regarded as a valuable translational model for human OA because the slow progression and the propensity for joint trauma in horses have allowed for meaningful pathophysiological comparisons to be made between horses and people [4-7].

In an attempt to restore articular homeostasis, clinicians treat arthritic joints in horses with a symptom modifying approach using systemic non-steroidal anti-inflammatory drugs and polysulfated proteoglycans and/or intra-articular cortico-steroids and viscosupplementing non-sulfated glycosaminoglycans such as hyaluronic acid [8].

As joint disease progresses, the development of cartilage deterioration eventually leads to persistent articular pain which is often ineffectively addressed by the above mentioned therapeutics. In an attempt to seek more effective alternatives for this frustrating disease, scientists and clinicians have been exploring the use of acellular and cell based biological therapeutics to enhance the repair of damaged cartilage [9,10].

Mesenchymal Stromal Cells (MSCs), have been the subject of research inquiry because they are an easily obtainable, self-renewing cellular therapy for musculoskeletal repair [10,11] MSCs have been shown to display disease modifying characteristics by preventing or slowing articular cartilage destruction [12,13] and promoting articular cartilage defect repair through formation of hyaline-like reparative tissue [14-16]. To maximize efficiency of treatment, research efforts have been made to understand the implications of using donor allogeneic MSCs for cartilage repair strategies [17]. The use of banked donor cells offers attractive possibilities such as reduced cell propagation times of a few days compared to several weeks needed to harvest and culture autologous cells. Allogeneic cells may also be carefully characterized prior to clinical use to ensure that these cells possess the most desirable phenotypic and functional characteristics for the intended therapeutic purpose. Furthermore, although all reactivity concerns remain, evidence would suggest that allogeneic MSCs evoke minimal immune responses and therefore can survive transplantation in part due to a differential expression of Major Histocompatibility Complexes (MHC) on their cell surface [18-21].

The direct effects of articular injection of MSCs to treat joint disease has been evaluated [22,23], however, when dealing with focal cartilage deficits, such as osteochondral trauma or developmental abnormalities, it is more desirable to implant MSCs specifically to the site of injury. Surgical implantation of MSCs requires co-delivery of these cells with a suitable matrix or scaffold to assist with cellular retention within the cartilage defect [24]. A recent study showed that cartilage repair of human osteoarthritic knees was improved with direct implantation of MSCs into cartilage defects compared to MSC injection into the joint alone [24]. As a result, it is appropriate to investigate the biological relationship of a cellular suspension of MSCs and a provisional matrix such as Platelet Rich Plasma (PRP) that can be activated upon delivery to enhance cellular retention into cartilage defects.

Platelet Rich Plasma is a blood concentrate that can play a pivotal role in wound repair because of its high concentration of growth factors and its ability to coagulate and form a provisional fibrinous scaffold [25]. Exposure of chondrocytes and MSCs to PRP has been shown to enhance cellular proliferation, viability, and the expression of genes important for repair or regeneration of the articular cartilage matrix [26-30]. Additionally, PRP decreases the release of inflammatory cytokines from chondrocytes and synoviocytes [31,32] and enhances synoviocyte hyaluronic acid production [33,34]. For these reasons, PRP has been suggested as a direct intra-articular therapeutic [35,36] and as a bioscaffold for MSC implantation in articular cartilage defects [37].

The overall goal of this study was to further clarify the effects of combining PRP with MSCs *in vitro*. Very few studies have evaluated the effects of this combination on MSC viability, proliferation and function and to further complicate matters the literature reports a wide range of PRP preparation techniques, final composition, mode of activation, and volume of supplementation making it difficult to draw definitive conclusions on the biological interactions between MSCs and PRP [28,38-41]. As an example, low volumes of homologous PRP have been used as MSC culture media substitutes to minimize the introduction of xenogenic elements prior to the clinical use of stem cell preparations [42]. Conversely, studies have shown a dose dependent effect of high volumes of PRP on cellular proliferation in MSC cultures [43]. This contrasting evidence does not help clarify the appropriate combination to use for clinical application of PRP as a cellular suspension media for intra-articular administration or direct implantation of MSCs [40,43]. Regardless of the many unexplored details regarding PRP-MSC combination therapy, clinical data would suggest that the administration of MSCs suspended in PRP leads to clinical improvement in naturally occurring [35,44] and in experimentally-induced [36] osteoarthritis. Furthermore, in a study involving 165 horses with naturally occurring arthritis of various joints, clinical scores improved following intra-articular administration of allogeneic peripheral blood MSCs suspended in allogeneic PRP [35].

These encouraging clinical reports combined with a lack of research investigating the combination of MSCs and PRP prompted this study in which our overall goal was to evaluate the outcome of culturing MSCs with autologous and allogeneic PRP. We hypothesized that MSCs supplemented in culture with autologous PRP would demonstrate greater viability, proliferation, and chondrogenic differentiation potential than MSCs cultured in allogeneic PRP.

## Results

### BMSC characterization

A subset of BMSCs from all of the six horses used in this study successfully exhibited osteogenic, chondrogenic and adipogenic differentiation potential. Compared to BMSCs treated with control media, plastic adherent cells exposed to differentiation media exhibited positive uptake of von Kossa and 7% Oil-Red-O staining to confirm osteogenic and adipogenic differentiation. Chondrogenic differentiation was confirmed by the formation of micromass cell aggregates that stained positively for 1% alcian blue (Figure 1).



**Figure 1:** BMSC tri-lineage differentiation. Tri-lineage differentiation of one representative BMSC line. BMSCs stained positive for Oil Red O (A), Von Kossa (B), and Alcian blue (C).

### Platelet counts and growth factors.

Concentrations of platelet rich preparations from each individual horse are displayed in table 1. The mean platelet concentration was  $2,668 \pm 209 \times 10^3$  cells/ $\mu$ l with a high of  $3,185 \times 10^3$  cells/ $\mu$ l and a low concentration of  $2000 \times 10^3$  cells/ $\mu$ l. We characterized growth factor concentrations from a subsample of our PRP preparations and growth factor concentration relative to platelet numbers was consistent across our preparations. Mean concentrations of growth factors relative to the platelet counts in the individual PRP samples were 9.4 ng/ml  $\pm$  1.8 ng/ml for TGF- $\beta$ 1, 5.2 ng/ml  $\pm$  2.2 ng/ml for PDGF-BB, and 14.6 ng/ml  $\pm$  16.0 ng/ml for VEGF-A.

PRP Line	Platelet Count ( $\times 10^3$ platelets/ $\mu$ l)
1	3,185
2	3,150
3	2,235
4	3,015
5	2,000
6	2,420

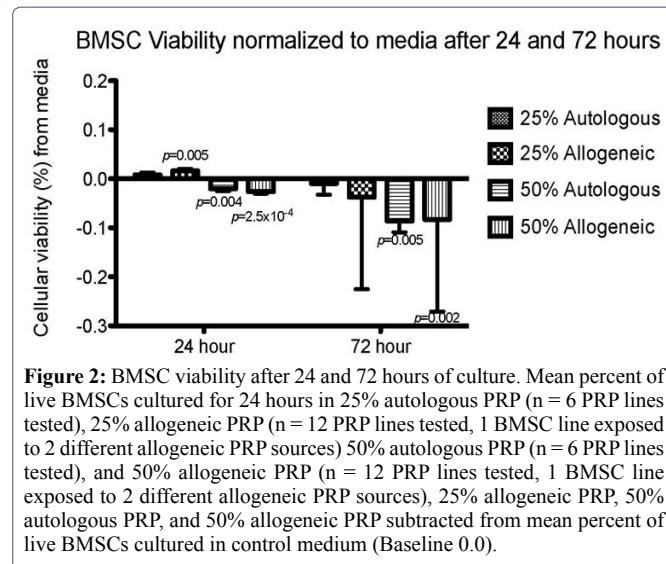
**Table 1:** Platelet counts. Platelet counts (cells/ $\mu$ l) for each individual PRP.

### BMSC viability

Compared to standard culture media, supplementation of BMSC cultures with 25% PRP significantly increased BMSC viability after 24 hours followed by a decline in viability at 72 hours regardless of the PRP source. Conversely, supplementing BMSC cultures with 50% PRP significantly decreased BMSC viability at 24 and 72 hours regardless of the PRP source (Figure 2).

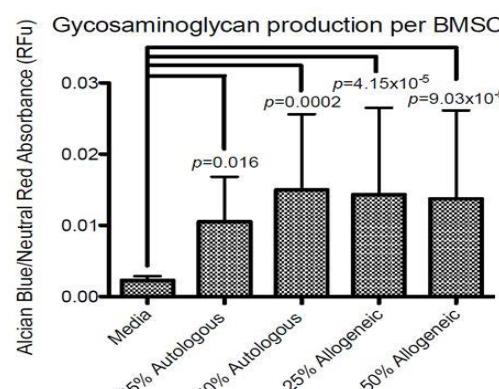
## Cellular Proliferation via EdU DNA incorporation

There was a significant decrease in cellular proliferation of BMSCs cultured in all PRP preparations compared to BMSCs cultured in media alone regardless of the source of the PRP (autologous or allogeneic). No significant differences between PRP preparations were found, but there was a trend for higher PRP concentrations to decrease the quantification of EdU suggesting a more pronounced decline in proliferation at the 50% PRP concentration (Figure 3).



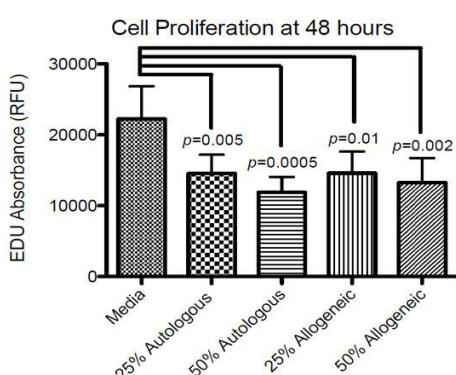
**Figure 2:** BMSC viability after 24 and 72 hours of culture. Mean percent of live BMSCs cultured for 24 hours in 25% autologous PRP (n = 6 PRP lines tested), 25% allogeneic PRP (n = 12 PRP lines tested), 1 BMSC line exposed to 2 different allogeneic PRP sources) 50% autologous PRP (n = 6 PRP lines tested), and 50% allogeneic PRP (n = 12 PRP lines tested, 1 BMSC line exposed to 2 different allogeneic PRP sources), 25% allogeneic PRP, 50% autologous PRP, and 50% allogeneic PRP subtracted from mean percent of live BMSCs cultured in control medium (Baseline 0.0).

was evaluated in relation to the percentage of viable BMSC, all PRP conditions produced significantly higher GAG per BMSC compared to chondrogenic medium, but no differences were detected between treatment groups (Figure 4).



**Figure 4:** Glycosaminoglycan content per viable BMSC. Ratio of alcian blue absorbance (glycosaminoglycan content) to neutral red absorbance (cell number) for BMSC micromass pellets cultured for 28 days in 25% autologous PRP (n = 6 PRP lines tested), 25% allogeneic PRP (n = 12 PRP lines tested, 1 BMSC line exposed to 2 different allogeneic PRP sources) 50% autologous PRP (n = 6 PRP lines tested), and 50% allogeneic PRP (n = 12 PRP lines tested, 1 BMSC line exposed to 2 different allogeneic PRP sources). Results that are significantly different ( $p < 0.05$ ) from media are displayed as p values overlying the treatment group from which a significant difference was seen.

No significant differences between treatment groups were identified.



**Figure 3:** Cellular proliferation after 48 hours in culture. Mean absorbance (RFU) for EdU for BMSCs cultured for 48 hours. Significant differences ( $p < 0.05$ ) from media are displayed. No significant differences between treatment groups were identified. Media, 25% autologous PRP, and 50% autologous PRP groups contained data from the 6 BMSC lines (n=6). Data for the 25% and 50% allogeneic groups was generated following exposure of 6 BMSC lines to 6 different PRP lines, but each BMSC line was exposed to 2 different allogeneic sources of PRP (n=12).

## Chondrogenesis

There were no differences in the quantity of viable cells contained within chondrogenic pellets cultured in control chondrogenic medium and all PRP sources as determined by neutral red staining.

All PRP preparations enhanced GAG production compared to control chondrogenic medium and GAG production was higher with the higher concentrations of PRP (50%). When GAG production

## Discussion

Platelet rich plasma is an easily obtainable blood derived biological product that serves as a scaffold and a source of growth factors shown to variably sustain or enhance the cellular proliferation, migration, and biological activity of progenitor cells from various tissue sources and species [45]. In this study we report a dose dependent effect of PRP on BMSC viability and proliferation, with increasing concentration of media supplementation of PRP causing reduced BMSC viability and proliferation. Contrary to our hypothesis, the PRP source (autologous vs. allogeneic) had no significant effect on cellular viability and proliferation. Interestingly and despite an overall decline in viability and proliferative capacity, chondrogenic differentiation of BMSC was enhanced by supplementing differentiation media with PRP irrespectively of the source or concentration.

The effect of PRP on the biological activity of various cell types has been the subject of a number of research papers [28,38,39,41,46]. Very few of these papers, however, have considered investigating if PRP preparations autologous or allogeneic to the MSC source would impact the biology and development of MSCs [47]. In this study, the viability, proliferation, and the chondrogenic differentiation potential of equine BMSCs were similar regardless of exposure of the cells to autologous or allogeneic PRP. It is worth noting that incubation of MSCs in allogenic serum has been shown to significantly enhance MSC cytotoxicity via the activation of complement and formation of membrane attack complexes compared to incubation of MSCs in autologous serum [48]. Plasma retains components of the complement system along with naturally occurring allo-antibodies and platelet micro particles/membranes which contain complex surface antigens

that could be recognized by the recipient immune system. It was beyond the scope of this study to address the potential immunogenicity of BMSCs following exposure to allogeneic PRP; however, mixed leukocyte reaction studies or co-incubation of media obtained from BMSCs exposed to allo-PRP could be investigated to further elucidate this important aspect of BMSC biology.

Numerous studies address cellular proliferation [43,49-53], but few studies address cellular viability following supplementation with platelet products *in vitro* [43,54]. These studies were primarily conducted to evaluate platelet products as an alternative growth supplement for MSCs, to address the concern that fetal bovine or calf serum contain xenogenic proteins that could enhance the immune response directed toward MSCs, leading to decreased therapeutic efficacy. Most of the aforementioned studies, evaluated low volumes (<20%) of media supplementation with platelet derived products but little is known about the effects of higher concentrations of PRP on MSCs. Higher media supplementation *in vitro* would more closely mimic a clinical scenario in which PRP and MSCs are admixed immediately prior to injection into a joint or cartilage defect [43,49,53].

In the present study the platelet counts achieved with our centrifugation technique (average of 14.3-fold increase from a baseline platelet count) are higher than those investigated in the literature (2-10 fold increase from baseline). The ideal concentration of platelets in a therapeutic PRP preparation is unknown, but ranges from 3 to 6 fold higher than peripheral blood are recommended in the literature for various clinical applications. Growth factor concentrations correlate to platelet numbers hence the desire to maximize platelet concentration in our clinical practice. The relatively high platelet counts may have influenced the results our study, however growth factor analysis characterizing our PRP preps showed very similar average concentrations of growth factors relative to platelet numbers. Nevertheless, our individual platelet concentrations differed and this may have played a role in the variability of our results. At the same time, we chose not to standardize platelet numbers in order to mimic as closely as possible the clinical scenario in which MSCs would be admixed with a preparation containing platelet concentrations that vary dependent on the donor and processing technique. PRP is a biological product that is subject to great variability due to differences in collection and processing as well as differences within and between subjects. This variability in PRP preparation is a major limitation of this study and makes it difficult to draw definitive conclusions about the effect of PRP on BMSCs when comparing to other studies.

After a 24-hour exposure to 25% PRP, BMSC viability was comparable (autologous) or slightly improved (allogeneic) to that seen with control medium whereas exposure to 50% PRP significantly reduced BMSC viability. This decline in cellular viability with increasing concentration of platelet derived product is consistent with the findings of Russell et al., [43] in which umbilical cord blood derived MSC viability declined with >30% supplementation of culture media with pooled allogeneic platelet lysate. Furthermore, Garvican et al., [53] showed significant reduction in BMSC viability following suspension in 100% allogeneic PRP and storage at 4-8°C. The reason for this reduced cellular viability following PRP exposure and/or suspension is unknown. Possible mechanisms include immune mediated cytotoxicity via the complement pathway as mentioned above or cytotoxicity associated with an acidic pH. In our study, BMSC viability may have been conditioned by the relatively acidic pH resulting from exposure to high concentration of platelets especially at the 50% concentration. There is evidence to suggest that buffering PRP prior to

therapeutic application may enhance cellular viability prior to therapeutic application [27].

We report a significant decrease in cellular proliferation at 25% and 50% PRP supplementation compared to media alone. This is in contrast, to a study by Del Bue et al., [49] which showed a dose dependent increase in adipose derived MSC proliferation up to 50% supplementation with platelet lysate and a study by Russell et al., [43] that showed no significant differences in umbilical cord blood derived MSC proliferation between the same volume of FBS and platelet lysate supplementation up to 30%. In the study by Russell, there was dose dependent increase in cellular proliferation with a trend for platelet lysate to have greater proliferation rates compared to supplementation with the same volume of FBS, but following supplementation of >30% platelet lysate umbilical cord derived MSC proliferation significantly declined while umbilical cord derived MSC proliferation continued to increase with increasing volume of supplementation. In the current study, BMSC proliferation was significantly decreased following supplementation with 25% and 50% PRP compared to standard culture media supplementation of 10% FBS. The trend for increasing PRP concentrations to cause reduction in BMSC proliferation and this is consistent with the findings of Russell et al., [43] in which supplementation with >30% platelet lysate resulted in a dose dependent decline in umbilical cord derived MSC viability. Reasons for the initial dose-dependent increase followed by a rapid and steep decline in MSC proliferation following exposure to platelet products is unknown, but could be related to the platelet concentration and/or protein (growth factor) composition of the platelet product. Additionally, direct comparison of platelet derived products is difficult given the varying cellular compositions that could also contribute to differences in MSC response, with platelet lysate being acellular and PRP containing white and red blood and platelet cellular fragments.

In the present study, direct contact of BMSCs with the PRP clot was not allowed to facilitate data measurement of the BMSCs. When PRP is activated it forms a gel-like matrix that incorporates platelet fragments, white blood cells, and red blood cells which could have contributed to the cellular viability and/or proliferation data in our experiment. Therefore, the PRP was activated within the culture system, but physically separated using a co-culture system and removed prior to performing viability and proliferation assays. The majority of studies that have evaluated the effects of platelet products on MSC viability, proliferation, or chondrogenesis use a PRP releasate or platelet lysate which contains released factors from activated platelets [38,39,40,43,46]. Creeper et al., [47] evaluated the effects of various concentrations of activated autologous and allogeneic PRP in direct contact during culture of gingival fibroblasts. No differences between autologous and allogeneic preparations on gingival fibroblast function were found [47]. Inhibition of direct contact between BMSCs and PRP in this study is a major limitation. However, the PRP clot was activated and remained within the culture system to ensure that the BMSCs were exposed to residual factors secreted from the PRP clot that may not be included in PRP releasate or platelet lysate in an attempt to mimic the clinical setting as best possible.

Platelet rich plasma had a significant effect on chondrogenic differentiation of BMSCs in comparison to chondrogenic medium alone, however a dose-dependent effect was not observed. The literature has shown that chondrogenesis of MSCs can be maintained or enhanced after supplementing culture medium with 5-10% platelet derived products [27,51,54-58]. The higher concentrations of PRP that we used in this study, however, are rarely evaluated in the literature.

In the current study, 25% and 50% PRP concentrations were shown to enhance GAG production of differentiated BMSCs over medium alone. These higher supplementation volumes more closely reflect the clinical reality in which BMSCs are implanted in cartilage defects suspended in 100% PRP suggesting that, PRP and BMSC combinations should be further evaluated for the purpose of cartilage defect delivery.

Measurement of GAG production of MSCs within micro mass pellet culture is a commonly used method to confirm chondrogenesis. Negatively charged GAGs are attached to the aggrecan molecules within the cartilage matrix and are responsible for cartilages compressive strength. Demonstration of type II collagen and GAG production within micro mass pellet culture and/or cartilage defect suggest the formation of a more hyaline-like tissue matrix resembling articular cartilage not fibro cartilage. PRP has been used as a cellular suspension for delivery into articular cartilage defects due to its important biological activity and its ability to form a provisional scaffold/matrix within the defect site. Goodrich et al., [59] showed that equine BMSCs suspended in platelet-enriched fibrin and implanted in experimentally induced chondral defects supported formation of hyaline-like repair tissue. However, platelet-enriched fibrin outperformed BMSCs in repair of the chondral defects and ectopic bone formation was found in the repair tissue of some of the defects in which BMSCs and platelet enriched fibrin were used to fill the defect [59]. This is in contrast to our findings in which PRP enhanced chondrogenesis, though its effects of BMSC osteogenesis were not evaluated. This further supports the need for evaluating the role of MSCs and more clinically relevant concentrations of PRP both *in vitro* and *in vivo*.

Our findings indicate that there is no difference in viability, proliferation, or chondrogenesis of BMSCs treated with either 25% or 50% autologous or allogeneic PRP. Higher supplementation of PRP (50%) reduces BMSC viability and proliferation, but enhances chondrogenic differentiation. Suspension of equine BMSCs in 25-50% autologous or allogeneic PRP for injection may cause an acute decrease in cellular viability and cellular proliferation with enhancement of chondrogenesis. Although a direct extrapolation of our results to the clinical setting in which BMSCs may be mixed or re-suspended in nearly 50-100% PRP may not be accurate, our data raises the concern that BMSCs viability may deserve further scrutiny when these combinations are used.

## Materials and Methods

### Animals

Bone marrow for the proliferation of MSCs and blood for the production of PRP were aseptically harvested on separate dates from 6 healthy adult horses aged 8-14 years old. All procedures involving collection of samples for this study were done with the approval of the Animal Care and Use Committee at The University of Georgia.

### Isolation of BMSCs

Bone marrow was aseptically harvested from the sternum of six healthy adult horses using an 8-gaugeX 4 in Jamshidi bone marrow biopsy needle. Approximately 20 cc of bone marrow were drawn from either the 4<sup>th</sup> or 5<sup>th</sup> sternebrae into 2-35cc syringes containing 2500 units of heparin sulfate<sup>b</sup> in each syringe. BMSCs were obtained using a plate adherency culture method. Cells were cultured in defined culture medium consisting of Dulbecco's Modified Eagles Medium with 4.5 g/L glucose and sodium pyruvate without L-Glutamine<sup>c</sup>

supplemented with 10% fetal bovine serum<sup>d</sup>, 4 mM L-Glutamine<sup>e</sup> and 50 µg/ml Streptomycin, 50 U/ml Penicillin<sup>f</sup> under standard cell culture conditions (37°C and 5% CO<sub>2</sub>). Initial BMSC colonies were manually dissociated to allow re-distribution prior to trypsinization. Once the cells reached 70-80% confluence, they were harvested with 0.05% trypsin-EDTA<sup>g</sup> and reseeded at 5000 ccells/cm<sup>2</sup> for further expansion.

### BMSC characterization

For osteogenic and adipogenic differentiation, 36,000 MSCs per cm<sup>2</sup> from each of the 6 lines were plated in 35 mm plates. The cells were differentiated using Hyclone Advance STEM osteogenic differentiation medium<sup>h</sup> with 50 µg/ml Streptomycin, 50 U/ml Penicillin, and Hyclone Advance STEM adipogenic differentiation medium<sup>i</sup> with 50 µg/ml Streptomycin, 50 U/ml Penicillin respectively. The medium was changed every third day for 28 days. For osteogenic differentiation, cells were stained with Von Kossa<sup>j</sup> and for adipogenic differentiation cells were stained with 7% O<sup>k</sup>. Chondrogenesis was performed using micromass pellets formed in 15 ml polypropylene tubes. 1 × 10<sup>6</sup> cells were pelleted at 400 g and Advance STEM chondrogenic differentiation medium<sup>l</sup> was changed every third day for 28 days. Micro masses were stained with 1% alcian blue<sup>m</sup>.

### Platelet rich plasma processing

Approximately 1 liter of blood was aseptically harvested from the left jugular vein of the same 6 adult horses from which BMSCs lines were developed and collected in 2 blood collection bags containing 63 mls of citrate phosphate dextrose adenine solution (CPDA-1)<sup>n</sup>. The blood was subsequently processed aseptically under a direct laminar flow hood using standard cellular processing techniques. The blood was centrifuged at 1800 rpm at 4°C for 10 minutes without automated deceleration. Plasma above the buffy coat was harvested and then centrifuged at 2400 rpm at 4°C for 5 minutes with automated deceleration. The supernatant was decanted and then centrifuged at 3300 rpm at 4°C for 5 minutes with automated deceleration. The platelet poor supernatant was decanted and discarded. The cellular pellets from the second and third centrifugation cycles were re-suspended in the remaining plasma after decantation and combined to produce PRP. A 200 µl sample was obtained from the PRP to perform an automated platelet count. This method of PRP processing yielded approximately 10-15 mls of PRP from 1 liter of blood. To allow for analysis, BMSCs were kept separate from the PRP through the use of trans-well inserts with a 0.04 µM pore size. BMSC groups were organized so that each of the 6 BMSC lines were exposed to autologous PRP and two allogeneic PRP samples. Treatment groups were as follows: defined culture medium, defined culture medium with 25% autologous PRP, defined culture medium with 50% autologous PRP, defined culture medium with 25% allogeneic PRP, and defined culture medium with 50% allogeneic PRP. The PRP was activated by the addition of 10% CaCl<sub>2</sub> to each of the trans-well inserts. PRP was activated to more closely reflect the clinical scenario for filling of a articular cartilage defect where MSCs and PRP would be injected together with an activating agent to allow clot formation of the PRP within the defect. Growth factor release from platelets is influenced by activation, therefore it was important to resemble this clinical scenario as closely as possible *in vitro*. However, direct contact with the PRP clot was not allowed to facilitate diffusion of reagents for cellular assays and facilitate data measurement.

PRP aliquots from each horse were analyzed using immuno assays

(Quantikine Immunoassay, R&D Systems, MN, USA) for platelet derived growth factor-BB (PDGF-BB) and transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) and immunoassay (Vet Set, Kingfisher, MN, USA) for Vascular Endothelial Growth Factor-A (VEGF-A) following the manufacturer's instructions.

### Proliferation assay

BMSCs were plated at 3500 cells/cm<sup>2</sup>. Proliferation of BMSCs in the presence of autologous or allogeneic PRP was assessed using the Clik-iT® EdU Microplate Assay<sup>o</sup>. 5-ethynyl-2'-deoxyuridine (EdU) was added at a concentration of 10  $\mu$ M to the medium of all treatment groups and cells were cultured for 48 hours in the presence of EdU. Six hours prior to initiating the Clik-iT® EdU microplate assay the medium was removed from the plates and 150  $\mu$ l defined medium containing type IV collagenase (1 mg/mL)<sup>p</sup> and 50  $\mu$ l of 0.05% trypsin-EDTA were added to each well of the 96 well plate to allow dissolution of cells from the protein matrix that eluded from the trans well. The plates were then centrifuged and the assay was performed according to package insert instructions with the addition of centrifugation between steps. The plate was analyzed using a microplate reader set to read at 495 with correction at 519.

### Viability assay

BMSCs were plated at 3500 cells/cm<sup>2</sup>. Viability of BMSCs was assessed using the LIVE/DEAD® viability/cytotoxicity kit for mammalian cells q after 24 and 72-hour incubation in autologous or allogeneic PRP. DNA quantification was performed with bis-Benzimidazole H33342 tri-hydrochloride<sup>r</sup>. Six hours prior to performing the assay cells were separated from the protein matrix as described above. The cells were then centrifuged and the assay was performed according to package insert instructions. The plate was then assessed for absorbance at 528 with correction set at 617 nm on a microplate reader.

### Chondrogenic assay

BMSCs were seeded at density of 200,000 BMSCs/well in 96 well "V" bottom plates<sup>s</sup> for chondrogenesis. The plates were centrifuged at 400 g for 10 min to form micro mass pellets. 12 hours later the micro masses were transferred to trans well plates for an additional 24 hours prior to exposure to PRP and chondrogenic differentiation medium supplemented with 50  $\mu$ g/ml Streptomycin and 50 U/ml Penicillin. Treatment groups were as follows: chondrogenic medium, chondrogenic medium supplemented with 25% activated autologous PRP, chondrogenic medium containing 50% activated autologous PRP, chondrogenic medium containing 25% activated allogeneic PRP, and chondrogenic medium containing 50% activated allogeneic PRP. 50% medium changes were performed every other day. After 28 days in culture the transwell insert was removed and the micromass pellet was harvested from the protein matrix encasing the micromass pellet on the bottom of the insert and transferred to the correspondent well of the plate. The micromass pellets were rinsed 3 times with PBS and then fixed with 100% methanol for 10 minutes at -20°C. A 0.2% Alcian Blue 8GX in 0.1 M HCl solution was applied to the micromass pellets for 2 hours at room temperature. The pellets were then washed three times with PBS and alcian blue stain that had been taken up by the pellets was extracted by exposing the pellets to 6 M guanidine/HCl<sup>t</sup> overnight at room temperature. The optical density of the extracted alcian blue was measured at 650 nm on a micro plate reader. The quantity of viable cells within the pellets was measured by staining the pellets in parallel with Neutral Red<sup>o</sup>. The detection of neutral red content was measured at an optical density of 550 nm.

Using this method cell viability and chondrogenesis were simultaneously quantified. The total GAG/cell content was measured by dividing the fluorescence alcian blue by the fluorescence of neutral red for each micro mass pellet.

### Statistical Analysis

All analyses were performed using SAS V 9.2<sup>v</sup>. Control medium values were averaged over the three replicates for proliferation and chondrogenesis data to obtain a single control value for each horse. Control values were subtracted from viability, proliferation and chondrogenesis data prior to analysis to obtain change from control values for analysis.

A repeated measures model that recognized multiple observations as belonging to the same horse was used to test for differences in viability, proliferation and chondrogenesis change from control values between groups and PRP lines. The full model included fixed factors for group, PRP line, and an interaction effect of group and PRP line as well as a random factor of horse. If a significant interaction ( $p < 0.10$ ) of PRP line and group was found, then paired group differences were examined separately for each PRP line. An unstructured covariance structure was used in all repeated measures models. Student's t-tests were performed to test the hypothesis that change from control values were significantly different than 0, which indicated a significant difference from control. All hypothesis tests were 2-sided and the significance level was  $\alpha = 0.05$ . Tukey's test was used to adjust for multiple paired comparisons.

### Author Contributions

LB, JM, and JP conceived and designed experiments. LB, MT, JM performed experiments. RG assisted with experimental design and data interpretation. LB and JP wrote the manuscript.

### Conflicts of Interest

The authors declare no conflict of interest.

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