THE USE OF AUTOLOGOUS PLATELET LYSATE IN THE EXPANSION OF EQUINE BONE MARROW DERIVED MESENCHYMAL STEM CELLS

An Undergraduate Research Scholars Thesis

by

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TABLE OF CONTENTS

ABSTRACT

The Use of Autologous Platelet Lysate in the Expansion of Equine Bone Marrow Derived Mesenchymal Stem Cells. (May 2015)

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Fetal bovine serum (FBS) has a been standard supplement for mesenchymal stem cell (MSC) culture mediums. Unfortunately the use of this supplement can cause xenogenic reactions when contaminated MSCs are injected into the patient. Researchers have begun searching for alternative autologous and allogenic growth supplements and found hope in platelet lysate. Platelets and their abundant growth factors might allow for cell proliferation. Use of platelet lysate (PL) will eliminate the problem of xenogenic reaction. The use of autologous platelet lysate as a viable medium supplement for equine bone marrow derived MSCs was assessed through this research. This was accomplished by comparing the MSC proliferation in 10% serum supplemented media. FBS and PL were used as the serum sources and a 10% FBS containing heparin medium was also tested as a control for heparin in the platelet lysate supplement. MSC proliferation was assessed using cell counts and colony forming unit assays. Preliminary data showed greater MSC proliferation in the PL and FBS heparin supplemented mediums compared to FBS alone. Flow cytometry was performed to assay for the presence of cell surface markers. The 10% PL group were positive for cell surface markers CD-90 and negative for CD-45 and MHC-II, while the 10% FBS and 10% FBS groups were negative for CD-45 but showed variable results with regards to CD-90 and MHC-II. Each group will be further assessed on its ability to

undergo trilineage differentiation. Unaltered MSCs should possess the ability to differentiate into osteocytes, chondrocytes, and adipocytes. ELISA tests will also be performed to determine the concentration of platelet-derived growth factor (PDGF) in platelet lysate samples.

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NOMENCLATURE

bFGF – Basic Fibroblast Growth Factor

CFU – Colony Forming Unit

DMEM – Dulbecco's Modified Eagle's Medium

FBS – Fetal Bovine Serum

FITC - Fluorescein isothiocyanate

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

MSC – Mesenchymal Stem Cell

PDGF – Platelet Derived Growth Factor

PL – Platelet Lysate

PRP – Platelet Rich Plasma

Tris – (Hydroxymethyl)-aminomethane

CHAPTER I

INTRODUCTION

Mesenchymal stem cells (MSCs) have shown great promise in the world of regenerative medicine. These cells are defined by the International Society for Cellular Therapy (ISCT) as multipotent progenitor cells with the ability to adhere to plastic, display specific surface markers, and differentiate into osteocytes, chondrocytes, and adipocytes. A human cell must display CD73, CD90, and CD105 and be negative for HLA-DR, CD11b, CD14, CD34, CD31, and CD45 surface markers in order to be classified as an MSC under the ISCT definition. It should be noted that the surface markers expressed by MSC populations in other species may show slight variations. Recent studies have shown equine MSCs to be positive for CD29, CD44 and CD90, while being negative for CD14, CD34, CD45, CD79α and MHC-II[1,2]. These cells can be derived from bone marrow, umbilical cord, adipose tissue, muscle tissue, and various other tissues[3].

There is a great bit of debate over the best formulation of MSC isolation and growth mediums. The gold standard for MSC medium supplementation is Fetal Bovine Serum (FBS) because it is the only FDA approved serum supplement. This supplement is an important for a source of growth factors, chemokines, hormones and other factors. Since FBS is obtained from fetal calves, there are low levels of globulins and thus minimal interference with cell growth. However due to various health concerns, many researchers have begun searching for alternative medium supplements. One such concern is that FBS is from a bovine source and use for other species can lead to xenogenic immune reaction to bovine proteins[4,5]. Bovine derived diseases, such as bovine spongiform encephalopathy (BSE), may also be transmitted through the use of

FBS[6]. Use of an autologous product to supplement cell culture medium would alleviate concerns of immune reaction as well as disease transmission. Platelet lysates have shown promise as suitable replacements of FBS[7,8]. This platelet derivative contains platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), insulin-like growth factor I (IGF-I), transforming growth factor beta (TGF-β) and probably many other beneficial factors[9]. We hypothesize that autologous platelet lysate concentrate will be a suitable alternative to the more commonly used FBS in the in vitro expansion of MSCs, resulting in cell expansion and stimulated differentiation that is not different from cultures grown in FBS.

CHAPTER II

METHODS

Animals

Whole blood and bone marrow were collected from two young adult horses. The Institutional Animal Care and Use Committee (IACUAC) protocol for the collection of equine bone marrow and blood for laboratory research (2012-079) was used for this study.

Platelet Lysate Preparation

Two units of whole anti-coagulated blood were collected and brought to the laboratory. The whole blood sat undisturbed for 24 hours at room temperature, allowing for gravity separation of red blood cells and white blood cells from the platelets and plasma. Following gravity separation, plasma was collected and a complete blood count (CBC) including a platelet count was performed. Plasma was then aliquoted into 50 mL polypropylene conical tubes and centrifuged at 900 x G for 15 minutes at 0ºC to pellet the platelets. The appropriate volume of supernatant or platelet-poor-plasma was removed and platelets were resuspended in the remaining plasma at approximately $1x10^6$ platelets/µL, resulting in platelet rich plasma (PRP).

PRP produced was freeze-thawed three times. On the first two repetitions, PRP was frozen in an ethanol/dry-ice bath and thawed in a 35ºC water bath. On the third repetition, PRP was frozen in an ethanol/dry-ice bath, but thawed in a 4ºC refrigerator overnight to cryoprecipitate the fibrinogen. After the freeze-thaws, PRP was centrifuged at 1000 x G for 20 minutes at 0ºC to pellet the fibrinogen and the platelet lysate supernatant was pipetted into new 50 mL conical

tubes. Platelet lysate was centrifuged again at 1600 x G for 30 minutes to pellet platelet fragments and filtered using a 0.2µm filter. Then 1.5 mL of platelet lysate was aliquoted for a future ELISA to assay for PDGF, and stored in a -80˚C freezer. Remaining platelet lysate was aliquoted into 15 mL polypropylene conical tubes and stored at -20ºC. Platelet lysate aliquots were not thawed more than five times for culture.

Isolation and Culture of Bone Marrow Derived MSCs

Bone marrow was collected once autologous platelet lysate was prepared. Red blood cells in raw bone marrow were lysed with a Tris-NH_4Cl solution and nucleated cells were counted. Lysed bone marrow was divided into three equal volumes for each designated condition: 10% PL plus heparin, 10% FBS, or 10% FBS plus heparin. The equivalent of 1mL of raw bone marrow from each condition was plated on 10 cm tissue culture plates for colony forming unit (CFU) assay. The remaining bone marrow for each condition was plated on one T-75 treated flask per condition. Culture medium containing low-glucose (1g/dL glucose) DMEM, HEPES buffer, sodium pyruvate, and L-glutamine was supplemented with one of three serum supplements: 10% autologous PL and 1.0 units/mL heparin (to prevent gel formation), 10% FBS, or 10% FBS and 1.0 units/mL heparin. Culture mediums were changed every 48 hours for each medium condition. MSCs from each condition were grown to 70% confluence then passaged by lifting the cells with trypsin enzyme, counted, and re-plated at 5,000 cells/cm^2 until at least 4 million MSCs were present in each condition. When enough cells were cultured, approximately 3 million MSCs were set aside for flow cytometry and the remaining were cryopreserved for trilineage differentiation. Population doubling time was calculated after each passage. MSCs grown in 10% FBS and 10% FBS with heparin were cryopreserved in a solution of 95% FBS and

5% DMSO. MSCs grown in 10% PL were cryopreserved in a solution of 95% autologous serum and 5% DMSO.

Colony Forming Unit Assay

The medium in 10 cm tissue culture plates for each condition was replaced with respective culture mediums every other day for ten days. At ten days, medium was aspirated and the plates were rinsed with 1X PBS solution three times before being stained with 3% solution of crystal violet and methanol. Cell colonies were counted by gross examination.

Flow Cytometry for Cell Surface Markers

Approximately 500,000 to 1,000,000 fresh MSCs cultured in each medium condition were used to assay for CD90, CD45, and MHC-II. These cell surface markers were tested because they are recognized within the field as markers for equine MSCs. To assay for cell surface markers, MSCs were stained with antibodies conjugated with fluorescein isothiocyanate (FITC). MSCs were then analyzed using a FACSCalibur flow cytometer for their binding to the FITC conjugated antibodies.

Trilineage Differentiation

Cryopreserved MSCs were thawed from each of the three conditions to produce chondrocytes, osteocytes, and adipocytes. To produce chondrocytes, five hundred thousand MSCs were pipetted into three separate 15 mL polypropylene conical tubes for each condition. MSCs were centrifuged at 300g for 10 minutes to form pellets and maintained on 1 mL chondrogenic media in pelleted form with a loose lid. Chondrogenic medium was changed every 48 hours for 21

days. After 21 days, pellets were fixed in paraformaldehyde and placed in tissue cassettes and sent to the Texas A&M Histopathology laboratory for sectioning. Sectioned pellets on slides were stained with toluidine blue and examined for the presence of cartilage matrix accumulation. At a later date, MSCs were thawed and eight sets of MSCs for each condition were plated at a density of 100,000 cells per 10 cm tissue culture plate. Three of the eight plates were designated for osteogenic differentiation and another three plates were designated for chondrogenic differentiation. The remaining two 10 cm tissue culture plates were designated as controls which were fed their respective MSC isolation medium (10% PL plus heparin, 10% FBS, or 10% FBS plus heparin) throughout the induction periods. The 10 cm plates which were designated for differentiation were grown to 70% confluence before undergoing induction conditions for each differentiated cell type. Adipogenesis was induced when changed to adipogenic induction media and incubated 72 hours. Following 72 hour incubation, plates were switched to adipogenic maintenance media and incubated another 72 hours before being stained with Oil-Red-O and examined for the presence of adipocytes. One control plate, which was not exposed to the adipogenic mediums, was also stained with Oil-Red-O at this time. Osteogenesis was induced when changed to osteogenic media and maintained for 21 days. Osteogenic plates, and the second control plate, were fixed and stained with Alazarin Red after 21 days.

CHAPTER III

RESULTS

MSC Proliferation

Preliminary data collected from two horses provided higher MSC yields when cultured in a medium containing 10% platelet lysate than those cultured in 10% FBS (Table 1). Population doubling time (PDT) was calculated using the formula $PDT=CT*\log 2/(\log N_f-\log N_i)$, where CT is culture time, N_f is the final number of MSCs, and N_i is the initial number of MSCs. Nucleated cell counts at plating were elevated due to white blood cells present in the bone marrow, therefore the cell count at the first passage was used as the N_i value in the PDT formula (Table 2). Population doubling times for passage 2 were negative, indicating a loss of MSCs which was most likely due to other cell types from the bone marrow remaining in culture at the first passage. By the second passage, the majority of non-MSCs should have been removed from the culture giving a more accurate MSC count. Additionally, all conditions were scored on their morphology (Figure 1) and debris (Figure 2) prior to each passage. There appears to be no major differences in morphology between the three conditions, though 10% PL appears to have much more debris than the other two conditions.

Table 1. Cell counts at each passage (n=2)

Media Condition	Passage 1	Passage 2	Passage 3	Passage 4
10% FBS	525,000 ± 475,000	779,000 ± 79,000	1,450,000 ± 250,000	2,850,000 ± 150,000
10% PL	3,625,000 ± 3,575,000	1,292,000 ± 408,000	6,550,000 ± 4,250,000	17,850,000 ± 11,750,000
10% FBS and heparin	500,000 ± 400,000	912,500 ± 512,500	2,450,000 ± 1,050,000	8,475,000 ± 5,775,000

Table 2. Population doubling time (PDT) at each passage (n=2)

Media Condition	Passage 2 PDT	Passage 3 PDT	Passage 4 PDT
10% FBS	-3.277 ±4.496	5.553 ±4.613	5.816 ±3.954
10% PL	-0.597 ±1.804	8.507 ±6.878	3.803 ±2.076
10% FBS and heparin	-1.057 ±2.362	6.368 ±4.613	5.255 ±3.578

Figure 1. Morphology Scores at Each Passage

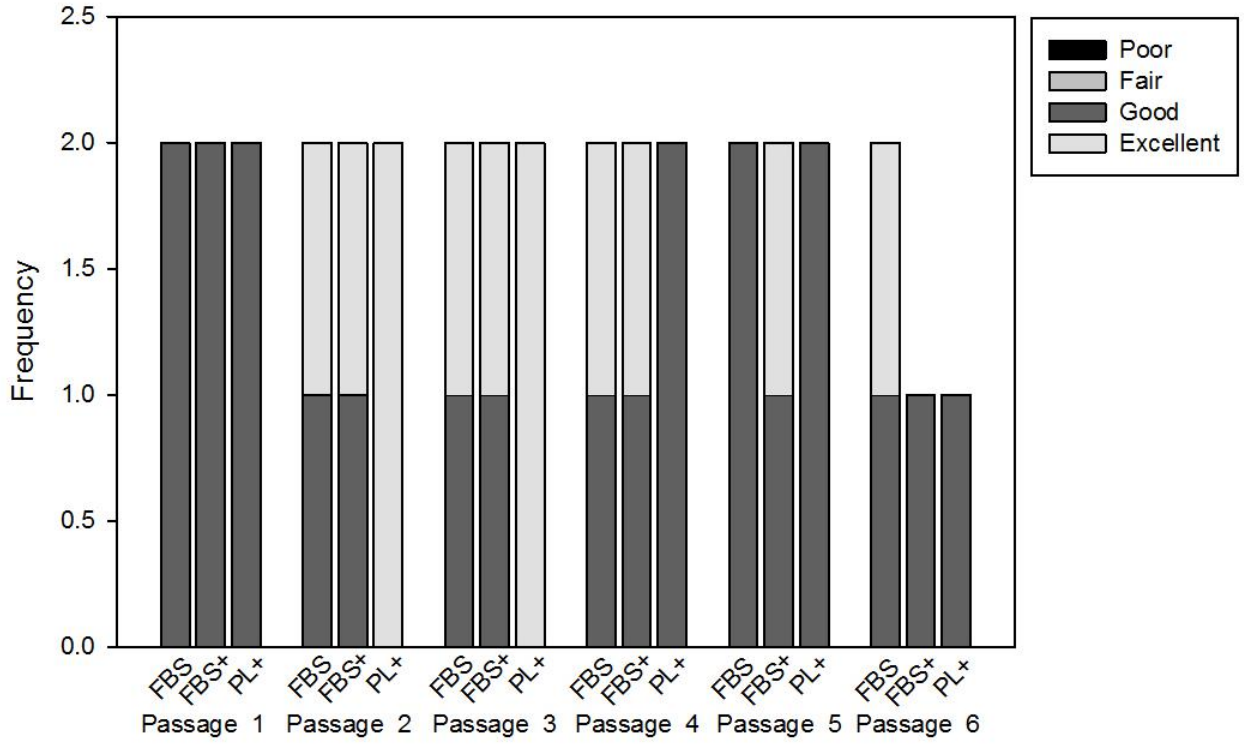
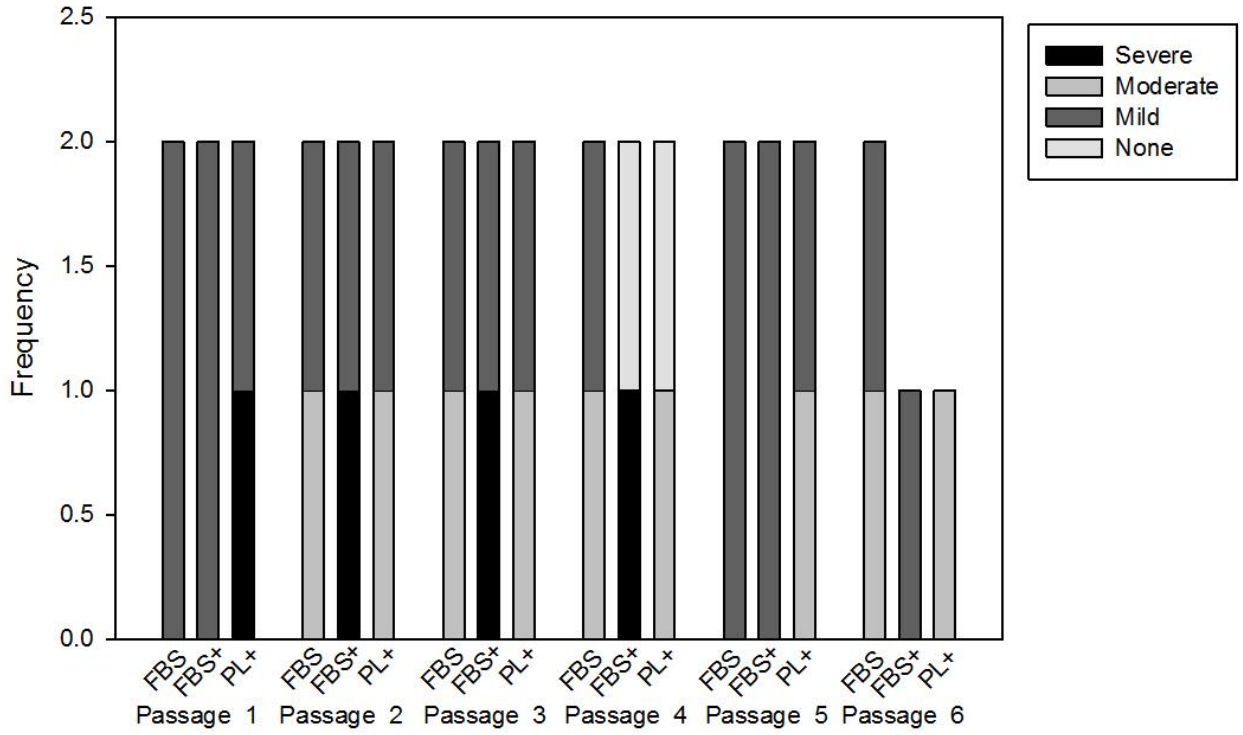


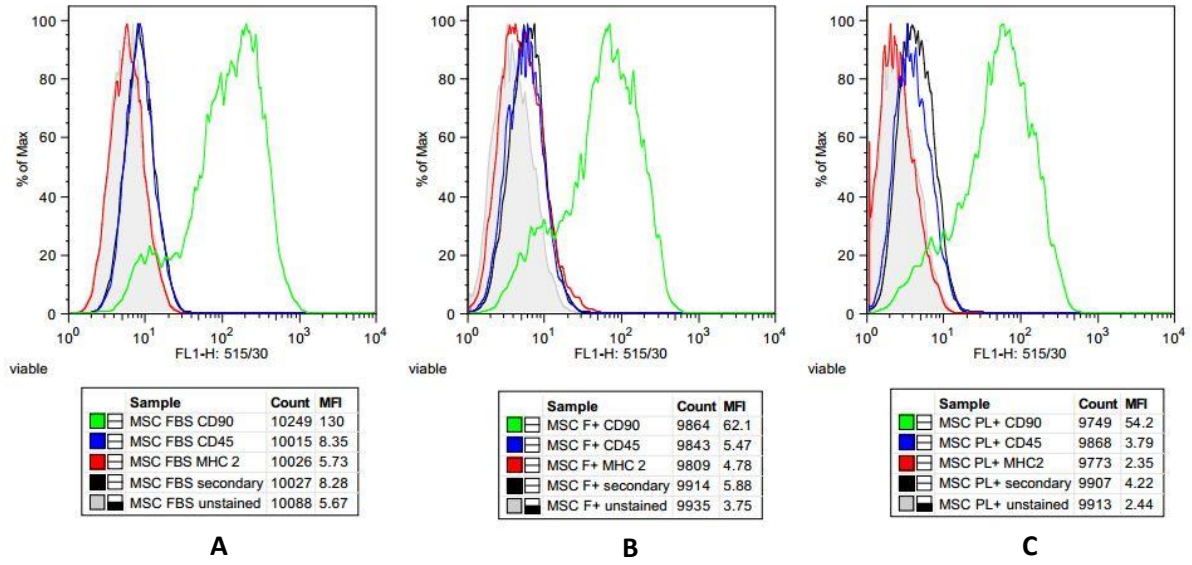
Figure 2. Debris Scores at Each Passage



Flow Cytometry

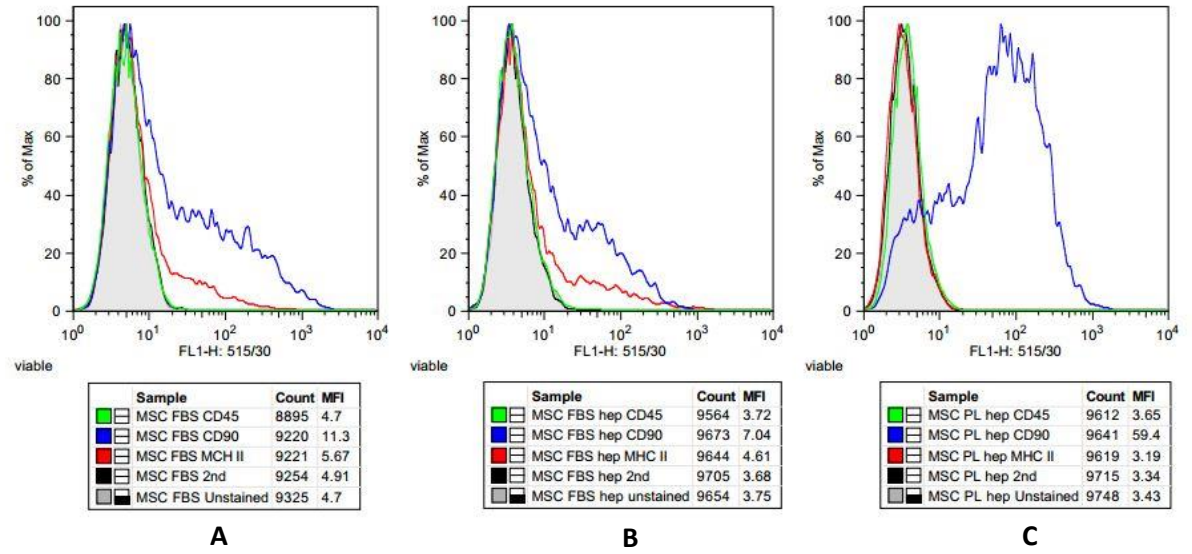
Flow cytometry was used to analyze cell surface markers. Data obtained from Horse 1 indicated all conditions were positive for CD-90, but negative for CD-45 and MHC-II (Figure 1), however data from Horse 2 shows 10% FBS and 10% FBS with heparin conditions to be slightly positive for CD-90 and MHC-II and negative for CD-45 (Figure 2). Furthermore, data from Horse 2 indicated MSCs grown in 10% PL were positive for CD-90, but negative for MHC-II and CD-45.

Figure 3. Flow cytometry data obtained from Horse 1. (A) Flow cytometry histogram for MSCs grown in 10% FBS. CD45 and MHC-II are negative while CD90 is positive. (B) 10% FBS with heparin flow cytometry histogram shows these MSCs to be positive only for CD90. (C) The histogram for 10% PL indicates these MSCs to be positive for CD90 and negative for MHC-II and CD45.



A **B** **C**

Figure 4. The below histograms display the data obtained from Horse 2. (A) The far left histogram shows data collected from 10% FBS. This group was slightly positive for both MHC-II and CD90 while being negative for CD45. (B) The middle histogram is from MSCs cultured in 10% FBS with heparin. This group was slightly positive for CD90 and MHC-II and negative for CD45. (C) The far right histogram shows the flow cytometry histogram for the 10% PL group. These MSCs were only positive for CD90.



A **B** **C**

ELISA and Trilineage Differentiation

Due to time constraints, serum samples were unable to be assessed for their PDGF content and trilineage differentiation was unable to be completed. MSCs which were cultured were cryopreserved to perform trilineage differentiation at a later date.

CHAPTER III

DISCUSSION

At the present time, data has been collected from two horses. In order to reach a definitive conclusion more replicates must be conducted, however it is obvious that platelet lysate supplemented MSC culture medium considerably affects the proliferation of equine bone-marrow derived MSCs. In these preliminary horses, CFU assays indicated that bone marrow allocated for 10% FBS supplemented growth medium had more MSCs per milliliter of bone marrow. In both horses, MSCs grown in media supplemented with 10% platelet lysate proliferated similarly as indicated by comparable population doubling times, but grew to a greater number than those grown in 10% FBS.

The effect of heparin on MSC proliferation is yet to be known, though it can be noted that MSCs grown in 10% FBS with heparin had lower population doubling times than those grown in 10% FBS indicating faster cell growth. Medium supplemented with FBS and heparin also yielded a higher number of MSCs than medium supplemented with FBS alone. These findings indicate that heparin may alter the proliferation of MSCs and the implications of adding heparin should be explored further before concluding that increased MSC yield is due to PL alone.

In order to reach a definitive conclusion on the effectiveness of platelet lysate as an autologous serum substitute for the culture of equine MSCs more replicates should be conducted. Further exploration into the use of platelet lysate as an autologous serum supplement includes completing trilineage differentiation of MSCs grown in each of the three medium types. MSCs

which are unaltered by their growth medium should be able to differentiate to osteocytes, chondrocytes, and adipocytes. Additionally, the PDGF content should be assessed using an ELISA assay. Further characterization of the platelet lysate could include assaying for various other growth factors, such as bFGF, TGF-β, or VEGF. Additionally, cell growth could further be investigated using CellTrace Violet Cell Proliferation Kit (Life Technologies) and performing flow cytometry to trace cell generations within the cell samples. This will give another method to quantify the growth rate of the MSCs in each media condition aside from population doubling time which is heavily dependent on nucleated cell counts which are merely estimates of the total number of cells and may be elevated due to other nucleated cell types at lower passages.

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