COMPARISON OF AUTOLOGOUS PLATELET RELEASATE AND FETAL BOVINE SERUM FOR IN VITRO EXPANSION OF EQUINE BONE MARROW-DERIVED MESENCHYMAL STEM CELLS

An Undergraduate Research Scholars Thesis

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ABSTRACT

Comparison of Autologous Platelet Releasate and Fetal Bovine Serum for In Vitro Expansion of Equine Bone Marrow-Derived Mesenchymal Stem Cells

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In human and veterinary medicine, mesenchymal stem cells (MSCs) have significant therapeutic benefits. MSCs can differentiate into a variety of cells including osteoblasts, chondrocytes, or adipocytes. Fetal bovine serum (FBS) is commonly used as a media supplement to support the proliferation of MSCs *in vitro*. Although FBS provides growth factors, hormones, and other valuable benefits to the cells, the ingredients are undefined, it varies between batches, and contains xenogens that could induce immune reactions. One alternative to FBS used in humans is platelet releasate (PR), which contains platelet-derived growth factors (PDGFs) that can be isolated from autologous or allogeneic blood. It was hypothesized that equine MSCs grown in autologous 10% PR will have the same or superior proliferation as those grown in 10% FBS. MSCs were obtained from raw equine bone marrow, expanded in media containing either autologous 10% PR or 10% FBS, and after reaching the appropriate confluence at passage three (P3) were cryopreserved. During the MSCs expansion in both medias, the number of colony forming units (CFUs), cell counts, growth rate, and confluence were documented. The FBS condition on average yielded higher numbers of colonies on the CFU plates as well as higher cell counts. The confluence over time and population doubling time showed that MSCs grown in

10% FBS proliferated more rapidly than in 10% PR. The MSCs grown in autologous 10% PR started senescing at passage two (P2) as shown with a gradual decline in proliferation. After performing a quantitative analysis, it can be concluded that MSCs grown in autologous 10% PR did not proliferate equal or superior to MSCs grown in 10% FBS. Further research needs to be conducted to conclude that PR is not a good alternative for FBS.

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NOMENCLATURE

DMEM	Dulbecco's modified Eagle's medium
DPBS	Dulbecco's phosphate-buffered saline
FBS	Fetal Bovine Serum
HBSS	Hank's Balanced Salt Solution
MEM	Minimum Essential Media
MSCs	Mesenchymal stem cells
PDGF	Platelet derived growth factor
PBS	Phosphate buffer solution
PR	Platelet Releasate
PL	Platelet Lysate
T75	Culture Flasks
T175	Culture Flasks
CFU	Colony Forming Unit; plate (10 cm dish)
P0	Passage zero
P1	Passage one
P2	Passage two
P3	Passage three

CHAPTER I

INTRODUCTION

Mesenchymal stem cells (MSCs) have the ability to differentiate which allows them to become cartilage, adipose tissue, muscle, tendon, and bone.[1] This ability along with self-renewal has prompted the use of MSCs for cell-based therapies and tissue engineering.[1,2] The main characteristics of MSCs include plastic adherence to tissue culture dishes, appropriate cell surface markers, and differentiation which allows for therapeutic benefits.[3] Treatments have been gravitating towards bone tissue engineering rather than bone grafting and other methods.[4] MSCs recently have been popular with equine orthopedic regenerative medicine. The horse is an excellent model for human joints and tissues due to the similarity in size, load on the joints, and types of injuries acquired.[5] Both humans and equine are highly active therefore requiring efficient methods of orthopedic therapies is essential for injuries.

MSCs are grown *in vitro* before being used for therapy to gain adequate numbers of cells and because they are rare to the tissue of origin.[1] In order for the MSCs to be grown in an *ex vivo* environment the media has to have the required nutrients for survival. The standard media supplement for expansion of MSCs is fetal bovine serum (FBS) which is rich in growth factors. However, FBS has many disadvantages such as high variability even when pooled, can induce immunological responses due to xenogens, and can transfer microbial contaminants to the patient receiving the MSCs. Also, the ingredients in FBS are not well defined.[6] These factors have deterred the use of FBS and has promoted the research of platelet derivatives.

A common platelet derivative used to grow MSCs in humans and equine is platelet lysate (PL).[7,8] Research has shown that there is a dose-dependent response with PL aiding in expansion

of equine MSCs.[9] This media supplement is produced by using a freeze/thaw method, and has to have an anti-coagulant called heparin to prevent fibrinogen contamination.[6] Heparin generally comes from a different species than the cells in the media.

Another platelet derivative called platelet releasate (PR) has the growth factors required to support the proliferation of MSCs. PR is activated using a physiological stimulus such as thrombin or $CaCl_2$ that releases the growth factors into the serum.[7] Thrombin and $CaCl_2$ also deplete the coagulation factors such as fibrinogen without needing an anti-coagulant.[7] PR has shown to be an adequate substitute to FBS[10] and has even produced higher proliferation rates of MSCs compared to PL or FBS.[7] PR has not yet been studied with equine MSCs possibly because equine contain a large amount of fibrinogen compared to humans. Research needs to be conducted with PR and equine MSCs to determine if PR is a suitable replacement for FBS in the expansion of equine MSCs.

Hypothesis

This research was conducted to determine if MSCs grown in 10% PR proliferated equally or greater than MSCs grown in 10% FBS. MSCs were grown in two conditions; media containing 10% PR and media containing 10% FBS. The cells were expanded to confluence at P3 then cryopreserved. Throughout expansion confluence and cell count was documented for each condition. At P1 CFU plates were fixed, stained, and counted.

Preliminary Data

Raw bone marrow used for this project was previously collected from five horses and cryopreserved. Autologous platelet releasate was also previously generated from whole blood

collected from the same five horses. Plasma was separated from the red blood cells. The platelets were activated and fibrinogen was depleted using $CaCl_2$. The concentration of the platelets was one million platelets per microliter. The PDGF-BB concentration was measured and recorded for the PR made from each horse.

CHAPTER II

METHODS

For this project, previously cryopreserved raw equine bone marrow was used to obtain MSCs. Each condition was plated on a tissue culture flask and CFU plate with the appropriate cell density required for this project. The two conditions consisted of culture medium containing either 10% FBS or 10% PR. The FBS condition consisted of FBS added to the standard culture medium for *in vitro* expansion. The PR condition consisted of previously generated autologous PR added to serum-free culture medium. The plasma used to create the PR was fibrinogen depleted therefore no additives were required to prevent clotting.

Media

Two different medias were used for this project. For MSCs expanded in FBS, MSC isolation media (DMEM 1 g/dl glucose supplemented with 10% FBS, 2.5% HEPES buffer, 100µg/ml Basic Fibroblast Growth Factor, and 1% antibiotic and antimycotic) was used. For MSC proliferation in PR, MSC isolation media (DMEM 1 g/dl glucose supplemented with 10% (autologous) PR, 2.5% HEPES buffer, 100µg/ml Basic Fibroblast Growth Factor, and 1% antibiotic and antimycotic) was used. The PR for each horse was previously made and stored in a -20ºC freezer. Both medias were used respectively when feeding (aspirate old media out and add new media) throughout the MSC proliferation in both conditions from P0-freezing.

Bone Marrow Plating from Raw Frozen Cells

The appropriate number of cryovials was located for the number of cells needed for both conditions. The cells were thawed slowly in a 37ºC water bath for three minutes. Under a flow hood, 1 mL of DPBS was slowly added to each cryovial. This mixture sat for 5 minutes. Each cryovial was then slowly added to separate 50 ml conical tubes containing 18 ml of DPBS. A 100µl cell solution sample was obtained from each conical tube and immersed in separate cell counting solution B (see cell count section). Cell counts were obtained from these solutions under a UV microscope to ensure the number of cells in each cryovial were accurate and to ensure the thawing process did not disrupt the cells viability. The cell count and viability of each cryovial was recorded. The conical tubes were centrifuged for 5 minutes at the setting 300g, acceleration 7, and deceleration 7. Pellets were formed in centrifugation allowing the supernatant to be aspirated. The cell pellets were resuspended in the appropriate amount of DPBS (10-40 ml). The appropriate amount was transferred to both a PR and FBS labeled conical tube and centrifuged for 5 minutes at the setting 300g, acceleration 7, deceleration 7. Lastly, the supernatants were aspirated and the cell pellets were resuspended with the appropriate media into a T75 flask and CFU plate. Both the flasks and CFU plates were rocked to evenly distribute the cells and placed on the flattest spot in the incubator set at 37°C, 95% relative humidity and 5% CO_2.

Expansion of MSCs

The MSCs in T75 or T175 flasks and CFU plates were fed media containing FBS or PR every two to three days throughout their expansion until cryopreservation. Prior to feeding, each flask was examined macroscopically and microscopically for qualitative traits as well as

confluence of the MSCs. As the cells proliferated they were passaged to either T75 flasks or T175 flasks depending on their cell count and the desired density (5,000-7,000 cells/cm^2). For T75 flasks being fed every two to three days, 10 or 15 ml is needed for a feeding respectively. For T175 flasks being fed every two to three days, 20 or 30 ml of media is required for the feeding respectively.

During P0, when the cells obtained tightly packed colonies they were passed (see passaging of MSCs section) to P1. At this time the CFU plates were stained and counted later (see CFU plate staining section). Throughout the rest of the MSC proliferation (P1- freezing) the MSCs were passed when they reached 70% confluence.

CFU Plate Staining

The media from the 10 cm dishes were aspirated and rinsed two times with 1X phosphate buffer solution (PBS). Next, 5 ml of crystal violet was added to the plates and they were allowed to sit for 10 minutes. They were gently rinsed with DI H_2O and left to dry overnight. The dishes were saved after the colonies were counted and recorded.

Passaging of MSCs

Before passaging, the MSCs were examined for qualitative traits and confluence. Photos were taken at 4X and 10X. The media was then aspirated from the flasks and rinsed two times with 10 ml HBSS. Next, 5 ml of 1X trypsin was added to the flask then put in the incubator set at 37°C, 95% relative humidity and 5% CO_2 for 3-5 minutes to lift the cells. Before looking for stuck cells under the microscope, the sides of the flasks were tapped to dislodge cells. Inactivation serum (10% Hyclone Equine Serum) was added in equal quantity to trypsin and

rocked gently to stop trypsinization. The cells were pipetted into 50 ml conical tubes and centrifuged for 5 minutes at the setting 300g, acceleration 7, deceleration 7. After the supernatant was aspirated, the cell pellets were resuspended in the appropriate amount of DPBS (10-40 ml). Then, 100 µl of cell solution was deposited into cell counting solution B (see cell count section). The cells were counted with a UV microscope while the 50 ml conical tubes were centrifuging for 5 minutes at the setting 300g, acceleration 7, deceleration 7. The supernatant was aspirated and the cells were resuspended with appropriate media before being plated to the desired density (5,000-7,000 cells/cm^2).

Cell Count

To perform a cell count, two cell counting solutions had to be made. Solution A contained 10 µl FDA stock solution and 2.5 ml DPBS. Solution B contained 60 µl Propidium Iodide stock solution, 100 µl solution A, 740 µl DPBS, and 100 µl of the cell suspension. The 100 µl of cell suspension was transferred from the conical tube under the flow hood with a sterile barrier pipette tip to solution B. Both solution A and B were mixed thoroughly and kept in a drawer to avoid light exposure. Once the solutions were made, 10 µl of solution B was added to each side of the hemocytometer. The cells were viewed at 10X using the UV microscope with the fluorescence set to green. The grid visible under the microscope has nine larger squares with smaller squares inside them but only the four corners and middle large squares were used for counting. The green cells were counted and recorded as live cells while the red cells were counted and recorded as dead cells. To determine the cell count Equ. (1) was used.

$$Cell\ Count = (live\ cells) * \left(10{,}000\,\frac{cells}{ml}\right) * \left(\frac{dilution\ factor=10}{\#\ squares\ counted=10}\right) * (total\ volume\ cell\ suspension\ in\ ml) \qquad (1)$$

Cryopreservation

For cryopreservation, the exact same procedure as passaging was followed until the cells were counted. The cell count determined the amount of freezing medium ((1 ml per 10e6 cells) (see media section)) needed. While counting, the cells were centrifuged in DPBS then the supernatant was aspirated. The cell pellets were resuspended in 1 ml MEM per 1e6 cells or at least 10 ml MEM and centrifuged for 5 minutes at the setting 300 g, acceleration 7, deceleration 7. After aspirating the supernatant, the cryopreservation (freeze) media was added to the pellets and resuspended gently. Next, 1 ml of suspension was transferred into each labeled cryovial. The cryovials were placed in a Mr. Frosty freezer container filled with isopropanol and placed in a -80ºC freezer for 24 hours. The cryovials were then transferred to a liquid nitrogen tank and their information was logged for future use.

Quantitative Analysis

The results of this paper are based off quantitative analyses. The colony forming units were counted and graphed using Excel to compare the amount of progenitor cells when using either condition. The cell counts at each passage was graphed to show the trend for each media condition for each horse over time. The confluence over time was generated using Equ. (2).

$$\frac{Confluence}{Time} = \frac{\%\ Confluence\ at\ P(n+1)}{Time\ in\ days\ to\ go\ from\ P\ (n)\ to\ P\ (n+1)} \qquad (2)$$

The population doubling time (PDT) was calculated with Equ. (3).

$$PDT = \frac{(days\ between\ passages) * \ln(2)}{\ln\big(final\ cell\ count\ P(n+1)\big) - \ln\big(initial\ cell\ count\ P(n)\big)} \qquad (3)$$

The confluence over time and population doubling time are both ways to show proliferation over time. Confluence over time uses the amount of time in between passages relative to the confluence where population doubling time is the amount of time it takes one cell to become two.

CHAPTER III

RESULTS

Colony Forming Unit

Raw equine bone marrow from five equine subjects was plated at a density of 20 million cells per 10 cm CFU plate for each media condition, 10% PR and 10% FBS. Simultaneously T75 culture flasks were plated with the appropriate density for each media condition. When the T75 flasks reached 70% confluence for P1, the CFU plates were fixed and stained. After the plates were allowed to dry overnight, the colonies were counted. In Figure 1 the number of colony forming units is displayed for each horse and each condition. The average number of colony forming units for MSCs grown in media supplemented with FBS was greater than the average number of colony forming units for MSCs grown in media supplemented with PR.

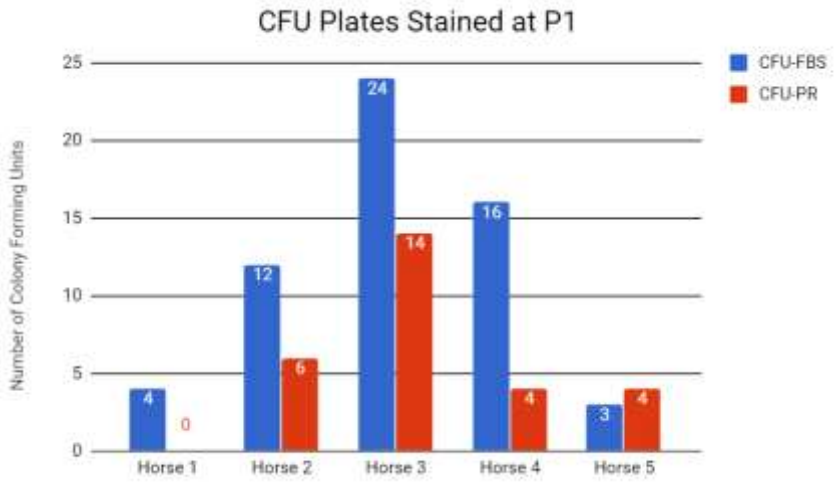

Figure 1: CFU Plates Stained at P1. Number of colony forming units for each horse and each media condition, 10% PR (red) and 10% FBS (blue).

Cell Count

A T75 flask for each media condition was plated with 250 million cells from raw equine bone marrow. The MSCs in each flask were passaged until P3 and then upon reaching confluence were frozen. At each passage the cells were counted and plated at an appropriate density for further cell proliferation. The cell counts were documented at each passage and are shown in Figures 2 and 3 for P1, P2, P3, and freeze. Figure 2 shows the cell counts for the media condition containing 10% PR and Figure 3 shows the cell counts for the media containing 10% FBS. Both figures display the passage number on the x-axis and the number of cells on the y-axis. Each colored line represents a different horse. The FBS condition resulted in higher cell counts than the PR condition.

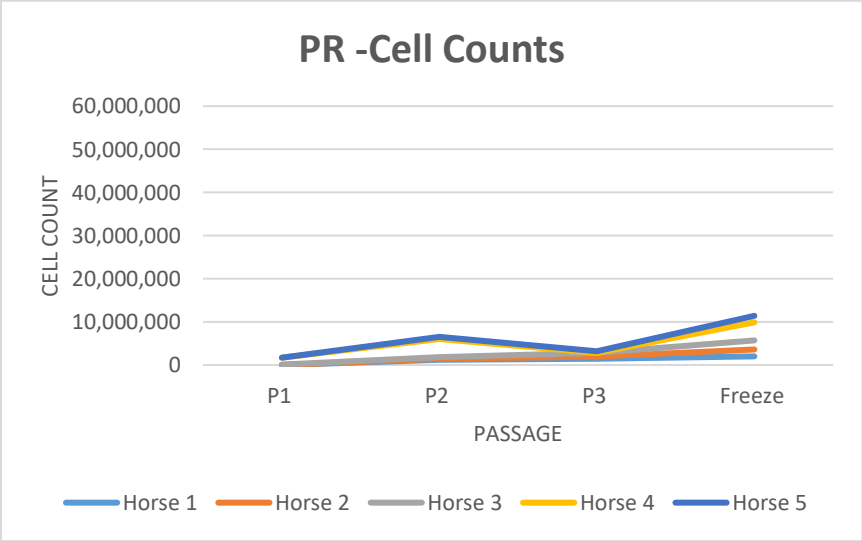

Figure 2: PR-Cell Counts. Cell counts for each horse at P1, P2, P3, and freeze grown in media containing 10% PR.

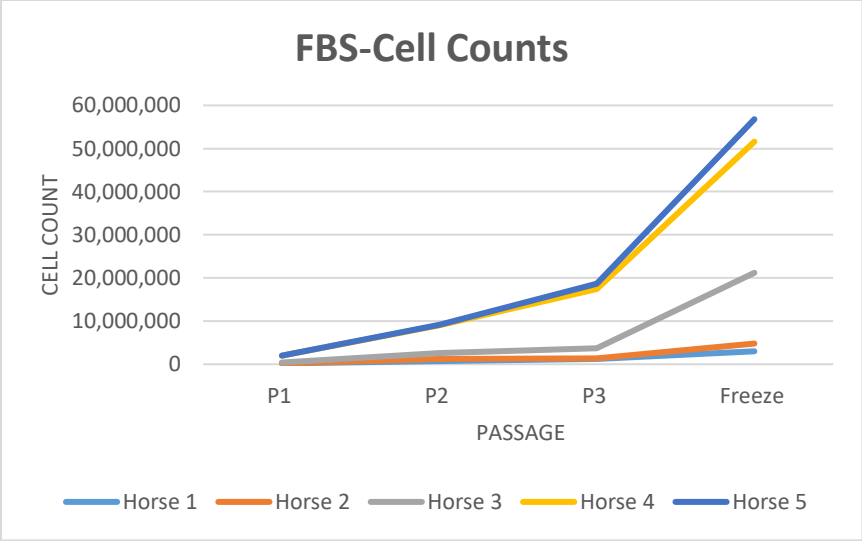

Figure 3: FBS-Cell Counts. Cell counts for each horse at P1, P2, P3, and freeze grown in media containing 10% FBS.

Confluence

The flasks were fed every two to three days by aspirating the old media and adding new media of the same condition. At these feedings, the cells were observed under a UV microscope and their confluence was recorded. Once the flasks reached at least 70% confluence the cells were passaged. On occasion, the MSCs in the PR condition would grow very slowly or not at all. In this case the flasks would not reach 70% confluence and after a prolonged period the cells were passaged in an attempt to aid proliferation. In Figures 4 and 5 the confluence over the time to passage is displayed. On the y-axis the confluence at each passage was divided by the amount of time it took for the cells to get to that passage. The x-axis shows the passage interval for which represents the time between each passage. Each line represents a different horse.

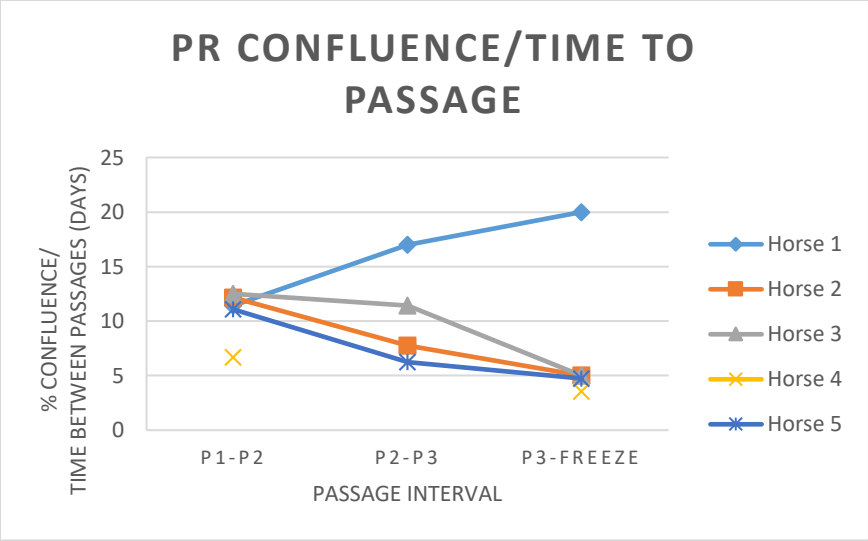

Figure 4: PR Confluence/ Time to Passage. The confluence for flasks containing MSCs and PR condition media divided by the amount of time from one passage to the next. Horse 4 ran out of PR to make more media so was not passaged at P3 instead went from P2 to freeze. This did not change the results of the experiment.

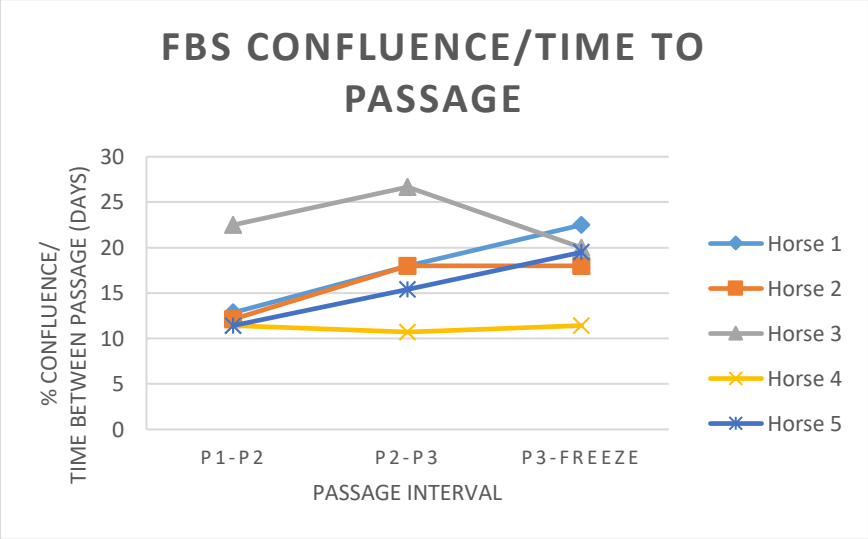

Figure 5: FBS Confluence/ Time to Passage. The confluence for flasks containing MSCs and FBS condition media divided by the amount of time from one passage to the next.

Over time, especially after P2, the confluence of MSCs in the flasks of the PR condition at the time of each passage started to decrease, and the days in between passages increased. The confluence for MSCs in the FBS condition flasks increased or stayed the same at the time of each passage, while the days between the passages started to decrease. FBS appears to proliferate

more rapidly than PR based on the confluence and the amount of time it took to reach each passage.

Population Doubling Time

The population doubling time for each horse is represented by the different colored lines in Figures 6 and 7. The two graphs represent how long it took the cells in each condition to go from one cell to two. The duration was measured in days from one passage to the next. The x-axis shows the interval at which the duration was measured for the population doubling time. The y-axis is the actual time it took the cells to double measured in days.

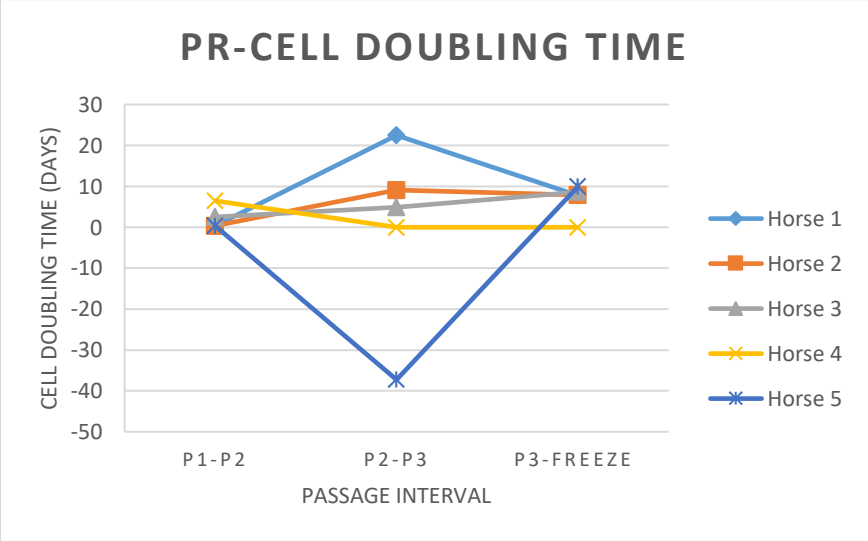

Figure 6: PR- Cell Doubling Time. The cell doubling time for each horse with MSCs grown in PR condition media. Horse 4 had no growth after P2. Horse 5 had a decrease in cell count at P3.

Figure 6 shows high variability from horse to horse and has higher population doubling times than in Figure 7. Based on this data it can be concluded that MSCs in the FBS condition media proliferate more rapidly and consistently than MSCs in the PR condition media.

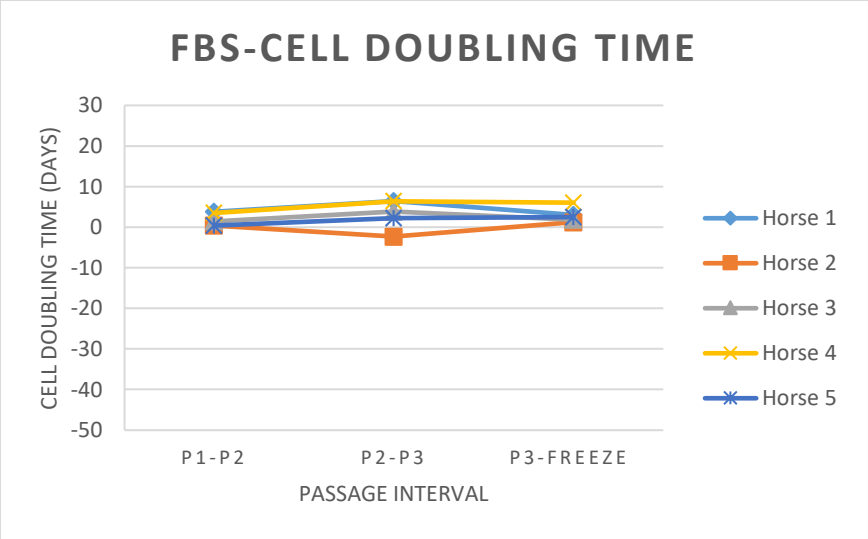

Figure 7: FBS- Cell Doubling Time. The cell doubling time for each horse with MSCs grown in FBS condition media. Horse 2 had a decrease in cell count at P3.

CHAPTER IV

CONCLUSION

Discussion

The results conclude that PR is not a suitable replacement for FBS in the culture of equine MSCs because the proliferation was not the same or superior. The CFU plates showed more colonies representing progenitor cells per ml for the FBS condition than the PR condition. The unknown composition of FBS does not help to determine why FBS has better colony forming properties. Previous research in humans has matched these CFU results. Researchers predict that FBS having a different composition than PR, contains growth factors that better support plastic adherence.[7] On the contrary, most human research on PR has proven it to be a suitable replacement for FBS.[7,10,11]

The FBS condition resulted in higher cell counts than PR. In the beginning, the PR condition yielded relatively high cell counts, but the rate at which the MSCs in the PR condition grew decreased. The confluence over time and population doubling time show that MSCs grown in the FBS condition proliferate more rapidly. It seems that the MSCs grown in the PR condition start to senesce after P2. A sign of MSC senescence includes a gradual decrease in proliferation.[12,13] The MSCs in the PR condition were less confluent at each passage, and took longer to reach each passage proving a decrease in proliferation. The calculated population doubling time shows that the FBS condition has a more consistent proliferation rate of MSCs compared to the PR condition. It also shows MSCs in the FBS condition overall proliferate more rapidly. When MSCs senesce their gene expression changes, such as telomere length shortening, consequently blocking function of cell cycles.[12] This experiment did not focus on the

morphology of the MSCs but the overall appearance did change following P2 for the MSCs in the PR condition. They lost their spindle like shape and started to look more flattened with jagged edges. This is another sign of cell senescence. [12,13]

Another theory to what could have caused PR to not be a suitable replacement for FBS is the amount of PDGFs in the PR for each horse. Prior to this experiment the concentration of growth factor PDGF-BB was measured in the PR of each horse. In this experiment, the concentration of PDGF-BB was between 7,300 pg/ml and 10,800 pg/ml for each horse. Horse 4 had the highest amount of PDGFs and proved to have the highest cell counts in the FBS condition. Cell counts in the PR condition were highest for Horse 4 but stopped proliferating after P2. The reason for the PR condition halting growth is unknown. In human research the amount of PDGF-BB is tremendously higher usually around 20,000 pg/ml.[11] The amount of PDGFs might have caused the decrease in proliferation of the PR condition.

Overall the proliferation of equine MSCs expanded in media supplemented with 10% PR had worse proliferation than equine MSCs expanded in media supplemented with 10% FBS. This contradicts the proposed hypothesis and more research needs to be conducted. This experiment did shed light on possible reasons for decreased proliferation of equine bone marrow derived MSCs in PR supplemented media.

Future Research

Some future research ideas include pooling the PR, increasing the platelet concentration, and changing the concentration of PR supplemented in the media. Previous research has shown that pooling can reduce the variability in PR.[10,11] This study used autologous PR, to keep the

future study autologous pooled batches would come from different samples from the same horse. This could help the variation in growth factors between batches of PR.

The PR used in this experiment used platelets at a concentration of one million platelets per microliter. Since the PDGF-BB concentrations were much lower than most human studies, it might prove beneficial to increase the platelet concentration to five million platelets per microliter. With the higher platelet concentration, there should be more PDGFs produced that can be diluted to different concentrations using fibrinogen depleted platelet poor plasma. A study should be done using different concentrations of PDGFs to see if there is an optimal concentration.

Lastly, it would be beneficial to see if 10% PR is the optimal concentration needed to support MSC proliferation. Different concentrations such as 5%, 10%, and 15% PR or FBS may prove different proliferation results. This experiment would help to test if PR is dose-dependent or not with equine MSCs.

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