# THE EFFECT OF AUTOLOGOUS PLATELET LYSATE ON THE EXPANSION AND ADHESION CHARACTERISTICS OF EQUINE BONE MARROW-DERIVED MESENCHYMAL STEM CELLS

An Undergraduate Research Scholars Thesis

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# ABSTRACT

The Effect of Autologous Platelet Lysate on the Expansion and Adhesion Characteristics of Equine Bone Marrow-Derived Mesenchymal Stem Cells

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Mesenchymal stem cells (MSCs) are undifferentiated cells that are being investigated as a therapy for equine musculoskeletal disease. Fetal bovine serum (FBS) is commonly used as a serum supplement when culturing MSCs. However, there is a risk of xenogenic reactions when MSCs that have incorporated bovine proteins are injected into a patient. Researchers have begun searching for autologous alternatives to FBS, including platelet lysate (PL). Platelets and their growth factors might allow for cell proliferation without the risk of xenogenic reactions. The impact of autologous platelet lysate supplementation on the expansion and tissue culture plastic adhesion of equine bone marrow-derived MSCs was assessed. MSC proliferation in 10% serum supplemented media was compared using PL and FBS as sources of serum. A 10% FBS medium containing heparin was also tested as a control for heparin in the PL supplement. MSC proliferation was assessed using cell counts and CellTrace™ Violet proliferation assays. Preliminary data showed greater overall population numbers in the PL and FBS with heparin supplemented mediums compared to FBS alone. Population doubling times were lower for PL and FBS heparin supplemented mediums compared to FBS alone early in cell culture time. However, at later cell culture times, population doubling times were higher for PL-supplemented cultures. The amount of cells lost during routine media removal and washes of the cell

monolayer was assessed through a DNA assay. Preliminary data showed greater cell loss in PL and FBS heparin supplemented cultures as compared to FBS alone, especially during times when PL population doubling times had increased. Flow cytometry was used to assay for the presence of cell surface markers. All groups were positive for cell surface markers CD-90 and negative for CD-45 and MHCII. The high cell losses in PL-supplemented cultures during times when PL population doubling times increased indicates that cell losses may be obscuring the population growth rates for these MSCs. If this is the case, then solving the adhesion problem in PL-supplemented MSC cultures may allow these cultures to grow to higher cell numbers at a higher rate.

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# NOMENCLATURE

bFGF – Basic Fibroblast Growth Factor

CDPA- Citrate Phosphate Dextrose Adenine

CFU – Colony Forming Unit

DMEM- Dulbecco's Modified Eagle's Medium

DMSO- Dimethyl Sulfoxide

FBS- Fetal Bovine Serum

FITC - Fluorescein isothiocyanate

HBSS- Hank's Balanced Salt Solution

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

MSC- Mesenchymal Stem Cell

PDGF – Platelet Derived Growth Factor

PL- Platelet Lysate

PPP- Platelet-Poor Plasma

PRP- Platelet Rich Plasma

Tris – (Hydroxymethyl)-aminomethane

# CHAPTER I

# INTRODUCTION

Mesenchymal stem cells (MSCs) are undifferentiated cells that can be recovered and enriched from bone marrow aspirations due to their property of adhering to tissue culture surfaces.[1,2] MSCs have been shown to be useful in the repair of cartilage, bone, tendon, and meniscus.[3] In equine medicine, MSCs are being investigated as a therapy for musculoskeletal disease, a common cause of reduced performance in horses.[4,5] The use of MSCs in clinical therapies requires the expansion of the cells to large numbers before use, because they are present in relatively low numbers in their tissue of origin.[1,6] To expand the cells to clinically significant numbers, media that provides essential nutrients to the cells is often enhanced with supplements.[1,6] Fetal bovine serum (FBS) is commonly used as a serum supplement when culturing MSCs because it provides attachment factors, nutrients, and growth factors.[6,7] However, cells cultured in FBS can incorporate bovine immunogenic proteins and infectious agents.[8] When cells that incorporate those proteins are injected into a patient, there is a risk of xenogenic reactions.[8-10] The risk of xenogenic reaction increases when repeated infusions are needed, as is required by some forms of cell therapy.[8,9]

Several autologous alternatives to fetal bovine serum are being investigated. These methods use supplements derived from the patient's blood, including serum, platelet rich plasma, platelet lysate, and platelet releasate.[1,3,9,11-13] Platelet lysate (PL) is formed by isolating platelets from blood samples, lysing the platelets, and diluting the lysate in a small volume of plasma.[6,14] Platelet lysate includes adhesive proteins, a variety of growth factors, coagulation factors,

protease inhibitors, mitogens, and proteoglycans.[1,7] Platelet growth factors are stored in the α-granules of platelets and are released when the platelets are lysed.[15,16] The most common platelet growth factors include platelet-derived growth factors, transforming growth factor-β1, basic-fibroblast growth factor, and insulin-like growth factor-1.[7,14-16] FBS has these same growth factors but at lower concentrations.[1,9] Investigation of the surface receptors on MSCs has revealed that MSCs have high levels of surface receptors for the most common platelet growth factors, and, therefore, are responsive to them.[1] This suggests that PL has the potential to support the expansion of MSCs.

Several studies of human MSCs have found that platelet lysate significantly enhances proliferation rates of MSCs when compared to fetal bovine serum.[1,7,10] However, recent studies on the expansion of equine bone marrow-derived MSCs supplemented with PL found that the proliferative capacity of equine MSCs was not significantly different from cultures supplemented with FBS.[6,15] Interestingly, studies on human and equine MSCs found that PL-supplemented MSCs tended to appear smaller and more spindle-shaped, grow in more dense clusters, and require less trypsinization to detach from plastic than FBS supplemented MSCs.[6,9] The reason for the reduced trypsinization time has not been elucidated. The rapid detachment of platelet lysate supplemented MSCs could be due to a difference in plastic adhesion potential, which could impact the expansion of MSCs due to loss of cells during routine media removal and washes of the cell monolayer. In addition, this would result in the appearance of a higher population doubling time and lower total cell number than platelet lysate supplemented MSCs are capable of.

Further investigation into the impact of platelet lysate supplementation on the characterization of equine MSCs is necessary before this alternative can be successfully applied to therapeutic uses. An improved understanding of the impact of platelet lysate supplementation could be instrumental in improving culture performances in terms of quantity and quality of MSCs, as well as providing an autologous, xenogenic reaction-free method of expanding MSCs for therapeutic applications.

# CHAPTER II

# METHODS

## Animals

Whole blood and bone marrow were collected from three young adult horses. The Institutional Animal Care and Use Committee (IACUAC) protocol for the collection of bone marrow for equine stem cell laboratory projects (2015-0038) was used for this study.

## Platelet lysate preparation

Blood was collected in two blood collections bags containing citrate phosphate dextrose adenine (CDPA), an anticoagulant. The anti-coagulated blood was brought to the laboratory where it 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. After gravity separation, plasma was collected and swirled aggressively for 2 minutes to make a homogenous solution of platelets. A sample was collected and submitted to the Texas A&M Clinical Pathology laboratory for a complete blood count (CBC), including a platelet count. Plasma was aliquoted into 50 mL polypropylene conical tubes and centrifuged at 900 x G for 15 minutes at 10°C to pellet the platelets. The pellets were resuspended in 5 mL of supernatant or platelet-poor-plasma (PPP), resulting in platelet rich plasma (PRP), and aliquoted into 50mL polypropylene conical tubes. The remaining PPP was aliquoted into 50 mL polypropylene conical tubes.

The PRP and PPP were freeze-thawed three times. On the first two freeze-thaw cycles, they were frozen in an ethanol/dry ice bath and thawed in a 35°C water bath. On the last freeze-thaw cycle,

both PRP and PPP were frozen in an ethanol/dry ice bath, but thawed in a 4°C refrigerator overnight to cryoprecipitate out the fibrinogen. When nearly thawed, PRP and PPP were centrifuged at 1000 x G for 20 minutes at 0°C to pellet the fibrinogen. The platelet lysate supernatant was pipetted into new 50 mL conical tubes. Using the platelet count, an appropriate amount of PPP was added to the platelet lysate to produce an approximate concentration of $1x10^6$ platelets/μL. Platelet lysate was filtered using a 0.2μm filter. A 1.5 mL sample 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 -20°C until use. Platelet lysate aliquots were not thawed more than three times for culture.

**Isolation and culture of bone marrow derived MSCs**

Bone marrow was collected after autologous platelet lysate was prepared. A Tris-$NH_4Cl$ solution was used to lyse red blood cells in raw bone marrow. Lysed bone marrow was divided into three equal volumes for each condition: 10% PL plus heparin, 10% FBS, or 10% FBS plus heparin. Nucleated cells were counted for each condition. The equivalent of 1 mL of raw bone marrow from each condition was plated onto 10 cm tissue culture plates for colony forming unit (CFU) assays. The remaining bone marrow was plated on one T-75 flask for each condition. Culture medium containing low glucose (1g/dL glucose) DMEM, sodium pyruvate, L-glutamine, bFGF, and HEPES buffer was supplemented with one of three serum supplements: 10% autologous PL and 1.0 units/mL heparin, 10% FBS, or 10% FBS and 1.0 units/mL heparin. Culture mediums were changed every Monday, Wednesday, and Friday. At each media change, the old media for each condition was collected and analyzed for cells through cell count and DNA content. MSCs from each condition were grown to 70% confluence then passaged by rinsing the cells with

HBSS, lifting the cells with trypsin enzyme, counting the cells, and replating at 5,000 cells/$cm^2$ for a total of five passages. At each passage, HBSS rinses were collected for each condition and were analyzed for cells through a cell count and DNA content. Population doubling time was calculated after each passage. At passage three, at least 1 million MSCs from each condition were set aside for CellTrace Violet staining when they reached 70% confluence. When each condition had at least 8 million unstained cells, approximately 5 million MSCs were set aside for flow cytometry for cell surface markers and the remaining were cryopreserved for trilineage differentiation. MSCs cultured in 10% FBS and 10% FBS plus heparin were cryopreserved in a solution of 95% FBS and 5% DMSO. MSCs cultured 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 every 48 hours for ten days. At each medium change, the old media for each condition was collected and analyzed for cells through a cell count and DNA content. At ten days, medium was collected for DNA assay 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 condition were used to assay for CD90, CD45, and MHC-II. MSCs were at passage five except for the 10% FBS plus heparin condition for one horse which was assayed at passage seven after a lab error necessitated further passages to culture the cells to sufficient population numbers. MSCs were stained with

antibodies conjugated with fluorescein isothiocyanate (FITC). The FACSCalibur™ flow cytometer at the Texas A&M Flow Cytometry Core Laboratory was then used to analyze the MSCs for their binding to the FITC conjugated antibodies.

**CellTrace™ Violet cell proliferation assay**

Cells were set aside as passage three. When each condition reached 70% confluence, the cells were lifted with trypsin enzyme and centrifuged at 300 x G for 5 min at 4°C. Cells were resuspended in 1 mL DPBS for every $10^6$ cells or at least 1 mL DPBS. The CellTrace™ Violet Cell Proliferation Kit for flow cytometry (Molecular Probes™) was used to stain the cells. For each 1 mL cell solution, 1 μL of a 5mM solution of CellTrace™ Violet dye and DMSO was added. The stained cells were incubated at room temperature and centrifuged at 300 x G for 5 min at 4°C to pellet cells. The supernatant was aspirated, and the cell pellet for each condition was resuspended in DPBS. Half of the cell solution was used for the zero hour flow cytometry sample. The Astrios™ High-Speed Cell Sorter at the Texas A&M Flow Cytometry Core Laboratory was used to analyze cells for concentration of CellTrace™ Violet dye. The remaining cell solution was replated onto one T-175 flask per condition and maintained for 72 hours. At 72 hours, the cells were lifted with trypsin enzyme and taken to flow cytometry. Just before leaving to take the samples to flow for each timepoint, 1 μL of propidium iodide was added to each sample.

**DNA purification and quantification**

Samples of media or HBSS rinses were centrifuged at 300 x G for 5 minutes at 4°C to pellet any cells present. The supernatant was aspirated, and a cell count was performed. For the first two

horses, the PerfectPure™ DNA Cultured Cell Kit (5 Prime™) was used to purify and isolate DNA. For the third horse, the Purelink® Genomic DNA Kit (Invitrogen™) was used to purify and isolate DNA. After the cell count, the samples were pelleted by centrifuging at 300 x G for 5 minutes at 4°C. Lysis solution from the DNA kits was added to the cell pellets, and the pellets were frozen at -80°C until they could be processed.

Samples were removed from -80°C and allowed to thaw at room temperature. The cell lysates were prepared according to instructions in the PerfectPure™ DNA Cultured Cell Kit or Purelink® Genomic DNA Kit. Both kits used a spin-column procedure to isolate purified DNA. The purified DNA was collected in 1.5 mL collection tubes. A Thermo Scientific NanoDrop™ 1000 Spectrophotometer was used to measure the ng per μL of DNA in each sample.

# CHAPTER III

# RESULTS

## MSC Proliferation

Preliminary data collected from three horses indicated higher MSC yields when cultured in a medium containing 10% platelet lysate than when cultured in 10% FBS (Table 1). Starting at passage three, 10% FBS with heparin supplemented cultures had higher cell numbers than either of the other two conditions.

*Table 1. Cell counts at each passage (n=3)*

| Media Condition | Passage 1 | Passage 2 | Passage 3 | Passage 4 | Passage 5 |
|---|---|---|---|---|---|
| 10% FBS | 500,000 ± 529,150 | 1,500,000 ± 700,000 | 4,400,000 ±3,520,000 | 7,500,000 ± 6,240,000 | 15,700,000 ± 13,800,000 |
| 10% PL | 867,000 ± 833,000 | 2,550,000 ± 1,630,000 | 6,520,000 ± 3,460,000 | 11,200,000 ± 7,390,000 | 22,900,000 ± 25,600,000 |
| 10% FBS and heparin | 417,000 ± 419,000 | 2,500,000 ± 1,610,000 | 9,150,000 ± 3,680,000 | 15,800,000 ± 8,420,000 | 34,400,000 ± 20,200,000 |

Population doubling time 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 (Table 2). A population doubling time was not calculated for passage one due to high numbers of other nucleated cell types from the bone marrow present at plating. All conditions had comparable population doubling times for passage two. At passage three, 10% PL and 10% FBS with heparin supplemented cultures both tended to have lower population doubling times than FBS supplemented cultures. The population doubling times for MSCs cultured in media containing

10% platelet lysate were much higher at passages 4 and 5 than either of the other two media conditions, indicating a slower rate of expansion.

*Table 2. Population doubling time (PDT) at each passage (n=3)*

| Media Condition | Passage 2 PDT | Passage 3 PDT | Passage 4 PDT | Passage 5 PDT |
|---|---|---|---|---|
| 10% FBS | 2.893 ±1.171 | 4.114 ±1.970 | 5.672 ±4.024 | 2.731 ±0.737 |
| 10% PL | 2.952 ± 1.494 | 3.691 ±0.927 | 10.486 ±6.888 | 13.911 ±10.187 |
| 10% FBS and heparin | 2.125 ±0.728 | 2.678 ±1.473 | 5.361 ±2.460 | 3.699 ±2.443 |

All conditions were scored on their morphology (Figure 1) and debris (Figure 2) prior to each passage. MSCs cultured in media containing 10% FBS with heparin tended to have a more elongated, spindle-like appearance and tended to score higher on morphology. Cultures supplemented with 10% PL tended to have more debris at later passages.

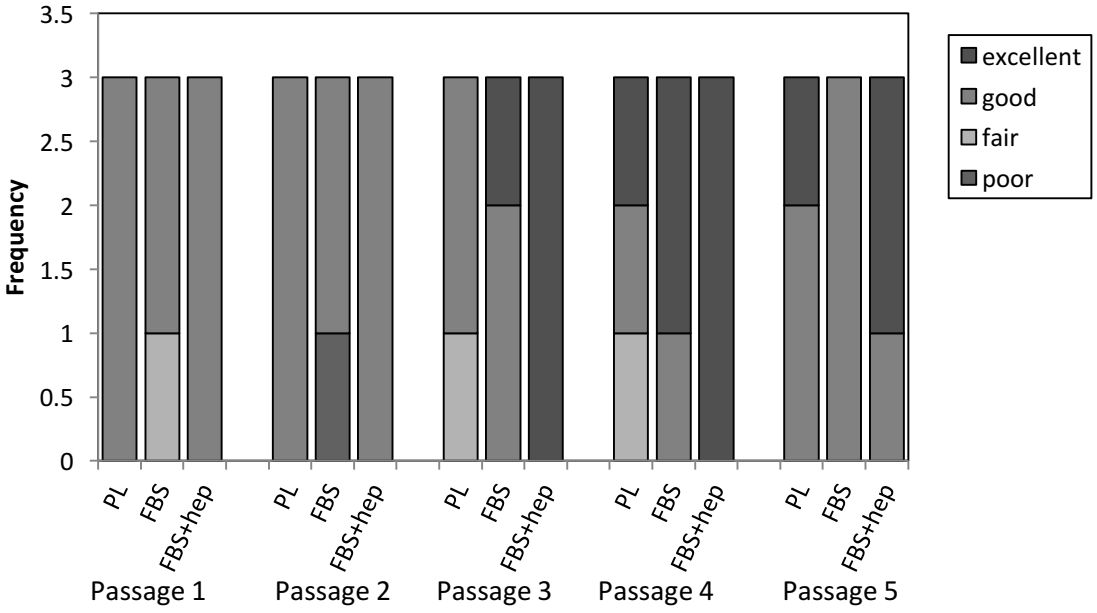


*Figure 1. Morphology scores at each passage.*

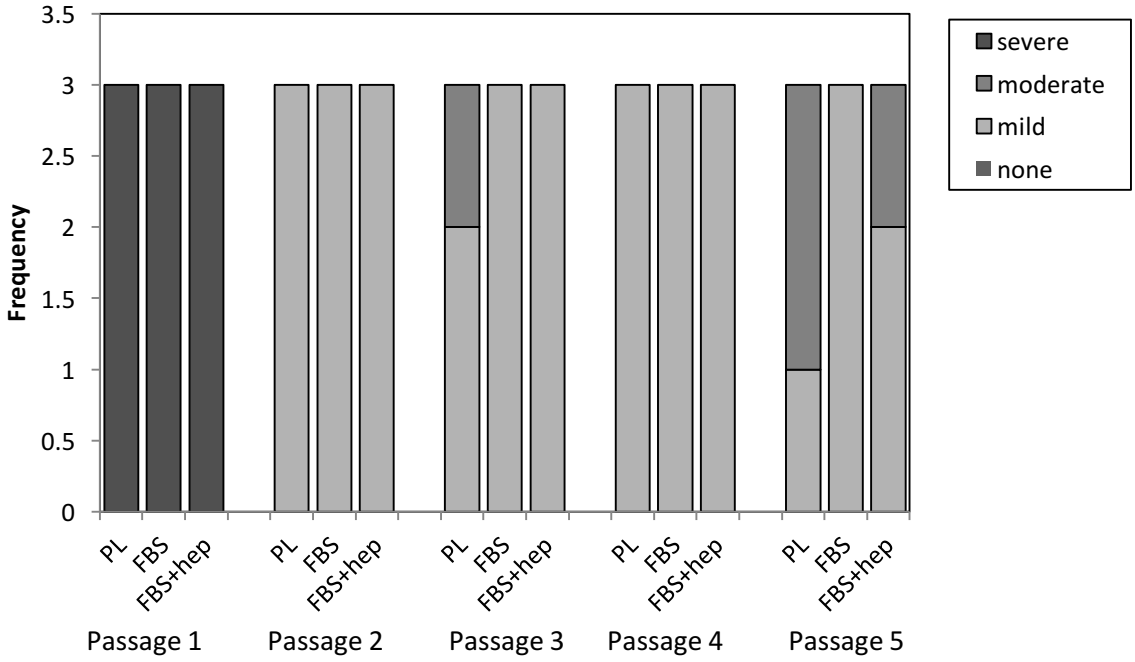


*Figure 2. Debris scores at each passage.*

**Flow cytometry**

Flow cytometry was used to analyze cell surface markers. Data from Horse 1 indicated all conditions were positive for CD-90, but negative for CD-45 and MHCII (Appendix A Figure 3). Similarly, data from Horse 2 indicated all conditions to be positive for CD-90, but negative for CD-45 and MHC-II (Appendix A Figure 4). All conditions for Horse 3 were negative for CD-45 and MHC-II, while 10% PL was mildly positive for CD-90 and 10% FBS and 10% FBS with heparin were strongly positive for CD-90 (Appendix A Figure 5).

**CellTrace™ Violet cell proliferation assay**

Flow cytometry was used to analyze a cell sample for each condition for concentration of CellTrace Violet dye. The samples were analyzed immediately after staining and 72 hours after staining. The resulting data was analyzed to determine the number of times the cells had divided

and the percentage of each generation present was calculated for Horse 1 (Figure 6), Horse 2 (Figure 7), and Horse 3 (Figure 8).

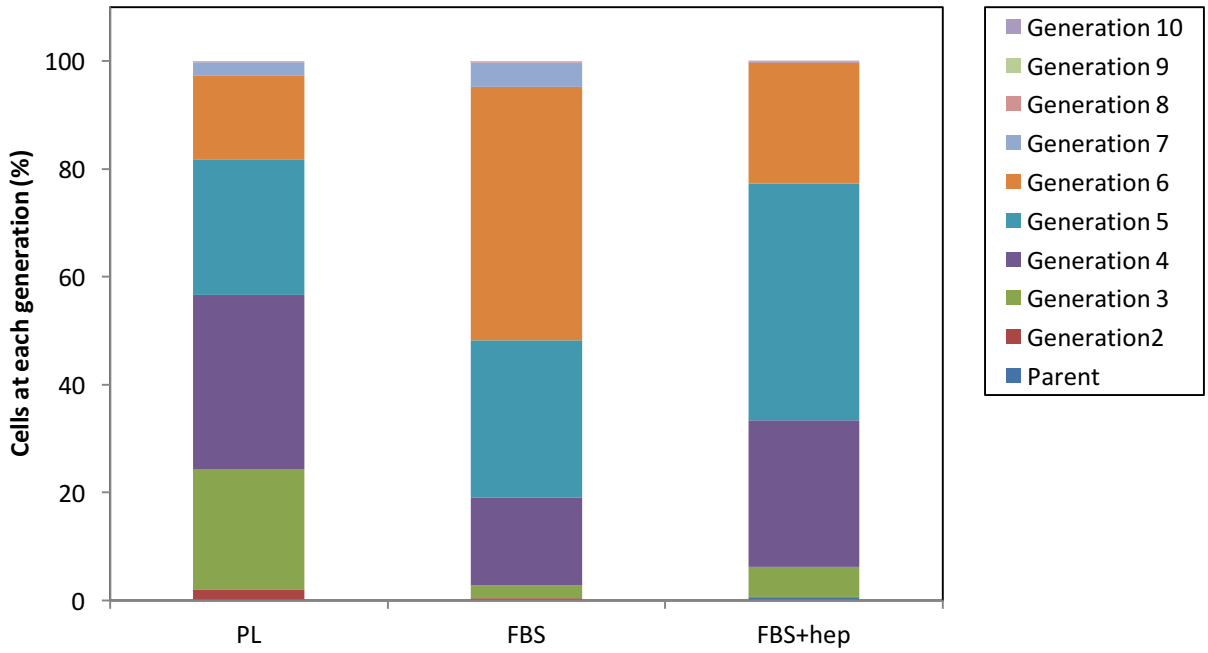


*Figure 6. Generation data obtained from CellTrace™ Violet assay for Horse 1.*

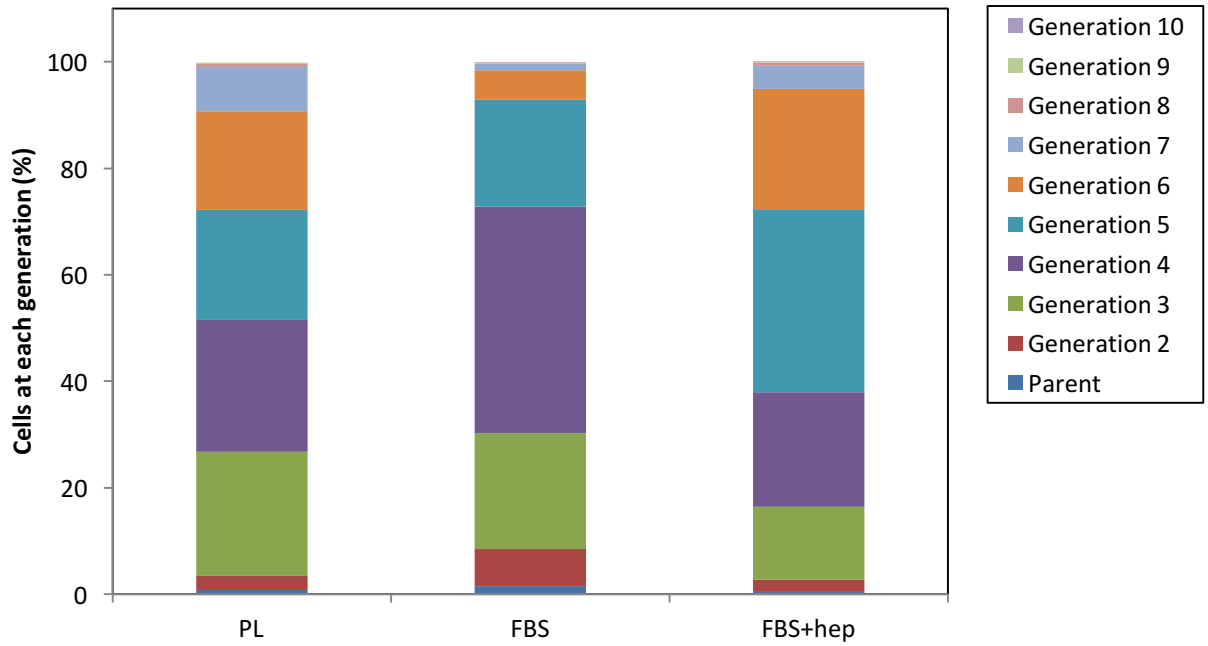


*Figure 7. Generation data obtained from CellTrace™ Violet assay for Horse 2.*

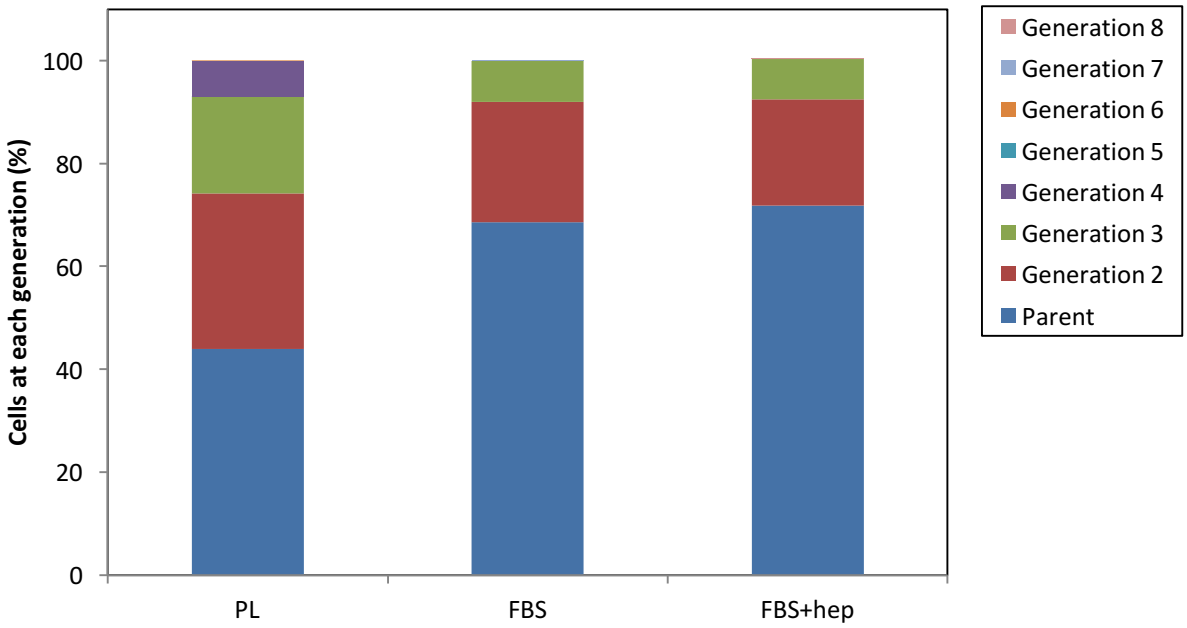


*Figure 8. Generation data obtained from CellTrace™ Violet assay for Horse 3.*

An upper generation proliferation index was calculated based on the 72 hour cell generation data for cells at generations above the parent generation (Table 3). Higher proliferation indexes indicate a faster rate of proliferation and more cells at later generations. For Horse 1, 10% FBS had the highest proliferation index and 10% PL had the lowest proliferation index. The ranking of 10% PL as the lowest rate of expansion was consistent with the observed population doubling times at passage five, the passage that coincided with the CellTrace™ Violet assay (Table 4). For Horse 2, 10% FBS with heparin had the highest proliferation index and 10% FBS had the lowest. This was not consistent with the observed population doubling times at passage 5, which showed 10% PL to have the lowest expansion rate (Table 4). The proliferation index was highest for 10% PL for Horse 3 with 10% FBS and 10% FBS with heparin being very similar. Once again, this was not consistent with the observed population doubling times at passage 5, which showed 10% PL to have the lowest expansion rate (Table 4).

*Table 3. Upper generation proliferation indexes calculated from Cell Trace Violet assay*

| Media Condition | Proliferation index Horse 1 | Proliferation index Horse 2 | Proliferation index Horse 3 |
|---|---|---|---|
| 10% FBS | 16.18 | 6.29 | 4.05 |
| 10% PL | 7.88 | 8.11 | 4.62 |
| 10% FBS and heparin | 12.01 | 9.76 | 4.04 |

*Table 4. Passage 5 population doubling times (PDT) for each horse*

| Media Condition | Passage 5 PDT Horse 1 | Passage 5 PDT Horse 2 | Passage 5 PDT Horse 3 |
|---|---|---|---|
| 10% FBS | 3.53 | 2.59 | 2.07 |
| 10% PL | 24.16 | 13.79 | 3.79 |
| 10% FBS and heparin | 2.47 | 6.51 | 2.12 |

**MSC adhesion characteristics**

During routine media removal and washes of the cell monolayer, the removed media and washes were analyzed for detached cells by determine the DNA content. The cell counts were too low in the media removals after passage one and all of the washes to obtain an accurate cell count and viability. Therefore, the DNA content was used to approximate relative cell losses. HBSS was used to rinse the cell monolayer at each passage and the resulting cell loss was quantified by determining the total DNA content in the HBSS rinses (Table 5). Cultures grown in 10% PL media had a higher DNA content in HBSS rinses than cultures grown in 10% FBS or 10% FBS with heparin.

*Table 5. Total ng DNA in HBSS rinses of cell monolayer at each passage (n=3)*

| Media Condition | Passage 1 | Passage 2 | Passage 3 | Passage 4 | Passage 5 |
|---|---|---|---|---|---|
| 10% FBS | 34.1667 ±59.178 | 20.833 ±36.084 | 72.5 ±94.967 | 168.333 ±85.049 | 114.167 ±116.306 |
| 10% PL | 212.500 ± 143.549 | 265.833 ± 167.357 | 125.833 ± 81.866 | 297.5 ±172.064 | 138.333 ±122.381 |
| 10% FBS and heparin | 200 ±346.410 | 210 ±130.120 | 20 ±34.641 | 201.667 ±53.929 | 124.167 ±111.980 |

The DNA content in the removed media was analyzed by looking at the combined ng of DNA from all media changes in the interval between passages (Table 6). For the time before the first passage, called passage zero, the first media removal was not analyzed for DNA because there were too many cells for the DNA purification kits used. In the media changes that were analyzed for DNA content before the cells reached 70% confluence for the first time, 10% PL had a much lower DNA content than the other two conditions. For the interval after passage one until the subsequent passage, 10% PL tended to have more DNA in the media than the other two conditions. The conditions followed a similar pattern for the culture time after passage two. For the interval after passage 3 until the subsequent passage, both 10% PL and 10% FBS with heparin had a much higher DNA content than 10% FBS supplemented cultures. After the first passage, cultures supplemented with 10% FBS maintained a similar level of DNA content in the media at the interval between each passage. Following passage four, 10% FBS with heparin tended to have the highest DNA content, although 10% PL supplemented cultures still had a high DNA content compared to 10% FBS supplemented cultures.

*Table 6. Total ng DNA in removed media at all feedings after each passage (n=3)*

| Media Condition | Passage 0 | Passage 1 | Passage 2 | Passage 3 | Passage 4 |
|---|---|---|---|---|---|
| 10% FBS | 31,773.833 ±27,784.564 | 399.833 ±160.499 | 476 ±420.967 | 596.167 ±574.899 | 588.5 ±258.228 |
| 10% PL | 11,241.5 ± 4,873.341 | 803 ± 352.756 | 721.667 ± 379.114 | 5,198.333 ±7,193.376 | 1,697.5 ±701.173 |
| 10% FBS and heparin | 31,856.33 ±36,326.06 | 586.333 ±301.088 | 347.5 ±297.584 | 4,474.5 ±5,471.64 | 2,632.5 ±3,072.28 |

# CHAPTER IV

# DISCUSSION

At the present time, data has been collected from three horses. While more replicates must be conducted before a definitive conclusion can be drawn, it appears that platelet lysate supplemented MSC culture medium has an impact on the proliferation and tissue culture plastic adhesion of equine bone marrow-derived MSCs. In all horses, MSCs grown in media supplemented with 10% platelet lysate grew to a greater number than those grown in 10% FBS, although population doubling times indicated that they grew at a lower rate later in the culture time.

The effect of heparin on MSC proliferation has yet to be elucidated. In all three horses, MSCs grown in 10% FBS with heparin grew to a higher cell number than either of the other two conditions. They also had comparable population doubling times to MSCs grown in 10% FBS, indicating a similar rate of cell growth. This indicates that heparin may alter the proliferation of MSCs. Studies in endothelial cells have shown that exposure to heparin can cause the release of basic fibroblast growth factor from the extracellular matrix and, therefore, increase endothelial cell proliferation. [17] The increased MSC yield of MSCs supplemented with heparin indicates that a similar process may occur in MSCs. The implications of adding heparin should be explored further before concluding that PL alone is responsible for the increased MSC yield in PL-supplemented cultures.

MSC cultures supplemented with 10% PL and 10% FBS with heparin tended to have higher cell losses from routine media removal and washes. This preliminary data indicates that heparin may have an effect on MSC adhesion to tissue culture plastic surfaces. However, platelet lysate tended to have consistently higher cell losses than the 10% FBS with heparin media condition for all of the washes and during media removals after the first passage. This suggests that platelet lysate itself may have an impact on MSC adhesion to tissue culture surfaces, in addition to heparin alone.

Cell losses from PL-supplemented cultures may be obscuring the population growth rates for these MSCs. The times at which the platelet lysate population doubling times increased, indicating a slower growth rate, tended to coincide with times of high cell losses for the PL-supplemented cultures. Since population doubling times are highly dependent on nucleated cell counts at the beginning and end of a time period, loss of viable cells in removed media could make the growth rates appear to be lower than the cultures were actually growing at. The possibility that the PL-supplemented cultures were actually growing at a rate higher than the PDTs indicated is supported by the fact that the CellTrace™ Violet cell proliferation assay showed that MSCs grown in 10% PL media had a higher proliferation rate than those grown in 10% FBS for two of the three horses. More replicates need to be performed to definitively conclude if an adhesion problem is the reason for the increased PDTs in platelet lysate supplemented cultures. If this is the case, then solving the adhesion problem could allow for platelet lysate to support the expansion of MSCs to even higher population numbers and at an increased rate.

Further exploration into the use of platelet lysate as an autologous serum supplement includes completing trilineage differentiation on MSCs grown in each of the three medium types. MSCs should be able to differentiate into osteocytes, chondrocytes, and adipocytes. Additionally, the PDGF content in the platelet lysate for each horse should be assessed using an ELISA assay.

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# APPENDIX A

# CELL SURFACE MARKER HISTOGRAMS

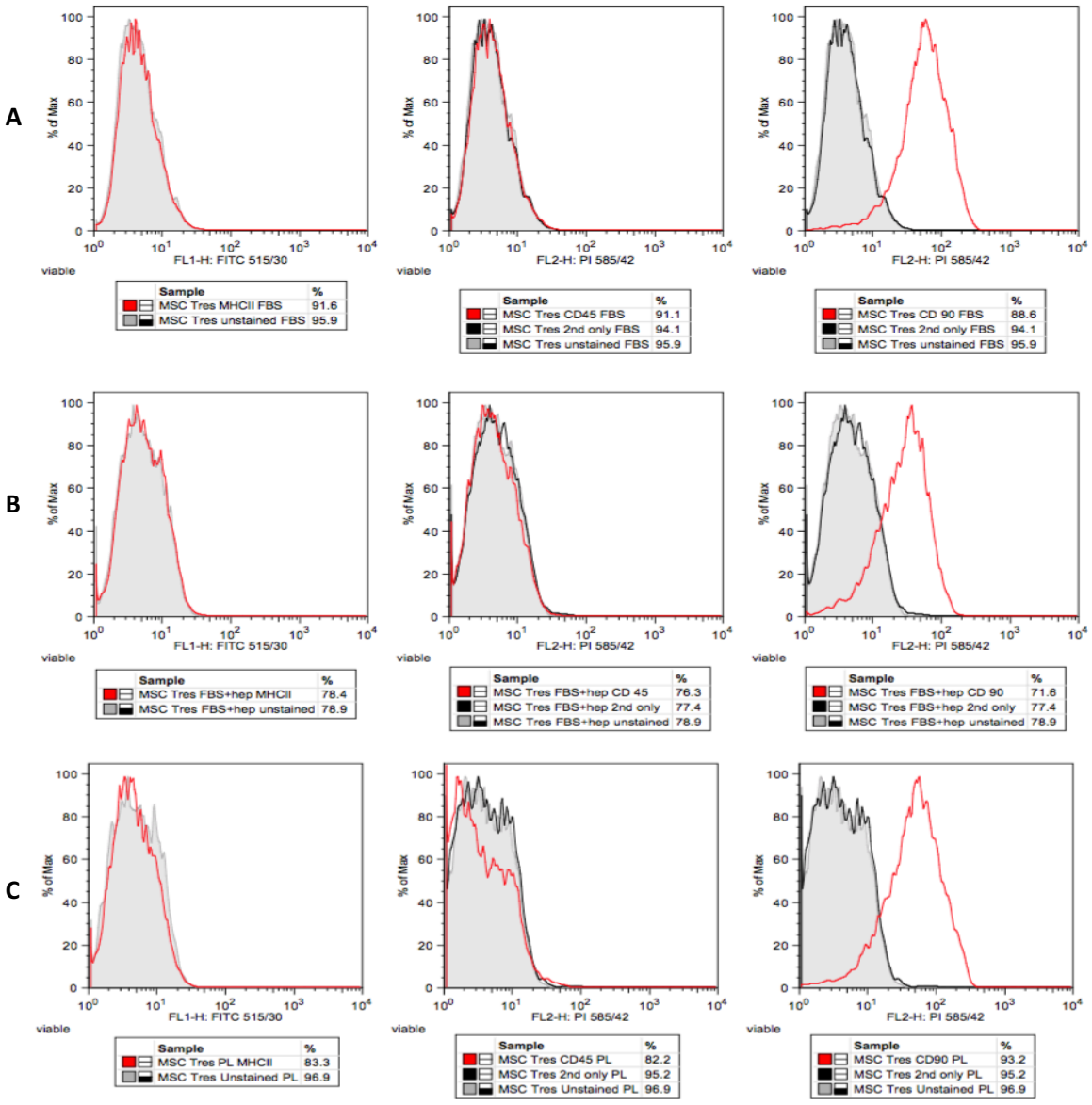


*Figure 3. Flow cytometry data obtained from Horse 1. (A) Flow cytometry histograms for MSCs grown in 10% FBS. MHCII and CD45 are negative while CD90 is positive. (B) 10% FBS with heparin flow cytometry histogram shows these MSCs to be positive for CD90 and negative for MHCII and CD45. (C) Histograms for 10% PL shows these MSCs to be positive for CD90 and negative for MHCII and CD45.*

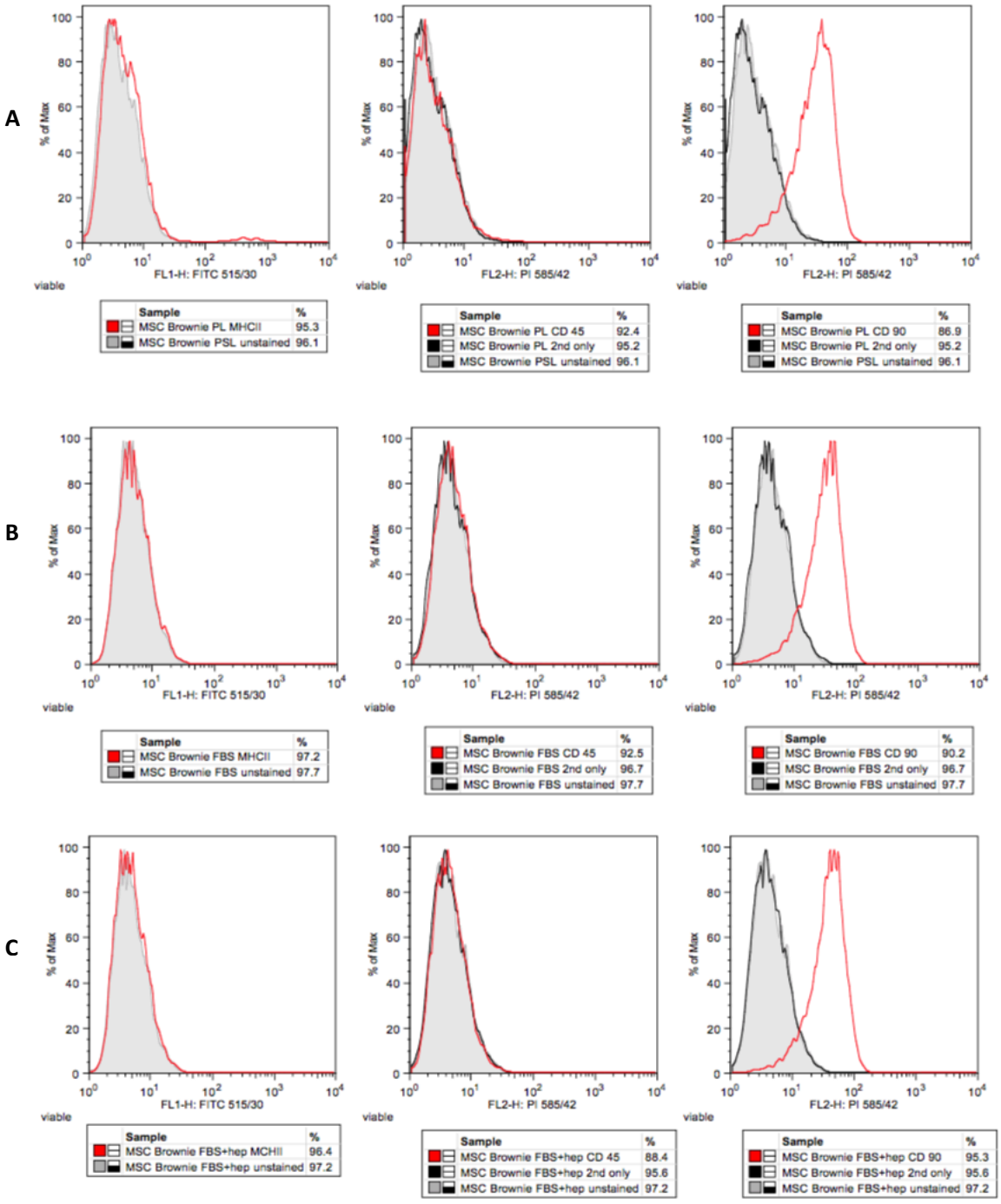


*Figure 4. Flow cytometry data obtained from Horse 2. (A) Flow cytometry histograms for MSCs grown in 10% FBS. MHCII and CD45 are negative while CD90 is positive. (B) 10% FBS with heparin flow cytometry histogram shows these MSCs to be positive for CD90 and negative for MHCII and CD45. (C) Histograms for 10% PL shows these MSCs to be positive for CD90 and negative for MHCII and CD45.*

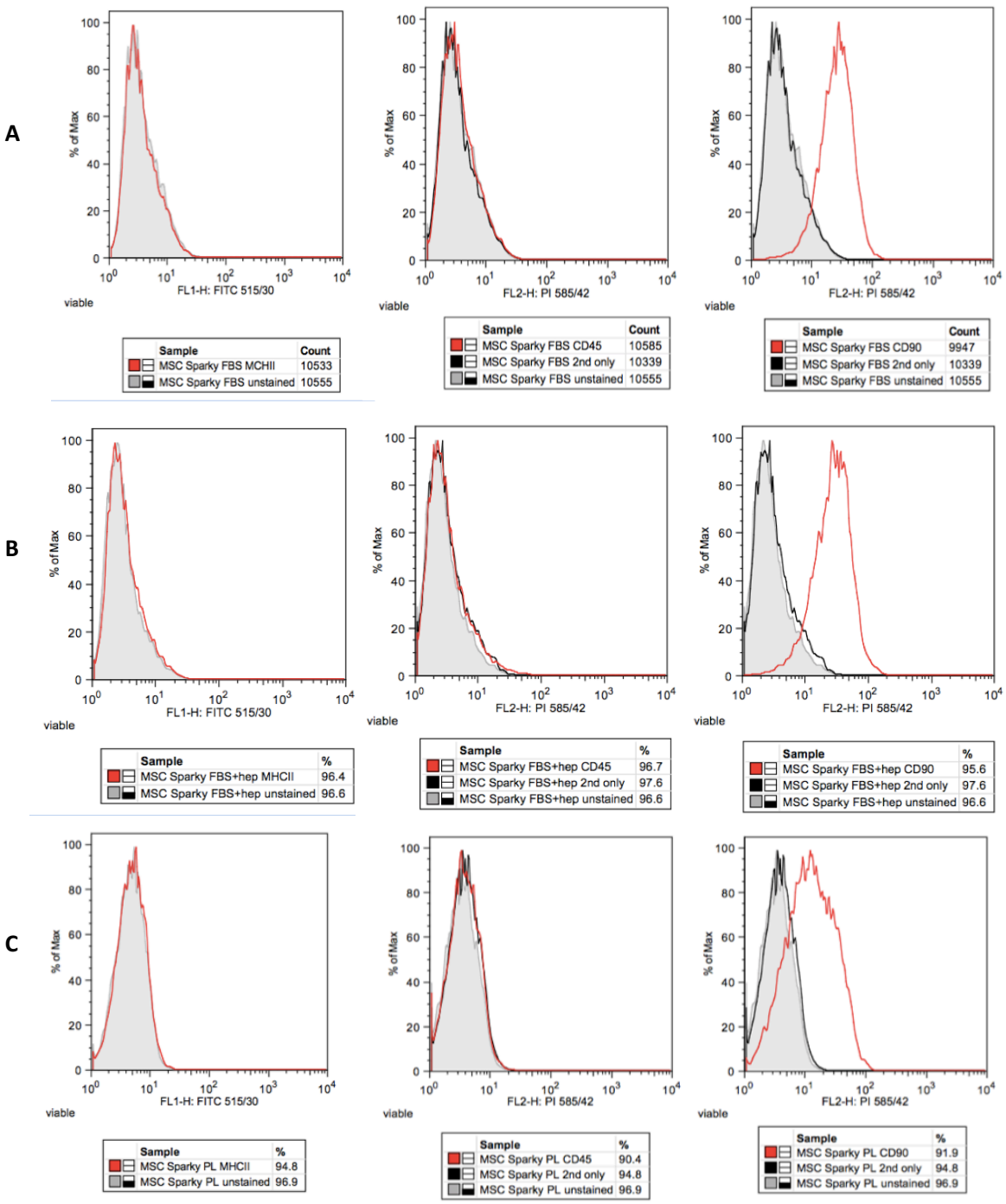


*Figure 5. Flow cytometry data obtained from Horse 3. (A) Flow cytometry histograms for MSCs grown in 10% FBS. MHCII and CD45 are negative while CD90 is positive. (B) 10% FBS with heparin flow cytometry histogram shows these MSCs to be positive for CD90 and negative for MHCII and CD45. (C) Histograms for 10% PL shows these MSCs to be positive for CD90 and negative for MHCII and CD45.*