

**OPTIMIZATION OF EQUINE MESENCHYMAL STEM CELL
CRYOPRESERVATION FOR CLINICAL USE**

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Equine Mesenchymal Stem Cells: Determination of the Optimal Freezing Protocol Prior to Intra-articular Injection for the Reduction of Synovitis. (May 2014)

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Synovitis is the inflammation of the synovial membrane and is a common condition in horses. Synovitis can occur with many other conditions such as rheumatoid arthritis and can lead to joint degeneration if left untreated. The purpose of this study is to determine the optimal freezing condition to cryopreserve equine mesenchymal stem cells (MSCs) prior to use for synovitis control by intra-articular injection. In this study, MSCs were isolated from nine different horses and cryopreserved in the six conditions for each horse. After at least 24 hours, the cells were thawed and their cytoplasm was stained with standard CellTrace™ violet dye. The cells were then plated on two T25 flasks at a concentration of 10,000 cells/cm² per condition and one colony formation assay at 10 cells/cm² per condition. The remainder of the cells were set aside for flow cytometry as the baseline because they had not had time to undergo mitosis. The cell fluorescence from the dye was analyzed 24hr and 72hr post-plate respectively and the distribution of the dye was analyzed. With the colony formation assay, cell morphology, confluency, and data from the flow cytometer, a holistic idea of which condition produces the most viable cells post-thaw was hoped to be obtained. Upon a one-way analysis of variance (one-way ANOVA), it was found that there was no significant difference in cell viability post-thaw.

This conclusion is further supported by statistical analysis of the CFU, cell counts, and post-thaw viabilities, which all produced large p-values indicating insignificance.

DEDICATION

To my mom and dad, none of this would be possible without your endless support. Thank you for everything you have done for me

ACKNOWLEDGMENTS

I would like to thank Dr. Ashlee Watts. You have gone above and beyond in furthering my education and for that, I am forever thankful.

I would also like to thank the members of the Comparative Orthopedic and Regenerative Medicine Laboratory. I could not have asked for a better group of people to work with.

Finally, Dr. Roger Smith, thank you for making time in your busy schedule for us to use your flow cytometer.

CHAPTER I

INTRODUCTION

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent, self-renewing stem cells that are traditionally found in bone marrow [5]. Mesenchymal stem cells have the capability to differentiate into many different orthopedic tissues including adipocytes (fat), chondrocytes (cartilage), and osteocytes (bone) [5]. Currently, osteoarthritis and other forms of cartilage damage are typically treated by invasive joint replacement surgeries [5]. However, current research has demonstrated that mesenchymal stem cells have the capability of generating cartilage upon localized injections, leading to the possibility of alternative, more effective treatments [5]. MSCs have also demonstrated the ability to significantly reduce inflammation in fibroblast-like synoviocytes in vitro [4]. Fibroblast-like synoviocytes (FLS) are found in the synovial membrane of joints and play a crucial role in inflammatory response due to chronic disease and injury such as osteoarthritis [4]. In order to maximize the findings of future research, a reliable method to effectively culture and prepare mesenchymal stem cells for experimentation should be developed. The current methods of cryopreserving MSC include freezing the cells in a medium with varying concentrations of DMSO (dimethyl sulfoxide), MEM (Minimum Essential Medium Eagle) and either autologous serum or fetal bovine serum (FBS) [1][2][3].

Purpose of flow cytometry

An interesting tool that will be used in this study is the flow cytometer. Originally, this study was going to be performed strictly based on cell count post-culture, cell morphology, and colony

formation by the colony formation assay. However, we hope that the flow cytometer will provide a more sophisticated idea of how often the cells proliferate post-thaw and thus, how actively and effectively they will control synovitis in the joint. In order to measure this quantitatively, flow cytometry will measure the generations of cells stained with CellTrace violet dye immediately after staining, 24 hours post-culture, and 72 hours post-culture. As the cells undergo mitosis, the dye staining the cytoplasm will further dissipate with each generation of MSC. This will be performed for each of the six conditions for each of the nine horses.

Importance and possible outcome

When organizing a treatment for synovitis with stem cell injections, the viability of the cells directly affect the efficacy of the injection. Thus, it is important to determine the cryopreservation condition that will maximize the cell viability post-thaw. The six conditions that will be tested are as follows:

Condition #1 → 70% MEM, 20% autologous serum, 10% DMSO

Condition #2 → 70% MEM, 20% FBS, 10% DMSO

Condition #3 → 95% autologous serum, 5% DMSO

Condition #4 → 95% FBS, 5% DMSO

Condition #5 → 70% MEM, 20% HyClone™ equine serum, 10% DMSO

Condition #6 → 95% HyClone™ equine serum, 5% DMSO

A possible outcome of this study is to come significantly closer to determining the optimal cryopreservation condition for clinical use of MSCs. Further research will need to be done following the results of this study, but we will be closer nonetheless. If the results are significant, this could help normalize how clinically used MSCs are cryopreserved.

CHAPTER II

METHODS

Thawing

A cryovial containing previously frozen MSCs was removed from the liquid nitrogen and placed in the water bath for 3 minutes to thaw. In the meantime, 10 mL of DPBS (Dulbecco's Phosphate-Buffered Saline) was pipetted into a 50-mL conical vial under the hood. After the cells completely thawed, a p1000 was used to add 1 mL of DPBS to the vial of cells and they were left to stand for 2 minutes to make sure that the cells reached an isotonic state. The cells were then removed from the cryovial and placed in the conical tube. Finally, 100 microliters of the cell solution was removed and placed in the previously prepared cell counting solution and the remainder of the conical tube was centrifuged at 300g and 4° C for 5 minutes. While spinning, the cells were counted for an initial count.

Staining

The cells were resuspended in DPBS at a concentration of 1e6 cells/mL to prepare to be stained. The CellTrace™ violet dye stock was then added at a concentration of 1 microliter of dye per 1 milliliter of cell suspension (if there were 9 milliliters of cell suspension, 9 microliters of dye would be added). Upon addition of the dye, the cells were transferred to a light-free area of the lab (inside a drawer) where they were incubated for 20 minutes and inverted every 3 minutes to insure a consistent distribution of the dye. After the first incubation period, 5 mL of MSC ISO 10% FBS + Fungizone was added to the suspension in order to inactivate any of the excess dye and the suspension was incubated for another 5 minutes. The suspension was then centrifuged at

300g and 4° C for 5 minutes. After centrifugation, the cells were resuspended in 5 more milliliters of MSC ISO 10% FBS + Fungizone and incubated for 10 minutes while inverting every 3 minutes.

Plating

Prior to plating the cells, a portion (0.5 mL) of the cell suspension was removed for flow cytometry in order to establish a baseline to compare data from 24 hours post-plate and 72 hours post-plate. The 0.5 mL that was removed was placed in a flow cytometry tube and the methods discussed in the “collecting to flow” section are applied here. The remainder of the cells were used to plate two T25 flasks at a concentration of 10,000 cells/cm² (250,000 cells total) and one colony formation assay on a 10 cm plate at 1,000 cells. The cell density and morphology was documented for every day including the same day the cells were plated. The media used was MSC ISO 10% FBS + Fungizone.

Collecting to flow

After 24 hours, the 24-hour flask of cells was ready to be flowed. The media was aspirated off of the cells and 2 mL of HBSS (Hank’s Balanced Salt Solution) was added to aid in the removal of the media. The HBSS and remaining media was aspirated and an additional HBSS rinsing was performed. One milliliter 10% trypsin at 35° C was then added to the media-free flask and placed in the incubator for no more than 5 minutes so the trypsin could detach the cells from the flask. The flask was then tapped gently to aid in the dislodgement of the cells. Finally, two milliliters of inactivation serum (10% equine serum + HBSS solution) was added to the cell suspension in order to inactivate the trypsin. At this point, the cells should be detached from the flask and

ready to be transferred to a 15-mL conical vial. Once successfully transferred, the cell suspension was centrifuged at 300g and 4° C for 5 minutes and the supernatant was aspirated. One half of a milliliter of DPBS was added to the cells and they were then transferred to a flow cytometry tube. One hundred microliters of cell suspension was removed for a cell count and 2 microliters of propidium iodine was added just prior to flow cytometry. The cells were quickly vortexed and finally taken down to Dr. Roger Smith's Flow Cytometry Core Lab to be analyzed. Repeated for the 72-hour flask and this entire protocol was followed for each of the nine horses.

CHAPTER III

RESULTS

According to the data collected, there was no significant difference in cell viability post-thaw across the 6 conditions. The median cell viability ranged from 83% to 88% (figure 1) and the median number of colonies formed from the colony forming unit (CFU) ranged from 72 to 106 (figure 2).

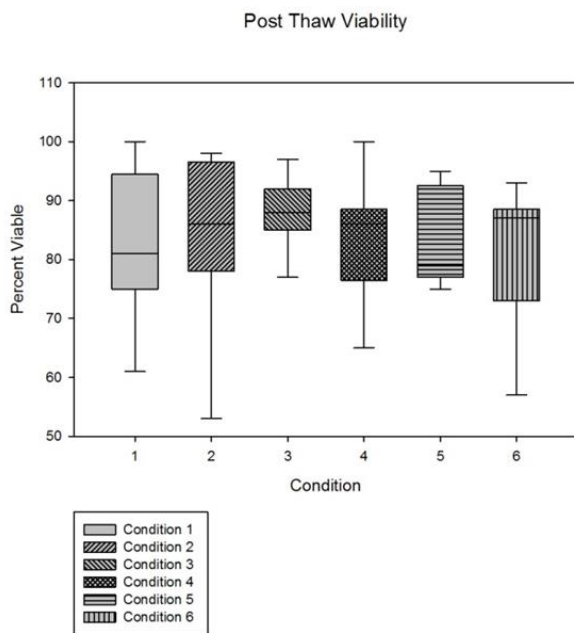


Figure 1. Post-thaw Viability

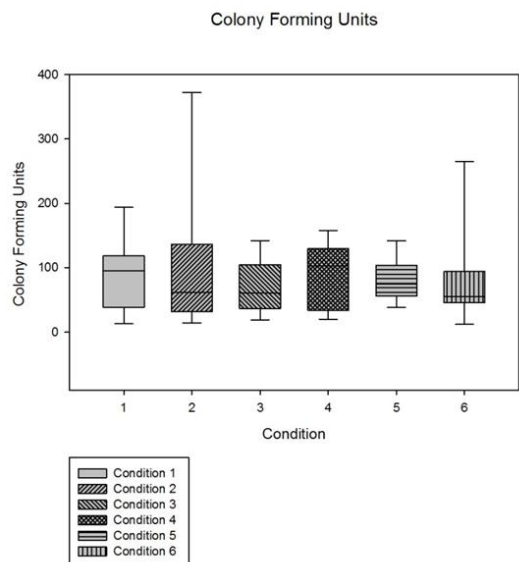


Figure 2. Colony Forming Unit

Given this data, a one-way ANOVA was performed for post-thaw viability and the number of colonies from the CFU. A p-value of 0.8747 was determined for post-thaw viability and 0.9306 for the CFU indicating no significant difference among the conditions in each of the nine horses. Additionally, data from the flow cytometer also indicated a lack of significance among the cells from different conditions. Figure 3 shows the fluorescence measured by the flow cytometer on day 0 in order to obtain a baseline parental peak. At 24 hours post-thaw, 96.8% of the cells were still in their parental generation. However, at 72 hours post-thaw (figure 4), 55.84% were in the 4th generation of daughter cells.

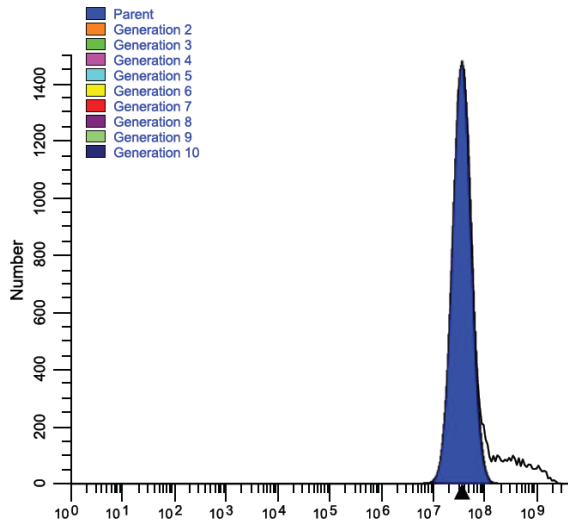


Figure 3. Baseline Fluorescence Obtained on Day 0.

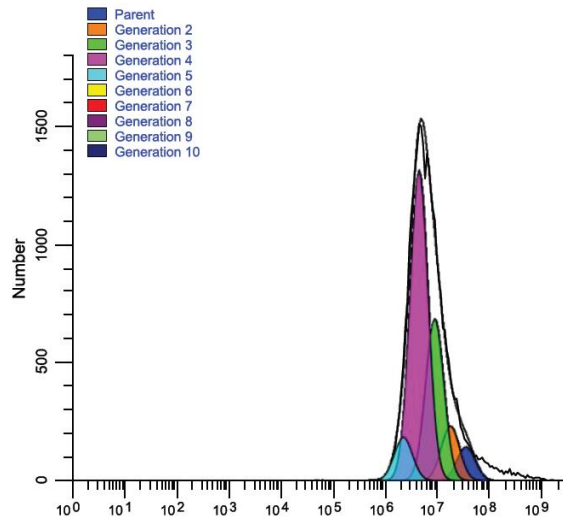


Figure 4. Fluorescence 72 Hours Post-Thaw.

CHAPTER IV

DISCUSSION

Expectations

The results from this study were highly unexpected, though very interesting. Expectations were that one of the conditions would prove to have a higher viability post-thaw, but this was simply not the case. According to the data collected, there appeared to be no significant difference among the conditions. Though this was unexpected, it can have beneficial implications. If further studies support the data, researchers can turn away from the freezing mediums containing FBS, and use a more cost-friendly medium that does not require FBS without sacrificing cell viability and proliferation. This was a residual finding, however, as the purpose of the study was to determine the optimal freezing medium for use *in-vivo*. The only reason conditions with FBS were included was to monitor its effect as a growth factor.

Limitations

One of the limitations to this study is the number of horses that were used. Unfortunately, due to limited resources and time, analysis of only 9 horses was conducted. Additionally, data collection ceased after 72 hours. If this study were to be performed again, data should be taken after 72 hours in order to detect minor differences in the proliferation of the cells between the conditions. By ceasing the data collection at 72 hours, variation in cell proliferation could go undetected if it were to occur after this time. However, the clinical application of such a minor variation (if one was found) would need to be a subject of further research.

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