EVALUATION OF BONE MARROW ASPIRATION TECHNIQUES FROM THE EQUINE STERNUM

AN UNDERGRADUATE RESEARCH SCHOLARS THESIS

by

RACHEL BROOKS

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Research Advisor: Dr. Ashlee Watts

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ABSTRACT

Evaluation of bone marrow aspiration techniques from the equine sternum (May 2013)

Rachel Brooks

Department of

Animal Science

Texas A&M University

Research Professor: Dr. Ashlee E. Watts

Department of

Large Animal Clinical Sciences

Many methods are used in the aspiration of bone marrow from the equine. The two common aspiration sites include the ileum and the sternum. The location used in this study was the equine sternum, where 60mls were withdrawn using two different methods. In the first two syringes, the needle is inserted and 60mls are aspirated without advancements. The second two aspirations are made with an advancement of the needle after every 15 mls of mesenchymal stem cell recovery. While there were no statistically significant conclusions to be drawn in the study, there are strong

trends supporting the theory that multiple advancements of the needle during aspiration yield higher numbers of more viable stem cells.

There is strong support for the theory that multiple advancements (syringe 2) during aspiration will result in higher numbers of more viable mesenchymal stem cells. Syringe 2 cells tended to be more confluent—this was routinely observed in all samples. Initial red blood cell counts also show that syringe 2 does tend to yield a higher number of cells than syringe 1.

The top two plates in each photograph are from syringe 1 of each horse, and the bottom two plates are from syringe 2 of each horse. There appears to be more numerous, denser colonies in the bottom plates. This is especially noticeable in Groups 1, 2, and 4. Group 1 was plated with an original, higher volume for 14 days instead of 10. The colonies were too dense to work with in this cases.

DEDICATION

I dedicate this to my parents, who have pushed me to try new things and supported me throughout all my endeavors—no matter what—always believing in my abilities.

ACKNOWLEDGEMENTS

I would like to thank Dr. Watts for allowing me to take on this project, and for providing me with guidance every step of the way. I would also like to thank Kathy Rector, for being in the lab every day to answer my questions and train me to perform the necessary protocols. Cody Munson also helped calculate dilutions and prepare the first batch of crystal violet.

CHAPTER I

INTRODUCTION

There has been a lot of interest in regenerative medicine in veterinary orthopedics. While muscle, tendon, cartilage, and bone tissue are efficiently built during embryonic development, post-natal healing of these tissues is incomplete. Regenerative medicine improves this tissue repair using techniques that are continually being improved upon.

There has also been a lot of use in bone marrow-derived stem cells. Stem cells are self-renewing and are also particularly useful for treating such issues as joints and tendons. Joint and tendon tissue lack regenerative ability due to a deficiency in stem cell response (Bone marrow-derived stem cell sources compared).

Throughout the stem cell transplantation process, there are procedures with several options for execution. One of these is the optimum procedure for collecting bone marrow. Stem cell therapy is being used more and more frequently and there is a need to lay out a safe, effective, and consistent technique for the reliable recovery of these mesenchymal stem cells.

CHAPTER II

METHODS

Bone marrow will be collected from the sternum of 12 horses under mild sedation. Two separate sites will be used. From the first site, the bone marrow biopsy needle will be placed and the full collection of 60 ml of marrow made without needle advancement. From the second site, the bone marrow biopsy needle will be placed and the needle will be advanced after each 15 ml of marrow is collected to a total of 60 ml. Marrow from each syringe will be plated separately using standard tissue culture techniques. The adherent population of cells will be isolated through aspiration of supernatant every other day. The adherent cells from syringe 1 and syringe 2 will be observed daily, scored for cell morphology and degree of confluence and digitally photographed. At each passage, total cell number will be assessed and recorded and cells will be re-plated at 20,000 cells/cm^2.

Scores and total cell numbers isolated at each stage of culture will be compared between syringe 1 and syringe 2 within each horse through paired non-parametric analysis. There were two different calculation points: initial RBC counts and final colony size after plating.

Plating of red blood cells

30 mls of raw marrow were plated with 30 mls MSC ISO 10%. The MSC ISO was added and the flask was rocked back and forth. 5mls of the 60mls were withdrawn and plated to a 60mm plate. There were four plates per horse: two from syringe one (S1) and two from syringe two (S2). These cells were passaged every 72 hours. During passaging, they were rinsed with 5mls HBSS

and fed with 5 mls of fetal bovine serum. This process was performed under the tissue culture hood in a sterile environment to prevent contamination.

Lysis of red blood cells

Cell counting solution was used to take an initial count of cells after aspiration. First, one tube of cell counting solution A was prepared. This glass tube contained 10microliters of FDA stock solution and 2.5 mls Dulbecco's Phosphate Buffered Saline. Then, one tube of cell counting solution B for each sample to be counted was prepared. Solution A and Solution B were stored in a closed container to be protected from the light. Solution B contained 100 microliters of FDA solution (solution A), 60 microliters of Propidium Iodide, 740 microliters of media, and 100 micr Pellet the 2ml cell sample (300g/5 min, aspirate and discard) and re-suspend in 40mls Tris-NH4Cl working solution (0.1ml packed cells/ml Tris NH4Cl). The solution was then held at room temperature for two minutes and centrifuged again at 300g for 10 minutes. The supernatant was aspirated and discarded. There were still red blood cells in the pellet, so this process was repeated once. 5 mls HBSS with phenol red were added. 100ml of this cell solution from each sample were added to its own solution B tube using a sterile pipette tip each time. This solution B was mixed by pipetting the solution up and down 5 times. The tubes were kept in a closed drawer in a tube rack. 10 microliters of this counting solution B with cells were added to both sides of a hemocytometer. This hemocytometer was immediately brought under the microscope and focused. The dead and live cells were counted on each side of the hemocytometer.

Preparation of crystal violet

PBS, 0.5% Crystal Violet, Methanol, ddH2O were used. First, 0.5% Crystal Violet was prepared. Sigma, 3% in 100% methanol was added to 3 g crystal violet in 100 ml 100% methanol. This solution was filtered with Whatman paper and then stored at room temperature.

Staining MSCs with Crystal Violet

After the 10 day passaging period, the media was aspirated and washed three times with 10mls warm PBS. 5 mls of the crystal violet solution (prepared above) were used to stain each plate for 5-10 minutes at room temperature. The plates were then washed two times each with mqH2O. The plates were then photographed and stored at room temperature.

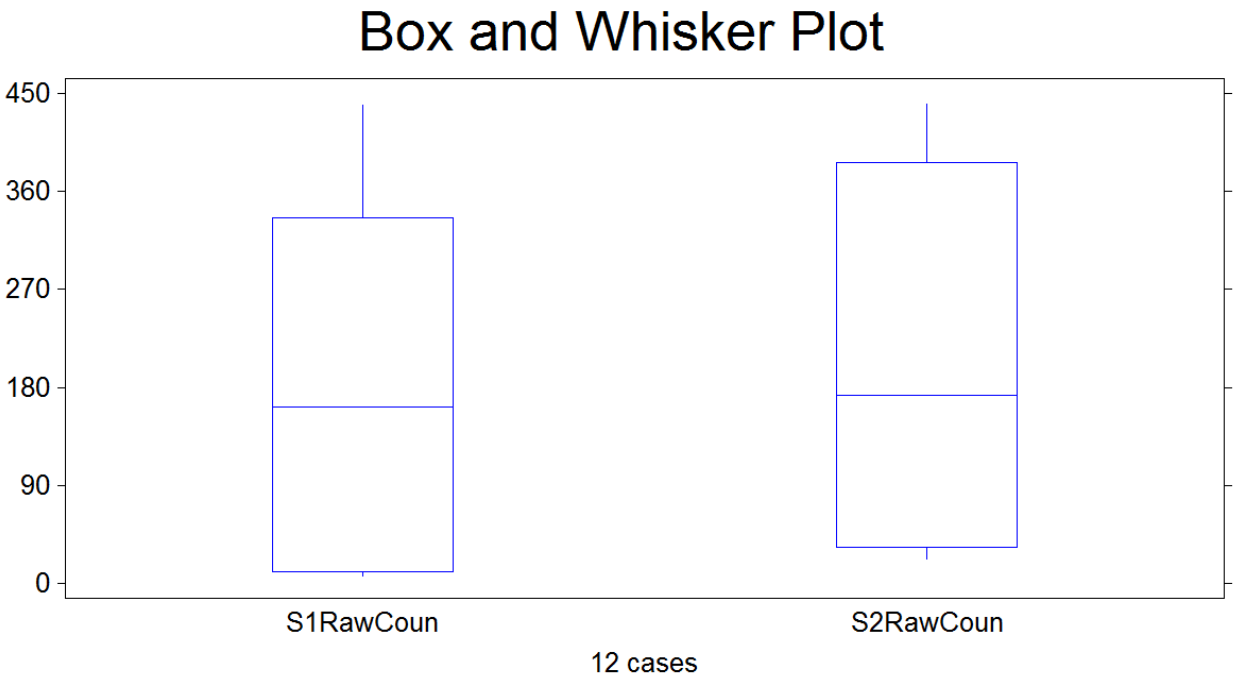
CHAPTER III

RESULTS

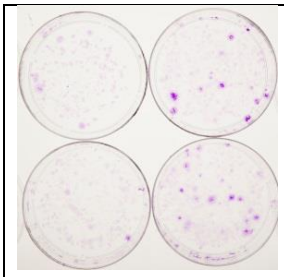
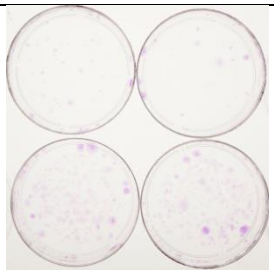
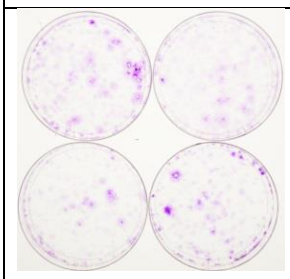
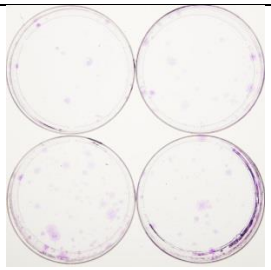
There is strong support for the theory that multiple advancements (syringe 2) during aspiration will result in higher numbers of more viable mesenchymal stem cells. Syringe 2 cells tended to be more confluent—this was routinely observed in all samples.

Initial red blood cell counts show that syringe 2 does tend to yield a higher number of cells than syringe 1 (see figure below).

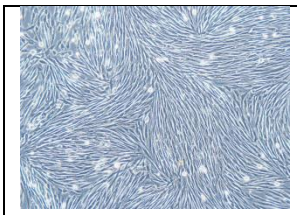
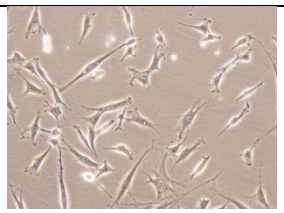
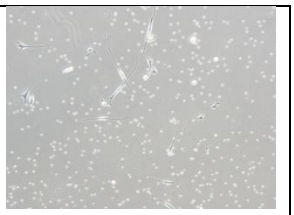
	Raw count and first passage mean	median	Min/max values	p-value
Raw count S1	180.67	162.25	7.000	0.07
Raw count S2	202.42	32.625	22.500	
First passage S1	8.766E+06	343336	338345	0.42
First passage S4	8.766E+06	1.539E+06	956250	

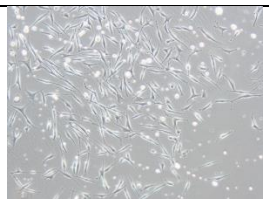
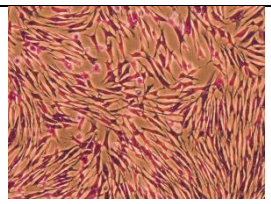

Box and Whisker Plot
450
360
270
180
90
0
S1RawCoun
S2RawCoun
12 cases


Box and Whisker Plot
Axis scaled by 10E7
2.4
1.6
0.8
0.0
S1firstpa
S2firstpa
12 cases

	
Group 1	Group 2
	
Group 3	Group 4

The top two plates in each photograph are from syringe 1 of each horse, and the bottom two plates are from syringe 2 of each horse. There appears to be more numerous, denser colonies in the bottom plates. This is especially noticeable in Groups 1, 2, and 4. Group 1 was plated with an original, higher volume for 14 days instead of 10. The colonies were too dense to work with in this cases.

			
Chill S2 day 10	Carlito S1 12-11-21	Chill S1 96h	Chill S1 120h

			
Chill S1 144h	Friday S2 day 14		

CHAPTER IV

CONCLUSIONS

While there are strong trends in the data, the protocols for this project were not consistent enough to draw any publishable conclusions. The beginning horses were plated at a much higher density and the colonies were too thick to count after the 2 week period. Half-way through the data collection process, the protocol was changed to plate half the initial cell volume for 10 days instead of 14 days.

There is a strong correlation between syringe 2 aspiration techniques and higher cell numbers and more viable cells. This is in accordance with the theory that because the first 5mls extracted from the aspiration site usually yield the highest number of viable mesenchymal stem cells, the second method of aspiration will yield a higher number of stem cells that are also more viable. The first p value (0.0781) for S1 raw and S2 raw count calculated in the *Wilcoxon Signed Rank Test* is not statistically significant, but there is a trend of increased numbers in S2 compared to S1.

REFERENCES

KASASHIMA, Y., UENO, T., TOMITA, A., GOODSHIP, A. E. and SMITH, R. K. W. (2011), Optimisation of bone marrow aspiration from the equine sternum for the safe recovery of mesenchymal stem cells. Equine Veterinary Journal, 43: 288–294. doi: 10.1111/j.2042-3306.2010.00215.x

Can J Vet Res. 2012 Jan;76(1):52-6.

Comparison of bone marrow aspiration at the sternum and the tuber coxae in middle-aged horses.

Delling U, Lindner K, Ribitsch I, Jülke H, Brehm W.

Large Animal Clinic for Surgery, Faculty of Veterinary Medicine, University of Leipzig, An den Tierkliniken 21, 04103 Leipzig, Germany. delling@vetmed.uni-leipzig.de