THESIS

CRYOPRESERVATION OF COOLED SEMEN FOR EQUINE INTRACYTOPLASMIC SPERM INJECTION

Submitted by

Bradford W. Daigneault

Department of Biomedical Sciences

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Spring 2010

COLORADO STATE UNIVERSITY

December 16th, 2009

WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY BRADFORD WILLIAM DAIGNEAULT ENTITLED CRYOPRESERVATION OF COOLED SEMEN FOR EQUINE INTRACYTOPLASMIC SPERM INJECTION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

Committee on Graduate Work

Jason E Bruemmer

James K Graham

David J Denniston

Advisor Elaine M Carnevale

Acting Department Head Thomas R Hansen

ABSTRACT OF THESIS

CRYOPRESERVATION OF COOLED SEMEN FOR EQUINE INTRACTYOPLASMIC SPERM INJECTION

The development of intracytoplasmic sperm injection (ICSI) for use in the horse has resulted in unique needs for semen. Modifications of ways to handle sperm for ICSI-related procedures were investigated through a series of three experiments. In Experiment 1, semen was cooled for 24 h and then frozen to provide a viable population of sperm for ISCI. The objectives of this study were to compare the efficacy of: (1) two extenders for cooling semen prior to freezing and (2) four extenders for freezing cooled semen. Stallion semen was extended in two commercial cooling extenders (CST and INRA96) and cooled for 24 h before freezing in four extenders. Freezing extenders were a modified- French (FR8), Lactose-EDTA (LAC10), glycerol (G), and glycerol egg yolk (GEY). Extenders were added directly to cooled semen and diluted to a final concentration of 20 x 10^6 sperm/mL before freezing. After thawing, sperm were evaluated at 0, 45 and 75 min. Cooling extender did not affect sperm motility after freezing. Total motility and progressive motility was similar ($p>0.05$) for all freezing extenders at 0 and 45 min, but sperm frozen in glycerol egg yolk (GEY) yielded the highest total and progressively motile sperm at 75 min. Differences in motility over time

were not detected except for sperm frozen in glycerol (G) in which total and progressive motility declined from 0 to 75 min. For all extenders, some motile sperm were obtained from semen cooled for 24 h and then frozen.

In Experiment 2, five media were evaluated for holding sperm to determine which best maintained the motility of stallion sperm over time. The experiment was designed to determine appropriate media in which to handle sperm for ICSI selection procedures. The media compared were: 1) GIVF, 2) FDCM, 3) TALP, 4) Emcare (EM) and 5) Mare Mojo (MM). The only media designed for holding sperm was TALP; the remaining media were formulated as holding media for oocytes and embryos. Motility of sperm was analyzed every hour for 5 h. No differences in total or progressive motility were detected among groups until 5 h. At 5 h, sperm held in TALP had higher ($p<0.05$) total and progressive motility than all other groups. Sperm can be held in any of the five media evaluated and remain motile for ICSI. However, TALP resulted in the best sperm motility over time.

Experiment 3 was performed to evaluate a hyaluronic acid (HA) binding assay for the selection of sperm for ICSI. Sperm binding to hyaluronic acid has been characterized for many species. Sperm that bind to HA have been shown to exhibit more normal chromosomal structures and improve fertilization. The objective of Experiment 3 was to determine if fresh and frozen stallion sperm bind to HA when introduced to premanufactured hyaluronic acid binding kits (HBA, Biocoat Inc., Horsham, PA). Fresh semen pooled from two stallions exhibited approximately 15% of sperm bound to HA. Four frozen samples from Experiment 1 were thawed and pooled. Frozen sperm did not bind to the assays. For fresh and frozen samples, sperm displayed some hyperactivation,

uncharacteristic circling, and tails bent at the mid-piece. Sperm were observed to agglutinate in congregations of fifty or more sperm. Hyaluronic acid seemed to have an effect on stallion sperm that has not been described for other species. Further investigation of HA binding for selection of sperm for ICSI is warranted.

Bradford W. Daigneault
Department of Biomedical Sciences
Colorado State University
Fort Collins, Colorado 80523
Spring 2010

ACKNOWLEDGMENTS

The work that I have accomplished herein would not have been possible without the advising and friendship of the many people that I have encountered over two years at this laboratory. My gratitude is extended toward my advisor Dr. Elaine Carnevale and my committee members Dr. Jason Bruemmer, Dr. James K. Graham, and Dr. David J. Denniston. I wish to thank all those people who have helped me through my work, including those who may not have played a direct role in research, but rather supported me through friendship and advice.

This journey would not have been a success without prior preparation from years past. I wish to formally thank coaches, friends, immediate and extended family, as well as employers who gave me the chance to succeed through their generous and unselfish gifts that helped me to grow as a person.

Finally, I owe all of my accomplishments to my wife Sarah, who has persevered with me for over six years through the good and the bad as I worked to further my education. I have only succeeded through her selfless support and undying love.

TABLE OF CONTENTS

FIGURES AND TABLES

TABLE OF APPENDICES

CHAPTER I

REVIEW OF LITERATURE

Introduction

Intracytoplasmic sperm injection (ICSI) is an assisted reproductive technology that has gained a great amount of success in recent years when applied to the mare. The use of ICSI in the horse has resulted in oocyte cleavage rates of 60% and blastocyst development of up to 30% (Squires et al. 2003). When compared to other assisted reproductive procedures, ICSI provides the most conservative use of sperm (Carnevale 2008). The frequency of using frozen sperm for ICSI is as high as 85% (Carnevale 2008). Therefore, improving upon the efficiency of using frozen-thawed sperm for ICSI is an important objective. A means of processing shipped, cooled semen for freezing would provide a more cost-effective method for obtaining sperm for clinical programs.

Methods to process and cryopreserve shipped, cooled semen for use in an equine ICSI program have yet to be established. The processing and packaging of cooled semen for freezing at low concentrations could provide for extended use of limited amounts of semen. Packaging semen by means of individually segregated portions will allow for utilization of smaller amounts of semen without liquid nitrogen exposure to the unused portion. Methods developed to accomplish these goals will provide a more efficient means of utilizing equine semen for ICSI.

Determining which freezing extender will provide the highest post-thaw motility for individual stallions is difficult unless test freezes are performed. Although performing a test freeze is beneficial for freezing semen from stallions with unknown freezing potential, a test freeze may be unrealistic when considering semen that has been cooled and shipped unless performed at a very low concentration.

Cooled Semen

Improved methods for processing cooled semen to ensure maximal survival and motility are necessary in the equine industry. Semen used for insemination at a location distant to the stallion is often shipped in a passive cooling device that cools semen to 5°C. Semen can be held for a period of 24 to 48 h while maintaining acceptable motility for most stallions (Jasko et al. 1992). Cooled semen for insemination is commonly shipped at concentrations of 50 x 10^6sperm/mL (Crockett et al. 2001).

One effect of cooling semen to 5°C is a change in sperm membranes from a liquid-crystalline state to a gel state (Squires et. al 1999). Byproducts of metabolism such as CO_2 and lactic acid can cause permanent damage to sperm if they remain at room temperature. The occurrence of peroxidation of lipid membranes, or oxidation of lipids in the cell membrane, can lead to membrane damage and reduced fertilization capability after a period of 12 to 24 h (Squires et al. 1999). Every 10°C reduction in temperature of sperm results in a 50% decrease in metabolic activity. Consequently, semen held at 5°C metabolize at about 10% of what they would when stored at 38°C, the normal body temperature of a horse (Squires et al. 1999). Due to a decrease in metabolic activity, the lifespan of sperm can be lengthened. Decrease in metabolism allows storage of sperm for

a prolonged period of time when cooled at a controlled rate in a medium that has membrane-protective properties (Squires et al. 1999).

The viability of cooled stallion sperm for future use is stallion dependent (Aurich 2008). Not all stallion semen can be cooled and preserved to provide acceptable parameters for insemination (Brinsko et al. 2000). Quality of semen, composition of seminal plasma and integrity of plasma membranes are contributing factors influencing the ability to maintain cooled semen (Aurich 2005). The sensitivity of individual stallions' semen to cooling may render the use of cooled semen from individual stallions unrealistic for insemination regardless of cooling extender (Aurich 2008). However, enough sperm cooled from these stallions may remain viable for ICSI.

Cooling Extenders

Cooling extenders provide nutrients, a balance of minerals, and ensure proper cellular osmotic pressure. Extenders help to neutralize byproducts of metabolism that can be toxic, buffer temperature changes, and help to stabilize enzyme systems as well as membrane integrity (Squires et al. 1999). The addition of a cooling extender to semen can also be a useful means of introducing antibiotics.

The most common semen extenders include milk, milk by-products, and buffers to help regulate pH and osmolarity. In 1975, Kenney et al. reported the successful use of dried skim milk extender for stallion sperm. Subsequent additives to stallion sperm have attempted to investigate the efficacy of additives to cooling extenders for improving fertility and survivability. The addition of glutamine to INRA82 cooling extender significantly increased motility compared to samples without glutamine (49% versus

46%) but did not alter fertility (Vidament et al. 2001). Bedford et al. (1994) noted a detrimental effect on progressively motile sperm when egg yolk was added to semen containing seminal plasma before cooling. Upon removal of seminal plasma, the addition of egg yolk or glycerol added to a nonfat dried skim milk-glucose (NDSMG) extender did not depress motility of sperm but did negatively affect fertility. Although many additives have been applied to cooling extenders, there has yet to be defined a superior cooling extender that works for all stallions.

Milk or milk-based extenders continue to be commonly used for diluting and storing stallion semen. Milk contains more than 100,000 complex molecules. These molecules have been speculated to be both beneficial and deleterious to sperm (Battelier et al. 2001). Milk can be fractionized by microfiltration, ultrafiltration, and freeze-drying which allows for purification of fractions. The equine cooling extender INRA96 was developed based on this science (Battelier et al. 2001).

INRA96 is supplemented with native phosphocaseinate (NPCC) and formulated based on Hanks' salts, a collective group of salts with bicarbonate ions that helps to buffer pH (Battelier et al. 2001). Native phosphocaseinate is a milk fraction composed of micellar caesins and has been found to be protective to stallion sperm along with β-lactoglobuline, a major soluble protein. The protein, α-lactoglobuline, has been identified as harmful to sperm and has been removed from some sperm diluents (Battelier et al. 1997). Native phosphocaseinate was reported to be the most efficient fraction at 15°C in aerobic conditions when compared to milk-based extenders held at 4°C for maintaining sperm motility (Battelier et al. 1997). These findings ultimately led to the development of INRA96, often referred to as a chemically-defined extender. Sperm held at 15°C in

INRA96 extender in aerobic conditions resulted in higher motility than milk-based extender. However, sperm held at 4°C in anaerobic conditions with a milk-based extender was superior to INRA96 at the same conditions when evaluated for motility after 4 days of storage (Battelier et al. 1997). Although sperm can be cooled and maintained for several days, motility of sperm could be improved upon with a shorter cooling time such as 24 h.

Cold Shock for Cooled and Frozen Semen

Methods to prevent cold shock, or cooling stress, reduce cellular damage and ensure post-thaw survivability during the cooling process. Cooling semen at a rapid rate can cause membranes to rupture, resulting in permanent cellular damage that can be observed after cooling and thawing (Squires et al. 1999). Abnormal sperm motility patterns can include circling, backward motility, and loss of motility. Acrosomal and plasma membrane damage as well as the absence of cellular components can also be seen with sperm subjected to cold shock (Squires et al. 1999). Lipids and lipophilic molecules are additives that help to protect the plasma membrane during the transition from the fluid phase to the solid phase during cooling. Ethylenediaminetetraacetic acid (EDTA) is utilized to bind calcium, magnesium, and other ions which likely function to stabilize ion diffusion across membranes during transition phases (Squires et. al 1999). Egg yolk is commonly used as a lipid additive in semen extenders to aid in the protection of the lipid membrane. Phospholipids, such as low-density lipoproteins present in egg yolk, aid in maintaining the sperm membrane during cooling. Lipoproteins in skim-milk extenders are commonly used to prevent cold shock and are thought to function similary to the

lipoproteins found in egg yolk (Squires et al. 1999). The phospholipids that are abundant in milk and egg yolk have been proposed to produce structural modifications to cell membranes that could help to prevent temperature-related damage (Varner et al. 1987). However, the amount of energy that would be necessary for lipid exchange is unlikely present (John Parrish; personal communication). Caseins, that are present in milk and have been purified for use in the cooling extender INRA96 (IMV Technologies, Normandy, France), have been shown to bind strongly with Ca^{++}. It has been postulated that they prevent accumulation of intracellular Ca^{++} that can be toxic to sperm at high concentrations (Quinn and White 1967).

Cooling Rates

Different species have varying responses to cooling rates for sperm. The response of sperm to cold shock affected by cooling rate may be attributed to differences in plasma membrane compositions (Varner et al. 1987). Plasma membranes characterized by high levels of cholesterol, or an increased ratio of saturated to unsaturated phospholipids, are better able to withstand cold shock (Varner et al. 1987). The use of passive cooling devices for storage and transport of semen has been studied in the stallion to address these concerns.

Cooled stallion sperm survive best when stored at a temperature range of 4 to 6°C (Moran et al. 1992). Membrane damage of stallion sperm is often associated with improper cooling rates. During cooling, stallion sperm are susceptible to cold shock between temperatures of 24 to 4°C (Moran et. al 1992). Stallion sperm can be cooled rapidly (-7°C/minute) to 19°C without increased cold shock damage when compared to

slow cooling (Moran et al. 1992). Rapid cooling did not induce cold shock when sperm reached a temperature of 8°C (Moran et al. 1992). The hypothesis for the above experiment was that slow cooling at a rate of -0.05°C/min would allow adequate time for membrane reconstruction, which has presumably taken place during this phase transition stage (Moran et al. 1992). Stallion sperm are most susceptible to cold shock induced by rapid cooling from 19 to 8°C. Sperm should be cooled at a slow cooling rate during this time interval (Moran et al. 1992).

Freezing Extenders

Stallion sperm was first frozen in 1950 (Smith and Polge 1950). The first equine pregnancy from frozen sperm was reported in 1957 (Barker and Gandier 1957). Glycerol is the most common cryoprotectant used for freezing sperm in a number of species, including the horse. The concentration of glycerol observed in stallion freezing extenders ranges from 2.5 to 6% (Vidament et al. 2001). However, glycerol toxicity could be a reason for variations observed with individual stallion freezability (Alvarenga et al. 2005). Furthermore, glycerol has been shown to have contraceptive effects for some species. Although glycerol functions as a moderator of cellular osmolarity, it can also cause damage to sperm that has already been compromised during the freezing process.

Protein denaturation, changes in actin interactions, and changes in cytoplasmic properties are a few byproducts of glycerol that can induce damage to cells (Alvarenga et al. 2005). Due in part to these findings and the need to increase the success of freezing stallion sperm, alternative cryoprotectants, such as amides, have been investigated and

utilized in a commercial industry (Alvarenga et al. 2005). These alternative cryoprotectants have a lower molecular weight and greater membrane permeability than glycerol, which help to minimize osmotic damage to sperm (Scherzer et al. 2009).

Scherzer et al. (2009) designed a randomized 2 x 3 block experiment to compare differences in motility when using a skim-milk glucose extender (EZ Mixin Original Formula) or a milk-free extender (INRA96). These extenders were used in combination with 2, 3 or 4% glycerol for freezing. There was a difference between the two extenders in motility of sperm after cryopreservation, with INRA96 being superior. When glycerol was added at a concentration of 4%, the highest post-thaw motility was observed. The addition of 4% glycerol was significantly different than 2% glycerol. In conclusion, INRA96 when combined with 4% glycerol provided the highest average post-thaw motility (Scherzer et al. 2009). However, sperm for this experiment was cooled for 1 h to 4°C and then centrifuged at 4°C to remove seminal plasma prior to freezing. This method is not common practice when cooling stallion sperm for commercial purposes. Motility may be improved by centrifuging semen at room temperature rather than after cooling. The alternative method has been shown to be deleterious to sperm (Backman et al. 2004).

Although egg yolk has been added to extenders at concentrations ranging from 2 to 20%, the optimal concentration has not been clearly defined for a number of freezing extenders (Vidament et al. 2001). An additional class of additives shown to have positive effects on improving post-thaw motility is the addition of amino acids. Glutamine is an example of an amino acid that improves frozen semen parameters across a variety of species including the stallion (Vidament et al. 2001).

Cooling Rates for Freezing Sperm

The use of a cryoprotectant is essential to ensure survivability when freezing stallion sperm, as is the rate of temperature decline at which semen is cooled. In order to fully understand the effects of freezing on stallion sperm, measuring the biophysical response, water transport, and intracellular ice formation in sperm during freezing must be addressed (Devireddy et al. 2002).

The addition of cryoprotectant helps to ensure that ice nucleation is formed in the extracellular space. A cryoprotectant, such as glycerol, creates an osmotic gradient across the plasma membrane to draw water out of intracellular solution that was initially isotonic. A fast or slow cooling rate can determine if intracellular water will move across the cell membrane to become part of the extracellular ice phase, or if it will remain inside the cell and freeze as ice crystals (Devireddy et al. 2002).

There are differences in membrane permeability between cell types. Intracellular ice formation is dependent on different cooling rates between cells and media. Therefore, knowing the cell membrane's permeability to water in the presence of extracellular ice and cryoprotectants is essential (Devireddy et al. 2002). Generally, cells that undergo intracellular ice formation become osmotically inactive or lysed due to the loss of cell membrane integrity (Mazur 1984). The opposite occurs, but with the same effect, when too much water leaves the cell because of exposure to high solute concentrations (Lovelock 1953). Therefore, the cooling rate must be optimized to ensure cell survival.

The best cooling rate to optimize the freeze/thaw results of any cellular system has been defined as the fastest cooling rate in a given medium that precludes the formation of damaging intracellular ice (Mazur et al. 1972). Stallion sperm viability

remains constant when cooled at a rate between 20°C/min and 130°C/min (Devireddy et al. 2002). If cooling is too rapid, cells freeze intracellularly because an insufficient amount of water has left the cell (Mazur 1984). In contrast, slow freezing can cause ice to form outside the cells, and the remaining unfrozen medium forms channels that shrink in size and consequently increase in solute concentration. The cells trapped inside these channels also begin to shrink due to an osmotic response elicited by the high concentration of solute in the gradient. Ultimately, these changes can cause harm to the cells (Mazur 1984).

Semen cooled for transport is not cooled below 4°C; because between -5°C and -15°C, ice begins to form in the external holding medium. The cell contents are not frozen, but they are supercooled due to the protective properties of the plasma membrane (Mazur 1984). Changes elicited in sperm depend on the velocity of cooling. When subjected to a sufficient slow cooling process, the cells can adequately remove enough water to maintain equilibrium between intracellular and extracellular water. The cell dehydrates and does not freeze intracellularly. However, if the cell is cooled too fast, it becomes increasingly supercooled, and the only way to obtain equilibrium is by freezing intracellulary because water is not able to leave the cell at a fast enough rate (Mazur 1984). Consequently, similar results can be observed when thawing semen. Slowly frozen sperm cells are sensitive to rapid thawing due to osmotic shock. Solute loading into the cell during slow cooling may not have enough time to diffuse out if a cell is subjected to a rapid thaw, causing the cell to swell and ultimately lyse (Mazur 1984).

A common misconception is that cells are most challenged when stored at low temperatures. An intermediate zone of approximately -15°C to -60°C has the most

potential lethality, because cells must pass through that zone twice (Mazur 1984). Sperm are commonly stored in liquid nitrogen at -196°C, because liquid water does not exist below -130°C. Only crystalline or glassy states exist below this temperature in which the viscosity is too high for diffusion. Sperm is protected indefinitely when stored in liquid nitrogen. At -196°C, an insufficient amount of thermal energy exists for chemical reactions. The only reactions that occur at such a temperature are photophysical reactions. These reactions are breaks in macromolecules, which are caused by ionizing radiation or cosmic rays. Such reactions could ultimately cause enough breaks in DNA to be detrimental to sperm after thawing (Mazur 1984).

The properties of cell membranes play critical roles during the freezing process. As previously discussed, the structure of the surface membrane functions to protect the cell during the cooling process and allows it to supercool. This membrane most likely determines the ice-nucleation temperature for a particular cell. These two major components ultimately determine if cells will equilibrate with extracellular medium via dehydration or by intracellular freezing (Mazur 1984). Membranes on the surface of cells as well as the interior are main subjects to injury. Furthermore, membranes allow permeability to the cell allowing access to protective solutes during the freezing process. Depending on the cell type, most cryoprotectants and solutes are low in molecular weight, allowing permeabilization into the cell. If additives are not able to permeabilize, they have the potential to induce osmotic stress. Lastly, damage caused to intracellular organelles such as mitochondria and microtubules are detrimental to cell survivability (Mazur 1984).

Seminal Plasma

Seminal plasma has both detrimental and beneficial effects on frozen stallion semen (Jasko et al. 1991, Dawson et al. 1999). Several studies have been conducted to investigate the significance of removing seminal plasma from semen and the concentrations of seminal plasma that can be added back to sperm for freezing. Seminal plasma must be diluted to obtain maximum sperm motility and survivability after cooling. When seminal plasma is removed, egg yolk in an extender is beneficial when cooling for 24 h (Bedford et al. 1995). Similar results were obtained when egg yolk extenders were compared to skim milk for a 24 h cooling period (Jasko et al. 1992). Completely removing seminal plasma before cooling has been shown to be detrimental. A concentration of 5 to 10% seminal plasma in semen maintains sperm motility and acrosome integrity (Jasko et al. 1991, Dawson et al. 1999).

The speed, timing, and temperature of sperm during centrifugation have been investigated. When semen is cooled before freezing, optimal survival is achieved by centrifuging semen upon collection before cooling to 5°C at 200 x 10^6sperm/mL (Backman et al. 2004). Sperm processed in this manner without the addition of glycerol for 18 h before freezing provided the best results regarding post-thaw motility of previously cooled sperm (Backman et al. 2004). Also, sperm survived better when frozen at 200 rather than 400 x 10^6sperm/mL (Backman et al. 2004).

Semen should not be centrifuged after cooling (Backman et al. 2004). Warming semen after cooling and centrifuging before freezing has been shown to result in detrimental effects on motility of frozen sperm (Backman et al. 2004). Low-level forces

of centrifugation (370 to 829 x g) are necessary to avoid detrimental effects on equine sperm with the presence of 10% seminal plasma (Pickett et al. 1975).

Freezing stallion sperm that has been previously cooled can be accomplished (Crockett et al. 2001). An appropriate extender for cooling, packaging time, removal of seminal plasma, concentration and length of time after collection are described (Crockett et al. 2001). Semen was first centrifuged at 300 x g for 8 min then cooled to 5°C. The sample was held for 24 h in a skim milk extender before cryopreservation. Post-thaw motilities were similar for skim milk-egg yolk-sugar extender and skim milk-egg yolk-salt extender (Crockett et al. 2001). All extenders contained 10% glycerol. Progressive motilities averaged 10% and were considered unacceptable for insemination. The overall conclusions for this experiment were: semen could not be held at 5°C for 24 h prior to freezing; the addition of egg yolk aided post-thaw motility; and packaging cooled samples into 0.25-mL versus 0.5-mL straws did not change motility (Crockett et al. 2001). Furthermore, freezing semen that was centrifuged before cooling resulted in improved motility when compared to centrifuging after cooling (Crockett et al. 2001). Similar results have been obtained by other researchers (Backman et al. 2004). Based on the above-described experiments, conventional methods exist for shipping cooled semen for the purpose of freezing.

Optimal concentrations for freezing stallion sperm combined with the absence or presence of seminal plasma have been described (Crockett et al. 2001). The effects on sperm cooled to 5°C for 12 h at three different concentrations followed by freezing were examined. Egg yolk helped to maintain sperm motility after freezing and thawing and stallion sperm should be centrifuged to remove seminal plasma at 25°C. Sperm should

be resuspended to 50 x 10^6sperm/mL or 250 x 10^6/mL for shipping up to 12h. If sperm are to be stored for more than 2.5 h before freezing, they must be centrifuged to remove seminal plasma at 25°C (Crockett et al. 2001). This information provides insight to diluting shipped, cooled semen into small aliquots to be frozen for later use in an ICSI program.

Processing Sperm for ICSI

Methods of processing sperm for ICSI have been explored with fresh, cooled, and frozen equine sperm. Sperm have been thawed and refrozen at low concentrations to help preserve limited amounts of sperm (Choi et al. 2005). Choi and colleagues thawed frozen sperm from a fertile stallion and diluted the sample 1:100 (EZ Mixin ^{TM} MFR5 and LE; Animal Reproduction Systems, Chino CA). Sperm were refrozen in 0.5-mL straws at a final concentration of 2 million sperm/mL. Refrozen sperm diluted with both extenders provided motile sperm that could fertilize oocytes using intracytoplasmic sperm injection. Thawed and refrozen samples did not contain enough sperm for a swim-up; therefore, the samples were washed, centrifuged, and resuspended in the remaining medium after aspiration of the supernatant. Motile sperm were then placed on top of Chatot-Ziomek-Bavister medium modified for sperm (Sp-CZB) containing 10% polyvinylpyrrolidone (PVP). This method allowed sperm that swam into the droplet to be visualized and selected for ICSI. The dilution of semen to 2 million sperm/mL allowed harvesting of sufficient numbers of motile, functional sperm for ICSI. The same results were obtained for both a fertile and subfertile stallion, albeit the subfertile stallion had sperm with 3% total motility after thawing (Choi et al. 2005). This data is

convincing evidence that sperm may be diluted to low concentrations and cryopreserved to provide adequate numbers of motile sperm for ICSI.

Frozen sperm have been prepared for ICSI using swim-up procedures (Altermatt et al. 2009). The following method describes utilization of a minimal number of sperm frozen in a 0.5-mL straw by cutting a portion (~5mm) of the straw under liquid nitrogen. This technique preserves the remaining portion of the straw (Altermatt et al. 2009). The cut portion of the straw is introduced into a round-bottom tube containing 1 mL of the holding medium F-CDM (CDM (Olsen and Seidel 2000) supplemented with 0.5% fatty acid-free bovine serum albumin 2 mM caffeine and 2μg mL^{-1} heparin). The tube is placed into incubation at 38.5°C at a 45° angle for 20 min in 6% CO_2 and air. A total of 500 μl of supernatant is aspirated from the top of the tube and placed into 2 mL of pre-equilibrated F-CDM for centrifugation at 300 x g for 5 min at room temperature. Following centrifugation, 1μl of sperm pellet is placed in 5μl of G-MOPS (Vitrolife AB, Goteborg, Sweden) containing 0.5% bovine serum albumin and 5% polyvinylpyrrolidone. Sperm is then selected for injection based on morphology and motility characteristics (Altermatt et al. 2009).

The use of a Percoll gradient to select frozen sperm for ICSI has been successfully employed in the horse (Lazzari et al. 2002). The protocol includes thawing frozen sperm 1 hr prior to injection followed by separation on a 45-90% Percoll gradient. The sperm pellet is diluted to a concentration of 4 million sperm/mL and then 1:1 with a 12% PVP solution in synthetic oviductal fluid. The medium is supplemented with BSA, amino acids, heparin and penicillamine, hypotaurine, and epinephrine. One motile sperm is then selected in PVP medium after immobilization by Piezo pulses (Lazzari et al. 2002).

The description of methods to freeze semen for ICSI from taxonomic species related to the horse has potential application. Such information has been described for the rhesus monkey (Yeoman et al. 2005). Semen was collected via penile electroejaculation from nine rhesus monkeys and subjected to four freezing treatments. Prior to freezing, semen was allowed to liquefy for 10-15 min followed by two washes in HEPES-buffered TALP containing 0.3% BSA. The four treatments consisted of: 1) extension in a medium consisting of 6 % glycerol and 0.1 M sucrose followed by cooling to 4°C for 90 min and floating over liquid nitrogen (LN_2), 2) dilution in a 30% egg yolk, 20% skim milk, 0.06 M glucose medium and 3% glycerol followed by 60 min equilibration at 4°C and frozen in LN_2, 3) same as treatment two with the addition of a post-thaw density gradient centrifugation using Percoll, 4) modified freezing protocol using dry ice before plunging into LN_2 for storage. Reduced fertilization unrelated to post-thaw motility was observed when sperm was frozen in LN_2 vapors; however, blastocyst development was not different among treatments (Yeoman et al. 2005). Freezing sperm in drops on dry ice resulted in similar fertility rates to fresh semen. The slower cooling rates of -60 to -85°C/min, compared to LN_2 vapor of -150°C/min is attributed to the different success rates (Yeoman et al. 2005). A total of 23 pregnancies have been achieved to date using this method of cryopreservation (Yeoman et al. 2005).

The acrosomal status of fresh sperm is thought to affect fertilization rates achieved by ICSI (Mansour et al. 2008). Increasing the incubation time of human sperm perhaps increases the rate of acrosome reaction, thus increasing fertilization rates (Mansour et al. 2008). Acrosome reacted sperm may have decreased sperm chromatin decondensation rates (Mansour et al. 2008). When acrosome reaction is induced,

electron microscopy shows the rate of acrosome reaction is time dependent (Mansour et al. 2008). Fertilization achieved by ICSI is most successful when sperm are incubated in tissue culture medium for 3 hours (Mansour et al. 2008).

Fertilization Capability of Frozen Sperm for ICSI

Changes that could affect the ability of a frozen-thawed sperm to fertilize an oocyte after ICSI are not well defined and are theoretical in nature. Exempting physical and cellular changes that have already been described, specific fertilization factors could be impacted during the freezing process. Certain sperm-borne, oocyte-activating factors that are generally insoluble may be compromised during freezing and thawing (Yeoman et al. 2005).

Equine oocytes in metaphase II of meiotic arrest require a number of factors to initiate fertilization. These factors include a decrease in maintenance of maturation promotion factor (MPF) composed of a complex of $p34^{cd2}$ kinase and cyclin B (Meng and Wolf 1997). The entry or injection of a sperm into the oocyte results in cyclin B degredation and a decrease in MPF activity allowing the oocyte to activate (Yeng and Wolf 1997). Sperm-induced changes in intracellular calcium concentrations function to initiate oocyte activation (Meng and Wolf 1997). Sperm-induced calcium changes in the oocyte could be initiated when sperm bind to the oocyte membrane receptor, resulting in G-protein and phospholipase C activation and the production of inositol 1,4,5-triphosphate, a calcium-releasing agent (Meng and Wolf 1997).

A second hypothesis that may be relative to ICSI with frozen sperm involves a soluble cytostolic factor, oscillin, which has been characterized in several mammalian

species (Meng and Wolf 1997). The protein, oscillin, has been shown to induce Ca^{2+} oscillations that could aid in oocyte activation (Meng and Wolf 1997). The loss of structural integrity to oscillin during freezing and thawing of sperm could implicate fertilization.

Incomplete or premature chromosome decondensation of sperm contribute to possible failure of oocyte activation (Sousa and Tesarik 1994). Although ICSI circumvents oocyte activation via the sperm-oocyte binding process, micromanipulation of the sperm and the physical act of injection into the oocyte are thought to aid in the release of sperm factors contributing to oocyte activation and fertilization (Yeoman et al. 2005).

Freezing and thawing of sperm could reduce chromosomal compaction of sperm, possibly contributing to fertilization failure (Hammadeh et al. 1999). Fertilization can still be achieved after intentional manipulation of protamines reducing chromosomal compaction (Ahmadi and Ng 1999). Sperm exposed to varying levels of gamma radiation prior to insemination in a mare were verified for DNA-breakage via a TUNNEL assay. No significant differences were detected for fertilization rates between the control group and those exposed to varying degrees of radiation. However, blastocyst development was 50% in the control group and 20% for sperm exposed to gamma radiation at the lowest treatment level. Like correlations for implantation and fetal development were observed (Ahmadi and Ng 1999). The study demonstrated that DNA-damaged sperm have the ability to fertilize oocytes, but embryonic development is highly related to the degree of sperm DNA damage. These findings attribute to the ability of the oocyte to repair DNA of sperm with less than 8 % damage (Ahmadi and Ng 1999).

HspA2 and Hyaluronic Acid Binding Glycoprotein

The testis-expressed heat shock protein chaperone (HspA2) is a 70 kDa protein formerly known as creatine kinase M isoform. This protein can be used as a measure of sperm cellular maturity, function and fertility in humans. The testis-expressed heat shock protein is expressed in elongating spermatids and is associated with the formation of hyaluronic acid (HA) and zona pellucida binding sites as well as plasma membrane remodeling. When the HA receptor of mature human sperm are introduced to HA-coated slides and/or Petri dishes, microscopic observation reveals sperm that are bound to HA, thus providing for selection of mature sperm that can be selected for use in ICSI. HA-selected sperm for ICSI exhibited a 4 to 6-fold reduction in the frequency of chromosomal disomy and diploidy compared to sperm not selected using this method (Huszar 2007).

The testis-expressed heat shock protein exhibits a two-wave expression. The first wave is seen in spermatocytes as a meiotic component within the synaptonemal complex. The second wave is exhibited during terminal spermiogenesis in elongating spermatids (Huszar et al., 2000). In human and stallion sperm, all CK and HspA2-related maturational events are completed in sperm located in the caput epididymis (Huszar et al., 1998b). Elongated spermatids that express a substantial amount of HspA2 exhibit fully formed binding sites for hyaluronic acid (Huszar 2007). These sperm are indicative of cells that have undergone a normal maturation process and are capable of fertilizing an oocyte.

The presence of a cell surface hyaluronic acid binding glycoprotein (HABP) in sperm was identified for several species including the rat, mice, bull, and human

(Ranganathan et al. 1994). The protein was found to be present on the head, midpiece, and tail depending upon the species. These proteins function as receptors that bind to the surface of cells. The HA-binding protein described has been shown to specifically bind to hyaluronan (Ranganathan et al. 1994). The localization of HA-binding protein on the sperm surface was detected by indirect immunofluorescence using anti-HA-binding protein antibodies. The protein was found to be present on the head and tail of rat sperm and less prominent on the midpiece. Human sperm were found to have varying intensities of staining, differing from sperm to sperm from the same ejaculate, with relatively more intense staining on the midpiece of human as compared to the rat sperm. Bull sperm had similar staining patterns as rat sperm, described as head and tail staining (Ranganathan et al. 1994).

Similarly, stallion sperm express a surface-associated hyaluronidase that can be found on the posterior head of ejaculated sperm (Meyers 2001). The protein is similar to proteins that have been characterized in other species and is known as PH-20 (Meyers 2001). Glycerolphosphatidylinositol-linked hyaluronidase (PH-20) translocates during final epididymal maturation in the stallion (Meyers 2001). When the acrosome of stallion sperm is exocytosed, the inner acrosomal membrane fluoresces when hyaluronidase is stained. The author suggested that hyaluronidase plays a role in zona penetration by sperm (Meyers 2001). Although stallion sperm have not been shown to bind in vitro to hyaluronic acid assays, post-thaw motility is increased when hyaluronan is added to cooled semen before freezing (Bruemmer et al. 2009). The characterization and localization of HA receptors on stallion sperm coupled with the positive effects of hyaluronan warrant further investigation as possible methods of sperm selection for ICSI.

The testis-expressed heat shock protein has a high ATP content (Huszar et al. 2000). Associated with HspA2 in sperm is B-type creatine kinase (CK) that is found in significantly higher content in sperm of subfertile men (Huszar et al. 1998a,b and Cayli et al. 2003a). High sperm CK activity was directly correlated with increased cytoplasmic protein and CK concentrations, which indicate the amount of cytoplasmic retention in immature sperm (Huszar and Vigue 1992). The finding suggested a defect in sperm development during the latter parts of spermiogenesis, at which time excessive cytoplasm is commonly extruded from elongating spermatids (Clermont 1963). The HspA2 ratio (formerly known as the CK-M ratio) reflects the proportion of mature to immature sperm within a semen sample. Furthermore, proportions of mature and immature sperm, regardless of normozoospermic or oligozoospermic producing men, have resulted in day-to-day and man-to-man variations between semen samples (Huszar et al. 1988b).

The content of HspA2 in sperm associated with zona-binding capabilities has helped to demonstrate the importance of mature sperm for fertilization. Sperm bound to the zona pellucida of halved unfertilized human oocytes did not retain cytoplasm. Immature sperm exhibiting retained cytoplasm and low expression of HspA2 were deficient in the zona-binding site (Huszar et al. 1994).

Due to the above findings, investigation of a plasma membrane remodeling step followed. Remodeling of the plasma membrane occurs only during spermiogenesis. The enzyme B1,2-galactosyltrasferase is present only in the plasma membrane. The membrane density of B1,2-galactosyltransferase and cytoplasmic concentrations of HsPA2 or CK were significantly related (Huszar et al. 1997). Further analysis concluded that the formation of the hyaluronic acid (HA) binding site also occurs at this time,

providing a stepping-stone for fertility testing and ICSI sperm selection in men (Huszar 2007).

The sperm-hyaluronan-binding assay (HBA) uses the hylauronidase (HA) receptor present on the intact acrosome of the sperm membrane surface to test for HA binding ability (Hong et al. 2006). When sperm are placed in 10-ul droplets into the well of pre-manufactured HBA kits (Biocoat Inc., Horsham, PA) and allowed to incubate for 15 minutes, they can be visualized and counted for the total number of motile sperm bound to HA versus unbound motile sperm to gain a percentage. Sperm bound to HA can be detected due to the increase in tail cross-beat frequency as opposed to unbound sperm which remain swimming freely (Huszar et al. 2003). In addition to sperm selection, medium containing HA has been shown to increase sperm velocity, long-term motility, and viability of freshly ejaculated sperm as well as cryopreserved/thawed human sperm (Huszar et al. 1990b, Sbracia et al. 1997).

Sperm that bind to hyaluronic acid are viable; non-viable sperm do not bind (Huszar et al. 2003). Sperm that bind to HA do not exhibit cytoplasmic retention, persistent histones, DNA fragmentation or capase 3, an apoptotic marker (Irvine et al. 2000 and Sati et al. 2004). These findings are significant because immature sperm with DNA fragmentation are known to be a hindrance to fertilization (Huszar et al. 2003).

Use of Hyaluronic Acid for ICSI

Apart from motility, there is not a universally accepted method for distinguishing a fully functional sperm capable of fertilizing an oocyte from a less-capable sperm. However, HA binding score zones that have been established for human clinical

laboratories have been applied to ICSI with some success. The binding scores have been shown to correlate to fertilization capability of sperm in the human (Huszar et al. 2002). Three binding zones indicate the percentage of sperm within a sample that bind to HA. A score of >80% is indicative of excellent binding. Intermediate binding is designated to samples binding 60 to 80% of sperm. Men with sperm in this category are advised that intrauterine insemination of their partner may be an option to enhance fertilization rates. Sperm exhibiting less than 60% binding to HA are considered to have diminished binding capabilities, and intrauterine insemination or ICSI is an option for couples wishing to reproduce (Huszar et al. 2002).

The observance of chromosomal aneuplodies and diploidies in humans has been investigated in HA-bound sperm for potential use with ICSI (Jakab et al. 2005). In summary, HA- bound sperm were subjected to fluorescence in situ hybridization (FISH). Except for Y disomy, all other aneuploidies and diploidies declined 4 to 6 fold, resulting in HA-selected sperm being within the range of normozoospermic men. When sperm is selected by visual assessment, a direct correlation has been observed comparing the 5-fold decline seen in X, Y and XY disomies to chromosomal abnormalities seen in ICSI-derived children (Huszar 2007, Simpson and Lamb 2001, Bonduelle et al. 2002).

No adverse effects on fertilization or embryonic development have been observed in laboratories currently utilizing HA-selected sperm (Huszar 2007). HA-selected sperm used for ICSI in humans resulted in significantly higher pregnancy rates than visually selecting sperm (Worrilow et al. 2006). Furthermore, miscarriage rates were higher ($p=0.013$) in the visually selected group of embryos versus selecting sperm bound to HA (Worrilow et al. 2006).

The use of HA-selected sperm has been reported in other species, including pigs (Park et al. 2005). More normal embryos were produced with HA selected sperm and ICSI versus visual selection of sperm for ICSI and IVF. Using HA-selected sperm reduced the number of early embryonic mortalities and chromosomal abnormalities (Park et al. 2005). Fluorescence in situ hyrbridization (FISH) analysis was used to analyze chromosome aneuploidy in sperm and embryos using chromosome 1 submetacentric probe. Although no significant differences were observed in blastocysts rates among embryos produced from IVF, ICSI, and ICSI-HABS (hyaluornic acid binding sperm), embryos derived from ICSI-HABS had a lower ($p<0.05$) chromosome abnormality rate (Park et al. 2005). Chromosomal abnormality in 4-cell porcine embryos derived from IVF, ICSI, and ICSI-HABS procedures were 43.0, 31.8, and 24.5%, respectively. The authors concluded that the selection of sperm with a hyaluronic acid binding assay may help to reduce early embryonic mortality in the pig. The conclusion is based on decreased incidence of chromosomal aneuploidy in HA-selected sperm and embryos produced by ICIS-HABS. Furthermore, ICSI-HABS is superior to conventional ICSI for higher production of porcine embryos with normal chromosomal complement (Park et al. 2005).

Nasr-Esfahani et al. (2008) compared the efficiency of routine sperm selection methods to HA-selection procedures for human sperm. The researchers' objectives were to observe fertilization rates, embryo development, implantation and pregnancy rates, and the relationship between HA-binding ability with sperm protamine deficiency. The percentage of sperm that bind to HA was statistically significant when compared to those with protamine deficiency, DNA fragmentation, and abnormal sperm morphology.

Although pregnancy and implantation rates were not shown to increase with HA procedures, oocytes injected with HA-selected sperm had significantly higher fertilization rates (Nasr-Esfahani et al. 2008). Sperm chromatin dispersion testing and chromomycin A3 staining were used to evaluate DNA fragmentation and protamine deficiency for human sperm (Nasr-Esfahani et al. 2008). In agreement with similar work conducted by Huszar et al. (2003), this study revealed that sperm samples with chromatin immaturity exhibited lower HA-binding ability. Sperm motility was not directly correlated with DNA fragmentation and abnormal morphology.

Although HA selection improved fertilization rates, it did not have a significant effect on cleavage and embryo quality on day 2 and 3 after ICSI compared to routine sperm selection (Nasr-Esfahani et al. 2008). The authors suggest that embryo quality was not different between the HA and control group due to the possibility of paternal DNA anomalies occurring during post genomic activation and peri-implantation.

Current methods of sperm selection for ICSI using motility and morphology versus HA-binding have been explored. Sperm hyaluronan-binding assay (HBA) scores are highly and significantly correlated with sperm motility and normal morphology (Hong et al. 2006). Commercial HBA kits (Biocoat Inc., Fort Washington, PA) were utilized on 175 human patients participating in an IVF study. Objectives were to determine the relationship between HBA selected sperm and routine sperm analysis results and fertilization rates with conventional IVF (Hong et al. 2006). In contrast to previously mentioned studies, results from this experiment indicate that normal sperm morphology was more significant than HBA sperm in relation to fertilization rates, although HBA methods were significantly correlated with fertilization rate in conventional IVF (Hong et

al. 2006). The use of HBA's was not shown to clearly identify patients with low and high fertilization rates. Furthermore, in contrast to other findings, the authors conclude that HBA is not superior to the selection of sperm through routine semen analysis as a predictor of fertility due to the lack of a distinct threshold value as a predictor of sperm-fertilizing ability (Hong et al. 2006).

Methods of Sperm Evaluation

Sperm Chormatin Dispersion (SCD) is an assay for sperm DNA fragmentation. Sperm are subjected to an acid denaturing solution and then stained for fluorescence or brightfield microscopy to allow for detection of single-stranded DNA from DNA breaks (Fernandez et al. 2003). This assay demonstrates that sperm with fragmented DNA do not produce the normal halo of dispersed DNA loops. A halo is characteristic of normal sperm presenting non-fragmented DNA after acid denaturation and the removal of nuclear proteins (Fernandez et al. 2003). Several tests are available to assess sperm DNA fragmentation including the TUNNEL assay, the comet assay, chromomycin A3 testing, DNA Breakage Detection-Fluorescence In Situ Hybridization (DBD-FISH) test, and the sperm chromatin structure assay (SCSA) (Fernandez et al. 2003).

Normal sperm with non-fragmented DNA can be monitored by fluorescence microscopy using specific DNA flurochromes to visualize halos of DNA dispersion. These halos can be evaluated after sperm are immersed in an agarose matrix and exposed to lysing solutions. The deproteinized nuclei, exibiting relaxed DNA loops or halos, are then referred to as "nucleoids." DNA damage can be assessed by looking for breaks that will facilitate the expansion of the halo. Sperm nuclei that have fragmented DNA can be

detected by the absence or minimal observance of the halo when treated with an acid solution before the addition of a lysis buffer using the SCD test (Fernandez et al. 2003). Acid or alkaline solution acts as a denaturing solution for somatic cells with fragmented DNA and allows for increased spreading of DNA fragments. The fragmentation is much more recognizable than when observed without a denaturing solution (Fernandez et al. 2002).

The SCSA is a flow cytometer assay that measures the susceptibility of sperm DNA to low pH-induced denaturation *in situ* (Larson et al. 2000). This test utilizes Triton X-100 which is a low pH solution used to permeate cell membranes allowing for access of arcidine orange to DNA. The solution acts to partially denature DNA in sperm that have abnormal chromatin structures. When passed through a flow cytometer, DNA fluorescing red indicates single-stranded (denatured) DNA that has associated with arcidine orange while green indicates normal double-stranded DNA. The results of DNA denaturation can be quantified by using an Ortho Diagnostic Cytoflurograf II (Larson et al. 2000). Results of one experiment utilizing SCSA for ICSI in humans revealed that no pregnancies occurred if >27% of sperm within a given sample showed DNA denaturation (Larson et al. 2000). SCSA is one of the most popular and widely used tests independent of sperm morphology that can be used as a predictor of pregnancy rates in human IVF clinics.

Conclusions

Semen collected from stallions can be used for a variety of assisted reproductive techniques. Stallion sperm can be inseminated into a mare immediately after collection, cooled for use within 48 h, or cryopreserved for an indefinite period. Stallion sperm can also been cooled prior to freezing with some success. However, these techniques can be improved upon by conserving the volume and number of sperm needed to fit a specific application. Intracytoplasmic sperm injection is one such technique that requires a unique set of sperm parameters. ICSI differs from all other assisted reproductive techniques in that only a single sperm is required for injection into an oocyte. The cooling and cryopreservation of sperm can be modified to preserve low concentrations of sperm specifically designed for ICSI. Freezing sperm in smaller volumes and numbers than what is needed for insemination would allow for conservation of limited quantities of stallion sperm. The amount of time that semen can be cooled before freezing can be further evaluated to fit less strict parameters regarding overall motility and sperm numbers that are necessary for success with ICSI.

The selection of sperm for ICSI involves techniques such as swim-up procedures and Percoll gradients with the objective to select a fully functional sperm capable of fertilization. Other than a visual assessment of motility, after initial selection procedures stallion sperm cannot be selected for fertilizing potential. Selecting sperm through an alternate method such as hyaluronic acid binding, would utilize a biochemical marker to help determine fertilizing potential of sperm. A method such as HA binding would help differentiate sperm that are more likely to lack chromosomal aberrations and improve fertilization, blastocyst, and pregnancy rates after ICSI. Advances such as those

described would ultimately aid to improve clinical ICSI programs. Therefore, a series of experiments were conducted help facilitate advances in the above described areas of interest:

Experiment 1

In Experiment 1 we determined if semen could be cooled for 24 h and then frozen to provide sufficient motility for ICSI selection. The objectives of Experiment 1 were to compare: 1) two commonly used commercial diluents for cooling semen before freezing and 2) compare four freezing diluents added to semen that has been cooled for 24 h prior to freezing.

Experiment 2

Maintaining the viability of sperm in a holding medium is essential for many assisted reproductive techniques. Sperm must survive the time required for processing and injection into an oocyte and any unforeseen circumstances that would increase processing time. A medium that does not require an altered environment, such as humidity and CO_2, would be optimum for maintaining sperm over time. The objective of Experiment 2 was to determine which of five holding media best maintains the motility of stallion sperm over time.

Experiment 3

Experiment 3 tested the hypothesis that stallion sperm could bind to a commercially available HA-binding assay (HBA, Biocoat Inc., Horsham, PA). The

objective of this experiment was to determine if fresh and frozen stallion sperm bind to HA when introduced to pre-manufactured HBA kits.

CHAPTER II

CRYOPRESERVATION OF COOLED SEMEN FOR EQUINE INTRACTOPLASMIC SPERM INJECTION

Introduction

Frozen sperm that have been previously cooled are of potential use for equine intracytoplasmic sperm injection (ICSI). Improving the efficiency of frozen sperm for ICSI is of importance when considering the frequency of use (85%) in recent years by one clinic (Carnevale 2008). Progressive motility is a parameter of successful freezing and is an indication of fertilizing ability. Although the motility of sperm that have been cooled and later frozen may be inadequate for insemination, there may be sufficient numbers of motile sperm for ICSI.

Semen can be frozen after various cooling periods with acceptable post-thaw motility and fertility after insemination (Backman et al., 2004; Crockett et al., 2001). Results depended on the length of time sperm were cooled and the type of extender used. Cooling semen without glycerol at concentrations of 200 x 10^6sperm/mL before freezing provided numerically higher post-thaw motility rates (Backman et al. 2004). Semen cooled for 1 h at 4°C and then centrifuged to remove seminal plasma survived best when 4% glycerol was added to INRA96 prior to freezing.

Glycerol is the most common cryoprotectant utilized in stallion freezing extenders, but concentrations varying from 2.5 to 6% have proven to be effective (Vidament et al. 2001). The addition of 4% glycerol did not prove to be significantly different when compared to 3%, which is a common industry standard (Scherzer et al. 2009). When combined, a positive effect is obtained with the addition of glycerol and egg yolk to cooling extenders for up to 24 h prior to freezing (Crockett et al. 2001). Similarly, egg yolk has been added to freezing extenders at different concentrations ranging from 2% (Palmer, 1984) to 20% (Martin et al. 1979). Although several commercial freezing extenders are available for the stallion, the optimal concentration of egg yolk that should be present within an extender is not clear.

Stallion sperm can be frozen at several concentrations that preserve post-thaw motility equally well. Semen can be cooled and then frozen at lower than industry standard concentrations (50 x 10^6sperm/mL) and still provide similar post-thaw motility to industry standard concentrations of 250 x 10^6sperm/ml (Crockett et al. 2001). Although semen can be frozen at concentrations of 2 x 10^6sperm/mL for ICSI, this concentration does not provide enough sperm that can be used for swim-up selection as the concentration of sperm is too dilute (Choi et al. 2005).

The overall goal of Experiment 1 was to develop a method for freezing stallion sperm that have been previously cooled for 24 h, while maintaining sufficient motility for ICSI selection. We tested the hypothesis that semen can be cooled for 24 h and then frozen to provide a viable population of sperm for ISCI. The objectives of this study were to compare: (1) two diluents for cooling semen prior to freezing and (2) four diluents for freezing cooled semen.

Materials and Methods

A two-by-four factorial design was used to compare two cooling diluents and four freezing diluents at three time points after thawing semen (Figure 1). Eight stallions were chosen at random from approximately 18 stallions housed on the facility for research and commercial uses. Stallions were of light-horse breeds and ranged in age from 11 to 18 yr. Prior to the beginning the experiment, five stallions were on an inconsistent collection schedule. These stallions were collected twice, approximately 1 h apart, to minimize extra-gonadal reserves of sperm. They were rested for 1 d prior to the collection for this experiment. Three stallions were being collected for a commercial breeding program, and semen for the project was collected in accordance with their normal collection schedule.

Semen was collected using a CSU Model artificial vagina with an in-line gel filter (Animal Reproduction Systems; Chino, CA) and evaluated within 10 min of collection. The concentration was evaluated using a Densimeter (Model #543B, Animal Reproduction Systems; Chino, CA). Total and progressive motility was observed using a computer-automated sperm analysis system (CASA; Hamilton-Thorn HT-IVOS Motility Analyzer, Hamilton-Thorn Research, Danvers, MA) by placing a 5.5-µl droplet of raw semen, diluted to 50 x 10^6sperm/ml with CST (E-Z Mixin®- CST Animal Reproduction Systems, Chino, CA), onto a preheated (37°C) glass slide with a cover slip. Three random frames on each slide were obtained and averaged to count a minimum of 100 sperm. System parameter settings were: 15 frames acquired at 30 frames/sec; magnification factor 1.95; minimum contrast 75, minimum size 4 pixels; lower and upper

static size limits of 0.7 and 7.75, respectively; minimum velocity of the average path of motile sperm (VAP) was 20 μm/sec.

Ejaculates were split and the semen was extended to 50 x 10^6 total sperm/mL in a total volume of 23 mL using two cooling diluents: (1) INRA96 (IMV International Corporation, Minneapolis, MN) and (2) CST (E-Z Mixin®- CST Animal Reproduction Systems, Chino, CA). The samples were cooled in a passive cooling device (EST Equine Semen Transporter™, Baraka Enterprises Inc., Arkansas, USA) and held at 5°C for 24 h.

After 24-h, 3 mL of sperm were removed from each sample to be used as the cooled control; the samples were further diluted to 20 x 10^6 total sperm/mL in their respective diluents and placed in an incubator at 37°C for 10 min prior to being evaluated with the CASA at 0, 45 and 75 min (Figure 2).

The remaining sperm in INRA96 and CST diluents were diluted with their respective diluents to a concentration of 40 x 10^6 total sperm/mL before being assigned in 3-mL aliquots to one of the following groups for freezing: (1) FR8, diluted with 0.75 mL of a modified French extender (Wilhelm et al. 1996) containing 0% glycerol then diluted 1:1 (3.75 mL) with FR8 (the modified French extender containing 8% glycerol) for a final concentration of 4% glycerol and 20 x 10^6 total sperm/mL; (2) LAC10, diluted with 0.75 mL lactose EDTA extender (Cochran et al. 1984) containing 0% glycerol and then diluted 1:1 with LAC10 (lactose EDTA extender containing 10% glycerol); (3) G, diluted with 0.75 mL of CST and then 1:1 with 8% glycerol solution; and (4) GEY, diluted with 0.75 mL of the respective cooling extender, then diluted 1:1 with a solution containing 8% glycerol and 8% egg yolk. The G solution was composed by diluting CST with 8% glycerol by volume. Solutions for GEY were composed with respective cooling

extenders with the addition of 8% glycerol and 8% egg yolk by volume (see Appendix I for composition of freezing extenders).

Sperm were packaged into 0.5-mL polyvinylchloride straws (IMV International Corporation, Minneapolis, MN). Samples in FR8 were loaded into a cell programmable freezer (Kryo 10 Series III, Planer, Middlesex, UK) and cooled at a rate of -10°C/min from 20 to -15°C, at -15°C/min from -15 to -120 °C, then plunged into liquid nitrogen. Samples in LAC, G and GEY were floated 3 cm above liquid nitrogen for 10 min at approximately -160°C and then submerged.

One straw from each treatment was thawed in a water bath at 37°C for 30 sec. A 5.5-µl sample of thawed semen was analyzed immediately for motility using the CASA. Remaining sperm from samples were stored in an incubator at 37°C in 5-mL polypropylene tubes and evaluated at 45 and 75 min (Figures 1 and 2).

Statistical Analyses

All analyses were conducted using SAS 9.2 (SAS Institute Inc, Cary, NC); prior to analyses, data were arcsine transformed. In Experiment 1, total and progressive motility were compared between cooling diluents and among freezing treatments using ANOVA at 0, 45 and 75 min. No effect of cooling diluents (CST and INRA96) was detected; therefore, data from both diluents were combined for further analyses of freezing treatments. Freezing treatments were compared among groups at each time point by ANOVA, and if significant ($p<0.05$), differences were determined between treatments by least significant differences. The effect of time was evaluated for each freezing treatment by comparing the three time points using ANOVA.

Results

Mean total and progressive motility were not different ($p>0.05$) for sperm cooled in CST versus INRA96 when evaluated at 0, 45 and 75 min. Total and progressive motilities among groups of frozen semen were not different ($p>0.05$) for cooling diluents; therefore, freezing treatments with different cooling diluents were combined.

Mean total motility of sperm was not different among freezing groups after thawing for 0 and 45 min. However, by 75 min, differences were detected among freezing treatments ($p=0.03$), with motility for GEY higher ($p<0.05$) than LAC10 and G, and motility for FR8 higher ($p<0.05$) than G (Figure 3).

Mean progressive motility of sperm followed a similar pattern as total motility, with approximately half as many progressively motile sperm for GEY and FR8 as for LAC10 and G at each time. No differences were observed at 0 and 45 min among groups, although groups tended ($p<0.08$) to differ at 45 min. A difference ($p=0.01$) was present among groups at 75 min, with motility for G being lower ($p<0.05$) than for FR8 and GEY (Figure 4).

Mean total and progressive motilities were not different for the three time points when analyzed across time for any group except G. Mean total and progressive motility for G were lower ($p=0.02$) for 75 versus 0 min after thawing (Figure 5).

Discussion

Frozen sperm are frequently used for equine ICSI (Carnevale, 2008). Sperm used for ICSI is often packaged at concentrations that are common for insemination. The need to have a large population of motile sperm for insemination is not relevant, as only one

sperm is required for injection into an oocyte. The concentration of frozen sperm stored for ICSI can be improved upon by freezing a minimal number of sperm. Any method of freezing sperm for ICSI must result in a viable population of sperm after thawing.

Cooled semen has the potential to be frozen and later used for ICSI. Stallion semen can be cooled for various time lengths before freezing (Backman et al., 2004; Crockett et al., 2001). Previous groups have conducted experiments to determine if stallion sperm can survive prolonged cooling before freezing and still provide adequate sperm for future insemination (Backman et al., 2004; Crockett et al., 2001). Our study differs from those described in that viable sperm with acceptable motility parameters for ICSI differs from that of what is required for insemination. Selection of sperm for ICSI requires swim-up and centrifugation; therefore, sperm were evaluated at 45 and 75 min after thawing. These time points were selected to simulate the time required for sperm preparation for ICSI (Altermatt et al. 2009).

The removal of seminal plasma is not feasible for every facility that ships cooled semen. Seminal plasma has been shown to be detrimental when freezing stallion sperm (Jasko et al. 1991, Dawson et al. 1999, Crockett et al.2001). We have demonstrated that the removal of seminal plasma is not necessary for sperm being frozen for ICSI. This finding is significant because cryoprotectant can be directly added to cooled semen without the removal of seminal plasma and then frozen for ICSI. The methods described herein for freezing semen containing seminal plasma may be of application for facilities lacking the ability to remove seminal plasma before shipping. Although seminal plasma has the potential to be removed upon arrival, centrifugation would be an additional insult

to sperm survival. However, further investigation is needed to determine the effects on freezing sperm with seminal plasma and implications for ICSI.

Differences in the composition of freezing diluents have likely contributed to the results described. Egg yolk is commonly used as a lipid additive in semen extenders to aid in the protection of the lipid membrane. Phospholipids, such as low-density lipoproteins present in egg yolk, aid in maintaining the sperm membrane during cooling (Squires et al. 1999). The highest motilities were observed with the addition of FR8 and GEY. These results are probably attributed to the egg yolk present in both diluents. When GEY was added to cooling diluents, the combination resulted in the GEY diluent being very similar in composition of ingredients to FR8. Our results are in agreement with other studies in which egg yolk has been shown to aid in preservation of motility (Crockett et al. 2001). Cooled sperm frozen with only the addition of glycerol resulted in the lowest motilities among groups. However, sperm frozen with only the addition of glycerol still resulted in motile sperm for ICSI.

Freezing sperm at a concentration of 20 x 10^6sperm/mL has allowed for the cryopreservation of a low number of sperm. Low concentrations of frozen sperm are beneficial to preserving limited quantities of stallion sperm. Freezing sperm at this concentration in a 0.5-mL straw, results in a total population of approximately one tenth of industry standard concentrations. Conventional methods of selecting sperm for ICSI require cutting a portion of a straw with frozen sperm under liquid nitrogen (Altermatt et al. 2009). The total number of sperm obtained by freezing sperm in one straw at 20 x 10^6sperm/mL is comparable to numbers of sperm obtained from a single cut portion of straw using current methods (Altermatt et al.2009). Therefore, freezing sperm at 20 x

10^6sperm/mL eliminates the need of liquid nitrogen for cutting a straw, eliminates contamination to an unused portion of straw, and is more efficient in a laboratory setting and conservative in quantity.

The use of two different cooling diluents did not have any bearing on freezing ability of sperm or differences in controls after cooling. This finding is significant when considering that facilities shipping stallion semen may only have access to one commonly used cooling diluent at a given time. Sperm frozen at a slow cooling rate (FR8) did not perform better than sperm frozen at a faster rate (GEY). Motility rates after 24 h of cooling as well as after freezing may be improved if the length of cooling time is shortened. The ability of sperm to survive a 24 h cooling period before freezing is beneficial for shipments arriving overnight at a facility that lacks the personnel to freeze sperm immediately. The above-described methods for freezing semen that has been cooled for 24 h without the removal of seminal plasma demonstrate the ability to maintain sufficient numbers of motile sperm for ICSI. Clinical changes in mare management, such as a change in procedure from ooycyte transfer to ICSI, would potentially result in a standard insemination dose of semen that is no longer needed at such a quantity. The remaining sperm not used for ISCI could be frozen in this circumstance and others of unforeseen nature. Due to the observation of variability in stallion sperm surviving a cooling process, further investigation is warranted to characterize differences in seminal plasma between stallions. The ability to predict cooling and freezing potential for individual stallions based on characterization of seminal plasma would be of great benefit for a number of assisted reproductive techniques.

Figure 1. Diagram of experimental design for Experiment 1

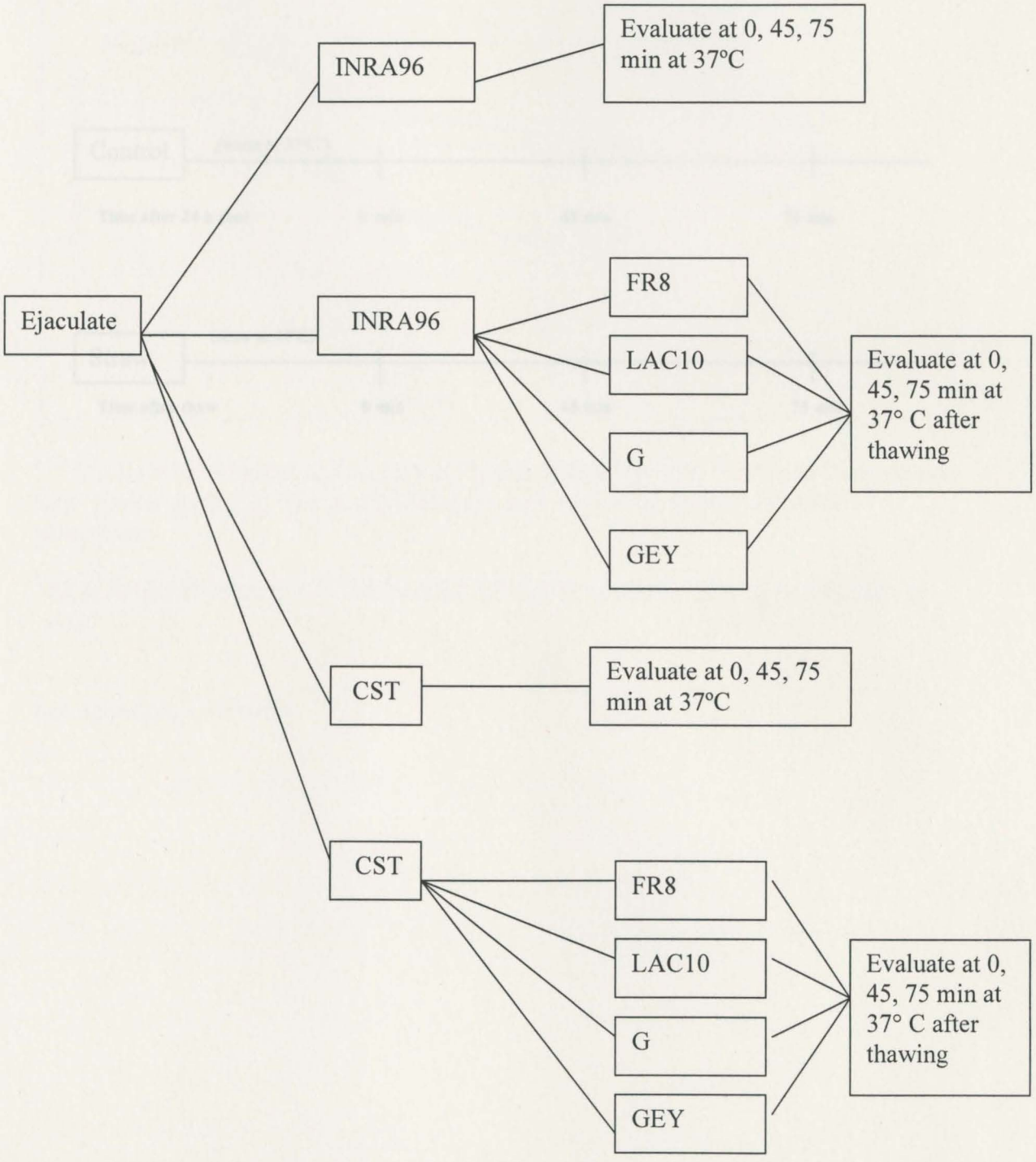


Figure 2. Time points for evaluation of controls and frozen straws

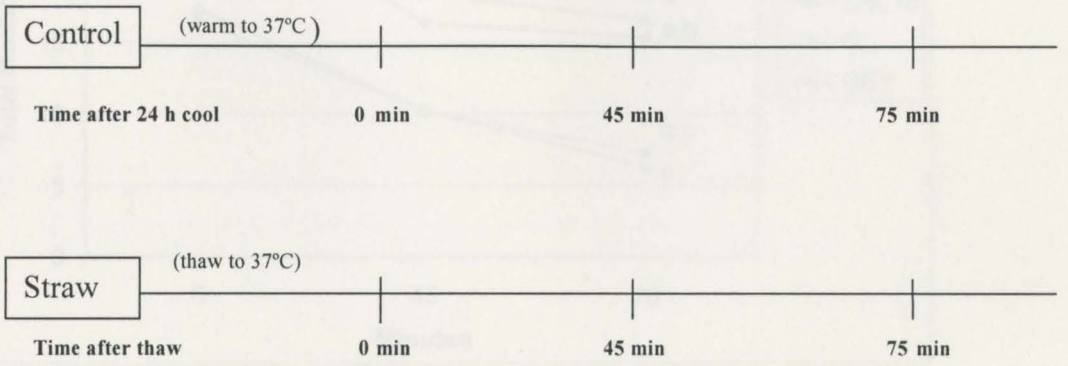
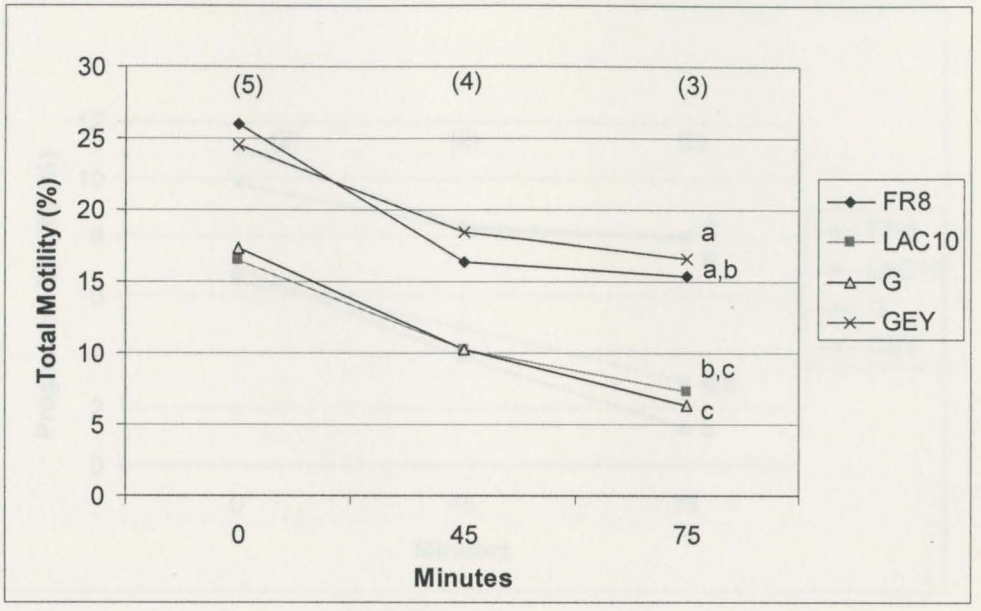


Figure 3. Mean total motility of semen cooled for 24 h, frozen in four extenders* and warmed for evaluation of motility at 0, 45 and 75 min (n = 16 samples).

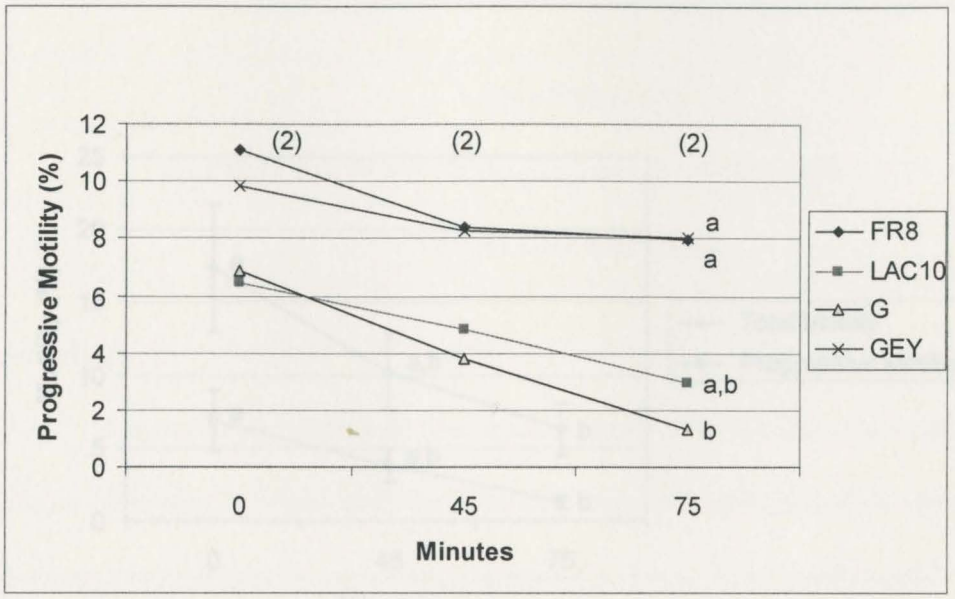


[a,b,c] Values with different superscripts at 75 min differed (p=.03) Values at 0 and 45 min were similar (p>0.05). The pooled SEM for each time point appears between parentheses.

* FR8: Modified French, LAC10: Lactose-EDTA, G: glycerol, GEY: glycerol and egg yolk

See Appendix I for table

Figure 4. Mean progressive motility of semen cooled for 24 h, frozen in four extenders* and warmed for evaluation of motility at 0, 45 and 75 min (n = 16 samples).

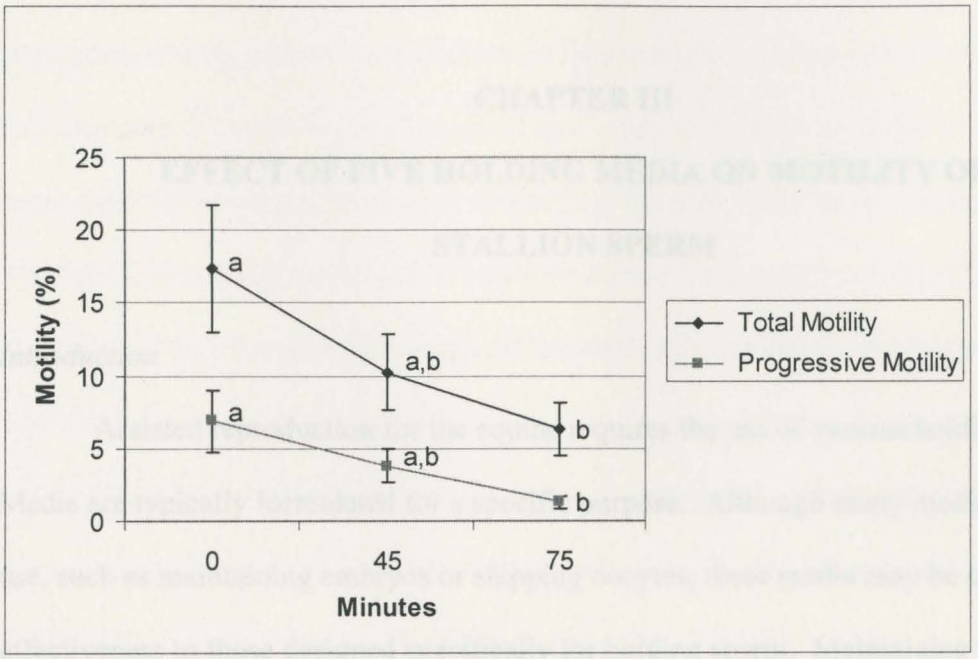


[a,b] Values with different superscripts at 75 min differed ($p=0.01$) Values are similar ($p>0.05$) at 0 min. A trend towards significance is present at 45 min ($p<0.08$). The pooled SEM for each time point appears between parentheses.

*FR8: Modified French, LAC10: Lactose-EDTA, G: glycerol, GEY: glycerol and egg yolk

See Appendix I for table

Figure 5. Means ± SEM of total and progressive motility over time for sperm frozen in glycerol (G) and evaluated at 0, 45 and 75 min post thaw (n = 16 samples).



[a,b] Values with different superscripts for total or progressive motility differed over time ($p<0.05$).

CHAPTER III

EFFECT OF FIVE HOLDING MEDIA ON MOTILITY OF STALLION SPERM

Introduction

Assisted reproduction for the equine requires the use of various holding media. Media are typically formulated for a specific purpose. Although many media specify a use, such as maintaining embryos or shipping oocytes, these media may be comparable in effectiveness to those designed specifically for holding sperm. Maintaining the viability of stallion sperm for a prolonged period of time is beneficial for many assisted reproductive techniques. Holding sperm in a medium that does not require altered environments, such as maintenance of humidity and CO_2, would provide alternative sources of media that may be more compatible for use in research or a clinical equine program.

Swim-up procedures are common practice when selecting sperm for intracytoplasmic sperm injection (ICSI);(Altermatt et al. 2009). Sperm must be able to survive and maintain motility over a period of time in order to be selected by swim-up. Swim-up techniques often involve centrifugation and washing of sperm which increases the length of time before sperm selection and injection into an oocyte (Altermatt et al. 2009, Choi et al. 2005). The objective of Experiment 2 was to determine which of five holding media best maintains the motility of stallion sperm over time.

Materials and Methods

Single ejaculates were collected from seven randomly chosen stallions out of a population of approximately 18 stallions of light-horse breeds housed on the facility. Stallions were between 4 and 15 years of age. After evaluation of initial concentration, semen was diluted in a skim-milk extender (E-Z Mixin®- CST Animal Reproduction Systems, Chino, CA) and placed into five media at a final concentration of 50 x 10^6 total sperm/mL. For 5 h, semen was analyzed for total and progressive motility at hourly intervals.

Semen was collected and evaluated as described in Experiment 1. Five media were evaluated: 1) GIVF (Vitrolife; Sweden AB, Kungsbacka, Sweden), 2) FCDM [CDM (Olson and Seidel 2000) supplemented with 0.5% fatty acid-free bovine serum albumin 2 mM caffeine and 2μg mL^{-1} heparin], 3) TALP [Tyrodes Albumin Lactate Pyruvate; (see Appendix II)], 4) EM (EmCare™ Embryo Holding Solution; AgTech Inc., Manhattan, KS), and 5) MM (Mare Mojo Embryo Holding Medium; Jason Abraham, Canadian, TX). See Appendix II for TALP recipe.

TALP, EM and MM were warmed to 37°C prior to the addition of sperm. GIVF and FCDM require CO_2 for the maintenance of pH; therefore, they were equilibrated in an incubator at 38.5°C in 6.0% CO_2 and air for approximately 2 h before addition of sperm. Sperm were added to 5-mL polystyrene round bottom tubes containing 1.5 mL of each of the five media to achieve a final concentration of 50 x 10^6 total sperm/mL. After the addition of sperm, GIVF and FCDM samples were held in an incubator at 38.5°C in a 6.0% CO_2 atmosphere. EmCare, TALP and MM were maintained at 37°C in an incubator without CO_2 and capped to prevent evaporation. An aliquot of each sample

was removed at hourly intervals for a total of 5 h for evaluation of total and progressive sperm motility using the CASA.

Statistical Analyses

All analyses were conducted using SAS 9.2 (SAS Institute Inc, Cary, NC); prior to analyses, data were arcsine transformed. Total and progressive motility were compared among groups at each time point by ANOVA. When significant differences were detected, differences between groups were determined by least significant differences. Differences were considered significant at $p<0.05$.

Results

The mean ±SEM total motility for semen from the seven stallions at the time of collection was 85 ± 3%. Mean total motility was not different among groups from 1 through 4 h, although means tended to differ at 3 h and 4 h ($p = 0.12$ and 0.09, respectively), with TALP having the highest numerical motility among all groups. Total motility was different ($p = 0.03$) among groups at 5 h, with sperm in TALP having a higher ($p<0.05$) total motility than sperm in other media (Figures 6 and 8).

The mean ±SEM total progressive motility for semen from the seven stallions at the time of collection was 33 ± 5%. Mean progressive motility approached significance ($p=0.055$) at 1 h with progressive motility of sperm in TALP higher ($p<0.05$) than FCDM and GIVF. Differences among groups were not different at 2 h. At 5 h, groups differed ($p=0.004$), with TALP having higher ($p<0.05$) progressive motility than all other groups (Figures 7,and 9).

Discussion

Sperm subjected to five holding media were analyzed every hour for 5 h. These time points were selected to ensure the adequate time necessary for sperm selection and preparation before ICSI. Although sperm preparation does not usually require more than 1 h, we evaluated the motility of sperm for 5 h to replicate a situation in which unforeseen circumstances would necessitate the maintenance of sperm in media for a prolonged time.

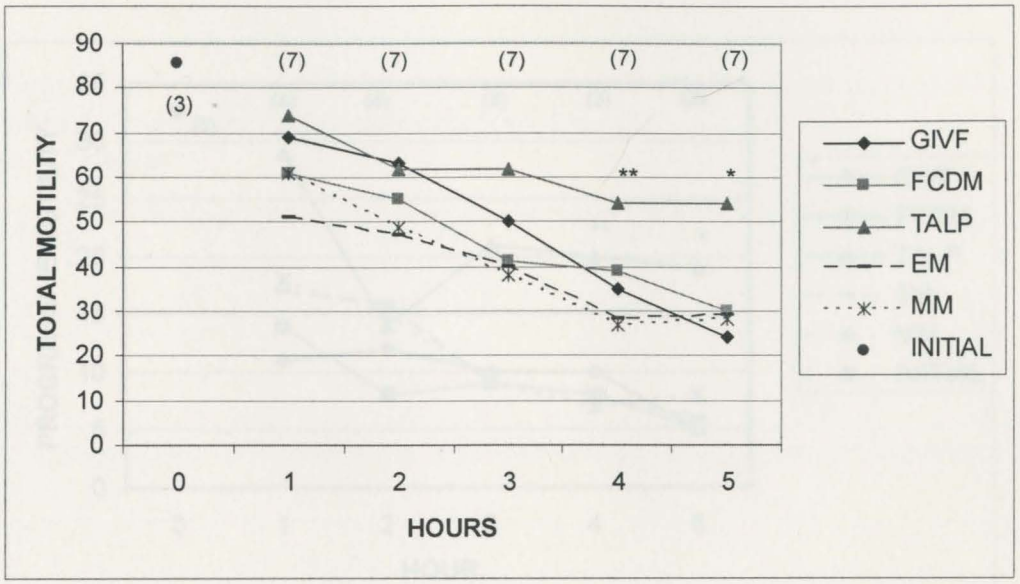
Cell culture mandates the maintenance of pH. The incubation of five media required that GIVF and FCDM be maintained in an exogenous CO_2 atmosphere. Both GIVF and FCDM are formulated with HEPES (N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid) buffer. Media used for cell culture is often formulated with HEPES or sodium bicarbonate to maintain pH. Buffers are most effective when the pH is equal to the pKa (acidic strength) of that buffer. Therefore, when a higher pH is desired, HEPES if often added to a media to maintain a pH of 7.2 to 7.6 (Good et al. 1966). Although HEPES-buffered media such as TALP can be maintained without CO_2, extracellular CO_2 is thought to play a novel, but not fully understood, role in cell proliferation (Chakrabarti R, 2001). Exogenous CO_2 is used by one clinic for oocyte incubation and may be compatible for holding sperm (Altermatt et al. 2009). Apart from buffer, the incubation temperature of GIVF and FCDM was also different (38.5°C) than for the remaining three media (37°C). Discrepancy in temperatures is attributed to GIVF and FCDM being held in an incubator used for oocyte culture. Adjusting the temperature of incubation for holding sperm would not be practical for a clinical program. Although holding sperm in a cooler environment would have increased survival time, motility

patterns would have been depressed. Motility is the only indication of sperm fertilizing potential and is essential to repeatedly successful ICSI.

The comparison of five media for maintaining sperm motility resulted in TALP being superior over time. TALP was the only medium evaluated that is specifically designed for holding sperm. TALP was initially formulated in response to the sensitivity of hamster sperm to culture media (Bavister and Yanagimachi 1977). The composition of energy substrates within a solution (lactate, pyruvate, and glucose) were found to effect motility of sperm at different time points with varying concentrations. The ingredients in TALP have since been modified for use with other species (Bavister and Yanagimachi 1977, Graham et al. 1986). Furthermore, TALP is a clear medium and the use of a clear medium is essential for clear observation of sperm during a selection process.

Although differences in media approached significance at 1 h, it is important to note that no significant differences were detected until analyses at 5 h. The preparation of sperm for ICSI may not require a full hour. Therefore, media that are not designed for holding sperm may be of acceptable use. Holding sperm in TALP is beneficial, because TALP does not require an altered atmosphere. TALP is relatively simple to make and can be frozen for later use.

Figure 6: Mean total motility for sperm held in five media* for 5 h (n = 7 stallions).

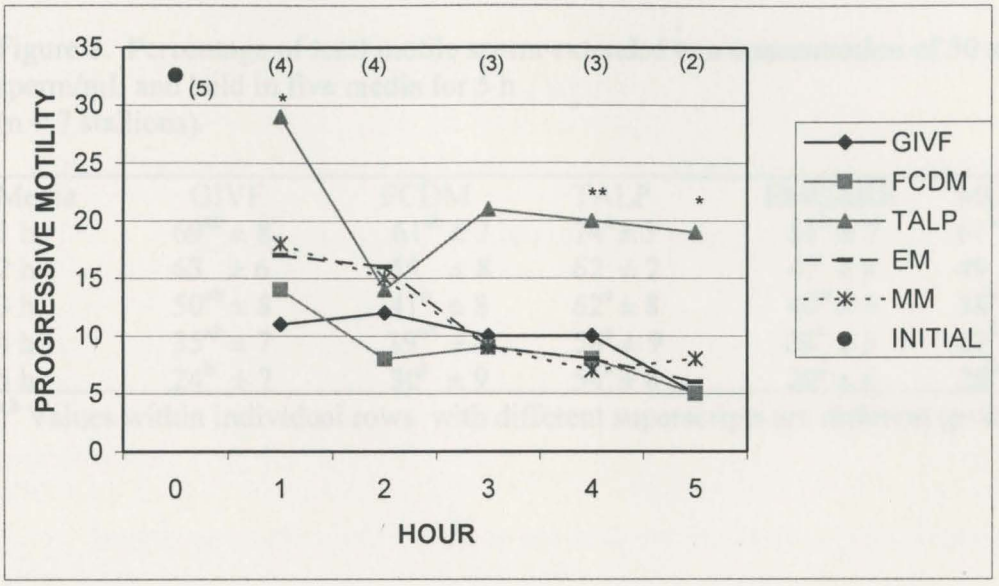


• Initial total motility after collection
* $p < 0.05$ among groups
** $p < 0.09$ among groups

The pooled SEM for each hour appears between parentheses.

* GIVF (Vitrolife; Sweden AB, Kungsbacka, Sweden)
FCDM (Fertilization Chemically Definied Medium with BSA, Caffeine and Heparin)
TALP (Tyrodes Albumin Lactate Pyruvate)
EM (EmCare™ Embryo Holding Solution; AgTech Inc., Manhattan, KS)
MM (Mare Mojo Embryo Holding Medium; Jason Abraham, Canadian, TX)

Figure 7: Mean progressive motility for sperm held in five media* for 5 h (n = 7 stallions).



• Initial progressive motility at collection
* $p = 0.004$ among groups with TALP differing ($p<0.05$) from all other media
** $p < 0.09$ among groups

The pooled SEM for each hour appears between parentheses.

* GIVF (Vitrolife; Sweden AB, Kungsbacka, Sweden)
FCDM (Fertilization Chemically Defined Medium with BSA, Caffeine and Heparin)
TALP (Tyrodes Albumin Lactate Pyruvate)
EM (EmCare™ Embryo Holding Solution; AgTech Inc., Manhattan, KS)
MM (Mare Mojo Embryo Holding Medium; Jason Abraham, Canadian, TX)

Figure 8. Percentage of total motile sperm extended to a concentration of 50 x 10^6 total sperm/mL and held in five media for 5 h
(n = 7 stallions).

Media	GIVF	FCDM	TALP	EMCARE	MOJO
1 h	$69^{ab} \pm 8$	$61^{ab} \pm 7$	$74^{a} \pm 5$	$51^{b} \pm 7$	$61^{ab} \pm 8$
2 h	63 ± 6	55 ± 8	62 ± 2	47 ± 8	49 ± 9
3 h	$50^{ab} \pm 8$	$41^{b} \pm 8$	$62^{a} \pm 8$	$40^{b} \pm 5$	$38^{b} \pm 7$
4 h	$35^{ab} \pm 7$	$39^{ab} \pm 8$	$54^{a} \pm 9$	$28^{b} \pm 5$	$27^{b} \pm 5$
5 h	$24^{b} \pm 7$	$30^{b} \pm 9$	$54^{a} \pm 8$	$29^{b} \pm 6$	$28^{b} \pm 5$

[a,b] Values within individual rows with different superscripts are different ($p<0.05$).

Figure 9. Percentage of progressively motile sperm extended to a concentration of 50 x 10^6 total sperm/mL and held in five media for 5 h
(n = 7 stallions).

Media	GIVF	FCDM	TALP	EMCARE	MOJO
1 h	$11^{b} \pm 4$	$14^{b} \pm 3$	$29^{a} \pm 5$	$17^{ab} \pm 4$	$18^{ab} \pm 3$
2 h	12 ± 3	8 ± 3	14 ± 3	16 ± 4	15 ± 6
3 h	$10^{b} \pm 3$	$9^{b} \pm 2$	$21^{a} \pm 6$	$9^{ab} \pm 2$	$9^{b} \pm 3$
4 h	$10^{ab} \pm 2$	$8^{b} \pm 3$	$20^{a} \pm 5$	$8^{b} \pm 2$	$7^{b} \pm 2$
5 h	$5^{b} \pm 1$	$5^{b} \pm 1$	$19^{a} \pm 4$	$6^{b} \pm 2$	$8^{b} \pm 2$

[a,b] Values within individual rows with different superscripts are different ($p<0.05$).

CHAPTER IV

EVALUATION OF A HYALURONIC ACID SPERM BINDING ASSAY FOR EQUINE INTRACYTOPLASMIC SPERM INJECTION

Introduction

Intracytoplasmic sperm injection involves the selection of a single sperm chosen from a population of viable and non-viable sperm. Apart from motility, there is not a universally accepted method of selecting and distinguishing a fully functional sperm from one that may not be as likely to fertilize an oocyte. Current methods of selecting equine sperm include centrifugation and swim-up techniques (Altermatt et al. 2009). Others have described similar techniques that mandate swim up or Percoll gradients for selection of stallion sperm for ICSI (Choi et al. 2005, Lazarri et al. 2002). However, aside from visual assessment of motility, sperm cannot be selected for fertilization capability.

Some human in vitro clinics implement a method of sperm selection that helps to distinguish a more fully functional and mature sperm from one that is not mature (Huszar 2007). Hyaluronic acid binding assays (HBA) are used to distinguish sperm that are mature, and are likely to have a 4 to 6-fold decrease in chromosomal aberrations (Jakab et al. 2005). Similarly, others have shown that a 5-fold decrease in chromosomal abnormalities has been observed when sperm are selected with HA binding techniques

rather than visual assessment (Huszar 2007, Simpson and Lamb 2001, Bonduelle et al. 2002).

The sperm-hyaluronan-binding assay uses the hylauronidase receptor present on the intact acrosome of the sperm membrane surface to test for hyaluronic acid (HA) binding ability (Hong et al. 2006). The presence of a cell surface hyaluronic acid binding glycoprotein (HABP) in sperm was identified for several species including the rat, mouse, bull and human (Ranganathan et al. 1994). Sperm obtain the HA receptor during spermiogeneis. This event occurs at the same time as plasma membrane remodeling (Huszar et al. 1997). Therefore, sperm that will bind to hyaluronidase are indicative of cells that have undergone a normal maturation process and are capable of fertilizing an oocyte. Sperm that are bound to HA can be distinguished by cessation of head movement, and an increase in tail cross-beat frequency, as opposed to unbound sperm which remain swimming freely (Huszar et al. 2003).

Porcine sperm have also been shown to bind to HA and have been selected for ICSI using this technique (Park et al. 2005). The localization of HA-binding protein on the sperm surface has been detected by indirect immunofluorescence using anti-HA-binding protein antibodies for many species (Ranganathan et al. 1994). Similarly, stallion sperm express a surface-associated hyaluronidase that can be found on the posterior acrosome of ejaculated sperm (Meyers 2001). Our hypothesis is that stallion sperm can bind to a commercially available HA-binding assay (HBA, Biocoat Inc., Horsham, PA). The objective of this experiment is to determine if fresh and frozen stallion sperm bind to HA when introduced to pre-manufactured HBA kits (Biocoat Inc., Horsham, PA).

Methods and Materials

Two preliminary studies were designed to test a hyaluronic-binding assay (HA) as a means to select stallion sperm for intracytoplasmic sperm injection in the mare. Boar sperm were used as a control to observe sperm binding to assays. Semen from two boars were pooled and subjected to a hyaluronic acid binding assay (HBA® Biocoat Inc., Horsham, PA) and visualized for binding of sperm. Shipped semen from two boars was cooled and extended at a distant facility. The boar semen arrived to our lab within 24 h of collection. Samples were pooled, analyzed for motility, and centrifuged at 600 x G for 5 minutes. The supernatant was removed and the sperm pellet resuspended in TALP (Tyrodes Albumin Lactate Pyruvate) and washed by centrifugation a second time at 600 x G for 5 minutes. After removal of the supernatant, the pellet was resuspended, and the concentration of sperm obtained by spectrophotometer following dilution of sperm to 20 x 16^6 total sperm/mL. A 10-µl sample of the extended semen was placed onto the center of premanufactured hyaluron coated slides with a coverslip. A separate sample was placed onto a glass slide with a coverslip and incubated simultaneously to serve as a control. Sperm were incubated for 10 min on the slide at room temperature (20 -25°C) for optimum binding to hyaluron. After 10 minutes, the slide was placed under microscope for visualization of binding. Binding was determined as cessation of head movement of the sperm and increased cross-beat frequency of the tail. The slide was then placed back into the humidified incubator and analyzed once more for binding 10 minutes later.

Upon confirmation that boar sperm would bind to the assay, two random stallions were collected; the semen pooled and analyzed for motility. Each sample was diluted to

200 x 10^6 sperm/mL in TALP (Experiment 2). The pooled sample contained 4000 x 10^6 total sperm. The sample was centrifuged at 1000 X G for 20 minutes. The supernatant was removed, and sperm were washed by centrifugation at 1000 x G for 20 minutes. The sperm pellet was resuspended in 5 mL of TALP after removal of the supernatant. The concentration of sperm was obtained using a Nucleocounter (Nuclecounter™, New Brunswick Scientific; Edison, NJ). The sample was extended to 50 x 10^6sperm/mL, and a 10-µl droplet was placed onto the center of the premanufactured hyaluron coated slides with a coverslip. Slides were placed into a humidified chamber at room temperature and incubated for 10 minutes. Sperm were analyzed microscopically for visual confirmation of binding and then returned to incubation for 10 more minutes followed by a final binding evaluation. Controls were placed onto glass slides with coverslips as described for boar semen.

Upon confirmation that fresh stallion semen bound to hyaluronic acid, four frozen samples of stallion semen from Experiment 1 were analyzed for binding to assays. Stallions were selected by eliminating the two stallions with the highest and lowest post-thaw sperm motilities at 75 min, and then randomly selecting from the remaining six stallions. Straws frozen in GEY were selected for thawing by randomly choosing two samples that had been previously cooled in INRA 96 and two samples cooled in CST. Straws were placed in a 37°C water bath for 30 sec and pooled for a total volume of 2 mL containing 40 x 10^6 total sperm at a concentration of 20 x 10^6sperm/mL. TALP held at 37°C was added to the pooled sample bringing the total volume to 5 mL. Sperm were washed by centrifugation at 1000 x g for 10 min. Upon completion of centrifugation, no sperm pellet was observed, so a 100-µl sample was obtained from the bottom of the

centrifugation tube and visualized microscopically for confirmation of sperm. TALP was then added to the 100-µl sample to bring the total concentration to approximately 50 x 10^6sperm/ml. A 10-µl droplet of sperm was then placed onto the hyaluron assay, and incubated as described above for a total of 20 min. Samples placed on glass slides with coverslips served as controls.

Results

Pooled boar semen that arrived cooled had an initial total and progressive motility of 35 and 20%, respectively. Sperm were introduced to the hyaluron assay and visualized for binding after a 10-min incubation, which did not result in any bound sperm. The assay was placed back in the humidified chamber for an additional 10 minutes. Approximately 15% of boar semen was observed bound to the slide after 20 min of incubation time.

Fresh semen pooled from two stallions exhibited total and progressive motility of 65 and 26%, respectively. The semen displayed approximately 5% bound sperm after incubation on hyaluron-coated assays for 20 min. Frozen sperm were pooled and had an initial post-thaw total and progressive motility of 69 and 33%, respectively. Frozen sperm that were washed by centrifugation, as described above, did not exhibit any binding during the total 20-min incubation period. Frozen stallion sperm were agglutinated in numerous congregations of up to 50 or more sperm. The sperm seemed to be attached to one another at the head with an increased affinity, when compared to controls on glass slides. Furthermore, uncharacteristic circling, tails bent at the midpiece, and some hyperactivation was observed. Stallion sperm seemed to be affected by the

hyaluron-coated slide, but did not behave in the same manner as described for other species.

Discussion

Reports indicating that stallion sperm may have the potential to bind to hyaluronic acid are vague and unclear at best (Huzar et al. 1998b). However, stallion sperm have been shown to express a surface-associated hyaluronidase on the posterior acrosome (Meyers 2001). Binding characterization and localization of HA receptors on sperm have been described for other species (Ranganathan et al. 1994, Park et al. 2005). Boar sperm selected for ICSI using HA-binding techniques results in more normal embryos than visual selection of sperm for IVF and ICSI (Park et al. 2005). Chromosomal abnormalities in 4-cell porcine embryos are lower when sperm is selected using HA (Park et al. 2005). No adverse effects on fertilization or embryonic development have been observed in human laboratories currently utilizing HA-selected sperm (Huszar 2007). Sperm binding scores have been developed for use in human clinics and have been shown to be directly correlated to fertilization capability (Huszar et al. 2002). The success of sperm selected for ICSI using HA could have the potential to be replicated for the equine as demonstrated for other species.

The objective for this study was to see if fresh and then frozen stallion sperm could bind to HA assays. Results from pooling fresh semen from two stallions indicated that a small percentage (~5%) of sperm was bound to HA after 20 min of incubation. Sperm that had been previously cooled and then frozen did not bind to the assay. Because frozen sperm used for this experiment had been subjected to a 24 h cool prior to freezing, this process may have had a negative impact on binding ability. Sperm that

have advanced levels of capacitation or a disrupted acrosome are not able to bind to HA (Huszar et al. 2003). However, when stallion sperm are in vitro capacitated and the acrosome is shed, the inner acrosomal membrane stains positive for hyaluronidase (Meyers 2001). During capacitation, seminal proteins covering the sperm membrane are lost as well as receptors that aid in binding the sperm to the oocyte. Although capacitation of sperm was not induced, sperm acrosomal integrity may have been compromised during cooling and freezing for this experiment.

Fresh and frozen sperm exposed to HA exhibited uncharacteristic circling, tails bent at the midpiece, and some hyperactivation that needs further clarification. Hyaluronic acid containing medium has been show to increase sperm velocity and the retention of long-term motility of fresh and frozen human sperm (Huszar et al. 1990b, Sbracia et al. 1997). Hyaluronan also increased the post-thaw motility of stallion sperm when added to sperm before cooling (Bruemmer et al. 2009). Based on our observations, stallion sperm are affected by hyaluronic acid, but behave differently from what has been described for other species. Although fresh sperm pooled from two stallions appeared to bind to HA assays, the experiment should be repeated with a larger population of stallion sperm for clarification. Also, sperm appeared to be sticking to slides provided in the HBA kits. Glass or silicon slides could be used for comparative analysis.

Clearly, further evaluation of stallion sperm is necessary in order to elucidate HA binding ability. A biochemical marker that would serve to clearly distinguish a more fully functional and mature sperm from within a viable population of sperm would be of great application to ICSI in the mare.

CHAPTER V

Summary

Intracytoplasmic sperm injection is a viable assisted reproductive technique for the horse. Successful fertilization using ICSI requires a single viable sperm. The motility of stallion semen and the concentration at which sperm can be stored are different for ICSI than what is common for artificial insemination. Due to the less stringent requirements for ICSI, sperm could be held for a prolonged period of time and subjected to further processing that may otherwise result in inadequate motility and viability for other reproductive practices. We have demonstrated that stallion semen can be cooled for 24 h and frozen to provide viable sperm for ICSI. Although others have described less than adequate survival of semen subjected to cooling (Brinsko et al. 2000), the motility of cooled then frozen sperm obtained for this experiment was acceptable for ICSI.

Freezing stallion sperm at industry standard concentrations of 200 x10^6sperm/mL would be a higher concentration than what is needed for ICSI. If sperm could be frozen in very low numbers, a stallion would only need to be collected once in order to obtain billions of sperm with the potential use for ICSI. Therefore, freezing stallion sperm at the concentration of 20 x 10^6 total sperm/mL in this experiment was a practical method to conserve sperm. Stallion sperm used for ICSI by one group was typically frozen at 200 x 10^6sperm/mL (Altermatt et al. 2009). By freezing sperm at 20 x 10^6/mL, one 0.5-mL

straw contains approximately one tenth of the total amount of sperm in the same straw using current practices. The lower concentration of sperm is conservative, more user friendly, and eliminates possible contamination to unused portions of frozen semen. Potentially, with further validation, sperm can be frozen at even lower concentrations and volumes followed by swim-up selection for ICSI (see Appendix IV).

Results from the second experiment determined that sperm could be held in a medium that does not require a modified atmosphere. A medium formulated specifically for sperm performed better than those designed for other purposes. However, sperm survive equally well in all five holding media for up to 4 h. Therefore, sperm could be held in any of the five media evaluated for this experiment and maintain adequate motility to evaluate selection procedures for ICSI.

Our results using a hyaluronic acid assay to bind stallion sperm were inconclusive. The binding potential of sperm to HA has been described in other species and has been applied to sperm selection for ICSI (Ranganathan et al. 1994, Park et al. 2005). Based on our observations, it is clear that HA has an effect on stallion sperm. However, sperm behaved differently than described for other species. The lack of stallion sperm binding to HA, as determined in Experiment 3, should not be a complete surprise. As with most areas concerning reproductive physiology, the equine continues to remain elusive.

REFERENCES

Alvarenga MA, Papa FO, Landim-Alvarenga FC, Medeiros ASL. Amides as cryoprotectants for freezing stallion semen: A review. Anim Reprod Sci 2005;89:105-13.

Ahmadi A, Ng SC. Fertilizing ability of DNA-damaged spermatozoa. J Exp Zool 1999;284:696-704.

Altermatt JL, Suh TK, Stokes JE, Carnevale EM. Effects of age and equine follicle-stimulating hormone (eFSH) on collection and viability of equine oocytes assessed by morphology and developmental competency after intracytoplasmic sperm injection (ICSI). Reprod Fertil and Dev 2009;21:615-623.

Aurich C. Factors affecting the plasma membrane function of cooled-stored stallion spermatozoa. Anim Reprod Sci 2005;89:65-75.

Aurich C. Recent advances in cooled-semen technology. Anim Reprod Sci 2008;107:268-275.

Backman T, Bruemmer JE, Graham JK, Squires EL. Pregnancy rates of mares inseminated with semen cooled for 18 hours and then frozen. J Anim Sci 2004;82:690-94.

Barker CAV, Gandier JCC. Pregnancy in mare resulting from frozen epididymal spermatozoa. Can J Comp Med Vet Sci 1957;2:47-51.

Batellier F. Identification, purification et mécanismes d'action d'éléments contenus dans le lait agissant sur les spermatozoïdes équins. Thesis, François Rabelais University, Tours, France.

Batellier F, Magistrini M, Fauquant J, Palmer E. Effect of milk fractions on survival of equine spermatozoa. Theriogenology 1997;48:391-410.

Battelier F, Vidament M, Fauquant J, Duchamp G, Arnaud G, Yvon JM, Magistrini M. Advances in cooled semen technology. Anim Reprod Sci 2001;68:181-90.

Bavister BD, Yanagimachi R. The effects of sperm extracts and energy sources on the motilityand acrosome reaction of hamster spermatozoa in vitro. Biol Reprod 1977;16:228-37.

Bedford SJ, Jasko DJ, Graham JK, Amann RP, Squires EL, Pickett BW. Effect of seminal extenders containing egg yolk and glycerol on motion characteristics and fertility of stallion spermatozoa. Theriogenology 1995;43:955-67.

Bonduelle M, Van Assche E, Joris H, Keymolen K, Devroey P, Van Steirteghem A, Liebaers I. Prenatal testing in ICSI pregnancies: incidence of chromosomal anomalies in 1586 karyotypes and relation to sperm parameters. Hum Reprod 2002;17:2600-14.

Brinsko SP, Crockett EC, Squires EL. Effect of centrifugation and partial removal of seminal plasma on equine spermatozoa motility after cooling and storage. Theriogenology 2000;54:129-136.

Bruemmer JE, Wilson CH, Coutino da Silva M, Squires EL. Effects of hyaluronan supplementation on cryopreserved equine spermatozoa hyaluronan and cryopreserved equine spermatozoa. JEVS 2009;29:223-28.

Carnevale EM. Clinical considerations regarding assisted reproductive procedures in horses. JEVS 2008;28:686-690.

Cayli S, Jakab A, Ovari L. Delpiano E, Celik-Ozenci C, Sakkas D, Ward D, Huszar G. Biochemical markers of sperm function: male fertility and sperm selection for ICSI. Reprod Biomed Online 2003;7:462-68.

Chakrabarti R, Chakrabarti R. Novel role of extracellular carbon dioxide in lymphocyte proliferation in culture. J Cell Biochem 2001;83:200-3.

Choi YH, Love CC, Varner DD, Hinrichs K. Equine blastocyst development after intracytoplasmic injection of sperm subjected to two freeze-thaw cycles. Theriogenology 2005;65:808-19.

Clermont Y. The cycle of the seminiferous epithelium in man. Am J Anat 1963;7:462-68.

Cochran JD, Amann RP, Froman DP, Pickett BW. Effects of centrifugation, glycerol level, cooling to 5°C, freezing rate and thawing rate on post-thaw motility of equine sperm. Theriogenology 1984;22:25-38.

Crockett EC, Graham JK, Bruemmer JE, Squires EL. Effect of cooling of equine spermatozoa before freezing on post-thaw motility: preliminary results. Theriogenology 2001;55:793-803.

Dawson GR, Webb GW, Pruitt JA, Loughlin TM, Arns MJ. Effect of different processing techniques on motility and acrosomal integrity of cold-stored stallion spermatozoa. Proc ENPS, 1999;77-82.

Devireddy RV, Swanlund DJ, Olin T, Vincente W, Troedsson MHT, Bischof JC, Roberts KP. Cryopreservation of equine sperm: optimal cooling rates in the presence and absence

of cryoprotective agents determined using differential scanning calorimetry. Biol Reprod 2002;66:222-31.

Fernandez JL, Gosalvez J. Application of FISH to detect DNA damage: DNA breakage detection-FISH (DBD-FISH). Methods Mol Biol 2002;203:203-16.

Fernandez JL, Muriel L, Rivero MT, Goyanes V, Vazquez R, Alvarez JG. The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. J Androl 2003;24:59-66.

Good NE, Winget GD, Winter W, Connolly TN, Izawa S, Singh RM. Hydrogen ion buffers for biological research. Biochemistry 1966;5:467-77.

Graham JK, Foote RH, Parrish JJ. Effect of dilauroylphosphatidylcholine on the acrosome reaction and subsequent penetration of bull spermatozoa into zona-free hamster eggs. Biol Reprod 1986;35:413-424.

Hammadeh ME, Askari AS, Georg T, Rosenbaum P, Schmidt W. Effect of freeze-thawing procedure on chromatin stability, morphological alteration and membrane integrity of human spermatozoa in fertile and subfertile men. Int J Androl 1999;22:155-62.

Hong Y, Huang G, Gao Y, Liu DY. Relationship between human sperm-hyaluronan binding assay and fertilization rate in conventional *in vitro* fertilization. Hum Reprod 2006;21:1545-50.

Huszar G, Corrales M, Vigue L. Correlation between sperm creatine phosphokinase activity and sperm concentrations in normozoospermic and oligozoospermic men. Gamete Res 1988a;19:67-75.

Huszar G, Vigue L, Corrales M. Sperm creatine phosphokinase activity as a measure of sperm quality in normozoospermic, variablespermic, and oligozoospermic men. Biol Reprod 1988b;38:1061-66.

Huszar G, Willetts M, Corrales M. Hyaluronic Acid (Sperm Select) improves retention of sperm motility and velocity in normozoospermic and oligozoospermic specimens. Fertil and Steril 1990b;54:1127-34.

Huszar G, Vigue L, Morshedi M. Sperm creatine phosphokinase M-isoform ratios and fertilizing potential of men: a blinded study of 84 couples treated with in vitro fertilization. Fert and Steril 1992;57:882-88.

Huszar G, Vigue L, Oehninger S. Creatine kinase immunocytochemistry of human sperm-hemizona complexes: selective binding of sperm with mature creatine kinase-staining pattern. Fetil and Steril 1994;61:136-142.

Huszar G, Sbracia M, Vigue L. Miller DJ, Shur BD. Sperm plasma membrane remodeling during spermiogenetic maturation in men: relationship among plasma membrane beta 1,4-galactosyltransferase, cytoplasmic creatine phosphokinase, and creatine phosphokinase isoform ratios. Biol Reprod 1997;56:1020-24.

Huszar G, Patrizio P, Vigue L, Willets M, Wilker C, Adhoot D, Johnson L. Cytoplasmic extrusion and the switch from creatine kinase B to M isoform are completed by the commencement of epididymal transport in human and stallion spermatozoa. J Androl 1998b;19:11-20.

Huszar G, Stone K, Dix D, Vigue L. Putative creatine kinase M-isoform in human sperm is identified as the 70-kilodalton heat shock protein HspA2. Biol Reprod 2000;63:925-32.

Huszar G, Celik-Ozenci C, Vigue L. Sperm maturity and fertility: testing by hyaluronic acid binding. Abstracts of the 18th Annual Meeting of the ESHRE, Vienna, Austria. Hum Reprod 2002;17 (suppl. 1):O-024.

Huszar G, Ozenci CC, Cayli S, Zavaczki K, Hansch E, Vigue L. Hyaluronic acid binding by human sperm indicates cellular maturity, viability, and unreacted acrosomal status. Fertil Steril 2003;79:1616-24.

Huszar G. Fertility testing and ICSI sperm selection by hyaluronic acid binding: clinical and genetic aspects. Reprod BioMedicine Online 2007;14:650-63.

Irvine DS, Twigg JP, Gordon EL, Fulton N, Milne PA, Aitken RJ. DNA integrity in human spermatozoa: relationships with semen quality. J Androl 2000;21:33-44.

Jakab A, Sakkas D, Delpiano E, Cayli S, Kovanci E, Ward D, Revelli A, Huszar G. Intracytoplasmic sperm injection: a novel selection method for sperm with normal frequency of chromosomal aneuploidies. Fertil Steril 2005;84:1665-73.

Jasko DJ, Hathaway JA, Schaltenbrand VL, Simper WD, Squires EL. Effect of seminal plasma dilution or removal on spermatozoal motion characteristics of cooled stallion semen. Theriogenology 1991;35:1059-67.

Jasko DJ, Moran DM, Farlin ME, Squires EL. Effect of seminal plasma and egg yolk on motion characteristics of cooled stallion spermatozoa. Theriogenology 1992;37:1241-52.

Kenney RM, Bergman RV, Cooper WL, Morse GW. Minimal contamination techniques for breeding mares: Technique and preliminary findings. Proc. 21st Ann.Conv. A.A.E.P. 1975;327-36.

Larson KL, DeJonge CJ, Barnes AM, Jost LK, Evenson DP. Sperm chromatin structure assay as predictors of failed pregnancy following assisted reproductive techniques. Hum. Reprod 2000;15:1717-22.

Lazzari G, Crotti G, Turini P, Duchi R, Mari G, Zavaglia G, Barabcini S, Galli C. Equine embryos at the compacted morula and blastocyst stage can be obtained by intracytoplasmic sperm injection (ICSI) of in vitro matured oocytes with frozen-thawed spermatozoa from semen of different fertilities. Theriogenology 2002;58:709-12.

Lovelock JE. Haemolysis of human red blood-cells by freezing and thawing. Biochim Biophys Acta 1953;10:414-26.

Mansour RT, Serour MG, Abbas AM, Kamal A, Tawab NA, Aboulghar MA, Serour GI. The impact of spermatozoa preincubation time and spontaneous acrosome reaction in intracytoplasmic sperm injection: a controlled randomized study. Fertil Steril 2008;90(3):584-91.

Martin JC, Klug E, Gunzel A. Centrifugation of stallion semen and its storage in large volume straws. J Reprod Fertil Suppl 1979;27:47-51.

Mazur P, Leibo SP, Chu EHY. A two-factor hypothesis of freezing injury. Exp Cell Res 1972;71:345-55.

Mazur P. Freezing of living cells: mechanisms and implications. Am J Physiol 1984;247:C125-42.

Meng Li, Wolf DP. Sperm-induced oocyte activation in the rhesus monkey: nuclear and cytoplasmic changes following inracytoplasmic sperm injection. Hum Reprod 1997;12:1062-68.

Meyers SA. Equine sperm-oocyte interaction: the role of sperm surface hyaluronidase. Anim Reprod Sci. 2001;68:291-303.

Moran DM, Jasko DJ, Squires EL, Amann RP. Determination of temperature and cooling rate which induce cold shock in stallion spermatozoa. Theriogenology 1992;38:999-1012.

Nasr-Esfahani MH, Razavi S, Vahdati AA, Fathi F, Tavalaee M. Evaluation of sperm selection procedure based on hyaluronic acid binding ability on ICSI outcome. J Assist Reprod Genet 2008;25:197-203.

Olson SE, Seidel GE Jr. Reduced oxygen tension and EDTA improve bovine zygote development in a chemically defined medium. J Anim Sci 2000;78:152-57.

Palmer E. Factors affecting stallion semen survival and fertility. In: Proceedings of the 10th International Congress on Animal Reproduction, AI, USA;1984:377–78.

Park CY, Uhm SJ, Song SJ, Kim KS, Hong SB, Chung KS, Park C, Lee HT. Increase of ICSI efficiency with hyaluronic acid binding sperm for low aneuploidy frequency in pig. Theriogenology 2005;64:1158-69.

Pickett BW, Sullivan JJ, Byers WW, Pace MM, Remmenga EE. Effect of centrifugation and seminal plasma on motility and fertility of stallion and bull spermatozoa. Fertil Steril 1975;26:167-74.

Quinn PJ, White IG. The effect of pH, cations and protective agents on the susceptibility of ram spermatozoa to cold shock. Exp Cell Res 1968;49:31-39.

Ranganathan S, Ganguly AK, Datta K. Evidence for presence of hyaluronan binding protein on spermatozoa and its possible involvement in sperm function. Mol Reprod Dev 1994;38:69-76.

Sati L, Ovari L, Demir R, Ward DC, Bray-Ward P, Huszar G. Persistent histones in immature sperm are associated with DNA fragmentation and affect paternal contribution of sperm: A study of aniline blue staining, fluorescence *in situ* hybridization (FISH) and DNA nick translation. Fertil Steril 2004;82(suppl. 2):O-129.

Sbracia M, Grasso J, Sayme N, Stronk J, Huszar G. Hyaluronic acid substantially increases the retention of motility in cryopreserved/thawed human spermatozoa. Hum Reprod 1997;12:1949-54.

Scherzer J, Fayrer-Hosken RA, Aceves M, Hurley DJ, Ray LE, Jones L, Heusner GL. Freezing equine semen: the effect of combinations of semen extenders and glycerol on post-thaw motility. Aust Vet J 2009;87:275-9.

Simpson JL, Lamb DJ. Genetic effects of intracytoplasmic sperm injection. Semin Reprod Med 200;19:239-49.

Sousa M, Tesarik J. Ultrastructural analysis of fertilization failure after intracytoplasmic spermatozoa injection. Hum Reprod 1994;9:2374-80.

Smith AU, Polge C. Survival of Spermatozoa at Low Temperatures. Nature 1950;166:668-9.

Squires EL, Pickett BW, Graham JK, Vanderwall DK, McCue PM, Bruemmer JM. Cooled and Frozen Stallion Semen. Colorado State University, 1999.

Squires EL, Carnevale EM, McCue PM, Bruemmer JE. Embryo technologies in the horse. Theriogenology 2003;59(1):151-70.

Varner DD, Blanchard TL, Love CL, Garcia MC, Kenney RM. Effects of cooling rate and storage temperature on equine spermatozoal motility parameters. Theriogenology 1987;29:1043-54.

Vidament M, Ecot P, Noue P, Bourgeois C, Magistrini M, Palmer E. Centrifugation and addition of glycerol at 22°C instead of 4°C improve post-thaw motility and fertility of stallion spermatozoa. Theriogenology 2000;54:907-19.

Vidament M, Yvon JM, Couty I, Arnaud G, Nguekam-Feugang J, Noue P, Cottron S, Le Tellier A, Noel F, Palmer E, Magistrini M. Advances in cryopreservation of stallion semen in INRA82. Anim Reprod Sci 2001;68:201-18.

Wilhelm KM, Graham JK, Squires EL. Comparison of the fertility of cryopreserved stallion spermatozoa with sperm motion analyses, flow cytometric evaluation, and zona-free hamster oocyte penetration. Theriogenology 1996;46:559-578.

Worrilow KC, Huynh HT, Bower JB, Peters AJ, Johnston JB. The clinical impact associated with the use of PICSI-derived embryos. Abstracts of the Scientific Oral and Poster Sessions, 62nd Annual Meeting of the American Society for Reproductive Medicine, New Orleans, LA. Fertil Steril 2006;86 (suppl. 2):O-146.

Yeoman RR, Mitalipov S, Gerami-Naini B, Nusser KD, Wolf DP. Low temperature storage of rhesus monkey spermatozoa and fertility evaluation by intracytoplasmic sperm injection. Theriogenology 2005;63: 2356-71.

APPENDIX I: EXPERIMENT 1

RECIPES FOR FREEZING DILUENTS FOR EXPERIMENT 1 AND RAW DATA

Modified-French (FR8)

Solution A:

Glucose	277mM
Lactose	8mM
Raffinose	5mM
Na Citrate	1.7mM
K Citrate	2.5mM
HEPES	60mM

Solution B:

Sanalac (Dry Skim Milk)	144mg/ml
Glucose	55mM

300-310 mOsM, pH = 7.2

Add solution A and solution B 1:1 (v:v)
Add 3% egg yolk by volume
Centrifuge 7000 x g for 30 min and filter
Add glycerol for cryoprotectant by volume

Lactose-EDTA (LAC10)

Lactose	153mM
Glucose-EDTA	25% by volume
Egg Yolk	20% by volume
Equex STM	0.5% by volume

Centrifuge at 7000 x g for 30 min and filter
Add glycerol for cryoprotectant by volume

Glycerol Egg Yolk (GEY)
Add 8% egg yolk by volume
Add glycerol for cyoprotectant by volume

Add egg yolk and glycerol to CST and INRA96
cooling diluents, respectively

Glycerol (G)

Add 8% glycerol by volume to CST and INRA96
cooling diluents, respectively

CST8 and INRA8
Add 8% glycerol by volume respectively to each cooling diluent

Total and progressive motility of stallion semen cooled for 24 h in CST and INRA96 (n = 8 stallions).

CST (TM)	Stallion	0	45	75	INRA	0	45	75
	Premiero	38	57	35		22	60	23
	Scotti	45	71	80		50	71	64
	Tinman	91	64	57		83	82	67
	Sammy	85	68	50		88	85	70
	HR Dually	37	20	35		36	37	42
	Capote	47	34	22		48	23	13
	Sylekt	40	58	66		38	47	49
	L Merada	74	77	64		64	77	70
	Mean	57	56	51		54	60	50

CST (PM)	Stallion	0	45	75	INRA	0	45	75
	Premiero	10	29	20		7	38	13
	Scotti	8	45	70		17	55	32
	Tinman	38	22	33		68	13	9
	Sammy	5	16	10		15	37	20
	HR Dually	2	7	14		7	12	20
	Capote	2	2	3		10	7	3
	Sylekt	10	17	44		13	44	41
	L Merada	3	41	40		17	49	57
	Mean	10	22	29		19	32	24

Percentage of post-thaw total motility for individual stallions cooled in CST from Experiment 1 at 0, 45 and 75 min for four freezing treatments.

FR8	0		45		75	
Stallion	TM	PM	TM	PM	TM	PM
Premiero	2	2	6	6	7	1
Scotti	23	15	13	11	24	16
Tinman	25	19	8	0	11	2
Sammy	82	36	61	26	31	11
HR Dually	8	2	5	3	4	1
Capote	18	8	7	1	0	0
Sylekt	7	2	23	18	18	19
L Merada	30	9	33	17	28	17

LAC10	0		45		75	
Stallion	TM	PM	TM	PM	TM	PM
Premiero	5	2	4	3	6	2
Scotti	28	13	19	12	14	10
Tinman	42	19	9	0	2	0
Sammy	42	10	11	6	13	1
HR Dually	1	0	1	0	3	2
Capote	2	2	0	0	0	0
Sylekt	2	1	2	2	6	0
L Merada	25	10	15	6	18	10

G	0		45		75	
Stallion	TM	PM	TM	PM	TM	PM
Premiero	3	3	0	0	0	0
Scotti	10	5	8	2	4	0
Tinman	25	10	7	0	13	2
Sammy	63	11	14	4	18	1
HR Dually	7	1	6	0	4	2
Capote	10	0	0	0	0	0
Sylekt	7	3	0	0	0	0
L Merada	19	7	22	8	23	1

GEY	0		45		75	
Stallion	TM	PM	TM	PM	TM	PM
Premiero	6	2	11	2	12	0
Scotti	26	15	25	18	14	9
Tinman	40	24	15	3	19	5
Sammy	36	2	34	7	22	13
HR Dually	5	0	7	4	5	1
Capote	9	0	3	0	6	0
Sylekt	6	4	12	7	4	1
L Merada	44	11	36	15	36	18

Percentage of post-thaw total motility for individual stallions cooled in INRA96 from Experiment 1 at 0, 45 and 75 min for four freezing treatments.

FR8	0		45		75	
Stallion	TM	PM	TM	PM	TM	PM
Premiero	7	5	4	13	0	0
Scotti	35	20	20	16	42	30
Tinman	32	16	9	2	12	5
Sammy	78	24	36	11	29	5
HR Dually	10	3	9	3	5	2
Capote	12	2	0	0	0	0
Sylekt	9	1	6	3	5	1
L Merada	37	14	22	13	29	17

LAC10	0		45		75	
Stallion	TM	PM	TM	PM	TM	PM
Premiero	3	1	1	1	0	0
Scotti	10	9	9	6	10	4
Tinman	26	4	3	0	2	1
Sammy	57	17	53	28	25	7
HR Dually	4	2	4	0	2	1
Capote	2	1	0	0	0	0
Sylekt	2	1	1	0	3	3
L Merada	12	11	31	13	13	6

G	0		45		75	
Stallion	TM	PM	TM	PM	TM	PM
Premiero	5	4	11	5	0	0
Scotti	43	33	22	12	12	6
Tinman	13	5	7	0	0	0
Sammy	38	10	33	12	11	4
HR Dually	3	0	3	3	4	2
Capote	6	0	0	0	0	0
Sylekt	3	2	3	2	2	0
L Merada	23	16	28	13	10	3

GEY	0		45		75	
Stallion	TM	PM	TM	PM	TM	PM
Premiero	26	16	3	2	0	0
Scotti	46	20	30	19	26	16
Tinman	16	10	6	2	13	7
Sammy	68	29	45	21	33	13
HR Dually	11	5	14	4	10	6
Capote	3	0	0	0	0	0
Sylekt	6	4	11	5	5	5
L Merada	44	15	42	23	41	34

Combined post thaw total motility at 0, 45 and 75 min for sperm frozen in FR8, LAC10, G, and GEY from Experiment 1 (n = 16 samples). Cooling diluent (CST or INRA96) is shown for each sample.

FR8

	0	45	75	
Stallion				
Premiero	2	6	7	CST
	7	4	0	INRA
Scotti	23	13	24	CST
	35	20	42	INRA
Tinman	25	8	11	CST
	32	9	12	INRA
Sammy	82	61	31	CST
	78	36	29	INRA
HR Dually	8	5	4	CST
	10	9	5	INRA
Capote	18	7	0	CST
	12	0	0	INRA
Sylekt	7	23	18	CST
	9	6	5	INRA
L Merada	30	33	28	CST
	37	22	29	INRA
Average	26	16	15	
SEM	6	4	3	

Combined post thaw total motility at 0, 45 and 75 min for sperm frozen in FR8, LAC10, G, and GEY from Experiment 1 (n = 16 samples). Cooling diluent (CST or INRA96) is shown for each sample.

LAC10

	0	45	75	
Stallion				
Premiero				
	5	4	6	CST
Scotti	3	1	0	INRA
	28	19	14	CST
Tinman	10	9	10	INRA
	42	9	2	CST
Sammy	26	3	2	INRA
	42	11	13	CST
HR Dually	57	53	25	INRA
	1	1	3	CST
Capote	4	4	2	INRA
	2	0	0	CST
Sylekt	2	0	0	INRA
	2	2	6	CST
L Merada	2	1	3	INRA
	25	15	18	CST
	12	31	13	INRA
Average	16	10	7	
SEM	5	4	2	

Combined post thaw total motility at 0, 45 and 75 min for sperm frozen in FR8, LAC10, G, and GEY from Experiment 1 (n = 16 samples). Cooling diluent (INRA96 or CST) is shown for each sample.

G

	0	45	75	
Stallion				
Premiero	3	0	0	CST
	5	11	0	INRA
Scotti	10	8	4	CST
	43	22	12	INRA
Tinman	25	7	13	CST
	13	7	0	INRA
Sammy	63	14	18	CST
	38	33	11	INRA
HR Dually	7	6	4	CST
	3	3	4	INRA
Capote	10	0	0	CST
	6	0	0	INRA
Sylekt	7	0	0	CST
	3	3	2	INRA
L Merada	19	22	23	CST
	23	28	10	INRA
Average	17	10	6	
SEM	4	3	2	

Combined post thaw total motility at 0, 45 and 75 min for sperm frozen in FR8, LAC10, G, and GEY from Experiment 1 (n = 16 samples). Cooling diluent (INRA96 or CST) is shown for each sample.

GEY

	0	45	75	
Stallion				
Premiero	6	11	12	CST
	26	3	0	INRA
Scotti	26	25	14	CST
	46	30	26	INRA
Tinman	40	15	19	CST
	16	6	13	INRA
Sammy	36	34	42	CST
	68	45	33	INRA
HR Dually	5	7	5	CST
	11	14	10	INRA
Capote	9	3	6	CST
	3	0	0	INRA
Sylekt	6	12	4	CST
	6	11	5	INRA
L Merada	44	36	36	CST
	44	42	41	INRA
Average	25	18	17	
SEM	5	4	4	

Combined post thaw progressive motility at 0, 45 and 75 min for sperm frozen in FR8, LAC10, G, and GEY from Experiment 1 (n = 16 samples). Cooling diluent (INRA96 or CST) is shown for each sample.

FR8

	0	45	75	
Stallion				
Premiero	2	6	1	CST
	5	4	0	INRA
Scotti	15	11	16	CST
	20	16	30	INRA
Tinman	19	0	2	CST
	16	2	5	INRA
Sammy	36	26	11	CST
	24	11	5	INRA
HR Dually	2	3	1	CST
	3	3	2	INRA
Capote	8	1	0	CST
	2	0	0	INRA
Sylekt	2	18	19	CST
	1	3	1	INRA
L Merada	9	17	17	CST
	14	13	17	INRA
Average	11	8	8	
SEM	3	2	2	

Combined post thaw progressive motility at 0, 45 and 75 min for sperm frozen in FR8, LAC10, G, and GEY from Experiment 1 (n = 16 samples). Cooling diluent (INRA96 or CST) is shown for each sample.

LAC10

	0	45	75	
Stallion				
Premiero	2	3	2	CST
	1	1	0	INRA
Scotti	13	12	10	CST
	9	6	4	INRA
Tinman	19	0	0	CST
	4	0	1	INRA
Sammy	10	6	1	CST
	17	28	7	INRA
HR Dually	0	0	2	CST
	2	0	1	INRA
Capote	2	0	0	CST
	1	0	0	INRA
Sylekt	1	2	0	CST
	1	0	3	INRA
L Merada	10	6	10	CST
	11	13	6	INRA
Average	6	5	3	
SEM	2	2	1	

Combined post thaw progressive motility at 0, 45 and 75 min for sperm frozen in FR8, LAC10, G, and GEY from Experiment 1 (n = 16 samples). Cooling diluent (INRA96 or CST) is shown for each sample.

G

	0	**45**	**75**	
Stallion				
Premiero	3	0	0	CST
	4	5	0	INRA
Scotti	5	2	0	CST
	33	12	6	INRA
Tinman	10	0	2	CST
	5	0	0	INRA
Sammy	11	4	1	CST
	10	12	4	INRA
HR Dually	1	0	2	CST
	0	3	2	INRA
Capote	0	0	0	CST
	0	0	0	INRA
Sylekt	3	0	0	CST
	2	2	0	INRA
L Merada	7	8	1	CST
	16	13	3	INRA
Average	7	4	1	
SEM	2	1	1	

Combined post thaw progressive motility at 0, 45 and 75 min for sperm frozen in FR8, LAC10, G, and GEY from Experiment 1 (n = 16 samples). Cooling diluent (INRA96 or CST) is shown for each sample.

GEY

	0	45	75	
Stallion				
Premiero	2	2	0	CST
	16	2	0	INRA
Scotti	15	18	9	CST
	20	19	16	INRA
Tinman	24	3	5	CST
	10	2	7	INRA
Sammy	2	7	13	CST
	29	21	13	INRA
HR Dually	0	4	1	CST
	5	4	6	INRA
Capote	0	0	0	CST
	0	0	0	INRA
Sylekt	4	7	1	CST
	4	5	5	INRA
L Merada	11	15	18	CST
	15	23	34	INRA
Average	10	8	8	
SEM	2	2	2	

Effect of freezing diluent on total and progressive motility parameters of equine sperm after thawing for semen cooled for 24 h (CST and INRA96) and frozen (means ± SEM).

	Time Post Thaw (min)					
Treatment	**0**		**45**		**75**	
	TM	PM	TM	PM	TM	PM
FR8	26 ± 6	11 ± 3	16 ± 4	8 ± 2	15^{a} ± 3	8^{a} ± 2
LAC10	16 ± 4	6 ± 2	10 ± 4	5 ± 2	$7^{b,c}$ ± 2	$3^{a,b}$ ± 1
G	17 ± 4	7 ± 2	10 ± 3	4 ± 1	6^{c} ± 2	1^{b} ± 0.4
GEY	25 ± 5	10 ± 2	18 ± 4	8 ± 2	17^{a} ± 4	8^{a} ± 2

Abbreviations: TM, total motility; PM, progressive motility.

^{a,b,c} Values in the same column with different superscripts are different ($P < 0.05$).

APPENDIX II: EXPERIMENT 2

RECIPE FOR TALP DILUENT FOR EXPERIMENT 2 AND RAW DATA

Composition of media used (mM)

Ingredient	
NaCl	97.4
KCl	3.1
Na_2HPO4	0.3
Na HCO3	24.9
$CaCl_2$	2.0
$MgCl_2$	0.4
Na Pyruvate	0.2
Glucose	5
Lactate	3.60mL
HEPES	10
BSA	3g

Adjust Osmolality 301 and pH 7.0

Appendix 2a. Percentages of total motile (TM) sperm from individual stallions from Experiment 2 held in five different mediums for 1 h incubation at 37°C.

Stallion	GIVF	FCDM	TALP	EMCARE	MOJO
1	82	65	78	63	64
2	81	62	90	79	92
3	66	40	68	40	67
4	85	66	83	48	52
5	72	80	68	41	59
6	26	33	52	26	21
7	72	83	78	60	71
Mean	69	61	74	51	61
SEM	8	7	5	7	8

$p = 0.21$ among groups at 1 hr

Appendix 2b. Percentages of progressively motile (PM) sperm from individual stallions from Experiment 2 held in five different mediums for 1 h incubation at 37°C.

Stallion	GIVF	FCDM	TALP	EMCARE	MOJO
1	12	15	41	20	24
2	7	14	47	22	20
3	5	0	7	9	14
4	35	27	33	21	24
5	2	14	17	7	5
6	8	6	23	6	11
7	8	21	36	34	26
Mean	11	14	29	17	18
SEM	4	3	5	4	3

$p = 0.06$ among groups at 1 h

Appendix 2c. Percentages of total motile (TM) sperm from individual stallions from Experiment 2 held in five different mediums for 2 h incubation at 37°C.

Stallion	GIVF	FCDM	TALP	EMCARE	MOJO
1	68	76	64	62	51
2	65	63	63	68	80
3	72	45	65	45	52
4	78	67	51	68	75
5	73	58	69	20	20
6	48	12	55	20	15
7	33	65	66	49	49
Mean	63	55	62	47	49
SEM	6	8	2	8	9

$p = 0.45$ among groups at 2 h

Appendix 2d. Percentages of progressively motile (PM) sperm from individual stallions from Experiment 2 held in five different mediums for 2 h incubation at 37°C.

Stallion	GIVF	FCDM	TALP	EMCARE	MOJO
1	7	10	10	20	17
2	10	8	12	21	21
3	8	1	7	10	7
4	27	15	13	29	47
5	15	0	9	3	1
6	11	4	26	6	6
7	5	18	19	24	8
Mean	12	8	14	16	15
SEM	3	3	3	4	6

$p = 0.42$ among groups at 2 h

Appendix 2e. Percentages of total motile (TM) sperm from individual stallions from Experiment 2 held in five different mediums for 3 h incubation at 37°C.

Stallion	GIVF	FCDM	TALP	EMCARE	MOJO
1	51	79	61	53	50
2	80	52	87	48	64
3	68	31	41	46	34
4	50	42	83	38	49
5	50	21	64	41	18
6	31	14	33	14	13
7	21	47	65	43	35
Mean	50	41	62	40	38
SEM	8	8	8	5	7

$p = 0.12$ among groups at 3 h

Appendix 2f. Percentages of progressively motile (PM) sperm from individual stallions from Experiment 2 held in five different mediums for 3 h incubation at 37°C.

Stallion	GIVF	FCDM	TALP	EMCARE	MOJO
1	5	6	15	15	14
2	25	6	36	14	20
3	1	1	4	6	4
4	20	11	43	8	12
5	7	10	5	3	2
6	11	6	18	6	2
7	3	20	28	14	6
Mean	10	9	21	9	9
SEM	3	2	6	2	3

$p = 0.12$ among groups at 3 h

Appendix 2g. Percentages of total motile (TM) sperm from individual stallions from Experiment 2 held in five different mediums for 4 h incubation at 37°C.

Stallion	GIVF	FCDM	TALP	EMCARE	MOJO
1	43	51	46	8	33
2	64	76	87	24	46
3	36	33	71	42	27
4	11	33	56	30	24
5	40	20	35	30	25
6	41	11	20	15	6
7	8	49	60	49	28
Mean	35	39	54	28	27
SEM	7	8	9	5	5

$p = 0.09$ among groups at 4 h

Appendix 2h. Percentages of progressively motile (PM) sperm from individual stallions from Experiment 2 held in five different mediums for 4 h incubation at 37°C.

Stallion	GIVF	FCDM	TALP	EMCARE	MOJO
1	12	17	13	2	15
2	11	7	42	5	15
3	4	3	12	4	4
4	6	15	26	17	7
5	5	1	3	9	3
6	20	1	12	3	2
7	1	10	30	13	4
Mean	10	8	20	8	7
SEM	2	3	5	2	2

$p = 0.08$ among grops at 4 h

Appendix 2i. Percentages of total motile (TM) sperm from individual stallions from Experiment 2 held in five different mediums for 5 h incubation at 37°C.

Stallion	GIVF	FCDM	TALP	EMCARE	MOJO
1	26	54	54	19	32
2	58	69	92	25	50
3	33	11	69	46	31
4	9	29	52	20	31
5	15	18	36	23	17
6	21	7	26	15	15
7	8	21	52	54	19
Mean	24	30	54	29	28
SEM	7	9	8	6	5

p = 0.03 among groups at 5 h

Appendix 2j. Percentages of progressively motile (PM) sperm from individual stallions from Experiment 2 held in five different mediums for 5 h incubation at 37°C.

Stallion	GIVF	FCDM	TALP	EMCARE	MOJO
1	9	5	28	1	14
2	9	12	31	3	8
3	2	1	16	10	6
4	3	7	29	7	16
5	2	2	2	3	3
6	5	3	12	4	4
7	2	7	13	14	2
Mean	5	5	19	6	8
SEM	1	1	4	2	2

p = 0.004 among groups at 5 h

APPENDIX III

PROPOSAL AND PROTOCOL FOR APPLICATION OF A SEPHADEX/GLASS WOOL COLUMN TO SELECT FROZEN SPERM FOR ICSI

Specific Aim 1: Determine if a population of motile sperm can be obtained using a sephadex/glass wool column for frozen sperm previously subjected to a 24 h cool.

Hypothesis A: Cryopreserved stallion sperm frozen at low concentrations with seminal plasma following a 24 h cool can be further processed using a sephadex/glass wool technique in order to obtain a sub-population of viable sperm for ICSI.

Specific Aim 2: Evaluate the total number of sperm that can be obtained from frozen semen after further processing using a sephadex/glass wool technique.

Hypothesis B: Frozen-thawed stallion sperm can be subjected to further processing and result in an adequate number of sperm for ICSI.

Experimental Design

Previously frozen sperm from three stallions utilized in Experiment 1 were selected for use in the following experiment. One 0.5ml straw of sperm frozen in FR8 medium and one 0.5-ml straw of sperm frozen in GEY medium were thawed and subjected to a sephadex/glass wool column for each of the three stallions.

Protocol

1. A solution of 20% sephadex-gel was prepared by incubating 10 g of sephadex beads (SIGMA-ALDRICH® St. Louis, MO) in 50 mL of TALP (Appendix II). Ticarcillin was added to control bacterial growth. The gel was incubated for 24 h at 4°C allowing beads to settle before adding sperm.
2. Cut portions (1 cm^2) of glass wool were placed at the bottom of 3-mL plastic columns with mesh filters and layered until the wool reached 0.3 mL high on the plastic column.
3. Glass wool was overlayed with 1.5 mL of 20% sephadex-gel warmed to 37°C.
4. One 0.5-mL straw of sperm from Experiment 1 containing 10 $x10^6$ total sperm at a concentration of 20 x 10^6/sperm/ml was thawed at 37°C for 30 sec. Thawed sperm was placed over the top of the sephadex gel.

5. TALP (1 to 1.5 mL) held at 37°C was added over the sperm to aid in filtration of sperm through the sephadex/glass wool column, out through the filter and into 0.5 mL plastic collection tubes.
6. Sperm (0.5 mL) were collected through filtration and evaluated under microscope to confirm. If no sperm were present, TALP was added to the column to aid in filtration, and 0.5 mL of additional sample was collected through filtration.

Preliminary Results

Sperm cooled for 24 h and then frozen without the removal of seminal plasma were obtained through a sephadex/glass wool column as confirmed by microscopic evaluation. However, no more than approximately 5 sperm were seen after filtration, so samples were centrifuged. After centrifugation at 320 x G for 5 min, the concentration of sperm were increased approximately 10 fold but all sperm were dead as indicated by detached heads, tails, and midpieces. This observation could have been caused by sephadex beads that escaped the mesh filter located at the base of the plastic conical. The beads could have caused mass destruction to sperm during the centrifugation process.

APPENDIX IV

PROTOCOL TO SELECT SPERM FROZEN AT A LOW CONCENTRATION FOR ICSI USING A MODIFIED SWIM-UP TECHNIQUE

For sperm frozen at 20 x 10^6/sperm/mL in 0.5-mL straws:

1. Place 1 ml of TALP at 37°C in a 3-mL glass tube to prepare for addition of sperm.
2. Thaw straw of sperm and slowly expel 200 µl of sperm below TALP at the base of tube, being careful to keep as much sperm as possible at the base of the tube.
3. Place sample containing sperm and TALP at a 45° angle inside an incubator held at 37°C for 30 min to allow sperm time to swim up into supernatant.
4. Aspirate 50 µl of sample from top of the media and count concentration of sperm that has swum up during the incubation period using the NucleCounter™.
5. Aspirate 500 µl of media from the top layer of the swim up sample while taking care not to contaminate aspirated sample with sperm that has not swum up. This technique should be done while maintaining the 3 mL tube at the 45° angle.
6. Place 500-µl sample into a 1.6-mL micro centrifugation tube and centrifuge at 320 x G for 5 min. Centrifugation at a controlled speed can be accomplished by placing the micro centrifugation tube into a 50 mL conical tube in a centrifuge with adjustable speed.
7. After centrifugation, aspirate a 5.5µl sample of media containing sperm from the bottom of the micro centrifugation tube and evaluate using the CASA™.

Preliminary Results

A viable population of sperm was obtained from sperm that had been previously cooled for 24 h before freezing without the removal of seminal plasma. The concentration obtained after centrifugation appeared adequate for ICSI. Sperm aspirated from the top of the swim-up tube were analyzed using the NucleoCounter. Sperm that were not motile and considered dead after a 24 h cool were used as a control. Occasionally, the NucleoCounter gave a positive reading indicating the presence of sperm aspirated from the top of some of the control samples from sperm that did not survive the cooling process. This method of analyzing concentration may not be accurate due to possible contamination when sperm is initially added before incubation and swim up.