IMPROVING THE POST-THAW VIABILITY OF CRYOPRESERVED STALLION SPERMATOZOA

by

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(Under the Direction of Richard Fayrer-Hosken)

ABSTRACT

The present study was to improve the success for the cryopreservation of stallion spermatozoa, especially as the costs of using low post-thaw motility or sub-optimal fertility deters owners and breeders from using frozen semen. The aim was to develop a freezing extender/process that supports cell survivability throughout the freezing. Eleven stallions were collected and frozen in INRA 96 with two different concentrations of glycerol added. The mean post-thaw motility as assessed by percent alive (57.93% and 66.50%) was significantly ($p<0.05$) higher for two experimental glycerol concentrations (3.5% and 6.0%) when compared to the control, Minitube Cryoguard egg-yolk extender (39.71%). The Minitube semen freezing protocol has been one of the processes that we typically use for freezing equine spermatozoa. The addition of glycerol (6.0% concentration) to INRA 96 had the highest survivability and was used in the fertility trials. To evaluate the fertility of the frozen semen, eight mares were bred over two cycles with both fresh and frozen semen. The pregnancy rate of mares bred with frozen semen (55.56%) was not statistically ($p<0.05$) different from the pregnancy rate of mares bred with fresh semen (55.56%). INRA 96 with 6.0% glycerol improved the survivability of stallion spermatozoa through the cryopreservation process, and subsequent fertility was not statistically ($p<0.5$) different from fresh, extended semen.

INDEX WORDS: Stallion, semen freezing, INRA 96

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

In 1776, the first freezing of spermatozoa occurred by a scientist named Spallanzani. He noted sperm cells cooled in the snow became inactive, but were revived when warmed. It wasn't until 1866, however, that the first real interest in cryopreservation of human spermatozoa occurred, and the first successful protocols weren't seen until the 1940s. The 20th century also showed an adaptation of cryopreservation for the animal sciences. In the 1950s, glycerol was first introduced as a successful cryoprotectant for bull spermatozoa and in 1951 the first calf was born using AI frozen-thaw semen. In 1957 the first foal was born using the same technique. Since then, much research has been done to improve post-thaw survival and fertility (Samper 2001).

Cryopreservation is the storage of cells or tissues at -196^{O}C. Fundamental cryobiology is an understanding of what effect low temperatures have on cellular systems. The use of this knowledge leads to an improvement of freezing protocols. Cryopreservation of reproductive cells and tissues has brought about significant advancements in the field of agriculture, including international exchange of germ cells of genetically superior animals, storage of scientifically important murine lines, and conservation of endangered species (Woods 2004).

Cryopreservation has been more widely used in the equine industry during the past few decades, including recent acceptance of frozen semen use by Arabian, Quarter Horse, and Paint Horse Associations. There are many advantages to the use of frozen semen. As with any form of artificial insemination, the mare and stallion are protected against injury that could occur during live cover. Overuse of the stallion is minimized because the freezing process can occur in the off-season. The stallion's show schedule does not need to be interrupted for a collection. International shipment is

available since delays will not affect the sperm's viability. The semen can also be available anytime prior to insemination. And lastly, problem stallions can be frozen and then gelded (Alvarenga, Papa et al. 2005).

The Equine Spermatozoon

Spermatogenesis

Spermatogenesis is the formation of spermatozoa within the seminiferous tubules of the testes. The germ cells are located within the epithelium of the seminiferous tubules, and are where sperm cell development occurs. Cross-sections of the seminiferous tubules will show multiple levels of sperm cell development due to the continuous division of germ cells. Spermatogenesis can be divided into three stages: spermatocytogenesis, meiosis, and spermiogenesis (Johnson 2000).

Spermatocytogenesis is also known as the proliferative phase (Abou-Haila 2000). The germ cells divide by mitosis, producing diploid A-type spermatogonia. A-type spermatogonia contain no heterochromatin. Heterochromatin is highly condensed areas of DNA and proteins, causing transcriptional inactivity. The absence of heterochromatin allows for rapid cell proliferation, therefore the A-type spermatogonia can either replenish the germ cell population or further develop into one of five different types of spermatogonia, A1-3, B1 or B2 (Samper 2000). These spermatogonia have heterochromatin and will develop into primary spermatocytes. The primary spermatocytes undergo the first meiotic division, and upon completion of meiosis become secondary spermatocytes. Throughout this entire process, each of the spermatogonia and spermatocytes produced by a single germ cell are linked together via cytoplasmic bridges and are embedded within the epithelium.

The secondary spermatocytes are round, haploid sperm cells. They will now undergo spermiogenesis, forming spermatids. This leads to the development of a functionally mature sperm cell through structural and biochemical changes (Abou-Haila 2000). At the same time, the cellular organelles (nucleus, endoplasmic reticulum, Golgi, mitochondria, and centriole) undergo the same changes. The

cell starts to differentiate into the head, midpiece, and tail of the sperm cell. The axoneme, cytoskeletal elements, and acrosome are formed by cellular organelles. As the spermatids are deposited into the central lumen of the seminiferous tubules, the cytoplasmic bridges between the spermatids are broken, leading to synchronous release of the sperm cells.

In late spermiogenesis, elongation of the sperm head occurs, the DNA condenses, and the acrosome becomes compact. The plasma membrane changes with the elimination of certain proteins, the modification of existing glycoproteins, and proteolysis (the activation of enzymatic proteins). At this point the spermatids are spermatozoa and are transported into the epididymis for maturation.

The lumen of the epididymis provides an environment for functionally immature spermatozoa to undergo the morphological and biochemical modifications for sperm cell maturation (Abou-Haila 2000). Specific modifications occur within the plasma membrane, the acrosome, and the tail. Modifications in the epididymis provide the sperm cells with the ability to move and fertilize an oocyte. Spermatozoa in the proximal epididymis, or caput, exhibit no signs of forward movement. Rotary movement is seen in the medial, or corpus, epididymis. Forward movement of the sperm cell is not seen until the caudal epididymis, or cauda, has been entered and spermatozoa found here are considered functionally competent. The addition of the seminal plasma to the spermatozoa during ejaculation allows for the final maturation of the sperm cells.

The process of testicular spermatogenesis, prior to the release of spermatozoa into the epididymis, takes between 55-57 days (Card 2005). Spermatogenesis and meiosis takes approximately 19.4 days each, whereas spermiogenesis occurs in approximately 18.6 days. Nine days are required for transport through the epididymis; therefore 64-66 days are required for a new population of spermatozoa to be ejaculated. Due to the continuous production of spermatozoa within the testes, stallion ejaculates can be collected more than once per day. It is recommended that stallions be collected once every other day, but

dependant on the fertility of the stallion, they can be collected once a day or multiple times per day (Sieme 2004). Sixteen million spermatozoa per gram of stallion testicular tissue are produced per day.

Stallions regulate daily sperm production on a seasonal basis. The breeding season for the stallion in North America typically ranges from January to August and is based on the length of daylight, which coincides with the estrous cycle of the mare, but can differ with respect to region, owner preferences, stallion breed, and individual stallion production (Blanchard 2003). Spermatozoa are produced year round, but the number of sperm cells produced is influenced by season. The number of A-type spermatogonia is increased by 50% in breeding season compared to non-breeding season. The combined number of A- and B-type spermatogonia is 71% higher in the breeding season compared to non-breeding season. The daily number of sperm production within each testis is 84% higher in the breeding season than the non-breeding season (Johnson 2000).

Seminal plasma

Seminal plasma is a mixture of secretions from the epididymis and the accessory sex glands that supports the survival of the sperm cells within the female reproductive tract (Topfer-Petersen, Petrounkina et al. 2000). This ensures that functional spermatozoa reach and fertilize the oocyte. The seminal plasma contains several different protein types that protect the cells from premature capacitation, assist in capacitation and the acrosome reaction, and helps facilitate sperm-oocyte binding.

Once the elongated spermatid enters the epididymis, its DNA is almost completely condensed and transcription has stopped (Sostaric 2008). The proteins found in the seminal plasma must then complete the maturation of the sperm cells. The majority of these proteins are added in the cauda epididymis (Dacheux 2003). The major proteins found in stallion seminal plasma can be divided into three groups: Fn-2 type proteins, cysteine-rich secretory proteins (Crisp), and spermadhesins.

The Fn-2 type proteins HSP-1 and HSP-2 comprise 70-80% of the seminal plasma proteins (Topfer-Petersen 2005). They are heparin-binding proteins that bind the spermatozoa plasma membrane

at the acrosome and equatorial segment. HSP-1 and HSP-2 assist in the facilitation of capacitation by binding heparin to the plasma membrane. CRISP-3 in stallion seminal plasma plays a role in sperm-oocyte fusion. HSP-7 is an equine spermadhesin that is involved in the sperm interaction with the zona pellucida.

Other components of equine seminal plasma include lactoferrin, leptin, growth factors, enzymes, glycoproteins, and acrosome-stabilizing proteins (Eddy 1994; Dacheux 2003). These components coat the spermatozoa. Leptin and growth factors, for example spermine, promote sperm cell life longevity. Other components such as enzymes and acrosome-stabilizing proteins prevent premature capacitation and acrosome reaction.

Spermatozoal Morphology

The equine sperm cell can be broken down into three parts, the plasma membrane, the head, and the tail. The plasma membrane is broken down into regional domains that differ in composition and function. The head contains the acrosome, nucleus, cytoskeletal structures, and the cytoplasm. The tail is a flagellum that contains the connecting piece, middle piece, principle piece, and the end piece (Eddy 1994).

The plasma membrane

The plasma membrane is a heterogeneous structure. Its composition is different between the head and tail, and in regions within each domain (Pesch 2006). The different compositions reflect specialized functions suited for that region. The composition of the plasma membrane changes during the life-span of the cell, during epididymal maturation and during capacitation. Presently the focus will be on epididymal-matured spermatozoa (Abou-Haila 2000).

Over the acrosome, the plasma membrane is divided into three regions, the marginal segment, the principal segment, and the equatorial segment. The marginal segment is the most apical surface of the

cell. The principal segment covers the majority of the acrosome. These segments together cover the acrosome cap, and the two membranes fuse during capacitation, allowing for enzyme release. The equatorial segment covers the equatorial region of the acrosome, and the fusion of the two during capacitation renders the sperm cell capable of binding to the plasma membrane of the oocyte. The interaction between the plasma membrane and the acrosome regulate adhesion factors and cell signaling (Gadella, Rathi et al. 2001).

The post-acrosomal region of the plasma membrane lies in close proximity to the nuclear membrane, separated by a small area of cytoplasm and cytoskeletal components. The interaction between the plasma membrane and the nuclear membrane in this region make up the nuclear envelope.

The plasma membrane forms a tight seal at the posterior ring, the junction between the head and tail. This tight seal is attached to the nuclear envelope and prevents interaction between the different intracellular environments of the head and tail. The plasma membrane forms another tight seal at the posterior end of the midpiece, preventing mitochondrial displacement caudally during movement. Over the tail, the plasma membrane contains proteins that help anchor the fibrous sheath to the tail (Eddy 1994).

Overall, the molecular make-up of the equine sperm cell plasma membrane is 57% phospholipids, 37% cholesterol, and 6% glycolipids (Gadella, Rathi et al. 2001). The cholesterol content in equine spermatozoa is higher compared to other species, causing decreased membrane fluidity and longer time for the completion of capacitation. The phospholipid distribution in the sperm plasma membrane is similar to that of somatic cells. Sphingomyelin and phosphatidylcholine are concentrated in the outer leaflet of the membrane, while phosphatidylserine and phosphatidylethanolamine are concentrated in the inner leaflet (Thomas 2006). The glycolipid concentration within the plasma membrane is similar to those seen in other species, and may aid in the fertilization process (Gadella, Rathi et al. 2001). Overall,

the high cholesterol content and the asymmetric phospholipid distribution allow the sperm cell to be stored in the cauda epididymis until ejaculation. (Sostaric 2008)

The sperm head

The sperm head can be described as a nucleus and acrosome surrounded by plasma membrane (Eddy 1994). Cytoplasm and cytoskeletal proteins are found in the subacrosomal, post-acrosomal, and the para-acrosomal spaces, and differ in components with respect to the region. The subacrosomal space is located between the nucleus and the acrosome, and contains adhesive proteins that bind the nucleus to the acrosome. The post-acrosomal space is located between the nucleus and the plasma membrane posterior to the acrosome, and contains adhesive proteins that bind the nucleus to the plasma membrane. The para-acrosomal space is located between the acrosome and the plasma membrane, and contains proteins involved in the fusion of the two membranes during capacitation and the acrosome reaction.

The nucleus contains haploid, highly condensed DNA enclosed by a nuclear membrane. The nuclear membrane is closely associated with the inner acrosomal membrane, and the two together forms part of the nuclear envelope. There are few nuclear pores in the nuclear membrane that are focused on the posterior edge of the nucleus, in contact with the neck of the sperm (Fawcett 1975). The majority of the DNA is highly condensed and inactive, only in the posterior region of the nucleus is there active DNA, which produces proteins necessary for tail movement. The posterior nuclear region contains the implantation fossa, which attaches to the neck of the sperm.

The acrosome is a membrane bound, cap-like structure covering the majority of the nucleus. It is analogous to a lysosome. It consists of two membranes, the inner and outer acrosomal membranes, surrounding the acrosomal space. The two membranes merge at the distal end of the acrosome. The acrosome can be divided into two areas, the acrosomal cap and the equatorial segment (Eddy 1994).

The acrosomal cap is anterior and contains hydrolytic enzymes. The most studied enzymes are acrosin and hyaluronidase, but there are many other hydrolases and esterases involved. These enzymes

are released during the acrosome reaction and are essential for lysis of the zona pellucida and penetration of the oocyte. The equatorial segment contains no enzymes, however the protein composition allows for sperm binding to the zona pellucida upon fertilization. (Eddy 1994) Soluble *N*-ethylmalmeide-sensitive factor attachment protein receptors (SNARE) proteins are the most studied fusion protein found in equine sperm cells (Gadella, Rathi et al. 2001).

The sperm tail

The sperm tail consists of four regions, the connecting piece, the middle piece, the principle piece, and the end piece (Eddy 1994). The structural components of the tail are the axoneme, mitochondrial sheath, outer dense fibers, and the fibrous sheath. The propulsive force initiated by the tail is used for reaching the site of fertilization and penetration of the egg.

The connecting piece, or the neck, is a short area connecting the head to the tail. The most anterior region of the neck is the basal plate. A dense fibrous structure known as the capitulum attaches the basal plate to the implantation fossa of the nucleus. Proteins synthesized in the nucleus enter the tail region at this junction. Posterior to the capitulum is the centriole of the flagellum. Fibrous proteins surrounding the centriole differentiate into the nine outer dense fibers of the principle piece.

The middle piece of the sperm tail contains the mitochondrial sheath. This sheath surrounds the axoneme and the outer dense fibers. Sperm cells containing higher numbers of mitochondria have a higher likelihood for survival (Pesch 2006). Differentiation of the axoneme into the 9+2 formation occurs in the middle piece. The axoneme consists of two central microtubules surrounded by nine microtubule doublets. The outer dense fibers are arranged peripherally around the axoneme, providing elastic recoil and additional strength to prevent damage. The caudal end of the middle piece contains the annulus, which is firmly bound to the plasma membrane, preventing posterior displacement of the mitochondria during tail movement.

The principle piece is the longest segment of the tail. It contains the axoneme and the outer dense fibers surrounded by the fibrous sheath. This sheath lies underneath the plasma membrane and regulates

flagellar beat and direction of motion. ATP from the mitochondria is moved along the axoneme via dynein. The dynein transport ATP between the doublets, and the ATPase on the dynein arm creates a kinetic energy, causing the doublets to slide. This generates axoneme bending and therefore flagellar movement (Pesch 2006).

The fibrous sheath ends at the junction of the principle piece and the end piece. The axoneme looses its 9+2 conformation. The doublets separate to form 18 separate microtubules. The two central tubules and the outer dense fibers are sometimes absent in the end piece (Pesch 2006).

Capacitation and the acrosome reaction

At the time of ejaculation, the sperm cell is not capable of fertilizing the oocyte (Gadella, Rathi et al. 2001). Inside the female reproductive tract, the spermatozoa undergo physiological changes to the plasma membrane that renders the cell able to fertilize the oocyte, called capacitation (Eddy 1994). Capacitation includes membrane cholesterol efflux, cAMP/PKA pathway activation, calcium influx, and protein kinase phosphorylation (Neild, Gadella et al. 2005). Once capacitation has occurred, the cell is able to undergo the acrosome reaction, rendering the cell capable of penetrating the zona pellucida and fusing with the plasma membrane of the oocyte.

Capacitation

When the spermatozoa are deposited into the female reproductive tract, they accumulate in the lower region of the oviductal isthmus until ovulation (Eddy 1994). The cells will attach to the mucosa of the isthmus and is the site of capacitation. The timing of sperm ascent and oocyte descent within the oviduct is simultaneous. The events during capacitation lead to alterations in plasma membrane composition and morphology, as well as metabolic changes.

The exact steps of the capacitation process remain unknown (Eddy 1994). An important process during capacitation is increased membrane fluidity due to the efflux of cholesterol. Albumin, a major protein found in the female reproductive tract, facilitates the removal of fatty acids and cholesterol from

the plasma membrane, increasing membrane fluidity and permeability. The amount of cholesterol in the plasma membrane differs between species and between individuals, and is thought to determine the length of time for capacitation to occur (Gadella, Rathi et al. 2001).

Activation of the cAMP/PKA pathway occurs parallel with the efflux of cholesterol, however how the two pathways are connected remains unknown (Topfer-Petersen, Petrounkina et al. 2000). Bicarbonate activates cAMP, which in turn activates protein kinase A receptors, resulting in an influx of calcium ions. The increase of intracellular calcium and the efflux of cholesterol lead to the activation of phospholipid scramblase. This activation causes reorganization of membrane phospholipids, causing and internalization phosphatidylcholine and sphingomyelin and the externalization of phosphtidylserine and phosphatidylethanolamine. Phospholipid scrambling further assists in the complete efflux of cholesterol from the plasma membrane (Gadella, Rathi et al. 2001).

The phosphorylation of tyrosine kinase receptors seems to play an essential role in capacitation. Inhibition of phosphorylation causes the spermatozoa to loose the ability to respond to extracellular events (Aitken 1996). Phosphorylation of the tyrosine kinase receptors occurs after cAMP activation, and occurs in the plasma membrane covering the head and tail. This results in increased binding affinity to the zona pellucida and hyperactivity (Gadella, Rathi et al. 2001).

The shedding of protective proteins and glycoproteins as well as the reorganization of surface molecules prepares the sperm cells for the acrosome reaction (Eddy 1994). The protective coat that is removed is collectively termed as decapacitation factors. These are the peripheral proteins embedded in the plasma membrane from the seminal plasma in the epididymis. The removal of decapacitation factors and the increased membrane fluidity allows for the redistribution of intramembraneous proteins over the head and tail region. Rearranging of these proteins starts the fusion of the acrosome with the plasma membrane, and caused hyperactivation of the sperm tail.

Acrosome Reaction

As the spermatozoa reach the oocyte, a capacitated sperm cell enters the cumulus of the egg and attaches to the zona pellucida. Spermatozoa that have not undergone capacitation are unable to penetrate the cumulus, and therefore initiate the acrosome reaction (Eddy 1994). Due to the unstable plasma membrane caused by capacitation, the sperm cells are now sensitive to the smallest extracellular environmental changes (Gadella, Rathi et al. 2001). Sperm-oocyte interactions induce the acrosome reaction, allowing fusion of the two cells and fertilization to occur.

Hyperactivation of the spermatozoa that occurs as a result of capacitation facilitates the sperm cells' ascent towards the oocyte and initiates primary contact (Topfer-Petersen, Petrounkina et al. 2000). Sperm-ooctye interaction initiates the acrosome reaction (Neild, Gadella et al. 2005). Physiological inducers of the acrosome reaction include progesterone, follicular fluid, and cumulus cell secretions including prostaglandins and glycosaminoglycans (Abou-Haila 2000). Zona pellucida glycoprotein 3 (ZP3) is the sperm receptor, located on the surface of the oocyte. This is the primary initiator of the acrosome reaction. ZP3 acts as a ligand for cell-surface receptors located on the spermatozoa, initiating a cascade of events that lead to fusion of the two cells.

Several events occur due to ZP3 binding. Guanine nucleotide binding proteins (G-proteins) are activated on the sperm surface due to ZP3 binding, opening ion channels, therefore leading to an increase of intracellular calcium ions within the sperm cell (Gadella, Rathi et al. 2001). The G-protein activates the phospholipase C (PLC) pathway, releasing calcium from intracellular stores and causing an influx of calcium from the extracellular environment. G-protein activation also leads to the activation of phospholipase A (PLA) and phospholipase D (PLD). PLA cleaves phosphatidylcholine into lysophosphatidylcholine and arachidonic acid, both of which are highly fusogenic compounds. PLD cleaves phosphatidylcholine into choline and phosphatidic acid, both of which also contain fusogenic properties (Eddy 1994).

The increase of intracellular calcium and the release of fusogenic compounds lead to the fusion of the spermatozoa's plasma membrane and outer acrosomal membrane (Eddy 1994). Fusion of the two membranes leads to fenestrations within the membrane, leading to the release of the acrosomal enzymes. Hyaluronidase is released, dissolving the cumulus matrix around the spermatozoa, creating more room for tail movement to allow penetration into the oocyte. Binding of the spermatozoa occurs before penetration. ZP3 on the oocyte is the primary receptor that binds the outer acrosome of the spermatozoa. There is also a secondary receptor, ZP2, which binds the inner acrosomal membrane.

There are two theories to the entry mode of spermatozoa into the oocyte (Eddy 1994). The mechanical theory states that the inner acrosomal membrane forms a sharp perforatorium, and the strong thrusts from the sperm tail forces the cell through the zona pellucida. No acrosomal enzymes play a role in this theory. The enzymatic theory states that the presence of acrosin on the inner acrosomal membrane surface hydrolyzes the zona, allowing penetration of the zona. Since neither theory can be accurately proven, the combination of the two theories may prove to be the resolution (Green 1987).

Cryopreservation Procedure

Correct use of the freeze-thaw procedure is critical in the survivability of the sperm cell. In 1976, Demick et al adapted the use of glycerol to stallion semen cryopreservation from the bovine procedure. However, the results proved sub-optimal. The addition of glycerol to chilled, extended semen decreased the pregnancy rates by 50%. The extender used was a primitive mixture of butterfat, casein, albumin and water, and a glycerol concentration of 7% was used.

In 1984, the first moderately successful freeze-thaw procedure was developed based on centrifugation speed, glycerol concentration, and the freezing and thawing rates (Cochran 1984). This procedure increased post-thaw motility from 22-33%, and the amount of sperm meeting the post-thaw requirements for insemination increased from 34-51%. Despite the increase, these numbers are still extremely low. Now several different freezing regimens are used depending on the freezing facility or

the individual stallion, but all follow a basic outline (Samper and Morris 1998). The basic procedure developed is as follows:

1. Dilute gel-free semen to 20ºC in a citrate-EDTA solution
2. Centrifuge at 400xg for 15 minutes, remove supernatant
3. Resuspend sperm in lactose-EDTA-egg yolk extender containing 4% glycerol
4. Package at 20ºC and freeze

The following is a description and background of the procedure used in the present study.

Initial dilution

The initial dilution occurs after the gel-free semen is evaluated for volume, percent motility, and concentration. The citrate-EDTA solution binds free calcium, maintains the solution at a pH of 7.0, provides energy to the cells, and contains antimicrobials (Moore, Squires et al. 2005). To minimize cold shock damage, the extended semen is slowly cooled to 5^{o}C over one hour. It has been shown that rapid cooling to 5^{O}C is more detrimental to the sperm cell's plasma membrane than slow cooling (Vidament, Ecot et al. 2000; Crockett, Graham et al. 2001). Equilibration over an hour and the components in the extender helps to prevent cold shock damage (Crockett, Graham et al. 2001).

Centrifugation

The process of centrifugation leads to the removal of seminal plasma prior to the addition of glycerol. It is arguable whether seminal plasma is detrimental to the survivability of the sperm cell during the freezing process. Depending on the composition of the extender, the complete removal of seminal plasma can have either beneficial or detrimental effects on the cryopreservation of stallion spermatozoa (Rigby 2001). It has been shown that equine spermatozoa frozen in an egg-yolk based extender has higher post-thaw motility with the removal of the seminal plasma (Bedford, Graham et al. 1995). It is

also believed that certain skim milk extenders alone are adequate to support sperm cell life through the cryopreservation process (Jasko 1991). Also, the seminal plasma of individual stallions can affect the survivability of the spermatozoa (Aurich 1996; Moore, Squires et al. 2005).

The speed and time least detrimental to the sperm cells is 400 x g for 10-15 minutes (Cochran 1984). In the present study, the freezing extender used was skimmed milk; therefore the majority of the seminal plasma was removed prior to the addition of glycerol.

Resuspension

Resuspension with the freezing medium occurs immediately after the supernatant is removed. Another period of equilibration is needed to further protect from cold shock during the addition of glycerol to the sperm cells. The freezing medium contains cryoprotectants. There are two types of cryoprotectants, non-penetrating and penetrating. Non-penetrating cryoprotectants are sugars and lipoproteins that help protect the cell from osmotic stress, reduce cell shrinking and swelling, and protect membrane viability. Penetrating cryoprotectants minimize intracellular ice crystal formation. Two examples are glycerol and DMSO. Glycerol is more widely used in stallion cryopreservation (Cristanelli 1985).

Freezing and Packaging

There are many packaging techniques used for cryopreservation, including straws, pellets, aluminum tubes, and in a few documented cases, a bag (Samper and Morris 1998). Straws are used in the equine industry, and include many different sizes such as 0.5 ml, 2.5 ml, and 5 ml. When inserted with a thermometer, it has been seen that 0.5ml straws cool and freeze more evenly than the larger straws (Crockett, Graham et al. 2001). Freezing can occur by either a moderate or rapid freezing protocol. Moderate freezing is done with a programmable freezer that decreases the temperature a few degrees over a given amount of time, and is more expensive. The rapid freezing protocol involves a Styrofoam cooler filled half way with liquid Nitrogen, then placing the straws 5cm above the liquid nitrogen vapors for ten minutes and then plunging them into the liquid nitrogen. Studies performed show that neither procedure

is more effective than the other (Cristanelli 1985). The straws are then stored at -196^{o}C immersed in liquid nitrogen.

Thawing

The thawing rate is also important in preventing cold shock damage. The rate is dependant on several factors, including the packaging type and stallion preferences. For example, with the 0.5 ml straws used in our research, they can be thawed at 37^{O}C for 30 seconds, or at 50^{O}C for 15 seconds, or at 75^{O}C for exactly 7 seconds and then at 37^{O}C for at least 5 seconds. Evaluation of post-thaw motility occurs immediately after thawing.

INRA96

INRA 96 has proven an effective extender for chilled semen by decreasing the detrimental effects of cold shock. Skim milk has components beneficial to the survival of sperm cells at low temperatures as well as components detrimental to the cell membrane (Garcia MA 1987). INRA 96 was specially formulated to contain only components beneficial to the survival of sperm cells (Batellier F 1996). This extender contains specified concentrations of native phosphocaseinate (NPPC), Hanks' salts, glucose, lactose supplemented with milk purified fraction, and antibiotics. NPPC is an efficient component of skim milk for the preservation of spermatozoa stored at 4^{O}C (Leboeuf 2003). Hanks' salts maintain appropriate calcium and magnesium levels in and surrounding the cells. Glucose and lactose provide additional supportive proteins and lipids as well as supplying energy to the cells. All together, these ingredients support the survivability of cells at low temperatures.

Glycerol

An important advance in the field of cryopreservation was the introduction of glycerol as a cryoprotective agent in bull spermatozoa (Polge C. 1949). Glycerol replaces water within the sperm cell cytoplasm and thereby minimizes intracellular ice crystal formation. However, the use of glycerol has not

proven as successful in the cryopreservation of stallion spermatozoa, and a great variability of success is seen between individual stallions (Vidament M 1997), (Guay, Rondeau et al. 1981). Glycerol toxicity has a more detrimental effect on stallion spermatozoa than on bull spermatozoa (Alvarenga, Landim-Alvarenga et al. 2000). Other cryoprotectants have been tested, however have proven only equal or less capable than glycerol (Alvarenga, Papa et al. 2005), (Squires, Keith et al. 2004). Glycerol concentrations have been tested at multiple concentrations in egg-yolk extenders, and lower concentrations of glycerol are more effective in the survivability of sperm cells, (Cochran 1984; Cristanelli 1985), as compared to bull spermatozoa which requires a glycerol concentration of 7% (DeJarnette 2000). The current concentration seen in egg-yolk stallion freezing extenders is 4% (Alvarenga, Papa et al. 2005). However, the pairing of glycerol with the appropriate freezing medium could increase the survivability of the cells during the freezing process.

Insemination

Timing of the insemination is crucial when using frozen semen. Daily ultrasound exams are needed during estrus, and as ovulation nears, exams are performed every 6 hours. Insemination occurs when ovulation is detected. Variability in ovulation timing between breeds and individual mares can make this a difficult process. Some programs use exogenous hormonal aids such as hCG or deslorelin to help in ovulation timing.

Human chorionic-gonadotropin (hCG) is a large glycoprotein that is purified from the urine of pregnant women. Its LH-like activity is used to induce ovulation in mares displaying estrus. A single intramuscular injection of 1500-3000 IU is effective in inducing ovulation in mares showing signs of heat and with a follicle larger than 35mm (Wilson GG 1990). At a follicle size of at least 35 mm and maximum uterine edema, a dose of hCG given intramuscularly will induce ovulation within 12 to 48 hours, with most ovulations seen close to 36 hours (Evans 2006).

Negative effects of cryopreservation

There are portions of the freeze-thaw process that leads to decreased spermatozoal viability, bringing on many negative effects and therefore a decrease in pregnancy rates with frozen semen use. These disadvantages include cold shock intolerance, glycerol toxicity, osmotic fluctuations, and plasma membrane alterations.

Cold shock intolerance

Cold shock is the irreversible damage due to rapid cooling. There is species variability to cold shock tolerance. Humans and birds are unaffected, ruminants are slightly sensitive, dogs and stallions are more sensitive, and boars are the most sensitive. Prevention of this damage is achieved by slow cooling and effective extender components. The critical temperature is between OºC and -50ºC and is when ice crystals form in the cytoplasm. When ice crystal formation occurs, a hypo-osmotic solution is created and further cell shrinking occurs. The cryoprotectants in the freezing medium minimize the effects of cold shock damage (Braun, Hochi et al. 1995).

Glycerol toxicity

Glycerol is the permeable cryoprotective agent most commonly used in stallion semen freezing. Cells exposed undergo initial dehydration followed by replacement with glycerol. The critical concentration must be met to prevent intracellular ice formation. Impermeable sugars in the extender prevent excessive swelling upon removal. The toxic effects of glycerol include protein denaturation, the alteration of actin interactions, membrane blisters, and changes in cytoplasmic events. Studies show that lower amounts of glycerol are more effective in cell survivability, however a wide variety of concentrations are used due to species variability (Alvarenga, Papa et al. 2005).

Osmotic fluctuations

Viable spermatozoa are perfect osmo-regulators and can readily adjust to different environmental conditions found throughout the male and female reproductive tracts. However, proper freezing and thawing techniques are imperative to maintain cell membrane integrity and therefore the ability to maintain osmotic changes. Rapid shrinking and swelling of the sperm cell can lead to cell death. Osmotic stress due to freezing too slowly will lead to excessive membrane destruction, but freezing too rapidly will lead to incomplete water removal and intracellular ice crystal formation. Osmotic stress due to thawing too slowly will lead to the reformation of intracellular ice crystals, leading to organelle and plasma membrane destruction. On the other hand, thawing too rapidly will lead to unbalanced glycerol efflux and water influx, causing cell swelling (Woods 2004).

Cryocapacitation

The alterations to the plasma membrane caused by cryopreservation leads to decreased post-thaw viability. It was originally believed that the freeze-thaw process induces capacitation of sperm cells; however recent studies show that the cells merely undergo similar changes to the plasma membrane as in capacitation. This is called cryocapacitation. The similarity between the two reactions is that plasma membrane fluidity is increased, however the pathways are different. Like capacitation, cryocapacitation decreases the length of the cell's viability within the female reproductive tract (Thomas 2006).

Phospholipid scrambling occurs in both capacitation and cryocapacitation, but the pathway used in capacitation is cAMP/PKA signaling dependant, while the pathway induced in cryopreservation is not.

Protein tyrosine phosphorylation occurs through different pathways in capacitated and cryocapacitated cells (Thomas 2006). The increased fluidity caused by capacitation is needed for the acrosome reaction to occur. Capacitation-like changes in the plasma membrane speeds the induction of the acrosome reaction. After initiation of the acrosome reaction, the sperm cell will die within 6-8 hours. This is why timing of the insemination is crucial when using frozen semen.

Conclusions

Despite variability between breeds, each individual stallion is different; therefore adjustments to the protocols are used when determining the best method in freezing a stallion's semen. A survey in 1996 administered to 25 laboratories and breeding programs that used frozen semen and the techniques were compared (Samper and Morris 1998). It showed that a basic procedure was used; however variations to the steps were seen between all programs. The lack of a standard method is one reason why breeders are hesitant to use frozen semen. The differences include extenders used, centrifugation techniques, freezing rates, packaging, and insemination timing. Less than optimal efficacy and repeatability of semen quality also discourages the use of frozen semen.

Every species has different cryobiological properties with respect to manipulation, osmotic tolerance, and cold-shock sensitivity, not to mention the variety of success between individuals. For a successful fertilization to occur, high initial quality is needed, post thaw requirements must be met, proper freezing and thawing protocols must be followed, and timing of insemination is crucial. A better understanding of the effects of cryopreservation on the sperm cells will lead to increased survivability and viability of the cells, therefore increasing the likelihood of use from breeders.

The study presented here is an extension of preliminary studies in 2004 performed by Richard Fayrer-Hosken. The aim was to test the capability of INRA 96 to be a successful freezing extender with the addition of glycerol, and this has now been extended with the addition of egg yolk. To determine a compatible concentration of glycerol for INRA 96, two concentrations were tested in this double-blind study against a control egg-yolk extender (Minitube Cryoguard, Minitube of America, Inc.). Mean post-thaw motility was evaluated to determine the optimal sperm cell survivability and the preferred glycerol concentration. Fertilizing capability of the optimal methods was evaluated against the fertilizing capability of fresh-extended semen.

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CHAPTER 2

CRYOPRESERVATION OF STALLION SPERMATOZOA WITH INRA 96 AND GLYCEROL.

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Abstract

The aim of the present study was to improve the success for the cryopreservation of stallion spermatozoa. Semen from eleven stallions were collected and frozen in INRA 96 with two different concentrations of glycerol (3.5% and 6.0%) which were compared to a control freezing process. The mean post-thaw motility for the eleven stallions was 57.93% (3.5% glycerol) and 66.50% (6.0% glycerol) was statistically higher ($p<0.05$) when compared to the mean post thaw motility (39.71%) of controls, Equipro® CryoGuard™ Complete (Minitube) egg-yolk extender. The Equipro® CryoGuard™ Complete is a commercial semen freezing protocol has been one of the standard processes that is typically used in our laboratory for freezing equine spermatozoa. INRA 96 with 6% added glycerol was used in the fertility trial as it provided the highest spermatozoa survival. To evaluate the fertility of the frozen semen, eight mares were bred over two cycles with both fresh and frozen semen. The pregnancy rate of mares bred with frozen semen (55.56%) was not statistically different ($p>0.05$) from the pregnancy rate of mares bred with fresh semen (55.56%). INRA 96 with 6.0% glycerol improved the survivability of stallion spermatozoa through the cryopreservation process, and subsequent fertility was not different ($p>0.05$) from fresh, extended semen.

Keywords: Stallion, semen freezing, INRA96.

Introduction

The need for the cryopreservation of reproductive cells and tissues has brought about significant advancements in the field of agriculture and research[1]. It allows for the international exchange of germ cells, the storage of scientifically important lines, and the conservation of endangered species. In equids, the genetics of the mare and stallion can be preserved, semen can be shipped and stored prior to insemination, international shipment is possible, the stallion's show schedule does not need to be interrupted, and stallions with behavioral problems can have their semen frozen and then be gelded [2].

An important advance in the field of cryopreservation was the introduction of glycerol as a cryoprotective agent in bull spermatozoa [3]. Glycerol replaces water within the sperm cell cytoplasm and thereby minimizes intracellular ice crystal formation. However, the use of glycerol has not proven as successful in the cryopreservation of stallion spermatozoa, and a great variability of success is seen between individual stallions [4]. Ethylene glycol toxicity has a more detrimental effect on stallion spermatozoa than on bull spermatozoa [5]. Other cryoprotectants have been tested, however have proven only equal or less efficient than glycerol[2, 6]. Glycerol concentrations have been tested at multiple concentrations in egg-yolk extenders, and lower concentrations of glycerol are more effective in the survivability of sperm cells, [7, 8], compared to bull spermatozoa which requires a glycerol concentration of 7% [9]. The range of glycerol concentrations is present in many egg-yolk stallion freezing extenders ranging from 2-5% [2, 10]. However, the pairing of glycerol with the appropriate freezing medium could increase the survivability of the cells during the freezing process.

INRA 96 has proven an effective extender for chilled semen. Skimmed milk[11] powder added to semen extender has beneficial effects on the survival of equine sperm cells at 5°C and there is a reduction in the components detrimental to the cell membrane [12]. INRA 96 was custom formulated to contain only components beneficial to the survival of sperm cells[13]. This extender contains specified concentrations of native phosphocaseinate (NPPC), Hanks' salts, glucose, lactose supplemented with milk-purified fraction, antibiotics and fungicide. NPPC is an defined component of skim milk for the preservation of

spermatozoa stored at 4ºC [14]. Hanks' salts maintain physiologic levels of calcium and magnesium levels in and surrounding the cells, glucose and lactose supply energy, penicillin, gentamycin and amphotericin B reduce contamination.

Damage to the sperm cell's membrane during the freezing process can lead to capacitation-like changes in the cell [15], and this is termed cryocapacitation. The effect of cryocapacitiation makes the sperm more likely to undergo the acrosome reaction, this decreases the length of time the spermatozoa is viable. Therefore, the timing of insemination is most critical when using frozen semen [16]. Increasing the frequency of inseminations when using frozen semen improves the conception rate [17], however this can be time consuming and expensive. Reducing the damage caused by cold-shock sensitivity would improve spermatozoa post-thaw viability.

The aim of this study was to test the capability of INRA 96 to be a successful freezing extender with the addition of glycerol. To determine a compatible concentration of glycerol for INRA 96, two concentrations were tested in this double-blind study against a control commercial egg-yolk extender (Equipro® CryoGuard™ Complet, Minitube of America, Inc.). Mean post-thaw motility was evaluated to determine the optimal sperm cell survivability and the preferred glycerol concentration. To determine the fertilizing capability of the frozen semen breedings were compared to fresh-extended semen. The project was an extension of preliminary studies in 2004 and 2005 (Fayrer-Hosken personal communication).

Materials and Methods

Animals

Ejaculates were collected from eleven mature, proven stallions over two breeding seasons (2007-2008). Each breeding season lasted from January to July [18]. Stallions were collected using a Missouri-model artificial vagina. Eight stallions were collected and housed at the University of Georgia, College of Veterinary Medicine, and three stallions were housed and collected at the University of Georgia Animal

and Dairy Science Equine Breeding Facility. Nine quarter horse mares, ranging in age from 3-15 years, of proven fertility were used in the fertility trials during the 2008 breeding season. All mares were housed and bred at the University of Georgia Animal and Dairy Science Equine Breeding Facility. All animals were treated humanely and use of these animals followed procedure set forth by the Federation of Animal Science Societies (FASS). Both facilities were inspected and followed protocol approved by the Institutional Animal Care and Use Committee (IACUC).

Semen freezing procedure

Immediately following collection, the gel-free portion of the ejaculate was evaluated for volume and progressive motility, and concentration was determined with a densimeter. The semen was then extended 1:1 (semen:extender) in INRA96 that had been warmed to 38ºC and the progressive motility re-evaluated. All ejaculates that were considered for freezing had to have at least 60% progressively motile sperm after extension. The diluted samples were placed into 50 ml tubes and were then cooled slowly to 5ºC over one hour under aerobic conditions[19].

Prior to centrifugation, the sample was divided into three aliquots and assigned either INRA A, INRA B, or control. The samples were then centrifuged in a refrigerated centrifuge for 10 minutes at 400 x g [7]. At least 95% of the supernatant was removed [20-22]. Each pellet was then diluted with either INRA A (3.5% glycerol), INRA B (6.0% glycerol), or Equipro® CryoGuard™ Complete egg-yolk extender (control) to a final sperm concentration of 100 x 10^6 motile sperm/ml. Each aliquot was then incubated at 5ºC for one hour.

The extended semen was drawn into 0.5 ml straws (Minitube of America, Inc., cat. #13408/0010) and sealed with a sealing powder. The straws were placed in a closed freezer chest on a float that was 3 cm above liquid nitrogen surface. The straws were allowed to freeze in the vapors for ten minutes [8] and then were plunged into the liquid nitrogen. The straws were then stored in goblets on canes, and kept immersed in liquid nitrogen.

To evaluate post-thaw motility, two straws per treatment were warmed in a water bath at 50ºC for 15 seconds. Each sample was evaluated for progressive motility immediately and then one minute after being placed on a slide warmer. The evaluator was unaware at the time of analysis or until the completion of the manuscript which treatment was being evaluated to avoid bias, and the same observer evaluated each sample.

Insemination procedure

At the beginning of the 2008 breeding season, all mares were monitored for signs of heat. At first signs of estrus, mares were monitored every 48 hours until a dominant follicle size of 35 mm was detected. At this time, the mare was randomly assigned either Group I or II. Group I mares were to be first inseminated with frozen semen, and then fresh extended semen on the second cycle. Group II mares were to be first inseminated with fresh semen, and then by frozen semen. If pregnancy was detected after insemination, 10 mg of prostaglandin-$F_{2\alpha}$ (Lutalyse, Pfizer Inc.) was administered intra-muscularly.

For insemination with frozen semen, the mares were administered 2,500 IU human chorionic gonadotropin (hCG, Chorulon, Intervet Inc.) intra-muscularly after the detection of 35 mm follicle [23] and clear signs of estrous behavior. Thirty hours post-administration, the mares were monitored for ovulation every six hours via transrectal ultrasonography. Upon detection of ovulation, the mares were inseminated deep in the ipsilateral uterine horn with a pipette (Minitube of America, Inc., cat. #17207/2075). The insemination dose was 500 x 10^6 motile sperm determined by post-thaw motility. Pregnancy was determined via ultrasonography at 15 days post-ovulation. Due to the limited amount of mares available for insemination, INRA B (6.0% glycerol) was used in the fertility study based on the post-thaw motility compared to INRA A (3.5% glycerol).

Mares inseminated with fresh semen were monitored at signs of heat for follicle size and uterine edema via ultrasonography[24]. Upon detection of a follicle ≥35 mm and clear signs of estrous behavior, the mares were artificially inseminated with 500 x 10^6 motile sperm. Mares were then monitored every

48 hours for ovulation. If the mare had not ovulated, she was re-inseminated. Pregnancy was determined via ultrasonography at 15 days post-ovulation.

Statistical Analysis

To evaluate the freezing capability of the treatments used, 11 stallions were used with a total number of 48 ejaculates. The mean post-thaw motility percentage was determined for each stallion in each treatment using Microsoft Office Excel 2003. Two stallions were only available for a single collection, so statistical analysis of mean was not available. A chi-square test was used to compare differences of means. Nine mares were used in the fertility trial. Statistical significance was considered at $P<0.05$.

Results

Post-thaw motility

For the eleven stallions collected, 48 ejaculates were frozen and observed for post-thaw motility. The mean post-thaw motilities for each stallion with each treatment are reported in Table 1. Most of ($n=11$, $p<0.05$) the stallions had a higher mean post-thaw motility when frozen with either INRA B when compared to Equipro® CryoGuard™ Complete. INRA B had the statistically highest mean post-thaw motility of 66.5% when compared to the mean post-thaw motility for INRA A (57.93%) and for Equipro® CryoGuard™ Complete (39.71%).

Fertility trials

Each of nine mares was bred with fresh and frozen semen from one of two the most fertile stallions. Stallion A had 60% frozen semen pregnancies compared to 80% fresh semen pregnancies (Table 2). Stallion B had 50% frozen semen pregnancies compared to 25% fresh semen pregnancies. 75% of the mares bred with fresh semen conceived on the first breeding (Table 3). 50% of the mares bred with frozen semen conceived on the first breeding. On the second insemination, 60% of the mares bred to frozen semen conceived compared to 40% of conceptions with fresh semen.

Discussion

Stallion spermatozoa have a lower tolerance to the detrimental effects of cold shock and glycerol toxicity [25]. INRA 96 was formulated to support cell survival at 15ºC, preventing damage to the cells caused by cold shock[13]. However, after extension with INRA96, no significant difference was seen between semen stored at 15ºC and at 4ºC [13]. Preliminary studies we performed suggested that INRA 96 could further support cell survivability through the freezing process with the addition of glycerol. Two different concentrations of glycerol were added to INRA 96 to test the glycerol concentration most compatible with sperm cell survivability. While both concentrations of glycerol improved post-thaw motility when compared to the conventional freezing extender, INRA 96 with glycerol (6.0%) added had the highest overall post-thaw motility.

Post-thaw motility is not an accurate indicator of fertility; therefore nine mares were bred with the frozen semen to test the fertilizing capacity. Two stallions were chosen based on availability for collection, amount of semen collected, and post-thaw motility. INRA 96 with glycerol (6.0%) was used based on higher post-thaw survivability. Compared with 80% (4/5 mares) conception rate with fresh semen for stallion A, the conception rate for frozen semen was 60% (3/5 mares). Compared with the 25% (1/4 mares) conception rate with fresh semen for stallion B, the conception rate for frozen semen was 50% (2/4 mares). Overall, INRA 96 with 6.0% glycerol had a conception rate of 55.56% (5/9 mares).

Based on these results, INRA 96 has proven to be an efficient freezing extender with the addition of 6.0% glycerol. This is based on 0.5ml straws packaged at 50×10^6 sperm per straw. To test the effects of straw concentration on the viability of the spermatozoa, more research will need to be done. The effect of concentration and freezability may also vary between individual stallions. With the ability of INRA 96 to protect the plasma membrane at low temperatures, this new freezing formulation may prove to extend the sperm cell's fertility. The results seen in this study can help increase acceptance of frozen semen use. These preliminary studies provide encouraging results for further studies.

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Table 1. Mean post-thaw motility (+ SEM) of each stallion using three experimental freezing protocols. Each of 11 stallions were collected and frozen in INRA A (3.5% glycerol), INRA B (6.0%glycerol), and Minitube Cryoguard (control).

Stallion #	Treatment INRA A	Treatment INRA B	Control Minitube Cryoguard
1	42.00	62.00	38.00
2	45.50	65.50*	34.50*
3	64.00	73.00*	47.00*
4	72.00	72.00	59.00
5	73.00*	72.00*	41.00*
6	70.63	86.25**	45.63**
7¶	40.00	50.00*	20.00*
8	40.00	42.40	40.00
9	71.67	75.00*	45.00*
10¶	60.00*	70.00**	30.00*,**
11	58.33	63.33	36.67
Mean	57.93(2.52)	66.50(2.12)	39.71(2.28)

¶Stallion was only available for one collection

Statistical significance within a single row. Repeated measures ANOVA.

$p < 0.05$*

$p < 0.01$**

Table 2. Fertility rates using semen frozen in INRA B compared to fertility rates inseminating with fresh semen.

	Frozen semen pregnancies %	Fresh semen pregnancies %
Stallion A	60 (n=5)	80 (n=5)
Stallion B	50 (n=4)	25 (n=4)

No statistical difference between fresh and frozen conception rate.

Table 3. Mares inseminated and conceptions (%) with frozen and fresh semen.

	First Cycle %	Second Cycle %
Fresh	75 (n=4)	40 (n=5)
Frozen	50 (n=5)	60 (n=4)

No statistical difference between fresh and frozen conception rate.

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CHAPTER 3

CONCLUSION

Stallion spermatozoa have a lower tolerance to the detrimental effects of cold shock and glycerol toxicity than bull spermatozoa. The use of alternative cryoprotectants and different freezing protocols has not shown improvement in survivability. Advances in cooled semen technology seen in the past decade can prove beneficial in improving the technology for frozen semen.

INRA 96 was formulated to support cell survival at 15ºC, preventing damage to the cells caused by cold shock. However, after extension with INRA96, no significant difference was seen between semen stored at 15ºC and at 4ºC. Therefore, we tested if INRA 96 could further support cell survivability through the freezing process with the addition of glycerol. Two different concentrations of glycerol were added to INRA 96 to test which was more compatible with the extender in cell survivability. While both concentrations of glycerol improved post-thaw motility when compared to the conventional freezing extender, INRA 96 with glycerol (6.0%) added had the highest overall post-thaw motility ($p<0.05$).

Post-thaw motility is not an indicator of fertility, therefore nine mares were bred with the frozen semen to test viability. Two stallions were chosen based on availability for collection, amount of semen collected, and post-thaw motility. INRA 96 with glycerol (6.0%) was used based on higher post-thaw survivability. Compared with 80% (4/5 mares) conception rate with fresh semen for stallion A, the conception rate for frozen semen was 60% (3/5 mares). Compared with the 25% (1/4 mares) conception rate with fresh semen for stallion B, the conception rate for frozen semen was 50% (2/4 mares). Overall, INRA 96 with 6.0% glycerol had a conception rate of 55.56% (5/9 mares).

Based on these results, INRA 96 has proven to be an efficient freezing extender with the addition of 6.0% glycerol. This is based on 0.5ml straws packaged at 50×10^6 sperm per straw. To test the effects

of straw concentration on the viability of the spermatozoa, more research will need to be done. The effect of concentration may also vary between individual stallions. The thawing temperature of 50ºC for 15 seconds was used based on thawing instructions provided by Minitube of America, Inc. Therefore, different thawing temperatures may also prove effective with this extender.

With the ability of INRA 96 to protect the plasma membrane at low temperatures, this new freezing formulation may prove to extend the sperm cell's life by maintaining membrane integrity. This is beneficial in the timing of insemination, providing a larger window around ovulation, therefore saving time, resources, and money. The next step will be to test the ability of this extender to support post-thaw sperm viability when inseminated further from ovulation.

The results seen in this study can help increase acceptance of frozen semen use. With an increase of pregnancy rates due to the survivability of semen through the freezing process, this technology could lead to a more widespread use of frozen semen. With the increase of spermatozoa survivability through the freezing process, less time and resources are spent, reducing the cost of the procedure. Veterinarians, breeders and horse owners would be more willing to endorse the use of frozen semen.