The effect of hydroxypropyl-β-cyclodextrin and egg yolk concentration on cryopreservation and post-thaw parameters of jack and stallion semen

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LIST OF ABRREVIATIONS

AI	Artificial Insemination
β-CD	Hydroxypropyl-β-Cyclodextrin
BSA	Bovine Serum Albumin
cAMP	3', 5'-cyclic monophosphate
CASA	Computer Assisted Sperm Analysis
EG	Ethylene Glycol
EY	Egg Yolk
GLYC	Glycerol
hCG	Human Chorionic Gonadotropin
HCO_3^-	Bicarbonate
HEY	High Egg Yolk
LEY	Low Egg Yolk
R-PE-PNA	Phycoerythrin peanut agglutinin
PI	Propidium Iodide
PIP_2	Phosphoinositol-bisphosphate
PKA	Protein Kinase A
ROS	Reactive Oxygen Species
VS.	Versus
ZP	Zona Pellucida

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ABSTRACT

Pregnancy rates following artificial insemination with frozen-thawed jack spermatozoa have been relatively low compared to those attained in other species. Cholesterol is known to interfere with post-thaw fertility of jack and stallion semen. Altering the amount of cholesterol in the freezing extender may improve the fertility of frozen-thawed jack semen. Here we report clinical work completed with semen collected from a single jack; extended in EZ Mixin® OF and slowly cooled to 5°C. Extended semen samples were then centrifuged at 400G for 10 minutes and the supernatant removed. The spermatozoa were frozen in liquid nitrogen vapor after resuspension in the appropriate freezing medium to a final concentration of 400 x 10^6 cells/mL. Freezing extender treatments were: 1) 20% Egg yolk (EY); 2) 5% EY; and 3) 20% EY + 60mM hydroxypropyl-β-cyclodextrin (β-CD). A total of 28 mares 2 to 18 years in age were utilized over 5 breeding seasons (82 total cycles). Mares were administered human chorionic gonadotropin (hCG) to induce ovulation when the dominant follicle was ≥ 35 mm as assessed by ultrasonography and were inseminated within 6 hours pre-ovulation and again within 6 hours post-ovulation. Pregnancy rates for each treatment were as follows: 1) 6.25% (1 pregnancy, 15 matings), 2) 46.5% (20 pregnancies, 43 matings), 3) 58.5% (14 pregnancies, 24 matings). These data support the theory of cholesterol interfering with post-thaw fertility of jack semen. We have established that mule pregnancies can be achieved at acceptable rates with frozen-thawed jack semen cryopreserved in 5%EY and 20% EY + 60 mM β-cyclodextrin transferred directly post-thaw.

Cyclodextrins are reported to improve post-thaw viability and motility in boar semen. Cyclodextrin mediates cholesterol efflux and subsequent acrosome reaction and capacitation in the sperm cells of several species *in vitro*. The second objective of this study was to

evaluate whether or not addition of hydroxypropyl-β-cyclodextrin to freezing extenders containing low or high concentrations of egg yolk (cholesterol source) would improve laboratory indicators of fertility for jack and stallion semen and to evaluate post-thaw effects of cyclodextrin on acrosome reaction. Post-thaw motility was improved ($p<.05$) for jack semen cryopreserved in freezing extender containing 20% egg yolk and 60 mM β-cyclodextrin compared to 5% egg yolk freezing extender, with all other treatments for jack and stallions being similar. Post-thaw viability was not different for species or freeze treatments. Post-thaw addition of cyclodextrin to jack and stallion sperm for 90 minutes induced the acrosome reaction 93.5±5.94% and 22.5 ± 4.66% of viable cells, respectively, as measured by a triple stain procedure and subsequent analysis via flow cytometry. Here we have demonstrated that hydroxy-propyl-β-cylcodextrin can be utilized as a powerful agent of induction of acrosome reaction for the jack and stallion semen.

CHAPTER 1. GENERAL INTRODUCTION

STATEMENT OF THE PROBLEM

The development of the technology of artificial insemination has its origin in stories of Indian and Arab tribes stealing stallion semen from other enemy tribes and carrying it home in a sponge or piece of cotton (1, 27). Further development of modern artificial insemination began in Russia and China during the early 20th century with horses (1, 27). The discovery of the protective effects of glycerol in the late 1940's on chicken and bull semen has been identified as being a serendipitous event and from then the dairy and beef AI industries were revolutionized (5, 86, 99). In 1957, Barker and Gandier reported the first equine pregnancy from the use of frozen-thawed stallion semen (10). Ten years later, Krause and Grove reported the first successful mule pregnancy from frozen-thawed jack semen (59). Unfortunately, the techniques that have been so successful for the cryopreservation of bovine semen have not yielded a universal method of cryopreservation for semen from all animal species (49).

Until recently, there has been very little interest from the equine industry with regards to cryopreservation of stallion or donkey semen. Much of this lack of interest was due to restrictions on foal registry in many of the breed associations and was also related to the low pregnancy/conception rates associated with the use of frozen semen. Over the past 10 years many breed associations have lifted the restrictions on foal registration with respect to foals derived from frozen-thawed semen. This has mainly been attributed to the ease with which DNA parent identification can occur in resultant foals.

Even though the advances in cryopreservation of stallion and donkey semen still lag behind the success achieved with bovine semen; the techniques in cryopreservation and in insemination that are available have led to an increase in the use of frozen-thawed semen in the equine species. However, use of cryopreserved donkey semen is minuscule to that of stallion semen. There are distinct advantages to using frozen-thawed donkey semen. First the use of frozen-thawed donkey semen on a mare for the creation of a mule foal eliminates the physical interaction of the two species during the copulation act, which can prove to be quite challenging at best as the loud boisterous braying of a jack can intimidate and scare a mare. Additionally, use of frozen-thawed donkey semen can eliminate the physical limitations of a small jack when the mare to be mated is a large draft breed. Further more, frozen jack semen can more easily be exported or imported into third world countries where mules are still used as a primary mode of transportation. And finally, successful cryopreservation of donkey semen with successful subsequent pregnancies would ensure preservation of endangered donkey species such as the Poitou jackass.

Traditional methods utilizing glycerol as the cryoprotectant for stallion semen have resulted in poorly fertile post-thaw donkey semen. To date, few comparisons have been made between the two species. Ethylene glycol has been utilized as a substitute cryoprotectant for cryopreserving stallion semen. Hydroxypropyl-β-cyclodextrin has been utilized to improve post-thaw parameters of cryopreserved semen from several species.

The first objective of this project was to establish a freezing protocol for jack semen that would successfully result in pregnancies with a direct thaw and transfer method of insemination. The second objective of this study was to evaluate whether or not addition of hydroxypropyl-β-cyclodextrin to freezing extenders containing low or high concentrations of

egg yolk (cholesterol source) would improve laboratory indicators of fertility for jack and stallion semen and to evaluate post-thaw effects of cyclodextrin on acrosome reaction.

LITERATURE REVIEW

A. Comparison of stallion and jack ejaculates

Table 1 contains information comparing ejaculate parameters from jacks and stallions (13, 66, 72, 89). Jack and stallion seminal plasma and spermatozoa have unusually high superoxide dismutase activity compared to other species such as boar, rabbit, rooster, ram, bull and man (72). Further, the superoxide dismutase activity in seminal plasma and spermatozoa of jacks is 3 to 5 fold higher than that of stallions (72). Superoxide dismutase is an enzyme found in cells and micro-organisms that possess an aerobic form of metabolism and is thought to protect cells from the deleterious effects of superoxide anions during electron-transfer reactions (72).

Table 1. Comparison of jack and stallion ejaculate parameters

	Jack	**Stallion**
Volume (ml)	10 to 120	17 to 80
Density (million cells/ml)	100 to 250	100 to 475
Total sperm / ejaculate (billion)	4 to 18	4 to 12
pH	7.0 to 7.8	7.2 to 7.7
Osmolarity (mOsm)	273 to 373	273 to 373
Superoxide dismutase activity in seminal plasma (unit/ml)	5260 ± 1830	1600 ± 260
Superoxide dismutase activity in spermatozoa (units/10^9cells)	1430 ± 390	266 ± 9

B. Insemination methods with frozen-thawed equine semen

Because the cryopreservation process imparts damage to the sperm, the longevity of sperm on post-thaw is shortened as compared to freshly collected sperm and, therefore, it makes sense that the window of opportune use of frozen-thawed semen would be shorter than when using fresh semen. With this in mind it then becomes critical to manage artificial insemination with frozen-thawed semen appropriately. Previous to recent research, in commercial settings it was and still is common practice to examine mares 4 to 6 times per day and inseminate the mares just prior to ovulation or just post ovulation. By doing so, the limited number of inseminations eliminates opportunity for post-breeding endometritis to develop and also places motile sperm in the reproductive tract at or near the time of ovulation (102). One early protocol studying fertility with frozen-thawed stallion semen involved inseminating mares every 24 hours beginning on day 2 of estrus until ovulation (63). Another early study by Pace and Sullivan reported that a higher percentage of mares became pregnant if insemination occurred within 12 h of ovulation (80). Due to the shortened life-span of frozen-thawed semen, the standard recommendation is to inseminate mares within 12 h prior to and within 6 h post-ovulation (64, 71, 92, 93, 95). Researchers have shown that fertility was improved when mares were inseminated more than once in a cycle with frozen-thawed equine semen (92, 113, 114). Metcalf also showed that the incidence of post-insemination endometritis did not increase with the use of frozen-thawed semen (73). Current research has shown that similar pregnancy rates for mares inseminated once within 6 h post-ovulation (83.3%) versus those inseminated with a “timed insemination” protocol of insemination at 24 and 40 h after hCG administration (86.6%) (102). Therefore, rather than

ultrasonically evaluating the mare every 3 to 4 hours as ovulation approaches it has become standard to evaluate the reproductive tract of the mare daily until the dominant follicle attains >35-mm in size (64). Ovulation is then induced with deslorelin acetate or hCG and artificial insemination is completed at 24 and 40 hours post-injection or other variations in timing (1, 9, 64, 74, 92). Pregnancy rates vary, but have been reported to be between 60 and 90% when this highly managed protocol has been instituted and mares were inseminated with approximately 600 to 800 million total cells with a minimum of 35% post-thaw motility (62, 74). The daily ultrasound evaluation allows for the diagnosis of ovulation and any fluid accumulations, which can be treated, if necessary (92). A retrospective study of hCG use by Barbacini et al. indicated that only 9% of all cycles did not result in an ovulation and therefore non-response should not be of concern to equine practitioners (9). McCue et al. reported that only 78.4% of mares ovulated within 48 hours of administration of 2500 IU hCG and clearly showed a trend towards non-response with repeated use in a season (69). These Colorado researchers suggested using a GnRH agonist such as deslorelin acetate as an alternative ovulating agent after two cycles of hCG use within a breeding season (69).

C. Capacitation and acrosome reaction

In order to appropriately understand capacitation and acrosome reaction, it is first necessary to have a basic understanding of the sperm cell and its membrane organization. The sperm cell can be divided into lateral domains and subdomains based on the topology of surface molecules (39, 42). A mature sperm cell is comprised of lateral domains: 1) a sperm head which contains the DNA and the acrosome; 2) a midpiece which contains mitochondria involved in energy production; 3) and a flagellum which is involved in motility (29, 39, 42).

The sperm head contains the nucleus where the DNA is stored, a small amount of cytoplasm, and the acrosome (42). The acrosome is essentially a large vesicle that contains the hydrolytic enzymes required for penetration of the zona pellucida (ZP) (42).

There are carbohydrates bound to membrane integral proteins and some lipids on the plasma membrane; forming what is formally known as the glycocalyx (42). Essentially the plasma membrane of a sperm cell is a fluid bilayer of amphipathic lipids containing integral and associated peripheral membrane proteins, very much identical to the fluid mosaic model of cell membranes (42, 82, 98). The bulk of the bilayer is composed of phospholipids, but glycolipids and sterols are also present (82, 98). For the stallion the lipid content of the sperm membrane is approximately 57% phospholipids, 37% cholesterol and 6% glycolipids. These amounts vary by species (42). Predominant phospholipids include choline, ethanolamine phosphoglycerides, and sphingomyelin (82, 83). Research with human sperm suggests that sphingomyelin influences rate of capacitation by slowing release of sterol (30). The primary sterol present in the sperm membrane is cholesterol and it has been extensively studied (28, 82, 122). It is thought that sperm cells obtain cholesterol from their environment and that freshly ejaculated sperm may obtain additional cholesterol from seminal plasma (28). The seminal plasma of stallions and men is notably high in protasomes rich in cholesterol secreted by the prostate (6, 42). The cholesterol: phospholipid ratio of the sperm membrane is lowest in boar sperm (~0.2), followed by the rooster (0.30), stallion (0.36), bull (~0.40), ram (0.43) and highest for human sperm (0.83) (28, 82, 83). Cholesterol appears to be more densely distributed over the sperm head rather than the plasma membrane over the midpiece or tail (28). Integral membrane proteins are unevenly distributed among different

regions of the sperm head, midpiece and tail (42). Furthermore, other lipids are also organized into distinct domains (42).

Mammalian spermatozoa are not motile when they leave the testis and enter the epididymis; motility is gained during epididymal transport (121). Austin and Chang first demonstrated that freshly ejaculated mammalian sperm cells are not capable of fertilizing an ovum until they dwell in the reproductive tract of the female for a defined period of time (8, 17). As the sperm cells travel through the male reproductive tract and then the female reproductive tract, a series of biochemical changes occur known as capacitation. Capacitation occurs in the uterus in many species *in vivo*, but can be induced *in vitro* when well defined media are utilized (including a source of lipoprotein with an affinity for sterols such as bovine serum albumin (BSA), bicarbonate and calcium) (33, 47, 105, 109, 110, 119, 120). These changes allow the sperm cell to eventually fuse with the (ZP) (12, 24, 40, 42, 119). Capacitation is a multi-step process involving major sperm plasma membrane changes that include: removal or inactivation of decapacitation factors on the sperm surface; changes in localization, molecular structure, and lateral mobility of integral proteins; adsorption onto the sperm plasma membrane of proteins from the female reproductive tract; alterations in membrane lipid composition, in particular in the cholesterol:phospholipid ratio; ionic deregulation manifested as increases in internal Ca, Na, and pH; hyperpolarization of the sperm plasma membrane; generation of reactive oxygen species (ROS) and an increase in cAMP and protein tyrosine phosphorylation (11, 24, 71, 79, 110, 120, 121). Capacitation is irreversible (11).

The so called “decapacitation factors” (mainly proteins and lipids) are present on the extracellular side of the plasma membrane (24, 42, 47, 71, 82). Some of these are derived

from the male reproductive tract and are lost or removed from sperm when they enter the female reproductive tract or are incubated in its secretions (24, 42, 71, 82). Proteins from the female reproductive tract are also adsorbed onto the sperm surface (71). During capacitation repositioning of glycolipids occurs and leads to removal of the decapacitation factors, thus altering the glycocalyx (40, 42, 110). These alterations of the sperm head glycocalyx lead to tyrosine phosphorylation of transmembrane proteins which in turn form active tyrosine kinases (see below) (42, 88).

Loss of cholesterol from the plasma membrane is an all important step and one of the beginning steps in the capacitation process. Researchers have shown that addition of substances that sequester cholesterol away from sperm cells helps induce capacitation, while addition of supplemental cholesterol complexes causes a decrease in the amount of sperm cells undergoing capacitation and gaining fertilization capabilities (see sections titled cholesterol and cyclodextrin for further discussion) (33, 42, 71). Cholesterol efflux leads to lipid scrambling in the plasma membrane and an increase in membrane fluidity (12, 24, 105, 121). Addition of 15 mM $NaHCO_3$ *in vitro* caused a 45% drop in membrane cholesterol content within 30 minutes, after the inner and outer leaflet plasma membrane phospholipids were scrambled (24). The scrambling of phospholipids has been demonstrated in boar semen by flow cytometry as an increase in plasma membrane staining with merocyanine 540 (40). Gadella et al. noted that addition of $NaCO_3$ to boar semen *in vitro* caused sperm plasma membrane changes suitable for albumin mediated cholesterol efflux (41). Notably, the concentration of bicarbonate is negligible in epididymal fluids, but can reach concentrations up to 25 mM in the ampulla of the mare's oviduct, and other species as well, therefore bicarbonate may be an all important capacitation inducer *in vivo* as well as *in vitro* (24, 108,

119, 121). It has been suggested that the movement of HCO_3^- may in part be responsible for the increase in pH observed to take place during capacitation and that the HCO_3^- and calcium regulate adenylate cyclase and the production of adenosine 3', 5'-cyclic monophosphate (cAMP) (12, 39, 109, 119, 121). The elevated cAMP causes activation of protein kinase A (PKA) (12, 38, 39, 88, 121). The activated PKA then causes tyrosine phosphorylation of proteins (12, 39, 88, 121). Tyrosine phosphorylation of flagellar proteins leads to motility changes and may also be involved in phosphorylation of other proteins necessary for the completion of capacitation and acrosome reaction (12, 121). It has also been documented that the presence of albumin, which efficiently binds cholesterol, can induce cholesterol loss and therefore capacitation (28, 42, 121). Cholesterol is thought to slowly diffuse from the sperm cell and is transferred to high density lipoproteins or albumin *in vivo* as this has been demonstrated when bovine sperm cells were incubated with oviductal fluid (28). Flesh et al. demonstrated that HCO_3^- caused a redistribution of cholesterol in the sperm cells from a generalized distribution to the apical site, which makes it more readily available for extraction by albumin (38). During *in vitro* experiments tyrosine phosphorylation will not occur without the presence of BSA, HCO_3^- or Ca^{2+}; BSA is necessary to act as a sink for cholesterol, cholesterol loss leads to an influx of HCO_3^- and together with the separate influx of Ca^{2+} adenylate cyclase is activated to form cAMP, which in turn activates PKA (79, 117, 118, 121). The more cholesterol present in the sperm membrane, the greater the time for capacitation, i.e. t = time for capacitation = $t_{man} > t_{rabbit} > t_{rat} > t_{bull} > t_{boar}$ (32). Coincidently, Brinsko et al. recently reported an elevated cholesterol:phospholipid ratio of 0.97:1 to 1.96:1 in a group of subfertile stallions with apparently normal breeding soundness evaluations compared to 0.61:1 to 1.2:1 for fertile

stallions (15). They reported acrosome reaction failure within this group of subfertile stallions after a 2 hour incubation period in capacitation medium (15). It is suggested that loss of cholesterol from the sperm plasma membrane causes an increase in membrane fluidity and permeability (especially to calcium) and leads to the elevation in cAMP, activation of protein kinase A and eventually the activation of tyrosine kinases (12, 24, 38, 40, 70, 76, 82, 87, 119, 127). Loss of cholesterol also causes destabilization of the plasma membrane which may assist in its ability to eventually undergo the acrosome reaction (48). Phosphorylation of tyrosine is crucial for sperm-zona binding, hyperactivity and eventually the acrosome reaction (24, 40, 42). Cholesterol efflux from the sperm membrane has a direct role in the membrane fusion events that lead to the acrosomal exocytosis (12, 70). Belmonte et al. were able to demonstrate this by addition of the antibiotic Amphotericin B to human sperm under capacitating conditions (12). The Amphotericin B binds and anchors cholesterol in the sperm membrane and inhibits acrosomal exocytosis (12).

Reactive oxygen species (ROS) such as, superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and nitric oxide (NO), play an active role in the events of capacitation (70, 119). Sperm actually secrete ROS that results from flagellar activity (42). During capacitation, intracellular ionic changes precede generation of the reactive oxygen species, which is required for protein tyrosine phosphorylation and O_2^- induces sperm hypermotility and increases their affinity for the ZP, presumptively by increasing tyrosine phosphorylation of several proteins (42, 70, 119). However, the reactive oxygen species are also detrimental to sperm, as they cause ionization and inactivation of proteins, DNA damage, and peroxidation of unsaturated lipids (42). The effects of reactive oxygen species are negated by the presence of the enzymes superoxide dismutase, catalase, peroxidases and the reducing agents

glutathione, ascorbic acid, taurine, and hypotaurine (70). Presence of these substances is species specific (70).

The presence of progesterone receptors has been identified on the plasma membrane of the sperm cell head in several species, including the stallion (18, 42, 76). Typically, progesterone exerts its effects on protein expression via intracellular receptors (18, 42). Addition of progesterone *in vitro* will induce the acrosome reaction in the sperm of humans, boars, and stallions (16, 42, 91). Notably, progesterone concentrations are quite high in the follicular fluid surrounding an ovulated oocyte (mare: ~0.5 μM in follicular fluid versus <6 nM in serum) (42). Apparently these progesterone receptors have a low affinity for progesterone when compared to the cytosolic progesterone receptors of somatic cells (42). Evidence exists suggesting that the progesterone receptors are somehow masked on the plasma membrane until cholesterol leaves the membrane (76). Rathi et al. showed that progesterone mediates induction of the acrosome reaction by activating a sperm protein tyrosine kinase (PTK) that is coupled to phopholipase C, which in turn activates protein kinase C by generating diacylglycerol (DAG) from phosphoinositol-bisphosphate (PIP_2) (91). In capacitated sperm, progesterone causes an influx of Ca^{2+}, theoretically via GABA receptor Cl^--channel complex and it also primes the sperm plasma membrane to acrosome react more readily in response to ZP binding (42, 76). Progesterone will not bind to freshly ejaculated sperm cells, thereby suggesting that some "decapacitation factors" must be removed before progesterone can bind to the receptors (42).

Binding of the sperm to the oocyte induces further cellular changes collectively referred to as the acrosome reaction (42). The zona binding triggers an influx of Ca^{2+} which is required for the following acrosomal reaction steps to occur: 1) point fusions between the

plasma membrane and the outer acrosomal membrane, 2) followed by formation of mixed membrane vesicles and extrusion of the acrosomal enzymes onto the ZP for digestion of the ZP, and 3) exposure of inner acrosomal membrane antigens (12, 42, 47, 70, 110). This leaves the inner acrosomal membrane as the new outer acrosomal membrane and it then binds to the ZP (secondary ZP-binding), followed by ZP penetration and entrance into the perivitelline space (42). Here a single sperm will bind to the oolemma (egg plasma membrane), followed by fusion of the two membranes and incorporation of the sperm into the oocyte (42). This activates the oocyte and the block to polyspermy is initiated (42). If the acrosome reaction is initiated before ZP binding, the sperm cell looses its enzymes and is no longer capable of zona penetration and fertilization (42).

In vitro, the acrosome reaction has also been induced with the use of the calcium ionophore A23187 and heparin (21, 47, 110, 128). In bulls, the binding affinity of bull sperm to heparin or induction of *in vitro* acrosome reaction has been highly correlated with pregnancy rates (21). Stallion semen also will acrosome react in the presence of A23187 or heparin, however with less efficiency than bull semen (21).

D. Cellular changes during cryopreservation

Changes that occur during cooling, cryopreservation, and thawing of semen include: partial dehydration, cryoprotectant penetration of sperm cells, reorganization and distortion of membrane lipids and proteins (membrane damage), exposure to high salt concentrations and exposure to inter- and intracellular ice crystals (5, 64, 78, 82). Membrane damage caused by cold-shock is the primary cryoinjury that occurs during the cryopreservation process (124). Indications of the presence of cold-shock include spermatozoa swimming in a

circular pattern, premature loss of motility, decreased energy production, increased membrane permeability and loss of intracellular molecules and ions (5, 78, 122). As the temperature decreases, a thermotropic phase transition of the phospholipids occurs in the membrane from liquid-crystalline to a gel phase (5, 70). The gel phase imparts more rigidity to the membrane structure and membrane damage commonly occurs when this transition phase occurs (49). The different phospholipids and other components in the membrane have different phase transition temperatures, which leads to shifts, migrations and rearrangements of the membrane components (5, 72). These alterations eventually lead to altered membrane permeability to water and solutes, which can damage the cells (5, 72). The combination of membrane elements such as cholesterol:phospholipid ratio, content of non-bilayer preferring lipids, degree of hydrocarbon chain saturation and protein:phospholipid ratio in the membrane all dictate the extent of damage that will occur, because the fluidity of the membrane is determined by its composition (5, 70). That is to say that different species have different sperm membrane makeup and therefore respond differently to cooling. It has been suggested that the more cholesterol present in a membrane or area of a membrane the less fluid that membrane will be (5). However, sensitivity to cold shock is highest for the boar; followed by the bull, ram and stallion; next the dog and cat are somewhat sensitive to cold shock; and the rabbit, human and rooster display the least sensitivity to cold shock (31, 70). Sensitivity to cold shock is inversely related to cholesterol content; that is the higher the sensitivity to cold shock the lower the cholesterol content of the sperm membrane. Parks and Lynch suggest that the phase behavior of the sperm glycolipids may also impart sensitivity to cold shock, for example rooster sperm do not possess a high melting point glycolipid as do other species (83). This comment is further supported by the work of Darin-Bennett and

White, where these workers determined that the ratio of polyunsaturated: saturated fatty acids also influences sensitivity to cold shock (31). They determined that ram and bull sperm have a higher ratio of polyunsaturated: saturated fatty acids vs. rabbit and human sperm (31). Membrane cholesterol causes fatty acids to condense and that polyunsaturated phospholipids form expanded films, so cholesterol and saturated phospholipids create a more impermeable cell membrane with stronger cohesiveness and therefore more resistance to cold shock (31, 122). Conversely, sperm cells with high polyunsaturated fatty acids and low cholesterol (bull and ram) have higher membrane fluidity, but this imparts more susceptibility to cold shock (31).

Intracellular and extracellular water stay unfrozen at temperatures around -5°C (70). However, as the temperature drops between -5°C and -15°C, intracellular water becomes supercooled without ice formation, but ice crystals begin to form in the extracellular liquid (5, 70, 82). The plasma membrane acts as a barrier to prevent ice crystal growth in the cells (5, 82). Cellular dehydration then begins, because water moves out of the sperm cell to high salt concentrations that are excluded from the ice crystals (5, 70, 82). During this time, the cooling rate must be at a rate slow enough to allow cellular dehydration so as to avoid intracellular ice formation and fast enough to avoid solution effect dehydration damage (5, 70, 82). Solution effect dehydration damage includes denaturation of molecules and extreme shrinkage of the cell, which can lead to irreversible membrane collapse (70). The quickest freezing rate that allows extracellular water freezing without intracellular ice formation is optimal (5, 50, 70). Intracellular ice formation can lead to cell damage and death and its formation is a function of cooling and thawing rates (82). Deleterious effects of ice crystal formation are when the crystals are large (82). Slow cooling from body temperature to 5°C

is beneficial to sperm cells in that it can decrease the effects of cold shock (~ -0.3°C/min.) (5, 122). The cooling/freezing rate in liquid nitrogen vapor when a conventional extender and 0.5 ml straws are used is approximately 60°C/min. (5). Moore et al. recently showed that stallion semen can tolerate many different freezing rates from 5 to 45° per minute when 4% glycerol was used as the cryoprotectant (75).

"The reverse occurs during the thawing process…..Rehydration of the sperm cells occur when water moves back into the cell across the plasma membrane to balance the osmotic imbalance created when extracellular ice melts" (64). Cryoprotectants move out of the cells and the plasma membrane proteins and lipids reorganize (64). Some of the membrane shift/rearrangements are irreversible after warming (5). Research has shown that a thaw rate of 2000 to 4000°C/min or submersion of a straw in a 75°C water bath for 7 seconds is superior to a thaw rate of 700°C/min or submersion in a 37°C water bath for 30 seconds (5, 23). However, the precision of 7 seconds for a thaw time may preclude this thaw procedure from some veterinary practices and breeding farms.

Frozen-thawed sperm have characteristics similar to those acquired through the capacitation process, such as a decreased survival time and decreased time required and decreased dose of capacitating agent to develop the capacity to penetrate oocytes (70, 82, 87). Neild et al. showed that there was a 4 fold increase in live acrosome reacted sperm cells during the freeze-thaw process (78). However, the cryopreservation process causes a reduction in the amount of live non-capacitated cells through all of the cryopreservation steps (78). Neild et al. further indicate that there is some question as to whether this acrosome reaction is truly a result of capacitation or merely cell membrane deterioration and go on to suggest that determination of tyrosine phosphorylation after freeze-thawing would be better

means of assessing true capacitation (78). Bedford et al. also concluded that the freeze-thaw process caused acrosome changes that could interfere with fertility and were not necessarily associated with capacitation owing to the fact that there was little *in vitro* response to the well known capacitation agent calcium ionophore A2317 over a 2 hour time period (11). By monitoring equine sperm cell membrane changes, utilizing chlortetracycline fluorescence after each step during the cryopreservation process, Schrembi et al. showed that these capacitation-like changes mostly occur as a result of removing the seminal plasma with additional changes occurring during the freezing and thawing steps (97). Furthermore, Dobrinski et al. showed that fewer frozen-thawed sperm cells bound to oviductal epithelial cells and to ZP *in vitro* than sperm cells that had been stored at room temperature (36). These studies would further support and explain the reduced fertility observed when cryopreserved semen is used vs. fresh semen.

The goals of cryopreservation are to limit damage during the cooling and cryopreservation process by controlling the cooling rate and adding protective compounds to the freezing extenders and to arrest or slow cellular processes, as sperm cells have very limited biosynthetic activity (64,70). By doing this, some of the sperm cells will retain the following necessary functions: metabolism/production of energy; progressive motility; enzymes within the acrosome; proteins on the plasma membrane (5).

E. Generalities of the stallion semen cryopreservation process

Cryopreservation of spermatozoa irregardless of species, involves several basic steps (13, 61, 70):

1) Immediate dilution of semen after collection (37).

2) Cooling from ambient temperatures to 4°C (37).

3) Increasing the concentration of the spermatozoa by centrifugation. Semen is mixed with an appropriate centrifugation medium to prevent cell damage.

4) Adding cryopreservation medium that generally consists of varying amounts of egg yolk, milk, various sugars, buffers, electrolytes, antibiotics and a cryoprotectant (1, 5, 37).

5) Packaging the semen. Equine semen is most frequently packaged in 0.5 ml to 5 ml straws.

6) Freezing the semen in liquid nitrogen vapor or in a programmable nitrogen freezer.

7) Storing the semen. The packaged dormant semen is submerged in the liquid nitrogen at -196°C.

8) Thawing the semen is accomplished by quickly removing the straw from the liquid nitrogen storage tank and submerging it in a warm water bath. Thawing temperatures and times vary from one facility to the next.

9) Insemination of the mare should occur immediately after thawing. That is to say that frozen semen should not be thawed until the mare is ready to be inseminated.

Freezing extenders have as previously stated been mostly composed of egg yolk, milk, sugars, electrolytes and a cryoprotectant (5, 37). The confounding factors of stallion, donkey, packaging system, cooling and warming rates, the subjective nature of visual evaluation of post-thaw motility, and the huge numbers of mares/cycles needed to complete

fertility trials have made comparison of data very difficult (5, 37, 51, 63, 94). Additionally, many different processing protocols have been followed (94). The order in which the above listed steps have been performed varies and the steps have also been performed at varying temperatures (14, 37, 115). Examples of variance are as follows: 1) Centrifugation and addition of cryoprotectant have been performed at room temperature and at 4°C; 2) Cooling from room temperatures to 4°C has been performed without caution, controlled at a slow rate, and included in the freezing rate (37). Ecot et al. found that there are differences in tolerance of spermatozoa from individual stallions to different cooling treatments and some stallions also had improved motility in some freezing extenders (37). These workers also observed an improvement in sperm motility when Kenney extender was used with 4% egg yolk and 3.4% glycerol (37). Vidament et al. observed that post-thaw motility was improved when centrifugation occurred at 22°C rather than 4°C (114).

Heitland et al. indicate that their results show the appropriate freezing protocol includes centrifugation at 23°C at 400 *g* for 14 to 16 min., resuspension in skim milk-egg yolk-glycerol freezing medium to a final concentration of 400 x 10^6 cells/ml, followed by slow cooling to 5°C, packaging in 0.5 ml straws, freezing in liquid nitrogen vapor (-160° C), and finally thawing at 37°C for 30 s just prior to insemination (50). Vidament et al. reported a similar freezing protocol after summarizing data over a 20 year time span (111).

Blottner et al. reported similar cryosurvival rates for stallion semen cryopreserved in December versus the breeding season (northern hemisphere) (14). Janett et al. reported improved post-thaw motility of Warmblood stallion semen in the autumn season versus other

seasons and suggested that cryopreservation of stallion semen should occur in autumn (also northern hemisphere) (55).

F. Jack semen cryopreservation

In 1964, Polge and Minotakis reported good post-thaw motility of jack semen with freezing solutions containing glucose and glycerol (7.5 to 10%) and with freezing in an alcohol bath with the addition of dry ice and storing at -79°C (85). Later, Krause and Grove reported in 1967 of successfully attaining 1 pregnancy out of 2 mares and only 2 cycles covered with frozen-thawed jack semen in a mare when glycerol was used as the cryoprotectant and semen frozen in pellet form on dry ice and finally, stored in liquid nitrogen (-196°C) (59). The thawing method included thawing the pellets into sterile milk, possibly diluting the deleterious effects of glycerol on post-thaw fertility (59). In recent years, workers in France have studied cryopreservation of semen from the Poitou Jackass, which is an endangered species (105, 106, 107). The workers indicated that there were improvements in post-thaw motility when 80 mM glutamine was added to the freezing medium and also when quail egg yolk was used instead of chicken egg yolk (106, 107, 108). The cryoprotectant used for this series of studies was 4% (v/v) glycerol (106, 107, 108). However, no pregnancies were obtained with a direct thaw and artificial insemination procedure (108). Conversely, a 62% pregnancy rate (8/13) was achieved when jennies were inseminated with frozen-thawed semen that was further extended with skim milk extender, consequently diluting the glycerol concentration (108).

Santos et al. suggest that the phase transition for donkey sperm membrane phospholipids may be higher than that of stallions based on the data they obtained indicating that faster cooling rates could be used to preserve semen at 5°C (96).

Cotorello et al. reported that donkey semen cooled and maintained at 0°C, 5°C or 10°C had better motility when the extender contain 3 or 10% egg yolk versus Kenney extender (26).

G. Cryoprotective agents

Cryoprotective agents are added to freezing extenders to aid in avoidance of intracellular ice formation (70). Addition of glycerol or most other cryoprotective agents lowers the salt concentration and raises the percentage of unfrozen water at any given temperature (5). Intracellular ice formation will physically destroy cell organelles. Barker and Gandier used 10% glycerol in their freezing media of heated, whole milk extender to cryopreserve the stallion epididymal spermatozoa that resulted in the first reported foal from frozen-thawed semen and today it is the most commonly used cryoprotectant cryopreservation of semen (5, 10, 27). Since then, it has been demonstrated that high concentrations of glycerol are detrimental to the fertility to stallion, donkey and boar semen (2, 5, 27, 34, 80, 108). Vidament et al. utilized a range of 1.5 to 4.5% glycerol in the freezing extender and did not demonstrate any differences in post-thaw motility (115). They did note variability in responses with individual stallions and others have noted this phenomena as well (61, 80, 104, 113, 115). Pace and Sullivan showed that fertility of mares inseminated with frozen thawed semen was improved when glycerol level was reduced from 7% to 2% and limiting exposure to glycerol to 15 minutes prior to freezing also improve fertility (80).

It has thus been fairly typical to use only 3 to 4% glycerol as the cryoprotectant in stallion and donkey extender to avoid the toxic effects glycerol has on spermatozoa and yet still maintain the protection needed during the freezing and thawing processes (1, 5, 23, 70). Research has also shown that glycerol need only be present for a few seconds prior to freezing for it to be beneficial to the sperm cells (5).

Other cryoprotectants have also been utilized with stallion or donkey semen in attempts to eliminate the use of glycerol and thus avoid interference with post-thaw fertility; EDTA, ethylene glycol, dimethyl sulfoxide (DMSO), and dimethyl-formamide to name a few have been used with variable results (3, 5, 19, 47, 49, 52, 67, 103, 112). Sugars such as lactose, mannose, and trehalose have also been utilized as cryoprotectants (5, 52, 103). Since these sugars are unable to penetrate the sperm cells, "it is thought that they exert their effect by altering the phase transition properties of the plasma membranes by raising the percentage of unfrozen water at a given temperature, or by reducing the concentration of salt in the unfrozen portion of the extracellular water (5)." Khlifaoui et al. found that addition of 50 mM glutamine and 2.5% glycerol to equine freezing extender resulted in better post-thaw motility (46.4%) than 2.5% glycerol alone (38.3%) (57). Squires et al. conducted a series of experiments that resulted in similar post-thaw motilities for methyl formamide, dimethyl formamide and ethylene glycol when compared to stallion sperm frozen with glycerol as the cryoprotectant (103). Alvarenga et al. also concluded that post-thaw motility of stallion semen was similar when ethylene glycol, dimethyl formamide, or glycerol was utilized as the cryoprotectant (4). Vidament et al. concluded that 2% dimethyl formamide alone is a suitable cryoprotectant for stallion semen (112). Because of their lower molecular weights, ethylene glycol and other cryoprotectants may permeate the sperm membrane more readily

than does glycerol (48, 112). Therefore, spermatozoa frozen with ethylene glycol as the cryoprotectant theoretically will undergo less osmotic swelling when the sperm is thawed and inseminated than will sperm cryopreserved with glycerol as the cryoprotectant (48, 112).

Henry et al. concluded that ethylene glycol, ethylene glycol with glycerol or acetamide combined with trehalose and methyl cellulose could all be utilized as appropriate cryoprotectants for stallion semen as no differences in post-thaw motility were indicated (52). However, they did not report on fertility data in this project (52). Kotajagina et al. reported that 3 of 5 mares became pregnant with stallion semen that had been cryopreserved with 6% ethylene glycol (58). Conversely, Moore et al. reported that better post-thaw total motility was obtained when 4% glycerol was utilized as the cryoprotectant as opposed to 4% ethylene glycol or 4% dimethyl formamide (75). Alvarenga et al. observed no differences in post-thaw motility of stallion semen cryopreserved in 5% glycerol, 5% ethylene glycol, or 3% ethylene glycol and 2% glycerol together; 34.25, 36.5 and 34.75% respectively (3). Additionally, these researchers also noted no difference for acrosome integrity for these groups; 28.0, 22.5, 22.5%, respectively (3). Mantovani et al. also concluded that ethylene glycol could be substituted for glycerol at low concentrations for cryopreservation of stallion semen (67). Work completed by Vincent et al. confirms that glycerol is deleterious to the fertility of fresh or cryopreserved jack semen and this fertility loss was not observed when dimethylformamide or ethylene glycol replaced glycerol in fresh extended jack semen (116).

H. **Cholesterol and phospholipids in the cryopreservation process**

For many years, it has been common knowledge that the low-density lipoproteins present in egg yolk are effective in protecting sperm from cold shock during cryopreservation processes and are commonly used in freezing extenders today (82, 122). The exact

mechanism of protection is unknown, but speculated that the lipoproteins from egg yolk assist in preventing sperm membrane phospholipids loss during cooling or by occupying sites on the plasma membrane and therefore forming a loose association with the sperm membrane that assists in stabilizing the membrane (82, 90, 122). Colorado workers were able to demonstrate an improvement in sperm motility when stallion semen was stored at 5°C or cryopreserved in skim milk egg yolk extender containing supplemental liposomes composed of phosphatidylserine and cholesterol (123). Prior work with the same liposomes did not improve post-thaw motility, but pregnancy rates with semen frozen with glycerol and liposomes were similar to pregnancy rates with fresh extended semen (50).

Supplemental cholesterol has been demonstrated to inhibit acrosome reaction of human sperm and other species and to regulate the acrosome response to progesterone (28, 77). In rabbits, supplemental cholesterol has been demonstrated to inhibit fertilization of oocytes *in vitro* (33).

Colorado workers were unable to show an actual increase in sperm membrane cholesterol content when cholesterol or phosphatidylserine was added to freezing media (48). However, there was almost a 10% increase in post-thaw motility (48). Denniston et al. reported no difference in pregnancy rates when mares were inseminated with stallion semen treated with liposomes composed of cholesterol and phosphatidylserine (45%) versus control (48%) with a total of 80 estrous cycles reported (35).

The seminal plasma of stallions contains protasome structures that contain cholesterol, proteins and phospholipids (6). Aurich et al. noted an improvement in post-thaw motility

and sperm membrane integrity for spermatozoa from stallions noted to have poor post-thaw motility when freezing extender was supplemented with 30% seminal plasma harvested from stallions noted to have good post-thaw motility (7). They also demonstrated the reverse case when the same amount of seminal plasma was supplemented from stallions noted to have poor post-thaw motility (7). These researchers did not report on any measurements or differences in seminal plasma contents. Conversely, Moore et al. showed no difference in post-thaw motility, viability or acrosome integrity after a 15 minute exposure of equine semen to 5 or 20% equine seminal plasma prior to cryopreservation (76). In a second experiment, these researchers showed a deleterious effect of seminal plasma on post-thaw motility with prolonged exposure times of 2, 4 or 6 hours (76). Kawano et al. also showed that exposure of miniature pig spermatozoa to seminal plasma prior to freezing caused reduced post-thaw motility (56).

I. Cyclodextrins and its uses in the cryopreservation process and capacitation

Cyclodextrins are macrocyclic oligosaccharides that contain a hydrophobic center or pocket that can accommodate nonpolar substances and are soluble in water (12, 29, 84). The pore (center) size varies and are designated α, β, or γ. The pore size of the β-cyclodextrins accommodates or accepts cholesterol easily to form an inclusion complex (29, 84). Once bound the molecule is then referred to as an inclusion complex (12, 124). Cyclodextrins have been heavily used in the pharmaceutical, food, cosmetic and pesticide industries; primarily as a carrier that creates a water soluble inclusion complex even when complexed

with insoluble reagents (84, 120). Cholesterol has been effectively removed from homogenized milk utilizing β-cyclodextrin (60).

In recent years, cyclodextrins have been used in several facets in the reproductive industry as well. Cyclodextrins have been used to form inclusion complexes with cholesterol and have been proven to actually increase membrane cholesterol content of cells, including sperm cells and oocytes (22, 25, 52, 124). Most recently, Horvarth and Seidel showed that a 1 hour preincubation of bovine oocytes in a defined medium containing 2 mg/mL cholesterol loaded methyl-β-cyclodextrin improved cleavage and the number of eight cell embryos recovered after vitrification of oocytes and subsequent IVF (53). Supporting the fact that incorporation of cholesterol into gamete membranes can minimize temperature related injuries during cryopreservation. The complexed cholesterol is known to exchange with the cellular cholesterol and if a high enough concentration of complexed cholesterol is used, the cellular cholesterol mass increases (22, 48, 124). Combes et al. reported improvements in post-thaw motility and plasma membrane integrity of stallion semen with the addition of cyclodextrin-cholesterol complex (25). Spizziri et al. reported higher total post-thaw motility for stallion semen treated with 1.5 mg/mL cholesterol-loaded-cyclodextrins (51%) versus control semen (37%) from the same stallion and same ejaculate (101). When 0.125 mM cholesterol complex was added to equine freezing extender, Zahn et al. observed an increase in plasma membrane integrity as measured by fluorescent probes, but a noticeable decrease in pregnancy rates (75 vs. 25%) (124). Zahn et al. also observed that supplemental cholesterol inhibited acrosome reaction when calcium ionophore was added *in vitro* (124). Addition of cyclodextrin-cholesterol complex to bull sperm also improved post-thaw motility but not fertility (48, 71). Recent research utilizing complexed cyclodextrin-cholesterol

yielded the highest post-thaw viability of porcine semen when calcium was not supplemented to the freezing media (45). These researchers measured a decrease in tyrosine phosphorylation, thereby reporting a decrease in freeze-thaw induced capacitation (45). Galantino-Homer et al. were also able to demonstrate higher viability after cold-shock treatment when porcine sperm were incubated in medium containing both 2-hydroxy-β-cyclodextrin (β-CD) and cholesterol sulfate and reduced numbers of premature acrosome reacted cells (43). Current research raises the question, if we add cholesterol to freezing media to improve post-thaw "viability", but are impeding fertility or capacitation; then aren't we going backwards rather than forward? Methods to negate the negative fertility effect of supplemental cholesterol must be developed.

In several mammalian species addition of cyclodextrins *in vitro* mediated cholesterol efflux from the sperm membrane, thus inducing capacitation and the acrosome reaction (12, 20, 54, 71, 77, 87, 100). Belmonte et al. showed that addition of 1 mM cyclodextrin to human sperm *in vitro* facilitated normal membrane fusion processes and cholesterol efflux as opposed to nonspecific mechanisms (12). Cross showed that addition of methyl-β-cyclodextrin to human sperm caused a dose dependent loss of up to 89% of membrane cholesterol in 30 min., compared to ~30% loss in 24 h in a traditional medium and also increased the sperm responsiveness to P_4 (29). Parinaud et al. were able to demonstrate an increase in sperm binding to ZP and an increase in spontaneous acrosome reaction, when human ejaculates were exposed to hydroxypropyl-β-cyclodextrin (80). However, Choi et al. were unable to show a difference in methyl-β-cyclodextrin treated mouse sperm and controls with reference to spontaneous acrosome reaction (20). Van Gestel et al. showed that methyl-

β-cyclodextrin induced sperm cell deterioration rather than capacitation in porcine sperm (109). Visconti et al. showed that addition of β-cyclodextrin to mouse and bovine *in vitro* was able to induce tyrosine phosphorylation, capacitation and acrosome reaction in the absence of BSA (120). This same phenomenon was demonstrated by Osheroff et al. for human sperm as well (79). Mao et al. reported that use of methyl-β-cyclodextrin to increase acrosome reacted porcine spermatozoa vs. caffeine reduced the incidence of polyspermy in embryos derived by IVF (68).

Finally, various forms of β-cyclodextrins have been directly added to the freezing extender for cryopreservation of boar semen (125, 126, 127). Zeng and Terada were able to show an improvement in post-thaw acrosome integrity and certain parameters of motility when either 2-hydroxypropyl-β-cyclodextrin or methyl-β-cyclodextrin were added to freezing extender during a 3 hour cooling process (125, 126, 127). Zeng and Terada further suggested that the protective mechanism involved by the addition of β-cyclodextrin to the freezing medium was that cholesterol efflux from the sperm cell membranes causes an increase the fluidity of the cell membrane and this fluidity imparts a resistance to cold shock during the cryopreservation process (127). Pennsylvania researchers also observed an increase in post-thaw viability of boar semen cryopreserved with 40 or 60 mM 2-hydroxypropyl-β-cyclodextrin (44). However, they reason that this improvement is based on the 2-hydroxypropyl-β-cyclodextrin acting as a cholesterol shuttle to increase sperm plasma membrane cholesterol:phospholipid ratio and thus reducing cold shock sensitivity of the sperm cells (44).

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CHAPTER 2. Case Report: Establishing Pregnancies with Frozen-Thawed Semen from a Standard Jack

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ABSTRACT

Pregnancy rates following artificial insemination with frozen-thawed jack spermatozoa have been relatively low compared to those attained in other species. Cholesterol is known to interfere with post-thaw fertility of jack and stallion semen. Altering the amount of cholesterol in the freezing extender may improve the fertility of frozen-thawed jack semen. Here we report clinical work completed with semen collected from a single jack, extended in EZ Mixin® OF, and slowly cooled to 5°C. Extended semen samples were then centrifuged at 400G for 10 minutes and the supernatant removed. The spermatozoa were frozen in liquid nitrogen vapor after resuspension in the appropriate freezing medium to a final concentration of 400 x 10^6 cells/mL. Freezing extender treatments were: 1) 20% egg yolk (EY); 2) 5% EY; and 3) 20% EY + 60mM hydroxypropyl-β-cyclodextrin (β-CD). A total of 28 mares 2 to 18 years in age were utilized over 5 breeding seasons (82 total cycles). Mares were administered human chorionic gonadotropin (hCG) to induce ovulation when the dominant follicle was ≥ 35 mm as assessed by ultrasonography and were inseminated within 6 hours pre-ovulation and again within 6 hours post-ovulation. Pregnancy rates for each treatment were: 1) 6.25% (1 pregnancy, 15 matings), 2) 46.5% (20 pregnancies, 43 matings), 3) 58.5% (14 pregnancies, 24 matings). These data support the theory of cholesterol interfering with post-thaw fertility of jack semen. We have established that mule pregnancies can be achieved at acceptable rates with frozen-thawed jack semen cryopreserved in 5% EY and 20% EY + 60 mM β-cyclodextrin transferred directly post-thaw.

KEYWORDS: Donkey; semen; cryopreservation; cyclodextrin; cholesterol

INTRODUCTION

In 1964, Polge and Minotakis reported up to 70% post-thaw motility for jack semen frozen in freezing media containing glucose and 7.5% glycerol and 3.75% egg yolk after freezing in an alcohol bath with the addition of dry ice and storing at -79°C (20). Later in 1967, Krause and Grove reported successfully attaining 1 pregnancy out of 2 mares inseminated with frozen-thawed jack semen when glycerol was used as the cryoprotectant and semen was frozen in pellets on dry ice and stored in liquid nitrogen (-196°C) (15). They reported thawing the pellets into sterile milk, possibly diluting the deleterious effects of glycerol on post-thaw fertility (15). In recent years, researchers in France have studied cryopreservation of semen from the Poitou Jackass, which is an endangered species (22, 23, 24). The researchers indicated that optimal improvements in post-thaw motility were obtained when 80 mM glutamine was added to the freezing medium and also when 10% quail egg yolk was used instead of chicken egg yolk (22, 23, 24). The cryoprotectant used for this series of studies was 4% (v/v) glycerol (22, 23, 24). However, no pregnancies were obtained when jennies were artificially inseminated directly with this frozen-thawed jack semen (24). Conversely, a 62% pregnancy rate (8/13) was achieved when the same group of jennies were inseminated with frozen-thawed semen that was post-thaw extended with skim milk extender, presumably diluting the glycerol concentration (24).

In 1957 Barker and Gandier used 10% glycerol in their freezing media of whole milk extender to cryopreserve the stallion epididymal spermatozoa resulting in the first reported foal from frozen-thawed equine semen (3). Today glycerol is the most commonly used cryoprotectant for the cryopreservation of semen (2, 6). Since then, it has been demonstrated that high concentrations of glycerol are detrimental to the fertility of stallion, donkey and

boar semen (1, 2, 6, 8, 18, 24). Other cryoprotectants including ethylene glycol have also been utilized with stallion semen in attempts to eliminate the use of glycerol and thus avoid interference with post-thaw fertility (2, 5, 12, 13, 14, 16, 21, 25). Squires et al. (2004) conducted a series of experiments that resulted in similar post-thaw motilities for methyl formamide, dimethyl formamide and ethylene glycol when compared to stallion sperm frozen with glycerol as the cryoprotectant (21). Work completed by Vincent et al. (2004) confirms that glycerol is deleterious to the fertility of fresh or cryopreserved jack semen and this fertility loss was not observed when dimethyl formamide or ethylene glycol replaced glycerol in fresh extended jack semen (26). Because of their lower molecular weights, ethylene glycol and other cryoprotectants may permeate the sperm membrane more readily than does glycerol (13, 21). Therefore, spermatozoa frozen with ethylene glycol as the cryoprotectant will theoretically undergo less osmotic swelling when the sperm are thawed and inseminated than will sperm cryopreserved with glycerol as the cryoprotectant (13, 21).

Cyclodextrins are macrocyclic oligosaccharides that contain a hydrophobic center or pocket that can accommodate nonpolar substances and are soluble in water (4, 7, 16). The pore (center) size varies and are designated α, β, or γ. The pore size of the β-cyclodextrins accommodates or accepts cholesterol easily to form an inclusion complex (7, 16). Various forms of β-cyclodextrins have been directly added to the freezing extender for cryopreservation of boar semen (28, 29, 30). Zeng and Terada were able to show an improvement in post-thaw acrosome integrity and certain parameters of motility when either 2-hydroxypropyl-β-cyclodextrin or methyl-β-cyclodextrin were added to freezing extender during a 3 hour cooling process (28, 29, 30). Zeng and Terada further suggested that the protective mechanism involved by the addition of β-cyclodextrin to the freezing medium was

that cholesterol efflux from the sperm cell membranes causes an increase the fluidity of the cell membrane and this fluidity imparts a resistance to cold shock during the cryopreservation process (30). Pennsylvania researchers also observed an increase in post thaw viability of boar semen cryopreserved with 40 or 60 mM 2-hydroxypropyl-β-cyclodextrin (7). However, they reason that this improvement is based on the 2-hydroxypropyl-β-cyclodextrin acting as a cholesterol shuttle to increase sperm plasma membrane cholesterol:phospholipid ratio and thus reducing cold shock sensitivity of the sperm cells (7). When 0.125 mM cyclodextrin-cholesterol complex was added to equine freezing extender, Zahn et al. observed an increase in plasma membrane integrity as measured by fluorescent probes, but a noticeable decrease in pregnancy rates (75 vs. 25%) (27). Zahn et al. also observed that supplemental cholesterol inhibited acrosome reaction when calcium ionophore was added *in vitro* (27). Addition of cyclodextrin-cholesterol complex to bull sperm also improved post-thaw motility but not fertility (13, 17).

In the spring of 1999, a client-owned jack was presented to the Iowa State University College of Veterinary Medicine for semen collection and semen freezing with the resultant frozen semen to be utilized for insemination of the client owned mares; of which several were draft breed mares. Concern for potential injury to the standard sized jack during natural mating was the primary reason for this request. The main objective of this project was to establish a freezing protocol for jack semen that would successfully result in pregnancies with a direct thaw and transfer method of insemination.

MATERIALS AND METHODS

At the beginning of the clinical work, the standard jack was 5 years old and weighed approximately 400 kg. This jack was known to be fertile with several mule offspring on the ground as a result of natural matings. Semen was collected from the jack with the use of an ovariectomized, hormonally stimulated and sedated mount mare. The collection technique involved allowing the jack to tease the mare while she was restrained in palpation stocks. After an erection developed, the jack's penis was gently washed with warm water and dried with a paper towel. The mount mare was removed from the stocks during which time the jack was tethered to a sturdy post. The mare was moved around the collection area for several minutes to gain the jack's attention. Once an erection again developed, the mare was twitched and the jack would be allowed to mount and a collection was obtained. Occasionally, if an erection did not develop during the initial teasing phases, false mounts were also allowed to occur. Semen was collected with the use of a Missouri-type artificial vagina (AV).

Cryopreservation Process

Once the semen had been analyzed for motility, morphology and concentration, the temperature of the raw sample was assessed and the semen was extended in EZ Mixin® OF (Animal Reproduction Systems, Chino, CA) at the same temperature as the raw semen to a final concentration of approximately 50 x 10^6 to 75 x 10^6 cells per ml extended semen. The extended semen was placed in 50 ml conical centrifuge tubes (Corning Incorporated, Corning, NY); cooled slowly to 5°C in a water bath; and centrifuged in a refrigerated centrifuge (Beckman Model TJ-6) at 400G for 10 minutes. The supernatant was siphoned off and discarded. The remaining sperm cell pellet was re-suspended in the appropriate freezing

extender to a final concentration of 400 x 10^6 cells per ml and the spermatozoa were packaged in appropriately labeled ½ cc PVC straws. Total equilibration time, including the time necessary to hand fill the straws, was 30 minutes in the freezing extender. The straws were placed on stainless steel racks, counted and then placed in liquid nitrogen vapor (-160°C) for a minimum of 20 minutes, at which time the straws were packaged in canes and plunged into liquid nitrogen until the time of analysis or use for insemination. For post-thaw analysis and artificial inseminations, each straw of semen was removed from the liquid nitrogen storage tank and immediately placed in a 37°C water bath for 30 seconds. Freezing extender treatments were: 1) 20% egg yolk (EY), 4% glycerol (GLYC), Merck II (used in 1999); 2) 20% EY, 2% ethylene glycol (EG), Merck II (used in 2000); 3) 5% EY, 2% EG, EZ Mixin® OF (used in 2000, 2001, 2002, and 2003); 4) 20% EY, 2% EG, EZ Mixin® OF + 60 mM hydroxypropyl-β-cyclodextrin (β-CD) (used in 2002 and 2003).

Establishment of Hydroxypropyl-β-Cyclodextrin Concentration

In the fall of 2001, a small trial utilizing the client-owned jack was conducted to establish an appropriate concentration of hydroxypropyl-β-cyclodextrin (β-CD) to supplement in the freezing extender that was being utilized in the clinic. The collection and processing procedures were as previously described. The following concentrations of β-CD were examined in the freezing medium (20% EY + 2% EG + EZ mixin ® OF) : 0 mM, 10 mM, 20 mM, 40 mM, 60 mM, and 80 mM. A total of 8 ejaculates was collected, and each ejaculate was split and frozen in each of 2 or 3 different concentrations of β-CD in a balanced incomplete block design. Each treatment was replicated a total of 3 times.

Post-thaw sperm viability was determined by flow cytometry and a dual-stain technique described by Garner et al. (10, 11) and Squires et al. (21). LIVE/DEAD Sperm Viability Kit (L-7011; SYBR-14 and propidium iodide (PI)) was purchased from Molecular Probes, Inc. (Eugene, OR). HEPES, NaCl and BSA were all purchased from Sigma-Aldrich Co. (St. Louis, MO). A 1:50 diluted working solution of SYBR-14 (Component A of LIVE/DEAD® Sperm Viability Kit) was prepared using HEPES buffered saline solution (10 mM HEPES, 145 mM NaCl, 1% BSA pH 7.4). The PI (component B of LIVE/DEAD® Sperm Viability Kit) was utilized unchanged. The HEPES buffer was stored at 4°C until needed and then warmed to room temperature, filtered with a 0.22 μM filter, and warmed to 37°C before use. The frozen samples were thawed as previously described and diluted 1:50 in the warmed HEPES buffer. A total of 5 μl of the SYBR-14 working solution was added to a 1 ml sample of the diluted semen for a final concentration of 100 nM SYBR-14. These samples were incubated for 5 minutes and then 5 μl of PI solution was added to the samples (final concentration of 12 μM PI) and the samples were incubated for an additional 10 minutes before analysis by flow cytometry.

The samples were analyzed utilizing a Beckman Coulter XL-MCL flow cytometer. The machine was set on low flow rate and excitation of the dyes was with a 488 nm argon laser. Fluorescence detector 1 (505-545nm) detected the viable cells staining green with SYBR-14, while fluorescence detector 2 (605-635 nm) detected the dead cells staining red with PI. A total of 10,000 cell events was counted. The flow cytometer was calibrated once weekly using Coulter Flow Check Beads. Prior to beginning the analysis of samples, the

protocol was verified by staining frozen thawed samples with each of the dyes separately and together. Additionally, blank (unstained) cells were also analyzed.

Motility characteristics of spermatozoa after thawing were analyzed using computer assisted sperm analysis (CASA) (Hobson SpermTracker version7V2B, Hobson Vision Ltd., Baslow, Derbyshire, UK).

Inseminations

Twenty-eight mares were utilized for the fertility trials over 5 breeding seasons. The mares were 2 to 18 years of age, ranged in weight from 454 to 818 kg, were mostly client-owned and included 7 Quarter horses, 1 American Paint horse, 4 Thoroughbreds, 11 Belgians, 2 Percherons, 2 Warmbloods, and 1 Standardbred. All mares were housed at Iowa State University College of Veterinary Medicine Teaching Hospital during the estrous period for the purpose of artificial insemination.

Mares were brought to the Teaching Hospital for an initial exam to determine the stage of the estrous cycle or when they displayed estrus. Follicular assessment was made by rectal palpation and ultrasonography. Estrus was diagnosed when the mare had at least one follicle measuring greater than 30 mm in diameter with evidence of uterine edema on ultrasound and $\pm$ estrous behavior (squatting, urinating and general acceptance of a stallion). The mares were examined daily until the dominant follicle measured 35 mm or more in diameter, at which time assessment intervals were every 12 h. Once the dominant follicle had attained a minimum measurement of 35 mm, 2500 to 3500 IU of Chorulon® (Intervet, Millsboro, DE, USA) was administered intravenously. Administration of Chorulon® occurred following ultrasonographic examination in the morning. At 24 hours post-administration of Chorulon®, ovarian status of mares was assessed every 6 hours. Mares

were inseminated by the standard technique of uterine body deposition with approximately 300 x 10^6 motile sperm cells within 6 hours pre-ovulation and again 6 hours post-ovulation. Insemination typically occurred at approximately 10 p.m. and again at 8 a.m the next day. Semen was thawed at 37°C for 30s prior to insemination. Pregnancy diagnosis was performed at 14 to 18 days post-ovulation using transrectal ultrasonography.

RESULTS

Ejaculate parameters are summarized in Table 1.

Table 1. Range of ejaculate parameters from client-owned jack from 1999 through 2003

MORPHOLOGY (% NORMAL)	PRE-FREEZE MOTILITY (%)[1]	VOLUME (mL)	POST-THAW MOTILITY (%)[1]
75 - 99	70 - 90	28 – 114	10 – 30

[1]Visual assessment.

As shown in Table 2, post-thaw viability and motility for this jack were not different for the varying concentrations of β-CD in the freezing extender. However, a trend toward improved viability is observed with increasing concentration of β-CD.

Table 2. Viability of post-thaw jack spermatozoa frozen in various concentrations of hydroxypropyl-β-cyclodextrin (β-CD)

CONCENTRATION OF β-CD (MM)	VIABILITY (%)[1] x ± SEM	MOTILITY (%)[2] x ± SEM
0	27.25 ± 4.32	20.17 ± 5.80
10	28.34 ± 4.32	24.33 ± 5.80
20	27.20 ± 4.32	26.20 ± 5.80
40	31.50 ± 4.32	31.80 ± 5.80
60	43.47 ± 4.32	31.13 ± 5.80
80	37.10 ± 4.32	20.60 ± 5.80

[1]Prob > F = 0.1090
[2]Prob > F = 0.6046

As shown in table 3, pregnancy rates for freezing extenders containing high egg yolk were lower than for freezing extenders containing low egg yolk or high egg yolk plus 60 mM β-CD.

Table 3. Fertility data with frozen-thawed donkey semen.

Freezing Treatment	No. of Matings	Number of Pregnancies	Conception Rate (%)
High Egg Yolk [1]	15	1	6.25[a]
Low Egg Yolk[2]	43	20	46.5[b]
High Egg Yolk + β-CD[3]	24	14	58.3[b]

[1] 20% Egg yolk (EY), base extender and either 4% glycerol (GLYC) or 2% Ethylene glycol (EG)

[2] 5% EY, EZ Mixin® OF and either 5% GLYC or 2% EG

[3] 20% EY, 2% EG, EZ Mixin® OF + 60 mM hydroxypropyl-β-cyclodextrin (β-CD)

[a, b] Means with unlike superscripts are different ($p<.06$), χ^2 analysis

DISCUSSION

These data represent work completed in a clinical setting and occurred over a span of 5 breeding seasons. As illustrated in Table 1 the ejaculates that were obtained over the five year period were all acceptable for cryopreservation, but did vary to some degree. The original intent of the project was to successfully achieve pregnancies for the client. Economics dictated that, if a particular freezing extender did not achieve acceptable pregnancies rates after 6 to 8 matings, a new "direction" was sought; therefore, in contrast to well controlled non-clinical studies very broad comparisons are made in this report. The"high egg yolk" treatment (table 2) represents the initial research with the jack which resulted in a pregnancy rate of only 6.25%. Two different cryoprotectants, 5% glycerol or 2% ethylene glycol, were used. Squires et al. noted that post-thaw motility of stallion semen

was not different for freezing extenders containing ethylene glycol or glycerol as the cryoprotectant (21). Vincent et al. reported that fertility of fresh extended jack semen was not decreased when dimethyl formamide or ethylene glycol replaced glycerol as the cryoprotectant (26).

We were able to achieve a pregnancy rate of 46.5% over the subsequent four breeding seasons with freezing extenders that contained 5% egg yolk when mares were inseminated using a direct transfer method. Trimeche et al. were unable to establish pregnancies in jennies inseminated with a direct transfer method utilizing frozen-thawed Poitou Jackass semen cryopreserved with 10% quail egg yolk and 4% glycerol (24). However, they achieved a 62% pregnancy rate when the frozen-thaw semen was diluted with skim milk prior to insemination. Therefore, we were able to eliminate the step of post-thaw semen extension, a finding of great practical significance.

Post-thaw viability (Table 2) was not different for jack semen cryopreserved in varying concentrations of β-CD. Although these data are from only a single jack (and inferences can not be made for other jacks), a clear but non-significant trend towards improved viability was observed with increasing concentrations of β-CD with the highest post-thaw viability occurring with the freezing extender containing 60 mM β-CD. Other researchers have noted an improvement in post-thaw viability of porcine semen when the freezing extender contained 40 or 60 mM β-CD (7, 28, 29, 30). Even though statistical differences were not observed between varying concentrations of β-CD, we felt the trend in improvement, coupled with data reported by others, were sufficient to proceed with inseminations with frozen-thawed jack semen that had been cryopreserved with freezing

extender containing 20% egg yolk and 60 mM β-CD. The overall pregnancy rate was 58.3% for the High Egg Yolk + β-CD freezing extender when using a direct thaw and transfer method for insemination. There was no difference in pregnancy rate between the Low Egg Yolk group or the High Egg Yolk + β-CD. These data would suggest that high concentrations of egg yolk (and possibly the increased amount of cholesterol) inhibit the fertilizing capacity of jack semen post-thaw, and the addition of β-CD sequestered enough cholesterol to decrease this inhibitory effect. Zahn et al. reported improvement in post-thaw plasma membrane integrity for equine semen cryopreserved with supplemental complexed cholesterol in 20% egg yolk extender, but a decrease in pregnancy rates (27).

These data indicate that successful pregnancy rates can be achieved with jack semen cryopreserved in a freezing extender that contains low egg yolk or a high egg yolk extender containing β-CD when it is thawed and directly transferred into a mare. Clearly, further research is warranted.

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CHAPTER 3. The effect of Hydroxy-Propyl-β-Cylcodextrin on cryopreservation and post-thaw parameters of jack and stallion semen

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ABSTRACT

Cyclodextrins are reported to improve post-thaw viability and motility in boar semen. Cyclodextrin mediates cholesterol efflux and subsequent acrosome reaction and capacitation in the sperm cells of several species *in vitro*. The objective of this study was to evaluate whether or not addition of hydroxypropyl-β-cyclodextrin to freezing extenders containing low or high concentrations of egg yolk (cholesterol source) would improve laboratory indicators of fertility for jack and stallion semen and to evaluate post-thaw effects of cyclodextrin on acrosome reaction. Post-thaw motility was improved ($p<.05$) for jack semen cryopreserved in freezing extender containing 20% egg yolk and 60 mM β-cyclodextrin compared to 5% egg yolk freezing extender, with all other treatments for jack and stallions being similar. Post-thaw viability was not different for species or freeze treatments. Post-thaw addition of cyclodextrin to jack and stallion sperm for 90 minutes induced the acrosome reaction in 93.5±5.94% and 22.5 ± 4.66% of viable cells, respectively, as measured by a triple stain procedure and subsequent analysis via flow cytometry. Here we have demonstrated that hydroxy-propyl-β-cylcodextrin can be utilized as a powerful agent of induction of acrosome reaction for the jack and stallion semen.

KEYWORDS: stallion, jack, donkey, cryopreservation, spermatozoa, cyclodextrin

INTRODUCTION

In 1957, Barker and Gandier reported the first equine pregnancy and live birth from the use of frozen-thawed stallion semen (3). Then in 1964, Polge and Minotakis reported good post-thaw motility of cryopreserved jack semen and three years later, in 1967, Krause and Grove reported the first successful pregnancy and live birth of a mule foal from frozen-

thawed jack semen (16, 24). Improvements in the techniques for cryopreservation and artificial insemination have led to an increase in the use of frozen-thawed semen in the equine species. However, use of cryopreserved jack semen lags behind that of stallion semen.

Advantages associated with the use of frozen-thawed jack semen are numerous and include: 1) eliminate physical interaction of mares and jacks during the copulation act, which can prove to be quite challenging at best as the loud boisterous braying of a jack can intimidate and scare a mare; 2) eliminate physical limitations of a small jack when the mare to be covered is a large draft breed; 3) cryopreserved jack semen could more easily be transported than live animals to 3rd world countries where mules are still a primary mode of transportation; and 4) successful cryopreservation of jack semen with successful establishment of pregnancies would assist preservation of endangered donkey species such as the Poitou Jackass.

In recent years, workers in France have studied cryopreservation of semen from the Poitou Jackass, which is an endangered species (29, 30, 31). The workers indicated that there were improvements in post-thaw motility when 80 mM glutamine was added to the freezing medium and also when quail egg yolk was used instead of chicken egg yolk (29, 30, 31). The cryoprotectant used for this series of studies was 4% (v/v) glycerol (29, 30, 31). However, no pregnancies were obtained with a direct thaw and artificial insemination procedure (31). Conversely, a 62% pregnancy rate (8/13) was achieved when jennies were inseminated with frozen-thawed semen that was diluted with skim milk extender, consequently diluting the glycerol concentration and other freezing constituents (31). Workers have shown glycerol to be moderately inhibitory to fertilization in the stallion (2).

Ethylene glycol has been utilized as a successful cryoprotectant for cryopreserving stallion semen and has shown to have a lower toxicity effect than glycerol on cooled jack semen (28, 33).

Cyclodextrins have been utilized in cryopreservation and capacitation studies in a number of species. Cyclodextrins are macrocyclic oligosaccharides that contain a hydrophobic center or pocket that can accommodate nonpolar substances (e.g., cholesterol) and are soluble in water (4, 8, 23). Cyclodextrins have been complexed with cholesterol and utilized as a source of supplemental cholesterol for cryopreserved spermatozoa of several species (6, 7, 9, 11, 12, 14, 18, 35). Supplemental cholesterol in this form has been shown to improve post-thaw motility and viability, but inhibits post-thaw acrosome reaction and fertility of post-thaw equine and bovine semen (7, 12, 18, 27, 35). Media have been supplemented with cyclodextrins to induce cholesterol efflux which has led to sperm capacitation in many species (4, 5, 15, 18, 19, 22, 25, 26, 34). In addition, cyclodextrin has been added directly to freezing extender and has improved post-thaw parameters of cryopreserved semen from boars (10, 36, 37, 38).

The objective of this study was to evaluate whether or not addition of hydroxypropyl-β-cyclodextrin to freezing extenders containing low or high concentrations of egg yolk (cholesterol source) would improve laboratory indicators of fertility for jack and stallion semen and to evaluate post-thaw effects of cyclodextrin on acrosome reaction.

MATERIALS & METHODS

Animals and semen collection

Jacks

Three healthy jacks of proven fertility were utilized for the project. The client-owned jacks, ranging from 5 to 13 years in age and 273 to 432 kg in body weight, were housed at the Iowa State University College of Veterinary Medicine Teaching Hospital during the collection period.

Semen was collected from the jacks with the use of a sedated, ovariectomized, and hormonally stimulated mount mare. Estradiol cypionate (ECP®, Pharmacia & Upjohn Company, Kalamazoo, MI 49001, USA) was administered intramuscularly (IM) at a rate of 4 to 6 mg every 2 to 3 weeks, as deemed necessary when estrous behavior began to diminish. Sedation with 5 mg detomidine hydrochloride IV (Dormosedan®, manufactured by Orion Corp., Espoo, Finland and distributed by Pfizer Animal Health, Exton, PA 19340, USA) was utilized as a precautionary measure to prevent handler or animal injury. Semen was collected with the use of a Missouri-type artificial vagina (A.V.) (Nasco, Fort Atkinson, WI). Each ejaculate was taken to the lab immediately for processing where the majority of the gel fraction was physically removed by siphoning with a catheter-tipped 60 cc syringe prior to filtration with Miracloth® (Calbiochem, LaJolla, CA) into a pre-warmed graduated cylinder. The gel-free volume was recorded, and the initial semen analysis was completed.

Stallions

Five healthy stallions, ranging from 8 to 18 years of age and from 454 to 591 kg of body weight were utilized. The stallions, including two Quarter horses, two American Paint horses and one Thoroughbred, were either privately owned or owned by Iowa State

University Department of Animal Science. All stallions were housed at the Iowa State University Horse Barns or the Iowa State University College of Veterinary Medicine Teaching Hospital for the entire reproductive season during which the project/collection period took place. All stallions had been used for breeding purposes prior to the start of the experiment.

The stallions were all trained to serve a phantom. Semen was collected with the use of a Missouri-type A.V. (Nasco, Fort Atkinson, WI), which had a pre-placed gel filter inside of the collection container. The gel filter was removed and discarded, and the semen was transferred into a pre-warmed graduated cylinder for measurement of gel-free volume and subsequent analysis.

Semen Analysis

Immediately after determination of the gel-free ejaculate volume, motility was visually assessed by placing a 5 µl drop of semen on a pre-warmed (37°C) microscope slide with a coverslip. Semen samples with greater than 70% motility (visual assessment) were retained for cryopreservation (1 jack [not included in the description above] was excluded for failing to meeting this criterion). Sperm cell concentration was determined after diluting raw semen 1:100 using a commercial white-cell diluent (Unopette®, Becton-Dickenson Co., Franklin Lakes, NJ) via the use of a hemocytometer. Morphology of spermatozoa was assessed by placing a 10 µl drop of semen on a slide with an equal amount of eosin/nigrosin stain (13).

Cryopreservation Process

After the preliminary semen analysis was completed, the raw samples were extended with EZ Mixin® OF (Animal Reproduction Systems, Chino, CA) to a concentration of 50 x 10^6 cells per ml extended semen. The extended semen was placed in 50 ml conical centrifuge tubes (Corning Incorporated, Corning, NY), which were placed in a 35°C water bath, which was immediately placed in a 5°C refrigerator to cool. Once the extended semen had reached 5°C (2 to 3 hours), the tubes were centrifuged in a refrigerated centrifuge (Beckman Model TJ-6) at 400G for 10 minutes. The supernatant was siphoned off and discarded. The sperm cell pellet was re-suspended in the designated experimental freezing extender to a final concentration of 400 x 10^6 cells per ml. The samples were then placed in labeled 0.5 ml polyvinylchloride straws (IMV International, Maple Grove, MN). Total equilibration time in the freezing extender, including the time necessary to hand fill the straws, was 30 minutes. The straws were placed on stainless steel racks, counted and then placed in liquid nitrogen vapor (-160°C) for a minimum of 20 minutes, followed by packaging in canes and plunging into liquid nitrogen, where they were stored until thawing.

Freezing Extenders

Experimental freezing extender components are listed in Table 1. The base freezing medium consisted of EZ Mixin® OF containing 2% (v/v) ethylene glycol. Treatments included egg yolk (5% or 20%) and β-CD (0 or 60 mM; 2-Hydroxypropyl-β-Cyclodextrin; Sigma-Aldrich Company, St. Louis, MO MW = 1576) in a 2x2 factorial treatment arrangement. Each stallion and jack was collected a total of four times for the study, and each ejaculate was split into four equal aliquots (one for each experimental freezing extender).

Table 1. Freezing extender components

	Treatment*			
Component	LEY	LEY+β-CD	HEY	HEY+β-CD
Egg Yolk (%)	5	5	20	20
β-CD	-	+	-	+
Ethylene Glycol (%)	2	2	2	2
Base Medium (%)[1]	93	93	78	78

*LEY = low egg yolk; β-CD = 60 mM β-cyclodextrin; HEY = high egg yolk
[1]Base Medium = EZ Mixin® OF

Semen Thawing

For all post-thaw analyses, each straw of semen was removed from the liquid nitrogen dewar and immediately placed in a 37°C water bath for 30 seconds.

Post-thaw Semen Analysis

Computer Assisted Sperm Analysis (CASA)

Motility characteristics of spermatozoa were analyzed after thawing using computer assisted sperm analysis (CASA; IVOS Sperm Analyzer; Hamilton-Thorne Research, Inc., Beverly, MA). The samples were thawed as previously described and subsequently diluted with pre-warmed (37°C) skim milk extender (VP-BB-USA904 equine breeding buffer, IMV International Corp., Maple Grove, MN) to a final concentration of 20 x 10^6 cells/ml. The samples were warmed for 5 minutes at 37°C, and 4 μl of diluted semen was placed into one

end of a disposable 20 μm depth chamber (Leja 4 Chamber). The percentage of motile sperm, the straight line (VSL) and the average path (VAP) velocities were recorded.

Plasma membrane and acrosomal membrane integrity assessment

Plasma membrane and acrosomal membrane integrity were assessed after exposure to calcium ionophore A23187 and/or β-CD via a modified triple stain flow cytometric protocol described by Nagy et al. (20). Reagents included a LIVE/DEAD Sperm Viability Kit (L-7011; SYBR-14 and propidium iodide (PI); Molecular Probes, Inc., Eugene, OR), peanut agglutinin conjugated with phycoerythrin (stock solution of 1 mg/ml in a buffer composed of 50 mM sodium phosphate, 0.05% sodium azide, and containing 1 mM Ca2+ and Mg2+ ions; Phycoprobe R-PE-PNA, P44; Biomeda Corp.;Foster City, CA), calcium ionophore A23187 (C-5149), β-CD (C-0926), and PI (P4170; all from Sigma-Aldrich Co., St. Louis, MO), and Dulbecco's Phosphate Buffered Saline (PBS 1X; Gibco BRL, Invitrogen Corp.,Carlsbad, CA).

The following working solutions were prepared: 1) 5 mM Calcium Ionophore A23187 in dimethylsulfoxide (DMSO) stored at room temperature in an amber vial; 2) Component A (SYBR-14, 1 mM in DMSO) from LIVE/DEAD sperm viability kit diluted 10-fold with DMSO appropriately labeled and stored in the freezer in 50 μl aliquots for later use; 3) 2 mg/ml PI in ultra-pure water, stored in 250 μl aliquots in the freezer; 4) R-PE-PNA was utilized unchanged and stored at 5°C; and 5) 0.15 M solution of β-CD in ultra-pure water stored at room temperature.

Each day the stains were thawed and mixed in bulk, and 500 µl aliquots were placed into 0.5 ml microcentrifuge tubes and held in the dark at 37°C until time of staining. The final concentrations of stains were: 12 µM PI, 100nM SYBR-14 and 2.5 µg/ml PE-PNA.

Semen samples were thawed, and 100 µl of frozen-thawed sample were placed in an appropriately labeled 0.5 ml microcentrifuge tube. Samples that were frozen in the presence of β-CD (LEY+β-CD and HEY+β-CD) were treated post-thaw as follows: 1) nothing added, control (C) or 2) addition of calcium ionophore A23817 (CaI) to a final concentration of 1 µM. Samples that were frozen in the absence of βCD (LEY and HEY) were treated post-thaw as follows: 1) nothing added, control (C), or 2) addition of CaI to a final concentration of 1 µM, or 3) addition of β-CD to a final concentration of 60 mM, or 4) addition of β-CD +CaI to a final concentration of 60 mM and 1 µM, respectively (see Table 2). The treated samples were maintained at 37°C until sub-samples were taken for the staining procedure at time 1) 0 minute, 2) 60 minutes and 3) 90 minutes post-treatment.

Staining was performed in an incubator (5% CO_2 in humidified air at 37°C) for 15 minutes, and the stained cells were analyzed with a Beckman Coulter XL-MCL flow cytometer for analysis. The machine was set on low flow rate, and excitation of the stains was achieved with a 488 nm argon laser. Fluorescence detector 1 (505-545nm) detected the viable cells (staining green with SYBR-14), while fluorescence detector 2 (560-590 nm) detected the acrosome reacted cells (staining orange with PE-PNA). Fluorescence detector 3 (605-635 nm) detected the dead cells (staining red with PI). A total of 20,000 cell events were counted. The flow cytometer was calibrated weekly using Coulter Flow Check Beads.

Table 2. Experimental Design for Post-thaw Semen Treatments

	Post-thaw treatments*			
	C	CaI	β-CD	β-CD+CaI
Freezing Treatment:				
LEY	Yes	Yes	Yes	Yes
LEY+β-CD	Yes	Yes	No	No
HEY	Yes	Yes	Yes	Yes
HEY+β-CD	Yes	Yes	No	No

***C** = Control, nothing added; **CaI** = 1 μM Calcium ionophore A23187; **β-CD** = 60 mM β-CD; **β-CD+CaI** = 1 μM Calcium ionophore A23187 and 60 mM β-CD

Statistical Analysis

Differences in post-thaw treatments within and between species were examined by analysis of variance using the least squares procedure and general linear models procedure of JMP (SAS Institute, Inc.). Mean separation tests were performed using a Tukey's HSD test.

RESULTS

Post-thaw motility assessments obtained via CASA are shown in Table 3. Motility for jack semen in HEY+β-CD was higher ($p<.05$) than that in LEY, but differences amongst other treatments were not significant. The average path velocity (VAP) was faster for jack

semen in LEY compared with stallion semen in LEY+ β-CD and for stallion and jack semen in both HEY and HEY+β-CD. Straight line (progressive) velocity (VSL) was faster for jack semen in LEY compared to all other freeze treatments, except for jack semen in LEY+β-CD, which was intermediate.

Table 3. Least square means (± s.e.m.) for post-thaw semen traits as measured by CASA

Species	Freeze Treatment	Motility (%)	VAP* (μm/sec)	VSL* (μm/sec)
Jack	LEY	17.8 (3.34) [b]	93.2 (4.89) [a]	84.1 (4.84) [a]
Stallion		15.7 (2.60) [a, b]	69.3 (3.81) [a, b, c]	56.0 (3.77) [c]
Jack	LEY+β-CD	21.2 (3.34) [a, b]	89.9 (3.81) [a, b]	80.2 (4.84) [a, b]
Stallion		17.5 (2.59) [a, b]	67.1 (3.79) [b, c]	54.5 (3.75) [c]
Jack	HEY	22.2 (3.36) [a, b]	80.5 (4.92) [b, c]	70.2 (4.86) [b, c]
Stallion		14.2 (2.60) [a, b]	68.7 (3.81) [b, c]	56.2 (3.77) [b, c]
Jack	HEY+β-CD	24.2 (3.35) [a]	77.1 (4.91) [c]	65.2 (4.85) [c]
Stallion		16.6 (2.59) [a, b]	66.9 (3.80) [b, c]	53.7 (3.76) [c]

*VAP = Average Path Velocity; VSL = Straight Line (Progressive) Velocity
[a, b, c] means within a column with unlike superscripts are different $p<.05$; Tukey HSD analysis

Post-thaw viability of the sperm cells was not different between species; therefore the data were pooled for subsequent data analysis. Post-thaw viability was not different ($p>.20$) for the freeze treatments: LEY (15.7 ± 1.75%), LEY+ β-CD (16.0 ± 1.84%), HEY (16.9 ±1.75%), HEY+β-CD (18.5 ± 2.00%); therefore, data were pooled across freeze treatments for subsequent analysis.

All post-thaw treatments caused a decrease ($p<.05$) in viability [β-CD (15.8 ± 1.89%), CaI (15.9 ± 1.62%), β-CD +CaI (16.5 ± 1.72%)] compared with the control (C 19.0 ± 1.62%); however they were not different from each other. Post-thaw viability decreased

($p<.05$) from time 1 (17.9 ± 1.70%) to time 3 (15.9 ± 1.70%) with time 2 (16.5 ± 1.70%) being intermediate. A species by time interaction was observed ($p<.01$), stallion semen viability remaining relatively constant, whereas viability of jack semen decreased by nearly 30% of the viable cells from time 1 to time 3.

The proportion of sperm cells that underwent the acrosome reaction post-thaw is presented in Table 4. A post-thaw treatment by species by time interaction was observed ($p<.01$). In general, the proportion of stallion sperm cells that acrosome reacted changed very little over time irrespective of post-thaw treatment. In contrast, post-thaw treatment of jack sperm cells with β-CD accelerated acrosome reaction particularly between time 1 and time 2.

Table 4. Acrosome reacted sperm cells as measured by triple stain procedure

Species	Post-thaw Treatment	Time	Acrosome Reacted Cells (% of Viable Cells (± S.E.M.))
Jack	C	1	11.5 (3.64) [g, j, k]
		2	24.9 (3.64) [e, f, h, i]
		3	31.0 (3.64) [c, d]
Stallion	C	1	3.20 (2.82) [i, k]
		2	4.29 (2.82) [f, i, j, k]
		3	5.30 (2.82) [f, i, j, k]
Jack	CaI	1	12.8 (3.64) [g, j, k]
		2	27.6 (3.64) [c, d, e, f, h]
		3	32.5 (3.64) [c]
Stallion	CaI	1	3.44 (2.82) [i, k]
		2	6.00 (2.82) [f, i, j, k]
		3	8.00 (2.82) [d, f, j]
Jack	β-CD	1	12.6 (5.94) [e, f, g, h, i, j, k]
		2	82.2 (5.94) [a, b]
		3	93.5 (5.94) [a]
Stallion	β-CD	1	3.27 (4.66) [f, h, i, j, k]
		2	14.0 (4.66) [c, d, e, f, g, h, i, j, k]
		3	22.5 (4.66) [c, e, g]
Jack	β-CD+CaI	1	12.5 (4.54) [g, j, k]
		2	72.7 (4.54) [b]
		3	82.4 (4.54) [a, b]
Stallion	β-CD+CaI	1	3.84 (3.55) [f, i, j, k]
		2	11.0 (3.55) [c, d, e, f, g, h, i, j, k]
		3	18.5 (3.55) [c, e, g, h]

[a, b, c, d, e, f, g, h, i, j, k] means within a column with unlike superscripts are different $p<.05$; Tukey HSD analysis

DISCUSSION

The 60 mM concentration of β-CD was previously established in our laboratory (Jepsen et. al, submitted for publication) as being efficacious as a freeze treatment additive with mule foals resulting from artificial insemination of jack semen cryopreserved in HEY+β-CD. Various forms of β-cyclodextrins have been directly added to freezing extenders for cryopreservation of boar semen (36, 37, 38). Zeng and Terada were able to show an improvement in post-thaw acrosome integrity and motility parameters when either 2-hydroxypropyl-β-cyclodextrin or methyl-β-cyclodextrin were added to freezing extender during a 3 hour cooling process (36, 37, 38). Pennsylvania researchers also observed an increase in post-thaw viability of boar semen cryopreserved with 40 or 60 mM 2-hydroxypropyl-β-cyclodextrin (10). They suggest this improvement is based on the cyclodextrin acting as a cholesterol shuttle to increase the sperm plasma membrane cholesterol:phospholipid ratio and thus reducing cold shock sensitivity in the sperm cells (10). Motility for jack semen in HEY+β-CD was higher ($p<.05$) than jack semen in LEY, but differences amongst other freeze treatments or for stallions were not significant. Post-thaw motility did not approach the industry standard of 30% for any of the freezing treatments for either species. Individual responses to cryopreservation could attribute for a portion of this observation. Additionally, all of the ejaculates were centrifuged at 5° C instead of room temperature (22°C) to treat all samples in a similar manner due to the transport time involved to return ejaculates to the laboratory for the stallions. Vidament et al. observed that post-thaw motility was improved when centrifugation occurred at 22°C rather

than 4°C (32). Utilizing EZ Mixin OF® as the base for the freezing extenders may not be the best for freezing of jack and stallion semen. Post-thaw viability was not different for jack or stallion semen for the various freezing treatments. Viability of jack semen decreased ($p<.05$) by nearly 30% of viable cells from time 1 to time 3, whereas, stallion semen viability remained relatively constant. Further study of post-thaw viability of jack semen is warranted.

Post-thaw treatment of jack semen with β-CD caused the majority viable sperm cells to undergo the acrosome reaction in as little as 60 minutes. Stallion sperm cells responded to post-thaw treatment with β-CD in a similar fashion, but to a lesser extent. Cyclodextrins are safe sugars as compared to the potential health hazards associated with the use of CaI. Others have demonstrated that addition of cyclodextrin *in vitro* mediates cholesterol efflux from the sperm membrane, thus inducing capacitation and the acrosome reaction for several species (4, 5, 15, 18, 19, 22, 25, 26). Belmonte et al. showed that addition of 1 mM cyclodextrin to human sperm *in vitro* facilitated normal membrane fusion processes and cholesterol efflux as opposed to nonspecific mechanisms (4). Cross showed that addition of methyl-β-cyclodextrin to human sperm caused a dose dependent loss of up to 89% of membrane cholesterol in 30 min., compared to ~30% loss in 24 h in a traditional medium and also increased the sperm responsiveness to P_4 (8). Parinaud et al. were able to demonstrate an increase in sperm binding to zona pellucida and an increase in spontaneous acrosome reaction, when human ejaculates were exposed to hydroxypropyl-β-cyclodextrin (22). Visconti et al. showed that addition of β-CD to mouse and bovine *in vitro* was able to induce tyrosine phosphorylation, capacitation and acrosome reaction in the absence of BSA (34) and this same phenomenon was demonstrated by Osheroff et al. for human sperm as well (21).

Presumably, stallion and jack semen undergo the acrosome reaction by a mechanism similar to that described in other species, when β-CD is added to *in vitro* sperm cells.

Ethylene glycol (2% v/v) was utilized as the cryoprotectant for this project. Ethylene glycol has been shown to a successful replacement for glycerol for cryopreservation of stallion (1, 17, 28). Vincent et al. showed that glycerol is deleterious to the fertility of fresh or cryopreserved jack semen and this fertility loss was not observed when dimethylformamide or ethylene glycol replaced glycerol in fresh extended jack semen (33). We have previously established (Jepsen et al., submitted for publication) that jack semen cryopreserved in the presence of ethylene glycol can be utilized with successful conception of mule foals. To the author's knowledge, published research is unavailable indicating successful use of ethylene glycol with cryopreservation of jack semen.

In summary we showed that β-CD is powerful sperm acrosome reaction inducing agent for jacks and a moderate inducing agent for stallions. Additional studies are required to elucidate the specific biological mechanism through which β-CD enhances sperm acrosome reaction for jacks and stallions. Fertility studies are necessary to verify that *in vitro* findings translate to viable pregnancies.

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CHAPTER 4. SUMMARY

Utilizing a direct thaw and transfer method for insemination, there was no difference in pregnancy rates between the LEY (46.5%) group or the HEY + β-CD (58.3%), but the pregnancy rates for these two groups were higher ($p<.06$) than the HEY group (6.25%). These data would suggest that high concentrations of egg yolk (possibly cholesterol) inhibit the fertilizing capacity of jack semen post-thaw and the addition of cyclodextrin sequestered enough cholesterol to decrease this inhibitory effect on the fertility of jack semen. These data indicates that successful pregnancies rates can be achieved with frozen-thawed jack semen when it is thawed and directly transferred into a mare with freezing extender that contains low egg yolk or a high egg yolk extender containing β-CD.

Motility for jack semen in HEY+β-CD was higher ($p<.05$) than jack semen in LEY, but differences amongst other freeze treatments were not significant and none of the freeze treatments were significant for the stallions. Post-thaw motility for the *in vitro* experiment did not approach the industry standard of 30% for any of the freezing treatments for either species. Individual responses to cryopreservation could attribute for a portion of this observation. Additionally, all ejaculates were centrifuged at 5° instead of room temperature (22°C) to treat all samples in a similar manner due to the transport time involved to return ejaculates to the laboratory for the stallions. Post-thaw viability was not different for jack or stallion semen for the various freezing treatments. Viability of jack semen decreased ($p<.05$)

by nearly 5% from 0 to 90 minutes post-thaw, whereas, stallion semen viability remained relatively constant. Thought must be given to the biological significance of a 5% loss in a 90 minute test period. This loss over time could suggest that insemination with frozen-thawed jack semen needs to occur very close to ovulation time in the female. Further study of post-thaw viability of jack semen is warranted.

We clearly demonstrated that β-CD is a powerful sperm acrosome reaction inducing agent for jacks and a moderate inducing agent for stallions. Additional studies are required to elucidate the specific biological mechanism through which β-CD enhances sperm acrosome reaction for jacks and stallions. Additional fertility studies are necessary to verify how in vitro findings translate to viable pregnancies.

APPENDIX: Detailed Materials & Methods

Introduction

In the spring of 1999 a client owned jack was presented to the Iowa State University College of Veterinary Medicine for semen collection and semen freezing with the resultant frozen semen to be utilized for insemination of the client owned mares. Concern for potential injury to the jack during natural mating was the primary reason for this request. This simple request eventually led to the development of this thesis project. Essentially, three areas developed over time and are described in this section: 1) A clinical breeding trial developed utilizing the initial jack and 26 mares over a total of 4 breeding seasons; 2) during this period of time, review of the literature led to the development of a small trial to determine the appropriate concentration of β-CD to be added to freezing extender of jack semen; and 3) finally, a trial was conducted to study the effect of β-CD on cryopreservation of jack and stallion semen.

A. Animal Selection

Breeding Trials - Jack

At the onset of the project, the single standard jack was 5 years old and weighed approximately 400 kg. This jack was subsequently utilized for the establishment of 2-Hydroxypropyl-β-Cyclodextrin concentration and was also utilized during the *in vitro* effect of β-cyclodextrin trial.

Breeding Trials - Mares

Twenty-six mares were utilized for the fertility trials over 4 breeding seasons. The mares were 2 to 18 years in age, ranged from in weight 454 to 818 kg, were mostly client owned and included 7 Quarter horses, 1 American Paint horse, 4 Thoroughbreds, 9 Belgians, 2 Percherons, 2 Warmbloods, and 1 Standardbred. All mares were housed at Iowa State University College of Veterinary Medicine Teaching Hospital during the estrous period for the purposes of artificial insemination.

Effect of Cyclodextrin - Jacks

Three healthy jacks were utilized for this portion of the project. The jacks ranged from 5 to 13 years in age and ranged 273 to 432 kg in weight. These animals were all client-owned and were housed at the Iowa State University College of Veterinary Medicine Teaching Hospital for the collection period only. All of the jacks had been utilized for breeding purposes by the owner prior to the collection period.

Effect of Cyclodextrin - Stallions

Five healthy stallions were also utilized for part of this study. The stallions ranged in age from 8 to 18 years and weighing 454 to 591 kg included 2 Quarter horses, two American Paint horses and one Thoroughbred. The stallions were either privately owned or owned by Iowa State University Department of Animal Science. All of the stallions were housed at the Iowa State University Horse Barns or the Iowa State University College of Veterinary Medicine Teaching Hospital for the entire reproductive season during which the project/collection period took place. All of the stallions had been used for breeding purposes prior to the collection period.

B. Semen Collection, Analysis and Freezing

Semen Collection

Jacks

Semen was collected from the jacks with the use of an ovariectomized, hormonally stimulated and sedated mount mare. Estradiol cypionate (ECP®, Pharmacia & Upjohn Company, Kalamazoo, MI 49001, USA) was administered at a rate of 4 to 6 mg intramuscularly (IM) every 2 to 3 weeks, as deemed necessary when estrous behavior began to diminish. Sedation with 5 mg detomidine hydrochloride IV (Dormosedan®, manufactured by Orion Corp., Espoo, Finland and distributed by Pfizer Animal Health, Exton, PA 19340, USA) was utilized as a precautionary measure to prevent handler or animal injury. The collection technique involved allowing the jack to tease the mare while she was restrained in stocks. After an erection developed, the jack's penis was gently washed with warm water and dried with a paper towel. The mount mare was next removed from the stocks during which time the jack was tethered to a sturdy post. The mare was then moved around the collection area for several minutes to gain the jack's attention. Once an erection again developed, the mare was twitched and the jack would be allowed to mount and a collection was obtained. Occasionally, false mounts were also allowed to occur, if an erection did not develop during the initial teasing phases. Semen was collected with the use of a Missouri-type artificial vagina (A.V.). Each collection was taken to the lab immediately for processing, including determination of volume, motility, morphology, and concentration. If a

large quantity of gel fraction was present in the ejaculate collected, it was physically removed by siphoning with a catheter-tipped 60 cc syringe. Next, the ejaculate was filtered with Miracloth® (Calbiochem, LaJolla, CA) into a pre-warmed graduated cylinder and the volume was recorded.

Stallions

The stallions involved in the project were all trained to a phantom. Therefore, semen collection involved teasing the stallion with an estrous mare to an erection. The erect penis was gently cleansed with warm water and paper towels. Afterwards, the stallion was once again allowed to tease the mare and once an erection was obtained the stallion was allowed to mount the phantom for semen collection. Semen was collected with the use of a Missouri-type A.V., which had a pre-placed gel filter inside of the collection container. The gel filter was removed and discarded and the semen was transferred into a pre-warmed graduated cylinder for measurement of volume and subsequently motility, morphology, and concentration.

Motility of Spermatozoa

Determination of motility was initially made by placing 5 µl drop of semen on a pre-warmed (37°C) microscope slide with a coverslip. A minimum of 4 visual fields were viewed at 100X and 400X, using a phase-contrast microscope (with a stage warmer (37°C)). For the purpose of this study, motile sperm were those considered to be independently moving within a microscope field. The assessments were given in 10% increments. Motility assessments were completed by two individuals, compared and agreed upon.

Morphology of Spermatozoa

Morphology of spermatozoa was completed by placing a 10 µl drop of semen on a slide with an equal amount of eosin/nigrosin stain (3). The two drops were gently mixed together with the use of a second slide and then drawn out as a thin film across the slide. The slide was air-dried and then a total of 100 spermatozoa was morphologically assessed using a phase contrast microscope (1,000X, oil immersion). The sperm were categorically assigned to the following: normal sperm, abnormal sperm heads, proximal cytoplasmic droplets, distal cytoplasmic droplets, and abnormal/bent tails.

Concentration of Spermatozoa

Concentration determination was accomplished by diluting raw semen to a 1:100 ratio using a white-cell Unopette® (Becton-Dickenson Co., Franklin Lakes, NJ). The diluted semen was slowly agitated to thoroughly mix the spermatozoa and interrupt motility. The sample solution was then placed on each side of a hemacytometer. The hemacytometer was placed in a humidified chamber for 5 minutes to allow the spermatozoa to settle into one visual plane. All spermatozoa within 5 large squares on the diagonal of the central 1-mm square were counted under 400X and repeated on the other side of the hemacytometer. The final count obtained from both sides had to be within 10% of one another; otherwise the process was repeated. If the counts were within 10%, the two counts were then averaged. The number of cells counted represents the concentration of the diluted sample in a 1/50 mm^3 volume. The concentration per ml was calculated by multiplying the average number in 5 squares by 100 (the dilution factor) x 50 (the 1/50 mm^3 in the 5 squares) and x 1000 (the conversion of mm^3 to cm^3). The concentration per ml was then multiplied by the volume in

milliliters of semen harvested to acquire the total number of sperm cells contained in the ejaculate.

Cryopreservation Process

Once the semen had been analyzed for motility, morphology and concentration, the temperature of the raw sample was assessed and the semen was then extended in EZ Mixin® OF (Animal Reproduction Systems, Chino, CA) at the same temperature as the raw semen to a final concentration of approximately 50 x 10^6 to 75 x 10^6 cells per ml extended semen. The extended semen was placed in 50 ml conical centrifuge tubes (Corning Incorporated, Corning, NY), which were then placed in a same temperature water bath container that was then placed in a 5°C refrigerator to cool slowly to 5°C over 2 to 3 hours duration. Once the extended semen had reached 5°C, the tubes were centrifuged in a refrigerated centrifuge (Beckman Model TJ-6) at 400G for 10 minutes. The supernatant was siphoned off and discarded. The remaining sperm cell pellet was re-suspended in the appropriate freezing extender to a final concentration of 400 x 10^6 cells per ml. The samples were then placed in appropriately labeled (animal identification, date) ½ cc straws. Colored straws were utilized to identify the freezing extender used. Total equilibration time including the time necessary to hand fill the straws was 30 minutes in the freezing extender. The straws were placed on stainless steel racks, counted and then placed in liquid nitrogen vapor (-160°C) for a minimum of 20 minutes, at which time the straws were packaged in canes and plunged into liquid nitrogen until the time of analysis.

Semen Thawing

For all post-thaw analysis and artificial inseminations, each straw of semen was removed from the liquid nitrogen storage tank and immediately placed in a 37°C water bath for 30 seconds.

Freezing Extenders

Breeding Trials

Initial collections were frozen in freezing extender containing 20% egg yolk (EY), 4% glycerol (GLYC) and a standard freezing medium (Merck II). Because of the poor pregnancy rates with the laboratory standard equine freezing procedure, the following treatments were tested in the fertility trials: 1) 20% EY and 2% ethylene glycol (EG), 2) 10% EY and 2% EG, 3) 5% EY and 5% GLYC, 4) 5% EY and 2% EG, and 5) 20% EY, 2% EG and 60 mM hydroxypropyl- β-Cyclodextrin (β-CD). The freezing extender base for the majority of the breeding trials was EZ Mixin® OF skim milk extender. However, some of the initial work utilized Merck II as the base.

Establishment of 2-Hydroxypropyl-β-Cyclodextrin Concentration

The following concentrations of 2-Hydroxypropyl-β-Cyclodextrin were utilized in the freezing medium (EZ Mixin® + 5% egg yolk + 2% ethylene glycol) : 0 mM, 10 mM, 20 mM, 40 mM, 60 mM, and 80 mM.

Effect of 2-Hydroxypropyl-β-Cyclodextrin (*in vitro* trial)

Freezing extender components for the *in vitro* trial are listed in Table I. The base freezing medium consisted of EZ Mixin® OF and 2% ethylene glycol. Treatment groups included 5% and 20% egg yolk with and without 60 mM β-CD.

Table 1. Freezing extender components

Component	TRT 1	TRT 2	TRT 3	TRT 4
Egg Yolk (%)	5	5	20	20
Ethylene Glycol (%)	2	2	2	2
β-CD* (mM)	--	60	--	60
Base (%)**	93	93	78	78

*2-Hydroxypropyl-β-Cyclodextrin (Sigma-Aldrich Company, St. Louis, MO MW = 1576)
**Base = EZ Mixin® OF

C. Breeding Trials

Generally, the mares were brought to the Teaching Hospital when they displayed estrus or initially to establish stage of cycle. Follicular assessment was made by rectal palpation and ultrasonography. Estrus was diagnosed when the mare had at least one follicle measuring greater than 30 mm with evidence of uterine edema on ultrasound ± estrous behavior (squatting, urinating and general acceptance of a stallion). The mares were examined daily until the dominant follicle measured 35 mm, at which time assessment intervals were q12 hours. Once the dominant follicle had attained the minimum measurement of 35 mm, 2500 to 3500 IU of Chorulon® (Intervet, Millsboro, DE, USA) was administered intravenously. Administration of Chorulon® occurred in the morning following

after examination. At 24 hours post administration of Chorulon, mares were assessed every 6 hours. Mares were inseminated with approximately 500 x 10^6 motile sperm cells within 6 hours pre-ovulation and again 6 hours post-ovulation. Insemination typically took place at approximately 10 p.m. and again at 8 a.m. At the time of insemination the mare's tail was wrapped and tied away from the perineal area. The perineal area was cleansed using Nolvasan® scrub and water. Next, 3 to 5 straws of frozen semen were thawed in 37°C water for 30 seconds, the straw content was transferred to a small plastic test tube and then transferred into a sterile insemination catheter (IMV International, Maple Grove, MN) attached to a 12 cc syringe. A sterile glove was donned, lubricated with sterile non-spermicidal lubricant and the catheter was taken into the vagina. The catheter was then passed through the cervix so that the tip was approximately 3 cm past the internal os of the cervix and the semen was deposited slowly.

Pregnancy diagnosis was performed at 14 to 18 days post-ovulation by transrectal ultrasonography.

D. Establishment of 2-Hydroxypropyl-β-Cyclodextrin Concentration

In the fall of 2001, a small trial utilizing a single jack was conducted to establish an appropriate concentration of 2-Hydroxypropyl-β-Cyclodextrin (β-CD) to supplement in the freezing extender that was being utilized in the clinic. The collection and processing procedures are previously described. The following concentrations of β-CD were established in the freezing medium (EZ mixin OF® + egg yolk + ethylene glycol) : 0 mM, 10 mM, 20 mM, 40 mM, 60 mM, and 80 mM. A total of 8 ejaculates were collected and each ejaculate was split and frozen in each of 2 or 3 different

concentrations of β-CD. Each treatment was repeated a total of 3 times. Samples were analyzed be CASA utilizing a Hobson Tracker 7V2B and by viability tests using flow cytometry, both described later.

E. Effect of 2-Hydroxypropyl-β-Cyclodextrin in different freezing extenders (*in vitro* trial)

Jacks

Initially, each jack was collected once and the semen was assessed and frozen for client use prior to the collection period and to determine that the jack semen would have post-thaw motility. Each jack was collected a total of 4 times for the study and each ejaculate was split into 4 equal aliquots for the 4 freezing extenders involved in the project. A 4th jack was collected and evaluated, but initial motility was 50% or less. A 5th jack had been collected on one occasion with the ejaculate split and frozen as described above. However, libido difficulties led to the animal being eliminated from the trial. This initial frozen ejaculate was utilized to complete the final verification for the triple stain protocol as described later.

Stallions

As with the jacks, the stallions were each collected 4 times in total for the study and each ejaculate was split into 4 equal aliquots for the 4 freezing extenders involved in the project.

F. Post-thaw Semen Analysis

CASA

Motility characteristics of spermatozoa after thawing were analyzed using computer assisted sperm analysis (CASA) (IVOS Sperm Analyzer; Hamilton-Thorne Research, Inc., Beverly, MA and Hobson Tracker 7V2B). The samples were thawed as previously described and then diluted with pre-warmed (37°C) VP-BB (USA904 equine breeding buffer, IMV International Corp., Maple Grove, MN 55369) to a final concentration of ~20 x 10^6 cells/ml. The samples were warmed for 5 minutes at 37°C and 4 μl diluted semen was placed into one end of a disposable 20 μm depth chamber (Leja 4 Chamber). A minimum of 45 fields were evaluated for each sample. The following parameters were measured:

ALH: amplitude of lateral head displacement in μm

BCF: beat cross frequency

STR: straightness

VAP: average path velocity

VSL: straight line (progressive) motility

VCL: curvilinear velocity

LIN: linearity

Dual Stain Viability Test

Viability of frozen-thawed samples was assessed via a dual staining and flow cytometry protocol described by Garner et al. and Love et al. (1, 2, 4). LIVE/DEAD Sperm Viability Kit (L-7011; SYBR-14 and propidium iodide (PI)) was purchased from Molecular Probes, Inc. (Eugene, OR). HEPES, NaCl and BSA were all purchased from Sigma-Aldrich

Co. (St. Louis, MO). A 1:50 diluted working solution of SYBR-14 (Component A of LIVE/DEAD® Sperm Viability Kit) was prepared using HEPES buffered saline solution (10 mM HEPES, 145 mM NaCl, 1% BSA pH 7.4). The PI (component B of LIVE/DEAD® Sperm Viability Kit) was utilized unchanged. The HEPES buffer was stored at 4°C until needed and then warmed to room temperature and filtered with a 0.22 μM filter and finally warmed to 37°C before use. The frozen samples were thawed as previously described and diluted 1:50 in the warmed HEPES buffer. A total of 5 μl of the SYBR-14 working solution was added to a 1 ml sample of the diluted semen for a final concentration of 100 nM SYBR-14. These samples were incubated for 5 minutes and then 5 μl of PI solution was added to the samples (final concentration of 12 μM PI) and the samples were incubated for an additional 10 minutes before analysis by flow cytometry.

The samples were analyzed utilizing a Beckman Coulter XL-MCL flow cytometer. The machine was set on low flow rate and excitation of the dyes was with a 488 nm argon laser. Fluorescence detector 1 (505-545nm) detected the viable cells staining green with SYBR-14 and fluorescence detector 2 (605-635 nm) detected the dead cells staining red with PI. A total of 10,000 cell events were counted. The flow cytometer was calibrated once weekly using Coulter Flow Check Beads. This protocol was completed in 1 day. Prior to beginning the analysis of samples the protocol was verified by staining frozen thawed samples each of the dyes separately and together. Additionally, blank (unstained cells) were also analyzed.

Assessing plasma membrane integrity and acrosome membrane integrity post-thaw in response to capacitation induction reagents

Plasma membrane integrity and acrosome membrane integrity in response to the addition of calcium ionophore A23187 and/or 2-Hydroxypropyl-β-Cyclodextrin was assessed via a modified triple stain flow cytometric protocol described by Nagy et al. (2003) (5). LIVE/DEAD Sperm Viability Kit (L-7011; SYBR-14 and propidium iodide (PI)) were purchased from Molecular Probes, Inc. (Eugene, OR). Peanut agglutinin conjugated with phycoerythrin (stock solution of 1 mg/ml in a buffer composed of 50 mM sodium phosphate, 0.05% sodium azide, and containing 1 mM Ca2+ and Mg2+ ions) (Phycoprobe R-PE-PNA, P44) was purchased from Biomeda Corp. (Foster City, CA), stored in a 5°C refrigerator until use. Calcium ionophore A23187 (C-5149), 2-Hydroxypropyl-β-Cyclodextrin (C-0926), and PI (100 mg powder) were all purchased from Sigma-Aldrich Co. (St. Louis, MO). Dulbecco's Phosphate Buffered Saline (PBS) (1X) was purchased from Gibco BRL, Invitrogen Corp. (Carlsbad, CA).

The following working solutions were prepared: 1) 5 mM Calcium Ionophore A23187 in dimethylsulfoxide (DMSO) stored at room temperature in an amber colored vial; 2) Component A (SYBR-14, 1 mM in DMSO) from LIVE/DEAD sperm viability kit diluted 10-fold with DMSO appropriately labeled and stored in the freezer in 50 µl aliquots for later use; 3) 2 mg/ml PI in ultra-pure water, stored in 250 µl aliquots in the freezer; 4) R-PE-PNA was utilized unchanged; and 5) 0.15 M solution of 2-Hydroxypropyl-β-Cyclodextrin in ultra-pure water.

Each day the stains were thawed and mixed in bulk and then 500 µl aliquots were placed into 0.5 ml microcentrifuge tubes and held in the dark at 37°C until time of staining. The final concentration of dyes was as follows: 12 µM PI, 100nM SYBR-14 and 2.5 µg/ml PE-PNA.

The staining procedure was conducted in an incubator (5% CO_2 humidified air) for 15 minutes and then the samples were run through a Beckman Coulter XL-MCL flow cytometer for analysis. The machine was set on low flow rate and excitation of the dyes was with a 488 nm argon laser. Fluorescence detector 1 (505-545nm) detected the viable cells staining green with SYBR-14; fluorescence detector 2 (560-590 nm) detected the acrosome damaged/acrosome changed cells staining orange with PE-PNA; and fluorescence detector 3 (605-635 nm) detected the dead cells staining red with PI. A total of 20,000 cell events were counted. The flow cytometer was calibrated once weekly using Coulter Flow Check Beads.

Verification of the protocol was conducted first by collecting a fresh ejaculate of stallion semen. This ejaculate was initially extended with EZ Mixin®OF to a concentration of approximately 50 x 10^6 sperm cells per ml. The extended semen was then transferred to the laboratory where it was immediately centrifuged and resuspended in each of the freezing extenders. The goal of this portion of the verification was to determine that each of the dyes involved in the triple stain would actually work with the stallion semen and help to eliminate the machine error of identifying egg yolk particles as sperm cell material, therefore, eliminating the need to dilute the semen post-thaw for analysis. Each of the 4 extender samples was analyzed as blank (no dye added), individually with each of the above listed dyes (SYBR-14, RPE-PNA, and PI) and finally with all 3 dyes together. The initial scatter plots appeared to be within normal limits of the reference article (5). For further verification

of this protocol, 10 ml of the original extended semen sample was "killed" by freezing in liquid nitrogen and thawing rapidly in hot water (~50°C) 3 times. The freeze/thaw cycles were completed to cause sperm membrane damage. This "killed" sample was then added back to the original live samples at the following dead:live ratios: 5:0, 4:1, 3:2, 2:3, 1:4, 0:5. This process was completed to verify that the stains were properly monitoring sperm membrane damage.

The protocol was further verified utilizing the semen frozen from a donkey that did not complete the entire collection period (as previously identified). Each sample from the initial frozen ejaculate was thawed as previously described. Then 100 µl of frozen-thawed sample was placed in an appropriately labeled 0.5 ml microcentrifuge tube. Samples that were frozen in the presence of βCD (TRT2 and TRT4) were post-thaw treated as follows: 1) control (C) (nothing added) and 2) addition of calcium ionophore A23817 (I) to a final concentration of 1 µM. Samples that were frozen in the absence of βCD (TRT1 and TRT3) were post-thaw treated as follows: 1) control (C) (nothing added), 2) addition of ionophore to a final concentration of 1 µM calcium ionophore A23817 (I), 3) B (a final concentration of 60 mM βCD, and 4) B+I (a final concentration of 1 µM calcium ionophore A23817 and 60 mM βCD) (See Table for clarification). The samples were maintained at 37°C and samples were analyzed at time 0, 30 minutes and 90 minutes post-staining/treatment.

Table 2. Experimental Design for Post-thaw Semen Treatments

	Post-thaw treatments*:			
Freezing Treatment:	C	I	β	β+I
LEY	Yes	Yes	Yes	Yes
LEY + β-CD	Yes	Yes	No	No
HEY	Yes	Yes	Yes	Yes
HEY + β-CD	Yes	Yes	No	No

***C** = Control, nothing added; **I** = 1 μM Calcium ionophore A23187; **β** = 60 mM 2-Hydroxypropyl-β-Cyclodextrin; **β+I** = 1 μM Calcium ionophore A23187 and 60 mM 2-Hydroxypropyl-β-Cyclodextrin

Once these verification steps were completed, the procedure was completed on the project samples as described above.

G. **Statistical Analysis**

Differences in post-thaw treatments within and between species were examined by analysis of variance using the least squares procedure and general linear models procedure of JMP (SAS Institute, Inc.). Mean separation tests were performed using a Tukey's HSD test. Fertility data was analyzed by Chi Square Analysis.

H. References

1 Garner,Duane L; Johnson,Lawrence A (1995): Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol. Reprod. 53, 276-284.

2 Garner,DL; Johnson,LA; Yue,ST; Roth,BL; Haugland,RP (1994): Dual DNA staining assessment of bovine sperm viability using SYBR-14 and propidium iodide. J Androl 15(6), 620-629.

3 Hancock,JL (1951): A staining technique for the study of temperature-shock in semen. Nature 167, 323-324.

4 Love,CC; Thompson,JA; Brinsko,SP; Rigby,SL; Blanchard,TL; Lowry,VK; Varner,DD (2003): Relationship between stallion sperm motility and viability as detected by two fluorescence staining techniques using flow cytometry. Theriogenology 60, 1127-1138.

5 Nagy,Szabolcs; Jansen,Johannes; Topper,Einko K; Gadella,Barend M (2002): A triple-stain cytometric method to assess plasma- and acrosome membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. Biol. Reprod. 68, 1828-1835.