The utilization of the prostacyclin analogue iloprost in the cryopreservation of stallion spermatozoa

by

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Table of Contents

Abstract

The progress in the stallion semen cryopreservation industry has been slow, creating the need for further research and development directed toward improving post thaw parameters. Most producers utilize glycerol as the primary cryoprotectant when freezing stallion semen, fully knowing that the cell undergoes significant damage during the cryopreservation process as well as from the exposure to the cryoprotectant itself. The current study evaluated the potential of the prostacyclin analogue as a protective additive to stallion semen during cryopreservation. Collections were taken from 12 stallions and divided into four samples, one to be designated as control and the other three to be exposed to various levels of Iloprost (0.001 μg, 0.1 μg, and 1.0 μg). All samples were frozen using a mechanical controlled rate freezer. Semen was analyzed for standard parameters post thaw using a computer assisted semen analyzer at 0 hr, 3 hr, and 4 hr time points. Morphology and acrosome slides were also made. Analysis showed significant decreases in post thaw motility and rapid cell percentages associated with increasing concentrations of Iloprost, suggesting the Iloprost imposes damage to the cell that is detrimental to function.

List of Tables

List of Figures

Chapter I

Literature Review

Introduction

The frozen semen industry is constantly changing and evolving. Many recent research developments have created potential opportunities to expand the use of semen cryopreservation beyond the current limited applications. Cryopreservation is currently a necessary process in order to contribute to more widespread genetics as well as to preserve desired genetics among species. However, the use of frozen semen in the equine industry has progressed significantly slower than that of other species such as human and cattle. Cryopreservation has also proven to be much less efficient in establishing pregnancy in the horse than the industry of transported cooled semen (Loomis, 2001). Further, horses are typically selected for their performance rather than their reproductive efficiency. Individuals wishing to breed particular stallions and continue their lineage rarely consider the reproductive potential of said stallion, potentially making the cryopreservation of their semen more difficult. Although problems have arisen, there has been some success with the utilization of frozen/thawed semen in the horse. The first successful cryopreservation of equine spermatozoa was reported by Smith and Polge in 1950. Only a few years later in 1957, the first equine pregnancy was documented with the use of frozen/thawed semen (Barker and Gandier, 1957). Stallion semen cryopreservation is a frequently utilized practice in today's horse industry in order to preserve the genetics of desirable bloodlines (Loomis, 2001). In 2001, both the American Quarter Horse Association (AQHA) and the American Paint Horse Association (APHA) approved the use of frozen-thawed semen (Loomis, 2001). This had a significant impact

on the industry because the AQHA and the APHA are two of the largest breed associations in the country. Despite this, the equine industry still faces multiple challenges in regard to efficiency.

As stated above, frozen/thawed semen has significantly higher fertility issues than that of fresh or transported cooled semen. Previous research suggests this reduction in fertility is the result of alterations in the membrane structure and function that occur during the cooling, freezing, and thawing process (Parks and Graham, 1992). Overall fertility of frozen/thawed semen can also be affected by semen quality, insemination dose, mare selection, and mare management (Loomis, 2001). There is a general feeling in the industry that the quality of both frozen semen and transported cooled semen is variable around the United States. However, unlike the cattle industry, there is no national network of A.I. centers and therefore no centralized system that compiles results. There is also no certification process for semen production facilities, A.I. centers, or semen handling technicians. This lack of structure does not allow for proper recording of success rates with the utilization of frozen/thawed semen or transported cooled semen (Loomis, 2001). However, it could be expected that with further research and improvement upon the quality of frozen/thawed semen, its utilization would increase further, greatly expanding the industry as a whole.

Frozen/thawed semen presents greater challenges in regard to labor requirements compared to other insemination methods. More frequent ultrasounds are required on mares in order to properly time insemination with ovulation. It has been established that the longevity of cryopreserved semen within the female tract is relatively low, so timing with ovulation is critical in order to produce a pregnancy (Loomis, 2001). Although

difficult, there are many advantages to the utilization of frozen/thawed semen. Stallions do not need to be as readily available and can be shown throughout the breeding season. It could also be used to prevent permanent removal of a particular stallion's genetics from circulation due to illness, injury, or death. Frozen/thawed semen can make shipments easier to schedule, and opens the opportunity for international distribution. The widespread use of frozen/thawed semen would also significantly lower the waste of unused or discarded semen in comparison to the current method of transported cooled semen.

Stallion Reproduction and Endocrine Control

In a similar manner to mares, stallions have been shown to be seasonal breeders with their reproductive efficiency being reported at its highest during the long days of summer months (McKinnon and Voss, 1993). When selecting a stallion for breeding purposes, traditionally many producers look at both age and testicular size. Oftentimes, young stallions are overused before they have had the chance to reach their optimal age for reproductive efficiency. This tends to result in the underutilization of older stallions. False assumptions have been made that older stallions cannot produce the quantity or quality of semen seen in the younger stallions, but this is solely determined on an individual basis. It has been shown that each stallion is different and will have varying semen qualities and parameters (Loomis, 2001). This has not deterred producers from gravitating toward younger stallions because of their endurance and performance along with reproductive potential. It has been well established that testicular size is a good indicator of potential sperm output (McKinnon and Squires, 1988). Larger testicles in a

stallion are being associated with a higher sperm output and greater overall reproductive efficiency than smaller testicles.

As in most higher order species, the testes have been established as the site of both sperm and testosterone production in the stallion (McKinnon and Squires, 1988). Testosterone, the primary sex hormone from the male, is produced by the Leydig cells in the testes and is regulated by luteinizing hormone (LH) from the anterior pituitary. The LH is regulated by gonadotropin releasing hormone (GnRH) from the hypothalamus. Spermatogenesis, the process of sperm production, takes place in the seminiferous tubules over a time period of around 57 days in the stallion (McKinnon and Squires, 1988). It is divided into two phases including spermatocytogenesis, and spermiogenesis. Spermatocytogenesis is divided into both mitotic and meiotic cell divisions. The first stage includes the multiple developmental changes involved in mitosis as well as the differentiation of spermatogonia. Meiosis divisions involve the production of haploid spermatids through the exchange of genetic material between homologous chromosomes. Spermiogenesis is the final stage of spermatogenesis and is the final differentiation of the spermatids into spermatozoa, at which point they are released into the lumen within the seminiferous tubules (McKinnon and Voss, 1993). The process has been shown to progress continuously with a cohort of germinal cells entering the beginning stages about every twelve days. All cells within each cohort develop at a synchronous rate through the stages of spermatogenesis. Sperm production has also been shown to be constant and not dependent on ejaculation frequency. It has been shown that if ejaculation frequency is drastically increased, the number of sperm per ejaculate will decrease, suggesting there

are only a certain number of spermatozoa available on a daily basis (McKinnon and Voss, 1993).

Upon completion of sperm production, the sperm leave the testes and proceed to the epididymis. However, the sperm that leave the testis are infertile and they must undergo a series of changes that allow for further maturation and the eventual development of fertilization capabilities (McKinnon and Voss, 1993). The sperm cells travel through the caput and corpus epididymis before finally reaching the cauda epididymis, where they are stored until ejaculation. The cells that are stored within the cauda epididymis have gained biochemical processes, which make them both motile and fertile. At this point, the spermatozoa are mature and ready for ejaculation.

The accessory sex glands, which include the prostate gland, the bulbourethral glands, and the vesicular glands, play a critical role in semen production (McKinnon and Voss, 1993). They contribute the majority of the fluid to the ejaculate. The prostate gland secretes the majority of the seminal plasma fluid, with the bulbourethral glands secreting only a minor portion. The vesicular glands secrete what is known as the gel portion of the ejaculate. Sperm cells that are stored in the cauda epididymis are only slightly motile until they are mixed with the fluids produced by the accessory sex glands.

As stated above, the complex process of sperm production is highly regulated through endocrine control. The hypothalamus produces GnRH, which acts as the trigger for the stimulation of production and subsequent release of follicle stimulating hormone (FSH) and LH (McKinnon and Voss, 1993). The release of GnRH prompts the pituitary gland to release FSH and LH. The Leydig cells in the testes produce testosterone, but this production is controlled by the LH release. The testosterone that is produced enters

circulation and eventually travels back around to the hypothalamus and pituitary gland. This activates a feedback loop that further regulates the secretion of GnRH and LH. For example, if there is high LH in circulation, there will be a very low GnRH output by the hypothalamus. Sertoli cells located in the seminiferous tubules produce inhibin and activin (McKinnon and Voss, 1993). Inhibin production acts to directly regulate the production of FSH, which works in conjunction with testosterone to stimulate spermatogenesis. Activin has the opposite effect, directly promoting FSH release.

Stallion Semen Biology and Development

The biology of stallion semen is critical for its fertilizing potential. Each spermatozoa must possess a specific number of qualities in order for it to be classified as having the ability to fertilize an oocyte. A few of these characteristics include: the production of energy, progressive motility, the presence of enzymes within the acrosome, the proper arrangement of lipids within the plasma and acrosomal membranes, and the presence of proper proteins within the plasma membrane that are needed for the survival of the spermatozoa within the female reproductive tract (McKinnon and Voss, 1993). One of the most crucial structures to a spermatozoa in regards to its survivability as well as its cryopreservation potential is its plasma membrane. The plasma membrane is the outermost component of a spermatozoa that encompasses the entire structure. It consists of three major parts: the lipid bilayer, the phospholipid-water interface, and the glycocalyx. The lipid bilayer is primarily composed of polar phospholipids with fatty acyl chains. Within the lipid bilayer, phospholipids and cholesterol are the primary lipid components. There are also proteins dispersed within the lipid bilayer that can serve as channels or surface receptors. Overall membrane fluidity is determined by the ratio of

cholesterol to phospholipids and the nature of the phospholipids within the lipid bilayer (McKinnon and Voss, 1993).

The head of a spermatozoa contains the nucleus and the acrosome (McKinnon and Voss, 1993). The shape of the head is greatly determined by the shape of the nucleus, which, for stallions, is primarily broad and flat. The acrosome overlays the nucleus and contains glycolipids and enzymes. The primary enzymes stored within the acrosome are hyaluronidase, proacrosin/acrosin, and lipases. These enzymes are necessary in order to break down the zona pellucida of the oocyte for fertilization, but this process cannot take place until the cells have undergone a remodeling called the acrosome reaction. The acrosome reaction changes the plasma membrane to allow leakage of the enzymes from under its cap and fusion of the sperm cell to the zona pellucida.

Each sperm cell requires energy in order to maintain proper function including motility. Most of the energy is obtained from extracellular substrates such as the breakdown of glucose by the mitochondria (McKinnon and Voss, 1993). The mitochondria's major function is to supply the cell with energy; however, it also plays a major role in the regulation of cell death (Ott et al., 2007). It has been demonstrated that the mitochondria play a critical role in the regulation of apoptosis and that decreased mitochondrial membrane potential can be associated with the induction of apoptosis (Ortega-Furrusola et al., 2008).

Earlier research has shown the sperm must undergo a series of changes before they are capable of fertilizing an oocyte. Capacitation is a necessary process each sperm cell must go through in order for it to have fertilizing capabilities (Neild et al., 2003). This process is accompanied by a reduction in the sperm's life span and eventual cell

death. Capacitation involves changes in the sperm cell that result in hyperactivated motility and the ability to undergo the acrosome reaction (McKinnon and Voss, 1993). No clear morphological change occurs during the process of capacitation.

Stallion Semen Cryopreservation

The cryopreservation of stallion semen has been shown to be extremely variable on a stallion-to-stallion basis (Alvarenga et al., 2005). It has been estimated that only somewhere between 30-40% of stallions produce semen that meet the requirements for cryopreservation (Alvarenga et al., 2005). With the current technology available, some stallions do not produce high enough quality of semen to withstand the physical forces encountered during the freezing process. From a management standpoint, other stallions' genetics do not warrant the highly increased costs associated with the use of frozen/thawed semen (Loomis, 2001). Mare management when utilizing frozen/thawed semen is much more demanding and costly because of the exact timing of ovulation that is necessary for success. It requires more frequent ultrasounds, which increases costs. Further, studies have shown there is a significantly shortened life span associated with frozen/thawed semen than with cooled or fresh semen. It has been suggested that mares be inseminated with frozen/thawed semen within twelve hours prior to eight hours after ovulation (Loomis, 2001).

Evaluation of post-thaw quality has been shown to be critical to the success of freeze/thaw programs and spermatozoal motion is one of the parameters most frequently studied when evaluating sperm viability post-thaw. Motility is used so often because of its accessibility and ease of performance (Katila, 2001). It is typically evaluated as total motility and progressive motility. Total motility includes any type of motion and

progressive motility only includes actual forward motion. Spermatozoal motion was evaluated by Blach et al., in 1989 through a variety of parameters using the computer assisted semen analyzer (CASA). His observations included: percentage of motile sperm, percentage of progressively motile sperm, curvilinear velocity, straight line velocity, linearity, mean amplitude of lateral head displacement, and radius of the average path for circularly swimming sperm (Blach et al., 1989). Each of these parameters was evaluated over the steps of cryopreservation. The results showed a decrease in percentage of motile sperm over centrifugation, removal of seminal plasma, and resuspension of sperm in the freezing extender. The greatest difference in motion, however, was demonstrated from freezing and thawing. There was a lower percentage of motile sperm in the frozen/thawed sample and the sperm that was motile showed less head movement and moved slower than those in fresh samples. The motion parameters were not statistically different between semen stored at -196°C for 1-3 hours and semen stored at -196°C for 10-20 days. These results further support the findings that stallion semen sustains significant damage throughout the cryopreservation process, most of which is detrimental to function.

Another parameter frequently utilized when evaluating frozen/thawed semen is the percentage of live cells. With frozen/thawed semen, it has been shown that there is generally a significant decrease in live spermatozoa percentage post-thaw (Ortega-Ferrusola et al., 2008). The steps in the cryopreservation process have been shown to contribute to this decrease in live cell percentage post-thaw (Neild et al., 2003). The study demonstrated an overall increase in sperm death throughout each step of sperm processing for cryopreservation. The pre-freezing cooling rates are a large factor in

survivability. Sperm have been shown to be more resistant to injury during the cryopreservation process when exposed to slower pre-freeze cooling rates in comparison to faster pre-freeze cooling rates (Salazar et al., 2011), possibly due to changes in lipid structure due to peroxidation (Goolsby et al., 2014).

Apoptosis is a required process in the testis that ensures proper spermatogenesis (Caselles et al., 2014). This process is often evaluated with semen cryopreservation because there is a correlation between the number of apoptotic bodies and total motility and viability post-thaw. Sometimes cells that are destined for apoptosis escape the testis, which causes a significantly higher concentration of apoptotic or abnormal spermatozoa in the ejaculate. Although the correlation exists between apoptotic bodies and post-thaw viability, there appears to be no correlation between the number of apoptotic bodies and fresh semen parameters, including: volume, concentration, total motility, progressive motility, and viability. However, a number of apoptotic markers have been utilized as a means of forecasting freezability in stallions (Ortega-Ferrusola et al., 2009). They include membrane changes, mitochondrial membrane potential, and caspase activity.

Membrane integrity continues to be a major concern of researchers when exposing sperm cells to the cryopreservation process because of the stresses they undergo (Parks and Graham, 1992). The addition of cryoprotectants can be detrimental because of the induced volumetric changes. Intracellular ice formation is a large concern because of it deleterious effects it can have on sperm cell structure and therefore function. There is also a substantial amount of dehydration that occurs during the process along with phase transitions of the phospholipids located within the membrane. The acrosomal membrane has also been shown to undergo some harmful effects during the process.

Previous research suggests that there is an overall decrease in the percentage of sperm with intact acrosomal membranes over the entire cryopreservation process (Blach et al., 1989). In that study, cells were evaluated over four steps, including: within fifteen minutes of collection, prior to freezing, after 1-3 hours of storage at -196°C and after 10-20 days of storage at -196°C.

It has been suggested the overall membrane integrity is also adversely affected by the reordering of these membrane lipids during the cryopreservation process (Parks and Graham, 1992). The data suggests cryopreservation processes potentially disturb the lipid-lipid and lipid-protein interactions that are essential for normal membrane function. As the temperature is reduced during cryopreservation, lipids undergo a phase change from a fluid like state to a more gel like state in which they are more rigid and parallel to one another (McKinnon and Voss, 1993), and their fluidity steadily decreases as the temperature decreases (Oldenhof et al., 2012). Further, the extent and rate of the membrane dehydration, a complicating factor in membrane integrity, is highly dependent on the media in which the cells are frozen. It was found that the addition of cholesterol could be beneficial to sperm cells because of its role in maintaining membrane integrity post-thaw (Oliveira et al., 2010). It was suggested that the addition of cholesterol protected the membranes from freezing induced damage. Additionally, overall freezability was shown to have some variability between different stallions based on the composition of fatty acids or plasmalogens in the plasma membrane (García et al., 2011), and a correlation was found between percent of unsaturated fatty acids with intact sperm membranes post-thaw.

A study was conducted utilizing glycerol as a cryoprotectant to determine membrane characteristics during the freezing process (Oldenhof et al., 2012). A programmable-rate freezer was used and the cells were cooled to 5°C during a time span of two hours then they were cooled to -140°C at 60°C per minute. All straws were thawed in a 37°C water bath. It was found that the membranes are in a fluid phase at 37°C with fluidity slowly decreasing as temperature decreases. They undergo a phase transition from 30°C-10°C, which is most likely the result of the membrane components undergoing some reorganization. Cholesterol removal from the membrane was shown to increase fluidity especially when the temperatures were a little higher. Treatment of the membranes with methyl-β-cyclodextrin removes the cholesterol and increases the main phase transition temperature from around 24°C to around 32°C, but both the quantity and rate of overall membrane dehydration are highly dependent on what the sperm cells are frozen in (Oldenhof et al., 2012).

Mitochondrial activity has been tracked throughout the stallion semen cryopreservation process because of the role mitochondria play in providing the sperm cell's energy. Without a proper supply of energy, cellular function would be impeded and the ability to fertilize an ovum compromised. Mitochondrial function has been evaluated by measuring mitochondrial membrane potential. The results of one experiment showed a significant decrease in mitochondrial membrane potential throughout centrifugation, cooling, and the freezing-thawing process (Ortega-Ferusola et al., 2008).

Freezing methods are an important factor to consider when evaluating the viability of stallion semen post-thaw. Programmable freezers are often used because they provide a more consistent and reliable freeze than some other methods (Clulow et al., 2008). They have the ability to precisely control cooling rates to better protect the cells from intracellular ice formation and osmotic shock. However, they are extremely expensive and are not always financially feasible for some operations.

Cryoprotectants must be added to the semen prior to the actual freezing process in order to help protect the cells against freezing induced damage. There are many different kinds of cryoprotectants available and the ability of a compound to act as an effective cryoprotectant has been shown to largely be dependent upon its ability to protect the cell during freezing while limiting its own toxicity toward the cell while not frozen (Squires et al., 2004). To date, glycerol has been the most widely used cryoprotectant across species for the freezing of spermatozoa (Alvarenga et al., 2005). However, it causes some injury to cells during the freezing process. Various concentrations of glycerol have been tested to determine which one is the most ideal for cryopreservation. In one experiment with equine semen, the post thaw motility was shown to be higher in 4% glycerol in comparison to 2% and 6% (Cochran et al., 1984).

Numerous chemical characteristics have been examined in order to precisely determine what properties make an effective cryoprotectant. An ideal cryoprotectant has been suggested to have low molecular weight, good water solubility, and low levels of toxicity associated with it. Amides are a fairly new class of cryoprotectants that have a lower molecular weight than glycerol. Recent studies have suggested they can enhance

post-thaw motility and preserve membrane integrity more effectively than glycerol (Alvarenga et al., 2005).

The various cryoprotectants have been compared in order to determine which is the superior. Glycerol and various amides have often been tested against one another. Sperm cryopreserved in the presence of glycerol have shown higher percentages of total motility and progressive motility in comparison to sperm cryopreserved in the presence of formamide, acetamide, or methyl acetamide (Squires et al., 2004). However, methyl formamide and dimethyl formamide have been demonstrated to yield similar results post-thaw to that of glycerol, suggesting that they can be used successfully as cryoprotectants for stallion semen. Further, antidotal evidence suggests it also is possible that beside the chemical differences between cryoprotectants, there is also a difference between stallions, suggesting the choice of cryoprotectant may be stallion specific with some being more effective on particular stallions than others.

BotuCrio is a new cryoprotectant, which contains both glycerol and methylformamide that is being utilized at an increasing rate in horses (Pukazhenthi et al., 2014). Studies have shown the glycerol concentration is relatively low while the methylformamide acts as the primary cryoprotectant (Melo et al., 2007). Because methylformamide has a lower molecular weight than glycerol, it may induce a lower level of osmotic stress upon the sperm cell during the freezing process. Recently, the use of amides in combination was studied against glycerol and BotuCrio (Pukazhenthi et al., 2014). Dimethylformamide and methylformamide were combined and their incorporation yielded higher motility values post thaw than that of just glycerol. Similar to methylformamide, dimethylformamide has a lower molecular weight than glycerol and

therefore a higher permeability to the sperm cell than glycerol (Maziero et al., 2013). The BotuCrio yielded similar motility results to that of the amide combination (Pukazhenthi et al., 2014). This study demonstrates the potential of BotuCrio as well as the superiority of amides in regard to post thaw motility.

BotuCrio was used as the primary cryoprotectant in a study examining the effect of sperm concentration and straw volume (Nascimento et al., 2008). The sperm frozen in BotuCrio at a concentration of $100x10^6$ sperm/mL had greater motion characteristics, a higher percentage of intact plasma membranes, and higher mitochondrial membrane potential than sperm frozen at a concentration of $400x10^6$ sperm/mL. Not only did this research demonstrate the effectiveness BotuCrio as a cryoprotectant in the horse but it also suggests a correlation between motility values and concentration. A later experiment with BotuCrio again demonstrates the potential benefits of the addition of seminal plasma and the potential for improved fertility in cryopreserved stallion semen (Andrade et al., 2011). Further studies compared fertility and post-thaw characteristics between sperm collected from the epididymis and sperm collected using an artificial vagina (Monteiro et al., 2011). BotuCrio was utilized as the cryoprotectant for all samples in the trial. The total sperm count was found to be higher in samples recovered from the epididymis than when collected using an artificial vagina, but the total and progressive motility was higher in sperm collected using the AV. However, the overall conception rate was higher when using the epididymal sperm than freshly ejaculated sperm.

Other studies compared BotuCrio to previously used cryoprotectants. In a study comparing BotuCrio to a glucose-EDTA-lactose extender with glycerol in regard to fertility and post thaw parameters (Samper and Garcia, 2008) it was concluded that the

BotuCrio appeared to improve quality and fertility of stallions post thaw; including those that may previously have been considered poor freezers. It was determined the post-thaw progressive motility was higher in samples cryopreserved in the BotuCrio. Further the overall fertility was significantly higher in stallions that were once considered poor freezers when utilizing the BotuCrio over the glucose-EDTA-lactose extender with glycerol. Another study examined the effectiveness of various cryoprotectants on stallion epididymal sperm (Papa et al., 2008). The total and progressive motility were significantly higher in semen cryopreserved in BotuCrio in comparison to semen cryopreserved in EDTA-Lactose of INRA82. The BotuCrio performed the best out of the three freezing extenders tested when looking at post-thaw motion parameters, and the semen cryopreserved in BotuCrio produced a 66.6% pregnancy rate.

Milk and egg yolk are the most frequently used protein sources for the cryopreservation of semen (Papa et al., 2011). They act as a source of lipoprotein that protects the cells from cold shock during the freezing process. However, there has been some concern with their continued use due to the fact that there is a potential biosecurity issue concerning their use in international transport and the fact they are animal-source additives. Therefore alternative protein sources have been exampled. A study was done to determine if soybean lecithin would be a valid substitute for the egg yolk (Papa et al., 2011). BotuCrio was utilized as the cryoprotectant, but the egg yolk in one group was replaced with soybean lecithin. Both sperm motility and plasma membrane integrity were found to be the same in both groups, but fertility and pregnancy rates were increased for sperm frozen directly in BotuCrio without the soybean lecithin addition.

BotuCrio was used as a cryoprotectant in a study aimed toward comparing the effect of volume on storage, Following preparation, semen was frozen in 0.25 ml straws and 0.5 ml straws (Maziero et al., 2013). Although BotuCrio produced successful parameters for both straw sizes post thaw, the sperm frozen in the 0.5 ml straws demonstrated higher sperm kinetics. Studies were also conducted with BotuCrio to determine if cooled storage time had any long-term effect on stallion semen fertility and viability (Melo et al., 2007). The results demonstrated that there was no change in the total or progressive motility when the semen was cooled for 24 hours prior to freezing. However, membrane integrity was higher in sperm cells that were frozen immediately after collection. Further, progressive motility was also shown to be higher in the BotuCrio containing glycerol and methylformamide in comparison to BotuCrio containing glycerol and dimethylformamide or a control cryoprotectant consisting of glycerol and dimethylacetamide.

BotuCrio has been utilized as a cryoprotectant for studies involving species other than the horse; including the Baird's Tapir (Pekazhenthi et al., 2011). For this animal, the BotuCrio was compared to INRA 96. The results showed no difference in sperm motility between the BotuCrio and INRA 96 up to 3 hours of incubation post-thaw. However, at 4 hours of incubation post-thaw, the sperm cells cryopreserved in BotuCrio had higher motility than those cryopreserved in INRA 96, reporting the first use of BotuCrio as a successful cryoprotectant for an endangered species and a means of characterizing its semen traits. BotuCrio was also used in a study to compare its effectiveness against other cryoprotectants when freezing semen from Mangalarga Marchador stallions (Candeias et al., 2012). The Mangalarga Marchador stallions have

previously been reported as relatively poor freezers. In the study, semen parameters were compared post-thaw after cryopreservation using BotuCrio, INRA82 with 4% glycerol addition, and Merck Gema with 4% glycerol addition. The total and progressive motility post-thaw was significantly higher in sperm frozen in BotuCrio in comparison to other treatments.

BotuCrio was utilized in an experiment that was attempting to remove seminal plasma with a filter prior to cryopreservation (Ramiers Neto et al., 2013). The presence of seminal plasma can cause damage to the sperm when undergoing the cryopreservation process. Study results demonstrated the filtering technique was effective in removing the seminal plasma prior to freezing and marked yet another successful use of BotuCrio as a cryoprotectant.

Together, these studies support the use of BotuCrio as a cryoprotectant for both the epididymal sperm and ejaculated sperm in the equine as well as it potential use in endangered species.

Iloprost Properties

Prostacyclin is a member of the prostanoid family, a subclass of the eicosanoids. It is the main product of the metabolism of arachidonic acid that occurs both in endothelial and smooth muscle cells (Lundblad et al., 2008). Prostacyclin synthase is the primary enzyme involved in the synthesis of prostacyclin. Its method of action is still relatively unclear but it works primarily as a vasodilator (McCalden et al., 1984) although it has also been shown to have some permeability-reducing properties (Bentzer and Grände, 2004). It is the main prostanoid produced by the vascular endothelium and causes blood vessel dilatation while inhibiting platelet aggregation (Smyth and

FitzGerald, 2002). There has also been evidence suggesting its potential anti-apoptotic qualities (Pakrasi and Jain, 2008).

The prostacyclin analogue Iloprost is not a new compound. It has been generally thought to be involved with the maintenance of vascular homeostasis, but its use has further developed and adapted. It has been shown over the years to possess some cytoprotective qualities and a number of studies have been done to investigate its use in this capacity (Bursch and Schulte-Hermann, 1986; Fitzl et al., 1999; Johnson et al., 1984; Palaoglu et al., 1990). It has also been extensively used in works involving embryos (Huang et al., 2003; Huang et al., 2004; Kim et al., 2010; Song et al., 2009).

When exposing cells to a compound such as Iloprost, the toxicity of the compound must be investigated in order to ensure that the benefits of its addition outweigh any toxic effects that may occur. The toxicity of Iloprost was examined on the reproductive performance of rats, rabbits, and monkeys (Battenfeld et al., 1995). The results showed no clear negative effects regarding the addition of Iloprost to male or female rats. Similar results were found on both the rabbits and the monkeys except for some slight retardations in the rabbit fetuses when exposed to high concentrations of the Iloprost.

Recent Works with Iloprost

Although originally thought to be primarily involved with the maintenance of vascular homeostasis, Iloprost has been utilized for multiple other purposes since its development. Its cytoprotective qualities have been examined on a variety of different tissues and cells including embryos, sperm, adrenal medullary grafts, cortical brain tissue grafts, platelets, liver, and myocardial myocytes (Bursch and Schulte-Hermann, 1986;

Caglar et al., 2007; Davies et al., 1992; Fitzl et al., 1999; Huang et al., 2003; Huang et al., 2004; Johnson et al., 1984; Kim et al., 2010; Palaoglu et al., 1990; Song et al., 2009). Its use in cryopreservation is limited but the potential of a prostacyclin analogue in that regard was demonstrated on platelet concentrates (Johnson et al., 1984). The addition of a prostacyclin analogue was shown to help maintain platelet function post-thaw. This suggests the possibilities of using a prostacyclin analogue such as Iloprost through the cryopreservation process of reproductive tissues.

The protective qualities of Iloprost in cortical brain tissue grafts from rats were examined in 1990 (Palaoglu et al., 1990). The graft tissues were stored in either Iloprost or saline for various time periods including: thirty minutes, three hours, six hours, and twenty-four hours. The results show significantly better preserved cellular components of the graft tissues stored in the Iloprost versus those stored in the saline. The tissue stored in the saline showed greater tissue dissolution and nuclear degeneration. By comparison, the organelles, nucleus, and chromatin were all well preserved in the grafts exposed to Iloprost. Iloprost was also utilized on adrenal medullary grafts (Caglar et al., 2007). Graphs maintained in Iloprost prior to transplantation into patients suffering from Parkinson's disease were shown to better maintain viability. This particular study also suggested the potential role of Iloprost in the prevention of apoptosis, a quality that would be extremely beneficial in regard to the culture and storage of reproductive tissues.

Earlier studies had demonstrated the protective effect of Iloprost in liver cells (Bursch and Schulte-Hermann, 1986). Treatment with Iloprost preserved normal hepatocellular morphology in tissues previously exposed to carbon tetrachloride and bromobenzene, two compounds known for their toxicity. Further, it reduced the release

of hepatocellular enzymes that are associated with toxin exposure into the bloodstream. Both of these results suggest the cytoprotective qualities of Iloprost in regard to rat hepatocytes. Iloprost was also shown to prevent paracetamol toxicity in hamster hepatocytes (Davies et al., 1992). Paracetamol is a widely used analgesic that in large doses can cause a loss of viability in liver cells. The hepatocytes were further protected from this toxicity when exposed to Iloprost at a concentration of 10^{-10}M. The study further suggests that the protective effects of Iloprost could be demonstrated at low concentrations; between 10^{-8}M and 10^{-14}M, which might possibly allow its use while avoiding any toxicity associated with higher concentrations.

Originally thought to be only useful for maintenance of vascular homeostasis, Iloprost has also been shown to have protective qualities in the heart (Fitzl et al., 1999; Smith et al., 1984). It was shown to preserve the myocardial integrity during periods of ischemia in rabbit hearts, those not receiving enough blood supply (Smith et al., 1984); as demonstrated by a significantly greater recovery of left ventricular pressure and an increase in coronary resistance in those hearts exposed to Iloprost. Iloprost was also utilized to protect the ultrastructure of myocardial myocytes in dogs (Fritzl et al., 1999). The experiment was performed in order to determine the protective effect of Iloprost toward the myocardium during periods of ischemia and reperfusion and it was found it restored blood flow. Special attention was paid to the damage to the mitochondria during the trial. The cells not exposed to Iloprost showed significantly greater damage to the mitochondria than those treated with Iloprost; as indicated by less mitochondrial edema and structural degenerations in those hearts treated with Iloprost.

The prostacyclin analogue Iloprost has also been shown to have a positive effect on embryo development (Huang et al., 2003; Huang et al., 2004; Kim et al., 2010; Song et al., 2009). Exposure to Iloprost restored mouse embryo hatching after subjection to a COX-2 inhibitor (Huang et al., 2004), which had previously been shown to inhibit embryo hatching. Although the mechanism is not known, the restoration of hatching suggests the protective capabilities of Iloprost. In a subsequent study done by Huang et al., Iloprost also was shown to enhance embryo implantation rates in mice (Huang et al., 2004). More implantation sacs were found in the embryos treated with Iloprost than those that were not. In the same study, it also was found that exposure to Iloprost increased the live birth rates of the mice.

Although many preliminary embryo trials were done on mice, Iloprost has also been recently utilized on cattle and pig embryos (Kim et al., 2010; Song et al., 2009). Exposure to Iloprost showed to stimulate embryonic development in cattle (Song et al., 2009). The embryos were exposed to Iloprost at varying concentrations including 0, 0.1, 1, and 10 μM in order to determine optimum concentration for effectiveness and potential toxicity. The rate of development to the blastocyst stage was greater in the embryos exposed to Iloprost than the control. Further, progression to the blastocyst stage was the greatest in embryos exposed to Iloprost at a concentration of 1 μM. Subsequent analysis demonstrated a decrease in the number of apoptotic nuclei in the Iloprost treated blastocysts. This further supports the potential anti-apoptotic qualities of Iloprost. The addition of Iloprost demonstrated similar results in pigs by stimulating meiotic maturation and embryonic development (Kim et al., 2010). This study utilized Iloprost at concentrations of 1, 5, and 10 μM; consistent with the study done

in cattle (Song et al., 2009). Embryonic development to the blastocyst stage was greatest when utilizing Iloprost at a concentration of 1 μM. The study also demonstrated that the highest number of embryos that progressed through the cleavage stages were those that had been exposed to Iloprost, with those embryos having consistently higher mitochondrial membrane potentials than the controls. Further, there was also a significantly lower quantity of apoptotic nuclei in the treatment groups in comparison to non-treated embryos. Together, this data suggest treatment with Iloprost potentially results in higher quality embryos with an enhanced ability to implant.

Limited studies have been done in regard to the use of Iloprost on sperm cells. Its previous use on other tissues in various animals would suggest its potential for use on spermatozoa. It has been shown that exposure of human spermatozoa to Iloprost had no affect on overnight survival or motility (Huang et al., 2003). The percentages of motile sperm and hyperactivated sperm were not affected by the addition of Iloprost, suggesting a lack of toxicity of Iloprost toward spermatozoa. Further research is necessary in order to completely understand the potential of Iloprost toward the cytoprotection of spermatozoa. The successful application of Iloprost toward spermatozoa, especially in the equine species, would greatly impact the industry allowing for potentially higher survival rates of spermatozoa post thaw. A cost-effective additive that results in improved post-thaw parameters would be a great development for the stallion semen cryopreservation industry. Therefore, the objective of the present study is to determine the effects of Iloprost addition to stallion semen during cryopreservation in regard to post-thaw motion parameters, morphology, and acrosome integrity.

Chapter II

Materials and Methods

Experimental Design

Previous research has shown Iloprost to be membrane protective in myocardium during periods of ischemia (Smith et al., 1984). While previous research has suggested Iloprost has no effect on sperm motility (Huang et al., 2003), it is unclear if its protective qualities might help stabilize stallion semen during cryopreservation. To test this hypothesis, semen from 12 stallions were prepared for cryopreservation using standard cryoprotectants supplemented with one of three concentrations of Iloprost or a non-Iloprost containing control.

Equine Semen Collection

All semen samples were collected from stallions housed and maintained at the 6666's Ranch located in Guthrie, Texas. Samples were collected between 5/27/2014 and 6/12/2014; a period when all stallions had been adequately collected and prepared for the breeding season. Each stallion was housed and maintained in similar environments with a proper feed regiment established by the facility.

Stallions were collected using standard collection methods with a Missouri model artificial vagina and jump phantom. The gel free portion was taken for immediate computer assisted semen analysis (CASA -- IVOS, Hamilton Thorn; Beverly, MA), suspended in INRA-96T (IMV Technologies, Maple Grove, MN), transferred to large centrifuge tubes, and cooled for transport back to the Texas Tech Health Sciences Center using ice packs and a Styrofoam box. Temperatures were continuously monitored using a temperature probe and shown to remain between 12-17^{o}C during transport. Transport

and packing was done following standard procedures of the facility.

Upon arrival at the Texas Tech Health Sciences Center (Lubbock, TX), the semen samples were reanalyzed for concentration and motility using the lab's CASA system (IVOS). Samples with fewer than 500 million cells/milliliter were centrifuged at 5000 RPM for 10 minutes, and a portion of the supernatant removed to adjust the final pre-cryoprotected concentration to a minimum of 500 million cells/mL (confirmed by CASA).

Media Preparation

All media was prepared prior to semen collection. The Iloprost solutions were prepared to contain Iloprost (Santa Cruz Biotechnology; Dallas, Texas) quantities of 1.0, 0.1, and 0.001 micrograms. The original Iloprost was suspended in methyl acetate. The methyl acetate was removed by dehydration using a steady flow of liquid nitrogen vapor across the top of the bottle. Upon complete dehydration, the Iloprost was weighed and separated into small vials for dilution into the three desired concentrations, resuspended in Dimethyl Sulfoxide (DMSO; Sigma-Aldrich; St. Louis, MO), and stored frozen (-18^{o}C) until use.

The cryoprotectant selected for this study was BotuCrio (State University of São Paulo, Brazil), a fairly new cryoprotectant, which has been demonstrated to provide superior post-thaw motility recovery in previous trials with equine semen (Samper and Garcia, 2008; Candeias et al., 2012). The cryoprotectant arrived pre-mixed and was stored frozen (-18^{o}C) until use.

Semen Cryopreservation

Prior to cryopreservation, the semen from each sample was divided into four groups, one to be used as a control and the other three to be exposed to the Iloprost treatments. All semen samples were suspended in a 1:1 ratio of semen to BotuCrio all at once and not in a stepwise fashion while still in their 50 ml polypropylene conical tubes. Each 50 ml conical tube containing the semen in the cryoprotectant was assigned as Control, Iloprost A, Iloprost B, or Iloprost C. Based on previous calculations, the Iloprost dilutions had been mixed so that 27 µL of each could be added to their respective sample. Using a pipette, 27 µL of the Iloprost A (0.001 µg) solution was added to the conical tube containing semen sample designated as Iloprost A. The same procedure was repeated for Iloprost B (0.1 µg) and Iloprost C (1.0 µg). The Control group contained no Iloprost, and was therefore not altered beyond the addition of the cryoprotectant. All tubes were then placed into the refrigerator to rest for about 45 minutes.

The prepared semen was loaded into 0.5 ml straws utilizing a straw filler system (Animal Reproduction Systems; Chino, CA) and heat-sealed, producing five straws per treatment. All straws regardless of treatment were frozen using the Planar Electronic Controlled Rate Freezing system (TS Scientific; Perkasie, PA) using a pre-programmed freezing curve developed for equine semen. Upon reaching a final temperature of approximately -196°C, the straws were loaded into goblets and canes and stored in liquid nitrogen containing cryotanks for at least 7 days prior to thawing.

Semen Thawing

Straws were removed from the cryotanks at random and transferred to a styrofoam box containing liquid nitrogen. Each straw was held in the air for 30 seconds before being submerged in a 37°C waterbath to thaw for 2.5 minutes. Once completely thawed, straws were removed from the waterbath and the semen pushed into 5 ml polypropylene round-bottom tubes (BD Falcon; Franklin Lakes, NJ) with 0.5 mL of pre-warmed Ham's F-10 Media (Irvine Scientific; Santa Ana, CA) already added. Each sample was then centrifuged for five minutes at 1200 RPM in order to remove the cryoprotectant. The supernatant was removed after completion of centrifugation and the sperm cell resuspended in 0.5 mL of fresh, pre-warmed Ham's F-10 Media. Once prepared, the tube was held at 37°C to await analysis.

Post-thaw Semen Evaluation

Once thawed and prepared, each sample was analyzed for motility parameters using the CASA. A portion of the sample was transferred to a Leja 20 micron slide (Leja; Nieuw-Vennep, Netherlands) and 500 cells were counted. The system provided information for concentration, motility, rapid cells, path velocity, progressive velocity, track speed, elongation, lateral displacement, beat cross frequency, straightness, and linearity. The path velocity refers to the average velocity the cell reaches during its path, while progressive velocity is the velocity reached when the cell progresses forward. Beat cross frequency is the frequency at which the sperm cell's head crosses its path and elongation measures the actual elongation of the sperm cell's head. Straightness is an estimation of the cell's path to a straight line while linearity is an estimation of the cell's path in proximity to a straight line, and track speed measures the velocity of the cell over

its point to point track (Verstegen et al., 2001). Lateral displacement is the mean width of the sperm cell's head oscillation as it moves (Kathiravan et al., 2008). These measurements were taken immediately after sample thaw and preparation (0 hr post-thaw), 3 hr post-thaw, and 4 hr post-thaw.

In addition, morphology and acrosome slides were prepared simultaneously at each time-point. All morphology and acrosome slides were made on standard glass slides (Fisher Scientific; Waltham, MA). The morphology slides were prepared by placing a drop of the sample at one end of the slide and performing a smear using another unused glass slide. The acrosome slides required the addition of 3 μL of 3% formalin. The formalin was added to the sample on the slide and the combination mixed prior to performing the smear. The slides were set out to dry prior to loading them in slide boxes for storage at room temperature until it was time for analysis.

After collection of the initial (0 hr post-thaw) date, the tubes were transferred to a 37°C 95% humidity incubator (Forma Instruments; Marietta, OH) where they were held until the 3 and 4 hr time-points.

Staining and Counting of Morphology and Acrosome Slides

Morphology slides were stained using a Stat III Stain: Three Step Andrology Stain kit (Origio; Mt. Laurel, NJ). All slides were prepared following stain kit instructions, which included a series of dips in a fixative solution, an eosin solution, an azure solution, and a final dip in deionized water. The solutions were replaced every 100 slides to ensure adequate staining. All slides were allowed to dry for at least 24 hours prior to analysis on the Lietz microscope (Leica Microsystems; Buffalo Gobe, IL) equipped with a ProgResC_{14} Plus Camera (Jentoik; Jena, Germany). Morphology slides

were read under a 100X oil emersion lens. A total of 100 cells were counted per slide and designated as having normal morphology, abnormal morphology, or abnormal morphology with curled tails based on the criteria outlined by Varner (2008).

Acrosome slides were stained using a saturation chlortetracycline hydrochloride (Sigma-Aldrich; St. Louis, MO) stain (Neild et al., 2003). The stain was prepped by adding 5 grams of the chlortetracycline hydrochloride to about 30 mL of deionized water. The solution was mixed well in a 50 ml polypropylene conical tube (BD Falcon; Franklin Lakes, NJ) that was made light tight by covering it in its entirety in aluminum foil. The solution was filtered prior to slide staining. All slides were stained by exposure to the chlortetracycline solution for approximately one minute and rinsed briefly with chilled phosphate buffered saline (Irvine; Santa Ana, CA). Slides were set out to dry completely before analysis. The staining, drying, and analysis of the acrosome slides were completed in a dark room due to the light sensitivity of the chlortetracycline.

Acrosome analysis was also completed on the Lietz microscope equipped with fluorescent optics and the ProgResC_{14} Plus Camera under a 100X oil emersion lens. A total of 100 cells were counted per slide and evaluated as having an intact acrosome, a partially intact acrosome, or a non-intact acrosome. The nature of the stain allows it to interact more intensely with the acrosomal membrane and causes the cell to light up intensely under the microscope when the membrane is intact. A partially intact acrosome will still light up, but not to the extent of the intact acrosomes. It will be much dimmer and less noticeable. A non-intact acrosome will not light up and is difficult to distinguish under the microscope.

All data was statistically analyzed using the Statistical Package for the Social Sciences Version 12 (SPSS; Chicago, IL). Treatment and time were compared for all parameters including: concentration, motility, rapid cells, path velocity, progressive velocity, track speed, elongation, lateral displacement, beat cross frequency, straightness, linearity, normal morphology, abnormal morphology, abnormal morphology with curled tails, intact acrosomes, partially intact acrosomes, and non-intact acrosomes, using a 2-way analysis of variance (ANOVA). Data was considered to be statistically significant for values of P that were less than 0.05 ($P<0.05$). Any parameter that demonstrated to have statistical significance was reanalyzed utilizing Tukey's HSD test and a one-way ANOVA.

Chapter III

Results

A total of 12 collections were performed on 10 stallions at the 6666's Ranch in Guthrie, Texas. Twenty straws per ejaculate were assigned to the treatments and controls and prepared as described, resulting in a total of 240 straws being analyzed in the study, One straw was lost during the trial and sixteen straws were utilized for preliminary trials. All samples were analyzed for motion parameters, morphology, and acrosome intactness immediately post-thaw, 3 hours post-thaw, and 4 hours post-thaw. All results were analyzed for statistical significance between treatments for the three designated post thaw times.

Concentration

As expected, there was no change in concentration over treatment or time (Table 1). It remained relatively consistent across treatments (p= 0.097) and at each of the three time-points (P =0.148).

Table 1: Mean concentrations over the post-thaw time points of 0 hr, 3 hr and 4 hr for Control, Iloprost A (0.001 μg), Iloprost B (0.1 μg), and Iloprost C (1.0 μg).

Parameter	**Concentration (M/mL)**	**0 hr**	**3 hr**	**4 hr**	**P-value Time:** 0.148
Treatment	Control	125.36	124.70	129.84	**P-value Treatment:** 0.097
	Iloprost A	131.15	133.85	136.54	
	Iloprost B	139.78	128.96	143.98	
	Iloprost C	118.21	129.48	135.17	

Motility

While a previous study in fresh human semen demonstrated no effect of Iloprost on motility (Huang et al., 2003), in the present study there was a stairstep decrease in motility and a number of other motility parameters with increasing concentrations of Iloprost in the cryoprotectant (Figure 1, $P < 0.001$). However, recovered motility appeared relatively stable over time regardless of Iloprost treatment ($P = 0.583$). Overall, the Control group containing no Iloprost demonstrated the highest average recovered motility values over the three time points (13.52±9.86). The lowest Iloprost concentration found in Iloprost A (0.001 μg) produced similar motility values (12.58±10.35) to that of Control with the highest Iloprost concentration found in Iloprost C (1.0 μg) showing the lowest average motility values over the three time-points (9.28±7.02). A Tukey HSD test was utilized to determine which treatments showed statistically significant differences with other treatments. There were significant differences between the treatment Iloprost C and the treatment Iloprost A and Control. However, the treatment Iloprost B was not significantly different from any other treatment or Control.

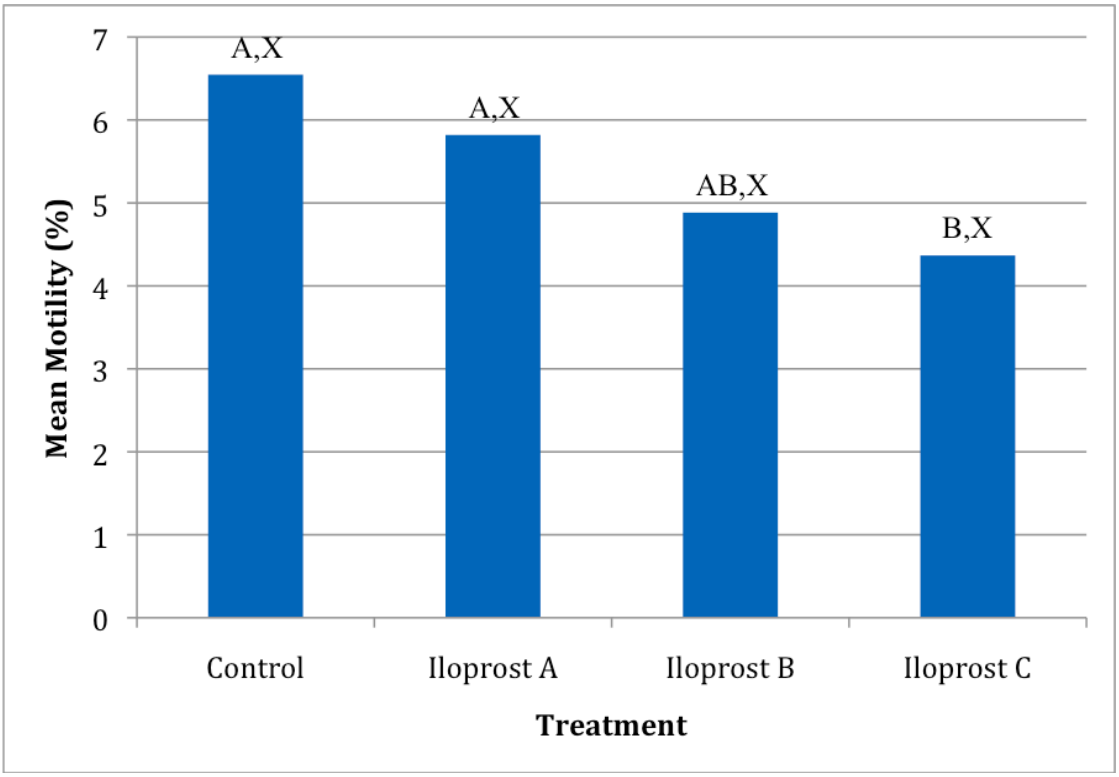

Figure 1: A comparison of recovered average motility values among the Control, Iloprost A (0.001 µg), Iloprost B (0.1 µg), and Iloprost C (1.0 µg) samples demonstrate a decrease in motility with increasing Iloprost concentrations. Differences between treatments are denoted by different subscripts ($P < 0.001$).

Rapid Cells

Along with motility, the percentage of rapid cells has been shown to be a good indication of potential fertility. While there were no differences detected over time (Figure 2; P = 0.982), there was a statistically significant difference between treatments (P < 0.001). Control produced the highest rapid cell percentage (6.55±5.82) while Iloprost C showed the lowest rapid cell percentage (4.37±3.90). Tukey's HSD test was utilized again to determine where the differences arose between treatments. The treatment Iloprost C demonstrated significantly different results from that of Control and Iloprost A while Iloprost B only differed from Control.

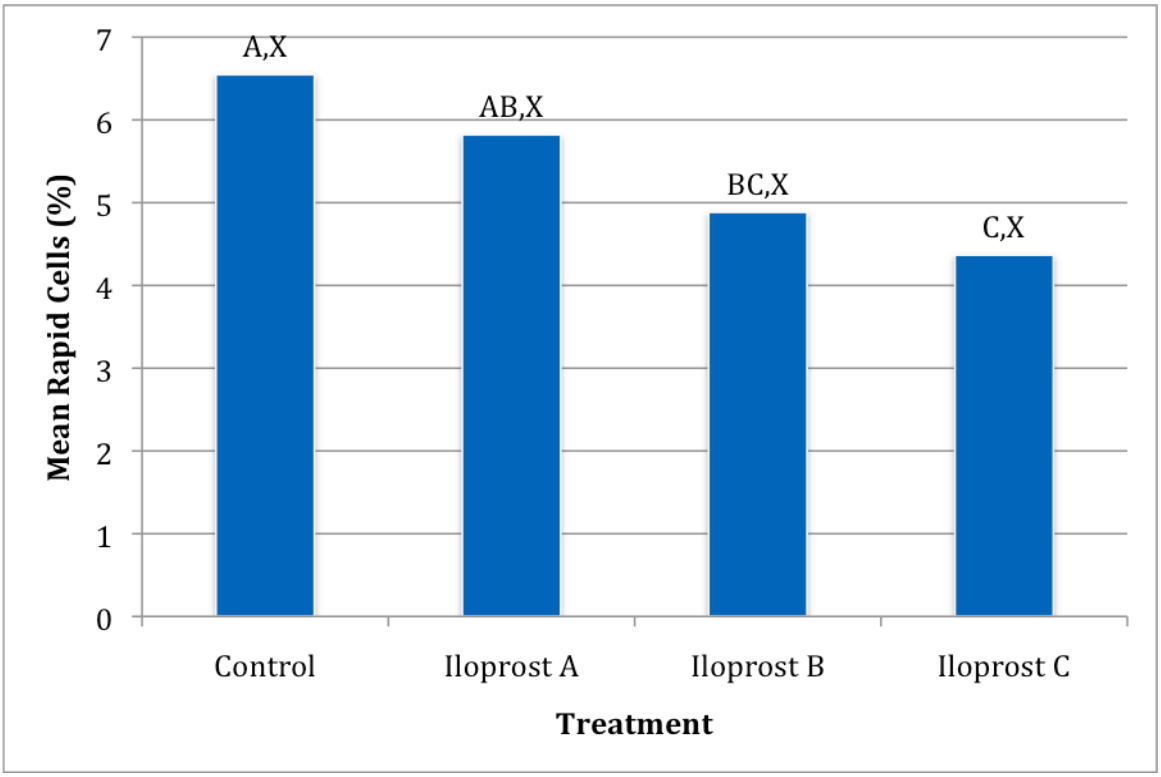

Figure 2: A comparison of recovered average rapid cell values among the Control, Iloprost A (0.001 µg), Iloprost B (0.1 µg), and Iloprost C (1.0 µg) samples. Differences between treatments are denoted by different subscripts (P<0.001).

Motion Parameters: Speed and Movement

The parameters Path Velocity (μm/s), Progressive Velocity (μm/s), and Track Speed (μm/s) all measure the speed of the sperm cell in relation to forward movement, while Lateral Displacement (μm/s) was analyzed to determine sideways motion. The results showed no significant difference in any of these parameters for treatment (Table 2; $P = 0.292$), but a difference did arise in regard to time for Lateral Displacement. While there was no significant difference in Path Velocity, Progressive Velocity, or Track Speed over time ($P = 0.063$), there was a trend toward increasing values between the 0 and 3 hr post-thaw time point. This relationship was also seen in the sperm head Lateral Displacement ($P < 0.001$), where increasing values were seen over all three time-points regardless of treatment. Together, these finding suggest premature capacitation possibly due to cryo-damage.

Table 2: The effect of varying concentrations of Iloprost (Control, Iloprost A (0.001 µg), Iloprost B (0.1 µg), and Iloprost C (1.0 µg)) over time for the various sperm cell speed and movement parameters including: Path Velocity, Progressive Velocity, Track Speed, and Lateral Displacement. Values of $P<0.05$ were considered statistically significant.

Parameter	**Path Velocity (µm/s)**	**0 hr**	**3 hr**	**4 hr**	**P-value Time:** 0.629
Treatment	Control	62.36	65.79	65.60	
	Iloprost A	62.27	64.21	61.98	
	Iloprost B	63.14	63.18	60.19	**P-value Treatment:** 0.372
	Iloprost C	64.35	64.88	65.67	

Parameter	**Progressive Velocity (µm/s)**	**0 hr**	**3 hr**	**4 hr**	**P-value Time:** 0.633
Treatment	Control	44.14	45.00	46.64	
	Iloprost A	45.21	44.72	44.28	
	Iloprost B	46.23	42.92	42.45	**P-value Treatment:** 0.292
	Iloprost C	46.29	45.03	48.67	

Parameter	**Track Speed (µm/s)**	**0 hr**	**3 hr**	**4 hr**	**P-value Time:** 0.063
Treatment	Control	123.77	133.97	130.41	
	Iloprost A	121.71	130.73	124.92	
	Iloprost B	122.50	127.98	120.64	**P-value Treatment:** 0.393
	Iloprost C	125.99	130.68	128.65	

Parameter	**Lateral Displacement (µm/s)**	**0 hr**	**3 hr**	**4 hr**	**P-value Time:** 0.001
Treatment	Control	6.95	7.73	7.75	
	Iloprost A	6.75	7.32	7.42	**P-value Treatment:** 0.263
	Iloprost B	7.30	7.46	7.17	
	Iloprost C	6.44	7.06	7.74	

Motion Parameters: Movement

In addition to motion parameters, pattern forms of sperm cell movement were also analyzed including: Elongation (%), Straightness (%), Linearity (%), and Beat Cross Frequency (Hz). The data shows no significant differences in any of the parameters due to treatment (Table 3; P = 0.249). However, differences were found regarding the post-thaw time points for Straightness ($p<0.003$) and Linearity ($p<0.001$) which were consistent with possible early capacitation in these cells.

Table 3: The effect of varying concentrations of Iloprost (Control, Iloprost A (0.001 μg), Iloprost B (0.1 μg), and Iloprost C (1.0 μg)) over time for the various motion parameters including Elongation, Straightness, Linearity, and Beat Cross Frequency. Values of $P<0.05$ were considered statistically significant.

Parameter	**Elongation (%)**	**0 hr**	**3 hr**	**4 hr**	**P-value Time:** 0.737
Treatment	Control	54.68	53.48	54.57	
	Iloprost A	53.32	52.57	52.55	
	Iloprost B	54.08	54.02	53.86	**P-value Treatment:** 0.254
	Iloprost C	54.02	54.22	53.38	

Parameter	**Straightness (%)**	**0 hr**	**3 hr**	**4 hr**	**P-value Time:** 0.003
Treatment	Control	65.70	64.80	66.21	
	Iloprost A	66.83	64.29	65.21	
	Iloprost B	67.47	64.80	66.61	**P-value Treatment:** 0.352
	Iloprost C	67.76	63.84	69.22	

Parameter	**Linearity (%)**	**0 hr**	**3 hr**	**4 hr**	**P-value Time:** 0.001
Treatment	Control	37.17	36.38	37.91	
	Iloprost A	38.83	35.88	36.66	
	Iloprost B	39.23	36.79	37.88	**P-value Treatment:** 0.249
	Iloprost C	38.85	35.93	40.31	

Parameter	**Beat Cross Frequency (Hz)**	**0 hr**	**3 hr**	**4 hr**	**P-value Time:** 0.586
Treatment	Control	37.38	36.38	35.91	
	Iloprost A	35.93	35.63	36.42	**P-value Treatment:** 0.444
	Iloprost B	36.85	36.37	36.37	
	Iloprost C	36.95	37.10	36.62	

Morphology

Normal morphology is an excellent indication of potential reproductive efficiency as well as a potential indicator of cryo-damage. Results demonstrated a consistent decrease in normal morphology percentages over the three post thaw time points (Figure 3; $P < 0.001$). However, the Iloprost C treatment, the treatment with the highest level of Iloprost (1.0 µg), consistently had the highest average morphology values (76.85±7.91) over time (Figure 3) in comparison to Control (74.86±8.22), Iloprost A (75.32±7.93), and Iloprost B (76.03±8.36).

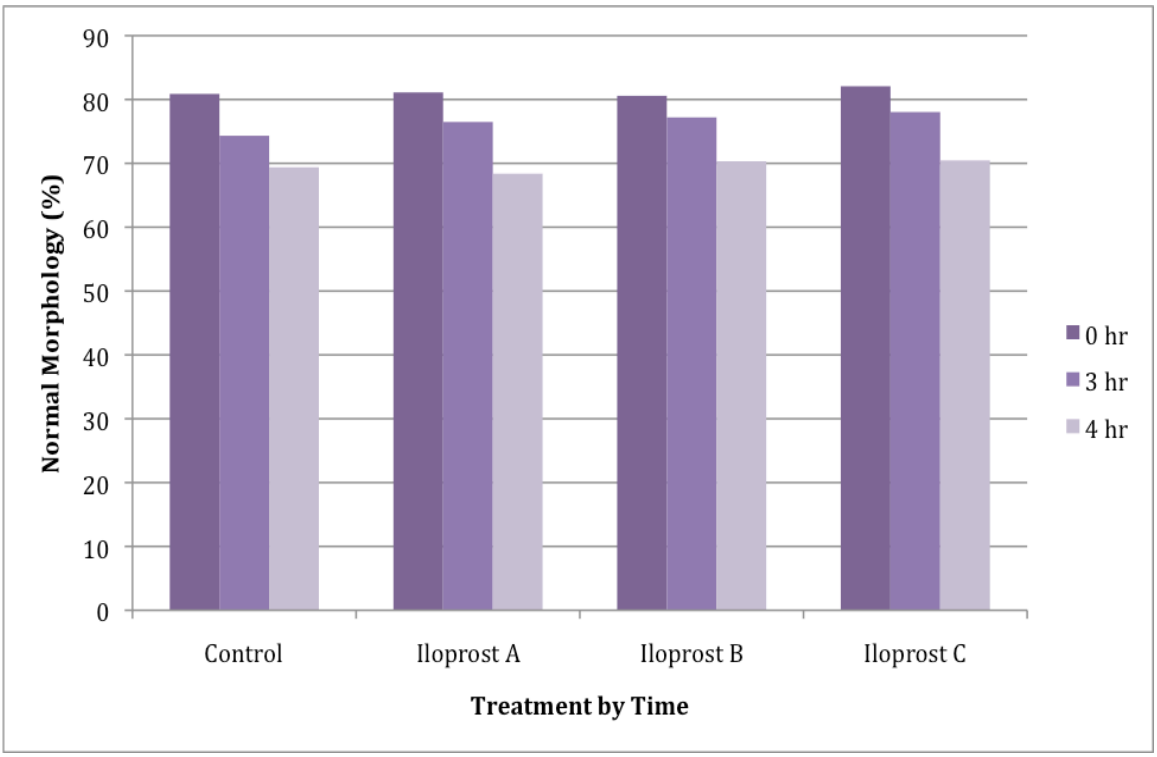

Figure 3: A comparison of the recovered equine sperm cells with normal morphology over time ($P < 0.001$) from cryopreserved equine semen frozen using standard techniques (Control) or with the addition of varying levels of Iloprost (Iloprost A (0.001 µg), Iloprost B (0.1 µg), and Iloprost C (1.0 µg)) ($P < 0.033$). .

Focusing on the types of abnormal forms, abnormal morphology can include a number of very specific defects affecting the head, midpiece, and tail. In the present experiment morphology was examined for defects in these three major areas as well as specific observation of the presence of curled tails. No statistically significant differences could be demonstrated in morphological defects due to the treatments (Table 4; $P = 0.051$), but Iloprost treatment C consistently had the fewest average defects at all time points (Figure 4). However, the number of morphological differences increased in all treatments over time (Table 4; $P < 0.001$) suggesting cryo-damage. While cryo-damage can be manifested as curled tails due to osmotic shock and there were both treatment ($P < 0.035$) and time ($P < 0.027$) differences in the present data (Table 5), the relatively low number of curled tails seen in any treatment (< 1%) suggests this effect is of little significance.

Table 4: The mean number of abnormal cells recover over time from post-thaw equine semen samples treated with varying concentrations of Iloprost [Control, Iloprost A (0.001 µg), Iloprost B (0.1 µg), and Iloprost C (1.0 µg] Values of $P<0.05$ were considered statistically significant.

Parameter	**Abnormal Morphology (%)**	**0 hr**	**3 hr**	**4 hr**	**P-value Time:** 0.000
Treatment	Control	18.64	25.16	30.36	
	Iloprost A	18.30	23.00	31.29	
	Iloprost B	19.11	22.46	29.43	**P-value Treatment:** 0.051
	Iloprost C	17.49	21.69	29.22	

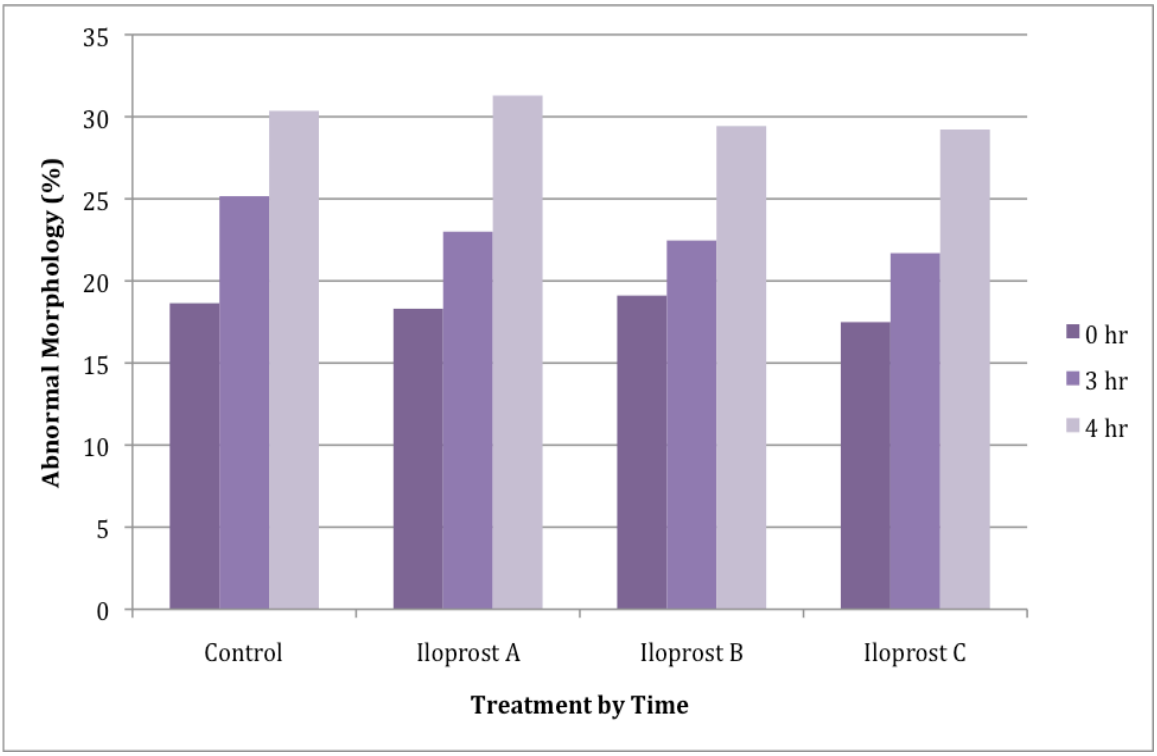

Figure 4: A comparison of the recovered equine sperm cells with abnormal morphology over time ($P < 0.001$) from cryopreserved equine semen frozen using standard techniques (control) or with the addition of varying levels of Iloprost {Iloprost A (0.001 µg), Iloprost B (0.1 µg), and Iloprost C (1.0 µg)] ($P < 0.051$).

Table 5: The mean number of abnormal cells with the presence of curled tails recover over time from post-thaw equine semen samples treated with varying concentrations of Iloprost [Control, Iloprost A (0.001 µg), Iloprost B (0.1 µg), and Iloprost C (1.0 µg] Values of $P<0.05$ were considered statistically significant.

Parameter	**Abnormal Morphology – Curled Tails (%)**	**0 hr**	**3 hr**	**4 hr**	**P-value Time:** 0.027
Treatment	Control	0.46	0.50	0.27	
	Iloprost A	0.52	0.70	0.32	**P-value Treatment:** 0.035
	Iloprost B	0.32	0.34	0.25	
	Iloprost C	0.44	0.27	0.33	

Together, the morphological data suggest the potential for maintaining higher levels of normal morphology post-thaw with high levels of Iloprost. However, given the negative impact of the concentration of Iloprost on cell motility, the overall impact on cell preservation appears negative.

Intact Acrosomes

All spermatozoa must undergo the acrosome reaction at the proper time in order to be viable for fertilization. However, having intact acrosomes are a critical component when determining the reproductive capabilities of a sperm cell and loss of the acrosome as a result of the cryopreservation/thaw process would be detrimental to breeding operations. There are many factors during the cryopreservation process that can alter the intactness of the acrosome. While there were differences in the number of partially intact and completely reacted cells over time (Table 6; $P < 0.001$), data from the present study shows no significant difference in the number of cells with intact acrosomes for either treatment ($P = 0.896$) or time ($P = 0.290$) and suggest Iloprost has little impact on maintenance of the acrosomal membrane during cryopreservation

Table 6: The effects of varying levels of Iloprost on the maintenance of the acrosomal membrane during the freeze/thaw of equine semen undergoing cryopreservation [Control, Iloprost A (0.001 μg), Iloprost B (0.1 μg), and Iloprost C (1.0 μg)]. Values of $P<0.05$ were considered statistically significant.

Parameter	**Acrosomes - Intact (%)**	**0 hr**	**3 hr**	**4 hr**	**P-value Time:** 0.290
Treatment	Control	83.89	82.48	82.16	
	Iloprost A	82.11	84.00	82.70	
	Iloprost B	82.52	82.89	82.38	**P-value Treatment:** 0.896
	Iloprost C	80.25	83.22	83.87	

Parameter	**Acrosomes – Partially Intact (%)**	**0 hr**	**3 hr**	**4 hr**	**P-value Time:** 0.000
Treatment	Control	9.45	8.89	7.46	
	Iloprost A	9.96	8.48	7.20	**P-value Treatment:** 0.527
	Iloprost B	10.25	8.52	7.14	
	Iloprost C	11.38	8.89	9.20	

Parameter	**Acrosomes – Non Intact (%)**	**0 hr**	**3 hr**	**4 hr**	**P-value Time:** 0.000
Treatment	Control	6.68	8.55	10.43	
	Iloprost A	7.89	7.48	10.11	
	Iloprost B	7.21	8.54	10.48	**P-value Treatment:** 0.922
	Iloprost C	8.55	7.98	8.82	

Chapter IV

Conclusion

The cryopreservation of stallion semen has been a long studied practice, with the first live foal being produced from frozen/thawed semen by Barker and Gandier in 1957 (Barker and Gandier, 1957). While the potential for widespread use of semen freezing within the horse industry is great, current limitations of the technique make further study crucial toward its progress. With improvement, the ability to freeze and transport equine semen across longer distances would contribute to the redistribution of genetics on a global scale. It would also enable producers to preserve the desired traits of specific horse more effectively (Loomis, 2001). Stallions that are no longer capable of being active within the breeding operation due to death, illness, or injury could potentially have their genetics preserved for future use. The use of frozen/thawed semen also broadens possibilities from a management standpoint. Transport of frozen/thawed semen has proven significantly easier to schedule than that of cooled transported semen or live animals, and frozen/thawed semen can be shipped much farther distances.

Despite all the recent advances in the cryopreservation industry, the art of freezing stallion semen still has limitations. This is at least partly due to the management practices in equine programs. Unlike cattle, horses are not selected for their breeding potential, but instead are selected based on performance. Horse breeders want to produce offspring of the horses that are successful in the show pen, performance event, race-track or work environment regardless of whether or

not their future semen will be of adequate quality for cooled transport or cryopreservation. This has led to significantly more fertility-related issues working with frozen/thawed equine semen compared to cooled transported (Loomis, 2001). Further, unlike the cattle industry, there are no national A.I. centers or certification process for semen handling equine technicians; which has resulted in highly variable pregnancy rates and inconsistent record keeping (Loomis, 2001).

Alvarenga et al. (2005), stressed the importance of understanding how semen parameters fluctuate between stallions and demonstrated that their reproductive efficiency is highly variable. Further, their work suggested only 30-40% of all stallions produce semen that is of adequate quality to withstand the freezing process given current technologies (Alvarenga et al., 2005). Additionally, because of expenses associated in processing semen for cryopreservation, there can also a significant cost difference between the use of frozen/thawed semen and cooled transported semen. However, the processing costs can become inconsequential given the value of the genetics of some stallions (Loomis, 2001). Therefore the main limitation preventing the current widespread use of cryopreservation in the equine industry remains the poor recovery rates of viable (motile) semen due to the damage done to sperm cells by the actual freezing process. Blach et al. (1989) demonstrated the deleterious effects cryopreservation had on equine sperm cells; especially in regard to post-thaw motility (Blach et al., 1989). They also demonstrate the process can have negative impacts on acrosome integrity, which is critical for fertilization (Blach et al., 1989).

The present study was designed to examine the effects of the compound Iloprost on the maintenance of cell function during cryopreservation. Iloprost, a prostacyclin analogue, was selected given recent findings demonstrating its ability to enhance embryo growth, potentially by stabilizing cellular function. In mice, the use of Iloprost increased the implantation rate of embryos (Huang et al., 2004) and enhanced hatching (Huang et al., 2004). It increased the rate of development to the blastocyst stage in cattle (Song et al., 2009) and promoted embryonic development in pigs (Kim et al., 2010).

These studies suggest two reasons to investigate Iloprost and its effects on sperm. First, if Iloprost were to stabilize cellular function in sperm, especially during cryopreservation, as it has in embryos, it could have direct effects on increasing fertility. Second, as Iloprost has been demonstrated to be beneficial to embryo development, it is reasonable to assume it will be considered for inclusion in culture medias in the future. While the only previous study with sperm suggested Iloprost had no effect on motility in human sperm (Huang et al., 2003), there is not literature to suggest it might not stabilize cellular function and prevent loss of motility or other biochemical processes (the acrosome reaction) when used for cryopreservation. Therefore, in the present study sperm cells were exposed to four concentrations of Iloprost consistent with those reported previously in embryo work. The Iloprost was added into the cryoprotectants used as part of the preparatory work prior to cryopreservation. Post-thaw analysis examined a number of parameters, including: concentrations, motion analysis, morphology and acrosome intactness, but focused primarily on motility and rapid cells due to their

critical importance to the fertilization process. All these parameters were evaluated immediately post-thaw (0 hr), 3 hrs post-thaw, and 4 hrs post-thaw. The 4 hrs time-point was added due to the recent findings of Pukazhenthi et al. that suggested a possible decrease in acrosome integrity post-thaw with the use of the cryoprotectant BotuCrio (Pukazhenthi et al., 2011).

The premise of the present study was that Iloprost might serve to stabilize cellular function during the cryopreservation process and thus increase post-thaw motility, not by increasing overall sample motility, but by preventing its loss during processing. However, both overall motility and the percentage of rapid cells decreased significantly with higher Iloprost concentrations. As the concentrations of Iloprost used were consistent with those showing enhanced embryo viability, the data presented suggest even these extremely low concentrations might be toxic to sperm cells and lead to lower rates of fertilization. Further, while none of the Iloprost appeared to have any effect on cell morphology, there did appear to be a trend toward fewer intact acrosomes with increasing Iloprost concentration.

Collectively, this data suggests Iloprost, if not directly toxic to sperm cells, at least interferes with normal sperm processes necessary for fertility (ie. motility and possibly the acrosome reaction among others). While not the original goal of the experiment, the data strongly suggests the use of Iloprost in fertility culture be limited to embryos and not be included at any stage where sperm cells are present.

In conclusion, while the present study suggests Iloprost does not enhance, and may in fact be detrimental to sperm cell function, further study is needed with other compounds and techniques directed at improving all aspects of male fertility

and reproductive efficiency in the horse; especially in the area of cryopreservation. Further, given the positive results in other species and the increase use of assisted reproductive technologies in the horse, Iloprost research with fertility in the horse should not be abandoned, but shifted to embryos to determine if enhanced growth, development, and pregnancy rates are found.

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