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Effects of Resveratrol on Post-Thaw Quality of Stallion Sperm

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By

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Current equine sperm cryopreservation methods fail to reliably prevent damages to important cellular structures such as the cell membrane and DNA. The objective of this study was to determine the effects of supplementing a stallion semen extender with 1 or 10 mM resveratrol on post-thaw sperm characteristics. Results showed that sperm death was increased with 10 mM compared to both the control and 1 mM ($P < 0.05$). DNA fragmentation was increased in the 1 mM treatment compared to the control ($P < 0.05$). ROS activity was reduced the most in the 10 mM with differences between all groups ($P < 0.05$). Membrane integrity was not different between groups ($P > 0.05$). Motility of the control was higher than the treatment groups ($P < 0.05$). Resveratrol was able to reduce ROS but was unable to preserve motility or viability at the concentrations tested.

Key Words: stallion, sperm, cryopreservation, resveratrol

DEDICATION

I would like to dedicate this thesis to my parents, Daron and Christy Matheny. Your unconditional love and support gives me the courage to reach my goals and chase new ones.

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CHAPTER I

REVIEW OF LITERATURE

History of Cell Cryopreservation

Cryopreservation, or the freezing of cells, tissues, and other substances for long-term storage, has aided the progression of many fields of interest including that of reproduction, medicine, and conservation. Reports of cell cryopreservation date back to the 1600's, however, it was not until the 1900's that the cryopreservation of cells became a major topic of scientific investigation (Sherman, 1964). With previous attempts unable to maintain cell viability post-thaw, Polge et al. (1949) achieved the first successful cryopreservation of any cell type resulting in live chicken sperm upon thawing. Since then, cryopreservation of other cell types has proven beneficial in other areas of reproduction. By transferring frozen-thawed mice morulae, Whittingham et al. (1972) performed the first cryopreservation of mammalian embryos that resulted in the birth of live offspring. In 1977, it was found that cryopreserved mouse oocytes could be fertilized post-thaw leading to live births (Whittingham, 1977).

Cell cryopreservation has also aided in the progression of medicine. Because of their ability to differentiate into a variety of cell types, mesenchymal stem cells (MSCs) have become an important topic in medical research. Lee et al. (2004) sought to determine the presence of MSCs in human umbilical cord blood as well as their ability to differentiate into various cell phenotypes after cryopreservation. They found that the

MSCs present in human umbilical cord blood could be recovered with 90% viability after having been cryopreserved for 0.1-5 years. Cells that were viable upon thawing were able to differentiate into osteoblasts, adipocytes, and chondrocytes *in vitro*. The ability to use cryopreserved MSCs provides convenience over the use of fresh samples and has aided in the progression of stem cell research and therapeutic uses including the regeneration of bone, cartilage, and other tissues for the treatment of various diseases.

Process of Cell Cryopreservation

Cell cryopreservation is typically achieved through the use of a manual protocol or a machine specialized for cell freezing. Both methods involve the gradual cooling of cells until reaching the storage temperature of -196°C, the temperature of liquid nitrogen. Using the cryopreservation of equine sperm as an example, generally, semen is collected from the stallion via an artificial vagina, and an extender is added. Samples are then centrifuged in order to remove the seminal plasma, and sperm pellets are again suspended in an extender. Following packaging in straws, the samples are cooled to 5ºC at a rate of -0.25ºC per minute and allowed to equilibrate for 20-120 minutes. Then, the samples are gradually cooled to a final temperature of -196ºC and stored in liquid nitrogen (de Oliveira, 2013).

During frozen storage, the cells are metabolically inactive and can be stored for long periods of time (Karlsson and Toner, 1996). All cells undergo similar changes during the process of cryopreservation. During the cooling process, extracellular ice formation occurs creating a hypertonic environment. As the cell attempts to maintain equilibrium between the internal and external spaces, water rushes out of the cell

resulting in cellular dehydration. During the thawing process, extracellular ice crystals melt causing a reverse in osmotic activity and the uptake of water (Oldenhof et al., 2013).

The rate at which cells are cooled during the cryopreservation process has a profound effect on cell viability. It has been shown that the use of an optimal cooling rate can result in increased cell survival; however, this ideal rate varies between cell types (Mazur, 1970). Generally, the cooling rate must be slow enough to avoid the formation of intracellular ice crystals, a major contributor to cell damage (Leibo et al., 1978). However, it is also known that the cooling rate must be fast enough to prevent the exposure of the cells to a hypertonic environment for too long a time. Subjecting cells to hypertonic solutions, or solutions of high salt concentrations, is known to cause the removal of membrane proteins from the cell surface, thus disrupting membrane order leading to weakening of the plasma membrane (Holt, 2000).

The rate at which cryopreserved cells are thawed is also an important factor in cell survival. Cells that undergo the thawing process at too rapid a rate are at risk of experiencing osmotic shock. Before freezing, cells are commonly extended in a freezing medium containing various cryoprotectants that permeate the cell membrane in order to prevent intracellular ice crystal formation. If thawing occurs too rapidly, these cryoprotectants could lack the time needed to diffuse back out of the cell resulting in swelling and lysis of the cell membrane as the extracellular environment becomes increasingly hypotonic (Mazur, 1984).

History of Sperm Cryopreservation

Being the first cells to be cryopreserved, mammalian sperm have an extensive history in the field of cryobiology. Interest in cryopreservation techniques for mammalian

sperm followed the dramatic increase in the popularity of artificial insemination (AI). Although AI had been routinely used during the 1800's in Europe and Russia, the need for a method of extended storage did not arise until the United States dairy industry popularized the use of AI in the 1930's (Herman, 1981). The American Society of Animal Production (ASAP), founded in 1939, was established by researchers from various universities in response to the rapidly growing dairy industry. The ASAP encouraged research to be done in the area of bull sperm cryopreservation in an effort to develop efficient methods of genetic improvement focused on increasing milk yield in dairy herds. With the ability to ship cryopreserved semen of bulls with desirable genetics, producers would be able to breed more cows with semen from superior bulls that they did not have access to when using fresh semen (Foote, 1998).

The discovery of glycerol's role as an effective cryoprotectant helped to propel further advancements in sperm cryopreservation (Polge et al., 1949). In 1951, the first calf was born from the use of frozen-thawed bull sperm (Stewart, 1951). Davis et al. (1963) later discovered that the lipids in egg yolk could convey even greater protection to sperm when used in conjugation with glycerol, leading to the commonly used egg yolk-glycerol extender. Today, sperm cryopreservation is heavily utilized in the breeding programs of both companion animals and livestock species.

Equine Sperm Cryopreservation

In 2001, two major North American equine breed registries, the American Quarter Horse Association and the American Paint Horse Association, began allowing registration of foals produced through the use of AI with cryopreserved semen. This acceptance has led to investigation into the improvement of cryopreservation techniques

for stallion sperm. Motivation for better cryopreservation methods can also be accredited to the system's many benefits to the equine industry. These benefits include the preservation of the genetics of valuable sires, the ability to ship sperm nationally and internationally, and the capability to conserve the genetics of endangered or wild equines. Through the use of sperm cryopreservation techniques pioneered primarily in bulls, the first foal resulting from the use of frozen-thawed stallion sperm was born in 1957 (Barker and Gandier, 1957). Since then, artificial insemination using cryopreserved stallion spermatozoa has become an integral part of the equine industry.

Challenges of Stallion Sperm Cryopreservation

While the use of stallion sperm cryopreservation has increased, the higher pregnancy rates observed in cattle have not been consistently replicated in horses (Curry, 2000). Also, pregnancy rates obtained using fresh sperm continue to be higher than those of cooled or cryopreserved stallion sperm. In a study by Jasko et al. (1992) comparing fertility rates of fresh, cooled, and frozen sperm from the same stallions, it was found that a per cycle pregnancy rate of 76% was obtained using fresh sperm while pregnancy rates of only 65% and 56% were recorded for cooled and frozen-thawed sperm, respectively. In various studies conducted between the years of 1987 and 2000, pregnancy rates of mares inseminated with cryopreserved semen have ranged from 32-73% per estrous cycle with no trend of improvement over the years observed (Loomis, 2001). Loomis and Squires (2005) conducted a retrospective study on the pregnancy rates of mares inseminated with frozen-thawed semen during the breeding season of 2003. The study incorporated pregnancy data of 217 mares inseminated using either a single dose AI protocol or a multi-dose AI protocol. They found that 58.1% of mares conceived on the

first cycle of breeding with an overall average conception rate of 52.7% per estrous cycle. However, there was no difference found between the conception rates per estrous cycle of the two insemination protocols. As it is common practice in the equine industry to sell cryopreserved semen by the dose and because veterinary care can become costly when utilizing multi-dose AI protocols, it is important to understand why the variability in conception rates exists.

Reasons for Variability in Stallion Sperm Freezability

It has been found that the survival rate of stallion sperm after cryopreservation depends greatly on the individual. Tischner (1979) estimated that approximately 20% of stallions could be considered "good freezers", 60% could be considered "fair/sufficient freezers", and 20% of stallions produced spermatozoa that classified them as "poor/insufficient freezers". Stallions classified as good freezers produced sperm with post-thaw motility of >40%, fair freezers produced sperm that had 40-20% post-thaw motility, and poor freezers produced sperm with <20% motility. Age of the stallion also plays a significant role in the quality of equine sperm before and after cryopreservation. Dowsett and Knott (1996) conducted a study to determine the effect of age on stallion sperm quality that included 222 stallions ages 2-26 years old over the course of four breeding seasons. They discovered that stallions under the age of three and over the age of 11 featured a decrease in total semen volume, sperm concentration, and total sperm numbers as well as an increase in sperm with morphological abnormalities. Similarly, in a study by Kalmar et al. (2013), it was found that the post-thaw motility of stallions ages 11-15 years was decreased compared to that of younger stallions, and motility continued to decrease in stallions of increasing age. This decrease in sperm quality of stallions

under three years of age is thought to be attributed to the fact that stallions do not reach sexual maturity until five years of age. Alternatively, stallions over the age of 11 are suspected to have decreased sperm quality due to aging effects like testicular degeneration (Dowsett and Knott, 1996).

Season and climate can also affect stallion sperm quality and freezability. In a study by Janett et al. (2003), sperm from 10 stallions were collected and cryopreserved every other week for one year, then analyzed for post-thaw quality. Results indicated that post-thaw motility was significantly higher for sperm collected in autumn when compared to collections from spring and summer. Also, viability of sperm collected in summer was significantly lower than collections from any other season. Data from the Kalmar et al. (2013) study was also analyzed to determine the seasonal effects on post-thaw motility. It was found that motility was greater in thawed samples collected in the fall and winter, possibly indicating an effect of heat stress on sperm quality during the spring and summer.

There is some debate as to the effects of collection frequency on stallion sperm quality. Pickett et al. (1975) performed a study in which nine stallions were collected at frequencies of either one time per week, three times per week, or six times per week over the course of four weeks. They found that stallions collected one and three times per week had a higher number of sperm per ejaculate as compared to the group collected six times per week; however, there was no difference in weekly sperm output between stallions collected three and six times per week. When motility was analyzed, there were no differences found between any of the collection frequencies. Sieme et al. (2004) compared post-thaw viability of stallion sperm collected either once per day or twice

every other day and found that the first collection on the double collection days contained a larger amount of viable sperm than both the second collection of that day or the samples collected once per day.

Osmotic Damage

Cryoinjury damage can be divided into two main categories, the first being osmotic damage. During the freezing and thawing process, sperm are subjected to large fluctuations in cellular volume as the result of osmotic activity. Pommer et al. (2002) found that stallion sperm were able to regain functionality after exposure to only a small range of change in osmolality. Functions that were impaired as a result of both hypertonic and hypotonic insult included motility, mitochondrial membrane potential, and sperm viability. This study supported the previous discovery by Windsor and White (1995) that mitochondrial membrane potential is decreased following the freeze-thaw process. As mitochondrial membrane potential is crucial for the production of ATP and thus, motility, these studies help to explain the decrease in sperm motility and conception rates seen in the equine industry. It has also been found that rearrangement of the sperm actin cytoskeleton occurs in response to osmotic stress, which causes an inability to regulate osmotic activity as well as inducing morphological changes of the flagellum leading to decreased motility (Correa et al., 2007).

Oxidative Damage

The second type of cryoinjury sperm undergo can be characterized as oxidative damage. Oxidative stress occurs when the number of free radicals, or reactive oxygen species (ROS), exceeds the cell's antioxidant capacity leading to membrane and DNA

damage (Agarwal et al., 2003). The primary source of ROS generation in stallion sperm is electron leakage from the mitochondrial electron transport chain resulting in the reduction of oxygen to form a ROS known as superoxide (Sabeur and Ball, 2006). ROS are thought to have physiological roles in sperm function. Baumber et al. (2003a) recorded that stallion sperm incubated with a ROS inducing substrate showed an increased incidence of capacitation and acrosome reaction. However, excess levels of ROS generation can lead to debilitating cellular damage. Peroxidation of plasma membrane lipids, or lipid peroxidation (LPO), was detected in frozen-thawed stallion sperm and featured a negative correlation with the number of membrane-intact sperm (Ferrusola et al., 2009).

Spermatozoal DNA has also shown susceptibility to oxidative damage. Baumber et al. (2003b) demonstrated an increase in DNA fragmentation in stallion sperm that were incubated with a ROS inducing agent and in sperm that were cryopreserved, suggesting that ROS produced during the cryopreservation process are responsible for subsequent DNA damage. Similarly, in a study analyzing the effects of exposing chilled stallion sperm to hydrogen peroxide (H_2O_2), a type of ROS, Burns and Herickoff (2014) found a significant increase in DNA fragmentation.

The effects of DNA damage are becoming increasingly concerning as assisted reproductive technologies such as *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) allow the possibility of fertilization with DNA damaged sperm. While the outcome of fertilization with sperm containing known DNA damage have yet to be elucidated in the horse, Morris et al. (2002) found that DNA damage was associated with a decrease in in the cleavage rates of human embryos. In mice, the long-term

developmental effects of fertilization with DNA fragmented sperm have been documented. It was found that of the mice resulting from DNA fragmented sperm, 20% did not survive past the first five months of life while premature aging was symptomatic in 25% of the remaining live mice (Fernandez-Gonzalez et al., 2008).

ROS can not only cause direct cellular damage by way of oxidation of cell components like DNA and membrane constituents but can also cause sperm death indirectly. Ortega-Furrusola et al. (2008) detected the activity of apoptotic factors, caspases 3, 7, and 9, in cryopreserved stallion sperm. Further investigation in this area discovered that inhibition of the mitochondrial permeability transition pore in cryopreserved stallion sperm resulted in decreased caspase activity and increased mitochondrial membrane potential, suggesting that the intrinsic or mitochondrial pathway is responsible for apoptosis in cryopreserved equine sperm (Ferrusola et al., 2010).

Proposed Solutions to Cryopreservative Damage

Due to the wide variation in conception rates observed when using cryopreserved stallion sperm, much research has been devoted to the investigation of various cryoprotectants in order to improve post-thaw sperm viability and quality. Focusing on the prevention of oxidative damage, antioxidants have been the center of many studies. Superoxide dismutase (SOD) is an antioxidant found in stallion seminal plasma and sperm that catalyzes the dismuation of superoxide to H_2O_2 (Baumber and Ball, 2005). In a study done by Baumber et al. (2005), sperm of two collections from each of five stallions were cryopreserved in extender supplemented with 200 IU/ml SOD. Results from the study reported no significant improvement in motility, viability, acrosome integrity, or mitochondrial membrane integrity, and DNA fragmentation was significantly

increased. However, a recent study by Cocchia et al. (2011) chilled the sperm of five stallions, 16 collections each to 5°C in an extender utilizing lower concentrations of SOD, 25 IU/ml and 50 IU/ml, and saw significant improvement in aspects such as stallion sperm motility and viability. However, differences in variables such as storage temperature present difficulty in comparing the results of the two studies.

Another antioxidant found in stallion seminal plasma and sperm, glutathione (GSH), has a scavenging role in that it reduces the harmful H_2O_2 to water and oxygen (Baumber and Ball, 2005; Luberda, 2005). Zhandi et al. (2013) chilled stallion sperm from three stallions, four collections each in extenders supplemented with 5 and 10 mM GSH and found significant improvement in motility and plasma membrane integrity for the 5 mM GSH treatment group. Another study, by Oliveira et al. (2013), extended and cryopreserved stallion sperm from 12 stallions, three collections each with a wider range of concentrations, 2.5, 5.0, 7.5, and 10 mM GSH, finding better motility, plasma membrane integrity, and viability for the lower concentration of 2.5 mM GSH; and yet, there were more acrosome intact sperm in the 5.0 mM GSH treatment. However, differences in factors such as the type of extender used in these studies make it difficult to compare the results.

Catalase is another antioxidant of stallion seminal plasma and sperm and plays a similar role to GSH in that it reduces H_2O_2 to water and oxygen (Ball et al., 2000). Baumber et al. (2003b) incubated fresh sperm from four stallions, two collections each with the ROS inducing xanthine-xanthine oxidase system as well as 200 U/ml catalase and found that DNA fragmentation was significantly reduced. However, Gibb et al. (2013) cryopreserved sperm from three stallions, three collections each with the same

concentration of catalase and found a significant increase in DNA fragmentation as well as no significant increase in motility, acrosome integrity, or viability.

Other forms of cryoprotectants have been used in an attempt to improve stallion sperm cryopreservation methods. Glycerol is a commonly used cyroprotectant that permeates the cell to increase intracellular solute concentration, thus reducing osmotic stress by decreasing fluctuations in water volume during the freezing and thawing processes. Garcia et al. (2012) tested the effects of different concentrations of glycerol in stallion semen extenders and found that inclusions above 3.5% were toxic to sperm. Although it is known that species with higher levels of cholesterol in their sperm plasma membranes have increased success with cryopreservation (Darrin-Bennett and White, 1977), incorporating cholesterol into the plasma membrane of stallion sperm has only recently been examined. Knowing that stallion sperm plasma membrane has relatively low cholesterol content when compared to other species (Parks and Lynch, 1992), Hartwig et al. (2014) studied the effects of incorporating cholesterol-loaded cyclodextrin into the sperm membranes of stallions known to have either "good" or "bad" success rate when chilled. They found that the incorporation of cholesterol into the sperm plasma membrane increased fertility rates of "bad cooler" stallions, but the fertility rates of "good cooler" stallions was unaffected.

The Future of Stallion Sperm Cryopreservation

While many cryoprotectants have been researched in the cryopreservation of stallion sperm, the need for a supplement that can consistently decrease cellular damages still remains. The antioxidant resveratrol is a polyphenol found in the skin of red grapes at an average concentration of 4.12 μg/g and has been discovered to have anti-

inflammatory, anti-tumorigenic, and antioxidant properties as well as the ability to increase the lifespan of certain animals (Okuda and Yokotsuka, 1996; Smoliga et al., 2011). When supplemented in the cryopreservation extender of human sperm, resveratrol was seen to decrease DNA fragmentation (Branco et al., 2010) as well as decrease lipid peroxidation and increase SOD and CAT activity (Garcez et al., 2010). Alternatively, when ram semen extender was supplemented with resveratrol no significant improvement was observed in motility, acrosome integrity, or plasma membrane integrity (Silva et al., 2012). Mojica-Villegas et al. (2014) conducted a study in which intracellular ROS levels were significantly reduced in mouse sperm cryopreserved with 15 μg/mL resveratrol. While resveratrol has yet to be utilized in the cryopreservation of stallion sperm, its ability to reduce ROS levels and potential to decrease oxidative damages could be beneficial to post-thaw quality.

Cryopreservation of stallion sperm features the combination of both osmotic and oxidative stress, making it difficult to develop a highly successful preservation protocol. Discoveries such as the use of antioxidants to combat oxidative stress and permeable cryoprotectants, like glycerol, to minimize osmotic damage have proven beneficial to sperm quality. However, the high individual variability of stallions limits consistency of success rates of certain extenders and protocols. Further research should investigate methods to predict this individual variability in order to determine the practicality of tailoring cryopreservation protocols to fit a particular stallion's need. Also, more research should be done to determine the most effective cryoprotectants to use as well as their concentrations to find the most appropriate way to limit cellular damage.

References

Agarwal, A., R. A. Saleh, and M. A. Bedaiwy. 2003. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil. Steril. 79:829-843.

Ball, B. A., C. G. Gravance, V. Medina, J. Baumber, and I. K. M. Liu. 2000. Catalase activity in equine semen. Am. J. Vet. Res. 61:1026-1030.

Barker, C. A. V., and J. C. C. Gandier. 1957. Pregnancy in a mare resulting from frozen epididymal spermatozoa. Can. J. Com. Med. Vet. S. 21:47-51.

Baumber, J., and B. A. Ball. 2005. Determination of glutathione peroxidase and superoxide dismutase-like activities in equine spermatozoa, seminal plasma, and reproductive tissues. Am. J. Vet. Res. 66:1415-1419.

Baumber, J., K. Sabeur, A. Vo, and B. A. Ball. 2003a. Reactive oxygen species promote tyrosine phosphorylation and capacitation in equine spermatozoa. Theriogenology. 60:1239-1237.

Baumber, J., B. A. Ball, and J. J. Linfor. 2005. Assessment of the cryopreservation of equine spermatozoa in the presence of enzyme scavengers and antioxidants. Am. J. Vet. Res. 66:772-779.

Baumber, J., B. A. Ball, J. J. Linfor, and S. A. Meyers. 2003b. Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. J. Androl. 24:621-628.

Burns, P. D., and L. A. Herickoff. 2014. DNA fragmentation as an early indicator of extender efficacy: A preliminary study. J. Equine Vet. Sci. 34:375-379.

Cocchia, N., M. P. Pasolinia, R. Mancinib, O. Petrazzuoloc, I. Cristofarod, I. Rosapanea, A. Sicaa, G. Tortoraa, R. Lorizioa, G. Paraggioe, and A. Mancinib. 2011. Effect of sod (superoxide dismutase) protein supplementation in semen extenders on motility, viability, acrosome status and ERK (extracellular signal-regulated kinase) protein phosphorylation of chilled stallion spermatozoa. Theriogenology. 75:1201-1210.

Correa, L. M., A. Thomas, and S. A. Meyers. 2007. The Macaque sperm actin cytoskeleton reorganizes in response to osmotic stress and contributes to morphological defects and decreased motility. Bio. Reprod. 77:942–953.

Curry, M. R. 2000. Cryopreservation of semen from domestic livestock. Rev. Reprod. 5:46-52.

Darin-Bennett, A., and I. G. White. 1977. Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold-shock. Cryobiology. 14:466-70.

Davis, I. S., R. W. Bratton, and R. H. Foote. 1963. Livability of bovine spermatozoa at 5, −25 and −85°C in Tris-buffered and citrate-buffered yolk-glycerol. J. Dairy Sci. 46:333.

Dowsett, K. F., and L. M. Knott. 1996. The influence of age and breed on stallion semen. Theriogenology. 46:397-412.

de Oliveira, R. A., C. A. Wolf, M. A. de Oliveira Viu, and M. L. Gambarini. 2013. Addition of Glutathione to an extender for frozen equine semen. J. Equine Vet. Sci. 33:148-1152.

Ferrusola, O. C., L. G. Fernandez, J. M. Morrell, C. S. Sandoval, B. M. Garcia, H. Rodriguez-Martinez, J. A. Tapia, and F. J. Pena. 2009. Lipid peroxidation, assessed with BODIPY-C11, increases after cryopreservation of stallion spermatozoa, is stallion-dependent and is related to apoptotic-like changes. Reproduction. 138:55-63.

Ferrusola O. C., L. G. Fernandez, C. S. Sandoval, B. M. Garcia, H. R. Martinez, J. A. Tapia, and F. J. Pena. 2010. Inhibition of the mitochondrial permeability transition pore reduces “apoptosis like” changes during cryopreservation of stallion spermatozoa. Theriogenology. 74:458-65.

Foote, R. H. 1998. Artificial Insemination to Cloning: Tracing 50 Years of Research. Cornell University Resource Center, Ithaca, New York.

Garcia, M. B., C. O. Ferrusola, I. M. Aparicio, A. Miro-Moran, A. M. Rodriguez, J. M. G. Bolanos, L. G. Fernandez, C. M. B. da Silva, H. R. Martinez, J. A. Tapia, and F. J. Pena. 2012. Toxicity of glycerol for the stallion spermatozoa: Effects on membrane integrity and cytoskeleton, lipid peroxidation and mitochondrial membrane potential. Theriogenology. 77:1280-1289.

Gibb, Z., T. J. Butler, L. H. A. Morris, W. M. C. Maxwell, and C. G. Grupen. 2013. Quercetin improves the postthaw characteristics of cryopreserved sex-sorted and nonsorted stallion sperm. Theriogenology. 79:1001-1009.

Hartwig, F. P., F. P. Lisboa, F. P. Hartwig, G. A. Monteiro, R. R. D. Maziero, C. P. Freitas-Dell`Aqua, M. A. Alvarenga, F. O. Papa, J. A. Dell`Aqua Jr.. 2014. Use of cholesterol-loaded cyclodextrin: An alternative for bad cooler stallions. Theriogenology. 81:340-346.

Herman, H. A. 1981. Improving Cattle by the Millions: NAAB and the Development and Worldwide Application of Artificial Insemination. University of Missouri Press, Columbia.

Holt, W. V. 2000. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenology. 53:47-58.

Janett, F., R. Thun, K. Niederer, D. Burger, and M. Hassig. 2003. Seasonal changes in semen quality and freezability in the Warmblood stallion. Theriogenology. 60:453-461.

Jasko, D.J., Moran, D.M., Farlin, M.E., Squires, E.L., Amann, R.P., Pickett, B.W. 1992. Pregnancy rates utilizing fresh, cooled and frozen-thawed stallion semen. In: Proceedings of the 38th Annual Convention of American Association on Equine Practice. Orlando, Florida. 649‑660.

Kalmar, J. J., B. A. Ball, M. H. T. Troedsson, J. Skaife, P. I. Loomis, and E. L. Squires. 2013. Factors impacting freezability of stallion sperm. J. Equine Vet. Sci. 33:5. Abstract.

Karlsson, J. O. M., and M. Toner. 1996. Long-term storage of tissues by cryopreservation: critical issues. Biomaterials. 17:243-256.

Lee, M. W., J. Choi, M. S. Yang, Y. J. Moon, J. S. Park, H. C. Kim, and Y. J. Kim. 2004. Mesenchymal stem cells from cryopreserved human umbilical cord blood. Biochem. Bioph. Res. Co. 320:273-278.

Leibo, S. P., J. J. McGrath, and E. G. Cravalho. 1978. Microscopic observation of intracellular ice formation in unfertilized mouse ova as a function of cooling rate. Cryobiology. 15:257–271.

Loomis, P. R. The equine frozen semen industry. Anim. Reprod, Sci. 2001. 68:191-200.

Loomis, P. R. and E. L. Squires. 2005. Frozen semen management in equine breeding programs. Theriogenology. 64:480-491.

Luberda, Z. 2005. The role of glutathione in mammalian gametes. Reprod. Biol. 5:5-17.

Mazur, P. 1970. Cryobiology: the freezing of biological systems. Science. 168:929-949.

Mazur, P. 1984. Freezing of living cells: mechanisms and implications. Am. J. Physiol. 247:C125-C142.

Okuda, T., and K. Yokotsuka. 1996. Trans-resveratrol concentrations in berry skins and wines from grapes grown in Japan. Am. J. Enol. Vitic. 47:93-99.

Oldenhof, H., M. Gojowsky, S. Wang, S. Henke, C. Yu, K. Rohn, W. F. Wolkers, and H. Sieme. 2013. Osmotic stress and membrane phase changes during freezing of stallion sperm: Mode of action of cryoprotective agents. Bio. Reprod. 88:1-11.

Ortega-Furrusola, C., Y. Sotillo-Galan, E. Varela-Fernandez, J. M. Gallardo-Bolanos, A. Muriel, L. Gonzalez-Fernandez, J. A. Tapia, and F. J. Pena. 2008. Detection of “apoptosis-like” changes during the cryopreservation process in equine sperm. J. Androl. 29:213-21.

Parks, J. E., and D. V. Lynch. 1992. Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. Cryobiology. 29:255-66.

Pickett, B. W., J. J. Sullivan, and G. E. Seidel Jr. 1975. Reproductive physiology of the stallion. V. Effect of the frequency of ejaculation on seminal characteristics and spermatozoal output. J. Anim. Sci. 40:917-923.

Polge, C., A. U. Smith, and A. S. Parkes. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature. 164:666.

Pommer, A. C., J. Rutlant, and S. A. Meyers. 2002. The role of osmotic resistance on equine spermatozoal function. Theriogenology. 58:1373-1384.

Sabeur, K., and B. A. Ball. 2006. Detection of superoxide anion generation by equine spermatozoa. Am. J. Vet. Res. 67:701-706.

Sherman, J. K. 1964. Low temperature research on spermatozoa and eggs. Cryobiology. 2:103-29.

Stewart, D. L. 1951. Storage of bull spermatozoa at low temperatures. Vet. Rec. 63:65-66.

Tischner, M. 1979. Evaluation of deep-frozen semen in stallions. J. Reprod. Fert. Suppl. 27:53-59.

Vidament, M., A. M. Dupere, P. Julienne, A. Evain, P. Noue, and E. Palmer. 1997. Equine frozen semen: freezability and fertility field results. Theriogenology. 48:907-917.

Whittingham, D. G., S. P. Leibo, and P. Mazur. 1972. Survival of mouse embryos, frozen to -196^{o}C and -289^{o}C. Science. 178:411-414.

Whittingham, D. G. 1977. Fertilization in-vitro and development to term of unfertilized mouse oocytes previously stored at -196^{o}C. J. Reprod. Fertil. 49:89-94.

Windsor, D. P., and I. G. White. 1995. Mitochondrial injury to ram sperm during procedures associated with artificial insemination or frozen storage. Anim. Reprod. Sci. 40**:**43-58.

Zhandi, M., and V. Ghadimi. 2013. Effect of glutathione-supplemented INRA82 extender on miniature caspian stallion Sperm quality during storage at 5^{o}C. J. Equine Vet. Sci. 34:606-610.

CHAPTER II

EFFECTS OF SUPPLEMENTING EXTENDER WITH RESVERATROL ON STALLION SPERM VIABILITY

Introduction

Sperm cryopreservation became a focus in reproductive technology in the mid-1900's following the dramatic increase in the popularity of artificial insemination (AI) in the United States dairy industry (Sherman, 1964; Herman, 1981). The acceptance of AI in many equine breeding registries led investigation into the improvement of cryopreservation techniques for stallion sperm. During the process of cryopreservation, sperm cells undergo dramatic changes in volume that result in osmotic stress. These volumetric changes include shrinking and swelling of the plasma membrane during the freezing and thawing process as the cell attempts to maintain equilibrium between the intra- and extracellular space (Oldenhof, 2013). During frozen storage, the sperm are metabolically inactive and can be stored for long periods of time (Karlsson and Toner, 1996). The benefits of cryopreservation are numerous and include allowing for the preservation of genetic material of desirable stallions, the shipment of sperm long distance, and the avoidance of disease transmission and physical injury to the mare or stallion (Loomis and Squires, 2005).

As a result, many studies have been conducted involving the use of various levels and types of cryoprotectants including antioxidants. Although some studies have yielded

promising results, the wide variety of damages experienced by stallion spermatozoa as a result of the cryopreservation process provide a challenge when formulating extenders with effective levels and types of cryoprotectants (Pena et al., 2011).

Stallion sperm undergo a variety of damages resulting from the cryopreservation process. The first type of damage, osmotic damage, is caused by large fluctuations in cell volume and results in a decrease in sperm motility, viability, and mitochondrial membrane potential (Pommer et al., 2002). The second major type of cell damage, oxidative damage, results from the production of reactive oxygen species (ROS) and leads to membrane lipid peroxidation, DNA fragmentation, and apoptosis (Ferrusola et al., 2009; Baumber et al., 2003b; Ortega-Ferrusola et al., 2008). The prevention of oxidative damages has been the primary focus of recent research, and specifically, through the supplementation of semen extenders with a variety of antioxidants. Antioxidants of high importance in stallion sperm, superoxide dismutase (SOD) and catalase (CAT), have been utilized in semen extenders but failed to improve quality parameters such as DNA integrity, motility, and viability in frozen-thawed sperm (Baumber et al., 2005; Gibb et al., 2013). The antioxidant glutathione (GSH) was shown to improve motility, viability, and plasma membrane integrity of frozen-thawed sperm at low concentrations; however, there were no improvements in these parameters at a higher concentration (de Oliveira et al., 2013; Baumber et al., 2005).

Resveratrol is a polyphenol that has been isolated from the skin of red grapes and found to not only act as an antioxidant, but to also exhibit anti-inflammatory and anti-tumorigenic properties (Smoliga et al., 2011). When resveratrol was evaluated as a possible supplement in the cryopreservation of human sperm, a decrease in DNA

fragmentation (Branco et al., 2010) as well as decrease lipid peroxidation and increase SOD and CAT activity (Garcez et al., 2010) were observed. Alternatively, ram sperm cryopreserved with resveratrol supplementation did not express improvement in quality parameters such as motility, acrosome integrity, or plasma membrane integrity (Silva et al., 2012).

The aim of this study was to improve post-thaw stallion sperm quality parameters including membrane integrity, viability, DNA integrity, and motility through the supplementation of a semen extender with the antioxidant resveratrol. The central hypothesis of this study was that supplementation of a stallion semen extender with the antioxidant resveratrol improves sperm viability and motility by protecting against sperm membrane and DNA damages caused by reactive oxygen species (ROS).

Materials and Methods

All procedures in this study were approved by the Institutional Animal Care and Use Committee of Mississippi State University.

Collection and cryopreservation of sperm

Sperm from four healthy stallions between the ages of 5-13 years old were collected three times each totaling 12 collections using a Missouri type artificial vagina at a temperature of 37-48°C. Each stallion was collected every two to three days and all collections took place within a period of one month. Additional information on each stallion can be found in Appendix A. The semen samples were then filtered to remove any contaminants. The sperm concentration was determined using a hemocytometer, and motility was determined by visual analysis. Only collections with a pre-freeze motility of

60% or higher were used in the study. The sperm were aliquoted and diluted in a Modified Kenney extender supplemented with one of three treatments: 1) control (no resveratrol), 2) 1 mM resveratrol, or 3) 10 mM resveratrol. The resveratrol was considered ≥99% pure (Sigma, St. Louis, MO). Samples were then allowed to equilibrate at 5°C for two hours. Then, $1x10^8$ cells/ml were loaded into 0.5 ml straws, sealed, and gradually frozen until reaching a temperature of -196ºC. The straws were then stored at -196ºC until time of analysis.

Sperm isolation

For each analysis, cryopreserved sperm ($50x10^6$ cells/straw) were thawed at 37ºC for one minute followed by removal of extender by centrifuging the sperm through 30 µl of 90% Percoll overlaid with 200 µl of 45% Percoll at 800 g at 4ºC for 10 minutes. Then, the sperm pellets were washed with PBS once at 800 g at 4ºC for 10 minutes followed by one of four analyses: sperm viability, DNA integrity, ROS content, or membrane integrity.

Determining sperm viability and membrane integrity using LIVE/DEAD assay and flow cytometry

According to the manufacturer's recommendations, sperm from all treatment groups were classified as either viable or nonviable using dual DNA staining (LIVE/DEAD Sperm Viability Kit, Molecular Probes, Eugene, OR). Samples were isolated as described above, diluted 1:10 in HEPES-buffered saline solution containing bovine serum albumin (10 mM HEPES, 150 mM NaCl, 5% BSA, pH 7.4), and incubated with 5 µL of diluted SYBR-14 (50-fold dilution of a 1 mM stock solution) at 36°C for 10 minutes. Then, cells were incubated with 5 µL of propidium iodide (2.4 mM stock

solution) for 10 minutes at 36°C followed by classification based on the fluorescence detected by flow cytometry.

Measuring sperm DNA integrity using TUNEL and flow cytometry

Sperm samples from each treatment group were analyzed for DNA integrity using the Terminal Deoxynucleotide Transferase dUTP Nick End Labeling or TUNEL method (APO-BrdU TUNEL Assay Kit, Molecular Probes, Eugene, OR). Sperm were isolated as described above and fixed using 1% (w/v) paraformaldehyde in PBS. Each sample was then incubated with 50 µL of DNA labeling solution (10 µL reaction buffer, 0.75 µL TdT enzyme, 8.0 µL BrdUTP, 31.25 µL dH_2O) at 37°C for 60 minutes. Next, the samples were rinsed with rinse buffer and incubated with 100 µL of antibody staining solution containing 5.0 µL of Alexa Fluor 488 dye-labeled anti-BrdU antibody and 95 µL of rinse buffer at room temperature for 30 minutes which was followed by analysis of amount of DNA fragmentation based on fluorescence using flow cytometry.

Ascertaining Reactive Oxygen Species content of sperm using flow cytometry

Sperm from each treatment group were analyzed for intracellular ROS content using the carboxy derivative of fluorescein, carboxy-H_2DCFDA kit according to manufacturer's recommendations. Sperm were isolated as described above and $5.5x10^6$ cells per sample were incubated with 1µL of carboxy-H_2DCFDA (50 µM carboxy-H_2DCFDA) at 37°C for 30 minutes. Samples were then analyzed for amounts of intracellular ROS detected by fluorescence using flow cytometry.

Determining osmotic activity using the Hypoosmotic Swelling Test (HOST)

Sperm from all of the three collections from the four stallions were analyzed using hypo-osmotic swelling test (HOST) as described by Neild et al. (2000). Samples were isolated as described above and re-suspended in 250 µl of PBS. Fifty microliters of the sperm suspension were transferred into 450 µl of pre-equilibrated hypo-osmotic solution (150 miliosmol containing 7.35 grams of Sodium citrate and 13.51 grams of Fructose in 1000 ml of distilled water) at 37^{o}C for 30 min. Under the 400X objective of a light microscope, 200 spermatozoa were counted in triplicate for each sample. As depicted in Figure 1, coiled-tailed spermatozoa were considered membrane intact while the straight tailed spermatozoa were considered membrane damaged.

Assessing motility using Computer Assisted Sperm Analysis (CASA)

Sperm from each treatment group were analyzed for motility characteristics using computer-assisted sperm analysis (Hamilton Thorne Biosciences IVOS instruments). Straws from each treatment were thawed and 100,000 sperm in 2 µl of each sample were analyzed. At least 200 cells per sample were analyzed for parameters including total motility and progressive motility.

Statistical Analyses

Histograms were used to visually assess if the data were normally distributed using PROC UNIVARIATE in SAS for Windows 9.3 (SAS Institute, Inc., Cary, NC). The data were not found to be sufficiently normally distributed. Accordingly, to determine if there were differences in results among the three treatment levels, a method similar to the non-parametric Friedman's test was conducted. Each collection within a

stallion was considered a block. For each outcome, the data was first ranked within each stallion-collection block. An analysis of variance using PROC GLM in SAS for Windows was then conducted on the ranked data with treatment and block as the explanatory variables. If treatment was found to have a significant effect, differences in least squares means with Tukey adjusted p-values were used to make pairwise comparisons among the treatment levels. An alpha level of 0.05 was used to determine statistical significance for all methods.

Results

Data for each analysis and treatment classified by stallion are exhibited in Appendix B.

Sperm viability and membrane integrity

Significant differences were found for spermatozoa viability between all three groups (Table 1). The control contained significantly more viable sperm than both Treatment 1 ($P < 0.0001$) and Treatment 2 ($P < 0.0001$). Treatment 1 contained significantly more viable sperm than Treatment 2 ($P < 0.0001$).

Sperm DNA integrity

Treatment 1 exhibited sperm with significantly more DNA fragmentation than the control ($P = 0.0113$); however, no differences were found between the control and Treatment 2 ($P = 0.6188$) or Treatment 2 and Treatment 1 ($P = 0.1163$) (Table 2).

Reactive Oxygen Species content of sperm

Significant differences in ROS content were observed between all three treatment groups (Table 3). Spermatozoa in Treatment 1 contained significantly less ROS than

those in the control ($P < 0.0001$). The ROS content was found to be significantly lower in Treatment 2 than in the control ($P < 0.0001$). When comparing the two treatments, Treatment 2 yielded significantly less ROS than Treatment 1 ($P < 0.0001$).

Osmotic activity and membrane integrity

No significant differences were observed for osmotic activity and membrane integrity between the control and Treatment 1 ($P = 0.2440$) or Treatment 2 ($P = 0.2532$) as well as between Treatments 1 and 2 ($P = 0.9997$). (Table 4).

Sperm motility

As seen in Table 5, total sperm motility was significantly higher for the control as compared to Treatment 1 ($P < 0.0001$) and Treatment 2 ($P < 0.0001$); however, there was no significant difference in total sperm motility between Treatments 1 and 2 ($P = 0.1661$). There were significantly higher numbers of progressively motile sperm for the control as compared to Treatment 1 ($P < 0.0001$) and Treatment 2 ($P < 0.0001$); however, there was no difference in progressive motility between the two treatment groups ($P = 0.1869$).

Discussion

Stallion semen cryopreservation is an essential factor in the success of AI, preserving the genetics of valuable stallions, and in the ability to ship semen worldwide. However, current methods of cryopreservation result in damages to sperm and decreased quality resulting in a reduction in post-thaw sperm viability and thus, fertilizing capacity.

In the current study, the addition of resveratrol was unable to prevent a reduction in sperm viability defined by Graham (2001) as the amount of sperm with intact plasma membranes and actually increased sperm death with increasing resveratrol concentration.

Collodel et al. (2011) also observed an increase in cell death when incubating human sperm with increasing resveratrol concentrations. However, when analyzing the results of HOST, no significant differences in plasma membrane damage were observed with the addition of either 1 mM or 10 mM resveratrol when compared to the control. Although the current study utilized higher concentrations of resveratrol, Silva et al. (2010) also reported no significant differences in plasma membrane integrity detected by visual differential staining when ram sperm were cryopreserved in a semen extender supplemented with 0, 5, 10, 15, or 20 μg/mL resveratrol. The differences in membrane intact sperm between the differential staining and HOST could be due to the increased handling and processing time required for flow cytometric analysis, causing increased cell death. However, both total and progressive motility were higher for the control when compared to the resveratrol treatments. This result supports the finding by Garcez et al. (2010) in which human sperm cryopreserved with 1 and 10 mM resveratrol featured significantly lower total motility. Similarly, Collodel et al. (2011) observed a complete lack of motility in human sperm incubated with 100 μM resveratrol. The clear reduction in motility with resveratrol supplementation along with the reduction in viability with increasing resveratrol concentration point to a possible toxic effect of resveratrol at the concentrations tested. The lack of differences in membrane damage detected by HOST could be due to the test's subjective nature as compared to the objective nature of the flow cytometric analyses. In another study, an increase in progressive motility was observed with lower concentrations of resveratrol, indicating that lower doses could be beneficial in preserving sperm motility (Collodel et al., 2011).

There was a significant increase in DNA fragmentation in Treatment 1 when compared to the control; however, no differences existed between Treatment 2 and either the control or Treatment 1. Alternatively, in a study on human semen, Branco et al. (2010) saw reduced DNA fragmentation in spermatozoa of both fertile and infertile men when cryopreserving with an extender supplemented with 10 mM resveratrol. The addition of resveratrol significantly reduced intracellular ROS generation with increasing concentrations of resveratrol. These results support previous findings by Mojica-Villegas et al. (2014) in which intracellular ROS levels were significantly reduced in mouse sperm cryopreserved with 15 μg/mL resveratrol. From a physiological point of view, reduced levels of intracellular ROS are expected to prevent excessive damage to cell membranes and DNA.

Because of resveratrol's powerful antioxidant capacity, it was expected that intracellular ROS production would be reduced (Petruska et al., 2014). This reduction may not only be attributed to resveratrol's direct scavenging ability, but to its potential to increase the activity of other antioxidants including SOD (Garcez et al., 2010). Because high doses of resveratrol have been known to induce apoptosis and chromatin damage in cells, resveratrol toxicity may also be another factor causing sperm death (Mukherjee et al., 2010).

The current study made use of a similar number of stallions and collections used by other studies. Age of the stallion has been shown to play a significant role in the quality of equine sperm before and after cryopreservation with a decrease in quality outside the age range of 3-11 years old (Dowsett and Knott, 1996). Therefore, the current study included three stallions within this age range. The use of the stallion included

outside of this age range was justified by ensuring a similar pre-freeze motility percentage between all stallions used. Effect of breed on post-thaw sperm motility has also been observed. In one study, stallion sperm of three different breeds was cryopreserved under the same conditions. Results showed that 11% of Mangalarga stallions, 50% of Quarter horse stallions, and 53% of Warmblood stallions exhibited an acceptable post-thaw motility of at lease 40% motile sperm (Alvarenga et al., 2005). By using a limited number of stallions with similar qualities such as age, breed, sperm motility, and collection schedule, we reduced variation that could have been introduced by incorporating a large number of diverse stallions under different management practices.

Because oxidative damage is a major contributor to the reduction in stallion sperm quality after cryopreservation, further research needs to focus on reducing oxidative stress. This study supports the claim that high concentrations of resveratrol are deleterious to cells. Future studies should investigate the efficacy of supplementing stallion semen extenders with lower concentrations of resveratrol. Although other studies have found the concentrations used in the current study to be beneficial to human sperm, perhaps concentrations of resveratrol within the physiological range of plants (0.00219 - 0.0613 mM) would be more effective in stallion sperm (Okuda and Yokotsuka, 1996). Discovery of an optimal concentration of resveratrol could be the key to improving cryopreservation methods for stallion spermatozoa. Because it is a plant-based substance, resveratrol is also more readily available than other animal-derived antioxidant supplements (Smoliga et al., 2011). Analysis the fertilization capacity and effects on embryo development of resveratrol treated sperm using techniques such as *in vitro*

fertilization (IVF) and intracytoplasmic sperm injection (ICSI) would help to generate additional knowledge on the effects of resveratrol on sperm physiology involving fertilization and early embryonic development. Once sperm cryopreservation techniques are improved for stallion sperm, this will enable more efficient cryopreservation of sperm with higher post-thaw quality and fertilization rates.

Summary

While resveratrol supplementation was able to reduce ROS content in post-thaw stallion sperm, it was not able to prevent a reduction in viability or motility. The concentrations of resveratrol used in this study appear to have a cytotoxic effect on stallion sperm; therefore, lower concentrations used singly or in combination with antioxidants of differing mechanisms of action may be more effective in preventing oxidative damages such as membrane and DNA damage during the process of cryopreservation. As a plant-derived substance, resveratrol could serve as a readily available antioxidant source as opposed to other animal-derived antioxidants. The need for a supplement that provides consistent protection against oxidative damages to cryopreserved stallion sperm still exists, therefore, future research should investigate optimum concentrations and combinations of supplements.

Table 1 Percent live sperm

	Percent live
Control	2.0 ± 2.0[a]
1 mM Resveratrol (Treatment 1)	1.0 ± 0.00[b]
10 mM Resveratrol (Treatment 2)	0.0 ± 0.00[c]

Different letters in the same column indicate differences ($P < 0.05$)

Table 2 DNA fragmentation

	*MFI
Control	193.20 ± 101.0[a]
1 mM Resveratrol (Treatment 1)	232.02 ± 127.37[b]
10 mM Resveratrol (Treatment 2)	226.26 ± 142.82[a,b]

*Mean fluorescent intensity
Different letters in the same column indicate differences ($P < 0.05$)

Table 3 Reactive oxygen species content

	*MFI
Control	65.44 ± 16.76[a]
1 mM Resveratrol (Treatment 1)	57.25 ± 11.05[b]
10 mM Resveratrol (Treatment 2)	37.24 ± 16.98[c]

*Mean fluorescent intensity
Different letters in the same column indicate differences ($P < 0.05$)

Table 4 Percent membrane intact sperm

	Percent membrane intact
Control	12 ± 7.0[a]
1 mM Resveratrol (Treatment 1)	10 ± 7.0[a]
10 mM Resveratrol (Treatment 2)	10 ± 5.0[a]

Different letters in the same column indicate differences ($P < 0.05$)

Table 5 Percent total and progressive motility

	Percent total motility	Percent progressive motility
Control	25 ± 13.0[a]	13 ± 8.0[a]
1 mM Resveratrol (Treatment 1)	5 ± 4.0[b]	1 ± 1.0[b]
10 mM Resveratrol (Treatment 2)	3 ± 3.0[b]	1 ± 1.0[b]

Different letters in the same column indicate differences ($P < 0.05$)

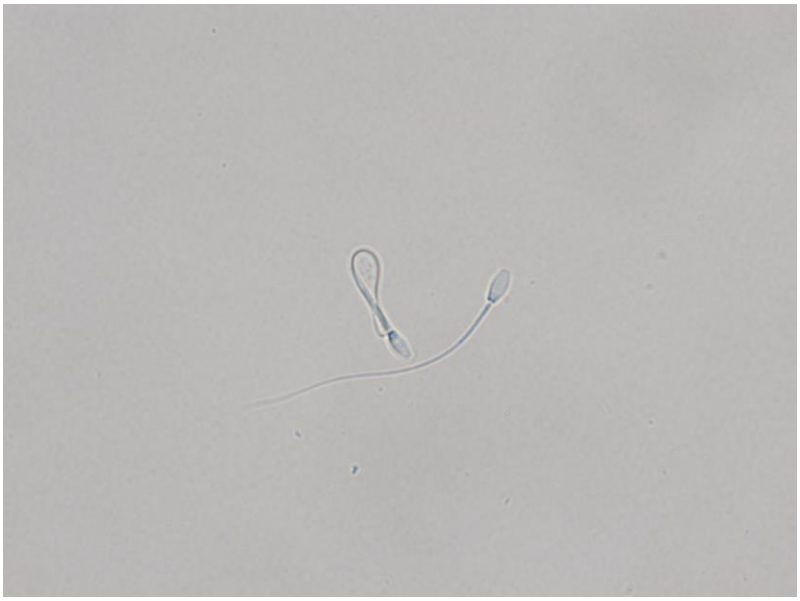

Figure 1 HOST positive and HOST negative sperm

Membrane intact sperm (HOST positive) is depicted on the left and membrane damaged sperm (HOST negative) is depicted on the right.

References

Alvarenga, M. A., F. O. Papa, F. C. Landim-Alvarenga, and A. S. L. Medeiros. 2005. Amides as cryoprotectants for freezing stallion semen: A review. Anim. Reprod. Sci. 89:105-113.

Baumber, J., B. A. Ball, and J. J. Linfor. 2005. Assessment of the cryopreservation of equine spermatozoa in the presence of enzyme scavengers and antioxidants. Am. J. Vet. Res. 66:772-9

Baumber, J., B. A. Ball, J. J. Linfor, and S. A. Meyers. 2003b. Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. J. Androl. 24:621-628.

Branco, C. S., M. E. Garcez, F. F. Pasqualotto, B. Erdtman, and M. Salvador. 2010. Resveratrol and ascorbic acid prevent DNA damage induced by cryopreservation in human semen. Cryobiology. 60:235-237.

Collodel, G., M. G. Federico, M. Geminiani, S. Martini, C. Bonechi, C. Rossi, N. Figura, and E. Moretti. 2011. Effect of trans-resveratrol on induced oxidative stress in human sperm and in rat germinal cells. Reprod. Toxicol. 31:239-246.

de Oliveira, R.A. 2013. Addition of glutathione to an extender for frozen equine semen. J. Equine Vet. Sci. 33:1148-1152.

Dowsett, K. F, and L. M. Knott. 1996. The influence of age and breed on stallion semen. Theriogenology. 46:397-412.

Ferrusola, C. O., L. G. Fernandez, J. M. Morrell, C. S. Sandoval, B. M. Garcia, H. Rodriguez-Martinez, J. A. Tapia, and F. J. Pena. 2009. Lipid peroxidation, assessed with BODIPY-C11, increases after cryopreservation of stallion spermatozoa, is stallion dependent and is related to apoptotic-like changes. Reproduction. 138:55-63.

Garcez, M. E., C. S. Branco, L. V. Lara, F. F. Pasqualotto, and M. Salvador. 2010. Effects of resveratrol supplementation on cryopreservation medium of human semen. Fertil. Steril. 94:2118-2121.

Gibb Z., T. J. Butler, L. H. A. Morris, W. M. C. Maxwell, and C. G. Grupen. 2013. Quercetin improves the postthaw characteristics of cryopreserved sex-sorted and nonsorted stallion sperm. Theriogenology. 79:1001-1009.

Graham, J. K. 2001. Assessment of sperm quality: a flow cytometric approach. Anim. Reprod. Sci. 68:239-247.

Herman, H. A. Improving Cattle by the Millions: NAAB and the Development and Worldwide Application of Artificial Insemination. University of Missouri Press, Columbia.1981.

Karlsson, J. O. M., and M. Toner. 1996. Long-term storage of tissues by cryopreservation: critical issues. Biomaterials. 17:243-256.

Loomis, P. R. and E. L. Squires. 2005. Frozen semen management in equine breeding programs. Theriogenology. 64:480-491.

Mojica-Villegas, M. A., J. A. Izquierdo-Vega, G. Chamorro-Cevallos, and M. Sanchez-Gutierrez. 2014. Protective effect of resveratrol on biomarkers of oxidative stress induced by iron/ascorbate in mouse spermatozoa. Nutrients. 6:489-503.

Mukherjee, S., J. I. Dudley, and D. K. Das. 2010. Dose-dependency of resveratrol in providing health benefits. Int. Dose-Response Soc. 8:478-500.

Neild, D. M., M. G. Chaves, M. Flores, M. H. Miragaya, E. Gonzalez, and A. Aguero. 2000. The HOS test and its relationship to fertility in the stallion. Andrologia. 32:351-355.

Okuda, T., and K. Yokotsuka. 1996. Trans-resveratrol concentrations in berry skins and wines from grapes grown in Japan. Am. J. Enol. Vitic. 47:93-99.

Oldenhof, H., M. Gojowsky, S. Wang, S. Henke, C. Yu, K. Rohn, W. F. Wolkers, and H. Sieme. 2013. Osmotic stress and membrane phase changes during freezing of stallion sperm: Mode of action of cryoprotective agents. Bio. Reprod. 88:1-11.

Ortega-Ferrusola, C., Y. Sotillo-Galan, E. Varela-Fernandez, J. M. Gallardo-Bolanos, A. Muriel, L. Gonzalez-Fernandez, J. A. Tapia, and F. J. Pena. 2008. Detection of "apoptosis-like" changes during the cryopreservation process in equine sperm. J Androl. 29:213-221.

Petruska, P., M. Capcarova, and P. Sutovsky. Antioxidant supplementation and purification of semen for improved artificial insemination in livestock species. DOI: 10.3906/vet-1404-82.

Pena, F. J., B. M. Garcia, J. C. Samper, I. M. Aparicio, J. A. Tapia, and C. O. Ferrusola. 2011. Dissecting the molecular damage to stallion spermatozoa: The way to improve current cryopreservation protocols? Theriogenology. 76:1177-1186.

Pommer, A. C., J. Rutllant, and S. A. Meyers. 2002. The role of osmotic resistance on equine spermatozoal function. Theriogenology. 58:1373-1384.

Sherman, J. K. 1964. Low temperature research on spermatozoa and eggs. Cryobiology. 2:103-29.

Silva, E. C. B., J. F. P. Cajueiro, S. V. Silva, P. C. Soares, and M. M. P. Guerra. 2012. Effect of antioxidants resveratrol and quercetin on *in vitro* evaluation of frozen ram sperm. Theriogenology. 77: 1722-1726.

Smoliga, J. M., J. A. Baur, and H. A. Hausenblas. 2011. Resveratrol and health: A comprehensive review of human clinical trials. Mol. Nutr. Food Res. 55:1129-1141.

CHAPTER III

CONCLUSIONS AND FUTURE STUDIES IN CRYOPRESERVATION

The cryopreservation process inflicts a variety of damages on spermatozoa. These damages can be classified as either osmotic or oxidative in nature. Osmotic damages result from large fluctuations in cell volume as the cell attempts to maintain equilibrium with the hypertonic environment experienced during the freezing process and the hypotonic environment it is exposed to during the thawing process (Ball, 2008). Osmotic damages include detachment of the cytoskeleton from the plasma membrane, detachment of the acrosomal membrane from the sperm head (Gonzalez-Fernandez et al., 2012) as well as rearrangement of the actin cytoskeleton (Correa et al., 2007). Osmotic damages impair functions such as motility, mitochondrial membrane potential, and viability (Pommer et al., 2002).

Several factors can influence stallion sperm quality. Stallions ages 3-11 years old have been shown to have increased sperm quality compared to stallions outside this range (Dowsett and Knott, 1996). To minimize variation, three of the stallions used in the current study fell into this age range. While the remaining stallion in the study fell outside of this range, its use was justified by exhibiting similar pre-freeze sperm motility to the other stallions. However, because many factors can influence sperm motility, it would be useful to analyze fresh sperm motility of the stallions at various time points other than immediately before cryopreservation to ensure consistency between stallions. While the

stallions used had different average numbers of foals produced per year, these variances were not the results of proven differences in fertility, but were the results of varying degrees of promotion and breeding advertisement between each stallion. As breed can also cause differences in sperm quality (Alvarenga et al., 2005), the study was limited to include only one breed of horse. Season has been shown to affect stallion sperm quality (Kalmar et al., 2013); therefore, the current study worked to minimize these effects by establishing a collection schedule in which each stallion was collected two to three times per week, and all collections took place during the month of May. However, a study by Kalmar et al., (2013) suggests that the highest post-thaw sperm motility can be obtained when collections take place during the fall and winter months. Therefore, better post-thaw motility results may have been observed in the current study if stallions were collected during these months. By limiting the study to include stallions of similar age, breed, management, and sperm motility, the stallion variation introduced into the study was minimized in order to better evaluate resveratrol's effect on sperm quality.

There are a number of methods to detect various damages in spermatozoa. Cell viability, or the presence of an intact plasma membrane, is commonly determined through the use of the fluorescent DNA-binding stain propidium iodide (PI). PI enters cells with damaged plasma membranes and can then be detected using microscopy or flow cytometry to quantify the number of cells with damaged membranes (Graham, 2001). In the current study, the cell permeating stain Syber-14 was also used to more clearly discriminate the live cells from debris and dead cells while flow cytometry allowed for the objective analysis of larger numbers of cells than would visual analysis. One problem with the approach of this study, however, is that the viability of a sample was consistently

underestimated when compared to the amount of live cells detected with the motility analysis. This could be due to mechanical stress the cells were exposed to during processing and flow cytometric analysis. While analysis of viability using flow cytometry is a more objective approach than visual analysis, it should be noted that the amount of viable cells detected could be underestimated, and the flow cytometric settings used should aim to inflict the least amount of damage possible. The hypo-osmotic swelling test (HOST) can be used to determine plasma membrane integrity by determining osmotic activity. In HOST, cells are incubated in a hypo-osmotic solution that causes a cellular intake of water. Using microscopy, cells are classified as either osmotically active with an intact plasma membrane (coiled tail) or osmotically inactive with a damaged plasma membrane (straight tail) (Colenbrander et al., 2003). While it is a simple and fast way to determine membrane integrity, the HOST results in our study contradicted the numbers of membrane intact cells found in our other analyses. This could be due to its subjective nature, and the differing degrees of tail coiling could lead to an overestimate of the actual number of membrane intact cells. For these reasons, this study enlisted the use of flow cytometry and the differential staining techniques described previously.

DNA integrity can also be analyzed using a variety of techniques including the sperm chromatin structure assay (SCSA) in which a fluorescent DNA binding stain is used to determine the degree of DNA denaturation (Graham, 2001). Another method to determine sperm DNA integrity is the terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling (TUNEL) assay in which a fluorescent antibody marks DNA strand breaks (Martins et al., 2007). Both SCSA and TUNEL can be utilized through flow cytometry allowing for a more objective measurement of a large number of cells.

However, SCSA measures the susceptibility of DNA to denature by utilizing an acid-induced denaturation method while TUNEL provides a direct measurement of existing DNA fragmentation. TUNEL also has drawbacks in that it does not yet have a clearly established standardized scale to measure the severity of DNA damage in equine sperm. This study was only able to compare the degree of fluorescence detected in each treatment with the control (Bungum et al., 2011). It would be beneficial in future studies on equine DNA fragmentation to establish a scale to which fluorescent intensity could be compared to better evaluate the amount of fragmentation in sperm and its relation to fertility.

Functional characteristics such as sperm motility can be evaluated using computer assisted sperm analysis (CASA) in which each individual cell's motility path and velocity among other parameters is recorded (Colenbrander et al., 2003). By using CASA to measure motility instead of visual analysis, we were able to more precisely determine the total motility of a sample as well as the number of sperm that were progressively motile. Not only cell damages themselves can be detected but also molecular indicators of cellular damage such as ROS activity and LPO through the use of assays that detect the oxidative activity of ROS and lipid peroxides. This study utilized a carboxy-H_2DCFDA ROS assay which allowed for measurement of all ROS activity in a sample rather than limiting the measurement to ROS responsible for lipid peroxidation. By using a ROS assay in combination with flow cytometry, this study was able to detect the amount of oxidative activity taking place in each cell in order to directly determine the effectiveness of the supplemented antioxidant, resveratrol.

Aside from the negative effects cryopreservation has on sperm functionality such as decreased motility, viability, and increased DNA damage; cryopreservation can also affect fertilization and embryo development. It has been found that cryopreserved stallion sperm have a decreased ability to bind to the zona pellucida *in vitro* when compared to fresh spermatozoa (Dobrinski et al., 1995). When fertilization by DNA-damaged sperm does occur, embryonic development is reduced and there is an increase in early embryonic death (Ball, 2008). Morris et al. (2002) found that DNA damage was associated with a decrease in embryo cleavage rates in humans. Long-term developmental effects of fertilization with DNA fragmented sperm have been documented in mice. It was found that 20% of mice resulting from DNA fragmented sperm did not survive past the first five months of life while premature aging was symptomatic in 25% of the remaining live mice (Fernandez-Gonzalez et al., 2008). While these analyses were not conducted in the current study, fertilization assays and the study of long-term development of the resulting offspring would provide additional information about the functionality of the treated sperm.

Several antioxidants found in stallion sperm and seminal plasma have been the focus of research in preventing oxidative damage to sperm. Superoxide dismutase has been found to improve aspects such as stallion sperm motility and viability when supplemented in semen extender (Cocchia et al., 2011). Glutathione was found to improve post-thaw motility, plasma membrane integrity, and viability when supplemented in semen extender (Oliveira et al., 2013). Catalase was found to reduce DNA fragmentation in fresh sperm incubated with a ROS inducing agent (Ball et al., 2000). Antioxidants of exogenous sources have also been researched for the prevention

of oxidative damages to sperm. Resveratrol, a polyphenol found in the skin of red grapes, was found to decrease DNA fragmentation (Branco et al., 2010), decrease lipid peroxidation as well as increase SOD and CAT activity in cryopreserved human sperm (Garcez et al., 2010). However, the current study observed an increase in the number of nonviable stallion sperm with the addition of resveratrol to the cryopreservation extender indicating a cytotoxic effect. An increase in DNA fragmentation was observed for one of the treatments in our study, contradicting findings by Branco et al. (2010) in which DNA fragmentation was reduced in human sperm cryopreserved with resveratrol. In the current study, there were no differences detected for plasma membrane damage with the addition of resveratrol to the semen extender. Similarly, Silva et al. (2012) reported no differences in plasma membrane integrity for ram sperm cryopreserved with resveratrol supplemented semen extender. Resveratrol supplementation reduced ROS production in post-thaw stallion sperm. These results support findings by Mojica-Villegas et al. (2014) in which intracellular ROS levels were reduced in mouse sperm cryopreserved in resveratrol supplemented semen extender.

Although the concentrations of resveratrol used in this study appear to exert negative affects on stallion sperm quality, lower concentrations could prove beneficial in preventing oxidative damage during the cryopreservation process. The concentrations used in the current study are much larger than the physiological level of resveratrol found in grape skins (0.00219-0.0613 mM); therefore, concentrations within the physiological range could prevent resveratrol toxicity in stallion sperm (Okuda and Yokotsuka, 1996). At an optimum concentration, resveratrol's ability to decrease ROS activity could help improve DNA and membrane integrity in frozen-thawed sperm. Specifically,

resveratrol's superoxide scavenging ability along with its ability to increase the activity of SOD could prove beneficial at a suitable concentration either as a single supplement or in combination with antioxidants such as catalase or glutathione that target other types of ROS. Also, because resveratrol is plant-derived, it could provide a readily available antioxidant source as opposed to many animal derived antioxidants. Another possible mechanism resveratrol could improve sperm quality is through nutritional supplementation. In rats, dietary resveratrol supplementation has been shown to increase testosterone levels and sperm output (Juan et al., 2005). Because there remains a need for a supplement that can consistently reduce cellular damages to cryopreserved stallion sperm, more research should be done to identify beneficial concentrations and combinations of additives.

References

Alvarenga, M. A., F. O. Papa, F. C. Landim-Alvarenga, and A. S. L. Medeiros. 2005. Amides as cryoprotectants for freezing stallion semen: A review. Anim. Reprod. Sci. 89:105-113.

Ball, B. A. 2008. Oxidative stress, osmotic stress and apoptosis: Impacts on sperm function and preservation in the horse. Anim. Reprod. Sci. 107:257-267.

Ball, B. A., C. G. Gravance, V. Medina, J. Baumber, and I. K. M. Liu. 2000. Catalase activity in equine semen. Am. J. Vet. Res. 61:1026-1030.

Branco, C. S., M. E. Garcez, F. F. Pasqualotto, B. Erdtman, and M. Salvador. 2010. Resveratrol and ascorbic acid prevent DNA damage induced by cryopreservation in human semen. Cryobiology. 60:235-237.

Bungum, M., L. Bungum, and A. Giwercman. 2011. Sperm chromatin structure assay (SCSA): a tool in diagnosis and treatment of infertility. Asian J. Androl. 13:69-75.

Cocchia, N., M. P. Pasolinia, R. Mancinib, O. Petrazzuoloc, I. Cristofarod, I. Rosapanea, A. Sicaa, G. Tortoraa, R. Lorizioa, G. Paraggioe, and A. Mancinib. 2011. Effect of sod (superoxide dismutase) protein supplementation in semen extenders on motility, viability, acrosome status and ERK (extracellular signal-regulated kinase) protein phosphorylation of chilled stallion spermatozoa. Theriogenology. 75:1201-1210.

Correa, L. M., A. Thomas, and S. A. Meyers. 2007. The Macaque sperm actin cytoskeleton reorganizes in response to osmotic stress and contributes to morphological defects and decreased motility. Bio. Reprod. 77:942–953.

Colenbrander, B., B. M. Gadella, and T. A. E. Stout. 2003. The predictive value of semen analysis in the evaluation of stallion fertility. Reprod. Dom. Anim. 38:305-311.

de Oliveira, R.A. 2013. Addition of glutathione to an extender for frozen equine semen. J. Equine Vet. Sci. 33:1148-1152.

Dobrinski, I., P. G. A. Thomas, and B. A. Ball. 1995. Cryopreservation reduces the ability of equine spermatozoa to attach to oviductal epithelial cells and zonae pellucidae *in vitro*. J. Androl. 16:536-542.

Dowsett, K. F, and L. M. Knott. 1996. The influence of age and breed on stallion semen. Theriogenology. 46:397-412.

Fernandez-Gonzalez, R., P. N. Moreira, M. Perez-Crespo, M. Sanchez-Martin, M. A. Ramirez, E. Pericuesta, A. Bilbao, P. Bermejo-Alvarez, J. de Dios Hourcade, F. R. de Fonseca, and A. Gutierrez-Adan. 2008. Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring. Bio. Reprod. 78:761-772.

Garcez, M. E., C. S. Branco, L. V. Lara, F. F. Pasqualotto, and M. Salvador. 2010. Effects of resveratrol supplementation on cryopreservation medium of human semen. Fertil. Steril. 94:2118-2121.

Graham, J. K. 2001. Assessment of sperm quality: a flow cytometric approach. Anim. Reprod. Sci. 68:239-247.

Gonzalez-Fernandez, L., J. M. Morrell, F. J. Pena, and B. Macias-Garcia. 2012. Osmotic shock induces structural damage on equine spermatozoa plasmalemma and mitochondria. Theriogenology. 78:415-422.

Juan, M. E., E. Gonzalez-Pons, T. Munuera, J. Ballester, J. E. Rodriguez-Gil, and J. M. Planas. 2005. Trans-Resveratrol, a natural antioxidant from grapes, increases sperm output in healthy rats. J. Nutr. 135:757-760.

Kalmar, J. J., B. A. Ball, M. H. T. Troedsson, J. Skaife, P. I. Loomis, and E. L. Squires. 2013. Factors impacting freezability of stallion sperm. J. Equine Vet. Sci. 33:5. Abstract.

Martins, C. F., M. N. Dode, S. N. Bao, and R. Rumpf. 2007. The use of the acridine orange test and the TUNEL assay to assess the integrity of freeze-dried bovine spermatozoa DNA. Genet. Mol. Res. 1:94-104.

Mojica-Villegas, M. A., J. A. Izquierdo-Vega, G. Chamorro-Cevallos, and M. Sanchez-Gutierrez. 2014. Protective effect of resveratrol on biomarkers of oxidative stress induced by iron/ascorbate in mouse spermatozoa. Nutrients. 6:489-503.

Morris, I. D., S. Ilott, L. Dixon, and D. R. Brison. 2002. The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. Human Reprod. 17:990-998.

Okuda, T., and K. Yokotsuka. 1996. Trans-resveratrol concentrations in berry skins and wines from grapes grown in Japan. Am. J. Enol. Vitic. 47:93-99.

Pommer, A. C., J. Rutlant, and S. A. Meyers. 2002. The role of osmotic resistance on equine spermatozoal function. Theriogenology. 58:1373-1384.

Silva, E. C. B., J. F. P. Cajueiro, S. V. Silva, P. C. Soares, and M. M. P. Guerra. 2012. Effect of antioxidants resveratrol and quercetin on *in vitro* evaluation of frozen ram sperm. Theriogenology. 77: 1722-1726.

APPENDIX A

STALLION INFORMATION

Table 6 Stallion information

Stallion	Breed	Age (yrs old)	Weight (kg)	Height (m)	*Pre-Freeze Sperm Motility	Average number of foals per year	Year of first breeding
Ace	Quarter Horse	13	454.5	1.44	66.7 ± 5.77	7	2004
Smokey	Quarter Horse	9	409.1	1.43	70 ± 10.0	3	Unknown
Roany	Quarter Horse	6	454.5	1.44	73.3 ± 5.77	5	2013
Oakley	Quarter Horse	5	500.0	1.54	66.7 ± 5.77	10	2013

*Data presented as percent mean ± standard deviation of motile sperm

APPENDIX B

DATA

Table 7 Data for each horse by treatment and analysis

Stallion	Treatment	*ROS content	*DNA fragmentation	% Live	% HOST positive	% Motile	% Progressively Motile
Ace	Control	62.5 ± 27.87	198.9 ± 76.13	4.7 ± 1.29	22.6 ± 4.00	17.6 ± 6.56	8.1 ± 3.39
	1 mM	62.5 ± 15.69	279.0 ± 146.69	0.9 ± 0.54	20.2 ± 2.60	2.9 ± 2.26	0.8 ± 0.65
	10 mM	32.7 ± 17.78	302.4 ± 150.00	0.3 ± 0.15	16.8 ± 3.62	2.7 ± 2.62	0.7 ± 0.65
Oakley	Control	64.3 ± 7.13	186.5 ± 87.09	1.2 ± 0.52	11.0 ± 4.36	20.5 ± 6.33	8.6 ± 3.29
	1 mM	53.8 ± 4.98	238.0 ± 155.62	0.3 ± 0.16	7.9 ± 4.15	2.3 ± 1.53	0.6 ± 0.24
	10 mM	31.5 ± 10.31	209.4 ± 141.76	0.2 ± 0.08	9.7 ± 1.75	3.4 ± 2.38	0.8 ± 0.52
Roany	Control	68.3 ± 11.68	180.3 ± 46.52	2.4 ± 0.53	6.1 ± 1.76	35.9 ± 10.28	18.5 ± 6.73
	1 mM	59.1 ± 7.18	197.5 ± 59.25	0.4 ± 0.28	5.1 ± 1.83	6.6 ± 4.06	1.8 ± 1.45
	10 mM	39.6 ± 23.02	220.6 ± 149.32	0.2 ± 0.16	7.7 ± 3.06	4.2 ± 3.45	0.9 ± 0.75
Smokey	Control	66.7 ± 15.56	207.1 ± 169.10	1.4 ± 0.78	7.3 ± 1.50	25.7 ± 16.62	15.6 ± 10.64
	1 mM	53.7 ± 12.21	213.5 ± 132.25	0.6 ± 0.46	7.8 ± 2.30	7.5 ± 4.05	1.7 ± 0.89
	10 mM	45.2 ± 13.08	172.6 ± 119.26	0.3 ± 0.20	5.3 ± 1.79	2.8 ± 1.91	0.9 ± 0.79

*units are mean fluorescent intensity
Data are presented as mean ± standard deviation