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The molecular and cellular dynamics of Holstein bull spermatozoa associated with cryotolerance

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The objective of this study was to uncover molecular and cellular dynamics in spermatozoa from Holstein bulls with Good (11 bulls) and Poor (5 bulls) cryotolerance. Post-thaw sperm kinetics, membrane integrity, and DNA fragmentation were assessed. Data was analyzed using principal component analysis. The spermatozoa from Good bulls had a higher number of cells with intact membranes ($P = 0.029$), non-fragmented DNA ($P = 0.018$), post-thaw viability ($P < 0.001$) in comparison to those of the Poor bulls. The Good bulls also had a faster average path velocity (VAP; $P = 0.017$), straight-line velocity (VSL; $P = 0.036$), a greater distance average path (DAP; $P = 0.006$) and distance straight line (DSL; $P = 0.011$). No differences were found in total antioxidant capacity (TAC), number of live cells or other kinetic parameters between spermatozoa from Good and Poor groups, showing that no one characteristic can be used to determine cryotolerance.

DEDICATION

I would like to dedicate this thesis to my parents, Eric and Tracye Gilmore. Your unconditional love and support have given me the strength and ambition to follow my dreams. Even though pursing my dreams has taken me far from home, you both have been my number one cheerleaders through all the challenges that life has thrown my way. I am truly blessed to call you my parents.

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CHAPTER I

LITERATURE REVIEW

Male Reproduction and Development

Male reproductive efficiency is one of the most important factors influencing the livestock industry today. Male fertility is defined as the ability of the spermatozoa to fertilize and activate the oocyte and support subsequent embryonic development (Martins et al., 2018). Poor fertility and reproductive inefficiency can greatly impact the economic success of a bovine herd. Bull fertility is crucial for determining the reproductive performance of a herd but is often overlooked as the reason for low reproductive efficiency and/or reproductive failure within a herd. Infertility rates are estimated to be between 15 to 25% of potential sires in the U.S. cattle industry. This is why breeding soundness exams (BSE) are essential for a successful breeding program. The current BSE consists of a physical exam of the animal including, an exam of the reproductive organs, and measurement of scrotal circumference and evaluation of the semen. Semen evaluation is used to identify sperm parameters such as concentration, motility, morphology, organelle integrity, and DNA integrity (Roldan, 2007). While reproductive management of both beef and dairy cattle has been greatly advanced in the last twenty years, there is still much room for improvement as new information is found and innovative techniques are developed.

Cryopreservation or freezing of spermatozoa was first introduced in the 1960's and has become an effective way to manage and preserve male fertility in both animals and humans. The first recorded attempt to cryopreserve semen dates back to 1776, when Lazaro Spallanzani endeavored to preserve stallion spermatozoa in snow. In the following decades, great scientific strides were made in the progression of cryopreservation techniques. In 1949, glycerol was found by Polge and colleagues to have cryoprotectant properties, which was a turning point in this field of study. Since this discovery, many advancements have been made in the cryopreservation process. The first bovine offspring produced from cryopreserved spermatozoa was reported in 1951. This process is widely used for artificial insemination (AI) to manage or accelerate genetic improvement by using semen from genetically superior males on females that would not normally be an option for breeding due to location of animals (Hezavehei et al., 2018). Though the cryopreservation process has been greatly improved and is widely used, it is a well-known fact that the freezing and thawing processes of cryopreservation damage spermatozoa due to the rapid change in temperature, osmotic stress, and the formation of ice crystals. On average, only about 50% of spermatozoa survive the freezing and thawing processes. This process can cause alterations to the plasma membrane, acrosome, mitochondria, DNA integrity, increase production of reactive oxygen species (ROS), as well as a marked reduction to spermatozoa motility and viability (Gürler et al., 2015).

Cryoprotectants are widely used to counteract the negative effects of cryopreservation. Glycerol is the most commonly used cryoprotectant because it diminishes the mechanical damage the spermatozoa may incur during the freezing process, but glycerol causes osmotic damage due to its slow passage rate through the plasma membrane. Other cryoprotectants such as dimethyl sulfoxide (DMSO) and ethylene glycol (EG) have been shown to be just as

protective as glycerol and cause less damage to the cells. More research needs to be done to better understand the optimum extender composition, type and concentration of cryoprotectant, and cryopreservation protocol to best preserve bovine spermatozoa (Taşdemir et al., 2013)

Artificial insemination (AI) is an assisted reproductive technique in which semen is collected from a male using an artificial vagina or electro-ejaculation, and then, manually deposited into the reproductive tract of a female by a trained technician using the rectovaginal technique that is the most extensively used practice in the industry. This method is guided by a gloved hand in the rectum that is above the reproductive tract and requires the use of a sterile disposable catheter containing the semen sample that is inserted into the vagina, and then, passed through the cervix into the body of the uterus where the semen is deposited (Webb, 2003). Artificial insemination has been proven to be effective at increasing genetic gain by increasing selection pressure on males and allowing for an increased number of offspring from males who have been proven to pass on desirable traits to their progeny per year. AI is heavily used within the dairy industry and in many countries the milk yield per cow has doubled in the last 40 years due to the rapid genetic progression made through the use of AI. Some advantages to using AI in cattle is the reduced risk of sexually transmitted diseases, maximum use of genetically superior sires, early detection of sub-fertile bulls, the ability to use bulls in a breeding program for a longer period, and the option of not having an on-farm bull. Some of the disadvantages of the use of AI are that proper implementation of an AI program requires special training for a technician to breed females, accurate heat detection to find females in estrus, the cost and time to synchronize the estrus cycles of females, and the potential reduction in the gene pool by only utilizing a few select sires (Mohammed, 2018). Another disadvantage of using AI on a large scale is the danger of spreading a genetic defect that was not known among a breed or species.

While the process of cryopreservation can impact male fertility, semen quality is influenced by a number of genetic and environmental factors. Bull age is a key component in reproductive performance due to the variability of the onset of puberty within and among breeds. Age of puberty and maturity level are important factors in semen characteristics. As the bull matures, semen volume and quality increase. This is believed to be due to an increase in body mass and the concurrent growth of the testis and accessory sex glands (Barth et al., 2008). Murphy et al. (2018) investigated the effects of bull age, ejaculate number, and season of collection on semen production parameters of 176 Holstein Friesian bulls between the ages of nine months and eight years of age housed at the National Cattle Breeding Centre over a four-year period. A total of 8,983 ejaculates were assessed to determine volume, sperm concentration, total motility, and gross motility to ensure that all samples were of commercial standard. Following preliminary evaluation, samples were cryopreserved, and then, post-thaw motility was assessed. It was found that there was an age effect on ejaculate volume, total sperm number (TSN), sperm concentration, and pre- and post-thaw motility. Bulls that were under a year old had the poorest semen production and motility values in comparison to bulls that were over a year old similar to the findings of Al-Kanaan et al. (2015). The first ejaculates collected had greater semen volume, total number of spermatozoa and pre-freeze motility rates than second consecutive ejaculates. There was no statistical difference in post-thaw motility among the different age groups. Furthermore, ejaculates collected in the winter had the poorest values of sperm concentration and TSN. It was concluded that a second ejaculate can be collected without decreasing the post-thaw motility, which can be used as a strategy to increase semen availability from highly sought-after bulls.

Along with the impact of age, studies have shown that bulls exposed to heat stress have been shown to have reduced testis weight and a shift in testosterone production along with sperm cell abnormalities (Setchell, 2006). The production of ROS is a frequent consequence of heat stress in males, and it is believed this kind of stress induces OS in spermatozoa. Houston et al. (2018) explored the negative impact of heat stress on the molecular structure and function of spermatozoa in relation to decreased fertility seen in the mouse model after exposure to acute or chronic heat stress. In this study there were three treatment groups: control, acute heat stress (24 hrs. at 35ºC), and chronic heat stress (8 hrs. per day at 35º for two weeks). Mice exposed to acute heat stress had elevated sperm mitochondrial ROS generation, increased sperm membrane fluidity, and increased DNA damage in the form of single strand breaks ($P<0.05$) that are all characteristics of OS. No such decline was seen in the chronic heat stress group. It was concluded that exposure to acute heat stress resulted in significant alterations to sperm cell development impacting overall quality.

The effects of heat stress on semen quality have been studied via scrotal insulation. This technique is not the most accurate method. It does not indicate the changes in sperm quality that might occur in warm climates because it supersedes the bull's natural testicular temperature regulation (Hansen, 2009). Sabès-Alsina et al. (2017) explored the possible influence of climate on semen quality of Holstein bulls maintained in northern Spain, where the summers are warm and the winters are mild. For this study, semen samples were collected from eleven Holstein bulls aged one to six years old, and then, cryopreserved. The samples were collected during the winter, spring, and summer. Daily and monthly average temperature and humidity were calculated, and then, correlated with the semen quality parameters. Motility, morphology, plasma membrane integrity, acrosome status, mitochondrial membrane potential, DNA fragmentation

index (DFI), and ROS were evaluated for all samples post-thaw. Semen samples collected in the spring months had significantly higher mean values of total progressive motility, proportion of viable sperm cells, and portion of live cells that did not produce superoxide or hydrogen-peroxide than samples that were collected during the winter months ($P \leq 0.05$). There were no statistical differences found in sperm cell morphology or DFI % between the seasons. The authors concluded that sperm cell quality of Holstein bulls living in northern Spain was affected by the different seasons.

It is of great economic importance that bull fertility is accurately predicted because obtaining high conception rates is essential to the success of the beef and dairy industries. If bull fertility can be accurately predicted, then, the use of semen from subpar bulls can be reduced, decreasing the number of open cows. If the number of open cows in a herd is reduced, then, the profitability of the herd is increased by the larger number of calves born. Successful fertilization is key in reducing open cows, and fertilization success can begin with the proteins of the seminal plasma and spermatozoa. The proteins that are present in seminal plasma and spermatozoa can influence the function and fertilization ability of the cell. Kumaresan et al. (2017) investigated thirty-seven sperm characteristics that make the sperm cell viable for fertilization in frozen-thawed spermatozoa from twenty dairy breeding bulls with different adjusted nonreturn rates (NRR). This study also endeavored to identify a combination of sperm parameters that could be used to accurately predict bull fertility. The twenty bulls were divided into three groups, above-average fertility, average fertility, and below-average fertility, based upon their 56-day NRR. Bulls were classified as below-average if their NRR was ≤94 and bulls with a NRR ≥103 were classified as above-average, with the remaining bulls classified as average. Significant differences were found in sperm viability, acrosomal integrity, ROS production, and DFI %

among the above-average, average, and below-average fertility bulls. The proportion of live spermatozoa was significantly lower, and the proportion of dead spermatozoa was significantly higher in the below-average fertility group in comparison with the average and above-average fertility groups. In fact, several studies have shown that sperm viability is related to bull fertility level (Rodríguez-Martínez, 2007). It was concluded that a combination of number of live and dead spermatozoa, live hydrogen peroxide-negative spermatozoa, and percentage DFI could be used to distinguish below-average and above-average fertility bulls in a fertility prediction model.

Reproductive performance of cattle is prominently influenced by pregnancy loss. Pregnancy success is affected by maternal, parental, embryonic, and environmental factors (Kropp et al., 2014). The success of early pregnancy events in cattle is heavily influenced by the sire's genetic contribution. Currently, sire conception rates (SCR) are used as an indicator of bull fertility in AI programs. Sire conception rate is defined as the probability of a semen sample from a bull resulting in a pregnancy in comparison with the mean semen sample of other bulls based on the number of confirmed pregnancies by day 70 after AI. In 2018, Ortega et al. studied the sire influence on pregnancy establishment in dairy cattle. Semen from ten Holstein bulls (five low SCR bulls and five high SCR bulls) were utilized in a field trail. Pregnancy rates at first insemination showed no differences between the high SCR and low SCR bulls. A significant difference was found in pregnancy loss among high SCR bulls and low SCR bulls between day 19 and day 33 post insemination. On day 19 after insemination, pregnancy rates for the high SCR bulls was 89.6% and 68% for low SCR bulls ($P < 0.029$). By day 33 after insemination pregnancy rates were 79.0% for high SCR bulls and 49.0% for low SCR bulls ($P < 0.0001$). For the next component of the study, semen from three low SCR and two high SCR bulls were

utilized in evaluating *in vitro* embryo production. No effect of SCR was observed in embryo cleavage rates, but bulls with low SCR produced less day eight blastocysts in comparison to high SCR bulls (42.6% *vs.* 35.6%). The researchers also found that low SCR bulls produced a higher proportion of unfertilized oocytes compared to the high SCR bulls (3.7% *vs.* 20.7%). This study emphasized the importance of understanding the influence of the sire's genome on a range of early pregnancy events from fertilization to embryogenesis is vital to increasing reproductive efficiency in dairy cattle.

Currently, bull fertility is mainly predicted based off of semen characteristics such as volume, concentration, motility, morphology, and ability to survive the freezing and thawing process. Fertility rates have been shown to differ greatly among bulls and the variations are not accounted for in routine semen evaluation parameters (Larson et al., 2000). It has been reported that proteins, osteopontin, prostaglandin D heparin-binding protein (HBP) synthase, and bovine seminal plasma proteins can be used as indicators of bull fertility. Karunakaran et al. (2017) evaluated twenty-two breeding dairy bull semen samples for the presence of a fertility-associated protein, 28-30 kDa heparin-binding protein and the effects that it may have on *in vitro* sperm characteristics and fertility. The semen samples were collected via an artificial vagina, and then, cryopreserved. The samples were thawed, and sperm proteins were extracted and characterized. Bulls were then split into two groups, those positive for the 28-30 kDa HBP (group I) and those negative for the 28-30 kDa HBP (group II). The samples were also analyzed for *in vitro* characteristics (motility, membrane integrity, DNA integrity, apoptosis, mitochondrial membrane potential, lipid peroxidation, and capacitation status) at four different time periods (immediately post thaw, 60 minutes, 120 minutes, and 180 minutes post thaw). Results indicated that 50% of the semen from bulls screened were 28-30 kDa HBP positive. Semen from these HBP-positive

bulls had better *in vitro* sperm characteristics such as motility, membrane integrity, mitochondrial membrane potential, and DNA integrity in comparison to 28-30 kDa HBP negative bulls. Bulls positive for the 28-30 kDa HBP protein also had a 13% higher conception rate in comparison to bulls lacking the 28-30 kDa HBP protein. It was concluded that screening bulls for the existence of 28-30 kDa HBP could be used as a potential biomarker of fertility.

Spermatozoa, the unique and specialized cells exerting paternal influence

Spermatogenesis is the process in which spermatozoa develop from germ cells within the seminiferous tubules of the testis. Spermatogenesis can be divided into three main phases, the proliferation phase, meiotic phase, and differentiation phase. The first phase of spermatogenesis is the proliferation phase in which all mitotic division of spermatogonia takes place. Spermatogonia develop from stem cells, undergo mitosis, and produce B-spermatogonia, which then enter the second phase of spermatogenesis called the meiotic phase. At this point, primary spermatocytes undergo meiotic division forming secondary spermatocytes. These secondary spermatocytes then undergo a second meiotic division producing haploid spermatids. Genetic diversity is achieved during this phase by DNA replication and crossing over, making no two sperm cells alike. The spermatids then enter the third and final phase of spermatogenesis called the differentiation phase where they develop into mature sperm cells. No cell division takes place during this phase (Senger, 2003). After the completion of spermatogenesis, the immobile spermatozoa are transported from the Sertoli cells to the lumen of the seminiferous tubules. The spermatozoa then pass through the testis onto the epididymis where they fully mature. As the spermatozoa pass through the epididymis, they attain their motility and ability to fertilize (Henkel et al., 2018).

The mature spermatozoon consists of a head, neck, and tail enclosed by a plasma membrane. The head is comprised of the acrosome and nucleus. The acrosome is a cap-like structure that encases two-thirds of the sperm cell head. It consists of an inner and outer membrane that is divided into three segments: the apical, main, and equatorial segments. The nucleus contains condensed chromatin with genetic information, which is the potential parental contribution of genetic material. The neck of the sperm cell is a short segment of the cell that connects the head and flagellum, composed of a dense fibrous capitulum and segmented columns. The longest part of the sperm cell is the flagellum and/or tail. It is comprised of three sections, the midpiece, principal piece, and end piece. The midpiece contains the mitochondria, which provides the energy for the sperm cell to swim (Pesch et al., 2006).

The success of embryonic development is highly dependent upon the quality of the genetic and epigenetic contributions from both the paternal and maternal contributors. Only viable spermatozoa have the ability to interact and fertilize the oocyte in the natural fertilization process. Previous research suggested that fertilization and embryonic development failure are of seminal origin (Saacke et al., 2000). Research has also shown that when low-fertility bulls were used for *in vitro* fertilization (IVF) procedure, the cleavage rates were decreased and the pronuclear formation was delayed (Walters et al., 2004). It has also been found that prolonged gamete coincubation of the oocyte and sperm cells may have a damaging effect on the embryonic development. It has been proposed that the longer that the gametes are incubated together may result in an excess amount of dead sperm cells that produce elevated quantities of ROS, inducing the zona pellucida to harden and compromising the fertilization process and embryo viability (Berland et al., 2011).

Sperm molecular and cellular characteristics associated with bull fertility

Oxidative stress can have a negative effect on sperm cell morphology. The acrosome is a membrane structure that covers two-thirds of the sperm head. It formed from the Golgi apparatus during spermatogenesis. A fully formed acrosome is composed of three main parts: outer acrosomal membrane (OAM), acrosomal matrix, and inner acrosomal membrane (IAM). The acrosome is highly susceptible to OS because it is a specialized structure composed of many proteins. The acrosome plays a major role in the binding between the sperm cell and the zona pellucida of the oocyte. In order for the acrosomal reaction to take place, the acrosome of the cell must have a normal morphology. El-Taieb et al. (2014) investigated the correlation between acrosomal anomalies and OS levels. Semen samples from 30 infertile and 20 fertile men were collected and tested for basic semen parameters, MDA levels via Thiobarbituric Acid (TBA) method and acrosomal anomalies via electron microscopy examination. MDA levels were found to be much higher in the infertile group in comparison to the fertile group. A positive correlation was found between acrosomal abnormalities and MDA levels. It was concluded that abnormalities of the acrosome lead to higher levels of ROS and OS causing infertility in men.

Sperm chromatin is another important structure associated with bull fertility and if damage is incurred then fertility is reduced. Its integrity is vital to normal sperm physiology and healthy early embryonic development. Mature sperm cell chromatin is composed of DNA enveloping nucleoproteins. Oliveira et al. (2011) investigated sperm chromatin status, expression levels, and cellular localization of histones within spermatozoa from bulls of both high fertility (HF) and low fertility (LF). Aniline blue (ANBL) staining was used to determine chromatin condensation, while western blotting was used to measure protein concentrations. Immunofluorescence microscopy was used to determine the localization of histones in individual

sperm cells. There was a significant difference between the number of positive ANBL cells in the HF and LF bulls ($P < 0.0001$); however, there were no significant differences in histone levels among the two groups. Immunocytochemical analysis showed that the majority of sperm cells demonstrated a low level of histone retention (78% in HF bulls; 71% in LF bulls) confirming the ANBL test results. It was concluded that abnormal chromatin condensation associated with retention of histones is related to bull fertility.

Sperm kinematics such as motility and velocity parameters are also related to bull fertility and can be used to help identify sub-par fertile bulls from fertile bulls. Kumaresan et. al. (2017) analyzed several sperm functions required for fertility, including progressive motility, velocity, and DNA damage in cryopreserved spermatozoa of 20 dairy bulls to discriminate bulls of different fertility levels. Sperm kinematics were measured using Sperm Vision CASA Software. A total of 12 parameters were analyzed including total and progressive motility, average path distance (DAP), curvilinear distance (DCL), straight line distance (DSL), average path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL), straightness (STR), linearity (LIN), wobble (WOB), amplitude of lateral head deviation (ALH), and beat cross frequency (BCF). No significant differences were observed in any of the CASA parameters; however, there was a significant difference in viability, acrosomal integrity, and DNA damage among the different fertility groups. It was it concluded that sperm kinematics alone were not good indicators of bull fertility and need to be paired with other sperm functions.

Roots of production and consequences of oxidative stress in the body

Oxidative stress (OS) is the imbalance between the excess creation of ROS and the inability of the antioxidant system in the seminal plasma and sperm cells to neutralize the excessive production of ROS (Barranco et al., 2015). ROS are free radical derivatives of oxygen-

containing molecules. They are a byproduct of aerobic metabolism that are generated through oxidative phosphorylation of adenosine triphosphate (ATP) within the mitochondria via the electron transport chain. Two sources of spermatic ROS are cytoplasmic glucose-6-phosphate dehydrogenase and spermatic leucocytes (Wagner et al., 2018).

The ROS are an essential part of male fertility. In small quantities, ROS play a vital role in normal sperm physiology. They stimulate sperm hyperactivation, capacitation, and acrosomal reaction. They also promote the binding of the sperm cell to the zona pellucida of the oocyte by oxidizing the plasma membrane (Henkel et al., 2018). The physiological and pathological roles of ROS in sperm cells are depicted in figure 1. When an excessive amount of ROS is produced or when antioxidant activity fails due to the inadequate levels of antioxidants available to counter act ROS production, there is a disruption between balance of ROS and antioxidant activity, resulting in OS. Spermatozoa are extremely susceptible to OS because they contain very low levels of enzymatic antioxidants (Majzoub and Agarwal, 2018). Oxidative stress can cause damage to sperm cells in four main ways: damage to the DNA, lipid peroxidation of the cell membrane, oxidation of amino acids, and oxidative deactivation of specific enzymes by oxidation of cofactors. This type of damage can lead to a variety of disorders and diseases within the male (Henkel et al., 2018). OS of spermatozoon membranes threatens sperm survival by impairing essential functions such as motility, membrane integrity, and fertilizing ability of individual sperm cells.

Another reason spermatozoa are susceptible to high levels of ROS is because of their large quantities of polyunsaturated fatty acids (PUFAs) within their membranes and the low amounts of antioxidants present within the cell due to the meniscal cytoplasmic space. Koppers et al. (2008) evaluated the importance of sperm cell mitochondria in creating OS associated with

faulty sperm function in human males. Extracellular H_2O_2 was measured using chemiluminescence and intracellular generation of O_2, lipid peroxidation and spontaneous ROS production was estimated with flow cytometry. Sperm parameters were also measured using Computer-assisted Sperm Analyses (CASA). The researchers found that defective spermatozoa spontaneously generate mitochondrial ROS in higher quantities than normal spermatozoa, which negatively affected motility. It was concluded that sperm mitochondria were a significant source of ROS production contributing to OS in defective human sperm cells.

Cryopreservation causes detrimental changes to sperm cell structure and function, as well as increase the production of ROS. This damage is caused by thermal stress due to the rapid change in temperature, the high amount of cryoprotectants used, and the formation of ice crystals inside the plasma membrane. Khalil et al. (2018) studied the damaging effects of cryopreservation on the structure and function of bull spermatozoa. Semen samples were collected from five Friesian bulls via an artificial vagina. All samples were evaluated before dilution and at hours two and four post-dilution. The samples were then cryopreserved and evaluated post thaw. Motility, structural abnormalities, and viability were assessed via microcopy. Structural damage was assessed using an electron microscope and ultrastructural damage was assessed using a transmission electron microscope (TEM). Chromatin integrity was assessed via Toluidine blue staining and sperm chromatin structure assay (SCSA). It was found that as the cooling process began, there was a significant drop in sperm motility and viability, along with an increase in abnormalities such as coiled, bent, or cracked tails and detached heads. These figures were exacerbated in the post thaw evaluation. Results of the cryopreserved samples showed that the plasma membrane was negatively affected, especially surrounding the sperm head, which seemed to be the most susceptible to damage during the cryopreservation

process. Following equilibration and freezing, approximately 50% of sperm cells incurred plasma membrane damage. There was a significant increase in mitochondrial cristae damage and chromatin damage due to DNA breaks after freezing. The conclusion of this study was that the most pronounced damage incurred by the sperm cell after cryopreservation was observed in the plasma membrane and protocols should be improved by focusing on preventing plasma membrane damage.

Effects of oxidative stress on reproduction and male fertility

Males with lower fertility tend to have lower total antioxidant capacity (TAC) than males that are fertile. Having a lower TAC leads to an increased amount of ROS and lipid peroxidation of the plasma membrane, ultimately decreasing the viability of the semen sample (Barranco et al., 2015). Antioxidants are natural compounds that scavenge free radicals, neutralizing their effects and halting chain reactions that lead to OS in spermatozoon (Majzoub et al., 2018). Antioxidants are classified as either enzymatic or non-enzymatic reducing agents. The most significant enzymatic agents in male reproduction are superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX). SOD hunts for superoxide anion $\cdot O_2$- and speeds up its conversion to hydrogen peroxide. Catalase transforms hydrogen peroxide into water and molecular oxygen. GPX uses glutathione to donate electrons to catalyze hydrogen peroxide and superoxide anion $\cdot O_2$-. The major nonenzymatic agents are vitamin E and C. Vitamin E is a fat-soluble antioxidant that prevents the production of ROS when fat undergoes oxidation. Vitamin C is a water-soluble antioxidant that donates its electrons to ROS to neutralize them (Henkel et al., 2018). Sperm cells have limited antioxidant defenses leaving the seminal plasma (SP) as the main source of antioxidants to keep ROS levels within physiological range (Barranco et al., 2015).

The SP has both enzymatic and non-enzymatic antioxidant components that help protect sperm cells from OS. These antioxidant components together determine seminal plasma's TAC. The enzymatic system is naturally occurring in sperm cells and SP and originates from the prostate. The non-enzymatic system is composed of compounds that are consumed in a male's diet (Majzoub et al., 2018). The determination of TAC levels has proven to be relevant in humans, bulls, and stallions for fertility assessment because low levels of TAC have been associated with male infertility and abnormal semen parameters. TAC may be used as a specific biomarker for assessing OS in sperm cells. TAC is a measurement of Superoxide dismutase, Catalase, Glutathione peroxidase, Vitamin-C (ascorbic acid), Vitamin-E (tocopherol), and Zinc levels in seminal plasma. TAC plays a major role in the viability of cryopreserved semen. Semen samples with a higher TAC levels undergo less OS during the cryopreservation process, resulting in better sperm recovery as measured in terms of viable, motile spermatozoa with intact membranes evaluated post-thaw. TAC should be considered as a prospective biomarker of fertility in males.

The SP is a multifaceted fluid that consists of inorganic ions, sugars, organic salts, lipids, enzymes, prostaglandins, and proteins that are secreted from the seminal vesicles, prostate, bulbourethral glands, seminiferous tubule lumen, epididymis, and vas deferens. The main function of SP is to serve as a medium to transport, protect, and nourish spermatozoa after ejaculation to fertilization and as a modulator of sperm function. The SP contact at the site of conception helps activate the endometrial gene expression and immune cell alterations that are essential for implantation, which greatly influences the quality of pregnancy and embryo development (Camargo et al., 2018). Proteins are a major component of SP and have been shown

to impact sperm cell maturation, plasma membrane stabilization, and capacitation. These proteins also interact with the oviduct and oocyte (Druart et al., 2013).

The SP can be both beneficial and detrimental to sperm cells. The effect that SP has on sperm cells seems to be related to the duration of contact between the sperm cells and SP. With natural breeding the sperm cells only interact with SP for a brief amount of time, whereas with cryopreservation the contact time is extended. The SP contains many proteins that participate in the regulation of cholesterol and phospholipid efflux from the plasma membrane. That is why extended exposure to SP may damage the sperm membrane. The composition of SP varies greatly among bulls and can be affected by the type of collection. It has been shown that semen samples collected using electroejaculation differ greatly in terms of quantity and quality along with the ratio of sperm to SP. Campanholi et al. (2017) investigated the effects of SP removal from semen collected via electroejaculation from Nellore bulls. A total of 38 bulls were used for this study. Each bull was collected, and the ejaculate was divided into three equal aliquots to compare three different treatment groups. The control group underwent conventional cryopreservation. The centrifugation group had the SP removed using centrifugation. The filtration group had the SP removed using filtration. The semen was collected and immediately cryopreserved after processing. Two straws per bull were evaluated for sperm kinetics using CASA, plasma and acrosome membrane integrity, mitochondrial membrane potential, and OS with flow cytometry. Researchers also assessed *in vitro* embryo development, but only utilized 31 randomly selected bulls. It was found that the average path velocity (VAP) and the straight-line velocity (VSL) were higher in the centrifugation group (84.35 μm/s; 71.06 μm/s) and the filtration group (86.00 μm/s; 74.15 μm/s) without SP in comparison with the control (79.65 μm/s; 66.97 μm/s). The beat cross frequency (BCF), straightness percentage (STR), and linearity

percentage (LIN) were higher in the filtration group (33.80 Hz; 86.16%; 55.54%) compared to the control (32.2 Hz; 84.32%; 52.70%) and centrifugation groups (31.96 Hz; 84.48%; 53.40%). Results also showed that the percentage of sperm cells with damage to both the plasma and acrosome membrane was much lower in the control group (30.61%) than the centrifugation (38.49%) and filtration groups (42.20%). While the percent of sperm cells with both membranes intact was lowest in the filtration group (18.04%), the centrifugation group had the lowest percentage of sperm with plasma membrane damage (38.49%). No differences were found in mitochondrial membrane potential between groups. Embryo development rates to the blastocyst and hatched blastocyst stages were higher in the control (31.30% and 21.22%, respectively) and filtration groups (32.30% and 24.00%, respectively) compared to the centrifugation group (26.55% and 18.83%, respectively). In conclusion, filtration of pre-frozen semen was found to be an effective alternative to centrifugation in terms of freezability and embryo production.

The freezing and thawing processes associated with cryopreservation have detrimental effects on sperm cells due to the rapid change in temperature, exposure to osmotic stress, and the formation of ice crystals within the cell. These changes contribute to alterations to the plasma membrane, acrosome, mitochondria, ROS synthesis, and the reduction of motility. Gürler et al. (2015) examined the relationship between the alterations that take place within spermatozoa induced by cryopreservation. For this study, six Simmental bulls around six years of age were utilized. Four ejaculates were collected via an artificial vagina from each bull, diluted, and then, equally divided into two aliquots. One aliquot was immediately cooled to 4ºC and kept at this temperature for twenty-four hours before being frozen. The other aliquot was diluted using Tyrode's medium and kept at 37ºC. The samples were analyzed for percentage of progressive motile sperm (PMS) with CASA. Seven flow cytometry assays were used to analyze the samples

for plasma and acrosomal membrane integrity, mitochondrial membrane potential, ROS synthesis, and DNA fragmentation. The sperm samples were evaluated at the following time periods: immediately after thawing, hour three, hour six, hour twelve, and hour twenty-four. The other aliquot was evaluated at the following time periods: immediately after dilution, hour three, hour six, hour twelve, and hour twenty-four. The results showed that cryopreservation adversely affected all sperm parameters (PMS, plasma and acrosomal membrane intactness, percentage of DNA fragmentation, ROS content, and mitochondrial membrane potential) in comparison to the non-frozen sample at every time interval. It was concluded that the changes in DNA integrity between the two treatment groups seemed to be correlated to the synthesis of H_2O_2, but not viability or the synthesis of other ROS.

Contemporary methods for the study of oxidative stress in sperm

The OS in sperm cells can be measured using both direct and indirect methods in the laboratory. Direct techniques measure free radicals, ROS and reactive nitrogen species (RNS). Indirect laboratory techniques measure lipid peroxidation, antioxidants, cofactors, and DNA damage within sperm cells (Agarwal et al., 2018).

Chemiluminescence assay can be used to directly measure the ROS in a semen sample. Two of the common probes used for this assay are luminol and lucigenin. Luminol measures both intracellular and extracellular ROS since it is not charged and can permeate the plasma membrane. Lucigenin only measures extracellular ROS because it is positively charged and cannot permeate the plasma membrane. Chemiluminescence is the light that is emitted when a chemical reaction takes place. When luminol is used for the assay, it becomes oxidized and leads to the formation of a free radical that reacts with ROS to generate an unstable endoperoxide that decomposes when electronically excited and releases a photon. When lucigenin is used for the

assay, it is first reduced to a cation radical that reacts with ROS to produce dioxetane, which then breaks down to produce a photon. The light emitted can be used as a direct measure of free radicals produced. This technique is highly sensitive and specific, but it is time-consuming and requires expensive equipment (Henkel et al., 2018).

For the chemiluminescence assay, the semen sample is collected, and test tubes are labeled blank [400 µL Phosphate-buffered saline (PBS)], negative control (400 µL PBS and 10 µL luminol), test sample (400 µL semen and 10 µL luminol), and positive control (400 µL PBS, 10 µL luminol, and 50 µL H_2O_2). Next, the luminometer is set up by entering initial data such as bull name and sample size. Right after adding the probes to the test tubes, the readings are started. The reading time is 15 to 20 minutes. (Pande et al., 2017).

Nitroblue Tetrazolium Test is a straightforward technique that provides data on intracellular oxidative balance and neutrophil activity. The assay uses nitroblue tetrazolium (NBT), a yellow nitroditetrazolium chloride that is an electron acceptor, to target cells to take up NBT into their cytoplasm where it is converted by superoxide radicals to a purple-blue color insoluble formazan crystals. This assay measures the intracellular ROS-generating ability, allowing the identification cellular ROS (Henkel et al., 2018). This test quantifies the formation of superoxide radicals within the cell membrane of the sperm cell by formazan formation.

The NBT test is ran by dissolving NBT salt into PBS that contains Dimethyl sulfoxide (DMSO), and then, adding to sperm cells, and incubating for an hour. After incubation, the sperm cells are washed with PBS and centrifuged for 8 minutes. The sperm cells are then resuspended in DMSO that contains Potassium hydroxide (KOH) and placed in a microplate to be read by an ELISA reader. This test is user friendly, relatively inexpensive to run, and highly

sensitive. The formation of formazan within the cells shows that the cell has undergone OS (Pande et al., 2017).

The colorimetric assay is dependent on the ability of antioxidants in the seminal plasma to impede the oxidation of 2, 20-Azino-di-[3- ethylbenzthiazoline sulphonate] (ABTS) to ABTS·+. The antioxidants present in the sample suppress the absorbance of the induced reaction to a level equal to their concentration. A comparison is made between the ability of the antioxidants in the seminal plasma verses the standard (Trolox) to prevent the oxidation of ABST (Henkel et al., 2018). This assay measures all antioxidant activity of the SP sample such as vitamins, minerals, proteins, and lipids.

This assay is used on SP that has been defrosted and centrifuged. The clear SP is removed and diluted with assay buffer in separate tubes. Seven standard tubes are prepared with Trolox and assay buffer. Then, 10 µL of Trolox or SP sample is added to 10 µL of metmyoglobin and 150 µL of chromogen per well of the plate. This should be done in duplicates. The reaction is induced by adding 40 µL of hydrogen peroxide. The plate is then covered and placed on a shaker for 5 minutes. Next, the cover is removed and the absorbance of each well is read using a plate reader (Redi, 2013).

The Ferric Reducing Antioxidant Power (FRAP) test can be used to measure the antioxidant capacity of all the antioxidants in a semen sample (Pahune et al., 2013). This assay utilizes the colorless oxidized $Fe^{3}+$, which is converted to a blue-colored $Fe^{2}+$ caused by an antioxidant donating an electron (Henkel et al., 2018). The FRAP assay is analyzed by measuring the absorbance that is emitted when $Fe^{3}+$ is oxidized into $Fe^{2}+$ giving the total antioxidant capacity of the sample.

To run this assay, a standard FRAP reagent solution of 2,4,6 Tri (2-Pyridyl), 1,3,5-Triazine (TPTZ), Acetate Buffer, Hydrochloric Acid (HCL), and Ferric Chloride ($FeCl_3$) is prepared. 50 µL of Ferrous Sulphate ($FeSO_4$) is dissolved in one mL of the FRAP reagent. 50 µL of SP is added to one mL of FRAP reagent and mixed. A spectrophotometer is then used to measure the absorbance against a blank of FRAP reagent and distilled water (Pahune et al., 2013).

Oxidation-reduction potential (ORP) is a new direct measurement of the overall balance/imbalance of oxidants and antioxidants within a semen sample, thereby providing a comprehensive measure of OS. It assesses all known and unknown oxidants and antioxidants within a sample. If the ORP value is greater than the established reference values, it can be assumed that the sample has undergone OS. Two parameters are measured, static ORP (sORP) and capacity ORP (cORP). sORP is the current OS in the sample, while cORP is the estimate of antioxidant reserves within the sample (Agarwal et al., 2017).

This test is completed by using the Male Infertility Oxidative System (MiOXSYS™) or RedoxSYS. The analyzer has an ultrahigh impedance electrometer and sensor that contains three electrodes. It works by measuring the transfer of electrons from antioxidants and/or reactants to oxidants and/or ROS. ORP can be calculated using the Nernst equation. It only requires a small amount of semen (30 µl) to perform the test. Once the sample is loaded into the analyzer, it is evaluated for two minutes where the ORP will be calculated from the average of the readings from the last ten seconds of the run (Henkel et al., 2018).

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labeling (TUNEL) assay measures the level of DNA damage within a sperm cell induced by ROS. DNA damage can involve single-stranded or double-stranded breaks, additions or

deletions, along with base modifications of the DNA. This test is extremely sensitive and specific, giving an accurate prediction of embryo viability. There are many elements involved in the processing for this assay, such as the permeabilization of the DNA with the probe. This permeabilization can be impeded due to how dense and tightly packed the chromatin are within the nucleus, which, in turn, can prevent the probe from interacting with the DNA (Redi, 2013). This assay utilizes a DNA polymerase called terminal deoxynucleotidyl transferase (TdT), which adds deoxyribonucleotides to 3′ hydroxyl to the DNA strand. The 3′ hydroxyl free ends are labeled using a fluorescent label, which on passing through a flow cytometer generates fluorescence. This is directly proportional to the number of breaks in the DNA strand. The more breaks that are present in the DNA, the more of the probe is integrated into the cell (Agarwal et al., 2017).

To run this assay, cryopreserved spermatozoa are thawed and washed twice with PBS. Spermatozoa are then fixed to slides and permeabilized using 0.1% Triton X-100 containing 0.1% sodium citrate. Next, the slides are incubated in 50 µL of TUNEL reagent containing fluorescein conjugated dUTP to label the DNA breaks for an hour in a dark, humidified environment. After incubation, slides are washed with PBS three times, and then, analyzed under a fluorescence microscope for positive TUNEL stained cells (Takeda et al., 2015).

Comet assay, also known as single-cell gel electrophoresis, measures DNA strand breaks in individual spermatozoa. It is one of the most straightforward methods to measure both single and double stranded DNA breaks. This assay uses the influence of an electric field to separate the broken DNA strands within the sperm cell. After this separation takes place, the fragmented DNA will travel to the tail of the cell, while the intact DNA will remain restricted to the head of

the sperm cell forming what is called a comet's head. The larger the size of the comet indicates that less DNA fragmentation is present within the cell (Agarwal et al., 2017).

In this assay, spermatozoa are thawed, mixed with agarose, and layered onto a slide. A detergent with a high salt concentration is added to lyse the sperm cell membranes and eliminate protamines and histones. This causes the DNA structure to relax. The DNA strands are then exposed to an electrophoretic field causing the migration of the broken strands of DNA through the agarose to the tail of the sperm cell. This assay can operate in both a neutral and alkaline environment, but the neutral buffer only measures the double strand breaks, while the alkaline buffer measures both single and double stranded breaks. A fluorescent dye is used to visualize the cell under the microscope. The longer the tail the of the comet, the more DNA damage is present within the cell (Redi, 2013).

The thiobarbituric acid (TBARS) assay is used to assess the changes in Malonaldehyde (MDA), which is a reactive compound formed during lipid peroxidation. Thiobarbituric acid (TBA) and MDA react together and form the MDA-TBA adduct. The formation of this adduct can be measured colorimetrically or fluorometrically to determine lipid peroxidation (Agarwal et al., 2017). TBARS assay needs to be carried out under high temperatures and acidic environment (Redi et al., 2013).

For this assay, spermatozoa are thawed and diluted in PBS. Then, 100 µL of the spermatozoa is mixed with 200 µL of 5% trichloroacetic acid, 0.375% thiobarbituric acid, and 0.25 N HCl reagent. Once it is fully blended, the mixture is heated to 90ºC for 15 minutes to induce the reaction. The reaction is stopped by transferring the tubes to an ice-water bath for five minutes. After the tubes have cooled, they are centrifuged at 1500 x g for 15 minutes. The

supernatant is then collected and transferred to a well plate and absorbance is read on a microplate reader to measure MDA concentration (Domínguez-Rebolledo et al., 2010).

Innovative approaches for preventing oxidative stress and improving fertility

Antioxidants are the primary defense against OS in semen. Sperm cells are naturally protected by numerous kinds of antioxidants within the SP and the sperm cell itself that prevents oxidative damage. Antioxidants prevent the excessive production of free radicals or ROS by disrupting the oxidative chain reaction by donating electrons to the unstable oxygen molecules. There is an assortment of biological and chemical antioxidants that scavenge and suppress ROS being currently researched. A recent study showed that the supplementation of semen extenders with antioxidants before and after cryopreservation has been shown to provide protection to the spermatozoa and improving semen parameters post-thaw such as motility and membrane integrity (Bansal and Bilaspuri, 2011).

Vitamin E is a fat-soluble antioxidant that is located in the plasma membrane. It destroys free hydroxyl radicals and superoxide anion that, in turn, reduces lipid peroxidation of the plasma membrane of the sperm cell that is initiated by ROS. Vitamin C is a water-soluble antioxidant that naturalizes hydroxyl, superoxide and hydrogen peroxide radicals providing fortification from endogenous oxidative stress. Carnitines are water soluble antioxidants that play an important role in sperm metabolism. They scavenge superoxide anions and hydrogen peroxide radicals, which inhibits lipid peroxidation. CoQ10 is a fat-soluble antioxidant that is present throughout body and found in high levels within sperm cell mitochondria and plays a vital role in energy production in the cell. It inhibits superoxide formation, which, in turn, protects sperm cells from lipid peroxidation (Majzoub et al., 2018).

Other organic compounds have been shown to have antioxidant properties such as some amino acids and certain trace minerals. For example, the amino acid N-acetyl cysteine (NAC) assists with preventing OS by scavenging for hydroxyl radicals and hypochlorous acid and neutralizing -+with NAC. The trace mineral zinc plays an essential role in the metabolism of DNA and RNA. Its antioxidant properties inhibit the production of hydrogen peroxide and hydroxyl radicals protecting the sperm cell structure (Majzoub et al., 2018).

Reducing the concentration of sperm cells in each insemination dose of semen and the removal of leukocytes, which are major sources of ROS production, before cryopreservation have also been investigated as a way of preventing OS. Murphy et al. (2013) found that decreasing the number of sperm cells per insemination dose below the average of five million cells had a favorable effect on the post-thaw viability of the spermatozoa. The fewer immature or dead spermatozoa present in a sample decreased the amount of ROS being produced by such cells. Using sperm separation techniques like glass-wool filtration and density centrifugation gradient will remove leukocytes from the sample eliminating a major production sites for ROS (Agarwal et al., 2005).

Arachidonate 15-lipoxygenase (Alox15), a member of the non-heme iron-containing lipoxygenase enzyme, is believed to play a central role in the maintenance of the redox status of sperm cells, which is an important factor of male fertility. Walters et al. (2018) hypothesized that the selective inhibition of Alox15 could reduce OS levels in mature human spermatozoa. To test the impact of Alox15 in the oxidative stress cascade, semen samples were incubated with an Alox15 inhibitor, PD146176, and H_2O_2 and the production of mitochondrial ROS was then measured. Exposure to PD146176 during the H_2O_2 challenge led to a significant reduction in

mitochondrial ROS production. The experimental evidence confirmed that the inhibition of Alox15 was effective in reducing damage caused by OS.

Despite considerable amounts of research, recovery of viable post-thawed spermatozoa has been poor and differs greatly among males. Molecular and cellular mechanisms underlying cryopreservation and oxidative stress are not well-studied. These gaps in the knowledge base translate into a loss of millions of dollars of economic impact for livestock industries. Thus, there is an urgent need for developing reliable markers to evaluate semen and predict bull fertility and improve freezability rates or post-thaw viability of spermatozoa.

CHAPTER II

THE MOLECULAR AND CELLULAR DYNAMICS OF HOLSTEIN BULL SPERMATOZOA IS ASSOCIATED WITH CRYOTOLERANCE

Introduction

Reproductive efficiency is an indispensable determinate of the profitability and sustainability of both the dairy and beef industries. The male influence on reproductive efficiency is often overlooked, even though it is a major contributor to the overall success of a breeding program. The cattle industry relies heavily upon service sire conception rate (SCR) data to calculate a standardized non-return rate for each bull used for artificial insemination (AI). This, in turn, eliminates the naturally occurring variation in fertility among bulls and leads producers to only select for female fertility. Male fertility is defined as the ability of the sperm cell to fertilize the oocyte and maintain healthy embryonic development that produces the birth of a healthy calf. There are a number of factors impacting the fertility of bulls, including the volume, morphology, motility, and physical normality of the semen sample (Whittier, 2000). Genomic selection and the use of AI has reduced the generation interval and increased the rate of genetic enhancement of important production traits within the dairy industry (Taylor et al., 2018).The spermatozoon is composed of several membrane-bound sections, consisting of the plasma membrane, acrosome membrane, and mitochondrial membrane, that must be intact to ensure the viability of the spermatozoa to fertilize an oocyte. Any damage incurred by these

membranes is detrimental to the sperm cell health, freezability, and fertilizing ability (Graham et al., 1990), and thus, understanding this damage is a crucial step in improvement of fertility.

Morphology is an important spermatic trait that has been widely accepted as a vital determinant of sperm quality. In 1990, Menkveld et al. endeavored to provide a scientific definition for a morphologically normal spermatozoon. The head of a normal spermatozoon, according to this definition, must be a smooth oval shape with a well-defined acrosome covering 40-70% of the spermatozoon head. The head length should be three to five μM and a width between two to three μM. The mid-piece should be axially attached to the head and approximately one- and one-half times the length of the head. The cytoplasmic droplet may be no larger than half of the sperm head and the tail must be uniform, slightly shorter than the mid-piece, uncoiled, and approximately 45 μM in length. Over the years, numerous studies have shown that any abnormalities to the structure of the sperm cell will assist with predicating infertility in males (Barth et al., 1989; Parkinson et al., 2004; Kastelic et al., 2008).

Sperm motility and morphology are intricately linked because any abnormalities will cause sperm to swim slower and less effectively (Katz et al., 1982). Motility is crucial for sperm transportation within the female reproductive tract and oocyte penetration. The numbers of spermatozoa that show forward progressive motility and are able to navigate the barriers of the female has been proven to be positively associated with fertility and freezability (DeJarnette et al., 1992; Nadir et al., 1993). Sperm samples are heterogenous, meaning that sperm cells from the same ejaculate have different motility within which subpopulations coexist. The concept of sperm heterogeneity has brought around the development of computerized methods for evaluating sperm kinematics. Computer Assisted Sperm Analyses (CASA) is one of the most popular methods. A study by Muino et al. (2007) revealed that sperm cells with the highest

velocity and progressive motion were positively correlated with their resilience post-cryopreservation, indicating the importance of motility to determining fertility.

For producers to use their bull of choice in an AI program, spermatozoa from these bulls must be cryopreserved. This is the process of preserving the biological function of living cells by freezing and storing the organic material below -80ºC with liquid nitrogen. The process of cryopreservation involves extension, temperature reduction, addition of cryoprotectants, and freezing and thawing of sperm cells (Medeiros et al., 2002). The rapid change in temperature alters the physical characteristics of the sperm plasma membrane. The addition of cryoprotectants causes a considerable change in the volume of the cells and requires a quick recovery. The freezing process further alters the structure of the plasma membrane by forming water crystals within the cell, which causes physical damage and the loss of the acrosomal cap. Spermatozoa are not designed to withstand rapid changes in temperature and experience cold shock during freezing. This causes disruption and rearrangement of membrane constituents, resulting in loss of plasma membrane integrity (Khalil et al., 2018). The thawing process requires the sperm cell to rapidly recover, rehydrate, and expand back into its normal shape in a brief timeframe, resulting in alteration of membrane function (Hammerstedt et al., 1990).

One way that sperm cells can become damaged and negatively influence the fertility of a bull is through oxidative stress (OS; Aitken et al., 2015), which is defined as the imbalance between oxidants produced by the spermatozoa, immune cells, and exogenous sources compared to the antioxidants present within the sperm and seminal plasma to protect the integrity of these cells. All cells that are actively respiring generate reactive oxygen species (ROS). These ROS molecules result from electron leakage from the mitochondrial electron transport chain. ROS are also generated by the presence of dead spermatozoa by way of an aromatic amino acid oxidase

catalyzed reaction along with the contributions of leukocytes and immature spermatozoa (Bansal et al., 2009). Small quantities of ROS are needed by spermatozoa during the fertilization process, including to initiate capacitation, acrosome reaction, hyperactivation, and binding to the oocyte. However, high levels of ROS can overwhelm the antioxidant system and cause detrimental damage to the spermatozoa through OS. When oxidants outnumber the antioxidants, protein damage, lipid peroxidation, DNA fragmentation, and apoptosis will occur (Makker et al., 2009). OS-induced damage negatively impacts the viability of spermatozoa via plasma membrane damage, which in turn reduces the fertility of the male (Sariözkan et al., 2009). The fluctuations in temperature and dehydration that a sperm cell undergoes during the cryopreservation process causes lateral-phase separation of the lipids present in the plasma membrane and reordering of its constituents, along with the loss of cholesterol and polyunsaturated fatty acids. These changes can modify the plasma membrane surface, thereby, altering the ability of water, cryoprotectants, and ions to cross this membrane (Leahy et al., 2009)

While research has improved cryopreservation over the years, today's producers are commonly facing post-thaw viability that is less than 50% (Ballester et al., 2007). Thus, objective of this study was to uncover molecular and cellular physiognomies in spermatozoa from Holstein bulls with Good (11 bulls) and Poor (5 bulls) cryotolerance. The central hypothesis was that the molecular and cellular dynamics of bull spermatozoa are associated with sperm cryotolerance. For this study we measured molecular and cellular attributes in sperm samples from bulls with reliable sperm freezability data using a systems biology approach. The rationale behind this research was that with a more complete understanding of the molecular and cellular phenotypes in sperm cells, we will be able to improve the viability and freezability of cryopreserved spermatozoa.

Material and Methods

Determination of sperm freezability and sample processing

Alta Genetics (Watertown, WI, USA) provided cryopreserved sperm samples that were collected from 16 mature Holstein bulls. All bulls were housed and fed identically during the collection period. Bulls had contrasting freezability phenotypes based upon post-thaw viability with 11 bulls categorized as having good freezability (average post-thaw viability 62.16%; Good) and 5 bulls with poor freezability (average post-thaw viability 52.59%; Poor). Sperm collection was done using an artificial vagina after false mounting of a teaser animal. The spermatozoa were separated from seminal plasma by centrifugation (700 g, 4°C, 10 min). Then, the pellet of spermatozoa was washed twice (700 g; 4°C; 15 min.) with cold phosphate-buffered saline (PBS). Following the second centrifugation, spermatozoa were snap-frozen in liquid nitrogen, as reported by Velho et al (2018). A total of five straws from each bull were used to conduct the following experiments.

Computer Assisted Sperm Analyses (CASA)

CASA was used to evaluate sperm cell motility and kinetic parameters. Frozen sperm cells were thawed in a 37ºC water bath for 30 sec, transferred to a 1.5 mL tube containing 1 mL of PBS, and centrifuged at 3700 rpm for 10 min. The supernatant was aspirated, and this process was repeated 3 times. Sperm cells were diluted with PBS to a concentration of $20x10^6$ and gently agitated for 1 min. Five µL of each sample was loaded into a prewarmed chamber slide and 400 spermatozoa were evaluated per chamber immediately to prevent evaporation. A total of 12 parameters were assessed according to Ibanescu et al. (2016). These parameters included the following: total motility (TM), progressive motility (PM), linearity (LN), straightness (ST), wobble (WB), curvilinear velocity (VCL), straight line velocity (VSL), beat cross frequency

(BCF), average path velocity (VAP), amplitude of lateral head displacement (ALH), distance average path (DAP), distance straight line (DSL), and distance curved line (DCL).

Hypoosmotic Swelling (HOS) Test

The HOS test was used to analyze the membrane integrity of the sperm cells according to the previously reported method by Gadkar et al. (2002). In a 1.5 mL tube, 30 µl of 90% Percoll was layered under 200 µl of 45% Percoll. Sperm cells were thawed in a 37ºC water bath for 30 sec and 500 µl of sperm cells were layered onto the Percoll gradient and centrifuged at 3700 rpm for 10 min. The supernatant was aspirated, and 1 mL of PBS was added to the tube and centrifuged at 3700 rpm for 10 min. This process was repeated twice. The sperm pellet was resuspended in 250 µl of PBS. Fifty µl of the sperm sample was transferred into 450 µl of HOS test solution (150 mOsm/kg pre-equilibrated at 37oC for 1 hr.) and gently mixed by hand. The mixture was then incubated in a 37oC water bath for 30 min upon when 10 µl of sample were transferred onto a clean glass slide and spread using a coverslip. Each slide was evaluated for HOS-positive (presence of coiled tail) or HOS-negative (absence of coiled tail) sperm by counting a total of 200 spermatozoa/sample using 40 x objective of a phase-contrast microscope.

Eosin-Nigrosin Staining

Eosin-Nigrosin staining was used to assess the viability of sperm cells according to the method reported by Dogan et al (2013). Frozen sperm cells were thawed in a 37ºC water bath for 30 sec, transferred to a 1.5 mL tube that contained 1 mL of PBS, and centrifuged at 3700 rpm for 10 min. The supernatant was aspirated, and this process was repeated 3 times. One mL of pre-equilibrated PBS was added to the tube and gently agitated by hand. 10 µl of sperm suspension and 10 µl of eosin-nigrosin solution (0.67 g eosin Y and 0.9 g of sodium chloride in 100 mL of

distilled water) was transferred into another tube and gently mixed together by hand. 10 µL of this solution was smeared on a glass slide using a coverslip and air dried for evaluation of 200 sperm cells per slide via light microcopy.

HaloSperm experiment

The Halosperm G2 test kit (Halotech DNA, SL San Diego, CA) is an in vitro diagnostic kit that measures DNA fragmentation in sperm cells. The experiment was carried out according to the manufacturer's instructions. An agarose screw tube (ACS) was melted using a 100ºC water bath for 5 min. One hundred µL of the melted agarose was transferred into a 1.5 mL tube. Sperm cells were thawed in a 37ºC water bath for 30 sec, transferred to a 1.5 mL tube that contained 1 mL of PBS, and centrifuged at 3700 rpm for 10 min. The supernatant was removed, and 1 mL of pre-equilibrated PBS was added to the tube and mixed gently by hand. Fifty mL of the sperm sample was transferred to an Eppendorf tube containing 100 µL of the agarose and mixed together with a micropipette. Eight µL of the cell suspension was placed in the center of a sample well and covered with a coverslip. Next, slides were placed on a plate precooled to 4ºC, and then, put into the fridge for 5 min. to solidify the agarose. Slides were kept in a horizontal position throughout the entire process. Solution 2 (LS) was applied until the sample well was fully immersed and incubated for 20 min., and then, washed with distilled water. The slides were dehydrated by flooding slides with 70% ethanol and incubating for 2 min. The 70% ethanol was drained off and 100% ethanol was applied for 2 min. Excess ethanol was drained off slides and slides were allowed to air dry horizontally on filter paper. Slides were then transferred into a Petri dish and Solution 3 (SSA) was applied until sample well was completely immersed, incubated for 7 min. and then, the excess stain was drained off. Solution 4 (SSB) was then applied until sample wells were fully immersed, incubated for another 7 min., and then, the

excess stain was drained off. Slides were dried at room temperature, and then, evaluated under bright field microscopy, counting 300 cells per slide.

Trolox Equivalent Antioxidant Capacity (TEAC) Assay

The TEAC experiment was conducted according to the method reported by Roychoudhury et al. (2016) to measure the total antioxidant capacity of sperm cells utilizing the Total Antioxidant Capacity (TAC) Colorimetric Assay kit (Cat # 709001; Cayman Chemical, Ann Arbor, Michigan). Frozen sperm cells were thawed in a 37ºC water bath for 30 sec, transferred to a 1.5 mL tube containing 1 mL of PBS, and centrifuged at 3700 rpm for 10 min. The supernatant was aspirated, and this was repeated 3 times. One mL of pre-equilibrated PBS was added to the tube and gently agitated. 0, 4, 8, 12, 16, 20 µl of the Trolox standard were added to individual wells of the plate. Fifty µL of samples were added to individual wells. One hundred µL of Cu^{2+} working solution was added to all wells on the plate. The plate was then covered and incubated at room temperature for 90 min. Following the incubation, the absorbance was read at 570 nm using a microplate reader.

Mitochondrial Function Assessment by Flow Cytometry

Following the method reported by Baumber et al. (2000), flow cytometry was employed to assess the mitochondrial function of the sperm cells using JC-1 staining. Frozen sperm cells were thawed in a 37ºC water bath for 30 sec. Spermatozoa were layered onto a 45% and 90% Percoll gradient in a 1.5 mL tube and centrifuged at 3700 rpm for 10 min., and then, the supernatant was aspirated. The pellet was washed and resuspended with 300 µL of PBS. One and half µM of JC-1 were added to the sample and incubated in the dark for 15 min. After the incubation period the samples were centrifuged for 10 min. at 6,000 g and the supernatant

removed. The pellet was then resuspended with 300 µL of PBS and analyzed using the flow cytometer.

Statistical Analysis

Sixteen Holstein bulls (n = 11 Good, n = 5 Poor) were used for statistical analysis. Principal component analysis (PCA) as Jolliffe et al. (2016) described was used to reduce sperm population variables (POP). The number of live cells, cells with intact membranes, cells without fragmented DNA, total motility, progressive motility, linearity (LIN), straightness (STR), wobble (WOB), and post-thaw viability (PTV) were divided into 2 principal components (POP1 and POP2), while preserving total variance in the data. The correlation coefficients of POP variables with POP1 and POP2 were used to map these variables into a biplot. The principal component analysis was also used to reduce sperm cell characteristic variables (CELL), including curvilinear velocity (VCL µm/s), straight line velocity (VSL µm/s), beat cross frequency (BCF Hz), average path velocity (VAP µm/s), amplitude of lateral head (ALH µm), distance average path (DAP µm), distance straight line (DSL µm), and distance curved line (DCL µm) into two principal components CELL1 and CELL2, while preserving the total variance within the data. The correlation coefficients of CELL variables with CELL1 and CELL2 were used to map these variables in another biplot. The scores of bulls based on these two PCAs were used to map the bulls onto both biplots. Additionally, correlation coefficients of TAC with the scores were determined by the CORR procedure (SAS version 9.4; SAS Inst. Inc., Cary, NC) and were used to map TAC variable on both biplots. The CORR procedure was also used to determine Spearman's correlation coefficients between the POP and CELL variables. Univariate analysis of variance was performed by the GLIMMIX procedure of SAS 9.4 with freezability phenotype being the fixed effect in a generalized linear mixed model. The degree of

freedom was estimated by the Kenward–Roger approximation method and means were separated by a protected t-test. Actual probability values were reported with statistical comparisons ($\alpha \leq 0.05$).

Results

Sperm population dynamics

Principal component analysis designated that the total variance of sperm population characteristics was largely explained by two principal components, POP1 (60.6% of total variance) and POP2 (39.4% of total variance; Figure 2). Factor pattern analysis indicated a strong correlation between POP1 and the number of live cells (LC; $r = 0.70$; $P = 0.002$), LIN ($r = 0.91$; $P < 0.001$), STR ($r = 0.87$; $P < 0.001$), and WOB ($r = 0.87$; $P < 0.001$); whereas POP2 was correlated with cells with non-fragmented DNA (NF; $r = -0.57$; $P = 0.020$), total motility (MT; $r = 0.91$; $P < 0.001$), progressive motility (PM; $r = 0.94$; $P < 0.001$; Figure 2). The PCA scores of individual bulls on the population characteristic biplot (Figure 2) showed that there was slight separation between Good and Poor groups; however, there was an overlap between the two populations. The Good bulls clustered within the proximity of the center of the biplot in quadrant II and III with two bulls in quadrant IV. The Poor bulls did not cluster well, locating in quadrant I and II. The Good bulls were in the close proximity of IM, NF, and PTV and scored positively by POP1 variables (ST, LN, and WB); whereas the Poor bulls were in the close proximity of TM, PM, and PTV. One Poor bull was scored negatively by POP1 variables. Univariate analysis revealed that the Good bulls had 9% greater IM ($P = 0.029$), 20% greater NF ($P = 0.018$), and 10% greater PTV ($P < 0.001$).

Sperm cellular characteristics

The total variance of sperm cellular characteristics was also explained by two principal components, CELL1 (72.9%) and CELL2 (27.1%; Figure 3). Factor loadings on the biplot revealed a strong correlation between CELL1 and VCL ($r = 0.92$; $P < 0.0001$), VSL ($r = 0.94$; $P < 0.0001$), VAP ($r = 0.98$; $P < 0.0001$), DAP ($r = 0.95$; $P < 0.0001$), DSL ($r = 0.76$; $P = 0.001$), and DCL ($r = 0.73$; $P = 0.001$); whereas, CELL2 was correlated with BCF ($r = 0.82$; $P < 0.001$) and ALH ($r = -0.80$; $P < 0.001$; Figure 3). The PCA scores of each bull on the cellular characteristic biplot displayed a slight partition between the Good and Poor groups, although there was an overlap among the two groups. The Good bulls clustered into quadrants I, II, and III (Figure 3). The Poor bulls did not cluster very well, localizing into quadrants I and IV. The majority of the Good bulls were in close proximity and scored positively with CELL1 variables (DSL, VSL, VAP, and DAP); whereas, the Poor bulls were not in close proximity to any of the sperm cellular characteristic variables. Four of five Poor bulls were negatively scored by the CELL1 variables. The univariate analysis showed that spermatozoa from Good bulls was faster for VSL (8.17 μm/s; P = 0.017) and VAP (7.33 μm/s; P = 0.036) compared to spermatozoa from Poor bulls. In addition, spermatozoa from Good bulls traveled 4.21 μm further as measured by DAP ($P = 0.006$) and 5.29 μm further as measured by DSL ($P = 0.011$) compared to spermatozoa from Poor bulls.

Total antioxidant capacity

The TAC was in close proximity to the origin of both POP and CELL biplots (Figure 2 and 2), but not in close proximity to any sperm population or cellular characteristics. This indicated that TAC is not correlated with POP1 and POP2 or CELL1 and CELL2. Univariate

analysis of variance indicated that TAC was similar between Good (0.182 nm) and Poor (0.260 nm) groups.

Discussion and Conclusions

Male fertility is highly dependent upon the success of a spermatozoon fertilizing an oocyte that results in pregnancy with the ultimate birth of a viable offspring. There are many deterrents to this process that can reduce the chance of a viable offspring. For example, oxygen is needed for the aerobic metabolism of spermatogenic cells but can lead to the production of ROS that in high amounts can be damaging to spermatozoa. Sperm cells are exceedingly susceptible to ROS-induced harm during some of the critical phases of spermiogenesis. The stage of sperm chromatin condensation when sperm cells do not have DNA repair mechanisms, during passage through the epididymis, sperm cells produce ROS and spend extended periods of time secluded in the reproductive tracts of both the male and female (Sabeti et al., 2016). Therefore, we hypothesized that bulls with good cryotolerance would have a higher TAC and better cellular attributes in comparison to the poor cryotolerance bulls. To test our hypothesis, we carried out a study evaluating sperm kinematics, viability, plasma membrane and DNA integrity, TAC, and mitochondrial membrane potential.

In our study, of the CELL parameters analyzed, sperm from Good freezability bulls were significantly higher in the VSL and VAP versus sperm from Poor bulls, showing that these sperm travel at quicker speeds. There was also an increase in the DAP and the DSL for spermatozoa from Good bulls compared to Poor bulls (Table 2). These results confirm that bulls with good freezability have a higher number of spermatozoa that traveled longer distances and at swifter speeds post-thaw in comparison to the bulls with poor freezability. Motility is one of the most crucial sperm characteristics linked to the fertility of spermatozoa signifying their viability

and membrane integrity. Motility is essential for successful sperm transport and fertilization *in vivo* and *in vitro*. Our results found to be in accordance with Nagy et al. (2015) and Bollwein & Bittner (2018). The higher velocity can in part be attributed to the higher number of cells with intact plasma membranes in the Good bulls, which ensures the viability of the cell.

Among the POP parameters measured, membrane and DNA integrity, along with post-thaw viability, differed among the bulls with different cryotolerance and showed a significant relationship with sperm cryo-survival (Table 1). The Good bulls had a higher average percentage of sperm cells with intact plasma membranes and non-fragmented DNA. Furthermore, sperm membrane is composed of a large amount of polyunsaturated fatty acids, which are extremely susceptible to OS damage. Lipid peroxidation is a pathological process that affects the organization and function of the membrane, changing its fluidity. When sperm cells are cryopreserved, they undergo thermal stress, which results in protein denaturation, shrinkage, and collapse of the membrane, gravely damaging the viability of the spermatozoon (Kasimanickam et al., 2007; Khalil et al., 2018). Additionally, DNA damage can severely affect sperm cell viability. DNA fragmentation can occur due to many factors, but most concerning is OS. The oxidization of 8 hydroxy2deoxyguanosine (8OHdG) can weaken *de novo* methylase function, which, in turn, results in hypomethylation, compromising DNA stability. Not only does DNA integrity affect the reproductive health of the male, but it also reduces the possibility of a viable pregnancy by the oxidation of guanine, thus, impairing DNA replication and transcription (Dada, 2017).

The ROS are known to result in significant DNA fragmentation to both nuclear and mitochondrial genome of spermatozoa (Aitken et al., 2010). This fragmentation is brought about when ROS attack DNA bases and phosphodiester backbones, which destabilizes the molecule. It

has been proposed that sperm DNA is primarily damaged by mitochondrial ROS deriving from defective or immature spermatozoa. (Gharagozloo et. al., 2011) Sperm DNA fragmentation was indicative of low AI success in bulls (Karoui et al., 2012). DNA fragmentation was significantly higher in the Poor bull group in our study. Thus, we propose that assessment of sperm DNA, in addition to conventional semen analysis, may offer additional insight into identifying poor cryotolerance bulls. Our conclusions are supported by multiple studies where DNA fragmentation is much higher in infertile males compared to fertile males (Saleh et al., 2003; Alkhayal et al., 2013; Oleszczuk et al., 2013).

Both spermatozoa and seminal plasma contain an antioxidant system to protect against OS, but due to the small size of spermatozoa, their antioxidant capacity is very limited. The previous reports on TAC of semen are contradictory. Studies conducted by Smith et al. (1996) and Lewis et al. (1995, 1997) revealed that infertile men demonstrated a lower TAC than fertile men and lower levels of individual antioxidants. Siciliano et al. (2001) showed that the TAC did not differ among fertile and infertile men. Similarly, the TAC levels in our study did not differ significantly among the Good and Poor bulls. During the cryopreservation process the naturally occurring antioxidants lose their strength. The relationship between TAC and cryotolerance is highly variable partly due to the varying amount of antioxidants in commercial semen extenders used for cryopreservation (Numan et al., 2010). Thus, these inconsistencies between studies show that TAC alone may not be used to predict freezability phenotype of bulls. Instead, prediction of freezability phenotype of bulls needs along with TAC at least one other independent variable that is more correlated with POP1 and CELL1 to better predict freezability of bull spermatozoa.

In conclusion, the comprehensive assessment of varying sperm functions and the subsequent analysis of these functions indicated that semen from bulls with good cryotolerance differed in post-thaw viability, plasma membrane, DNA integrity, VSL, VAP, DAP, and DSL in comparison to the bulls with poor cryotolerance. The PCA also indicated that spermatozoa from Good bulls was strongly correlated with a higher percentage of cells with intact plasma membrane and DNA, and post-thaw viability, along with higher levels of certain sperm kinematic parameters (VSL, VAP, DAP, and DSL) compared to spermatozoa from Poor bulls. There is no one specific sperm function variable alone that can accurately predict cryotolerance of bull spermatozoa, and thus, a combination of sperm cell attributes and kinematics needs to be utilized by the AI industry in differentiating between freezability of spermatozoa between bulls.

CHAPTER III

CONCLUSIONS AND FUTURE DIRECTIONS FOR ADVANCEMENT IN BULL FERTILITY AND SPERM CRYOPRESERVATION

Bull fertility is highly variable and can be influenced by a number of different factors. Differences in fertility levels among bulls classified as fertile have been demonstrated (Saacke et al, 1980; DeJarnetteet al, 1992), though the causes of these differences are still unclear. Low breeding pressure and multiple sire breeding programs can delay the identification of sub-fertile bulls. On the other hand, the use of artificial insemination (AI) and single-sire breeding programs can promote sub-fertile bull identification. Nevertheless, the increased uses of AI in the dairy and beef industries have amplified the importance of predicting bull fertility and freezability of spermatozoa. Cryopreserved spermatozoa have been widely used for AI. However, the freezing and thawing processes have been proven to be damaging to the ultrastructure of the sperm cell and promote the excess generation of reactive oxygen species (ROS), leading to oxidative stress (OS). Sperm cells are especially susceptible to OS because they have minimal cytoplasmic defenses due to their small size (Saleh and Agarwal, 2002). In addition, sperm plasma membranes contain large amounts of polyunsaturated fatty acids, which are vulnerable to attack by ROS, causing lipid peroxidation (Zalata et al, 2004). In the presence of ROS, DNA can also be damaged causing deletions or mutations in the genome (Tominaga et al, 2004). Thus, these damaging effects of OS can have a significant financial impact on breeding programs in the dairy and beef industries.

The use of OS analysis brings male reproduction and cryotolerance of spermatozoa to the forefront of new advancements in biomedical and agricultural fields. This area of study is essential for the continued growth and development of AI technologies to enhance human and animal reproductive technologies and abilities. The evaluation of OS status is imperative to the research of fertility and sperm cell cryotolerance at the molecular level to improve post-thaw viability and conception rates. In this study, it was ascertained that sperm kinematics can aid in the estimation of sperm viability poth-thaw, which could impact fertility.

One of the most logical places to begin in ways to prevent damage to the sperm during cryopreservation is through use of antioxidants. The addition of antioxidants to freezing extenders has been shown to have protective effects on cryopreserved spermatozoa. Numan et al. (2010) conducted a study to determine the effects of different inclusion rates of antioxidants, methionine, inositol, and carnitine to Tris-based extender on sperm and OS parameters. Ejaculates from three Simmental bulls were equally split into seven groups (methionine 2.5 and 7.5 mM, inositol 2.5 and 7.5 mM, carnitine 2.5 and 7.5 mM, and control) cryopreserved, and then, assessed. All the antioxidants, at both inclusion rates, resulted in a lower number of sperm cells with DNA damage in comparison to the control group ($P < 0.05$). No significant differences were observed in computer-assisted sperm analysis (CASA) motility or motion characteristics or in levels of lipid peroxidation. It was concluded that the supplementation of these antioxidants before freezing can protect DNA integrity.

In another study, Hu et al. (2010) investigated the effects of ascorbic acid (vitamin C) supplementation to bovine semen cryoprotective medium (2.42 g Tris, 1.48 g citric acid, 1.00 g fructose, 6.6 ml glycerol, 20 ml egg yolk, 25 mg gentamicin, and 50,000 UI penicillin for 100 ml deionized water) on frozen-thawed spermatozoa. Ejaculates were collected from six bulls and

split into five aliquots (control, 2.5, 4.5, 6.5, and 8.5 mg/ml ascorbic acid) each. The results showed improved sperm motility and motion (straight linear velocity, VSL; linearity index, LIN; average path velocity, VAP; wobble coefficient, WOB; and lateral head displacement, ALH) in the group with extender supplemented with 4.5 mg/ml ascorbic acid in comparison to the other treatment groups ($P < 0.05$).There was also a significant improvement in acrosome and plasma membrane integrity in the 4.5 mg/ml ascorbic acid group than the control group. Researchers concluded that the supplementation of ascorbic acid to extender can reduce OS induced by cryopreservation.

In 2017, Motemani et al. examined the effects of different inclusion levels of α-tocopherol (vitamin E) in Bioxcell extender on sperm cell quality post-thaw. Semen samples were collected from 6 Holstein bulls and divided into four equal aliquots, one for each treatment group (control, 1.2, 2.4, and 4.8 mM α-tocopherol). After the thawing process, motion characteristics, viability, plasma membrane functionality, lipid peroxidation and H_2O_2 status were measured. The results showed 2.4 and 4.8 mM α-tocopherol groups had the highest motility (74.2±1.6%, 75.9±1.6%), viability (78.2±1.8%, 76.1±1.8%) and membrane functionality (73±1.6%, 70.5±1.6%) compared to the other groups. The 4.8 mM group also had the lowest H_2O_2 concentrations. It was concluded from these results that α-tocopherol at the concentration of 4.8 mM can be an efficient antioxidant additive in Bioxcell extender for cryopreservation of bull spermatozoa.

Despite the advances made, viability of post-thawed sperm has continued to be poor and inconsistent between males according to Numan et al. (2010), Hu et al. (2010), and Motemani et al. (2017). Furthermore, molecular and cellular mechanisms underlying cryopreservation and OS are still not well-understood, and thus, studies such as the one presented in this thesis assist to

further address ways, we can advance cryopreservation. Nevertheless, in this study, we did experience some disadvantages from the experimental design. One of the major limitations was the small sample size of bulls (n =16). Preferably, a greater number of bulls could be utilized for more thorough statistical analysis. While our desire to find optimum samples for our study helped to reduce variability within our research samples, statistical analysis was limited with our current numbers, thus, suggesting larger sample sizes should be implemented in future studies. Additionally, the sperm samples that were evaluated did not differ greatly in cryotolerance. That is to say, we did not have samples from bulls with extremely low cryotolerance. The samples came from select commercial AI bulls that all had relatively good fertility across the board. Nevertheless, this sample population is common in competitive breeding programs employing common culling practices, and so, while it represents the standards of the industry, it also limits the potential of significance between groups.

As for specifics of the samples utilized, all of the samples used in this study came from Holstein bulls. Utilizing one breed assisted in limiting the potential of variability introduced by different breeds, and yet, it would be of interest to the dairy and beef cattle industries to look at the parameters studied in other breeds due to the variety of breeds utilized in these breeding programs. Of particular interest would be looking at the potential differences in *Bos taurus* and *Bos indicus* species due to the phenotypical differences these breeds are known for. *Bos taurus* breeds thrive in temperate climates and breeds are known for their carcass and production traits. On the other hand, *Bos indicus* breeds are known for their heat tolerance due to their shorter hair coats, larger ears, and dewlap. These characteristics allow these breeds to survive in harsher and subtropical environments, but could there also be differences that impact their cryopreservation success rate? While we found significant differences between sperm from poor and good fertility

bulls for most of the variables measured in this study, could other breeds not demonstrate the same differences or can other variables associated with OS be more of an indication of OS than what was measured in this study? In 2016, Ntemka et al. investigated the quality difference of post-thawed semen from bulls of different breeds (Holstein, Brown Swiss, Limousin, Belgian Blue, and Blonde d' Aquitaine). Twenty-six bulls from each breed were used. Each semen sample was exposed to thermal resistance test (TR), hypo-osmotic swelling test (HOST), and kinetics by CASA. All samples were also evaluated for morphometry, morphology, viability, and DNA fragmentation. Results showed that quality characteristics of frozen-thawed semen was influenced by breed. The curvilinear velocity was higher in the Holstein and Limousin bulls compared to Belgian Blue and Blonde d' Aquitaine bulls ($P < 0.05$). The number of progressive spermatozoa was found to be significantly higher in Holstein and Brown Swiss bulls when compared to Belgian Blue and Blonde d' Aquitaine bulls ($P < 0.05$). Lower morphological abnormalities were found in Holstein bulls compared to Limousin, and in Belgian Blue bulls ($P < 0.05$). Based off these results, researchers concluded that semen from different bull breeds need to be handled specific to the breed when being cryopreserved, and thus, suggesting future research building off our current study should consider breed type and comparative measures of breeds when using similar analysis techniques as that done in this study. Furthermore, Morrell et al. (2018) explored the differences in sperm quality of cryopreserved beef and dairy bull semen. All samples were accessed for morphology, kinematics, membrane integrity, and chromatin structure. Significant differences were found in several parameters between the two groups. Beef bull semen contained a lower percentage of normal morphological and membrane intact spermatozoa in comparison to dairy bulls ($P < 0.05$; $P = 0.053$; respectively). Most kinematics (velocity curved line, velocity straight line, velocity average path, amplitude of lateral head

displacement, straightness, linearity, and wobble) were significantly higher for dairy bull spermatozoa than for beef bulls ($P < 0.001$). Chromatin damage was significantly higher ($P < 0.001$) for beef bulls than for dairy bulls. It was concluded that different sperm quality parameters may be needed when evaluating fertility in the two types of bulls, suggesting the results of our study may be limited to dairy bulls with future studies needing to be focused on beef cattle sperm.

Bull spermatozoa have limited endogenous antioxidants because of their small size. The main source of antioxidant protection is in the seminal plasma (Bilodeau et al., 2000). This may explain why we did not find a significant difference in TAC between the good and poor cryotolerance bulls in our study. The seminal plasma had been removed prior to the freezing process, which potentially depleted the antioxidant availability. Nevertheless, it would be interesting to test the TAC of the seminal plasma from the bulls used in this study to see if the Good bulls had a higher TAC initially, and thus, resulting in the increase in cryotolerance. Despite the limitations to this study, what we learned about the molecular and cellular characteristics associated with cryotolerance will be useful in the improvement of the cryopreservation process and post-thaw viability of spermatozoa. In the future, the characteristics found to be of importance in cryotolerance could be used to enhance the evaluation process of bull sperm both before and after cryopreservation. If sperm cryotolerance and post-thaw viability can be improved, it will advance preservation biotechnologies greatly, which will have a significant impact not only in the cattle industry, but also for other species.

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APPENDIX A

TABLES AND FIGURES

Table 1 Average sperm population characteristic values of bulls with good and poor cryotolerance

	Phenotype			
Variable	**Good**	**Poor**	**Standard Error**	**P-value**
Intact membranes	27.955[a]	19.000[b]	3.059	0.029
Live cells	43.636[a]	37.950[a]	4.854	0.348
Non-fragmented DNA	63.454[a]	43.266[b]	6.277	0.018
Motility	44.955[a]	45.560[a]	4.731	0.917
Progressive motility	29.482[a]	29.240[a]	3.405	0.954
Linearity	58.373[a]	56.700[a]	1.637	0.411
Straightness	88.164[a]	86.340[a]	1.058	0.175
Wobble	64.955[a]	64.260[a]	1.199	0.638
Post-thaw viability	62.161[a]	52.158[b]	1.137	<.0001

a,b Means within a row with different superscripts differ ($P \leq 0.05$)

Table 2 Average sperm cellular characteristic values of bulls with good and poor cryotolerance

	Phenotype			
Variable	**Good**	**Poor**	**Standard Error**	**P-value**
Curvilinear velocity (VCL; µm/s)	118.250[a]	108.400[a]	4.704	0.104
Straight line velocity (VSL; µm/s)	67.546[a]	59.380[b]	2.502	0.017
Beat cross frequency (BCF; Hz)	30.982[a]	28.800[a]	1.192	0.151
Average path velocity (VAP; µm/s)	75.146[a]	67.820[b]	2.619	0.036
Amplitude of lateral head (ALH; µm)	4.927[a]	4.840[a]	0.268	0.791
Distance average path (DAP; µm)	31.527[a]	27.320[b]	1.078	0.006
Distance straight line (DSL; µm)	29.191[a]	23.900[b]	1.500	0.011
Distance curved line (DCL; µm)	47.782[a]	43.840[a]	2.663	0.240

a,b Means within a row with different superscripts differ ($P \leq 0.05$)

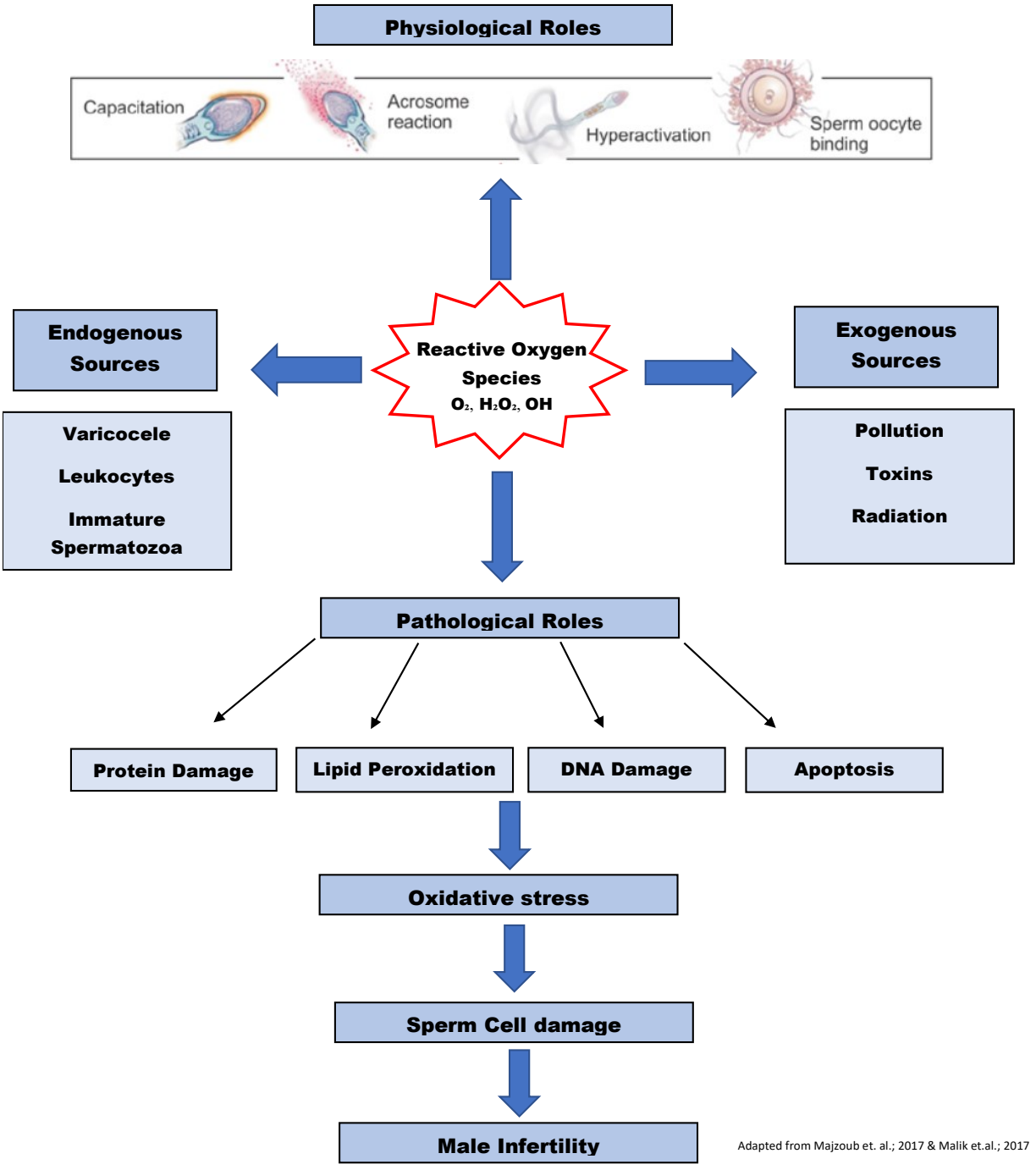

Figure 1 Effects of Reactive Oxygen Species on Spermatozoa

Association of physiological and pathological roles of reactive oxygen species (ROS) with male infertility

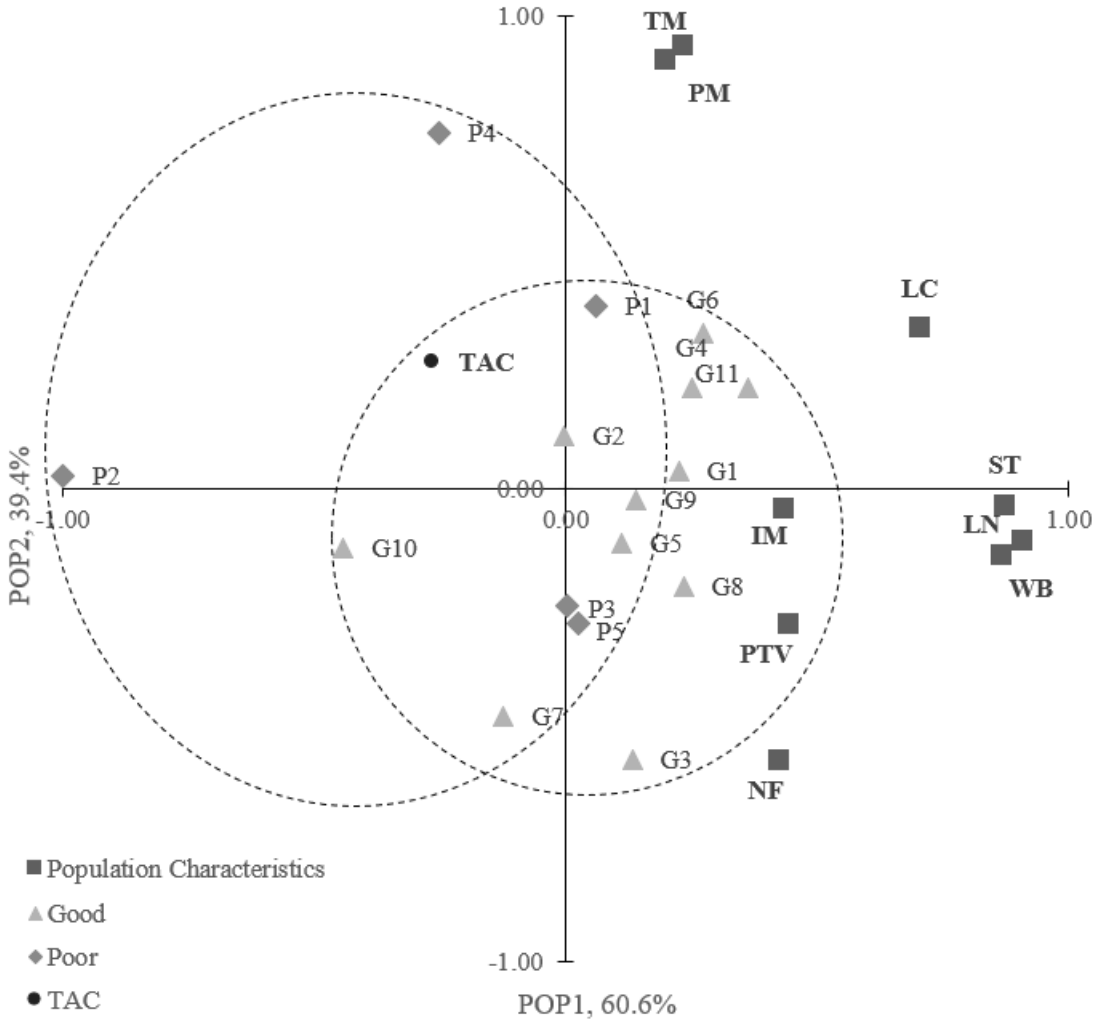

Figure 2 Sperm Population Variables PCA

Principal component analysis of sperm population variables (POP) of bulls with good and poor cryotolerance: percentage of total motility (TM), percentage of progressive motility (PM), percentage of live sperm (LC), percentage of sperm that travel straight (ST), percentage of sperm with linear movement (LN), percentage of deviation of the sperm head from the path of progression (WB), percentage of sperm with intact membranes (IM), post-thaw viability (PTV), percentage of sperm with non-fragmented DNA, and total antioxidant capacity (TAC).

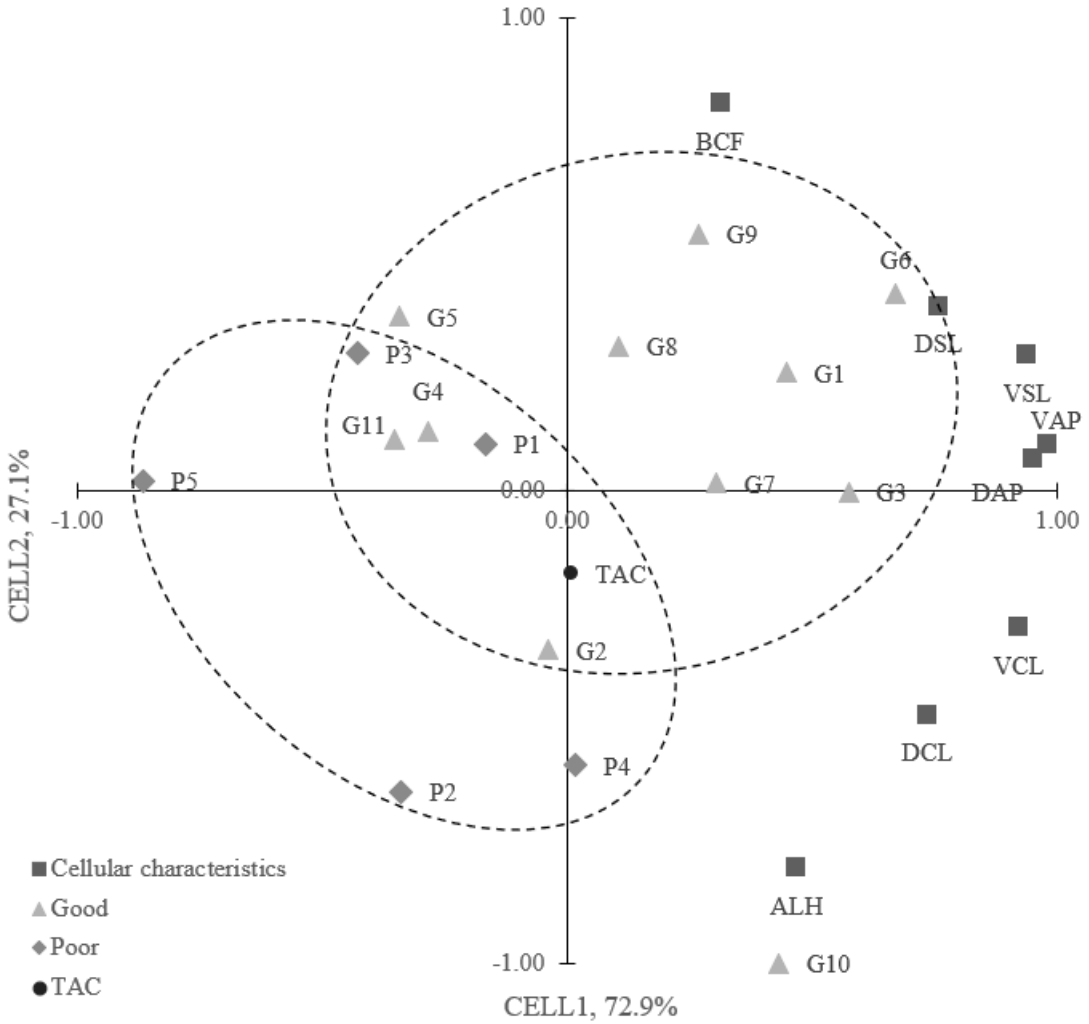

Figure 3 Sperm Cellular Characteristic Variable PCA

Principal component analysis of sperm cellular characteristic variables (CELL) of bulls with good and poor cryotolerance: curvilinear velocity (VCL; µm/s), straight line velocity (VSL; µm/s), beat cross frequency (BCF; Hz), average path velocity (VAP; µm/s), amplitude of lateral head (ALH; µm), distance average path (DAP; µm), distance straight line (DSL; µm), distance curved line (DCL; µm), and total antioxidant capacity (TAC).