8-2011

Effects of Ergot Alkaloids and Antioxidants on Bovine Sperm Motility

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Animal Science

By

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Bachelor of Science in Animal Science, 2009

August, 2011
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ABSTRACT

The studies that comprise this thesis were performed in an attempt to identify and eliminate stressful conditions that may lead to infertility in the male bovine. The first project was performed to determine if ergot alkaloids directly affect bovine sperm motility. Percentage of motile spermatozoa was affected ($P = 0.015$) by a three-way interactions between time, concentration, and alkaloid. Ergotamine and dihydroergotamine decreased ($P = 0.01$) sperm motility in a concentration and time dependant manner and ergonovine had a minimal effect on overall sperm motility. The number of static spermatozoa also was affected ($P < 0.01$) by a three way interaction and increased as ET and DHET concentrations increased. Percentages of progressively motile and rapidly motile spermatozoa decreased ($P < 0.01$) in a two way interaction between alkaloid and concentration. Overall, sperm motility was decreased by ET and DHET. Furthermore, the qualities of motility as represented by progressive, rapid, path velocity (VAP), progressive velocity (VSL), track speed (VCL), beat frequency (BCF), lateral amplitude (ALH), straightness (STR), elongated, and area were decreased by those alkaloids. These data verify that ergot alkaloids commonly found in toxic tall fescue are detrimental to bovine spermatozoa. In the second study two antioxidants (alpha-tocopherol and ascorbic acid) were added to bovine sperm culture media and cryopreservation extender. The antioxidant capabilities of these two vitamins could help reduce free radical production and help preserve sperm viability. There was an interactive effect between ascorbic acid concentration and storage method on the bovine sperm motility characteristics: motile, progressive, rapid, track speed (VCL), and straightness ($P < 0.05$). A bull by ascorbic acid interaction ($P < 0.05$) was observed for motile, progressive, rapid, path velocity (VAP),

progressive velocity (VSL), and VCL characteristics. Alpha tocopherol had no affects on sperm motility characteristics. Lipid peroxidation was affected ($P < 0.01$) by bull and ascorbic acid. Malondialdehyde concentrations for ascorbic acid treatments (0, 5, 10, 20 mM) were 3.25, 4.2, 2.96, and 2.15 µM respectively. Results from the second study indicate that the addition of ascorbic acid may reduce sperm motility, but help prevent lipid peroxidation.

This thesis is approved for recommendation
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ACKNOWLEDGMENTS

I appreciate the positive environment and educational assistance that the University of Arkansas provided. I would also like to thank Toby Lester along with multiple Animal Science graduate students for their assistance with daily animal management and data collection.

I owe a special thanks to Dr. Rick Rorie, Dr. Michael Looper, and Dr. Nick Anthony for serving on my Masters committee. They have been instrumental in the completion of my research and degree. I consider all three of these individuals great educational mentors.

Lastly, I would like to extend my gratitude to Dr. Charles Rosenkrans for his willingness to accept me as a graduate student. He is truly passionate about his profession and thoroughly enjoys seeing his students succeed. Dr. Rosenkrans served as the committee chair and my major advisor; therefore, he devoted much of his time after hours assisting me with research and data analysis and for that I am very grateful.

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Introduction

Reproductive efficiency is often ranked as the top selection criteria for beef cattle producers and heavily influences profit margins (Cliff et al., 2009). It is estimated that in the United States producers lose billions of dollars annually due to decreased calf crops because of infertility (Cliff et al., 2009). There are many environmental factors that influence reproductive capabilities including nutrition, climate, and stress. Cattle that encounter stressful conditions often suffer from abnormal reproductive deficiencies (Dobson and Smith, 2000). Most of the previous research concerning reproduction has focused on the female, but more recently scientists have shifted their attention to the male (Jones et al., 2004; Schuenemann et al., 2005; Looper et al., 2009).

Sperm motility measurements are commonly used in veterinary practice during breeding soundness exams to predict sperm viability (Eilts, 2005); however, it is not the only determining factor that influences the fertilizing capabilities of spermatozoa (Claassens et al., 1991; Liu et al., 1991). Many other criteria contribute to male fertility, such as morphology (Claassens et al., 1991) and track speed (Liu et al., 1991). Past investigations have linked toxic tall fescue and semen processing techniques to many of these aforementioned sperm defects (Jones et al., 2004; Schuenemann et al., 2005; Looper et al., 2009). Because tall fescue is the most abundant grazing option for many producers in the eastern United States, it appears to be a constant concern within the cattle industry. Also, the current demand for assisted reproductive technology (ART), such as *in vitro* fertilization, is becoming more popular; therefore, further investigations must be performed to help eradicate stress induced sperm defects.

CHAPTER 1

LITERATURE REVIEW

Tall Fescue. Tall fescue (*Festuca arundinacea Schreb.*) is a deep rooted, cool-season, perennial forage grass that is commonly grazed throughout the southeastern United States. Its extended grazing season and ease of establishment make it a viable grazing option for many livestock producers, but its hardiness, pest resistance, and sustainability during overgrazing help distinguish it from other popular forages (Stuedemann and Hoveland, 1988). It is highly tolerant to varying moisture conditions and low fertility soils. Because of its adaptability to a variety of different soil types and climate conditions, it is found in over one half of the United States and occupies millions of acres (Stuedemann and Hoveland, 1988).

Endophyte. An endophyte is any organism that lives inside a plant, along with an epiphyte living on the exterior surface of the plant (Clay and Schardl, 2002). Endophytes spend the entirety of their existence inside the host plant, and while doing so, form a symbiotic relationship with the plant that improves the overall fitness (Bacon and Siegel, 1988; Malinowski and Belesky, 2000). Through this relationship the endophyte not only gains the habitat and nutrients necessary for survival, but the infected forage also experiences increased nutrient intake, enhanced stress tolerance, improved pest protection, and greater root system and plant growth (West et al., 1990; Latch, 1997; Clay and Schardl, 2002). Growth of the endophyte occurs in the aerial portion of the host forage and is located in the intercellular regions where there is an abundance of sugars and amino acids (Clay and Schardl, 2002). Because of its many agronomic consequences, the majority of current research focuses on the fungal endophyte family known as Clavicipitaceae (Clay and Schardl, 2002). Many common forages grazed by livestock play host to fungal endophytes, but perhaps endophyte infected (EI) tall fescue

has the greatest economic impact. It is estimated that tall fescue with an endophyte level of 60% or more accounts for nearly three-fourths of all fescue stands in the United States (Oliver et al., 2000).

Ergot Alkaloids. Ergot alkaloids have been studied to a great degree and it is well established that ergot alkaloids located within the endophytic fungus (*N. coenophialum*) have been associated with fescue toxicosis (Bacon et al., 1977; Cornell et al., 1990). These ergot alkaloids can be separated into four primary groups: the clavines, lysergic acids, simple lysergic acid amides, and ergopeptines (Berde, 1980). Ergovaline seems to be the most abundant and most active alkaloid isolated from EI fescue (Porter et al., 1979; Lyons et al., 1986) and comprises the majority of the ergopeptine alkaloids (Lyons et al., 1986); however, many other alkaloids have been extracted from EI fescue including several loline alkaloids (N-acetyl and N-formyl loline) (Jones, 1981; Bush et al., 1982), peramine (Siegel et al., 1990), ergotamine (ET) (an ergopeptine), and ergonovine (EN) (simple lysergic acid amide) (Porter, 1995) along with a number of others (Bacon and Siegel, 1988). Alkaloids have been produced in vitro by the endophytic fungus, therefore, it is likely that alkaloids are generated by the fungus rather than the grass (Porter et al., 1979).

Understanding the chemical structures of ergot alkaloids have helped scientists to better understand the toxicity of each alkaloid and has also led to the use of some alkaloids for pharmacological treatments. The ergopeptine alkaloids all contain an ergoline ring but differ in their radical groups. Because they have the ergoline ring, they share structural commonalities with norepinephrine, dopamine, and serotonin (Berde, 1980) and have been greatly utilized in the world of pharmacology. Both ET and DHET

are agonists for serotonin (5-HT) receptors (Silberstein, 1997) and are highly effective when treating migraines (Lipton, 1997). These two alkaloids along with many others also have vasoconstrictor activity and have been investigated for treatment of postural hypotension (Oliver et al., 1993; Lipton, 1997; Madlom, 2002; Villalon et al., 2002). Other alkaloids, such as bromocriptine, have been used for the clinical treatment of human Parkinson disease (Hoehn and Elton, 1985; Shiraishi et al., 2004) and were used in previous years in obstetrics and gynecology because of their uterotonic effects (Albert-Puleo, 1979; de Groot et al., 1998). Many synthetic alkaloids have been derived from natural alkaloids to yield different pharmacological responses than their parent alkaloids (Madlom, 2002). Generally, these dihydrogenated derivative's agonistic characteristics are less potent than the natural alkaloids (Madlom, 2002).

Toxic Affects on Animal Physiology and Reproduction. The consumption of EI fescue has induced negative responses in a broad array of animal species ranging from small laboratory rodents to cattle and horses. Even though there is some conflicting literature, it is evident that animals consuming toxic fescue can suffer from many physiological consequences. The ruminant has been the most heavily studied because it accounts for the majority of grazed livestock in production agriculture, and is highly effective at retrieving ergot alkaloids from ingested plant tissue (Greatorex and Mantle, 1974). Depending on the time of year and severity of the issue, symptoms of fescue toxicosis can often be detected from external behavioral characteristics such as increased respiration rates, excessive salivation, less time spent grazing, and more time in the shade (Schmidt et al., 1982). Past studies have shown that cattle grazing infested pastures and fungus infected hay can have reduced daily gains and decreased intake (Hoveland et al.,

1980 a,b; Schmidt et al., 1982). One possible explanation for this is that cattle simply spend less time grazing due to altered behavioral characteristics. In one instance where steers were exposed to a toxic line of fescue, they tended to spend more time in the shade and in water than other cattle (Bond et al., 1978, 1979).

Fescue toxicosis also has been linked to alterations in endocrine profiles. The most commonly observed alteration seems to be in serum prolactin (PRL) levels. Recent studies have consistently observed declines in PRL levels in livestock consuming EI fescue (Hurley et al., 1980; Schillo et al., 1988; Porter and Thompson, 1992, Looper et al., 2009). Unlike other symptoms, declines in PRL have been observed within a few days following consumption of toxic fescue (Bolt et al., 1982). Several *in vitro* studies also have shown that ergot alkaloids can have a negative impact on the production of pituitary PRL (Porter et al., 1990b; Strickland et al., 1992). Changes in just this one hormone can cause a multitude of problems for livestock because PRL has been linked to a plethora of biological functions. In the case of female mammalian species, PRL has been linked to mammary function and milking ability (Bartke, 1980) and also aids in sexual maturity (Stroud et al., 1985). In humans, there also has been evidence indicating that PRL may play a role in the maturation of oocytes (Oda et al., 1991). In regards to the male, PRL can affect the release of gonadatropins from the pituitary and influence the development of accessory sex glands (Bartke, 1980). In a past study conducted on rats, Prasad et al. (1989) demonstrated that PRL also can inhibit testicular functions. Aside from PRL, there have been instances in which toxic fescue influenced cortisol concentration (Browning et al., 1998a) and also plasma melatonin in steers (Porter et al., 1990a). Other publications indicate that toxic agents associated with EI fescue can

impact blood progesterone concentrations in mares and luteinizing hormone (LH) concentrations in cattle (Browning et al., 1997). Decreases in LH production in the bull could possibly inhibit the stimulation of leydig cells. As a result, testosterone production would decline and the spermatogenesis process could be impaired (Jones et al., 2004). Other literature also has indicated that alterations in progesterone and estrodiol concentrations can be linked to toxic fescue consumption (Burke et al., 2001). These previously mentioned changes in hormone profiles due to toxic agents help explain reproductive deficiencies in livestock consuming EI fescue.

In cattle, decreased conception and calving rates have resulted from ingesting EI fescue (Gay et al., 1988). The reason for this can stem from a multitude of changes that occur in both the male and female reproductive process. In cows, toxic fescue has been linked to delayed onset of puberty (Washburn et al., 1989; Washburn and Green, 1991) and alterations in estrous cycles (Jones et al., 2003). According to Burke et al. (2001), follicular size and diameter was reduced when cattle consumed EI fescue. In bulls, scrotal circumference (SC) is commonly used in breeding soundness exams to evaluate reproductive competency, and changes in scrotal temperatures can result in greater morphological abnormalities and decreased motility (Skinner and Luow, 1966; Rhynes and Ewing, 1973). Recent research showed that both SC and scrotal temperatures declined after ingesting toxic fescue (Jones et al., 2004; Schuenemann et al, 2005). Toxic fescue has also been associated with declines in sperm motility which could provide a possible explanation for decreased conception and calving rates. In a previous study with Brahman-influenced bulls, sperm motility tended to be lower amongst bulls consuming EI fescue rather than bulls grazing novel endophyte pastures (Looper et al., 2009).

Coinciding with this data, Jones and her colleagues (2004) observed a decline in bovine sperm motility during the final two weeks of a 60 d trial, indicating that length of exposure to EI fescue may influence levels of toxicity within the animal. Furthermore, Schuenemann et al. (2005) supplemented bulls with an ergot alkaloid diet and evaluated their fertilizing capabilities *in-vitro*. They discovered that spermatozoa produced from bulls consuming ergotamine tartrate were less likely to fertilize an oocyte.

Sperm Anatomy. All mammalian spermatozoa contain a head and a tail which are connected to each other by the capitellum and the basal plate (Mortimer, 1997). A plasma membrane coats the entire sperm cell and has two distinguishable parts (Koehler, 1966; Mortimer, 1997). The sperm head contains a homogeneous flattened nucleus which is surrounded by a cap containing the acrosome and post-acrosomal regions (Saake and Almquist, 1964; Koehler, 1966). This cap is approximately 0.2 to 0.3 µ thick and is rich in poly-unsaturated fatty acids (Saake and Almquist, 1964). The primary responsibility of the sperm head is to transfer a haploid set of chromosomes to the female gamete (Mortimer, 1997). The tail contains the mid-piece, principal piece, and the end piece (Fawcett, 1965) and is responsible for sperm movement which is regulated by the flagellum (Mortimer, 1997). The first two segments of the flagellum are organized into a structure known as an axoneme, which contains microtubules that contribute to motility. There are two microtubules located in the center of the flagellum, and they are linked to nine outer dense fibers (ODF) by a variety of dynein arms and radial spokes (Gibbons and Grimstone, 1960; Hopkins, 1970). The dynein arms are responsible for linking the outer microtubules to one another, and the radial spokes attach the nine outer doublets to the central microtubules. When movement of the flagella occurs, there is a sliding

process that takes place within adjacent microtubule doublets (Mortimer, 1997). During this process, the dynein arms experience a cycle in which they continuously attach and release the adjacent doublets, propagating axonemal bending (Marchese-Ragona and Johnson, 1990). The mid-piece, located within the axoneme, houses the mitochondria. The mitochondria is the site of energy production necessary for flagellar movement (Curry and Watson, 1995). The end piece marks the final piece of the flagellum as it narrows down to a single microtubule (Mortimer, 1997).

Sperm Motility. Sperm motility is one of the primary measurements used for predicting sperm viability during a breeding soundness exam. The motility of spermatozoa is derived within the flagellum (Mortimer, 1997). The sliding of the doublets, located within the axenome of the flagellum, propagates sperm movement (Mortimer, 1997). The energy that stimulates sperm motility originates from the dynein-ATPase catalyzed hydrolysis of ATP. Regeneration of ATP occurs by the breakdown of glucose and fructose to lactate during the process of glycolysis and the citric acid cycle (Ford and Reese, 1990).

Cyclic adenosine monophosphate (cAMP) is an important biochemical contributor to sperm motility. Cyclic AMP activates cAMP-dependant protein kinases which contribute to axonemal sliding and flagellar movement (Tash et al., 1982; Horowitz et al., 1988). The production of cAMP is caused by adenylate cyclase, which originates from bicarbonate ions during maturation in the epididymas (Morisaw and Morisaw, 1990; Mohri, 1993). Sperm motility is stimulated by cAMP during epididymal maturation (Hoskins et al., 1974, 1975). Lindemann (1978) reported that cAMP increased motility in demembraned spermatozoa. Cyclic AMP phosphodiesterase

inhibitors also have proven beneficial for enhancing motility of washed bovine spermatozoa (Garbers et al., 1971).

Calcium also appears to be associated with sperm motility, but its role remains less clear. It does have some regulatory duties in regards to flagellar beating, but excessive concentrations can be detrimental to sperm movement (Mohri, 1993). It is believed that external calcium aids at inducing cAMP production and is necessary for hyperactivation (Marquez and Suarez, 2004). However, after one episode of freezing and thawing, increases in intracellular calcium levels seem to damage motility parameters and reduces fertilizing capabilities (McLaughlin and Ford, 1994). Furthermore, elevated concentrations of intracellular calcium also leads to the formation of spermicidal residues, 2', 4'-dichlorobenzamil (Patni et al., 2001). Recent research by Saberwal et al. (2002) suggests that calcium hinders dynein ATPase due to dephosphorylate mechanisms. Another study indicates that calcium reduces intracellular cAMP by stimulating phosphodiesterases (Wasco et al., 1989).

Cryopreservation and Cryoprotectants. Cryopreservation is a technique used to preserve spermatozoa for extended periods of time for use at a later date. The entire cryopreservation process can be simplified into six broad processing steps: extension and cooling, cryoprotectant application, straw loading, freezing, storage, and thawing (Hammerstedt et al., 1990). The extender ingredients typically include a buffer (citrate, TRIS), divalent metal ions, glucose or fructose, and a form of crude lipid (milk, egg yolk) (Ahmad and Foote, 1986; Hammerstedt et al., 1990). It has been documented that the addition of antibiotics and detergents may have beneficial affects on sperm survival (Ahmad and Foote, 1986). Spermatozoa are then cooled at a moderate rate to 4º C

(Hammerstedt et al., 1990). During this steady cool down, the spermatozoa transition through a temperature phase (15-20^{o} C), in which the physical integrity of the membrane begins to change (Hammerstedt et al., 1990). After spermatozoa are loaded into straws, they are lowered into liquid nitrogen vapor for a period of time before being plunged into liquid nitrogen for storage (Ahmad and Foote, 1986). The cooling process and freezing rate is a critical step. It is important that decreases in temperature (approximately 20^{o} C/min) be monitored at a steady rate that will allow movement of water and cryoprotectant without inducing intracellular ice crystal formation (Hammerstedt et al., 1990).

The cryopreservation process itself can be stressful to spermatozoa (Hammerstedt et al., 1990), even more, preparation procedures that are required to prepare sperm to go into extender also may induce cellular damage (Agarwal et al., 1994; Foote et al., 2002). Frozen-thawed bull spermatozoa experience peroxidation more easily than fresh (Trinchero et al., 1990), and it was demonstrated that up to eight times more live and motile spermatozoa are required for fertilization after cryopreservation as compared to fresh semen (Shannon and Vishwanath, 1995). Perhaps this is because cryopreserved sperm often experience capacitation-like changes (Cormier et al., 1997) and typically have decreased life expectancy (Watson, 1995). If the sperm do survive long enough to reach the oviduct, the alterations in the physical integrity of the sperm head plasma membrane may hinder the ability of sperm to interact with oviductal epithelial cells (Goldman et al., 1998). Spermatozoa may also encounter problems with swelling and breaking (Pace et al., 1981) and losses in membrane fluidity and permeability (Buhr et al., 1989). Studies also indicate that leakage and aggregation of

phospholipids and proteins may occur along with reduced enzymatic activity (Watson, 1981; Parrish et al., 1988; Thundathil, 1999). The excess exposure to oxygen and light during processing procedures and centrifugation during the washing phase can cause increased formation of reactive oxygen species (ROS), leading to reduced sperm motility as well as damaged genomic integrity (VanDemark et al., 1949; Agarwal et al., 1994; Foote et al., 2002).

Capacitation. Capacitation is a maturation process in which acrosome-intact sperm experience a preparation process, which allows spermatozoa to fertilize an oocyte (Yanagimachi, 1994). In 1995, Storey updated the definition for capacitation and described the process as a period when mature spermatozoa gain reaction pathways, which enables them to undergo the exocytotic acrosome reaction in response to an inducing signal sent from the zona pellucida. Once capacitation occurs, the sperm membrane begins to destabilize and eventually results in cell death (Harrison, 1996). Sperm plasma membranes undergo several modifications during epididymal transport to help protect against untimely induced capacitation (Mortimer, 1997). The primary changes that occur during this point appear to be an increase in net negative charge (Langlais and Roberts, 1985), glycosylation of surface contents, and addition of cholesterol into plasma membrane (Yanagimachi, 1994). In natural mating environments when spermatozoa are ejaculated *in-vivo*, they undergo a period in the female reproductive tract which allows ample time and conditions for capacitation to occur (Galantino-Homer et al., 1997). Scientists also have found ways to mimic the oviductal fluid for *in-vitro* studies by forming solutions that contain electrolytes, metabolic energy sources, and serum albumin (Yanagimachi, 1994). In many cases, heparin also has been

used to induce capacitation (Parrish et al., 1988). The exact locations and mechanisms that control capacitation are still unknown, but recently a multitude of biochemical contributors have been linked to the process. The previously mentioned modifications that occur during epididimyl transit are reversed (Voglmayr and Sawyer, Jr., 1986), the sialic acid residues are removed (Langlais and Roberts, 1985), and respiration rates increase (Hamner and Williams, 1963). Cholesterol is removed as well, and changes occur in sperm intracellular ion concentrations, membrane fluidity, calcium concentration, and cAMP level (Singh et al., 1978; Langlais et al., 1988; White and Aitken, 1989).

The influx of intracellular calcium concentrations caused by capacitation (Adeoya-Osiwua and Fraser, 1993) appears to be quite similar to the increase that O'Flaherty and his colleagues (1999) observed during the freezing of bull spermatozoa. This information serves as a possible explanation for why the cryopreservation process induces premature capacitation. Glycosaminoglycans also have been associated with capacitation due to their ability to alter the properties of the plasma membrane by binding seminal plasma proteins that are absorbed though the plasma membrane (Miller et al., 1990; Therien et al., 1995). Further, cyclic nucleotide metabolism and protein phosphorylation also have multiple responsibilities in regulating sperm functions, one of them being capacitation (Stein and Fraser, 1984; Parrish et al., 1994). The capacitation process induces hyperactivity of the sperm (Yanagimiachi and Usui, 1974) and then is terminated by an acrosome reaction (AR) (Roldan and Harrison, 1993). The AR is believed to be induced by zona pellucida glycoprotein 3 (ZP3) and occurs on the surface

of the zona pellucida (Wassarman, 1995). The resulting AR allows the spermatozoa to penetrate the zona pellucida (Mortimer, 1997).

Lipid peroxidation. Bovine semen is rich in lipids, and the lipid content within the sperm membrane plays an active role in processes associated with fertilization (Foote et al., 2002; Agarwal et al., 2008). Under normal physiological conditions sperm are mostly exposed to anaerobic conditions, which limits the opportunity for oxidative damage (Foote et al., 2002), but in the presence of excess oxygen, damage may occur due to lipid peroxidation induced by H_2O_2 (Bansal and Bilaspuri, 2010). The poly unsaturated fatty acids that partially make up the sperm membrane are very unstable due to their double bonds (Bansal and Bilaspuri, 2010). When free radicals attack the double bonds, the lipid peroxidation cascade occurs, and lipid peroxides begin to accumulate in the sperm plasma membrane (Sharma and Agarwal, 1996). Phospholipase A_2 then causes the release of lipid peroxides and results in cellular damage (Twigg et al., 1998; Bansal and Bilaspuri, 2010).

Reactive Oxygen Species. Reactive oxygen species (ROS) result from free radicals that are generated from enzymatic reactions that occur during cellular signaling (Bansal and Bilaspuri, 2010). Free radicals occupy only one electron while leaving at least one unpaired electron (Ho, 1996). Because the free radical has only one electron in orbit, it is highly reactive. In an effort to become more stable the resulting ROS begin to scavenge and bind to other molecules, directly causing oxidation that can lead to cellular damage (Bansal and Bilaspuri, 2010).

Reactive oxygen species can form from many different sources, but the electron transport chain in the mitochondria seems to be a common derivative amongst

mammalian spermatozoa (Ho, 1996). Typically O^2 is reduced to H^2O, but this process can be disrupted by a slow leakage of superoxide anions and cause free radical production (Ho, 1996). In bovine semen, dead spermatozoa are a primary source of ROS. They release an aromatic amino acid oxidase catalyzed reaction which generates free oxygen species (Sariozkan et al., 2009). Also, leukocytes are primary producers of ROS in semen (Sharma et al., 2001).

The production of ROS is a necessary process for many physiological functions and occurs regularly throughout the male reproductive system. Reactive oxygen species produced from sperm cells are critical for capacitation, acrosome reaction, oocyte fusion, fertilization, and maintenance of the mitochondrial capsule (Goncalves et al., 2010; Desai et al., 2009; Agarwal et al., 2008; Rivlin et al., 2004). Formation of ROS is, in part, responsible for sperm motility as well. Low levels promote cAMP-PKA signaling cascade, which stimulates sperm motility (O'Flaherty et al, 2006; Wang et al, 2009); however, under certain conditions, ROS production may exceed the antioxidant scavenging availability and severely compromise sperm function (Desai et al., 2010). Bovine spermatozoa contain high levels of polyunsaturated fatty acids and lipid dense membranes, which are highly susceptible to oxidative stress (Agarwal et al., 2008; Foote et al., 2002). As suggested by Bansal and Bilaspuri (2010), the ROS attack may cause a sudden loss of intracellular ATP, leading to a decrease in sperm viability. When this occurs, spermatozoa may suffer from multiple morphological defects (Aziz et al., 2004), induced lipid peroxidation (Fraczek and Kurpisz, 2005), and decreases in acrosome reactions (Lemkecher et al., 2005). There also is growing support that ROS formation could possibly damage genomic material (Foote et al., 2002; Bilodeau et al., 2001).

These alterations could explain declines in conception rates during recent IVF studies (Zorn et al., 2003) and support the assumption that ROS generation can cause male infertility (Sharma and Agarwal, 1996).

Antioxidants. Antioxidants have shown the ability to reduce ROS production, therefore, addition of vitamins to sperm cultures is one possible solution to treat this issue (Foote, 1967; Beconi et al., 1993; O'Flaherty et al., 1997). The scavenging ability of antioxidants helps neutralize the deleterious effects of free radical production (Askari et al., 1994; Dalvit et al., 1998; Bansal and Bilaspuri, 2009), and because of their ability to occupy excess radicals that are in orbit, antioxidants have chain breaking capabilities that eliminate oxidative stress (Miller et al., 1993; Kumar and Mahmood, 2001).

Antioxidants can be categorized into two primary groups, enzymatic and non-enzymatic. The enzymatic category contains catalase, glutathione peroxidase (GPx), superoxide dismutase (SOD), and glutathione reductase (GR) (Bansal and Bilaspuri, 2010). The SOD yields O_2 and H_2O_2 via spontaneous dismutase of superoxide anion (O^-_2·), while the catalase operates by transferring H_2O_2 to O_2 and H_2O (Sikka, 1996). Glutathione reductase assists with control of hydrogen peroxides by reducing hydrogen peroxides to water and alcohol (Agarwal et al., 2005). The non-enzymatic category is comprised primarily of ascorbic acid, α-tocopherol, zinc, taurine, glutathione, and butylated hydroxytoluene (BHT), along with numerous other vitamins and minerals (Agarwal et al., 2005; Bansal and Bilaspuri, 2010). In the presence of sperm, these non-enzymatic antioxidants act as scavengers, reducing agents, and divalent chelators (Tarin et al., 1998; Bansal and Bilaspuri, 2010).

Spermatozoa and seminal plasma are naturally equipped with an antioxidant defense mechanism that helps prevent oxidative stress (Kim and Parthasarathy, 1998). Although both seminal fluid and sperm contain antioxidants, they are typically more abundant in seminal fluid because spermatozoa have little capacity for antioxidants and protein synthesis (Zini et al.; 1993). Unfortunately, because of the presence of seminal plasma motility inhibitor generated from semenogelin I and II (Murakami et al., 1998; Yoshida et al., 2003), most cryopreservation techniques require the removal of the seminal plasma, leaving spermatozoa vulnerable to excess free radicals. The primary detoxification antioxidants found in bovine semen are catalase, GPx, and SOD (Bilodeau et al., 2000). However, several others, such as α-tochopherol (Beconi et al., 1991 and 1993), albumin (Alvarez and Storey, 1983), cysteine (Meister and Tate, 1976), and butylated hydroxytoluene (Graham and Hammerstedt, 1992) have proven beneficial for combating cellular damage. Under normal conditions, antioxidant concentrations found in sperm are abundant enough to counter oxidative stress, but semen processing techniques promote oxidative stress, and diminishe the natural antioxidant supply (Anghel et al., 2009). Research suggests that antioxidant supplementation may benefit sperm viability by reducing the cellular damage (Foote et al., 2002; O'Flaherty et al., 1997; Beconi et al., 1993). For example, albumin is linked to transitional metal ions and has the ability to decrease the production of OH^- radicals, therefore, it has been identified as a useful extracellular antioxidant for eliminating lipid peroxidation (Alvarez and Storey, 1983). Similarly, cysteine has a protective role against toxic oxygen metabolites (Meister and Tate, 1976). Graham and Hammerstedt (1992) reported that BHT serves as a useful antioxidant when used during cryopreservation of bovine spermatozoa. Also,

numerous studies recommend adding tocopherol and ascorbate to sperm culture media at manageable concentrations (Beconi et al., 1993; Uysal et al., 2007; Bansal and Bilaspuri, 2009). However, not all instances of antioxidant supplementation have been beneficial, because excess antioxidants may lead to hypertonic media (Anghel et al., 2009). Several recent studies indicated that vitamin E and C may lead to sperm DNA damage (Hughes et al., 1998; Donnelly et al., 2000), or at the minimum, have no beneficial effects (Yenilmez et al., 2006). Dalvit et al. (1998) reported that co-incubation with vitamin E and C resulted in decreased conception rates during *in vitro* fertilization. It appears that the benefits are antioxidant dependant and species specific, and must be kept within certain thresholds.

Vitamin E. Vitamin E is the name assigned to a group of lipid soluble tocopherols and tocotrienols that have antioxidant capabilities (Brigelius-Flohe and Traber, 1999; Horton et al., 2002). It serves as the primary membrane protector in spermatozoa (Yousef et al., 2003) and is believed to be the initial defense mechanism against the peroxidation of polyunsaturated fatty acids (Liebler, 1993). Vitamin E performs its antioxidant duties by attracting the unoccupied radicals, such as peroxyl and alkoxyl that result from ferrous ascorbate-induced peroxidation (Bansal and Bilaspuri, 2009), and yields a non-radical product, along with the tocopheroxyl radical (Liebler, 1993). Typically, vitamin E concentrations found in seminal plasma vary from 0.3-0.5 µm/l (Anghel et al., 2009). When spermatozoa encounter stressful circumstances, lipid radical production may exceed this level (Anghel et al., 2009).

Vitamin E has a protective role on lipid membranes found in bovine spermatozoa (Beconi et al., 1993; Uysal et al., 2007). Addition of vitamin E to cryoprotectants

benefited post-thaw measurements such as motility and improved membrane integrity of bovine spermatozoa (Uysal et al., 2007). Bansal and Bilaspuri (2009) reported that 2 mM of vitamin E is the best concentration to help preserve sperm motility. According to Askari et al. (1994), concentrations as high as 10 mM still benefit post-thaw sperm parameters. Several studies also have suggested that vitamin E supplementation can provide protection against sperm DNA damage (Lopes et al., 1998; Russo et al., 2006).

Vitamin C. Vitamin C is a unique and versatile biomolecule that appears to have an important role in preserving the male gamete. It is very unstable and is negatively influenced by increases in pH and temperature (Leung et al., 1981). It is unique in part because it is a water soluble antioxidant that can still interact with sperm membranes (Leung et al., 1981; Bascetta et al., 1983), and also because it's functional role seems to change at various concentrations (Reese and Slater, 1987). Ascorbic acid protects proteins from experiencing damage when exposed to free radicals by serving as an antioxidant (Gecha and Fagan, 1992). The antioxidant capabilities of vitamin C is credited to its redox potential (Niki, 1991). According to Dalvit et al. (1998), the antioxidant potential of ascorbic acid also could be dependent on vitamin E regeneration. It also has prooxidant properties because of its ability to reduce ferrous ions (Niki, 1991).

There has been contrasting reports regarding the ideal concentration of ascorbic acid for preventing ROS (Reese and Slater, 1987). At low concentrations it is believed to serve as a pro-oxidant, and it gains its antioxidant capabilities at higher concentrations. According to Reese and Slater (1987), 1 to 2 mM is the optimal threshold for ascorbic acid supplementation in sperm cultures. On the other hand, Liu and Foote (1995)

reported that concentrations of 5 mM or greater had minimal effects on the degradation of sperm membranes during incubation.

Multiple studies have shown that ascorbic acid is beneficial to spermatozoa during semen processing techniques. Ascorbic acid offers defense mechanisms to help preserve metabolic activity and cellular viability in cryopreserved bull sperm (Beconi et al., 1991, 1993). It also has a protective effect against lipid peroxidation during cryopreservation, leading to less capacitated spermatozoa (Askari et al., 1994). Ascorbic acid also protects against endogenous oxidative damage in human sperm and improves sperm DNA integrity (Fraga et al., 1991). In contrast, when ascorbic acid was added to fertilization media without vitamin E, it had no beneficial effect on fertilization rates (Dalvit et al., 1998). Furthermore, addition of ascorbic acid along with vitamin E appeared to decrease fertilization rates when added to IVF media (Dalvit et al., 1998). Even though Dalvit and his colleagues reported these observations during IVF, this data does not disprove that vitamin E and C could be beneficial during cryopreservation.

Relationship between Vitamin E and C. Both vitamins have antioxidant activities, but work in a different fashion. Vitamin E acts as a radical scavenger (Bansal and Biluspari, 2009), and ascorbic acid serves as a donor antioxidant. Together, the two form a relationship that benefits one another by creating a cycle that regenerates each other. The phenol group of tocopherol is known as the antioxidant mechanism of vitamin E. The phenol group is located within the water interface membrane of the biological membrane, which makes for any easy interaction with the water soluble ascorbate (Bisby and Ahmed, 1989; Van Acker et al., 1993). When ascorbate and tochopheral interact, ascorbate restores the tocopheroxyl radical of tocopheral and refurbishes the chain

breaking capabilities of vitamin E (Buettner, 1993). Because ascorbic acid aids in the regeneration of α-tochopherol, it is believed that the two can benefit each other in sperm media.

Summary

Bulls are typically responsible for breeding multiple cows. Therefore, male reproductive incompetence could result in significant economic losses. Spermatozoa that encounter stressful environments associated with plant toxins and semen processing techniques can severely limit fertilizing capabilities. Not only has toxic tall fescue been linked to sperm defects, but semen processing techniques can cause stressful conditions that decrease sperm viability. Complete eradication of toxic tall fescue is highly unlikely, and the demand for alternative reproductive techniques continues to be a relevant breeding option. Therefore, discovering alternative ways to combat this issue seems to be a more feasible option. These concepts serve as the primary reason for my investigations in eliminating stress on spermatozoa and preserving sperm viability.

Literature Cited

Adeoya-Osiwua, S. A., and L. R. Fraser. 1993. A biphasic pattern of $^{45}Ca^{2+}$ uptake by mouse spermatozoa in vitro correlates with changing functional potential. J. Reprod. Fertil. 99:187-194.

Agarwal, A., I. Ikemoto, and K. R. Loughlin. 1994. Effect of sperm washing on levels of reactive oxygen species in semen. Arch. Androl. 33:157-162.

Agarwal, A., S. Gupta, and R. K. Sharma. 2005. Role of oxidative stress in female reproduction. Reprod. Bio. Endocrin. 3, article 28.

Agarwal, A., K. Makker, and R. Sharma. 2008. Clinical relevance of oxidative stress in male factor infertility: and update. Am. J. Reprod. Immun. 59:2-11.

Ahmad, K., and R. H. Foote. 1986. Postthaw survival and fertility of frozen bull spermatozoa treated with antibiotics and detergent. J. Dairy Sci. 69:535-541.

Albert-Puleo, M. 1979. The obstetrical use in ancient and early modern times of convolvulus scammonia or scammony: Another non-fungal source of ergot alkaloids? J. Ethnopharmacol. 1:193-195.

Alvarez, J. G., and B. T. Storey. 1983. Taurine, hypotaurine, epinephrine, and albumin inhibit lipid peroxidation in rabbit spermatozoa and protect against loss of motility. Biol. Reprod. 29:548-555.

Anghel, A., S. Zamfirescu, D. Coprean, and E. Sogorescu. 2009. The effects of cystein, bovine serum albumin and vitamin E on the calitative parameters of frozen-thawed ram semen. Annals of RSCB. 14:97-103.

Askari, H. A., J. H. Check, N. Peymer, and A. Bollendorf. 1994.Effect of natural antioxidants tocopherol and ascorbic acids in maintenance of sperm activity during freeze-thaw process. Arch. Androl. 33:11-15.

Aziz, N., R. A. Saleh, R. K. Sharma, I. Lewis-Jones, N. Esfandiari, A. J. Thomas, Jr., and A. Agarwal. 2004. Novel association between sperm reactive oxygen species production, sperm morphological defects, and the sperm deformity index. Fertil. Steril. 81:349-354.

Bacon, C. W., J. K. Porter, J. D. Robbins, and E. S. Lutrell. 1977. *Epichloe typhina* from toxic tall fescue grasses. Appl. Environ. Microbiol. 34:526-581.

Bacon, C. W., and M. R. Siegel. 1988. Endophyte parasitism of tall fescue. J. Prod. Agric. 1:45-46.

Bansal, A. K., and G. S. Bilaspuri. 2009. Antioxidant effect of vitamin E on motility, viability and lipid peroxidation of cattle spermatozoa under oxidative stress. Anim. Sci. Papers and Reports. 27:5-14.

Bansal, A. K., and G. S. Bilaspuri. 2010. Impacts of oxidative stress and antioxidants on semen functions. Review article. Vet. Medicine Int. 2011:1-7.

Bartke, A. 1980. Role of prolactin in reproduction in male mammals. Fed. Proc. 39:2577-2581.

Bascetta, E., R. D. Gustone, and J. C. Walton. 1983. Electron spin resonance study of the role of vitamin E and vitamin C in the inhibition of fatty acid oxidation in a model membrane. Chem. Phys. Lipids. 33: 208-210.

Beconi, M., M. Affranchino, L. M. Schang, and N. Beorlegui. 1991. Influence of antioxidants on SOD activity in bovine sperm. Biochem. Int. 3:545-553.

Beconi, M. T., C. R. Francia, N. G. Mora, M. A. Affranchino. 1993. Effect of natural antioxidants of frozen bovine semen preservation. Theriogenology. 40:841-851.

Berde, B. 1980. Ergot Compounds: A synopsis. In: M. Goldstein, A. Lieberman, D. Calne, and M. Thorner (ed.). Ergot compounds and brain function: Neuroendocrine and neuropsychiatric Aspects. Raven Press, New York. p. 3-24.

Bilodeau, J., S. Chatterjee, M. Sirard, and C. Gagnon. 2000. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. Mol. Reprod. Dev. 55:282-288.

Bilodeau, J., S. Blanchette, C. Gagnon, and M. Sirard. 2001. Thiols prevent H_2O_2-mediated loss of sperm motility in cryopreserved bull semen. Theriogenology. 56:275-286.

Bisby, R. H., and S. Ahmed. 1989. Transverse distribution of α-tochopherol in bilayer membranes studied by fluorescence quenching. Free Radical Biol. Med. 6:231-239.

Bolt, D. J., G. P. Bond, G. P. Lynch, and T. Elsasser. 1982. Concentrations of PRL, LH, FSH, and TSH in plasma and pituitary of ewes grazing tall fescue and orchardgrass pastures. J. Anim. Sci. 55 (Suppl. 1):4 (Abstr.).

Bond, J., J. B. Powell, D. J. Undersander, L. I. Colbert, and R. R. Oltjen. 1978. Behavioral activity of steers grazing fescue. J. Anim. Sci. 47(Suppl. 1): 83.

Bond, J., J. B. Powell, and L. I. Colbert. 1979. Behavioral activity of steers grazing grass containing a toxin. J. Anim. Sci. 49(Suppl. 1): 146.

Brigelius-Flohe, R., and M G. Traber. 1999. Vitamin E: function and metabolism. The FASEB J. 13:1145-1155.

Browning, R., Jr., F. N. Thompson, J. L. Sartin, and M. L. Leite-Browning. 1997. Plasma concentrations of prolactin, growth hormone, and luteinizing hormone in steers administered ergotamine or ergonovine. J. Anim. Sci. 75:796-802.

Browning, R., Jr., M. L. Leite-Browning, H. M. Smith, and T. Wakefield, Jr. 1998a. Effect of ergotamine and ergonovine on plasma concentrations of thyroid hormones and cortisol in cattle. J. Anim. Sci. 76:1644-1650.

Buettner, G. R. 1993. The pecking order of free radicals and antioxidants: lipid peroxidation, α-tocopherol, and ascorbate. Arch. Biochem. Biophys. 300:535-543.

Buhr, M. M., A. T. Canvin, and J. L. Bailey. 1989. Effects of semen preservation on boar spermatozoa membranes. Gamete Res. 23:441-449.

Burke, J. M., D. E. Spiers, F. N. Kojima, G. A. Perry, B. E. Salfen, S. L. Wood, D. J. Patterson, M. F. Smith, M. C. Lucy, W. G. Jackson, and E. L. Piper. 2001. Interaction of endophyte-infected fescue and heat stress on ovarian function in the beef heifer. Biol. Reprod. 65:260-268.

Bush, L. P., P. L. Cornelius, R. C. Buckner, D. R. Varney, R. A. Chapman, P. B. Burrus, C. W. Kennedy, T. A. Jones, and M. J. Saunders. 1982. Association of N-acetylloline derivative and N-formylloline derivative with epichloe thyhina in tall fescue. Crop Sci. 22:941.

Claassens, O. E., R. Menkveld, D. R. Franken, E. Pretorius, Y. Swart, C. J. Lombard, and T. F. Kruger. 1991. The acridine orange test: determining the relationship between sperm morphology and fertilization *in vitro*. Hum. Reprod. 7:242-247.

Clay, K., and C. Schardl. 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. The American Naturalist. 160 (Suppl):99-127.

Cliff, G., C. Dahlen, and M. Maddox. 2009. What is the economic impact of infertility in beef cattle? University of Florida IFAS Ext. AN208. Accessed May 14, 2011. http://edis.ifas.ufl.edu/pdffiles/AN/AN20800.pdf.

Cormier, N., M. Sirard, and J. L. Bailey. 1997. Premature capacitation of bovine spermatozoa is initiated by cryopreservation. J. Androl. 18:461-468.

Cornell, C. N., J. V. Lueker, G. B. Garner, and J. L. Ellis. 1990. Establishing ergovaline levels for fescue toxicosis, with and without endoparasites, under controlled climatic conditions. New Orleans, LA. International Symposium on Acremonium/Grass Interactions. 75-79.

Curry, M. R., and P. F. Watson. 1995. Sperm structure and function. In J. G. Grudzinskas and J. L. Yovich. (ed.). Gametes-The Spermatozoon. Cambridge University Press. Cambridge, UK. 45-69.

Dalvit, G. C., P. D. Cetica, and M. T. Beconi. 1998. Effect of α-tocopherol and ascorbic acid on bovine in vitro fertilization. Theriogenology. 49:619-627.

de Groot, A. N., P. W. van Dongen, T. B. Vree, Y. A. Hekster, and J. van Roosmalen. 1998. Ergot alkaloids. Current status and review of clinical pharmacology and therapeutic use compared with other ocytocics in obstetrics and gynaecology. Drugs. 56:523-535.

Desai, N., R. Sharma, K. Maker, E. Sabnegh, and A. Agarwal. 2009. Physiological and pathological levels of reactive oxygen species in neat semen of infertile men. Fertil. Steril. 92:1626-1631.

Desai, N. R., R. Mahfouz, R. Sharma, S. Gupta, and A. Agarwal. 2010. Reactive oxygen species levels are independent of sperm concentration, motility, and abstinence in a normal, healthy, proven fertile man: a longitudinal study. Fertil. Steril. 94:1541-1543.

Dobson, H., and R. F. Smith. 2000. What is stress, and how does it affect reproduction? Anim. Reprod. Sci. 60:743-752.

Donnelly, E. T., N. McClure, and S. E. Lewis. 2000. Glutathione and hypotaurine in vitro: effects on human sperm motility, DNA integrity and production of reactive oxygen species. Mutagenesis. 15-61-68.

Eilts, B. E. 2005. Bull breeding soundness examination. Accessed May 14, 2011. http://www.vetmed.Isu.edu/elitslotus/theriogenology5361/bull.htm

Fraga, C. G., P. A. Metchnik, M. K. Shigenaga, H. J. Helbock, R. A. Jacob, and B. N. Ames. 1991. Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. Proc. Natl. Acad. Sci. 88:11003-11006.

Fawcett, D. W. 1965. The anatomy of the mammalian spermatozoa with particular reference to the guinea pig. Z Zellforsch. 67:279-296.

Foote, R. H. 1967. Influence of light and agitation on bovine spermatozoa stored with protective agents. J. Dairy Sci. 50:1468-1474.

Foote, R. H., C. C. Brockett, and M. T. Kaproth. 2002. Motility and fertility of bull sperm in whole milk extender containing antioxidants. Anim. Reprod. Sci. 71:13-23.

Ford, W. C. L., and J. M. Reese. 1990. The bioenergitics of mammalian sperm motility. In Gagnon, C. (ed.). Controls of sperm motility: Biological and clinical aspects. CRC Press. Boca Raton. 175-202.

Fraczek, M., and M. Kurpisz. 2005. The redox system in human semen and peroxidative damage of spermatozoa. Postepy. Hig. Med. Dasw. (Online) 59:523-534.

Galantino-Homer, H. L., P. E. Visconti, and G. S. Kopf. 1997. Regulation of protein tryrosine phosphorylation during bovine sperm capacitation by cyclic adenosine 3',5'-monophosphate-dependent pathway. Biol. Reprod. 56:707-719.

Garbers, D. L., W. D. Lust, N. L. First, and H. A. Lardy. 1971. Effects of phosphodiesterase inhibitors and cyclic nucleotides on sperm respiration and motility. Biochem. 10:1825-1831.

Gay, N., J. A. Boling, R. Dew, and D. E. Miksch. 1988. Effects of endophyte-infected tall fescue on beef cow-calf performance. Appl. Agric. Res. 3:182-186.

Gecha, O. M., and J. M. Fagan. 1992. Protective effect of ascorbic acid on the breakdown of proteins exposed to hydrogen peroxide in chicken skeletal muscle. J. Nutrition. 122:2087-1293.

Gibbons, I. R., and A. V. Grimstone. 1960. On flagellar structure in certain flagellates. J. Biophys. Biochem. Cytol. 7:697-716.

Goldman, E. E., J. E. Ellington, and R. H. Foote. 1998. Reaction of fresh and frozen bull spermatozoa incubated with fresh and frozen bovine oviduct epithelial cells. Reprod. Nutr. Dev. 38:281-288.

Goncalves, F., L. S. Barretto, R. P. Arruda, S. H. Perri, and G. Z. Mingoti. 2010. Effect of antioxidants during bovine in vitro fertilization procedures on spermatozoa and embryo development. Reprod. Domestic Anim. 45:129-135.

Graham, J. K., and R. H. Hammerstedt. 1992. Differential effects of butylated hydroxytoluene analogs on bull sperm subjected to cold induced membrane stress. Crybiol. 29:106-117.

Greatorex, J. C., and P. G. Mantle. 1974. Effect of Rye ergot on the pregnant sheep. J. Reprod. Fertil. 37:33-41.

Hammerstedt, R. H., J. K. Graham, and J. P. Nolan. 1990. Cryopreservation of mammalian sperm: What we ask them to survive. J. Androl. 11:73-87.

Hamner, C. E., and W. L. Williams. 1963. Effect of the female reproductive tract on sperm metabolism in the rabbit and fowl. J. Reprod. Fertil. 5:143-150.

Harrison, R. A. P. 1996. Capacitation mechanisms, and the role of capacitation as seen in eutherian mammals. Reprod. Fertil. Dev. 8:581-594.

Ho, L. 1996. The protective role of vitamin E and vitamin C as antioxidants in semen. Nutrition Bytes. 2:1-4.

Hoehn, M. M., and R. L. Elton. 1985. Low dosages of bromocriptine adds to levodopa in parkinson's disease. Neurology. 35:199-206.

Hopkins, J. M. 1970. Subsidiary components of the flagella of *chlamydomonas reinhardii*. J. Cell. Sci. 7:823-839.

Horowitz, J. A., W. Wasco, M. Leiser, and G. A. Orr. 1988. Interaction of the regulatory subunit of a type II cAMP-dependant protein kinase with mammalian sperm flagellum. J. Biol. Chem. 263:2098-2104.

Horton, H. R., L. H. Moran, R. S. Ochs, J. D. Rawn, and K. G. Scrimglour. 2002. Principals of biochemistry. 3rd Edition, Prentice Hall, Upper River Saddle, NJ 07458. 221-222.

Hoskins, D. D., D. T. Stephens, and M. L. Hall. 1974. Cyclic adenosine 3',5'-monophosphate and protein kinase levels in developing bovine spermatozoa. J. Reprod. Fertil. 37:131-133.

Hoskins, D. D., M. L. Hall, D. Munsterman. 1975. Induction of motility in immature bovine spermatozoa by cyclic AMP phosphodiesterase inhibitors and seminal plasma. Biol. Reprod. 13:168-176.

Hoveland, C. S., R. L. Haaland, C. C. King, Jr., W. B. Anthony, E. M. Clarke, J. A. McGuire, L. A. Smith, H. W. Grimes, and J. L. Holliman, 1980a. Association of Epichloe typhina fungus and steer performance on tall fescue pasture. Agron. J. 72:1064.

Hoveland, C. S., R. L. Haaland, C. C. King, Jr., W. B. Anthony, E. M. Clarke, J. A. McGuire, L. A. Smith, H. W. Grimes, and J. L. Holliman, 1980b. Steer performance on AP-2 phalaris and Kentucky 31 tall fescue pasture. Agron. J. 72:375.

Hughes, C. M., S. E. Lewis, V. J. McKelvey-Martin, and W. Thompson. 1998. The effects of antioxidant supplementation during Percoll preparation on human sperm DNA integrity. Hum. Reprod. 13:1240-1247.

Hurley, W. L., E. M. Convey, K. Leung, L. A. Edgerton, and R. W. Hemken. 1980. Bovine prolactin, TSH, T4 and T3 concentrations as affected by tall fescue summer toxicosis and temperature. J. Anim. Sci. 51:374-379.

Jones, T. A. 1981. The association of an endophytic fungus *Spachelia typhina* (Pers.) Sacc. with the production of pyrrolizidine alkaloids in tall fescue. M. S. Thesis. Univ. of Kentucky, Lexington.

Jones, K. L., S. S. King, K. E. Griswold, D. Cazac, and D. L. Cross. 2003. Domperidone can ameliorate deleterious reproductive effects and reduced weight gains associated with fescue toxicosis in heifers. J. Anim. Sci. 81:2568.

Jones, K. L., C. R. Mccleary, S. S. King, G. A. Apgar, and K. E. Griswold. 2004. Case Study: Consumption of toxic fescue impairs bull reproductive parameters. Prof. Anim. Sci. 20:437-442.

Kim, J. G., and S. Parthasarathy. 1998. Oxidation and the spermatozoa. Seminars in Reprod. Endocrin. 16:235-239.

Koehler, J. K. 1966. Electron microscope observations on the postnuclear region of bovine spermatozoa. Correlation between frozen-etched and chemically fixed material. Anat. Rec. 154:370-371. (Abstr.).

Kumar, H., and S. Mahmood. 2001. The use of fast acting antioxidants for the reduction of cow placental retention and subsequent endometritis. Indian J. Anim. Sci. 71:650-653.

Langlais, J., and K. D. Roberts. 1985. A molecular membrane model of sperm capacitation and the acrosome reaction of mammalian spermatozoa. Gamete Res. 12:183-224.

Langlais, J., F. W. K. Kan, L. Granger, L. Raymond, G. Bleau, and K. D. Roberts. 1988. Identification of sterol acceptors that stimulate cholesterol efflux from human spermatozoa during in vitro capacitation. Gamete Res. 20:185-201.

Latch, G. C. M. 1997. An overview of neotyphodium-grass interactions. In: C. W. Bacon and N. S. Hill (ed.). Neotyphodium/Grass interactions. Plenum Press, NY. p. 1-11.

Lemkecher, T., S. Dartigues, J. Vaysse, O. Kulski, V. Barraud-Lange, L. Gattegno, and J. P. Wolf. 2005. Leucocytospermia, oxidative stress and male fertility: facts and hypotheses. Gynecol. Obstet. Fertil. 33:2-10.

Leung, H. W., M. J. Vang, and R. D. Mavis. 1981. The cooperative interaction between vitamin E and vitamin C in suppression of peroxidation of membrane phospholipids. Biochem. Biophys. Acta. 664:266-272.

Liebler, D. C. 1993. The role of metabolism in the antioxidant function of vitamin E. Crit. Rev. Toxicol. 23:147-169.

Lindemann, C. B. 1978. A cAMP-induced increase in the motility of demembranated bull sperm models. Cell. 13:9-18.

Lipton, R. B. 1997. Ergotamine tartrate and dihydroergotamine mesylate: Safety profiles. Headache. 37 (Suppl 1):33-41.

Liu, D. Y., G. N. Clarke, and H. W. Baker. 1991. Relationship between sperm motility assissed with the Hamilton-Thorne motility analyzer and fertilization rates in-vitro. J. Androl. 4:231-239.

Liu, Z., and R. H. Foote. 1995. Development of bovine embryos in KSOM with added superoxide dismutase and taurine and with 5 and 20% O_2. Biol. Reprod. 53:786-790.

Looper, M. L., R. W. Rorie, C. N. Person, T. D. Lester, D. M. Hallford, G. E. Aiken, C. A. Roberts, G. E. Rattinghaus, and C. F. Rosenkrans, Jr. 2009. Influence of toxic endophyte-infected fescue on sperm characteristics and endocrine factors of yearling Brahman-influenced bulls. J. Anim. Sci. 87:1184-1191.

Lopes, S., A. Jurisicova, J. G. Sun, and R. F. Casper. 1998. Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. Hum. Reprod. 13:896-900.

Lyons, P. C., R. D. Plattner, and C. W. Bacon. 1986. Occurrence of peptide and clavinet ergot alkaloids in tall fescue grass. Science 232:487-489.

Madlom, Z. 2002. The origin of drugs in current use: The ergot alkaloids story. Accessed June 11, 2010. http://www.world-of fungi.org/Mostly_Medical/Ziad_ Medlom/ Ergot_ alkaloids.htm

Malinowski, D. P., and D. P. Belesky. 2000. Adaptations of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. Crop Sci. 40:923-940.

Marchese-Ragona, S. P., and K. A. Johnson. 1990. Structural and biochemical studies of the dynein ATPase. In Gagnon, C. (ed.). Controls of sperm motility: Biological and Clinical Aspects. CRC Press. Boca Raton. 203-217.

Marquez, B., and S. S. Suarez. 2004. Different signaling pathways in bovine sperm regulate capacitation and hyperactivation. Biol. Reprod. 70:1626-1633.

McLaughlin, E. A., and W. C. Ford. 1994. Effects of cryopreservation on the intracellular calcium concentration of human spermatozoa and its response to progesterone. Mol. Reprod. Dev. 37:241-246.

Meister, A, and S. S. Tate. 1976. Glutathione and related gamma-glutamyl compounds; biosynthesis and utilization. Ann. Rev. Biochem. 45:559-604.

Miller, D. J., M. A. Winer, and R L. Ax. 1990. Heparin-binding proteins from seminal plasma bind to bovine spermatozoa and modulate capacitation by heparin. Biol. Reprod. 42:899-915.

Miller, J. K., E. Brzezinkska-Slebodzinska, and F. C. Madsen. 1993. Oxidative stress, antioxidants, and animal function. J. Dairy Sci. 76:2812-2823.

Mohri, H. 1993. Role of tubulin and dynein in spermatozoan motility. Mol. Reprod. Dev. 36:221-223.

Morisaw, M., and S. Morisaw. 1990. Acquisition and initiation of sperm motility. In Gagnon, C. (ed.). Controls of sperm motility: Biological and clinical aspects. CRC Press. Boca Raton. 137-151.

Mortimer, S. T. 1997. A critical review of the physiological importance and analysis of sperm movement in mammals. Human Reprod. Update. 3:403-439.

Murakami, J., M. Yoshiike, M. Satoh, Y. Fucuichi, and T. Owamoto. 1998. Characterization of recombinant precursor proteins of the human seminal plasma sperm motility inhibitor synthesized in insect cells. Int. J. Mol. Med. 2:693-700.

Myles, D. G., P. Primakoff, and A. R. Bellve. 1981. Surface domains of the guinea pig defined with monoclonal antibodies. Cell. 23:433-439.

Nicolson, G. L., and R. Yanagimachi. 1974. Mobility and the restriction of mobility of plasma membrane lectin-binding components. Science. 184:1294-1296.

Niki, E. 1991. Action of ascorbic acid as a scavenger of active and stable oxygen radicals. American J. Clin. Nutrition. 54:1119S-1124S.

Oda, T., Y. Yoshimura, Y. Takehara, S. Kohriyama, Y. Sano, K. Tanabe, T. Kobayashi, Y. Nakamura, T. Ohno, and S. Nozawa. 1991. Effects of prolactin on fertilization and cleavage of human oocytes. Horm. Res. 35 (Suppl 1):33-38.

O'Flaherty, C. M., M. Beconi, and N. Beorlegui. 1997. Effect of natural antioxidants, superoxide dismutase and hydrogen peroxide on capacitation of frozen-thawed bull spermatozoa. Andrologia. 29:269-275.

O'Flaherty, C. M., N. B. Beorlegui, and M. T. Beconi. 1999. Reactive oxygen species requirements for bovine sperm capacitation and acrosome reaction. Theriogenology. 52:289-301.

O'Flaherty, C., E. de Lamirande, and C. Gagnon. 2006. Reactive oxygen species modulate independent protein phosphorylation pathways during human sperm capacitation. Free Radical Biol. Med. 40:1045-1055.

Oliver, J. W., L. K. Abney, J. R. Strickland, and R. D. Linnabary. 1993. Vasoconstriction in bovine vasculature induced by the tall fescue alkaloid lysergamide. J. Anim. Sci. 71:2708-2713.

Oliver, J. W., A. E. Schultze, B. W. Rohrbach, H. A. Fribourg, T. Ingle, and J. C. Waller. 2000. Alterations in hemograms and serum biochemical analytes of steers after prolonged consumption of endophyte-infected tall fescue. J. Anim. Sci. 78:1029-1035.

Pace, M. M., J. J. Sullivan, F. I. Elliott, E. F. Graham, and G. H. Coulter. 1981. Effects of thawing temperature, number of spermatozoa, and sperm quality on fertility of bovine spermatozoa packaged in 0.5 mL French straws. J. Anim. Sci. 53:693-701.

Parrish, J. J., J. L. Susko-Parrish, C. Uguz, and N. L. First. 1994. Differences in the role of cyclic adenosine 3',5'-monophosphate during capacitation of bovine sperm by heparin or oviduct fluid. Biol. Reprod. 51:1099-1108.

Parrish, J. J., J. Susko-Parrish, M. A. Winer, and N. L. First. 1988. Capacitation of bovine sperm by heparin. Biol. Reprod. 38:1171-1180.

Patni, A. K., S. Gupta, A. Sharma, A. K. Tiwary, and S. K. Garg. 2001. Role of intracellular calcium in the spermicidal action of 2',4'-dichlorobenzamil, a novel contact spermicide. J. Pharm. Pharmacol. 53:1387-1392.

Porter, J. K., C. W. Bacon, and J. D. Robbins. 1979. Ergosine, ergosinine, and chanoclavine I from epichloe typhina. J. Agric. Food Chem. 27:595-598.

Porter, J. K., J. A. Stuedemann, F. N. Thompson, H. A. Buchanan, H. A. Tucker, and L. B. Lipham. 1990a. Plasma melatonin suppression in steers on endophyte infected fescue. Toxicologist. 10:243 (Abstr.)

Porter, J. K., J. A. Stuedemann, F. N. Thompson, Jr., and L. B. Lipham. 1990b. Neuroendocrine measurements in steers grazed on endophyte infected fescue. J. Anim. Sci. 68:3285-3292.

Porter, J. K., and F. N. Thompson, Jr. 1992. Effects of fescue toxicosis on reproduction in livestock. J. Anim. Sci. 70:1594-1603.

Porter, J. K. 1995. Anslysis of endophyte toxins: Fescue and other grasses toxic to livestock. J. Anim. Sci. 73:871-880.

Prasad, M., G. Uma Devi, and S. Govindappa. 1989. Effect of hypo and hyperprolactinemia on the testicular maturation of wistar rats during puberal transition. Arch. Int. Physiol. Biochem. 97:347-353.

Reese, S. and T. F. Slater. 1987. Ascorbic acid and lipid peroxidation; the crossover effect. Acta. Biochem. Biophys. Hung. 22:241-249.

Rhynes, W. E., and L. L. Ewing. 1973. Testicular endocrine function in Hereford bulls exposed to high ambient temperatures. Endocinology. 92:509-515.

Rivlin, J., J. Mendel, S. Rubinstein, N. Etkovitz, and H. Breitbart. 2004. Role of hydrogen peroxide in sperm, capacitation and acrosome reaction. Biol. Reprod. 70:518-522.

Roldan, E. R. S., and R. A. Harrison. 1993. Diacylglycerol in the exocytosis of the mammalian sperm acrosome. Biochem. Soc. Transactions. 21:284-289.

Russo, A., N. Troncoso, F. Sanchez, J. A. Garbarino, and A. Vanella. 2006. Propolis protects human spermatozoa from DNA damage caused by benzo[a]pyrene and exogenous reactive oxygen species. Life Sci. 78:1401-1406.

Saake, R. G., and J. O. Almquist. 1964. Ultra-structure of bovine spermatozoa. I. The head of normal ejaculated sperm. Am. J. Anatomy. 115:143-161.

Saberwal, G. S., M. K. Sharma, N. Balasinor, J. Choudhary, and H. S. Juneja. 2002. Estrogen receptor, calcium mobilization and rat sperm motility. Mol. Cell. Biochem. 237:11-20.

Sariozkan, S., M. N. Bucak, P. B. Tuncer, P. A. Ulutas, and A. Bilgen. 2009. The influence of cysteine and taurine on microscopic-oxidative stress parameters and fertilizing ability of bull semen following cryopreservation. Cryobiology. 58:134-138.

Schillo, K. K., L. S. Leshin, J. A. Boling, and N. Gay. 1988. Effects of endophyte-infected fescue on concentrations on prolactin in blood sera and the anterior pituitary and concentrations of dopamine and dopamine metabolites in brains of steers. J. Anim. Sci. 66:713-718.

Schmidt, S. P., C. S. Hoveland, E. M. Clark, N. D. Davis, L. A. Smith, and H. W. Grimes. 1982. Association of an endophytic fungus with fescue toxicity in steers fed Kentucky 31 tall fescue seed or hay. J. Anim. Sci. 55:1259-1263.

Schuenemann, G. M., J. L. Edwards, M. D. Davis, H. E. Blackmon, F. N. Scenna, N. R. Rohrbach, A. M. Saxton, H. S. Adair, F. M. Hopkins, J. C. Waller, and F. N. Schrick. 2005. Effects of administration of ergotamine tartrate on fertility of yearling beef bulls. Theriogenology. 63:1407-1418.

Shannon, P., and Vishwanath, R. 1995. The affect of optimal and suboptimal concentration of spermatozoa on the fertility of fresh and frozen bovine semen and a theoretical model to explain the fertility differences. Anim. Reprod. Sci. 39:1-10.

Sharma, R. K., and A. Agarwal. 1996. Role of reactive oxygen species in male infertility. Urology. 48:835-850.

Sharma, R. K., F. F. Pasqualotto, D. R. Nelson, A. J. Thomas, Jr., and A. Agarwal. 2001. Relationship between seminal white blood cell counts and oxidative stress in men treated at an infertility clinic. J. Androl. 22:575-583.

Shiraishi, M., T. Kamo, M. Hotta, S. Nemoto, J. Oshima, H. Sugihara, S. Yasaki, M. Kawakami, Y. Takahashi, and S. Shimojo. 2004. Usefulness of switching to cabergoline from other dopamine agonists in patients with advanced parkinson's disease. J. Neural Transm. 111:725-732.

Siegel, M. R., G. C. M. Latch, L. P. Bush, F. F. Fannin, D. D. Rowan, B. A. Tapper, C. W. Bacon, and M. C. Johnson. 1990. Fungal endophyte-infected grasses: Alkaloid accumulation and aphid response. J. Chem. Ecol. 12:3301-3315.

Sikka, S. C. 1996. Oxidative stress and role of antioxidants in normal and abnormal sperm function. Frontiers of Biosciences. 1:78-86.

Silberstein, S. D. 1997. The pharmacology of ergotamine and dihydroergotamine. Headache. 37 (Suppl 1):15-25.

Singh, J. P., D. F. Babcock, and H. A. Lardy. 1978. Increased calcium ion influx is a component of capacitation of spermatozoa. Biochem. J. 172:549-556.

Skinner, J. D., and G. N. Luow. 1966. Heat stress and spermatogenesis in Bos Indicus and Bos Taurus cattle. J. Apply. Physiol. 21:1784-1790.

Stein, D. M., and L. R. Fraser. 1984. Cyclic nucleotide metabolism in mouse epididymal spermatozoa during capacitation in vitro. Gamete Res. 10:283-299.

Storey, B. T. 1995. Interaction between gametes leading to fertilization: the sperm's eye view. Reprod. Fertil. Dev. 7:927-942.

Strickland, J. R., D. L. Cross, T. C. Jenkins, R. J. Petroski, and R. G. Powell. 1992. The effect of alkaloids and seed extracts of endophyte-infected tall fescue on prolactin secretion in an *in vitro* rat pituitary perfusion system. J. Anim. Sci. 70:2779-2786.

Stroud, C. M., P. F. Noden, and J. M. Whitsett. 1985. Bromoergocryptine decreases serum prolactin and delays sexual maturation in male golden hamsters. Biol. Reprod. 32:1191-1199.

Stuedemann, J. A., and C. S. Hoveland. 1988. Fescue endophyte: History and impact on animal agriculture. J. Prod. Agric. 1:39-44.

Tarin, J. J., J. Brines, and A. Cano. 1998. Is antioxidant therapy a promising strategy to improve human reproduction? Hum. Reprod. 13:1415-1424.

Tash, J. S., and A. R. Means. 1982. Regulation of protein phosphorylation and motility of sperm by cyclic adenosine monophosphate and calcium. Biol. Reprod. 26:745-763.

Therien, I., G. Bleau, and P. Manjunath. 1995. Phosphatidylcholine-binding proteins of bovine seminal plasma modulate capacitation of spermatozoa by heparin. Biol. Reprod. 52:1372-1379.

Thundathil, J., J. Gil, A. Januskauskas, B. Larsson, L. Soderquist, R. Mapletoft, and H. Rodriguez-Martinez. 1999. Relationship between the proportion of capacitated spermatozoa present in frozen-thawed bull semen and fertility with artificial insemination. Inter. J. Androl. 22:366-373.

Trinchero, G. D., M. A. Affranchino, L. M. Schang, and M. T. Beconi. 1990. Antioxidant effect of bovine spermatozoa on lipid peroxidation. Com. Biol. 8:339-350.

Twigg, J., N. Fulton, E. Gomez, D. S. Irvine, and R. J. Aitken. 1998. Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation, and effectiveness of antioxidants. Hum. Reprod. 13:1429-1436.

Uysal, O., M. N. Bucak, and I. Yavas. 2007. Effect of various antioxidants on the quality of frozen-thawed bull semen. J. Anim. Vet. Advances. 6:1362-1366.

Van Acker, S., L. Koymans, and A. Bast. 1993. Molecular pharmacology of vitamin E. Structural aspects of antioxidant activity. Free Radical Biol. Med. 15:311-328.

VanDemark, N. L., G. W. Salisbury, and R. W. Bratton. 1949. Oxygen damage to bull spermatozoa and its prevention to catalase. J. Dairy Sci. 32:353-360.

Villalon, C. M., D. Centurion, L. F. Valdivia, P. De Vries, and P. R. Saxena. 2002. An introduction to migraine: From ancient treatment to functional pharmacology and antimigraine therapy. Proc. West. Pharmacol. Soc. 45:199-210.

Voglmayr, J. K., and R. F. Sawyer Jr. 1986. Surface transformation in ram spermatozoa in uterine, oviduct and cauda epididymal fluid in vitro. J. Reprod. Fertil. 78:315-325.

Wang, H., M. L. Looper, Z. B. Johnson, R. W. Rorie, C. F. Rosenkrans, Jr. 2009. Involvement of signaling pathways in bovine sperm motility, and effect of ergot alkaloids. In Vitro Cell. Dev. Biol. 45:483-489.

Wasco, W. M., R. L. Kincaid, and G. A. Orr. 1989. Identification and characterization of calmodulin-binding proteins in mammalian sperm flagella. J. Biol. Chem. 264:5104-5111.

Washburn, S. P., J. T. Green, Jr., and B. H. Johnson. 1989. Effects of endophyte presence in tall fescue on growth, puberty, and conception in Angus heifers. In: Tall fescue toxicosis workshop. November 13-14. p. 80. Southern Region Information Exchange Group 37 (SRIEG-37). Atlanta, GA.

Washburn, S. P., and J. T. Green, Jr. 1991. Performance of replacement beef heifers on endophyte-infected fescue pastures. In: Proc. 40th Annu. Conf. North Carolina State Cattlemen's Assoc. Ferbruary 25-26. North Carolina State Univ. Raleigh.

Wassarman, P. M. 1995. Sperm-egg recognition mechanisms in mammals. Curr. Top. Dev. Biol. 30:1-19.

Watson, P. F. 1981. The roles of lipids and protein in the protection of ram spermatozoa at 5º C by egg yolk lipoprotein. J. Reprod. Fertil. 62:483-492.

Watson, P. F. 1995. Recent developments in concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod. Fertil. Dev. 7:871-891.

West, C. P., E. Izekor, R. T. Robbins, R. Gergerich, and T. Mahmood. 1990. *Acremonium coenophialum* effects on infestations of Barley Yellow Dwarf Virus and soil-borne nemotodes and insects in tall fescue. p. 196-198. In: S. S. Quisenberry and Joost (Ed.) Proc. Intl. Symp. On *Acremonium*/Grass interactions. Baton Rouge, LA. 3 Nov. 1990. Louisiana Agric. Exp. Stn. Baton Rouge.

White, D. R., and R. J. Aitken, 1989. Relationship between calcium, cyclic AMP, ATP and intracellular pH and the capacity of hamster spermatozoa to express hyperactivated motility. Gamete Res. 22:163-177.

White, D. R., J. S. Clarkson, W. D. Ratnasooriya, and R. J. Aitken. 1995. Complementary effects of propranolol and nonoxynol-9 upon human sperm motility. Contraception. 52:241-247.

Yanagimachi, R., and N. Usui. 1974. Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. Exp. Cell. Res. 89:161-174.

Yanagimachi, R. 1994. Mammalian fertilization. In Knobil, E. and Neill, J. D. (eds). The Physiology of Reproduction. 2nd edn. Raven Press Ltd. New York. 189-317.

Yenilmez, E., S. Yildirmis, E. Yulug, S. Aydin, Y. Tekelioglu, E. Erdem, M. Topbas, and H. Arvos. 2006. Ham's F-10 medium and Ham's F-10 medium plus vitamin E have protective effect against oxidative stress in human semen. Urology. 67:384-387.

Yoshida, K., T. Yamasaki, M. Yoshiike, S. Takano, I. Sato, and T. Iwamoto. 2003. Quantification of seminal plasma motility inhibitor/semenogelin in human seminal plasma. J. Androl. 24:878-884.

Yousef, M. I., G. A. Abdallah, and K. I. Kamel. 2003. Effect of ascorbic acid and vitamin E supplementation on semen quality and biochemical parameters of male rabbits. Anim. Reprod. Sci. 76:99-111.

Zini, A., E. de Lamirande, and C. Gagnon. 1993. Reactive Oxygen species in semen of infertile patients: levels of superoxide dismutase- and catalase-like activities in smeinal plasma and spermatozoa. Int. J. Androl. 16:183-188.

Zorn, B., G. Vidmar, and H. Meden-Vrtovec. 2003. Seminal reactive oxygen species as predictors of fertilization, embryo quality and pregnancy rates after conventional in-vitro fertilization and intracytoplasmic sperm injection. Int. J. Androl. 26:279-285.

CHAPTER 2

EFFECTS OF ERGOT ALKALOIDS ON BOVINE SPERM MOTILITY

ABSTRACT

Toxic tall fescue grass has been associated with reduced reproductive rates in cattle. This study was conducted to determine the direct effects of the ergot alkaloids [ergonovine (EN), ergotamine (ET), and dihydroergotamine (DHET)] on motility of bovine spermatozoa. Spermatozoa were collected from mature Angus (n = 2) and Balancer (n = 4) bulls. Spermatozoa were washed once and resuspended in modified sperm medium (mSPTL). The experimental design was a randomized complete block, with bull serving as the block. Treatments were structured as a 3x5 factorial with three alkaloids (EN, ET, DHET) and five concentrations of each drug (0 µM, 33 µM, 66 µM, 100 µM, 200 µM). Spermatozoa (25×10^6) were incubated in 1 mL of mSPTL with treatment at 39° C. Sperm motility characteristics were evaluated at 0 h, 3 h, and 6 h using CASA (Hamiliton Thorne IVOS, Beverly, MA). Initial sperm motility was (69 ± 1.7 %) and declined to (35 ± 2.6 %) at 6 h. Percentage of motile spermatozoa was affected ($P = 0.015$) by a three way interaction between time, concentration, and alkaloid. Sperm motility decreased ($P < 0.01$) over time and with increasing concentrations of alkaloid with the exception of EN. The number of static spermatozoa also was affected ($P < 0.01$) by a three way interaction and increased as ET and DHET concentrations increased. Percentages of progressively motile and rapidly motile spermatozoa decreased ($P < 0.01$) in a two way interaction between alkaloid and concentration. Overall, sperm motility was decreased by ET and DHET. Furthermore, the qualities of motility as represented by progressive, rapid, path velocity (VAP), progressive velocity (VSL), track speed (VCL), beat frequency (BCF), lateral amplitude (ALH), straightness (STR),

elongated, and area were decreased by those alkaloids. These data verify that ergot alkaloids commonly found in toxic tall fescue are detrimental to bovine spermatozoa.

Introduction

It has been well documented that cattle consuming toxic tall fescue may suffer from numerous physiological consequences, resulting in significant economic losses for cattle producers annually (Paterson et al., 1995). Three-fourths of all tall fescue pastures in the Unites States are infested with endophyte at a level of at least 60% (Oliver et al., 2000). Ergot alkaloids located within the endophytic fungus have been associated with toxic effects on livestock (Lipton, 1997; Jones et al., 2003). More specifically, they have been linked to depressed reproductive performance (Porter and Thompson, 1992; Browning et al., 1998; Burke et al., 2001; Jones et al., 2003). Much of the previous research has focused on the reproductive parameters of females, such as altered endocrine profiles (Browning et al., 1998; Burke et al., 2001), changes in the estrous cycle (Jones et al., 2003) and decreased progesterone concentrations (Peters et al., 1992). More recently, more attention has been shifted toward the effects of endophyte infected (EI) fescue on male reproduction (Schuenemann et al., 2005; Looper et al., 2009).

Jones et al. (2004) showed that bulls supplemented with toxic fescue seed had increased scrotal temperatures and decreased scrotal circumference (SC). Also, Brahman-influenced bulls consuming EI fescue had decreased sperm motility during the summer months (Looper et al., 2009), and the consumption of ergot alkaloids reduced the fertilizing capabilities of spermatozoa (Schuenemann et al., 2005). The previously stated studies were structured around whole animal trials, and they suggest that toxic fescue can alter sperm function and fertilizing capabilities. However, they do not fully illustrate

what influence ergot alkaloids may have on the male gamete. Wang et al. (2009) showed certain ergot alkaloids do use specific signaling pathways to interact with spermatozoa. Therefore, the objective of this study was to further investigate the effects ergot alkaloids have on bovine sperm motility characteristics by using computer assisted sperm analysis (CASA).

Materials and Methods

Semen Collection and Preparation

Semen was collected from mature Angus (n = 2) and Balancer (n = 4) bulls at 0700 h via electro-ejaculation (Electroejac IV) and placed in a 15-mL conical centrifuge tube. Ejaculates were transported to the lab in a 39º C water bath where spermatozoa were centrifuged at 750 x g for 10 min. Seminal plasma was removed, and spermatozoa were washed once and re-suspended in modified sperm TALP (mSPTL) (Parrish et al., 1988). The mSPTL was prepared prior to collection and consisted of: NaCL (49.5 mM), KCL (1.5 mM), NaH_2PO_4 (0.17 mM), $CaCL_2 \cdot 2H_2O$ (0.10 mM), $MgCL_2 \cdot 2H_2O$ (0.055 mM), 5.25% $NaHCO_3$ (0.16 ml), HEPES (10 mM), Na-pyruvate (1 mM), 60% Na-lactate syrup (21.6 mM), gentamicin (0.05 mg), EGTA (2 mM), and PVA (0.05 mg) with pH adjusted to 7.4 and an osmolarity of ~300 mOsm. Spermatozoa were diluted 25:1, counted using an integrated visual optical system [(IVOS) Hamilton-Thorne Biosciences, Beverly, MA], and placed in experimental treatments (25 x 10^6 sperm/ml).

Preparation of alkaloid Treatments

All alkaloids were prepared directly prior to incubation with spermatozoa. Methanol (100%) was used as the solvent to prepare each alkaloid [ergonovine (EN), ergotamine (ET), dihydroergotamine (DHET)]. The stock solutions were aliquoted into

experimental concentrations (0, 33, 66, 100, 200 µM) in sterile flat-bottom 24-well tissue culture plates and evaporated. Alkaloids were resuspended in mSPTL.

Experimental Design

Experimental design was a randomized complete block, with bull serving as the block. Treatments were structured as a 3 x 5 factorial with three alkaloids (EN, ET, DHET) and five concentrations of each alkaloid (0, 33, 66, 100, 200 µM). Spermatozoa (25×10^6) were incubated in 1 mL of mSPTL with treatment at 39º C in an atmosphere of humidified air. Sperm motility characteristics were evaluated at 0, 3, and 6 h of incubation. Spermatozoa were evaluated by placing them on a warm slide and assessed using a Hamilton Thorne IVOS (Hamilton-Thorne Biosciences, Beverly, MA) utilizing Animal Motility Software, version 12.1.

Statistical Analysis

Sperm motility characteristics were analyzed using mixed model procedure (SAS). Bull served as the block, experimental unit was the concentration within alkaloid, and time was the repeated measure. If the F-test were significant ($P < 0.05$), means were separated using multiple t-tests.

Results

Sperm motility was inhibited by a three-way interaction between hour, alkaloid, and concentration. Both ET and DHET reduced sperm motility in a concentration and time dependent manner ($P < 0.05$). When compared to control spermatozoa (0 µM alkaloid), spermatozoa exposed to ET (≥ 33 µM) for 3 h and (≥ 66 µM) for 6 h were less motile ($P < 0.05$; Figure 2.1). Similar affects were observed for DHET, as concentrations of 66 µM and above reduced ($P < 0.05$) sperm motility (Figure 2.2). Ergonovine had

minimal effects on sperm motility until the 6 h observation at 200 µM ($P < 0.05$). Similarly, static spermatozoa also were affected by a three-way interaction between alkaloid, concentration, and time. As ET and DHET concentrations increased, the number of static spermatozoa also increased (Table 2.2). For progressive and rapid spermatozoa, a two-way interaction was observed. Both ET and DHET reduced ($P < 0.05$) the percentage of progressive (Figure 2.3) and rapid (Figure 2.4) spermatozoa when concentrations reached 66 µM and greater. Further, sperm motility characteristics VAP, VSL, VCL, ALH, BCF, STR, elongated, and area (as described in Table 2.1) also were inhibited. We observed a three-way interaction for both VAP and VCL, and the others were involved in two-way interactions (Table 2.3, 2.4, 2.5).

Discussion

There has been conflicting data published showing the effects of toxic agents found in EI fescue on the male gamete, but our results demonstrate that ergot alkaloids can directly affect bovine sperm motility. More specifically, ET and DHET reduced motile, progressive, rapid, and static spermatozoa and altered multiple other sperm characteristics associated with sperm viability. These data provide a possible explanation for decreased conception rates and declined reproductive performance amongst cattle grazing toxic fescue. As we try to discover different alternatives to combat fescue toxicosis, it is important that we understand all mechanisms and specific alkaloids that could possibly reduce the animals reproductive capabilities.

The altered sperm parameters observed in our study were somewhat similar to Wang et al. (2009); however, their method of evaluating sperm motility utilized subjective measures. According to Farrell et al., (1998), the repeatability and consistency

within each sperm evaluation is likely to be more accurate using CASA rather than subjective measures. In fact, Farrell's group reported a repeatability of 0.99 when using CASA. This also could provide a valid explanation for the contrasting results encountered with many other trials. For example, with the use of a microscope, Schuenemann et al., (2005) documented that sperm motility and morphology was not affected when bulls were supplemented with ET in their diet. However, in the current study, ET proved to significantly reduce sperm motility.

The use of CASA allowed us to evaluate both quality and quantity of sperm motility. Although sperm movement is important, it is not the only criteria necessary for a sperm to fertilize an oocyte. The ability of the sperm to progress forward into the reproductive tract in an efficient manner also is critical to achieve conception (Hinting et al., 1988). Results, in this report, not only showed declines in overall motility, but we also observed reductions in progressive and rapid spermatozoa. These results confirm our earlier work where we observed the average velocity of the smoothed sperm path as well as the average velocity measured over the actual point to point track became slower with elevated temperatures and increased *in-vivo* exposure to ergot alkaloids (Looper et al., 2009). We also demonstrated that the percentages of static spermatozoa increased due to the effects of ET and DHET. It appears that ET and DHET exasperate the intracellular energy of the sperm, and thereby, accelerate the pace at which sperm undergo apoptosis. We also observed morphological changes in the size and shape of the sperm head as exposure time and alkaloid concentration increased.

The previously mentioned alterations in scrotal temperature and SC along with changes in prolactin concentrations are just a few of the physiological changes that occur

after ingestion of EI fescue (Hurley et al., 1981; Jones, 2004), and these changes may be, in part, responsible for reducing sperm viability under normal physiological conditions. It is known that thermal regulation of the testis and prolactin levels are both important factors that can regulate the development of sperm (Waites et al., 1970; Barth and Oko, 1989; Karabinus et al., 1997; Hair et al., 2002).

In two of the more current whole animal studies, both Looper et al., (2009) and Jones et al., (2004) observed a small decline in sperm motility. Although it is important to understand how cattle react to toxic agents under normal grazing conditions, it is important to note that breed type and exposure period may have affected their results. Looper's trial utilized Brahman-influenced bulls which are known for their heat tolerance (Blackshaw and Blackshaw, 1994). Therefore, it may be that Brahman-influenced bulls withstand the toxic effects of EI fescue better than other breed types. Even though sperm motility was not greatly affected over the entire length of the study, Jones's article did state that motility decreased during the final two weeks of the 60 d trial. Perhaps, bulls with a longer exposure period may begin to respond differently. It is also known that elevated environmental temperatures can magnify the effects of toxic fescue (Burke et al., 2001). Ultimately, there are many factors that could possibly influence whole animal trials such as breed type, exposure period, toxicity levels, temperature, and body weight. By taking an *in-vitro* approach to this study we were able to determine if ergot alkaloids directly interact with spermatozoa.

It is still unknown exactly what mechanisms ergot alkaloids use to inhibit sperm motility. The chemical structures of the three alkaloids used in this study could possibly explain the observation differences amongst the alkaloids. Both ET and DHET are

classified as ergopeptines (Yates and Powell, 1988). In theory, it seems logical that inhibitory effects of DHET were slightly less intense than ET because DHET was originally synthesized to be a more stable version of ET for use in the pharmacological realm (Madlom, 2002). Ergonovine, the smallest structure of the three, is a simple lysergic acid amide that doesn't contain a peptide group (Madlom, 2002). Ergonovine decreased sperm motility when exposure occurred during cryopreservation (Gallagher and Senger, 1989), and increased rate of sperm transport when placed in the vagina of ewes (Hawk et al., 1984). Even though a reduction in sperm motility was observed when exposed to large amounts of EN (200 μM), overall, EN had a minimal affect on bovine sperm motility. Ergot alkaloids are lipid soluble and presumably can permeate readily across sperm membranes. Sperm motility is dependent on many cellular functions including cAMP and calcium concentrations (Lindemann, 1978; Wasco et al., 1989; Saberwal et al., 2002). It is plausible that ergot alkaloids can directly affect sperm motility by altering cAMP and calcium levels within the germ cell. Fertilizing capacity and motility also may be compromised by ergot alkaloid interaction with plasma membrane receptors on spermatozoa (Wang et al., 2009).

It seems highly unlikely that producers will eradicate EI fescue stands because of its persistence and agronomic benefits, so establishing a method to help overcome the negative consequences toxic fescue has on reproductive performance seems to be the more feasible option. To do this, scientist must retrieve more knowledge of the toxic agents and their mechanism of action. This study provides a better understanding of the role ergot alkaloids have on male reproduction. It is also one of the few studies that took an *in-vitro* approach suggesting that ergot alkaloids can directly affect bovine sperm

motility. Unfortunately, ergot alkaloid concentrations are not yet known under normal physiological conditions, but with the knowledge that these toxic agents can directly hinder sperm motility, we can further address the mechanisms used to inhibit sperm function.

Literature Cited

Barth, A. D., and R. J. Oko. 1989. Abnormal morphology of bovine spermatozoa. Iowa State University Press, Ames.

Blackshaw, J. K., and A. W. Blackshaw. 1994. Heat stress in cattle and the effect of shade on production and behavior. Australian J. of Exp. Agric. 34:285-295.

Browning, R. Jr., F. N. Schrick, F. N. Thompson, and T. Wakefield. 1998. Reproductive hormonal responses to ergotamine and ergonovine in cows during the luteal phase of the estrous cycle. J. Anim. Sci. 76:1448-1454.

Burke, J. M., D. E. Spiers, F. N. Kojima, G. A. Perry, B. E. Salfen, S. L. Wood, D. J. Patterson, M. F. Smith, M. C. Lucy, W. G. Jackson, and E. L. Piper. 2001. Interaction of endophyte-infected fescue and heat stress on ovarian function in the beef heifer. Biol. Reprod. 65:260-268.

Farrell, P. B., G. A. Presicce, C. C. Brockett, and R. H. Foote. 1998. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. Theriogenolgy. 49:871-879.

Gallagher, G. R., and P. L. Senger. 1989. Effect of phenylephrine, oxytocin, and norepinephrine as an extender ingredient on viability of bovine spermatozoa. J. Anim. Sci. 67:1573-1576.

Hair, W. M., O. Gubbay, H. N. Jabbour, and G. A. Lincoln. 2002. Prolactin receptor expression in human testis and accessory tissues: localization and function. Mol. Hum. Reprod. 8:606-611.

Hawk, H. W., B. S. Cooper, and H. H. Conley. 1984. Improvement by ergonovine of sperm transport, fertilization and pregnancy rates in ewes in natural or prostaglandin-induced estrus. J. Anim. Sci. 59:754-763.

Hinting, A., F. Comhaire, and F. Schoonjans. 1988. Capacity of objectively assessed sperm motility characteristics in differentiating between semen of fertile and subfertile men. The American Fertil. Soc. 50:635-639.

Hurley, W. L., E. M. Convey, K. Leung, L. A. Edgerton, and R. W. Hemken. 1981. Bovine prolactin, TSH, T4 and T3 concentrations as affected by tall fescue summer toxicosis and temperature. J. Anim. Sci. 51:374-379.

Jones, K. L., C. R. Mccleary, S. S. King, G. A. Apgar, and K. E. Griswold. 2004. Case Study: Consumption of toxic fescue impairs bull reproductive parameters. Prof. Anim. Sci. 20:437-442.

Jones, K. L., S. S. King, K. E. Griswold, D. Cazac, and D. L. Cross. 2003. Domperidone can ameliorate deleterious reproductive effects and reduced weight gains associated with fescue toxicosis in heifers. J. Anim. Sci. 81:2568-2574.

Karabinus, D. S., C. J. Vogler, R. G. Saacke, D. P. Evenson. 1997. Chromatin structural changes in sperm after scrotal insulation of Holstein bulls. J. Androl. 18:549-555.

Lindemann, C. B. 1978. A cAMP-induced increase in the motility of demembranated bull sperm models. Cell. 13:9-18.

Lipton, R. B., 1997. Ergotamine tartrate and dihydroergotamine mesylate: Safety profiles. Headache. 37(Suppl 1):33-41.

Looper, M. L., R. W. Rorie, C. N. Person, T. D. Lester, D. M. Hallford, G. E. Aiken, C. A. Roberts, G. E. Rattinghaus, and C. F. Rosenkrans, Jr. 2009. Influence of toxic endophyte-infected fescue on sperm characteristics and endocrine factors of yearling Brahman-influenced bulls. J. Anim. Sci. 87:1184-1191.

Madlom, Z. 2002. The origin of drugs in current use: The ergot alkaloids story. Accessed June 11, 2010. http://www.world-of fungi.org/Mostly_Medical/Ziad_ Medlom/ Ergot_ alkaloids.htm

Oliver, J. W., A. E. Schultze, B. W. Rohrbach, H. A. Fribourg, T. Ingle, and J. C. Waller. 2000. Alterations in hemograms and serum biochemical analytes of steers after prolonged consumption of endophyte-infected tall fescue. J. Anim. Sci. 78:1029-1035.

Parrish, J. J., J. Susko-Parrish, M. A. Winer, and N. L. First. 1988. Capacitation of bovine sperm by heparin. Biol. Reprod. 38:1171-1180.

Paterson, J., C. Forcherio, B. Larson, M. Samford, and M. Kerley. 1995. The effects of fescue toxicosis on beef cattle productivity. J. Anim. Sci. 73:889.

Peters C. W., F. K. Brazle, and L. R. Corah. 1992. Effect of endophyte fungus infection of tall fescue and oxytetracycline on performance, puberty, and reproductive parameters in crossbred beef heifers. J. Anim. Sci. 70:21.

Porter, J. K., and F. N. Thompson, Jr. 1992. Effects of fescue toxicosis on reproduction in livestock. J. Anim. Sci. 70:1594-1603.

Saberwal, G. S., M. K. Sharma, N. Balasinor, J. Choudhary, and H. S. Juneja. 2002. Estrogen receptor, calcium mobilization and rat sperm motility. Mol. Cell. Biochem. 237:11-20.

Schuenemann, G. M., J. L. Edwards, M. D. Davis, H. E. Blackmon, F. N. Scenna, N. R. Rohrbach, A. M. Saxton, H. S. Adair, F. M. Hopkins, J. C. Waller, and F. N.

Schrick. 2005. Effects of administration of ergotamine tartrate on fertility of yearling beef bulls. Theriogenology. 63:1407-1418.

Waites, G. M. H., A. D. Johnson, W. R. Gomes, and N. L. S. Vandemark. 1970. Temperature regulation and the testis. In The Testis. Academic Press, New York.

Wang, H., M. L. Looper, Z. B. Johnson, R. W. Rorie, C. F. Rosenkrans, Jr. 2009. Involvement of signaling pathways in bovine sperm motility, and effect of ergot alkaloids. In Vitro Cell. Dev. Biol. 45:483-489.

Wasco, W. M., R. L. Kincaid, and G. A. Orr. 1989. Identification and characterization of calmodulin-binding proteins in mammalian sperm flagella. J. Biol. Chem. 264:5104-5111.

Yates, S. G., and R. G. Powell. 1988. Analysis of ergopeptine alkaloids in endophyte-infected tall fescue. J. Agric. Food Chem. 36 (2):337-340.

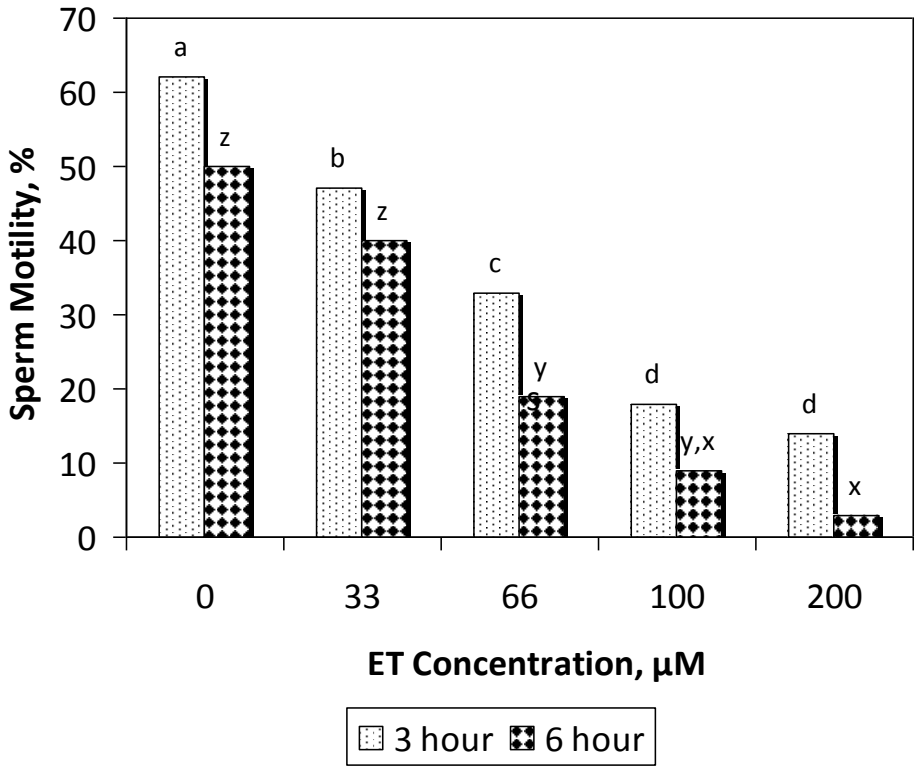

Figure 2.1 Effects of ergotamine (ET) concentration and incubation time on bovine sperm motility. Spermatozoa (25 x 10^6 sperm/ml) were incubated with ET at various concentrations (0-200 µM) in modified sperm TALP (mSPTL). Sperm motility characteristics were evaluated at 0, 3, 6 h. Initial motility was 68% and SEM = 4.1. Superscripts a,b,c,d are designated to the 3 h column and superscripts z,y,x are designated to the 6 h column. Values in columns (within an evaluation time) without a common superscript letter differ ($P < 0.05$).

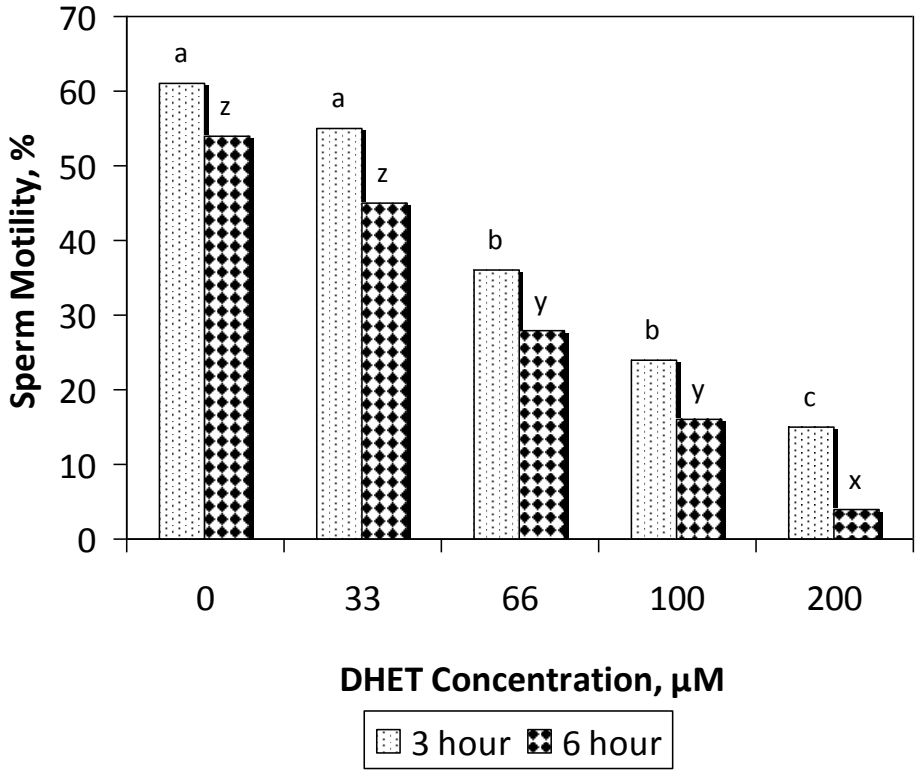

Figure 2.2 Effects of dihydroergatamine (DHET) concentration and incubation time on bovine sperm motility. Spermatozoa (25 x 10^6 ml^{-1}) were incubated with DHET at various concentrations (0-200 µM) in modified sperm TALP (mSPTL). Sperm motility characteristics were evaluated at 0, 3, 6 h. Initial sperm motility was 67% and SEM = 4.1. Superscripts a,b,c are designated to the 3 h column and superscripts z,y,x are designated to the 6 h column. Values in columns (within an evaluation time) without a common superscript letter differ ($P < 0.05$).

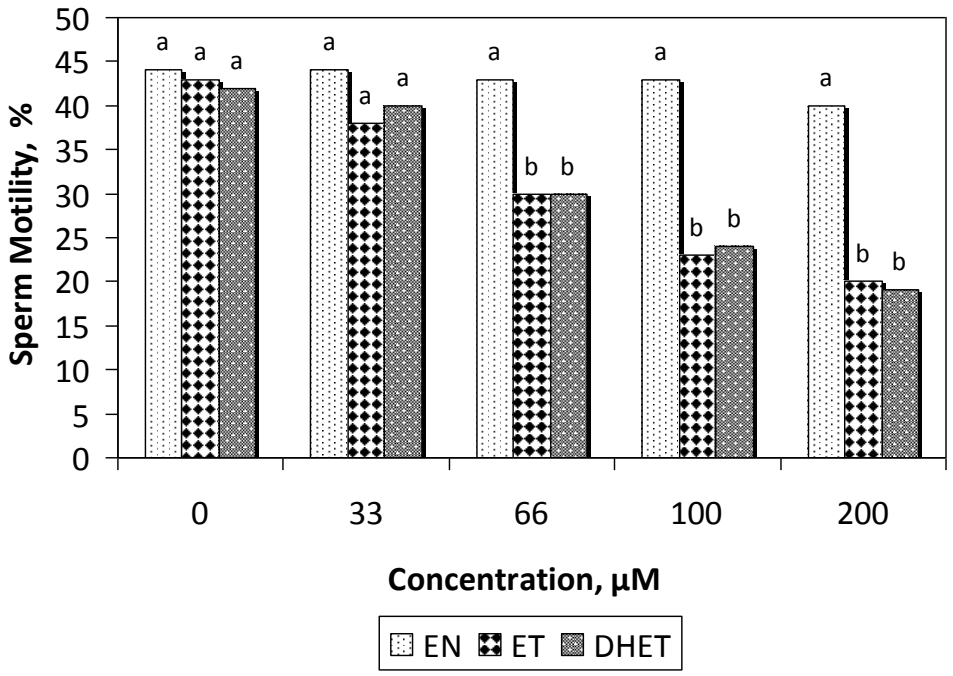

Figure 2.3 Effects of alkaloid and concentration interaction on progressive sperm motility. Spermatozoa (25 x 10^6 ml^{-1}) were incubated with alkaloid [ergonovine (EN), ergotamine (ET), dihydroergotamine (DHET)] at various concentrations (0-200 µM) in modified sperm TALP (mSPTL). SEM = 3.0. Values in columns (within concentration) without a common superscript letter differ ($P < 0.05$).

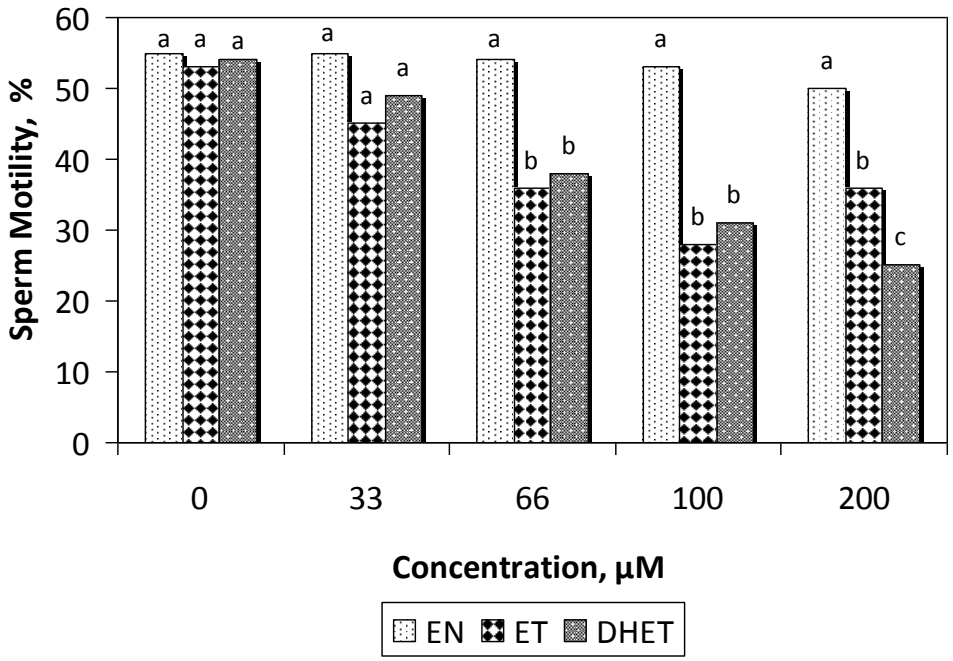

Figure 2.4 Effects of alkaloid and concentration interaction on rapid sperm motility. Spermatozoa (25 x 10^6 ml^{-1}) were incubated with alkaloid [ergonovine (EN), ergotamine (ET), dihydroergotamine (DHET)] at various concentrations (0-200 µM) in modified sperm TALP (mSPTL). SEM = 3.5. Values in columns (within concentration) without a common superscript letter differ ($P < 0.05$).

Table 2.1 Sperm Variables measured by Hamilton-Thorne IVOS (Hamilton-Thorne Biosciences, Beverly, MA)

Variable	Description
Motile	% of total sperm moving at a track velocity ≥30 µm/sec and progressive velocity ≥15 µm/sec
Progressive	% of total sperm moving at a track velocity ≥50 µm/sec and straightness ≥70%
Rapid	Progressive % with path velocity >50 µm/sec
Medium	Progressive % with path velocity <50 µm/sec but >30 µm/sec
Slow	% of total sperm moving at a path velocity <30 µm/sec and progressive velocity <15 µm/sec
Static	Sperm with no movement at all
Path Velocity (VAP)	Average velocity of the smoothed cell path (µm/sec)
Progressive Velocity (VSL)	Average velocity measured in a straight line from the beginning to the end of the track
Track Speed (VCL)	Average velocity measured over the point-to-point track
Lateral Amplitude	Mean width of the head oscillation as the sperm swims
Beat Frequency (BCF)	Frequency of sperm head crossing the sperm average path in either direction
Straightness	Measures departure of average sperm path from straight line (ratio of VSL/VAP)
Linearity	Measures departure of actual sperm path from straight line (ratio of VSL/VCL)
Elongation	Ratio of head width to head length (%)
Area	Average size in square microns of all sperm heads

Table 2.2 Three-way interaction between incubation time, alkaloid, and concentration on static bovine sperm

Time (h)	Conc. (μM)	ET	DHET	EN
3	0	21.5 [a]	25.1 [a]	22.1 [a]
3	33	30.5 [ab]	25.0 [a]	22.3 [a]
3	66	48.3 [dc]	45.6 [bc]	21.8 [a]
3	100	60.3 [ed]	55.6 [cd]	24.1 [a]
3	200	63.1 [e]	59.8 [d]	24.8 [a]
6	0	28.8 [ab]	27.5 [a]	27.0 [a]
6	33	40.8 [bc]	34.6 [ab]	23.8 [a]
6	66	61.3 [e]	46.5 [bc]	24.5 [a]
6	100	76.5 [f]	54.8 [cd]	33.5 [a]
6	200	80.5 [f]	78.0 [e]	25.6 [a]
SEM	-	4.4	4.4	4.4
P value	-	0.003	0.003	0.003

a,b,c,d,e,f superscripts within motility characteristic; means without common superscripts differ ($P < 0.05$)
Time 0 h measurements: ET (19.7), DHET (16.8), EN (15.8)

Table 2.3 Two way interaction between incubation time and concentration on bovine sperm motility characteristics

Time (h)	Conc. (µM)	VAP	VSL	VCL	BCF	STR	Elong	Area
3	0	103.7 ^{a}	85.6 ^{a}	170.8 ^{a}	31.2 ^{abc}	80.9 ^{ab}	46.7 ^{a}	5.9 ^{ab}
3	33	99.1 ^{ab}	84.8 ^{a}	159.7 ^{ab}	33.9 ^{a}	83.0 ^{a}	42.5 ^{abc}	5.9 ^{ab}
3	66	94.4 ^{ab}	78.4 ^{ab}	158.4 ^{ab}	31.4 ^{ab}	80.5 ^{ab}	42.8 ^{abc}	5.8 ^{b}
3	100	88.9 ^{b}	72.1 ^{bc}	148.9 ^{bc}	27.3 ^{cde}	79.5 ^{ab}	41.0 ^{c}	5.2 ^{b}
3	200	87.9 ^{b}	71.9 ^{bc}	144.8 ^{bc}	25.1 ^{e}	78.0 ^{ab}	41.7 ^{bc}	5.6 ^{b}
6	0	95.5 ^{ab}	79.6 ^{ab}	156.0 ^{ab}	31.1 ^{abc}	81.3 ^{ab}	45.4 ^{ab}	5.8 ^{b}
6	33	92.4 ^{b}	76.5 ^{ab}	156.1 ^{ab}	31.8 ^{ab}	80.8 ^{ab}	44.0 ^{abc}	6.5 ^{a}
6	66	88.3 ^{b}	72.5 ^{bc}	148.4 ^{bc}	29.3 ^{bcd}	81.3 ^{ab}	41.6 ^{bc}	5.8 ^{b}
6	100	75.6 ^{c}	62.3 ^{c}	131.2 ^{c}	26.6 ^{de}	75.3 ^{b}	40.3 ^{c}	5.4 ^{b}
6	200	53.4 ^{d}	43.5 ^{d}	88.1 ^{d}	23.4 ^{e}	62.0 ^{c}	32.4 ^{d}	4.4 ^{c}
SEM	-	4.0	3.9	6.6	1.4	2.5	1.6	0.23
P value	-	0.001	0.005	0.0001	0.05	0.003	0.02	0.001

a,b,c,d,e superscripts within motility characteristic; means without common superscripts differ ($P < 0.05$)

Table 2.4 Two way interaction between alkaloid and concentration on bovine sperm motility characteristics

Alkaloid	Conc. (μM)	VAP	VCL	BCF
ET	0	101.8 [a]	166.8 [a]	31.9 [ab]
ET	33	99.1 [ab]	162.9 [a]	33.5 [a]
ET	66	95.3 [ab]	157.7 [a]	30.8 [ab]
ET	100	82.0 [cd]	135.1 [b]	26.0 [de]
ET	200	71.1 [d]	120.7 [b]	26.4 [cde]
DHET	0	103.4 [a]	170.6 [a]	31.6 [ab]
DHET	33	100.3 [ab]	165.9 [a]	32.8 [a]
DHET	66	96.4 [ab]	163.0 [a]	30.4 [abc]
DHET	100	90.1 [bc]	157.7 [a]	28.1 [bcd]
DHET	200	79.9 [cd]	128.0 [b]	22.6 [e]
EN	0	105.2 [a]	172.4 [a]	32.2 [ab]
EN	33	103.2 [a]	169.9 [a]	32.8 [a]
EN	66	102.1 [a]	169.0 [a]	32.9 [a]
EN	100	103.7 [a]	170.4 [a]	33.2 [a]
EN	200	101.8 [a]	167.2 [a]	32.8 [a]
SEM	-	4.0	6.6	1.4
P value	-	0.02	0.02	0.01

Ergotamine (ET), Dihydroergotamine (DHET), Ergonovine (EN)
a,b,c,d,e superscripts within motility characteristic; means without common superscripts differ ($P < 0.05$)

Table 2.5 Two way interaction between alkaloid and incubation time on bovine sperm motility characteristics

Alkaloid	Time (h)	VAP	VCL	ALH
ET	0	109.8 ^{ab}	181.3 ^{ab}	6.6 ^{a}
ET	3	87.8 ^{d}	146.0 ^{d}	5.8 ^{a}
ET	6	72.0 ^{e}	118.2 ^{e}	4.6 ^{b}
DHET	0	111.3 ^{a}	184.8 ^{a}	6.5 ^{a}
DHET	3	94.3 ^{cd}	155.1 ^{cd}	5.9 ^{a}
DHET	6	76.5 ^{e}	131.3 ^{e}	5.9 ^{a}
EN	0	112.7 ^{a}	182.5 ^{ab}	6.4 ^{a}
EN	3	102.2 ^{bc}	168.5 ^{bc}	6.3 ^{a}
EN	6	94.6^{cd}	158.4^{cd}	6.2 ^{a}
SEM	-	3.1	5.1	0.29
P value	-	0.02	0.004	0.04

Ergotamine (ET), Dihydroergotamine (DHET), Ergonovine (EN)
a,b,c,d,e within motility characteristic; means without common superscripts differ ($P < 0.05$)

CHAPTER 3

ADDITION OF ALPHA TOCOPHEROL AND ASCORBIC ACID TO BOVINE SPERM CULTURE MEDIA AND CRYOPRESERVATION EXTENDER

ABSTRACT

The purpose of this study was to determine if ascorbic acid and α-tocopherol, both individually and in combination, prevent sperm damage during different storage methods; and if so, what are the optimal concentrations at which they should be supplemented. Semen was collected from mature Angus bulls (n = 4), washed once, and placed into experimental treatments. The experimental design was a randomized complete block, with bull serving as the block. Treatments were structured as a 4x4 factorial with four concentrations of ascorbic acid (0, 5, 10, 20 mM) and four concentrations of α-tochopherol (0, 0.05, 0.5, 5 mM). Spermatozoa were either incubated with treatment in modified sperm TALP (mSPTL) or cryopreserved in liquid nitrogen using egg yolk extender. Sperm motility characteristics were evaluated using computer assisted sperm analyses (CASA) (Hamiliton Thorne IVOS, Beverly, MA) at 0, 4, and 8 h of incubation with treatment and post cryopreservation. Initial sperm motility was (79.8% $\pm$ 1.6) and decreased to (4.9 %$\pm$ 1.6) after cryopreservation. There was an interactive effect between ascorbic acid concentration and storage method on the bovine sperm motility characteristics: motile, progressive, rapid, track speed (VCL), and straightness ($P < 0.05$). A bull by ascorbic acid interaction ($P < 0.05$) was observed for motile, progressive, rapid, path velocity (VAP), progressive velocity (VSL), and VCL characterstics. Alpha tocopherol had no affects on sperm motility characteristics. Lipid peroxidation was affected ($P < 0.01$) by bull and ascorbic acid. Malondialdehyde concentrations for ascorbic acid treatments (0, 5, 10, 20 mM) were 3.25, 4.2, 2.96, and 2.15 µM respectively (Figure 3.1). These data suggest that ascorbic acid can alter sperm motility and lipid peroxidation during storage of bovine spermatozoa.

Introduction

Current semen processing techniques have been linked to decreased sperm viability, and therefore, are associated with declines in bovine conception rates (Sharma and Agarwal, 1996; Agarwal et al., 2006). The ability of spermatozoa to navigate the female reproductive tract and penetrate the oovum is essential for fertilization to occur. Semen processing techniques associated with artificial reproductive techniques (ART) reduce sperm motility and induce cellular damage (Agarwal et al., 2006). Bilodeau et al. (2000) reported that cryopreservation depletes spermatozoa of their natural antioxidant supply, which results in increased cellular damage. Under these circumstances, free radical production is increased resulting in heightened oxidative stress (Trinchero et al., 1990; Agarwal et al., 1994). Research is needed to help identify possible storage modifications that may prevent sperm damage.

Antioxidants have scavenging abilities that help quench free redicals (Askari et al., 1994; Dalvit et al., 1998; Bansal and Bilaspuri, 2009). Therefore, previous research has utilized antioxidant supplementation in sperm cultures mediums and cryopreservation extenders to help prevent oxidative stress (Foote et al., 2002; O'Flaherty et al, 1997; Beconi et al., 1993). For example, cysteine has shown to have beneficial affects on sperm function (Meister and Tate, 1976), and superoxide dismutase (SOD) was found to be a useful antioxidant for preventing premature capacitation (O'Flaherty et al., 1997). Supplementation of other natural antioxidants, such as ascorbic acid and α-tocopherol, have produced conflicting results (Dalvit et al., 1998; Uysal et al., 2007; Anghel et al., 2009; Bansal and Bilaspuri, 2009), suggesting there is and optimal threshold at which antioxidant supplementation is beneficial. The purpose of this study was to determine if

ascorbic acid and α-tocopherol, both individually and in combination, prevent sperm damage; and if so, what are the optimal concentrations at which they should be supplemented.

Materials and Methods

Semen Collection and Preparation

Semen was collected from mature Angus bulls (n = 4) at 0700 hours via electro-ejaculation (Electroejac IV) and placed in 15 mL conical centrifuge tube. Ejaculates were transported to the lab in 39º C water bath and centrifuged at 750 x g for 10 min. Seminal plasma was removed, and spermatozoa were washed once and re-suspended in either modified sperm TALP (mSPTL) (Parrish et al., 1988) or fraction A of the cryopreservation extender, depending on storage method. All culture media and extender was prepared prior to semen collection. The previously prepared mSPTL consisted of: NaCL (49.5 mM), KCL (1.5 mM), NaH_2PO_4 (0.17 mM), $CaCL_2 \cdot 2H_2O$ (0.10 mM), $MgCL_2 \cdot 2H_2O$ (0.055 mM), 5.25% $NaHCO_3$ (0.16 ml), HEPES (10 mM), Na-pyruvate (1 mM), 60% Na-lactate syrup (21.6 mM), gentamicin (0.05 mg), EGTA (2 mM), and PVA (0.05 mg) with pH adjusted to 7.4 and an osmolarity of ~300 mOsm. Spermatozoa (25 x 10^6) placed in mSPTL were incubated with treatments (1 ml) in 5 ml tubes and evaluated at 0, 4, and 8 h post exposure to treatment. Spermatozoa used for cryopreservation were placed in fraction A (1 ml) of the extender and cooled to 5º C.

Cryopreservation

A stock solution of sterile extender containing TRIS Base (200 mM), citric acid monohydrate (6.6 mM), D-fructose (55.5 mM) was made. Two fractions of sperm extender were prepared with a pH of 6.56 and an osmolarity of ~290 mOsm. Fraction A

consisted of sterile extender stock solution and egg yolk (20%). Fraction B contained heat treated organic milk, and glycerol (14%). Spermatozoa (2×10^8) were placed in 5 ml screw top tubes containing fraction A and treatment (1 ml). Treatments were stored in the cool room until their temperature reached 5º C. Fraction B (5º C), containing experimental treatment, was added step wise every 10 min [10% (100 µl), 20% (200 µl), 30% (300 µl), 40% (400 µl)] until the final volume of extender reached 2 ml (1 ml fraction A, 1 ml fraction B). The samples were left to equilibrate for 3 h. The extended spermatozoa (50×10^6/0.5 ml) were loaded into 0.5 ml straws and placed in a rack to prepare for cryopreservation. The heat sealed straws were lowered into liquid nitrogen vapor at -15º C/min (1 inch/min) starting at -10º C and then held for 10 min at -100º C. Straws were then plunged into liquid nitrogen and stored for evaluation at a later date.

Preparation of Antioxidant Treatments

All antioxidant were purchased from Sigma Aldrich and treatments were prepared prior to incubation with spermatozoa. Antioxidants (ascorbic acid and α-tochopherol) were diluted in mSPTL or cryopreservation extender, both individually and in combination, to form stock solutions. Sixteen stock solutions were prepared for each mSPTL, fraction A, and fraction B.

Experimental Design

Treatments were structured as a 4x4 factorial with four concentrations of ascorbic acid (0, 5, 10, 20 µM) and four concentrations of α-tochopherol (0, 0.05, 0.5, 5 µM). Sperm motility characteristics were assessed at 0 h, 4 h, and 8 h of incubation with treatment and immediately after thawing cryopreserved spermatozoa. Sperm motility characteristics were evaluated by placing them on a warm slide and assessed using a

Hamilton Thorne IVOS (Hamilton-Thorne Biosciences, Beverly, MA) utilizing Animal Motility Software, version 12.1. Following motility measurements, cryopreserved spermatozoa were assessed for lipid peroxidation.

Lipid Peroxidation Assay

Lipid peroxidation was measured using thiobarbituric acid reactive substances kit (TBARS Assay Kit, Cayman Chemical Company, Ann Arbor, MI). Spermatozoa were thawed in a water bath (39º C), diluted in warm phosphate-buffered saline (PBS) (1 ml), and centrifuged at 1000 x g for 5 min. The cryopreservation extender was removed and spermatozoa were re-suspended in PBS. Samples, containing 25 x 10^6 cells, were sonicated utilizing a Vibra Cell (Sonics and Materials Inc., Baton Rouge, LA) three times for 5 sec intervals at 40 V setting over ice. Samples were boiled for 1 h and then placed on ice for 10 min. Samples were then centrifuged for 10 min at 1,600 x g at 4º C. Duplicates of each sample (150 µl) were loaded into a clear 96-well plate and malondialdehyde (MDA) concentrations were determined using a Spectra Max 250 plate reader (Molecular Devices, Sunnyvale, CA). Absorbance was read at 530 nm.

Statistical Analysis

Sperm motility characteristics were analyzed using mixed model procedure (SAS). Bull served as the block, experimental unit was the antioxidant concentration, and time was the repeated measure. If the F-test were significant ($P < 0.05$), means were separated using multiple t-tests.

Results

There was an interactive effect between ascorbic acid concentration and storage method on sperm motility characterstics: motile, progressive, rapid, VCL and straightness

($P < 0.05$) (Table 3.2). As concentrations of ascorbic acid increased, sperm motility characteristics decreased. Initial sperm motility was 79.8% $\pm$ 1.6% and declined to 4.9% $\pm$ 1.6% following cryopreservation. Spermatozoa were less ($P < 0.05$) progressive and slower moving when exposed to ascorbic acid. When 5 mM of ascorbic acid was added during cryopreservation, the track speed declined (109.9 $\pm$ 4.1 µm/sec) and then rebounded to at 10 mM (124.5 µm/sec $\pm$ 4.1). A bull by ascorbic acid interaction ($P < 0.05$) was observed for motile, progressive, rapid, VAP, VSL, and VCL characterstics, indicating that spermatozoa from different bulls may react differently to adverse conditions. Alpha tocopherol had no affects on sperm motility characteristics and there was no interaction between antioxidants observed. Lipid peroxidation was affected ($P < 0.01$) by bull and ascorbic acid. Malondialdehyde concentrations for ascorbic acid treatments (0, 5, 10, 20 mM) were 3.25, 4.2, 2.96, and 2.15 µM respectively. It appears that ascorbic acid acted as a pro-oxidant at 5 mM, but served as an antioxidant at concentrations 10 mM and above.

Discussion

Semen processing techniques can reduce the natural antioxidant supply normally found in semen (Alvarez and Storey, 1992; Bilodeau et al. 2000). Multiple studies have been performed to determine if antioxidant supplementation can help counter this issue. We wanted to focus on multiple sperm motility characteristics along with the lipid membrane of the sperm, while exploring several storage options. Our results indicate that the addition of ascorbic acid has an interactive affect on bovine sperm motility characteristics, but α-tocopherol on the other hand, showed no effects on sperm motility. Previous work by Beconi et al., (1993) indicated that 5 mM of ascorbic acid can benefit

bovine sperm motility, but our data was inconsistent on which concentrations were beneficial and which were detrimental. Furthermore, α-tocopherol has shown to be beneficial to ram spermatozoa at concentrations of 0.1 mM and 1.0 mM (Anghel et al., 2009) and to bovine spermatozoa at 2 mM (Bansal and Bilaspuri, 2009), which contradicts our results. It's possible that ram sperm reacts differently to α-tocopherol than bovine spermatozoa. Also, part of these observation differences may be due to differences in evaluation techniques.

There is evidence that ascorbic acid can serve both as a pro-oxidant and an antioxidant (Niki, 1991). We are in agreement with Reese and Slater (1987) that ascorbic acid serves as a pro-oxidant at lower concentrations and gains its antioxidant capabilities at higher concentrations, but their optimum threshold was between 1 to 2 mM for sperm culture and ours were much higher. In our study, ascorbic acid didn't uphold its antioxidant responsibilities until 10 mM and higher. Ascorbic acid tended to promote lipid peroxidation at 5 mM, but then reduced lipid peroxidation at both 10 mM and 20 mM.

It is intriguing that ascorbic acid decreased sperm motility characteristics but prevented lipid peroxidation. We have proof that ascorbic acid acted as a scavenger and reduced free radicals, but the net result still lead to reduced sperm motility during extended storage methods. This could mean that free radical production is not the only major issue to be concerned with during sperm storage. It also may be that the ascorbic acid alone wasn't capable of eliminating all free radical induced damage.

According to our results, individual bulls react differently to ascorbic acid. It could be that bulls are naturally equipped with different concentrations of antioxidants. It

could also be due to the difference in initial sperm quality. Previous studies have shown that higher quality semen benefits more from antioxidant supplementation than lower quality semen (Beconi et al., 1993). We evaluated initial motility differences directly after ejaculation, but we did not perform other initial evaluations that are linked to sperm viability. Further research should be conducted to determine if bulls poor quality semen react differently to ascorbic acid.

Although sperm cryopreservation and ART are already commonly used throughout the United States, finding ways to eliminate stressful conditions for spermatozoa during storage could significantly benefit the entire cattle industry. Further investigations must be performed to find alternative ways to protect the male gamete.

Literature Cited

Agarwal, A., I. Ikemoto, K. R. Loughlin. 1994. Effect of sperm washing on levels of reactive oxygen species in semen. Arch. Androl. 33:157-162.

Agarwal, A., T. M. Said, M. A. Bedaiwy, J. Banerjee, and J. G. Alvarez. 2006. Oxidative stress in an assisted reproductive techniques setting. Fertil. Steril. 86:503-513.

Alvarez, J. G., and B. T. Storey. 1992. Evidence for increased lipid peroxidation damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. J. Androl. 13:232-241.

Anghel, A., S. Zamfirescu, D. Coprean, and E. Sogorescu. 2009. The effects of cystein, bovine serum albumin and vitamin E on the calitative parameters of frozen-thawed ram semen. Annals of RSCB. 14:97-103.

Askari, H. A., J. H. Check, N. Peymer, and A. Bollendorf.1994.Effect of natural antioxidants tocopherol and ascorbic acids in maintenance of sperm activity during freeze-thaw process. Arch. Androl. 33:11-15.

Bansal, A. K., and G. S. Bilaspuri. 2009. Antioxidant effect of vitamin E on motility, viability and lipid peroxidation of cattle spermatozoa under oxidative stress. Anim. Sci. Papers and Reports. 27:5-14.

Beconi, M. T., C. R. Francia, N. G. Mora, M. A. Affranchino. 1993. Effect of natural antioxidants of frozen bovine semen preservation. Theriogenology. 40:841-851.

Bilodeau, J., S. Chatterjee, M. Sirard, and C. Gagnon. 2000. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. Mol. Reprod. Dev. 55:282-288.

Dalvit, G. C., P. D. Cetica, and M. T. Beconi. 1998. Effect of α-tocopherol and ascorbic acid on bovine in vitro fertilization. Theriogenology. 49:619-627.

Foote, R. H., C. C. Brockett, and M. T. Kaproth. 2002. Motility and fertility of bull sperm in whole milk extender containing antioxidants. Anim. Reprod. Sci. 71:13-23.

Graham, J. K., and R. H. Hammerstedt. 1992. Differential effects of butylated hydroxytoluene analogs on bull sperm subjected to cold induced membrane stress. Crybiol. 29:106-117.

Meister, A, and S. S. Tate. 1976. Glutathione and related gamma-glutamyl compounds; biosynthesis and utilization. Ann. Rev. Biochem. 45:559-604.

Niki, E. 1991. Action of ascorbic acid as a scavenger of active and stable oxygen radicals. American J. Clin. Nutrition. 54:1119S-1124S.

O'Flaherty, C. M., M. Beconi, and N. Beorlegui. 1997. Effect of natural antioxidants, superoxide dismutase and hydrogen peroxide on capacitation of frozen-thawed bull spermatozoa. Andrologia. 29:269-275.

Parrish, J. J., J. Susko-Parrish, M. A. Winer, and N. L. First. 1988. Capacitation of bovine sperm by heparin. Biol. Reprod. 38:1171-1180.

Reese, S. and T. F. Slater. 1987. Ascorbic acid and lipid peroxidation; the crossover effect. Acta. Biochem. Biophys. Hung. 22:241-249.

Sharma, R. K., and A. Agarwal. 1996. Role of reactive oxygen species in male infertility. Urology. 48:835-850.

Trinchero, G. D., M. A. Affranchino, L. M. Schang, and M. T. Beconi. 1990. Antioxidant effect of bovine spermatozoa on lipid peroxidation. Com. Biol. 8:339-350.

Uysal, O., M. N. Bucak, and I. Yavas. 2007. Effect of various antioxidants on the quality of frozen-thawed bull semen. J. Anim. Vet. Advanves. 6:1362-1366.

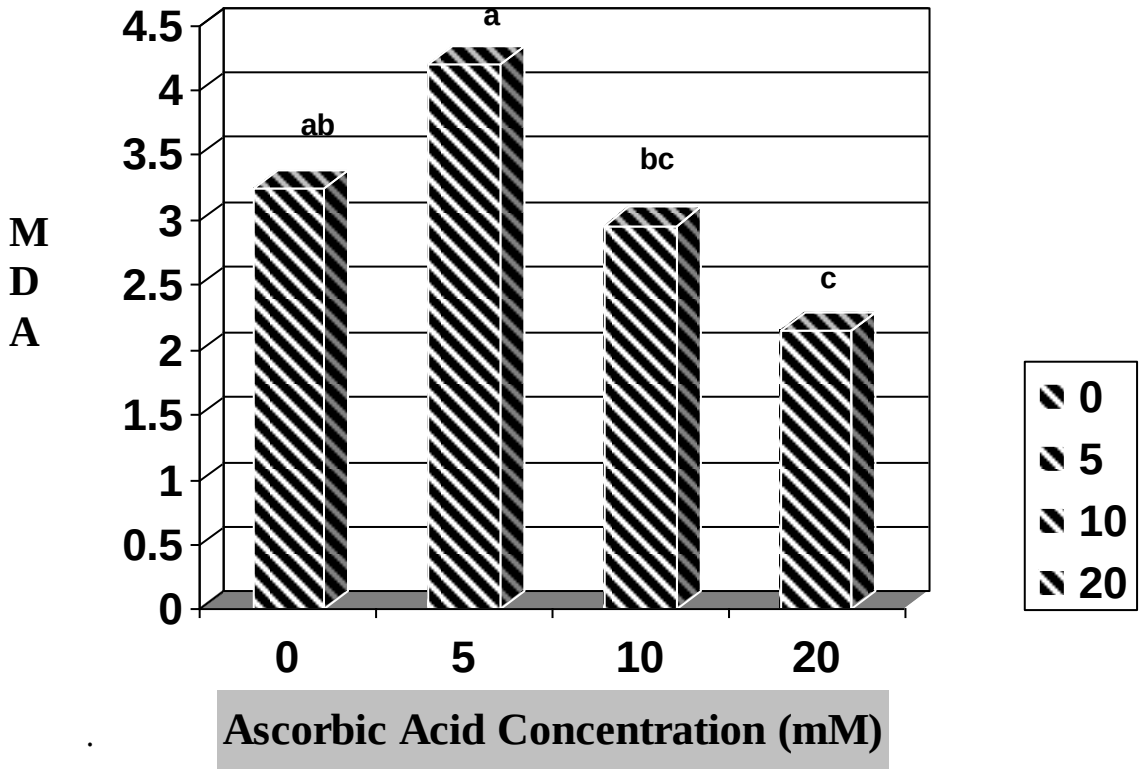

Figure 3.1 Effects of ascorbic acid on lipid peroxidation. Spermatozoa were cryopreserved in the presence of ascorbic acid (0, 5, 10, 20 mM) and Malondialdihyde (MDA) concentrations were measured to determine lipid peroxidation. Columns without common superscripts differ by ($P < 0.05$).

Table 3.1 Interactive effects of ascorbic acid concentration and storage method on sperm motility characteristics.

		Sperm Motility Characteristic				
Ascorbic Acid, mM	Storage*	Motile, %	Progressive, %	Rapid, %	vcl	str
0	0	79.8^{a}	63.3^{a}	76.0^{a}	199.3^{a}	83.3^{a}
0	4	41.4^{b}	29.5^{b}	33.3^{b}	137.9^{b}	81.3^{ab}
0	8	15.4^{d}	11.3^{d}	12.8^{e}	92.7^{f}	62.5^{d}
0	C	8.2^{ef}	3.8^{e}	5.4^{g}	135.6^{bc}	78.1^{c}
5	0	79.8^{a}	63.3^{a}	76.0^{a}	199.3^{a}	83.3^{a}
5	4	37.8^{bc}	27.3^{bc}	30.4^{bc}	124.3^{cd}	82.2^{ab}
5	8	11.8^{de}	8.9^{d}	9.5^{ef}	91.4^{f}	61.9^{d}
5	C	4.4^{f}	2.4^{e}	2.6^{g}	109.9^{e}	85.1^{a}
10	0	79.8^{a}	63.3^{a}	76.0^{a}	199.3^{a}	83.3^{a}
10	4	40.1^{b}	25.7^{bc}	28.5^{cd}	117.8^{de}	83.2^{a}
10	8	6.1^{f}	3.6^{e}	4.0^{g}	78.3^{g}	61.1^{d}
10	C	7.0^{f}	3.7^{e}	4.6^{g}	124.5^{cd}	80.9^{abc}
20	0	79.8^{a}	63.3^{a}	76.0^{a}	199.3^{a}	83.3^{a}
20	4	33.9^{c}	23.4^{c}	25.3^{d}	114.2^{de}	84.1^{a}
20	8	6.8^{f}	3.3^{e}	3.6^{g}	70.2^{g}	62.9^{d}
20	C	4.9^{f}	2.1^{e}	2.8^{g}	121.5^{cd}	80.3^{bc}
SEM	-	1.6	1.4	1.5	4.1	0.95
P-value**	-	0.015	0.05	0.03	0.001	0.002

*Storage: 0 (0 h of incubation in mSPTL), 4 (4 h of incubation in mSPTL), 8 (8 h of incubation in mSPTL), C (Post cryopreservation)
**F-test probability for interaction of ascorbic acid concentration and time.
^{abcdefg}Means within column without a common superscript differ ($P < 0.05$).

Table 3.2 Sperm Variables measured by Hamilton-Thorne IVOS (Hamilton-Thorne Biosciences, Beverly, MA)

Variable	Description
Motile	% of total sperm moving at a track velocity ≥30 µm/sec and progressive velocity ≥15 µm/sec
Progressive	% of total sperm moving at a track velocity ≥50 µm/sec and straightness ≥70%
Rapid	Progressive % with path velocity >50 µm/sec
Medium	Progressive % with path velocity <50 µm/sec but >30 µm/sec
Slow	% of total sperm moving at a path velocity <30 µm/sec and progressive velocity <15 µm/sec
Static	Sperm with no movement at all
Path Velocity (VAP)	Average velocity of the smoothed cell path (µm/sec)
Progressive Velocity (VSL)	Average velocity measured in a straight line from the beginning to the end of the track
Track Speed (VCL)	Average velocity measured over the point-to-point track
Lateral Amplitude	Mean width of the head oscillation as the sperm swims
Beat Frequency (BCF)	Frequency of sperm head crossing the sperm average path in either direction
Straightness	Measures departure of average sperm path from straight line (ratio of VSL/VAP)
Linearity	Measures departure of actual sperm path from straight line (ratio of VSL/VCL)
Elongation	Ratio of head width to head length (%)
Area	Average size in square microns of all sperm heads

Conclusion

These studies focused on stressful conditions that may lead to infertility in the male bovine. We determined that ergot alkaloids can directly affect bovine sperm motility. More specifically, we found that ergotamine and dihydroergotamine can impair sperm motility. Ergonovine was found to have minimal effects on bovine sperm motility. We also concluded that supplementing sperm cryopreservation extender with ascorbic acid can help reduce lipid peroxidation of sperm membranes. However, increasing concentrations of ascorbic acid threatens multiple sperm variable associated with motility. Even though there have been reports that alpha-tocopherol may benefit sperm viability during storage, we observed no significant differences in sperm motility due to alpha-tocopherol. Both of these studies should have major implications in the beef industry as it relates to stress induced infertility, but more research must be performed to determine what mechanisms of the spermatozoa are attacked by ergot alkaloids and also to evaluate other possible combinations of antioxidants.