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Comparison of epididymal and ejaculated sperm collected from the same Holstein bulls

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COMPARISON OF EPIDIDYMAL AND EJACULATED SPERM
COLLECTED FROM THE SAME HOLSTEIN BULLS

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The School of Animal Sciences

by

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ABBREVIATIONS USED IN DISSERTATION

ABP - Androgen Binding Protein
AI - artificial insemination
ART - assisted reproductive techniques
AV - artificial vagina
bFGF - basic Fibroblast Growth Factor
BSA - bovine serum albumin
BSP - bovine seminal proteins
cAMP - cyclic Adenylyl Monophosphate
CLC - cholesterol loaded cyclodextrins
COC - cumulus oocyte complex
DF - Decapacitation Factor
DF-R - Decapacitation Factor Receptor
DSC - differential scanning calorimeter
EE - electro-ejaculation
EGF - Epidermal Growth Factor
Ejac - ejaculated
Epi - epididymal
FBS - fetal bovine serum
FITC - fluorescein isothiocyanate
FSH - Follicle Stimulating Hormone
GAG - glycosaminoglycan
GnRH - Gonadotropin Releasing Hormone
Hep - heparin
IGF-1 - Insulin-like Growth Factor-1
IGF-2 - Insulin-like Growth Factor-2
IVF - in vitro fertilization
IVM - in vitro maturation
LH - Luteinizing Hormone
LHRH - Luteinizing Hormone Releasing Hormone
LN₂ - liquid nitrogen
NGF - Nerve Growth Factor
PDGF - Platelet-derived Growth Factor
PI - propidium iodide
PIF - Preimplantation Factor
PKA - Protein Kinase A
PNA - *Arachis hypogaea* (peanut) agglutinin
PVA - polyvinyl alcohol
PVP - polyvinylpyrrolidone
sAC - soluble Adenylyl Cyclases
TALP - Tyrodes Albumin Lactate Pyruvate medium
TF - Transferrin
TGF- α - Transforming Growth Factor-alpha
TGF- β 1 - Transforming Growth Factor-beta1

ABSTRACT

Epididymal sperm can be collected from injured or deceased animals and allows for the propagation of favorable traits from genetically superior males. Techniques developed in domestic species can also serve as the foundation for the collection, cryopreservation and utilization of epididymal sperm in exotic breeds. The need to preserve and utilize epididymal sperm in the most efficient manner is of the utmost importance. In a series of experiments, ejaculated and epididymal sperm from the same Holstein bulls were collected, cryopreserved, cultured and used for in vitro fertilization. In Experiment I, epididymal sperm was found to have higher post-thaw motility compared to ejaculated sperm. During cryopreservation, the membrane permeability of ejaculated and epididymal sperm was found to be similar. In addition, the membrane permeability of both ejaculated and epididymal sperm were decreased by the inclusion of glycerol during freezing. The optimal cooling rate for ejaculated and epididymal sperm was determined to be between 50 and 60°C/minute. In Experiment II, we demonstrated that following castration circulating concentration of plasma cholesterol increased. In Experiment III, the percentage of post-thaw auto-acrosome reacted ejaculated sperm was found to be higher than epididymal sperm. During in vitro culture, the percentage of auto-acrosome reacted ejaculated sperm remained relatively stable compared with epididymal sperm that significantly increased over time. The percentage of capacitation significantly increased over time for ejaculated sperm but not epididymal sperm, which only slightly increased following 6 hours of culture. In Experiment IV, cryopreserved ejaculated Holstein bull sperm was unaffected by the inclusion of preimplantation factor to the culture medium. PIF was also unable to improve or inhibit the in vitro fertility of cryopreserved ejaculated bull sperm. In Experiment V, ejaculated sperm with heparin and epididymal sperm with and without heparin were similar in their ability to fertilize oocytes in vitro and cleavage rates compared with ejaculated sperm without heparin, which was significantly lower. The number of embryos developing to the 8-cell stage was higher for the epididymal sperm plus heparin group compared with epididymal sperm without heparin. The number of blastocyst were higher for both ejaculated and epididymal sperm, when heparin was added to the fertilization medium, compared with when heparin was not included in the fertilization medium.

CHAPTER I INTRODUCTION

Genetically superior bulls contribute greatly to the genetic improvement of the producers herd. The sudden injury and or death of a valuable bull carrying genetically beneficial traits can be a marked loss to cattle producers and the cattle industry. Since the epididymides function as in vivo storage vessels, epididymal spermatozoa (sperm) could be collected from injured or deceased bulls and could serve as an indispensable resource for saving the germplasm for genetically valuable males. Epididymal sperm are a finite genetic resource and should be utilized in the most efficient manner possible to maximize the number of progeny per male. Furthermore, epididymal sperm could be cryopreserved for use in assisted reproductive techniques such as artificial insemination (AI), in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). Epididymal bull sperm have been shown to be viable for AI (Barker, 1954) and IVF (Goto et al., 1989), and both of these procedures could be used to increase the production of genetically valuable progeny and further the impact of these bulls.

Some unique challenges arise when working with epididymal sperm. First, the majority of research has focused on increasing the effectiveness of cryopreservation and assisted reproductive techniques only utilizing ejaculated sperm. Secondly, ejaculated and epididymal sperm are different with regards to their exposure to seminal plasma. Epididymal sperm has been shown to have different respiration and metabolism rates, heparin binding sites, motility characteristics and ability to undergo capacitation (Miller et al., 1990; Hammerstedt, 1993; Goovaerts et al., 2006).

The objective of this research was to compare ejaculated and epididymal sperm collected from the same bulls before, during and after cryopreservation. Little information is available as to the difference between ejaculated and epididymal sperm collected from the same bulls with regard to their ability to endure cryopreservation. An important aspect of sperm cryopreservation is membrane permeability. A new technique, differential scanning calorimetry (DSC) has been utilized to evaluate the water transport response of sperm during the freezing process. This technique has been reported in a variety of mammalian species including: mouse (Devireddy et al., 1999), human (Devireddy et al., 2000), stallion (Devireddy et al., 2002), dog (Thirumala

et al., 2003), boar (Devireddy et al., 2004), bull (Li et al., 2006), rhesus monkey (Alapati et al., 2008) and rat (Hagiwara et al., 2009).

In conjunction with LSU Mechanical engineering, the DSC technique was be utilized to determine the water transport response of ejaculated and epididymal bovine sperm collected from the same 4 Holstein bulls at a cooling rate of 20°C/minute in 2 different media: the first without cryoprotective agents (CPAs) and the second with 0.7 M glycerol. The experimental water transport response of ejaculated and epididymal bovine sperm were then utilized to determine the cooling behavior and membrane permeability parameters. These results were then used in determining the optimal freezing rates for ejaculated epididymal bovine sperm with or without glycerol.

The concentration of cholesterol in sperm membranes has been known to affect the ability of bovine sperm to endure cryopreservation. Little information is available as to the ability to effect the concentration of cholesterol in epididymal bull sperm. Testosterone has been noted as being able to influence the concentration of cholesterol in the blood. Castration increases the cholesterol concentration in the blood plasma of the rabbit (Hussein et al., 1999), rat (Cinci et al., 2000), pig (Yao et al., 2011) and man (Bo et al., 2011). Supplementation with exogenous testosterone was noted to reverse the increase in cholesterol induced by castration and returned cholesterol concentration to precastration concentrations in the rat, rabbit and man (Takahashi et al., 1986; Hussein et al., 1999). Before and after castration, blood plasma was taken from the same Holstein bulls to determine if the concentration of cholesterol is affected by the castration induced loss of testosterone. If the concentration of circulating plasma cholesterol is androgen dependent this may prove useful in devising strategies for improving the cryopreservation of bull sperm.

The birth of live young following the storage of epididymal sperm within the epididymides for up to 24 hours at 22°C before collection allows for the possibility to collect epididymal sperm from other animals that suddenly die (Songsasen et al., 1998). Few studies have compared the in vitro culture dynamics of cryopreserved ejaculated and epididymal sperm collected from the same bulls. Ejaculated and epididymal sperm may respond differently due to the lack of exposure to seminal plasma. The addition of seminal plasma has profound effects on epididymal sperm including removal of

cholesterol from the sperm membrane to the addition of heparin binding sites. Understanding the difference between ejaculated and epididymal sperm collected from the same bulls during in vitro culture will allow for the modification of existing assisted reproduction protocols to maximize the use of epididymal sperm. Ejaculated and epididymal sperm from the same 4 Holstein bulls will be cultured under capacitating conditions for 6 hours to determine viability, acrosomal integrity and percentage of capacitation.

Bovine ejaculated and epididymal sperm respond to various factors in their environment such as peptides, proteins and small molecules. Preimplantation factor (PIF), a peptide recently reported to be released from viable bovine embryos, was evaluated to determine how bovine cryopreserved sperm react upon PIF exposure. During in vitro culture of uterine endometrial cells, PIF has been shown to affect receptors, signaling pathways, enzymes and membrane ion channels (Paidas et al., 2010). Ejaculated and epididymal sperm undergo many biological processes during their trek to fertilize an oocyte. These processes include intracellular signaling, binding of receptors and an influx and efflux of ions through ion channels (Leclerc et al., 1996; Dragileva et al., 1999; Newton et al., 2010). The possibility to affect sperm processes such as capacitation, the acrosome reaction, motility or increase the viability by exposure to PIF may have implications in sperm preservation or in assisted reproductive techniques.

The effects of exposing ejaculated and epididymal sperm to PIF have yet to be investigated. These studies will give us a better understanding of the effect, if any, of PIF on cryopreserved bull sperm viability, its ability to induce or inhibit capacitation and the auto-acrosome reaction during in vitro culture. Cryopreserved bull sperm will also be compared following in vitro fertilization with or without exposure to PIF.

In vitro fertility of ejaculated and epididymal sperm collected from the same bulls may be different due to the lack of exposure to seminal plasma. The major protein components of seminal plasma are the bovine seminal proteins (BSP) proteins, comprising ~65% of all seminal plasma proteins (Bergeron et al., 2004). These BSP proteins are secreted by the seminal vesicles and bind to choline phospholipids on the sperm plasma membrane (Therien et al., 1995). BSP proteins also bind to the

glycosaminoglycan heparin, a known inducer of capacitation (Miller et al., 1990). One of the major aspects of capacitation is the efflux of cholesterol from the sperm plasma membrane. Exposure of epididymal sperm to BSP proteins induces an efflux of cholesterol from the plasma membrane (Therien et al., 1998). Ejaculated and epididymal sperm collected from the same 4 Holstein bulls will be compared as to their in vitro fertility with and without the capacitating agent heparin.

Discarding epididymal sperm from an injured or deceased animal is a loss of not only great genetic value but also economic value. Due to the known variability among males, it is important to compare ejaculated and epididymal sperm collected from the same males to minimize such issues. Further research comparing ejaculated and epididymal sperm collected from the same males is needed to increase the efficient and effective use of epididymal sperm. Our research findings comparing ejaculated and epididymal sperm from the same bulls can then be adapted to fit other economically valuable animals and possibly endangered species.

This research was conducted to better understand the difference between ejaculated and epididymal sperm collected from the same males. From collection to cryopreservation and from in vitro culture to in vitro fertilization epididymal sperm may react completely different compared with ejaculated sperm, therefore it is important to understand the difference between ejaculated and epididymal sperm. This will allow for increased efficiency and better utilization of epididymal sperm in producing progeny of high genetic value that would otherwise be lost.

CHAPTER II LITERATURE REVIEW

FUNCTION OF THE TESTIS

The testes are located outside of the body cavity in a specialized pouch called the scrotum. The scrotum serves to protect and regulate the temperature of the testes, which is required for normal spermatogenesis. The testis functions in the production of hormones through the process of steroidogenesis and spermatozoa through the process of spermatogenesis. The hormonal functions of the testes can influence and can be influenced by the hormones of the hypothalamus and the pituitary gland. The two functions of the testis are linked in that adequate concentrations of testosterone are necessary for spermatozoa (sperm) production. Testes are compartmentalized, consisting of an interstitial compartment and the basal and adluminal compartments of the seminiferous tubules. The division between the basal and adluminal compartments is formed by specialized junctions (called tight junctions) between Sertoli cells. These tight junctions form an immune privileged area that provides protection for developing sperm. The Sertoli cells also play a key role in regulating the microenvironment of the adluminal compartment that is required for regulation of germ cell development (Amann and Schanbacher, 1983).

Hormone Production

In the bull, hormone production is regulated by the interaction of the hypothalamus, anterior pituitary and the testes (Ball and Peters, 2004). The 2 major cells involved in hormone production in the testis are the Leydig and Sertoli cells. Both Leydig and Sertoli cells are stimulated by the gonadotrophins: Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) that are secreted by the anterior pituitary in response to Gonadotrophin Releasing Hormone (GnRH). Gonadotrophin releasing hormone is secreted by the hypothalamus and can be inhibited by increased concentrations of testosterone from the Leydig cells.

The Leydig cells, in response to LH, convert cholesterol to the androgen testosterone. This conversion is initiated in the mitochondria by enzymatic cleavage of the cholesterol side chain to form pregnenolone. Pregnenolone is then transferred to the

smooth endoplasmic reticulum where it is converted to testosterone through a series of intermediates steps (Amann and Schanbacher, 1983).

LH and testosterone in the male are secreted in a pulsatile manner (Katongole et al., 1971). The secretion of LH is followed by an increase in testosterone ~1-hour later (Katongole et al., 1971). This release of LH is not only under hormonal control but can be influenced by environmental stimuli also. Visual and auditory sexual stimulation has been reported to cause an immediate release of a LH pulse in the bull (Katongole et al., 1971). Thompson et al. (1994) reported testosterone concentrations in the bull at 0, 30 and 150 minutes following a GnRH challenge (100 µg im) of 7.26, 10.0 and 14.5 ng/ml, respectively. Byerley et al. (1990) also reported an increase in testosterone concentrations from 2 to 5 ng/ml before GnRH challenge (10 ng/kg of body weight IV) to 17 to 22 ng/ml after ~90 minutes. Aspden et al. (1998) used deslorelin, an LH releasing hormone (LHRH) agonist, to induce an increase circulating testosterone from 2.4 to 11.8 ng/ml in the bull. These studies show the interaction of the hormones from the brain to the testis.

Testosterone and inhibin are transported to the brain and regulate the GnRH production from the hypothalamus and LH and FSH from the anterior pituitary by way of negative feedback (Amann and Schanbacher, 1983). Following bilateral castration, the negative feedback is eliminated resulting in a marked increase of both LH and FSH secretion (Amann and Walker, 1983). These hormones all interact and regulate each other to keep the reproductive system functioning properly. In a study by Aspden et al. (1998), bulls with chronically elevated testosterone concentrations were not able to respond to LHRH challenge compared with controls showing the negative feedback response of testosterone. The LH concentration before and after LHRH challenge for bulls with high testosterone were noted as 0.55 and 0.50 ng/ml compared with control bulls 0.51 and 6.92 ng/ml, respectively.

There are two major sources of cholesterol involved in the production of testicular steroids: cholesterol, transported in the blood to the testis from elsewhere in the body; or from acetate, from the blood or formed from acetyl-coenzyme-A during glucose metabolism (Setchell, 1978). The main source of cholesterol utilized in steroid production is species specific as in the rat where ~40% of all cholesterol utilized in the

testis originated from the plasma, but in the guinea pig only 13% is from plasma (Morris and Chaikoff, 1959; Setchell, 1978). To our knowledge, there are no reports as to the utilization of plasma cholesterol in the production of testicular testosterone in the bull.

The concentration of testosterone varies over time in the blood. Testosterone concentrations leaving the testis of beef and dairy bulls are the highest concentration at 75 to 575 ng/ml and decreases to the circulating concentration of 2 to 20 ng/ml (Katongole et al., 1971; Amann and Ganjam, 1976). Kawate et al. (2011) compared testosterone concentrations of beef bull calves (n=15) at 0, 28, 56 and 84 days of age with average circulating concentrations of 0.1, 0.5, 1.0 and 1.1 ng/ml, respectively. Significant increases were noted between days 0 to 56 days, but no difference was detected between days 56 and 84 (Kawate et al., 2011). Amann and Walker (1983) reported the average circulating testosterone concentration for Holstein bull calves at 4 to 7, 11 to 13, 15 to 17, 18 to 20, 21 to 23 and 25 to 28 weeks of age at 0.39, 0.53, 0.94, 1.80, 2.67 and 5.76 ng/ml, respectively. Cross et al. (1984) reported an age related increase in testosterone (2.22, 2.95, 9.09 and 3.13 ng/ml) for beef calves at 6, 9, 12 and 15 months of age, respectively. Kawate et al. (2011) noted a significant increase in mean testosterone concentrations from prepubertal beef bull calves (3 to 6 months) compared with early (6 to 12 months), late (12 to 18 months) and post-pubertal (18 to 22 months) bulls: 2, 4, 5 and 4 ng/ml, respectively. A difference has also been noted for circulating testosterone concentrations (ng/ml) between different beef breeds (Simmental 5.21, Charolais 4.31, Herford 2.90 and Angus 4.97 ng/ml) (Cross et al., 1984). These studies revealed the gradual increase in testosterone from birth to the high concentrations around the time of puberty and subsequent decrease of mean circulating testosterone concentration of bulls with age.

Higher concentrations of testosterone are partially maintained in the bull testis by transfer of testosterone from the testicular vein to the testicular artery along the pampiniform plexus, with testosterone concentration in the testicular artery roughly double that of circulating blood (Amann and Ganjam, 1976).

Within the testis, testosterone is transferred to the lumen of the seminiferous tubules through the Sertoli cells. Sertoli cells produce the hormones estrogen, inhibin and dihydrotestosterone (DHT). Sertoli cells also secrete androgen binding protein

(ABP) that binds testosterone and is taken up into the circulation and/or carried with the sperm into the epididymis (Cosentino and Cockett, 1986). Androgen binding protein maintains high concentration of androgens in the proximal epididymis, which is necessary for its normal testicular function (Cosentino and Cockett, 1986). In the bull, testosterone concentration of rete testis fluid has been reported at 33 ng/ml (Amann and Ganjam, 1976). Androgen binding protein has also been found in the seminal plasma of the bull (Jegou and Le Gac-Jegou, 1978).

Removal of the testes has long been known to have profound physiological effects on the male. Castration of 16 to 32-week-old Holstein bulls calves results in a rapid decrease in circulating testosterone concentrations (Amann and Walker, 1983). Disappearance rates ($t_{1/2}$ minute) for circulating testosterone have been calculated at a half-life of ~8 minutes for the first 8 minutes following castration, 29 minutes for 8 to 30 minutes following castration, and then 49.7 minutes for 30 to 60 minutes following castration and continued to decrease over 4 to 6 hours (Haynes et al., 1976; Amann and Walker, 1983). Testosterone concentrations for castrated beef steers (6 to 18 months of age) has been reported as being consistently below <0.5 ng/ml (Cross et al., 1984).

Sperm Production

In the testis, sperm are produced on a continual basis. The production of sperm occurs within the lining of the seminiferous tubules. These seminiferous tubules are located within the parenchyma of the testis and are connected to the rete testis by the straight tubules. Seminiferous tubules consist of two main compartments; the basal and adluminal compartments (Amann and Schanbacher, 1983). The basal compartment is covered by a layer of smooth muscle, this layer not only aides in sperm movement by way of peristaltic contractions but also comprises the first line of defense in preventing an immune reaction to meiotically dividing spermatocytes. Within the tubule, the basal and adluminal compartments are defined by the Sertoli cells. These sertoli cells form tight junctions between two adjacent cells establishing the functional division between these two compartments. The tight junctions also serve as the second barrier in preventing an immune response. These two barriers form an immune privileged area.

In the basal compartment, beneath the smooth muscle layer, lay the spermatogonia. They are divided into the spermatogonia A_0 , which are not part of the dividing pool of cells and are resistant to radiation and toxic substances, followed by A_1 , A_2 , A_3 , I_n , B_1 and B_2 -spermatogonia that have committed to dividing and differentiation, (Amann and Schanbacher, 1983). These cells divide by mitosis to produce spermatocytes that are transferred to the adluminal compartment. The divisions between the germ cells occur in unison but are not complete. Following division, the cytoplasm remains connected by intercellular bridges (Dym and Fawcett, 1971). These intercellular bridges form a chain that links the cohort of dividing germ cells and only breaks at points where the germ cells die (Amann and Schanbacher, 1983).

The adluminal compartment is the site of meiosis and spermiogenesis, which comprise all the division and morphological changes that must occur to change round diploid spermatogonia into highly specialized motile, haploid spermatozoa. Once sperm are produced, they are released into the lumen of the seminiferous tubule. The whole process takes ~61 days in the bull (Amann and Schanbacher, 1983). The scrotum serves to protect and regulate the temperature of the testes. Temperature regulation is important in the production of sperm in the bull. Normal spermatogenesis can only occur at temperatures 2 to 3°C below body temperature (Kastelic et al., 1996). Increasing the temperature of the testes by insulation results in decrease in motility, increase in abnormal morphology and disruption of nuclear protamination (Rahman et al., 2011).

FUNCTION OF THE EPIDIDYMIS

The epididymides are specialized structures attached to the exterior of the testes. The epididymis is a highly convoluted tightly coiled tube that connects the efferent ducts of the testicle to the vas deferens. The epididymis is composed of three main regions: the caput (head), corpus (body) and the cauda (tail). The bovine epididymis can reach a length of 40 meters (Cosentino and Cockett, 1986). Epididymal transit time varies among bulls but averages 8 days, with a range of 4 to 15 days (Amann et al., 1974).

The main functions of the epididymides are in sperm maturation, transport, concentration, protection and storage that results in a heterogeneous sperm population that become motile and capable of fertilizing oocytes (Amann and Almquist, 1962; Igboeli and Foote, 1968; Amann and Griel, 1974; Amann et al., 1974). The epithelium of

the epididymis is composed of different cell types that function in secretion, absorption, endocytosis, luminal fluid acidification, protection from an immune response, phagocytosis and antioxidant production (Caballero et al., 2010).

Maturation

While in transit through the caput and corpus epididymides, sperm undergo the process of maturation (Bedford, 1966). Maturation consists of morphological and biochemical modifications, that results in sperm acquiring forward motility and the ability to fertilize an oocyte (Amann and Griel, 1974). As sperm progress through the epididymides, their ability to fertilize an oocyte increases, with the sperm in the cauda region displaying the highest fertility. Sperm are transcriptionally inactive due to the highly condensed nature of their DNA, making them dependent upon the epididymis during the maturational process. The epididymis releases various factors that function in the maturation process. Some sperm motility is gained while traveling through the epididymis. Sperm have some intrinsic movement but progressive motility is acquired while maturing in the cauda epididymis (Cosentino and Cockett, 1986).

Transport and Storage

Sperm are transported through the caput and corpus epididymides by smooth muscle peristaltic contractions. In contrast, the cauda functions in sperm storage and remains relatively quiescent unless stimulated to contract. The nervous system plays a role in sperm transport. Stimulation of the nerves of the cauda epididymides results in the movement of sperm from the cauda during times of sexual stimulation. Disturbance of the sympathetic nerves originating from the inferior mesenteric ganglion of the bull has been shown to cause sperm accumulation and an increase in abnormal sperm morphology (Barth, 2007). Hormonal influences from oxytocin and vasopressin also play a role in regulating contractility of the duct system and sperm movement (Barth, 2007). As sperm accumulate in the epididymides the surplus is evacuated via the urine and is referred to as spermaturia (Cosentino and Cockett, 1986).

Epididymal sperm collected from the bull, rat, hamster, human and guinea pig are all stored in an immotile state in the cauda epididymides (Cascieri et al., 1976; Acott and Carr, 1984; Carr and Acott, 1984; Turner and Reich, 1985). Although this may not be the case for the rabbit, whose epididymal sperm have been reported to be highly

motile during their storage in the epididymides (Turner and Reich, 1985). It has been shown that a low pH plays a critical role in keeping epididymal sperm in an immotile state. The pH of the epididymis has been reported to be 5.8 in the bull (Acott and Carr, 1984). Bicarbonate has been shown to activate the motility of epididymal sperm. The bicarbonate concentration of the caudal epididymis is ~5mM in the bull (Hess et al., 2005). Upon exposure to seminal plasma, that has a bicarbonate concentration of ~25mM, sperm motility is activated.

The ion composition and osmolarity of the bull cauda epididymal plasma has been reported to be different than the circulating blood plasma. Verberckmoes et al. (2001) determined the ion composition of epididymal plasma and it compared with earlier reports of epididymal (Crabo, 1965; Wales et al., 1966) and blood plasma (Kaneko, 1989). Ion composition for epididymal and blood plasma were reported for: sodium (50.5 and 142.0 mEq/l), potassium (33.5 and 4.9 mEq/l), chloride (31.5 and 104.0 mEq/l), inorganic phosphorus (25.1 and 6.1mg/dl), magnesium (3.6 and 2.1 mg/dl), bicarbonate (0.0 and 23.0 mEq/l), Na:K ratio (1.5 and 29.0) and osmolarity (354.3 and 285.0 mOsm), respectively.

Protection

The epididymis protects sperm by forming a selective barrier from the blood. This barrier consists of an anatomical and physiological component. The anatomical components consist of the basolateral membrane, apical membrane and the tight junctions between epithelial cells. These structures restrict the entrance and exit of molecules, such as immunoglobulins, sperm antigens and immune cells (Mital et al., 2011). The physiological barrier consists of specific transporters along the basolateral and apical membranes; this regulates the passage of molecules in and out of the lumen. This selective passage creates a favorable environment for the maturation of sperm and changes as sperm progress through the epididymis. These changes reflect the different microenvironments required for proper function of the epididymis. These components work together to form an immunological privileged area preventing the body from recognizing and attacking the sperm within the epididymis (Mital et al., 2011).

Cholesterol Regulation

During epididymal transit, sperm are subjected to numerous changes, one of which is the modification of cholesterol concentration. Cholesterol plays a crucial part during the preparation of sperm in the epididymides. Bull and mouse epididymides have been noted to have the ability to synthesize cholesterol from [$1\text{-}^{14}\text{C}$] acetate (Hamilton et al., 1969; Evans and Johnson, 1975). This synthesis of cholesterol has been shown to be androgen dependent (Hamilton et al., 1969). The epididymis was also noted as having an active cholesterol metabolism as both [$1\text{-}^{14}\text{C}$] acetate and [$4\text{-}^{14}\text{C}$] cholesterol were converted to cholesterol esters.

Sperm cholesterol concentration has been shown to change during transit through the epididymis. From the caput to the cauda, the concentration of cholesterol in epididymal sperm decreases ~50% in the ram (~9 and ~6 nmol/ 10^8 sperm) and rat (237 and 122 $\mu\text{g/ml}$), but was unchanged in the mouse (0.24 and 0.26 $\mu\text{mol} \times 10^6$). The cholesterol to phospholipid ratio also changes from the caput to the cauda epididymis of the ram (0.26 to 0.44) but remained relatively stable in the rat (0.59 to 0.53), and mouse (0.26 to 0.29) (Parks and Hammerstedt, 1985; Hall et al., 1991; Awano et al., 1993; Rejraji et al., 2006).

In the epididymides, principal cells of the lumen secrete small membranous vesicles, called epididymosomes. These secretions or “blebs” have been noted in the epididymides of the hamster (Legare et al., 1999), bull (Frenette et al., 2002), mouse (Rejraji et al., 2006), rat (Fornes et al., 1995), ram (Gatti et al., 2005) and human (Frenette et al., 2005). Epididymosomes have their own cholesterol to phospholipid ratio varying among species from 0.5 in the mouse (Rejraji et al., 2006) to 2.0 in the bull (Sullivan et al., 2007). During transport through the epididymides, epididymosomes have been shown to transfer proteins to the plasma membranes of epididymal sperm. It has also been proposed that epididymosomes transfer cholesterol to epididymal sperm. Although in the mouse, cholesterol to phospholipid ratio of the epididymosomes rises from the caput (0.26) to the cauda epididymis (0.48), but as stated earlier, remains relatively constant in epididymal sperm raising some question as to their influence on sperm cholesterol concentrations (Rejraji et al., 2006).

COLLECTION OF SPERM

Ejaculated and epididymal sperm can be collected and used in assisted reproductive techniques. Both types yield fertile sperm but methods of collection are very different. Ejaculated sperm can be collected by artificial vagina (AV), electro-ejaculation, massaging the pelvic genital tract (ampulla) or recovered from the vagina of a naturally mated cow. The latter two methods are not normally used for semen collection. In contrast, epididymal sperm are most often removed by flushing, mincing, incising or cannulating the epididymis.

Ejaculated

Ejaculated sperm can be collected from bulls on a routine basis and cryopreserved for use in assisted reproductive techniques. The usual method of collecting ejaculated sperm is by artificial vagina (AV) or by stimulation with an electrical probe termed electro-ejaculation (EE) (Austin et al., 1961). Collection is preceded by sexual stimulation and preparation. Sexual stimulation of the bull is accomplished with visual and auditory sexual stimuli and by repeatedly allowing the bull to mount a teaser steer. These preparatory steps lead to increased quantity and quality of ejaculates. The AV is used to closely mimic the natural mating process. This method provides a means of collecting an ejaculate in nearly the same state as in natural mating. Electro-ejaculation method involves inserting an electric probe into the rectum of the bull and stimulating the extension and emission nerve centers (Dziuk et al., 1954; Marden, 1954). This method also provides good quality semen but is used in cases where the temperament or physical condition of the bull makes it impossible or unsafe to collect semen by way of an AV.

Epididymal

Epididymal sperm are collected directly from the cauda epididymides thus, circumventing the ejaculation process. Epididymal sperm can be collected from the testes of bulls following castration by flushing, incising and mincing, also epididymal sperm can be collected by cannulation of the epididymides of live intact bulls (Bialy and Smith, 1959; Amann and Almquist, 1961a; Igboeli and Foote, 1968; Deutscher et al., 1974; Ellington et al., 1993; Graham, 1994; Kershaw-Young and Maxwell, 2011; Turri et al., 2011). Flushing is the process by which a catheter is threaded into the vas deferens

and isotonic solution is used to push, by way of retrograde flow, sperm from the cauda epididymis into a collection vessel. A second method is mincing of a cauda epididymis with subsequent filtering and isolation of sperm (Amann and Almquist, 1961a). Another method of collecting epididymal sperm is making incisions along the cauda epididymis that allows sperm to be released and collected into a collection vessel for subsequent processing. Collection of epididymal sperm from the live bull is performed by surgically inserting a small tube (cannula) into the vas deferens of the intact male and allowing the end to extend through the scrotum to the exterior for collection of epididymal sperm into a small vial (Deutscher et al., 1974).

Ejaculated and Epididymal Sperm Collection

Amann and Almquist (1961b) assessed ejaculated semen collection from 8 Holstein bulls and noted average values of: volume (3.9 ml), concentration of sperm ($1.36 \times 10^9/\text{ml}$), motile (70%), %live (85%), daily sperm output ($4.58 \times 10^9/\text{ml}$), normal sperm morphology (92.7%). Austin et al. (1961) compared ejaculated sperm from 12 Hereford bulls collected by AV or EE with average values of: volume (2.8 ml and 6.3 ml), sperm concentration ($0.63 \times 10^9/\text{ml}$ and $0.28 \times 10^9/\text{ml}$), sperm concentration per ejaculate ($1.69 \times 10^9/\text{ejaculate}$ and $1.83 \times 10^9/\text{ejaculate}$), pH (6.7 and 7.3) and progressive motility (64 and 67, respectively). Almquist and Amann (1961) evaluated 55 dairy bulls and reported an average testis and epididymis weight of (351 g and 36.1 g, respectively).

Amann and Almquist (1961a) minced the cauda epididymides of dairy bulls with a recovery rate to be 13.4×10^9 . Amann and Almquist (1962) collected epididymal sperm from 40 dairy bulls and reported the live population to be 72.5% and 32.1% motile following dilution in saline. Amann and Griel (1974) compared ejaculated, cauda epididymal and testicular sperm from the same seven mature Holstein bulls. Ejaculated, epididymal and testicular sperm were compared by their volume (4.7, 0.71 and 39.3 ml/day); concentration (1.24 , 5.12 and 0.078×10^9 sperm/ml); progressive motility (68%, 65% and 0%) and %live (84%, 82% and 79%), respectively (Amann and Griel, 1974). Deutscher et al. (1974) collected ejaculated sperm from 10 Angus bulls followed by epididymal sperm from vas deferens cannulation. Comparison of epididymal and ejaculated values for motility (66% and 74%), live (87% and 80%), concentration

($3.81 \times 10^9/\text{ml}$ and $0.27 \times 10^9/\text{ml}$) and volume (0.13 ml and 6.95 ml), respectively (Deutscher et al., 1974). Goovaerts et al. (2006) compared ejaculated and epididymal sperm from beef and dairy bulls of various breeds to determine the differences among breeds. They also compared epididymal sperm parameters from the same bulls. Ejaculated and epididymal sperm collected from beef and dairy bulls were compared with averages of: motility (79.9% and 48.7%), progressive motility (58.4% and 34.4%), linear motility (84.5% and 80.5%), amplitude (5.0 μm and 6.1 μm) and curve linear velocity (156.4 $\mu\text{m/s}$ and 173.5 $\mu\text{m/s}$). Average epididymal collection values were calculated for 25 bulls: testis weight (308 g), cauda epididymis weight (12.6 g), volume (0.9 ml), concentration (31×10^9) and %live (84%). Variation in sperm concentration, morphology and motility parameters between epididymides were noted within bulls. The testis and cauda epididymis weight, %live, sperm volume, linear sperm movement and amplitude of epididymal sperm collected between epididymides were reported to be consistent within bulls.

Gábor et al. (1995) reported the average testis weight, %live and motile sperm for 5- to 6-year old Holstein bulls to be 371.2 g, 46.9% and 76.6%, respectively. Thompson et al. (1994) reported the average scrotal circumference for 30 crossbreed beef bulls, 12- to 13-months of age, to be 32.2 cm.

Turri et al. (2011) compared two collection methods for bovine epididymal sperm: retrograde flush and the incising (float-up) method. It was determined retrograde flush yielded sperm of higher motility and viability compared to incising but neither method affected the concentration of epididymal sperm collected.

SEMINAL PLASMA

Seminal plasma is expressed from the accessory sex glands. In the bull, these accessory sex glands consist of the seminal vesicles, prostate and bulbourethral glands. The functions of the accessory sex glands are androgen dependent. Exposure to the accessory sex gland fluids constitutes the principal difference between epididymal and ejaculated sperm. Accessory sex gland fluids are expressed during the ejaculation process exposing epididymal sperm to these factors effectively making them ejaculated sperm.

Seminal fluids provide bull sperm with nutrients, and biochemical factors such as citric acid, fructose, glycoproteins, proteins, glycerylphosphorylcholine and prostaglandins (Barth and Oko, 1989). These fluids also alter the surface composition of sperm, which has an effect on the ability of sperm to fertilize an oocyte.

Ejaculated sperm in the bull have been reported to have fewer distal droplets compared with epididymal sperm (Cooper, 2005). This indicates that the distal droplets are lost just prior to or during ejaculation. The addition of seminal vesicle fluid has been shown to decrease the amount of distal droplets found in bovine epididymal sperm (Bialy and Smith, 1958a). Epididymal bull sperm incubated with seminal plasma for 30 minutes prior to cryopreservation has been shown to have significantly better normal sperm morphology than those without seminal plasma before and after cryopreservation (Graham, 1994). Bull-to-bull variation was also noted with 70% of bulls showing significant distal droplet detachment while the other 30% showing no effect following exposure to seminal plasma (Guerrero, 2006).

Reports on the effects of seminal plasma when added to epididymal sperm are inconsistent and controversial. Some reports are positive showing beneficial effects (Dott et al., 1979; Okazaki et al., 2012) while others indicate a negative effect (Shannon, 1965; Way et al., 2000). Bovine epididymal sperm collected from 25 beef and dairy bulls were reported to have lower progressive motility compared with ejaculated sperm collected from 53 beef and dairy bulls (Goovaerts et al., 2006). Also, bull epididymal sperm incubated with seminal plasma showed higher post-thaw motility when compared with sperm not incubated with seminal plasma (Guerrero, 2006). Although contrary to the latter reports, Way et al. (2000) compared fresh epididymal sperm incubated with or without seminal plasma collected from the same 6 Holstein bulls. Results indicated that epididymal sperm incubated with or without seminal plasma did not significantly differ in motility. However, the inclusion of seminal plasma did decrease the amount of live acrosomal intact sperm (Way et al., 2000). Seminal plasma addition has been reported to have no effect on post-thaw motility of washed ejaculated and epididymal bull sperm (Graham, 1994).

Seminal plasma has also been reported to affect sperm during in vitro fertilization. Katska et al. (1996) compared bovine epididymal and ejaculated sperm

collected from different males as to their ability to fertilize oocytes in vitro and their subsequent embryo development. Bovine epididymal sperm utilized in IVF resulted in significantly better fertilization, cleavage and blastocyst development at 83.7%, 80.2% and 30.6% compared with ejaculated sperm at 50.1%, 39.9% and 10.6%, respectively. The removal of seminal plasma from ejaculated bovine sperm at the time of collection also increased the fertilization, cleavage and blastocyst development following IVF: without seminal plasma at 63.3%, 52.0% and 16.2% and with seminal plasma at 50.1%, 39.9% and 10.6%, respectively (Katska et al., 1996).

Bovine Seminal Plasma Proteins

During ejaculation, sperm are mixed with fluid secreted by the accessory sex glands to form semen. The seminal vesicles, 1 of 3 accessory sex glands found in the bull, secretes a family of closely related proteins designated as BSP-A1, BSP-A2, BSP-A3, and BSP-30-kDa and are collectively referred to as BSP proteins (Therien et al., 1998). BSP-A1 and BSP-A2 together are considered to be a single chemical entity also referred to as BSP-A1/-A2, gonadostatins or PDC-109 (Therien et al., 1998).

BSP proteins bind to choline phospholipids of the sperm plasma membranes during ejaculation (Desnoyers and Manjunath, 1992). Once bound, BSP proteins stimulate the initial efflux of cholesterol and phospholipids from sperm plasma membranes reducing the structural stability, and facilitate the binding to the oviductal epithelium (Therien et al., 1998, 1999; Gualtieri et al., 2010).

Exposure of bull sperm to BSP proteins for 4 hours has been reported to reduce membrane cholesterol concentrations ~25% (Moreau and Manjunath, 2000). Cooling cells incubated with BSP proteins below 22°C did not prevent cholesterol efflux suggesting the removal of cholesterol is not due to intracellular mobilization (Moreau and Manjunath, 2000). In the bull, BSP proteins also bind to the glycosaminoglycan heparin, that has been shown to participate in sperm capacitation (Therien et al., 1998).

Srivastava et al. (2012) used anti-bodies to sequester PDC-109 (BSP-A1/A2) upon collection of an ejaculate from dairy crossbreed bulls. Sequestered sperm was then compared to sperm collected without sequestering PDC-109 before and after cryopreservation. Both viability and acrosomal integrity were improved by sequestering PDC-109 at the time of collection; 84% and 81% compared with 75% and 73%,

respectively (Srivastava et al., 2012). Post-thaw, viability, acrosomal integrity, motility and hypo-osmotic response of PDC-109 antibody treated sperm (70%, 75%, 56% and 69%) were improved compared with non-treated sperm (60%, 62%, 47% and 58%).

The exposure to seminal plasma has also been implicated in the binding of bull sperm to the oviduct epithelium. Ejaculated and epididymal bull sperm are both able to bind to the oviductal epithelium but epididymal sperm bind at a reduced rate (Gualtieri et al., 2010). This binding of bull sperm to the oviductal epithelium is mediated by BSP proteins and forms the oviductal reserve (Gwathmey et al., 2003; Gwathmey et al., 2006). The release of sperm from the oviductal epithelium is accompanied by the reduction of sperm surface proteins binding sites from disulfide to sulfhydryls (Talevi et al., 2007).

Fresh epididymal bull sperm has been reported to maintain its initial sperm motility (90%) following an 8-hour incubation without BSP proteins 80% compared with 50% when BSP proteins were added, respectively (Gwathmey et al., 2006).

Seminal Plasma Lipid Concentration

Komarek et al. (1964) reported the lipid composition (g of lipid /100 g of dry matter) of dairy bull seminal plasma to be 1.35%, and is composed of phospholipids (68.6%), cholesterol (20.2%), diglycerides (4%), triglycerides (4%) and wax esters (3.2%). Beer-Ljubić et al. (2009) reported the seminal plasma total cholesterol of 19 Simmental bulls to be 0.47 to 1.19 mmol/L. Boar seminal plasma composition was similar to bull seminal plasma. Komarek et al. (1965) reported a lipid content of 0.23% of which phospholipids composed 64%, cholesterol 17.7%, diglycerides 5.8%, triglycerides 5.3% and wax esters 6.5%.

Decapacitation Factors

Seminal plasma contains a factor or factors that when exposed to sperm blocks their ability to capacitate and fertilize oocytes. These factors are referred to as “decapacitation factors” and may function in prevention of premature capacitation (Bedford and Chang, 1962).

CRYOPRESERVATION OF SPERM

Cryopreservation of bovine sperm has become a routine procedure that supports the cattle artificial reproduction industry. A significant advancement in the ability to

cryopreserve sperm came about with the discovery of the protective effects of glycerol (Polge et al., 1949). This discovery has led to the routine collection and cryopreservation of sperm. Maximizing the production of genetically superior bulls can be accomplished by housing them in climate controlled barns and routinely collecting and cryopreserving their semen. Semen cryopreservation allows for the proliferation of genetically important agricultural traits through the use of artificial reproductive techniques such, as AI and IVF.

Cryopreservation induces considerable changes to and causes a great amount of stress on sperm plasma membranes (Bailey et al., 2000). During the freezing and thawing phases, sperm are exposed to alterations in solution osmolality, which induces changes in cell water volume. This movement of water across the membrane applies considerable mechanical stress on the cell. Also, following cryopreservation the sperm membrane composition is changed. Plasma membrane proteins have random distributions that are disrupted by the freezing process, leaving the sperm membranes with regions free of any proteins. Cryopreservation also induces intracellular changes in sperm that are induced in all sperm compartments (Medeiros et al., 2002). This damage is responsible for the loss of motility, acrosome integrity, viability and fertilizing ability of sperm (Holt, 2000).

During capacitation of bovine sperm, intracellular calcium concentrations increase from 25 to 160 nM (Breitbart, 2002). This increase in intracellular calcium has also been noted following cryopreservation of sperm. Kadirvel et al. (2009) reported the percentage of buffalo ejaculated sperm with high membrane fluidity and live sperm with increased calcium concentration before and after cryopreservation at 26% and 12% compared with 57% and 44%, respectively. This increase intracellular calcium concentration post-thaw implies an impairment of the selective membrane permeability mechanism of the sperm plasma membrane.

The ratio of cholesterol to phospholipids has an effect on membrane lipid phase transition. The lower the concentration of cholesterol the more sensitive the sperm cell is to cooling. Also the percentage of unsaturated fatty acids affects the susceptibility of sperm to the effects of cooling. Bull sperm are considered to be sensitive with a higher ratio of unsaturated fatty acids and a lower concentration of cholesterol in their sperm

membranes when compared with the rabbit and human sperm that is highly resistant to damage induced by cryopreservation (Bailey et al., 2000). Changes to sperm membranes caused by cryopreservation affects the fertility of cryopreserved semen, with a decrease in fertility when compared with fresh semen (Watson, 1995, 2000).

Cholesterol

Sperm membranes at body temperature are a fluid mixture of lipids (mostly phospholipids and cholesterol), and proteins (integral and peripheral) that can move laterally within the membrane (Amann and Pickett, 1987). The function of the membrane is dependent on the phospholipid protein arrangement (Hammerstedt et al., 1990). Phase transition of phospholipids from the fluid state to the crystalline-gel state occurs during the cooling process of cryopreservation. This phase transition results in a more orderly and packed membrane that restricts movement of proteins and lipids (Amann and Pickett, 1987; Hammerstedt et al., 1990). Integral proteins are excluded from the phospholipid crystalline-gel domains, resulting in proteins clustering together. Functionality and stability of the membrane is lost when protein exclusion and subsequent protein clustering occurs (Amann and Pickett, 1987).

Overall sperm membrane fluidity is determined by the ratio of cholesterol to phospholipids and the amount of phospholipids that are polyunsaturated fatty acyl chains (Amann and Pickett, 1987). Different species have been noted as having varying susceptibility to cold shock (Watson, 1981). The bull, boar, ram and stallion are all noted as being very susceptible to cold shock; whereas rabbit, fowl and human are noted as being very resistant to cold shock. Membrane composition revealed, that generally, cold shock resistant species contained higher ratios of cholesterol to phospholipids and an increased in polyunsaturated fatty acids compared with cold shock susceptible species (Watson, 1981). Darin-Bennett and White (1977) reported the molar ratio of cholesterol to phospholipids of ram and bull (cold shock sensitive) to be 0.38 and 0.45 compared with the rabbit and human (cold shock resistant) at 0.88 and 0.99, respectively. These values were in close agreement with the ratios later reported by Parks and Lynch (1992) with molar ratio of cholesterol to phospholipid of the cold shock sensitive species to be low, bull (0.45), stallion (0.36) and boar (0.26).

At low temperatures, the cholesterol to phospholipid ratio is also important in determining sperm membrane fluidity and stability. Cholesterol interacts with fatty acyl chains of the phospholipids, modulating the fluidity of the membrane. Cholesterol also maintains phospholipids in a random laminar arrangement during the decline in temperature (Amann and Pickett, 1987). Cryopreserved sperm have been noted as having lower cholesterol concentrations compared with fresh sperm. Kadirvel et al. (2009) compared cholesterol concentrations of ejaculated buffalo sperm before and after cryopreservation. Cryopreserved sperm had significantly lower cholesterol concentrations compared with fresh sperm 12.89 and 21.67 $\mu\text{g}/10^8$ sperm, respectively. Increasing the cholesterol to phospholipid ratio also broadens the phase transition, reduces membrane leakage and lowers membrane phase separation in model membranes (Drobnis et al., 1993). Therefore, the addition of cholesterol to sperm membranes before cryopreservation may increase, a sensitive bull's sperm, ability to survive the cryopreservation process.

Cholesterol, not being soluble in aqueous semen diluents, requires a carrier to facilitate its transfer to cell membranes. Cyclodextrins can be used to transport cholesterol due to their hydrophilic face and hydrophobic core, which can bind cholesterol. The addition of methyl or hydroxypropyl groups to cyclodextrins enhances solubility in water and their ability to dissolve hydrophobic compounds (Yancey et al., 1996). By pre-loading cyclodextrins with cholesterol, these molecules "cholesterol loaded cyclodextrins" (CLCs) are able to insert cholesterol directly into cell membrane (Purdy and Graham, 2004a). Concentration of cholesterol transferred to sperm increases linearly with the concentration of CLCs incubated with sperm (Purdy and Graham, 2004a). The ratio of cholesterol to phospholipids in cold shock sensitive bull sperm (0.45) can be brought to the concentration of highly cold shock resistant species such as the rabbit (0.88) or human (0.99) (Darin-Bennett and White, 1977; Purdy and Graham, 2004a). However, there appears to be a threshold of ~4 to 5 times normal concentrations, that when crossed, cholesterol becomes detrimental to bull sperm survival (Purdy and Graham, 2004a). The addition of cholesterol to bull sperm that are to be sex-sorted has also been reported as yielding unacceptable results following staining and dilution (De Graaf et al., 2007).

Epididymal sperm having not been exposed to seminal plasma may have an increased concentration of lipids and cholesterol in the plasma membrane. Zittle and O'dell (1941) noted that washed bovine epididymal sperm have a lipid content of 13%. The lipid content of the major sections of the sperm cell were broken down into the head (7%), midpiece (6%) and tail (23%). Komarek et al. (1964) reported ejaculated bull sperm have a lipid content of 12% consisting of phospholipids (73.3%) and cholesterol (14.5%). In the boar, Pickett et al. (1967) reported the lipid content of epididymal sperm is greater than ejaculated sperm collected from the same boars at $2.57 \mu\text{g}/10^6$ sperm compared to $2.25 \mu\text{g}/10^6$ sperm, respectively. Matsumoto (1996) also reported in the bull that epididymal sperm and ejaculated sperm differed in lipid content at 21.2% and 9.7%, respectively cited by Pickett et al. (1967). Pamornsakda et al. (2011) reported no difference in sperm cholesterol concentration of equine epididymal sperm ($0.24 \mu\text{g}/10^6$) and ejaculated sperm ($0.23 \mu\text{g}/10^6$).

Although, Manjunath and Thérien (2002) reported the removal of 7% to 15% of cholesterol within 15 to 30 minutes of exposure to seminal plasma and a 25% loss after 4 hours of incubation of bovine epididymal sperm with seminal plasma. Moreau et al. (1998) also noted an increase in cholesterol efflux from bovine epididymal sperm when incubated with or without BSP-A1/A2; 17.46% compared with 6.5%, respectively. A 3.7 fold increase in cholesterol loss has also been noted when fibroblast cells were incubated with BSP proteins compared with controls (Moreau et al., 1999). This loss of cholesterol from sperm induced by seminal plasma may decrease the ability of ejaculated sperm to endure the stresses applied during the cooling and cryopreservation process.

The ratio of protein to phospholipid in the plasma membrane has also been suggested to have an effect on the relative sensitivity of sperm to cold shock (Parks and Lynch, 1992). With species such as the boar (1.26) that are considered to be cold shock sensitive having higher concentrations of protein to phospholipid ratios in their sperm plasma membranes compared to the bull (0.86), stallion (0.86) or rooster (0.46) that are considered to be more cold shock resistant.

Epididymal bull sperm have been reported to be cold shock resistant compared with ejaculated sperm (Bialy and Smith, 1959). It was also reported the motility of

epididymal sperm collected from slaughterhouse bulls did not significantly differ before (77.5%) or after (76.2%) cold shock (0°C for 10 minute); but exposure of epididymal bull sperm to the ampulla before cold shock did result in significant decreases in motility compared with epididymal sperm not exposed; 42% and 76%, respectively (Bialy and Smith, 1959). Lasley and Bogart (1944) collected epididymal and ejaculated sperm from the same 20 boars and reported, epididymal sperm survived (%live) a cold shock of 10 minutes at 0°C significantly better (65%) compared with ejaculated sperm (12.5%). Boar epididymal sperm also survived a 16 day in vitro storage better than ejaculated sperm at 51.2% and 9.6% live sperm, respectively.

The addition of cholesterol to sperm has been shown to increase the survival of sperm following cryopreservation in the: bull (Purdy et al., 2005; Amorim et al., 2009; Moraes et al., 2010), ram (Mocé et al., 2010), stallion (Moore et al., 2005) and boar (Tomás et al., 2011). Purdy and Graham (2004a) reported an increase in bull sperm motility and viability following cryopreservation with addition of cholesterol (60% and 55%) compared with without addition of cholesterol (42% and 46%). Amorim et al. (2009) also reported an increase in bull sperm motility following cryopreservation from 40% without the addition of cholesterol to 54% with the addition of cholesterol prior to cryopreservation.

The time required for sperm to capacitate may be influenced by their cholesterol to phospholipid ratio. Species having higher cholesterol to phospholipid ratios such as the human (0.99) and rabbit (0.88) take longer to capacitate compared with species with lower cholesterol to phospholipid ratios such as the boar (0.26) and ram (0.38). In a report by Davis (1981), it was noted that the time required for sperm to capacitate closely followed their cholesterol to phospholipid ratios; ram (1.5 hours, 0.34), boar (2 hours, 0.35), bull (3 hours, 0.45), rat (3.75 hours, 0.58), rabbit (6 hours, 0.88) and man (7 hours, 0.99).

Cryopreservation of Epididymal Sperm

Successful cryopreservation of sperm allows for the wide spread distribution of favorable genetics. A problem arises, when an animal becomes permanently incapacitated or suddenly dies, making the collection of semen impossible. Before animal genetics are lost, epididymal sperm can be collected, giving the producer a last

chance at obtaining progeny from this animal. Ejaculated and epididymal sperm may be different due to the lack of exposure to seminal plasma.

The cryopreservation of bovine epididymal sperm was first reported with the collection of epididymal sperm from a 9-year-old Jersey bull in 1953 and later with a Holstein bull of known fertility (Barker, 1954). Following collection, epididymal sperm was cryopreserved and stored at -79°C . This epididymal sperm was used to successfully artificially inseminate cows, showing that cryopreserved bovine epididymal sperm are capable of fertilizing ova in vivo with comparable pregnancy rates (63.6%) to frozen thawed ejaculated sperm (60.0%).

Goto et al. (1989) collected and cryopreserved (-196°C) epididymal bovine sperm from 5 slaughterhouse bulls for use in in vitro fertilization (IVF). Bovine epididymal sperm collected from different bulls was reported to give results that were more consistent compared with previous studies using ejaculated bull sperm. The addition of seminal plasma during ejaculation was implied to be the source of variation in IVF rates using ejaculated bull sperm since the only difference between the ejaculated and epididymal sperm was the exposure to seminal plasma.

There are conflicting reports as to the effects of seminal plasma on ejaculated sperm (Graham, 1994). The addition of seminal plasma to bovine epididymal sperm has been reported not to affect the motility of sperm following cooling to 5°C or post-thaw; 78% compared with 73% and 66%, respectively (Graham, 1994). However, the seminal plasma from different bulls did have an effect. Seminal plasma may also affect the membrane permeability of sperm due to the addition and or removal of factors from the plasma membrane.

The differential scanning calorimeter (DSC) technique allows for the measurement of water transport during the freezing of sperm and has been used to study bovine sperm (Li et al., 2006). Evaluation of ejaculated and epididymal sperm for differences in membrane permeability were determined and used to calculate the optimal cooling rates for cryopreservation. It was determined that bovine epididymal and ejaculated sperm membrane permeability were not different and could likely be optimally cryopreserved in the same manner (Alapati et al., 2009).

Motility before and after cryopreservation, of bovine ejaculated (79% and 60%, respectively) compared with epididymal sperm (82% and 63%, respectively) have been reported as being similar (Graham, 1994). These results were similar to the results noted by Martins et al. (2007), with a loss of ~10% motility following cryopreservation of bovine ejaculated (90% and 80%, respectively) compared with epididymal sperm (75% and 65%, respectively). Ejaculated and epididymal sperm collected from 8 stallions resulted in ejaculated sperm having higher pre-freeze total motility (79.6%) compared with epididymal sperm (29.4%). Although, no difference was noted in the post-thaw total motility ejaculated sperm (63.4%) compared with epididymal sperm (73.4%); it was suggested that epididymal sperm endure cryopreservation better than ejaculated sperm (Monteiro et al., 2011). Magistrini et al. (1988) reported that the post thaw motility of stallion epididymal sperm (37%) was greater than ejaculated sperm (31.6%) as cited by Braun et al. (1994). This was also noted by Braun et al. (1994) with epididymal sperm having higher post-thaw motility (40%) compared with ejaculated sperm (35%) collected from the same stallions. In the ram, significant differences in motility before and after cryopreservation, of ejaculated sperm (71% and 26%) compared with epididymal sperm (77% and 3%) have been reported (Graham, 1994). The addition of seminal plasma to epididymal ram sperm significantly increased its post-thaw motility from 3% without seminal plasma to 34% with seminal plasma (Graham, 1994). Although, this is in contrast to the Black Manchega ram, whose fresh epididymal sperm motility (77%) was not different than ejaculated sperm (74%), and whose post-thaw epididymal sperm motility (58%) was significantly better than ejaculated sperm post-thaw (37%) (García-Álvarez et al., 2009).

Chromosomal integrity was also reported as not being different between ejaculated and epididymal bovine sperm following cryopreservation (Martins et al., 2007). Garcia-Macias et al. (2006) also reported the chromosomal integrity (mean DNA fragmentation index) to be similar between fresh ejaculated and cauda epididymal sperm collected from Iberian Red deer (184 and 178), ram (174 and 163) or the domestic dog (184 and 174). García-Álvarez et al. (2009) also found no difference in percentage of high DNA stability post-thaw between ejaculated sperm (26%) or epididymal sperm (28%) collected from 6 black Manchega rams.

Acrosomal integrity of bovine ejaculated sperm, collected from a bull of known fertility, has been reported to be significantly better (88%) than epididymal sperm (65%), collected from 3 slaughterhouse bulls, following cryopreservation (Martins et al., 2007). In contrast, Varisli et al. (2009) compared the acrosomal integrity of ejaculated and epididymal ram sperm as to their susceptibility to cryobiologically relevant stressors and found epididymal sperm more resilient. When exposed to hypo-osmotic (75 mOsm) stress, ram epididymal sperm was unaffected while ejaculated sperm had a 30% loss of acrosomal integrity. Acrosomal integrity of ram epididymal sperm was also unaffected by chilling stress but the acrosomal integrity of ejaculated sperm was reduced 40% to 50%.

Heise et al. (2011) collected ejaculated and epididymal sperm from the same 4 stallions and reported viable acrosome intact sperm at the time of collection was significantly greater for epididymal sperm (91%) compared with ejaculated sperm (84%). However, following cryopreservation there was no difference in the percentage of viable acrosome intact sperm noted between epididymal sperm (60%) or ejaculated sperm (64%). In the goat, acrosomal integrity of fresh and cryopreserved goat ejaculated (95% and 89%) and epididymal (100% and 84%) sperm has been noted as being similar (Blash et al., 2000). In the domestic cat, Tebet et al. (2006) also reported the percentage of acrosome reacted sperm between fresh and cryopreserved ejaculated (5.4% and 28.3%) compared with epididymal sperm (7.4% and 23.5%).

Temporary storage of epididymal sperm, within the epididymides, has been reported without detrimental effects. This will allow for transportation of epididymides to the laboratory for epididymal sperm collection possible. In the stallion, Monteiro et al. (2011) collected ejaculated and epididymal sperm from the same 4 stallions and compared ejaculated and epididymal sperm cryopreserved at the time of collection to epididymal sperm held at 5°C for 24 hours in the epididymides before subsequent cryopreservation. No difference was noted in the progressive motility of epididymal sperm stored (5°C, 24 hours) in the epididymides (32.4%) compared with ejaculated and epididymal cryopreserved following collection (36.2% and 33.0%, respectively).

The fertility following AI was also found to be similar for epididymal sperm held at 5°C for 24 hours (61.5%, 8/13) compared with ejaculated (61.5%, 8/13) and epididymal (92.3% 12/13) that were cryopreserved following collection.

In the Burchell's zebra, epididymal sperm stored in the epididymides at 4°C for 16 hours was able to maintain its motility (40%) but motility significantly decreased following 24 hours (20%) and 32 hours (10%) of storage (Bezuidenhout et al., 1995). In the Eland, live (40%) and motile (30%) epididymal sperm were collected following storage within the epididymides at 4°C for 72 hours (Bissett and Bernard, 2005). Bezuidenhout et al. (1995) stored Eland epididymal sperm at 4°C for 32 hours without significant drop in motility (70%), and was able to collect motile sperm for up to 5 days of epididymal storage.

Yu and Leibo (2002) found that dog epididymal sperm could be held at 4°C for 48 hours without affecting the acrosomes integrity (90%); and only decreased to 65% at 192 hours. Although, motility of dog epididymal sperm was noted as being more sensitive and significantly decline from 50% to 30% following 5 hours of refrigerated (4°C) storage. However, following 192 hours motile sperm were still able to be collected (Yu and Leibo, 2002).

Tamayo-Canul et al. (2011) compared the storage of ram epididymal sperm at 5°C in the epididymis or undiluted in vitro of 15 rams. Ram epididymal sperm total motility at 0 hour (67%) was significantly better following 24 hours of refrigerated storage (5°C) within the epididymides (49.2%) compared with undiluted in vitro storage (27.6%). The African buffalo epididymal sperm stored within the epididymides for 5-days at 4°C were compared as to their motility (Bezuidenhout et al., 1995). A significant decrease in motility was noted in the first 8 hours of storage (60% to 50%) but remained constant until 64 hours of refrigerated epididymal storage (40% to 30%). Following 5 days of storage within the epididymides collection of motile sperm (10%) was still possible.

When transporting testes and epididymides to the laboratory for epididymal sperm collection, it is important to take proper care to ensure the best collection results. In the ram, Lone et al. (2011) compared epididymal sperm collected from 12 pairs of testes obtained from slaughterhouse and transported to the laboratory at refrigerated

(4.9 to 6°C) or ambient temperatures (17.9 to 21.5°C). The different transportation temperatures (refrigerated and ambient) were compared at the time epididymal sperm collection resulting in differences in: motility (82.5% compared with 75%); %live (92.9% compared with 88.9%); %intact acrosome (98.5% compared with 90.6%). The transportation temperatures (refrigerated and ambient) were also compared following 72 hours of storage at 4°C resulted in motility (60% compared with 45.8%); %live (81.5% compared with 73.2%) and %intact acrosome (91.7% compared with 82.3%). Results were significantly different at the time of collection and following 72 hours of storage at 4°C, emphasize that although epididymal sperm are robust care needs to be take in their transportation.

Collection and cryopreservation of epididymal sperm has been performed in a wide variety of exotic animals in the hopes of conserving the genetic diversity of these species. These include: springbok, impala and blesbok (Chatiza et al., 2011), North American bison (Krishnakumar et al., 2011), Wood bison (Thundathil et al., 2007) common hippopotamus (Saragusty et al., 2010), black Manchega ram (García-Álvarez et al., 2009), eland (Bissett and Bernard, 2005), Spanish ibex (Santiago-Moreno et al., 2006), Iberian Red deer (Martínez-Pastor et al., 2006), African buffalo (Lambrechts et al., 1999), gazelle (Saragusty et al., 2006) Cantabric brown bear (Anel et al., 1999), Red hartebeest and Bruchell's zebra (Bezuidenhout et al., 1995), African Black rhinoceros (Stoops et al., 2011) and Black wildebeest (Herrick et al., 2004).

CAPACITATION OF SPERM

The obligatory process of capacitation was first discovered independently by Austin (1951) and Chang (1951). These studies revealed that sperm that resided in the female reproductive track for a period of time were able to fertilize oocytes in compared with freshly ejaculated sperm that was unable to fertilized oocytes. The following year, Austin (1951) coined the term "capacitation" to describe the changes sperm must undergo in the process of gaining the ability to fertilize an oocyte. Early descriptions of capacitation included the acrosome reaction as part of capacitation, but since capacitation is exclusive to mammals and the acrosome reaction is not, these processes are now considered to be separate, see the review by Bavister (2002). The

process of capacitation is not completely understood and research is ongoing to better understand the process.

Sperm are stored in the cauda epididymides until the time of ejaculation. Upon ejaculation, sperm are mixed with seminal plasma from the accessory sex glands. At this time, sperm are not able to fertilize an oocyte but must undergo the process of capacitation. Under normal mating, in vivo fertilization, sperm need to reside within the female reproductive tract for a species-specific time period in order to become capacitated (Austin, 1951; Chang, 1951). During in vivo fertilization, sperm are supplied the necessary factors by the female reproductive tract to undergo capacitation. This is in contrast to in vitro fertilization, in which sperm must be incubated in a capacitation media containing specific capacitation inducing factors to gain the ability to fertilize ova. Capacitation consists of distinct biochemical and physical changes that act to alter the sperm surface, change the intracellular pH, and stimulate signal transduction pathways. This leads to changes in motility, ability to recognize and adhere to the zona pellucida, and undergo the acrosome reaction (Reid et al., 2011).

Seminal plasma changes the environment with an increase in bicarbonate, calcium, and albumin along with other factors such as decapacitation factors and BSP proteins. These factors both stimulate and inhibit capacitation.

Albumin serves as a cholesterol acceptor during sperm capacitation. Efflux of cholesterol from the sperm plasma membrane marks one of the initial events that alter the sperm surface during capacitation (Visconti et al., 1999). Cholesterol has been shown to stabilize the plasma membrane and its loss results in an increase in membrane permeability and lateral movement of integral proteins. During in vitro capacitation, bovine serum albumin (BSA) is routinely used in capacitation media as a cholesterol acceptor but can be substituted with high density lipoproteins or β -cyclodextrins (Fraser, 2010). Polyvinylpyrrolidone (PVP) and polyvinyl alcohol (PVA) are also used in completely defined media to replace BSA (Parrish et al., 1989; Keskinetepe and Brackett, 1996; Kato and Nagao, 2009).

Bicarbonate is required to activate sperm motility, concentrations in the epididymis are held at low concentrations ($\sim 3\text{mM}$), upon ejaculation bicarbonate concentrations are increased to that of general body fluids ($\sim 30\text{mM}$) (Fraser, 2010).

Bicarbonate enters the sperm by way of a sodium bicarbonate co-transporter (Visconti, 2009). An initial response to increase bicarbonate concentration is the activation of sAC that leads to increases in cAMP and Protein Kinase A (PKA) dependent protein phosphorylation (Abou-Haila and Tulsiani, 2009). Increases in intracellular pH are also associated with increased bicarbonate concentration that is necessary for capacitation (Tardif et al., 2003). The characteristic asymmetry of the sperm plasma membrane is disrupted by a bicarbonate dependent enzyme, phospholipid scramblase, causing phospholipids to be flipped across the membrane bilayer (Gadella and Harrison, 2000). This flipping of phospholipids causes a reduction in the plasma membrane stability and makes it easier for cholesterol to be removed (Fraser, 2010).

Calcium, like bicarbonate and albumin, is also required for sperm capacitation. Sperm do not carry a complete intracellular load of calcium but rely on extracellular calcium, which is taken up by bovine sperm during capacitation (Parrish et al., 1999). There are many different plasma membrane calcium permeable channels that have been found in sperm (Darszon et al., 2006). One of which, Ca^{2+} -ATPase located on the sperm plasma membrane functions in the regulation of intracellular calcium concentration by actively removing calcium from the sperm. It has also been proposed to be involved in the loading of intracellular calcium stores utilized during the acrosome reaction (Parrish et al., 1999). During capacitation in vitro, intracellular calcium concentrations have been reported to increase 6-fold (from ~25 to ~160nM) (Dragileva et al., 1999; Abou-Haila and Tulsiani, 2009). Like bicarbonate, calcium is required for the activation of sAC, which increases cAMP and Protein Kinase A (PKA) activity (Jaiswal and Conti, 2003; Visconti, 2009). Increases in cAMP also activates CatSper Ca^{2+} channels that functions in sperm hyperactivated motility and capacitation by increasing calcium concentrations (Ren and Xia, 2010).

Decapacitation factors (DF) are inhibitory molecules that prevent sperm from undergoing capacitation (Fraser et al., 2006). The addition of DF to sperm occurs in a species specific manner and can be acquired in the epididymis or by seminal plasma. These factors bind to Decapacitation Factor Receptors (DF-R) and are attached to the sperm surface membrane by-way of glycosylphosphatidylinositol (GPI) anchors. The binding of DF to DF-R has been shown to be fucose-mediated with DF having fucose

moieties and DF-R having fucose binding sites. Decapacitation factors have been proposed to activate a calcium-ATPase, which maintains a low intracellular concentration of calcium. Also DF binding to capacitated cells causes conformational and functional changes to G-protein coupled receptors activating the stimulatory receptors and inactivating inhibitory receptors (Fraser et al., 2006). Some decapacitation factors that have been characterized include: HongrES1, DF glycoprotein, phosphatidylethanol-binding protein 1 and NYD-SP27 (Reid et al., 2011).

Capacitation of mammalian sperm functions through signal transduction pathways. Factors found in the seminal plasma and capacitation media serve as first messengers; these bind to specific receptors or are transported through the plasma membrane and directly or indirectly influence the production of cyclic AMP (cAMP) by adenylyl cyclases.

Multiple forms of adenylyl cyclase have been identified with the two major categories being: membrane bound and soluble adenylyl cyclases. All membrane bound adenylyl cyclases have been identified as being G protein regulated in response to multiple activating factors or ligands (Reid et al., 2011). Some specific factors, that have been identified in seminal plasma and bind to receptors on the plasma membrane, include Fertilization-promoting Peptide, adenosine, calcitonin and Angiotension-2 (Fraser et al., 2001). In contrast to membrane bound adenylyl cyclases, soluble adenylyl cyclases are stimulated by increases in intracellular bicarbonate, and calcium (Chen et al., 2000; Jaiswal and Conti, 2003; Litvin et al., 2003). Soluble adenylyl cyclases are found in the cytoplasm associated with various intracellular organelles (Zippin et al., 2003). Controversy has arisen as to the exact role each form of adenylyl cyclase plays in the regulation of sperm function. Although, it is likely that both membrane bound and soluble adenylyl cyclases participate in the production of cAMP (Reid et al., 2011).

Membrane bound adenylyl cyclases are dependent upon G protein coupled receptors for activation (Taussig and Gilman, 1995). There are multiple forms of G protein coupled receptors, both stimulatory and inhibitory. Stimulatory receptors are known to be active in decapacitated sperm whereas inhibitory receptors are active in capacitated sperm (Reid et al., 2011). This biphasic response of initial stimulation followed by inhibition of adenylyl cyclase has been proposed as a possible safe guard

against over capacitation, which leads to the spontaneous acrosome reaction and loss of fertilizing potential. Various reports show agents that are known to affect the activity of somatic cell trans-membrane adenylyl cyclases and also affect sperm membrane adenylyl cyclase activity (Fraser and Duncan, 1993; Fraser and Adeoya-Osiguwa, 1999; Leclerc and Kopf, 1999; Wade et al., 2003), although others reports have not reported this response (Forte et al., 1983; Hildebrandt et al., 1985).

Soluble adenylyl cyclases are responsive to increases in intracellular bicarbonate, and calcium, which are regulated by ion channels. The initial increase in bicarbonate following ejaculation causes an increase in intracellular bicarbonate by way of sodium bicarbonate cotransporters (Salicioni et al., 2007). This increase in bicarbonate causes an increase in intracellular pH, and the activation of the soluble adenylyl cyclase to produce cAMP and consequently increases PKA and activates CatSper Ca^{2+} channels (Chen et al., 2000; Salicioni et al., 2007; Ren and Xia, 2010). CatSper Ca^{2+} channels increase the concentration of calcium in the sperm that further activates soluble adenylyl cyclase activity (Jaiswal and Conti, 2003).

Phosphodiesterases are enzymes that convert cAMP to the nonreactive 5' AMP (Breininger et al., 2010). This conversion helps to regulate cAMP concentrations in sperm during capacitation and fertilization (Gadella and Harrison, 2000). Since increases in cAMP concentrations are required for sperm to undergo capacitation, the level of phosphodiesterases activity may influence the time required for sperm to undergo capacitation. The inhibition of phosphodiesterases can cause an increase in cAMP concentrations resulting in capacitation of sperm cells. This has been demonstrated by including caffeine, a non-selective inhibitor of phosphodiesterase, to in vitro capacitation medium causing an increase in sperm capacitation (Breininger et al., 2010).

The production of cAMP induces several signal transduction elements including Protein Kinase A, Protein Kinase C, and protein tyrosine kinases (Leclerc et al., 1996; Thundathil et al., 2002). These signal transducers phosphorylate multiple proteins and lead to the increase in protein tyrosine phosphorylation that has been shown to be associated with capacitation (Visconti et al., 1995).

Many methods such as high ionic strength solution, heparin, caffeine and nitric oxide have been employed in the attempt to efficiently capacitate sperm. High ionic strength solution was used by Brackett et al. (1982) to induce capacitation of bovine sperm in vitro. This method consisted of incubating sperm in hypertonic media (380 mOsm/kg) for 5 minutes prior to being added to oocytes. This study successfully produced the first bovine IVF calf ("Virgil"). Although, when Bondioli and Wright (1983) compared high ionic strength media and a standard media, they found no difference in fertilization rates using fresh and frozen bovine semen.

In the bull, heparin binds to BSP proteins attached to the sperm membrane, that has been shown to participate in sperm capacitation (Therien et al., 1998). Heparin is able to bind both ejaculated and epididymal bovine sperm. Although, Handrow et al. (1984) noted the number of heparin binding sites were lower for epididymal sperm ($10 \times 10^5/\text{sperm}$) compared with ejaculated sperm ($30 \times 10^5/\text{sperm}$).

Heparin a commercially available GAG was noted as being the most potent inducer of the acrosome reaction in the bull (Handrow et al., 1984). The addition of heparin to bull sperm stimulates an increase in intracellular calcium, pH and cAMP, necessary to start the signaling pathway associated with capacitation (Parrish et al., 1999). The addition of glucose to bovine capacitation media has been shown to inhibit capacitation in bovine ejaculated sperm by delaying the rise in intracellular pH, but glucose has no effect on mouse capacitation (Parrish et al., 1989).

Hyperactivated motility was associated with capacitation, but now has been shown to be induced through an independent signaling pathway (Marquez and Suarez, 2004). Hyperactivated motility is characterized by an increased in sperm velocity with a rapid, high arched head movements that facilitates fertilization by penetration of the zona pellucida (Marquez and Suarez, 2004).

Cryopreservation-Induced Capacitation

Cryopreservation has been noted as inducing a capacitation like state in sperm, and has been coined 'cryocapacitation' (Bailey et al., 2000). Cryocapacitation is characterized by a reduction in the time required for sperm to fertilize oocytes.

Cryopreservation causes a reduction in the heterogeneity of the sperm population by killing or incapacitating some, leaving some with a shortened lifespan and some partially or fully capacitated sperm.

Fresh ejaculated boar sperm require a pre-incubation period of 4 to 8 hours to become capacitated and fertilize oocytes in vitro (Ikeda et al., 2002). Cryopreservation seems to negate this need as frozen-thawed ejaculated and epididymal boar sperm are able to fertilize oocytes without pre-incubation (Ikeda et al., 2002).

EPIDIDYMAL SPERM IN ASSISTED REPRODUCTIVE TECHNIQUES

Assisted reproductive techniques, such as AI and IVF will allow for the efficient use of epididymal sperm (Barber, 1983).

Artificial Insemination (AI)

Barker (1954) reported the first AI pregnancies (n=7) using cryopreserved (-79°C) Jersey epididymal sperm. In his report, Barker showed that epididymal sperm could be collected, cryopreserved and utilized in assisted reproductive techniques to save the genetics of bulls. Epididymal sperm was also later harvested from a Holstein bull of known fertility that had become lame and was no longer able to be collected. Pregnancy rates following AI with epididymal sperm (63.6%, 7/11) and ejaculated sperm (59.6%, 216/362) collected from this bull were similar (Barker, 1954). This suggested that epididymal sperm may have pregnancy rates comparable to ejaculated sperm.

Bratton and Foote (unpublished data) collected epididymal sperm from a genetically superior bull following his unexpected death. The epididymal sperm was cooled (5°C) and stored for 30 to 60 hours prior to being used to AI 41 cows. Non-return rate of 871 cows artificially inseminated with ejaculated sperm collected shortly before the bulls unexpected death were compared with epididymal sperm. The resulting non-return rate for epididymal sperm (61%) and ejaculated sperm (72%) were similar and followed by uneventful pregnancies and birth of normal young (Foote, 2000b).

Igboeli and Foote (1968) compared cooled (5°C) epididymal and ejaculated sperm collected from 4 bulls (1 Holstein and 3 Angus) of known fertility. Following AI of 100 cows, a non-return rate of 69% was reported using cooled epididymal sperm, compared with previous non-return rates of 75% for 9,391 cows inseminated using ejaculated sperm from the same bulls.

It was noted that the non-return rate for epididymal sperm was only lower for 1 bull that had previously been culled for low fertility.

Amann and Griel (1974) collected fresh testicular, ejaculated and epididymal sperm from the same 12 Holstein bulls and utilized it to AI 141 dairy heifers and cows. Following insemination with fresh ejaculated and epididymal sperm, presumptive embryos were collected from females resulting in a fertilization rate of 0% for testicular, 94% for ejaculated sperm and 84% for epididymal. No significant difference was noted as to the ability of fresh ejaculated and epididymal sperm collected from the same bulls to fertilize ova following AI.

Guerrero et al. (2008) collected and cryopreserved (-196°C) epididymal sperm from 3 beef bulls. Testes were obtained from a local slaughterhouse and transported to the laboratory at ambient temperature (28.5°C). Frozen-thawed epididymal sperm were used to AI 6 cows resulting in the birth of 3 calves.

Barker and Gandier (1957) reported the birth of the first foal resulting from the use of cryopreserved epididymal stallion sperm. In this report, no attempt was made to estimate the fertility of epididymal sperm compared with ejaculated sperm.

Heise et al. (2010) compared the fertility of fresh and cryopreserved ejaculated and epididymal sperm collected from the same 3 stallions and the effects of exposure to seminal plasma. Insemination of 21 mares (over 5 consecutive estrous cycles), with epididymal and ejaculated sperm into the uterine body resulted in significantly better pregnancy rates for ejaculated, fresh (84%) and cryopreserved (64%) sperm compared with epididymal fresh (22%) and cryopreserved (6.7%) sperm. The addition of seminal plasma to epididymal sperm was noted to increased pregnancy rates in fresh epididymal sperm with (75%) and without (27%) seminal plasma and for cryopreserved epididymal sperm with (22%) and without (6.7%) seminal plasma following AI. Although, Monteiro et al. (2011) collected and cryopreserved ejaculated and epididymal sperm from the same 8 stallions to compared their fertility. Sperm from all 8 stallions were pooled for insemination at the tip of the uterine horn to compare fertilization rate of ejaculated and epididymal sperm. Following insemination, a fertilization rate of 61.5% (8/13) for ejaculated and 92.3% (12/13) for epididymal sperm was reported.

In the goat, Blash et al. (2000) collected and cryopreserved epididymal sperm from 25 bucks at necropsy for use in assisted reproductive techniques. Cryopreserved ejaculated sperm was noted as having significantly better pregnancy rates (39%, 7/18) compared with epididymal sperm (5%, 1/20) following AI (Blash et al., 2000).

Cryopreserved epididymal sperm has also been used to AI exotic breeds resulting in the production of young. In the Spanish ibex, Santiago-Moreno et al. (2006) used cryopreserved epididymal sperm collected from deceased rams to AI 6 ibex females, resulting in the birth of the first ibex conceived using AI.

In the Iberian Red deer, epididymal sperm has also been collected from 3 males postmortem and cryopreserved for use in AI. Cryopreserved epididymal sperm was then used to breed 66 females with 56% of all inseminations resulting in the birth of live young (Soler et al., 2003).

In Vitro Fertilization (IVF)

In vitro fertilization using epididymal sperm has been accomplished in a variety of animals, including the guinea pig (Yanagimachi, 1972), rat (Toyoda and Chang, 1974; Seita et al., 2009), monkey (Sankai et al., 1997), rabbit (Brackett et al., 1978), cat (Niwa et al., 1985), cow (Pavlok et al., 1988), llama (Del Campo et al., 1994), Hartmann's and Burchell's zebra (Meintjes et al., 1995), African buffalo (Shaw et al., 1995), swine (Rath and Niemann, 1997), Wood bison (Thundathil et al., 2007) and camel (Badr and Abdel-Malak, 2010).

The use of epididymal sperm gives the producer a last chance to save an animal's genetics for use in assisted reproductive techniques. In vitro fertilization may allow for the efficient use of this sperm by maximizing the number of subsequent progeny produced (Loskutoff et al., 1995).

The first report of bovine epididymal sperm utilized in IVF was by Ball et al. (1983). In this report, fresh epididymal sperm was collected from testes of bulls obtained from the slaughterhouse for use in IVF. Following IVF with fresh epididymal sperm, 40% of the 104 oocytes had both male and female pronuclei. In the same year, Lenz et al. (1983) described the effects of temperature during IVF using fresh epididymal bull sperm collected from testes of slaughterhouse bulls. At 39°C, epididymal sperm was noted to penetrate 58% (39/67) of oocytes in vitro, with 36% having both male and

female pronuclei. Later, Pavlok et al. (1988) compared fresh epididymal sperm collected from 7 bulls with fresh ejaculated sperm collected from 5 bulls. In this study, IVF with 203 oocytes resulted in a fertilization rate of 72% for epididymal sperm compared with 54% for ejaculated sperm. In spite of having a higher fertilization rate, epididymal sperm was reported as having an increased incidence of fertilization anomalies (32.6%) compared with ejaculated sperm (5.4%). Bovine epididymal sperm could be a potential source of sperm for use in IVF (Pavlok et al., 1988). These reports suggest epididymal sperm as a viable source for use in IVF.

Although the previous reports established epididymal sperm as a viable source for IVF, fresh epididymal sperm will not allow for the efficient use of this finite resource. The need to use cryopreserved epididymal sperm is necessary for the efficient dissemination of genetics from genetically superior bulls. The first report of cryopreserved epididymal sperm used in IVF was reported by Goto et al. (1989). In this report, cryopreserved epididymal sperm collected from 5 different bulls were used for IVF of bovine oocytes. Fertilization rate (male and female pronuclei) averaged 57% following IVF of 968 oocytes using cryopreserved epididymal sperm. Of the 2612 embryos cultured during this study, development to the 8-cell stage was reported to range from 21% to 31% on days 3 to 4 with 9.3% to 12% reaching blastocyst stage on days 7 to 8 post-insemination, respectively. It was proposed that individual variation among bulls was not a significant factor in the fertilization and development rates of bovine follicular oocytes when using epididymal sperm.

Katska et al. (1996) compared the effects of performing IVF with cryopreserved epididymal sperm (988 oocytes) or ejaculated sperm with or without the removal of seminal plasma at the time of collection (741 oocytes). Epididymal sperm was collected from the testes of 7 slaughterhouse bulls and ejaculated sperm was collected from 4 bulls of known fertility. Frozen-thawed epididymal sperm and ejaculated sperm collected with or without the removal of seminal plasma at the time of collection were compared as to their fertilization rate (84%, 63% and 50%, respectively), cleavage (80%, 52% and 40%, respectively) and blastocyst rate (31%, 16% and 11%, respectively). It was recommended that seminal plasma be removed from ejaculated bull sperm prior to cryopreservation if sperm was to be used for IVF. Martins et al. (2007) also performed

IVF with cryopreserved epididymal sperm from 3 bulls along with ejaculated sperm from a fourth bull of known in vitro fertility. Embryo development following IVF with epididymal sperm resulted in a cleavage rate of 80% and blastocyst rate 35%. It was also noted that the development of embryos fertilized with ejaculated sperm had similar cleavage (89%) and blastocyst (57%) rates. Although, variation was noted between bulls in this study, with epididymal sperm from 1 of the 3 bulls noted as having lower cleavage (42%) and blastocyst (26%) rates following IVF.

To date, there are no published reports of the birth of a live calf following IVF with bovine epididymal sperm. The longest reported development of bovine IVF embryos fertilized with epididymal sperm was reported by Graff et al. (1996) who noted ongoing pregnancies. Although, live births have been reported following the transfer of IVF embryos fertilized with ejaculated sperm. Brackett et al. (1982) reported the birth of the first calf following IVF with ejaculated sperm. Two years later, Brackett et al. (1984) reported 2 sets of twin pregnancies following the transfer of IVF-derived embryos to recipients. One set of twins was lost due to entanglement of their umbilical cords, while the other set was delivered resulting in 2 live bull calves.

In other species such as the human, mouse, rat and Golden hamster epididymal sperm has been successfully used to produce live young following IVF.

In humans, the use of epididymal sperm is necessary for men with obstruction or congenital absence of the excurrent ducts preventing the ejaculation of sperm. Temple-Smith et al. (1985) collected sperm from the corpus epididymides by microaspiration from a 42-year-old man with secondary obstructive azoospermia for use in IVF. Following IVF, 1 of the 5 ova was fertilized and subsequently transferred to the patient's wife at the two-cell stage. Pregnancy at 30 weeks of gestation was confirmed by ultrasound examination and changing hormone concentrations. Bladou et al. (1991) collected epididymal sperm from men with a congenital absence of the vas deferens or with secondary extended obstruction of spermatic ducts for use in IVF. The transfer of IVF-derived embryos fertilized with epididymal sperm resulted in 3 ongoing pregnancies at the time of this report. It was noted that epididymal sperm retrieved from the caput epididymis had a higher percentage of embryo degeneration (>50%) following embryo transfer.

Tzeng et al. (1996) reported the birth (3/3/1994) of a healthy female infant following IVF with epididymal sperm collected from a man with congenital absence of the vas deferens. IVF was performed on 12 oocytes resulting in 3 transferable embryos. Hovatta et al. (1993) reported the microsurgical epididymal sperm collection from 24 men diagnosed with obstructive azoospermia. IVF using epididymal sperm resulted in the birth of 2 healthy female twins and one ongoing twin pregnancy.

Songsasen et al. (1997) used cryopreserved mouse epididymal sperm, for IVF of mouse oocytes. Following IVF of 316 oocytes with epididymal sperm, 34% developed to the blastocyst stage. Embryos produced with cryopreserved epididymal sperm when transferred to recipients resulted in pregnancies in 12 of 15 recipients and the birth of live young (57 pups from 167 embryos transferred). It was also noted that the results obtained with frozen thawed epididymal sperm were comparable to those obtained from transfer of IVF embryos produced with fresh epididymal sperm (17 pregnancies from 19 recipients, 87 live young from 197 embryos). The next year, Songsasen et al. (1998) reported the viability and fertility of mouse epididymal sperm obtained at various postmortem intervals following storage at $\sim 22^{\circ}\text{C}$ for up to 24 hours. Following IVF of oocytes with sperm collected immediately after death or at 6, 12, 18, or 24 hours postmortem resulted in a cleavage rate of, 81%, 70%, 64%, 34% and 19%, respectively. Of the oocytes fertilized, more than 65% developed to the morulae/blastocyst stage. Transfer of embryos (n=166) produced in vitro with postmortem epididymal sperm resulted in the birth of 44 live pups (26.5%). Of the 44 pups produced, 3 resulted from the transfer of 11 embryos (27.3%) produced with epididymal sperm collected at 24 hours postmortem.

Barnett and Bavister (1992) used Golden hamster epididymal sperm to produce IVF embryos. It was determined that these embryos were able to develop in chemically defined, protein-free culture medium to the morulae and blastocyst stage (38/200), and produce normal offspring (22/200) following transfer to recipients.

Toyoda and Chang (1974) performed IVF using fresh rat epididymal sperm. Fertilization rate was determined to be 88.7% following IVF with 203 2-cell embryos transferred to 14 recipients. Of the 14 recipients, 9 were pregnant resulting in the birth of 43 live young.

Like in cattle epididymal sperm has been successfully utilized in IVF of various species. Unfortunately the birth of live young following IVF with epididymal sperm have yet to be reported in the scientific literature.

The first report of the birth of live young following IVF in mammals was reported in the rabbit by Chang (1959). In this study, ejaculated sperm was used to fertilize oocytes. Although, IVF was later performed with epididymal sperm by Brackett et al. (1978), embryos were not transferred to females. In this report, the in vitro fertilizing ability of testicular, epididymal, and ejaculated rabbit sperm from the same 10 bucks were compared. Epididymal sperm was noted as having a higher fertilizing ability, with 68 (73.1%) of the 93 oocytes fertilized compared with ejaculated sperm, which fertilized 34 (36.6%) of 93 oocytes or testicular sperm with <11% oocytes fertilized.

In the boar, Rath and Niemann (1997) compared ejaculated and epididymal sperm collected from the same boars for use in IVF. When evaluated side by side, frozen-thawed epididymal sperm fertilized (pronuclear formation) significantly more of the 161 oocytes (23%) compared with either fresh (8.9%) or frozen-thawed (2.2%) ejaculated boar sperm. These results were also confirmed by embryo development of the 485 oocytes with cleavage (≥ 2 cell) for epididymal sperm (59%) compared with fresh (14.6%) and frozen-thawed (16%) ejaculated sperm. Matás et al. (2010) also noted that over all treatments studied that fresh epididymal boar sperm resulted in better oocyte (716) penetration rates at 4 hours post-insemination (86.4%) compared with ejaculated sperm (45.3%) following IVF. As in the cow, there are no known reports of live births following the transfer of IVF embryos fertilized with epididymal sperm. Although, there are reports of live births following the transfer of IVF embryos fertilized with ejaculated sperm. Cheng et al. (1986) reported the first piglets born following the transfer of IVF-derived embryos inseminated with ejaculated sperm. Embryos were transferred to 15 recipient gilts resulting in 6 pregnancies and 19 piglets born. Abeydeera et al. (1998) reported pregnancies after transfer of pig embryos derived from IVF oocytes by X and Y chromosome-bearing fresh ejaculated sperm sorted by flow cytometry. Transfer of embryos fertilized with X-bearing sperm to 18 recipients resulted in 5 pregnancies and the delivery of 23 females and 1 male piglet. The transfer of embryos fertilized with Y-

bearing sperm to 10 recipients resulted in 3 pregnancies, and the delivery of 9 male piglets.

Song (1988) first used fresh and cryopreserved epididymal goat sperm to fertilize oocytes in vitro. Fertilization rates were similar following IVF with fresh (51%) and cryopreserved (57%) epididymal goat sperm. Pawshe et al. (1994) used fresh epididymal goat sperm for IVF of oocytes (n=289) collected from slaughterhouse ovaries. In this study, a cleavage rate of 65.6% and a blastocyst rate of 18.4% were reported following IVF. Blash et al. (2000) compared goat cryopreserved ejaculated and epididymal sperm. Cleavage and blastocyst development following IVF (oocytes n=337) using cryopreserved ejaculated (37% and 4%) compared with epididymal sperm (40% and 6%) were determined to be similar. Honda (1985) reported the first successful birth of a live goat kid following IVF as reported by De Smedt et al. (1992). In this report, the transfer of 12 2-cell IVF embryos to 5 recipients resulted in 1 pregnancy that successfully developed to term and the birth of 1 kid.

Wani et al. (2000) reported fresh ram epididymal sperm was able to penetrate IVM sheep oocytes collected by follicular puncture (38/73, 52%), slicing (55/101, 54.4%) and aspiration (39/76, 51.3%) following IVF. Wani et al. (2012) reported the utilization of fresh ram epididymal sperm collected from slaughterhouse testes to fertilize IVM oocytes (n=401) with and without cysteamine and EGF. Following IVF the percentage of fertilization (~74.2%) and cleavage (52.6%) were reported as being similar; although, development to the morula stage was determined to be greater when cysteamine (36.7%) and EGF (38.7%) were added compared with conventional IVF (22.1%). No known live births have resulted from the transfer of IVF embryos fertilized with epididymal sperm. However, live births have been reported from the transfer of IVF embryos fertilized with ejaculated sperm. Cheng et al. (1986) reported the first lambs born following the transfer of IVF embryos fertilized with ejaculated ram sperm. Embryos were transferred 16 hours following IVF to 16 recipients resulting in 7 pregnancies and 10 lambs born. Crozet et al. (1987) performed IVF on ovine tubal (87) and ovarian in vitro matured oocytes (99) fertilized with ejaculated sperm. The transfer of an embryo derived from an ovulated oocyte resulted in the birth of a lamb.

Flores-Foxworth et al. (1995) reported the transfer of 13 IVF-derived Red sheep embryos to 6 Rambouillet recipients resulting in 3 pregnancies and the live birth of 3 Red sheep lambs.

In the Dromedary camel, Badr and Abdel-Malak (2010) used epididymal sperm collected from males for IVF, following cold storage (4°C) for 0, 4 and 6 days. Following IVF, penetration (~56% of 167 oocytes), fertilization (~43% of 167 oocytes) and cleavage (~34% of 201 oocytes) rates were reported to be similar even though the time sperm were held in cold storage. Although, blastocyst rates were significantly higher when sperm were used on day 0 (16.3%) compared with day 6 (1.3%) of cold storage. No attempt was made to transfer embryos to recipients. To date, there are no known reports of live births following IVF with epididymal sperm in the Dromedary camel. Although there are reports of live births following the transfer of IVF embryos fertilized with ejaculated sperm. Khatir et al. (2007) reported the birth of 1 (8%; 1/13) calf following IVF of 120 in vivo-matured oocytes. Khatir and Anouassi (2006) reported the birth of dromedary calves following the transfer of embryos produced by IVM, IVF and IVC using slaughterhouse oocytes and fresh ejaculated sperm. Following IVF, a cleavage rate of 64% (425/664) and the percentage of oocytes reaching the blastocyst stage was 23% (155/664) was reported. Of the 18 pregnant females, 5 aborted between the 5th and 7th month of pregnancy and 13 females (20%) remained pregnant. At the time of the report, 5 calves were delivered with 8 females due to calf.

In the zebra, Meintjes et al. (1995) used fresh epididymal sperm collected from Burchell's stallions for use in IVF of oocytes collected from Burchell's (n=6) and Hartmann's (n=6) culled zebra females. Of the 85 oocytes, 13 developed to the morula and 1 developed to the blastocyst stage. No known transfers of IVF embryos fertilized with ejaculated or epididymal sperm resulting in the birth of live young have been reported. Although in the horse, Palmer et al. (1991) reported the birth of the first foal following IVF with fresh ejaculated sperm. Of the 60 oocytes IVF was performed on, 11 (18%) developed into embryos and 5 (8%) were fertilized without segmentation.

Thundathil et al. (2007) utilized Wood bison cryopreserved ejaculated sperm and epididymal sperm stored at 4°C for 24 hours for IVF. Ejaculated and epididymal sperm were compared as to their ability to fertilize oocytes (n=160) and the subsequent

embryo development in vitro. Utilizing ovaries collected from postmortem Wood bison cows, it was noted that the fertilization rate and blastocyst development of embryos fertilized with ejaculated sperm (64.4% and 7.5%, respectively) was lower compared with epididymal sperm (89.2% and 10%, respectively). To date, there are no known reports of live births of bison calves following IVF.

Del Campo et al. (1994) utilized fresh epididymal llama sperm for IVF of IVM slaughterhouse oocytes. Of 192 oocytes, 56 (29.2%) were penetrated by sperm with 57.1% (32/56) of the oocytes penetrated having both male and female pronuclei. Embryo development from the 2-cell to blastocyst stage was 32.1% (75/234). Berland et al. (2011) also using fresh epididymal llama sperm for IVF, fertilized TUGA collected super stimulated llama oocytes (n=308). Following IVF, embryo development resulted in a cleavage rate of 64%, morula of 44.4% and blastocyst 22.1% was reported. Conde et al. (2008) utilized fresh ejaculated sperm with and without capacitation agents (PHE and heparin) for IVF of TUGA collected llama oocytes (n=94). Cleavage rates were 40.8% (20/49) and 42.2% (19/45) for IVF with or without capacitating agents, respectively, and blastocyst rates were 35% (7/20) and 47.3% (9/19), respectively. Capacitation agents were noted as not being necessary in llama IVF. To date, there are no known reports of live births following IVF embryos to recipients.

Stoops et al. (2011) collected oocytes from postmortem African Black rhinoceros females and performed IVF with cryopreserved epididymal sperm collected from postmortem African Black rhinoceros males. Success was limited, with the production of a single 2-cell embryo from 95 total oocytes noted.

Pope et al. (2009) reported the birth of live kittens following IVF with sex-sorted cat ejaculated sperm. Following insemination, day-2 embryos (45) were transferred to the oviduct of 4 recipients. Subsequently, 3 females delivered litters of 1, 4 and 7 female kittens, respectively.

FACTORS ASSOCIATED WITH REPRODUCTION

Factors Associated with Embryonic Development

Embryos cultured in vitro have been shown to develop better in groups as opposed to individually (Doherty et al., 1997; Donnay et al., 1997; Jewgenow et al., 1999; Goovaerts et al., 2011). It's known that embryos secrete paracrine and autocrine

factors to communicate with the female. The goal of an embryo in vitro culture system is to provide all the necessary factors making an optimal environment resulting in increased embryo development. One of the strategies to attain this goal is by adding growth factors in an attempt to mimic the in vivo environment. Although, numerous factors have been added to culture medium with a range of results, none have completely overcome inefficiency of single embryo culture.

Jewgenow et al. (1999) cultured bovine embryos individually (n=391) and in groups (n=870) from maturation to blastocyst stage and significantly lower numbers of embryos developing into blastocyst following individual culture (5%) compared with group culture (25%).

Donnay et al. (1997) compared the development of bovine embryos cultured in vitro individually (n=48), in groups of 3 to 6 (n=96) or in groups of 20 (n=159). Results indicated significant differences in blastocyst development for individually cultured embryos (0%), groups of 3 to 6 (6%) and groups of 20 (23%) cultured embryos. Although, no significant difference was noted in blastocyst development when embryos were co-cultured with Buffalo Rat liver cells individually (12%), in groups of 3 to 6 (10.5%) or in groups of 20 (9%).

Doherty et al. (1997) compared individual and group bovine embryo culture with and without granulosa cell co-culture. Group size (1, 5, 10, 20 and 40 embryos/group) was also compared in co-culture. Blastocyst development was significantly better in group (7.5%, n=481) compared with individually (0.5%, n=552) cultured embryos. Granulosa cell co-culture was able to significantly improve embryo development in group (37.4%, n=371) compared with individually cultured embryos (10.1%, n=383). Blastocyst development of cleaved embryos for groups of 20 (40%, n=392) and 40 (42%, n=398) were significantly better than groups of 10 (29%, n=360), 5 (27%, n=355) or individual (19%, n=358).

Nagao et al. (1998) compared the number of bovine embryos (1, 5, 10, 25, 50, 100 and 250) per 50 µl culture drop. It was noted that blastocyst development was increased in an embryo to culture medium volume ratio of 0.5:1 (47%) to 2:1 (47%) compared with reduced blastocyst rates for ratios below 0.5:1 (16%) and above 2:1 (31%).

Stokes et al. (2005) compared the blastocyst development of in vivo derived porcine embryos (n=100) cultured in a 2 x 2 factorial arrangement to determine the effects of culturing embryos at increasing distances. Optimal embryo distance was determined to be from 81 to 168 μm apart and resulted in a blastocyst rate of 28.7%. Results showed that embryos cultured beyond 240 μm apart had significantly lower blastocyst development (~12%) compared with embryos touching (~19%) and with a continual decline to 0% beyond 640 μm apart. This demonstrates that paracrine factors secreted by accompanying embryos have a limit to their ability to diffuse causing a reduction in beneficial effects.

These studies demonstrate the need for autocrine and paracrine factors given off by cells in co-culture and or accompanying embryos in the development of embryos.

Various growth factors have been tested to compare their effects on bovine embryo development during in vitro culture (Flood et al., 1993; Keefer et al., 1994; Thibodeaux et al., 1995).

Flood et al. (1993) compared bovine embryo development (150 embryos/treatment) to the blastocyst stage following in vitro culture in; media alone (control, ~12%), Transferrin (TF, ~11%), Epidermal Growth Factor (EGF, ~17%), Transforming Growth Factor alpha (TGF- α , ~14%), Transforming growth factor-Beta1 (TGF- β 1, ~13%), Insulin-like Growth Factor-1 (IGF-1, ~12%), Insulin-like Growth Factor-2 (IGF-2, ~11%), Platelet-derived Growth Factor (PDGF, ~13%), Basic Fibroblast Growth Factor (bFGF, ~12%), and Nerve Growth Factor (NGF, ~12%). No significant difference was noted between the factors tested compared with media alone. Although not significant, EGF did increase the blastocyst development compared to all other treatments.

Keefer et al. (1994) also noted no increase in blastocyst development when bovine embryos were cultured individually or in groups with EGF and or TGF- β 1 compared with media alone. However, the inclusion of EGF was noted as significantly increasing the hatching of blastocyst (45%) when added to 8-cell embryos (n=26/treatment) individually cultured compared with TGF- β 1 (8%) or media alone (0%).

Thibodeaux et al. (1995) compared IVF results of bovine embryos cultured individually or in groups of 10 with or without PDGF-antibody. No difference in

blastocyst development was noted for individually cultured embryos (n=110/treatment) with (14%) or without (15%) PDGF-antibody. However, a significant decrease was reported when PDGF-antibody was included in the group culture (19%) compared with when PDGF-antibody was not included in the group culture (31%).

Lee and Fukui (1995) compared the development of bovine embryos following individual or group cultured with: Fibroblast Growth Factor (FGF), EGF, IGF-I, TGF- β 1. No effect was noted when factors were added to individually cultured 2-cell (~2.8%, n=62/factor) stage or 8-cell (~11%, n=60/factor) stage bovine embryos compared with embryos cultured in SOFM+human serum (37%, n=60/cell stage). Although, blastocyst development increased when FGF was added to individually cultured morule (73%, n=45) and when EGF was added to individually cultured early blastocyst (84%, n=45) compared with SOFM+HS (80% and 93%, respectively n=45/cell stage). Blastocyst development of bovine 2-cell embryos cultured in groups with FGF (27%, n=275) and EGF (27%, n=273) did not significantly affect blastocyst development compared with media alone (22%, n=250). However, when both FGF and EGF were added significantly more bovine embryos developed to the blastocyst stage (31%, n=236) compared with medium alone (22%).

Factors Associated with Sperm Function

Sperm undergo significant alterations from their storage in the epididymides to the fertilization of an oocyte. Changes to epididymal sperm are brought about by exposure to various factors found in the seminal plasma and female reproductive tract. Some of the various factors that have been shown to affect sperm include growth factors, peptides, proteins and small molecules.

Growth factor receptors have been located on sperm and shown to interact with their respective ligands to initiate a response. Sperm have been noted as having increased motility and viability; also their percentage of capacitation and acrosome reaction increased following the addition of various factors. The addition of various factors to cryopreservation or culture media may be beneficial in the preservation of sperm or increase embryo development.

Lax et al. (1994) identified a 170-kDa EGF-receptor (EGFR) on the head of ejaculated bull sperm. In addition, it was noted that EGF can induce the acrosome

reaction in capacitated bovine sperm (30%) compared with not including EGF (14%). It was also noted that PKC was involved in the mechanism in which EGF exerts its effect on acrosome reaction.

Henricks et al. (1998) demonstrated that IGF-1 present in the seminal plasma of 7 beef bulls can interact with a specific IGF-1 receptor on the acrosomal region of ejaculated sperm. In addition, it was noted that when physiological concentrations of IGF-1 (100 ng/ml) and IGF-2 (250 ng/ml) were added to sperm they maintained their motility better than without the additions of IGF-1 or IGF-2.

Li et al. (2010) identified the NGF receptor TrkA on the acrosomal and tail of bull sperm. In addition, it was determined that NGF binds bull sperm on the acrosomal cap and along the tail. Sperm viability following 2 hours of culture, was increased by the inclusion of 20 µg/ml NGF (72%) compared with sperm without the addition of NGF (62%)

Iyibozkurt et al. (2009) compared the effect of different concentrations (5, 10, 15, 20 ng/ml) of Vascular Endothelial Growth Factor (VEGF) on motility and survival of human sperm during 24 hours of in vitro culture. A positive effect was noted with the inclusion of VEGF on motility in a concentration-dependent manner. Maximal effect on progressive motility was observed at a concentration of 15 ng/ml, however, sperm viability was not prolonged at any concentration of VEGF.

In the mouse, Adeoya-Osiguwa and Fraser (1996) noted that inclusion of calmodulin stimulated Ca^{2+} -ATPase activity, preventing capacitation. In addition, it was noted that trifluoperazine (TFP), a calmodulin antagonist, accelerated capacitation of mouse sperm. It was proposed that a calmodulin-sensitive Ca^{2+} -ATPase is responsible for the maintenance of a low intracellular Ca^{2+} concentration in the sperm. As capacitation progresses, and DF are lost, Ca^{2+} -ATPase activity declined, with the decline of Ca^{2+} -ATPase, intracellular Ca^{2+} concentrations rises, promoting capacitation.

In the mouse, human and boar it has been noted that fertilization-promoting peptide (FPP), Angiotensin-II (A-II), calcitonin and adrenaline are able to regulate capacitation (Fraser et al., 2001; Fraser and Osiguwa, 2004; Fraser, 2008). Specific receptors for all these factors have been located on the acrosomal cap and tail plasma membrane of sperm. Binding of these factors to their appropriate ligands, leads to

modulation of membrane-associated adenylyl cyclase (mAC) activity and production of cAMP, stimulating cAMP production in uncapacitated sperm and inhibiting cAMP production in capacitated sperm. Receptors for all of these factors except for FFP have been determined to be G-protein coupled receptors. FFP are thought to operate in conjunction with adenosine receptors, although the mechanism is still under investigation. It has been noted that these factors were dose dependent with maximal capacitation (CTC) of uncapacitated human sperm, obtained with calcitonin (0.5–15 nmol/l), A-II (0.3–100 nmol/l) and FFP (100 nmol/l) (Fraser and Osiguwa, 2004). In the bull, A-II has been shown to stimulate the acrosome reaction in capacitated sperm (Gur et al., 1998). Receptors for A-II were found only on the tail of uncapacitated sperm, although following capacitation, receptors were identified on the head of the bull sperm.

Preimplantation Factor

Preimplantation factor (PIF), a 15 amino acid peptide, was first discovered in pregnant women as early as 4 days following embryo transfer and was suggested for use as a possible early pregnancy detection assay (Barnea et al., 1994). It was found that PIF was specific to females (human, mouse, monkey) whose embryos had implanted and absent in females whose pregnancies had aborted (Coulam et al., 1995; Roussev et al., 1995; Roussev et al., 1996a; Rosario et al., 2005). Isolation and partial characterization of PIF, collected from the human and mouse embryo culture media, revealed it to be a low molecular weight peptide that is expressed by viable embryos (Roussev et al., 1996b).

As discussed earlier, autocrine and paracrine factors released from the embryo have been shown to be essential for embryo development. With the release of PIF from viable embryos, it is plausible that PIF may have beneficial effects on developing in vitro cultured-embryos.

Detection of PIF from in vitro produced mouse embryos became possible starting at the morula and increasing at the blastocyst stages (Roussev et al., 1996b). PIF has also been suggested as initiating an immune tolerance state in the mother that facilitates the maternal tolerance and uterine receptivity to the embryo (Barnea, 2007). Not only are the effects of PIF found during pregnancy, but the ability of non-toxic low dose administration of PIF to reduce the effects of auto-immune disorders, such as

multiple sclerosis, diabetes mellitus and graft-vs.-host disease were also suggested for clinical testing (Barnea, 2007). Genomic and proteomic investigation of the effects of PIF revealed that PIF affected the many signaling pathways that included immune, adhesion and apoptosis processes (Paidas et al., 2010).

When PIF was administered to mice (non-obese diabetic murine model, NOD) used as Type-1 diabetes mellitus models, it was found that PIF preserved pancreatic islet form and function preventing the onset of diabetes with a lasting effect (Weiss et al., 2011a). PIF was also tested as to its effects on mice (clinically-relevant murine EAE-PLP acute and chronic models) used as multiple sclerosis models, it was found that PIF prevented paralysis and lowered mortality in acute cases, also reversing paralysis in chronic models (Weiss et al., 2011c).

CHAPTER III

MEMBRANE PERMEABILITY DURING CRYOPRESERVATION OF BOVINE EJACULATED AND EPIDIDYMAL SPERM FROM THE SAME HOLSTEIN BULLS

INTRODUCTION

Salvaging of epididymal sperm from injured or deceased animals allows for the propagation of favorable traits from these genetically superior males. Techniques developed in domestic species can also serve as the foundation for the collection, cryopreservation and utilization of epididymal sperm in exotic breeds. In an effort to improve the preservation of these finite resources, a better understanding of the underlying fundamental differences between ejaculated and epididymal sperm is needed. To date there is little if any information regarding the precise differences between bovine ejaculated and epididymal sperm water transport (membrane permeability) characteristics.

During ejaculation, epididymal sperm are mixed with seminal plasma as they travel through the male reproductive track. This makes the addition of seminal plasma a main contributing factor in the difference between ejaculated and epididymal sperm. Seminal plasma is composed of a multitude of components and has been reported to have marked effects on sperm (Bialy and Smith, 1958a; Dott et al., 1979; Desnoyers and Manjunath, 1992; Therien et al., 1995; Therien et al., 1998; Way et al., 2000). One of the main effects of seminal plasma is the modification of the cholesterol phospholipid ratio in the sperm plasma membrane. This alteration may change the ability of ejaculated and epididymal sperm to endure cryopreservation.

During cryopreservation, extracellular water freezes making the medium hypertonic, inducing the release of intracellular water from sperm exposed to this environment. The rate that these sperm can expel water is dependent on membrane permeability. The membrane permeability of ejaculated bovine sperm collected from mature Angus and Senepol bulls has been described (Li et al., 2006). However, little is known as to the difference in membrane permeability of ejaculated and epididymal bovine sperm collected from the bulls.

A new method, differential scanning calorimetry (DSC), has been utilized to evaluate the water transport response of sperm during the freezing process. This

technique has been reported in a variety of mammalian species including: mouse (Devireddy et al., 1999), human (Devireddy et al., 2000), stallion (Devireddy et al., 2002), dog (Thirumala et al., 2003), boar (Devireddy et al., 2004), bull (Li et al., 2006), rhesus monkey (Alapati et al., 2008) and rat (Hagiwara et al., 2009).

This DSC procedure was utilized in the present study to determine the water transport response of ejaculated and epididymal bovine sperm from the same 4 Holstein bulls at a cooling rate of 20°C/minute in two different media: the first without cryoprotective agents (CPAs), and the second with 0.7 M glycerol. The experimental water transport response of ejaculated and epididymal bovine sperm were then utilized to determine the cooling behavior and membrane permeability parameters. These results were then used to determine the optimal freezing rates for ejaculated and bovine sperm with or without glycerol.

Ejaculated and epididymal sperm from the same bulls were also compared by their sperm collection values, motility before and after cryopreservation. To our knowledge these are the first experiments comparing the membrane permeability of ejaculated and epididymal bovine sperm collected from the same 4 bulls. These studies will give us a better understanding of any difference between ejaculated and epididymal bovine sperm collected from the same bulls.

MATERIALS AND METHODS

Experimental Design

This experiment was designed to determine if differences exist between ejaculated and epididymal sperm collected from the same 4 healthy, mature Holstein bulls as to their collection, cryopreservation and water transport (membrane permeability) characteristics. Ejaculated and epididymal sperm were collected from the same mature Holstein bulls in 2 replicates (5 months/replicate) with 2 bulls in each replicate over the duration of this study. Ejaculated sperm was collected by artificial vagina and epididymal sperm collected by way of retrograde flush of the caudal epididymides.

Ejaculated and epididymal sperm collection values: motility, volume and concentration; were determined from ≥ 10 separate ejaculates and for both epididymides from each bull. Cryopreservation data were collected from 2 ejaculates per bull and 1

cauda epididymis from each of the 4 bulls. Total motility, before and after cryopreservation, for ejaculated and epididymal sperm were compared. Water transport of ejaculated and epididymal sperm was determined by DSC technique (Alapati et al., 2009). Water transport values were collected for ejaculated sperm from 10 separate ejaculates and 1 epididymis from each of the 4 bulls. Each sperm collection yielded 6 separate (3 with CPA, 3 without CPA) DSC experiments replicates.

Experimental Procedure

Animals

During this study, semen was collected in 2 replicates over a 10-month period from mature, healthy, fertile Holstein bulls (2 bulls/replicate) housed at Genex Custom Collection Service near the Louisiana State University (LSU) campus. Bulls were purchased from a local purebred dairy producer and were of separate pedigrees. The bulls were of sound conformation and in good body condition at the onset and throughout the study. Semen was collected biweekly, with no fewer than 3 days between collections. Bulls were maintained on pasture and supplemented with Bermuda hay and 12% protein concentrate feed. The average daily temperature for the first replicate of semen collection (bulls A and B) was 27°C with a maximum of 33°C and minimum of 22°C. And the average daily temperature for the second replicate of semen collection (bulls C and D) was 17°C with a maximum of 24°C and minimum of 11°C.

Ejaculated sperm collection

All ejaculates from each bull were collected by the same experienced professional technicians throughout the study. Two false mounts were made by the bulls atop a trained teaser steer, before actual semen collection. A total of >10 separate ejaculates from each of the bulls were collected. Ejaculated semen was collected by artificial vagina into a 15 ml polypropylene conical tube and volume noted. A sample was taken to determine the concentration and motility before the ejaculate was extended in standard bovine egg yolk-based diluents and then placed into a 37°C water bath for equilibration.

A sample from each ejaculate was diluted in a 2.9% (w/v) sodium citrated solution for calculation of sperm concentration and progressive motility, following standard commercial bull stud procedures. Motility was assessed by placing sperm onto

a pre-warmed glass slide and visually evaluation of ≥ 3 different fields of view using phase microscopy. Sperm concentration was calculated by a standard curve derived from measurements of optical density using a spectrophotometer.

Epididymal sperm collection

Epididymal sperm was collected from the same 4 mature Holstein bulls that ejaculated sperm was collected from. Collection of epididymal sperm was performed following isolation of the testes by dissecting the epididymides and a portion of the vas deferens from each testis in a sterile manner. Each bull was restrained, sedated and remained standing throughout the procedure. A licensed veterinarian surgically removed the testes by making an incision down the midline of the scrotum between the two testes. Testes were then removed taking care to prevent excessive loss of blood. The vas deferens and the cauda epididymides were then carefully dissected away from each testis. The vas deferens were catheterized using a modified tip of a plastic syringe. Cauda epididymal tubes were severed to harvest luminal sperm. Egg yolk-based extender was used to push epididymal sperm out of the epididymis by retrograde flow.

Epididymal sperm was collected in a 15 ml polypropylene conical tube, a sample was taken to determine the concentration and progressive motility before the sperm was extended in standard bovine egg yolk-based diluents and then placed into a 37°C water bath for equilibration. Concentration and progressive motility were evaluated in the same manner as with ejaculated sperm.

Cryopreservation

Extension and cryopreservation of ejaculated and epididymal sperm was performed with a standard bovine egg yolk-based Tris extender (Genex commercial extender). The extender was divided into two parts, Fraction-A (without glycerol) and Fraction-B (with 1.4M glycerol or 12% v/v glycerol). Fraction-A was first used to extend the sperm. The sperm/Fraction-A mixture was then placed in a 37°C water jacket and cooled to 5°C (~4 hours). Once equilibrated to 5°C, Fraction-B was slowly added to the sperm/Fraction-A mixture to a ratio of 1:1 (v/v). The extended sperm was then loaded into 0.5 ml plastic straws (IMV, International, Minneapolis, MN) at a concentration of 30×10^6 sperm/straw. The labeled straws were sealed and laid horizontally on racks over liquid nitrogen for freezing. The cooling rate was 15°C/minute from 5°C to -100°C.

Straws were then plunged into liquid nitrogen and stored at -196°C until subsequent evaluation.

Post-thaw evaluation

Ejaculated and epididymal sperm were thawed by removing a frozen straw from liquid nitrogen and immediately submerging it into a 37°C water bath for 40 seconds. Straws were removed from the water bath and excess water dried off, taken care not to cold shock the sperm. Sperm from the straw was then placed into a 0.5 ml microcentrifuge tube. A 10 µl sample of sperm was then placed onto a pre-warmed glass slide and evaluated by phase microscopy. Progressive sperm motility and sperm morphology were assessed on 3 straws of ejaculated and epididymal sperm from each bull.

Transport to LSU Bioengineering Laboratory

Following collection and evaluation of pre-freeze values the semen was extended 1:1 in Fraction-A (non-glycerated egg yolk-based Tris extender) and placed in a 15 ml polypropylene conical tube and sealed. The tube carrying the semen was held in a 50 ml water jacket (37°C) and placed in a Styrofoam container for transportation. The sample was delivered to the LSU Bioengineering Laboratory (~10 minutes) for in vitro DSC experiments.

Membrane permeability

All DSC assays performed were conducted at the LSU Bioengineering Laboratory by the same experienced scientist. Four ejaculates from each bull (1-ejaculate/week) were randomly selected for DSC evaluation. Three replicates of DSC evaluation were conducted for every sperm sample (ejaculated and epididymal) submitted.

Loading of glycerol for DSC

Differential scanning calorimeter experiments conducted in the absence of CPAs, required bovine sperm to be concentrated by centrifugation (300 x g, ~25°C) for 5 minutes. Following centrifugation, the sperm pellet was resuspended in residual supernatant. Ejaculated and epididymal bovine sperm were also evaluated in the presence of the permeating CPA, glycerol (0.7 M, or 6% v/v), due to its routine inclusion during cryopreservation (Li et al., 2006). Addition of CPA was performed in a stepwise

manner at 25°C to prevent osmotic shock and reduce volumetric changes of bovine sperm during the process loading of CPA. A stock of 1.4 M CPA was added to sperm in 5 equal volumes at 5-minute intervals resulting in a final concentration of 0.7 M CPA (Li et al., 2006). Samples were then centrifuged (300 x g, ~25 °C) for 5-minutes. Following centrifugation the sperm pellet was resuspended in residual supernatant in preparation for DSC experiments.

Differential scanning calorimeter (DSC)

Water transport measurements during the freezing of bovine ejaculated and epididymal sperm were performed using the DSC dynamic cooling protocol in the same manner that has been reported in the scientific literature (Devireddy et al., 1999; Devireddy et al., 2000; Devireddy et al., 2002; Thirumala et al., 2003; Devireddy et al., 2004; Li et al., 2006; Alapati et al., 2008). The DSC technique, measures the response of 2 heat releases from the same cell suspension during 2 cooling runs. The first measurement is taken during the freezing of osmotically active (“live”) cells in medium at a rate (20°C/minute) that allows intracellular water to diffuse across the membrane and freeze in the extracellular space. Sperm cells are then frozen at a high rate causing a disruption of the sperm membranes. The second measurement is then taken during the freezing of osmotically inactive (“dead”) cells in medium at the same rate as the first (20°C/minute). The temperature dependence of the difference in heat release between the 2 cooling runs has been correlated to water transport, as described in earlier studies (Devireddy et al., 1999; Devireddy et al., 2000; Devireddy et al., 2002; Thirumala et al., 2003; Devireddy et al., 2004; Li et al., 2006; Alapati et al., 2008) and is described as:

$$V(T) = V_o - \frac{\Delta q(T)_{dsc}}{\Delta q_{dsc}} * (V_o - V_b)$$
 (Equation, 1). The heat release measurements $\Delta q(T)_{dsc}$ and Δq_{dsc} were taken separately for ejaculated and epididymal sperm at a cooling rate of 20°C/minute with and without CPAs. The unknowns V_o (initial or isotonic cell volume) and V_b (osmotically inactive cell volume) were obtained from the literature (Van Duijn, 1960; Van Duijn and Voorst, 1971; Drevious, 1972; Hammerstedt et al., 1978; Cummins and Woodall, 1985; Guthrie et al., 2002; Révay et al., 2004).

Water transport model

During cryopreservation sperm lose cellular water, this reduction in volume has been thermodynamically modeled and is described by the following relationship

$$\frac{dV}{dT} = \frac{L_p A_c R T}{B} [C_i - C_o] \text{ (Equation, 2) (Mazur, 1963; Levin et al., 1976). Plasma membrane permeability, } L_p \text{ is defined as, } L_p = L_{pg}[cpa] \exp\left(-\frac{E_{Lp}[cpa]}{R}\left(\frac{1}{T} - \frac{1}{T_R}\right)\right) \text{ (Equation, 3).}$$

Where, L_{pg} or $L_{pg}[cpa]$ symbolize the reference membrane permeability ($\mu\text{m}/\text{minute-atm}$) at the reference temperature, T_R (273.15°K) with and without CPA; E_{Lp} or $E_{Lp}[cpa]$ represents the apparent activation energy (kJ/mol) or the temperature dependence of the cell membrane permeability with and without CPA; V is the sperm cell volume at temperature, T °K; A_c represents the effective membrane surface area for water transport, and is assumed to be constant during the freezing process; R stands for the universal gas constant; B is the constant cooling rate (°K/min); finally C_i and C_o symbolize the concentrations of intracellular and extracellular (unfrozen) solutions.

During this study, the ejaculated and epididymal sperm cells were modeled as long cylinders with a length (L) of 39.8 μm and a radius (r_o) of 0.4 μm (Cummins and Woodall, 1985) that translates to an initial (or isotonic) cell volume $V_o \sim 20 \mu\text{m}^3$ and $A_c \sim 100 \mu\text{m}^2$. The osmotically inactive cell volume, V_b , was taken to be 0.61 of V_o as reported by Guthrie et al. (2002). The unknown water transport parameters of the model, $L_{pg}[cpa]$ and $E_{Lp}[cpa]$ with the inclusion CPA or L_{pg} and E_{Lp} without CPA, were determined by curve-fitting the water transport model to the volumetric shrinkage data experimentally obtained during freezing.

Numerical methods

Water transport parameters that best fit the volumetric shrinkage data were determined using a nonlinear least squares curve fitting technique that was implemented using a computer program (Alapati et al., 2009). The optimal fit of L_p (plasma membrane permeability to water) to the experimental data was achieved by choosing a set of parameters that minimized the variance, x^2 , while maximizing the goodness of fit parameter, R^2 (Alapati et al., 2009). A good agreement between the experimental data points and the fit calculated using the estimated water transport

parameters was achieved with all curve fitting results presented having an R^2 value greater than or equal to 0.99.

Theoretical prediction of optimal cooling rate

As reported by Alapati et al. (2009), by comparing the published experimentally determined values of B_{opt} ($^{\circ}\text{C}/\text{minute}$) for a variety of biological systems it was noted that they agreed very closely to the value obtained using a Generic Optimal Cooling Rate Equation (GOCRE) that defines: $B_{opt} = 1009.5 \cdot \exp^{(-0.0546 \cdot E_{Lp})} \cdot (L_{pg}) \cdot \left(\frac{SA}{WV}\right)$ (Equation, 4). With equation symbols: L_{pg} and E_{Lp} representing the membrane permeability parameters ($\mu\text{m}/\text{min-atm}$ and kcal/mol , respectively); $SA/WV = V_o - V_b$ (μm^{-1}) signifying the ratio of available surface area for water transport ($SA = A_c$) to the initial volume of intracellular water ($WV = V_o - V_b$).

Based on the assumed values of V_b and cell dimension, the ratio of SA to WV for bovine sperm equals $12.5 \mu\text{m}^{-1}$. The prediction of optimal freezing rates is greatly simplified with the use of the GOCRE equation and is based on the assumption that the optimal rate of cryopreservation of any cellular system can be defined as the freezing rate at which 5% of the initial water volume is trapped inside the cell at -15°C . Following the determination of L_{pg} and E_{Lp} using the fitting procedure described above, the GOCRE equation was used to predict the optimal cooling rate to cryopreserve bovine sperm.

Statistical Analysis

Variances in semen collection volume, concentration, pre- and post-cryopreservation motility were statistically analyzed by ANOVA. The semen collection volume, concentration, pre- and post-cryopreservation total motility are expressed as mean \pm SEM per treatment group. A $P < 0.05$ was considered significant in this study. Data were analyzed using SigmaPlot Statistical Software Version 10.0.

The difference in measured water transport data between animals, treatments (with and without glycerol) and collection conditions (ejaculated and epididymal) were statistically analyzed by ANOVA. Data were analyzed using SigmaStat Statistical Software. A $P < 0.05$ was considered the level of statistical significance in this study.

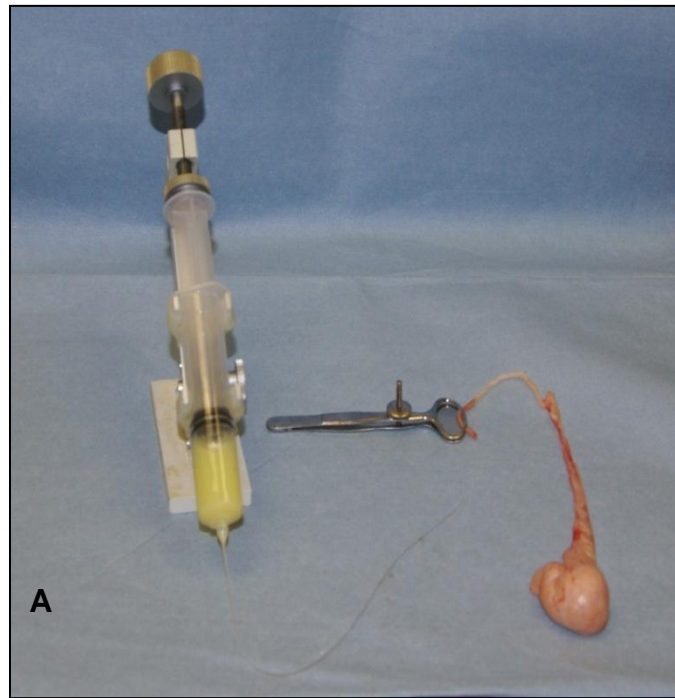


Figure 3.1. Epididymal sperm collection. (A) An isolated epididymis, the vas deferens is cannulated with the tip of a heat-modified syringe. (B) Epididymal sperm being collected by retrograde flush of the cauda epididymis.

RESULTS

Ejaculated and epididymal sperm from the same mature Holstein bulls (n=4) were collected and compared in this study. As noted in Table 3.1, all 4 bulls were of similar: age, weight, scrotal circumference, testis and caudal epididymis weight. Bulls were compared by semen collection values and sperm characteristics fresh and post-thaw (see Table 3.2). No significant difference in the sperm motility, either fresh or post-thaw, was noted between bulls ($P=0.361$) and ($P=0.135$), respectively. Likewise, no significant difference was detected between fresh ejaculated or epididymal sperm total motility ($P=0.425$). Although, a significant difference was detected between ejaculated and epididymal sperm post-thaw total motility ($P<0.05$). With epididymal sperm noted as having a higher post-thaw motility compared to the same ejaculated sperm. Concentration of sperm collected by way of epididymal flush was also noted to be significantly greater than collection by way of ejaculation ($P<0.001$). No bull exceeded the acceptable range for percent abnormal sperm for bulls collected in a commercial bull stud ($<30\%$).

The DSC experimentally measured water transport data of ejaculated sperm with and without CPAs were found to be statistically significantly different from each other over the whole temperature range of interest ($P<0.01$)(Figure 3.1, A and B). Epididymal sperm water transport data was also found to be significantly different when cooled with or without CPA ($P<0.01$)(Figure 3.2, A and B). Although, as noted in Figures 3.1 and 3.2 there was a lack of distinction between the freezing response of bull ejaculated and epididymal sperm. With the measured water transport response between bulls and between ejaculated and epididymal sperm noted as being both not significantly different ($P<0.99$) and significantly different ($P<0.01$).

The theoretically predicted optimal cooling rates for bovine ejaculated and epididymal sperm were determined by incorporating the best fit parameters of water transportation, listed in Tables 3.3 and 3.4, into equation-4. The optimal cooling rates are listed in Tables 3.3 and 3.4 and for bovine ejaculated and epididymal sperm the calculated rate is between 50 and 60°C/minute. No difference was noted in the water transport response between ejaculated and epididymal sperm and between bulls.

Table 3.1. Bull conformation values collected from mature Holstein bulls.

Bull ID	Age in months [†]	Average weight in kg (range [†])	Scrotal circumference (range [†])	Testis weight in g (range [‡])	Cauda epididymides weight in g (range [‡])
A	17 - 20	536 (494-576)	37.5 (37-38)	300 (298-303)	12.5 (12-13)
B	16 - 20	552 (515-585)	37.5 (37-38)	288 (279-296)	12.5 (12-13)
C	16 - 20	488 (453-522)	36.5 (36-37)	269 (256-273)	10.5 (10-11)
D	15 - 20	491 (435-549)	37.0 (36-38)	265 (259-279)	10.5 (10-11)
Overall average	18	517	37.1	280	11.5

[†] Noted at onset and conclusion of sperm collection interval.

[‡] Noted from the left and the right testis.

Table 3.2. Ejaculated and epididymal sperm collection values from the same mature Holstein bulls.

Bull ID	Collection type	Volume in ml ± SEM (range)	Concentration x10 ⁹ /ml ± SEM (range)	% Motility fresh ± SEM (range)*	% Motility post-thaw ± SEM (range)*	Loss in % motility
A	Ejaculated	7.8 ± 0.7 (4.0-13.0)	1.0 ± 0.08 (0.06-1.33)	72.5 ± 1.5 (60-75)	55.0 ± 2.9 (50-60)	17.5
	Epididymal	5.0 ± 0.8 (4.2-5.8)	1.7 ± 0.04 (1.66-1.74)	77.5 ± 2.5 (75-80)	58.8 ± 6.9 (45-75)	18.7
B	Ejaculated	7.4 ± 0.6 (5.0-10.0)	0.8 ± 0.09 (0.05-1.50)	53.0 ± 4.5 (30-70)	41.7 ± 1.7 (40-45)	11.3
	Epididymal	5.5 ± 0.5 (5.0-6.0)	1.1 ± 0.05 (1.06-1.15)	75.0 ± 2.0 (73-77)	56.7 ± 8.3 (40-65)	18.3
C	Ejaculated	6.5 ± 0.5 (3.0-11.6)	0.6 ± 0.05 (0.31-1.40)	68.0 ± 0.8 (65-75)	57.5 ± 5.2 (45-70)	10.5
	Epididymal	4.9 ± 0.05 (4.9-5.0)	1.1 ± 0.14 (0.92-1.20)	60.0 ± 3.0 (57-63)	55.0 ± 6.5 (40-70)	5.0
D	Ejaculated	4.6 ± 0.3 (1.5-7.5)	0.77 ± 0.05 (0.52-1.45)	72.1 ± 0.7 (65-75)	48.0 ± 3.0 (40-55)	21.4
	Epididymal	4.3 ± 2.3 (2.0-6.5)	1.1 ± 0.05 (1.05-1.15)	70.0 ± 8.0 (62-78)	63.0 ± 1.2 (60-65)	7.0
Overall average	Ejaculated	6.1 ± 0.29 ^a	0.76 ± 0.03 ^c	64.4 ± 2.7 ^a	50.9 ± 2.2 ^a	13.5
	Epididymal	4.9 ± 0.49 ^a	1.24 ± 0.11 ^d	70.6 ± 3.1 ^a	58.8 ± 2.6 ^b	11.8

^{a,b}Means with different letters in columns are significantly different P<0.05.

^{c,d}Means with different letters in columns are significantly different P<0.001.

Range is noted as the smallest and largest recorded values overall collections, respectively.

*Total motility determined by visual estimation using phase microscopy.

Table 3.3. Best fit water transport parameters for the volumetric shrinkage response of ejaculated Holstein sperm without CPA and with glycerol, at a cooling response of 20°C/min. All parameters a goodness of fit, $R^2 > 0.99$.

Bull ID	Freezing medium	$^{\dagger}L_{pg}$ or $L_{pg}[cpa]$ ($\mu\text{m}/\text{min-atm}$)	$^{\ddagger}E_{Lp}$ or $E_{Lp}[cpa]$ (Kcal/mol)	Optimal cooling rate ^a (°C/min)
A and B*	Without CPA	0.034	36.0	63
	With glycerol	0.020	27.2	59
C and D**	Without CPA	0.035	36.1	63
	With glycerol	0.020	27.4	59

^aObtained using Equation 4.

(Alapati et al., 2009)

*Bulls in Replicate-1, 4 ejaculates were randomly selected per bull.

**Bulls in Replicate-2, 4 ejaculates were randomly selected per bull.

CPA = cryoprotective agent.

[†]Reference membrane permeability at the reference temperature (273.15°K) with and without CPA.

[‡]Apparent activation energy or the temperature dependence of the cell membrane permeability with and without CPA.

Table 3.4. Best fit water transport parameters for the volumetric shrinkage response of ejaculated and epididymal Holstein sperm without CPA and with glycerol, at a cooling response of 20°C/minute. All parameters a goodness of fit was $R^2 > 0.99$.

Bull ID	Freezing medium	$^{\dagger}L_{pg}$ or $L_{pg}[cpa]$ ($\mu\text{m}/\text{min-atm}$)	$^{\ddagger}E_{Lp}$ or $E_{Lp}[cpa]$ (Kcal/mol)	Optimal cooling rate ^a (°C/min)
Ejaculated	Without CPA	0.035	36.1	63
	With glycerol	0.021	27.8	59
Epididymal	Without CPA	0.038	41.1	52
	With glycerol	0.024	31.4	56

^aObtained using Equation 4.

(Alapati et al., 2009)

CPA = cryoprotective agent.

[†]Reference membrane permeability at the reference temperature (273.15°K) with and without CPA.

[‡]Activation energy or the temperature dependence of the cell membrane permeability with and without CPA.

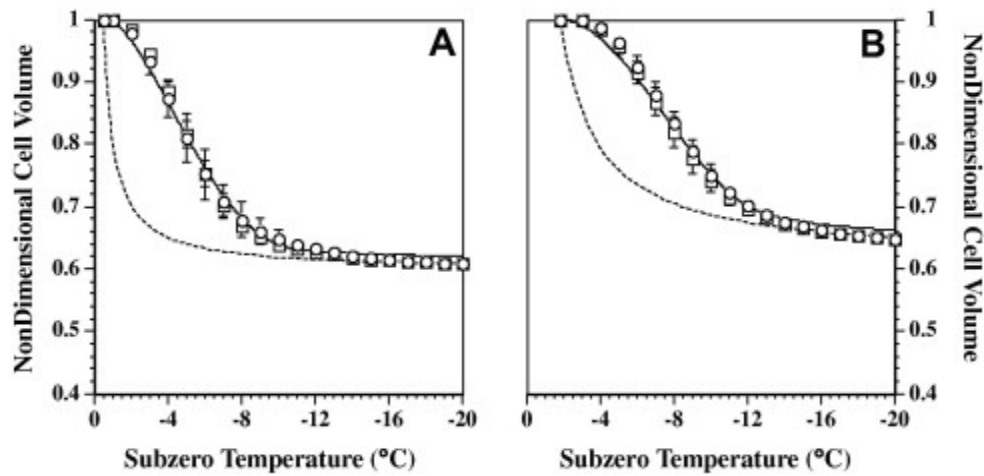


Figure 3.2. Comparative volumetric shrinkage response of ejaculated bovine sperm from the 4 bulls used in this study, shown as open circles (bull A & B) and open squares (bull C & D). DSC technique was used to obtain the volumetric response, at a cooling rate of 20°C/minute. Water transport data for bovine sperm with no CPAs (Figure, A) and pre-loaded with glycerol (Figure, B) are depicted. The model simulated dynamic cooling response is shown as a solid line (—) was attained using the best fit membrane permeability parameters (L_{pg} and E_{Lp}) shown in Table 3.3 (parameters obtained using the average volumetric shrinkage response), in the water transport equations (2 and 3). The model replicated equilibrium cooling response obtained and is shown as a dotted line (-----) in both the figures. The subzero temperatures are shown along the x-axis and the non-dimensional cell volume is plotted along the y-axis. The error bars symbolize the standard deviation in the data set (Alapati et al., 2009).

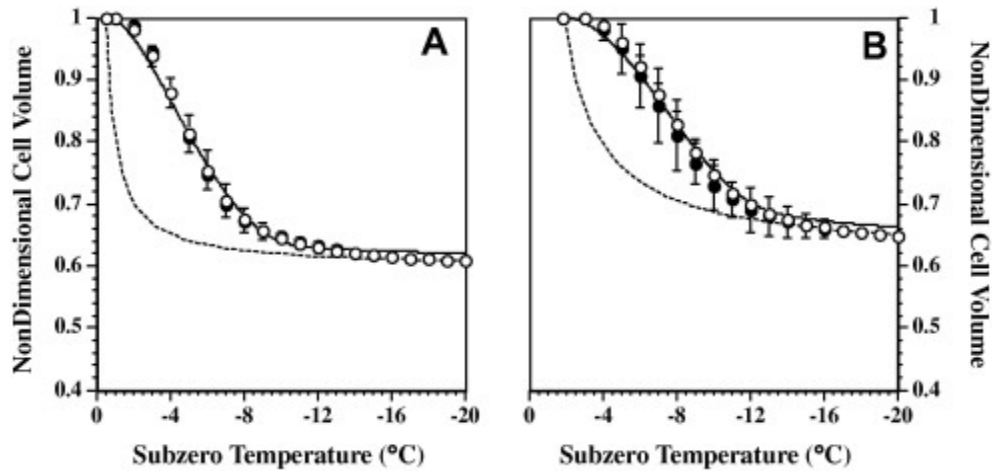


Figure 3.3. Comparative volumetric shrinkage response of ejaculated and epididymal bovine sperm, collected from the same Holstein bulls, obtained using the DSC technique at a cooling rate of 20°C/minute. Shown in figures A and B are the water transport data for bovine sperm without adding CPAs and pre-loaded with glycerol, respectively. Open circles are representative of the volumetric response of ejaculated sperm while closed circles represent epididymal sperm volumetric response. The solid line (—) represents the dynamic cooling response obtained using the best fit membrane permeability parameters (L_{pg} and E_{Lp}) shown in Table 3.4, in the water transport equations (2 and 3). The dynamic cooling response obtained using the best fit parameters for ejaculated and epididymal sperm are almost indistinguishable from each other so only the dynamic cooling curve (—) for ejaculated sperm best fit parameters are depicted in both figures. The dotted line (----) represents the model simulated cooling response. The subzero temperatures are shown along the x-axis and the non-dimensional cell volume is plotted along the y-axis. The error bars symbolize the standard deviation in the data set (Alapati et al., 2009).

DISCUSSION

Collection and cryopreservation of epididymal sperm from genetically valuable males will allow for the further dissemination of these favorable traits. The collection of epididymal sperm represents the “last chance” at collecting sperm from these males. This makes it imperative that epididymal sperm be collected, preserved and utilized in the most efficient manner possible. The majority of studies on bovine sperm cryopreservation have been performed using ejaculated sperm without addressing any possible differences between ejaculated and epididymal sperm. To better understand the difference between ejaculated and epididymal sperm, we must first have a better understanding of the fundamental differences between the 2-types of sperm. In this study, we compared the collection, water transport during cryopreservation and post-thaw motility of ejaculated and epididymal sperm collected from the same 4 Holstein bulls.

Sperm are stored in the cauda epididymides prior to ejaculation (Bialy and Smith, 1958b). As expected we found the concentration of sperm in the cauda epididymides ($1.24 \times 10^9/\text{ml}$) to be significantly greater than the concentration of an ejaculate ($0.76 \times 10^9/\text{ml}$). Our results are in close agreement with values reported by Deutscher et al. (1974) with concentration of epididymal sperm $3.81 \times 10^9/\text{ml}$ and ejaculated sperm $0.27 \times 10^9/\text{ml}$. This was expected due to multiple ejaculations being required to deplete the epididymal sperm reserves (Amann and Almquist, 1961a). Although, as reported by Amann and Almquist (1976) the concentration of the cauda epididymal sperm reserve can vary due to ejaculations within 72 hours of epididymal sperm collection as cited by Coulter et al. (1987).

As sperm progress through the epididymides they develop motility with cauda epididymal sperm having full motility following dilution (Pholpramool et al., 1985). Total motility of freshly collected ejaculated (65%) and epididymal (71%) sperm in our study did not significantly differ. This is in close agreement with values reported by Amann and Griel (1974), Graham (1994) and Martins et al. (2007). Amann and Griel (1974) noted the initial progressive motility of ejaculated (68%) and epididymal (65%) sperm from the same seven Holstein bulls to be similar. Graham (1994) comparing the effects of adding seminal plasma before cryopreservation on the same 5 Holstein bulls also

reported no significant difference between the ejaculated sperm (79%) and epididymal sperm exposed to seminal plasma (82%) or TALP (82%). Although, Way et al. (2000) reported a 16% decreased in the percentage of acrosome intact live sperm due to the addition of accessory sex gland fluid to epididymal sperm collected from 6 Holstein bulls.

Monteiro et al. (2011) compared ejaculated and epididymal sperm collected from the same 8 stallions before and after cryopreservation. In his study he noted that motility of ejaculated sperm was significantly greater (79.6%) than epididymal sperm (29.4%) at the time of collection. But when extended in cryopreservation medium, epididymal sperm motility (84%) increased to the percentage of ejaculated sperm. This was most likely due to the quiescent state that epididymal sperm are held in during storage in the epididymis, but by exposing these cells to factors, such as bicarbonate and calcium, in the cryopreservation medium, motility increased to the level of ejaculated sperm.

García-Álvarez et al. (2009) compared ejaculated and epididymal sperm before and after cryopreservation from the same 6 rams. No significant difference in motility or acrosomal integrity was noted between ejaculated and epididymal sperm upon collection.

Reports on motility following cooling and cryopreservation of ejaculated and epididymal sperm and the effects of seminal plasma have been inconsistent. Amann and Griel (1974) reported the progressive motility of epididymal sperm (43%) as being lower following 24 hours of storage at 5°C compared with ejaculated sperm (57%) collected from the same 7 Holstein bulls. Although, Martins et al. (2007) noted a similar decrease in motility (~10%) following cryopreservation of ejaculated sperm collected from a bull of known fertility compared with epididymal sperm collected from the testes of 3 slaughterhouse bulls.

Graham (1994) noted that re-suspension of washed ejaculated bull sperm in either seminal plasma or TALP had no effect on the motility of sperm after cooling to 5°C (73 vs. 75%) or after thawing (60 vs. 60%), respectively. In addition, seminal plasma had no beneficial effect on the motility of epididymal bull sperm when compared with that of TALP-treated sperm after cooling (75 vs. 72%) or after thawing (66 vs. 63%), respectively. Although, In the stallion, Braun et al. (1994) reported the removal of

seminal plasma from ejaculated sperm (from 25% to 5%) significantly increased the sperm motility over 72 hours of cold storage at 5°C.

In our study we found that the total motility of epididymal sperm (58.8%) was significantly greater than ejaculated sperm (50.9%) following cryopreservation (-196°C). Our results are in agreement with reports from Rath and Niemann (1997) and García-Álvarez et al. (2009).

Rath and Niemann, (1997) compared ejaculated and epididymal sperm collected from the same 3 boars and found the motility of frozen thawed ejaculated sperm (40.2%) was lower compared with frozen thawed epididymal sperm (72.2%). García-Álvarez et al., (2009) noted the motility following cryopreservation of epididymal sperm (58%) was significantly greater than ejaculated sperm (37%) collected from the same 6 rams.

Freezing rates during cryopreservation have been evaluated for a variety of mammals. In the ram, Byrne et al. (2000) compared the cooling rates of 0.5 to 5°C/minute for 6 mature rams and found that sperm cooled at a rate of 5°C/minute was significantly better able to fertilize oocytes when used in AI on FSH treated ewes. Fiser and Fairfull (1983) compared glycerol concentration and cooling rate of ram sperm and found the optimum post-thaw survival at a glycerol concentration to 4% to 6% at a cooling rate of 10 to 100°C/minute. In the bison, sperm collected by electroejaculation has been shown to have better post-thaw sperm parameters when cooled at a rate of 40°C/minute compared with 10 or 25°C/minute (Hussain et al., 2011).

A wide range of cooling rates have been reported for bovine sperm. With the optimal cooling rate for bovine sperm reported to range between 26 and 150°C/minute, but if sperm are cooled too fast (300°C/minute) or too slow (1°C/minute) a decrease in viability has been noted. (Rodriguez et al., 1975; Robbins et al., 1976; Woelders, 1997; Woelders et al., 1997; Kumar et al., 2003; Chaveiro et al., 2006).

Rodriguez et al. (1975) reported the optimal cooling rate for ejaculated sperm collected from beef bulls to be 39°C/minute compared to slower (3 to 7°C/minute) cooling rates. Robbins et al. (1976) reported findings that agreed with those of the previous report, finding the optimum cooling rate to range between 26 and 52°C/minute. A higher cooling rate was reported by Woelders et al. (1997), that noted the optimal

cooling rate of bovine sperm to be between 76 to 140°C/minute. In this report, it was also noted that rapid cooling rates (300°C/minute) caused significant decreases in post-thaw viability (Woelders et al., 1997). Using a controlled rate freezing machines Kumar et al. (2003) compared cooling rates, and found that bull sperm survived cryopreservation at a cooling rate of 30 to 50°C/minute significantly better than at 1°C/minute. Chaveiro et al. (2006) also using a controlled rated freezing machine found the optimum cooling rate for bull sperm to be between 80 and 150°C/minute. These studies reported a range of cooling rates for bovine ejaculated sperm but none addressed epididymal sperm.

In our study we utilized the DSC technique to determine the optimal cooling rate for ejaculated and epididymal sperm collected from the same mature Holstein bulls. This technique has been utilized in various species including bovine (Li et al., 2006). Li et al. (2006) compared the membrane water transport of bovine ejaculated sperm with and without CPA and the effects of the addition of cholesterol to the membranes by CLC. The addition of cholesterol to the sperm membranes may mimic the response seen in epididymal sperm as to their ability to endure cryopreservation better than ejaculated sperm. It was determined that the optimal cooling rate for bovine ejaculated sperm to be between 45 and 60°C/minute. The addition of cholesterol to the membrane may have increased the optimal freezing rate to 60°C/minute compared to ejaculated sperm 58°C/minute.

In our study it was noted that the predicted optimal rates of cooling for bovine ejaculated and epididymal sperm were 50 to 60°C/minute during cryopreservation, and were obtained by analyzing the water transport simulations. This predicted optimal cooling rate was in agreement (within 5%) with the values acquired using GOCRE (Equation 4). These results are also in close agreement with the optimal cooling rate for bovine ejaculated sperm with or without cholesterol added to the membranes (45 to 60°C/minute) previously reported by Li et al. (2006) utilizing the DSC technique. A lack of distinction was noted between ejaculated and epididymal sperm water transport response. This lack of distinction between ejaculated and epididymal sperm was also noted in the rhesus macaque using the DSC technique (Alapati et al., 2008).

In conclusion, our findings demonstrate ejaculated and epididymal sperm can be successfully collected and cryopreserved from the same Holstein bulls. That ejaculated and epididymal sperm have similar water permeability and the optimal freezing rates. This would make it possible to cryopreserve both in the same manner preventing the need to change existing cryopreservation protocols. The addition of glycerol significantly changes the water permeability of both ejaculated and epididymal sperm. And epididymal sperm were better able to endure cryopreservation as noted by a higher overall post-thaw motility and lower percent loss in motility.

CHAPTER IV

INFLUENCE OF CASTRATION-INDUCED TESTOSTERONE DEFICIENCY ON CIRCULATING PLASMA CHOLESTEROL LEVELS IN HOLSTEIN BULLS

INTRODUCTION

Cryopreservation of ejaculated semen has become routine and supports the assisted reproductive techniques in domestic cattle (Foote, 1982). One of the major problems that have been associated with cryopreservation of sperm is their susceptibility to the cooling and freezing process. This susceptibility is not equal among all bulls, as a significant amount of variability has been noted between bulls in the ability of their sperm to endure the stress of cryopreservation (Medeiros et al., 2002). This variability has giving rise to the “good and bad freezer” categories.

Species with increased concentrations of cholesterol in the membranes of their sperm have been noted to produce sperm with an increased resistance to cold shock and also increased post-thaw viability (Darin-Bennett and White, 1977; Watson, 1981; Parks and Lynch, 1992). By increasing the concentration of cholesterol in the membranes of sperm from species that are susceptible to cryopreservation, an increase in their survivability following cryopreservation can also be achieved. (Purdy and Graham, 2004b; Mocé and Graham, 2006; Mocé et al., 2010).

In the male reproductive system, cholesterol is utilized in a variety of ways. Cholesterol is incorporated into the cell membranes increasing their stability and also converted into steroid hormones for endocrine regulation (Setchell, 1978; Purdy and Graham, 2004a). Cholesterol can be obtained from a number of sources including cholesterol concentrated in the plasma membrane, circulating low- and high-density lipoproteins, cytoplasmic lipid droplets or by de novo synthesis (Azhar et al., 2003; Argov et al., 2007; Beer-Ljubić et al., 2009).

Testosterone may influence the concentration of cholesterol in the blood. Castration has been found to increase the cholesterol concentration in the blood plasma of the rabbit, rat, pig and man (Hussein et al., 1999; Cinci et al., 2000; Bo et al., 2011; Yao et al., 2011). Supplementation with exogenous testosterone was noted to reverse the increase in cholesterol induced by castration and returned cholesterol

concentrations to precastration concentrations in the rat, rabbit and man (Takahashi et al., 1986; Hussein et al., 1999).

Castration has also been noted to increase cholesterol concentration in the meat of goat (Madruga et al., 2001; Santos-Filho et al., 2005). Although when comparing bulls and steers, Clemens et al. (1973) noted the fatty acid composition to be similar. Eichhorn et al. (1986) also did not detect any difference in cholesterol concentrations from adipose or muscle tissue from bulls and steers raised in similar conditions. Drawing into question, the ability of testosterone or castration to influence the concentration of cholesterol in the male.

Seasonal changes in testosterone and lipids have been noted in the bull. The production of testosterone has been noted as decreasing in the blood during the winter and increased in the spring (Foote et al., 1976; Peirce et al., 1987). The concentrations of lipoproteins and fatty acids including cholesterol has been noted as also being seasonal in the bull and in the human (Argov et al., 2007; Beer-Ljubić et al., 2009). These changes in testosterone and lipoproteins may have an influence on bull sperm quality that has also been noted to be influenced by the season (Argov et al., 2007).

To our knowledge this is the first time the effect of castration on circulating cholesterol concentrations in the same mature Holstein bulls before and after castration has been evaluated. If the concentration of testosterone is able to affect the cholesterol concentration of the blood and subsequently the amount of cholesterol in the sperm membranes and seminal plasma, then this may prove useful in devising strategies to produce sperm with an increased ability to endure cryopreservation. The objective of the present study was to determine the effect of castration on the circulating concentrations of testosterone and cholesterol in mature Holstein bulls.

MATERIALS AND METHODS

Experimental Design

This experiment evaluated the effects of castration on the circulating testosterone and cholesterol concentrations of Holstein bulls. Four bulls were castrated and blood was collected by jugular vein-puncture from two of these mature Holstein bulls before and after bilateral castration. Blood was taken at least twice-a-week from 85 days before castration and daily for 28 days following bilateral castration. In addition,

immediately before castration and 6, 12 and 24 hours following castration blood was taken. Blood was collected at the same time of day by the same experienced technicians. The concentration of testosterone and cholesterol was determined from 82 blood samples and were measured in duplicate by radioimmunoassay and fluorometric assay, respectively.

Experimental Procedure

Blood plasma was taken before and after castration from the same fertile Holstein bulls, housed at Genex Custom Collection Service near the Louisiana State University (LSU) campus. Bulls were of sound conformation and in good body condition, averaging 490 kg throughout the study. Bulls were maintained on pasture and supplemented with Bermuda hay and 12% protein concentrate feed. Average daily temperature was 17°C with a maximum of 24°C and minimum of 11°C.

Blood plasma collection

Blood samples were obtained by way of jugular venipuncture with sterile, heparinized collection tubes (Monoject Vacutainers[®], Sherwood Medical, St. Louis, MO). Following collection, samples were centrifuged at 1000 x g for 10 minutes to isolate the blood plasma. Plasma supernatant was collected and frozen at -20°C in individual 7-ml plastic tubes for subsequent testing.

Castration

At the time of castration, each bull was restrained, sedated and remained standing throughout the procedure. A licensed veterinarian surgically removed the testes by making an incision down the midline of the scrotum between the 2 testes. Testes were then removed taking care to prevent excessive loss of blood.

Hormone detection

Sample blood plasma, stored at -20°C was thawed and used in radioimmunoassay (Diagnostic System Laboratories Inc., Webster, TX) to determine testosterone concentrations. Assay detection concentrations ranged from 0 to 25 µg/ml plasma. Standards (0, 0.1, 0.5, 2.5, 10 and 25 ng/ml) were pipetted into standard tubes and vortexed for 10 seconds. Labeled testosterone (250 µl) plus the sample plasma (25 µl) was also added to sample tubes and vortexed. Antibody (250 µl) was then added to sample tubes, vortexed and then incubated for 12 hours at 4°C. A precipitating agent

(250 µl) was then added to the sample tubes and incubated for an additional 18 hours. Chilled phosphate buffered saline (2 ml) was then added to the samples and centrifuged at 3,000 rpm for 30 minutes. Following centrifugation, samples were decanted and excess supernatants removed by blotting tubes on paper towels for 3 to 5 seconds. Tubes were then loaded into a gamma counter and analyzed for 1 minute.

Cholesterol detection

Sample blood plasma, stored at -20°C were thawed and used in a fluorescent assay (Cayman chemical Co., Ann Arbor, MI) to determine cholesterol concentrations. Standards were prepared by serial dilution (0, 2, 4, 6, 8, 12, 16, 20µM) and used as reference for measuring samples. Samples were diluted 1:400 in order for values to fall within the standard curve. Samples and standards were added to a 96-well plate in duplicate at 50 µl/well. The assay cocktail was prepared by combining the buffer, cholesterol detector, horseradish peroxidase, cholesterol oxidase and cholesterol esterase together. This assay cocktail was then added a 50 µl/well to initiate the reaction. The loaded plate was then covered and incubated for 30 minutes at 37°C protected from light. Following incubation, the sample fluorescent was evaluated by fluorescent reader (Cyto-Fluor, Perceptive Bio systems) using excitation wavelengths of 530-580 nm and emissions wavelengths of 585 to 595 nm.

Statistical Analysis

Differences between the concentration of plasma cholesterol before and after castration were determined by one way ANOVA. Pairwise comparisons between groups were performed using the Holm-Sidak method.

Differences between the concentration of plasma testosterone before and after castration were determined by Kruskal-Wallis one way ANOVA on ranks. Pairwise comparisons between groups were performed using the Dunn's method. All data were analyzed using SigmaPlot Software Version 10.0. For all analyses, $P < 0.05$ was regarded as statistically significant.

Table 4.1. Mean plasma testosterone and cholesterol concentration before and after bilateral castration of mature Holstein bulls.

Pre- and Post-Castration	Testosterone (ng/ml) ± SEM	Cholesterol (mg/dl) ± SEM
Pre-Castration [†]	10.21 ± 1.03 ^a	106.4 ± 4.57 ^c
Post-Castration [‡]	0.012 ± 0.0025 ^b	123.1 ± 3.52 ^d

[†]Blood collected at least twice a week for 85 days before castration.

[‡]Blood collected daily for 28 days following castration.

^{ab}Means with different letters within column significantly differ (P< 0.001).

^{cd}Means with different letters within column significantly differ (P< 0.01).

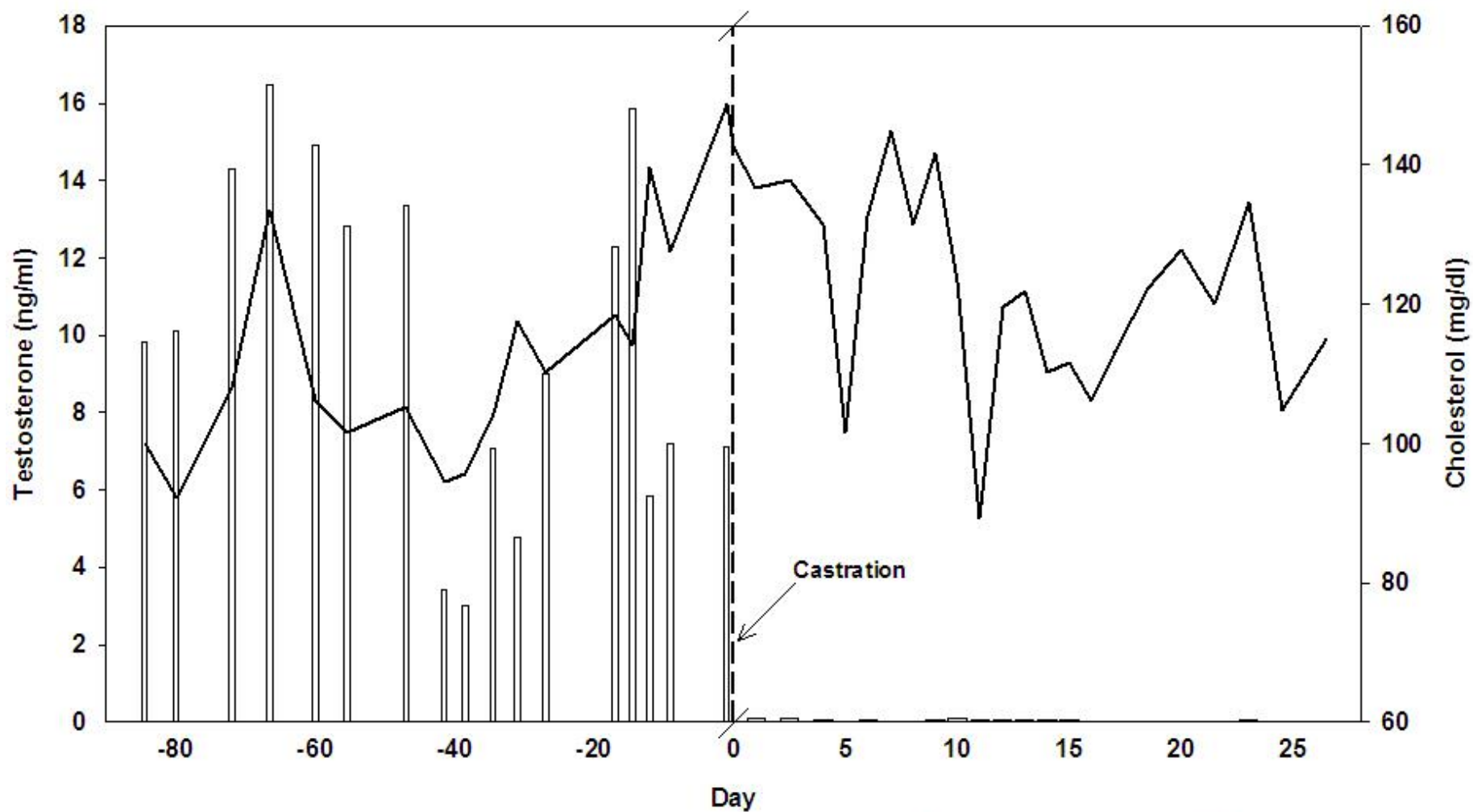


Figure 4.1. Mean plasma testosterone (bars) and cholesterol (line) levels from 85 days pre-castration to 28 days post-castration from mature Holstein bulls. Hyphenated line represents time of castration.

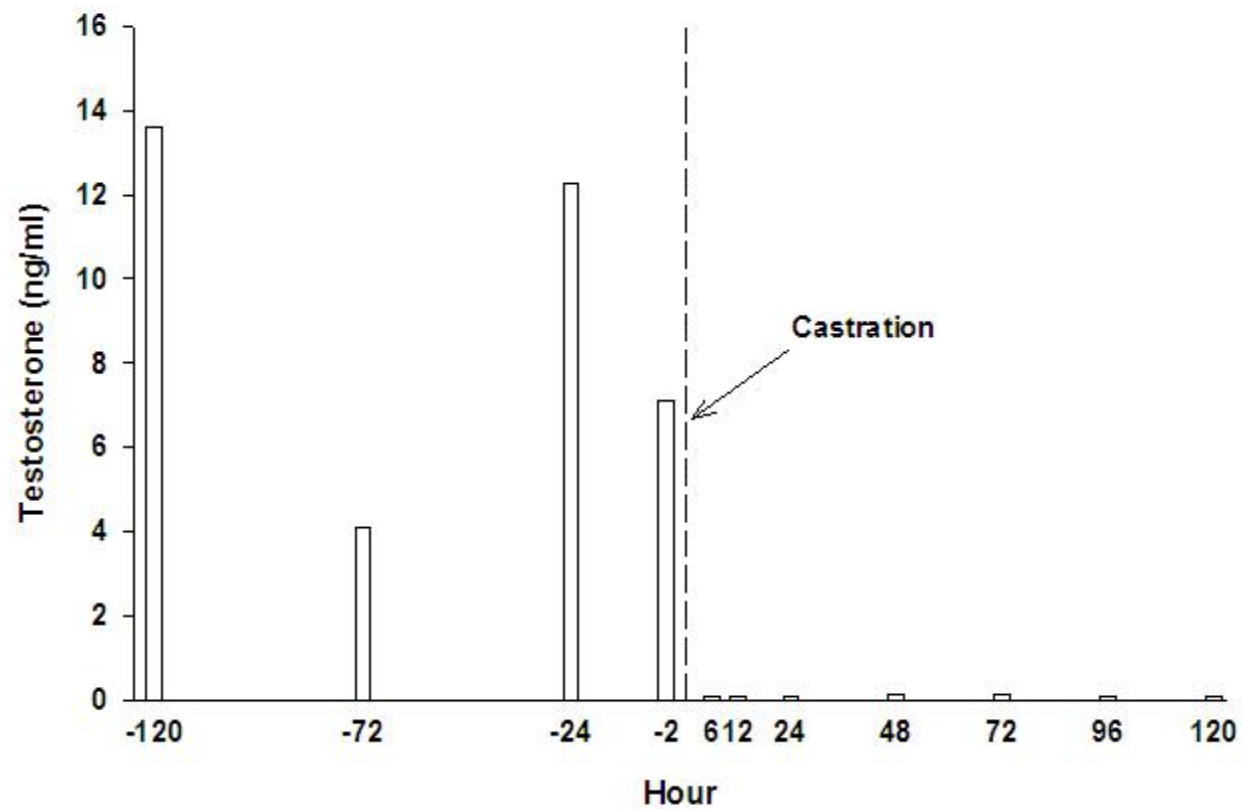


Figure 4.2. Mean plasma testosterone levels from 120 hours pre-castration to 120 hours post-castration from mature Holstein bulls. Hypenated line represents time of castration.

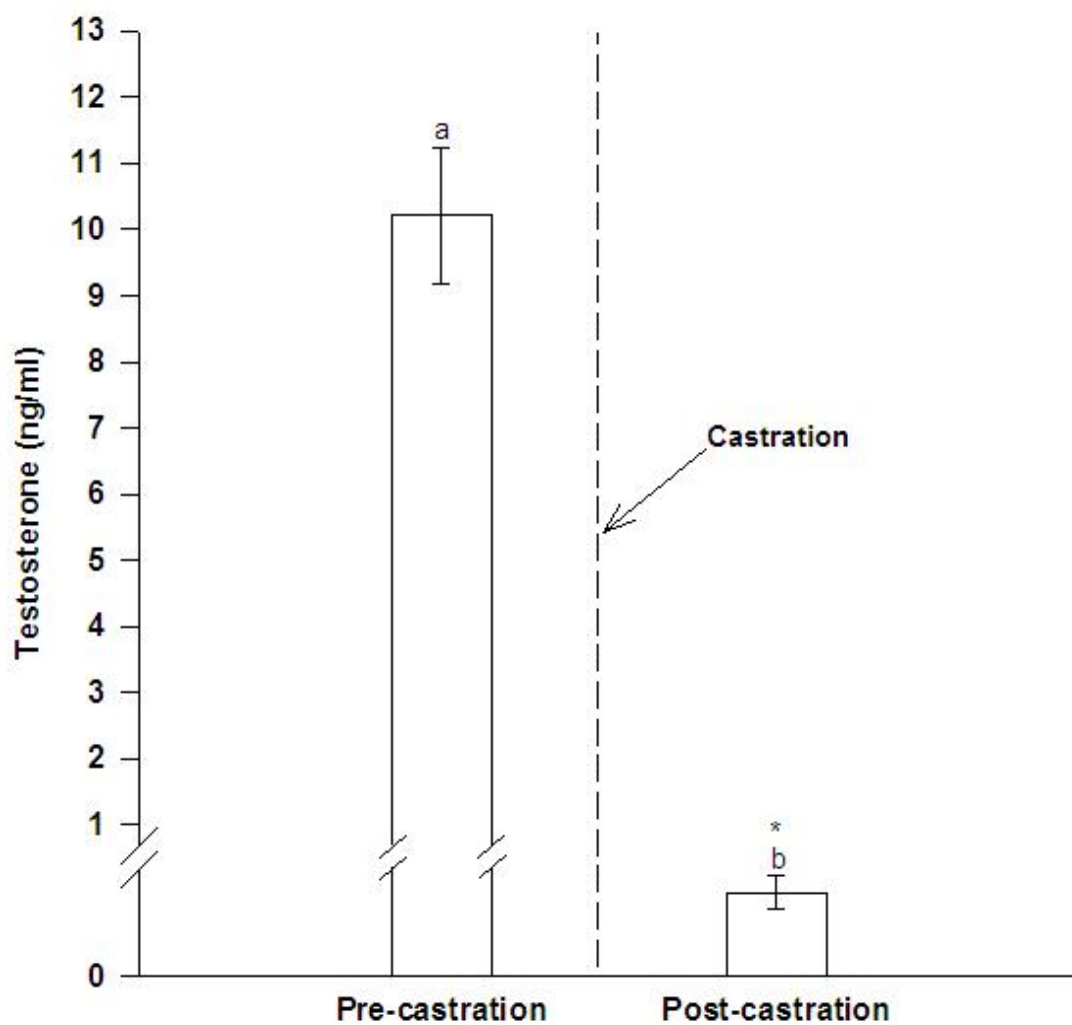


Figure 4.3. Mean plasma testosterone levels pre-castration and post-castration from mature Holstein bulls. ^{a,b}Mean values were significantly different ($P < 0.001$).

*Mean testosterone concentration for bulls at post-castration (0.012 ng/ml).

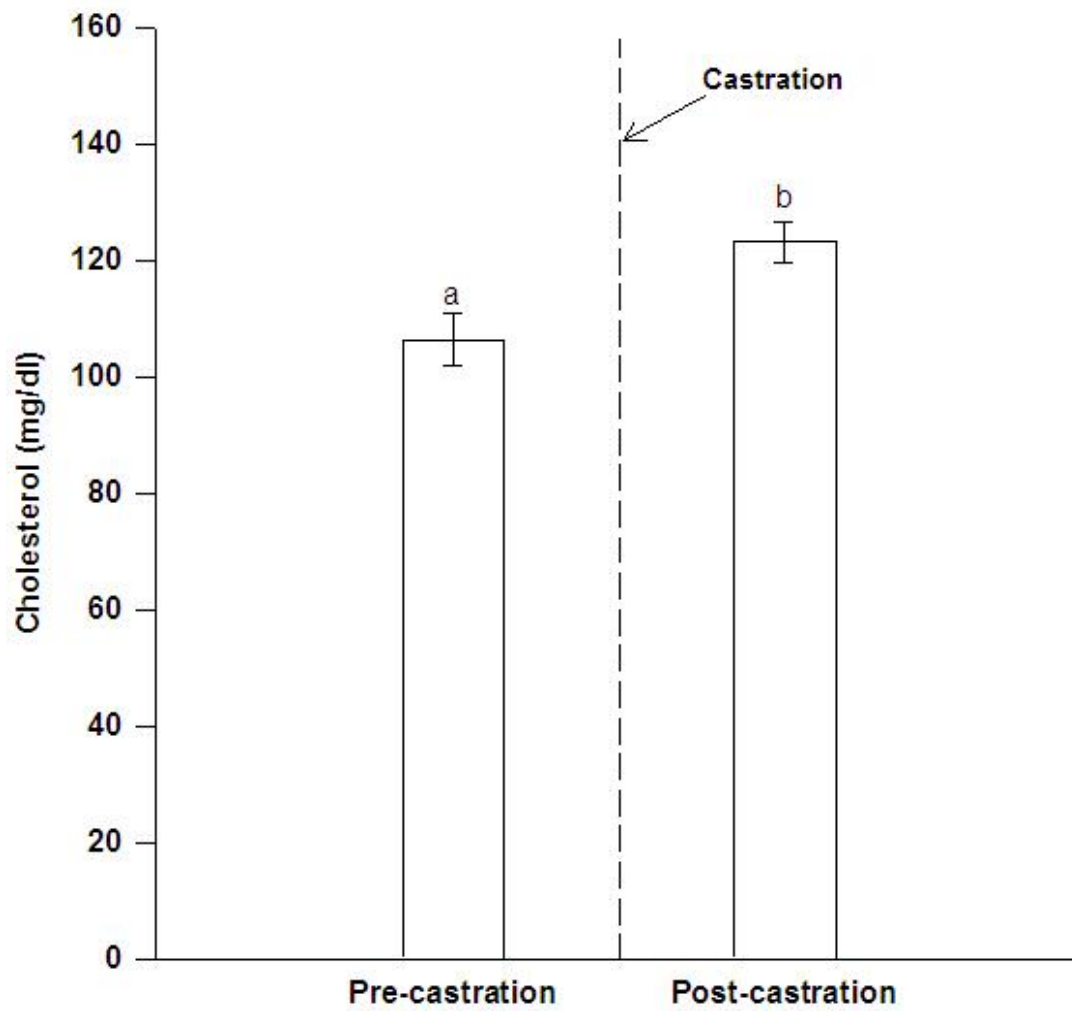


Figure 4.4. Mean cholesterol plasma level pre-castration (85 days) and post-castration (28 days) from mature Holstein bulls. ^{a,b} Mean values are significantly different ($P < 0.01$).

RESULTS

Pre-castration testosterone concentrations were within the reported normal range of 2 to 20 ng/ml (Figure 4.1). The concentration of testosterone significantly dropped ($P<0.001$) following castration see Table 4.1 and Figure 4.3. The concentration of circulating plasma cholesterol significantly increased ($P<0.01$) following castration as noted in Table 4.1 and Figure 4.4

DISCUSSION

The cryopreservation of sperm has become routine and supports the assisted reproduction techniques such as AI, IVF and ICSI. This use of cryopreserved sperm has greatly increased the genetic impact a single bull can have, and is substantiated by the potential calves a bull may sire in a year. In 1931 an average bull could sire only 50 calves compared with 1981 a bull has the potential to produce over 50,000 calves (Foote, 1982). This increase in the ability to disseminate the genetics of a superior bull is an integral part of the strategy for the improvement of animal production around the world. A problem arises when bull sperm has a reduced ability to endure the stresses of cryopreservation resulting in a low post-thaw yield of viable sperm (Watson, 1995, 2000).

Cholesterol has been noted as having an effect on the ability of sperm to endure cryopreservation. The lower the concentration of cholesterol the more sensitive the sperm is to cooling. The percentage of unsaturated fatty acids also affects the susceptibility of sperm to the effects of cooling. Bull sperm are considered to be sensitive with a higher ratio of unsaturated fatty acids and a lower concentration of cholesterol in their sperm membranes when compared with the more resistant rabbit or human sperm (Bailey et al., 2000).

Sperm membranes at body temperature are a fluid mixture of lipids, mostly phospholipids and cholesterol, integral and peripheral proteins that can move laterally within the membrane (Amann and Pickett, 1987). The function of the membrane is dependent on the phospholipid protein arrangement (Hammerstedt et al., 1990). Phase transition of phospholipids from the fluid state to the crystalline-gel state occurs during the cooling process of cryopreservation. This phase transition results in a more orderly and packed membrane that restricts movement of proteins and lipids (Amann and

Pickett, 1987; Hammerstedt et al., 1990). Integral proteins are excluded from the phospholipid crystalline-gel domains, resulting in proteins clustering together. Functionality and stability of the membrane is lost when protein exclusion and subsequent protein clustering occurs (Amann and Pickett, 1987).

The overall membrane fluidity is determined by the ratio of cholesterol to phospholipids and the amount of phospholipids, which are polyunsaturated fatty acyl chains (Amann and Pickett, 1987). Different species have been noted as having varying susceptibility to cold shock (Watson, 1981). The bull, boar, ram and stallion are all noted as being very susceptible to cold shock, while the rabbit, fowl and human are noted as being very resistant to cold shock.

Membrane composition revealed, that generally, cold shock resistant species contained higher ratios of cholesterol to phospholipids and an increased in polyunsaturated fatty acids compared with cold shock susceptible species (Watson, 1981). Darin-Bennett and White (1977) reported the molar ratio of cholesterol to phospholipids of the ram (0.38) and bull (0.45), cold shock sensitive species, to be lower than the rabbit (0.88) and human (0.99), that are considered to be cold shock resistant. These values were in close agreement with the ratios later reported by Parks and Lynch (1992) with molar ratio of cholesterol to phospholipid of the bull (0.45), stallion (0.36) and boar (0.26) all of which are considered cold shock sensitive species.

At low temperatures, the cholesterol to phospholipid ratio is also important in determining membrane fluidity and stability. Cholesterol interacts with fatty acyl chains of the phospholipids, modulating the fluidity of the membrane. Cholesterol also maintains phospholipids in a random lamellar arrangement during the decline in temperature (Amann and Pickett, 1987). Cryopreserved sperm have been noted as having lower cholesterol concentrations compared with fresh sperm. Kadirvel et al. (2009) compared ejaculated buffalo sperm before and after cryopreservation and noted significantly lower cholesterol concentrations following cryopreservation ($12.89 \mu\text{g}/10^8$ sperm) compared with fresh ($21.67 \mu\text{g}/10^8$ sperm). Increasing the cholesterol to phospholipid ratio also broadens the phase transition, reduces membrane leakage and lowers membrane phase separation in model membranes (Drobnis et al., 1993), therefore, an increase in cholesterol concentration of the sperm membranes may

increase the ability of sperm from a sensitive (“bad freezer”) bull to survive the cryopreservation process.

Currently, the only viable method to increase the cholesterol concentration in the membranes of sperm is by adding it via cholesterol loaded cyclodextrins following ejaculation. The concentration of cholesterol transferred to sperm increases linearly with the concentration of CLCs incubated with sperm (Purdy and Graham, 2004a). The ratio of cholesterol to phospholipids in cold shock sensitive bull sperm (0.45) can be increased to the concentration of highly cold shock resistant species such as the rabbit (0.88) or human (0.99) (Darin-Bennett and White, 1977; Purdy and Graham, 2004a). However, there appears to be a threshold of approximately 4 to 5 times normal concentrations, that when crossed, cholesterol becomes detrimental to cell survival (Purdy and Graham, 2004a). The addition of cholesterol to sperm that are to be sex-sorted has also been reported as yielding unacceptable results following staining and dilution (De Graaf et al., 2007).

The addition of cholesterol to sperm has been shown to increase the survival of sperm following cryopreservation in the: bull (Purdy et al., 2005; Amorim et al., 2009; Moraes et al., 2010), ram (Mocé et al., 2010), stallion (Moore et al., 2005) and boar (Tomás et al., 2011). Purdy and Graham (2004a) compared the effects of addition of cholesterol on bull sperm motility and viability following cryopreservation with (60% and 55%, respectively) and without (42% and 46%, respectively). Amorim et al. (2009) also reported an increase in bull sperm motility following cryopreservation from 40% without compared to 54% with the addition of cholesterol prior to cryopreservation.

The ability to increase the concentration of cholesterol in the membranes of sperm without adding cholesterol following ejaculation may be a strategy to increase the post-thaw viability of ejaculated sperm. There is little information available as to the ability to influence the cholesterol concentrations in the bull, but studies in the rabbit, rat, pig and man have all noted that the concentration of testosterone can influence the concentration of cholesterol in the blood of these species (Anderson et al., 1995; Hussein et al., 1999; Cinci et al., 2000; Bo et al., 2011; Yao et al., 2011).

In the present study, our objective was to determine if the removal of testosterone by castration would increase or decrease the concentration of cholesterol

in the circulation. If so, this would give reason to believe that the concentration of cholesterol in the blood plasma of bulls can be affected in the same manner as other species.

Yao et al. (2011) noted an increase in serum total cholesterol, triglycerides, high and low density lipoproteins and leptin concentrations along with the up-regulation of fatty acid synthase and acetyl-CoA carboxylase alpha genes in crossbreed barrows compared to boars. This was also the case in human males undergoing orchiectomy and non-orchiectomy treatment for androgen dependent prostate cancer (Bo et al., 2011). It was determined that total cholesterol concentrations in men who underwent orchiectomy were significantly higher than men who did not undergo orchiectomy or men in the control group. Hussein et al. (1999) also noted that when mature and immature rabbits were castrated their cholesterol concentrations increased.

Administering testosterone has been shown to reduce the concentration of cholesterol in the rabbit, rat and man. In mature and immature castrated rabbits, when administered exogenous testosterone, the cholesterol concentrations returned to precastration concentrations (Hussein et al., 1999). Takahashi et al. (1986) noted that the concentration of cholesterol of castrated rats decreased following the administration of testosterone. When Zgliczynski et al. (1996) treated hypogonadal men with exogenous testosterone, it was determined that their cholesterol concentrations also decreased.

In the bull, little information is available as to the influence of testosterone or castration has on plasma cholesterol concentration. Clemens et al. (1973) noted the fatty acid composition of adipose tissue in Angus bulls and steers to be similar. Eichhorn et al. (1986) also did not note any difference in cholesterol concentration from adipose or muscle tissue from steers and bulls raised in similar conditions. However, the castration of goats has been found to increase the cholesterol concentration of their meat (Madruga et al., 2001; Santos-Filho et al., 2005).

Information as to the seasonal effects on the bull may give us some insight into this problem. In the bull, sperm quality has also been noted to be influenced by the season that it was collected (Argov et al., 2007), with sperm collected during summer being of lower quality than sperm collected during the winter. Although, temperature has

been shown to have an effect, other parameters may also have an influence. Foote et al. (1976) found that the production of testosterone decreased in the winter and increased spring. Argov et al. (2007) and Beer-Ljubić et al. (2009) reported that the concentrations of lipoproteins and fatty acids including cholesterol as being seasonal in the bull, with increased concentrations of cholesterol and LDL lipoproteins during the winter months. This decrease in testosterone followed by an increase in cholesterol may cause sperm to have increased concentrations of cholesterol in their membranes in the winter resulting in better cryosurvivability.

The results of our study indicate that the concentration of cholesterol in the circulating blood plasma did significantly increase following castration. If the concentration of cholesterol can be influenced by the concentration of testosterone, then it may be possible to manipulate this, to change the concentration of cholesterol in the sperm membrane. By increasing the concentration of cholesterol in the sperm membranes of the so called “bad freezer” bulls, we may be able to increase the percentage of sperm able to survive cryopreservation from these bulls.

CHAPTER V

CAPACITATION AND ACROSOME REACTION OF EJACULATED AND EPIDIDYMAL SPERM POST-THAW AND THE EFFECTS OF HEPARIN DURING IN VITRO CULTURE

INTRODUCTION

The collection and cryopreservation of sperm from genetically valuable bulls has become routine and is the main source of sperm utilized in assisted reproductive techniques such as artificial insemination (AI) and in vitro fertilization (IVF).

Cryopreserved semen theoretically has the ability to be stored indefinitely and has played a significant role in improving animal genetics (Vishwanath and Shannon, 2000). Although in some animals, such as exotic bovine breeds, the collection of ejaculated sperm is not feasible. This makes the ability to collect, cryopreserve and utilize epididymal sperm necessary for species from which it is difficult or impossible to collect ejaculated sperm. In addition, the unexpected loss of a genetically valuable male due to injury or death will prove epididymal sperm indispensable in producing additional progeny that would otherwise be lost. Epididymal sperm, having not been exposed to seminal plasma, may potentially respond differently than ejaculated sperm with respect to cryopreservation, capacitation and their ability to fertilize oocytes in vitro. It is therefore important for us to understand the differences between ejaculated and epididymal sperm in an effort to utilize these finite sperm stores in the most efficient manner possible. This will maximize the number of progeny obtained from these genetically valuable males.

Our studies were conducted to compare ejaculated and epididymal sperm collected from the same Holstein bulls (n=4) by their percentage of viability, acrosomal integrity and capacitation post-thaw. Also, serial concentrations of heparin were added during a 6-hour in vitro culture to determine if the effects of the capacitation agent on epididymal sperm are consistent with the effects on ejaculated sperm.

During ejaculation, sperm are exposed to seminal plasma that contains many factors including ions, lipids, energy substrates and proteins (Moura et al., 2007). This exposure to seminal plasma has been noted as having both beneficial and detrimental effects on sperm (Bergeron et al., 2004). The major protein components of seminal plasma are the BSP proteins, comprising ~65% of all seminal plasma proteins

(Bergeron et al., 2004). These BSP proteins are secreted by the seminal vesicles and bind to choline phospholipids on the sperm plasma membrane (Therien et al., 1995). BSP proteins also bind to the glycosaminoglycan heparin, a known inducer of capacitation in bovine sperm (Miller et al., 1990). One of the major aspects of capacitation is the efflux of cholesterol from the plasma membrane. Exposure of epididymal sperm to BSP proteins induces an efflux of cholesterol from the plasma membrane (Therien et al., 1998).

Cholesterol has a stabilizing effect on membranes that increases their resistant to cold shock (Cross, 1998). By artificially adding cholesterol to the sperm plasma membrane, it has been shown that survival rates following cryopreservation also increases without effecting the ability of sperm to undergo capacitation (Purdy and Graham, 2004a). Epididymal sperm, having not been exposed to seminal plasma, may have higher cholesterol concentrations in their plasma membranes. In species, such as the human and rabbit, whose sperm contain high cholesterol to phospholipid ratios (>0.80) and are able to better endure cryopreservation compared to species such as the boar with low cholesterol to phospholipid ratios (0.20). Boar sperm has been noted as being very susceptible to cold shock and do not endure cryopreservation well (Cross, 1998). The efflux of cholesterol from epididymal sperm membranes following ejaculation may cause sperm to be more susceptible to cooling and cryopreservation induced damage.

Following cryopreservation, epididymal sperm may also have a higher percentage of viable acrosome-intact sperm that have not become capacitated. Cryopreservation has been shown to induce ultra-structural changes to sperm plasma membranes. These changes in the plasma membrane alter the sperm's ability to regulate cell functions such as capacitation. The capacitation like state that is induced by cryopreservation is termed (Cryocapacitation) (Bailey et al., 2000). Key components of capacitation are the regulation of intracellular calcium and bicarbonate concentrations that are dependent on the permeability and function of the plasma membrane. As previously stated, epididymal sperm having not been exposed to seminal plasma (high in both bicarbonate and calcium) may function differently following cryopreservation.

The ability of epididymal sperm to undergo the process of capacitation during in vitro culture may also be different compared with ejaculated sperm. Ejaculated and epididymal sperm differ with respect to the exposure of seminal plasma that contains BSP proteins. BSP proteins have been isolated and function in supporting; motility, binding of sperm to oviductal epithelium and binding of heparin (Gwathmey et al., 2006).

Heparin has been shown to increase the percentage of capacitation in bovine sperm during in vitro culture (Parrish et al., 1988). BSP proteins serve as the heparin binding sites on ejaculated sperm. Epididymal sperm also have heparin binding sites but at a lower level compared to ejaculated sperm. If epididymal sperm have a reduced capability to bind heparin, they may be inhibited from becoming capacitated. Seminal plasma also contains decapacitation factors that prevent capacitation. If epididymal sperm are not exposed to such factors, capacitation may be accelerated. If the time required for an epididymal sperm cell to become capacitated changes, it may necessitate a change in assisted reproduction protocols.

During these studies, we will compare ejaculated and epididymal sperm collected from the same bulls in an attempt to better understand the different dynamics that arise when utilizing them in assisted reproductive techniques. If epididymal sperm proves to be different, an adjustment in existing protocols may be warranted to better suite epididymal sperm to accomplish the most efficient use of this finite resource.

MATERIALS AND METHODS

Experimental Design

Experiment 5.1

This experiment was designed to examine the difference between ejaculated and epididymal sperm collected from the same mature Holstein bulls as to their percentage of viability, capacitation and acrosome reaction following cryopreservation. Ejaculated and epididymal sperm were collected from the same mature fertile Holstein bulls (n=4). Differences between ejaculated and epididymal sperm as to the percentage of viability and auto-acrosome reaction were determined with fluorescent staining using fluorescein isothiocyanate conjugated peanut agglutinin FITC-PNA and propidium iodide (PI). Capacitated cells were induced to undergo the acrosome reaction with

lysophosphatidylcholine (LPC). Fluorescent staining was assessed using flow cytometry.

Experiment 5.2

This experiment was designed to examine the effects of serial heparin concentrations on cryopreserved ejaculated and epididymal sperm collected from the same mature Holstein bulls. Ejaculated and epididymal sperm were incubated, for 2-, 4- and 6 hours, in capacitating medium in 1 of 4 treatments: Treatment 1 (Control) 0 µg/ml heparin; Treatment 2 (+5 Hep) 5 µg/ml heparin; Treatment 3 (+10 Hep) 10 µg/ml heparin and Treatment 4 (+20 Hep) 20 µg/ml heparin. Differences between ejaculated and epididymal sperm as to the percentage of viability and acrosomal integrity were determined with fluorescent staining using FITC-PNA and Pi. Capacitated cells were induced to undergo the acrosome reaction with LPC. Fluorescent staining was assessed using flow cytometry.

In both studies, 2 units of cryopreserved ejaculated sperm and 2 units of epididymal sperm were pooled for evaluation. The entire experiment was run in duplicate for each bull. Post-thaw viability, acrosomal integrity and capacitation were determined from 16 replicates. Effects of serial heparin concentration on ejaculated and epididymal sperm were determined from 16 replicates per treatment per hour at 2, 4 and 6 hours. Heparin treatments were pooled to compare ejaculated sperm with epididymal sperm over time, differences were determined from 64 replicates per hour at 2, 4 and 6 hours.

Experimental Procedure

Animals

Ejaculated and epididymal sperm was collected from the same mature, healthy, fertile Holstein bulls (n=4) housed at Genex Custom Collection Service near the Louisiana State University (LSU) campus. The bulls were of sound conformation and in good body condition at the time of semen collection.

Ejaculated sperm collection

All ejaculations from each bull were collected by the same experienced technicians throughout the study. Two false mounts were made by the bulls before actual semen collection. A total of at least 2 separate ejaculates from each of the bulls

were collected and cryopreserved. Ejaculated semen was collected by artificial vagina into a 15 ml polypropylene conical tube and volume noted. The ejaculate was extended in standard bovine egg yolk-based diluents and then placed into a water bath (37°C) for equilibration before evaluation of progressive motility. Sperm concentration was calculated by a standard curve from a spectrophotometer, following standard commercial bull stud procedures.

Epididymal sperm collection

Epididymal sperm was collected from the same mature Holstein bulls that from which ejaculated sperm was collected. Collection of epididymal sperm was performed by isolating the epididymides and a portion of the vas deferens from the testes in a sterile manner. The vas deferens was catheterized and egg yolk-based extender was used to push epididymal sperm out of the epididymis by retrograde flow. Epididymal sperm was collected into a 15 ml polypropylene conical tube, extended in standard bovine egg yolk-based diluents and then placed into a 37°C water bath for equilibration before evaluation of progressive motility. Sperm concentration was calculated by a standard curve from a spectrophotometer, following standard commercial bull stud procedures.

Cryopreservation

Extension and cryopreservation of the samples was performed with a standard bovine egg yolk-based Tris extender (Genex commercial extender). The extender was divided into 2-parts, with Fraction-A (without glycerol) and Fraction-B (with 1.4 M glycerol or 12% v/v glycerol). Fraction-A was first used to extend the sperm. The sperm/Fraction-A mixture was then placed in a 37°C water jacket and slowly (0.1°C/minute) cooled to 5°C. Once equilibrated to 5°C, Fraction-B was slowly added to the sperm/Fraction-A mixture in a ratio of 1:1 (v/v). The extended sperm was then placed into 0.5-ml plastic straws (IMV International, Minneapolis, MN) at a concentration of 30×10^6 sperm/straw. The labeled straws were sealed and laid horizontally on racks over liquid nitrogen vapor for freezing. The cooling rate was 15°C/minute from 5°C to -100°C. Straws were then plunged into liquid nitrogen and stored until subsequent evaluation.

Post-thaw sperm evaluation

Sperm thawing was conducted by placing the frozen straw into a 37°C water bath for 40 seconds. Straws were removed from the water bath and excess water dried off, taking care not to shock the sperm. A sample was then placed onto a pre-warmed glass slide and evaluated by phase microscopy. Total sperm motility and sperm morphology were assessed on three units of sperm from each ejaculate from each bull.

Sperm staining preparation

Sperm cryopreserved in egg-yolk glycerol extender were removed from the liquid nitrogen and thawed at 37°C for 40 seconds. Thawed sperm samples were then diluted in 500 µl of 2.9% sodium citrate buffer. Diluted sperm were then carefully layered over a discontinuous Percoll density gradient and centrifuged at 300 x g for 25 minutes to isolate a viable sperm population and remove egg yolk from the sample (Parrish et al., 1995). Following isolation, sperm were reconstituted in a modified Tyrodes glucose free capacitating medium (Parrish et al., 1988) at a concentration of 10×10^6 /ml, divided into treatment groups and incubated in humidified 5% CO₂ air at 39°C for 2, 4 and 6 hours.

Flow cytometry assay

Following incubation, 2 samples of sperm were taken, 100 µl each, from each treatment group and each added to 900 µl of sodium citrate in preparation for staining and evaluation by flow cytometer (Accuri Cytometers inc., Ann Arbor, MI). The acrosome integrity and viability of sperm sample was determined in the first sample by staining with fluorescent isothiocyanate conjugated peanut agglutinin (FITC-PNA, 5 µg/ml) and propidium iodide (Pi, 2.4mM), respectively for 5 minutes, followed by evaluation by flow cytometry. The second sample was exposed to 10 µg/ml of lysophosphatidylcholine (LPC) and incubated in the dark for 15 minutes to induce the acrosome reaction in capacitated sperm (Parrish et al., 1988). Following incubation with LPC, the second sample's percentage of acrosome reaction was determined by staining with FITC-PNA and Pi and evaluated by flow cytometry. Ten thousand events were evaluated for each sample analyzed. Capacitation was determined by calculating the difference between the auto- and LPC induced-acrosome reacted groups.

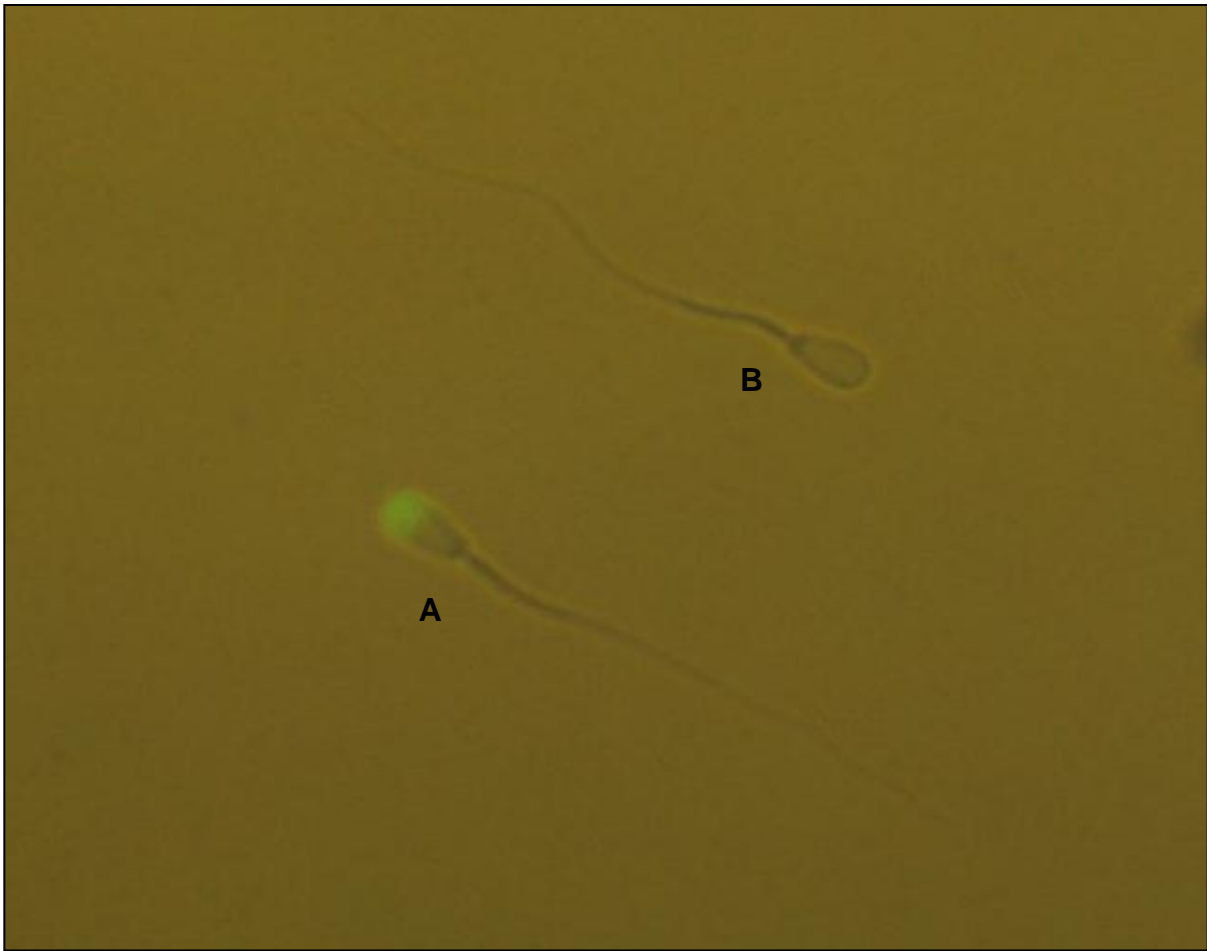


Figure 5.1. Acrosomal staining of bovine sperm cells, (A) lower left is stained positive for fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) (acrosome reacted). (B) Upper right is negative for FITC-PNA (acrosome intact).

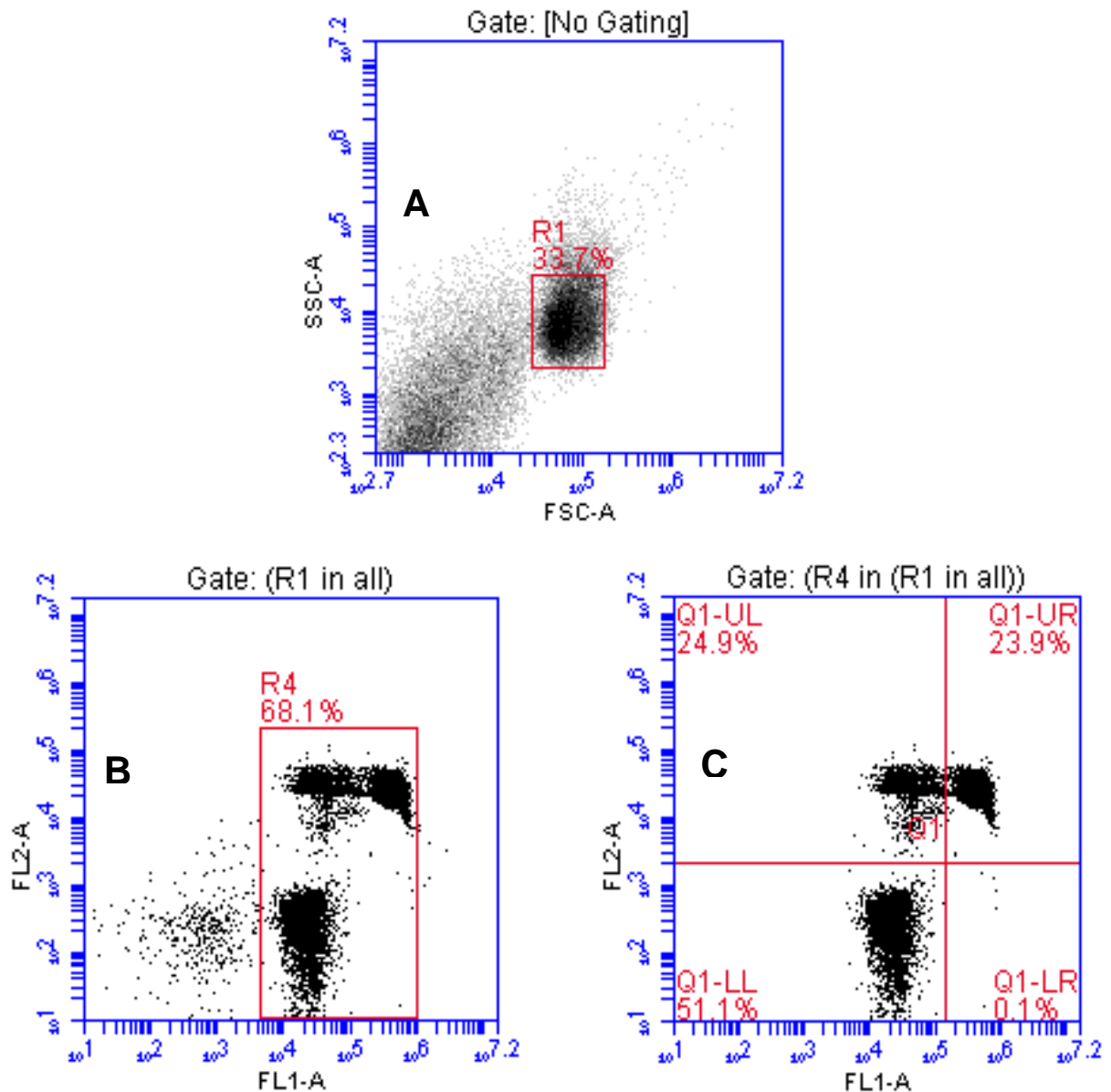


Figure 5.2. Scatter plots of sperm sample events obtained by flow cytometry. Sperm were stained with propidium iodide (PI) and fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) to evaluate viability and acrosomal status, respectively. Graph A depicts events separated by size (FSC) and density (SSC). Graph B and C depict events separated by fluorescent staining, FL1 (FITC-PNA, 515-545 nm) and FL2 (PI, 565-605 nm). Events were gated to isolate the sperm population. Graph A, events that are the size and density of sperm are gated in R1. Graph B, depicts events in R1 and are separated by fluorescent signal, and further gated in R4 to remove debris. Graph C are the events in R4 which are separated into quadrants: Q1-LL (- PI, - FITC-PNA), Q1-UL (+PI, -FITC-PNA), Q1-UR (+PI, +FITC-PNA), to quantify events with specific fluorescent staining.

Statistical Analysis

Differences among treatments were determined with ANOVA; Holm-Sidak test was used as a post-ANOVA pairwise multiple comparison. Non parametric data were analyzed by Kruskal-Wallis one way ANOVA on ranks. A $P < 0.05$ was considered significant in this study. All data were analyzed using SigmaPlot Software Version 10.0.

RESULTS

Experiment 5.1.

The percentage of sperm viability, acrosomal integrity and capacitation was compared between ejaculated and epididymal sperm collected from the same bulls following cryopreservation. The percentage of acrosome reacted sperm post-thaw was significantly higher for ejaculated sperm when compared with epididymal sperm ($P < 0.001$, Figure 5.3). Nonviable acrosome intact sperm did not differ between ejaculated and epididymal sperm ($P = 0.860$, Figure 5.4). The percentage of live sperm post-thaw did not significantly differ between ejaculated and epididymal sperm ($P = 0.094$). When ejaculated and epididymal sperm were compared as to the percentage of capacitation post-thaw, no difference was detected ($P = 0.628$, Figure 5.6).

Experiment 5.2.

The effects of in vitro culture and serial heparin concentrations on cryopreserved ejaculated and epididymal sperm as to their ability to affect sperm viability, acrosomal integrity and induce capacitation were compared. The effect of heparin concentration on ejaculated and epididymal sperm was compared within each time period during in vitro culture. Treatment groups were combined to determine the response and or difference between ejaculated and epididymal sperm during in vitro culture.

The effects of heparin on the percentage of auto-acrosome reaction are depicted in Figures 5.8 and 5.9. The inclusion of heparin, irrespective of concentration, did not significantly increase the percentage of auto-acrosome reaction between ejaculated or epididymal sperm ($P = 0.571$ and $P = 0.128$, respectively), but Figure 5.10 indicates that the percentage of auto-acrosome reacted ejaculated sperm are significantly higher than epididymal sperm ($P < 0.001$). The percentage of auto-acrosome reacted ejaculated sperm also does not significantly increase during the 6-hour in vitro culture, as was noted in epididymal sperm ($P < 0.05$, Figure 5.10).

The percentage of nonviable acrosome intact ejaculated and epididymal sperm was also not significantly affected by the inclusion of heparin during in vitro culture ($P=0.590$ and $P=0.521$, Figures 5.11 and 5.12, respectively), but Figure 5.13, indicated the percentage of nonviable acrosome intact ejaculated sperm was significantly greater than epididymal sperm ($P<0.001$). In addition, as noted earlier with auto-acrosome reaction in epididymal sperm, a significant increase was noted in the percentage of nonviable acrosome intact epididymal sperm over time ($P<0.001$, Figure 5.13). The percentage of nonviable acrosome intact ejaculated sperm also increased over time but was only significantly different from 2 to 4 hours ($P<0.05$, Figure 5.13).

The percentage of viable acrosome intact ejaculated or epididymal sperm was not significantly affected by the inclusion of heparin ($P=0.385$ and $P=0.325$, respectively) but as seen in Figure 5.14 and 5.15, all groups that included heparin did have reduced percentages of viable acrosome intact sperm. When ejaculated and epididymal sperm were compared as to their percentage of viable acrosome intact sperm, epididymal sperm was significantly greater than ejaculated sperm over all time periods ($P<0.001$, Figure 5.16). The same pattern (Figure 5.13) was also noted in the percentage of viable acrosome intact epididymal sperm, in which a significant decrease was noted over all time points ($P<0.001$) but ejaculated sperm only significantly decreased from 2 to 4 hours of culture ($P<0.05$, Figure 5.16).

The percentage of in vitro capacitation was compared among ejaculated and epididymal treatment groups. The concentration of heparin was unable to significantly increase the percentage of capacitation in either ejaculated or epididymal sperm ($P=0.807$ and $P=0.723$). Ejaculated sperm had significantly more capacitated sperm when compared with epididymal sperm over all time points ($P<0.001$, Figure 5.19). Capacitation was not significantly increased in epididymal sperm over any time points ($P=0.069$, Figure 5.19).

Table 5.1. Viability, acrosomal integrity and capacitation of cryopreserved ejaculated and epididymal sperm collected from the same four Holstein bulls exposed to varying concentration of heparin during 6 hours of in vitro culture.

Collection type	Hour	Heparin concentration (µg/ml)	% Live*	% Nonviable acrosome intact*	% Acrosome reacted*	% Live capacitated†
Ejaculated	0	0	63.1	22.2	14.4	13.0
Epididymal	0	0	74.2	21.4	4.1	10.4
Ejaculated	2	0	36.7	40.3	22.0	18.1
Epididymal	2	0	57.7	32.4	9.8	7.9
Ejaculated	2	5	29.9	45.3	24.4	21.6
Epididymal	2	5	50.8	36.9	11.8	8.2
Ejaculated	2	10	28.5	46.5	24.8	23.7
Epididymal	2	10	50.7	36.8	12.2	9.6
Ejaculated	2	20	29.4	46.6	24.5	27.0
Epididymal	2	20	46.8	39.6	13.3	12.9
Ejaculated	4	0	27.9	47.1	22.0	29.0
Epididymal	4	0	51.3	36.3	12.2	9.8
Ejaculated	4	5	22.3	52.0	25.4	38.5
Epididymal	4	5	40.9	43.5	15.2	12.1
Ejaculated	4	10	21.6	49.7	28.4	38.7
Epididymal	4	10	41.4	43.0	15.2	11.7
Ejaculated	4	20	23.5	53.3	25.8	54.2
Epididymal	4	20	40.2	44.6	14.8	15.9
Ejaculated	6	0	25.1	49.8	23.9	35.6
Epididymal	6	0	42.8	42.7	14.4	18.1
Ejaculated	6	5	19.0	53.8	27.0	54.2
Epididymal	6	5	34.7	47.7	17.2	16.9
Ejaculated	6	10	18.3	53.1	28.4	55.1
Epididymal	6	10	34.2	48.3	17.2	19.9
Ejaculated	6	20	19.6	54.3	26.9	63.9
Epididymal	6	20	32.4	49.0	18.2	24.9

*Sperm viability and acrosomal integrity determined by PI and FITC-PNA fluorescent staining, respectively. Sperm staining pattern evaluated by flow cytometer.

†Capacitation determined by inducing the acrosome reaction in viable capacitated sperm with lysophosphatidylcholine (10 µg/ml).

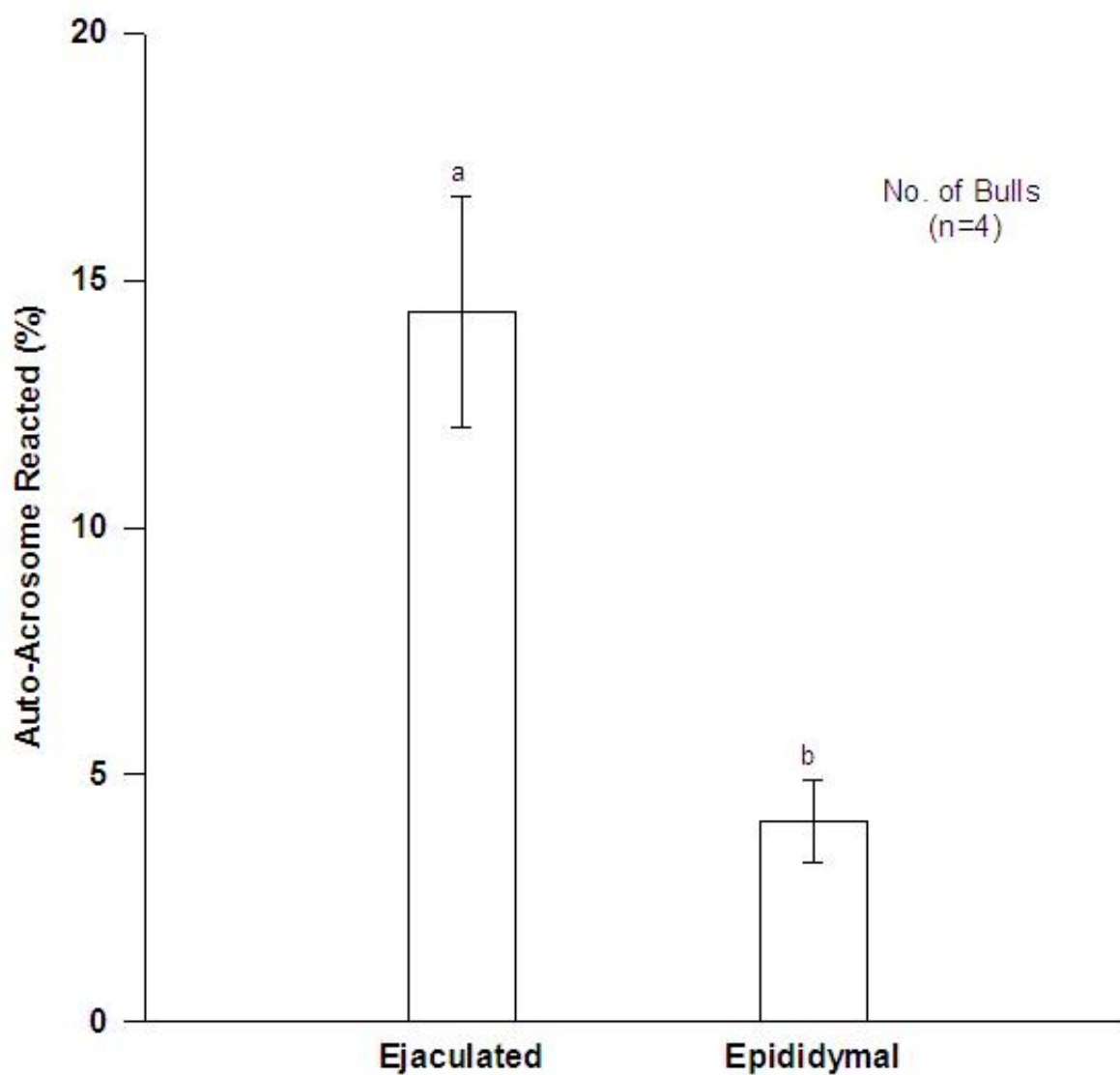


Figure 5.3. Percentage of auto-acrosome reaction of thawed cryopreserved ejaculated and epididymal bovine sperm (mean \pm SEM) collected from the same bulls. ^{a,b}Mean values with different letters are significantly different ($P<0.001$).

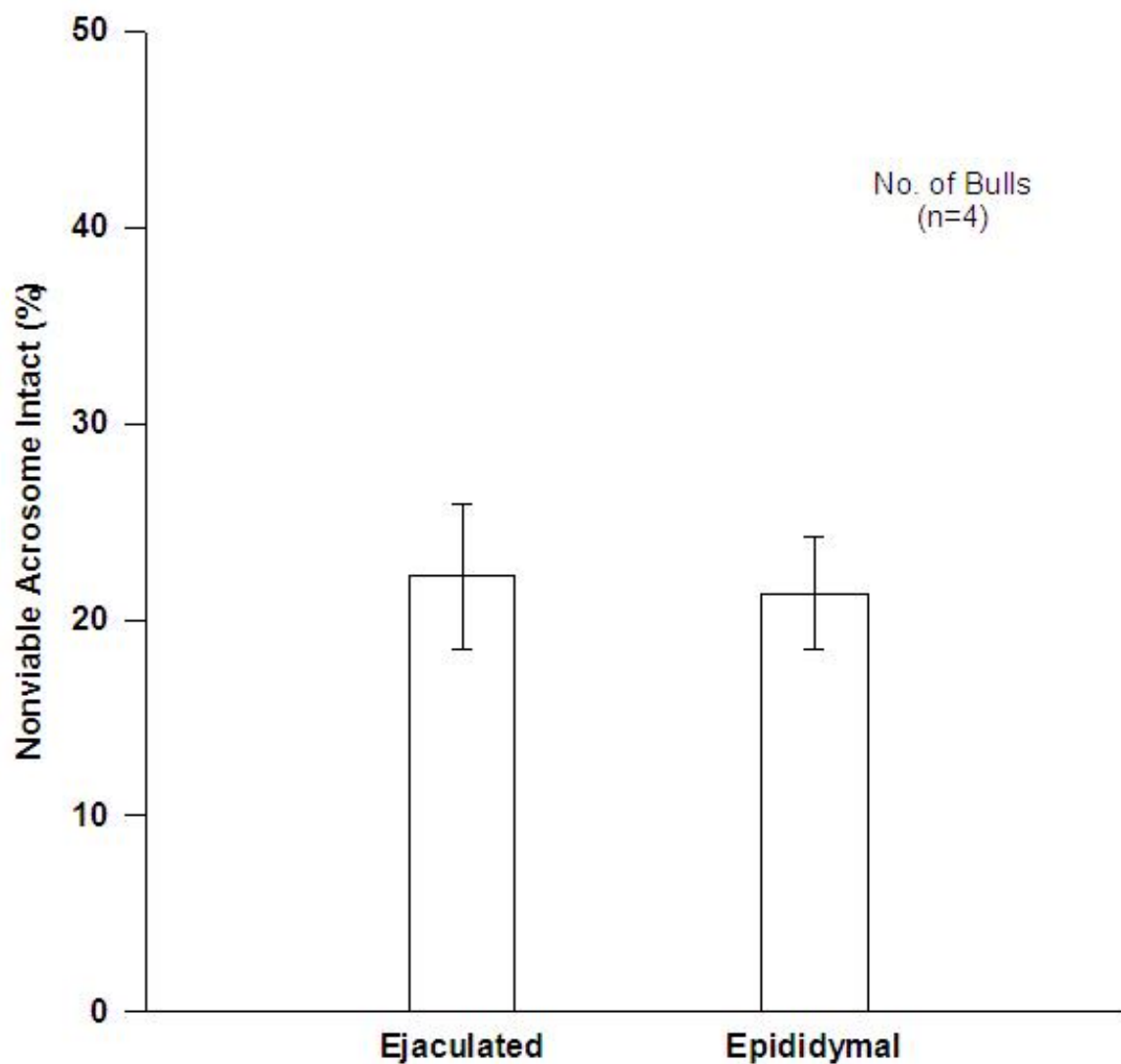


Figure 5.4. Percentage of nonviable acrosome-intact thawed cryopreserved ejaculated and epididymal bovine sperm (mean \pm SEM) collected from the same bulls. No difference was detected between ejaculated or epididymal sperm ($P=0.860$).

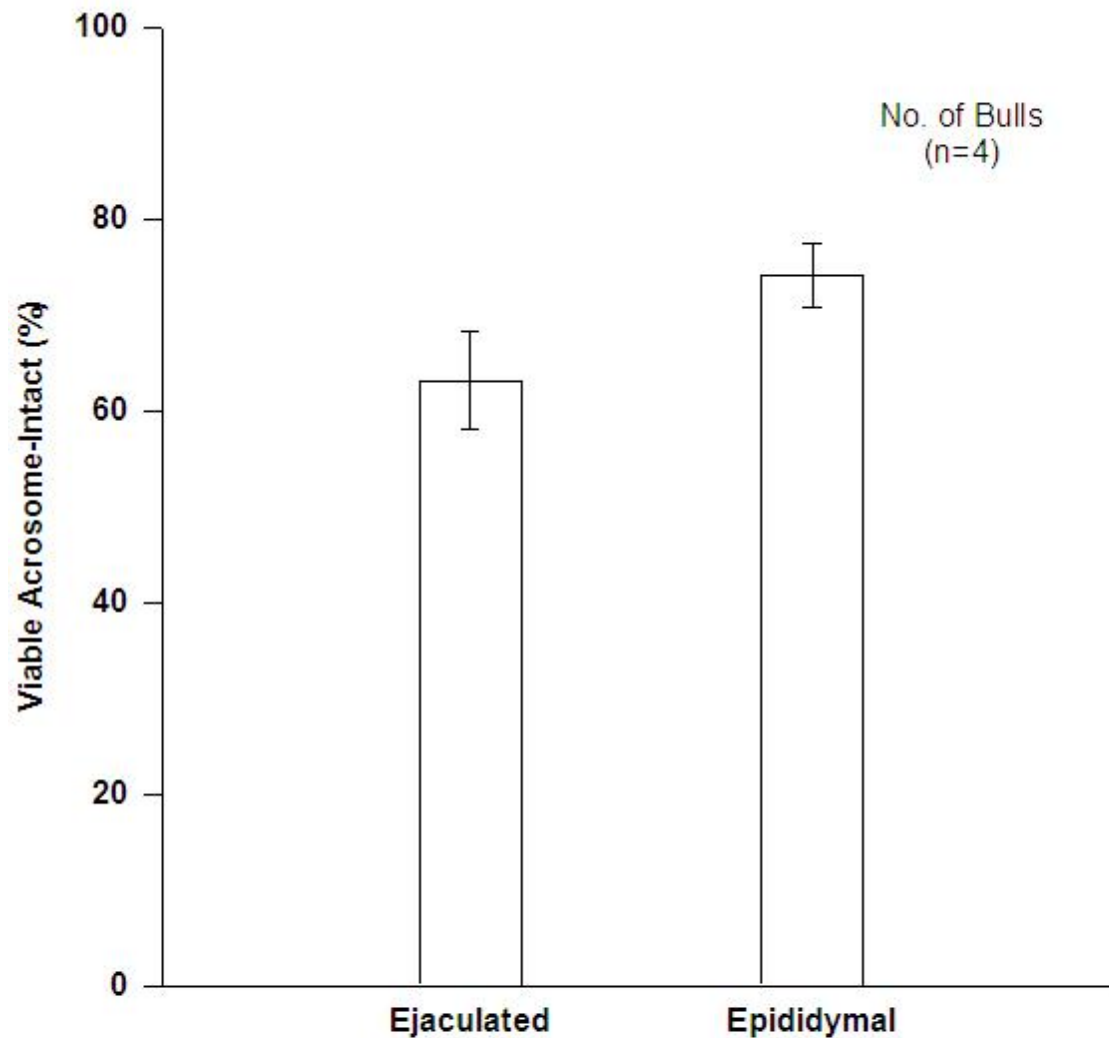


Figure 5.5. Percentage of viable acrosome-intact thawed cryopreserved ejaculated and epididymal bovine sperm collected from the same bulls. Sperm viability and acrosomal integrity were determined by staining with propidium iodide (PI) and fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) followed by evaluation with flow cytometer. No difference was noted between ejaculated and epididymal sperm ($P=0.094$).

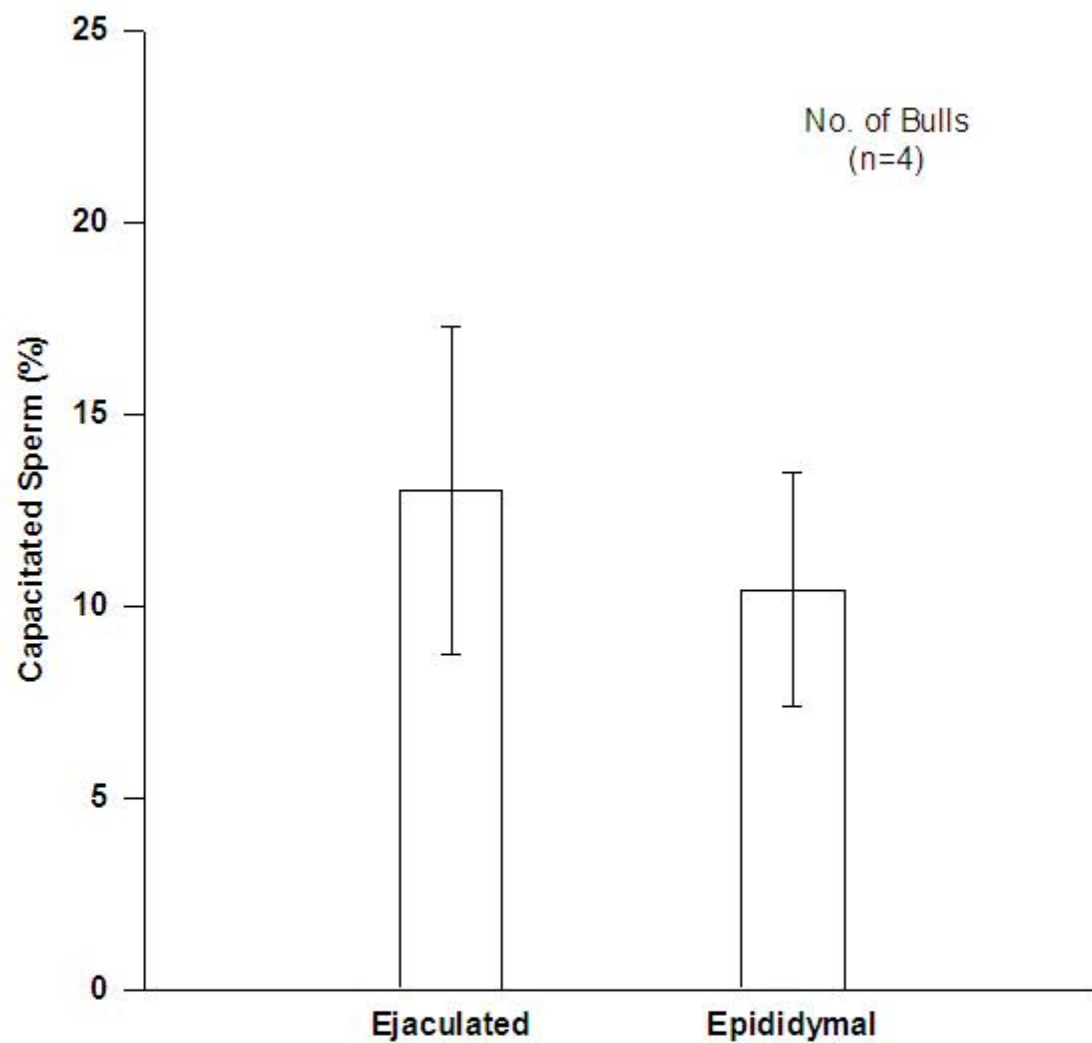


Figure 5.6. Percentage of capacitation post-thaw of live ejaculated and epididymal bovine sperm (mean \pm SEM) collected from the same bulls. Sperm viability and acrosomal status determined by propidium iodide (PI) and fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) fluorescent staining. Capacitated sperm were induced to acrosome react by exposure to lysophosphatidylcholine (LPC). No difference was detected between ejaculated and epididymal sperm ($P=0.628$).

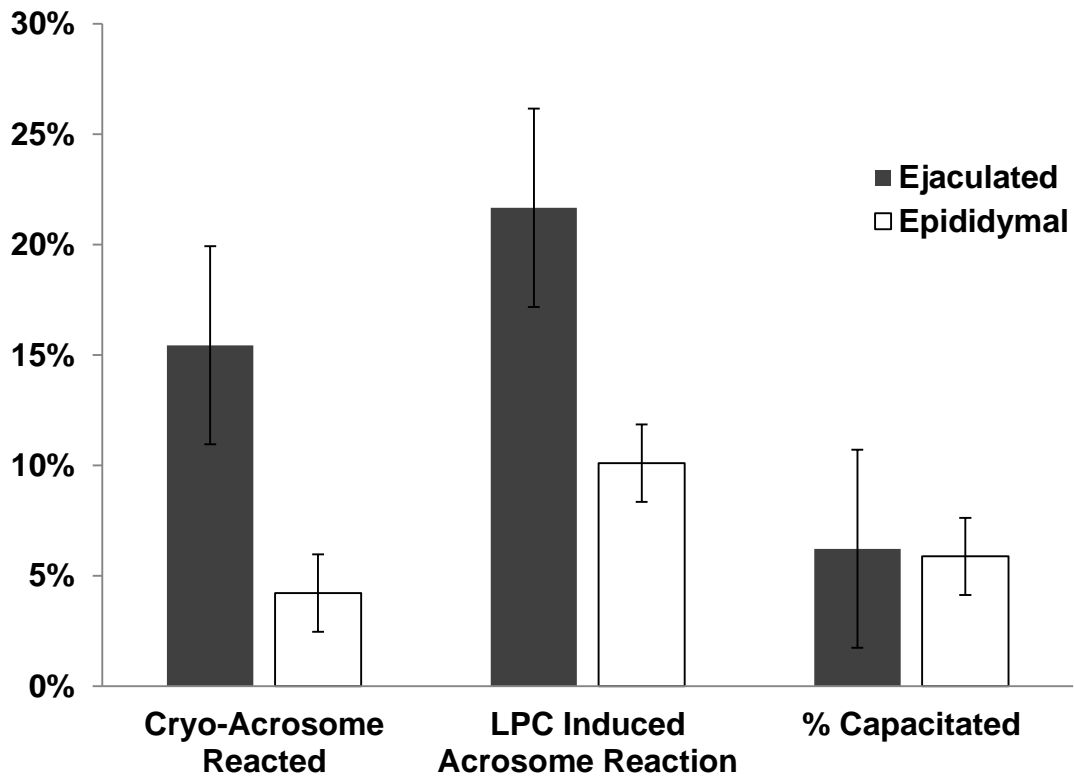


Figure 5.7. Effects of cryopreservation on ejaculated and epididymal sperm, measured by the percent of acrosome reacted and percent capacitation post-thaw. Ejaculated and epididymal sperm differed by percentage of acrosome reacted sperm post-thaw ($P < 0.001$). Percent capacitated was calculated by taking the difference between post-thaw acrosome reacted and lysophosphatidylcholine (LPC) induced acrosome reaction, resulted in no significant difference ($P = 0.628$) between ejaculated and epididymal sperm.

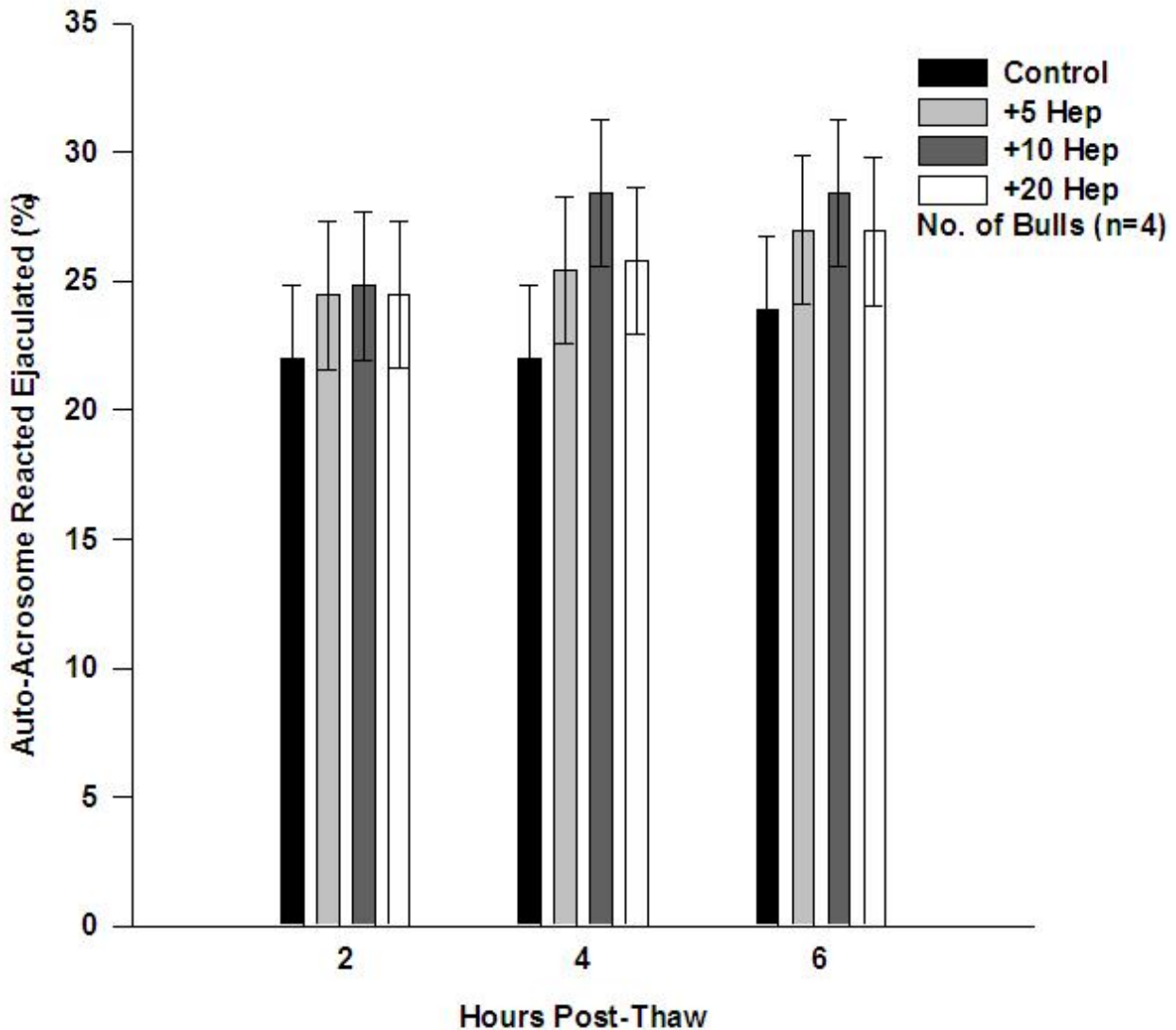


Figure 5.8. Effect of serial heparin concentration on the level of auto-acrosome reacted ejaculated bovine sperm (mean \pm SEM) during in vitro culture over time. Sperm viability and acrosomal status determined by staining with propidium iodide (PI) and fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) followed by evaluation with flow cytometer. No difference was detected between treatments per hour ($P=0.571$). Hep = heparin.

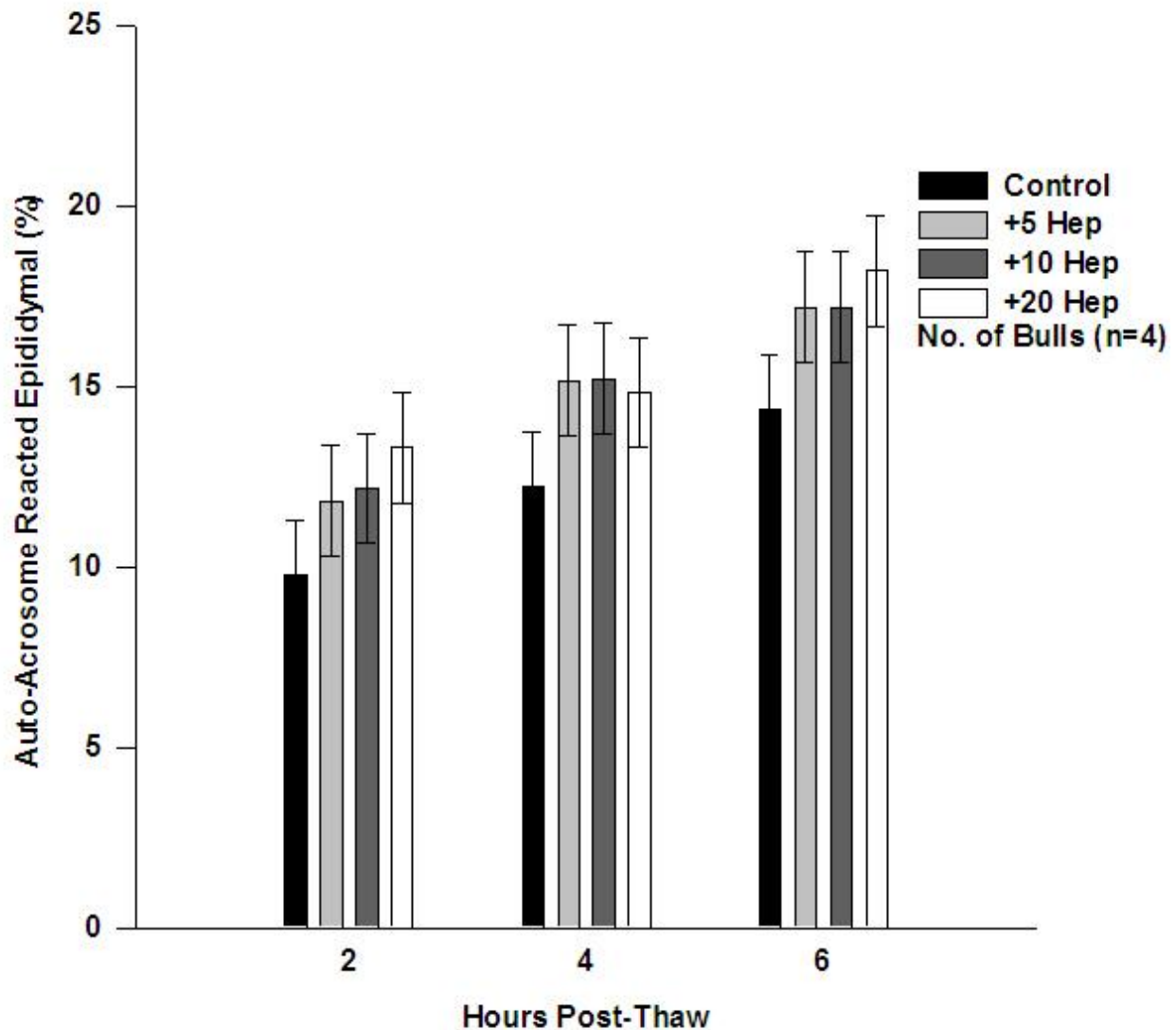


Figure 5.9. Effect of serial heparin concentration on the level of auto-acrosome reacted epididymal bovine sperm (mean \pm SEM) during in vitro culture over time. Sperm viability and acrosomal status determined by staining with propidium iodide (PI) and fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) followed by evaluation with flow cytometer. No difference was detected between treatments per hour ($P=0.128$). Hep = heparin.

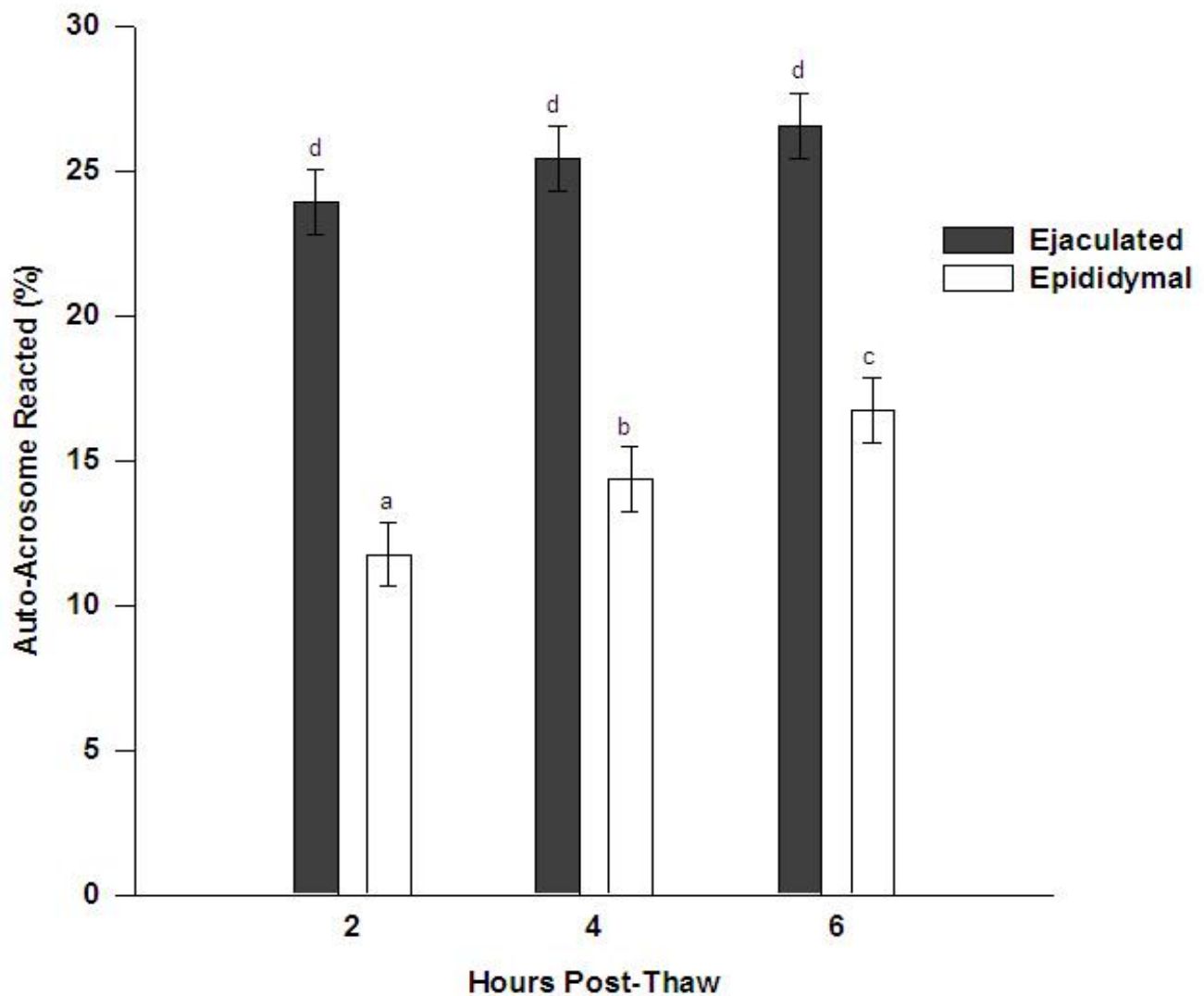


Figure 5.10. Percentage of auto-acrosome reacted cryopreserved ejaculated and epididymal bovine sperm (mean \pm SEM) during in vitro culture overtime. The percentage of auto-acrosome reacted ejaculated sperm was significantly greater than that of epididymal irrespective of time ($P < 0.001$). ^{a,b,c,d}Mean values with different letters are significantly different ($P > 0.05$).

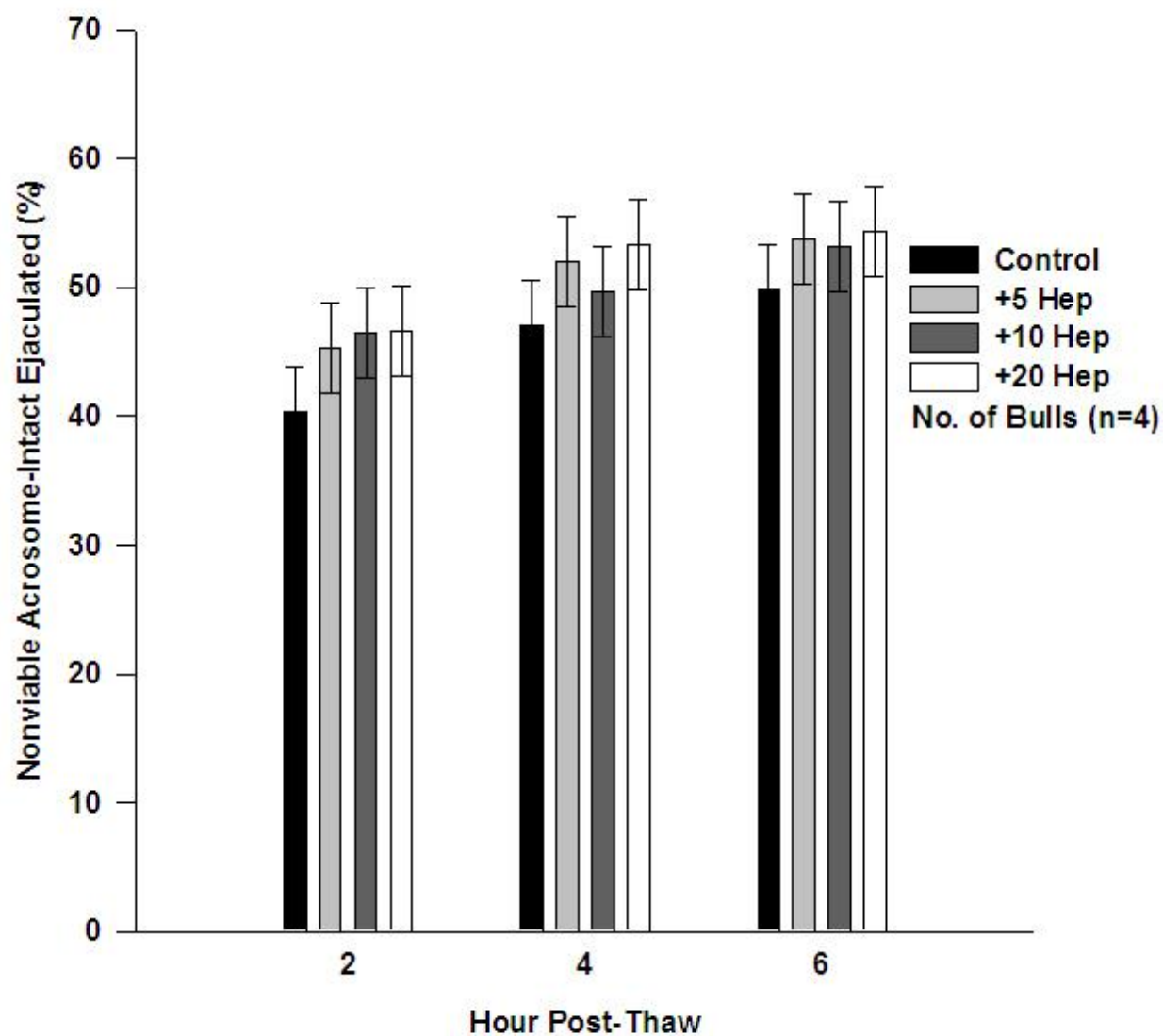


Figure 5.11. Effect of serial heparin concentration on the level of nonviable acrosome-intact ejaculated bovine sperm (mean \pm SEM) during in vitro culture over time. Sperm viability and acrosomal status determined by staining with propidium iodide (PI) and fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) followed by evaluation with flow cytometer. No difference was detected between treatments per hour ($P=0.590$). Hep = heparin.

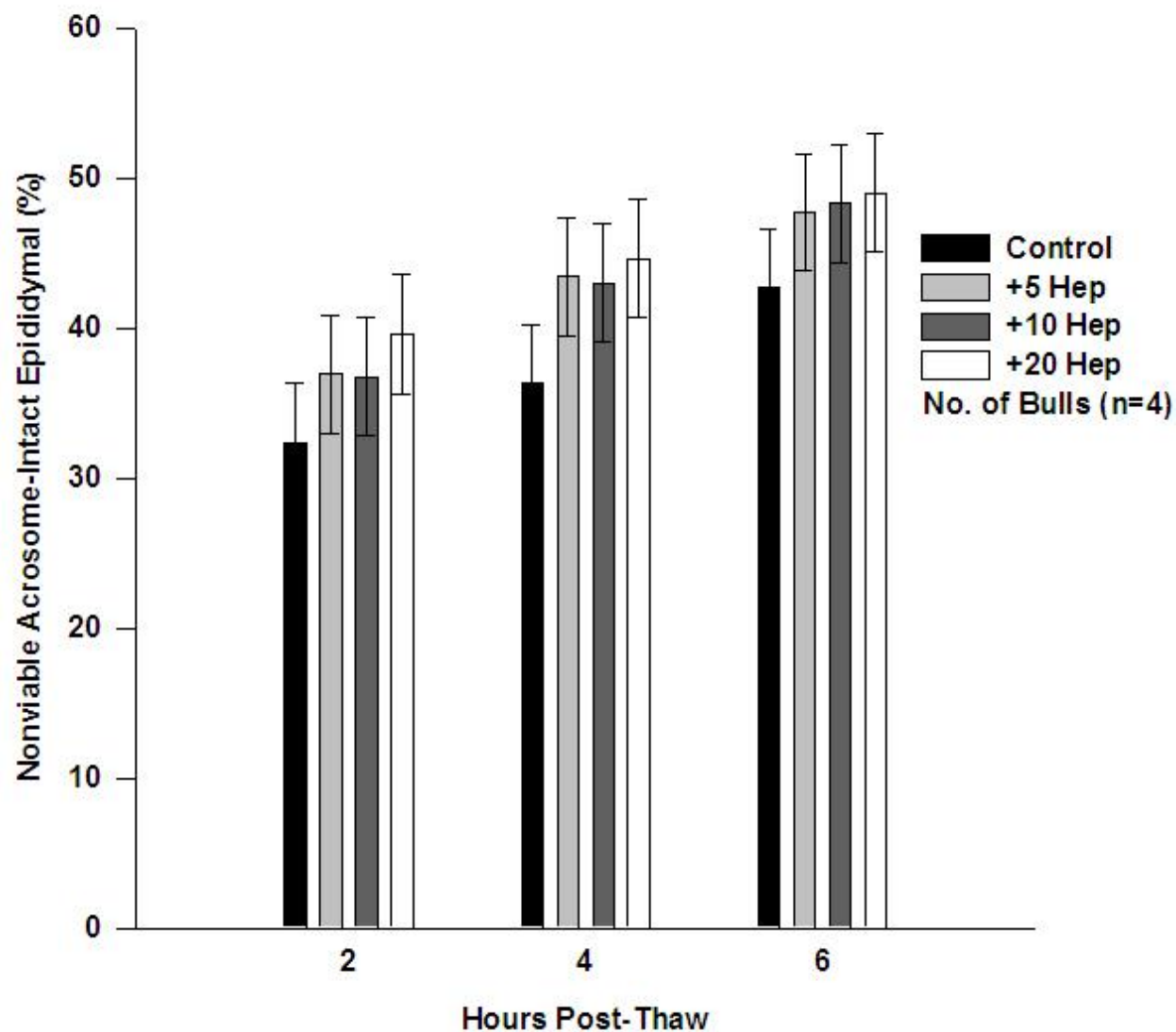


Figure 5.12. Effect of serial heparin concentration on the level of nonviable acrosome-intact epididymal bovine sperm (mean \pm SEM) during in vitro culture over time. Sperm viability and acrosomal status determined by staining with propidium iodide (PI) and fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) followed by evaluation with flow cytometer. No difference was detected between treatments per hour ($P=0.521$). Hep = heparin.

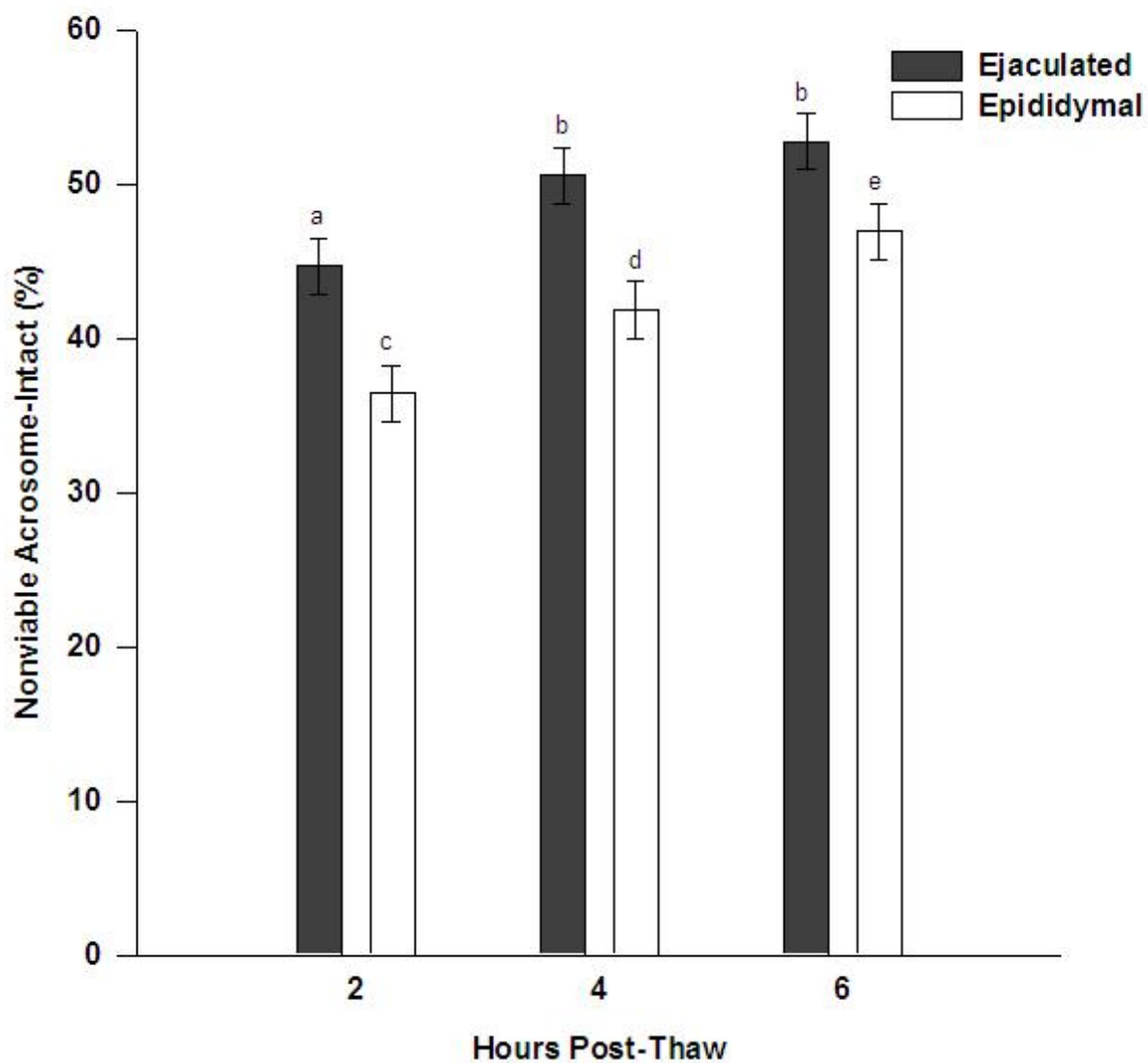


Figure 5.13. Percentage of nonviable acrosome-intact cryopreserved ejaculated and epididymal bovine sperm (mean \pm SEM) during in vitro culture over time. The percentage of nonviable acrosome-intact ejaculated sperm was significantly greater than that of epididymal irrespective of time ($P < 0.001$). ^{a,b}Mean values with different letters are significantly different ($P > 0.05$). ^{c,d,e}Mean values with different letters are significantly different ($P > 0.01$).

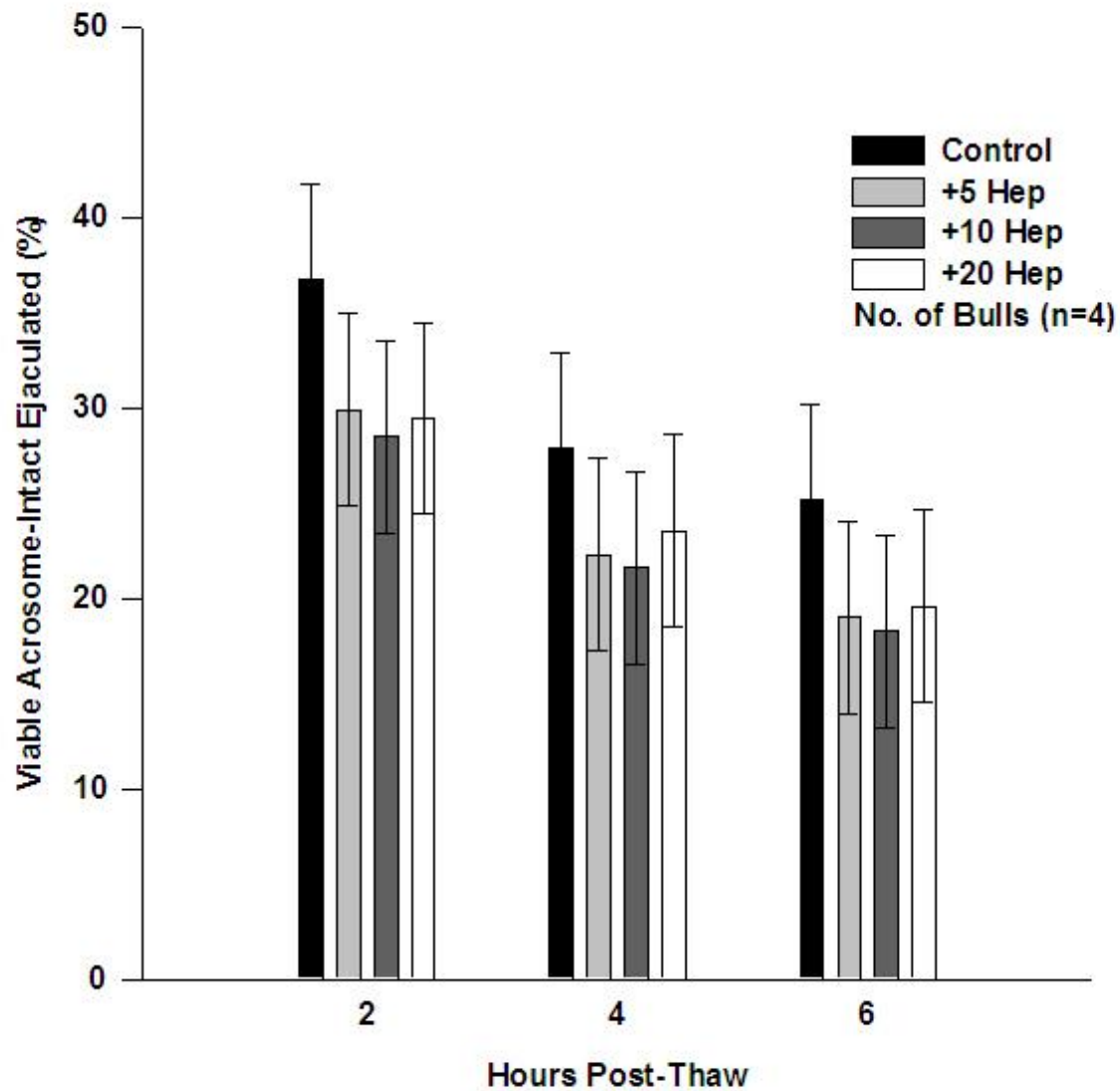


Figure 5.14. Effect of serial heparin concentration on the level of viable acrosome-intact ejaculated bovine sperm (mean \pm SEM) during in vitro culture over time. Sperm viability and acrosomal status determined by staining with propidium iodide (PI) and fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) followed by evaluation with flow cytometer. No significant difference was detected between treatments per hour ($P=0.385$). Hep = heparin.

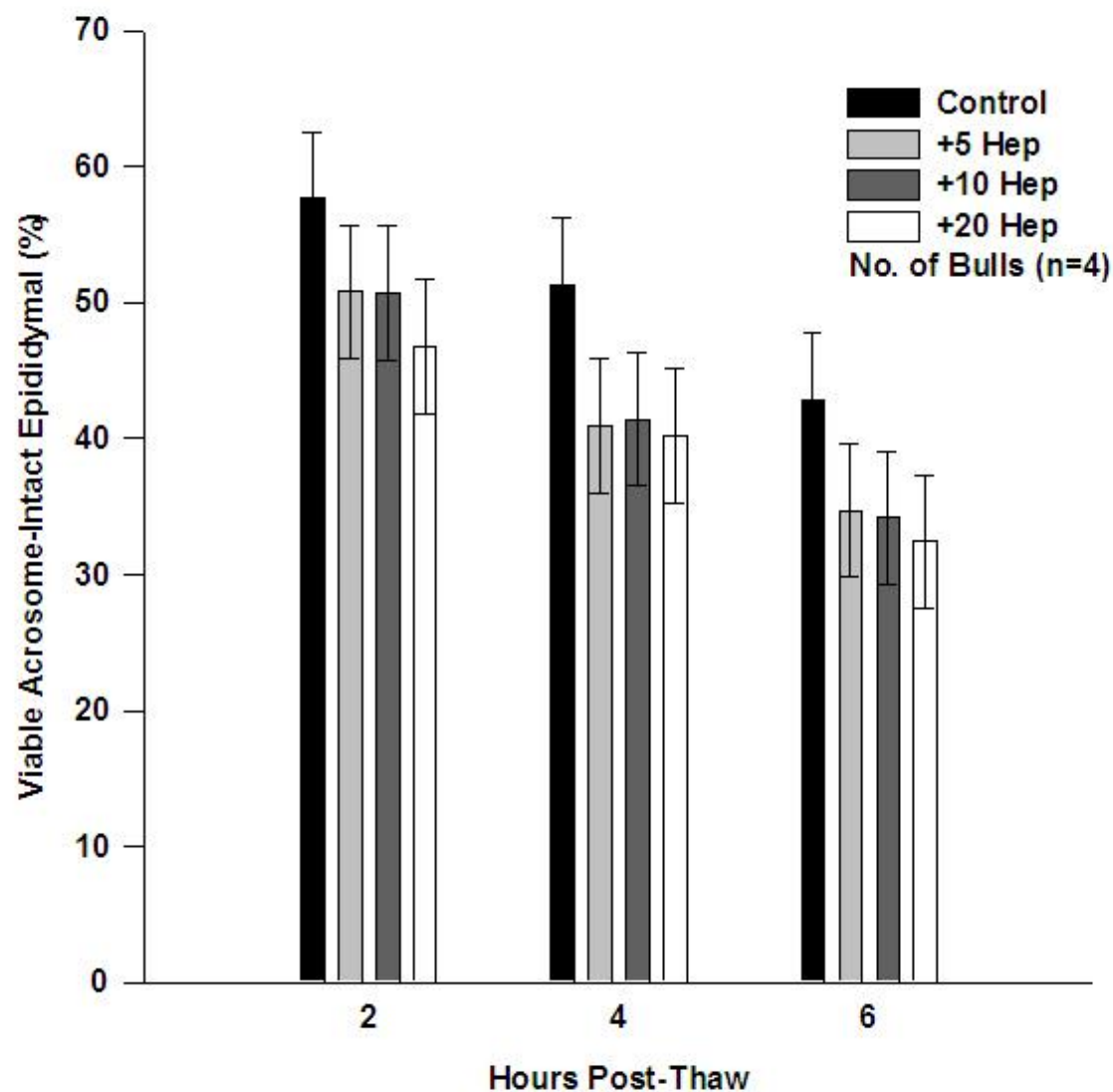


Figure 5.15. Effect of serial heparin concentration on the level of viable acrosome-intact epididymal bovine sperm (mean \pm SEM) during in vitro culture over time. Sperm viability and acrosomal status determined by staining with propidium iodide (PI) and fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) followed by evaluation with flow cytometer. No difference was detected between treatments per hour ($P=0.325$). Hep = Heparin.

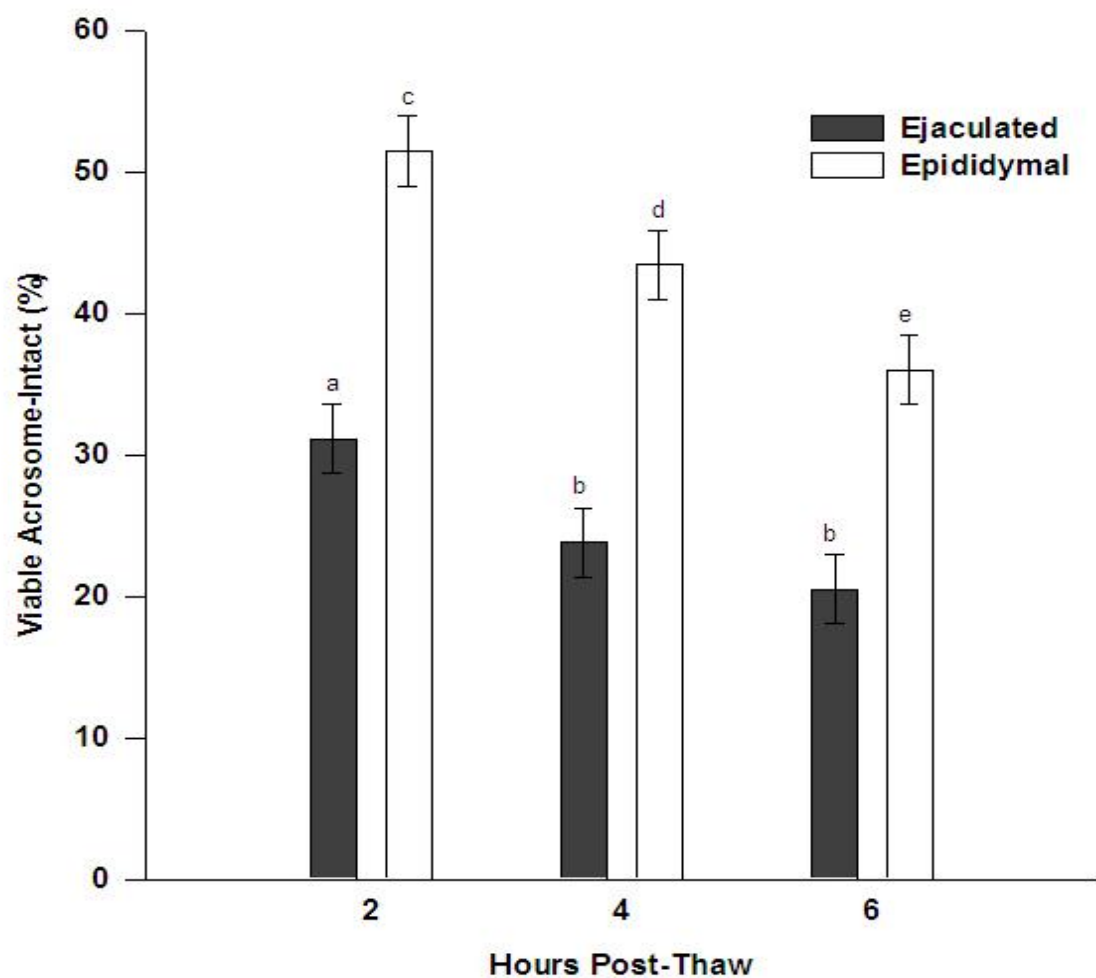


Figure 5.16. Percentage of viable acrosome-intact cryopreserved ejaculated and epididymal bovine sperm (mean \pm SEM) during in vitro culture overtime. The percentage of viable acrosome-intact epididymal sperm was significantly greater than that of ejaculated irrespective of time ($P<0.001$). ^{a,b}Mean values with different letters are significantly different ($P<0.01$). ^{c,d,e}Mean values with different letters are significantly different ($P<0.001$).

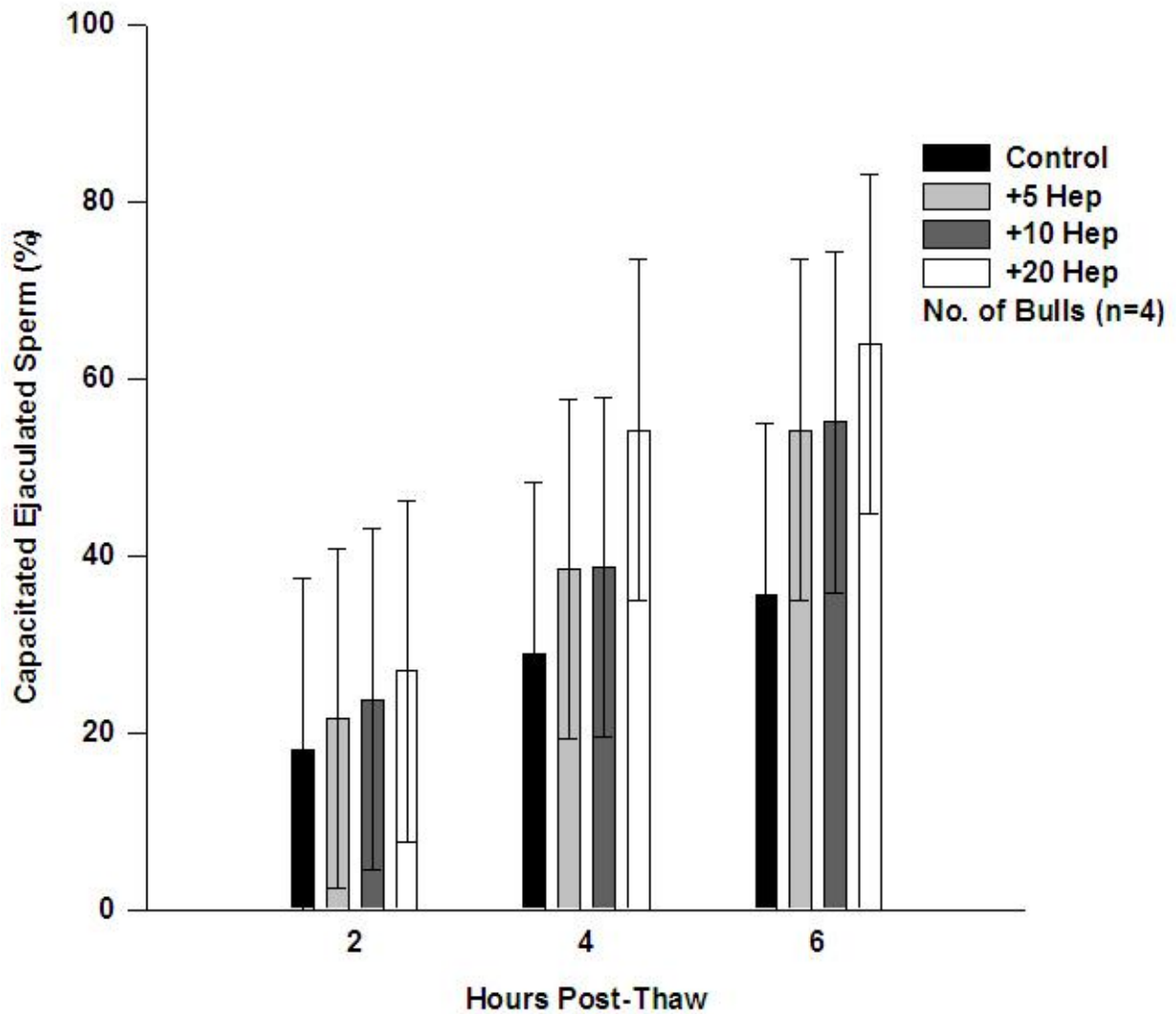


Figure 5.17. Effect of serial heparin concentration on the level of viable capacitated ejaculated bovine sperm (mean \pm SEM) during in vitro culture over time. Sperm viability and acrosomal status determined by staining with propidium iodide (PI) and fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) followed by evaluation with flow cytometer. No difference was detected between treatments per hour ($P=0.807$). Hep = heparin.

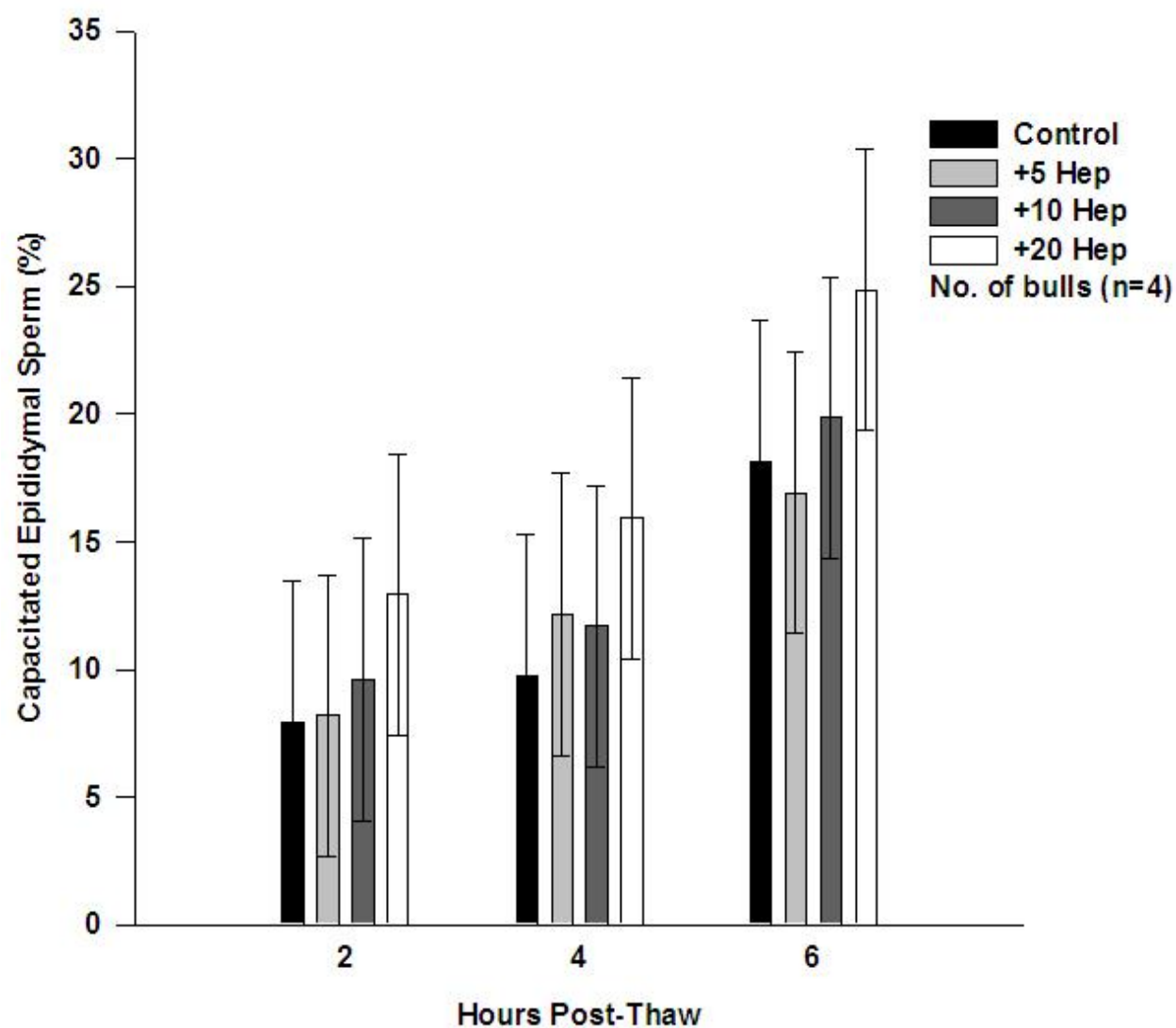


Figure 5.18. Effect of serial heparin concentration on the level of viable capacitated epididymal bovine sperm (mean \pm SEM) during in vitro culture over time. Sperm viability and acrosomal status determined by staining with propidium iodide (PI) and fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) followed by evaluation with flow cytometer. No difference was detected between treatments per hour ($P=0.723$). Hep = heparin.

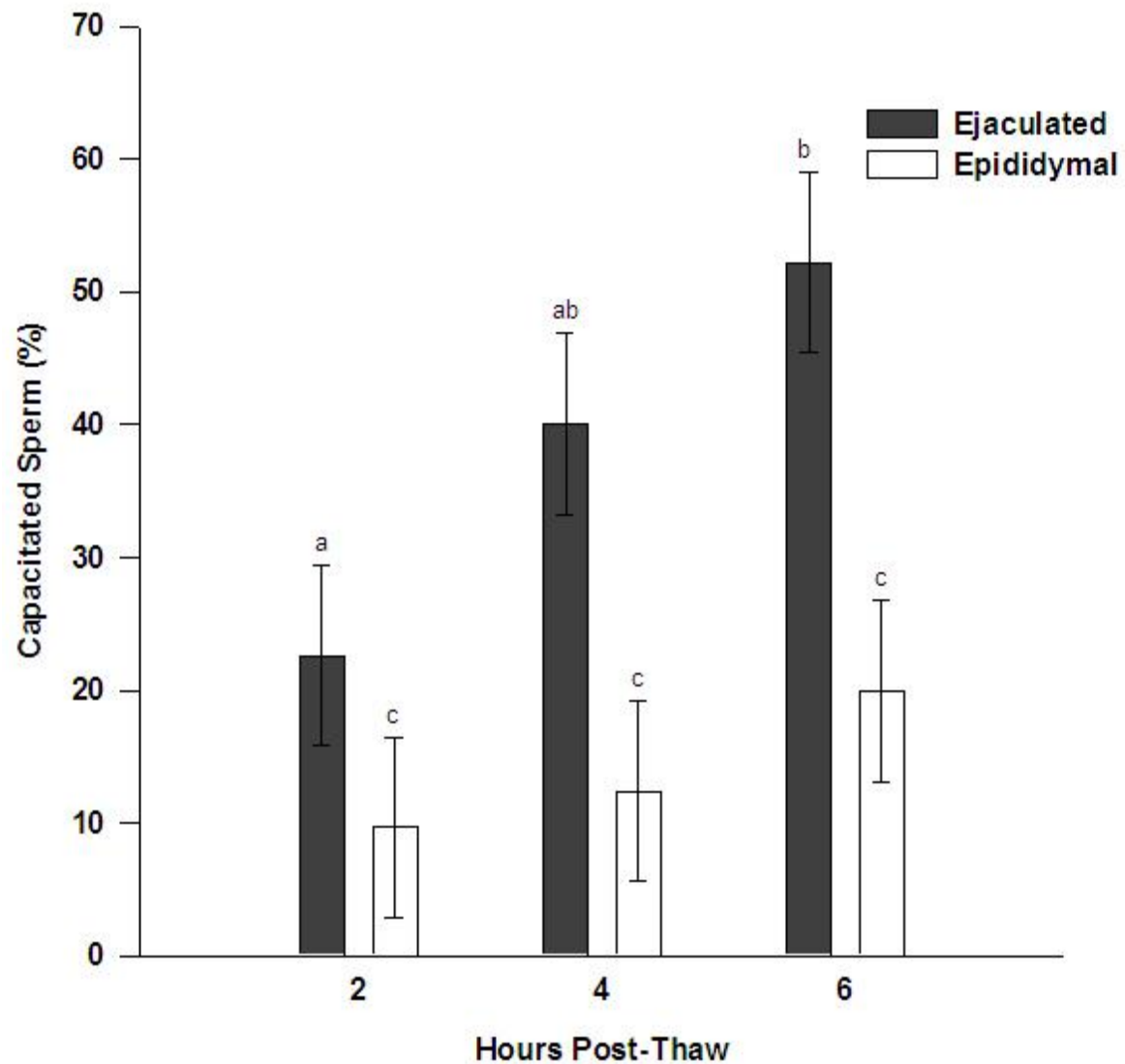


Figure 5.19. Percentage of capacitated viable cryopreserved ejaculated and epididymal bovine sperm (mean±SEM) post-thaw, collected from the same bulls. The percentage of capacitation for ejaculated sperm was significantly greater than that of epididymal ($P<0.001$). ^{a,b,c}Mean values with different letters are significantly different ($P<0.05$).

DISCUSSION

Genetically valuable males have the potential to significantly increase the production of agriculturally important species. These genetics can quickly be spread through the population with the use of cryopreserved sperm utilized in assisted reproduction techniques such as AI and IVF. A problem arises when these genetically valuable males become incapacitated due to injury or death. With the loss of the bull or the ability to collect sperm, it will be imperative to salvage the genetics of these bulls. Epididymal sperm are a viable source of sperm, in which genetics can be salvaged and will prove vital in the further dissemination of the genetics from these males (Barker, 1954; Martins et al., 2007). Epididymal sperm represents a “last chance”, making it of the utmost importance that this sperm be utilized in the most efficient manner possible.

Ejaculated sperm, unlike epididymal sperm, has been extensively studied and is routinely cryopreserved and utilized in assisted reproduction techniques. Cryopreserved bovine epididymal sperm was first utilized in artificial insemination by Barker (1954). Subsequent studies determined epididymal sperm to be comparable to ejaculated sperm when used for AI breeding (Igboeli and Foote, 1968; Amann and Griel, 1974; Foote, 2000a). The drawback to traditional AI is that 1 straw equals 1 calf at 100% efficiency. Realistically we can only expect ~65% pregnancy rates following AI (Demetrio et al., 2007; Schefers et al., 2010; Martinez et al., 2011). This leaves us with producing 1 calf for roughly every 2 straws of sperm. An alternative use for bovine epididymal sperm is IVF (Ball et al., 1983; Goto et al., 1989). Although, to utilize cryopreserved epididymal sperm in the most efficient manner possible we must first understand how it reacts comparable to ejaculated sperm in an in vitro environment. These experiments were conducted to better understand the difference between the 2-sources of sperm in an attempt to better utilize this finite resource.

Cryopreserved ejaculated and epididymal sperm collected from the same mature Holstein bulls were first compared for their viability, acrosomal integrity and capacitation post-thaw. The ability to endure cryopreservation dictates the amount of usable sperm post-thaw and may influence the concentration of sperm that is necessary per straw to achieve sufficient fertilization rates. Sperm from species with higher sperm membrane cholesterol to phospholipid ratios (human or rabbit) and in sperm whose membrane

cholesterol concentrations were artificially increased are better able to endure cryopreservation (Purdy and Graham, 2004a; Mocé and Graham, 2006; Amorim et al., 2009; Moraes et al., 2010). Cholesterol loaded cyclodextrins (CLCs) are able to insert cholesterol directly into sperm plasma membrane (Purdy and Graham, 2004a). Concentration of cholesterol transferred to sperm increases linearly with the concentration of CLCs incubated with sperm (Purdy and Graham, 2004a). The ratio of cholesterol to phospholipids in cold shock sensitive bull sperm (0.45) can be brought to the concentration of highly cold shock resistant species such as the rabbit (0.88) or human (0.99) (Darin-Bennett and White, 1977; Purdy and Graham, 2004a).

Epididymal sperm having not been exposed to seminal plasma, may have higher concentrations of cholesterol in their membranes. This was noted by exposing bull epididymal sperm to BSP proteins for 4 hours resulting in a ~25% reduction of membrane cholesterol concentrations (Moreau and Manjunath, 2000). Therefore, we examined whether epididymal sperm would better endure cryopreservation compared with ejaculated sperm collected from the same Holstein bulls.

Our results indicated that ejaculated sperm had increased percentages of acrosome reacted sperm post-thaw. Although following exposure of sperm to LPC, we found no difference in the percentage of capacitation between ejaculated or epididymal sperm. These results are similar to previous reports.

Way et al. (2000) compared fresh epididymal sperm incubated with or without seminal plasma collected from the same 6-Holstein bulls. Results indicated that the inclusion of seminal plasma decreased the amount of live acrosomal intact sperm.

Varisli et al. (2009) compared the acrosomal integrity of ejaculated and epididymal ram sperm as to their susceptibility to cryobiologically relevant stressors and found epididymal sperm more resilient. When exposed to hypo-osmotic (75 mOsm) stress, ram epididymal sperm was unaffected; while ejaculated sperm had a 30% loss of acrosomal integrity. Acrosomal integrity of ram epididymal sperm was also unaffected by chilling stress but the acrosomal integrity of ejaculated sperm was reduced ~45%.

Heise et al. (2011) collected sperm from the same 4 stallions and reported no difference in the percentage of viable acrosome intact sperm noted between epididymal sperm (60%) or ejaculated sperm (64%) following cryopreservation.

In the goat, acrosomal integrity of fresh and cryopreserved goat ejaculated (95% and 89%) and epididymal (100% and 84%) sperm has been noted as being similar (Blash et al., 2000).

We also wanted to determine the difference between ejaculated and epididymal sperm collected from the same bulls during in vitro culture and the effects of heparin. Due to the lack of exposure of epididymal sperm to seminal plasma, which adds factors such as BSP proteins that function as heparin binding sites and in the removal of cholesterol from sperm membranes, we compared ejaculated and epididymal sperm as to their viability and ability to become capacitated during in vitro culture and the effects of serial heparin concentration.

Ejaculated and epididymal sperm were compared as to their viability, acrosomal integrity and capacitation during a 6 hour in vitro culture and the effects of serial heparin concentration. During in vitro fertilization, sperm and oocytes are co-incubated in a medium that provides the necessary factors for sperm function and also facilitates capacitation and fertilization. One of these factors added to the fertilization medium is heparin. Heparin binds to sperm cells by way of BSP proteins (Therrien et al., 1995). BSP proteins are a component of the seminal plasma that is added during ejaculation. Epididymal sperm having not been exposed to seminal plasma have been noted as having fewer heparin binding sites (Handrow et al., 1984; Miller et al., 1990). This lack of heparin binding sites may alter the ability of epididymal sperm to become capacitated. When comparing the percentage of capacitation between ejaculated and epididymal sperm, it was noted that epididymal sperm displayed significantly lower percentages of capacitation and that epididymal sperm did not significantly increase over time (Figure 5.19). These results were concerning considering sperm must become capacitated in order to fertilize oocytes.

BSP proteins also function in the removal of cholesterol from the sperm plasma membrane. Manjunath and Thérien (2002) reported the removal of 7% to 15% of cholesterol within 15- to 30-minutes of exposure to seminal plasma and a 25% loss after 4 hours of incubation of bovine epididymal sperm with seminal plasma. Moreau et al. (1998) also noted an increase in cholesterol efflux from bovine epididymal sperm when incubated with BSP-A1/A2 (17.46%) compared with without BSP-A1/A2 (6.5%). A 3.7

fold increase in cholesterol loss has also been noted when fibroblast cells were incubated with BSP proteins compared with controls (Moreau et al., 1999). The time required for sperm to capacitate may be influenced by their cholesterol to phospholipid ratio. With species having higher cholesterol to phospholipid ratios such as the human (0.99) and rabbit (0.88) taking longer to capacitate than species with lower cholesterol to phospholipid ratios such as the boar (0.26) and ram (0.38). In a report by Davis (1981) it was noted that the time required for sperm to capacitate closely followed their cholesterol to phospholipid ratios; ram (1.5 hours, 0.34), boar (2 hours, 0.35), bull (3 hours, 0.45), rat (3.75 hours, 0.58), rabbit (6 hours, 0.88) and man (7 hours, 0.99), respectively. The lack of capacitation noted in epididymal sperm may be due to increased cholesterol to phospholipid ratio. If we compare the time required for epididymal bull sperm to increase in the percentage of capacitation (>6 hours) to that of the rabbit (6 hours) or man (7 hours) then it may be plausible that bull epididymal sperm may have a cholesterol to phospholipid ratio closer to that of the rabbit.

Sperm viability and acrosomal integrity are also of great importance as nonviable or acrosome reacted sperm are useless in an IVF program. Upon comparing epididymal and ejaculated sperm collected from the same mature Holstein bulls, we found that the percentage of viable acrosome intact sperm, as seen in the previous study, was significantly higher for epididymal sperm during in vitro culture.

In conclusion, epididymal sperm from an endangered or genetically superior male is a valuable and finite resource that should be utilized in the most efficient manner possible. In our studies, we note that epididymal sperm are better able to endure cryopreservation with higher percentages of viable acrosome intact sperm compared with ejaculated sperm collected from the same Holstein bulls. However, we also note epididymal sperm did not capacitate at the same rate as ejaculated sperm this may interfere in the fertilization process during IVF. Further studies comparing epididymal and ejaculated sperm as to their ability to fertilize oocytes in vitro will give a better understanding of the effects of this apparent lack of capacitation.

CHAPTER VI

EFFECTS OF PREIMPLANTATION FACTOR (PIF) ON BOVINE CRYOPRESERVED SPERM

INTRODUCTION

Epididymal sperm undergo significant modification from the time they reach maturity and are stored in the cauda epididymides compared with when they are fully capacitated, hyperactivated and undergoing the acrosome reaction in the process of fertilizing an oocyte. These changes to epididymal sperm are brought about by exposure to various factors found in the seminal plasma and female reproductive tract. Some of the various factors that have been shown to affect sperm include growth factors, peptides, proteins and small molecules. These growth factors and peptides include Epidermal Growth Factor (EGF), Insulin-like Growth Factor-1 (IGF-1), Insulin-like Growth Factor-2 (IGF-2), Nerve Growth Factor (NGF), Fertilization-promoting Peptide (FPP), calcitonin, Angiotensin-II (A-II) and relaxin.

Exposure of sperm to these growth factors and peptides has been associated with an increase in viability, stimulating motility, inducing capacitation and the acrosome reaction. Growth factors such as EGF have been shown to stimulate the acrosome reaction in bull (Lax et al., 1994; Daniel et al., 2010), mouse (Murase and Roldan, 1995) and boar (Oliva-Hernández and Pérez-Gutiérrez, 2008). IGF-1 and IGF-2 have been shown to maintain motility of ejaculated bull (Henricks et al., 1998) and Surti buffalo sperm (Selvaraju et al., 2009). IGF-1 also increases the viability of cool stored (15°C for 72 hours) boar sperm (Silva et al., 2011). Increased viability was also reported with the addition of NGF to ejaculated bull sperm (Li et al., 2010). Peptides such as FPP, calcitonin, A-II and relaxin have been proposed to be involved in regulating capacitation (Fraser et al., 2001; Vadnais et al., 2007; Miah et al., 2011).

These factors bind to various receptors on the sperm plasma membrane. Epidermal Growth Factor Receptor (EGFR) has been located on the acrosome of bull sperm. Activation of the EGFR is associated with Protein Kinase A (PKA), the SRC tyrosine kinase family and the inhibition of Na/K-ATPase (Daniel et al., 2010). With the activation of EGFR and the binding of EGF, an increase in internal Ca^{2+} concentration is noted leading to the acrosome reaction. IGF-1 receptors (IGF-1R) are transmembrane

tyrosine kinases and have been located on the acrosome of bull sperm. In addition, the Nerve Growth Factor Receptor (TrkA) was also localized to the acrosomal cap, nucleus and tail of ejaculated bull sperm. The TrkA receptor has also been identified as a tyrosine kinase (Kaplan et al., 1991).

Fertilization-promoting Peptide, calcitonin and A-II bind to sperm plasma membrane receptors and have been proposed to be involved in regulating capacitation by way of the membrane associated adenylyl cyclase (mAC)/cAMP pathway (Fraser et al., 2001; Vadnais et al., 2007). Relaxin, also binds to receptors on the bull sperm plasma membrane resulting in a stimulation of a signal transduction pathway. Relaxin accelerates the cholesterol efflux and increases the Ca^{2+} -influx, which stimulates the production of cAMP by way of a soluble adenylyl cyclase and protein tyrosine phosphorylation leading to capacitation (Miah et al., 2011). These examples display the cross talk between sperm and their environment and demonstrate the ability of small peptides to stimulate physiological processes within sperm cells.

Recently, it has been shown that preimplantation factor (PIF), a small peptide (15 amino acids), is expressed from viable bovine embryos during in vitro culture (Stamatkin et al., 2011a). PIF has also been reported to be secreted by viable human and mouse embryos (Roussev et al., 1996b; Stamatkin et al., 2011a). It is believed that PIF functions as a signal from the embryo to the dam preventing the immune system from aborting the embryo. Bovine embryos, cultured in vitro, have been reported to both express and take up PIF (Stamatkin et al., 2011a). In addition, it was reported that adding synthetic PIF to the culture medium, has a beneficial effect on embryo development (Stamatkin et al., 2011b).

Beneficial effects of PIF have also been demonstrated in various other systems, including the prevention of Type-1 diabetes mellitus and also in reversing nerve damage and promoting neural repair (Weiss et al., 2011b; Weiss et al., 2011c). Genomic and proteomic investigation of the effects of PIF revealed that PIF affected the many signaling pathways that influence the immune, adhesion and apoptosis processes (Paidas et al., 2010).

PIF has been shown to be beneficial to the embryo and to other systems, although its effects on sperm have yet to be investigated. In this study, we examine the

effect of PIF on cryopreserved bull sperm viability, and its ability to induce or inhibit capacitation and the auto-acrosome reaction during in vitro culture over time. PIF was also tested as to its effect on in vitro fertilization and subsequent embryo development. If PIF is able to influence sperm signaling, improving sperm viability, ability to capacitate or acrosome react, it may prove useful in the use of ejaculated and epididymal sperm in assisted reproductive techniques.

MATERIALS AND METHODS

Experimental Design

Experiment 6.1

This experiment was developed to examine the effect of PIF compared with heparin in its ability to induce or inhibit capacitation of cryopreserved sperm during in vitro culture. Cryopreserved ejaculated sperm from 9 fertile Holstein bulls (4 bulls from previous studies plus 5 obtained from Genex Custom Collection, LA) were evaluated in this study. Capacitation of ejaculated cryopreserved sperm was determined by staining with chlortetracycline. Sperm capacitation experiments were setup in factorial design arrangements with ejaculated sperm incubated in capacitating medium in 1 of 4 treatments for 0, 2, 4 and 6 hours. Treatment 1 (Control): Capacitation medium alone, Treatment 2 Preimplantation factor (PIF): addition of PIF (100 nM/ml), Treatment 3 (Heparin): addition of heparin (10 µg/ml), Treatment 4 (PIF + Heparin): addition of PIF and heparin (100 nM/ml and 10 µg/ml, respectively). The entire experiment was conducted in duplicate for each bull. Differences were determined from 18 replicates per treatment per hour.

Experiment 6.2

This experiment was designed to examine the effects of PIF compared with heparin on sperm viability and its ability to induce or inhibit the acrosome reaction or capacitation of cryopreserved ejaculated sperm during 6 hours of in vitro culture. Duplicate samples of cryopreserved ejaculated sperm obtained from 7 selected Holstein bulls (4 bulls from previous studies plus 3 bulls obtained from Genex Custom Collection, LA) were evaluated in this study. Acrosomal integrity and viability was determined by staining with FITC-PNA and Pi, respectively. Capacitation was determined by acrosome reacting, capacitated sperm with lysophosphatidylcholine. This experiment was setup in

factorial designs with ejaculated sperm being incubated in capacitating medium in 1 of 4 treatments for 0, 2, 4 and 6 hours. Treatment 1 (Control): Capacitation medium alone, Treatment 2 Preimplantation factor (PIF): addition of PIF (100 nM/ml), Treatment 3 (Heparin): addition of heparin (10 µg/ml), Treatment 4 (PIF + Heparin): addition of PIF and heparin (100 nM/ml and 10 µg/ml, respectively). Differences in viability, acrosomal integrity and capacitation were determined from 14 replicates per treatment per hour.

Experiment 6.3

This experiment was designed to examine the effects of PIF compared with heparin on embryo cleavage and subsequent development following in vitro fertilization with or without the addition of PIF and or heparin. The IVF experiment conducted in 6 replicates setup in a factorial design. Oocytes (n=580) and cryopreserved ejaculated sperm from a Holstein bull of known fertility were randomly assigned to a fertilization medium in 1 of 4 treatments; Treatment 1 (Control): Capacitation medium alone, Treatment 2 Preimplantation factor (PIF): addition of PIF (100 nM/ml), Treatment 3 (Heparin): addition of heparin (10 µg/ml), Treatment 4 (PIF + Heparin): addition of PIF and heparin (100 nM/ml and 10 µg/ml, respectively). Following IVF, embryos were cultured in the same medium to assess the effect of PIF on embryo development.

Experimental Procedure

Sperm preparation

Sperm cryopreserved in egg-yolk glycerol extender was removed from liquid nitrogen and thawed at 37°C for 40 seconds. Thawed sperm samples were then diluted in 500 µl of 2.9% sodium citrate buffer. Diluted sperm were then carefully layered over a discontinuous Percoll density gradient and centrifuged at 300 x g for 25 minutes to isolate a viable sperm population and remove egg yolk from the sample (Parrish et al., 1995). Following isolation, sperm was reconstituted in a modified Tyrodes glucose-free capacitating medium (Parrish et al., 1988) at a concentration of 10×10^6 /ml, divided into treatment groups and incubated in humidified 5% CO₂ air at 39°C for 0, 2, 4 and 6 hours.

Chlortetracycline assay

Once sperm incubated in their respective treatments for the designated time period (0, 2, 4, 6 hours), a sample was taken from each treatment group and stained

with chlortetracycline (CTC). The CTC assay was modified from (Collin et al., 2000) and carried out as follows, 100 μ l of sperm was taken from each treatment group and added to 150 μ l of CTC stock staining solution, which had been prepared the day of use and wrapped in foil and stored 4°C until use. Sperm were then fixed by adding 3 μ l of 12.5% glutaraldehyde in a 2.5 M Tris base. Ten microliters of stained sperm were placed on a slide, covered with a coverslip and stored in the dark at 4°C until evaluation. A total of 200 sperm were evaluated per slide as to their CTC staining pattern (Collin et al., 2000). Sperm in which their entire head fluoresced were considered decapacitated (F pattern). Sperm with a fluorescent acrosomal region and fluorescent free post-acrosomal region were considered capacitated (B pattern) and sperm with dull fluorescents over the entire head except for a thin bright band across the equatorial segment were considered acrosome reacted (AR pattern).

Flow cytometry assay

Following incubation, 2 samples of sperm were collected, 100 μ l each, from each treatment group and each added to 900 μ l of a 2.9% sodium citrate in preparation for staining and evaluation by flow cytometer (Accuri Cytometers Inc., Ann Arbor, MI). The percentage of auto-acrosome reacted and viable sperm were determined in the first sample by staining with fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA, 5 μ g/ml) and propidium iodide (PI, 2.4mM), respectively for 5 minutes, followed by evaluation with flow cytometry. The second sample was exposed to 10 μ g/ml of lysophosphatidylcholine (LPC) and incubated in the dark for 15 minutes to induce the acrosome reaction in capacitated sperm (Parrish et al., 1988). Following incubation with LPC, the second sample's percentage of acrosome reaction was determined by staining with FITC-PNA and PI and evaluated by flow cytometry. Capacitation was then determined by calculating the difference between the auto- and LPC-induced-acrosome reacted groups as percentage of total cells or total live cells to determine the percent total or live capacitated sperm.

In vitro fertilization

Bovine oocytes, obtained from a commercial supplier (Trans Ova Genetics, MN 56347), were matured in groups of 50 in a portable incubator at 39°C during overnight travel to the laboratory. Upon arrival to the LSU Embryo Biotechnology Laboratory,

shipping vials containing the oocytes were removed from the portable incubator and stored in a 5% CO₂ incubator in humidified air at 39°C until use.

Following 22 to 24 hours of in vitro maturation, the cumulus oocyte complexes (COCs) were washed 4 times through a modified glucose-free HEPES-TALP medium (Parrish et al., 1988), followed by washing 4 times through modified glucose-free IVF-TALP medium (Parrish et al., 1988). Cumulus oocyte complexes were then randomly assigned to a treatment group (25 oocytes/treatment). Oocytes in treatment medium (46 µl) were pipetted under mineral oil and 2 µl of PHE (Miller et al., 1994) and 2 µl of ejaculated sperm (final concentration of 1 x10⁶ sperm/ml) were added. The fertilization drops were placed into a modular incubator (Billups-Rothenberg, Del Mar, CA) with humidified 5% CO₂, 5% O₂, 90% N blood gas at 39°C and incubated for 18 hours.

Embryo culture

Following IVF, presumptive embryos were removed from fertilization medium and their remaining cumulous cells and accessory sperm removed by repeated pipetting through a 135 µm inside diameter pipette tip (Striper[®], Origio MidAtlantic Devices Inc. Mt. Laurel, NJ), Embryos were then added to a modified CR1aa culture medium (Rosenkrans et al., 1993; Rosenkrans and First, 1994) and placed in a modular incubator and incubated in humidified 5% CO₂, 5% O₂, 90% N blood gas at 39°C for 3 days. On the third day of culture, embryo development was assessed and embryos were transferred to a modified CR1aa plus 5% calf serum (Rosenkrans et al., 1993; Rosenkrans and First, 1994) culture medium and incubated in humidified 5% CO₂, 5% O₂, 90% N blood gas at 39°C until day 7. Development to the morula and blastocyst stages were determined on day 7 of embryo culture.

Definition of sperm status

Auto-acrosome reacted refers to a sperm cell that has undergone the acrosome reaction without an external stimulus. In the bull, sperm that have lost their acrosome are unable to bind to or penetrate the zona pellucida making them functionally infertile. Viability refers to the ability of the plasma membrane to regulate its internal environment. Loss of this ability prevents the cell from maintaining homeostasis and undergoing regulated processes.

Statistical Analysis

Experiments 6.1 and 6.2 differences among treatment groups were analyzed using SAS version 9.2 (SAS institute Inc. Cary, NC 27513) using Proc Glimmix. Tukey's pairwise comparison was used as a post-analysis multiple comparisons of differences.

Experiment 6.3 differences among treatment groups were analyzed by ANOVA in Sigma-Plot 10.0. A $P < 0.05$ was considered significant in both studies.

RESULTS

Experiment 6.1. CTC assay

The percentage of sperm that displayed the characteristic "B" pattern (capacitated), as determined by CTC assay, were compared among treatment groups. The overall comparison showed no difference between control and PIF treatments ($P = 0.9995$), also there were no differences between Heparin and the PIF + Heparin treatments ($P = 0.9344$). The addition of heparin did significantly increased the percentage of capacitation when compared with control ($P < 0.0001$). PIF showed no ability to enhance or suppress the percentage of in vitro capacitation of cryopreserved bovine ejaculated sperm. At 2, 4 and 6 hours of incubation the treatment groups that included heparin showed significantly more capacitated sperm when compared with treatments without heparin regardless of whether PIF was included or not in the culture medium (Figure 6.1).

Experiment 6.2. Flow cytometry

Percentages of sperm viability, acrosome integrity and capacitation were compared among treatment groups using flow cytometry (Figure 6.2). The percentage of viable acrosome intact sperm per treatment group were compared and no difference was noted between control and PIF treatments ($P = 0.7071$). There was no difference between the Heparin and PIF + Heparin treatments ($P = 0.9647$). The treatment groups that included heparin showed significantly less viable sperm with intact acrosomes compared with groups without heparin ($P < 0.0001$).

When comparing the percentage of auto-acrosome reacted sperm per treatment, it was noted that control and PIF treatments were not different ($P = 0.1523$) and also the Heparin and PIF + Heparin treatments showed no difference ($P = 0.9957$) as to the percentage of auto-acrosome reaction.

Table 6.1. The effects of preimplantation factor (PIF) and heparin on cryopreserved ejaculated bovine sperm during in vitro culture.

Treatment	Hour	% Viable acrosome intact* (±SEM)		% Auto-Acrosome reaction* (±SEM)		% Nonviable acrosome intact* (±SEM)		% Capacitated* [†] (±SEM)		% Capacitated **CTC (±SEM)	
Control	0	78.10	± 3.02	6.67	± 1.48	15.44	± 1.37	9.79	± 1.03	9.13	± 1.29
Control	2	71.94	± 3.60	9.58	± 2.06	17.48	± 1.51	6.52	± 0.71	17.42	± 2.18
Control	4	55.50	± 4.40	14.40	± 2.94	28.03	± 2.12	6.42	± 0.70	26.88	± 2.95
Control	6	41.17	± 4.28	17.50	± 3.40	37.10	± 2.45	5.00	± 0.55	41.90	± 3.64
Heparin	0	68.94	± 3.80	10.42	± 2.22	20.43	± 1.71	10.82	± 1.13	8.88	± 1.26
Heparin	2	53.85	± 4.39	15.38	± 3.10	28.92	± 2.16	4.40	± 0.49	23.81	± 2.73
Heparin	4	32.11	± 3.85	20.93	± 3.94	44.79	± 2.95	3.58	± 0.41	36.15	± 3.45
Heparin	6	21.48	± 2.98	23.64	± 4.30	52.29	± 2.62	3.68	± 0.42	47.43	± 3.72
PIF	0	77.70	± 3.06	7.36	± 1.63	15.20	± 1.35	10.12	± 0.11	8.72	± 1.24
PIF	2	68.83	± 3.79	11.34	± 2.40	18.82	± 1.60	6.30	± 0.69	17.28	± 2.17
PIF	4	53.48	± 4.39	15.70	± 3.15	28.32	± 2.13	4.95	± 0.55	27.84	± 3.02
PIF	6	40.36	± 4.25	18.34	± 3.57	36.97	± 2.44	4.50	± 0.50	42.77	± 3.66
PIF + Heparin	0	71.79	± 3.58	10.34	± 2.21	17.84	± 1.54	10.00	± 1.05	10.07	± 1.40
PIF + Heparin	2	52.98	± 4.40	15.97	± 3.21	29.25	± 2.17	4.34	± 0.49	22.52	± 2.63
PIF + Heparin	4	31.51	± 3.81	21.22	± 3.98	45.21	± 2.60	3.81	± 0.43	37.34	± 3.50
PIF + Heparin	6	22.38	± 3.07	23.54	± 4.30	51.15	± 2.62	3.72	± 4.21	47.57	± 3.72

*Sperm viability and acrosomal integrity determined by PI and FITC-PNA fluorescent staining, respectively. Sperm staining pattern evaluated by flow cytometer.

[†]Capacitation determined by inducing the acrosome reaction in viable capacitated sperm with lysophosphatidylcholine (10 µg/ml).

**Capacitation determined by chlortetracycline assay under fluorescent phase microscopy (40X).

Table 6.2. Effect of adding preimplantation factor (PIF) and or heparin (Hep) during bovine in vitro fertilization, on embryo cleavage and development.

Treatment	n	Percentage (%) \pm SEM			
		Cleaved ^a	8-Cell ^a	*Morula ^b	*Blastocyst ^b
+PIF +Hep	145	67.6 \pm 4.5	38.3 \pm 5.4	20.9 \pm 5.1	20.7 \pm 3.8 ^c
-PIF +Hep	130	62.6 \pm 5.0	34.3 \pm 9.8	22.3 \pm 5.0	20.6 \pm 5.2 ^c
+PIF -Hep	145	55.2 \pm 6.7	21.6 \pm 6.8	14.7 \pm 4.2	9.2 \pm 3.2 ^d
-PIF -Hep	160	55.5 \pm 6.0	19.9 \pm 7.3	21.3 \pm 6.0	8.7 \pm 2.9 ^d

^aMeasured at 72 hours post-insemination.

^bMeasured at 168 hours post-insemination.

*Percentage of cleaved embryos.

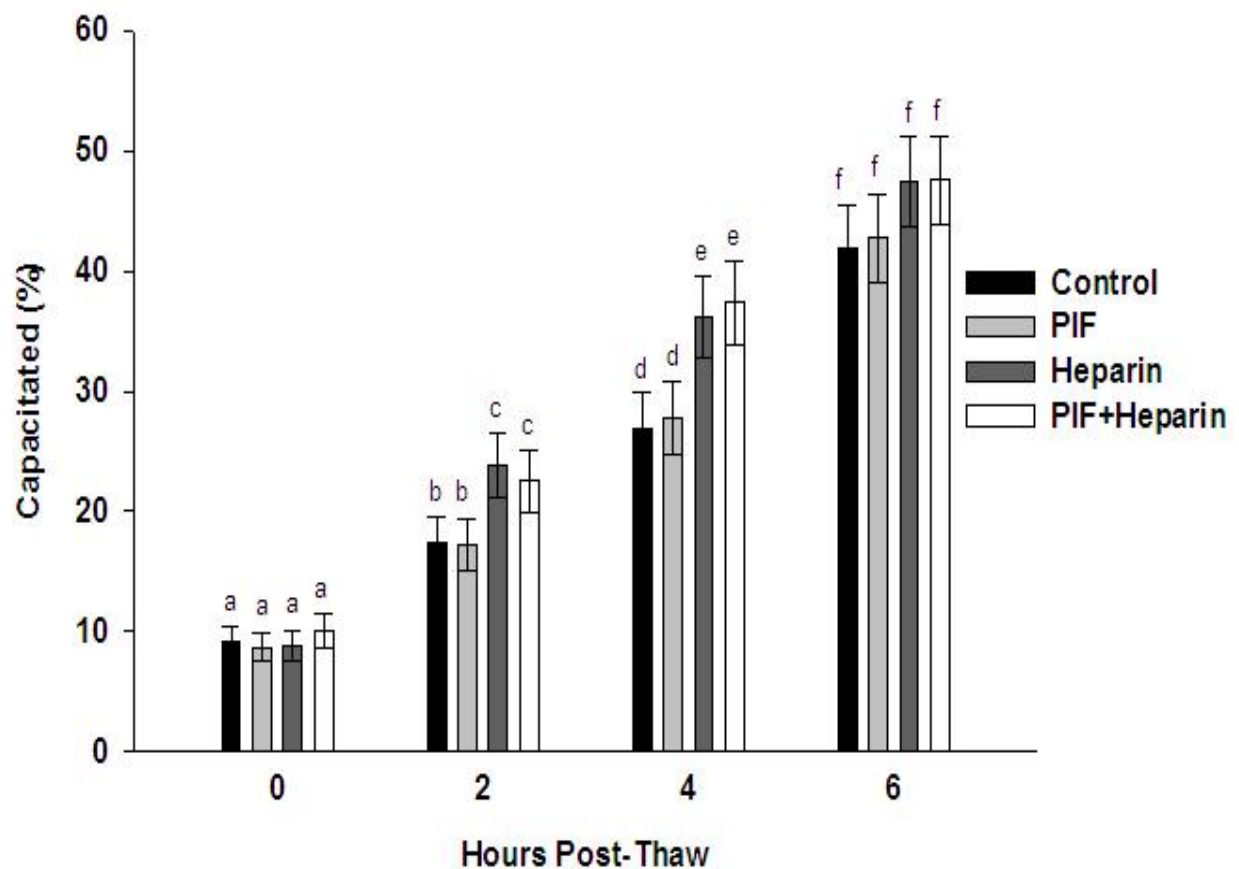


Figure 6.1. Percentage of sperm displaying the “B” (capacitated) CTC staining pattern (mean±SEM), over time for bull ejaculated cryopreserved sperm as affected by treatment. ^{a,b,c,d,e,f}Mean values with different letters within hour of treatment are significantly different (P<0.001). PIF = preimplantation factor.

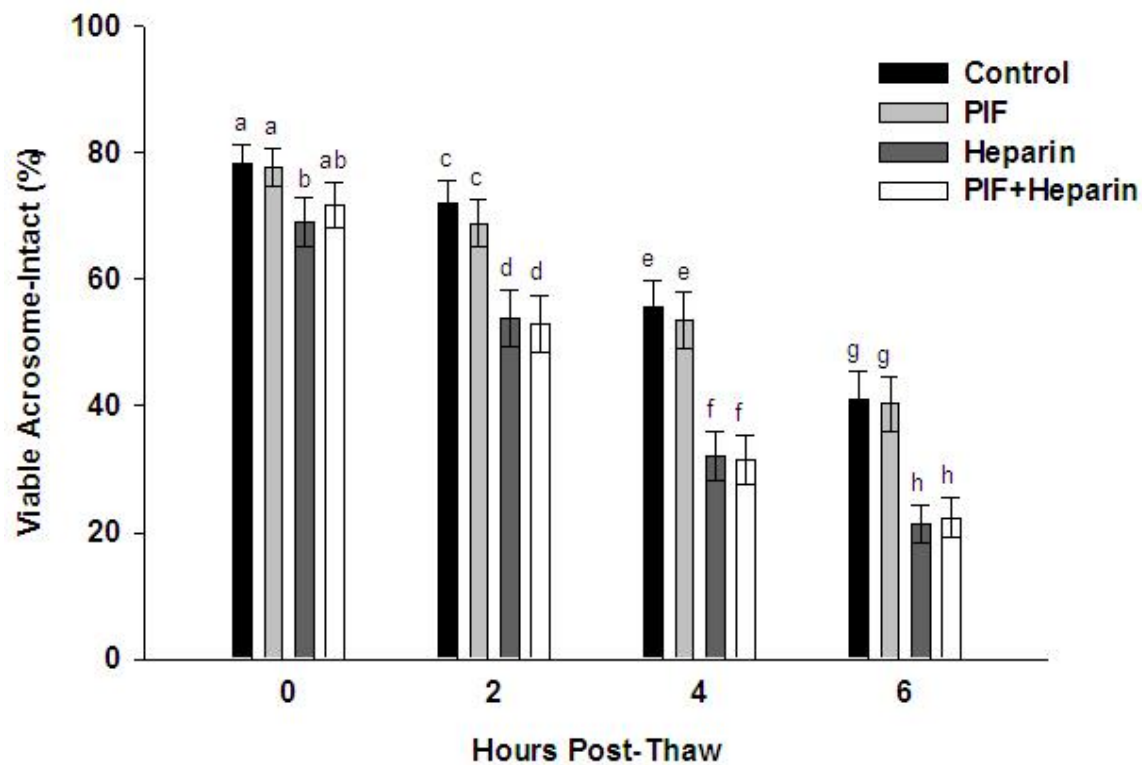


Figure 6.2. Percentage of viable acrosome-intact sperm (mean \pm SEM) over time as affected by treatment. Sperm viability and acrosomal status determined by staining with propidium iodide (PI) and fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) followed by evaluation with flow cytometer. ^{a,b,c,d,e,f,g,h} Mean values with different letters within hour of treatment are significantly different ($P<0.001$). PIF = preimplantation factor.

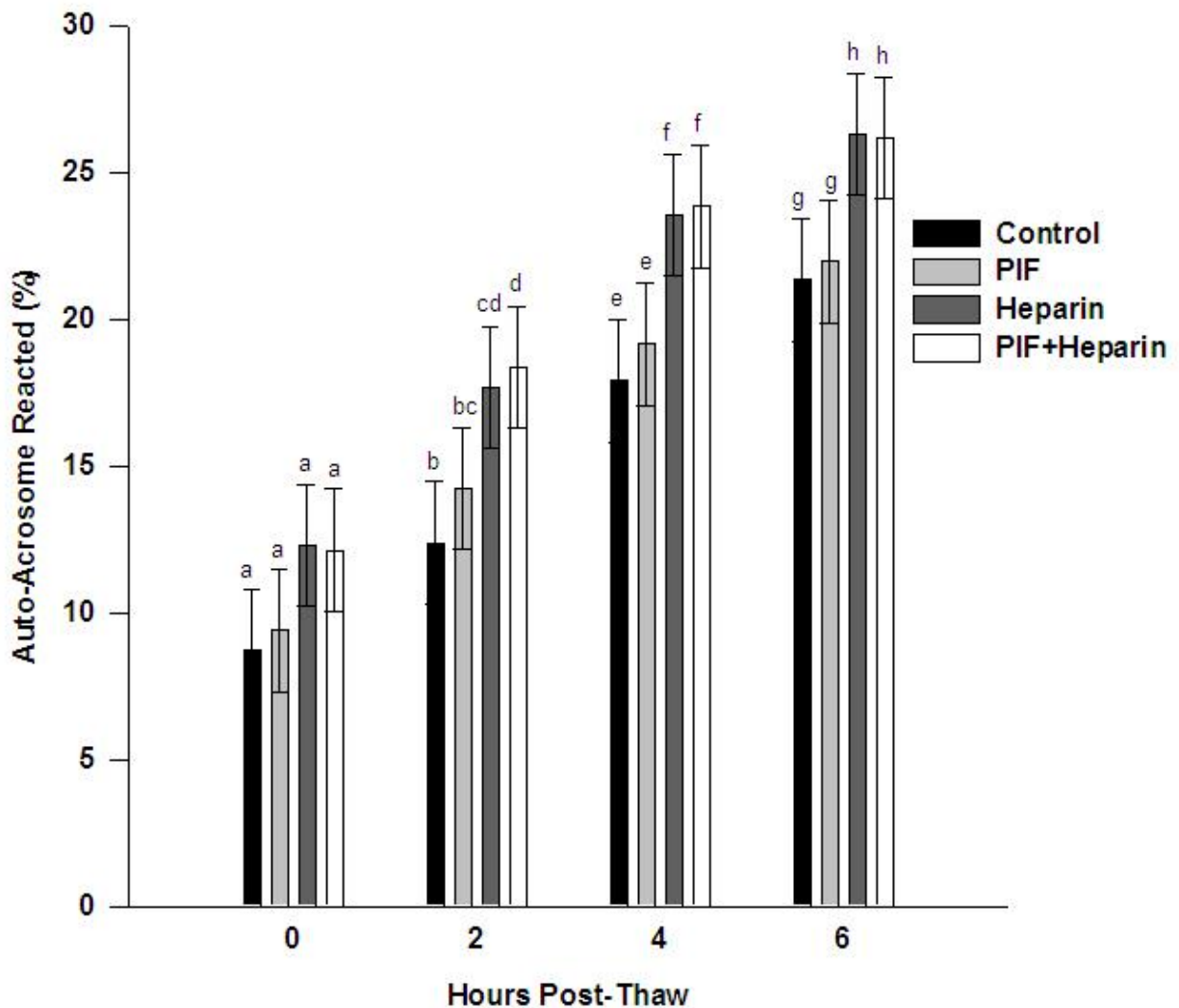


Figure 6.3. Percentage of auto-acrosome reacted sperm (mean \pm SEM) over time as affected by treatment. Sperm acrosomal integrity was assessed by staining with fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) and evaluated by flow cytometer. ^{a,b,c,d,e,f,g,h}Mean values with different letters within hour of treatment are significantly different ($P < 0.001$). PIF = preimplantation factor.

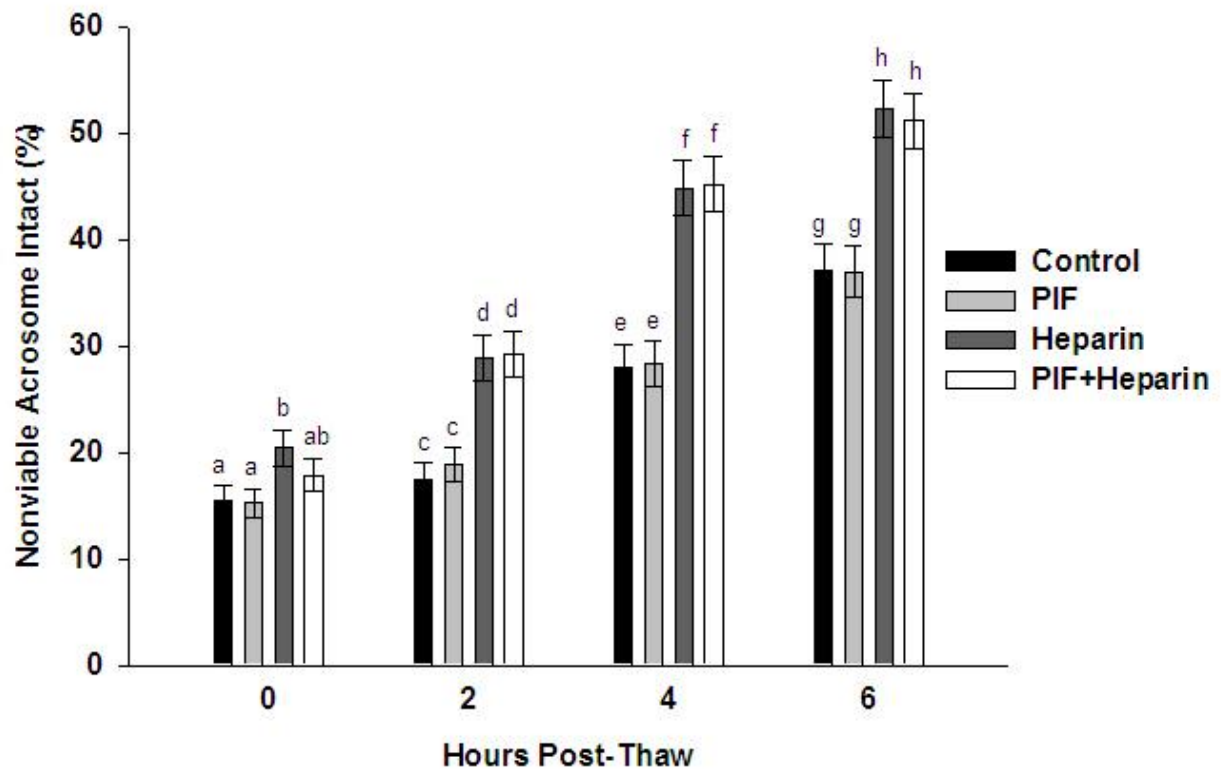


Figure 6.4. Percentage of nonviable sperm with intact acrosomes (mean \pm SEM) over time as affected by treatment. Sperm viability and acrosomal integrity was assessed by staining with propidium iodide (PI) and fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) followed by evaluation with flow cytometer. Inclusion of PIF did not change the proportion of sperm that were nonviable with intact acrosomes during their time in culture ($P=0.896$). The inclusion of heparin did increase the proportion during the 6-hour culture interval. ^{a,b,c,d,e,f,g,h}Mean values with different letters within each time period were significantly different ($P=0.0001$). PIF = preimplantation factor.

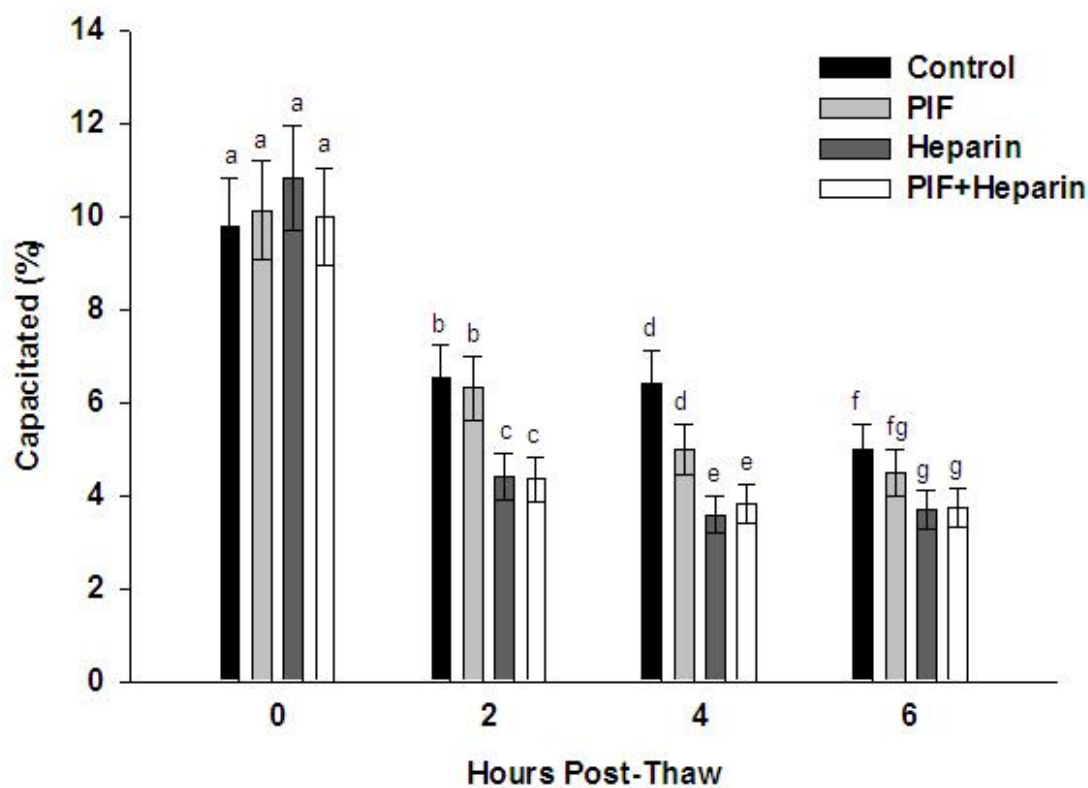


Figure 6.5. Capacitated sperm as a percentage of the total sperm count (mean \pm SEM) over time as affected by treatment. Capacitated sperm were induced to undergo the acrosome reaction by exposure to lysophosphatidylcholine. Sperm viability and acrosomal integrity was assessed by staining with propidium iodide (PI) and fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) followed by evaluation with flow cytometer. ^{a,b,c,d,e,f,g}Mean values with different letters within each time period were significantly different ($P<0.001$). PIF = preimplantation factor.

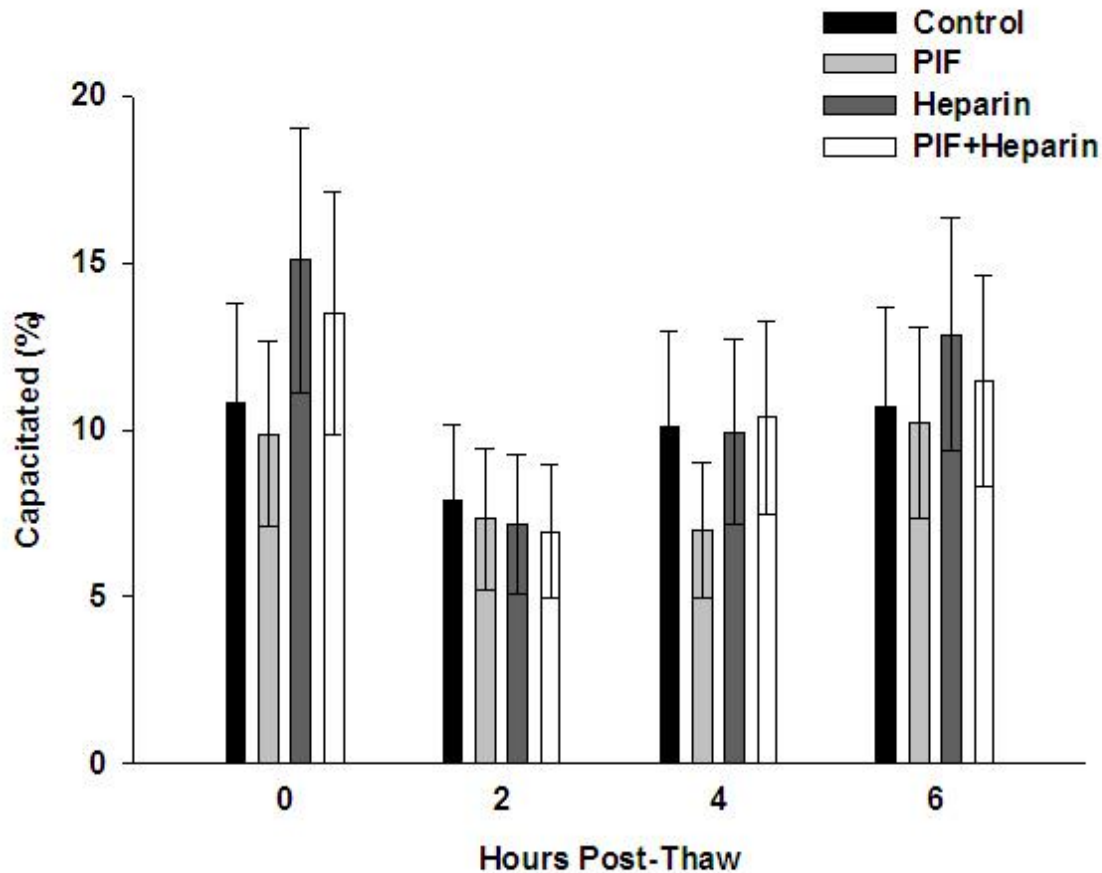


Figure 6.6. Capacitated sperm as a percentage of the viable sperm population (mean \pm SEM) over time as affected by treatment. Capacitated sperm were induced to undergo the acrosome reaction by exposure to lysophosphatidylcholine (LPC). Sperm viability and acrosomal integrity was assessed by staining with propidium iodide (PI) and fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) followed by evaluation with flow cytometer. No significant differences were detected between treatment groups at each time period ($P=0.80$). PIF = preimplantation factor.

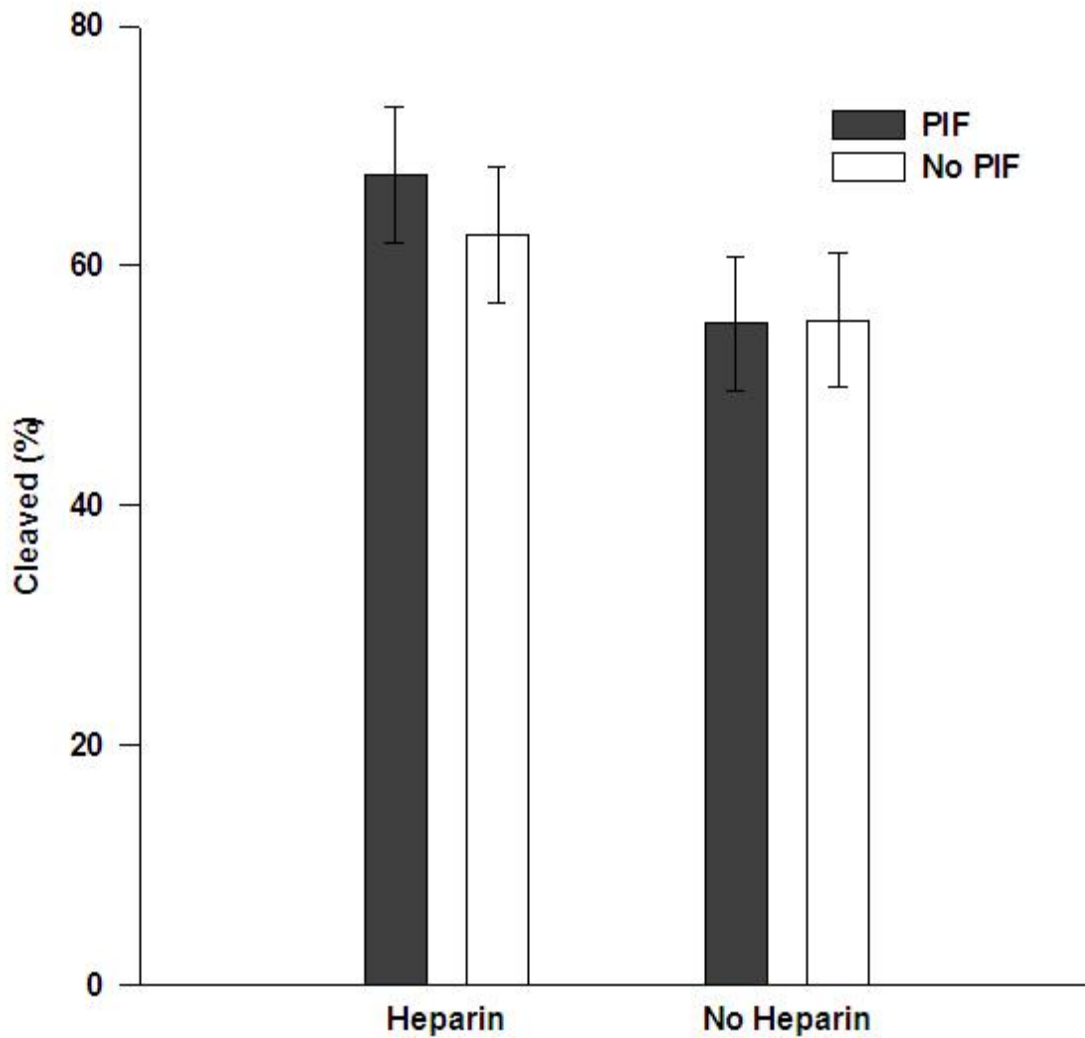


Figure 6.7. Percentage of oocytes (n=583) that cleaved (mean±SEM) following in vitro fertilization with and without preimplantation factor (PIF) and heparin. No significant differences were found for PIF (P=0.676) or heparin (P=0.098).

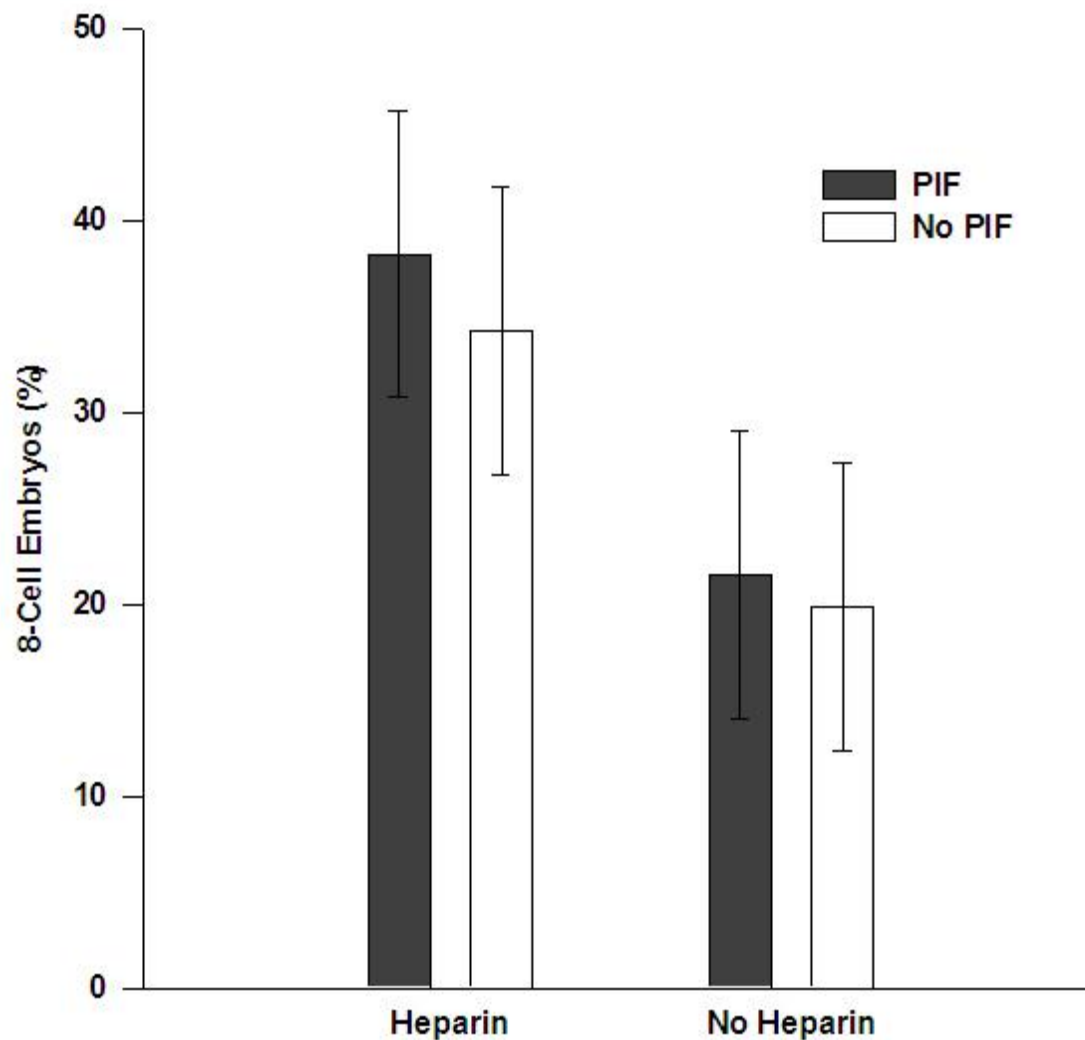


Figure 6.8. Percentage of embryos (n=583) that developed to the 8-cell stage (mean \pm SEM) following in vitro fertilization with or without preimplantation factor (PIF) or heparin. The inclusion of PIF had no effect on the percentage of embryos developing to the 8-cell stage ($P=0.71$). However, not significant the inclusion of heparin did increase the development to the 8-cell stage ($P=0.05$).

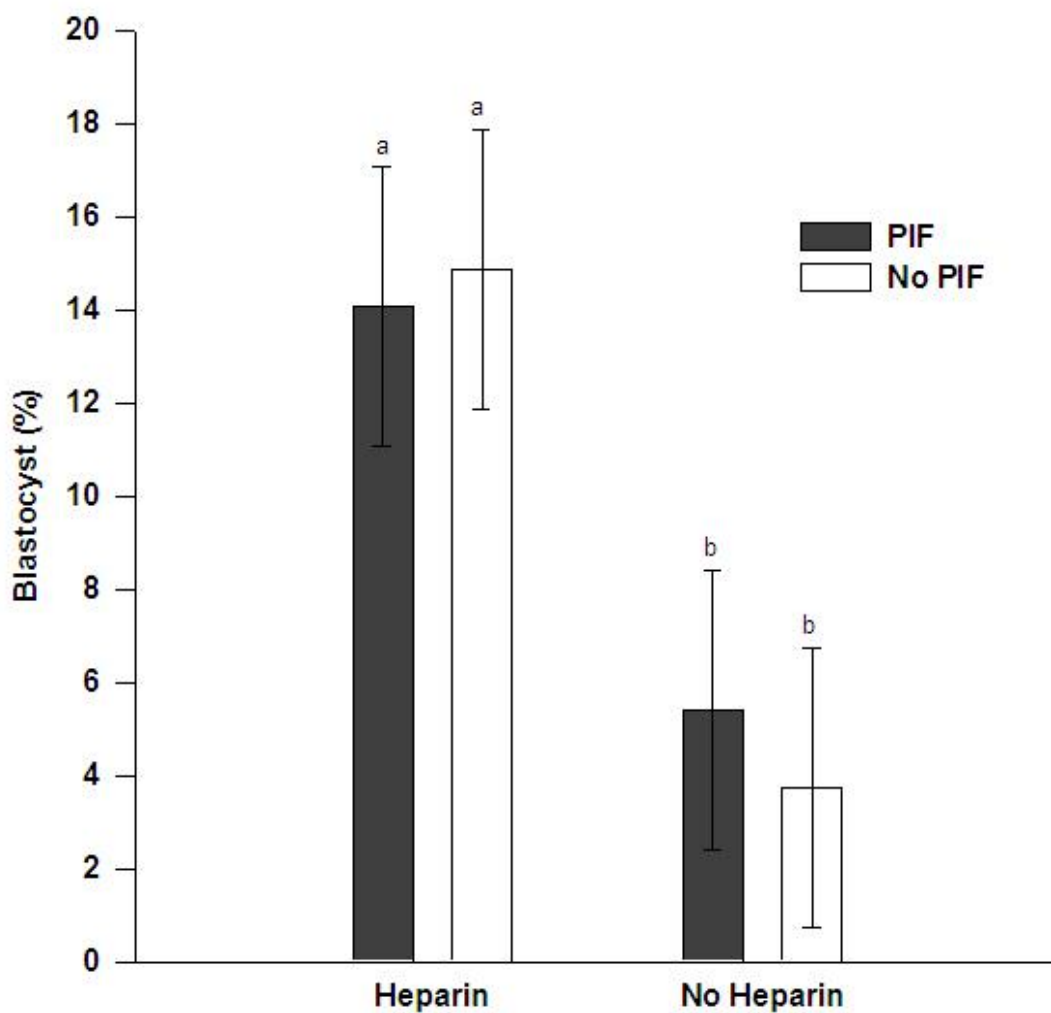


Figure 6.9. Percentage of embryos that developed to the blastocyst stage (mean \pm SEM) following in vitro fertilization with and without preimplantation factor (PIF) or heparin. Inclusion of PIF had no effect on the percentage of embryos developing to the blastocyst stage ($P=0.886$). ^{a,b}Significant differences were detected when heparin was included during IVF ($P<0.01$).

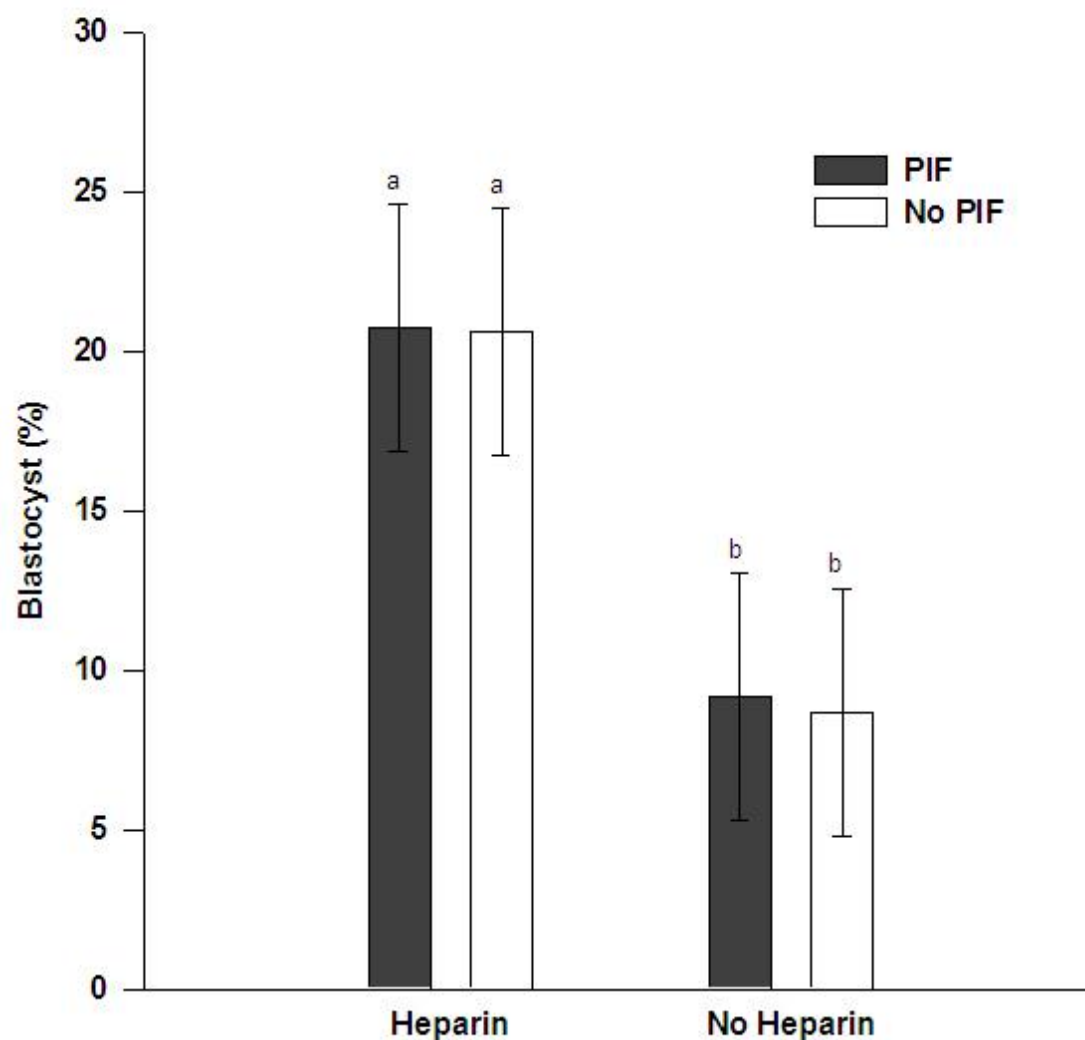


Figure 6.10. Percentage of cleaved embryos that developed to the blastocyst stage (mean±SEM) following in vitro fertilization with or without preimplantation factor (PIF) or heparin. The inclusion of PIF had no effect on the percentage of cleaved embryos developing to the blastocyst stage ($P=0.939$). ^{a,b}Significant differences were found when heparin was included during the IVF procedure ($P<0.01$).

However, the treatment groups that included heparin showed significantly more auto-acrosome reacted sperm compared with groups without heparin ($P<0.0001$, Figure 6.3).

The percentage of nonviable (“dead”) acrosome intact sperm per treatment group was compared over 6 hours of in vitro culture (Figure 6.4). No difference was noted between control and PIF treatments ($P=0.9895$) also the Heparin and PIF + Heparin treatments displayed no difference ($P=0.8960$) as to the percentage of nonviable acrosome intact sperm. The treatment groups, which included heparin, had significantly more nonviable acrosome intact sperm compared with groups without heparin ($P<0.0001$).

The percentage of LPC responsive sperm, taken as a measurement of total capacitation, was compared per treatment group (Figure 6.5). The percentage of reactive sperm was determined by taking the difference between the auto-acrosome reacted and the LPC-induced acrosome reacted sperm populations as a percentage of the total sperm population for each treatment group at each time period. Control and PIF treatments were not different ($P=0.4828$) also the Heparin and PIF + Heparin treatments showed no difference ($P=0.9996$) as to the percentage of reactive sperm. The treatment groups that included heparin compared with groups without heparin were significantly lower ($P<0.05$).

The percentage of LPC responsive sperm, as a measurement of viable sperm capacitation, was compared per treatment group (Figure 6.6). The percentage of viable reactive sperm was determined by taking the difference between the auto-acrosome reacted and the LPC-induced acrosome reacted sperm populations as a percentage of total viable sperm population for each treatment group at each time period. There was no difference found among treatment groups ($P>0.8$).

Experiment 6.3. In vitro fertilization

The effects of PIF compared with heparin when added to IVF medium on embryo cleavage and subsequent development were compared per treatment group. The percentage of oocytes that cleaved was unaffected by the inclusion of PIF or heparin ($P=0.860$) and ($P=0.109$), respectively (Figure 6.7). The percentage of embryos that developed to the 8-cell stage were also unaffected by the addition of PIF ($P=0.930$) but the inclusion of heparin in the fertilization medium significantly increased the percentage

of embryos that developed to the 8-cell stage ($P < 0.05$, Figure 6.8). The percentage of embryos that developed to the blastocyst stage were unaffected by the addition of PIF ($P = 0.910$) but the inclusion of heparin significantly increased the percentage blastocyst development ($P < 0.05$, Figure 6.9).

DISCUSSION

Sperm are dynamic cells that undergo many biochemical processes, from the quiescent state of epididymal sperm stored in the cauda epididymides compared with capacitated, hyperactivated ejaculated sperm undergoing the acrosome reaction during in vitro fertilization. These changes are brought about by epididymal sperm interacting with various factors such as growth factors, peptides and proteins within their environment. These factors are noted as binding to receptors on the sperm plasma membrane causing the removal of membrane components such as cholesterol and initiating signal transduction pathways leading to capacitation, hyperactivation and the acrosome reaction.

Recently, preimplantation factor, a small peptide, first identified in pregnant humans and reported as being secreted by viable embryos, has been found to be released by bovine embryos during in vitro culture (Stamatkin et al., 2011a). When tested on mice for treatment of neural disease, PIF was shown to affect their spinal cord protein profile (Weiss et al., 2011c). Of the proteins affected by PIF, several were sodium, potassium and calcium-ATPase transporters. PIF decreased the amount of these ATPase transporters (Paidas et al., 2010; Weiss et al., 2011c). Calcium is a key component in sperm signaling and function during capacitation and acrosome reaction (Breitbart, 2002). Control over intracellular sodium, potassium and calcium concentrations has been shown to affect the ability of sperm to become capacitated or undergo the acrosome reaction (Abou-Haila and Tulsiani, 2009; Newton et al., 2010).

During capacitation, bull sperm membranes become permeable to extracellular calcium due to the loss of cholesterol in the plasma membrane. Increases in the amount of intracellular calcium are controlled by actively pumping calcium into the acrosome byway of a calcium-ATPase (Breitbart and Naor, 1999; Dragileva et al., 1999; Haim, 2002; Abou-Haila and Tulsiani, 2009). Furthermore, decapacitation factors also initiate the active pumping of calcium from the cell by calcium-ATPases on the plasma

membrane (Dragileva et al., 1999; Abou-Haila and Tulsiani, 2009; Fraser, 2010). The inhibition of calcium-ATPases will result in an increase of intracellular calcium concentrations in sperm (Abou-Haila and Tulsiani, 2009; Fraser, 2010). If intracellular calcium concentrations become too high, the sperm will spontaneously auto-acrosome react (Dragileva et al., 1999). This loss of the acrosome effectively renders the sperm useless due to its inability to bind or penetrate the zona pellucida. On the other hand, if calcium is excluded or uptake is impaired, intracellular calcium concentrations needed for capacitation will be obstructed or not attained thus preventing or impeding capacitation and the acrosome reaction.

In the mouse, Adeoya-Osiguwa and Fraser (1996) noted that inclusion of calmodulin stimulated a Ca^{2+} -ATPase, preventing capacitation. In addition, it was noted that TFP, a calmodulin antagonist, accelerated capacitation of mouse sperm. It was proposed that a calmodulin-sensitive Ca^{2+} -ATPase is responsible for the maintenance of a low intracellular Ca^{2+} concentration in the sperm. As capacitation progresses, and DF are lost, Ca^{2+} -ATPase activity declines, with the decline of Ca^{2+} -ATPase, intracellular Ca^{2+} concentrations rises, promoting capacitation.

The effect of Na/K-ATPase activity was noted by Lax et al. (1994) who identified a 170-kDa EGFR on the head of ejaculated bull sperm. When bull sperm were exposed to EGF they were induced to undergo the acrosome reaction in a dose-dependent manner. Activation of the EGFR was found to be associated with Protein Kinase A (PKA), the SRC tyrosine kinase family and the inhibition of Na/K-ATPase (Daniel et al., 2010). With the activation of EGFR and the binding of EGF, an increase in internal Ca^{2+} concentration is noted, leading to the acrosome reaction. It was also noted that PKC was involved in the mechanism in which EGF exerts its effect on acrosome reaction.

Although, sperm are affected by Ca^{2+} -ATPase and Na/K-ATPase activity, PIF may also bind to specific receptors on the plasma membrane stimulating other signal transduction pathway. These signal transduction pathways are initiated by the binding of various factors to their ligands and include G-protein coupled receptors, soluble adenylyl cyclase and tyrosine kinase.

In the mouse, boar and human, it has been noted that FPP, A-II, calcitonin and adrenaline are able to regulate capacitation (Fraser et al., 2001; Fraser and Osiguwa,

2004; Fraser, 2008). Specific receptors for these factors have been located on the acrosomal cap and tail plasma membrane of sperm. Binding of these factors to their appropriate ligands, leads to modulation of membrane-associated adenylyl cyclase (mAC) activity and production of cAMP, stimulating cAMP production in uncapacitated sperm and inhibiting cAMP production in capacitated sperm. Receptors for all of these factors, except FFP, have been determined to be G-protein coupled receptors. FFP are thought to operate in conjunction with adenosine receptors, although the mechanism is still under investigation. In the bull, A-II has been shown to stimulate the acrosome reaction in capacitated sperm (Gur et al., 1998). Receptors for A-II were found only on the tail of uncapacitated sperm, although following capacitation, receptors were identified on the head of the bull sperm.

Relaxin, a peptide hormone, also has been found to bind to receptors on the bull sperm plasma membrane resulting in a stimulation of a signal transduction pathway (Miah et al., 2011). Relaxin accelerates the cholesterol efflux and increases the Ca^{2+} -influx, which stimulates the production of cAMP, by way of a soluble adenylyl cyclase and protein tyrosine phosphorylation, leading to capacitation.

Henricks et al. (1998) demonstrated that IGF-1 present in the seminal plasma of 7 beef bulls can interact with a specific IGF-1 receptor on the acrosomal region of ejaculated sperm. Moreover, it was noted that when physiological concentrations of IGF-1 (100 ng/ml) and IGF-2 (250 ng/ml) were added to sperm, they maintained their motility better than without the additions of IGF-1 or IGF-2. Hoeflich et al. (1999) also reported IGF-I (144 ng/ml) in bovine seminal plasma. Receptors for IGF-1 (IGF-1R), have been identified as transmembrane tyrosine kinases and are located on the acrosome of bull sperm.

Li et al. (2010) identified the NGF receptor (TrkA) on the acrosomal and tail of bull sperm. In addition, it was determined that NGF binds bull sperm on the acrosomal cap and along the tail. The TrkA receptor has been identified as a tyrosine kinase (Kaplan et al., 1991). Sperm viability following 2 hours of culture, was increased by the inclusion of 20 $\mu\text{g/ml}$ NGF (72%) compared with sperm without the addition of NGF (62%)

In our study, an increase concentration of capacitation in groups that included heparin according to the CTC assay were detected (see Figure 6.1). Although, no effect on capacitation was detected when PIF was included in the medium whether heparin was included or not. PIF alone was unable to induce capacitation, also PIF was unable to enhance or suppress the ability of heparin to capacitate sperm. The percentage of LPC reactive live sperm were compared per treatment. No difference was noted among treatment groups as to the ability of PIF to enhance or suppress the percentage of capacitation in live sperm cells.

The addition of NGF to bull sperm has been noted to increase viability during culture (Li et al., 2010). The ability of PIF to prevent an increase in the percentage of nonviable sperm over time during in vitro culture was investigated. The percentage of live acrosome intact sperm was measured by flow cytometry with the use of fluorescent probes. Preimplantation factor was unable to increase the amount of live sperm compared with the control group. Also, PIF was unable to prevent the decrease in sperm viability as seen when heparin was included in the culture medium. The percentage of nonviable acrosome intact sperm was then compared among treatment groups. As noted before, PIF was unable to decrease the amount of nonviable sperm or reduce the increase that was observed in the groups that included heparin.

The spontaneous auto-acrosome reaction eliminates the ability of sperm to fertilize an oocyte. The addition of FPP, A-II, calcitonin and adrenaline are able to prevent the auto-acrosome reaction in mouse, boar and human sperm (Fraser et al., 2001; Fraser and Osiguwa, 2004; Fraser, 2008). However, the addition of EGF and A-II has been noted to induce the acrosome reaction in bull sperm (Lax et al., 1994; Gur et al., 1998). In our study, the percentage of auto-acrosome reaction was compare among treatment groups. The addition of PIF when compared with control showed no difference, also PIF was unable to prevent the increase in auto-acrosome reaction noted in the groups in which heparin was included.

Including PIF in the culture medium may improve embryo development, but until now the effects of adding PIF to fertilization medium has not been evaluated. As noted (see Figure 6.7), PIF had no effect on the percentage of oocytes that cleaved following IVF ($P=0.860$). No effect was noted on the percentage of embryos that developed to the

8-cell stage ($P=0.930$) when PIF was included in the fertilization medium. PIF also had no effect on the percentage of embryos that developed to the blastocyst stage (Figure 6.9). PIF was unable to affect the ability of embryos that cleaved to developed to the blastocyst stage. Table 6.1 summarizes the embryo development following IVF with or without the inclusion of PIF to the fertilization medium.

In conclusion, during the course of our studies we were unable to demonstrate that PIF affected bovine sperm viability, ability to undergo capacitation, or the prevention or initiation of the auto-acrosome reaction of ejaculated sperm was affected by exposure to PIF. The inclusion of PIF in the fertilization medium also had no effect on the development of bovine embryos following IVF. Due to lack of response by ejaculated sperm, we did not expand this study to include epididymal sperm because of the limited availability of samples. Taken together, these results do not support any interaction of bovine cryopreserved sperm with PIF.

CHAPTER VII

THE EFFECTS OF EJACULATED AND EPIDIDYMAL SPERM COLLECTED FROM THE SAME HOLSTEIN BULLS ON IN VITRO FERTILIZATION RATES AND SUBSEQUENT EMBRYO DEVELOPMENT

INTRODUCTION

The collection and cryopreservation of ejaculated sperm from genetically valuable bulls has become routine and is the main source of sperm utilized in assisted reproductive techniques: such as artificial insemination (AI) and in vitro fertilization (IVF). Cryopreserved semen theoretically has the ability to be stored indefinitely and has played a significant role in improving animal genetics (Vishwanath and Shannon, 2000). Although in some animals, such as exotic bovine breeds, the collection of ejaculated sperm is not feasible. This makes the ability to collect, cryopreserve and utilize epididymal sperm necessary for species that are difficult or impossible to collect ejaculated sperm from. Also, the unexpected loss of a genetically valuable male due to injury or death will make the ability to collect epididymal sperm indispensable in producing additional progeny that would otherwise be lost.

Epididymal sperm having not been exposed to seminal plasma may potentially respond differently than ejaculated sperm with respect to cryopreservation, capacitation and their ability to fertilize oocytes in vitro. It is therefore important for us to understand the differences between ejaculated and epididymal sperm collected from the same males in an effort to utilize these finite sperm stores in the most efficient manner possible. This will maximize the number of progeny obtained from these genetically valuable males. In this study we compare ejaculated and epididymal sperm collected from the same Holstein bulls (n=4) by their ability to fertilize oocytes in vitro with and without the capacitating agent heparin and the subsequent embryo development.

During ejaculation, sperm are exposed to seminal plasma that contain many factors including ions, energy and proteins (Moura et al., 2007). This exposure to seminal plasma has been noted as having both beneficial and detrimental effects on sperm (Bergeron et al., 2004). The major protein components of seminal plasma are the BSP proteins, comprising ~65% of all seminal plasma proteins (Bergeron et al., 2004). These BSP proteins are secreted by the seminal vesicles and bind to choline phospholipids on the sperm plasma membrane (Therien et al., 1995). Bovine seminal

plasma proteins also bind to the glycosaminoglycan heparin, a known inducer of capacitation (Miller et al., 1990). One of the major aspects of capacitation is the efflux of cholesterol from the plasma membrane. Exposure of epididymal sperm to BSP proteins induces an efflux of cholesterol from the plasma membrane (Therien et al., 1998).

Cholesterol has a stabilizing effect on membranes that increases their resistant to cold shock (Cross, 1998). By artificially adding cholesterol to the sperm plasma membrane, it has been shown that survival rates following cryopreservation also increase without effecting the ability of sperm to undergo capacitation (Purdy and Graham, 2004a). Epididymal sperm, having not been exposed to seminal plasma, may have higher cholesterol concentrations in their plasma membranes. Species, such as human and rabbit, whose sperm contain high cholesterol to phospholipid ratios (>0.80) are able to better endure cryopreservation in comparison to species such as the boar with low cholesterol to phospholipid ratios (0.20). Boar sperm are very susceptible to cold shock and do not endure cryopreservation well (Cross, 1998). The efflux of cholesterol from epididymal sperm membranes following ejaculation may cause sperm to be more susceptible to cooling and cryopreservation induced damage.

Following cryopreservation, epididymal sperm may also have a higher percentage of viable acrosome-intact sperm that are not capacitated. Cryopreservation has been shown to induce ultra-structural changes to sperm plasma membranes. These changes in the plasma membrane alter the ability of sperm to regulate cell functions such as capacitation. The capacitation-like state that is induced by cryopreservation is termed 'Cryocapacitation' (Bailey et al., 2000). Key components of capacitation are the regulation of intracellular calcium and bicarbonate concentrations that are dependent on the permeability and function of the plasma membrane. As previously stated, epididymal sperm having not been exposed to seminal plasma (high in both bicarbonate and calcium) may function physiologically differently following cryopreservation.

The ability of epididymal sperm to undergo the process of capacitation during in vitro culture may also be different compared with ejaculated sperm. Ejaculated and epididymal sperm differ with respect to the exposure of seminal plasma that contains BSP proteins. Bovine seminal plasma proteins have been isolated and function in supporting; total motility, binding of sperm to oviductal epithelium, binding of heparin

and the removal of membrane cholesterol (Gwathmey et al., 2006). Heparin has been shown to increase the percentage of capacitation in bovine sperm during in vitro culture (Parrish et al., 1988). If epididymal sperm have a reduced capability or are unable to bind heparin this may prevent or inhibit them from becoming capacitated. On the other hand, seminal plasma also contains decapacitation factors that prevent sperm from becoming capacitated. If epididymal sperm are not exposed to such factors, capacitation may be accelerated. If the time required for a sperm cell to become capacitated changes, it may necessitate a change in assisted reproduction protocols.

During these studies, we will compare ejaculated and epididymal sperm from the same bulls in an attempt to better understand the different dynamics that arise when trying to utilize them in assisted reproductive techniques. If epididymal sperm proves to be different, an adjustment to existing protocols may be necessary to better suite epididymal sperm to accomplish the most efficient use of this finite resource.

MATERIALS AND METHODS

Experimental Design

Experiment 7.1.

This experiment was designed to examine ejaculated and epididymal sperm from the same bulls as to their ability to fertilize oocytes (n=705) with and without heparin. The experiment was set up in a factorial design in 1 of 4 treatments: Treatment 1 (Ejaculated + Heparin), ejaculated sperm with heparin (10 µg/ml); Treatment 2 (Ejaculated - Heparin), ejaculated sperm without heparin; Treatment 3 (Epididymal + Heparin), epididymal sperm with heparin (10 µg/ml); Treatment 4 (Epididymal - Heparin), epididymal sperm without heparin. Fertilization was assessed over 10 replications by pronuclear fluorescent staining at 18 hours post-insemination.

Experiment 7.2.

This experiment was designed to examine the effects of ejaculated and epididymal sperm from the same bulls with and without the capacitating agent heparin on the subsequent embryo development in vitro. The experiment was set up in a factorial design in 1 of 4 treatments: Treatment 1 (Ejaculated + Heparin), ejaculated sperm with heparin (10 µg/ml); Treatment 2 (Ejaculated - Heparin), ejaculated sperm without heparin; Treatment 3 (Epididymal + Heparin), epididymal sperm with heparin (10

µg/ml); Treatment 4 (Epididymal - Heparin), epididymal sperm without heparin. Ejaculated and epididymal sperm from the same 4 bulls were used in IVF. Embryo cleavage was assessed at 72 hours post-insemination over 16 replications with 1220 oocytes. Embryo development was determined from 2 of the 4 Holstein bulls due to lack of development. Embryo development was assessed over 5 replications with 265 oocytes.

Experimental Procedure

Animals

Ejaculated and epididymal sperm were collected from the same mature, healthy, fertile Holstein bulls (n=4) housed at Genex Custom Collection Service near the Louisiana State University (LSU) campus. The bulls were in good body condition and of sound conformation at the time of semen collection.

Ejaculated sperm collection

Ejaculates from each bull were collected by the same experienced technicians throughout the study. Two false mounts were made by the bulls before actual semen collection. A total of at least 2 separate ejaculates from each of the bulls were collected and cryopreserved. Ejaculated semen was collected by artificial vagina into a 15 ml polypropylene conical tube and volume noted. The ejaculate was extended in standard bovine egg yolk-based diluents and then placed into a 37°C water bath for equilibration before evaluation of progressive motility. Sperm concentration was calculated by a standard curve using a spectrophotometer, following standard commercial bull stud procedures.

Epididymal sperm collection

Collection of epididymal sperm was performed by isolating the epididymides and a portion of the vas deferens from the testes in a sterile manner. The vas deferens were catheterized and egg yolk-based extender was used to push epididymal sperm out of the epididymis by retrograde flow. Epididymal sperm was collected into a 15 ml polypropylene conical tube, extended in standard bovine egg yolk-based diluents and then placed into a 37°C water bath for equilibration before evaluation of progressive motility. Sperm concentration was calculated by a standard curve using a spectrophotometer, following standard commercial bull stud procedures.

Cryopreservation

Extension and cryopreservation of the samples was performed with a standard bovine egg yolk-based Tris extender (Genex commercial extender). The extender was divided into 2 parts, with Fraction-A (without glycerol) and Fraction-B (with 1.4 M glycerol or 12% v/v glycerol). Fraction-A was first used to extend the sperm to twice the desired concentration. The sperm/Fraction-A mixture was then placed in a 37°C water jacket and slowly (0.1°C/minute) cooled to 5°C. Once equilibrated to 5°C, Fraction-B was slowly added to the sperm/Fraction-A mixture in a ratio of 1:1 (v/v). The extended sperm was then placed into 0.5 ml plastic straws (IMV International, Minneapolis, MN) at a concentration of 30×10^6 sperm/straw. The labeled straws were sealed and laid horizontally on racks over liquid nitrogen for freezing. The cooling rate was 15°C/minute from 5°C to -100°C. Straws were then plunged into liquid nitrogen and stored until subsequent evaluation.

Post-Thaw Evaluation

Sperm thawing was conducted by removing a straw of semen and directly placing the frozen straw into a 37°C water bath for 40 seconds. Straws were removed from the water bath and excess water dried off, taking care not to cold shock the sperm. A sample was then placed onto a pre-warmed glass slide and evaluated by phase microscopy. Progressive sperm motility and sperm morphology were assessed on 3 units of sperm from each ejaculate from each bull.

Sperm separation

Cryopreserved epididymal and ejaculated sperm from the same bull was removed from liquid nitrogen storage and thawed. Sperm was then diluted in 500 µl of pre-warmed 2.9% sodium citrate solution. Post-thaw motility was assessed under phase microscope. Sperm was layered on a discontinuous Percoll gradient and centrifuged at 300 x g for 25 minutes. The sperm pellet was isolated and the concentration determined by hemocytometer. The sperm were then resuspended in IVF-TALP to a concentration of 25×10^6 sperm/ml (Parrish et al., 1995).

Oocyte maturation

Bovine oocytes were obtained from a commercial supplier (Trans Ova genetics, MN 56347) in groups of 50 and matured in a portable incubator at 39°C during overnight

travel. Upon arrival to the LSU Embryo Biotechnology Laboratory, shipping vials containing the oocytes were removed from the portable incubator and stored in a 5% CO₂ incubator in humidified air at 39°C.

In vitro fertilization

Following 22 ± 2 hours of in vitro maturation, the cumulus oocyte complexes were washed 4 times through modified glucose-free Tyrodes HEPES medium (Parrish et al., 1988). Cumulus oocyte complexes were then randomly assigned to a treatment group (25 oocytes/treatment), placed in pre-equilibrated 44 µl drop of modified glucose-free Tyrodes fertilization medium (Parrish et al., 1988) covered with mineral oil. Two microliters of heparin (10 µg/ml) or saline, 2 µl of PHE (Miller et al., 1994) and two microliters of ejaculated or epididymal sperm (final concentration of 1×10^6 sperm/ml) were added depending upon treatment. The fertilization drops were placed into a modular incubator (Billups-Rothenberg, Del Mar, Ca) with humidified 5% CO₂, 5% O₂, 90% N blood gas at 39°C and incubated for 18 hours

Fertilization rate

At 18 hours after addition of sperm, oocytes were removed from fertilization medium and their remaining cumulous cells and accessory sperm removed by repeated pipetting through a 135 µm inside diameter pipette tip (Striper[®], Origio MidAtlantic Devices Inc. Mt. Laurel, NJ). Oocytes were then stained with Hoechst 33342 (47 µg/ml) for 30 minutes for pronuclear formation. Following staining excess stain was removed by washing oocytes through 4 droplets of HEPES-TALP. Pronuclear formation was observed using a fluorescent inverted microscope fitted with Hoffman optics at 400 x magnification. Oocytes were then mounted and fixed in 1 part acetic acid and 3 parts ethanol for 48 hours, followed by staining with 1% aceto-orcein to verify the pronuclear formation (Goto et al., 1989).

Embryo culture

Following IVF, presumptive embryos were removed from fertilization medium and their remaining cumulous cells and accessory sperm removed as described above. Embryos were then added to a modified CR1aa day 0 to 3 (Rosenkrans et al., 1993; Rosenkrans and First, 1994) placed in a modular incubator and incubated in humidified 5% CO₂, 5% O₂, 90% N blood gas at 39°C for 3 days. On the third day of culture,

embryo development was assessed and embryos were transferred to CR1aa day 3 to 7 culture medium and incubated in humidified 5% CO₂, 5% O₂, 90% N blood gas at 39°C until day 7. Development to the morula or blastocyst stage was determined on day 7 of embryo culture.

Statistical Analysis

Differences among treatments were determined with ANOVA; Holm-Sidak test was used as a post-ANOVA pairwise multiple comparison. All data were analyzed using Sigma Stat Software Version 3.5. A $P < 0.05$ was considered significant during these studies.

RESULTS

Experiment 7.1.

In vitro fertilization of bovine oocytes following IVF using ejaculated or epididymal sperm with and without heparin were compared and results summarized in Table 7.1. Fertilization rate was significantly ($P < 0.001$) higher for epididymal sperm compared with ejaculated sperm. Ejaculated sperm without heparin had a significantly ($P < 0.001$) lower fertilization rate compared with ejaculated sperm with heparin. Epididymal sperm with and without heparin resulted in similar ($P > 0.05$) fertilization rates.

Experiment 7.2.

In vitro cleavage and embryo development of bovine oocytes/embryos following IVF with ejaculated or epididymal sperm with and without heparin were compared and results are summarized in Table 7.3.

The rate of embryo cleavage following IVF with ejaculated sperm without heparin was significantly lower compared with all other groups ($P < 0.05$). No significant difference ($P > 0.05$) was detected in the rate of cleavage among the epididymal sperm with or without heparin or ejaculated sperm with heparin treatment groups.

The percentage of embryos developing to the 8-cell stage when epididymal sperm was used was significantly influenced by inclusion of heparin. This was noted by significantly fewer ($P < 0.05$) embryos developing to the 8-cell stage when heparin was not included in the fertilization medium. In contrast no significant difference ($P > 0.05$) was noted between ejaculated sperm when heparin was or was not included in the

fertilization medium. In addition, no significant difference ($P>0.05$) was noted between ejaculated or epididymal sperm irrespective of the inclusion of heparin.

Blastocyst development following IVF with ejaculated and epididymal sperm was significantly ($P<0.01$) influenced by the inclusion of heparin in the fertilization medium. No difference ($P>0.05$) in blastocyst development was noted between ejaculated and epididymal sperm when heparin was included in the fertilization medium.

DISCUSSION

Genetically valuable males have the potential to significantly increase the production of agriculturally important species. These genetics can quickly be spread through the population with the use of cryopreserved sperm utilized in assisted reproduction techniques such as AI and IVF. A problem arises when these genetically valuable males become incapacitated due to injury or death. With the loss of the bull or the ability to collect sperm, it will be imperative to salvage the genetics of these males.

Epididymal sperm represents a viable source of sperm, in which genetics can be salvaged and will prove essential in the further dissemination of the genetics from these males. Epididymal sperm represents a “last chance”, so it is of the utmost importance that this sperm be utilized in the most efficient manner possible.

Unlike epididymal sperm, ejaculated sperm has been extensively studied and is routinely cryopreserved and utilized in assisted reproduction techniques. Cryopreserved bovine epididymal sperm was first utilized in artificial insemination by Barker (1954). Subsequent studies determined epididymal sperm to be comparable to ejaculated sperm when utilized in AI (Igboeli and Foote, 1968; Amann and Griel, 1974; Foote, 2000a). The challenge with traditional AI is that 1 straw equals 1 calf at 100% efficiency and we can realistically only expect ~65% pregnancy rates (Demetrio et al., 2007; Schefers et al., 2010; Martinez et al., 2011).

An alternative to AI for the use of bovine epididymal sperm is IVF. In vitro fertilization may allow for the efficient use of this sperm by maximizing the number of subsequent progeny produced (Loskutoff et al., 1995). These experiments were conducted to better understand the difference between ejaculated and epididymal sperm collected from the same mature fertile Holstein bulls in an attempt to better utilize this finite resource.

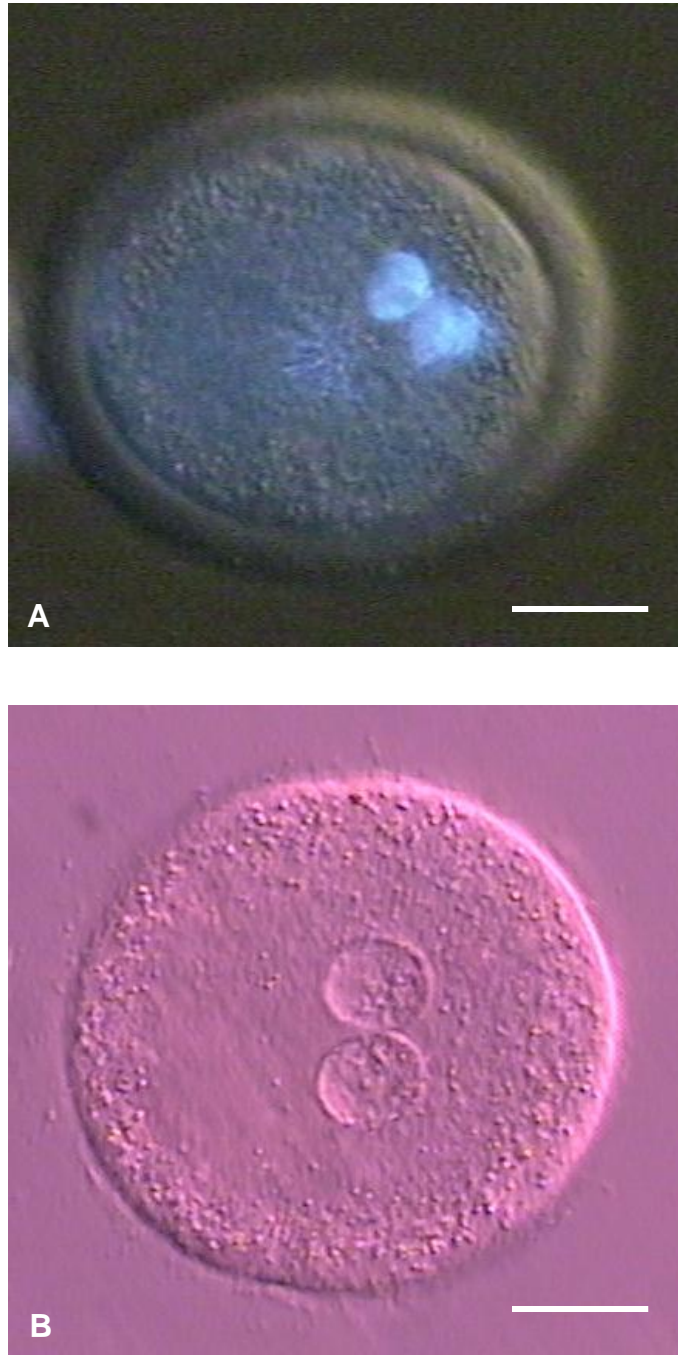


Figure 7.1. Pronuclear staining 18 hours following IVF with ejaculated and epididymal sperm collected from the same Holstein Bulls presumptive embryos were stained with Hoechst 33342 then fixed for 48 hours followed by staining with aceto-orcein. (A) Pronuclei stained with Hoechst 33342. (B) Pronuclei stained with aceto-orcein. Bar = 35 μ m.

Table 7.1. Fertilization rate following IVF with ejaculated or epididymal sperm from the same Holstein bulls with or without heparin.

Collection type	Treatment	n	% Fertilized (\pm SEM)
Ejaculated	No Heparin	180	43.4 \pm 4.3 ^a
Ejaculated	Heparin	170	67.6 \pm 2.4 ^b
Epididymal	No Heparin	170	76.0 \pm 3.6 ^c
Epididymal	Heparin	185	77.9 \pm 1.9 ^c

Fertilization rate assessed at 18 hours post-insemination by fluorescent staining of pronuclei.

^{abc}Mean values with different letters are significantly different ($P < 0.001$).

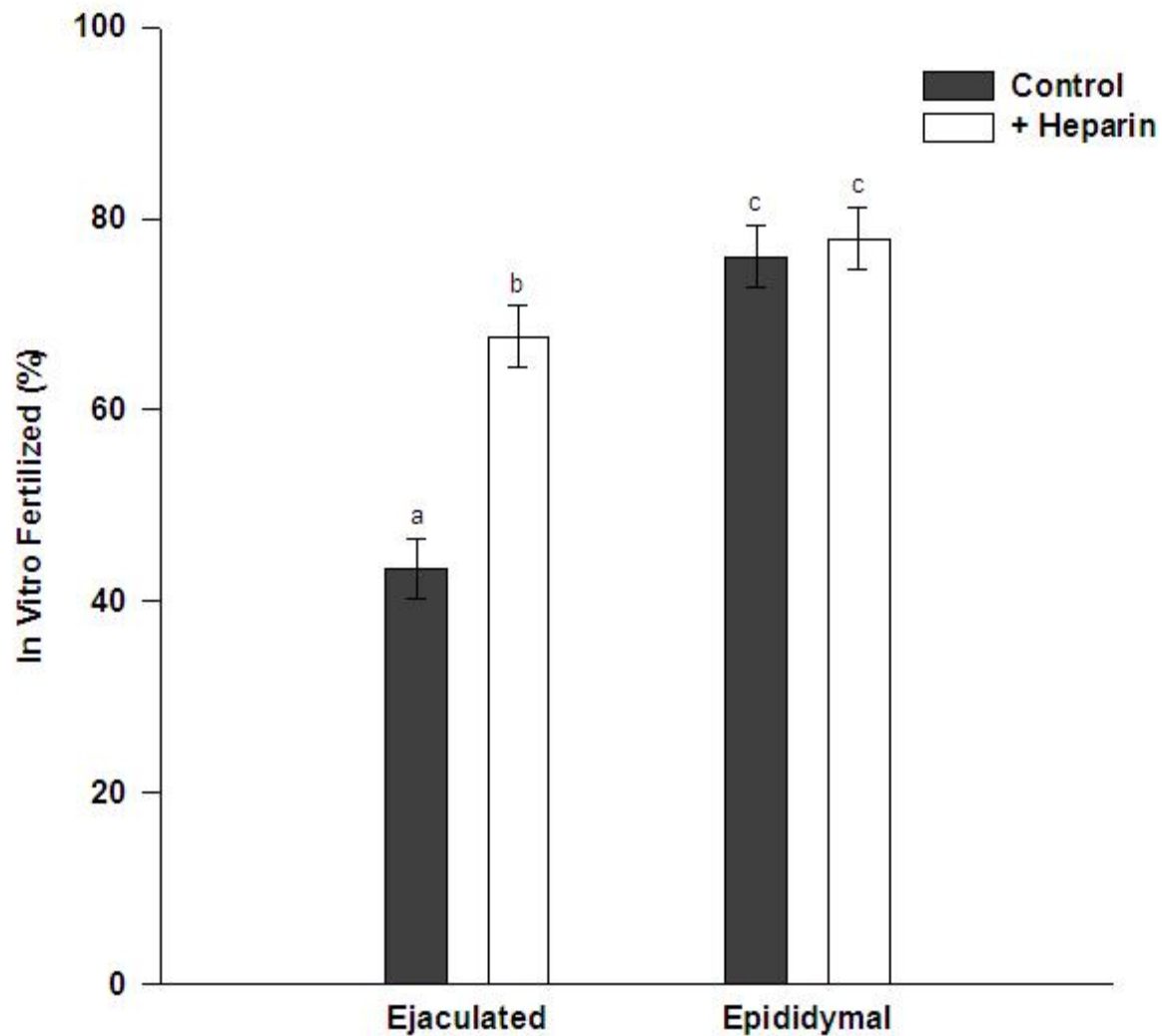


Figure 7.2. In vitro fertilization rate of ejaculated and epididymal sperm with and without heparin. Fertilization was determined by pronuclear staining with Hoechst 33342 at 18 hours post-fertilization followed by evaluation with epifluorescences. ^{abc}Mean values with different letters are significantly different ($P=0.001$).

Table 7.2. Cleavage and embryo development following IVF with ejaculated or epididymal sperm with or without heparin.

Collection type	Treatment	n	*Cleaved [†]	n	*8-Cell [‡]	*Morula [‡]	*Blastocyst [‡]
Ejaculated	No Heparin	305	45.2 ± 4.3 ^a	70	42.5 ± 11.9 ^{ab}	27.3 ± 7.1	9.3 ± 3.2 ^c
Ejaculated	Heparin	305	56.9 ± 3.7 ^b	65	49.2 ± 5.5 ^{ab}	20.1 ± 3.9	23.6 ± 5.5 ^d
Epididymal	No Heparin	305	57.5 ± 4.1 ^b	65	30.0 ± 10.6 ^a	24.3 ± 4.0	2.5 ± 1.6 ^c
Epididymal	Heparin	305	58.1 ± 4.3 ^b	65	63.5 ± 7.3 ^b	27.4 ± 6.9	23.3 ± 6.5 ^d

*Percent ± SEM

[†]Percent cleaved determined from all replicates including bulls A, B, C and D.

[‡]Embryo development determined from bulls A and D.

^{ab}Columns with different letters are significantly different (P<0.05).

^{cd}Columns with different letters are significantly different (P<0.01).

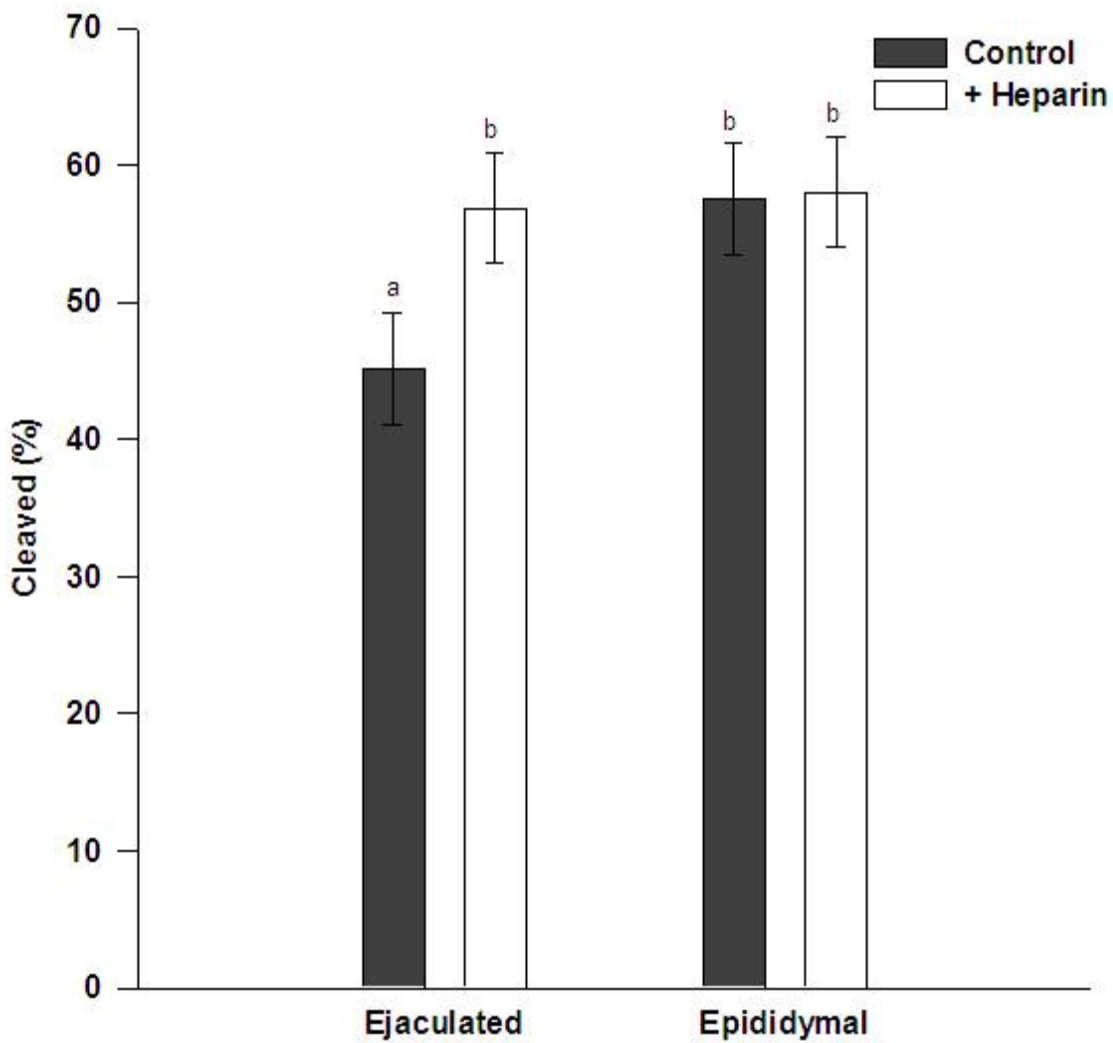


Figure 7.3. Percentage of cleaved oocytes following in vitro fertilization (mean \pm SEM) using ejaculated and epididymal sperm from the same Holstein bulls (n=4) with and without heparin. ^{a,b}Mean values with different letters are significantly different (P>0.05).

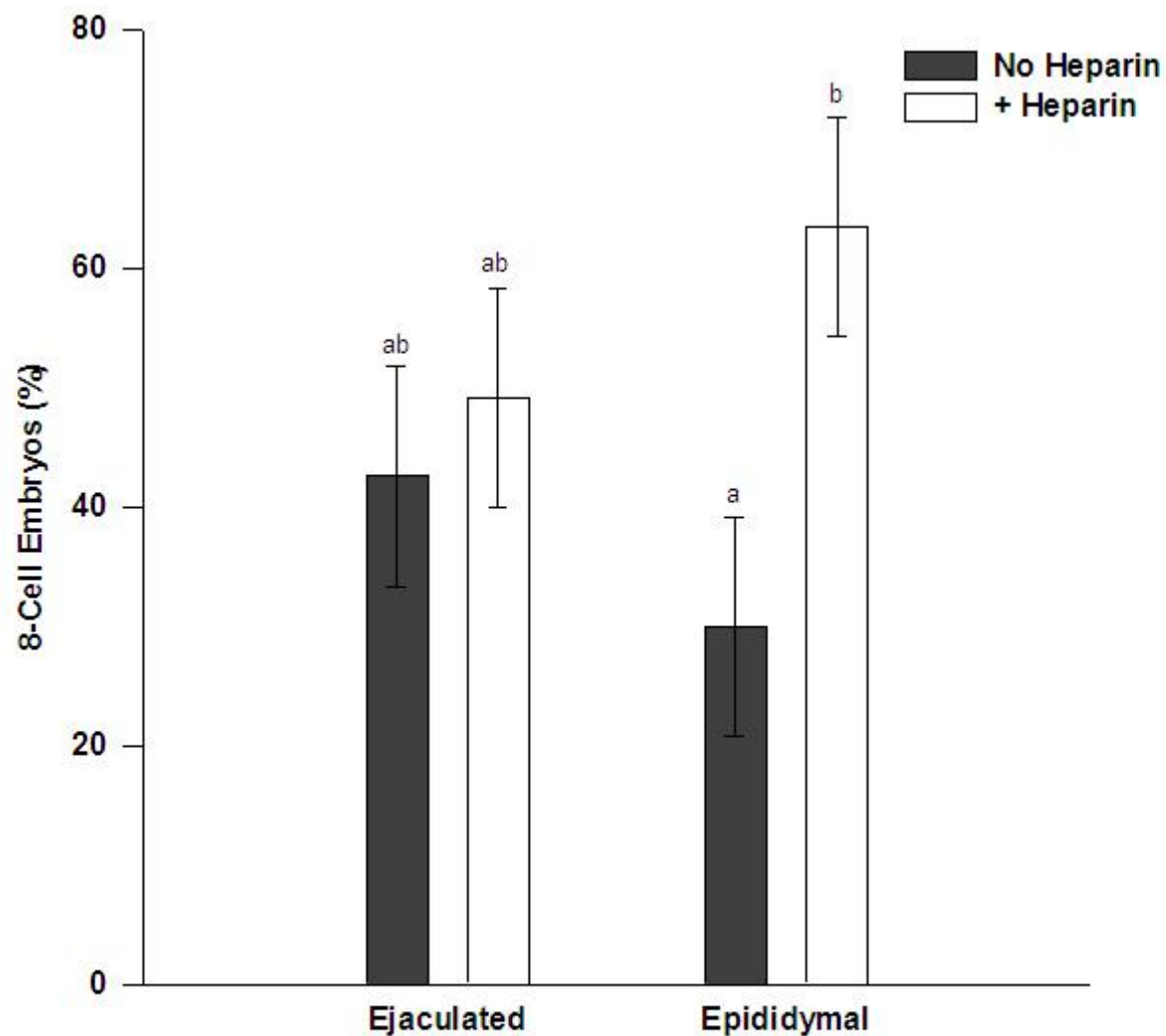


Figure 7.4. Percentage of embryos (mean \pm SEM) developing to the 8-cell stage following in vitro fertilization using ejaculated and epididymal sperm from the same Holstein bulls with and without heparin. ^{ab}Mean values with different letters are significantly different ($P<0.05$).

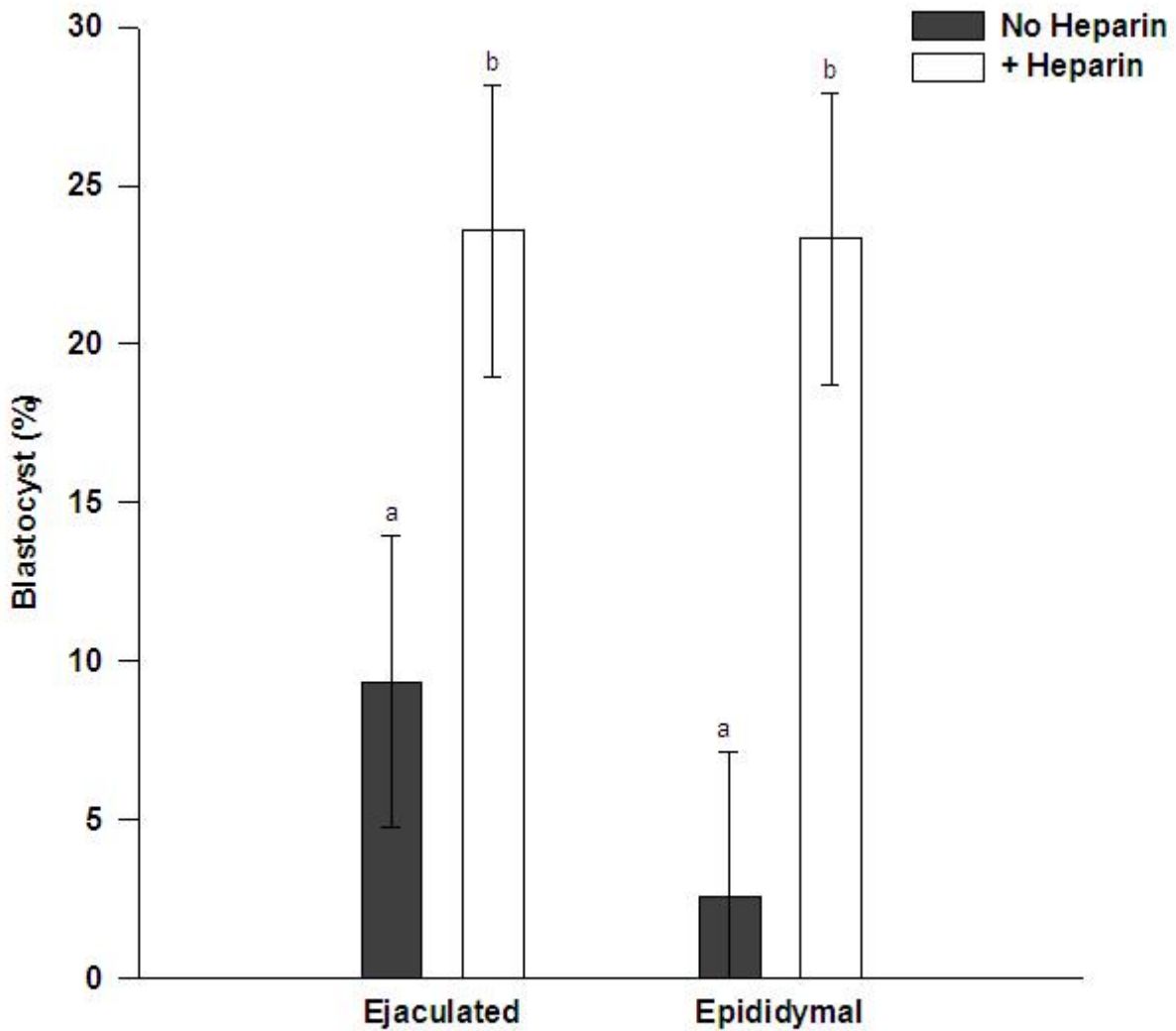


Figure 7.5. Percentage of embryos developing to the blastocyst stage following in vitro fertilization (mean±SEM) using ejaculated or epididymal sperm from the same Holstein bulls with and without heparin. ^{a,b}Mean values with different letters are significantly different ($P<0.01$)

The use of bovine epididymal sperm in IVF was first reported by Ball et al. (1983). Fresh epididymal sperm collected from slaughterhouse bull testes for use in IVF resulted in 40% of the 104 oocytes having both male and female pronuclei. In the same year, Lenz et al. (1983) reported the effects of temperature during IVF using fresh epididymal bull sperm obtained from the slaughterhouse testes. At 39°C (reported optimal temperature) epididymal sperm was noted to penetrate 58% (39/67) of oocytes, with 36% having both male and female pronuclei.

Pavlok et al. (1988) compared fresh epididymal sperm collected from 7 bulls with fresh ejaculated sperm collected from 5 bulls. IVF with 203 oocytes resulted in a fertilization rate of 72% for epididymal sperm compared with 54% for ejaculated sperm. In spite of having a higher fertilization rate, epididymal sperm was reported as having an increased incidence of fertilization anomalies (32.6%) compared with ejaculated sperm (5.4%). These reports established fresh epididymal sperm as a viable source of sperm for use in IVF.

The use of cryopreserved sperm allows for more efficient use of this finite resource. Goto et al. (1989) reported the use of cryopreserved epididymal sperm from 5 bulls for IVF. Fertilization rate (having both male and female pronuclei) averaged 57% following IVF of 968 oocytes using cryopreserved epididymal sperm. Of the 2612 embryos cultured, development to the 8-cell stage was reported to range from 21% to 31% on days 3 to 4 and a blastocyst rate of 9.3% to 12% on days 7 to 8 post-insemination. It was proposed that individual variation among bulls was not a significant factor in the fertilization and development rates of bovine follicular oocytes when using epididymal sperm.

Katska et al. (1996) compared the effects of performing IVF with cryopreserved epididymal sperm (988 oocytes) or ejaculated sperm with or without the removal of seminal plasma at the time of collection (741 oocytes). Epididymal sperm was collected from the testes of 7 slaughterhouse bulls and ejaculated sperm was collected from 4 bulls of known fertility. Frozen-thawed epididymal sperm and ejaculated sperm collected with or without the removal of seminal plasma at the time of collection were compared as to their fertilization rate (84%, 63% and 50%, respectively), cleavage (80%, 52% and 40%, respectively) and blastocyst rate (31%, 16% and 11%, respectively). It was

recommended that seminal plasma be removed from ejaculated bull sperm prior to cryopreservation if sperm was to be used for IVF.

Martins et al. (2007) performed IVF with cryopreserved epididymal sperm from 3 bulls along with ejaculated sperm from a fourth bull of known in vitro fertility. Epididymal sperm cleavage (80%) and blastocyst (35%) rates were similar to ejaculated sperm cleavage (89%) and blastocyst (57%) rates. Variation was noted between bulls, with epididymal sperm from 1 of the 3 bulls noted as having lower cleavage (42%) and blastocyst (26%) rates following IVF. These reports showed that cryopreserved epididymal sperm are similar to ejaculated sperm when used for bovine IVF.

These results were also noted in the boar (Rath and Niemann, 1997; Matás et al., 2010), goat (Blash et al., 2000) and Wood bison (Thundathil et al., 2007). Rath and Niemann (1997), compared ejaculated and epididymal sperm collected from the same boars in IVF. When evaluated side by side, frozen-thawed epididymal sperm fertilized (pronuclear formation) significantly more of the 161 oocytes (23%) compared with either fresh (8.9%) or frozen-thawed (2.2%) ejaculated boar sperm collected from the same boars. These results were also confirmed by embryo development of the 485 oocytes with cleavage (≥ 2 cell) for epididymal sperm (59%) compared with fresh (14.6%) and frozen-thawed (16%) ejaculated sperm. Matás et al. (2010) also noted that over all treatments studied fresh epididymal boar sperm had better oocyte ($n=716$) penetration rates at 4 hours post-insemination (86.4%) compared with ejaculated sperm (45.3%) following IVF. In the goat, Blash et al. (2000) noted the cleavage and blastocyst development following IVF (oocytes $n=337$) using cryopreserved ejaculated (37% and 4%) compared with epididymal sperm (40% and 6%) to be similar.

Thundathil et al. (2007) utilized Wood bison cryopreserved ejaculated sperm and epididymal sperm stored at 4°C for 24 hours for IVF. Ejaculated and epididymal sperm were compared as to their ability to fertilize oocytes ($n=160$) and the subsequent embryo development in vitro. Utilizing ovaries collected from postmortem Wood bison cows, it was noted that the fertilization rate and development to the blastocyst stage of embryos fertilized with ejaculated sperm (64.4% and 7.5%, respectively) were lower compared with epididymal sperm (89.2% and 10%, respectively).

In our study, we utilized ejaculated and epididymal sperm collected from the same Holstein bulls. We noted that ejaculated and epididymal sperm displayed no significant difference in their cleavage (56.9% and 58.1%, respectively) or blastocyst rates (23.6% and 23.3%, respectively) following IVF with heparin (Table 5.3.). Using our blastocyst rates and experience in IVF, we can approximate the effectiveness of the IVF system in producing future progeny. In preparation for IVF, a viable sperm population is isolated from a straw of cryopreserved epididymal sperm. We can prepare ~300 μ l of sperm fertilization suspension at a concentration of 25×10^6 /ml resulting in ~150, 2 μ l fertilization doses'. During IVF, 10 oocytes are added to 48 μ l of fertilization medium along with 2 μ l of sperm fertilization suspension. This results in one straw of epididymal sperm having the ability to fertilize 1,500 oocytes. At a blastocyst rate of 23%, we would expect ~345 blastocyst. If 10% of these blastocysts are carried full term, this would result in the production of ~35 calves per straw of cryopreserved sperm.

We also compared ejaculated and epididymal sperm collected from the same Holstein bulls following IVF with and without the capacitating agent heparin in the fertilization medium. The glycosaminoglycan heparin is a known inducer of capacitation in bovine ejaculated sperm (Parrish et al., 1985; Parrish et al., 1988). As seen in Table 5.3, the inclusion of heparin is essential to embryo development when both ejaculated or epididymal sperm are used for IVF. With the significant differences between groups with and without heparin beginning at the 8-cell stage and is evident at the blastocyst stage.

In conclusion, epididymal sperm from a genetically superior male is a valuable and finite resource that should be utilized in the most efficient manner possible. New technology utilizing ultrasound makes it possible to aspirate multiple oocytes from the follicles of a single donor for use in IVF (TUGA + IVF). One straw of cryopreserved epididymal sperm has the ability to fertilize thousands of oocytes resulting in the production of numerous embryos. These embryos can be transferred to recipient females increasing the production of offspring. The TUGA + IVF embryo production system is already commercially available utilizing ejaculated sperm and has reported improved embryo production over traditional embryo transfer and artificial insemination. Our studies have shown that epididymal sperm is viable and able to endure

cryopreservation, in vitro culture and fertilize oocytes in vitro comparable to ejaculated sperm collected from the same bulls utilizing current protocols. The ability of cryopreserved epididymal sperm to be utilized in the TUGA + IVF embryo production system could greatly increase the production potential of epididymal sperm collected from genetically valuable bulls following a debilitating injury or death.

CHAPTER VIII

SUMMARY AND CONCLUSION

The primary objectives of these experiments were to compare ejaculated and epididymal sperm collected from the same Holstein bulls. These experiments included the collection, cryopreservation, in vitro culture, in vitro fertilization and subsequent embryo development.

In the first experiment, ejaculated and epididymal sperm collected from the same Holstein bulls were collected and compared before and after cryopreservation and as to their membrane permeability during cryopreservation. The theoretical optimal cooling rate was also calculated. Results indicate that the concentration of sperm following epididymal collection was significantly greater than the concentration of sperm collected by ejaculation. Epididymal sperm total motility was also greater than ejaculated sperm following cryopreservation. During cryopreservation, a lack of distinction was noted between ejaculated and epididymal sperm collected from the same bulls in their water transport response using the DSC technique. The optimal cooling rate for bovine ejaculated and epididymal sperm was calculated to be between 50 and 60°C/minute. We concluded that epididymal sperm is better able to endure cryopreservation compared with ejaculated sperm collected from the same bulls. Ejaculated and epididymal bull sperm collected from the same bulls can be cryopreserved at the same rate.

In the second experiment, the effects of castration on the circulating concentration of plasma testosterone and cholesterol were determined in Holstein bulls. As expected, the concentration of testosterone significantly decreased following castration. Our results also indicated that the concentration of circulating plasma cholesterol significantly increased following castration. Taken together, we have demonstrated that the concentration of cholesterol in the bull can be influenced by testosterone.

In the third experiment, ejaculated and epididymal sperm collected from the same mature Holstein bulls were compared post-thaw and also during a 6-hour in vitro culture with varying concentrations of heparin as to their viability, percentage of capacitation and acrosomal integrity. Ejaculated sperm had significantly more acrosome

reacted sperm post-thaw compared to epididymal sperm but no difference was found in their percentage of capacitation.

During in vitro culture, heparin did not significantly increase the percentage of auto-acrosome reaction between ejaculated or epididymal sperm, although ejaculated sperm was significantly higher than epididymal sperm. Ejaculated sperm also did not significantly increase in its percentage of auto-acrosome reaction over time in culture, as was noted in epididymal sperm.

The percentage of nonviable acrosome intact ejaculated and epididymal sperm was also not significantly affected by the inclusion of heparin during in vitro culture, but the percentage of nonviable acrosome intact ejaculated sperm was significantly greater than epididymal sperm. We also noted a significant increase in the percentage of nonviable acrosome intact epididymal sperm over the 6-hour in vitro culture, ejaculated sperm also increased over time but was only significantly different from 2 to 4 hours of culture.

The percentage of viable acrosome intact ejaculated or epididymal sperm was not significantly affected within hour by the inclusion or concentration of heparin, but all groups that included heparin did have reduced percentages of viable acrosome intact sperm. When ejaculated and epididymal sperm were compared as to their percentage of viable acrosome intact sperm, epididymal sperm was significantly greater than ejaculated sperm at 2, 4 and 6 hours of in vitro culture. A significant decrease was noted in the percentage of viable acrosome intact epididymal sperm at 2, 4 and 6 hours, but ejaculated sperm only significantly decreased from 2 to 4 hours of in vitro culture.

The percentage of in vitro capacitation was compared among ejaculated and epididymal treatment groups. The concentration of heparin was unable to significantly increase the percentage of capacitation in either ejaculated or epididymal sperm. Ejaculated sperm had significantly more capacitated sperm when compared with epididymal sperm at 2, 4 and 6 hours of in vitro culture. This was in contrast to epididymal sperm that did not significantly increase in the percentage of capacitation during the 6 hours of culture.

In the fourth experiment, cryopreserved Holstein sperm was evaluated following exposure to peptide preimplantation factor (PIF). The viability, acrosomal integrity and

capacitation; also the ability of PIF to affect the ability of sperm to fertilize oocytes in vitro and their subsequent embryo development were determined.

The effects of PIF and heparin were compared by percentages of sperm, which displayed the characteristic “B” pattern (capacitated), as determined by the CTC assay. Results indicate that, PIF has no ability to enhance or suppress the percentage of in vitro capacitation of cryopreserved bovine ejaculated sperm. Although, heparin did significantly increase the percentage of capacitated sperm regardless of whether PIF was included or not in the culture medium.

No significant effect on the percentage of sperm viability, acrosome integrity or percentage of capacitation during 6 hours of in vitro culture were noted following exposure of sperm to PIF as determined by flow cytometry. The ability of heparin to induce a response in ejaculated bovine cryopreserved sperm was also unaffected by exposure to PIF.

The in vitro fertility of cryopreserved bull sperm following exposure to PIF compared with heparin when added to IVF medium on embryo cleavage and subsequent development were determined. Results showed that the percentage of oocytes that cleaved was unaffected by the inclusion of PIF or heparin. The percentage of embryos, which developed to the 8-cell and blastocyst stage were also unaffected by the addition of PIF. Although, the inclusion of heparin in the fertilization medium did significantly increase the percentage of embryos that developed to the 8-cell and blastocyst stage.

In the final experiment, ejaculated and epididymal sperm collected from the same Holstein bulls were compared by their ability to fertilize oocytes in vitro with and without heparin and their subsequent embryo development. Ejaculated sperm had a reduced ability to fertilize oocytes and cleave when heparin was not included in the fertilization medium, although epididymal sperm was unaffected by the removal of heparin. Embryo development of both ejaculated and epididymal sperm was significantly affected by the inclusion of heparin in the fertilization medium, with both having significantly lower blastocyst development when heparin was removed from the fertilization medium. Results indicate ejaculated and epididymal sperm are able to fertilize oocytes in vitro

and that the inclusion of heparin is necessary during IVF with both ejaculated and epididymal sperm for the development of blastocysts.

We conclude that ejaculated and epididymal sperm can be collected from the same genetically valuable males and cryopreserved in a similar manner for use in the in vitro production of embryos. Epididymal sperm are better able to endure cryopreservation compared to ejaculated sperm. Although, epididymal sperm may have a delay in becoming capacitated they are unaffected in their ability to fertilize oocytes in vitro compared with ejaculated sperm. The inclusion of heparin during IVF is essential to the production of blastocysts using both ejaculated and epididymal sperm. We also conclude that PIF was unable to increase the viability, induce or inhibit the acrosome reaction or percentage of capacitation in Holstein cryopreserved sperm during in vitro culture. PIF was also unable to influence the effects of heparin on cryopreserved bovine sperm. This is the first report as to the effects of PIF on sperm during in vitro culture.

Discarding epididymal sperm from an injured or deceased animal is a loss of not only great genetic value but also economic value. Due to the known variability among males, it was important to compare ejaculated and epididymal sperm collected from the same males to minimize such issues. This research will allow for increased efficiency and better utilization of epididymal sperm in producing progeny of high genetic value that would otherwise be lost.

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APPENDIX I

CR1 Stock

Component	Source	mM	Amount 100ml
NaCl	Sigma S-5886	114	670 mg
KCl	Sigma P-5405	3.1	23.1 mg
NaHCO ₃	Sigma S-8875	26	220 mg
Lactic Acid	Sigma L-4388	2.5	54.6 mg
Phenol Red	Sigma P-0290	---	100 µl
Milli Pore Water	Synergy UV	---	To 100 ml

(Modified from Rosenkrans et al.,1993 and Bondioli and Moore,1993)

Sterile filter (0.2 µm) store at 4°C up to 3 months.

Osmolarity 270-280 osmol/L.

CR1aa Embryo Culture Medium (Day 0 through Day 3)

Component	Source	mM	Amount/ 10ml
CR1 Stock	Lab Stock	---	9.3 ml
BME aa (50X)	Sigma B-6766	---	200 µl
MEM ne aa (100X)	Sigma M-7145	---	100 µl
Glycine Stock (100X)	Sigma G-8790	10.0	100 µl
Alanine Stock (100X)	Sigma A-7469	1.0	100 µl
L-Glutamine Stock (100X)	Sigma G-8540	1.0	100 µl
Pyruvic Acid	Lab Stock	0.4	200 µl
Penn/Strep	Gibco 15140-122	---	100 µl
BSA Fraction V	Sigma A-4503	---	60 mg

Prepare day of use, sterile filter (0.2 µm).

Equilibrate to 39°C in 5% CO₂ atmosphere 30 min prior to addition of embryos.

APPENDIX II

CR1aa Embryo Culture Medium (Day 3 through Day 7)

Component	Source	mM	Amount/ 10ml
CR1 Stock	Lab Stock	---	8.8 ml
BME aa (50X)	Sigma B-6766	---	200 μ l
MEM ne aa (100X)	Sigma M-7145	---	100 μ l
Glycine Stock (100X)	Sigma G-8790	10.0	100 μ l
Alanine Stock (100X)	Sigma A-7469	1.0	100 μ l
L-Glutamine Stock (100X)	Sigma G-8540	1.0	100 μ l
Pyruvic Acid	Lab Stock	0.4	200 μ l
Penn/Strep	Gibco 15140-122	---	100 μ l
Calf Serum	Hyclone 30073	---	500 μ l
BSA Fraction V	Sigma A-4503	---	60 mg

Prepare day of use, sterile filter (0.2 μ m).

Equilibrate to 39°C in 5% CO₂ atmosphere 30 min prior to addition of embryos.

10X Sperm-TALP Stock

Component	Source	mM	Amount/ 50ml
KCl	Sigma P-5405	31	115.56 mg
NaCl	Sigma S-5886	800	2.34 g
NaH ₂ PO ₄	Sigma S-5011	3	21.3 mg
HEPES	Sigma H-3375	100	1.19 g

Adjust pH to 7.3, sterile filter (0.2 μ m) and store at 4°C.

(Parrish et al.,1995)

APPENDIX III

45% Percoll Gradient

Component	Source	Unit	Concentration
90% Percoll	Lab Stock	ml	2
Sperm-TALP	Lab Stock	ml	2
Prepare day of use.			(Parrish et al.,1995)

90% Percoll Gradient

Component	Source	mM	Amount/ 40 ml
Percoll	Sigma P-4937	---	36 ml
10X Sperm-TALP Stock	Lab Stock	---	4 ml
CaCl ₂ **	Sigma C-7902	2.0	11.76 mg
MgCl ₂ **	Sigma M-2393	0.4	3.24 mg
Lactic acid**	Sigma L-7900	21.6	134.5 µl
NaHCO ₃ **	Sigma S-8875	25.0	84 mg

**Added after Percoll and 10X Sperm-TALP. (Parrish et al.,1995)

Store at 4°C.

2.9% Sodium Citrate Buffer

Component	Source	mM	Amount/ 200 ml
Sodium Citrate	Sigma C-3434	99	5.8 g
Milli Q Water	Synergy UV	---	To 200 ml

Sterile filter (0.2 µm) and store at 4°C for 3 months.

APPENDIX IV

Sperm-TL Stock

Component	Source	mM	Amount/ 50 ml
NaCl	Sigma S-5886	100.0	292.2 mg
KCl	Sigma P-5405	3.1	11.56 mg
NaHCO ₃	Sigma S-8875	25.0	105.01 mg
NaH ₂ PO ₄	Sigma S-5011	0.3	1.8 mg
HEPES	Sigma H-3375	10.0	119.16 mg
Lactic Acid	Sigma L-7900	21.6	168.75 µl
CaCl ₂ -2H ₂ O**	Sigma C-7902	2.0	14.7 mg
MgCl ₂ -6H ₂ O**	Sigma M-2393	0.4	4.07 mg
Phenol Red	Sigma P-0290	---	50 µl

**Mix separately before addition to solution. (Modified from Parrish et al., 1988)

Adjust pH to 7.4, Osmolarity 270-300 osmol/L,
Sterile filter (0.2 µm) and store at 4°C up to 1 month.

HEPES-TL Stock

Component	Source	mM	Amount/ 50 ml
NaCl	Sigma S-5886	114.0	333.12 mg
KCl	Sigma P-5405	3.1	11.56 mg
NaHCO ₃	Sigma S-8875	2.0	8.01 mg
NaH ₂ PO ₄	Sigma S-5011	0.3	1.8 mg
HEPES	Sigma H-3375	10.0	119.16 mg
Lactic Acid	Sigma L-7900	10.0	78.13 µl
CaCl ₂ -2H ₂ O**	Sigma C-7902	2.0	14.7 mg
MgCl ₂ -6H ₂ O**	Sigma M-2393	0.5	5.08mg
Phenol Red	Sigma P-0290	---	50 µl

**Mix separately before addition to solution. (Modified from Parrish et al., 1988)

Adjust pH to 7.4, sterile filter (0.2 µm) and store at 4°C up to 1 month.

APPENDIX V

IVF-TL Stock

Component	Source	mM	Amount/ 50 ml
NaCl	Sigma S-5886	114.0	333.12 mg
KCl	Sigma P-5405	3.2	11.56 mg
NaHCO ₃	Sigma S-8875	25.0	8.01 mg
NaH ₂ PO ₄	Sigma S-5011	0.3	1.8 mg
Lactic Acid	Sigma L-7900	10.0	78.13 µl
CaCl ₂ -2H ₂ O**	Sigma C-7902	2.0	14.7 mg
MgCl ₂ -6H ₂ O**	Sigma M-2393	0.5	5.08mg
Phenol Red	Sigma P-0290	---	50 µl

**Mix separately before addition to solution. (Modified from Parrish et al., 1988)

Adjust pH to 7.4, sterile filter (0.2 µm) and store at 4°C up to 1 month.

TALP media

Component	Source	Sperm-TALP*	HEPES-TALP*	IVF-TALP**
TL-Stock	Lab Stock	9.5 ml	9.9 ml	9.9 ml
Pyruvate	Lab Stock	500 µl	100 µl	100 µl
BSA	Sigma A-4503	60 mg	30 mg	---
BSA	Sigma A-6003	---	---	60 mg
Pen/Strep	Gibco 15140-122	100 µl	100 µl	100 µl

Prepare day of use, sterile filter (0.2 µm). (Modified from Parrish et al., 1988)

*Warm to 39°C in air prior to addition of gametes.

**Equilibrate to 39°C in 5% CO₂ atmosphere 30 min prior to addition of gametes.

APPENDIX VI

PHE Stock

Component	Source	mM	Amount/ 10 ml
Penicillamine	Lab Stock	2.0	5 ml
Hypotaurine	Lab Stock	1.0	5 ml
Epinephrine	Lab Stock	250	2 ml
Saline	Lab Stock	---	8 ml

Light sensitive, store at -20°C indefinitely.

APPENDIX VII

Laboratory Stock Solutions:

1. **BME amino acid solution (50X):** Sigma B-6766, Aliquot ~200 µl of freshly purchased solution and store at -80°C until use.
2. **MEM Non-Essential amino acid solution (100X):** Sigma M-7145, Aliquot ~200 µl of freshly purchased solution and store at -80°C until use.
3. **Glycine (100X):** Sigma G-8790, Dissolve 750 mg glycine to a final volume of 10 ml in CR1 stock. Aliquot ~200 µl and store at -20°C.
4. **Alanine (100X):** Sigma A-7469, Dissolve 80 mg alanine to a final volume of 10 ml in CR1 stock. Aliquot ~200 µl and store at -20°C.
5. **Glutamine (100X):** Sigma G-5763, Dissolve 146 mg L-Glutamine to a final volume of 10 ml in CR1 stock. Aliquot ~200 µl and store at -20°C.
6. **Na Pyruvate:** Sigma P-4562, Dissolve 22 mg of sodium pyruvate to a final volume of 10 ml in Mili Pore Water. Sterile filter (2 µm) into an aluminum-foil wrapped 15 ml polystyrene conical tube and store at 4°C for up to a week.
7. **Heparin:** Sigma H-3149, Dissolve 1 mg heparin to a final volume 5 ml in PBS without Ca and Mg. Aliquot ~200 µl store at -20°C.
8. **Hyaluronidase:** Sigma H-3506, Dissolve 10 mg hyaluronidase to a final volume of 10 ml in HEPES-TALP and adjust pH to 7.4. Aliquot ~1 ml and store at -80°C.
9. **Pen/Strep:** Gibco15140-122, Aliquot ~500 µl of freshly purchased solution and store at -20°C until use.
10. **Penicillamine:** Sigma P-4875, Dissolve 3 mg penicillamine to a final volume of 10 ml in saline.
11. **Hypotaurine:** Sigma H-1384, Dissolve 1.09 mg hypotaurine to a final volume of 10 ml in saline.
12. **Epinephrine:** Sigma E-1635, Dissolve 1.83 mg epinephrine to a final volume of 40 ml in Epinephrine diluent stock. Protect from light.
13. **Epinephrine Diluent:** Sigma S-9000 and L-7900, Dissolve 165 µl Na-lactate syrup and 50 mg of Na metabisulfite to a final volume of 50 ml in Mili Pore Water.

- 14. Lysophosphatidylcholine (LPC) Stock:** Sigma L-4129, Dissolve 100 mg lysophosphatidylcholine to a final volume of 10 ml in methanol (100µg LPC/10µl). Aliquot 50 µl store at -20°C.
- 15. Lysophosphatidylcholine Working Solution:** Evaporate 60 µl of LPC stock with nitrogen. Reconstitute LPC with 600 µl of sodium citrate; add 10 µl LPC/ ml of sample.
- 16. FITC-PNA Lectin from *Arachis hypogaea*:** Sigma L-7381, Dissolve 2 mg PNA to a final volume of 4 ml in PBS without Ca and Mg. Aliquot 1 ml store at -20°C.
- 17. Propidium Iodide:** Sigma P-4170, Dissolve 10 mg Propidium Iodide to a final volume of 6.23 ml in PBS without Ca and Mg. Aliquot 1ml store at -20°C.
- 18. Aceto-Orcein Stain:** Sigma O-7380, Dissolve 166.7 mg orcein powder in 9.15 ml boiling glacial acetic acid followed by carefully diluting in 15 ml Mili Pore Water. Solution store at room temperature and filter before use.
- 19. Acid Alcohol Fixative:** Sigma M-1775 & A-6283, Mix methanol and glacial acetic acid (3:1) into sealable glass container and store at room temperature.
- 20. Hoechst Stock:** Sigma B-2261, Dissolve 5.0 mg Hoechst to a final volume of 5 ml of PBS (without Ca and Mg). Aliquot ~15 µl and store at -80°C protected from light.
- 21. Hoechst Stain:** Sigma B-2261, Mix 14 µl Hoechst stock and 300 µl HEPES-TALP.
- 22. Saline:** Sigma S-5886, Dissolve 9 g NaCl to a final volume of 1000 ml in Mili Pore Water.

APPENDIX VIII

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VITA

Michael Alan Stout grew up in Erath, Louisiana. His parents are Mr. William Stout and Mrs. Linda Stout. Michael also has one sister and two brothers. His sister, Tellie Stout is a surgical nurse at Kaplan General Hospital. He has two brothers, Robert and John. Robert is a registered nurse and is currently attending Our Lady of the Lake in Baton Rouge pursuing a Masters of Nursing degree in anesthesia. John is an air-conditioning technician for Butcher Air Conditioning in Lafayette Louisiana.

Michael attended public school in Erath and was an active 4-H member, showing Red Brangus cattle. He was also an officer in the United Red Brangus Association.

Following graduation from High School, Michael attended the University of Louisiana at Lafayette from 1999-2004. While at ULL, he worked on the Desoto Cattle Project with Dr. Terry Clements and on a water quality project at the ULL Cade Farm with Dr. Lora Lana Goodeaux. Michael was a member of Alpha Zeta honor society, serving as treasurer for the 2002-2003 school year.

Michael attended Graduate School at Louisiana State University from 2005-2006. He studied Reproductive Physiology in pursuit of a Master of Science under the supervision of Dr. John Chandler in the Department of Dairy Science. While attending LSU, Michael worked in various capacities at Genex Cooperative. In 2006, Michael graduated Master of Science from LSU.

Upon attainment of his Master of Science degree, Michael served as interim agriculture teacher at Erath High School in 2007.

Michael attended the Graduate School at Louisiana State University from 2007-2012. He studied Reproductive Physiology in pursuit of a PhD under the direction of Dr. Robert A. Godke, Boyd Professor in the School of Animal Sciences. His research was conducted at the LSU Reproductive Biology Center, Embryo Biotechnology Laboratory and at Genex Cooperative. While at LSU, Michael was inducted into Gamma Sigma Delta honor society and Phi Kappa Phi honor society.

During this time, Michael served as a Wildlife Enforcement Agent, participating in the BP oil spill as a member of the Endangered Sea Turtle Rescue Team.

In 2010, Michael married Christine L. Boudreaux of Abbeville Louisiana, who has since given birth to a son, Everett James Stout.