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# Effects of Cryopreservation and Constituents of Semen Extenders on Mitochondrial Function of Bull Spermatozoa

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**EFFECTS OF CRYOPRESERVATION AND CONSTITUENTS OF SEMEN  
EXTENDERS ON MITOCHONDRIAL FUNCTION OF BULL SPERMATOZOA**

**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 Interdepartmental Program in Animal and Dairy Sciences**

**by  
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## LIST OF ABBREVIATIONS

AI-artificial insemination	RDR-resazurin reduction rate
RNA- ribonucleic acid	TM-total motility
mRNA-messenger RNA	PM-progressive motility
tRNA-transfer RNA	VAP-pathway velocity
DNA-deoxyribonucleic acid	PV-progressive velocity
MtDNA-mitochondrial DNA	VCL-curvilinear velocity
nDNA-nuclear DNA	PCR-polymerase chain reaction
OXPPOS-oxidative phosphorylation	GAPDH-glyceraldehyde-phosphate-dehydrogenase
ETC-electron transfer chain	AA-amino acid
ATP-adenosine triphosphate	ND1-NADH dehydrogenase subunit 1
GTP-guanidine triphosphate	ANOVA-analysis of variance
ADP-adenosine diphosphate	SE-standard error
P <sub>i</sub> -phosphate	LSM-least square mean
NAD-nicotinamide adenine dinucleotide	CytoCox-cytochrome c oxidase
FMN-flavin mononucleotide	CS-citrate synthase
FAD-flavin adenine dinucleotide	IOMM-integrity outer mitochondrial membrane
acyl-CoA-Acetyl-coenzyme A	IIMM- integrity inner mitochondrial membrane
ETF-electron transferring flavoprotein	nm-nanometer
LDL-low density lipoprotein	ng-nanogram
Linco-spectin-lincomycin-spectinomycin	mL-milliliter
CASA-computer assisted motility analysis system	nmole-nano mole
CP-Choline	bp-base pairs
EP-ethanolamine phosphoglycerides	NAAB-National Association of Animal Breeders
Bov-bovine	cm-centimeter
TAE-tris-acetate/EDTA	
V-volt	
RD-resazurin reduction	
µg-microgram	
mole/L-molar	
µg – microgram	
mg – milligram	
dl-deciliter	
IU – international unit	
min – minute	
g – gravity	
H <sup>+</sup> -hydrogen ion, proton	
vol/vol-volume by volume	
wt/vol-weight by volume pmole-pico mole	
%-percentage	
L-liter	
DTT- dithithreitol	
U-unit	
Min-minute	

## ABSTRACT

This study investigated the effects of semen extender constituents and cryopreservation on bovine spermatozoal mitochondrial function. Three yearling Holstein bulls were used. Two ejaculates per bull were collected and pooled on a weekly basis for five weeks and extended in four treatments: 1) sodium citrate egg yolk extender with antibiotics (lincomycin, spectinomycin, gentamicin and tylosin); 2) “1” with glycerol; 3) “2” without antibiotics; and 4) “1” without antibiotics. Each was divided into portions for analyses before freezing and after cryopreservation. The pre-freeze and thawed semen were transferred to a 37°C water bath, the same assays were performed. In assay 1, resazurin reduction (RD) was measured spectrophotometrically at sequential 25 minute intervals for 125 minutes. In assay 2, specific activities of cytochrome c oxidase (CytoCox) and citrate synthase (CS) were measured spectrophotometrically immediately post-thaw and after 125 minutes of incubation. In assay 3, ATP was measured using luciferin-luciferase assay simultaneously with RD. Total and progressive motilities (TM and PM), progressive (PV), curvilinear (VCL) and pathway (VAP) velocities were measured simultaneously with RD, ATP content and CytoCox and CS using computer assisted semen analysis system (CASA). In assay 4, the NADH dehydrogenase (ND1) gene of mtDNA was sequenced before and after cryopreservation using PCR. Data were analyzed by least square methods; mean differences were delineated by Tukey’s test.

In assay 1, RD differed among treatments ( $P < 0.05$ ). Cryopreservation decreased ( $P < 0.05$ ) RD, TM, PM, PV, VAP and VCL. Resazurin reduction correlated with PM ( $r = 0.45$ ,  $P < 0.05$ ) and TM ( $r = 0.2$ ,  $P < 0.05$ ). In assay 2, incubation time and incubation with Triton X100 were sources of variation in CytoCox and CS specific activities ( $P > 0.05$ ). Only CS from spermatozoa incubated with Triton X100 correlated with RD ( $r = 0.22$ ,  $P < 0.05$ ). CytoCox and CS did not

correlate with motility parameters. In assay 3, spermatozoal ATP was not different ( $P>0.05$ ) among treatments. However, cryopreservation decreased ( $P<0.05$ ) ATP. Spermatozoal ATP correlated with motility parameters ( $r\geq 0.65$ ) and RD ( $r=-0.30$ ) ( $P<0.05$ ). In assay 4, the frequency of amino acid change was higher ( $P<0.05$ ) post-thaw in the treatment containing only antibiotics. Cryopreservation, more than extender constituents impacted mitochondrial function of bovine spermatozoa.

## CHAPTER 1

### GENERAL INTRODUCTION

Sperm mitochondria, which are located in the midpiece (Bahr and Engler, 1970; Alcivar et al, 1989), must produce ample energy in the form of ATP to power the flagellar motion that propels the sperm to the site of fertilization. Spermatozoal ATP production occurs via the oxidative phosphorylation (OXPHOS) process in the respiratory chain within the mitochondria (Lamirande and Gagnon, 1992; Manfredi et al, 1997). This ATP produced must diffuse or be transported down the axoneme to the dynein ATPases, which produce motive force and subsequently sliding of doublet microtubules is translated to flagellar beating or sperm motility (Summers, 1974).

Mitochondria and the mitochondrial genome are maternally inherited and paternal mitochondria are eliminated after fertilization and prior to the 4-cell embryo stage is formed (Kaneda et al, 1995) by a mechanism involving ubiquitination of sperm mitochondria (Sutovski et al, 1999). The respiratory chain within the inner mitochondrial membrane is under the control of two separate genomes: mitochondrial DNA (mtDNA) and nuclear DNA (nDNA). The mtDNA encodes for 7 subunits of complex I, complex III, subunits I, II and III of complex IV and subunits 6 and 8 of the ATPase (Anderson et al, 1981). In human spermatozoa, it has been shown that mitochondrial enzymatic activities are correlated with spermatozoal motility (Ruiz-Pesini et al, 1998; Ruiz-Pesini et al, 2000a; Ruiz-Pesini et al, 2001). Mitochondrial DNA mutations caused defects in the OXPHOS system within the mitochondria and resulted in various clinical symptoms ranging from single organ involvement to multisystem disease (Wallace, 1992; Barroso et al, 1993; Leonard and Schapira, 2000; Sue and Schon, 2000; Fosslien, 2001). Several reports from human infertility clinics found an association between mtDNA point

mutations and male infertility (Kao et al, 1998; Kao et al, 2004). In previous studies, it has been shown that patients with primary mitochondrial disorders had reduced sperm motility evidenced by ultrastructural abnormalities in spermatozoal mitochondria (Folgero et al, 1993).

Within the mitochondrial matrix, the citrate synthase enzyme regulates the citric acid cycle which serves both in catabolism and anabolism as cycle intermediates can be drawn off and used as starting materials for a variety of biosynthetic products (Semeitink et al, 2004; Nelson and Cox, 2005). Also the citric acid cycle provides the respiratory chain with reducing equivalents or electron donor products (Ruiz-Pesini et al, 2000a).

The development of artificial insemination (AI) techniques has allowed the rapid dissemination of genetic material from a small number of superior sires to a large number of females (Salisbury et al, 1978). For all livestock species, semen traditionally has been stored either in the liquid form or in a cryopreserved state (Shannon, 1978; Johnson et al, 1981; Foote, 1982; Maxwell and Watson, 1996). Semen extenders were developed to provide an acceptable buffering capacity, osmolality, energy source, minimizing the capability of bacterial growth and provide protection due to the decrease in temperature (Salisbury et al, 1978). The widespread application of AI and realization of its full potential depends largely on the use of frozen semen. However, fertility from AI is lower than that resulting from the use of fresh semen in most species due to lower post-thaw spermatozoal survival and was reported to be 30 to 40% in the bull (Liu and Foote, 1998; Watson, 2000).

Major steps of cryopreservation, such as cooling and freezing/thawing, exert physical as well as chemical stresses on the sperm membrane (Chatterjee et al, 2001). Cryopreservation of spermatozoa is associated with an oxidative stress (Mazur et al, 2000).

The activity of superoxide dismutase, a scavenger of the destructive superoxide radical, is decreased in frozen/thawed spermatozoa (Chatterjee et al, 2001). Ultrastructural studies have shown altered spermatozoal mitochondria in the mouse (Nishizono et al, 2004), human (Woolley and Richardson, 1978) as well as in the bull by using fluorescent techniques (Thomas et al, 1998). Glycerol is the most commonly used cryoprotectant for use with bull semen and semen from other livestock species, it has been shown to adversely affect cells. This occurred via its osmotic effects and direct interaction with the plasma membrane proteins (Anchordoguy et al, 1987).

During the infancy of the AI industry, antibiotics in bull semen extenders were used to control both pathogenic and common contaminating microorganisms (Almquist, 1948). Most of the antibiotics used were found to be harmless to bovine spermatozoa (within ranges of concentrations) (Lein, 1986; Lorton, 1986; Shin, 1986; Ahmad et al, 1987). Improvements in fertility were obvious, as treating semen from infected bulls increased conception rates in cows (Salisbury et al, 1978). Overall, the antibiotics that the AI industry adopted and is currently used target bacterial ribosomes. All the antibiotics targeted bacteria, however some showed higher toxic effects on mitochondria than others (Ahmad et al, 1987).

As sperm motility depends on ATP production by the mitochondria (Zamboni, 1982), studying mitochondrial metabolic activity of spermatozoa provides valuable information about the metabolic activity of spermatozoa and semen quality. If cryopreservation is the only factor affecting spermatozoal metabolic activity reflected as lowered spermatozoal motility and therefore fertility, then why do certain populations of frozen spermatozoa survive the freeze-thaw processes? It is likely that other factors might affect spermatozoa during the freeze-thaw processes. The overall hypothesis for this study

was that mitochondrial function in bull spermatozoa would be influenced by the cryopreservation process and by extender constituents. To address these concerns, four assays were conducted. The first assay evaluated spermatozoal mitochondrial Complex 1 (NADH dehydrogenase) enzyme activity by using the reducible dye resazurin. The second assay evaluated spermatozoal mitochondrial Complex IV (cytochrome c oxidase) and citrate synthase enzyme specific activities. In addition, these activities were used to evaluate of the integrity of the outer and inner mitochondrial membranes of spermatozoa. The third assay evaluated the ATP production of spermatozoa using the luciferin-luciferase assay. The final assay sequenced the NADH dehydrogenase (ND1) gene of spermatozoal mitochondrial DNA using the polymerase chain reaction method.

## **CHAPTER 2**

### **REVIEW OF LITERATURE**

#### **2.1 Mitochondria**

##### **2.1.1 Definition**

Mitochondria are membrane-enclosed organelles distributed through the cytosol of eukaryotic cells. Their main function is the conversion of energy from food resources into ATP (Terada, 1990; Sue and Schon, 2000; Wakabayshi, 2002; Diez-Sanchez et al, 2003; Alberts et al, 2004; Nelson and Cox, 2005).

##### **2.1.2 Ultrastructure**

Mitochondria, like gram negative bacteria, have an outer and inner membrane (Margulis, 1970). The outer membrane encloses the entire mitochondria and is readily permeable to small molecules (Alberts et al, 2004; Nelson and Cox, 2005). It contains many complexes of integral membrane proteins that form channels through which a variety of molecules and ions move in and out of the mitochondrion. The inner membrane is elaborately folded with shelf-like cristae or invaginations projecting into the matrix. It is impermeable to most small molecules and ions, including protons ( $H^+$ ). The only molecules that cross this membrane do so through specific transporters (Alberts et al, 2004; Nelson and Cox, 2005). The inner membrane bears the components of the respiratory chain and ATP synthase. Mitochondria also have a matrix, enclosed by the inner membrane, that contains the pyruvate dehydrogenase complex and the enzymes of the citric acid cycle, the fatty acid  $\beta$ -oxidation pathway, and the pathways for amino acid oxidation (Alberts et al, 2004; Nelson and Cox, 2005). Thus, it has all the pathways for fuel oxidation except for glycolysis which takes place in the cytosol. Also within the

matrix is the mitochondrial genome, composed of circular double stranded DNA and ribosomes (Alberts et al, 2004; Nelson and Cox, 2005).

## **2.2 Mitochondria of Mammalian Spermatozoa**

Mitochondria, located within the sperm midpiece (Bahr and Engler, 1970; Alcivar et al, 1989), are energy generators that have been described as the combustion engine of the spermatozoa (Kao et al, 1998). Mammalian sperm flagella have a basic axoneme composed of a cylinder of nine parallel doublet microtubules (outer doublets) surrounding a central pair of single microtubules (Fawcett, 1970). These nine outer doublets microtubules are held together by various structures including nexin, radial spokes, and pairs of arms, which contain the ATPase protein dynein (Fawcett, 1970). Each of the outer doublets microtubules is composed of two subunits of tubules, one is designated as subunit A, and the other is subunit B (Fawcett, 1970). Subunit A is composed of 13 protofilaments while subunit B is composed of 10 protofilaments. Subunit A of a doublet interacts through the dynein arms with subunit B of a neighboring doublet, inducing the sliding activity of the these microtubules which is being translated to coordinated beating of the flagellum (Summers, 1974). The basic axoneme is supplemented by nine dense fibers (outer dense fibers) which lie parallel and adjacent to the nine doublet microtubules forming a second cylindrical layer in the flagellar structure (Fawcett, 1970; Summers, 1974; Setchell, 1978; Barth and Oko, 1989). The outer dense fibers are much larger than the doublet tubules near the head of the sperm cell and taper gradually until they disappear near the tip of the flagellum (Fawcett, 1970). The functions of the outer dense fibers are not defined, but it has been suggested that they are involved in the maintenance of elastic structures and the elastic recoil of sperm tails (Baltz et al, 1990). The microtubules and dense fibers are enclosed by a third cylindrical layer

consisting of a mitochondrial sheath near the sperm head and a fibrous sheath along the rest of the length of the flagellum (Summers, 1974; Setchell, 1978; Barth and Oko, 1989).

Several reports have shown that mitochondria of germ cells modify their morphological organization, number, and location during spermatogenesis. The morphological, histochemical and biochemical events of mitochondrial differentiation during rat spermatogenesis have been described (Fawcett, 1970; Domenech et al, 1972; Martino and Stefanini, 1974; Kaya and Harrison, 1976; Alcivar et al, 1989; Hecht, 1993). In the A-spermatogonial stage, mitochondria are ovoid in shape containing lamellar cristae and an electron translucent matrix. In some B-spermatogonia and more prominently during the transition to leptotene primary spermatocytes, the intracristal space begins to dilate (Martino and Stefanini, 1974; Martino et al, 1979). In the zygotene and early pachytene spermatocytes the mitochondria elongate and are located in close proximity to the outer nuclear membrane with their number increasing steadily (Martino and Stefanini, 1974; Martino et al, 1979). This is an indication of an active functional state of mitochondria during this phase of development (Martino et al, 1979).

Mitochondria form clusters in mid and late pachytene spermatocytes with an electron dense material, the nugae, between them (Fawcett, 1970; Russel and Frank, 1978). The mitochondria are round-shaped and relatively small with the an electron dense matrix flattened to the outer part of the organelle during this phase of meiosis (Martino and Stefanini, 1974; Martino et al, 1979). The intracristal space is clearly dilated and the functional status of condensed mitochondria is unclear (Martino and Stefanini, 1974; Martino et al, 1979).

In diplotene and secondary spermatocytes, the mitochondria are small, round-shaped and condensed but are not arranged in clusters (Martino and Stefanini, 1974; Martino et al, 1979). With the differentiation of germ cells to spermatids, a translocation of mitochondria beneath the plasma membrane is apparent, possibly due to a high energy demand and intense exchange of metabolites with the neighboring Sertoli cells (Martino et al, 1979). However, a group of elongating and dividing organelles is still visible inside the cell. From step 8 of spermiogenesis and onward, the condensed mitochondria develop crescent-shaped cristae and the matrix is less condensed. This is defined as the intermediate organelle form (intermediate spermatid) (Martino et al, 1979). Some of them show a tendency to move in the direction of the developing flagellum, whereas the remaining mitochondria progressively group together (Martino et al, 1979). These mitochondria leave the developing spermatozoa in the residual bodies and are probably phagocytosed by the Sertoli cells (Martino et al, 1979). Utilizing DNA analysis technologies, it has been found that mtDNA content is reduced to about a tenth of its initial value during the transition from the rounded spermatids to elongated spermatids (Hecht et al, 1984). In later stages of development, the expanded intracristal spaces of the flagellar mitochondria reduce their size, and the cristae distend themselves into the interior of the organelles (Martino et al, 1979). Simultaneously these mitochondria begin to elongate and fuse to a chain-like and twisted structure around the flagellum. In spermatozoa the cristae develop gradually forming a packed concentric system of membrane and matrix (Martino et al, 1979). In this manner, the middle piece mitochondria lose the morphological appearance typical for the condensed form of mitochondria. At this stage of sperm maturation, the mitochondria reach maximal elongation and assume a close relationship with the outer dense fibers of the middle piece

(Martino et al, 1979). Finally the mitochondria are arranged in a helix of 11-13 gyri, with two mitochondria per gyrus, and deliver ATP to the axoneme for flagellar propulsions (Martino et al, 1979).

Spermatozoal motility is dependent on an adequate supply of ATP produced by the oxidative phosphorylation process within the mitochondria (Zamboni, 1982). This ATP produced must diffuse or be transported down the axoneme to the dynein ATPases, which produce motive force (Schoff et al, 1989). Therefore, ATP-induced sliding of doublet microtubules is translated to flagellar beating or sperm motility (Summers, 1974). The mammalian spermatozoon contains approximately 72-80 mitochondria (72/bovine spermatozoa) in the mitochondrial sheath of the midpiece (Bahr and Engler, 1970; Alcivar et al, 1989).

### **2.3 Mitochondrial Genome**

Mitochondria , along with the mitochondrial genome are maternally inherited, and each mitochondrion carries its own multicopy genome, which is a circular double-stranded DNA molecule with 16.5 kilobases in humans (Giles et al, 1980; Anderson et al, 1981) and 16.338 kilobases in bovine (Anderson et al, 1982). Mitochondrial DNA (mtDNA) contains genes coding for 2 ribosomal RNAs and 22 transfer RNAs. The mitochondrial genome encodes for 13 essential subunits (structural proteins) out of the 67 subunits of the respiratory chain complexes (Wallace, 1992).

The function of the respiratory chain is unique in that it is under the control of two separate genomes: mtDNA and nDNA. The mtDNA encodes for 7 subunits of Complex I, one subunit of Complex III, subunits I, II and III of Complex IV and subunits 6 and 8 of the ATPase (Anderson et al, 1981). Paternal mitochondria are eliminated after

fertilization and prior to the formation of 4-cell embryo (Kaneda et al, 1995) by a mechanism involving ubiquitination of sperm mitochondria (Sutovski et al, 1999).

Somatic cells of mammals contain several thousand mitochondria, each of them having 1 to 10 copies of mtDNA. According to Cummins (1998), oocytes contain  $1 \times 10^5$  to  $2 \times 10^5$  mitochondrial genomes. The mouse oocyte mitochondrial content was the first to be estimated using electron microscopy, and an average of 92 500 mitochondria per mouse pronucleate oocyte were found (Piko and Matsumoto, 1976). Using dot blot techniques, Piko and Taylor (1987) found an average of 119 000 mtDNA copies per cell in mature pooled mouse oocytes. In pooled bovine oocytes, Michaels et al (1982) determined that bovine oocytes contain an average of 26 000 mtDNA copies per cell utilizing hybridization techniques. Utilizing the polymerase chain reaction, many researchers found that mtDNA content is variable in oocytes and found that it is variable even in oocytes collected from the same individual (Reynier et al, 2001). On average, mature mammalian spermatozoa contain 22 to 75 mitochondria (Bahr and Engler, 1970). Using the semiquantitative southern blot method, Hecht et al (1984) found 50 to 75 mtDNA copies per mouse spermatozoon, and Manfredi et al (1997) found about 1 500 molecules of mtDNA per human spermatozoon.

Each mitochondrion contains several copies of mtDNA and each cell has many mitochondria. There are hundreds of copies of mtDNA in one cell (polyplasmcy) compared to two copies of nuclear DNA (nDNA). Mitochondrial DNA coding sequences do not have introns as in nDNA, and mtDNA depends on nuclear genes for its enzymes for replication, translation, and repair (Taanman, 1999). Another difference between mtDNA and nDNA is that, at least in humans, mtDNA has a mutation rate 10 to 20 times over that of nDNA. This is attributed to the lack of proof-reading by mtDNA polymerase

(Leonard and Schapira, 2000). Normally, all mtDNA of an individual is identical (homoplasmy) but occasionally there is a sequence variation which creates a population of wild type and mutant mtDNA (heteroplasmy). Although heteroplasmy implies potentially harmful mutant type mtDNA, benign heteroplasmic polymorphisms can sometimes occur (Hauswirth and Laipis, 1982).

Mitochondrial DNA mutations include point mutations and rearrangements (deletions and duplications), and because there are no introns, there are no splice-site mutations (Leonard and Schapira, 2000). Mitochondrial DNA mutations of the pathogenic type are responsible for certain known diseases in humans, which depend not only on the site of mutation in the mtDNA but also on the proportion of the mutant to wild type molecules within cells and within mitochondria (Leonard and Schapira, 2000). Examples are diseases of brain and muscles, where these tissues are highly dependent on oxidative phosphorylation (OXPHOS), where neurological pathologies and myopathies are common features of mtDNA mutations (Wallace, 1992; Leonard and Schapira, 2000; Sue and Schon, 2000). Mitochondrial DNA mutations cause defects in the OXPHOS system within the mitochondria and result in various clinical symptoms depending on the organ involved. The striking feature of mtDNA mutations or disorders is their clinical heterogeneity, ranging from single organ involvement to multisystem disease (Wallace, 1992; Barroso et al, 1993; Leonard and Schapira, 2000; Sue and Schon, 2000; Fosslien, 2001). Leigh syndrome (multisystem disease: vomiting, seizures, respiratory failure, tendon problems and encephalomyopathies) is due to Complex I deficiency in infants and is caused by mtDNA mutations. While Leigh syndrome occurs during infancy, neuromuscular disorders like Alzheimer and Parkinson diseases result from

defective OXPHOS due to reduced Complex I activity with aging (Wallace, 1992; Leonard and Schapira, 2000; Sue and Schon, 2000).

Spermatozoal motility is required for the normal fertilization process and is one of the major determinants of male fertility (Hartman, 1965). Sperm motility is dependent on an adequate supply of ATP produced by the oxidative phosphorylation process within the mitochondria. Several reports from human infertility clinics have found an association between mtDNA point mutations and asthenospermia (reduced motility of spermatozoa), oligospermia (reduction in the number of spermatozoa), oligoasthnozoospermia (reduced number and absence of motility) and primary infertility in patients attending those infertility clinics (Kao et al, 1998; Kao et al, 2004).

#### **2.4 Mitochondrial Activity (Oxidative Phosphorylation and Electron Transfer Chain)**

Oxidative phosphorylation begins with entry of electrons into the respiratory chain. Most of these electrons arise from the action of dehydrogenases that collect electrons from catabolic pathways and funnel them into universal electron acceptors- nicotinamide nucleotides (NAD<sup>+</sup> or NADP<sup>+</sup>) or flavin nucleotides (FMN or FAD) (Wallace, 1992; Vishwanath and Shannon, 1997; Nelson and Cox, 2005).

The mitochondrial respiratory chain consists of a series of sequentially acting electron carriers, most of which are integral proteins with a prosthetic group capable of accepting and donating either one or two electrons (Leonard and Schapira, 2000; Alberts et al, 2004; Semeitink et al, 2004; Nelson and Cox, 2005). In addition to NAD and flavoproteins, three other types of electron-carrying molecules function in the respiratory chain: ubiquinone (coenzyme Q), cytochromes, and iron-sulfur proteins (Alberts et al, 2004; Nelson and Cox, 2005).

The electron carriers of the respiratory chain are organized into membrane-embedded supramolecular complexes designated as Complex I (NADH: ubiquinone oxidoreductase or NADH dehydrogenase), II (succinate dehydrogenase), III (cytochrome bc<sub>1</sub> complex or ubiquinone cytochrome c oxidoreductase) and Complex IV (cytochrome oxidase). Complexes I and II catalyze electron transfer to ubiquinone from two different electron donors NADH (Complex I) and succinate (Complex II). Complex III carries electrons from reduced ubiquinone to cytochrome c, and Complex IV completes the sequence by transferring electrons from cytochrome c to molecular oxygen (Jin and Bethke, 2002; Semeitink et al, 2004).

Other substrates for mitochondrial dehydrogenases pass electrons into the respiratory chain at the level of ubiquinone, but not through Complex II (Alberts et al, 2004; Nelson and Cox, 2005). The first step in the beta oxidation of fatty acetyl coenzyme A (acyl-CoA), catalyzed by the flavoprotein Acyl-CoA dehydrogenase, involves transfer of electrons from the substrate to the FAD of the dehydrogenase, then to electron transferring flavoprotein (ETF), which in turn passes electrons to ETF: ubiquinone oxidoreductase (Alberts et al, 2004; Nelson and Cox, 2005). This enzyme transfers electrons into the respiratory chain by reducing ubiquinone. Glycerol-3-phosphate formed either from glycerol released by triacylglycerol breakdown or by the reduction of dihydroxyacetone phosphate from glycolysis, is oxidized by glycerol-3-phosphate dehydrogenase (Jin and Bethke, 2002; Alberts et al, 2004; Nelson and Cox, 2005). This enzyme is a flavoprotein located on the outer face of the inner mitochondrial membrane, and like succinate dehydrogenase and acyl-CoA dehydrogenase it channels electrons into the respiratory chain by reducing ubiquinone. So glycerol-3-phosphate dehydrogenase has an important role in shuttling reducing equivalents from cytosolic

NADH into the mitochondrial matrix. The effect of each of these electron-transferring enzymes is to contribute to the pool of reduced ubiquinone which is then deoxidized by Complex III (Jin and Bethke, 2002; Semeitink et al, 2004; Nelson and Cox, 2005).

The energy of electron transfer is efficiently conserved in a proton gradient. Much of this energy is used to pump protons out of the mitochondrial matrix. For each pair of electrons transferred to molecular oxygen, four protons are pumped out by Complex I, four by Complex III, and two by Complex IV (Jain, 1988; Alberts et al, 2004). The electrochemical energy inherent in this difference in proton concentration and separation of charge represents a temporary conservation of much of the energy of electron transfer. The energy stored in such a gradient is termed the proton-motive force. This force has two components: the chemical potential energy due to the difference in concentration of a chemical species ( $H^+$ ) in the two regions separated by the membrane, and the electrical potential energy that results from the separation of charge when a proton moves across the membrane without a counterion (Mitchell, 1961; Mitchell, 1966; Mitchell, 1968; Mitchell and Moyle, 1969; Jain, 1988; Leonard and Schapira, 2000; Jin and Bethke, 2002; Alberts et al, 2004; Nelson and Cox, 2005). When protons flow spontaneously down their electrochemical gradient, energy drives the synthesis of ATP and therefore is made available to do work (Mitchell, 1961; Jain, 1988; Jin and Bethke, 2002; Semeitink et al, 2004; Nelson and Cox, 2005).

In human spermatozoa, mitochondrial enzymatic activities were shown to be correlated with spermatozoal motility (Ruiz-Pesini et al, 1998; Ruiz-Pesini et al, 2000a; Ruiz-Pesini et al, 2001). In previous studies, it has been shown that patients with primary mitochondrial disorders had reduced sperm motility evidenced by ultrastructural abnormalities in spermatozoal mitochondria (Folgero et al, 1993). However, in patients

with Parkinsons disease, no correlation has been found between the occurrence of this disease and activity of spermatozoal Complex I (Molina et al, 1999). In human patients with asthenozoospermia compared to normal individuals, it was shown using the polymerase chain reaction (PCR) method that spermatozoal mtDNA had a high incidence of deletions (Kao et al, 1995).

#### **2.4.1 Adenosine Triphosphate (ATP) Synthesis**

Mitochondrial ATP synthase is a large enzyme complex across the inner mitochondrial membrane that catalyzes the formation of ATP from ADP and Pi, accompanied by the flow of protons from outside the matrix to inside. ATP synthase, also called Complex V, has two distinct components: F<sub>1</sub>, a peripheral membrane protein, and F<sub>o</sub> (denoting oligomycin sensitive), which is integral to the membrane. This makes ATP synthase carry out rotational catalysis, in which the flow of protons through F<sub>o</sub> causes each of three nucleotide binding sites in F<sub>1</sub> to cycle from (ADP+Pi)-bound to ATP-bound to empty conformations (Jain, 1988; Gottlieb et al, 1991; Jin and Bethke, 2002; Nelson and Cox, 2005).

Although ATP synthase equilibrates ATP with ADP and Pi, in the absence of a proton gradient the newly synthesized ATP does not leave the surface of the enzyme. It is the proton gradient that causes the enzyme to release the ATP formed on its surface (it pushes ATP from its binding site on the synthase enzyme). For the continued synthesis of ATP, the enzyme must cycle between a form that binds ATP very tightly and a form that releases ATP (Nelson and Cox, 2005).

The summary of ATP synthesis involves the flow of electrons through Complexes I, III and IV which results in pumping of protons across the inner mitochondrial membrane making the matrix alkaline relative to the intermembrane space. This proton

gradient provides the energy (in the form of the proton-motive force) for ATP synthesis from ADP and Pi by ATP synthase ( $F_0F_1$  complex) in the inner membrane (Mitchell, 1966; Mitchell, 1968; Jin and Bethke, 2002; Nelson and Cox, 2005).

#### **2.4.2 The Citric Acid Cycle**

The citric acid cycle serves both in catabolism and anabolism as cycle intermediates can be drawn off and used as the starting material for a variety of biosynthetic products (Semeitink et al, 2004; Nelson and Cox, 2005).

Eukaryotic cells and many bacteria which live under aerobic conditions oxidize their organic fuels to carbon dioxide and water. Glycolysis is the first stage in the complete oxidation of glucose to pyruvate and can proceed anaerobically. The aerobic phase of catabolism is called respiration (cellular respiration) (Margulis, 1970; Jain, 1988; Matsuno-Yagi and Hatefi, 1993).

Cellular respiration occurs in three major stages. In the first stage, organic fuel molecules like glucose, fatty acids and some amino acids in the cytosol are oxidized to yield two-carbon fragments in the form of Acetyl-coenzyme A (Acetyl-CoA). In the second stage, the acetyl groups are fed into the citric acid cycle within the mitochondria, which enzymatically oxidizes them to carbon dioxide, and energy released is conserved in the reduced electron carriers NADH and FADH<sub>2</sub>. In the third stage of respiration, these reduced coenzymes are themselves oxidized, giving up protons (H<sup>+</sup>) and electrons.

#### **2.5 Spermatozoal Motility**

Spermatozoal motility is considered to be one of the most important characteristics for evaluating the fertility potential of ejaculated spermatozoa (Liu et al, 1991). The propulsive efficacy of spermatozoa is dependent on mitochondrial function (Auger et al, 1989). The mitochondria deliver the required energy in the form of ATP for

the generation and propagation of the flagellar wave (Auger et al, 1989). Male infertility can result from a significant decrease in the number of motile forms and/or from movement quality disorders (Auger et al, 1989). Visual estimation of the percentage of motile spermatozoa is the most common laboratory test of sperm quality (Budworth et al, 1988). However, it is subjective (Budworth et al, 1988) and differs among individuals and laboratories, and from 30 to 60% variation has been reported (Deibel et al, 1976). Subjectivity and human bias are undesirable, rendering visual estimation of spermatozoal motility a poor predictor of fertility (Saacke and White, 1972; Linford et al, 1976). Objective methods of evaluating spermatozoal motility gave a better prediction of fertility than visual estimates (Saacke, 1982). Photographic or computer assisted sperm analysis (CASA) systems have reduced the human bias in estimating the percentage of motile spermatozoa, but estimates from these procedures are time-consuming and usually evaluate fewer spermatozoa per sample than can be evaluated visually (Graham, 1994). Neither the visual estimation of spermatozoal motility (Graham et al, 1980) nor CASA (Budworth et al, 1988) were correlated with fertility in bulls. In addition to total and progressive motilities, CASA evaluations can also measure the velocity or motion parameters of motile spermatozoa. Velocity parameters of spermatozoa that CASA can evaluate include: curvilinear velocity (VCL), which is the average velocity measured over the actual point-to-point track followed by the cell ( $\mu\text{m}/\text{sec}$ ); progressive velocity (PV); velocity according to the straight path (VSL,  $\mu\text{m}/\text{sec}$ ); velocity according to the average smoothed pathway (VAP,  $\mu\text{m}/\text{sec}$ ); linearity (Lin, %); straightness (STR, %); amplitude of the lateral displacement of the sperm head (ALH,  $\mu\text{m}$ ) and the frequency of the flagellar beat (BCF, Hz) (Verstegen et al, 2002). Januskauskas et al (2003) found a significant correlation between CASA assessed spermatozoal motility and field fertility.

Similarly, Zhang et al (1998) found a correlation between postthaw spermatozoal linear motility and field fertility. However, in one study (Januskauskas et al, 2001), it was shown that spermatozoal velocity parameters obtained by CASA were not correlated to field fertility. In another recent study (Garcia-Macias et al, 2007), spermatozoal total and progressive motilities and velocity parameters were not correlated with fertility. However, the author noticed that velocity parameters were highest in the high-fertility group (Garcia-Macias et al, 2007). In a study where CASA motility and standard laboratory tests of semen quality were compared to fertility based on 66-day non-return rate, neither CASA nor any of the standard laboratory tests were significantly correlated with fertility (O'Connor et al, 1981). High CASA measurements of the velocity parameters VAP, VSL and VCI as well as low linearity have been reported to be associated with spermatozoal hyperactivation during the capacitation process (Verstegen et al, 2002). Using CASA, it has been shown that VCL and straightness were correlated with spermatozoal maturation within the epididymis in rats (Syntin and Robaire, 2001)

## **2.6 Mitochondrial Function Tests**

### **2.6.1 Adenosine Triphosphate Production**

The ATP generated by the oxidative phosphorylation process within the inner mitochondrial membrane is transferred to the microtubules to drive sperm motility (Zamboni, 1982). Therefore, the amount of ATP produced would reflect the metabolic status of mitochondria and subsequently the motility of spermatozoa. Reiger (1997) used a commercial luciferin-luciferase kit and scintillation counter to measure sperm ATP content in a semen sample to determine the metabolic activity of spermatozoa mitochondria. The technique involves ATP extraction from spermatozoa and mixing it with the luciferin-luciferase assay mixture.

The production of chemiluminescence by the firefly luciferin-luciferase reaction consists of an initial flash period, lasting a few seconds, followed by a gradual decay over an extended period which is proportional to the ATP content of cells. A luminometer is used to detect light emitted during the flash period and consequently each sample must be counted immediately after mixing the assay components. This assay is sensitive enough to measure the ATP content of a single oocyte, blastocyst, or approximately 1000 sperm (Reiger, 1997). However, this assay required several steps of addition of reagents which made the assay tedious, labor-intensive and time-consuming. With the advancement of technology, luminescent kits have been introduced which make the ATP assay simple, quick, and accurate. Currently, ATP kits employ only one reagent followed by a short period of sample incubation (15 minutes). Measurement of ATP is then performed using a luminometer (Orlando et al, 1982; Comhaire et al, 1989; Gottlieb et al, 1991; Orlando et al, 1994; Marin-Guzman et al, 2000).

Spermatozoal ATP content has been measured from a wide variety of species including the bull (Januskauskas and Rodriguez-Martinez, 1995; Reiger, 1997), boar (Johnson et al, 1981), domestic poultry (Wishart, 1982; Froman and Feltmann, 1998), rabbit (Minelli et al, 1999), carp (Perchec et al, 1995) and human (Levin et al, 1981; Calamera et al, 1982; Orlando et al, 1982; Comhaire et al, 1983; Irvine and Aitken, 1985; Pousette et al, 1986; Chan and Wang, 1987; Comhaire et al, 1987; Comhaire et al, 1989; Mieusset et al, 1989; Gottlieb et al, 1991; Bosman et al, 1994; Mahmoud et al, 1994; Orlando et al, 1994).

### **2.6.2 Resazurin Reduction Test**

Resazurin is a redox dye traditionally used for examining bacterial and yeast contamination of milk (Zhang et al, 2004), as well as for assessing semen quality in

mammals (Fuse et al, 1993; Dart et al, 1994; Rahman and Kula, 1997; Reddy et al, 1997; Carter et al, 1998; Wang et al, 1998; Reddy and Bordekar, 1999; Zrimesk et al, 2004). Resazurin is non-toxic to cells and does not necessitate killing the cells to obtain measurements that allows cell to be reused for further investigations (O'Brien et al, 2000). Resazurin is blue in color in the oxidized state, and reduced to resorufurin (pink) and then to hydroresorufin (colorless) by metabolically active cells (Rahman and Kula, 1997; Zhang et al, 2004).

The mechanism of resazurin reduction has been postulated to be due to oxygen consumption through metabolism or mitochondrial enzymes (oxidoreductases) (O'Brien et al, 2000; Zhang et al, 2004). Therefore, measurement of resazurin reduction is an indication of mitochondrial function. Resazurin reduction can be measured by visual color changes (Fuse et al, 1993; Dart et al, 1994; Carter et al, 1998), or by using spectrophotometry (Rahman and Kula, 1997; Reddy et al, 1997; Wang et al, 1998; Reddy and Bordekar, 1999; Zrimesk et al, 2004). Initially, resazurin reduction was measured visually and the time required for reduction to pink, the first endpoint, and reduction further to white was recorded (Erb and Ehlers, 1950). Time required for reduction of resazurin is dependent on the quality of semen (concentration and percentage of actively motile spermatozoa) and was highly correlated with the pink endpoint (Erb and Ehlers, 1950). In another study, the reduction of resazurin from blue to pink ( $\leq 3.5$  minutes) allowed identification of 88% of low and 94% of high fertility potential samples (Dart et al, 1994). In order to avoid the possibility of human error in assessing the quality of semen samples based on visual comparison of the colors due to resazurin reduction, spectrophotometry has been used. In this method, absorption spectra for resazurin and resorufin are scanned and wavelengths are selected (Rahman and Kula, 1997; Wang et

al, 1998; Reddy and Bordekar, 1999; Zrimesk et al, 2004). The use of resazurin dye has been shown to be accurate in the evaluation of human (Glass et al, 1991; Fuse et al, 1993; Rahman and Kula, 1997; Reddy et al, 1997; Reddy and Bordekar, 1999), bovine (Erb and Ehlers, 1950; Erb and Ehlers, 1952; Dart et al, 1994), ovine (Wang et al, 1998), equine (Carter et al, 1998) and porcine (Mesta et al, 1995; Zrimesk et al, 2004) semen.

## **2.7 Cryopreservation of Bull Semen**

The development of AI techniques has allowed the rapid dissemination of genetic material from a small number of superior sires to a large number of females. For all livestock species, semen traditionally has been stored either in the liquid form or in a cryopreserved state (Shannon, 1978; Johnson et al, 1981; Maxwell and Watson, 1996).

In normal, freshly ejaculated bull semen, the concentration of spermatozoa is high. Therefore, dilution is necessary to provide a convenient inseminate volume that will contain enough cells to ensure maximum fertilization rates without wasting spermatozoa. At the beginning of the bovine AI industry, semen was diluted in physiological media, split and used almost immediately for insemination (Coulter, 1992). This required that all females that had to be bred must be in close proximity to one another and to the donor bull, limiting the dissemination of superior germ plasm (Coulter, 1992). The development of liquid storage of semen permitted the use of collected bull semen for a short period of time and permitted the transportation of semen over moderate distances which allowed for a wider distribution of superior germ plasm. Spermatozoa progressively lose viability when stored in a supportive medium at ambient temperature (Shannon, 1978), so reducing storage temperature from body or ambient temperature to 5°C has been the primary means of slowing down chemical reactions and prolonging spermatozoal viability. However, after

rewarming to 37°C, the percentage of progressively motile cells decreases during storage. Furthermore, metabolism continues at a reduced rate at 5°C and fertility begins to decrease before day four of storage (Clamohoy and Foote, 1963).

## **2.8 Characteristics of Semen Extenders**

Semen extenders were developed to provide an acceptable buffering capacity, osmolality, energy in the form of metabolizable substrates, and to minimize bacterial growth and to provide protection due to decreases in temperature (Phillips, 1939; Phillips and Lardy, 1940; Salisbury et al, 1941; Foote and Berndtson, 1976; Salisbury et al, 1978; Shannon, 1978; Vishwanath et al, 1996; Vishwanath and Shannon, 1997).

### **2.8.1 Buffering Capacity**

Spermatozoa require protection from metabolic by-products, primarily lactic acid, especially when they are stored without refrigeration. Buffering solutions protect sperm cells from even minute changes in pH due to lactic acid formation. Phosphate buffer was included in the first semen extender, and satisfactory fertility resulted from its use (Phillips, 1939). However, phosphate buffer is seldom used because it produces an opaque mixture when added to egg yolk, thereby resulting in poor sperm visibility under the microscope. Two years later, citrate buffer solution replaced the phosphate buffer in semen extenders (Salisbury et al, 1941). Zwitter-ion buffers development led to their use for buffering egg yolk as extenders for bull semen. Those buffers were found to provide excellent buffering capacity over a suitable pH range. Bull spermatozoal survival at 5°C in Tris-buffered extenders was equal or superior to citrate-buffered extenders (Davis et al, 1963a; Davis et al, 1963b).

### **2.8.2 Energy Source**

Spermatozoa, requiring energy for motility, are capable of both aerobic and anaerobic metabolism (Ford, 2006). Semen extenders should supply a simple energy source such as glucose or fructose in order to protect the intracellular reserves of spermatozoa as well as cell components. Fructose is available in seminal plasma, while egg yolk contains some glucose and other compounds utilizable by bull spermatozoa (Salisbury et al, 1978).

### **2.8.3 Protection of Spermatozoa Against Cold Shock and Freezing**

Cold shock injury is damage to cell structure and function resulting from a sudden reduction in temperature. Spermatozoa incorrectly cooled to 5°C are subject to cold shock, which results in structural and biochemical damage. In particular, membranes lose their selective permeability with the result that many cellular components are released including lipids, proteins and ions (Salisbury et al, 1978). Additionally, sodium and calcium gain access to the interior of the cell and consequently, metabolic activities are diminished and further changes ensue. Cold shock can be prevented by cooling semen slowly in the presence of protective agents. A major breakthrough in semen extenders came when Phillips (1939) reported the value of hen's egg yolk. Initially, egg yolk was added to phosphate buffer extenders in equal volume (1:1) and was followed with a 1:1 (vol/vol) egg yolk-citrate extender that became the most widely used medium for preserving bull spermatozoa used for AI (Salisbury et al, 1941). In frozen semen, sperm survival was improved by reducing the egg yolk to 20 to 25% by volume (Dunn et al, 1954; Pickett and Berndston, 1978; Smith et al, 1979).

When 4% (vol/vol) of egg yolk was included in the extenders, spermatozoal survival following freezing and thawing was satisfactory and the percentage of normal acrosomes was highest when 2 to 8% (vol/vol) egg yolk was used (Smith et al, 1979). The

protective action of egg yolk is presumed to be largely due to low density lipoproteins (LDL). It has been suggested that LDL can adhere to cell membranes during the freeze-thaw process, thus preserving spermatozoa membranes (Foulkes, 1977; Graham and Foote, 1987). It is also known that loss of membrane phospholipids from sperm occurs during cold shock and egg yolk is generally assumed to prevent these losses or modulate their detrimental effects. The density of LDL is 0.982 g/mL and they are spherical molecules of 17-60 nm in diameter, with a lipid core of triglycerides and cholesterol esters surrounded by a phospholipid and protein film. Phospholipids are essential in the stability of the LDL structure because association forces are essentially hydrophobic. LDL contains 83-89% lipids and 11-17% proteins. Lipids of LDL are composed of approximately 69% triglycerides, 26% phospholipids and 5% cholesterol (Moussa et al, 2002).

Glycerol was found to minimize the harmful effects of freezing of rooster spermatozoa (Polge et al, 1949). Since then, semen from a broad range of domestic, laboratory, and wildlife species, as well as humans, has been frozen and used successfully for AI. Glycerol, the most commonly used cryoprotectant, may also adversely affect cells (Fahy, 1986). The mechanism by which glycerol protects spermatozoa from freeze-thaw injury is not completely understood. The major beneficial effect of glycerol may be extracellular (Amann and Pickett, 1987), however, it is clear that glycerol enters and resides in the cell membrane and cytoplasm of cells. Glycerol has osmotic effects on cells and appears to have a direct effect on the plasma membrane evident by the binding of glycerol to the phospholipids in the membrane of cells (Anchordoguy et al, 1987). Glycerol also interacts with membrane proteins and glycoproteins and causes clustering of intramembranous particles (Niedermeyer et al, 1977; Armitage, 1986). The addition of glycerol to cells reduces the membrane capacitance indicating large scale rearrangements

of membrane structure. This is possibly due to induced gap-junction-like structures in the plasma membrane of cells (Rudenko et al, 1984; Kachar and Reese, 1985). Glycerol was found to induce interdigitation of the two bilayer leaflets, altering membrane fluidity by increasing the order of the interior fatty acyl chains (Boggs and Rangaraj, 1985). It was concluded that glycerol-induced changes in the microtubular structure of the cytoskeleton can alter the interaction of microtubule-associated proteins with the cytoskeleton, thus altering membrane signal transduction or disrupting membrane domains (Keates, 1980).

Glycerol has an osmotic effect and is slow to permeate membranes of spermatozoa. Therefore, cell volume changes as a result of both the addition of glycerol and the loss of water during the freeze-thaw process are dramatic (Parks and Hammerstedt, 1985; Hammerstedt et al, 1990). Spermatozoa volumes reduce to about half that of the isotonic volume due to the addition of glycerol and water loss during freezing, and increase over two fold the isotonic volume when suspended in isotonic solution after thawing (Parks and Hammerstedt, 1985). These phenomena raised the question of how does the sperm cell respond to such volume changes without being lysed given that membrane thickness is unable to change appreciably (Hammerstedt et al, 1990). Hammerstedt et al (1992) suggested that volume reduction may be accomplished in a manner similar to that observed with plant protoplasts (Steponkus and Lynch, 1989) by a wrinkling of the membrane creating areas of invaginations or exvaginations. Evidence for such membrane modifications is lacking in the sperm and cellular expansion may be accommodated by increasing the cell volume to surface area ratio. This has been suggested to occur by altering sperm cell shape from its original shape to that of a sphere (Hammerstedt et al, 1992). These modifications cannot be accomplished easily for all sperm membrane compartments as the midpiece and tail regions of bull, ram and human spermatozoa swell

into a more spherical shape when exposed to hypotonic conditions (Drevious and Erickson, 1966; Jeyendran and Graham, 1980; Duncan and Watson, 1992).

### **2.8.3.1 Effects of Cryopreservation on Spermatozoa**

Reduced fertility of frozen semen is attributed largely to altered membrane structure and function during cooling, freezing and thawing. The nature of this damage remains unclear but it has been suggested that membranes are compromised due to reordering of membrane lipids during cooling and rewarming, thereby disturbing the lipid-lipid and lipid-protein associations required for normal membrane function (Poulos et al, 1973; Hammerstedt et al, 1990).

Cryopreservation of spermatozoa is associated with an oxidative stress (Mazur et al, 2000). Superoxide dismutase activity, a scavenger of the superoxide radical, is decreased in frozen/thawed spermatozoa (Bilodeau et al, 2000). Nishizono et al (2004) reported that cryopreservation induces abnormalities in mitochondrial morphology in the sperm of C57BL/6J mice. The morphological abnormalities included mitochondria with an increased relative area of the matrix, thickening of the outer membrane and swelling with loss of cristae (Nishizono et al, 2004). Woolley and Richardson (1978) studied the effect of freezing and thawing on the ultrastructure of human spermatozoa and found distinctive differences in the morphology of mitochondria from frozen semen samples compared with that seen in the unfrozen samples. In the frozen samples, mitochondria had rounder profiles, their cristae were more plate-like, with little swelling of the intracristal spaces and the intercrystal matrix had become heterogenous (Woolley and Richardson, 1978). Rhodamine 123 uptake was reduced significantly ( 36%) in the mitochondria of frozen human spermatozoa showing that not only did fewer sperm maintain functional mitochondria after freeze-thawing, but also activity within the mitochondria was similarly

damaged (O'Connell et al, 2002). Thomas et al (1998) used the mitochondrial probe JC-1 to study the effect of cryopreservation on bull spermatozoa and found the proportion of JC-1 aggregates was less than 1% after freezing, indicating a low to moderate membrane potential in all of the spermatozoa. Using the cationic lipophilic fluorochrome DiOC6 which accumulates in the inner mitochondrial membrane, Martin et al (2004) showed that cryopreservation induced a statistically significant increase in the proportion of bovine spermatozoa with low mitochondrial membrane potential (11.3% before and 44.9 after cryopreservation).

#### **2.8.4 Antibacterial Agents for Semen Extenders**

The AI industry relies upon the use of antibiotics in semen extenders to control contaminating organisms in bull semen (Almquist, 1948). These organisms are present in fertile and infertile bulls genitalia and even under sanitary collection procedures semen usually harbors these organisms. Egg yolk and other extending media for bull semen provide a good nutrient environment for the growth of these organisms. A wide variety of organisms has been isolated from bull semen (Salisbury et al, 1978). Although many of these organisms are not pathogenic, they compete with spermatozoa for nutrients and their by-products are toxic to spermatozoa. This reduces the livability of spermatozoa and reduces fertility (Foote and Berndtson, 1976). Some of these bacteria are pathogenic, and transmitting them to cows by AI, conception rates are reduced and fertility. Originally, the use of antibiotics was to control venereal diseases such as vibriosis (campylobacteriosis) as this disease was the primary factor in reducing fertility in highly fertile bulls (Almquist, 1948; Foote and Bratton, 1950; Morgan et al, 1959; Salisbury et al, 1978). The effect of antibiotics on improving fertility was obvious after treating semen from infected bulls with

antibiotics resulted in increased conception rate in cows. Therefore, the addition of antibiotics to extenders for bull semen has become a standard procedure.

Early sanitary attempts were to clean the sheath and the underline of the bull by placing a small apron on the chest of the bull and a polyurethane device at the entrance to the artificial vagina (Salisbury et al, 1978). Pathogenic bacteria such as *Pseudomonas aeruginosa* and *Clostridium pyogenes* are associated with low fertility in bulls as they occasionally infect the seminal vesicles and can be transmitted through frozen semen (Salisbury et al, 1978). Other organisms, which are contagious and infect the cows through insemination with infected semen, include *Brucella abortus*, *Vibrio fetus*, *Trichomonas fetus*, *Leptospira Pomona*, *Mycobacterium tuberculosis*, *Mycobacterium paratuberculosis*, *Mycoplasma bovine genitalium* and viruses such as infectious bovine rhinotracheitis and bovine viral diarrhoea viruses (Salisbury et al, 1978). AI organizations applied strict measures to completely eliminate such organisms from bull semen through testing of bulls, selection, isolation and treatment of infected or carrier bulls (Salisbury and Knodt, 1947; Foote and Bratton, 1950; Morgan et al, 1959; Salisbury et al, 1978; Ahmad and Foote, 1985; Ahmad et al, 1987).

Even with strict hygienic measures, variable numbers of organisms are still present in fresh bull semen, thereby necessitating the use of antimicrobials. The first antibiotic used in controlling bacteria in bull semen introduced by Salisbury in 1947 was a sulfanilamide. Egg yolk-citrate extender containing 0.3% (wt/vol) sulfanilamide improved fertility as it increased the nonreturn about 5% (Salisbury and Knodt, 1947). Later these researchers found that this antibiotic partially inhibited bacterial growth, however it damaged spermatozoa during freezing and it so it was excluded from extenders for semen to be frozen. Thus, it was not adopted for use in semen. Almquist (1948) reported that 500 to

1000 units of penicillin per mL of semen increased the fertility of low fertile bulls. A higher non-return rate was obtained when 1 000 units of penicillin or 1 000 µg streptomycin per mL or both were added to extended semen (Foote and Bratton, 1950). These antibiotics were harmless to spermatozoa and when combined, they inhibited a broad spectrum of microorganisms (Foote and Bratton, 1950; Salisbury et al, 1978). Many studies reported an increase in non-return rate by using these antibiotics in extended semen (Foote and Bratton, 1950; Almquist, 1951; Willett and Larson, 1952). Campbell and Edwards (1955) reported an increase in fertility when sulfanilamide was used singly or in combination with penicillin and streptomycin. The use of streptomycin with yolk phosphate buffers depressed fertility because it forms a precipitate (Campbell and Edwards, 1955). This problem has been solved by the use of dihydrostreptomycin (Easterbrooks et al, 1950). Many other antibiotics like the tetracycline family and chloramphenicol have been investigated and found to be ineffective in raising fertility (Salisbury et al, 1978).

The antibiotic polymyxin B was reported to increase fertility of frozen semen but failed to demonstrate an improvement when added to extenders containing both penicillin and streptomycin (Foote and Bratton, 1950). Therefore, penicillin and streptomycin became more popular and widely used as they are harmless to spermatozoa and control a broad spectrum of bacteria (Almquist, 1951). However, some reports showed that these antibiotics failed to control some specific pathogens like *Vibrio fetus* especially when used in egg yolk based extenders (Morgan et al, 1959). The prolonged use of penicillin and streptomycin lead to the development of bacterial resistance in addition to their questionable efficacy against other isolates such as mycoplasma and ureaplasma which survived the freezing process. These factors necessitated continued investigations of new antibiotics that are non-toxic to spermatozoa for control of contaminating organisms.

Clindamycin and tylosin were effective against several mycoplasma and ureaplasma isolates of bovine origin (Hamdy and Miller, 1971) . The antibiotics lincomycin and spectinomycin were effective in controlling most mycoplasma strains when used in combination (linco-spectin) and their efficacy was decreased when used singly (Hamdy, 1972). This combination showed a relatively high level of safety to spermatozoa in addition to its broad spectrum activity against gram positive bacterial contaminants in semen (Hamdy, 1972). In another study, effective control of mycoplasma strains, ureaplasma, *Haemophilus somnus* and *Campylobacter fetus* var. *venerealis* in processed bovine semen was achieved by the use of the combination of linco-spectin (300/600 µg/ml), tylosin (100 µg/ml) and gentamicin (500 µg/ml) (Lein, 1986; Shin, 1986). (Lorton, 1986) confirmed those results and found no significant differences in post-thaw measurements, compared to semen treated with routine antibiotics as described by the Certified Semen Services of the National Association of Animal Breeders (NAAB) (penicillin, dihydrostreptomycin and polymyxin B). This combination of antibiotics was effective with each extender used by the AI industry and was adopted by the AI industry and is currently used (Doak, 1986). Ahmad et al (1987) investigated the effects of five antibiotics (gentamicin, clindamycin, amikacin, minocin, tylosin and linco-spectin) on post-thaw motility of bull sperm frozen and thawed in egg yolk-citrate, egg yolk-tris and whole milk extenders. Results of their study (Ahmad et al, 1987) showed that the percentage of motile sperm was slightly but significantly depressed at the higher concentrations of clindamycin and linco-spectin tested in whole milk and with minocin in the two egg yolk extenders. Of the five antibiotics tested for fertility, only gentamicin reduced fertility on the basis of 59-day nonreturn rates (Ahmad et al, 1987).

The antibiotic cocktail which the AI industry adopted for use in extenders for bovine semen freezing is composed of lincomycin-spectinomycin (Lincospectin®), gentamicin and tylosin (Lein, 1986; Lorton, 1986; Shin, 1986). Lincomycin is a bacteriostatic antibiotic which inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit inhibiting peptidyl transferase enzyme activity (Gale et al, 1981; Rodriguez-Fonseca et al, 1995). Spectinomycin is a bacteriostatic antibiotic that also inhibits bacterial protein synthesis where it reversibly interferes with mRNA interaction with the 30S ribosomal subunit (Gale et al, 1981). Gentamicin belongs to the aminoglycoside family of antibiotics (bactericidal) which inhibits protein synthesis of bacteria by irreversibly binding to the 30S ribosomal subunit (Buss et al, 1984; Buss et al, 1985). This caused the inhibition of the initiation complex formation necessary for protein synthesis. Gentamicin also interferes with initiated protein synthesis by causing misreading of the mRNA (Buss et al, 1984; Buss et al, 1985). Tylosin is a bacteriostatic antibiotic belonging to the macrolide family of antibiotics which inhibits bacterial protein synthesis through binding to the 50S ribosomal subunit which inhibits translocation (Gale et al, 1981; Rodriguez-Fonseca et al, 1995).

Mitochondrial ribosomes of eukaryotes consist of large and small subunits bound together with proteins into one 55S subunit (Alberts et al, 2004). The antibiotics used for bovine semen freezing extenders target the ribosomes of bacteria. Most of the antibiotics that bind to the ribosome have been shown to interact directly with ribosomal RNA (Gale et al, 1981; Moazed and Noller, 1987; Fourmy et al, 1996).

Cytoplasmic ribosomes of eukaryotes are insensitive to ribosomal-targetted antibiotics while prokaryotes ribosomes are sensitive. However, in vitro mitochondrial ribosomes show a mixed susceptibility pattern (Kurtz, 1974). This mixed susceptibility has

been shown in humans where ototoxicity and nephrotoxicity has been reported after the use of the ribosomes-targeted antibiotics of the aminoglycoside family (streptomycin, gentamicin) (Kurtz, 1974; Gale et al, 1981). Isolated bovine mitochondrial ribosomes showed susceptibility to bacterial ribosome-targeted antibiotics like chloramphenicol, lincomycin, carbomycin, tylosin and vanamycin A (Denslow and O'Brien, 1978). Bovine mitochondrial ribosomes showed a low susceptibility to lincomycin and tylosin at normal doses while higher doses affected mitoribosomes (Denslow and O'Brien, 1978). The cocktail that the AI industry has adopted was tested and found to be harmless to spermatozoa at certain concentrations (Lein, 1986; Lorton, 1986; Shin, 1986). However, Ahmad et al (1987) showed that the percentage of motile spermatozoa was slightly but significantly depressed at the higher concentrations of linco-spectin tested in whole milk and with minocin in egg yolk citrate and egg yolk phosphate extenders. Of the cocktail, only gentamicin reduced fertility on the basis of 59-day nonreturn rates (Ahmad et al, 1987). Although all antibiotics in the cocktail target bacterial ribosomes, it appeared that there was selectivity of these drugs in regard to their toxicity. In rats treated with 2 mg/Kg gentamicin sulfate for eight days, spermatogonial division was blocked and spermatocyte I meiosis was partly or completely interrupted (Timmermans, 1974).

It appears that through the evolution of mammalian mitochondria, conservation of certain loci in the mitochondrial ribosomes from prokaryotes occurred. This appears to be responsible for the structure selectivity of antibiotics targeting mitoribosomes and their toxicity (Botteger et al, 2001).

## **2.9 Mitochondria as a Target for Cell Toxicity**

In addition to their critical role in energy generation in eukaryotes, mitochondria are also active participants in a variety of tissue-specific metabolic processes like urea

generation, heme synthesis, and fatty acid  $\beta$ -oxidation (Nelson and Cox, 2005). They are also structurally, electrochemically, and physiologically complex. The end result is that there are numerous possible mechanisms by which xenobiotics can interfere with the normal structure or function of this organelle. Inhibition of the respiratory chain occurs at any of the four protein complexes in the chain. However, effects on complex IV (cytochrome c oxidase) are the most severe because this is the step where oxygen is reduced to water. Inhibition at complex III can result in the generation of reactive oxygen species as the consequence of the inherent instability of the electron transfer process to this complex from reduced ubiquinone (Semeitink et al, 2004). Examples of toxicants which work by this mechanism are rotenone, cyanide, and antimycin (Fosslien, 2001; Nelson and Cox, 2005). Uncouplers of oxidative phosphorylation usually act to dissipate the proton gradient between the intramembrane space and the matrix (Terada, 1990; Semeitink et al, 2004; Nelson and Cox, 2005). Compounds such as 2, 4-dinitrophenol can act as direct protonophores to shuttle hydrogen ions into the matrix. They may also act as ionophores and exchange hydrogen ions for other mono or divalent cations, or may increase the permeability of the inner membrane in general. The dissipation of the proton gradient without ATP generation can result in the generation of heat (Terada, 1990; Fosslien, 2001; Semeitink et al, 2004; Nelson and Cox, 2005). The majority of mycotoxins such as oligomycin inhibits ATP synthase enzyme, thereby ATP synthesis is inhibited (Terada, 1990; Wallace and Starkov, 2000; Semeitink et al, 2004; Nelson and Cox, 2005).

Alternate electron acceptors like quinones (adriamycin and paraquat) are capable of extracting electrons from intermediates in the respiratory chain and competing with the natural substrates. Quinones may also redox cycle, by-passing electrons back to the

respiratory chain at a later point, by-passing points in the chain that are essential for energy generation (Wallace and Starkov, 2000; Fosslie, 2001).

Toxicity with t-butyl-hydroperoxide and the chronic hepatotoxicity with the non-steroidal anti inflammatory drugs (Lemasters, 1999) induce mitochondrial permeability transition. Mitochondrial permeability transition pore is a high conductance, nonspecific pore in the inner mitochondrial membrane that is composed of proteins that link the inner and outer mitochondrial membranes (Richelli et al, 1999). When opened as a result of exposure to high calcium or inorganic phosphate, depletion of NADPH and NADH, alkaline pH, or reactive oxygen species, and low molecular weight substrates can freely penetrate the mitochondrial matrix. These low molecular weight substrates induces mitochondrial swelling and rupture releasing cytochrome c to the cytosol (Richelli et al, 1999). Cytochrome c release triggers the cascade of events that lead to apoptosis (in ATP replete cells) or necrosis (in ATP-depleted cells) (Lemasters, 1999; Ravagnan et al, 2002).

The close similarity between bacterial and mitochondrial ribosomes makes the later a potential target for bacterial-targetted antibiotics. Therefore, bacteriostatic antibiotics such as chloramphenicol, aminoglycosides and oxazolidinones (Lemasters, 1999; Wallace and Starkov, 2000; Fosslie, 2001) inhibits mitochondrial protein synthesis.

## **2.10 Mitochondria and Apoptosis**

Apoptosis is a physiologically programmed cell death that affects single cells without any related inflammation in the surrounding tissue influencing the process (Kerr et al, 1972). Apoptosis is a complex phenomenon that can be divided into three phases: induction, execution, and degradation. Mitochondria are known to play a central role during the execution phase (Martin et al, 2004). After the induction phase, mitochondrial pores are opened which is characterized by decreased mitochondrial membrane potential.

Opening of these pores leads to the release of proapoptotic factors from the mitochondria (Ravagnan et al, 2002). In the cytoplasmic compartment, the proapoptotic factors, which include different proteases related to the caspase family, are activated leading to the degradation phase (Martin et al, 2004). During the degradation phase, changes at the level of the cell surface and nucleus occur. For example, phosphatidylserine which is usually sequestered in the plasma membrane inner leaflet, appears in the outer leaflet where it triggers a non-inflammatory phagocytic recognition of the apoptotic cell (Bratton et al, 1997). Spontaneous apoptosis has been reported in rat testis seminiferous epithelium, affecting spermatogonia, spermatocytes and spermatids (Lue et al, 1999). Using electron microscopy, ejaculated human spermatozoa have been shown to exhibit certain characteristics of apoptotic somatic cells like DNA fragmentation and chromatin condensation, lobulation of the acrosomal membrane and mitochondrial distension (Baccetti et al, 1996). In addition, the apoptotic spermatids phagocytosed by macrophages can be observed in ejaculated sperm (Baccetti et al, 1996). During apoptosis, the decrease in mitochondrial membrane potential results from the opening of membrane pores located in the mitochondrial membrane. The consequence is translocation and activation of various proapoptotic factors.

Cryopreservation of bovine spermatozoa induced a statistically significant increase in proportion of cells with active caspases (Martin et al, 2004). Cryopreservation of human spermatozoa induced caspase activity, activation of caspase-9 along with disruption of mitochondrial membrane potential, and the release of mitochondria-associated regulating proteins (Paasch et al, 2004). According to Martin et al (2004), 2.2% of fresh spermatozoa showed active caspases whereas after cryopreservation, this proportion increased to 12 %. Active caspases were found to be mainly detected in the intermediate piece of spermatozoa

(Martin et al, 2004). Martin et al (2004) showed that cryopreservation had a dramatic effect on sperm mitochondrial membrane potential. Mitochondrial ATP synthesis is dependent on high mitochondrial membrane potential and a decrease in this potential can result in mitochondrial dysfunction. This leads to depletion of ATP and subsequently lack of energy for spermatozoa and accounts for decreased motility after the freezing/thawing processes. During programmed cell death, the release of apoptotic factors located between the inner and outer membrane is another consequence of the loss of mitochondrial integrity. Then, disruption of mitochondrial membrane integrity induced by cryopreservation would be responsible for the entry of these apoptotic factors into the cytoplasm (Martin et al, 2004).

## **CHAPTER 3**

### **MATERIALS AND METHODS**

#### **3.1 Experimental Animals**

This study included three yearling Holstein bulls from the Dairy Improvement Center at Louisiana State University Agricultural Center (Baton Rouge, LA). During the study period (mid January to late May, 2006), the bulls were kept on pasture and maintained on hay and water ad libitum and concentrate.

#### **3.2 Semen Collection and Evaluation**

Semen samples were obtained from these bulls using an artificial vagina. Two ejaculates, 5 to 10 minutes apart, were collected from each bull on a weekly basis for 5 weeks. Immediately after semen collection, ejaculates were pooled together, evaluated for motility and cell concentration. Motility of spermatozoa from each bull was assessed using a computer assisted semen analysis system (CASA) (Hamilton-Thorn research, Beverly, MA, USA). Spermatozoal concentration was measured by using a spectrophotometer (Spectronic 20, Bausch and Lomb, Rochester, N.Y.).

#### **3.3 Semen Processing (Extension and Freezing)**

The semen freezing extender used in this study was an egg yolk- citrate glycerol extender (Table 3.1) (Salisbury et al, 1978). Immediately after semen evaluation, each ejaculate was split into 4 parts and each part was initially extended with part A (non-glycerolated portion) of the following assigned extender treatments:

Extender treatment 1: Semen extended with freezing extender (Table 3.1) without glycerol

Extender treatment 2: Semen extended with the freezing extender (Table 3.1)

Extender treatment 3: Semen extended with the freezing extender (Table 3.1) without antibiotics

Extender treatment 4: Semen extended with the freezing extender (Table 3.1) without glycerol and antibiotics.

Table 3.1. Components of egg yolk- citrate glycerol extender

Component	Part A (mL)*	Part B (mL)*
Sodium Citrate (2.9%, wt/vol, in distilled water)	100	77.5
Egg yolk	25	25
Glycerol (9%, vol/vol)	-	22.5
Linco-spectin (300/600 µg/mL)	0.09375	0.09375
Tylosin (100 µg/mL)	0.03125	0.03125
Gentamicin (500 µg/mL)	0.03125	0.03125

\* Quantities from parts A and B to make 250 mL of the extender

Semen extension was performed at 37°C. Then, the tube of extended semen was transferred in a water jacket, at 37°C to a cold room (5°C) and cooled to 5°C over two hours. The glycerolated portion (part B) of the extender corresponding to each treatment was then added to the cooled non-glycerolated extended portion of the semen (part A) and left in the cold room for 1.5 hours to equilibrate.

After equilibration, extended semen in each treatment was split into 2 portions: a portion to be used immediately in the resazurin reduction and ATP measurement assays (Pre-freeze semen) and an aliquot from each extender treatment stored at 0°C for sequencing of the NADH 1 (ND1) gene of spermatozoal mtDNA. The other portion was packaged in 0.5 mL French straws, frozen (Chandler et al, 1984) and stored in liquid nitrogen to be used later in the resazurin reduction and ATP measurement assays, electron transfer chain analysis and for sequencing of the NADH 1 (ND1) gene of spermatozoal mtDNA. The pre-freeze semen was transferred from the cold room to a water bath at 37°C before the beginning of the assay.

### **3.4 Spectrophotometric Measurement of Bovine Spermatozoal Metabolic Activity Using Resazurin Reduction Assay**

#### **3.4.1 Determination of the Amount of Resazurin in the Resazurin Reduction Assay**

The resazurin blue color exhibits an absorption peak at 600-650 nm, whereas the reduced form has an absorption maximum at 580 nm. To determine the concentration of resazurin that was to be used in the assay, several concentrations of resazurin dye (0, 20, 40, 60, 80, 100 and 120  $\mu\text{mole/L}$ ) were prepared by dissolving the resazurin dye (Sigma, San Jose, CA) in 2.9% sodium citrate solution. Then, spectrophotometric (Spectronic Genesis 5, Spectronic Instruments Inc., Rochester, NY) measurement of the absorbance of each concentration was performed at 600nm (absorption peak). All chemicals for this and other procedures, unless otherwise stated, were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

#### **3.4.2 Resazurin Reduction Assay**

Resazurin solutions were prepared on a weekly basis, as the dye is stable if protected from light and can be stored up to 6 months in the refrigerator (Glass et al, 1991). The resazurin test was performed by adding 5  $\mu\text{mole/L}$  of resazurin stock solution to a 1.5 mL spectrophotometer cuvette containing 1 mL sperm suspension ( $50 \times 10^6$  spermatozoa) to achieve a final concentration of 55  $\mu\text{mole/L}$  resazurin in the assay sample. The contents of the cuvette were then mixed gently and incubated in a water bath at 37°C for 125 minutes. Blanks were made by taking an equal amount of the sample to be assayed (1 mL), and spermatozoa were killed by imposing three freeze-thaw cycles. This allowed for excluding the background due to the presence of sperm cells and egg yolk of the extender. Spectrophotometric readings were obtained for each sample and its corresponding blank after 0, 25, 50, 75, 100 and 125 minutes of incubation.

At the end of each assay, the spectrophotometric readings of the blank treatments were subtracted from its corresponding treatment readings. The frozen portion of the semen in all treatments was thawed at 37°C for 20 seconds in a water bath (Chandler et al, 1984). Then, the resazurin assay was performed on these samples as described above.

### **3.5 Respiratory Chain Enzyme Activities in Cryopreserved Bovine Spermatozoa**

#### **3.5.1 Semen Thawing**

Frozen semen was thawed at 37°C for 20 seconds and motility was evaluated immediately by using CASA. The thawed semen was split into 2 portions: the first portion to be assayed for enzymatic activity immediately after thawing and the second portion was further incubated in a 37°C water bath for 125 minutes and assayed for enzymatic activities.

#### **3.5.2 Respiratory Chain Enzyme Activities**

##### **3.5.2.1 Sample Preparation**

The thawed semen from each treatment was diluted 1:1 (vol/vol) with 2.9% sodium citrate at 37°C. The diluted semen was centrifuged for 10 minutes at 600g at room temperature. The supernatant was discarded and the pellet was washed with 2.9% sodium citrate solution. Samples were centrifuged again for 10 minutes at 600g and the supernatants were eliminated. The pellets were resuspended in a sufficient volume of 20 mmole/L potassium phosphate buffer, pH 7.0, to achieve a final concentration of  $2 \times 10^{11}$  spermatozoa/L. Samples were then lysed by freeze-thawing before analysis (6 freeze-thaw cycles in liquid nitrogen) (Ruiz-Pesini et al, 1998). Prior to the lysis, each sample was split into 2 equal portions: one portion to be lysed and used for the cytochrome c oxidase (Complex IV) specific activity assay and the other portion kept in liquid nitrogen and lysed later for the citrate synthase specific activity assay.

### 3.5.2.2 Specific Activity of Cytochrome c Oxidase (Complex IV)

This assay is based on the decrease in absorbance at 550 nm of ferrocytochrome c (reduced cytochrome c) caused by its oxidation to ferricytochrome c (oxidized cytochrome c) by cytochrome c oxidase enzyme (Barroso et al, 1993; Brich-Machin et al, 1994; Ruiz-Pesini et al, 1998). The reaction assay (Ruiz-Pesini et al, 1998) was run in a 1 mL spectrophotometer cuvette using 100 mmole/L potassium phosphate buffer, high molecular grade water, sample (required aliquot containing  $4 \times 10^6$  spermatozoa) and 1% freshly prepared reduced cytochrome c which was added to the mixture immediately prior to the reaction assay. Cytochrome c was obtained in an oxidized state in a powdered form, diluted in high molecular grade water, pH 7 and then reduced with one mole/L dithithreitol (DTT) solution.

Prior to the assay, the semen sample was split into 2 portions: portion one portion was used in the assay mixture above (partial cytochrome c oxidase activity). Portion two was incubated with 1% Triton X-100 for 10 minutes at room temperature (total cytochrome c oxidase activity).

After the reaction mixture was made and before adding the required volume of reduced cytochrome c, the spectrophotometer cuvette was loaded into a water-jacketed cuvette holder. A continuous flow water bath kept the cuvette temperature at 38°C. Absorbance was measured every 10 seconds for one minute using a Spectronic Genesis 5 spectrophotometer. This measurement was considered the blank. After the blank reading was obtained, the required volume of reduced cytochrome c was added to the reaction mixture and the contents of the cuvette were mixed by inversion. Absorbance was measured using the Simple Kinetic option on the Biochemistry soft card of the Spectronic Genesis 5 spectrophotometer. The reaction was run for 1 minute at 550 nm, and the

absorbance was measured every 10 seconds with an initial lag of 15 seconds. The same procedure was applied to the portion of semen incubated with Triton X100. The reaction for each sample was run in duplicate. The UV option of the Spectronic Genesis 5 spectrophotometer was used to measure protein concentration (mg/dL) in each sample prior to the assay. The extinction coefficient between reduced and oxidized cytochrome c was used to calculate enzyme activity and protein content for calculation of enzyme specific activities. The following formula was used to calculate the enzyme activity:

$$\text{Units/mL (U/mL)} = (\Delta A/\text{min} \times \text{DF} \times 1) / (\text{sample vol}) \times 21.84$$

Where:

$\Delta A/\text{min} = A/\text{min} (\text{sample}) - A/\text{min} (\text{blank})$  - the change in the sample activity (absorbance/min) corrected for the change in blank activity (absorbance/min).

DF: dilution factor of sample

1: reaction volume in mL

21.84: the difference in extinction coefficient between reduced cytochrome c and oxidized cytochrome c at 550 nm.

Enzyme specific activity = enzyme activity/protein concentration

### **3.5.3 Specific Activity of Citrate Synthase**

#### **3.5.3.1 Sample Preparation**

Semen was prepared for this assay as described in the cytochrome c oxidase assay. This assay is based on the increase in absorbance at 412 nm due to the conversion of DTNB (5,5'-dithio-bis-2-nitrobenzoic acid) to TNB (5-thio-bis-2-nitrobenzoic acid) by the thiol group of Acetyl CoA (CoA-SH) in the reaction between acetyl coenzyme A and oxaloacetate. This reaction is catalyzed by citrate synthase (Barroso et al, 1993; Brich-

Machin et al, 1994; Ruiz-Pesini et al, 1998; Ruiz-Pesini et al, 2000a; Ruiz-Pesini et al, 2001).

The reaction assay (Ruiz-Pesini et al, 1998) was run in a 1 mL spectrophotometer cuvettes using 0.75 mole/L Tris-HCL pH 8, 10 mmole/L DTNB (5,5'-dithio-bis-2-nitrobenzoic acid in Tris-HCL 0.75 mole/L pH 8), 30 mmole/L Acetyl coenzyme A in Tris-HCL 0.75 mole/L, pH 8), high molecular grade water, sample (required aliquot containing  $4 \times 10^6$  spermatozoa), 10 mmole/L oxaloacetate in Tris-HCL 0.75 mole/L, pH 8, which was added to the mixture immediately prior to the reaction assay. Prior to the assay, the semen sample was split into 2 portions: one portion was used in the assay mixture above (partial citrate synthase activity) and the other portion was incubated with the detergent, Triton X100 (1%, vol/vol) for 10 minutes at room temperature (total citrate synthase activity).

After the reaction mixture was made and prior to adding the required volume of oxaloacetate, the spectrophotometer cuvette was loaded into a water-jacketed cuvette holder. A continuous flow water bath kept the cuvette temperature at 30°C (Barroso et al, 1993; Brich-Machin et al, 1994; Ruiz-Pesini et al, 1998; Ruiz-Pesini et al, 2000a; Ruiz-Pesini et al, 2001). Absorbance was measured every 10 seconds for 1.5 minutes using a Spectronic Genesis 5 spectrophotometer. This measurement was the blank. After the blank reading was obtained, the required volume of oxaloacetate was added to the reaction mixture and the contents of the cuvette were mixed by inversion. Absorbance was measured using the Simple Kinetic option on the Biochemistry soft card of the Spectronic Genesis 5 spectrophotometer. The absorbance was measured every 10 seconds with an initial lag of 20 seconds. The UV option of the Spectronic Genesis 5 spectrophotometer was used to measure protein concentration (mg/dL) in each sample prior to the assay.

The same procedure was applied to the portion of semen incubated with the detergent. The reaction for each sample was run in duplicate. The extinction coefficient of TNB was used to calculate enzyme activity and protein content for calculation of enzyme specific activities.

The following formula was used to calculate the enzyme activity:

$$\mu\text{mole/ml/min} = (\Delta A_{412})/\text{min} \times \text{Vol (ml)} \times \text{DF/EmM} \times \text{L (cm)} \times \text{Vol enzyme (mL)}$$

where:

$(\Delta A_{412}) = A/\text{minute (sample)} - A/\text{minute (blank)}$

DF - the dilution factor of the original sample

Vol (mL) – the reaction volume: for test in 1 mL cuvette = 1 mL

Vol enzyme (mL) – the volume of the enzyme sample in mL

EmM ((mmole/L)-1 cm<sup>-1</sup>) – the molar extinction coefficient of TNB at 412 nm is 13.6

L (cm) – pathlength for absorbance measurement:

for 1 mL cuvette, pathlength = 1 cm

enzyme specific activity = enzyme activity/protein concentration

### **3.6 Analysis of ATP Content of Bovine Spermatozoa Using Luciferin-Luciferase Assay**

#### **3.6.1 ATP and Spermatozoal Number Standard Curve Preparations**

At the beginning of the experiment, an ATP standard curve was generated in order to calculate the ATP content in spermatozoa. One  $\mu\text{mole/L}$  ATP stock solution was obtained (Promega®, Madison, WI) and different serial dilutions were prepared (0.001 pmole/L, 1 pmole/L, 0.1 nmole/L, 10 nmole/L and 1  $\mu\text{mole/L}$ ) in 2.9% sodium citrate buffer. One hundred  $\mu\text{mole/L}$  of each dilution were transferred to a multi-well plate (Promega®,

Madison, WI). Quantitative determination of ATP required the presence of the luciferin-luciferase which, when mixed with ATP, produced light according to the following reaction:

Luciferin+ATP+O<sub>2</sub>  $\xrightarrow{\text{Luciferase, Mg}^{2+}}$  Oxyluciferin+AMP+CO<sub>2</sub>+Light (Long and Guthrie, 2006). The intensity of the emitted light was proportional to the ATP concentration.

Equal volumes of the luciferin-luciferase solution from the assay kit (Promega®, Madison, WI, USA) were added to the ATP serial dilutions. The contents of the multi-well plate were mixed gently using an orbital shaker TekTator® V (Tekpro, Evanston, IL) for two minutes and then incubated for 15 minutes at room temperature. Measurement of bioluminescence was performed using the Veritas™ Microplate Luminometer (Turner Biosystems, Sunnyvale, CA, USA). The emitted light was measured by the luminometer in relative light units (RLU) and the Turner "Direct-to-Excel" based software reported data directly to a spreadsheet.

The number of spermatozoa needed to be used in the assay was determined by generating a standard curve. Different spermatozoa concentrations were prepared (0, 0.25, 0.5, 0.75 and 1 ×10<sup>6</sup> spermatozoa in 100 μL 2.9% sodium citrate solution). Then, these samples were transferred to a 96-well plate and mixed with equal volumes of the luciferin-luciferase solution and mixed gently on an orbital shaker for 2 minutes. After mixing, the contents of the plate were incubated for 15 minutes. The assay was run in duplicate. Bioluminescence was measured using the luminometer.

### **3.6.2 Evaluation of Cryopreservation and Semen Extender Constituents on Spermatozoal ATP Content and Production/utilization**

The pre-freeze semen was transferred from the cold room to a water bath at 37°C before the beginning of the assay. An aliquot of semen from each extender treatment was

further extended in 2.9% sodium citrate solution to achieve  $1 \times 10^6$  spermatozoa/0.2 mL final concentration. Then, two-100  $\mu$ L aliquots of the extended semen ( $1 \times 10^6$  spermatozoa/aliquot) from each treatment were transferred to a 96-well plate and mixed with equal volumes of the luciferin-luciferase solution. The contents then were mixed gently on an orbital shaker for 2 minutes and incubated for 15 minutes at 24°C. Bioluminescence readings were obtained for each sample at 0, 25, 50, 75, 100 and 125 minutes of incubation in a 37°C water bath. Bioluminescence was measured using the luminometer and the resulting light intensities were converted to ATP values from the ATP standard curve (Figure 4.1). The frozen portions of semen in each treatment were thawed at 37°C for 20 seconds in a water bath. Then, ATP measurements were performed as described above. Total motility (TM), progressive motility (PM), pathway velocity (VAP), progressive velocity (PV) and curvilinear velocity (VCL) were obtained using CASA (Hamilton-Thorn research, Beverly, MA) simultaneously with RDA spectrophotometric and ATP luminometric readings.

### **3.6.3 Statistical Analysis**

Resazurin reduction rate was analyzed by the least squares method using PROC GLM of the SAS Statistical Package (v.9.1, 2003, SAS Institute, Inc., Cary, NC). Slopes were generated for resazurin reduction over time and were analyzed using the least squares method. The ATP content of spermatozoa in the different extender treatment groups and the ATP reduction rate (utilization) were analyzed by the least squares methods. Slopes were generated for the ATP reduction over time and were analyzed using the least squares methods. Enzymatic activities and mitochondrial membrane integrity data were analyzed by the least squares methods using SAS PROC GLM.

The motility data were analyzed by the least squares methods (SAS PROC GLM). Slopes were generated for the decrease in motility over time and were analyzed using the least squares methods (SAS PROC GLM). Mean differences were tested by Tukey's Test using SAS Statistical Package (v. 9.1, 2003, SAS Institute, Inc., Cary, NC). Differences with a probability level (P) of 0.05 or less were considered significant in this study.

### **3.7 Analysis of the ND1 Gene of Mitochondrial DNA in Pre-freeze and Post-thaw Bovine Spermatozoa**

#### **3.7.1 Mitochondrial DNA Extraction**

Mitochondrial DNA (mtDNA) extraction was performed according to Avarindakshan et al. (1998). The extended semen samples from each treatment were transferred to 1.5 mL microcentrifuge tubes and centrifuged at 15 000g for 3 minutes. The supernatant was eliminated and pellets were resuspended in 0.5 mL Aravindakshan lysis solution warmed to 50°C prior to the resuspension process. The samples were incubated in a water bath at 50°C for 30 minutes. Five µL of Proteinase K (Qiagen Inc., Valencia, CA) were added to each sample, mixed gently and were incubated in a water bath at 50°C for 12 to 14 hours. Phenol:chloroform (0.5 mL) was added to each sample and the tubes were shaken often while incubating at room temperature for 15 minutes. The samples were then centrifuged at 15 000g for 3 minutes. Using a Pasteur pipette, the bottom layer was removed and discarded. The phenol:chloroform step was repeated and the bottom layer was discarded. To each sample, 0.5 mL of chloroform was added and the tubes containing samples were shaken while incubating at room temperature for 10 minutes. The samples were centrifuged for 2 minutes and again the bottom layer was removed and discarded using a Pasteur pipette. Ice cold absolute ethanol, 1 mL, was added to each sample and were spun in a circular motion by hand. The majority of samples revealed a small clump of

white cotton-type strands. The samples were then centrifuged for 1 minute and the ethanol was discarded. Samples were placed in a vacuum/desiccator until the pellet was dry (about 1 to 2 hours). The pellet was resuspended with 300  $\mu$ L of sterile water, and incubated in a water bath at 37°C until the pellet was completely dissolved. The DNA content of each sample was measured using a Spectronic Genesis 5 spectrophotometer (Spectronic Instruments, Rochester, NY) by measuring absorbances at 260 and 280 nm. Calf thymus DNA was used as a standard and the extinction coefficient between sample DNA and standard DNA measured at 260 nm was determined (0.022). Each sample DNA measurement at 260 nm was divided by the extinction coefficient (0.022) to determine the concentration of DNA (ng/ $\mu$ L) in each sample. The product was divided by 300 in order to determine the number of  $\mu$ Ls of each sample DNA required to give 300 ng of DNA (Avarindakshan et al,1998).

### **3.7.2 Primers Selection**

Only the ND1 sequence of the bovine mtDNA was sequenced because it codes for Complex I of the respiratory chain. The ND1 sequence used in this experiment contained the base pairs (bp) 3131 through 4055 of bovine mtDNA. The sequenced ND1 gene of mtDNA was compared to the mtDNA ND1 sequence available at the NCBI Gene Bank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) (Anderson et al., 1982).

The ND1 gene contains 925 bp and the PE Biosystems ABI 3700 DNA Analyzer 96 capillary electrophoresis system could not accommodate the 925 base pair sequence. The Gene Lab at Louisiana State University (personal communication) recommended that segments of 550 bp or less have to be used for accurate sequencing. Two overlapping segments were obtained from the 925 bp sequence to accommodate the electrophoresis system. Primers for each segment were selected from the Vector NTI computer Program

(InforMax Inc. US Headquarters, Frederick, MD). Based on primer length and annealing temperature, one primer set for each segment was selected. Shown below are the primers for both the segment 1 and 2 of the ND1 sequence (Table 3.2).

Table 3.2. Primers used in the mtDNA sequencing procedure

Segment	Size	Sense/antisense primer	Primer sequence
1	457 bp (3131 to 3588)	BOVNADH1S (sense)	ATTCCCATCCTATTGGCC
		BOVNADH1A(antisense)	GAGAGGGTAAAGGACCCACT
2	488 bp (3567 to 4055)	BOVNADH1S (sense)	TAAGTGGGTCCTTTACCCTC
		BOVNADH1A (antisense)	ATGTTTGTGGTGGGATGC

Primers were produced by the Gene Lab (Department of Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA). In order to align the two segments, the first segment of the ND1 sequence used the antisense primer (3' to 5'), and the second segment used the sense primer (5' to 3'). Because microcentrifuge tubes of extracted DNA contained both mitochondrial and nucleic DNA, these primers were selected specifically for mtDNA.

### 3.7.3 Conventional Polymerase Chain Reaction

In order to standardize conditions (including buffering capacity, ion concentrations and amount pipetted differences) of PCR component concentrations other than DNA and primers, PCR tubes (0.5 ml) containing Pure Taq Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, NJ) were used. A total of 25  $\mu$ L (containing DNA, primers, and sterile water) were placed in each tube. An amount of DNA, based on the spectrophotometric measurements, was placed in the tube. One  $\mu$ L of each primer for the first segment (457 bp [3131 - 3588]) BOVNADH1S1 and BOVNADH1A1, was placed in the tube. The specific amount of DNA that was placed in the tube combined with 2  $\mu$ L of

primers were subtracted from the 25  $\mu$ L total to determine how much sterile water to be added. An equal amount of DNA from the same sample was added to another PCR tube, and the primers for the second segment (488 bp [3567 - 4055]), BOVNADH1S2 and BOVNADH1A2, were added to that tube. A control tube was setup using a randomly selected sample, adding 1  $\mu$ L of each of the GAPDH primer sequences GAPDH S and GAPDH A and sterile water to bring the total volume to 25  $\mu$ L. Finally, 50  $\mu$ L of mineral oil were added to the PCR tubes to prevent evaporation during PCR procedure (Erlich et al, 1991; Erlich and Arnheim, 1992).

Thermocycler settings were one cycle at 95°C for 2 minutes, 53°C for 1 minute, 72°C for 2 minutes, and 0°C for zero minutes; 29 cycles at 94°C for one minute, 53°C for 2 minutes, 72°C for 2 minutes, and zero minutes at 0°C; one cycle at 72°C for 10 minutes. Finally, the samples were stored at 4°C for at least 60 minutes (Erlich et al, 1991; Erlich and Arnheim, 1992).

### **3.7.4 Gel Electrophoresis**

A 4% electrophoresis agarose gel (Agarose I, Amresco®, Solon, OH) was used. It was made by mixing 175 mL 1X Tris-acetate/EDTA electrophoresis buffer (TAE) with 7 g of agarose and heated in a microwave oven, until all of the agarose had been dissolved. Ethidium bromide (3  $\mu$ l) were added to the hot gel and swirled to mix. To avoid warping of the gel, it was allowed to incubate at 24°C to cool before being poured into a tray. The gel was made with either 30-well or 8-well combs, depending on the number of samples.

Microcentrifuge tubes (1.5 mL) were filled with 8  $\mu$ L of mtDNA product from the PCR tube for each sample and 5  $\mu$ L of tracking dye as well as for controls. A marker tube was also made containing 1  $\mu$ L of marker, 11  $\mu$ L of TAE buffer, and 5  $\mu$ L of tracking dye.

The electrophoresis machine (Hoefer Scientific Instruments, San Francisco, CA) was then set to 127 V for 1.5 hours.

The DNA marker was a molecular weight ladder to confirm that mtDNA which was produced during the PCR process and that GADPH was in the control tube. A band molecular weight of 281 was at the approximate location of the GADPH band which contained 293 bp. The bands appeared of both the first segment of ND1 gene containing 457 bp and the second segment containing 488 bp. Both bands were located between the molecular weight marker of 281 and 603 which confirmed that only mtDNA was produced, and not nDNA.

### **3.7.5 PCR**

The Millipore PCR Purification Kit (Millipore Corp., Billerica, MA) was used to extract the ND1 gene from the remaining PCR product. Only 16  $\mu\text{L}$  of product were placed into a sample reservoir of one of the two vials provided in the Millipore PCR purification kit. Sterile water, 364  $\mu\text{L}$ , were added to each reservoir to achieve a total amount of 400  $\mu\text{L}$ . Samples were centrifuged in a table top centrifuge at 562g for 27 minutes at 24°C. The reservoir was removed and transferred to a clean tube. The initial tube containing the filtrate was labeled and stored at refrigeration temperature until the sample was analyzed and the DNA content was confirmed. A volume of 20  $\mu\text{L}$  of sterile water was added to each reservoir and the contents were transferred to a new tube, inverted and tubes were centrifuged at 562g for four minutes. The supernatants were eliminated and the pellets were resuspended in 300  $\mu\text{L}$  sterile water. Samples were measured for DNA content using a Spectronic Genesis 5 Spectrophotometer. The amount of DNA in each sample was obtained by dividing the absorbance at 260 nm by the extinction coefficient (0.022). To allow 3 sequencing procedures to run on each sample, three-fold the amount of DNA

resulting from each measurement for each sample was shipped in siliconized tubes to Pennington Gene Lab (Pennington Biomedical Research Center, Baton Rouge, LA). Along with the samples to be sequenced, 20  $\mu$ L of each of the primers BOVNADH1A1 and BOVNADH1S2, each mixed with 5  $\mu$ L of sterile water were also shipped in siliconized tubes. Samples were sequenced at the Pennington Gene LAB using a PE Biosystems ABI 3700 DNA Analyzer 96 Capillary electrophoresis system (PE Biosystems, Foster City, CA).

### **3.7.6 ND1 Gene Sequence**

The software Chromas version 1.45 (Technelysium Pty Ltd., Helensvale, Australia) was used to open the output from the PE Biosystems ABI 3700 DNA Analyzer 96 Capillary electrophoresis system to compare it to the ND1 base sequence through the Blast search of Chromas on the Blast web site ([www.ncbi.nlm.nih.gov/blast](http://www.ncbi.nlm.nih.gov/blast)).

The base sequence of each unknown sequence was compared to the mtDNA code stored in GenBank for the *Bos taurus* sequence (Anderson et al, 1982). The position number where a mismatch occurred between the unknown sample and the Anderson sequence received a score of 1. A zero indicated that the base at that position in the Anderson sequence (GenBank) matched the unknown sequences. The amino acids for each sequence base triplicate was displayed. If an amino acid change occurred it was assigned a “0” meaning that a point mutation, deletion, or addition did not change the resulting amino acid, or a “1” to expressed changes. A denotation of “1” implied that the point mutation/deletion/addition had resulted in a change in the amino acid for that specific triplet. These results were recorded and only the positions where the amino acid changes occurred were considered as non-silent mutations. The binary data obtained using this scoring system were kept for statistical analysis.

### **3.7.7 Statistical Analysis**

The frequency of change in amino acids in each position in the different extender treatments were analyzed by the least squares methods using PROC GLM of the SAS Statistical Package (v. 9e, 2003, SAS Institute, Inc., Cary, NC). Least square means data generated were extender treatment by storage (pre-freeze or post-thaw) by position. The data were sorted by the least square means values. All means with values greater or equal to 0.5 were kept and the values of means lesser than 0.5 were deleted which created a consensus. The means which had values greater or equal to 0.5 had significant probabilities which resulted in retaining the data with significant probabilities to reduce the original data set. Mean differences were tested by Tukey's Test using SAS Statistical Package (v. 9.1, 2003, SAS Institute, Inc., Cary, NC). Differences with a probability level (P) of 0.05 or less were considered significant in this study.

## CHAPTER 4

### RESULTS

#### 4.1 Resazurin Reduction Assay

From the resazurin standard curve (Figure 4.1), the optimal concentration used in this assay was 55  $\mu\text{mole/L}$  and selected from the dye concentration standard curve. This concentration was selected because at lower concentrations, the spectrophotometer was unable to detect a change in resazurin due to its reduction by NADH dehydrogenase. While higher concentrations yielded inaccurate measurements and were out of range for the spectrophotometer.

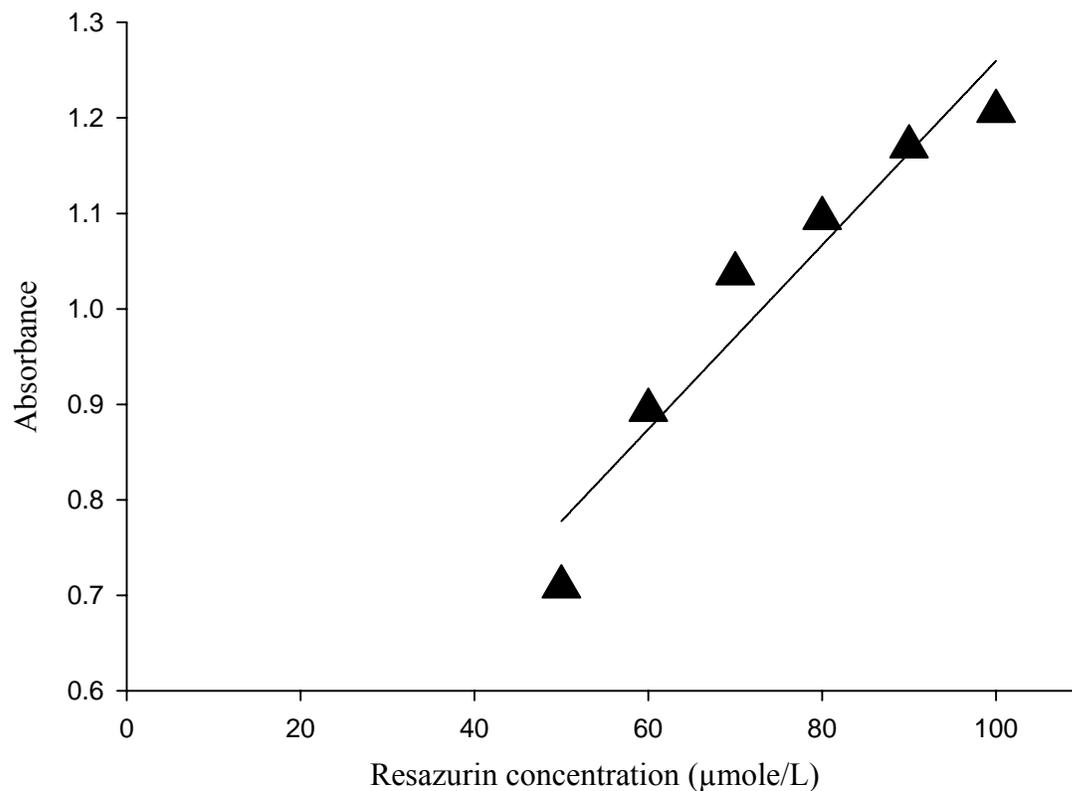


Figure 4.1. Resazurin standard curve. Absorbance of different resazurin concentrations measured using a Spectronic spectrophotometer at 600nm.

Shown in Table 4.1, extender, storage (pre-freeze vs. post-thaw) and incubation time main effects were significant sources of variation ( $P < 0.05$ ). However, the bull and the ejaculate within bull main effects were not significant sources of variation ( $P > 0.05$ ). Extender-storage and extender-storage-time interactions were significant sources of variation ( $P < 0.05$ ).

Table 4.1. ANOVA of resazurin reduction of bovine spermatozoa treated with extenders of various compositions‡ before and after cryopreservation

Source	Df	MS*	F	P-value
Bull	2	0.41	18.51	0.0004
Error a†	10.01	0.02		
Ejaculate(bull)	10	0.02	1.62	0.0980
Extender‡	3	0.12	8.93	<0.0001
Storage§	1	0.19	14.34	0.0002
Incubation time¶	5	0.27	20.34	<0.0001
Extender-storage	3	0.23	17.01	<0.0001
Extender-storage-incubation time	35	0.02	2.17	0.0002
Error: MS(error)	564	0.01		

\* Mean square.

† Error a:  $0.9992 \times \text{MS (ejaculate (bull))} + 0.0008 \times \text{MS (error)}$

‡ Extender 1 (sodium citrate egg yolk with antibiotics), extender 2 (extender 1 plus glycerol), extender 3 (extender 2 without antibiotics) and extender 4 (extender 1 without antibiotics).

§Pre-freeze vs. post-thaw.

¶ Sequential 25 minute intervals for 125 minutes at 37°C.

The resazurin reduction rate (the linear slopes of the individual treatment curves) decreases over incubation time (Figure 4.2) except for the cryopreserved portion of the spermatozoa in the extender treatments containing no glycerol (E1S2, E4S2). These results show that cryopreservation causes the most damage to spermatozoa, therefore decreased the dehydrogenase activity reflected in the resazurin reduction rates. There were differences between the extender treatments having glycerol and the extender treatments without glycerol in the frozen semen ( $P < 0.05$ ).

Bull main effect was not a significant source of variation ( $P > 0.05$ ) in the reduction rates, however, ejaculate within bull, extender and storage main effects and extender-storage interactions were significant sources of variation (Table 4.2) ( $P < 0.05$ ).

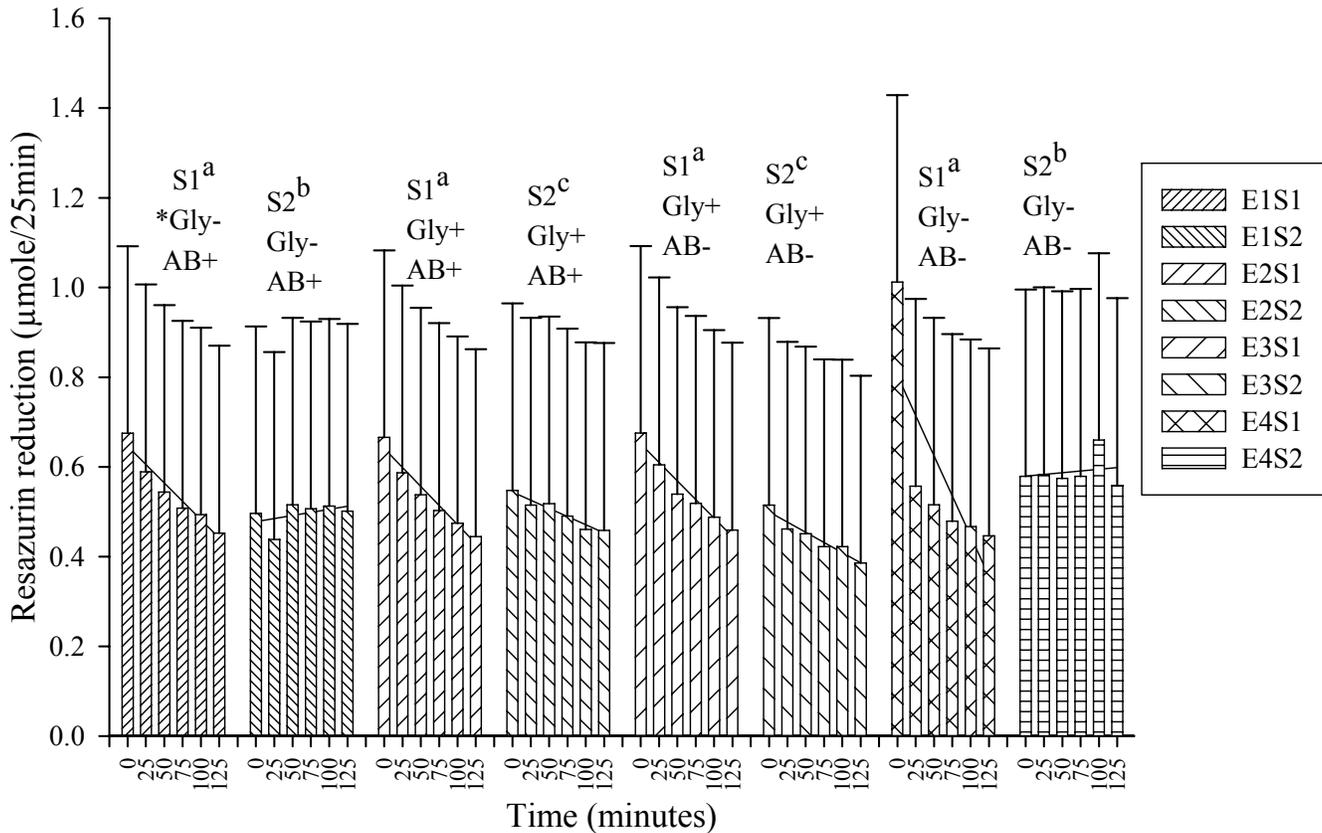


Figure 4.2. Least squares means for resazurin reduction ( $\pm$  SE) of bovine spermatozoa treated with extenders of various composition\* before and after cryopreservation. Gly-: contains no glycerol, Gly+: contains glycerol, AB+: contains antibiotics and AB-: contains no antibiotics E: extender, S1:Pre-freeze and S2: post-thaw. Means followed by same superscript are not different ( $P > 0.05$ ).

Regardless of glycerol inclusion in the extender, all the extender treatments were not different ( $P > 0.05$ ) from each other in the pre-freeze incubation (Figure 4.3). However, all the extender treatments did differ ( $P < 0.05$ ) after cryopreservation from their pre-freeze state. The extender treatments that had no glycerol were different ( $P < 0.05$ ) from the extender treatments that had glycerol after cryopreservation.

Table 4.2. ANOVA of resazurin reduction rates (slopes) of bovine spermatozoa treated with extenders of various composition<sup>‡</sup> before and after cryopreservation

Source	Df	MS*	F	P-value
Bull	2	0.001	0.59	0.5705
Error a <sup>†</sup>	10	0.001		
Ejaculate(Bull)	10	0.001	5.11	<0.0001
Extender <sup>‡</sup>	3	0.001	6.06	0.0009
Storage <sup>§</sup>	1	0.013	62.32	<0.0001
Extender-storage	3	0.001	3.88	0.0119
Error: MS(error)	84	0.0002		

\* Mean square.

<sup>†</sup> Error a: MS (ejaculate (bull))

<sup>‡</sup> Extender 1 (sodium citrate egg yolk with antibiotics), extender 2 (extender 1 plus glycerol), extender 3 (extender 2 without antibiotics) and extender 4 (extender 1 without antibiotics).

<sup>§</sup>Pre-freeze vs. post-thaw.

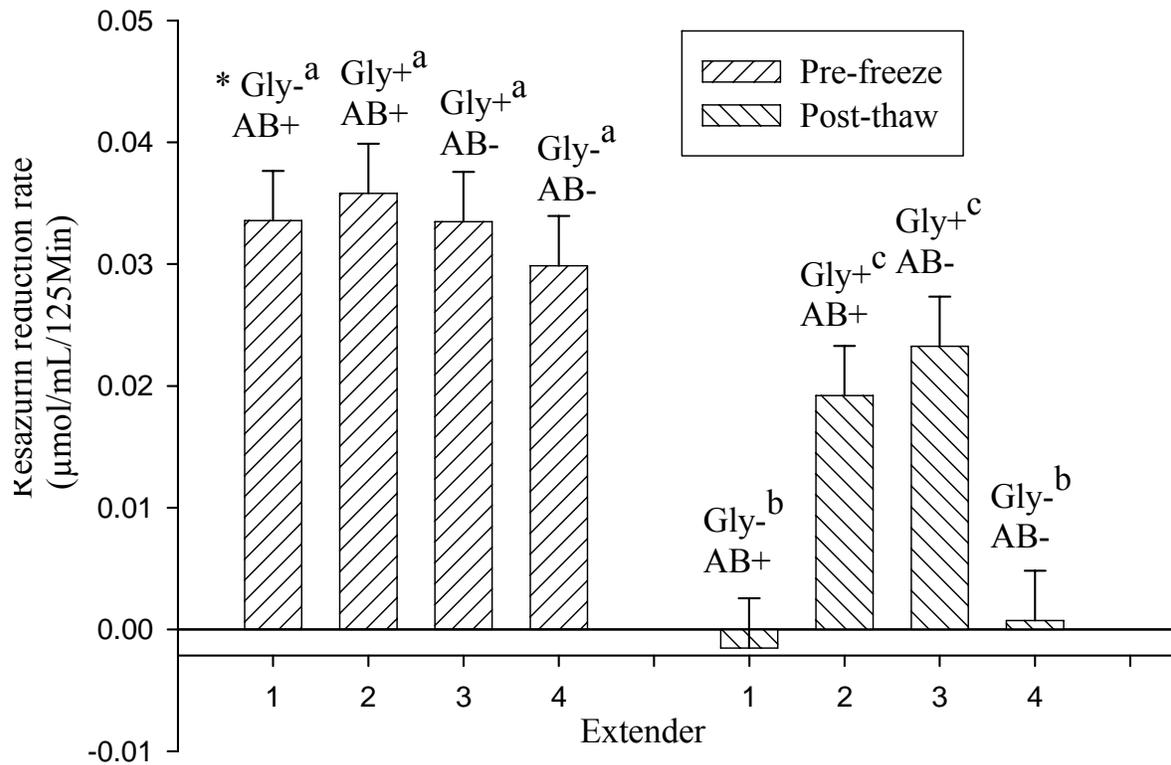


Figure 4.3. Least squares means for resazurin reduction rates (RDR,  $\pm$  SE) of bovine spermatozoa treated with extenders of various composition\* before and after cryopreservation. Gly<sup>-</sup>: contains no glycerol, Gly<sup>+</sup>: contains glycerol, AB<sup>+</sup>: contains antibiotics and AB<sup>-</sup>: contains no antibiotics. Means followed by the same superscript are not different ( $P > 0.05$ ).

Therefore, spermatozoal resazurin reduction rates were affected by the extender constituent glycerol and also by the cryopreservation process. There was no effect ( $P > 0.05$ ) of the extender constituent antibiotics on spermatozoal resazurin reduction rate either pre-freeze or post-thaw.

The starting points (intercepts) of the resazurin reduction assay did not differ ( $P > 0.05$ ). This means that spermatozoal resazurin reduction was the same at the beginning of the assay. Semen storage main effect was the only significant source of variation among the intercepts ( $P < 0.05$ ) (Table 4.3). This is due to the cryopreservation process, as shown in the extender treatments containing no glycerol as a cryoprotectant (Figure 4.4).

Table 4.3. ANOVA of resazurin reduction starting points (intercepts) of bovine spermatozoal treated with extenders of various composition‡ before and after cryopreservation

Source	Df	MS*	F	P-value
Bull	2	0.0060	0.37	0.7021
Error a†	10	0.0165		
Ejaculate(Bull)	10	0.0165	1.89	0.0579
Extender‡	3	0.0050	0.58	0.6299
Storage§	1	0.3004	34.39	<0.0001
Extender-Storage	3	0.0168	1.93	0.1316
Error: MS(Error)	84	0.0087		

\* Mean square.

† Error a: MS (ejaculate (bull))

‡ Extender 1 (sodium citrate egg yolk with antibiotics), extender 2 (extender 1 plus glycerol), extender 3 (extender 2 without antibiotics) and extender 4 (extender 1 without antibiotics).

§Pre-freeze vs. post-thaw.

#### 4.1.1 Spermatozoal Motility

Total motility (TM) analysis showed that ejaculate within bull, extender, storage, incubation time main effects and extender-storage as well as extender-storage-time interaction effects were significant sources of variance in TM ( $P < 0.05$ ) (Table 4.4).

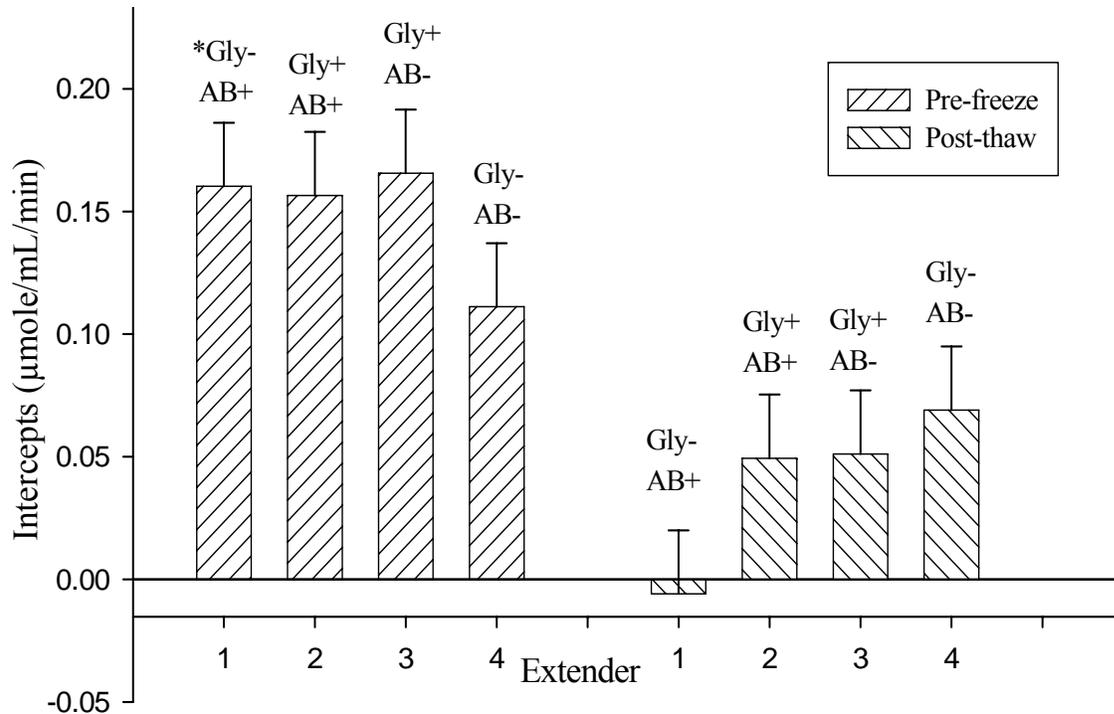


Figure 4.4. Least squares means for resazurin reduction starting points (intercepts,  $\pm$  SE) of bovine spermatozoa treated with extenders of various composition\* before and after cryopreservation. Gly-: contains no glycerol, Gly+: contains glycerol, AB+: contains antibiotics and AB-: contains no antibiotics.

Table 4.4. ANOVA of bovine spermatozoal total motility (TM) treated with extenders of various composition $\ddagger$  before and after cryopreservation

Source	Df	MS*	F	P-value
Bull	2	5122	0.77	0.4826
Error a $\dagger$	12.044	6615		
Ejaculate(Bull)	12	6944	28.01	<0.0001
Extender $\ddagger$	3	3619	14.60	<0.0001
Storage $\S$	1	663117	2674.35	<0.0001
Incubation time $\P$	5	2516	10.15	<0.0001
Extender-storage	3	7785	31.40	<0.0001
Extender-storage-incubation time	35	386	1.56	0.0224
Error: MS (Error)	610	247		

\* Mean square.

$\dagger$  Error a:  $0.9508 \times \text{MS (Ejaculate (Bull))} + 0.0492 \times \text{MS (Error)}$ .

$\ddagger$  Extender 1 (sodium citrate egg yolk with antibiotics), extender 2 (extender 1 plus glycerol), extender 3 (extender 2 without antibiotics) and extender 4 (extender 1 without antibiotics).

$\S$  Pre-freeze vs. post-thaw.

$\P$  Sequential 25 minute intervals for 1 25 minutes at 37°C.

During the incubation period, there was a reduction of TM over time and analysis of the reduction rate (slopes) in the percentages of TM revealed that extender main effect and extender-storage interaction effect were significant sources of variation in TM ( $P < 0.05$ ) (Table 4.5). Neither the ejaculate within bull nor storage main effects were significant sources of variation in the reduction rates of TM ( $P > 0.05$ ).

Table 4.5. ANOVA of bovine spermatozoal total motility (TM) reduction rates (slopes) treated with extenders of various composition ‡ before and after cryopreservation

Source	Df	MS*	F	P-value
Bull	2	0.8	0.08	0.9255
Error a†	10	10.7		
Ejaculate(bull)	10	10.7	1.73	0.0864
Extender‡	3	69.8	11.25	<0.0001
Storage§	1	0.3	0.05	0.8299
Extender-storage	3	53.9	8.69	<0.0001
Error: MS(Error)	84	6.2		

\* Mean square.

† Error a: MS (ejaculate (bull))

‡ Extender 1 (sodium citrate egg yolk with antibiotics), extender 2 (extender 1 plus glycerol), extender 3 (extender 2 without antibiotics) and extender 4 (extender 1 without antibiotics).

§Pre-freeze vs. post-thaw.

The reduction rates of TM (Figure 4.6) response to treatment were similar in the pre-freeze semen whereas  $3 = 2 > 1 = 4$  (ordered from highest to lowest) in the frozen semen. In the frozen semen, reduction rates in TM were similar in the extender treatments that had glycerol and there was no antibiotic effect on the rate of reduction in TM. In the extender treatments having no glycerol, slopes were zero because cryopreservation damaged unprotected spermatozoa and there was no motility post-thaw (Figure 4.6).

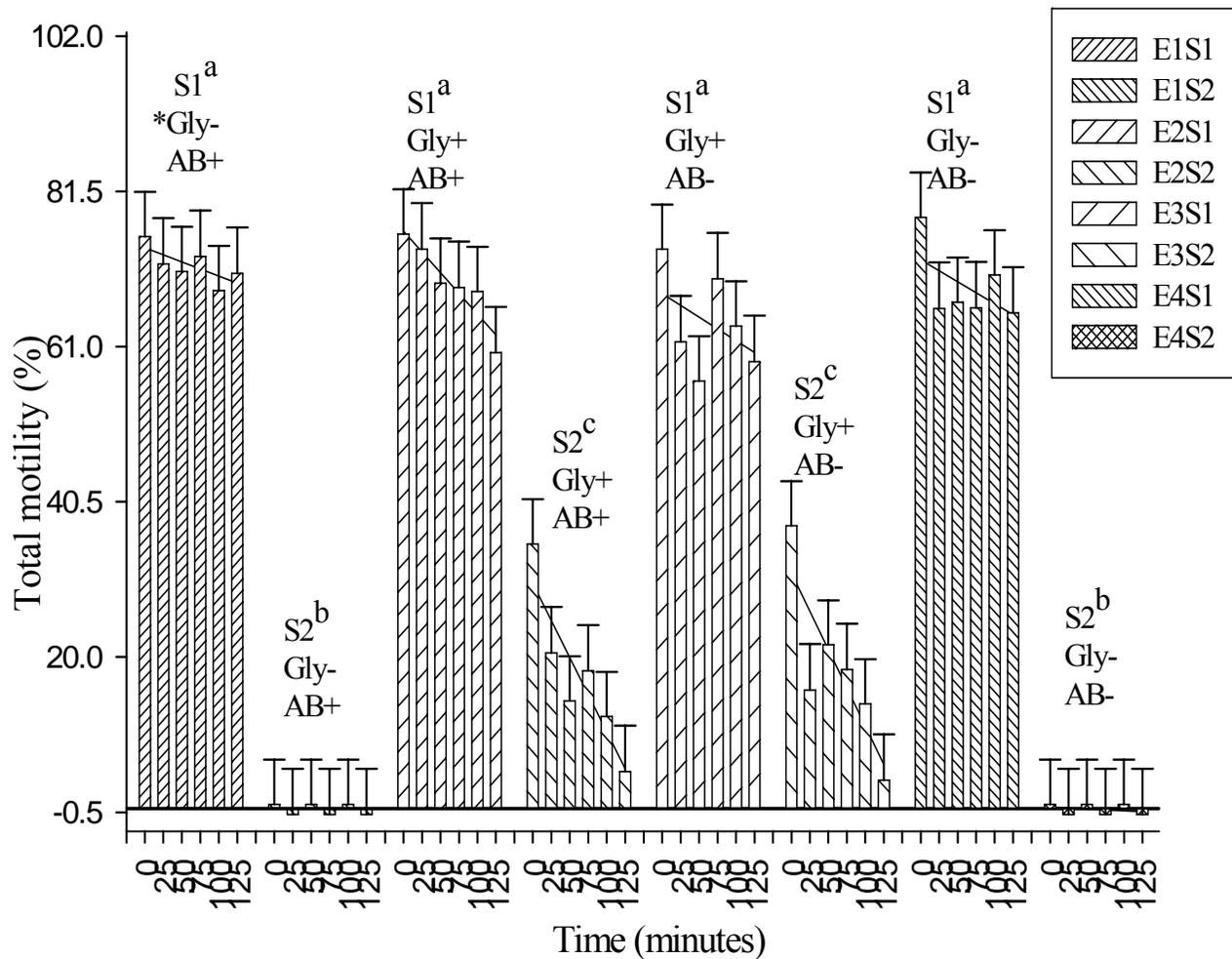


Figure 4.5. Least squares means for total motility (TM,  $\pm$  SE) of bovine spermatozoa treated with extenders of various composition\* before and after cryopreservation. Gly-: contains no glycerol, Gly+: contains glycerol, AB+: contains antibiotics, AB-: contains no antibiotics. E: Extender, S1: Pre-freeze and S2: post-thaw. Means followed by same superscript are not different ( $P > 0.05$ ).

There was a reduction in spermatozoal PM over time (Figure 4.7). Ejaculate within bull, storage and incubation time main effects as well as extender-storage and extender-storage-incubation time interactions were significant sources of variation in PM (Table 4.6) ( $P < 0.05$ ). Extender main effect was not a significant source of variation in PM (Table 4.6) ( $P > 0.05$ ).

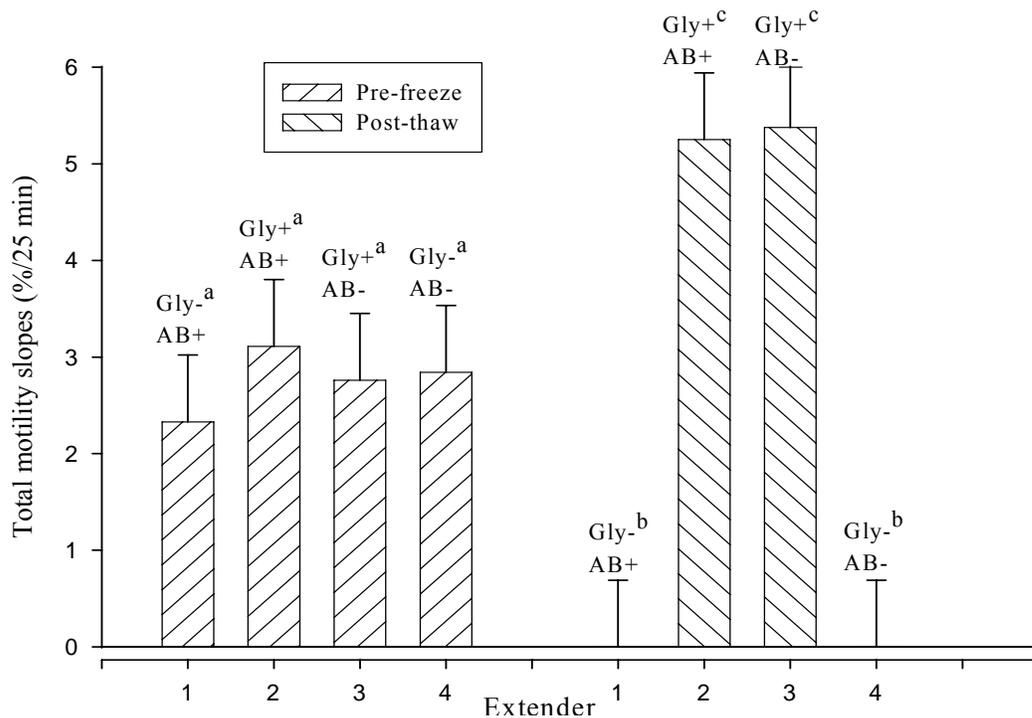


Figure 4.6. Least squares means for total motility (TM) reduction rates (slopes,  $\pm$  SE) of bovine spermatozoa treated with extenders of various composition\* before and after cryopreservation. Gly-: contains no glycerol, Gly+: contains glycerol, AB+: contains antibiotics and AB-: contains no antibiotics. Means followed by same superscript are not different ( $P > 0.05$ ).

Table 4.6. ANOVA of bovine spermatozoal progressive motility (PM) treated with extenders of various composition $\ddagger$  before and after cryopreservation

Source	Df	MS*	F	P-value
Bull	2	9427	4.95	0.0320
Error a $\dagger$	10.002	1904		
Ejaculate(bull)	10	1905	9.92	<0.0001
Extender $\ddagger$	3	282	1.47	0.2209
Storage $\S$	1	285963	1488.78	<0.0001
Incubation time $\P$	5	1747	9.10	<0.0001
Extender-storage	3	3495	18.20	<0.0001
Extender-storage-incubation time	35	389	2.03	0.0006
Error: MS(Error)	564	192		

\* Mean square.

$\dagger$  Error a:  $0.9992 \times \text{MS}(\text{ejaculate}(\text{bull})) + 0.0008 \times \text{MS}(\text{error})$

$\ddagger$  Extender 1 (sodium citrate egg yolk with antibiotics), extender 2 (extender 1 plus glycerol), extender 3 (extender 2 without antibiotics) and extender 4 (extender 1 without antibiotics).

$\S$  Pre-freeze vs. post-thaw.

$\P$  Sequential 25 minute intervals for 125 minutes at 37°C.

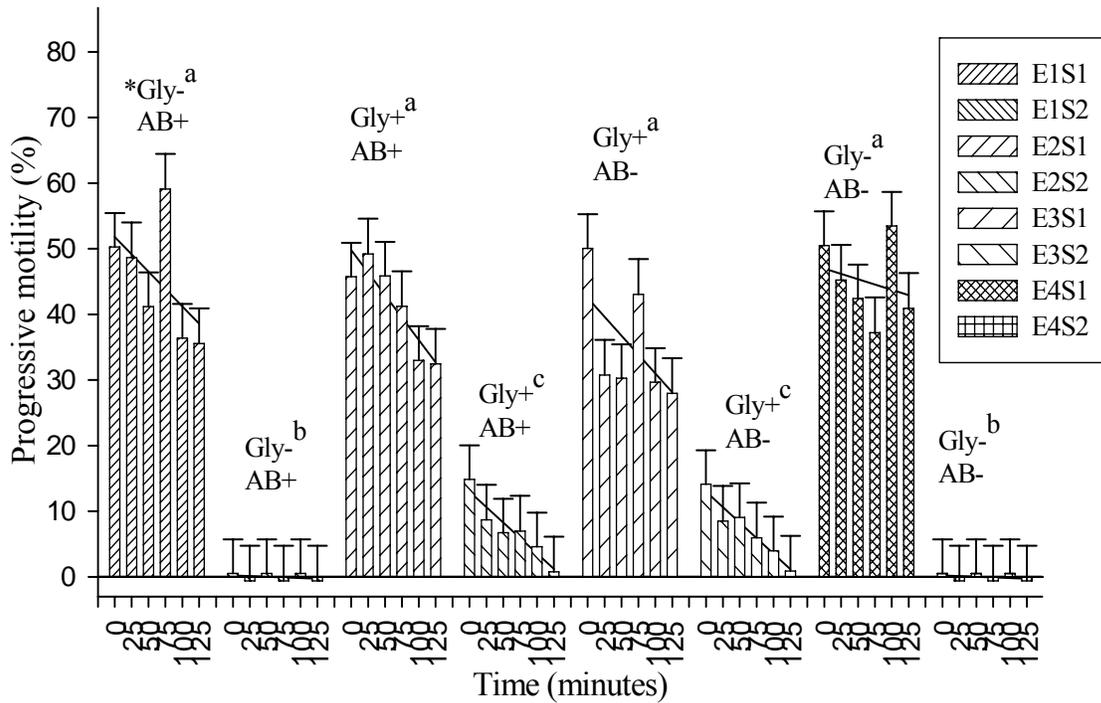


Figure 4.7. Least squares means for progressive motility (PM  $\pm$  SE) of bovine spermatozoa treated with extenders of various composition\* before and after cryopreservation. Gly-: contains no glycerol, Gly+: contains glycerol, AB+: contains antibiotics and AB-: contains no antibiotics, E: extender, S1: pre-freeze and S2: post-thaw. Means followed by same superscript are not different ( $P > 0.05$ ).

In table 4.7, extender treatment and storage (pre-freeze vs post-thaw) main effects were significant sources of variation in the reduction rates (slopes) of progressive motility ( $P < 0.05$ ). The ejaculate within bull main effect and extender storage interaction effect were not significant sources of variation in the reduction rate of progressive motility ( $P > 0.05$ ) (Table 4.7). In Figure 4.8, cryopreservation decreased the reduction rates (slopes) in the percentage of spermatozoal PM ( $P < 0.05$ ). In the pre-freeze semen, the reduction rate was similar in the extender treatments ( $P > 0.05$ ). The extender treatment that had neither glycerol nor antibiotics in the pre-freeze semen did not differ ( $P > 0.05$ ) from the extender treatments that had glycerol in the frozen semen regardless of their antibiotic content. In the extender treatments that had no glycerol, slopes were zero as cryopreservation damaged spermatozoa and there was no motility post-thaw.

Table 4.7. ANOVA of bovine spermatozoal progressive motility (PM) reduction rates (slopes) treated with extenders of various composition‡ before and after cryopreservation

Source	Df	MS	F	P-value
Bull	2	3.9	1.56	0.2581
Error a†	10	2.6		
Ejaculate(bull)	10	2.6	0.50	0.8888
Extender‡	3	30.8	5.95	0.0010
Storage§	1	110.8	21.40	<0.0001
Extender-Storage	3	3.9	0.76	0.5173
Error: MS(Error)	84	5.2		

\* Mean square.

† Error a: MS (ejaculate (bull)).

‡ Extender 1 (sodium citrate egg yolk with antibiotics), extender 2 (extender 1 plus glycerol), extender 3 (extender 2 without antibiotics) and extender 4 (extender 1 without antibiotics).

§Pre-freeze vs. post-thaw.

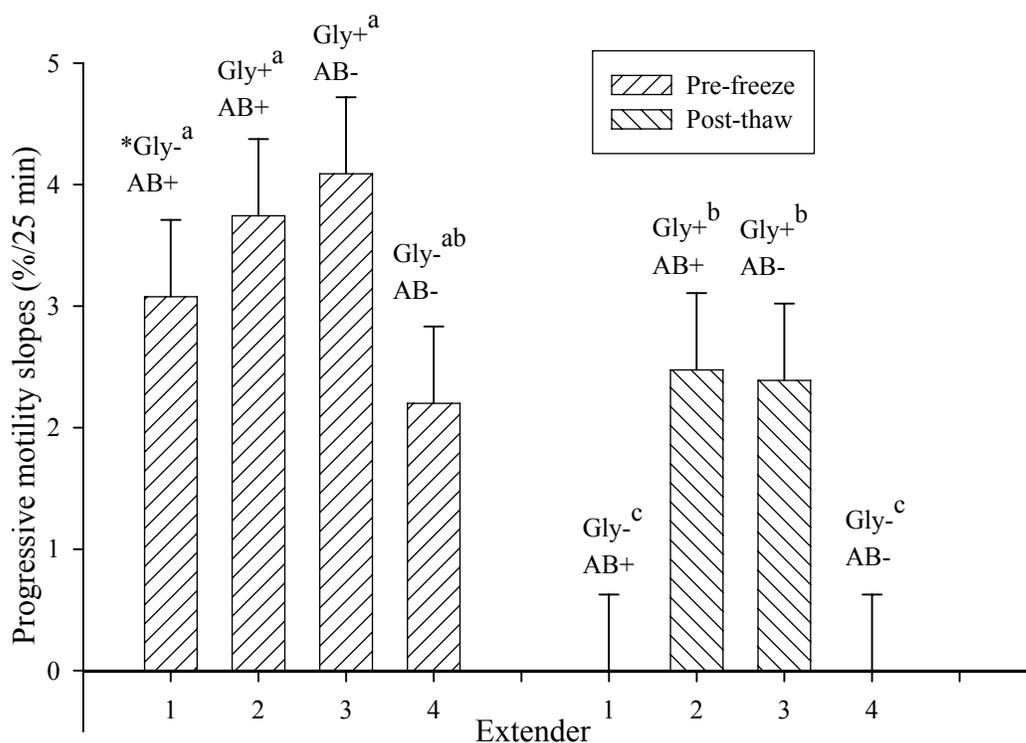


Figure 4.8. Least squares means for progressive motility (PM) reduction rates (slopes, ± SE) of bovine spermatozoa treated with extenders of various composition\* before and after cryopreservation. Gly-: contains no glycerol, Gly+: contains glycerol, AB+: contains antibiotics and AB-: contains no antibiotics. Means followed by same superscript are not different ( $P > 0.05$ ).

Ejaculate within bull, extender, storage (pre-freeze vs post-thaw) and incubation over time main effects and their interactions were significant sources of variation in PV of spermatozoa ( $P < 0.05$ ) (Table 4.8). There was a significant reduction in PV over time during the 125 min incubation. There was a significant difference between extender treatments (Table 4.8). This is clear when comparing those extender treatments in the pre-freeze and post-thaw semen (Figure 4.9). In addition, there was a significant effect of freezing on spermatozoal PV when comparing the extender treatments containing glycerol in the pre-freeze and post-thaw semen (Figure 4.9). Cryopreservation caused damage to a percentage of spermatozoa seen as a reduction in PV after the freezing process.

Table 4.8. ANOVA of bovine spermatozoal progressive velocity (PV) treated with extenders of various composition‡ before and after cryopreservation

Source	Df	MS*	F	P-value
Bull	2	2925.6	4.89	0.0121
Error a†	44.044	597.9		
Ejaculate(bull)	12	2701.6	8.27	<0.0001
Extender‡	3	5001.7	15.32	<0.0001
Storage§	1	412728.0	1263.78	<0.0001
Incubation time¶	5	8218.2	25.16	<0.0001
Extender-storage	3	17525.0	53.66	<0.0001
Extender-storage- incubation time	35	578.3	1.77	0.0051
Error: MS(Error)	470	326.5		

\* Mean square.

† Error a:  $0.1142 \times MS(\text{ejaculate (bull)}) + 0.8858 \times MS(\text{error})$ .

‡ Extender 1 (sodium citrate egg yolk with antibiotics), extender 2 (extender 1 plus glycerol), extender 3 (extender 2 without antibiotics) and extender 4 (extender 1 without antibiotics).

§ Pre-freeze vs. post-thaw.

¶ Sequential 25 minute intervals for 125 minutes at 37°C.

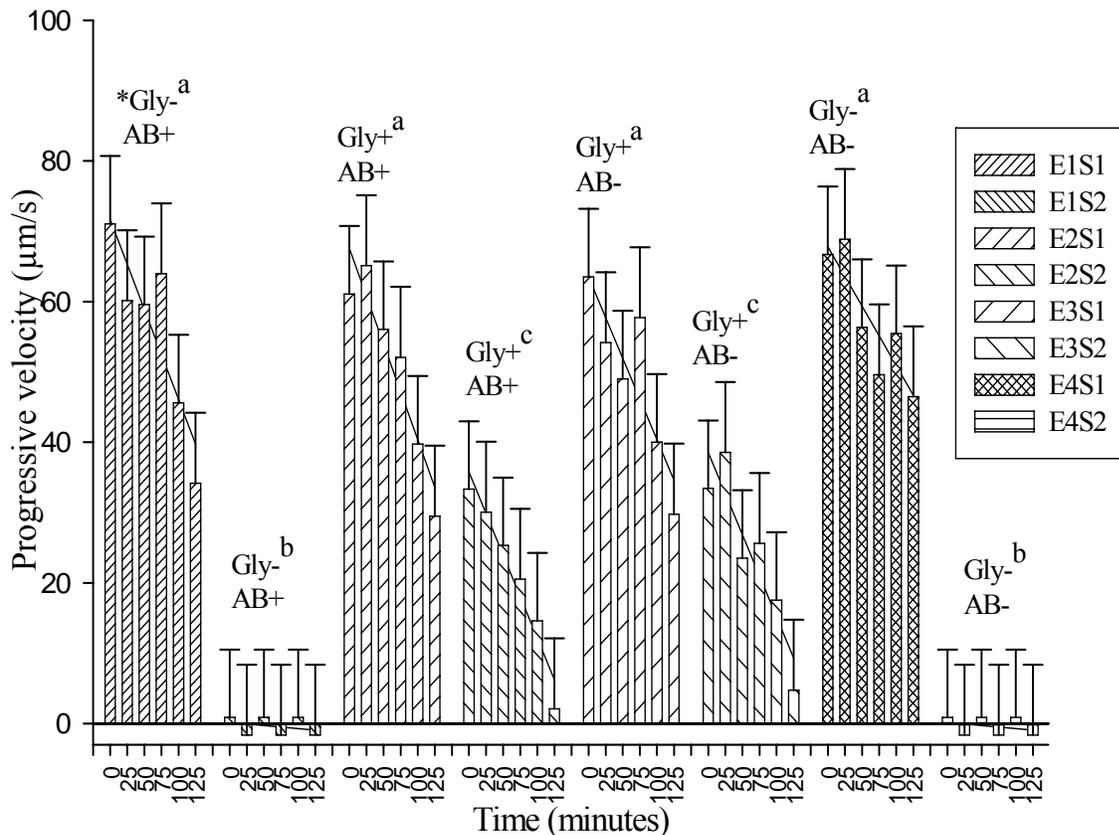


Figure 4.9. Least squares means for progressive velocity (PV,  $\pm$ SE) of bovine spermatozoa treated with extenders of various composition\* before and after cryopreservation. Gly-: contains no glycerol, Gly+: contains glycerol, AB+: contains antibiotics, AB-: contains no antibiotics, E: extender, S1: pre-freeze and S2: post-thaw. Means followed by same superscript are not different ( $P > 0.05$ ).

In the analysis of spermatozoal VAP data (Table 4.9), bull, ejaculate within bull, extender treatment, storage and time main effects as well as extender-storage, extender-incubation time and extender-storage-time interactions were significant sources of variation in spermatozoal VAP ( $P < 0.05$ ). The same scenario in the PV analysis is repeated in the VAP results (Table 4.9 and Figure 4.10) and in the VCL results (Tables 4.10 and Figure 4.11).

Table 4.9. ANOVA of bovine spermatozoal pathway velocity (VAP) treated with extenders of various composition‡ before and after cryopreservation

Source	Df	MS*	F	P-value
Bull	2	6742.1	7.10	0.0034
Error a†	26.319	949.2		
Ejaculate(bull)	12	5591.5	16.01	<0.0001
Extender‡	3	11826.0	33.87	<0.0001
Storage§	1	1064798.0	3049.65	<0.0001
Incubation time¶	5	22439.0	64.27	<0.0001
Extender-storage	3	45372.0	129.95	<0.0001
Extender-incubation time	15	1480.3	4.24	<0.0001
Extender-storage- incubation time	20	1426.6	4.09	<0.0001
Error: MS(Error)	1000	349.1		

\* Mean square.

† Error a:  $0.1145 \times \text{MS (ejaculate (bull))} + 0.8855 \times \text{MS (error)}$ .

‡ Extender 1 (sodium citrate egg yolk with antibiotics), extender 2 (extender 1 plus glycerol), extender 3 (extender 2 without antibiotics) and extender 4 (extender 1 without antibiotics).

§Pre-freeze vs. post-thaw.

¶ Sequential 25 minute intervals for 1 25 minutes at 37°C.

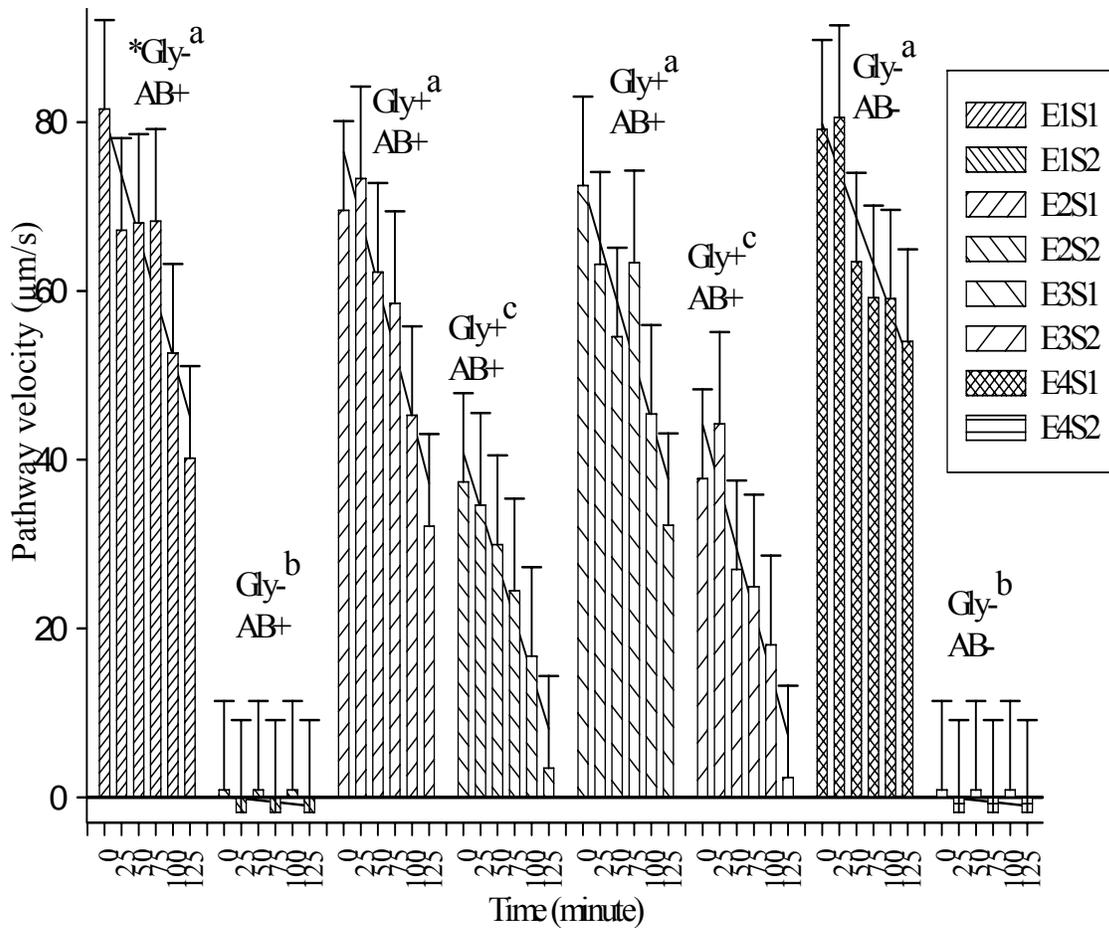


Figure 4.10. Least squares means for pathway velocity (VAP,  $\pm$ SE) of bovine spermatozoa treated with extenders of various composition\* before and after cryopreservation. Gly-: contains no glycerol, Gly+: contains glycerol, AB+: contains antibiotics, AB-: contains no antibiotics, E: extender, S1: pre-freeze and S2: post-thaw. Means followed by same superscript are not different ( $P > 0.05$ ).

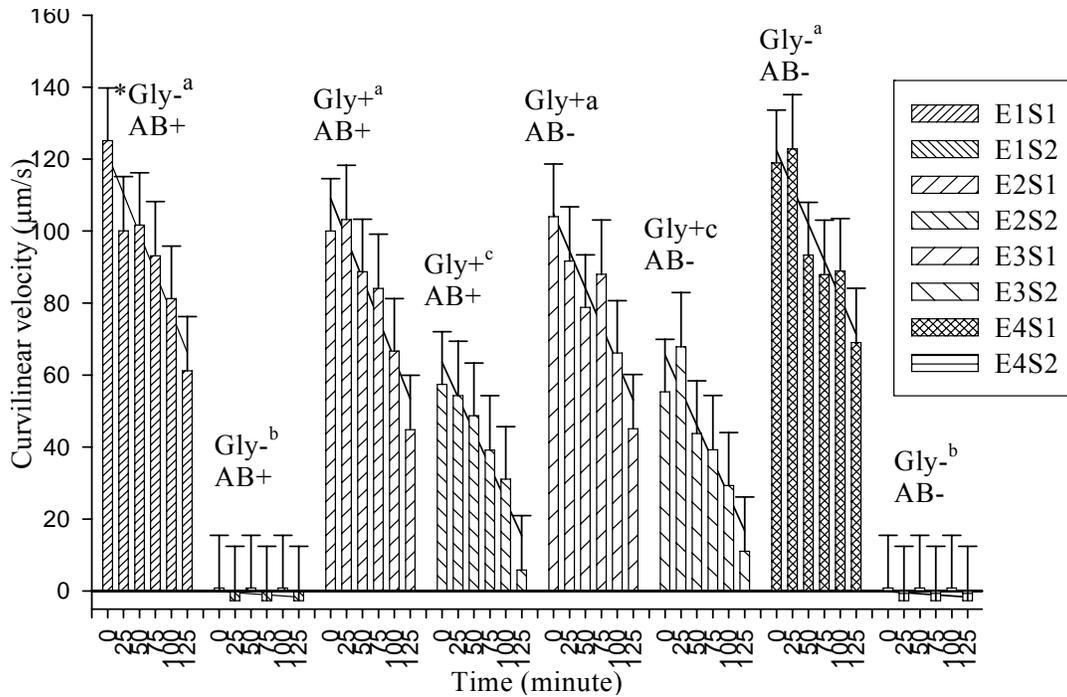


Figure 4.11. Least squares means for curvilinear velocity (VCL,  $\pm$ SE) of bovine spermatozoa treated with extenders of various composition\* before and after cryopreservation. Gly-: no glycerol, Gly+: contains glycerol, AB+: contains antibiotics, AB-: Contains no antibiotics, E: extender, S1: pre-freeze and S2: post-thaw. Means followed by same superscript are not different ( $P > 0.05$ ).

Table 4.10. ANOVA of bovine spermatozoal curvilinear velocity (VCL) treated with extenders of various composition‡ before and after cryopreservation

Source	Df	MS*	F	P-value
Bull	2	6173.2	6.16	0.0034
Error a†	70.071	1002.2		
Ejaculate (bull)	12	3525.1	5.21	<0.0001
Extender‡	3	13406.0	19.83	<0.0001
Storage§	1	1090355.0	1612.75	<0.0001
Incubation time¶	5	24503.0	36.24	<0.0001
Extender-storage	3	63538.0	93.98	<0.0001
Extender-incubation time	15	1207.8	1.79	0.0338
Extender-storage-incubation time	20	1786.9	2.64	0.0002
Error: MS (Error)	469	676.1		

\* Mean square.

† Error a:  $0.1145 \times \text{MS (ejaculate (bull))} + 0.8855 \times \text{MS (error)}$ .

‡ Extender 1 (sodium citrate egg yolk with antibiotics), extender 2 (extender 1 plus glycerol), extender 3 (extender 2 without antibiotics) and extender 4 (extender 1 without antibiotics).

§Pre-freeze vs. post-thaw.

¶ Sequential 25 minute intervals for 1 25 minutes at 37°C.

There was a significant correlation between resazurin reduction rate and TM as well as PM ( $P < 0.05$ ). However, there was no correlation between resazurin reduction rate and other motility parameters (PV, VAP and VCL) (Table 4.11).

Table 4.11. Pearson correlation coefficients between resazurin reduction rate and bovine spermatozoal motility parameters obtained by CASA

Spermatozoal motility parameter	r	Probability >  r  under H0: Rho=0
Total motility (TM)	0.21	0.0451
Progressive motility (PM)	0.45	<0.0001
Progressive velocity (PV)	0.04	0.6972
Curvilinear velocity (VCL)	0.10	0.3611
Pathway velocity	0.07	0.5204

## 4.2 Respiratory Chain Enzyme Activities

### 4.2.1 Specific Activity of Cytochrome c Oxidase (Complex IV)

The ejaculate within bull, incubation time and spermatozoal incubation with Triton X100 main effects were significant sources of variation in cytochrome c oxidase (CytoCox) specific activity ( $P < 0.05$ ) (Table 4.12). However, bull, extender main effects and extender by incubation time, extender by incubation with Triton X100, and extender by incubation with Triton X100 by incubation time interaction effects were not significant sources of variation in CytoCox specific activities ( $P > 0.05$ ) (Table 4.12).

Table 4.12. ANOVA of cryopreserved bovine spermatozoal cytochrome c oxidase specific activity (incubated with and without a detergent§§) treated with extenders of various compositions ‡

Source	Df	MS*	F	P-value
Bull	2	0.150571	0.39	0.6884
Error a†	10.004	0.388390		
Ejaculate (bull)	10	0.388582	3.56	0.0003
Extender‡	3	0.126933	1.16	0.3249
Incubation time§	1	5.552519	50.93	<0.0001
Detergent§§	1	8.021397	73.58	<0.0001
Extender-incubation time	3	0.081230	0.5985	0.5265
Extender-detergent	3	0.115561	1.06	0.3675
Extender-detergent-incubation time	4	0.208305	1.91	0.1107
Error: MS (Error)	174	0.109015		

\* Mean square.

† Error a:  $0.9993 \times \text{MS (Ejaculate (Bull))} + 0.0007 \times \text{MS (Error)}$

‡ Extender 1 (sodium citrate egg yolk with antibiotics), extender 2 (extender 1 plus glycerol), extender 3 (extender 2 without antibiotics) and extender 4 (extender 1 without antibiotics).

§ Immediately post-thaw (zero minutes) and after 125 minutes of incubation

§§ Triton X100

Incubation time significantly increased the specific activity of cytochrome c oxidase enzyme. In addition, the incubation of spermatozoa with Triton X100 significantly increased the specific activity of cytochrome c oxidase enzyme.

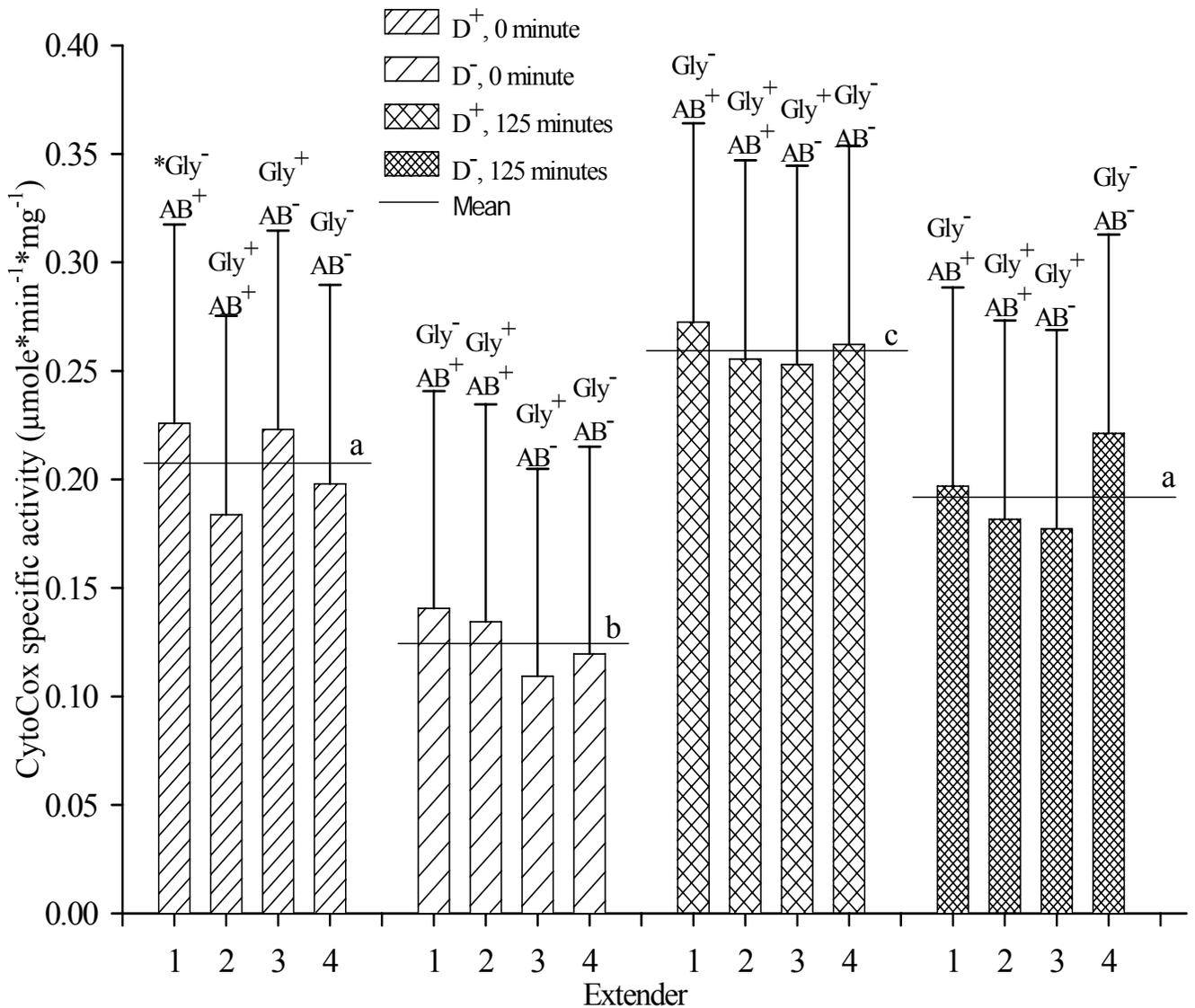


Figure 4.12. Least squares means for total cytochrome c oxidase specific activities (CytoCox,  $\pm$  SE) of cryopreserved bovine spermatozoa treated with extenders of various composition\* before and after (zero, 125 minutes) 37°C incubation. Gly<sup>-</sup>: contains no glycerol, Gly<sup>+</sup>: contains glycerol, AB<sup>+</sup>: contains antibiotics, AB<sup>-</sup>: contains no antibiotics, D<sup>+</sup>: incubated with Triton X100 and D<sup>-</sup>: incubated without Triton X100. Means followed by the same superscript are not different ( $P > 0.05$ ).

Spermatozoal CytoCox specific activity from spermatozoa incubated with and without Triton X100 increased ( $P < 0.05$ ) after incubation at 37°C for 125 minutes. Cytochrome c oxidase specific activity from spermatozoa that were incubated with Triton X100 was different ( $P < 0.05$ ) from spermatozoal CytoCox specific activity from spermatozoa that were incubated without Triton X100 either immediately post-thaw or

after 125 minutes 37°C incubation. Spermatozoal CytoCox specific activity from spermatozoa that were incubated without Triton X100 is activity due to already broken spermatozoal outer mitochondrial membranes. However, spermatozoal CytoCox specific activity from spermatozoa that were incubated with Triton X100 is the total Cytocox activity. Since the difference between both activities was significant ( $P < 0.05$ ), it conveyed information about the integrity of spermatozoal outer mitochondrial membranes. The means of spermatozoal CytoCox specific activity from spermatozoa incubated with Triton X100 immediately post-thaw and after 125 minutes of incubation at 37°C were 0.21 and 0.26  $\mu\text{mole}/\text{min}/\text{mg}$ , respectively, and from spermatozoa that were incubated without Triton X100 were 0.13 and 0.19  $\mu\text{mole}/\text{min}/\text{mg}$ , immediately post-thaw and after 125 minutes of incubation at 37°C, respectively. Based on the values of those means the integrity of outer mitochondrial membrane (IOMM) was calculated as follows:

percentage of spermatozoal mitochondrial with IOMM= ((mean CytoCox specific activity from spermatozoa incubated with Triton X100 - mean CytoCox specific activity from spermatozoa incubated without Triton X100) / mean CytoCox specific activity from spermatozoa incubated with Triton X100))  $\times 100$ .

Based upon that formula, the percentage of spermatozoal mitochondria with intact outer mitochondrial membrane (IOMM) immediately post-thaw was 38.1% and 26.9% after 125 minutes of incubation at 37°C. Thus, the percentage of spermatozoa with IOMM decreased after 125 minutes of incubation at 37°C.

#### **4.2.2 Specific Activity of Citrate Synthase**

The results of spermatozoal citrate synthase specific activity showed that the bull, ejaculate within bull, extender main effects and extender by incubation time, extender by

incubation with Triton X100 interactions were not significant sources of variation in spermatozoal citrate synthase specific activities ( $P > 0.05$ ) (Table 4.13). However, incubation time and incubation with Triton X100 main effects and extender by incubation time by incubation with Triton X100 interactions were significant sources of variation in spermatozoal citrate synthase specific activity ( $P < 0.05$ ) (Table 4.13).

Table 4.13. ANOVA of cryopreserved bovine spermatozoal citrate synthase specific activity (incubated with and without a detergent§§) treated with extenders of various composition‡

Source	Df	MS*	F	P-value
Bull	2	0.477394	2.12	0.1711
Error a†	10.013	0.225494		
Ejaculate (bull)	10	0.225572	1.53	0.1330
Extender‡	3	0.083284	0.56	0.6395
Incubation time§	1	1.750021	11.85	0.0007
Detergent§§	1	50.176526	339.71	<0.0001
Extender-incubation time	3	0.075798	0.51	0.6737
Extender-detergent	3	0.123242	0.83	0.4766
Extender-detergent-incubation time	4	0.670803	4.54	0.0016
Error: MS (Error)	174	0.109015		

\* Mean square.

† Error a:  $0.999 \times \text{MS (Ejaculate (Bull))} + 0.001 \times \text{MS (Error)}$

‡ Extender 1 (sodium citrate egg yolk with antibiotics), extender 2 (extender 1 plus glycerol), extender 3 (extender 2 without antibiotics) and extender 4 (extender 1 without antibiotics).

§ Immediately post-thaw (zero minute) and after 125 minutes of incubation

§§ Triton X100

Spermatozoal citrate synthase specific activity from spermatozoa incubated without Triton X100 decreased ( $P < 0.05$ ) after incubation at 37°C for 125 minutes compared to spermatozoal citrate synthase specific activity from spermatozoa incubated Triton X100 which did not change ( $P > 0.05$ ) (Figure 4.13). Spermatozoal citrate synthase specific activity increased ( $P < 0.05$ ) after spermatozoal incubation with Triton

X100 either immediately post-thaw or after 125 minutes incubation compared to the activity measured without incubation spermatozoa with Triton X100 (Figure 4.13).

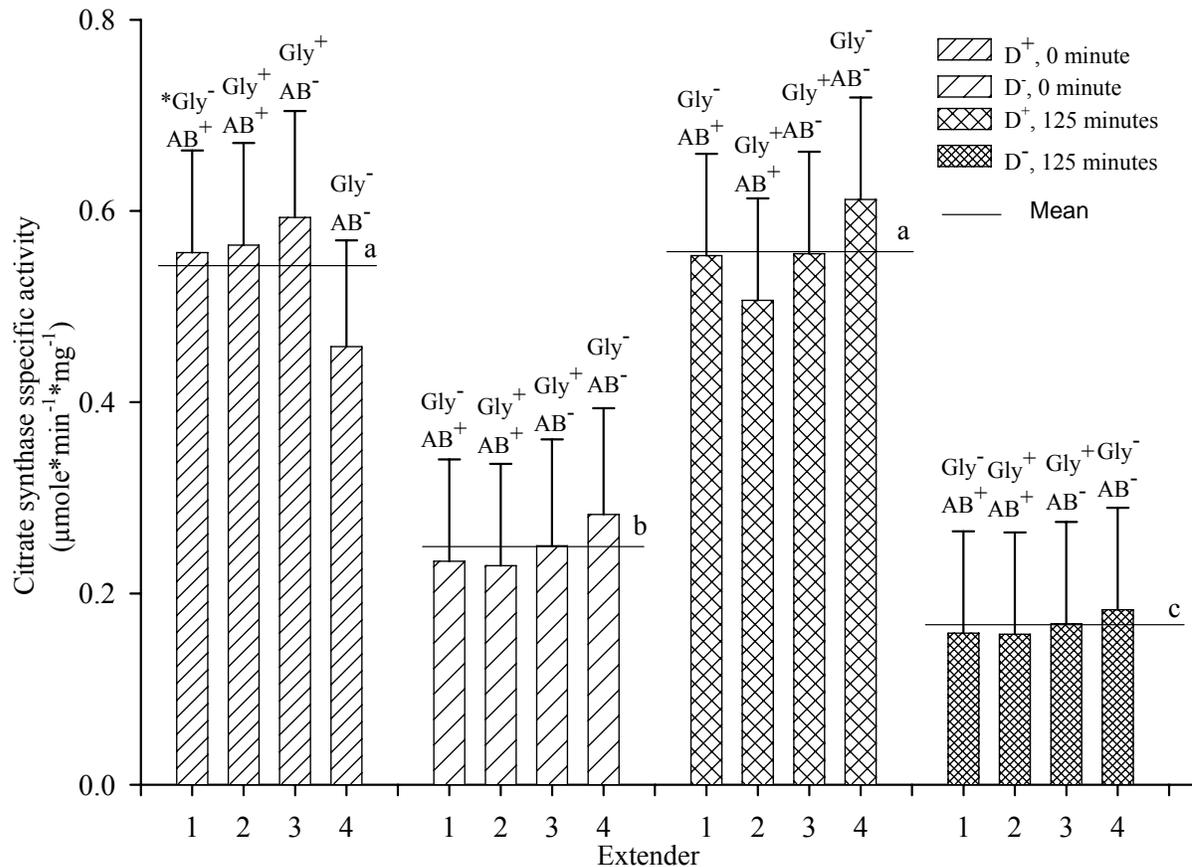


Figure 4.13. Least squares means for citrate synthase specific activities ( $\pm$  SE) of bovine spermatozoa treated with extenders of various composition\* before and after (zero, 125 minutes) 37°C incubation. Gly<sup>-</sup>: contains no glycerol, Gly<sup>+</sup>: contains glycerol, AB<sup>+</sup>: contains antibiotics, AB<sup>-</sup>: contains no antibiotics, D<sup>+</sup>: incubated with Triton X100 and D<sup>-</sup>: incubated without Triton X100. Means followed by the same superscript are not different ( $P > 0.05$ ).

Spermatozoal citrate synthase specific activity from spermatozoa that were incubated without Triton X100 is activity due to already broken spermatozoal inner mitochondrial membranes. However, spermatozoal citrate synthase specific activity from spermatozoa that were incubated with Triton X100 is the total citrate synthase specific activity.

Because the difference between both activities was significant ( $P < 0.05$ ) immediately

post-thaw and after 125 minutes of incubation at 37°C, it conveyed information about the integrity of spermatozoal inner mitochondrial membranes (IIMM). The means of spermatozoal citrate synthase specific activity from spermatozoa incubated with Triton X100 immediately post-thaw and after 125 minutes of incubation at 37°C were 0.54 and 0.56 µmole/ml/min, respectively. Means from spermatozoa that were incubated without Triton X100 were 0.25 and 0.17 µmole/ml/min, immediately post-thaw and after 125 minutes of incubation at 37°C, respectively. Based on the values of those means the integrity of the inner mitochondrial membrane (IIMM) was calculated as follows:

percentage of spermatozoal mitochondria with intact inner mitochondrial membrane =  
((mean citrate synthase specific activity from spermatozoa incubated with Triton X100 -  
mean citrate synthase specific activity from spermatozoa incubated without Triton X100)  
/ mean citrate synthase specific activity from spermatozoa incubated with Triton X100))  
×100.

Based upon that formula, the percentage of spermatozoal mitochondria with intact inner mitochondrial membranes immediately post-thaw was 53.7% and 69.6% after 125 minutes of incubation at 37°C.

#### **4.2.3 Spermatozoal Motility**

Extender treatment and incubation time main effects and extender by incubation time interaction effect were significant sources of variation in spermatozoal total and progressive motilities (TM and PM, respectively) ( $P < 0.05$ ) (Tables 4.14 and 4.15). Spermatozoal TM and PM were different ( $P < 0.05$ ) among the extender treatments that contained glycerol and the extender treatments that did not contain glycerol regardless of their antibiotic content (Figures 4.14 and 4.15). After incubation, spermatozoal TM and PM in the extender treatments that contained glycerol significantly decreased (Figures

4.14 and 4.15). The extender treatments containing no glycerol did not survive the cryopreservation process compared to the extender treatments which had glycerol (Figures 4.14 and 4.15).

Table 4.14. ANOVA for bovine spermatozoal total motility (TM) treated with extenders of various composition‡

Source	Df	MS*	F	P-value
Bull	2	991	4.37	0.0433
Error a†	10	226		
Ejaculate (Bull)	10	226	1.83	0.0667
Extender‡	3	7804	63.09	<0.0001
Incubation time§	1	5124	41.42	<0.0001
Extender-incubation time	3	1709	13.82	<0.0001
Error: MS (Error)	84	123		

\* Mean square.

† Error a: MS (Ejaculate (Bull)).

‡ Extender 1 (sodium citrate egg yolk with antibiotics), extender 2 (extender 1 plus glycerol), extender 3 (extender 2 without antibiotics) and extender 4 (extender 1 without antibiotics).

§ Immediately post-thaw (zero minutes) and after 125 minutes of incubation.

Table 4.15. ANOVA for bovine spermatozoal progressive motility (PM) treated with extenders of various composition‡

Source	Df	MS*	F	P-value
Bull	2	729	9.91	0.0042
Error a†	10	73		
Ejaculate(Bull)	10	73	1.13	0.3510
Extender‡	3	2484	38.08	<0.0001
Incubation time§	1	1344	20.62	<0.0001
Extender-incubation time	3	448	6.88	0.0003
Error: MS(Error)	84	65		

\* Mean square.

† Error a: MS (Ejaculate (Bull)).

‡ Extender 1 (sodium citrate egg yolk with antibiotics), extender 2 (extender 1 plus glycerol), extender 3 (extender 2 without antibiotics) and extender 4 (extender 1 without antibiotics).

§ Immediately post-thaw (zero minutes) and after 125 minutes of incubation.

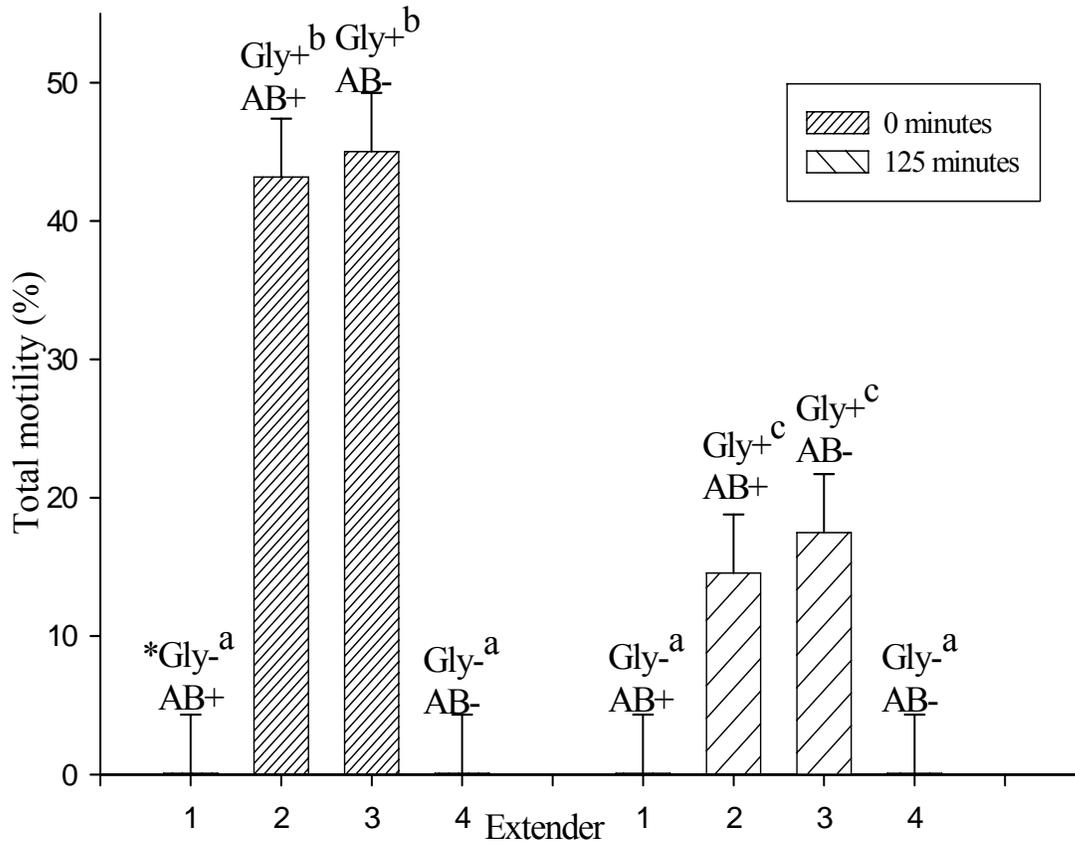


Figure 4.14. Least squares means for total motility (TM,  $\pm$  SE) of bovine spermatozoa treated with extenders of various composition\* before and after (zero, 125 minutes) 37°C incubation. Gly-: contains no glycerol, Gly+: contains glycerol, AB+: contains antibiotics, AB-: contains no antibiotics. Means followed by the same superscript are not different ( $P > 0.05$ ).

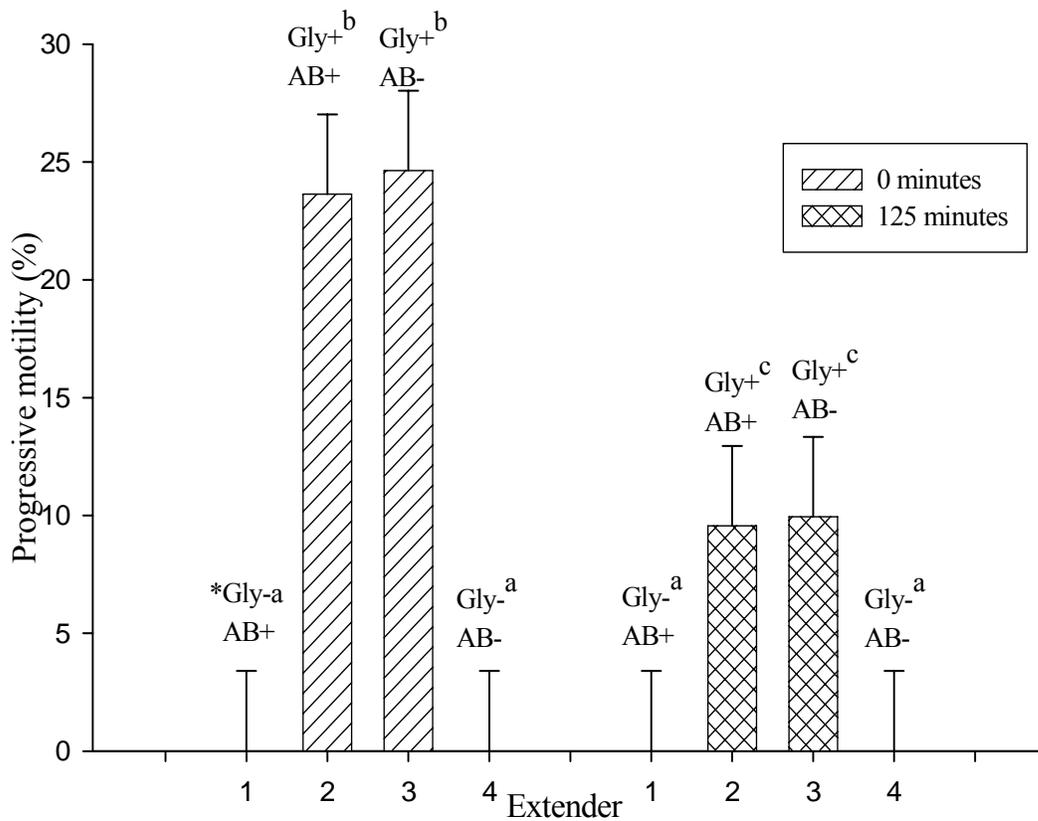


Figure 4.15. Least squares means for progressive motility (PM,  $\pm$  SE) of bovine spermatozoa treated with extenders of various composition\* before and after (zero, 125 minutes) 37°C incubation. Gly-: contains no glycerol, Gly+: contains glycerol, AB+: contains antibiotics, AB-: contains no antibiotics. Means followed by the same superscript are not different ( $P > 0.05$ ).

Ejaculate within bull, extender treatment, time main effects and extender by time interaction effect were significant sources of variation in PV and VAP ( $P < 0.05$ ) (Tables 4.16 and 4.17). However, the bull main effect was not significant ( $P > 0.05$ )

Table 4.16. ANOVA for spermatozoal progressive velocity (PV) treated with extenders of various composition‡

Source	Df	MS*	F	P-value
Bull	2	1705.2	3.43	0.0842
Error a†	8	497.8		
Ejaculate (Bull)	8	497.8	2.40	0.0237
Extender‡	3	5776.8	27.89	<0.0001
Incubation time§	1	5843.5	28.21	<0.0001
Extender-incubation time	3	1954.1	9.43	<0.0001
Error: MS (Error)	70	207.1		

\* Mean square.

† Error a: MS (Ejaculate (Bull)).

‡ Extender 1 (sodium citrate egg yolk with antibiotics), extender 2 (extender 1 plus glycerol), extender 3 (extender 2 without antibiotics) and extender 4 (extender 1 without antibiotics).

§ Immediately post-thaw (zero minutes) and after 125 minutes of incubation.

Table 4.17. ANOVA for spermatozoal pathway velocity (VAP) treated with extenders of various composition‡

Source	Df	MS*	F	P-value
Bull	2	1951.6	3.29	0.0906
Error a†	8	593.2		
Ejaculate (Bull)	8	593.2	2.42	0.0229
Extender‡	3	7153.5	29.15	<0.0001
Incubation time§	1	7798.2	31.78	<0.0001
Extender-incubation time	3	2602.5	10.61	<0.0001
Error: MS(Error)	70	245.3		

\* Mean square.

† Error a: MS (Ejaculate (Bull)).

‡ Extender 1 (sodium citrate egg yolk with antibiotics), extender 2 (extender 1 plus glycerol), extender 3 (extender 2 without antibiotics) and extender 4 (extender 1 without antibiotics).

§ Immediately post-thaw (zero minutes) and after 125 minutes of incubation.

Extender treatment, time main effects and extender time interaction were significant sources of variation in spermatozoal VCL ( $P < 0.05$ ) (Table 4.18). However, ejaculate within bull and bull main effects were not significant ( $P > 0.05$ ).

Table 4.18. ANOVA for spermatozoal curvilinear velocity (VCL) treated with extenders of various composition‡

Source	Df	MS*	F	P-value
Bull	2	4263.8	4.31	0.0536
Error a†	8	988.5		
Ejaculate(Bull)	8	988.5	2.02	0.0562
Extender‡	3	18229.0	37.28	<0.0001
Incubation time§	1	14742.0	30.15	<0.0001
Extender-incubation time	3	4961.3	10.15	<0.0001
Error: MS(Error)	70	488.9		

\* Mean square.

† Error a: MS (Ejaculate (Bull)).

‡ Extender 1 (sodium citrate egg yolk with antibiotics), extender 2 (extender 1 plus glycerol), extender 3 (extender 2 without antibiotics) and extender 4 (extender 1 without antibiotics).

§ Immediately post-thaw (zero minutes) and after 125 minutes of incubation.

Data analysis for spermatozoal PV, VAP and VCL showed significant differences among and within extender treatments (Figures 4.16, 4.17 and 4.18). In spermatozoal VCL data analysis, the ejaculate within bull was not a significant ( $P > 0.05$ ) source of variation compared to PV and VAP data where ejaculate within bull was a significant ( $P < 0.05$ ) source of variation in spermatozoal PV and VAP. The extender treatments without glycerol did not survive the cryopreservation process compared to the extender treatment groups which contained glycerol. When comparing the extender treatment group that contained glycerol and antibiotics to the extender treatment group that contained only glycerol, the results were similar.

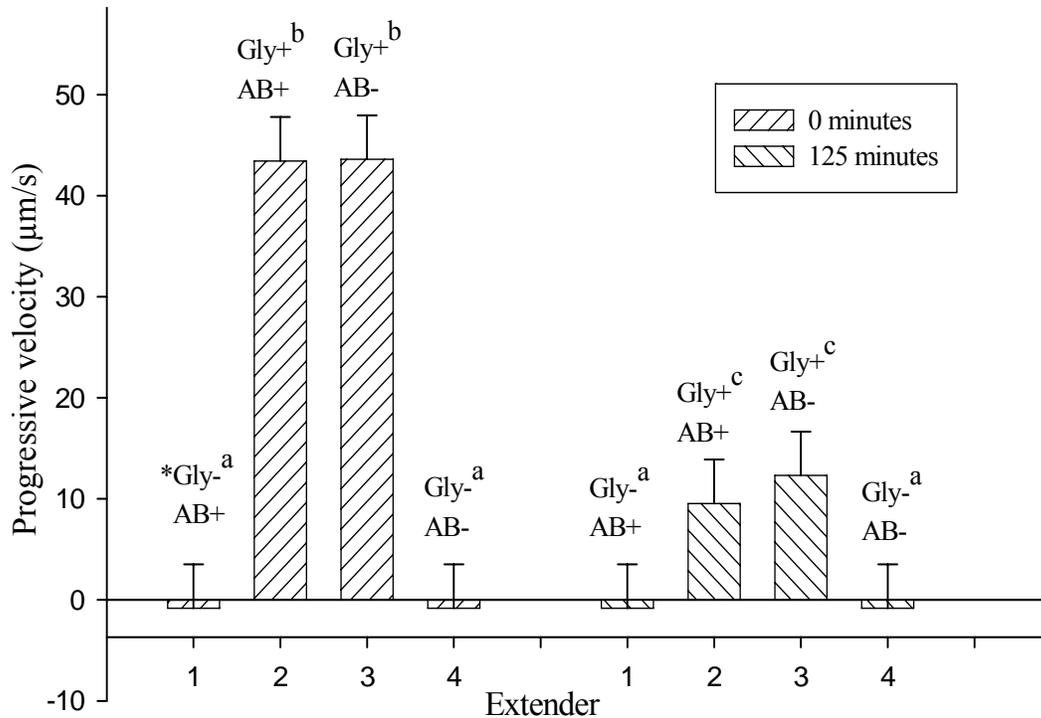


Figure 4.16. Least squares means for progressive velocity (PV,  $\pm$  SE) of bovine spermatozoa treated with extenders of various composition\* before and after (zero, 125 minutes) 37°C incubation. Gly-: contains no glycerol, Gly+: contains glycerol, AB+: contains antibiotics, AB-: contains no antibiotics. Means followed by the same superscript are not different ( $P > 0.05$ ).

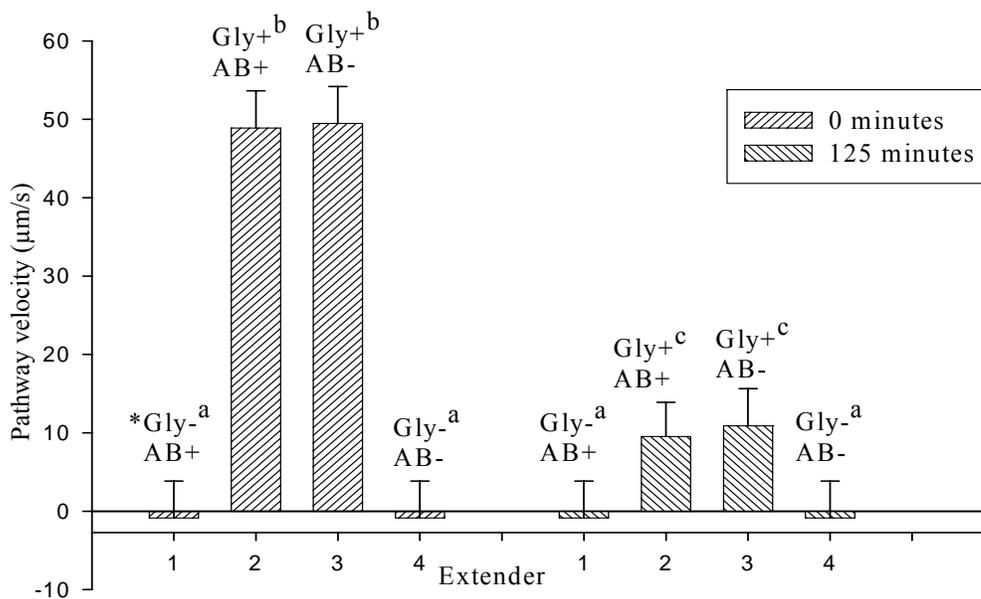


Figure 4.17. Least squares means for pathway velocity (VAP,  $\pm$  SE) of bovine spermatozoa treated with extenders of various composition\* before and after (zero, 125 minutes) 37°C incubation. Gly-: contains no glycerol, Gly+: contains glycerol, AB+: contains antibiotics, AB-: contains no antibiotics. Means followed by the same superscript are not different ( $P > 0.05$ ).

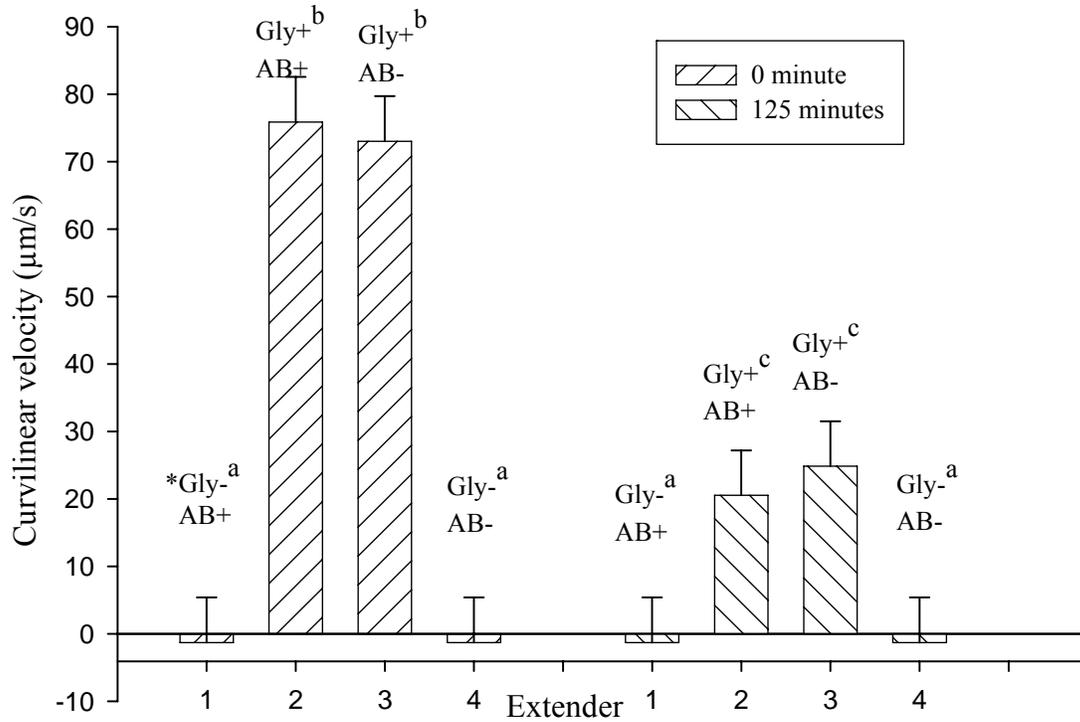


Figure 4.18. Least square means for curvilinear velocity (VCL,  $\pm$  SE) of bovine spermatozoa treated with extenders of various composition\* before and after (zero, 125 minutes) 37°C incubation. Gly-: contains no glycerol, Gly+: contains glycerol, AB+: contains antibiotics, AB-: contains no antibiotics. Means followed by the same superscript are not different ( $P > 0.05$ ).

There was a significant correlation between CytoCox specific activity from spermatozoa incubated without Triton X100 and CytoCox specific activity from spermatozoa incubated with Triton X100 ( $r = 0.70$ ,  $P < 0.05$ ) as well as with citrate synthase specific activity from spermatozoa incubated with triton X100 ( $r = 0.51$ ,  $P < 0.05$ ). There was significant correlation between CytoCox specific activity from spermatozoa incubated with Triton X100 and citrate synthase specific activity from spermatozoa incubated with Triton X100 ( $r = 0.76$ ,  $P < 0.05$ ). No correlation was found between both CytoCox specific activity and citrate synthase specific activity with total and progressive motilities or any velocity parameters.

### 4.3 Bovine Spermatozoal ATP Content

#### 4.3.1 ATP and Spermatozoal Number Standard Curve

The ATP standard curve generated is shown Figure 4.19. The number of spermatozoa needed used in the assay was  $1 \times 10^6$  spermatozoa (Figure 4.20).

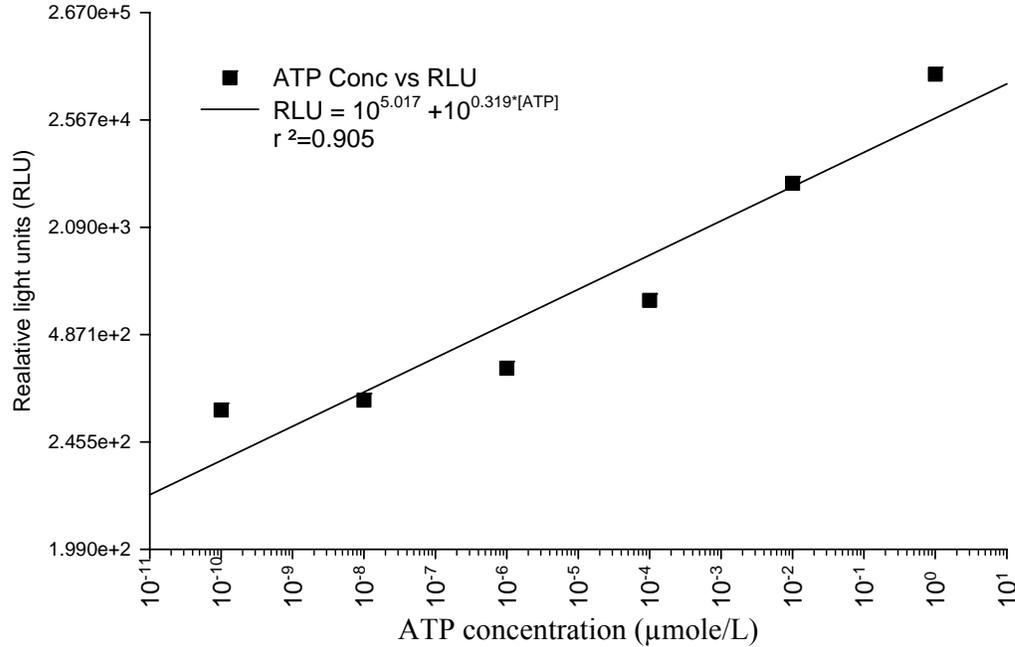


Figure 4.19. ATP standard curve. Different ATP concentrations and their relative light units (RLU) measured by using the luciferin-luciferase assay.

The ATP standard curve was used to calculate the ATP content of spermatozoa because the luminometer used in this assay measures ATP content of spermatozoa in terms of relative light units. The spermatozoal ATP content curve was used in order to determine the number of cells within the sensitivity range of the luminometer. Approximately one million spermatozoa was selected because the technique has been reported to be sensitive enough to determine ATP content of 1 000 spermatozoa (Reiger, 1997). Additionally, this number of cells was used in order to have enough material for the other three experiments performed on the same semen samples.

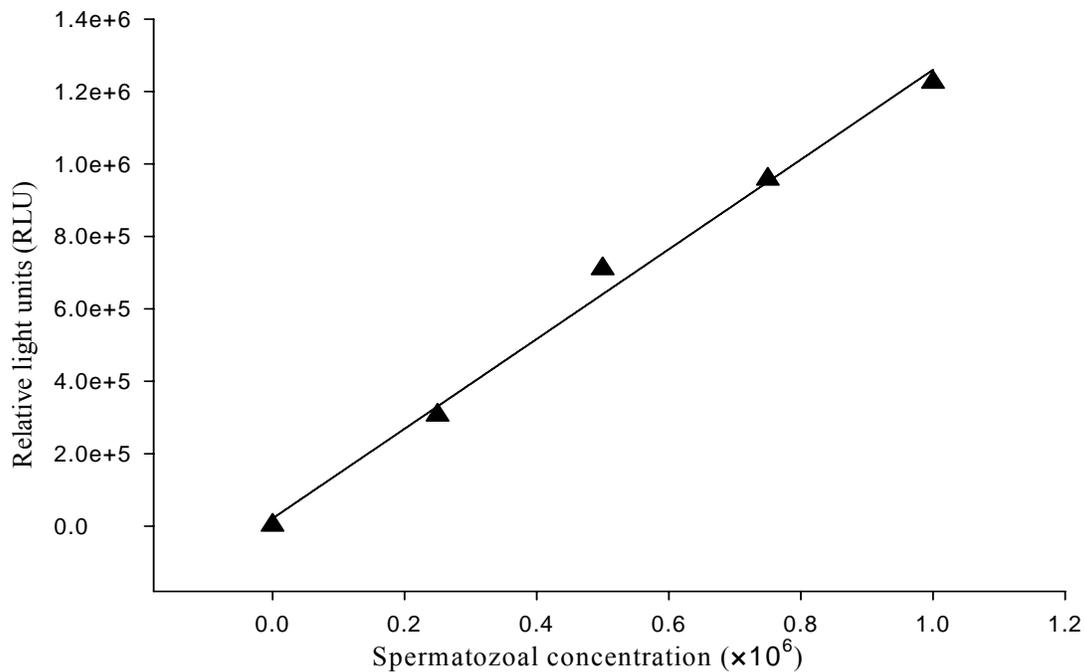


Figure 4.20. Different bovine spermatozoal concentrations and their ATP content (RLU) measured by using the luciferin-luciferase assay.

#### 4.3.2 Effects of Cryopreservation and Semen Extender Constituents on Spermatozoal ATP Content and Production/Utilization

The bull, ejaculate within bull, extender, storage (pre-freeze vs. post-thaw) and incubation time main effects and extender-storage interaction were significant sources of variation ( $P < 0.05$ ) (Table 4.19). The extender-incubation time and extender-storage-incubation time interactions effects were not significant ( $P > 0.05$ ) (Table 4.19).

In figure 4.21, cryopreservation (pre-freeze vs. post-thaw) was the main factor in reducing the amount of ATP and caused injury to spermatozoa thereby affecting the source of ATP production, the mitochondria. There was no significant effect of extender treatment on the ATP content of spermatozoa over incubation (Table 4.19).

Table 4.19. ANOVA of bovine spermatozoal ATP content ( $\mu\text{moles}/1 \times 10^6$  spermatozoa) treated with extenders of various composition $\ddagger$  before and after cryopreservation

Source	Df	MS*	F	P-value
Bull	2	0.0034689	19.06	<0.0001
Ejaculate (Bull)	10	0.005256	28.88	<0.0001
Extender $\ddagger$	3	0.026134	143.58	<0.0001
Storage $\S$	1	0.238022	1307.71	<0.0001
Incubation time $\P$	5	0.000628	3.45	0.0044
Extender-storage	3	0.041122	225.92	<0.0001
Extender-incubation time	15	0.0000645	0.35	0.9887
Extender-storage-incubation time	20	0.0000882	0.48	0.9722
Error: MS (Error)	564	0.000182		

\* Mean square.

$\dagger$  Error a:  $0.9992 \times \text{MS (ejaculate (bull))} + 0.0008 \times \text{MS (error)}$

$\ddagger$  Extender 1 (sodium citrate egg yolk with antibiotics), extender 2 (extender 1 plus glycerol), extender 3 (extender 2 without antibiotics) and extender 4 (extender 1 without antibiotics).

$\S$  Pre-freeze vs. post-thaw.

$\P$  Sequential 25 minute intervals for 125 minutes at  $37^\circ\text{C}$ .

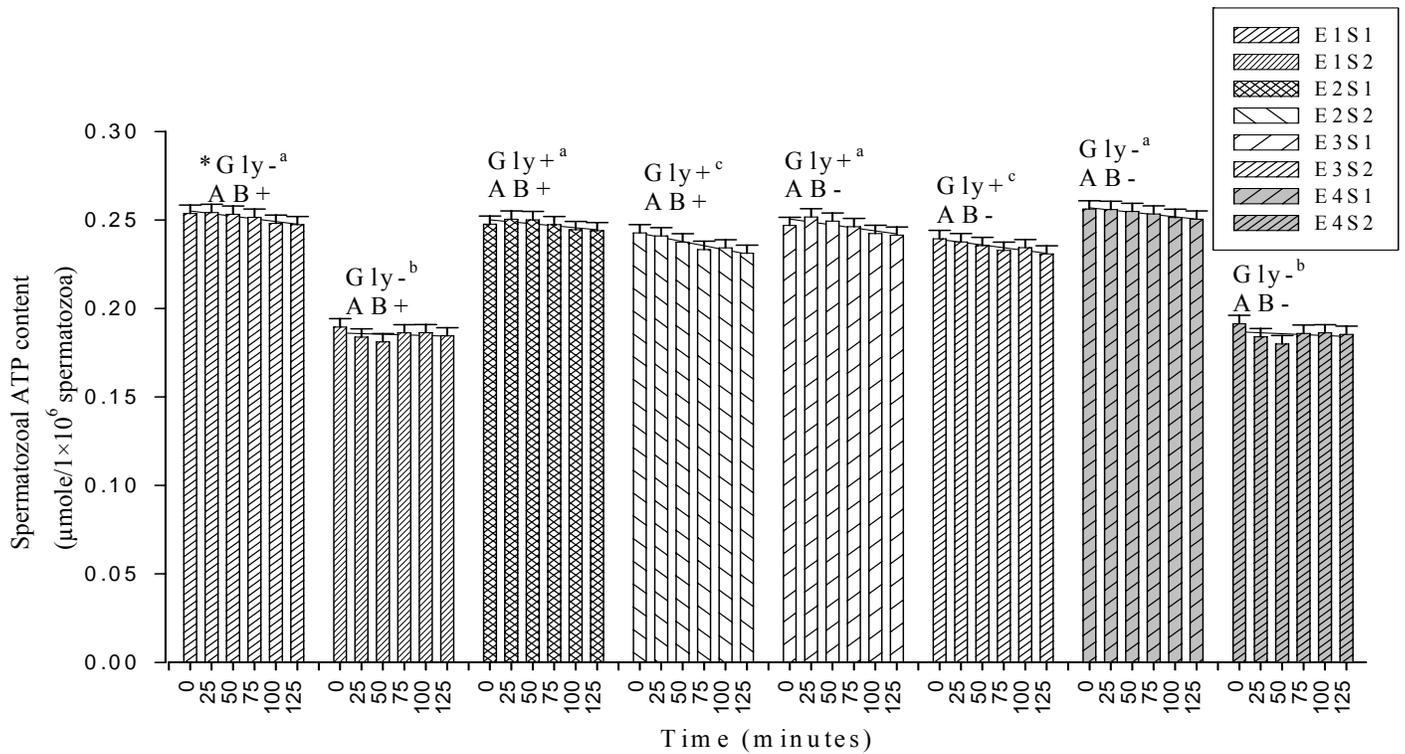


Figure 4.21. Least squares means for ATP content ( $\pm$  SE) of bovine spermatozoa treated with extenders of various composition\* before and after cryopreservation. E: extender, S: storage, S1: pre-freeze, S2: post-thaw, Glyc-: contains no glycerol, Gly+: contains glycerol, AB+: contains antibiotics and AB-: contains no antibiotics. Means followed by same superscript are not different ( $P > 0.05$ ).

Table 4.20. ANOVA for bovine spermatozoal ATP utilization ( $\mu\text{moles}/1 \times 10^6$  spermatozoa/min) (slopes) treated with extenders of various composition‡ before and after cryopreservation

Source	Df	MS*	F	P-value
Bull	2	$3.55 \times 10^{-8}$	0.17	0.8441
Error a†	10	$2.06 \times 10^{-8}$		
Ejaculate(Bull)	10	$2.06 \times 10^{-8}$	2.09	0.0337
Extender‡	3	$1.55 \times 10^{-9}$	1.58	0.1998
Storage§	1	$9.09 \times 10^{-9}$	9.24	0.0032
Extender-storage	3	$2.19 \times 10^{-8}$	2.23	0.0907
Error: MS(Error)	84	$9.83 \times 10^{-8}$		

\* Mean square.

† Error a: MS (Ejaculate (Bull))

‡ Extender 1 (sodium citrate egg yolk with antibiotics), extender 2 (extender 1 plus glycerol), extender 3 (extender 2 without antibiotics) and extender 4 (extender 1 without antibiotics).

§Pre-freeze vs. post-thaw.

Analysis of the ATP utilization (slopes) or ATP utilization showed no significant difference among extender treatment groups ( $P > 0.05$ ) (Table 4.20). However, ejaculate within bull and storage (pre-freeze vs. post-thaw) main effects were significant sources of variation in ATP utilization. Cryopreservation affected spermatozoal ATP utilization and Figure 4.22 shows the slopes of the different extender treatment groups in both the pre-freeze semen and post-thaw.

The cryopreservation process damaged a certain percent of spermatozoa and glycerol altered the membrane permeability, this reduced the spermatozoa's ability to produce ATP or decreased the efficiency of ATP production. This forced the spermatozoa to consume all the ATP produced and stored and caused an imbalance in the ATP produced/utilized.

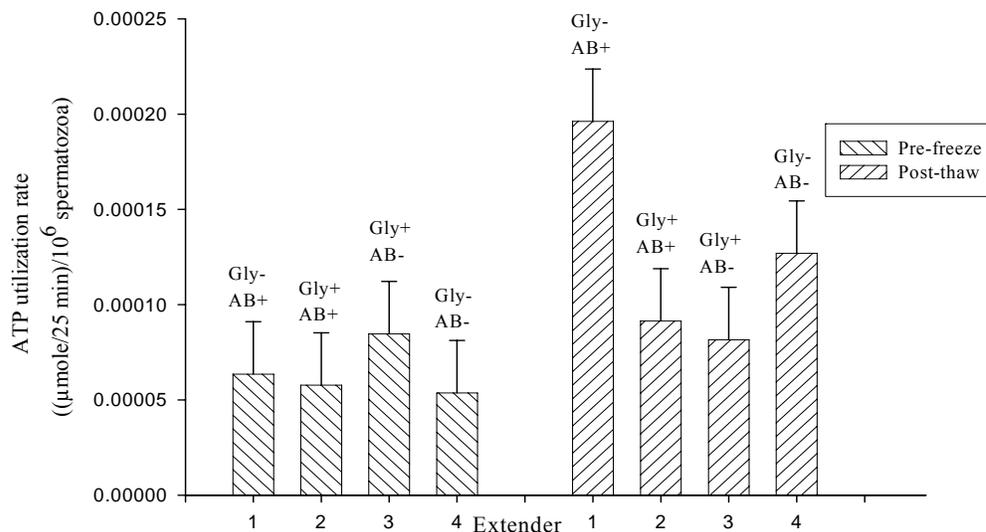


Figure 4.22. Least squares means for ATP utilization rates (slopes  $\pm$  SE) of bovine spermatozoa treated with extenders of various composition\* before and after cryopreservation. Glyc-: contains glycerol, Gly+: contains glycerol, AB+: contains antibiotics and AB-: contains no antibiotics.

In the analysis of the starting points (intercepts), ejaculate within bull, extender, storage (pre-freeze vs. post-thaw) main effects and extender-storage interaction effects were significant sources of variation ( $P > 0.05$ ) (Table 4.21).

Table 4.21. ANOVA for bovine spermatozoal ATP content ( $\mu\text{moles}/1 \times 10^6$  spermatozoa) starting points (intercepts) treated with extenders of various composition $\ddagger$  before and after cryopreservation

Source	Df	MS*	F	P-value
Bull	2	0.0002	0.15	0.8615
Error a $\dagger$	10	0.0012		
Ejaculate(Bull)	10	0.0012	20.71	<0.0001
Extender $\ddagger$	3	0.0052	90.12	<0.0001
Storage $\S$	1	0.0402	699.44	<0.0001
Extender-storage	3	0.0078	135.53	<0.0001
Error: MS(Error)	84	0.0001		

\* Mean square.

$\dagger$  Error a: MS (Ejaculate (Bull))

$\ddagger$  Extender 1 (sodium citrate egg yolk with antibiotics), extender 2 (extender 1 plus glycerol), extender 3 (extender 2 without antibiotics) and extender 4 (extender 1 without antibiotics).

$\S$ Pre-freeze vs. post-thaw.

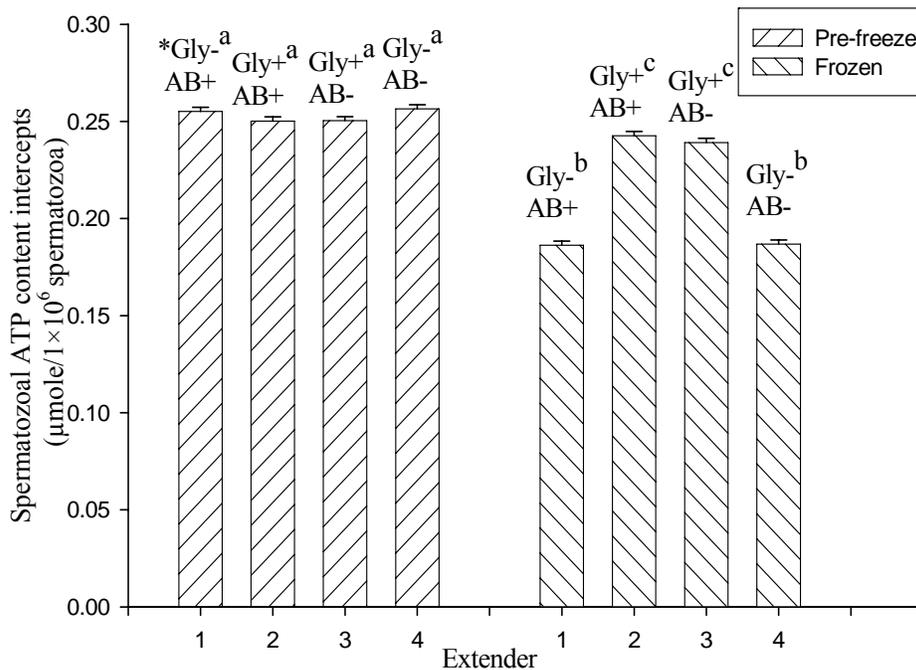


Figure 4.23. Least squares means for ATP content starting points (intercepts  $\pm$  SE) of bovine spermatozoa treated with extenders of various composition\* before and after cryopreservation. Glyc-: contains no glycerol, Gly+: contains glycerol, AB+: contains antibiotics and AB-: contains no antibiotics. Means followed by the same superscript are not different ( $P > 0.05$ ).

The ATP content varied between ejaculates within bulls. In Figure 4.23, the extender treatment groups in the pre-freeze semen had equal starting points (intercepts) of ATP content ( $P > 0.05$ ). However, the cryopreservation process altered spermatozoal ATP content and produced significant variation in the frozen starting points (Table 4.21).

### 4.3.3 Spermatozoal Motility

Results of spermatozoal motility (TM and PM) and other motility parameters (PV, VAP and VCL) obtained by CASA were presented earlier in section 4.1.1. There was a significant strong positive correlation between spermatozoal ATP content and all motility parameters (Table 4.22). The highest correlation was between ATP and TM and the lowest between ATP and PM (Table 4.22).

Table 4.22. Pearson correlation coefficients between spermatozoal ATP content and motility parameters obtained by CASA

Motility parameter	r	Probability >  r  under H <sub>0</sub> : Rho=0
Total motility (TM)	0.72	<0.0001
Progressive motility (PM)	0.66	<0.0001
Progressive velocity (PV)	0.70	<0.0001
Curvilinear velocity (VCL)	0.71	<0.0001
Pathway velocity (VAP)	0.71	<0.0001

### 4.4 Analysis of the ND1 Gene of Bovine Spermatozoal MtDNA

The bull and PCR main effects were not significant sources of variation in the frequency of amino acids change ( $P > 0.05$ ). However, the ejaculate within bull, extender treatment, plate within PCR, amino acid position and storage main effects were significant sources of variation in the frequency of amino acids change ( $P > 0.05$ ) (Table 4.23).

Storage by position and extender by storage interaction effects were significant sources of variation in the frequency of amino acid change ( $P < 0.05$ ). However, extender

by position and extender by storage by position interactions were not significant ( $P > 0.05$ ) (Table 4.23).

Table 4.23. ANOVA for the frequency of amino acids change of the ND1 gene of bovine spermatozoal mtDNA treated with extenders of various composition§ before and after cryopreservation

Source	DF	MS*	F	P-value
Bull	2	3.008	5.68	0.0853
Error a†	3.3029	0.529		
Ejaculate (bull)	3	0.918	16.65	<0.0001
Amino acid position‡	170	2.178	39.49	<0.0001
Extender§	3	0.335	6.08	0.0004
Storage§§	1	0.478	8.67	0.0032
Plate (PCR)	14	1.302	23.61	<0.0001
Extender- amino acid position	509	0.029	0.54	1
Storage-amino acid position	170	0.123	2.24	<0.0001
Extender-storage	3	2.275	41.24	<0.0001
Extender-storage- amino acid position	504	0.041	0.74	1
Error: MS (Error)	5739	0.055		
PCR	2	1.267	1.8	0.1991
Error b††	15.114	0.704		

\*Mean square

†Error a:  $0.5494 \times \text{MS (ejaculate (bull))} + 0.4506 \times \text{MS (Error)}$

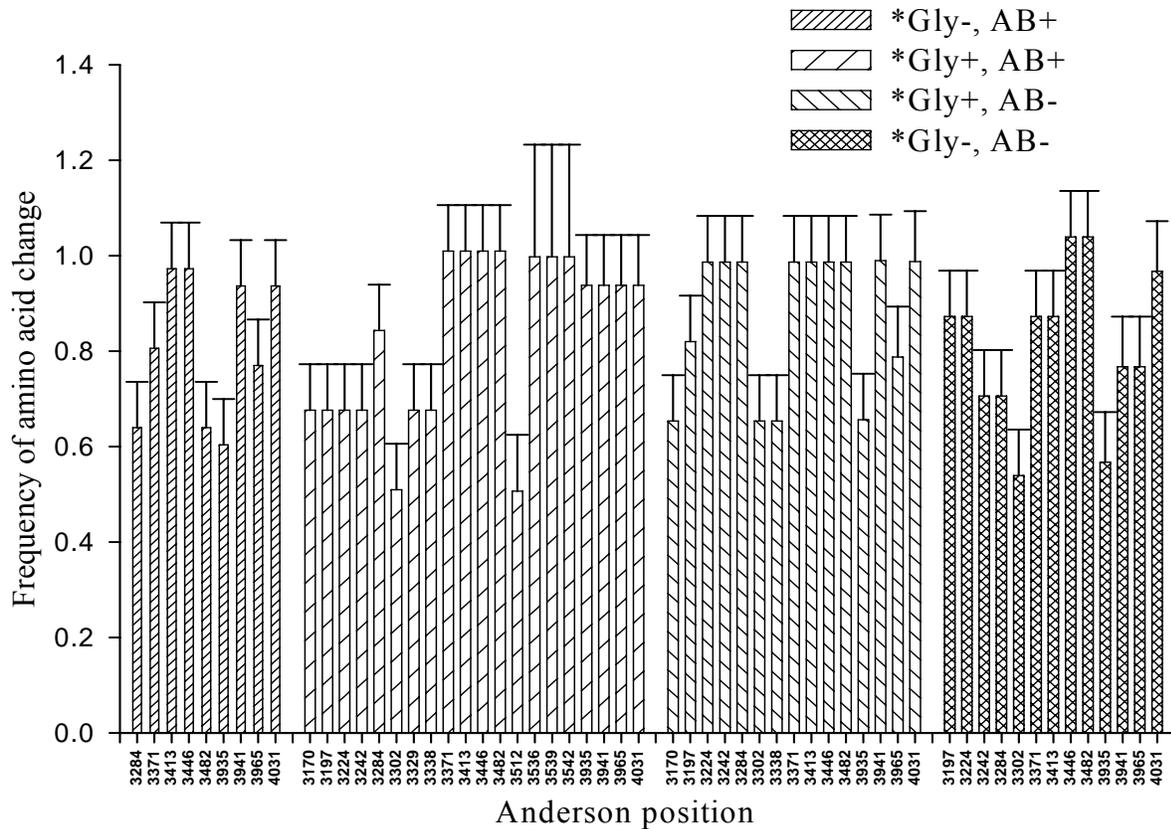
††Error b:  $0.5204 \times \text{MS (plate (PCR))} + 0.4796 \times \text{MS (Error)}$

§Extender 1 (sodium citrate egg yolk with antibiotics), extender 2 (extender 1 plus glycerol), extender 3 (extender 2 without antibiotics) and extender 4 (extender 1 without antibiotics).

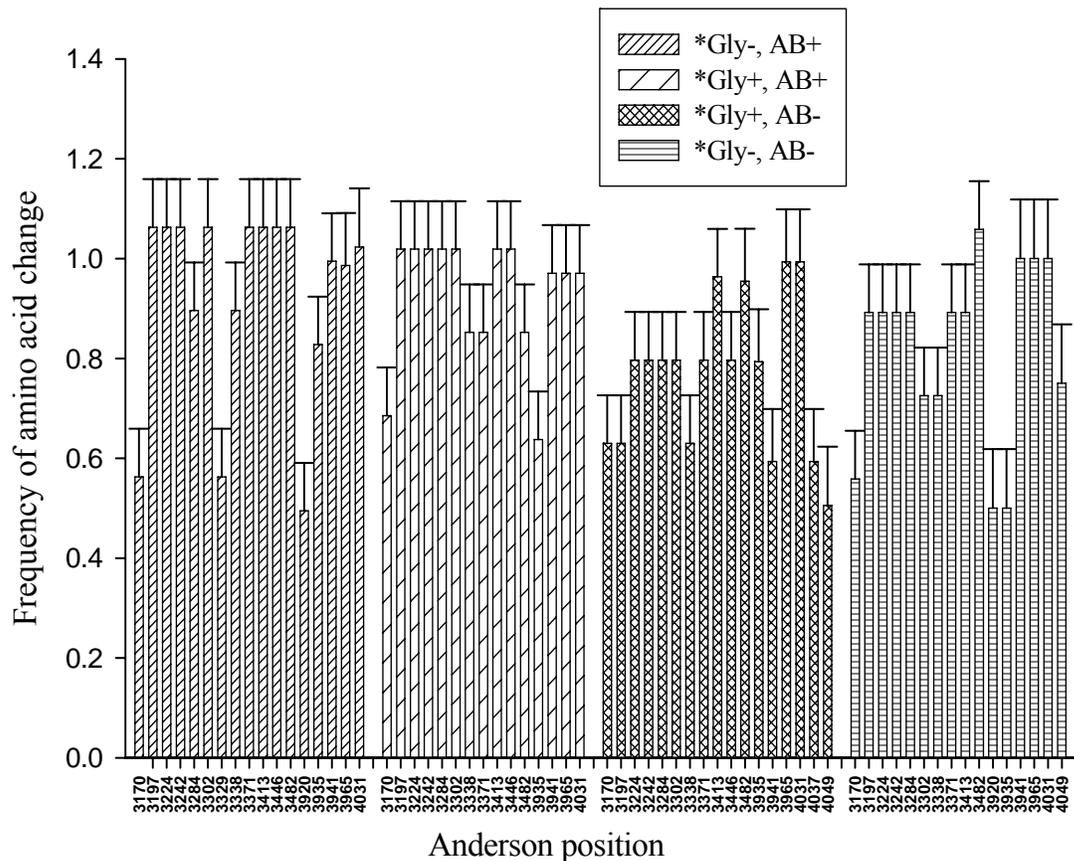
§§Pre-freeze vs. post-thaw.

Based upon the frequency of amino acid change of greater than 50%, before cryopreservation, the extender treatment that contained glycerol and antibiotics had the most positions (20) (Figure 4.24) with a frequency of amino acid change  $0.88 \pm 0.036$  (LSM  $\pm$  SE). This extender treatment was not different ( $P < 0.05$ ) from the extender treatments which contained glycerol and no antibiotics (15 positions (Figure 4.24) with a frequency of amino acid change of  $0.84 \pm 0.045$  (LSM  $\pm$  SE). The extender treatment that contained only antibiotics had nine positions with a frequency of amino acid change of  $0.62 \pm 0.036$  (LSM  $\pm$  SE). This extender treatment was not different from the extender

treatment that did not contain antibiotics and glycerol (13 positions (Figure 4.24) with a frequency of amino acid change of  $0.79 \pm 0.049$  (LSM  $\pm$  SE). The extender treatments which contained glycerol regardless of antibiotic content were different ( $P < 0.05$ ) from only the extender treatment which contained only antibiotics.



0.05). The extender treatment which contained only glycerol had a frequency of amino acid change of  $0.77 \pm 0.042$  over 17 positions while the extender treatment that contained neither antibiotics nor glycerol a frequency of amino acid change of  $0.78 \pm 0.43$  (LSM  $\pm$  SE) over 16 positions (Figure 4.25). Those extender treatments were not different ( $P > 0.05$ ) but were different ( $P < 0.05$ ) from the extender treatment that contained only antibiotics.



( $0.62 \pm 0.036$  vs.  $0.88 \pm 0.043$  (LSM  $\pm$  SE), pre-freeze vs. post-thaw, respectively)

(Figure 4.26).

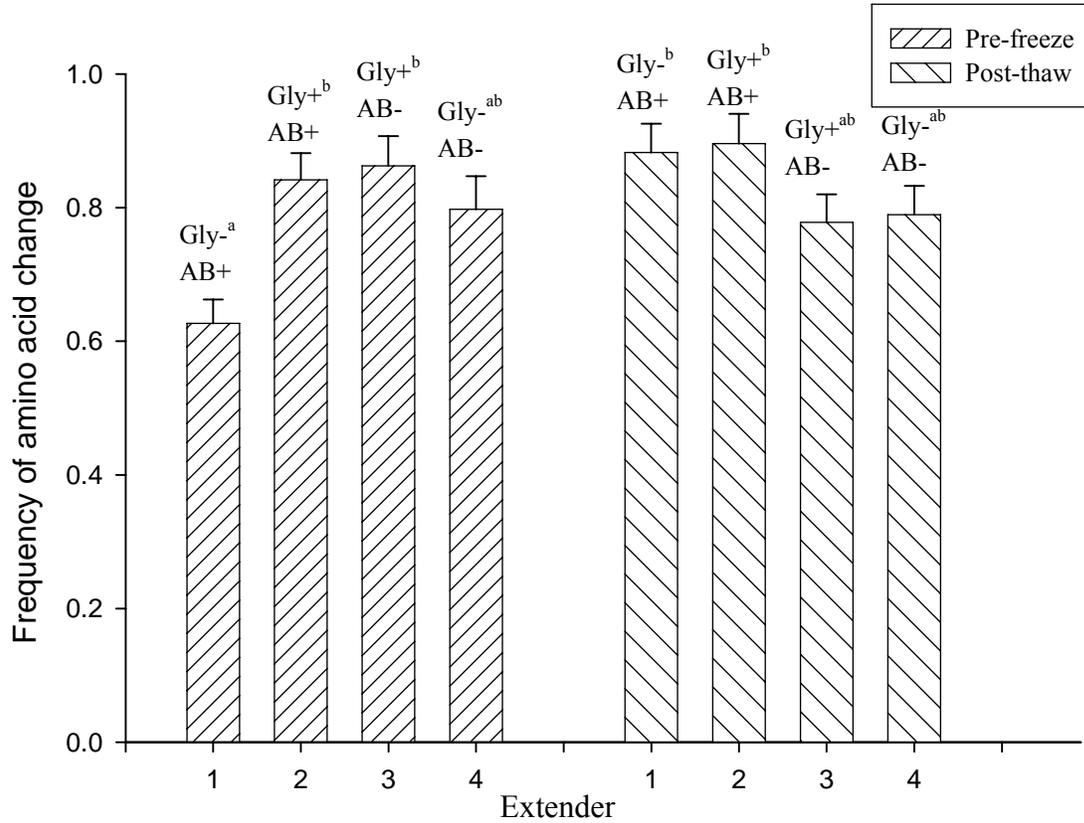


Figure 4.26. Least squares means for frequency of amino acid change ( $\pm$  SE) across Anderson positions at which there was a consensus of greater than 50% of the samples of bovine spermatozoal ND1 gene sequence. The semen was treated with extenders of various composition\* after cryopreservation. Glyc<sup>-</sup>: contains glycerol, Gly<sup>+</sup>: contains glycerol, AB<sup>+</sup>: contains antibiotics and AB<sup>-</sup>: contains no antibiotics

## CHAPTER 5

### DISCUSSION

#### 5.1 Resazurin Reduction Assay

In previous studies, the resazurin reduction assay used to assess semen quality had involved the unaided eye for the evaluation of color changes over a specified time period. Therefore, measurements were subjective because the detection of color changes varied between evaluators (Erb and Ehlers, 1950; Erb and Ehlers, 1952; Glass et al, 1991; Fuse et al, 1993; Dart et al, 1994; Reddy et al, 1997; Carter et al, 1998; Foote, 1999; Reddy and Bordekar, 1999). Later, authors developed and utilized the spectrophotometric evaluation of resazurin reduction, objectively assessed semen quality and measured sperm metabolic activity quantitatively. Most of these studies were in human medicine (Mahmoud et al, 1994; Zalata et al, 1995; Rahman and Kula, 1997; Zalata et al, 1998; Reddy and Bordekar, 1999) and few involved domestic animals like boar (Zrimesk et al, 2004) or ram (Wang et al, 1998). However, in the bull workers have used the unaided eye for assessing semen quality in the resazurin reduction assay. In addition, those assays were designed to test the whole ejaculate or the part of the ejaculate containing a high sperm cell concentrations, therefore, many false positive results were obtained (Erb and Ehlers, 1950; Erb and Ehlers, 1952; Glass et al, 1991; Dart et al, 1994). Although researchers were able to differentiate high fertility from low fertility bulls, their results were variable, especially when correlating the assay results with non return rates (Erb and Ehlers, 1950; Erb and Ehlers, 1952; Dart et al, 1994). In this study, a spectrophotometric method was developed that was based on fixed spermatozoal numbers and a known resazurin concentration so to measure spermatozoal metabolic activity quantitatively. Therefore, human error was not introduced, and the assay should now be standard

between laboratories. In addition, the experiment was designed so that effects of extender constituents and storage conditions could be tested for their possible impact spermatozoal mitochondrial function.

For both resazurin assay establishment and actual testing, a relatively low concentration of spermatozoa ( $50 \times 10^6$ /assay) equal to about three units of frozen bull semen was used rather than the entire ejaculate. Previous studies had shown that the use of the resazurin reduction assays as indicators of semen fertility was due to the time for dye reduction which was dependent on cell concentration (Erb and Ehlers, 1950; Erb and Ehlers, 1952; Glass et al, 1991; Fuse et al, 1993; Dart et al, 1994; Zalata et al, 1995; Rahman and Kula, 1997; Reddy et al, 1997; Carter et al, 1998; Wang et al, 1998; Zalata et al, 1998; Reddy and Bordekar, 1999; Zrimesk et al, 2004). In this experiment, both time and concentration were controlled thus avoiding such variability.

Glass et al (1991) reported that false-negative results increased with short reaction (incubation) times, whereas false positive results increased with long reaction times. In our experiment, the reaction time was two to three times longer (125 minutes) than those used in previous studies. Longer incubation times were used because the decrease in resazurin blue color was measured due to it being an irreversible reaction. In previous studies workers measured the increase in the resorufin (pink) due to reduction of resazurin; however, this is an intermediate and will change during longer incubation periods to dihydroresorufin. This is a reversible reaction, but the reduction of resazurin to resorufin is irreversible. Therefore, the latter is a more definitive measurement.

In this experiment, there was a significant correlation between the resazurin reduction and progressive motility ( $r = 0.5$ ,  $P < 0.05$ ) as well as total motility ( $r = 0.2$ ,  $P < 0.05$ ). This result is in agreement with previous studies (Erb and Ehlers, 1950; Erb and

Ehlers, 1952; Glass et al, 1991; Fuse et al, 1993; Dart et al, 1994; Mahmoud et al, 1994; Zalata et al, 1995; Rahman and Kula, 1997; Reddy et al, 1997; Carter et al, 1998; Wang et al, 1998; Zalata et al, 1998; Foote, 1999; Reddy and Bordekar, 1999; Zrimesk et al, 2004). There were significant extender main effect and extender-storage-time interactions with resazurin reduction, and significant extender main effect and extender-storage interactions in resazurin reduction rates. These results showed that metabolic activity of bovine spermatozoa was significantly influenced by extender type, as well as by the interaction of the extender with storage conditions and the incubation time of the reaction.

Reduction rates (slopes) (Table 4.2) in our experiment were significantly different between extenders and their constituents (glycerol in the pre-freeze vs. no glycerol). The results indicated that predominantly cryopreservation affected the resazurin reduction rates and therefore affected the metabolic activity of spermatozoa. There was also no significant effect of antibiotics on spermatozoal resazurin reduction rates.

The resazurin reduction assay directly reflected the dehydrogenase activity, which is complex I in the electron transfer chain. Complex I is the first enzyme of the electron transfer chain enzymes of the OXPHOS system and therefore may be a bottleneck in this system. Lowered activity of this enzyme resulted in less metabolic activity of spermatozoa as reflected in the resazurin reduction assay. However, the resazurin reduction assay was not sensitive enough to detect the potential effects of antibiotics on spermatozoal mitochondrial Complex I activity.

## **5.2 Respiratory Chain Enzyme Activities**

Specific activities of CytoCox from spermatozoa incubated with or without Triton X100 did not differ ( $P > 0.05$ ) among extender treatments. However, CytoCox specific

activities from spermatozoa incubated without Triton X100 were different ( $P < 0.05$ ) than those from spermatozoa incubated with Triton X100. Spermatozoal CytoCox specific activity which was measured without using Triton X100 represented the activity due to already ruptured outer mitochondrial membranes (Storrie and Madden, 1990; Hovius et al, 1990; Rasmussen and Rasmussen, 2000). Therefore, the increase in this specific activity meant the integrity of the outer mitochondrial membrane was not maintained. In addition, specific activities increased ( $P < 0.05$ ) after 125 minutes incubation at 37°C either in spermatozoa incubated with or without Triton X100. The increase in CytoCox specific activities from spermatozoa incubated without Triton X100 after 125 minutes incubation conveyed latent injury (Saacke and White, 1972) to the outer mitochondrial membrane which allowed more access of the substrate used in assaying the enzyme activity for CytoCox. This is evidenced from the negative correlation ( $r = -0.66$ ,  $P < 0.05$ ) between CytoCox specific activity from spermatozoa incubated without a Triton X100 and the percent of spermatozoal mitochondria with intact outer mitochondrial membranes (IOMM). Furthermore, CytoCox specific activities from spermatozoa incubated with Triton X100 immediately post-thaw were not different ( $P > 0.05$ ) from CytoCox specific activities from spermatozoa incubated without Triton X100 after 125 minutes of incubation. This result raised the question of whether the 125 minutes incubation revealed a latent injury to spermatozoal outer mitochondrial membranes or Triton X100 did not completely lyse spermatozoal outer mitochondrial membranes immediately post-thaw? It is possible that incubation revealed latent injury (Saacke and White, 1972) where the substrate used in assaying cytochrome c oxidase had more access to the enzyme and therefore CytoCox specific activity increased (Brich-Machin et al. 1994). Because there was a parallel increase ( $P < 0.05$ ) in CytoCox specific activities from spermatozoa either

incubated with or without Triton X100 after 125 minutes of incubation, latent injury to the outer mitochondrial membrane was revealed. Thus, the incubation or aging of semen at 37°C apparently conveys additional information concerning cell injury which has been described previously (Saacke and White, 1972).

In this experiment, spermatozoal CytoCox activity was measured as a marker for the outer mitochondrial membrane integrity. In previous studies, measurement of spermatozoal CytoCox was done without the use of a detergent and only six freeze-thaw cycles were imposed on spermatozoa for mitochondrial isolation (Ruiz-Pesini et al. 1998; Ruiz-Pesini et al. 2000; Ruiz-Pesini et al. 2001). However, in this study it was thought that measuring the specific activity of parallel samples with and without using a detergent would give an idea about the integrity of the outer mitochondrial membrane. This was based on previous studies where CytoCox specific activity was measured with the use of the detergent, dodecyl maltoside (N-dodecyl- $\beta$ -D-maltoside) (Brich-Machin et al. 1994). Because the detergent Triton X100 was used in this study to measure the specific activity of CytoCox as well as citrate synthase, this needed to be consistent with use of a detergent. Results of this study showed that spermatozoal CytoCox specific activities measured with and without spermatozoal incubation with a detergent were different ( $P < 0.05$ ). In other studies, a detergent was used to measure CytoCox activities in cells from tissues other than spermatozoa (Brich-Machin et al, 1994). Sonication was used in another study (Kraemer et al, 2005) while another did not use detergent (Wharton and Tzagoloff, 1967).

Spermatozoal citrate synthase specific activities from spermatozoa incubated without Triton X100 were different ( $P < 0.05$ ) from those incubated with Triton X100 either immediately post-thaw or after 125 minutes of incubation at 37°C. Spermatozoal

citrate synthase specific activities from spermatozoa incubated without Triton X100 decreased ( $P < 0.05$ ) after 125 minutes incubation at  $37^{\circ}\text{C}$  but did not change ( $P > 0.05$ ) when incubated with Triton X100. This decrease in specific activity following 125 minutes incubation was most likely due to the instability of citrate synthase incubated at  $37^{\circ}\text{C}$  (Barroso et al, 1993). This was evidenced from the positive correlation ( $r = 0.57$ ,  $P < 0.05$ ) between citrate synthase specific activities from spermatozoa incubated with Triton X100 and percent spermatozoa with intact inner mitochondrial membranes (IIMM). Another evidence was from the positive correlation between CytoCox specific activity from spermatozoa that were incubated with Triton X100 and IIMM ( $r = 0.31$ ,  $P < 0.05$ ) and the negative correlation between CytoCox and citrate synthase specific activities both from spermatozoa not incubated with Triton X100 ( $r = -0.27$ ,  $P < 0.05$ ). Spermatozoal citrate synthase specific activity from spermatozoa incubated without Triton X100 is the activity due to already broken spermatozoal inner mitochondrial membranes. However, the activity from spermatozoa incubated with Triton X100 is the total citrate synthase specific activity. Since the difference between both activities was significant ( $P < 0.05$ ) immediately post-thaw and after 125 minutes of incubation at  $37^{\circ}\text{C}$ , it conveyed information about the integrity of spermatozoal inner mitochondrial membranes.

Spermatozoal outer and inner mitochondrial membranes integrities were estimated based on the differences in mean specific activities for CytoCox and citrate synthase enzymes from spermatozoa incubated with and without Triton X100. Because only citrate synthase activity from spermatozoa incubated without Triton X100 decreased after 125 minutes of incubation, the validation of such estimation method for assessing the integrity of inner mitochondrial membrane is questionable.

Ruiz-Pesini et al (1998) found higher spermatozoal CytoCox and citrate synthase specific activities from fertile men compared to that in infertile ones as well as a positive correlation between those enzyme activities and spermatozoal motility. In this study, only CytoCox specific activity from spermatozoa incubated without Triton X100 was negatively correlated with TM ( $r = -0.22$ ,  $P < 0.05$ ) and specific activities of both enzymes were not correlated with spermatozoal TM, PM, PV, VCL and VAP.

Spermatozoal citrate synthase specific activities from spermatozoa incubated with Triton X100 were positively correlated with spermatozoal resazurin reduction ( $r = 0.22$ ,  $P < 0.05$ ). Citrate synthase is the regulatory enzyme in the citric acid cycle (Nelson and Cox, 2005). The citric acid cycle supplies the electron transfer chain involved in the OXPHOS process with reduced equivalents in the form of NADH and FADH<sub>2</sub> (Ruiz-Pesini et al, 1998; Nelson and Cox, 2005). This role is important in the generation of ATP which is required for spermatozoal function. Therefore, its importance as a player in the OXPHOS can not be ignored.

Complex IV is the final enzyme of the electron transfer chain enzymes of the OXPHOS system and is the site where molecular oxygen is converted to water (Semeitink et al. 2004). Therefore, reduction or impairment in the activity of this enzyme results in the reduction of the metabolic activity of spermatozoa as electrons passed from other complexes are pumped out to the mitochondrial inter membrane space and also used to convert oxygen to water.

### **5.3 Bovine Spermatozoal ATP Content**

Bovine spermatozoal ATP was highly correlated with spermatozoal motility parameters. These correlations are in agreement with previous studies which correlated spermatozoal ATP content with motility in the bull (Januskauskas and Rodriguez-

Martinez, 1995; Reiger, 1997), boar (Johnson et al, 1981), domestic poultry (Wishart, 1982; Froman and Feltmann, 1998) , rabbit (Minelli et al, 1999), carp (Perchee et al, 1995) and human (Levin et al, 1981; Orlando et al, 1982; Comhaire et al, 1983; Chan and Wang, 1987; Gottlieb et al, 1991; Bosman et al, 1994; Mahmoud et al, 1994).

Other researchers found a lacking correlations between human spermatozoal ATP content and motility (Calamera et al, 1982; Irvine and Aitken, 1985; Pousette et al, 1986) while some reported negative correlation (Schlegel et al, 1985). In previous studies, it was suggested that the concentration of ATP of spermatozoa is related to their energetic content and therefore could influence fertilizing potential (Comhaire et al, 1983; Irvine and Aitken, 1985; Chan and Wang, 1987; Comhaire et al, 1987; Comhaire et al, 1989; Gottlieb et al, 1991; Mahmoud et al, 1994). Therefore, ATP measurements were used as a parameter to assess sperm function and the fertilizing potential of human spermatozoa in infertility clinics (Comhaire et al, 1983; Irvine and Aitken, 1985; Chan and Wang, 1987; Comhaire et al, 1987; Comhaire et al, 1989; Gottlieb et al, 1991; Mahmoud et al, 1994). Some researchers found a significant positive correlation between ATP content of human spermatozoa and spermatozoal concentration, spermatozoal viability, motility (Orlando et al, 1982; Comhaire et al, 1983; Irvine and Aitken, 1985; Chan and Wang, 1987; Comhaire et al, 1989; Comhaire et al, 1989; Gottlieb et al, 1991; Orlando et al, 1994) and in vitro potential to penetrate zona-free hamster oocytes (Comhaire et al, 1983; Irvine and Aitken, 1985; Chan and Wang, 1987; Comhaire et al, 1989). However, researchers found that the fertilizing potential of spermatozoa assessed by the sperm penetration assay was due to sperm numbers and motility of spermatozoa used in the assay rather than ATP content. This was based on performing the assay using spermatozoa from fertile and infertile donors (Comhaire et al, 1983; Irvine and Aitken,

1985; Chan and Wang, 1987; Comhaire et al, 1989). They concluded that measuring ATP content of spermatozoa had a limited role in the laboratory evaluation of sperm function, yet the technique was able to differentiate between fertile and infertile men (Comhaire et al, 1983; Irvine and Aitken, 1985; Chan and Wang, 1987; Comhaire et al, 1989).

Results of several studies concerning the relation between spermatozoal ATP content and different sperm parameters have been contradictory (Levin et al, 1981; Calamera et al, 1982; Comhaire et al, 1983; Schlegel et al, 1985; Pousette et al, 1986; Mieusset et al, 1989). In this study, we showed that cryopreservation decreased spermatozoal ATP content. Cryopreservation killed spermatozoa and indirectly affected mitochondrial function by substances leaking to the inside of spermatozoa through the damaged sperm plasma membrane (Nath, 1972; Hammerstedt et al, 1990). Lowered spermatozoal ATP content produced by mitochondria was reflected in two forms: the first form occurred post-thaw in the extender treatments that contain glycerol regardless of their antibiotic content. In these extender treatments, glycerol protected a percentage of spermatozoa through the freeze-thaw process and they continued to make and utilize ATP. The second form occurred post-thaw in the extender treatments containing no glycerol regardless of their antibiotic content. Cryopreservation caused injury to all spermatozoa, and ATP production/utilization stopped. A previous study showed lowered human spermatozoal ATP post-thaw which occurred due to leakage because of cryolysis (Bosman et al, 1994). In this study, ATP content (starting points) were similar ( $P > 0.05$ ) in the pre-freeze semen, but decreased ( $P < 0.05$ ) after cryopreservation. Also the starting points not only decreased after cryopreservation, but they were different in the extender treatments that had glycerol compared to the extender treatments that did not have

glycerol. In the extender treatments that contained glycerol, cryopreservation killed a certain population of spermatozoa, and ATP content decreased likely due to ATP leakage from those killed. In the extender treatments that did not have glycerol, cryopreservation killed all spermatozoa and ATP decreased and was different from that in the extender treatments that had glycerol. Therefore, leakage of ATP due to cryolysis was the main factor in reducing the ATP content. This is in agreement with a previous study that showed a reduction in human spermatozoal ATP post-thaw which occurred due to leakage and was suggested due to cryolysis (Bosman et al, 1994)

In this experiment, using the luciferin-luciferase assay technique, we showed that cryopreservation affected bovine spermatozoal metabolic function, reflected by the decrease in spermatozoal ATP production/utilization. However, there was no effect of antibiotics on bovine spermatozoal ATP production/utilization.

#### **5.4 Analysis of the ND1 Gene of Bovine Spermatozoal MtDNA**

Results of this experiment showed that extender composition and cryopreservation significantly affected the change in amino acid frequency. Before cryopreservation, it appeared that the presence of glycerol and antibiotics resulted in an increase in the frequency in amino acid change in that extender treatment. However, the presence of only antibiotics resulted in a decrease in the frequency in amino acid change. After cryopreservation, there was an increase ( $P < 0.05$ ) in the frequency of amino acid change only in the extender treatment that had only antibiotics. Thus, there was no effect of antibiotics on the frequency of amino acid change in the pre-freeze semen, yet it was obvious in the frozen semen. Before cryopreservation and in the extender treatment which had only antibiotics, the majority of spermatozoal mitochondrial membranes were intact (based on IOMM and IIMM). Therefore, the antibiotics were unable to penetrate

the mitochondrial membranes and exert their toxicity by increasing the frequency of amino acid change. After cryopreservation and particularly in that extender treatment, spermatozoa were not protected by glycerol, and spermatozoal mitochondrial membranes were damaged during the freeze/thaw process. This damage rendered spermatozoal mitochondrial membranes permeable for antibiotics to enter inside the cells and exert their toxic effects.

The mismatch in the nucleotide base pairs in the analyzed bovine spermatozoal mitochondria from the known sequence refers to a nucleotide sequence that codes for a different amino acid and is therefore a mutation. Point mutations or multiple mtDNA deletions have been reported to be associated with infertility in asthenozoospermic (Folgero et al, 1993) and oligoasthenozoospermic (Lestienne et al, 1997) human patients. (Kao et al, 1995; Kao et al, 1998) reported that mtDNA deletions in human spermatozoa have been associated with a decline of motility and fertility. Also it has been shown that mtDNA point mutations, mtDNA single nucleotide polymorphisms, and mtDNA haplogroups can greatly influence semen quality (Holyoake et al, 1999; Ruiz-Pesini et al, 2000b; Holyoake et al, 2001; Sutarno et al, 2002). May-Panloup et al (2003) found that increased spermatozoal mtDNA content was associated with infertility in human patients when compared to spermatozoal mtDNA content from semen samples obtained from fertile individuals. To prove the relationship between mtDNA mutations and spermatozoal function, Spiropoulos et al (2002) fractionated semen from individuals according to spermatozoal motility using the Percoll fractionation method. Then, they analyzed spermatozoal mtDNA from those fractionated semen samples using the last cycle hot PCR method. Spiropoulos et al (2002) found a strong inverse correlation ( $r = 0.93$ ,  $P = 0.009$ ) between spermatozoal motility and A3243G mutation load. However,

Huang et al (1994) reported normal spermatozoal motility in an asymptomatic carrier of the A3243G mutation (38% A3243G mutation load) in a Chinese family. A more than 90% A3243G mutation load has been shown to be responsible for mitochondrial biochemical defects in hybrid cell lines carrying this particular mutation (Chomyn et al, 1992). In another study, Chinnery et al (2000) showed that very low levels of the A3243G mutation may cause a defect of mitochondrial energy metabolism in vivo.

In conclusion, antibiotics affected spermatozoal mitochondrial function as evidenced by an increased ( $P < 0.05$ ) frequency of amino acid change post-thaw. Although this effect was significant in the extender treatment that had only antibiotics, other treatments were affected but not significantly. Although in the extender treatments that were protected by glycerol, structural changes in spermatozoal membranes due to freeze/thaw processes and glycerol should not be ignored because the ultimate goal is to increase the efficiency of spermatozoal metabolic function.

Isolated bovine liver mitochondrial ribosomes showed susceptibility to the bacterial ribosome-targeted antibiotics lincomycin and tylosin, with low susceptibility to lincomycin and tylosin at normal doses while higher doses affected mitoribosomes (Denslow and O'Brien, 1978). Therefore, structural changes such as altered spermatozoal plasma membranes due to the freeze/thaw processes as well as the osmotic effect of glycerol could have permitted the entry of antibiotics to a certain subpopulations of spermatozoa and increased their concentration inside spermatozoa and impacted their metabolic function. The cocktail that the AI industry has adopted was tested and found to be harmless to spermatozoa at certain concentrations (Lein, 1986; Lorton, 1986; Shin, 1986). However, Ahmad et al (1987) showed that the percentage of motile spermatozoa was slightly but significantly depressed at the higher concentrations of linco-spectin tested

in whole milk and with minocin in egg yolk citrate and egg yolk phosphate extenders. Of the cocktail, only the antibiotic gentamicin reduced fertility on the basis of 59-d nonreturn rates (Ahmad et al, 1987). Although all antibiotics in the cocktail target bacterial ribosomes, there was selectivity of these drugs in regards to their toxicity. In rats treated with 2 mg/Kg gentamicin sulfate for eight days, spermatogonial division was blocked and spermatocytes I meiosis was partly or completely interrupted (Timmermans, 1974). Therefore, the use of antibiotics should be further addressed and further research is needed to determine the toxic effects of the antibiotic cocktail that the AI industry has adopted, particularly the antibiotic gentamicin.

## CHAPTER 6

### SUMMARY

In the first assay, there was a significant variation in reduction of resazurin among the extender treatments ( $P < 0.05$ ) in the pre-freeze semen. The bull main effect was not a significant source of variation ( $P > 0.05$ ). Spermatozoal total and progressive motilities (TM and PM, respectively), progressive, pathway and curvilinear velocities (PV, VAP and VCL, respectively) analyses were different among the extender treatments (except for PM) in the pre-freeze and frozen semen. There was significant correlation between resazurin reduction rate and PM ( $r = 0.5$ ,  $P < 0.05$ ) as well as TM ( $r = 0.2$ ,  $P < 0.05$ ). However, there was no correlation between resazurin reduction rate and PV, VAP and VCL.

In the second assay, cytochrome c oxidase specific activities from spermatozoa incubated with or without Triton X100 were not different among extender treatments ( $P > 0.05$ ). However, incubation time and incubation with Triton X100 were significant sources of variation ( $P < 0.05$ ). Overall, incubation time decreased ( $P < 0.05$ ) the percentage of spermatozoa with intact outer mitochondrial membranes. Spermatozoal citrate synthase specific activities from spermatozoa either incubated with or without Triton X100 were not significantly different among extender treatments ( $P > 0.05$ ). However, incubation time and incubation with Triton X100 were only significant sources of variation in citrate synthase specific activity ( $P < 0.05$ ). In spermatozoa incubated without Triton X100, there was a significant negative correlation between cytochrome c oxidase specific activity and citrate synthase specific activity ( $r = -0.27$ ,  $P < 0.05$ ). There was a significant negative correlation between total motility and cytochrome c oxidase

specific activity from spermatozoa incubated without Triton X100 ( $r = -0.21$ ,  $P < 0.05$ ). Overall, total and progressive motilities and progressive, pathway and curvilinear velocities were not correlated with cytochrome c oxidase and citrate synthase specific activities.

In the third assay, bull main effect was not a significant source of variation ( $P > 0.05$ ) in bovine spermatozoal ATP content. Spermatozoal ATP content was significantly different among extender treatments ( $P < 0.05$ ). However, cryopreservation significantly decreased spermatozoal ATP content. A significant ( $P < 0.05$ ) positive correlation was found between spermatozoal ATP content and TM, PM, PV, VAP and VCL ( $r = 0.72$ ,  $0.65$ ,  $0.70$ ,  $0.71$  and  $0.71$  for TM, PM, PV, VAP and VCL, respectively,  $P < 0.05$ ).

In the fourth assay, antibiotics increased the frequency of amino acid change in the extender treatment group which had only antibiotics. Overall, higher frequencies of amino acid change and an increased number of positions were found after cryopreservation

There was a negative correlation between spermatozoal ATP content and resazurin reduction ( $r = -0.30$ ,  $P < 0.05$ ). However, there was a positive correlation between citrate synthase specific activities and resazurin reduction ( $r = 0.22$ ,  $P < 0.05$ ). No correlation was found between ATP and both cytochrome c oxidase and citrate synthase specific activities.

Results of the first three assays showed no significant effect of the antibiotics on bovine spermatozoal mitochondrial function. Only assay 4 showed an effect of antibiotics on the ND1 gene. Therefore, the effects of antibiotics on bovine spermatozoal mitochondrial function should be further investigated and the use of antibiotics by the AI

industry should be addressed. Generally, the major factor that significantly reduced bovine spermatozoal mitochondrial function was cryopreservation.

## BIBLIOGRAPHY

- Ahmad K, Foote RH. Postthaw survival and fertility of frozen bull spermatozoa treated with antibiotics and detergent. *J Dairy Sci.* 1985;69:535-541.
- Ahmad K, Foote RH, Kaproth M. Antibiotics for bull semen frozen in milk and egg yolk extenders. *J Dairy Sci.* 1987;70:2439-2443.
- Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P, eds. Molecular biology of the cell. 4th ed. New York: Garland Science; 2004.
- Alcivar A, Hake L, Millette C. Mitochondrial gene expression in male germ cells of the mouse. *Dev Biol.* 1989;135:263-271.
- Almquist JO. A comparison of penicillin, streptomycin and sulfanilamide for improving the fertility of semen from bulls of low fertility. *J Dairy Sci.* 1951;34:819-822.
- Almquist JO. The effect of penicillin upon the fertility of semen from relatively infertile bulls. *J Dairy Sci.* 1948;31:681-682.
- Amann RP, Pickett BW. Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa. *Equine Vet Sci.* 1987;7:145-173.
- Anchordoguy TJ, Rudolph AS, Carpenter JF, Crowe JH. Modes of interaction of cryoprotectants with membrane phospholipids during freezing. *Cryobiology.* 1987;24:324-331.
- Anderson S, Bankier AT, Barrell BG, Bruijn MHd, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG. Sequence and organization of the human mitochondrial genome. *Nature.* 1981;290:457-465.
- Anderson S, Bruijn MHd, Coulson AR, Eperon IC, Sanger F, Young IG. The complete sequence of bovine mitochondrial DNA: conserved features of the mammalian mitochondrial genome. *J Mol Biol.* 1982;156:683-717.
- Armitage WJ. Osmotic stress as a factor in the detrimental effect of glycerol on human platelets. *Cryobiology.* 1986;23:116-125.
- Auger J, Ronot X, Dadoune JP. Human sperm mitochondrial function related to motility: a flow and image cytometric assessment. *J Androl.* 1989;10:439-448.
- Baccetti B, Collodel G, Piomboni P. Apoptosis in human ejaculated sperm cells (notulae seminologicae 9). *J Submicroscopic cytol Pathol.* 1996;28:587-596.
- Bahr GF, Engler WF. Considerations of volume, mass, DNA, and arrangement of mitochondria in the midpiece of bull spermatozoa. *Exp Cell Res.* 1970;60:338-340.

Baltz JM, Williams PO, A. CR. Dense fibers protect mammalian sperm against damage. *Bio Reprod.* 1990;43:485-491.

Barroso N, Campos Y, Huertas R, Esteban J, Molina J, Alonso A, Gutierrez-Rivas E, Arenas J. Respiratory chain enzyme activities in lymphocytes from untreated patients with parkinson disease. *Clin Chem.* 1993;39:667-669.

Barth A, Oko R, eds. Abnormal morphology of bovine spermatozoa. 1st ed. Ames: Iowa State University Press; 1989.

Bilodeau JF, Chatterjee S, Sirard MA, Gagnon C. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Mol Reprod Dev.* 2000;5:282-288.

Boggs JM, Rangaraj G. Phase transitions and fatty acid spin label behavior in interdigitated lipid phases induced by glycerol and polymixin. *Biochem Biophys Acta.* 1985;816:221-233.

Bosman E, Toit DD, Wessels PH, Bornman MS, Plessis DJD. Sperm adenosine triphosphate concentrations before and after freezing as a marker of cryolysis. *Arch Androl.* 1994;33:7-10.

Botteger E, Springer B, Prammananan T, Kidan Y, Sander P. Structural basis for selectivity and toxicity of ribosomal antibiotics. *Eur Mol Biol Org.* 2001;2:318-323.

Bratton D, Fadok V, Richter D, Kailey J, Guthrie L, Henson P. Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. *J Biol Chem.* 1997;272:26159-26165.

Brich-Machin M, Briggs H, Saborido A, Bindoff L, turnbull D. An evaluation of the measurement of the activities of complexes I-IV in the respiratory chain of human skeletal muscle mitochondria. *Biochem Med Met Biol.* 1994;51:35-42.

Budworth PR, Amann RP, Chapman PL. Relationships between computerized measurements of motion of frozen-thawed bull spermatozoa and fertility. *J Androl.* 1988;9:41-54.

Buss WC, Piatt MK, Robert K. Inhibition of mammalian microsomal protein synthesis by aminoglycoside antibiotics. *J Antimic Chemotherapy.* 1984;14:231-241.

Buss WC, Robert K, Piatt MK. Inhibitory effects of gentamicin and ethacrynic acid on mammalian microsomal protein synthesis. *J Antimic Chemotherapy.* 1985;15:105-113.

Calamera C, Brugo S, Vilar O. Relationship between motility and adenosine triphosphate (ATP) in human spermatozoa. *Andrologia.* 1982;14:239-241.

- Campbell C, Edwards J. The effect on conception rates of semen diluents containing citrate or phosphate buffer, with all combinations of sulfanilamide, streptomycin and penicillin. *J Agr Sci.* 1955;46:44-55.
- Carter RA, Ericsson SA, Corn CD, Weyerts PR, Dart MG, Escue SG, Mesta J. Assessing the fertility potential of equine semen samples using the reduction dyes methylene green and resazurin. *Arch Androl.* 1998;40:59-66.
- Chan SYW, Wang C. Correlation between semen adenosine triphosphate and sperm fertilizing capacity. *Fertil Steril.* 1987;47:717-719.
- Chandler JE, Ruiz CF, Adkinson RW, Koonce KL. Relationship between final temperature, thaw rate, and quality of bovine semen. *J Dairy Sci.* 1984;67:1806-1812.
- Chatterjee S, Lamirande ED, Gagnon C. Cryopreservation alters membrane sulfhydryl status of bull spermatozoa: protection by oxidized glutathione. *Mol Reprod Dev.* 2001;60:498-506.
- Chinnery PF, Taylor DJ, Brown DT, Manners D, Styles P, Lodi R. Very low levels of the mtDNA A3243G mutation associated with mitochondrial function in vivo. *Annal Neurol.* 2000;47:381-384.
- Chomyn A, Martinuzzi A, Yoneda M, Daga A, Hurko O, Johns D, Lai ST, Nonaka I, Angelini C, Attardi G. MELAS mutation in mtDNA binding site for transcription termination factor causes defects in protein synthesis and in respiration but no change in levels of upstream and downstream mature transcripts. Paper presented at: Proc Natl Acad Sci; 1992; USA.
- Clamohoy L, Foote RH. Effects of dyes on survival of bull spermatozoa stored in yolk and milk extenders at 5 and -79°C. *J Dairy Sci.* 1963;46:61-64.
- Comhaire F, Ir LV, Ghedira K, Irvine S, Callipolitis G. Adenosine triphosphate in human semen: a quantitative estimate of fertilizing potential. *Fertil Steril.* 1983;40:500-504.
- Comhaire F, Vermeulen L, Monsieur L. Determination of adenosine triphosphate in human semen to estimate the fertilizing potential and to quantify sperm antibodies. *J Biolumin Chemilum.* 1989;4:399-405.
- Comhaire F, Vermeulen L, Schoonjans S. Reassessment of accuracy of traditional sperm characteristics and adenosine triphosphate (ATP) in estimating the fertilizing potential of human semen *in vivo.* *Int J Androl.* 1987;41:653-662.
- Comhaire FH, Vermeulen L, Monsieur L. Determination of adenosine triphosphate in human semen to estimate the fertilizing potential and to quantify sperm antibodies. *J Biolum chemilum.* 1989;4:399-405.

- Coulter GH. Bovine spermatozoa in vitro: a review of storage, fertility estimation and manipulation. *Theriogenology*. 1992;38:197-207.
- Cummins J. Mitochondrial DNA in mammalian reproduction. *J Reprod Fertil*. 1998;3:172-182.
- Dart MG, Mesta J, Crenshaw C, Ericsson SA. Modified resazurin reduction test for determining the fertility potential of bovine spermatozoa. *Arch Androl*. 1994;33:71-75.
- Davis S, Bratton R, Foote R. Livability of bovine spermatozoa at 5, -25 and -85°C in tris-buffered and citrate-buffered yolk-glycerol extenders. *J Dairy Sci*. 1963a;46:333-336.
- Davis S, Bratton R, Foote R. Livability of bovine spermatozoa at 5°C in tris-buffered and citrate-buffered yolk-glycerol extenders. *J Dairy Sci*. 1963b;46:57-60.
- Deibel FC, Smith JEJ, Crabo BG, Graham EF. Evaluation of six assays of sperm quality by means of their accuracy, precision and sensitivity in separating known induced levels of damage. Paper presented at: Proc 8<sup>th</sup> Tech conf on AI and Reprod 1976; wisconsin.
- Denslow ND, O'Brien TW. Antibiotic susceptibility of the peptidyl transferase locus of bovine mitochondrial ribosomes. *Eur J Biochem*. 1978;91:441-448.
- Diez-Sanchez C, Ruiz-Pesini E, Montoya J, Perez-Martos A, Enriquez JA, Lopez-Perez MJ. Mitochondria from ejaculated human spermatozoa do not synthesize proteins. *Federation Eur Biochem Soc*. 2003;553:205-208.
- Doak GA. CSS implementation of new antibiotic combination. Paper presented at: Proc 11<sup>th</sup> Tech conf on AI and Reprod 1986; Wisconsin.
- Domenech EMd, Domenech E, Aoki A. Association of the testicular lactate dehydrogenase isozyme with a special type of mitochondria. *Biol Reprod*. 1972;6:136-147.
- Drevious LO, Erickson H. Osmotic swelling of mammalian spermatozoa. *Exp Cell Res*. 1966;42:136-156.
- Duncan AE, Watson PF. Predictive water loss curves for ram spermatozoa during cryopreservation: comparison with experimental observations. *Cryobiology*. 1992;29:95-105.
- Dunn O, Hafs H, Buckner P, Young G, Conrad E, Willett E, Larson G. A comparison of fertility of bovine spermatozoa stored at 5°C and -79°C. *J Dairy Sci*. 1954;37:1429-1434.
- Easterbrooks L, Heller P, Plastringer W, Jungherr E. Comparative fertility of diluted semen treated with calcium chloride complex streptomycin or dihydrostreptomycin sulfate. *J Dairy Sci*. 1950;33:

- Erb RE, Ehlers MH. Modified resazurin reduction test for estimating fertilizing capacity of bull semen. *J Dairy Sci.* 1952;35:881-888.
- Erb RE, Ehlers MH. Resazurin reducing time as an indicator of bovine semen fertilizing capacity. *J Dairy Sci.* 1950;33:853-864.
- Erlich HA, Arnheim N. Genetic analysis using the polymerase chain reaction. *Ann Rev Genet.* 1992;26:479-506.
- Erlich HA, Gelfand D, Sninsky JJ. Recent advances in the polymerase chain reaction. *Science.* 1991;252:1643-1651.
- Fahy GM. The relevance of cryoprotectant "toxicity" to cryobiology. *Cryobiology.* 1986;23:1-13.
- Fawcett W. A comparative view of sperm ultrastructure. *Biol Reprod (Suppl).* 1970;2:90-127.
- Folgero T, Bertheussen K, Lindal S, Torbergson T, Oian P. Mitochondrial disease and reduced sperm motility. *Hum Reprod.* 1993;8:1863-1868.
- Foote R. Cryopreservation of spermatozoa and artificial insemination: past, present, and future. *J Androl.* 1982;3:85-100.
- Foote RH. Resazurin reduction and other tests of semen quality and fertility of bulls. *Asian J Androl.* 1999;1:109-114.
- Foote RH, Berndtson WE. Antibacterial agents for bull semen; do they help? Paper presented at: Proc 6<sup>th</sup> Tech conf on AI and Reprod; 1976; Wisconsin.
- Foote RH, Bratton RW. The fertility of bovine semen in extenders containing sulfanilamide, penicillin, streptomycin and polymyxin. *J. Dairy Sci.* 1950;33:544-547.
- Foote RH, Bratton RW. The fertility of bovine semen in extenders containing sulfanilamids, penicillin, streptomycin and polymyxin. *J Dairy Sci.* 1950;33:544-547.
- Ford WC. Glycolysis and sperm motility: does a spoonful of sugar help the flagellum go around. *Hum. Reprod. Update.* 2006;12:269-274.
- Fosslien E. Mitochondrial medicine-molecular pathology of defective oxidative phosphorylation. *Annal Clin Lab Sci.* 2001;31:25-67.
- Foulkes J. The separation of lipoproteins from egg yolk and their effect on the motility and integrity of bovine spermatozoa. *J Reprod Fertil.* 1977;49:277-284.
- Fourmy D, Recht I, Blanchard C, Puglisi D. Structure of the A site of E.coli 16S rRNA complexed with an aminoglycoside antibiotic. *Science.* 1996;274:1367-1371.

- Froman DP, Feltmann AJ. Sperm mobility: a quantitative trait of the domestic fowl (*Gallus domesticus*). *Biol Reprod*. 1998;58:379-384.
- Fuse H, Okumura M, Kazama T, Katayama T. Comparison of resazurin test results with various sperm parameters. *Andrologia*. 1993;25:153-157.
- Gale F, Cundliffe E, Reynolds E, Richmond P, Waring M, eds. The molecular basis of antibiotic action. New York: John Wiley & Sons; 1981.
- Garcia-Macias V, Paz Pd, Martinez-Pastor F, Alvarez M, Gomes-Alves S, Bernardo J, Anel E, Anel L. DNA fragmentation assessment by flow cytometry and Sperm-Bos-Halomax (bright-field microscopy and fluorescence microscopy) in bull sperm. *Int J Androl*. 2007;30:88-98.
- Giles E, Blanc H, Cann M. Maternal inheritance of human mitochondrial DNA. Paper presented at: Proc Natl Acad Sci; 1980; USA.
- Glass RH, Ericsson SA, Ericsson RJ, Drouin MT, Marcoux LJ, Sullivan H. The resazurin reduction test provides an assesment of sperm activity. *Fertil Steril*. 1991;56:743-746.
- Gottlieb C, Svanborg K, Bygdeman M. Adenosine triphosphate (ATP) in human spermatozoa. *Andrologia*. 1991;23:421-425.
- Graham EF, Schmehl MKL, Nelson DS. Problems with laboratory assays. Paper presented at: Proc 11<sup>th</sup> Tech conf on AI and Reprod; 1980; Wisconsin.
- Graham JK. In vitro assays of bull fertility. Paper presented at: Proc 15<sup>th</sup> Tech conf on AI and Reprod; 1994; Wisconsin.
- Graham JK, Foote RH. Effect of several lipids, fatty acyl chain length and degree of unsaturation on the motility of bull spermatozoa after cold shock and freezing. *Cryobiology*. 1987;24:42-52.
- Hamdy AH. Linco-Spectin® as a bovine semen additive. Paper presented at: Proc 4<sup>th</sup> Tech conf on AI and Reprod 1972; Wisconsin.
- Hamdy AH, Miller C. Antibiotics for bovine mycoplasmas. *J Dairy Sci*. 1971;54:1541.
- Hammerstedt RH, Crichton EG, Watson PF. Comparative approach to sperm cryopreservation: does cell shape and size influence cryosurvival? *Proc Soc Theriogenology*; 1992:8-11.
- Hammerstedt RH, Graham JK, Nolan JP. Cryopreservation of mammalian sperm: what we ask them to survive. *J Androl*. 1990;11:73-88.
- Hartman G. Correlation among criteria of semen quality. *Fertil Steril*. 1965;16:632-644.

Hauswirth W, Laipis J. Mitochondrial DNA polymorphism in a maternal lineage of Holstein cows. Paper presented at: Proc Natl Acad Sci; 1982; USA.

Hecht B. The making of spermatozoon: a molecular perspective. *Dev Genet.* 1993;16:95-103.

Hecht B, Liem H, Kleene C, Distel J, Ho M. Maternal inheritance of the mouse mitochondrial genome is not mediated by a loss or gross alteration of the paternal mitochondrial DNA or by methylation of the oocyte mitochondrial DNA. *Dev Biol.* 1984;102:452-461.

Holyoake AJ, McHugh P, Wu M, O'Carroll S, Benny P, Sin IL, Sin FYT. High incidence of single nucleotide substitutions in the mitochondrial genome is associated with poor semen parameters in men. *Int J Androl.* 2001;24:175-182.

Holyoake AJ, Sin IL, Benny PS, Sin FYT. Association of a novel human mtDNA ATPase6 mutation with immature sperm cells. *Andrologia.* 1999;31:339-345.

Hovius R, Lambrechts H, Nicolay K, de Kruijff B. Isolation of subcellular organelles. *Biochim Biophys Acta.* 1990; 1021:217-226.

Huang CC, Chen RS, Chen CM, Wang HS, Lee CC, Pang CY, Hsu HS, Lee HC, Yei YH. MELAS syndrome with mitochondrial tRNA (Leu(UUR)) gene mutation in a chinese family. *J Neurol Neurosurg Psychiatry.* 1994;57:586-589.

Irvine DS, Aitken RJ. The value of adenosine triphosphate (ATP) measurements in assessing the fertilizing ability of human spermatozoa. *Fertil Steril.* 1985;44:806-813.

Jain MK, eds. Introduction to biological membranes. 2nd ed. New York: John Wiley & Sons Inc.; 1988.

Januskauskas A, Johannisson A, Rodriguez-Martinez H. Assessment of sperm quality through fluorometry and sperm chromatin structure assay in relation to field fertility of frozen-thawed semen from Swedish AI bulls. *Theriogenology.* 2001;55:947-961.

Januskauskas A, Johannisson A, Rodriguez-Martinez H. Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. *Theriogenology.* 2003;60:743-758.

Januskauskas A, Rodriguez-Martinez H. Assessment of sperm viability by measurement of ATP, membrane integrity and motility in frozen thawed bull semen. *Acta Veterinaria Scandinavia.* 1995;36:571-574.

Jeyendran RS, Graham EF. An evaluation of cryoprotective compounds on bovine spermatozoa. *Cryobiology.* 1980;17:458-464.

- Jin Q, Bethke C. Kinetics of Electron Transfer through the Respiratory Chain. *Biophys J*. 2002;83:1797–1808.
- Johnson L, Aalbers J, Willems C, Sybesma W. Use of boar spermatozoa for artificial insemination. I. Fertilizing capacity of fresh and frozen spermatozoa in sows on 36 farms. *J Anim Sci*. 1981;52:1130-1136.
- Kachar B, Reese TS. Rapid formation of gap-like structures induced by glycerol. *Anat Rec*. 1985;213:7-15.
- Kaneda H, Hayashi I, Takahama S. Elimination of paternal mitochondrial DNA in intraspecific crosses during early mouse embryogenesis. Paper presented at: Proc Natl Acad Sci; 1995; USA.
- Kao S, Chao H, Liu H, Liao T, Wei Y. Sperm mitochondrial DNA depletion in men with asthenospermia. *Fertil Steril*. 2004;82:66-73.
- Kao S, Chao H, Wei Y. Mitochondrial deoxyribonucleic acid 4977-bp deletion is associated with diminished fertility and motility of human sperm. *Biol Reprod*. 1995;52:729-736.
- Kao S, Chao H, Wei Y. Multiple deletions of mitochondrial DNA are associated with the decline of motility and fertility of human spermatozoa. *Mol Hum Reprod*. 1998;4:657-666.
- Kaya M, Harrison G. The ultrastructural relationships between sertoli cells and spermatogenic cells in the rat. *J Anat*. 1976;121:279-290.
- Keates RAB. Effects of glycerol on microtubule polymerization kinetics. *Biochem Biophys Res Comm*. 1980;97:1163-1169.
- Kerr J, Wyllie A, Currie A. Apoptosis: a basic biological phenomrnon with wide-ranging implications in tissue kinetics. *British J Cancer*. 1972;26:239-257.
- Kraemer K, Oglesbee D, Hartman S, Huey J, Anderson B, Magera M, Rinaldo P, Robinson B, Cameron J, Hahn S. Automated spectrophotometric analysis of mitochondrial respiratory chain complex enzyme activities in cultured skin fibroblasts. *Clin Chem*. 2005;51:2110-2116.
- Kurtz I. Fidelity of protein synthesis with chicken embryo mitochondrial and cytoplasmic ribosomes. *Biochemistry*. 1974;13:572-577.
- Lamirande ED, Gagnon C. Reactive oxygen species and human spermatozoa.II. Depletion of adenosine triphosphate plays an important role in the inhibition of spermatozoa motility. *J Androl*. 1992;13:379-386.

Lein DH. The current role of ureaplasma, mycoplasma, haemophilus somnus in bovine reproductive disorders. Paper presented at: Proc 11<sup>th</sup> Tech Conf on AI and Reprod; 1986; Wisconsin.

Lemasters J. Necrapoptosis and the mitochondrial permeability transition: shared pathways to necrosis and apoptosis. *Am J Phys*. 1999;276:1-6.

Leonard J, Schapira A. Mitochondrial respiratory chain disorders I: mitochondrial DNA defects. *The Lancet*. 2000;355:299-304.

Lestienne P, Reyneir P, Chretien MF, Penisson-Besnier I, Malthiery Y, Rohmer V. Oligoasthenozoospermia associated with multiple mitochondrial DNA rearrangements. *Mol Hum Reprod*. 1997;3:811-814.

Levin M, Shofer J, Wein J, Greenberg H. ATP concentration of human spermatozoa: Lack of correlation with sperm motility. *Andrologia*. 1981;13:468-472.

Linford E, Glover FA, Bishop C, Stewart DL. The relationship between semen evaluation methods and fertility in the bull. *J Reprod Fertil*. 1976;47:283-291.

Liu DY, Clarke GN, Baker HWG. Relationship between sperm motility assessed with the Hamilton-Thorn motility analyzer and fertilization rates in vitro. *J Androl*. 1991;12:231-239.

Liu Z, Foote RH. Osmotic effects on volume and motility of bull sperm exposed to membrane permeable and non permeable agents. *Cryobiology*. 1998;37:207-218.

Long JA, Guthrie HD. Validation of a rapid, large-scale assay to quantify ATP concentration in spermatozoa. *Theriogenology*. 2006;65:1620-1630.

Lorton SP. Laboratory and fertility studies using a combination of gentamicin, tylosin and lincospectin for the control of microorganisms in bovine semen. Paper presented at: Proc 11<sup>th</sup> Tech conf on AI and Reprod 1986; Wisconsin.

Lue Y, Hikim A, Swerdloff R, Im P, Taing K, Bui T, Leung A, Wang C. single exposure to heat induces stage-specific germ cell apoptosis in rats: role of intratesticular testosterone on stage specificity. *Endocrinology*. 1999;140:1709-1717.

Mahmoud AM, Comhaire FH, Vermeulen L, Andreou E. Comparison of the resazurin test, adenosine triphosphate in semen, and various sperm parameters. *Hum Reprod*. 1994;9:1688-1693.

Manfredi G, Thyagarajan D, Papadopoulo LC, Pallotti F, Schon EA. The fate of human sperm-derived mtDNA in somatic cells. *Am J Hum Genet*. 1997;61:953-960.

Margulis L, eds. Origin of eucaryotic cells. New Haven: Yale University Press; 1970.

Marin-Guzman J, Mahan DC, Whitmoyer R. Effect of dietary selenium and vitamin E on the ultrastructure of boar spermatozoa, and the efficacy of added sodium selenite in extended semen on sperm motility. *J Anim Sci.* 2000;78:1544-1550.

Martin G, Sabido O, Durand P, Levy R. Cryopreservation induces an apoptosis-like mechanism in bull sperm. *Biol Reprod.* 2004;71:28-37.

Martino CD, Floridi L, Marcante P. Morphological, histochemical and biochemical studies on germ cell mitochondria of normal rats. *Cell Tissue Res.* 1979;196:1-22.

Martino CD, Stefanini A. Morphological and functional modifications of mitochondrion in rat germ cells. *J Submicrosc Cytol.* 1974;6:115-116.

Matsuno-Yagi A, Hatefi Y. Studies on the mechanism of oxidative phosphorylation. *J Biol Chem.* 1993;268:1539-1545.

Maxwell W, Watson P. Recent progress in the preservation of ram semen. In: Stone G, Evans G, eds. Animal reproduction: research and practice. *Anim Reprod Sci*, 1996:55-67.

May-Panloup P, Chretien M-F, Savagner F, Vasseur C, Jean M, Malathiere Y, Reynier P. Increased sperm mitochondrial DNA content in male infertility. *Hum Reprod.* 2003;18:550-556.

Mazur P, Katkov I, Katkova N, Cristar J. The enhancement of the ability of mouse sperm to survive freezing and thawing by the use of high concentrations of glycerol and the presence of an Escherichia coli membrane preparation (oxyrase) to lower the oxygen concentration. *Cryobiology.* 2000;40:187-209.

Mesta J, Ericsson S, Dart M, Wansley R, Weyerts P. Assessment of fertility potential of porcine spermatozoa using the reducible dyes methylene green and resazurin. *J Anim Sci.* 1995;73:26-35.

Michaels S, Hauswirth W, Laipis J. Mitochondrial DNA copy number in bovine oocytes and somatic cells. *Dev Biol.* 1982;94:246-251.

Mieusset R, Parinaud J, Chap H, Pontonnier G. Absence of correlation between the levels of ATP and other seminal compounds in semen and the results of human in-vitro fertilization. *Int J Androl.* 1989;12:346-353.

Minelli A, Moroni M, Castellini C, Lattaioli P, Mezzasoma I, Ronquist G. Rabbit spermatozoa: A model system for studying ATP homeostasis and motility. *J Androl.* 1999;20:259-266.

Mitchell P. Chemiosmotic Coupling and Energy Transduction. *Glynn Research Ltd*, 1968: 1-111.

Mitchell P. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. *Biol Rev Camb Philos Soc.* 1966;41:445-502.

- Mitchell P. Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. *Nature*. 1961;191:144-148.
- Mitchell P, Moyle J. Estimation of membrane potential and pH difference across the crisyae membrane of rat liver mitochondria. *Eur J Biochem*. 1969;7:471-484.
- Moazed D, Noller F. Interaction of antibiotics with functional sites in 16S ribosomal RNA. *Nature*. 1987;327:389-394.
- Molina JA, Ruiz-Pesini E, Jimenez-Jimenez FJ, Lopez-Perez MJ, Alvarez E, Berbel A, Orti-Pareja M, Zurdo M, Tallon-Barranco A, Bustos Fd, Arenas J. Respiratory chain enzyme activities in spermatozoa from untreated parkinson's disease patients. *J Neur Transm*. 1999;106:919-924.
- Morgan B, Melrose D, Stewart D. The effect of streptomycin on the survival of vibrio fetus in semen and other diluents stored at 5 and -79°C. *J Comp Path*. 1959;69:257-267.
- Moussa M, Martinet V, Trimeche A, Tainturier D, Anton M. Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen-thawed bull semen. *Theriogenology*. 2002;57:1695-1706.
- Nath J. Correlative biochemical and ultrastructural studies on the mechanism of freezing damage to ram semen. *Cryobiology*. 1972;9:240-246.
- Nelson D, Cox M. Lehninger principles of biochemistry. New York: W. H. Freeman and Company, 2005:690-722.
- Niedermeyer W, Parish GR, Moore H. Reactions of yeast cells to glycerol treatment alterations to membrane structure and glycerol uptake. *Protoplasma*. 1977;92: Nishizono H, Shioda M, Takeo T, Irie T, Nakagata N. Decrease of fertilizing ability of mouse spermatozoa after freezing and thawing is related to cellular injury. *Biol Reprod*. 2004;71:973-978.
- O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the alamar blue (resazurin) fluorescent dye for the assessment of mammalian cell toxicity. *Eur J Biochem*. 2000;267:5421-5426.
- O'Connell M, McClure N, Lewis SEM. The effects of cryopreservation on sperm morphology, motility and mitochondrial function. *Hum Reprod*. 2002;17:704-709.
- O'Connor MT, Amann RP, Saacke RG. Comparisons of computer evaluations of spermatozoal motility with standard laboratory tests and their use for predicting fertility. *J Anim Sci*. 1981;53:1369-1376.

Orlando C, Caldini AL, Fiorelli G, Cuomo S, Serio M. ATP and ADP content of human ejaculated spermatozoa I. Relationship with semen physical parameters in normal donors and oligospermic patients. *Int J Androl.* 1982;5:497-502.

Orlando C, Krausz C, Fortli G, Casano R. Simultaneous measurement of sperm LDH, LDH-X, CPK activities and ATP content in normospermic and oligospermic men. *Int J Androl.* 1994;17:13-18.

Paasch U, Sharma R, Gupta A, Grunewald S, Mascha E, Thomas A, Glander H, Agarwal A. Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa. *Biol Reprod.* 2004;71:1828-1837.

Parks E, Hammerstedt H. Developmental changes occurring in the lipids of ram epididymal spermatozoa plasma membrane. *Biol Reprod.* 1985;32:653-668.

Perchec G, Jeulin C, Cossson J, Andre F, Billard R. Relationship between sperm ATP content and motility of carp spermatozoa. *J Cell Sci.* 1995;108:747-753.

Phillips PH. The preservation of bull semen. *J Biol Chem.* 1939;130:415.

Phillips PH, Lardy HA. A yolk-buffr pablum fo the preservation of bull semen. *J Dairy Sci.* 1940;23:399-404.

Picket W, Berndston W, eds. Principles and techniques of freezing spermatozoa. 2nd ed. San Francisco: W. H. Freeman and Co; 1978.

Piko L, Matsumoto L. Number of mitochondria and some propertirs of mitochondrial DNA in the mouse egg. *Dev Biol.* 1976;49:1-10.

Piko L, Taylor D. Amounts of mitochondrial DNA and abundance of some mitochondrial gene transcripts in early mouse embryos. *Dev Biol.* 1987;123:364-374.

Polge C, Smith AU, Parkes AS. Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature.* 1949;164:666.

Poulos A, Darin-Bennett A, White IG. The phospholipid-bound fatty acids and aldehydes of mammalian spermatozoa. *Comp Biochem Physiol.* 1973;46B:541-549.

Pousette A, Akerlof E, Lundin A, Rosenborg L, Fredricsson B. On the use of adenosine triphosphate for estimation of motility and energy status in human spermatozoa. *Int J Andrology.* 1986;9:331-340.

Rahman NA, Kula K. Enlarged spectrum of seminological diagnosis using the resazurin color reaction; a spectrophotometric application. *Int J Androl.* 1997;20:17-22.

- Rasmussen UF, Rasmussen HN. Human quadriceps muscle mitochondria: a functional characterization Improved methods to isolate and subfractionate rat liver mitochondria. *Mol Cell Biochem.* 2000; 208:37-44.
- Ravagnan L, Roumier T, Kroemer G. Mitochondria, the killer organelles and their weapons. *J Cell Physiol.* 2002;192:131-137.
- Reddy KVR, Bordekar AD. Spectrophotometric analysis of resazurin reduction test and semen quality in men. *Indian J Exp Biol.* 1999;37:782-786.
- Reddy KVR, Meherji PK, Gokral JS, SHahani SK. Resazurin reduction test to evaluate semen quality. *Indian J Exp Biol.* 1997;35:369-373.
- Reiger D. Batch analysis of the ATP content of bovine sperm, oocytes, and early embryos using a scintillation counter to measure the chemiluminescence produced by the luciferin-Luciferase reaction. *Anal Biochem.* 1997;246:67-70.
- Reynier P, May-Panloup P, Chretien M-F, Morgan CJ, Jean M, Savagner F, Barriere P, Malthiery Y. Mitochondrial DNA content affects the fertilizability of human oocytes. *Mol Hum Reprod.* 2001;7:425-429.
- Richelli F, Gobbo S, Moreno G, Salet C. Changes of the fluidity of mitochondrial membranes induced by the permeability transition. *Biochemistry.* 1999;38:9295-9300.
- Rodriguez-Fonseca C, Amils R, Garrett A. Fine structure of the peptidyl transferase center on 23S-like rRNAs deduced from chemical probing of antibiotic-ribosome complexes. *J Mol Biol.* 1995;247:224-235.
- Rudenko SV, Gapochenko SD, Bondarenko VA. Effect of glycerol on the capacitance and conductivity of bilayer lipid membranes. *Biophysics.* 1984;29:245-249.
- Ruiz-Pesini E, Alvarez E, Enriquez J, Lopez-Perez M. Association between seminal plasma carnitine and sperm mitochondrial enzymatic activities. *Int J And.* 2001;24:335-340.
- Ruiz-Pesini E, Diez C, Lapena AC, Perez-Martos A, Montoya J, Alvarez E, Arenas J, Lopez-Perez MJ. Correlation of sperm motility with mitochondrial enzymatic activities. *Clin Chem.* 1998;44:1616-1620.
- Ruiz-Pesini E, Lapena AC, Diez-Sanchez C, Alvarez E, Enriquez JA, Lopez-Perez MJ. Seminal quality correlates with mitochondrial functionality. *Clinica Chemica Acta.* 2000a;300:97-105.
- Ruiz-Pesini E, Lapena AC, Diez-Sanchez C, Perez-Martos A, Montoya A, Alvarez E, Enriquez JA, Diaz M, Urries A, Montoro L, Lopez-Perez MJ. Human mtDNA haplogroups associated with high or reduced spermatozoa motility. *Am J Hum Genet.* 2000b;67:682-696.

- Russel L, Frank B. Ultrastructural characterization of nugae in spermatocytes of the rat testis. *Anat Rec.* 1978;190:
- Saacke RG. Components of semen quality. *J Anim Sci.* 1982;55 Suppl:1-13.
- Saacke RG, White JM. Semen quality tests and their relationship to fertility. Paper presented at: Proc 4th Tech Conf on AI and Reprod; 1972; Wisconsin.
- Salisbury G, Fuller H, Willett E. Preservation of bovine spermatozoa in yolk-citrate diluent and field results from its use. *J Dairy Sci.* 1941;24:905-910.
- Salisbury G, Knodt C. The effect of aulfanilamide in the diluent upon the fertility of bull semen. *J Dairy Sci.* 1947;30:361-369.
- Salisbury GW, VanDemark NL, Lodge JR. Extenders and extension of unfrozen semen. In: Physiology of reproduction and artificial insemination in cattle. San Francisco: W. H. Freeman and Co, 1978:442-493.
- Schlegel W, Kusseler R, Nieschlag E. Significance of adenosine triphosphate in human sperm as clinical parameter. *Arch Androl.* 1985;14:171-176.
- Schoff P, Cheetham J, Lardy H. Adenylate kinase activity in ejaculated bovine sperm flagella. *J Biol Chem.* 1989;264:6086-6091.
- Semeitink JAM, Sengers RCA, Trijbels JMF, eds. Oxidative phosphorylation in health and disease. New York: Kluwer Academic/Plenum Publishers; 2004.
- Setchell B, eds. The mammalian testis. 1st ed. New York: Cornell University Press; 1978.
- Shannon P. Factors affecting semen preservation and conception rates in cattle. *J Reprod Fertil.* 1978;54:519-527.
- Shin SJ. The control of mycoplasmas, ureaplasmas, campylobacter fetus, haemophilus somnus in frozen bovine semen. Paper presented at: Proc 11<sup>th</sup> Tech conf on AI and Reprod; 1986; Wisconsin.
- Smith L, Berndston W, Unal M, Picket W. Influence of percent egg yolk during cooling and freezing on survival of bovine spermatozoa. *J Dairy Sci.* 1979;62:1297-1303.
- Spiropoulos J, Turnbull D, Chinnery P. Can mitochondrial DNA mutations cause sperm dysfunction? *Mol Hum Reprod.* 2002;8:719-721.
- Steponkus PL, Lynch DV. Freeze/thaw-induced destabilization of the plasma membrane and the effects of cold acclimation. *J Bioenerg Biomemb.* 1989;21:21-41.
- Storrie B, Madden EA. Isolation of subcellular organelles. *Methods Enzymol.* 1990;182:214-215.

- Sue CM, Schon EA. Mitochondrial respiratory chain diseases and mutations in nuclear DNA: a promising start? *Brain Path.* 2000;10:442-450.
- Summers K. ATP-induced sliding of microtubules in bull sperm flagella. *J Cell Biol.* 1974;60:321-324.
- Sutarno JM, Cummins JM, Greeff J, Lymbery AJ. Mitochondrial DNA polymorphisms and fertility in beef cattle. *Theriogenology.* 2002;57:1603-1610.
- Sutovski P, Moreno D, Ramalho-Santos J. Ubiquitin tag for sperm mitochondria. *Nature.* 1999;402:371-372.
- Syntin P, Robaire B. Sperm structural and motility changes during aging in the brown Norway rat. *J Androl.* 2001;22:235-244.
- Taanman W. The human mitochondrial genome: structure, transcription, translation and replication. *Biochem Biophys Acta.* 1999;1410:103-123.
- Terada H. Uncouplers of oxidative phosphorylation. *Environ Health Perspectives.* 1990;87:213-218.
- Thomas CA, Garner DL, DeJarnette JM, Marshall CE. Effect of cryopreservation on bovine sperm organelle function and viability as determined by flow cytometry. *Biol Reprod.* 1998;58:786-793.
- Timmermans L. Influence of antibiotics on spermatogenesis. *J Urol.* 1974;112:348-349.
- Verstegen J, Iguer-Ouada M, Onclin M. Computer assisted semen analyzers in andrology research and veterinary practice. *Theriogenology.* 2002;57:149-179.
- Vishwanath R, Pitt CJ, Shannon P. Sperm numbers, semen age and fertility in fresh and frozen bovine semen. *Proc New Zealand Soc Anim Prod.* New Zealand; 1996.
- Vishwanath R, Shannon P. Do sperm cells age? A review of physiological changes in sperm cells during storage at ambient temperature. *Reprod Fertil Dev.* 1997;9:321-331.
- Wakabayashi T. Megamitochondria formation - physiology and pathology. *J Cell Mol Med.* 2002;6:497-538.
- Wallace DC. Diseases of mitochondrial DNA. *Annu Rev Biochem.* 1992;61:1175-1212.
- Wallace K, Starkov A. Mitochondrial targets of drug toxicity. *Ann Rev Pharm Tox.* 2000;40:353-388.
- Wang S, Holyoak GR, Panter KE, Liu G, Liu Y, Evans RC, Bunch TD. Resazurin reduction assay for ram sperm metabolic activity measured by spectrophotometry (44223). *Soc Exp Biol Med.* 1998;217:197-202.

Watson PF. The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci.* 2000;60:481-492.

Wharton DC, Tzagoloff A. Cytochrome oxidase from beef heart mitochondria. *Meth Enzym.* 1967;10:245-250.

Willett EL, Larson GL. Fertility of bull semen as influenced by dilution level, antibiotics, spermatozoan numbers and the interaction of these factors. *J Dairy Sci.* 1952;35:899-905.

Wishart J. Maintenance of ATP concentrations in and of fertilizing ability of fowl and turkey spermatozoa in vitro. *J Reprod Fertil.* 1982;66:457-462.

Woolley DM, Richardson DW. Ultrastructural injury to human spermatozoa after freezing and thawing. *J Reprod Fertil.* 1978;53:389-394.

Zalata A, Hafez T, Mahmoud M, Comhaire F. Relationship between resazurin reduction test, reactive oxygen species generation and gamma-glutamyltransferase. *Hum Reprod.* 1995;10:1136-1140.

Zalata AA, Lammertijin N, Christophe A, Comhaire FH. The correlates and alleged biochemical background of the resazurin reduction test in semen. *Int J Androl.* 1998;21:289-294.

Zamboni L. The ultrastructure pathology of the spermatozoon as a cause of infertility-the role of the electron-microscopy in the evaluation of semen quality. *Fertil Steril.* 1982;48:711-734.

Zhang BR, Lundeheim N, Rodriguez-Martinez H. Sperm characteristics and zona pellucida binding in relation to field fertility of frozen-thawed semen from dairy AI bulls. *Int J Androl.* 1998;21:207-216.

Zhang H, Du G, Zhang J. Assay of mitochondrial functions by resazurin in vitro. *Acta Pharmacol Sin.* 2004; 25:385-389.

Zrimesk P, Kunc J, Kosec M, Mrkun J. Spectrophotometric application of resazurin reduction assay to evaluate boar semen quality. *Int J Androl.* 2004;27:57-62.

## VITA

Abdulhakeem Hashim Eljarah was born on December 11, 1975, in Irbid, Jordan. He grew up in Alal, a small town in Jordan, and completed his secondary education at Irbid Secondary School, Irbid, Jordan, in 1993. Dr. Eljarah attended Jordan University of Science and Technology, Irbid, Jordan, obtaining a bachelor of veterinary science (DVM) degree in 1994. He successfully completed his BVSc degree from Jordan University of Science and Technology, Irbid, Jordan, in 1999. In 1999, he started a two-year clinical diploma program in theriogenology and artificial insemination at Jordan University of Science and Technology, Irbid, Jordan. He successfully completed the diploma program and earned a clinical diploma in theriogenology and artificial insemination at Jordan University of Science and Technology, Irbid, Jordan, in 2001.

From 2001 to 2003, Dr. Eljarah completed a two-year residency program in theriogenology at Louisiana State University in Baton Rouge, Louisiana. In August 2004, Dr. Eljarah has been awarded the degree of Master of Science in veterinary medical Sciences at Louisiana State University School of Veterinary Medicine in Baton Rouge, Louisiana. Through his master's program, Dr. Eljarah met With Dr. John Chandler, Professor of Reproductive Physiology, in the School of Animal Sciences and registered for special problems in andrology with him. Dr. Eljarah then recognized the strength and value of Dr. Chandler's male reproductive physiology graduate program. In addition, Dr. Eljarah was interested in andrology and therefore he decided to pursue more graduate work in the field of male reproductive physiology and was accepted in Dr. Chandler's graduate program in January 2005. Through Dr. Eljarah's PhD program, he successfully passed the ACT certifying examination in theriogenology and became Diplomate by the American College of Theriogenologists in August 2005. Dr. Eljarah is currently enrolled

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