APOPTOSIS-LIKE CHANGES IN BULL SPERM AND THEIR EFFECTS ON FERTILITY

A Thesis Submitted to
the College of Graduate Studies and Research
in Partial Fulfillment of the Requirements
for the Degree of Masters of Science
in the Department of Veterinary Biomedical Sciences
University of Saskatchewan
Saskatoon, Saskatchewan, Canada

By
Lauren Erickson

PERMISSION TO USE

TITLE OF THESIS: Apoptosis-like changes in bull sperm and their effects on fertility

NAME OF AUTHOR: Lauren Erickson

Department of Veterinary Biomedical Sciences

DEGREE: Master of Science

In presenting this thesis in partial fulfillment of the requirements for a Master degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work, or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Veterinary Biomedical Sciences
Western College of Veterinary Medicine
52 Campus Drive
University of Saskatchewan
Saskatoon, Saskatchewan
S7N 5B4

ABSTRACT

The overall objective of this thesis was to evaluate the effects of apoptosis-like membrane and DNA changes in bull sperm, and to relate these changes to a bull's fertility potential. This thesis hypothesis is that apoptosis-like changes occurring in fresh or cryopreserved bull sperm have a negative effect on a bull's fertility potential.

Two studies were conducted, the objectives of study 1 were to confirm the relationship of apoptosis-related membrane and nuclear changes in bull sperm with fertility, to predict the fertility of beef bulls used for natural mating; and to evaluate the effect of sperm with nicked-DNA on cleavage and blastocyst formation *in vitro.* In Experiment 1, phosphatidylserine (PS) translocation, from the inner to the outer plasma membrane, and DNA nicks in the sperm from 50 dairy bulls were determined using Annexin-V/PI and TUNEL assays, respectively. Relationships between the parameters of the assays and the known fertility levels of the bulls were calculated. In Experiment 2, fertility levels of 15 beef bulls used for natural mating were estimated using a regression model of DNA nicks developed in Experiment 1. In Experiment 3, the effect of DNA nicked sperm on cleavage and blastocyst rates were evaluated in *in vitro* produced embryos, using high and low sperm concentrations (30,000 and 300,000 sperm per IVF droplet) to fertilize the mature oocytes. In Experiment 1, there were significant relationships of fertility with live sperm ($P<0.05$) and necrotic sperm ($P<0.01$) (Annexin-V/PI assay), and with DNA-nicked sperm ($P<0.001$) (TUNEL assay). In Experiment 2, the fertility level of bulls used for natural breeding was estimated and ranged from -7.3 to 2.4. In Experiment 3, the cleavage rate was significantly affected by the number of sperm with nicked DNA, regardless of sperm concentration. At the low sperm concentration, blastocyst rate was significantly lower when higher DNA nicked sperm were used (51% vs 32%; high vs low DNA nicks) ($P<0.05$). Blastocyst rate was non significant at the higher sperm concentration regardless of DNA nicks.

The second objective of this study was to evaluate the effect of apoptosis inhibitors added to post-thaw sperm samples on their longevity, to increase the availability of viable sperm to oocytes for fertilization. Frozen semen from seven bulls was used; six straws from

each bull were pooled. Samples included, untreated control (sperm remaining in extender), treated control (washed sperm), and four treatments (inhibitors) each at two concentrations. Apoptosis inhibitors assessed included; Bax channel blocker, z-VAD-FMK, Coenzyme Q_{10}, and XIAP. Motility related characteristics were evaluated using computer assisted sperm analysis (CASA). Membrane intactness and normal acrosomes were evaluated using fluorescein isothiocyanate-peanut agglutination (FITC-PNA)/propidium iodide (PI) assay. Mitochondrial membrane potential was evaluated using Mitotracker Deep Red (MtDR). Sperm parameters were evaluated at 0, 3, 6, and 12 hours of incubation. Our results showed, no significant effect of apoptosis inhibitors on post-thaw sperm motility and structural characteristics. The decline in sperm motility and structural characteristics at 6 h of incubation was lower ($P<0.05$) in treated control and treatment groups than untreated control group.

In conclusion, the presence of nicked DNA in sperm may be used as an estimate of the fertility level of a breeding bull. The levels of sperm with DNA nicks have a negative effect on cleavage rates and subsequent blastocyst development. The second conclusion indicates that the addition of an apoptosis inhibitor post-thaw to semen samples does not improve longevity or fitness, in any of the parameters evaluated. The simple removal of extender showed to be beneficial to sperm longevity and fitness. Further studies are needed to evaluate the cleavage and blastocyst rate of embryos fertilized with a single sperm known to carry DNA nicks. As well, the effect of the addition of apoptosis inhibitors before cryopreservation of bull semen needs to be evaluated.

ACKNOWLEDGEMENTS

First of all I would like to thank my supervisor Dr. Muhammad Anzar, for his guidance, patience, understanding and support. I would like to thank my committee member Dr. Carl Lessard for his guidance, helpfulness and input and Dr. Gillian Muir and Dr. Karen Machin for serving as my graduate chair.

I would like to thank Agriculture and Agri-Food Canada, Developing Innovative Agri-Products Initiation program and WestGen Endowment fund for their financial support.

I would like to send a grateful thank-you to the staff and fellow graduate students in Veterinary Biomedical Sciences and Agriculture and Agri-Food Canada for their daily support and motivation. To Lyle Boswall and Dr. Kosala Rajapaksha for all their guidance and help in the lab.

A huge thank-you to EastGen for access to their bulls and frozen semen. To Cargill in Calgary, AB for the shipment of ovaries, as well as to Drake Meats in Drake, Sk for their supply of ovaries. A huge thanks to the staff of Goodale Farms, University of Saskatchewan, and to the managers of the PFRA bulls, for all their assistance with the bulls and semen collections.

Lastly, I would like to thank my amazing family and friends for their never-ending support and encouragement throughout all the stressful times during my project. You have always taught me to never give up on my dreams and that life doesn't throw you more than you can handle. I would like to dedicate this thesis to my family, my parents Tannis and Brad, my little brother Brett Erickson, and to my best friend and boyfriend Gary Tiffin.

Thanks again to everyone for all your support and guidance.
Lauren Erickson

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABREVIATIONS

µl: Microliter

µg: Micrograms

AAFC: Agriculture and Agri-Food Canada

AI: Artificial insemination

Annexin V-FITC: FITC-conjugated Annexin-V

ATP: Adenosine triphosphate

Bax: Bax channel blocker

BCF: Beat cross frequency

bp: Base pairs

BO Media: Brackett-Oliphant media

BrdU: Bromolated deoxyuridine triphosphate nucleotide

BSA: Bovine Serum Albumin

BSPs: Bovine seminal plasma proteins

°C: Celsius

Ca^{2+}: Calcium

CASA: Computer assisted sperm analysis

CO_2: Carbon dioxide

COC's: Cumulous oocyte complexes

CS: Newborn calf serum

DED: Death effector domain

DISC: Death inducing signaling complex

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DPBS: Dulbecco's phosphate buffered saline

ETOH: Ethanol

FADD: FAS associated death domain protein

FASL: FAS ligand

Fig.: Figure

FITC: Fluorescein isothiocyanate

FSH: Follicle stimulating hormone

g: Gravitational force

h: Hour

H_20: Water

$\Delta\Psi m$: mitochondrial membrane potential

ICSI: Intracytoplasmic sperm injection

LPI: Lifetime profit index

IVF: *In vitro* fertilization

IVC: *In vitro* culture

IVM: *In vitro* maturation

LH: Luteinizing hormone

N or n: Number

N_2: Nitrogen

nm: Nanometer

nM: Nanomolar

NRR: Non-return rate

NS: Non significant

mg: Milligrams

Min: Minutes

ml: Milliliter

mM: Millimolar

MtDR: Mitotracker Deep Red

mW:Milliwatt

PBS: Phosphate buffered saline

P: Probability

PFRA: Prairie Farm Rehabilitation Administration

PI: Propidium iodide

PKA: Protein kinase A

PNA: Peanut agglutination

PS: Phosphatidylserine

PTK: Protein tyrosine kinase

R123: Rhodamine 123

RT: Room temperature

ROS: Reactive oxygen species

s: Second

SCD: Sperm chromatin dispersion test

SCSA: Sperm Chromatin Structure Assay

SNP: Single nucleotide polymorphism

TNF: Tumor necrosis factor

TNF-R: Tumor necrosis factor receptor

TRAIL: TNF-related apoptosis inducing ligand

TUNEL: Tdt-mediated deoxyuridine triphosphate nick end-labeling

U of G: University of Guelph

U of S: University of Saskatchewan

v/v: volume per volume

VAP: Average path velocity

VCL: Curvilinear velocity

VSL: Straight line velocity

w/v: weight per volume

WCVM: Western College of Veterinary Medicine

x: times

XIAP: X-linked inhibitor of apoptosis protein

ZP: Zona pellucida

CHAPTER 1: INTRODUCTION

Accessibility to bovine semen is no longer dependent on geographical location or time, due to the success in cryopreservation. Frozen semen is used in a variety of reproductive technologies including artificial insemination (AI), embryo transfer and *in vitro* fertilization (IVF) (Medeiros et al., 2002) and for banking of germplasm to maintain the genetic diversity.

Conservation of germplasm is in the interest of the international community to preserve livestock species, to maintain food security and act as an insurance against global climate change, disease threats, social changes and other unforeseen disasters (Andrabi and Maxwell, 2007). Conservation programs have focused efforts on cryopreservation of gametes, embryos, somatic cells, and gonadal tissues.

In the current agricultural production system, AI is extensively used for genetic improvement of dairy herds and has significant applications in other species. Today approximately 90% of Holsteins in Canada are bred by AI (Van Doormaal, 2013). Cryopreservation has allowed the Canadian dairy industry to export its superior genetics, and thus increased the genetic potential of the modern dairy animal worldwide. These animals produce genetically superior offspring, with high milk yields, and position Canadian dairy genetics as the best in the world.

Due to increased pressure on the dairy industry for more milk, there is a need to increase the availability of genetically superior sires worldwide. These sires have been deemed superior based on their daughters' performance, in terms of their milk production and fertility level. Unfortunately, the freezing process decreases the fertility potential of semen, as compared to fresh semen in natural breeding or the use of chilled semen. When frozen-thawed semen is used, the pregnancy rate is suboptimal, only 50% of the inseminations result in a full term pregnancy (Fricke, 1999). Any cow, which remains open longer than 100 days, increases a producer's costs. There is a lower rate of genetic improvement in the producer's herd, and decreased number of offspring produced during the lifetime of a cow and bull.

By the time bulls have enough data from their daughters performance to be 'proven', they are several years old. This is because many daughters need to be produced and data collected from these daughters lactations. These bulls will have younger sons, who may be genetically superior to them, but due to the lack of sufficient data on their families, they cannot be extensively used for AI. Therefore, there is a need for an accurate assay to detect sperm in relation to the fertility of young dairy bulls. This relationship between sperm fitness and fertility will benefit both the AI companies and the dairy producers. Moreover, the AI companies will minimize the cost of nutrition and management of low fertility bulls. Dairy producers will have faster access to better genetics and there will be higher selection pressure on young bulls.

In the future, semen will remain the most important vehicle for dairy genetic transport and improvement, due to its low cost and ease of transport. Therefore, we need to improve aspects of semen preservation and analysis, to maintain its importance in the dairy industry (Boettcher, 2001). There will be a need for further development of cryopreservation, AI techniques and protocols. Several of these techniques and protocols may lead frozen semen straws having a lower sperm concentration, therefore more AI doses can be made from a single bull collection. This will benefit the AI companies by generating greater profits and dairy producers will have a greater semen supply from superior bulls. The addition of an apoptotic inhibitor to semen straws may help to improve the longevity of frozen-thawed semen. An increase in the post thaw longevity by several hours may increase the chances of a cow conceiving and reduce the amount of sperm required in each AI dose.

CHAPTER 2: LITERATURE REVIEW

I. Artificial Insemination

2.1 History

Artificial insemination (AI) was the first great biotechnological improvement applied to reproduction and genetic advancement of animals. The primary reason for AI in farm animals is to speed up the rate of genetic improvement, through the selection of superior sires, therefore increasing the productivity of food producing offspring (Ombelet and Robays, 2010). Artificial insemination has an enormous impact on many species worldwide, both domesticated and wild, and has particularly influenced the modern dairy breeds (Foote, 2002). There is a history of AI research spanning two centuries and its commercial application now spans over 75 years (Vishwanath, 2003). There are tales from hundreds of years ago where Arabs would obtain sperm from mated mares belonging to rival groups and use the collected sperm, to inseminate their own mares (Foote, 2002), representing the first use of assisted reproduction.

Leeuwenhoek and his assistant were the first to identify the sperm, they referred to the single cell as “animalcules”(Leeuwenhoek, 1678). It was through this discovery that one of the first factors of reproduction was uncovered. It would be a century later before the first successful artificial insemination was reported. This insemination was executed by Spallanzani in 1784, and was performed on a dog, which resulted in 3 pups (Foote, 2002; Zorgniotti, 1975).

The use of AI in humans was first documented in London, in the 1770's by John Hunter (Ombelet and Robays, 2010). In Russia, Ivanov lead the pioneering efforts to establish the practicality of AI during the 19th century. They studied AI in domestic farm animals, dogs, foxes, rabbits and poultry, which lead to the development of modern day AI methods. This work in Russia stimulated the organization of a cooperative dairy AI network in Denmark during the 1930's. Shortly after this, the first AI cooperatives in the United States were developed. After the opening of these two AI cooperatives, there was a rapid increase in numbers across North America (Ombelet and Robays, 2010).

During the 1930-1960's there were numerous advances to the processing and handling of semen. Most notable was the discovery that glycerol could act as a cryoprotectant (Polge et al., 1949). This allowed semen to be extended and stored for several days, ultimately leading to the freezing and long-term storage of spermatozoa that is commonly practiced today (Holt, 2000).

2.2 AI in Cattle

AI in cattle, especially in the dairy industry, has remained the main tool for the rapid dispersal of valuable genes and has been the choice method of dairy farmers to improve the genetic worth of their breeding stock (Vishwanath, 2003). Although the use of AI is much lower in beef breeds compared to dairy breeds, primarily due to differences in breeding and production practices. The benefits for the use of AI are similar for both

dairy and beef breeds. In both industries, reproductive efficiency is a crucial factor when considering economic viability for producers. Bulls currently go through rigorous progeny and genetic testing to prove their high merit. Therefore, there is greater knowledge available to producers about these valuable bulls. These bulls are also more accessible to the average producer as their semen can be readily purchased and easily used on farm. The use of AI has become a more precise procedure, with the recent improvements in estrus synchronization and fixed timed AI protocols, helping to ensure that the producer receives the best pregnancy rates possible from each insemination (Johnson, 2005).

II. Fertilization Processes

2.3 Sperm Transport

During natural mating, bovine spermatozoa are deposited in the vagina along with the seminal plasma. As the spermatozoa rapidly move through the cervix the seminal plasma is left behind. The seminal plasma functions as a nutritive and protective transport medium, and supply bovine seminal plasma proteins (BSPs), for the spermatozoa as they travel through the vagina to the cervix (Juyena and Stelletta, 2012). However, BSP's in the seminal plasma sequester the plasma membrane of sperm (Manjunath et al., 1994). The cervix along with its mucosal contents function as a type of sperm sorter, removing abnormal spermatozoa that cannot swim properly or that possess abnormal morphology. The central portion of the cervix contains thick mucous, which removes microbes and

abnormal cells. There are also deep grooves in the cervix, which contain less dense mucus than the central portion, and the normal fully motile sperm travel in these grooves into the uterine body (Suarez and Pacey, 2006).

When AI is performed, the seminal plasma has been diluted and the sperm suspended in one of several types of extenders. The sperm are directly deposited into the body of the uterus, therefore bypassing the benefits of passing through the cervix (Suarez, 2007). Sperm move rapidly through the uterus towards the site of fertilization. Sperm transport through the uterus is probably aided by pro-ovarian contractions of the myometrium (Suarez and Pacey, 2006). Before the sperm reach the site of fertilization they must pass through the uterotubal junction, which presents as another physical barrier for the elimination of immotile or damaged spermatozoa, as well as foreign particles. Once through this narrow junction they become trapped within the first segment of the oviductal isthmus, forming a 'sperm reservoir'. This is due to the binding of sperm heads to the epithelial lining of the lumen. This is through the interaction of BSP, PDC-109, on the acrosome region of the sperm head, with the fructose containing ligands on the surface of the cilia on the oviductal epithelium (Hung and Suarez, 2012; Suarez, 2007).

2.4 Capacitation

Sperm gradually undergo the process of capacitation, which is characterized by changes to the plasma membrane, gaining the ability to bind to the oocyte and the zona pellucida (ZP) and hyperactivation of the flagella (Burkitt et al., 2012; Guidobaldi et al., 2012; Rodriguez-Martinez, 2007; Suarez and Pacey, 2006). Once capacitated, the sperm are

now capable of binding to the ZP, which then stimulates the acrosome reaction (Rodriguez-Martinez, 2007). The exact molecular mechanisms of the capacitation process are still unclear, but from many *in vitro* experiments numerous factors have been identified (Okabe, 2013). Capacitation is controlled by both intrinsic and extrinsic factors, with the major extrinsic factors commonly being sterol acceptors (Bovine serum albumin (BSA), bicarbonate and calcium). The intrinsic factors include, but are not limited to, pathways that are activated by the previously mentioned molecules, protein kinase A (PKA) and protein tyrosine kinase (PTK) pathways, these kinases cause protein phosphorylation (Harrison, 2004; Visconti et al., 1995a; Visconti et al., 1995b). Changes to the sperm membrane and hyperactivation cause the sperm to be released from the sperm reservoir, the sperm then travel to the site of the oocyte in the oviduct, here they bind with the oocyte mass and begin penetrating the cumulous cell layers.

2.5 Acrosome Reaction

Once the spermatozoa penetrate the remaining cumulous cells and reach the ZP they bind to it via ZP binding proteins and undergo the acrosome reaction, allowing the fertilization process to begin (Lin et al., 2007; Rodriguez-Martinez, 2007). The acrosome is a subcellular organelle at the tip of the sperm head, and contains many hydrolytic enzymes and ZP binding proteins. When the ZP binding is initiated, it causes a rise in cytosolic levels of calcium (Ca^{2+}), this rise in Ca^{2+} levels in the sperm easily triggers the acrosome reaction(Gadella and Luna, 2014). During the reaction the plasma membrane and the outer acrosome membrane fuse, with the involvement of phospholipase C and SNARE

proteins and the acrosomal contents are released (Okabe, 2013). The release of the lytic enzymes allows the sperm to penetrate the ZP; this is also aided by the capacitated sperm with hyperactive motility, which provides mechanical assistance, helping push the sperm through the ZP.

2.6 Fertilization

Once the spermatozoa has completely penetrated the ZP and reaches the perivitelline space, the plasma membrane of the oocyte fuses with the plasma membrane remnants on the sperm head, via IZUMO1 proteins on the sperm and CD9 proteins on the oocyte and the sperm is engulfed (Okabe, 2013). The oocyte also releases cortical granules, causing a zona block, which prevents other spermatozoa from penetrating the ZP. The release of cortical granules is stimulated by a calcium signaling pathway, which is induced by the fusion of the sperm plasma membrane with the oolemma (Liu, 2011). The sperm nucleus must now undergo removal of the strong disulfide bonds, causing the DNA to decondense, allowing for the pairing up with the chromosomes of the female pronucleus (Gadella and Luna, 2014; Okabe, 2013; Suarez, 2007; Tulsiani and Abou-Haila, 2012). Once this stage is complete fertilization has occurred.

III. Semen Assays

The main goal of semen analysis is to determine the quality of an ejaculate (normal motility, morphology and concentration), but it also has an underlying goal of providing

insight to a bull's fertility potential. A routine semen analysis begins with a physical assessment of the bull where testicular size and shape are evaluated, as well as his body condition and physical stature. The ejaculate undergoes a summary assessment in which, volume, concentration and sperm morphology are evaluated. This summary assessment is a good tool in the detection of major infertility cases. A more fine detailed assessment is needed to expose bulls with other subtle defects, not detected by a simple evaluation. Fertility potential depends on multiple parameters that require multi-parametric analysis, including evaluation of morphology, motility, membrane status, acrosome reaction and genome integrity, to provide a fuller picture of a bull's potential fertility (Bissonnette et al., 2009).

2.7 Motility

Motility is one of the main factors affecting the fertilizing potential of spermatozoa. Spermatozoa must be motile to pass through the complex female reproductive tract and end their journey in the fertilization of the oocyte (Gillan et al., 2008). Motility is the primary technique when wanting to determine the viability of an ejaculate. Motility can be evaluated by methods ranging from crude to highly accurate; methodologies include visual assessments to complex computer software and microscopes, respectively. Visual assessment is the quickest and simplest to perform. A practitioner can easily conduct the assessment in the field, as simple and inexpensive equipment is required. A drop of semen is placed on a slide and the amount of swirling seen in the drop is noted, giving a rough estimate of the actual motility. The complex computer software and microscope

systems seen today are referred to as CASA systems (Computer Assisted Sperm Analysis). These systems were pioneered over 40 years ago, by a group of individuals who wanted to develop an automated and accurate measurement of motion of individual sperm in a population. Allowing for the calculation of velocities and proportion of sperm exhibiting various motility parameters. Common sperm motion parameters evaluated include, curvilinear velocity (VCL) (velocity along a true trajectory), average path velocity (VAP) (velocity along an average path), straight line velocity (VSL) (first to last position of a sperm head), and beat-cross frequency (BCF) (points where the curvilinear and average path intersect) (Fig. 2.1) (Amann and Waberski, 2014). These systems also remove the bias seen in manual assessments. CASA allows for repeatability in assessment of many sperm motility parameters. There have been significant correlations found between many sperm motion parameters and *in vivo* fertility in several species. Subjectively assessed post-thaw motility correlates strongly with fertility as shown by several authors (Gillan et al., 2008; Januskauskas et al., 2003). CASA assessed motility had a significant positive correlation with bull fertility ($P<0.05$) (Januskauskas et al., 2003). Straight-line velocity had a strong positive correlation with the fertilizing capability of the spermatozoa ($P<0.05$) (Gillan et al., 2008; Oliveira et al., 2013). Motility analysis offers an insight into the *in vivo* fertility of a bull but should not be the only parameter evaluated when examining bull fertility.

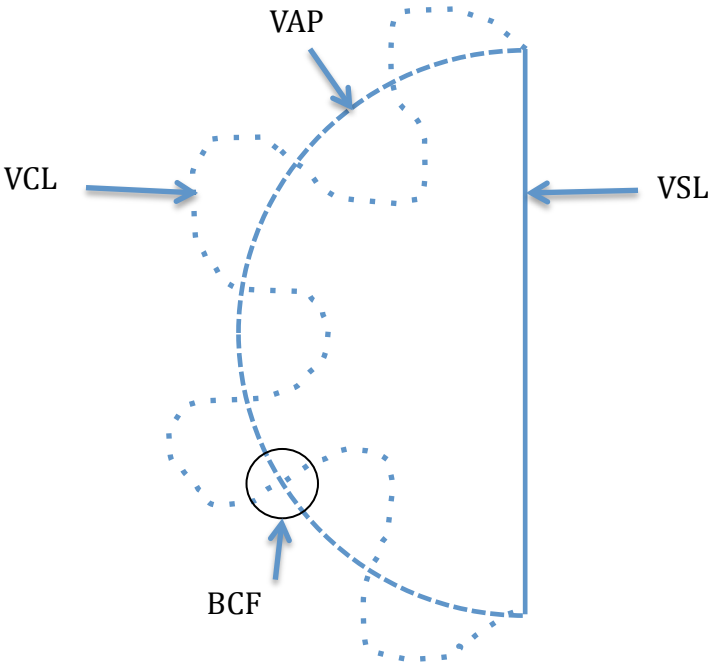


Figure 2.1. Illustration of CASA terminology. CASA determines the change in sperm location in successive time frames. Curvilinear path determines a sperms actual trajectory and curvilinear velocity is calculated from this path (VCL, μm/s). Average path is determined from the average of the curvilinear path and velocity along this trajectory is termed average path velocity (VAP, μm/s). Straight-line path is from the first and last sperm head position, velocity along this path is termed straight-line velocity (VSL, μm/s). Where the points of the curvilinear path and average path cross is termed beat-cross frequency (BCF, number per second). (Adapted from (Amann and Waberski, 2014)

2.8 Morphology

Parameters that should be evaluated include; physical shape of the spermatozoa, plasma membrane structure, acrosome integrity, DNA fragmentation and chromatin structure, as well as mitochondrial structure and function. A breeding soundness evaluation limits sperm head or nuclear defects at 15-20% and acrosomal or tail defects at 25%, and sets a minimum of 70% for normal sperm (Barth, 2000). Correlations have been found between several sperm parameters and fertility, therefore enhancing the need for analysis (Gillan et al., 2008; Hossain et al., 2011; Nagy et al., 2013). Sperm morphology has been shown to have a significant positive correlation with fertility (Gillan et al., 2008; Nagy et al., 2013; Oliveira et al., 2013) ($P = 0.010$, $P<0.05$, PLS regression <0.8, respectively).

2.8.1 Staining of Membranes

The sperm plasma membrane has several important roles; therefore its integrity is of utmost importance. These roles include acting as a cell boundary, enabling the maintenance of homeostasis and motility, a signaling surface, and cell to cell interactions. Important cell to cell interactions such as between the spermatozoa and the female reproductive tract and the oocyte and its vestments (Rodriguez-Martinez, 2003). The plasma membrane has several distinct regions, one that covers the acrosome membrane, one that covers the post acrosomal region down to the annulus and the last portion covering the tail and principal piece (Hossain et al., 2011). Using the modern fluorescent staining protocols damage to the plasma membrane and sperm viability can be rapidly

detected. Stains can be used in combination, to evaluate multiple sperm populations simultaneously. The premise of these stains is that sperm with intact and fully functional plasma membranes will exclude the stain, therefore remain unstained and are considered to be alive. Spermatozoa that allow the passage of the stains into the cell have damaged or disrupted membranes and are considered dead or undergoing apoptosis-like changes (Anzar et al., 2002; Silva and Gadella, 2006). Commonly used stains include; SYBR-14/Propidium iodide (PI), YO-PRO-1/PI and Annexin V-FITC/PI; these stains group cells into distinct populations, live sperm, dead sperm and apoptotic sperm. SYBR-14 is a membrane permeable stain, which binds to the nuclei of cells with intact membranes that stains cells with intact plasma membranes green (Garner and Johnson, 1995). YO-PRO-1 is a membrane impermeable stain that can detect early membrane destabilization. Annexin-V is a stain that binds to externalized phosphatidylserine (PS) and indicates a loss of membrane intactness. PI is a vital stain and stains cells with damaged plasma membranes. Annexin V/PI stains have been shown to be the most accurate of the assays mentioned, in detecting plasma membrane damage and viable cell populations. (Anzar et al., 2002; Farah et al., 2013; Hossain et al., 2011).

There have been confounding results between the relationship of membrane integrity and fertility. Several studies have found significant positive relationships between sperm with intact plasma membranes and fertility. One study conducted by Anzar (2002) and his team, found distinct populations of apoptotic, necrotic and live cells using the Annexin V/PI assay. Apoptotic sperm have PS exposed on their plasma membrane and necrotic cells have damaged and leaky plasma membranes. The relationship between fertility,

necrotic sperm and the number of viable sperm were statistically significant ($P<0.05$). A significant negative correlation of Annexin V-negative cells, to bull fertility was demonstrated ($r = -0.40$, $P<0.05$) (Januskauskas et al., 2003). Interestingly, no significant correlation between any membrane integrity parameters and field fertility in boars ($P<0.05$) was observed (Broekhuijse et al., 2012).

2.8.2 Staining of the Acrosome

As previously mentioned, an intact acrosome is an essential component to initiate the fertilization process. Any damages to or removal of the acrosome prior to the spermatozoon reaching the oocyte will render that spermatozoon infertile (Rodriguez-Martinez, 2007). Eosin-Nigrosin a non-fluorescent stain has been used in the past to assess the integrity of the acrosome (Barth, 2000). Today, fluorescent conjugated lectins are commonly used along with flow cytometry to evaluate the acrosome. Currently used stains include, but are not limited to: fluorescein isothiocyanate (FITC)-PNA, FITC-PSA, and FITC-ConA (Farah et al., 2013; Silva and Gadella, 2006). The lectin conjugates bind to the carbohydrate moieties of glycoproteins which are only located on the inner surface of the acrosome, thus only bind when the acrosome is reacted or damaged (Silva and Gadella, 2006). This is also used in combination with PI, acting as a counter stain to differentiate live and dead sperm populations. Several authors have not found a significant correlation between acrosome integrity and field fertility, in bulls and boars (Broekhuijse et al., 2012; Gillan et al., 2008).

2.8.3 DNA Integrity

Integrity of the sperm DNA is of outmost importance for the development of a fertilized oocyte. Slight DNA damage in spermatozoa can hinder embryo development and in severe cases lead to male infertility (Simoes et al., 2013). Several assays are used in assessing the degree of DNA fragmentation or chromatin integrity, currently the most commonly used assays include; sperm chromatin structure assay (SCSA), sperm chromatin dispersion (SCD) test, Comet assay, and terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) (Anzar et al., 2002; Farah et al., 2013; Hossain et al., 2011; Silva and Gadella, 2006).

The premise of the SCSA assay is based on knowledge that chromatin with DNA strand breaks have a tendency to denature when exposed to acid-detergents, where normal stable chromatin remains intact. This assay is performed using acridine orange dye. This dye differentially stains single and double stranded DNA, the single stranded DNA emits red fluorescence (650 nm) and the double stranded DNA emits green fluorescence (526 nm) (Evenson and Jost, 2000). SCSA has been significantly negatively correlated to percentage of normal sperm cells, a reported predictor of fertility ($r = -0.53$) (Fortes et al., 2012; Giwercman et al., 2010).

The SCD test is a novel assay for sperm DNA fragmentation in semen. This assay is similar to the SCSA assay in that the sperm cells are exposed to an acid solution to denature DNA containing fragmentation. After this step, the membranes and proteins are removed. This results in nucleoids expressed in a central core with a peripheral halo of

DNA loops. The assay is based on the principle that sperm with fragmented DNA fail to produce a characteristic halo, this halo is produced by the DNA loops which are present only in non-fragmented DNA. Recent data indicates that this assay is significantly correlated to pregnancy rates in humans ($P<0.05$) (Fernandez et al., 2005).

Comet assay allows for the analysis of single cells and involves electrophoresis and fluorescent stains. The assay is based on the principle that when DNA breaks are present in sperm the DNA supercoils become loose and negative charges are exposed. When these sperm cells are placed in an electrophoresis gel and exposed to an electric field, the DNA fragments will move out of the nucleus toward the positive gradient. This creates a comet like tail when exposed to fluorescent stains and evaluated by fluorescent microscopy. The comet assay is a very useful technique in assessing DNA damage, but the assay can underestimates the true frequency of DNA breaks, so therefore may not be a reliable assay to help in the prediction of fertility (Shamsi et al., 2010).

TUNEL assay identifies DNA strand breaks by labeling them with fluorescently tagged nucleotides. The strand breaks expose the 3'-OH end of the DNA fragments, this is the position where the nucleotides bind and can be detected by fluorescent microscopy or flow cytometry (Anzar et al., 2002; Farah et al., 2013; Gorczyca et al., 1993b). TUNEL can detect single and double strand breaks simultaneously, but a downside is its ability to only reveal total cells in a population exhibiting DNA damage, not the amount of DNA damage each cell has (Hossain et al., 2011). The DNA strand breaks identified by TUNEL are created by endogenous endonucleases, present in the sperm and are

characteristic of DNA changes seen in apoptotic somatic cells. This assay is specific to apoptotic cells as it allows for the discrimination of DNA strand breaks seen in apoptotic cells from necrotic cells (or the primary DNA stand breaks) (Gorczyca et al., 1993b). These breaks are differentiated through differing degrees of labeling by the cells. Indicating that DNA strand breaks at the 3'-OH end are more prevalent in cells that die through apoptotic like pathways and less prevalent in cells that die through the necrosis pathways (Gorczyca et al., 1993a). Several studies have been done in which a significant correlation has been found between the TUNEL assay and fertility and include several species (Anzar et al., 2002; Gillan et al., 2005; Ribas-Maynou et al., 2013; Waterhouse et al., 2006).

2.8.4 Staining of Mitochondria

Mitochondria activity is a key indicator of sperm function. It is the main powerhouse for generation of adenosine triphosphate (ATP), which is required for numerous sperm functions, most notability sperm motility (O'Connell et al., 2002). As well the mitochondria are considered to be the coordinators of apoptosis-like mechanisms in various cell systems (Rasola and Bernardi, 2007). There are several fluorescent stains commonly used to detect mitochondrial functionality rhodamine 123 (R123) and MitoTracker Green and MitoTracker Deep Red. These are often used in combination with PI. R123, MitoTracker Green and Deep Red stains function in very similar methods. They accumulate in functional mitochondria as a function of transmembrane potential and the intensity of fluorescence given an indication of the number of mitochondria that

are actively respiring (Gillan et al., 2005; Hossain et al., 2011). The following research indicates that there are discrepancies between studies determining the correlation of mitochondria function to fertility. A review by Amaral et al. clearly states that there are obvious correlations between human mitochondrial functionality and sperm functionality and fertilization ability (Gallon et al., 2006; Marchetti et al., 2004; Marchetti et al., 2002; Paoli et al., 2011; Troiano et al., 1998; Wang et al., 2012). In contrast, a study done by Broekhuijse and their lab, concluded that there was no significant relationship between the functionality of mitochondria and fertility in boars (Broekhuijse et al., 2012).

IV. Apoptosis

Apoptosis is one of the major types of cell death; it is a physiologically programed cell death that affects only single cells, without any negative effect on the surrounding cells or tissues (Kerr et al., 1972). Apoptotic cells undergo specific changes involving their structural and biochemical composition. Changes seen in sperm include; chromatin aggregation, cytoplasmic condensation exposure of PS on the outer plasma membrane, DNA degradation and indentation of nuclear and cytoplasmic membranes (Anzar et al., 2002; Kerr et al., 1972; Marti et al., 2008). Apoptosis is an important feature of spermatozoa development. It is a process that organizes the production and function of these specialized cells from the early stages of generation to the final stage of fertilization. Apoptosis regulates the germ cell:Sertoli cell ratio, in early development and removes damaged cells from the sperm population inside the testis and in the ejaculate, thereby protecting the genome of a species (Aitken and Baker, 2013). There are many

biological situations that can induce apoptosis in a cell including; cryopreservation, heat exposure, radiation, hydrogen peroxide, genetic perturbances, and endocrine disruptions (Aitken and Baker, 2013; Aitken and Koppers, 2011; Anzar et al., 2002).

Two major pathways for the initiation of apoptosis have been identified, stimuli originating from the outside of the cell (extrinsic pathway), or from inside the cell itself (intrinsic pathway) (Marti et al., 2008). The extrinsic pathway is activated by tumor necrosis factor family receptors (TNF-R) and is triggered by various pro-apoptotic ligands such as TNF, TNF-related apoptosis inducing ligand (TRAIL) and FASL binding to the respective receptors. These ligands are produced by cells of the immune system, and the receptors are located on the outer surface of cell membranes (Aitken et al., 2011). These receptors have an intracellular component of a small number of amino acids, called a death domain. FAS associated death domain (FADD) is the key transducer of the apoptotic signal by all receptors in the TNF family, TNF-R, TRAIL, and FAS (Vilmont et al., 2012). The FADD contains a death effector domain (DED) to which the pro-caspase 8 or 10 can bind. This complex of FAS, FASL, FADD and pro-caspase 8 or 10 is called death-inducing signaling complex (DISC), this complex can then initiate the cleavage of the initiator caspases. These active initiator caspases are then free to cleave pro-caspase 3 or other executioner caspases, leading to the apoptosis cascade (Fig. 2.2) (Aitken et al., 2011; Vilmont et al., 2012; Wajant, 2002).

The intrinsic apoptotic pathway is a pathway triggered by factors such, as reactive oxygen species (ROS), disruption of the extracellular matrix, radiation, DNA damage or

lack of pro-survival factors (Aitken et al., 2011). This pathway is primarily regulated by the mitochondria. There are several genes and molecules that are responsible for the initiation of this mode of apoptosis, BAX, BAK, p53, Bcl-2, and so on (Dogan et al., 2013). Activation of BAX or BAK proteins triggers a release of cytochrome c and other apoptogenic factors from the mitochondria. This results in the formation of an apoptosome, this complex facilitates the cleavage and activation of caspases, particularly initiator caspase 9. Caspase 9 subsequently activates effector caspases such as caspase 3 and 7, resulting in apoptotic death (Fig. 2.2).

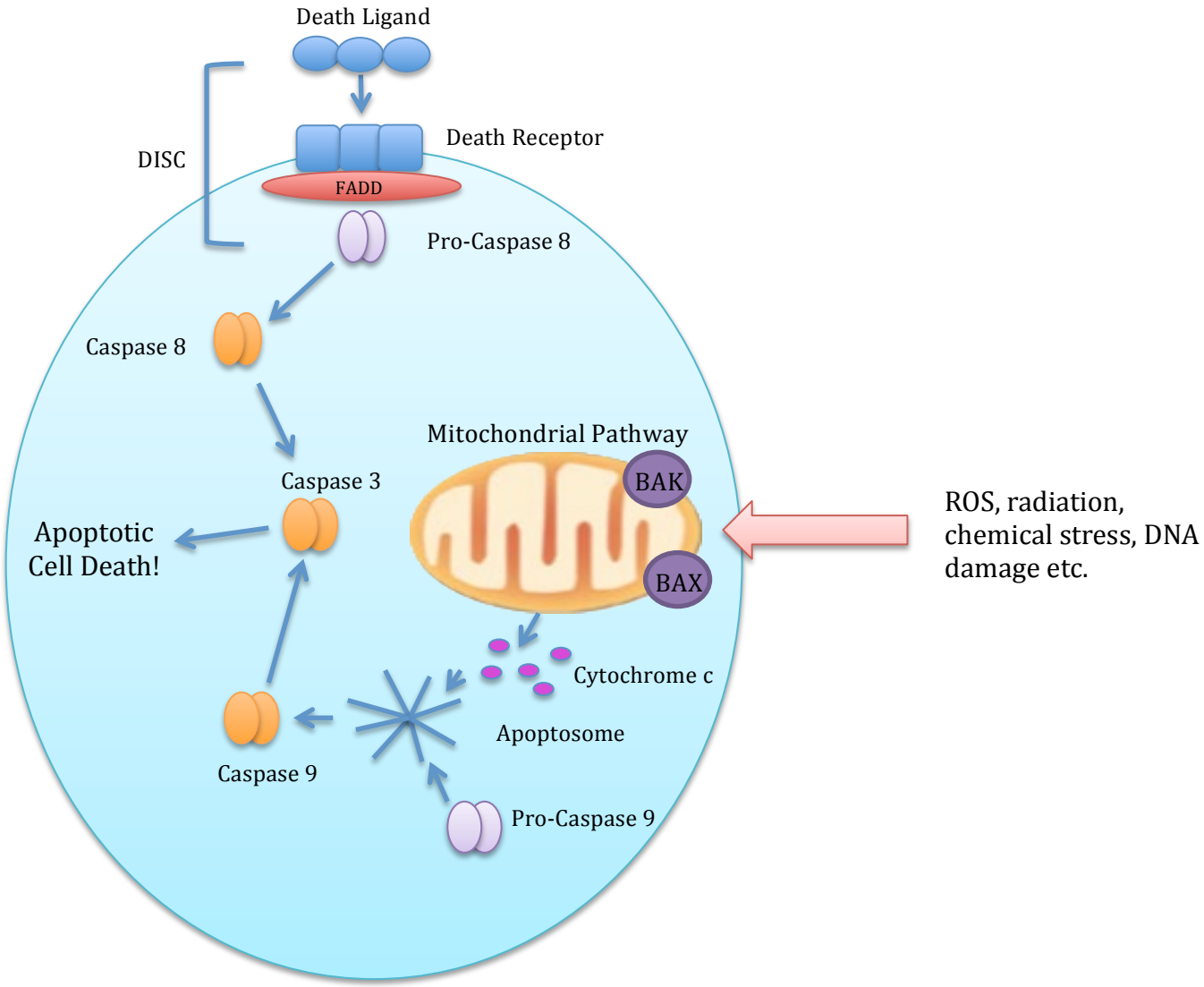


Figure 2.2. Simplified intrinsic and extrinsic pathways of apoptosis. Stresses to the cell cause the activation of the intrinsic pathway, which causes the mitochondria to release cytochrome c causing activation of caspases. Extracellular ligand binding to death receptors triggers the receptor-mediated pathway, which can cause activation of caspases without mitochondrial involvement. Both pathways lead to cellular death by apoptosis. Adapted from (Koul et al., 2004).

Mitochondria and external cells such as leukocytes can produce ROS (Oborna et al., 2009). Over production of ROS is commonly seen in defective spermatozoa. Oxidative stress in spermatozoa arises when the ROS production is in excess of the cells limited antioxidant capability. This is capable of inducing DNA damage and quite possibly leading to the activation of caspases and the apoptotic cascade ((Aitken and Koppers, 2011). Aitken and Koopers (2011) concluded that the activation of the apoptotic cascade in oxidatively stressed spermatozoa is an efficient method of removal of DNA damaged sperm from the germ line and the female tract without causing an inflammatory response, thereby preventing damaged cells form participating in the fertilization process.

Once the apoptosis process has progressed at a certain point, the two pathways (extrinsic and intrinsic) begin to show similarities. Motility is suppressed and spermatozoa begin to express the characteristic signs of apoptosis, such as PS exposure on the outer surface of the plasma membrane and caspase activation. The PS is translocated to the outer membranes as the caspases have inactivated the flippase, which normally maintains the PS on the inner leaflet of the plasma membrane. The caspase activation will lead to the activation of the endonucleases, either in the mitochondrial inner-membrane space or cytosol of the mitochondria. Activated endonucleases would then be translocated into the nucleus, where they would elicit DNA damage (Aitken and Baker, 2013). The externalization of PS can be detected by the Annexin-V assay and the DNA damage detected by TUNEL assay (Anzar et al., 2002; Dogan et al., 2013).

Apoptosis is a complex process that can be divided into three phases: induction, execution and degradation. The events occurring in these phases differ depending on the apoptotic path activated, extrinsic or intrinsic. Ultimately both phases result in the activation of caspases, externalization of PS and DNA fragmentation (Martin et al., 2004). In the degradation phase the cell blebs into apoptotic bodies and the nucleus undergoes fragmentation. This cellular debris is phagocytosed by neighboring cells or extruded into intracellular spaces (Anzar et al., 2002).

2.9 Fertilization and Embryo Development

Apoptosis is a very important process to ensure that only the healthiest sperm with intact DNA reach the oocyte and are able to complete fertilization. If there is a failure to remove the damaged cells from the testis or from the semen once in the female tract, there may be a decrease in the fertility of the semen or it may result in a plethora of early embryo development problems. Therefore bulls with normal cells and intact DNA have a much greater chance of producing viable offspring (Walters et al., 2005). Significant, inverse relationships between the level of apoptosis in bull spermatozoa and the proportion of fertilized oocytes developing into the blastocyst stage have been observed (Chaveiro et al., 2009). Also differences in the quality of the blastocysts produced was observed, assessed by the hatched blastocyst rates (Chaveiro et al., 2009).

CHAPTER 3: HYPOTHESES AND OBJECTIVES

Thesis Hypothesis:
Apoptosis-like changes occurring in fresh bull sperm have negative effects on bull fertility.

Hypothesis 1:

Apoptotic-like sperm present in fresh bull ejaculates have negative effect on the fertility potential of a breeding bull and on subsequent embryo formation.

Objective 1:

To confirm the relationship of apoptosis-associated membrane and nuclear changes in bull sperm with fertility on a large scale, and to evaluate the effect of DNA-nicked sperm on cleavage rate and blastocyst formation *in vitro*.

Hypothesis 2:

The addition of an apoptosis inhibitor in the extender will improve the longevity of frozen-thawed sperm; thus improving the availability of viable sperm to oocytes for fertilization.

Objective 2:

To study the addition of an apoptosis inhibitor in semen extender on post-thaw sperm longevity and fitness.

CHAPTER 4: APOPTOSIS-LIKE CHANGES IN BULL SPERM AND THEIR EFFECTS ON FERTILITY POTENTIAL OF BREEDING BULLS: *IN VIVO* AND *IN VITRO* MODELS

4.1 Abstract

Sperm plasma membrane asymmetry and intact DNA are essential attributes of a sperm to fertilize an oocyte and support early embryo development. The objectives of this study were to evaluate the relationship of apoptosis-like membrane changes and DNA intactness in sperm with the fertility of dairy bulls used for artificial insemination, to predict the fertility of beef bulls used for natural mating and to evaluate the effect of sperm with nicked DNA on cleavage and blastocyst rates in IVF produced embryos. Three experiments were conducted in this study. In Experiment 1, phosphatidylserine (PS) translocation, from the inner to the outer plasma membrane, and DNA nicks in the sperm from 50 dairy bulls were determined using Annexin-V/PI and TUNEL assays, respectively. Relationships between the parameters of the assays and the known fertility levels of the bulls were calculated. In Experiment 2, fertility levels of 15 beef bulls used for natural mating were estimated using a regression model of DNA nicks developed in Experiment 1. In Experiment 3, the effect of DNA-nicked sperm on cleavage and blastocyst rates were evaluated in *in vitro* produced embryos, using two sperm concentrations (30,000 and 300,000 sperm per IVF droplet) to fertilize *in vitro* matured oocytes. In Experiment 1, there were significant relationships of fertility with live sperm ($P<0.05$) and necrotic sperm ($P<0.01$) (Annexin-V/PI assay), and with DNA-nicked sperm ($P<0.001$) (TUNEL assay). In Experiment 2, the fertility level of bulls used for natural breeding was estimated and it ranged from -7.3 to 2.4. In Experiment 3, the

cleavage rate was significantly affected by the number of sperm with nicked DNA, regardless of sperm concentration. At the low sperm concentration, blastocyst rate was significantly lower when higher DNA nicked sperm were used (51% vs 32%; high vs low DNA nicks) ($P<0.05$). Blastocyst rate was non significant at the higher sperm concentration regardless of DNA nicks. In conclusion, the incidence of DNA-nicked sperm in semen is a useful marker to estimate the fertility potential of a breeding bull. The incidence of DNA nicked sperm has a negative effect on cleavage rates and subsequent early embryo development.

4.2 Introduction

In Canada, approximately 90% of dairy cows are bred by artificial insemination (AI) using frozen semen from genetically superior bulls (Van Doormaal, 2013). There is considerable bull to bull variation in fertility (Dogan et al., 2013). This variation leads to financial losses due to the cost involved in raising young bulls with poor fertility; as well as a reduction in genetic advancement, for producers and AI centers.

In vivo fertility testing is a time-consuming and costly procedure, as hundreds of inseminations are required. Andrologists and reproductive physiologists are direly looking for methods to predict fertility *in vitro.* Conventional *in vitro* evaluation of semen quality such as the assessment of concentration, motility and morphology have limited value when assessing field fertility (Dogan et al., 2013; Gillan et al., 2008; Rodriguez-Martinez, 2006). Thus it demands the need for a rapid, reliable and economical fertility assay.

Apoptosis naturally removes damaged or unnecessary cells and contributes to the maintenance of tissue homeostasis (Aitken and Baker, 2013; Aitken et al., 2011; Vaux and Korsmeyer, 1999). Approximately 25-75% of potential spermatozoa degenerate and die in adult mammalian testis following intrinsic or extrinsic pathway of apoptosis (Aitken et al., 2011). The failure to remove apoptotic sperm by the Sertoli cells, results in their release into the lumen of the seminiferous tubules, and causes an increase in abnormal sperm in an ejaculate (Yin et al., 1998).

Male infertility has become a major area of research in the last two decades. There still exists a knowledge gap between the basis of infertility mechanisms and their relationship to sperm DNA damage and apoptosis (Dogan et al., 2013). Several studies have found negative influences on the fertility of a bull with increased levels of spermatozoa exhibiting apoptosis-like changes (Anzar et al., 2002; Martin et al., 2007; Martin et al., 2004). It has been shown, that levels of apoptosis in bull sperm have a direct and negative effect on the proportion of fertilized oocytes developing into blastocysts (Chaveiro et al., 2009).

The objectives of this study were: 1) to evaluate the relationship of apoptosis-associated membrane and nuclear changes in bull sperm with fertility on a large scale, 2) to predict the fertility level of bulls used for natural breeding and 3) to evaluate the effect of DNA-nicked sperm on cleavage rate and blastocyst formation *in vitro.* In this study, it was hypothesized that a significant level of apoptotic-like sperm present in fresh bull

ejaculates have a negative effect on the fertility potential of a breeding bull and on subsequent early embryo development.

4.3 Materials and methods

4.3.1 Chemicals and Reagents

Dulbecco's phosphate buffered saline (DPBS), newborn calf serum (CS), TCM-199 and MEM non-essential amino acids were purchased from Life Technologies Inc. (Burlington, ON, Canada). Lutropin (LH) and folltropin (FSH) were supplied by Bioniche Animal Health, Inc (Belleville, ON, Canada). All other chemicals and reagents were purchased from Sigma- Aldrich (Oakville, ON, Canada), unless otherwise stated.

4.3.2 Experiment 1: Relationship between sperm apoptosis-like changes and fertility of breeding bulls

4.3.2.1 Bull Semen

Semen was collected from 50 bulls, stationed at EastGen, Guelph Ontario, during the months of November –December 2011 for 3 consecutive weeks. On each day of the experiment, a single ejaculate was collected from10 bulls, with a prewarmed artificial vagina. Samples were initially evaluated for visual motility, volume and concentration as per routine and were transported to University of Guelph (U of G), in a 37°C incubator and processed within an hour for further analysis. Each semen sample was divided into

two aliquots, one for detection of externalization of phosphatidylserine from the inner to the outer leaflet of the sperm plasma membrane using the Annexin V/PI assay, and the other for detection of sperm with DNA nicks using TUNEL assay. Annexin V/PI assay was conducted at U of G. For TUNEL assay, sperm were initially prepared at U of G as described below and transported to the Western College of Veterinary Medicine, (WCVM) University of Saskatchewan (U of S), Saskatoon, for the final analysis.

4.3.2.2.Annexin V/PI Assay

Translocation of PS from the inner to the outer leaflet of the plasma membrane in fresh sperm was detected by Annexin V Apoptosis Detection Kit II (BD Pharmingen Canada, Mississauga, ON), as recommended by the manufacturer with slight modifications (Anzar et al., 2002). Initially, sperm were diluted to $50x10^6$/ml in warm (37°C) phosphate buffered saline (PBS). Aliquots of $2x10^6$ sperm were further adjusted to $4x10^6$ sperm/ml with Annexin V Binding Buffer (0.01 M Hepes/NaOH [pH 7.4], 0.14 M NaCl, 2.5 mM $CaCl_2$)). Aliquots of $2x10^5$ cells were transferred to flow tubes. Sperm staining was completed as suggested by the manufacturer. Additional, Annexin V Binding Buffer was added to each tube bringing the final volume to 1 ml. Flow cytometric analysis was conducted within 15 min.

Sperm membrane asymmetry was analyzed with a FACSCalibur flow cytometer (BD Biosciences, Mississauga, Canada), located at U of G. Both, FITC and PI were excited with a 488 nm laser and their emission spectra were detected by photomultiplier detectors FL1 (350 voltage gain) and FL3 (425 voltage gain). The sperm population was located

using the forward and side scatters. A gating region was drawn around the sperm population. Data were collected using BD CellQuest Pro software (Version 4.0.4, 2002). Samples were run through a medium speed (75-125 events/s) and 5000 sperm were analyzed on a log scale. The typical 2-dimensional flow cytometric histograms of Annexin V/PI are presented in Appendix A.1.

4.3.2.3.TUNEL Assay

DNA nicks in fresh sperm were detected with APO-BRDU Kit (Millipore Bioscience Research Reagents, Temecula, CA), as recommended by the manufacturer with slight modifications. Fresh semen was initially diluted to $50x10^6$/ml in warmed PBS. The aliquot, of each semen mixture was further diluted in PBS to a final concentration of $4x10^6$ cells/ml. Sperm were then washed at 600x g for 10 min at RT. The supernatant was removed and the pellet was resuspended in paraformaldehyde (1% w/v). The samples were kept on ice for 30 min. Sperm were washed again at 600x g for 10 min at RT to remove the paraformaldehyde. The sperm pellet was resuspended in ice-cold methanol (100%). All samples were stored at -20°C until carried to WCVM, U of S, via airplane, on dry ice. Samples stayed on dry ice for approximately 6 h. At WCVM, samples were stored at -20°C until analysis. On the day of analysis, aliquots (approximately $1.5x10^6$ cells per sample) were transferred to flow tubes and methanol was removed by centrifugation (800x g for 15 min at RT). The sperm suspension was washed (1x) in Wash Buffer (APO-BRDU Kit) at 800x g for 15 min. The pellet was resuspended in freshly prepared DNA-labeling solution (APO-BRDU Kit) as suggested

by the manufacturer. The sperm suspensions were incubated at 37°C in the dark for 2 h. During the incubation, the sperm suspensions were gently mixed at 30 min intervals. After incubation, the sperm suspension was washed (800x g for 15 min at RT) in Rinse Buffer (APO-BRDU Kit). The pellet was resuspended in antibody solution as recommended by the manufacturer. The sperm suspensions were incubated at RT in the dark for 30 min. After incubation, PI/RNase A Solution (APO-BRDU Kit) was added. Flow cytometric analysis was conducted within 15 min.

Sperm DNA nicks were analyzed by CyFlow ® Space (Partec GmbH, Münster, Germany), located at WCVM, Saskatoon. A blue diode solid-state laser at 488 nm (200 mW) was used to excite both FITC and PI, their emission spectra were detected by photo-multiplier detectors at FL-1 and FL-3, respectively. The typical 1-dimensional flow cytometric histograms of TUNEL assay are presented in Appendix A.2.

The sperm population was located using the forward and side scatters. A gating region was drawn around the sperm specific events to isolate them from the background noise. Data were collected using FlowMax® software (version 2.6) for cytometry supplied by Partec GmbH. Simultaneous fluorescent data were collected from FITC and PI, from 10,000 sperm events on a log scale. All samples were passed through the flow cytometer at speed 1.3 µl/s. Flow cytometric results of both Annexin V/PI and TUNEL assays were also verified under fluorescent microscope (data not presented).

4.3.2.4 Bull Fertility Data

The fertility data were obtained from the Canadian Dairy Network (Guelph, ON, Canada). Bull fertility was based on 1st service insemination, expressed in non-return rate (56-day adjusted) and fertility deviation from population average. Non-return rate ranged from 61-70% and fertility deviation +3.9 to -5.2. In this study, the fertility deviation data were used to determine the relationships of fertility with apoptosis-like membrane and DNA changes in bull sperm.

4.3.3 Experiment 2: Estimation of fertility in beef bulls used for natural mating

Semen was collected from 60 beef bulls, stationed at Agriculture and Agri-Food Canada (AAFC) Community Pasture Program (Maple Creek, Saskatchewan), during a breeding soundness evaluation. An aliquot of each ejaculate was fixed in paraformaldehyde (1% w/v) and stored at 4°C for shipment to U of S, for subsequent TUNEL assay. The remaining ejaculate was diluted with Triladyl extender (1:1), kept in an electric cooler (4°C) and transported to U of S for evaluation and processing (12 h time span). Semen was placed on a prewarmed (37°C) Leja four chamber slide (20 microns, Leja Products, The Netherlands, provided by Gencor) and analyzed for sperm motility characteristics using a Computer-Assisted Sperm Analyzer (SpermVision 3.7, Minitube of America Inc.). Seven fields were assessed for total motility (%, all moving sperm).

4.3.3.1 TUNEL assay

Out of 60 bulls collected, 15 were selected for TUNEL assay based on their fresh sperm motility (8 high motility bulls and 7 low motility bulls, average motility 93±2% and 45±3% respectively). Semen aliquots ($2x10^6$ cells) were taken; and centrifuged at 500x g for 10 min at RT, to remove the paraformaldehyde. The cells were resuspended in 70% ice-cold ethanol and stored at -20°C until analysis.

On the day of analysis, ethanol was removed from the samples by centrifugation (500x g for 10 min at RT), and the sperm pellet was washed in PBS. Sperm membranes were permeabilized with Triton X-100 solution (0.1% v/v) on ice for 2 min and washed with PBS. The pellet was resuspended in the reaction mixture (In Situ cell Death Detection, Fluorescein Kit, Roche) and incubated at 37°C in the dark for 1 h. The reaction mixture was washed with PBS and samples were transferred to flow cytometry tubes. Additional PBS was added (500 µl) and flow cytometry was conducted as mentioned under TUNEL assay, Experiment 1.

4.3.4 Experiment 3: Role of sperm with nicked-DNA in fertilization and embryonic development

Composition of media used for oocyte and sperm washing, *in vitro* maturation, *in vitro* fertilization and *in vitro* culture are presented in Appendix B.

4.3.4.1 COC collection and initial processing

Bovine ovaries were collected from the slaughterhouse and transported to the laboratory at approximately 23°C within 3 h. Ovaries were washed in physiological saline, and follicles of size 5 to 8 mm were aspirated. Cumulus-oocyte complexes (COCs) of Grade 1 and 2 (oocytes with more than 3 layers of cumulus cells and uniform cytoplasm) were selected. These COCs were washed (3x) in DPBS with 5% CS before *in vitro* maturation procedure. A total of 704 COC's were used in this experiment.

4.3.4.2 In Vitro Maturation

COCs were washed (3x) in maturation medium (TCM-199, supplemented with 5% CS, 0.5 µg/ml FSH, 5 µg/ml LH and 0.05 µg/ml gentamycin). For *in vitro* maturation, 20 COC's were placed in 100 µl drops of maturation media under mineral oil and incubated at 38°C, 5% CO_2 in air with high humidity, for 24 h.

4.3.4.3 Semen Processing

Based on DNA nicks and estimated fertility in PFRA beef bulls, frozen-thawed sperm from above and below average fertility groups were analyzed for motility using CASA system. Semen was washed through Percoll (45%) (2000x g for 20 min at RT). Sperm were washed once again with sperm washing solution (1800x g for 5 min at RT). After the washes sperm concentration was calculated using a haemocytometer and a final

concentration was adjusted to $3x10^6$ by dilution with Brackett-Oliphant (BO) media. The detailed composition of these media are given in Appendix B.

4.3.4.4 In Vitro Fertilization and Embryo Culture

After 22 h, *in vitro* maturation (IVM) oocytes were washed (3x) in BO medium supplemented with 10% BSA (w/v). Twenty COCs were added to a 100 µl droplet of sperm with BO media under mineral oil. Sperm and COCs were co-incubated at 38.5 °C, 5% CO_2 with air and high humidity for 18 h. After incubation, cumulus cells and sperm were removed from COCs by pipetting. The presumptive zygotes were then washed (3x) in *in vitro* culture (IVC) medium, CR1aa containing 2% BME essential amino acids (v/v), 1% MEM nonessential amino acids (v/v), 1% L-Glutamic acid (v/v), 0.3% BSA (w/v), 0.05 µg/ml gentamycin and 5% CS (v/v). Twenty presumptive zygotes were then added to a 100 µl drop of IVC media under mineral oil and incubated at 38.5 °C with 5% CO_2, 90% N_2, 5% O_2 and high humidity. After 48 h culture, the cleavage rate was noted and culture was continued in the same drop until the blastocyst development was determined on day 8.

4.3.4.5 Treatment Groups

In vitro fertilization study was conducted using frozen-thawed semen from six beef bulls (3 from above-average and 3 from below-average fertility groups, estimated in Experiment #1.2). The COCs were divided into five groups based on number of sperm

per droplet: 1) no sperm (control group); 2) 300,000 sperm from above average fertility; 3) 300,000 sperm from below average fertility; 4) 30,000 sperm from above average fertility; and 5) 30,000 sperm from below average fertility. There were 20 COC's per droplet.

4.3.4.6 Statistical Analysis

In Experiment 1, bulls with <200 inseminations or <50% repeatability estimates were excluded from data analysis. The relationships of various sperm populations (Annexin-V/PI assay) and DNA-nicked sperm populations (TUNEL assay) with fertility were determined. A linear regression model was utilized to determine if there were significant relationships ($P<0.05$) between fertility deviation and Annexin-V/PI and TUNEL parameters. Data were analyzed using SPSS (IBM SPSS Statistics, Version 21, Armonk, NY).

In Experiment 2, fertility levels of 15 beef bulls were estimated using the regression model developed in dairy bulls (Experiment 1). An independent samples t-test was run in SPSS to determine the difference in sperm motility ($P<005$), the percentage of sperm with DNA nicks and fertility deviation, to validate the grouping of bulls.

In Experiment 3, cleavage rate was determined 48 h post-insemination, blastocyst rate was calculated on day 7 and 8 post-insemination. Cleavage and blastocyst rates were summed for all replicates. Chi-Square test was performed using SPSS. A critical value of $P<0.05$ was used to determine if the differences between the high and low DNA-nicked

groups was significantly different. Comparisons were done between groups of high and low DNA damage sperm within sperm concentrations, for cleavage and blastocyst rates.

4.4 Results

4.4.1 Experiment 1: Relationship between sperm apoptosis-like changes and fertility of breeding bulls

In the present study, four sperm populations were detected, live (AN-/PI-), early apoptotic (AN+/PI-), late apoptotic (AN+/PI+), and necrotic (AN-/PI+) sperm. These populations varied among bulls, i.e. live sperm 55-88%, early apoptotic sperm 0-5%, late apoptotic 0-2%, and necrotic 10-45% (Fig. 4.1). A significant relationship of fertility deviation with live sperm ($r = 0.32$; $P<0.05$) and necrotic sperm ($r = -0.40$; $P<0.01$) was observed.

TUNEL assay identified two sperm populations, nicked DNA (FITC-BrdU positive) and intact DNA (FITC-BrdU negative) sperm. Sperm population with nicked DNA varied among bulls and ranged 2-15%. Nicked DNA sperm population had a significant relationship with fertility ($r = -0.62$; $P<0.001$) (Fig. 4.2).

4.4.2 Experiment 2: Estimation of fertility in beef bulls used for natural mating

Sperm motility, the incidence of DNA nicks in sperm and estimated fertility deviation data are presented in Table 4.1. All three parameters varied between the high and low

motility groups (P<0.001), validating the grouping of bulls based on motility. The high motility group showed less number of sperm with DNA nicks (4±1%) as well as high estimated fertility deviation (1.2±0.3). The low motility group had high number of sperm with DNA nicks (15±2%) and a low estimated fertility deviation (-3.7±1.1).

4.4.3 Experiment 3: Role of sperm with nicked-DNA in fertilization and embryonic development

The cleavage rates were low in the high DNA nicked sperm group regardless of the sperm concentration used in the IVF droplet (P<0.001 in low sperm concentration; P<0.05 high sperm concentration, table 4.2). Blastocyst rate was also low in the high DNA nicked sperm group at the 30,000 sperm concentration only (P<0.05). The difference in blastocyst rates in the high sperm concentration was not statistically significant but there was a noticeable lower rate in the semen with high DNA nicked sperm. Typical blastocyst formation seen in our treatment groups is presented in Appendix C.1. Hatched blastocyst rates are given in Appendix C.2.

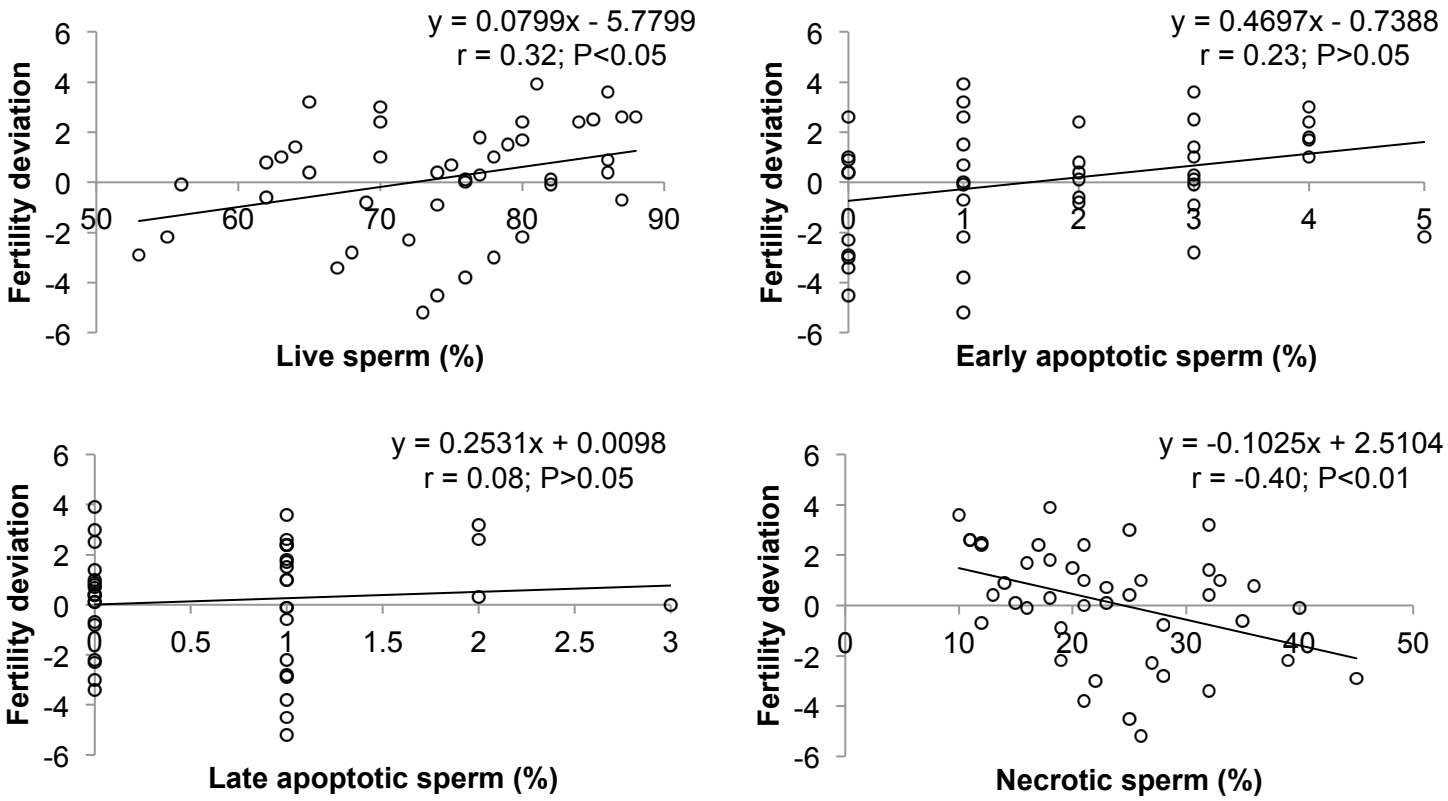


Figure 4.1. Relationships between bull fertility deviation and live, early apoptotic, late apoptotic and necrotic sperm populations in semen– Annexin-V/PI assay.

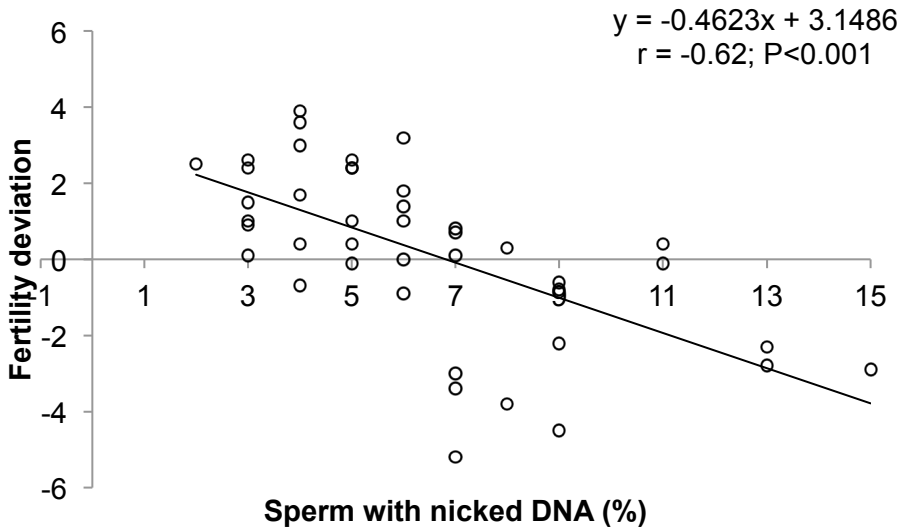


Figure 4.2. Relationship between bull fertility deviation and sperm with nicked DNA – TUNEL assay.

Table 4.1. Sperm motility, sperm with nicked DNA and estimated fertility deviation in bulls with high and low sperm motility (N=15)

	Bull #	Motility (%)	Nicked DNA Sperm (%)	Estimated Fertility Deviation
High Motility Bulls	1	96.5	4.6	1.0
	2	96.4	2.5	2.0
	3	95.8	4	1.3
	4	95.4	1.6	2.4
	5	93	7.3	-0.2
	6	92.9	4	1.3
	7	92.9	5.1	0.8
	8	84	4.3	1.2
Mean ± SEM		**93.4 ± 1.5**	**4.2 ± 0.6**	**1.2 ± 0.3**
Low Motility Bulls	9	52.7	3.6	1.5
	10	50.3	12.5	-2.6
	11	49.2	15.2	-3.9
	12	48.9	19	-5.6
	13	45.7	14	-3.3
	14	38	22.6	-7.3
	15	30.7	16.6	-4.5
Mean ± SEM		**45.1 ± 3.0***	**14.8 ± 2.3***	**-3.7 ± 1.0***

* $P<0.001$ between high and low motility bull groups

Table 4.2. Cleavage and blastocyst rates in low and high DNA nicked sperm samples with two concentrations (30,000 and 300,000 sperm per 100 µl IVF droplet).

DNA nick/sperm concentration	COCs	Cleavage rate* n (%)	P-value	Blastocyst rate** n (%)	P-value
Low DNA nick 30,000	176	112 (64%)		57 (51%)	
High DNA nick 30,000	177	60 (34%)	P<0.001	19 (32%)	P<0.05
Low DNA nick 300,000	182	139 (76%)		67 (48%)	
High DNA nick 300,000	169	112 (66%)	P<0.05	47 (42%)	NS

* Cleavage rate = (# of cleaved embryos/# of COC's) x 100

** Blastocyst rate = (# of blastocysts/# of cleaved embryos) x 100

4.5 Discussion

The relationship between sperm with DNA nicks and bull fertility level was higher than membrane associated apoptotic-like changes. Therefore, the TUNEL assay and the regression model we developed were selected to evaluate the DNA-nicked sperm in beef bull ejaculates, and to estimate the fertility-level of beef bulls used for natural mating. Assessing the IVF capabilities of bulls with varying fertility levels confirmed the fertility prediction and further supported our hypothesis that sperm with DNA nicks adversely affect the fertilization and early embryo development.

In apoptotic cells, DNA nicks are caused by endonucleases and produce characteristic ~180 bp DNA fragments, with exposed 3'-OH ends of DNA strands (Wyllie et al., 1980). TUNEL assay allows the detection of DNA nicks present at the 3'-OH end of the DNA, by labeling with fluorescent nucleotides (Gorczyca et al., 1993b). A significant positive correlation was found between level of DNA nicks and fertility deviation of bulls ($P<0.001$) and a regression model was formulated. A comparable study was performed on a smaller scale, and similar relationships were found (Anzar et al., 2002). A similar relationship between DNA fragmentation and fertility has been reported (Waterhouse et al., 2006). Simon et al. found a significant negative correlation between male fertility and DNA nicks, using the Comet assay (Simon et al., 2011). In contrast to our work, another study found no correlation between sperm DNA damage and fertility (Dogan et al., 2013). In this study, the use of frozen-thawed sperm may cause this lack of correlation. It has been shown that cryopreservation can cause structural changes to bull sperm

chromatin (Gravance et al., 1998). Also, an increase in chromatin condensation may cause the nicked DNA strands to be inaccessible to the fluorescent nucleotides (Anzar et al., 2002; Gravance et al., 1998).

Few bulls did not fit well in the regression model from our analysis; all these bulls have a negative fertility deviation and were included in the analysis. We hypothesize that these individuals have a reduced fertility due to factors not related to apoptosis-like changes. Factors may be related to faults in sperm transport, sperm capacitation, sperm-egg binding, acrosome reaction, penetration of the zona and fusion of sperm-egg membranes (Lessard et al., 2011). Further analysis of these bulls would be of interest to determine the exact the cause of their reduced fertility. One bull, who was excluded from our analysis due to low number of field inseminations (N=176) and as an outlier had a fertility deviation of -13. If this bull had been included in our analysis, it could further strengthen our correlation and regression coefficient.

Changes to the plasma membrane of cells are one of the earliest morphological events associated with the induction of apoptosis. During apoptosis, PS is translocated to the outer leaflet of the plasma membrane, resulting in loss of membrane symmetry (Vermes et al., 1995). Apoptotic sperm were present up to 5% in fresh semen, as characterized by PS detection on the outer plasma membrane (Fig 4.1). Apoptotic sperm present in an ejaculate represent a failure in the spermatogenesis process to remove these defective sperm from the normal population (Aitken and Baker, 2013).

In apoptotic sperm the plasma membrane remains intact. However in necrotic sperm the plasma membrane integrity is lost (Anzar et al., 2002). In this study, 10-45% of sperm present in a fresh ejaculate were necrotic (AN-/PI+) and have an adverse effect on a bull's fertility. The necrotic sperm population has previously not been studied in regards to bull fertility. The occurrence of live cell (AN-/PI-) population, exhibiting normal plasma membrane symmetry ranged 55-88% in an ejaculate and had a significant positive relationship to a bull's fertility. Early and late apoptotic sperm failed to relate with fertility. These results are in agreement with earlier reports in which the relationship between PS translocation and fertility was absent (Anzar et al., 2002; Dogan et al., 2013). The lack of correlation between PS translocation and fertility could be the result of the PS translocation being a compensable sperm defect which can be overcome by increasing the number of healthy sperm in an insemination dose (Saacke, 2008). It should be kept in mind that the dairy bulls used in this study were highly selected and well maintained breeding bulls. Therefore, their fertility range was quite narrow. It was anticipated that if bulls used for natural breeding were used there would be a broader range in parameters with stronger relationship of fertility.

To our knowledge, our study is the first to predict the fertility potential of beef bulls in relation to their DNA nick levels and to confirm their fertility potential *in vitro*.
Bull fertility is also expressed as a deviation from the population mean (Beavers and Van Doormaal, 2013). The regression model developed, in this study would be of great value in the identification of bulls with high levels of DNA nicks and therefore low fertility, during the selection process in breeding programs. Currently it takes several years to have

a good estimate of a bull's fertility through hundreds of inseminations. This is not only very time consuming also a very expensive process, through maintenance costs and the insemination of many cows.

The success of *in vitro* embryo production is primarily related to the quality of gametes used. Low *in vitro* fertility related to the bull can be associated with the quality and concentration of spermatozoa used (Alomar et al., 2006). In this study, the bulls exhibiting high number of sperm with DNA nicks expressed reduced capability to fertilize the oocytes, when compared to bulls exhibiting low levels of DNA nicks, as measured through cleavage rates. The ability of the fertilized oocytes to develop into blastocysts was adversely affected by the level of DNA-nicked sperm present at the lower concentration of sperm. The fate of a fertilized oocyte is influenced by the level of DNA fragmentation in the fertilizing sperm (Walters et al., 2005).

In conclusion, we have demonstrated that the live and necrotic cell populations as well as the level of DNA nicks present in an ejaculate, can be used to estimate a bull's fertility potential. The levels of DNA nicked sperm in a semen sample have a negative effect on cleavage rate and subsequent embryo competence and development. A regression model based on numbers of sperm with DNA damage can be used to estimate the fertility of a natural breeding bull.

CHAPTER 5: THE EFFECT OF THE ADDITION OF APOPTOSIS INHIBITORS ON POST-THAW SPERM LONGEVITY AND SPERM CHARACTERISTICS

5.1 Abstract

Sperm longevity while maintaining the characteristics required for oocyte fertilization is important, especially for cryopreserved semen. Sperm must remain motile and possess intact plasma and acrosome membranes for the fertilization process. The objective of this study was to evaluate the effect of apoptosis inhibitors added to post-thaw sperm samples on their longevity, to increase the availability of viable sperm to oocytes for fertilization. Frozen semen from seven bulls was used, six straws from each bull was pooled. Samples included: untreated control (sperm remaining in extender), treated control (washed sperm), and four treatments (inhibitors) at two concentrations each. Apoptosis inhibitors assessed included: z-VAD-FMK, Bax channel blocker, Coenzyme Q_{10}, and XIAP. Motility related characteristics were evaluated using computer assisted sperm analysis (CASA). Membrane intactness and acrosome presence were evaluated using fluorescein isothiocyanate-peanut agglutination (FITC-PNA)/propidium iodide (PI) assay. Mitochondrial membrane potential was evaluated using Mitotracker Deep Red (MtDR). Sperm parameters were evaluated at 0, 3, 6, and 12 hours of incubation at 37°C. Our results showed, no significant effect of apoptosis inhibitors on post-thaw sperm motility and structural characteristics. The decline in sperm motility and structural characteristics at 6 h of incubation was lower ($P<0.05$) in treated control and treatment groups than in untreated control group. Our results indicate that the addition of an apoptosis inhibitor to sperm samples post-thaw is not beneficial in improving sperm longevity.

5.2 Introduction

Cryopreservation of semen is routinely used in animal agriculture, to conserve the germplasm from endangered species and to preserve male fertility in human cancer patients. Although cryopreservation is widely used, it is generally accepted that approximately 40-50% of the sperm population do not survive the cryopreservation process (Watson, 2000). Despite improvements in the cryopreservation protocol, the post-thaw viability and fertility of semen is reduced due to osmotic and mechanical shock to the cells and apoptosis-like changes in sperm membranes and DNA (Anzar et al., 2002; Martin et al., 2007; Medeiros et al., 2002; Ortega-Ferrusola et al., 2008; Said et al., 2010). Sublethal changes in viable sperm, has similarities to apoptosis in somatic cells and has been deemed apoptosis-like changes (Ortega-Ferrusola et al., 2008). In cattle, increasing the sperm number per insemination dose can compensate for this reduction in viable cells, but does not fully remove the negative effect of the freezing procedures (Saacke, 2008). The longevity of frozen-thawed semen is shorter than fresh, diluted or cooled semen, therefore requiring intensive estrus detection and insemination close to ovulation in order to achieve optimum conception rates (Ahmad et al., 2014). If the total number of fully functional sperm decline below the threshold needed to achieve a high probability of fertilization, then fertility is reduced (Watson, 2000). Cryopreservation induces apoptosis-like changes including a decrease in mitochondrial membrane potential ($\Delta\Psi m$), caspase activation, and membrane changes (Martin et al., 2004).

The signaling events leading to apoptosis can be broken down into two distinct pathways i.e, the mitochondria (intrinsic pathway) or death receptor pathway (extrinsic pathway). Both pathways finally converge on the activation of cysteinyl aspartate proteases, called caspases (Ortega-Ferrusola et al., 2008). One of the mitochondrial functions is the regulation of cell death, through reactive oxygen species (ROS). ROS plays an important role in the release of pro-apoptotic factors (Ott et al., 2007), such as BAX, BAK, SMAC and cytochrome c (Gillies and Kuwana, 2014). Mitochondria is the most sensitive organelle to cryopreservation in sperm cells (Pena et al., 2003). The antioxidants and caspase inhibitors interfere with the apoptosis pathways, thereby inhibiting or delaying the negative effect of apoptosis-like changes in somatic cells (Hetz et al., 2005; Smolewski and Robak, 2011; Talevi et al., 2013). Currently, there is no common use of these compounds to slow down the apoptosis-like process in mammalian sperm.

The X-linked inhibitor of apoptosis (XIAP) is a potent inhibitor of caspase 3 and 7, and it blocks apoptosis in many cancerous cell types (Ekert et al., 1999; Saleem et al., 2013; Smolewski and Robak, 2011). The synthetic caspase inhibitor z-VAD-FMK blocks caspases 1-9, although its benefits have not been found in canine and equine semen (Peter et al., 2005; Peter and Linde-Forsberg, 2003). The antioxidant Coenzyme Q_{10} plays a crucial role in energy metabolism as well as blocking caspase-2, through mitochondrial pathways (Chen et al., 2013). Bax channel blocker has not previously been studied in mammalian sperm. The Bax protein allows the release of cytochrome c from the mitochondria, and therefore initiates the apoptosis cascade (Hetz et al., 2005). Bax channel blocker would then suppress the release of cytochrome c.

The objective of the present study was to evaluate the effect of apoptosis inhibitors (XIAP, Coenzyme Q_{10}, Bax channel blocker, and z-VAD-FMK) on the longevity of post-thaw sperm. Apoptosis inhibitors used in this study were selected based on their role in inhibiting apoptosis via mitochondrial or caspase dependent pathways.

5.3 Materials and Methods

5.3.1 Apoptosis inhibitors

A caspase-3 inhibitor (z-VAD-FMK), was purchased from SM Biochemicals LLC (Anaheim, CA); Bax channel blocker from EMD Chemicals Inc. (San Diego, CA); Coenzyme Q10, anti-oxidant from Sigma-Aldrich Co. (St. Louis MO); and XIAP, an apoptosis inhibitor protein from R&D Systems, Inc. (Minneapolis, MN). The stock solutions of z-VAD-FMK (1mM) and Bax channel blocker (1 mM) were prepared in dimethyl sulfoxide (DMSO). The stock solution of Coenzyme Q_{10} (1 mM) was prepared in chloroform, evaporated in nitrogen (N_2) stream before use and finally dissolved in PBS. XIAP stock (6.8 μM) was prepared by the supplier and used as such.

5.3.2 Sperm preparation

Frozen semen from seven bulls was used for this experiment. Each bull had six semen straws thawed at 37°C for 60s. Post-thaw motility was recorded using CASA, an aliquot (~$20x10^6$ sperm) remaining in extender was taken as an untreated control and remaining

semen was diluted 1:1 with Tris-Citric Acid (TCA) buffer (0.024 g/ml Tris base, 0.014 g/ml citric acid monohydrate, 0.01 g/ml fructose) washed at 500x g for 10 min. The supernatant was removed and sperm pellet was resuspended in TCA buffer. Sperm concentration was calculated by CASA system and adjusted to $20x10^6$/ml in TCA buffer. Sperm ($20x10^6$/ml) were then exposed to one of the four apoptosis inhibitors, i.e. z-VAD-FMK (5 or 10 μM, final concentration), Bax channel blocker (5 or 10 μM, final concentration), Coenzyme Q_{10} (5 or 50 μM, final concentration) and XIAP (50 or 100 nM, final concentration). The washed sperm without apoptosis inhibitors added was considered as treated control. After addition of apoptosis inhibitors, all semen samples were incubated at 37°C and evaluated at 0, 3, 6, and 12 h for sperm motility and sperm membrane characteristics as follows.

5.3.3 Sperm motility characteristics

An aliquot of semen was placed on a prewarmed (37°C) Leja four-chamber slide (20 microns, Leja Products, The Netherlands) and analyzed for sperm motility characteristics using CASA (SpermVision 3.7, Minitube of America Inc.) (Cell ID 25-200 μm^2, AOC<5, DSL<4.5). Seven fields were assessed for total motility (%), progressive motility (%), and curvilinear velocity (VCL) (μm/s).

5.3.4 Sperm structural characteristics

At each time point, 20,000 sperm were taken and incubated with fluorescent probes, 0.025 mg/ml FITC conjugated-peanut agglutinin (FITC-PNA; Sigma Aldrich, St. Louis,

MO; stock 1 mg/ml in PBS), 0.05 mg/ml Mitotracker Deep Red (MtDR; Life Technologies Inc. Burlington ON; stock 0.1 mM in DMSO) and 0.1 mg/ml propidium iodide (PI; Life Technologies Inc. Burlington ON; stock 2.4 mM in water) at 37°C in the dark for 20 min. After incubation, sperm were fixed with 10 µl formaldehyde (10% w/v) and flow cytometry was conducted within 15 min.

5.3.5 Flow cytometry

Sperm plasma membrane integrity, mitochondrial membrane potential and acrosome intactness were analyzed by CyFlow ® Space (Partec GmbH, Münster, Germany). A blue diode solid-state laser at 488 nm (200 mW) was used to excite both FITC-PNA and PI. FITC-PNA (emission wavelength 525 nm) was detected with photomultiplier tube FL1 (527 BP filter) and PI (emission wavelength 617 nm) was detected with photomultiplier FL3 (680 LP filter). A red laser diode at 638 nm (25 mW) was used to excite Mitotracker Deep Red, (emission wavelength 665 nm) and was detected with photomultiplier FL6 (670 BP filter). Data were collected using FlowMax® software (version 2.6) for cytometry supplied by Partec GmbH.

The sperm population was located using the forward and side scatters. A gating region was drawn around the sperm specific events to isolate them from the background noise. Fluorescent data were collected from all probes simultaneously, from 10,000 sperm events on a log scale. All samples were passed through the flow cytometer at a speed of 1.3 µl/s.

Sperm with compromised plasma membrane stained with PI and fluoresced red, whereas sperm with an intact plasma membrane remained unstained. Sperm with a damaged acrosome fluoresced green, and sperm with intact acrosome remained unstained. Sperm with a high mitochondrial membrane potential fluoresced orange and low mitochondrial membrane potential remained unstained. A 2-dimensional plot of MtDR/PI assay revealed four sperm subpopulations: sperm with intact plasma membrane and low mitochondrial membrane potential (ΔΨm); sperm with intact plasma membrane and high ΔΨm; sperm with damaged plasma membrane and low ΔΨm; sperm with damaged plasma membrane and high ΔΨm. Likewise, four sperm populations were observed on the 2-D plot of FITC-PNA/PI assay; sperm with intact plasma membrane and intact acrosomes, sperm with intact plasma membrane and damaged acrosomes, sperm with damaged plasma membrane and intact acrosomes, and sperm with both damaged plasma membrane and acrosomes. Typical flow cytometric histograms from all three fluorescent probes are presented in Appendix D.3.

5.3.6 Statistical analysis

Data on sperm motility, progressive motility and curvilinear velocity, sperm with high ΔΨm, and sperm possessing intact plasma membrane and intact acrosomes were used for statistical analysis. Completely randomized design was used to compare the treatment effect of these parameters. Within inhibitor, two concentrations were compared with paired t-test. To study the effect of treatment on post-thaw longevity of sperm, data were calculated as percent decline in sperm parameters at 6 h incubation periods at 37°C, for example:

$$\frac{Motility\ at\ 6h - Motility\ at\ 0h}{Motility\ at\ 0h} * 100$$

Percent decline in sperm characteristics at 6 h of incubation were compared for each treatment. Data were pooled due to the lack of concentration effect for each inhibitor group. Completely randomized design was used to compare the effect of inhibitors on post-thaw sperm longevity. In the case of $P<0.05$, the means were separated with Tukey's test. All analyses were conducted using the statistical program SPSS (IBM SPSS Statistics, Version 21). Data was analyzed at 6 h time point.

5.4 Results

The data on sperm longevity based on percent decline in sperm characteristics for 6 h of incubation, is presented in Table 5.1. Data from the 6 h time point for the untreated control sample revealed a decline in sperm motility (-55%), progressive motility (-69%), VCL (-49%), live cells (-36%) and high ΔΨm (-45%), this decline was significant ($P<0.05$) when compared to the treated control and treatment groups (Table 5.1). At 0 h of incubation motility ranged from 46 – 58% and ranged 26 – 41% after 6 h of incubation.

Table 5.1. Percent decline in post-thaw sperm characteristics from 0 – 6 h during incubation, untreated-control, treated-control and apoptosis inhibitor treated spermatozoa. (Mean ± SEM).

Treatments	**Motility (%)**	**Progressive Motility (%)**	**VCL[a] (μ/sec)**	**Live Cells (%)**	**High ΔΨm[b] (%)**
Untreated - Control	-55 ± 9*	-69 ± 9*	-49 ± 6*	-36 ± 9	-45 ± 8*
Treated - Control	-23 ± 9	-28 ± 12	-25 ± 8	-27 ± 8	2 ± 2
Bax[c]	-26 ± 12	-39 ± 15	-27 ± 7	-29 ± 7	1 ± 2
Vad[d]	-36 ± 11	-45 ± 13	-28 ± 9	-28 ± 7	-3 ± 3
Q10[e]	-24 ± 9	-29 ± 11	-25 ± 6	-27 ± 7	0 ± 1
XIAP[f]	-25 ± 9	-30 ± 11	-23 ± 5	-27 ± 7	-2 ± 2

[a] Curvilinear velocity
[b] High mitochondrial membrane potential
[c] Bax channel blocker
[d] z-VAD-FMK
[e] Coenzyme Q_{10}
[f] X-linked inhibitor of apoptosis
* $P < 0.05$, between controls and treatment groups

5.5 Discussion

In the present study, the effect of the addition of apoptosis inhibitors on post-thaw longevity of frozen-thawed sperm was evaluated. The removal of egg yolk containing extender by washing significantly improved post-thaw sperm longevity (motility and membrane characteristics) at 6 h. However, the addition of apoptosis inhibitors did not benefit the sperm longevity compared to the treatment control (washed) group.

Our results indicate that incubation of sperm diluted in extender causes a reduction in sperm motility parameters, plasma membrane integrity, normal acrosomes and sperm with high mitochondrial membrane potential. When semen is extended, the seminal plasma is not removed it is rather diluted. Seminal plasma contains proteins called bovine seminal plasma proteins (BSP's), which cause cholesterol and phospholipid efflux from the plasma membrane causing a loss of integrity (Manjunath et al., 1994). The presence of egg yolk in extender can cause the externalization of PS from the inner to the outer leaflet due to unknown mechanisms or through the addition of its own PS to the outer leaflet of the sperm plasma membrane (Ahmad et al., 2014). Egg yolk extender has been shown to be the best extender for cryopreservation of sperm cells, as it results in higher post-thaw longevity and survival, than soybean based extenders (Muino et al., 2007). When frozen-thawed sperm are used in AI, the sperm cells remain in the extender for a very short time, as the sperm quickly swim out of the extender, moving further into the uterine body. Therefore, the negative effect of prolonged incubation in egg yolk extender is minimized. This is exactly what was seen in our treatment control and treatment

groups, i.e. the removal of extender was beneficial in maintaining the motility, membrane symmetry, acrosome intactness and ΔΨm.

Sperm motility and velocity is important for their transport from the site of insemination or ejaculation to the site of fertilization (Suarez and Pacey, 2006). Reduction in sperm motility is mainly due to mitochondrial impairment (Bag et al., 2004) and the generation of reactive oxygen species (ROS) (Aitken and Baker, 2013). The reduction in motility in our study may be due to the decline in ΔΨm, as seen in the untreated control group (Fig. 5.1). This decline in ΔΨm is also a sign of apoptosis (Espinoza et al., 2009).

The addition of apoptosis inhibitors to post-thaw washed sperm did not improve the sperm longevity. The concentrations of apoptosis inhibitors used in this study may have been too low to elicit their biological effect (Peter et al., 2005). Improper inhibitor to sperm ratio may be involved in the lack of their beneficial effect (Pena et al., 2003). It is possible that the inhibitors did not cross the sperm plasma membrane, as sperm are resistant to the uptake of exogenous molecules (Anzar and Buhr, 2006). As well, since sperm are transcriptionally and translationally inactive, the inhibitors may not be able to regulate the death process (Aitken and De Iuliis, 2007).

To our knowledge, this is the first report on the incubation of sperm with X-linked inhibitor of apoptosis (XIAP). XIAP is a potent inhibitor of caspases 3 and 7 and blocks apoptosis in many cell types (Ekert et al., 1999; Saleem et al., 2013; Smolewski and Robak, 2011). The synthetic caspase inhibitor z-VAD-FMK has been used with limited

success in spermatozoa (Peter et al., 2005; Peter and Linde-Forsberg, 2003). Although z-VAD-FMK blocks caspases 1-9, its benefit has not been found in canine and equine semen (Peter et al., 2005; Peter and Linde-Forsberg, 2003). Coenzyme Q_{10}, an antioxidant and a crucial player in energy metabolism, blocked caspase-2 in ethanol-induced apoptosis through mitochondrial pathways (Chen et al., 2013). Recently, in contrast to our results, the incubation of human sperm with Coenzyme Q_{10} prevented a decrease in motility, increase in DNA fragmentation and lipid peroxidation (Talevi et al., 2013). To our knowledge, Bax channel blocker has not previously been studied in mammalian sperm. Bax is a member of the pro-apoptotic Bcl-2 family, and Bax channels allow a release of cytochrome c from the mitochondria (Hetz et al., 2005). Therefore the Bax channel blockers inhibit the release of cytochrome c from the mitochondria and thus maintain mitochondrial membrane potential, as seen in animal models and isolated mitochondria (Gomez-Crisostomo et al., 2013; Hetz et al., 2005). Inhibitors were selected for use in this study based on their method of inhibition of apoptosis in cells, whether it is mitochondrial or caspase based pathways.

In conclusion, the removal of extender from the semen may be more beneficial to post-thaw sperm longevity and fitness characteristics. A benefit of removing the extender was detected through the maintenance of motility, plasma membrane and acrosome intactness, and mitochondrial membrane potential in treatment control and inhibitor treatment-groups as compared with untreated control group. We found no benefit of the addition of apoptosis inhibitors on post-thaw longevity of bull sperm. Therefore, given the lack of

improvement in sperm post-thaw longevity, the addition of apoptosis inhibitors has no benefit to sperm samples at the concentrations we evaluated.

CHAPTER 6: DISCUSSION

When choosing a male for breeding purposes it is important to assess his semen quality, through several laboratory tests. One of the main goals of semen analysis is to determine the quality of an ejaculate to use it for further processing and to estimate a bull's fertility potential. The routine semen evaluation includes, ejaculate volume, sperm concentration, motility, which ultimately represents testicular and epididymal function and the accessory sex glands functions. The physical assessment of semen allows the elimination of poor quality ejaculates and definite cases of infertility and possibly subfertility (Rodriguez-Martinez, 2003; Rodriguez-Martinez, 2006). However, a more detailed assessment is needed to expose bulls with other sperm defects. Many reproductive biologists have reached the conclusion that fertility is a multi-faceted process that requires a multi-parametric analysis, including evaluation of morphology, motility, plasma membrane status, capacitation, acrosome reaction and genome integrity, to estimate a bull's potential fertility (Bissonnette et al., 2009). Ideally, semen assays should be rapid, inexpensive, accurate and repeatable (Moce and Graham, 2008). Currently there is little coherence amongst semen assays and amongst researchers attempting to predict fertility of bulls (Rodriguez-Martinez, 2006).

In this thesis, three studies were undertaken to understand the relationship between apoptosis-like changes in bull sperm plasma membranes and DNA integrity, with bull fertility potential, and to confirm these relationships *in vitro.* Lastly, the effect of the addition of apoptosis inhibitors on post-thaw sperm longevity and fitness was determined.

Apoptosis-like changes in plasma membranes and DNA of bull sperm have effects on the fertility potential of breeding bulls. In the first study, our results indicate a significant inverse relationship between sperm with DNA-nicks and the fertility potential of bulls. Through the regression model developed in this thesis, AI companies could estimate the fertility potential of a bull used for breeding. With the current system in use by AI and semen companies, a bull's fertility estimation normally takes several years, the process is time consuming as hundreds or thousands of inseminations are needed to collect sufficient data. This process also requires huge inputs of finances both by the AI and semen companies in the housing and maintenance of poor fertility bulls, and producers using the semen from a bull with poor fertility potential, ultimately resulting in long calving intervals (Gillan et al., 2008). Recent developments in molecular markers for genomic selection has allowed for the genetic selection of elite young bulls in AI programs (VanRaden et al., 2009). This new technology, which utilizes short nucleotide poylmorphism (SNP) identification, along with AI allows faster genetic advancement in the dairy industry. Although these bulls have been deemed superior based on their genetics, it still remains unknown if they will be fertile when used as AI sires. These bulls would benefit from TUNEL analysis to estimate their fertility value, before their extensive use in AI. The use of the regression model would simply require the evaluation of several ejaculates from a bull for the presence of sperm with nicked-DNA, using the TUNEL assay and flow cytometry, in a breeding soundness evaluation. The results of TUNEL assay can then be directly put into the regression model for fertility prediction.

The current analysis of bull fertility is dependent on several factors (mentioned below), which in turn can hinder the ability of an assay to accurately develop its correlations with the fertility potential of a bull. The definition of fertility is the first of these factors; which can be defined as percentage of females pregnant, number of females giving birth, or number of females that are not pregnant after insemination. The fertility of a bull increases with number of sperm per insemination dose. The technician inseminating cows can affect the fertility of a bull; if the semen is mishandled or an estrus heat is missed, a bull fertility value will subsequently decrease. If few females are inseminated from a given semen sample, the fertility results may be too variable to get a good estimation of fertility. All these factors on their own or in combination will have an effect on the fertility potential of a bull (Moce and Graham, 2008; Van Doormaal, 1993).

To our knowledge, this is the first time that the fertility level of beef bulls used for natural mating was predicted, based on the regression model of fertility index vs level of sperm DNA damage of dairy bulls. The estimation of fertility in bulls used for natural mating would have similar benefits as for AI bulls. If a natural breeding bull is found to have a reduced fertility, he could then be removed from the herd. The producer would then be able to replace him with a more fertile bull to maintain the best pregnancy rates, and to produce optimum numbers of calves, a main goal of beef producers.

The ability to distinguish bulls differing in fertility potentials using IVF procedures has previously been reported (Al Naib et al., 2011; Lonergan, 1994; Ward et al., 2003; Zhang et al., 1997). Through an IVF trial, we confirmed the predicted fertility potential of beef bulls. According to our hypothesis, the cleavage rates of embryos corresponded with the

predicted fertility potentials of bulls at both high and low sperm concentrations. Likewise, the development of embryos was significantly different between high and low DNA-nicked sperm at the low sperm concentration. It indicates if an oocyte is fertilized with a sperm possessing nicked-DNA, it may lead to failure in embryo development (Chaveiro et al., 2009; Fatehi et al., 2006).

Another objective of this thesis was to evaluate the potential benefit of the addition of apoptosis inhibitors to post-thawed bull sperm. Our results indicated the removal of extender from the sperm cells exhibited a positive effect on sperm longevity (Table 5.1), when compared to sperm cells remaining in extender. Moreover, there was no extra benefit of adding apoptosis inhibitors to post-thaw bull sperm on their longevity or fitness. The extender showed a negative effect on the sperm longevity and fitness, shown through significant declines in all sperm motility and membrane parameters evaluated. There was a severe reduction in motility parameters (motility, progressive motility and curvilinear velocity), as well as a decline in number of sperm with intact plasma membranes and acrosomes, and cells exhibiting a high mitochondrial membrane potential, during incubation. When AI is performed in cows, the sperm quickly swim out of the extender and make their way further into the female reproductive tract (Suarez, 2007; Suarez and Pacey, 2006; Vishwanath, 2003). So, prolonged incubation is not a major issue. The addition of an apoptosis inhibitor may be more beneficial if added, prior to freezing, in the extender or diluting media.

CHAPTER 7: FUTURE DIRECTIONS

Given the broad range of alterations that can be seen in spermatozoa undergoing apoptosis-like changes, there is a never-ending array of parameters that could be studied more in-depth than previously done. A future study to confirm the definite role of sperm with nicked-DNA in early embryo development would include an intracytoplasmic sperm injection trial using sperm with nicked-DNA. This trial should include sperm containing varying levels of DNA damage and the best quality oocytes. This study would allow a more in-depth analysis of the effect of DNA damaged sperm on cleavage, blastocyst and hatched blastocyst rates, levels of apoptosis in the produced embryos would be evaluated. A large-scale AI trial, using bulls with varying levels of DNA damage, would give insight into the *in vivo* effects of sperm with DNA nicks on pregnancy rates. Future studies regarding the addition of apoptosis inhibitors to semen extenders should include the evaluation of a greater range of concentrations for each inhibitor. As in our research we based our inhibitor concentration levels on previous work mainly completed in somatic cells. Inhibitors that target other various molecules involved in the apoptosis pathways should also be evaluated. As our research mainly focused on inhibiting caspases, providing antioxidant support and inhibiting Bax channel opening, in the inhibition of apoptosis. Research should also be conducted to evaluate the effect of the addition of the inhibitor in the extender or diluting media prior to freezing.

CHAPTER 8: GENERAL CONCLUSIONS

In conclusion, the results of this thesis indicate that apoptosis-like changes in bull sperm DNA can be related to the fertility potential of a bull. The addition of apoptosis inhibitors, post-thaw to bull semen have no benefit on sperm longevity or fitness. These results only offer a small insight into the relationship of bull fertility and apoptosis in spermatozoa and the role of apoptotic sperm (with nicked-DNA) in fertilization and early embryonic development.

REFERENCES

Ahmad M., Ahmad N., Riaz A., Anzar M. (2014) Sperm survival kinetics in different types of bull semen: progressive motility, plasma membrane integrity, acrosomal status and reactive oxygen species generation. Reprod Fertil Dev. DOI: 10.1071/rd13400.

Aitken R.J., Baker M.A. (2013) Causes and consequences of apoptosis in spermatozoa; contributions to infertility and impacts on development. Int J Dev Biol 57:265-72. DOI: 10.1387/ijdb.130146ja.

Aitken R.J., De Iuliis G.N. (2007) Origins and consequences of DNA damage in male germ cells. Reprod Biomed Online 14:727-33.

Aitken R.J., Findlay J.K., Hutt K.J., Kerr J.B. (2011) Apoptosis in the germ line. Reproduction 141:139-50. DOI: 10.1530/rep-10-0232.

Aitken R.J., Koppers A.J. (2011) Apoptosis and DNA damage in human spermatozoa. Asian J Androl 13:36-42. DOI: 10.1038/aja.2010.68.

Al Naib A., Hanrahan J.P., Lonergan P., Fair S. (2011) In vitro assessment of sperm from bulls of high and low field fertility. Theriogenology 76:161-7. DOI: 10.1016/j.theriogenology.2010.10.038.

Alomar M., Mahieu J., Verhaeghe B., Defoin L., Donnay I. (2006) Assessment of sperm quality parameters of six bulls showing different abilities to promote embryo development in vitro. Reprod Fertil Dev 18:395-402.

Amann R.P., Waberski D. (2014) Computer-assisted sperm analysis (CASA): Capabilities and potential developments. Theriogenology 81:5-17 e3. DOI: 10.1016/j.theriogenology.2013.09.004.

Andrabi S.M., Maxwell W.M. (2007) A review on reproductive biotechnologies for conservation of endangered mammalian species. Anim Reprod Sci 99:223-43. DOI: 10.1016/j.anireprosci.2006.07.002.

Anzar M., Buhr M.M. (2006) Spontaneous uptake of exogenous DNA by bull spermatozoa. Theriogenology 65:683-90. DOI: 10.1016/j.theriogenology.2005.06.009.

Anzar M., He L.W., Buhr M.M., Kroetsch T.G., Pauls K.P. (2002) Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. Biology of Reproduction 66:354-360. DOI: 10.1095/biolreprod66.2.354.

Bag S., Joshi A., Naqvi S.M., Mittal J.P. (2004) Effect of post-thaw incubation on sperm kinematics and acrosomal integrity of ram spermatozoa cryopreserved in medium-sized French straws. Theriogenology 62:415-24. DOI: 10.1016/j.theriogenology.2003.10.018.

Barth A. (2000) Bull breeding soundness evaluation. second ed. Western Canadian Association of Bovine Practitioners.

Beavers L., Van Doormaal B. (2013) Canada's semen fertility evaluation revamped Tech. Bull. Canadian Dairy Network.

Bissonnette N., Levesque-Sergerie J.P., Thibault C., Boissonneault G. (2009) Spermatozoal transcriptome profiling for bull sperm motility: a potential tool to evaluate semen quality. Reproduction 138:65-80. DOI: 10.1530/REP-08-0503.

Boettcher P.J. (2001) 2020 Vision? The future of dairy cattle breeding from an academic perspective. J. Dairy Sci. 84:E62-E68.

Broekhuijse M.L., Sostaric E., Feitsma H., Gadella B.M. (2012) Relationship of flow cytometric sperm integrity assessments with boar fertility performance under optimized field conditions. J Anim Sci 90:4327-36. DOI: 10.2527/jas.2012-5040.

Burkitt M., Walker D., Romano D.M., Fazeli A. (2012) Using computational modeling to investigate sperm navigation and behavior in the female reproductive tract. Theriogenology 77:703-16. DOI: 10.1016/j.theriogenology.2011.11.011.

Chaveiro A., Santos P., Carvalhais I., Faheem M., Habibi A., Moreira da Silva F. (2009) Sire Effect and Sperm Apoptosis on Bovine Embryonic Cleavage and Subsequent In Vitro Embryo Development. SRX Veterinary Science 2010.

Chen C.C., Liou S.W., Chen W.C., Hu F.R., Wang I.J., Lin S.J. (2013) Coenzyme Q10 rescues ethanol-induced corneal fibroblast apoptosis through the inhibition of caspase-2 activation. J Biol Chem 288:11689-704. DOI: 10.1074/jbc.M112.401844.

Dogan S., Mason M.C., Govindaraju A., Belser L., Kaya A., Stokes J., Rowe D., Memili E. (2013) Interrelationships between apoptosis and fertility in bull sperm. J Reprod Dev 59:18-26.

Ekert P.G., Silke J., Vaux D.L. (1999) Caspase inhibitors. Cell Death Differ 6:1081-6. DOI: 10.1038/sj.cdd.4400594.

Espinoza J.A., Paasch U., Villegas J.V. (2009) Mitochondrial membrane potential disruption pattern in human sperm. Hum Reprod 24:2079-85. DOI: 10.1093/humrep/dep120dep120 [pii].

Evenson D., Jost L. (2000) Sperm chromatin structure assay is useful for fertility assessment. Methods Cell Sci 22:169-89.

Farah O.I., Cuiling L., Jiaojiao W., Huiping Z. (2013) Use of Fluorescent Dyes for Readily Recognizing Sperm Damage. J Reprod Infertil 14:120-125.

Fatehi A.N., Bevers M.M., Schoevers E., Roelen B.A., Colenbrander B., Gadella B.M. (2006) DNA damage in bovine sperm does not block fertilization and early embryonic development but induces apoptosis after the first cleavages. J Androl 27:176-88. DOI: 10.2164/jandrol.04152.

Fernandez J.L., Muriel L., Goyanes V., Segrelles E., Gosalvez J., Enciso M., LaFromboise M., De Jonge C. (2005) Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. Fertil Steril 84:833-42. DOI: 10.1016/j.fertnstert.2004.11.089.

Foote R.H. (2002) The history of artificial insemination: Selected notes and notables. J Anim Sci 80:1-10.

Fortes M.R., Holroyd R.G., Reverter A., Venus B.K., Satake N., Boe-Hansen G.B. (2012) The integrity of sperm chromatin in young tropical composite bulls. Theriogenology 78:326-33, 333 e1-4. DOI: 10.1016/j.theriogenology.2012.02.007.

Fricke P. (1999) Management strategies for improving reproductive efficiency in lactating dairy cows. Advances in Dairy Technology 11:107-120.

Gadella B.M., Luna C. (2014) Cell biology and functional dynamics of the mammalian sperm surface. Theriogenology 81:74-84. DOI: 10.1016/j.theriogenology.2013.09.005.

Gallon F., Marchetti C., Jouy N., Marchetti P. (2006) The functionality of mitochondria differentiates human spermatozoa with high and low fertilizing capability. Fertil Steril 86:1526-30. DOI: 10.1016/j.fertnstert.2006.03.055.

Garner D.L., Johnson L.A. (1995) Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol Reprod 53:276-84.

Gillan L., Evans G., Maxwell W.M. (2005) Flow cytometric evaluation of sperm parameters in relation to fertility potential. Theriogenology 63:445-57. DOI: 10.1016/j.theriogenology.2004.09.024.

Gillan L., Kroetsch T., Maxwell W.M., Evans G. (2008) Assessment of in vitro sperm characteristics in relation to fertility in dairy bulls. Animal reproduction science 103:201-14. DOI: 10.1016/j.anireprosci.2006.12.010.

Gillies L.A., Kuwana T. (2014) Apoptosis regulation at the mitochondrial outer membrane. J Cell Biochem 115:632-40. DOI: 10.1002/jcb.24709.

Giwercman A., Lindstedt L., Larsson M., Bungum M., Spano M., Levine R.J., Rylander L. (2010) Sperm chromatin structure assay as an independent predictor of fertility in vivo: a case-control study. Int J Androl 33:e221-7. DOI: 10.1111/j.1365-2605.2009.00995.x.

Gomez-Crisostomo N.P., Lopez-Marure R., Zapata E., Zazueta C., Martinez-Abundis E. (2013) Bax induces cytochrome c release by multiple mechanisms in mitochondria from MCF7 cells. J Bioenerg Biomembr 45:441-8. DOI: 10.1007/s10863-013-9508-x.

Gorczyca W., Gong J., Darzynkiewicz Z. (1993a) Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. Cancer Res 53:1945-51.

Gorczyca W., Traganos F., Jesionowska H., Darzynkiewicz Z. (1993b) Presence of DNA strand breaks and increased sensitivity of DNA in situ to denaturation in abnormal human sperm cells: analogy to apoptosis of somatic cells. Exp Cell Res 207:202-5. DOI: 10.1006/excr.1993.1182.

Gravance C.G., Vishwanath R., Pitt C., Garner D.L., Casey P.J. (1998) Effects of cryopreservation on bull sperm head morphometry. J Androl 19:704-9.

Guidobaldi H.A., Teves M.E., Unates D.R., Giojalas L.C. (2012) Sperm transport and retention at the fertilization site is orchestrated by a chemical guidance and oviduct movement. Reproduction 143:587-96. DOI: 10.1530/rep-11-0478.

Harrison R.A. (2004) Rapid PKA-catalysed phosphorylation of boar sperm proteins induced by the capacitating agent bicarbonate. Mol Reprod Dev 67:337-52. DOI: 10.1002/mrd.20028.

Hetz C., Vitte P.A., Bombrun A., Rostovtseva T.K., Montessuit S., Hiver A., Schwarz M.K., Church D.J., Korsmeyer S.J., Martinou J.C., Antonsson B. (2005) Bax channel inhibitors prevent mitochondrion-mediated apoptosis and protect neurons in a model of global brain ischemia. J Biol Chem 280:42960-70. DOI: 10.1074/jbc.M505843200.

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

Hossain M.S., Johannisson A., Wallgren M., Nagy S., Siqueira A.P., Rodriguez-Martinez H. (2011) Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art. Asian J Androl 13:406-19. DOI: 10.1038/aja.2011.15.

Hung P.H., Suarez S.S. (2012) Alterations to the bull sperm surface proteins that bind sperm to oviductal epithelium. Biol Reprod 87:88. DOI: 10.1095/biolreprod.112.099721.

Januskauskas A., Johannisson A., Rodriguez-Martinez H. (2003) Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. Theriogenology 60:743-58.

Johnson S.K. (2005) Possibilities with today's reproductive technologies. Theriogenology 64:639-56. DOI: 10.1016/j.theriogenology.2005.05.033.

Juyena N.S., Stelletta C. (2012) Seminal plasma: an essential attribute to spermatozoa. J Androl 33:536-51. DOI: 10.2164/jandrol.110.012583.

Kerr J.F., Wyllie A.H., Currie A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239-57.

Koul A., Herget T., Klebl B., Ullrich A. (2004) Interplay between mycobacteria and host signalling pathways. Nat Rev Microbiol 2:189-202. DOI: 10.1038/nrmicro840.

Leeuwenhoek A. (1678) De natis è semine genital animalculis. Philos Trans. 12:1040-1043.

Lessard C., Siqueira L.G., D'Amours O., Sullivan R., Leclerc P., Palmer C. (2011) Infertility in a beef bull due to a failure in the capacitation process. Theriogenology 76:891-9. DOI: 10.1016/j.theriogenology.2011.04.020.

Lin Y.N., Roy A., Yan W., Burns K.H., Matzuk M.M. (2007) Loss of zona pellucida binding proteins in the acrosomal matrix disrupts acrosome biogenesis and sperm morphogenesis. Mol Cell Biol 27:6794-805. DOI: 10.1128/mcb.01029-07.

Liu M. (2011) The biology and dynamics of mammalian cortical granules. Reprod Biol Endocrinol 9:149. DOI: 10.1186/1477-7827-9-149.

Lonergan P. (1994) The application of in vitro fertilization techniques to the prediction of bull fertility. Repro Dom Anim 29:12-21.

Manjunath P., Chandonnet L., Leblond E., Desnoyers L. (1994) Major proteins of bovine seminal vesicles bind to spermatozoa. Biol Reprod 50:27-37.

Marchetti C., Jouy N., Leroy-Martin B., Defossez A., Formstecher P., Marchetti P. (2004) Comparison of four fluorochromes for the detection of the inner mitochondrial membrane potential in human spermatozoa and their correlation with sperm motility. Hum Reprod 19:2267-76. DOI: 10.1093/humrep/deh416.

Marchetti C., Obert G., Deffosez A., Formstecher P., Marchetti P. (2002) Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. Hum Reprod 17:1257-65.

Marti E., Perez-Pe R., Colas C., Muino-Blanco T., Cebrian-Perez J.A. (2008) Study of apoptosis-related markers in ram spermatozoa. Anim Reprod Sci 106:113-32. DOI: 10.1016/j.anireprosci.2007.04.009.

Martin G., Cagnon N., Sabido O., Sion B., Grizard G., Durand P., Levy R. (2007) Kinetics of occurrence of some features of apoptosis during the cryopreservation process of bovine spermatozoa. Human Reproduction 22:380-388. DOI: 10.1093/humrep/de1399.

Martin G., Sabido O., Durand P., Levy R. (2004) Cryopreservation induces an apoptosis-like mechanism in bull sperm. Biol Reprod 71:28-37. DOI: 10.1095/biolreprod.103.024281.

Medeiros C.M., Forell F., Oliveira A.T., Rodrigues J.L. (2002) Current status of sperm cryopreservation: why isn't it better? Theriogenology 57:327-44.

Moce E., Graham J.K. (2008) In vitro evaluation of sperm quality. Anim Reprod Sci 105:104-18. DOI: 10.1016/j.anireprosci.2007.11.016.

Muino R., Fernandez M., Pena A.I. (2007) Post-thaw survival and longevity of bull spermatozoa frozen with an egg yolk-based or two egg yolk-free extenders after an equilibration period of 18 h. Reprod Domest Anim 42:305-11. DOI: 10.1111/j.1439-0531.2006.00784.x.

Nagy S., Johannisson A., Wahlsten T., Ijas R., Andersson M., Rodriguez-Martinez H. (2013) Sperm chromatin structure and sperm morphology: their association with fertility in AI-dairy Ayrshire sires. Theriogenology 79:1153-61. DOI: 10.1016/j.theriogenology.2013.02.011.

O'Connell M., McClure N., Lewis S.E. (2002) The effects of cryopreservation on sperm morphology, motility and mitochondrial function. Hum Reprod 17:704-9.

Oborna I., Fingerova H., Novotny J., Brezinova J., Svobodova M., Aziz N. (2009) Reactive oxygen species in human semen in relation to leukocyte contamination. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 153:53-7.

Okabe M. (2013) The cell biology of mammalian fertilization. Development 140:4471-9. DOI: 10.1242/dev.090613.

Oliveira L.Z., de Arruda R.P., de Andrade A.F., Celeghini E.C., Reeb P.D., Martins J.P., dos Santos R.M., Beletti M.E., Peres R.F., Monteiro F.M., Hossepian de Lima V.F. (2013) Assessment of in vitro sperm characteristics and their importance in the prediction of conception rate in a bovine timed-AI program. Anim Reprod Sci 137:145-55. DOI: 10.1016/j.anireprosci.2013.01.010.

Ombelet W., Robays J.V. (2010) History of human artificial insemination. F, V & V in Obgyn:1-5.

Ortega-Ferrusola C., Sotillo-Galan Y., Varela-Fernandez E., Gallardo-Bolanos J.M., Muriel A., Gonzalez-Fernandez L., Tapia J.A., Pena F.J. (2008) Detection of "apoptosis-like" changes during the cryopreservation process in equine sperm. J Androl 29:213-21. DOI: 10.2164/jandrol.107.003640.

Ott M., Gogvadze V., Orrenius S., Zhivotovsky B. (2007) Mitochondria, oxidative stress and cell death. Apoptosis 12:913-22. DOI: 10.1007/s10495-007-0756-2.

Paoli D., Gallo M., Rizzo F., Baldi E., Francavilla S., Lenzi A., Lombardo F., Gandini L. (2011) Mitochondrial membrane potential profile and its correlation with increasing sperm motility. Fertil Steril 95:2315-9. DOI: 10.1016/j.fertnstert.2011.03.059.

Pena F.J., Johannisson A., Wallgren M., Rodriguez Martinez H. (2003) Antioxidant supplementation in vitro improves boar sperm motility and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. Anim Reprod Sci 78:85-98.

Peter A.T., Colenbrander B., Gadella B.M. (2005) Effect of caspase inhibitors on the post-thaw motility, and integrity of acrosome and plasma membrane of cryopreserved equine spermatozoa. Indian J Exp Biol 43:483-7.

Peter A.T., Linde-Forsberg C. (2003) Efficacy of the anticaspase agent zVAD-fmk on post-thaw viability of canine spermatozoa. Theriogenology 59:1525-32. DOI: S0093691X02012062 [pii].

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

Rasola A., Bernardi P. (2007) The mitochondrial permeability transition pore and its involvement in cell death and in disease pathogenesis. Apoptosis 12:815-33. DOI: 10.1007/s10495-007-0723-y.

Ribas-Maynou J., Garcia-Peiro A., Fernandez-Encinas A., Abad C., Amengual M.J., Prada E., Navarro J., Benet J. (2013) Comprehensive analysis of sperm DNA fragmentation by five different assays: TUNEL assay, SCSA, SCD test and alkaline and neutral Comet assay. Andrology 1:715-22. DOI: 10.1111/j.2047-2927.2013.00111.x.

Rodriguez-Martinez H. (2003) Laboratory semen assessment and prediction of fertility: still utopia? Reprod Domest Anim 38:312-8.

Rodriguez-Martinez H. (2006) Can we increase the estimated value of semen assessment? Reprod Domest Anim 41 Suppl 2:2-10. DOI: 10.1111/j.1439-0531.2006.00764.x.

Rodriguez-Martinez H. (2007) Role of the oviduct in sperm capacitation. Theriogenology 68 Suppl 1:S138-46. DOI: 10.1016/j.theriogenology.2007.03.018.

Saacke R.G. (2008) Sperm morphology: Its relevance to compensable and uncompensable traits in semen. Theriogenology 70:473-8. DOI: 10.1016/j.theriogenology.2008.04.012.

Said T.M., Gaglani A., Agarwal A. (2010) Implication of apoptosis in sperm cryoinjury. Reprod Biomed Online 21:456-62. DOI: 10.1016/j.rbmo.2010.05.011.

Saleem M., Qadir M.I., Perveen N., Ahmad B., Saleem U., Irshad T. (2013) Inhibitors of apoptotic proteins: new targets for anticancer therapy. Chem Biol Drug Des 82:243-51. DOI: 10.1111/cbdd.12176.

Shamsi M.B., Venkatesh S., Tanwar M., Singh G., Mukherjee S., Malhotra N., Kumar R., Gupta N.P., Mittal S., Dada R. (2010) Comet assay: a prognostic tool for DNA integrity assessment in infertile men opting for assisted reproduction. Indian J Med Res 131:675-81.

Silva P.F., Gadella B.M. (2006) Detection of damage in mammalian sperm cells. Theriogenology 65:958-78. DOI: 10.1016/j.theriogenology.2005.09.010.

Simoes R., Feitosa W.B., Siqueira A.F., Nichi M., Paula-Lopes F.F., Marques M.G., Peres M.A., Barnabe V.H., Visintin J.A., Assumpcao M.E. (2013) Influence of bovine sperm DNA fragmentation and oxidative stress on early embryo in vitro development outcome. Reproduction 146:433-41. DOI: 10.1530/rep-13-0123.

Simon L., Lutton D., McManus J., Lewis S.E. (2011) Sperm DNA damage measured by the alkaline Comet assay as an independent predictor of male infertility and in vitro fertilization success. Fertil Steril 95:652-7. DOI: 10.1016/j.fertnstert.2010.08.019.

Smolewski P., Robak T. (2011) Inhibitors of apoptosis proteins (IAPs) as potential molecular targets for therapy of hematological malignancies. Curr Mol Med 11:633-49.

Suarez S.S. (2007) Interactions of spermatozoa with the female reproductive tract: inspiration for assisted reproduction. Reprod Fertil Dev 19:103-10.

Suarez S.S., Pacey A.A. (2006) Sperm transport in the female reproductive tract. Hum Reprod Update 12:23-37. DOI: 10.1093/humupd/dmi047.

Talevi R., Barbato V., Fiorentino I., Braun S., Longobardi S., Gualtieri R. (2013) Protective effects of in vitro treatment with zinc, d-aspartate and coenzyme q10 on human sperm motility, lipid peroxidation and DNA fragmentation. Reprod Biol Endocrinol 11:81. DOI: 10.1186/1477-7827-11-81.

Troiano L., Granata A.R., Cossarizza A., Kalashnikova G., Bianchi R., Pini G., Tropea F., Carani C., Franceschi C. (1998) Mitochondrial membrane potential and DNA stainability in human sperm cells: a flow cytometry analysis with implications for male infertility. Exp Cell Res 241:384-93. DOI: 10.1006/excr.1998.4064.

Tulsiani D.R., Abou-Haila A. (2012) Biological Processes that Prepare Mammalian Spermatozoa to Interact with an Egg and Fertilize It. Scientifica (Cairo) 2012:607427. DOI: 10.6064/2012/607427.

Van Doormaal B. (1993) Linear model evaluations of non-return rates for dairy and beef bulls in Canadian AI. Can. J. Anim. Sci. 73:795-804.

Van Doormaal B. (2013) A.I. market share and most popular sires for 2012. Tech. Bull. Canadian Dairy Network.

VanRaden P.M., Van Tassell C.P., Wiggans G.R., Sonstegard T.S., Schnabel R.D., Taylor J.F., Schenkel F.S. (2009) Invited review: reliability of genomic predictions for North American Holstein bulls. J Dairy Sci 92:16-24. DOI: 10.3168/jds.2008-1514.

Vaux D.L., Korsmeyer S.J. (1999) Cell death in development. Cell 96:245-54.

Vermes I., Haanen C., Steffens-Nakken H., Reutelingsperger C. (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods 184:39-51.

Vilmont V., Tourneur L., Chiocchia G. (2012) Fas-associated death domain protein and adenosine partnership: fad in RA. Rheumatology (Oxford) 51:964-75. DOI: 10.1093/rheumatology/ker402.

Visconti P.E., Bailey J.L., Moore G.D., Pan D., Olds-Clarke P., Kopf G.S. (1995a) Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein tyrosine phosphorylation. Development 121:1129-37.

Visconti P.E., Moore G.D., Bailey J.L., Leclerc P., Connors S.A., Pan D., Olds-Clarke P., Kopf G.S. (1995b) Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Development 121:1139-50.

Vishwanath R. (2003) Artificial insemination: the state of the art. Theriogenology 59:571-84.

Wajant H. (2002) The Fas signaling pathway: more than a paradigm. Science 296:1635-6. DOI: 10.1126/science.1071553.

Walters A.H., Saacke R.G., Pearson R.E., Gwazdauskas F.C. (2005) The incidence of apoptosis after IVF with morphologically abnormal bovine spermatozoa. Theriogenology 64:1404-21. DOI: 10.1016/j.theriogenology.2005.03.011.

Wang M.J., Ou J.X., Chen G.W., Wu J.P., Shi H.J., O W.S., Martin-DeLeon P.A., Chen H. (2012) Does prohibitin expression regulate sperm mitochondrial membrane potential, sperm motility, and male fertility? Antioxid Redox Signal 17:513-9. DOI: 10.1089/ars.2012.4514.

Ward F., Rizos D., Boland M.P., Lonergan P. (2003) Effect of reducing sperm concentration during IVF on the ability to distinguish between bulls of high and low field fertility: work in progress. Theriogenology 59:1575-84.

Waterhouse K.E., Haugan T., Kommisrud E., Tverdal A., Flatberg G., Farstad W., Evenson D.P., De Angelis P.M. (2006) Sperm DNA damage is related to field fertility of semen from young Norwegian Red bulls. Reprod Fertil Dev 18:781-8.

Watson P.F. (2000) The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 60-61:481-92.

Wyllie A.H., Kerr J.F., Currie A.R. (1980) Cell death: the significance of apoptosis. Int Rev Cytol 68:251-306.

Yin Y., Stahl B.C., DeWolf W.C., Morgentaler A. (1998) p53-mediated germ cell quality control in spermatogenesis. Dev Biol 204:165-71. DOI: 10.1006/dbio.1998.9074.

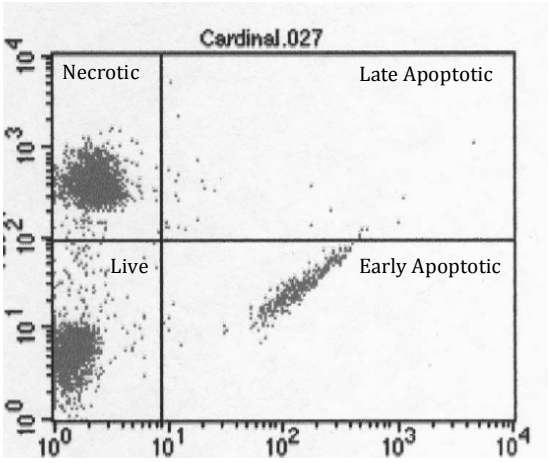
Zhang B.R., Larsson B., Lundeheim N., Rodriguez-Martinez H. (1997) Relationship between embryo development in vitro and 56-day nonreturn rates of cows inseminated with frozen-thawed semen from dairy bulls. Theriogenology 48:221-31.

Zorgniotti A.W. (1975) The spermatozoa count. A short history. Urology 5:673-4.

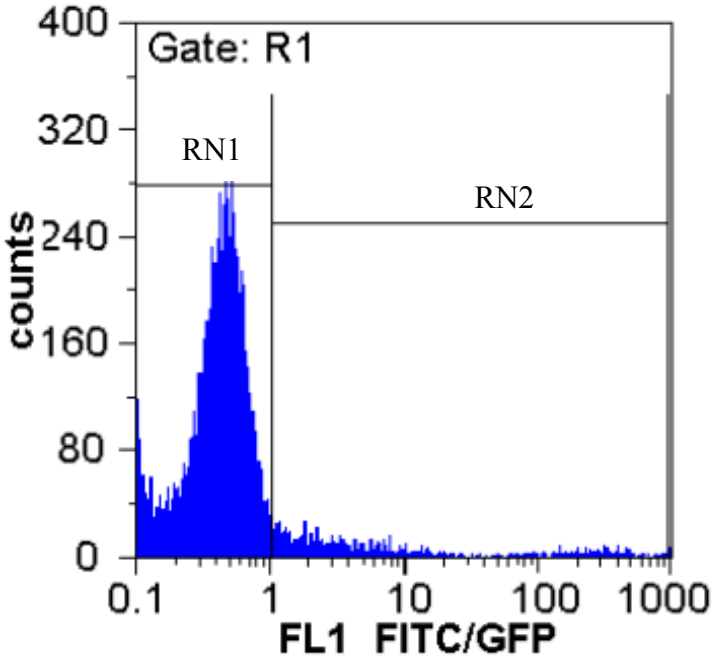
APPENDICES

A. Supplementary materials from Chapter 4

Appendix A.1 Typical two-dimensional flow cytometric histogram of FITC-Annexin V/PI. Quadrants represent various sperm populations.



Appendix A.2 Typical one-dimensional flow cytometric histogram of sperm distribution based on TUNEL staining, representing sperm with nicked DNA. Regions RN1incidate sperm with intact DNA (TUNEL negative) and RN2 indicates sperm with nicked DNA (TUNEL positive).



B. Media recipes from Chapter 4

Appendix B.1 Culture Media and Solutions for IVF protocol

TCM Media

Preparation volume (ml)	50 mL
TCM 199 (ml)	47.5
CS (ml)	2.5
FSH solution (μl)	12.5
LH solution (μl)	50
Gentamycin solution (μl)	50

BO Solution

Preparation volume (ml)	100mL
Stock solution A (ml)	76ml
Stock solution B (ml)	24ml
Sodium pyruvate (g)	0.01375g
Gentamycin solution (μl)	100μl

Sperm Washing Solution

Preparation volume (ml)	20ml
BO Solution (ml)	20ml
Hypotaurin (g): (antioxidant)	0.0218g
Heparin sodium solution (μl): (capacitation)	100 μl

Oocyte Washing Solution

Preparation volume (ml)	30ml
BO Solution (ml)	30ml
BSA (crystalized and lyophilized)	0.3g

Sperm Diluting Solution

Preparation volume (ml)	10ml
BO Solution (ml)	10ml
BSA (crystalized and lyophilized)	0.2g

BO Stock Solution A

Preparation volume (ml)	200ml
Sodium bicarbonate: $NaHCO_3$(g)	2.5873g
0.5% Phenol Red (µl)	40µl

BO Stock Solution B

Preparation volume (ml)	500 ml
Sodium chloride : NaCl (g)	4.3092g
Potassium chloride : KCl (g)	0.1974g
Calcium chloride dihydrate: $CaCl_2 \cdot 2H_2O$(g)	0.2171g
Sodium dihydrogen phosphate dihydrate: $NaH_2PO_4 \cdot 2H_2O$(g)	0.084g
Magnesium chloride hexahydrate: $MgCl_2 \cdot 6H_2O$(g)	0.0697g
0.5% Phenol Red (µl)	100 µl

45% Percoll Solution

Preparation volume (ml)	10ml
BO Solution (ml)	5ml
90 % Percoll Solution (ml)	5ml

10x BO Stock Solution A (for Percoll solution)

Preparation volume (ml)	50 ml
Sodium chloride : NaCl (g)	4.3092g
Potassium chloride : KCl (g)	0.1974g
Calcium chloride dihydrate: $CaCl_2 \cdot 2H_2O$(g)	0.2171g
Sodium dihydrogen phosphate dihydrate: $NaH_2PO_4 \cdot 2H_2O$(g)	0.084g
Magnesium chloride hexahydrate: $MgCl_2 \cdot 6H_2O$(g)	0.0697g
0.5% Phenol Red (μl)	100 μl

CR1aa Media

Preparation volume (ml)	50ml
Solution A (ml)	38ml
Solution B (ml)	10ml
BME Essential Amino Acids (50x) (ml)	1ml
MEM Nonessential Amino Acids (100x) (ml)	0.5ml
L-Glutamic acid (ml)	0.5ml
Bovine Serum Albumin (Fatty Acid-free) (g)	0.15g
Gentamycin solution (µl)	50µl

CR1aa + 5% CS

Preparation volume (ml)	50 ml
CR1aa (ml)	47.5 ml
CS (ml)	2.5 ml

CR1aa Stock Solution A

Preparation volume (ml)	760 ml	500 ml	250 ml	100 ml
Sodium chloride : NaCl (g)	6.7031g	4.4099g	2.2050g	0.8820g
Potassium chloride : KCl (g)	0.2311g	0.1520g	0.0760g	0.0304g
Sodium pyruvate (g)	0.0440g	0.02895g	0.0145g	0.0058g
Sodium bicarbonate : NaHCO3 (g)	2.2011g	1.4481g	0.7240g	0.2896g
0.5% Fenol Red (ml)	2 ml	1.3158 ml	0.6579 ml	0.2632 ml
HEPES(g)	0.4529g	0.2979g	0.1490g	0.0596g

CR1aa Stock Solution B

Preparation volume (ml)	200 ml	40 ml	20 ml
L(+)-Lactic acid Hemicalcium Salt (g)	0.5996	0.11992	0.05996

L-Glutamic acid solution

Preparation volume (ml)	30 ml
L-Glutamic acid (mg)	60mg

FSH (Folltropin) preparation for IVM

Folltropin: 400mg FSH/ vial

1. Add 80 mL TCM 199 (or 0.9% Nacl salin) to Folltropin (5000 µg/ml, 5 µg/µl)
2. Mix the solution by inverting several times
3. Filter (0.22 µm)-sterilize
4. Dispense into tubes
5. Store frozen

Final concentration: 0.5 µg/ml

Total IVM medium (ml)	**FSH solution**
10 ml	10 µl
30 ml	30 µl
50 ml	50 µl

LH (Lutropin-V) preparation for IVM

1. Add 5 ml TCM199 to LH (Lutropin, Lot#:E092A)
2. Mix the solution by inverting the container several times
3. Filter (0.22 µm)- sterilize
4. Dispense into tubes
5. Store frozen

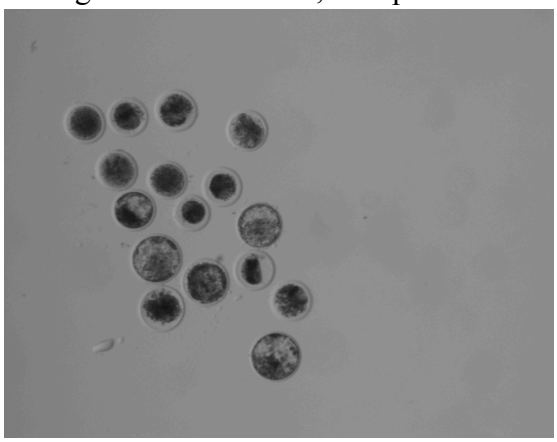
At the time of using:
Stock solution: 5000 µg/ml
Final concentration: 5 µg/ml

Total IVM medium (ml)	**LH solution**
10 ml	10 µl
30 ml	30 µl

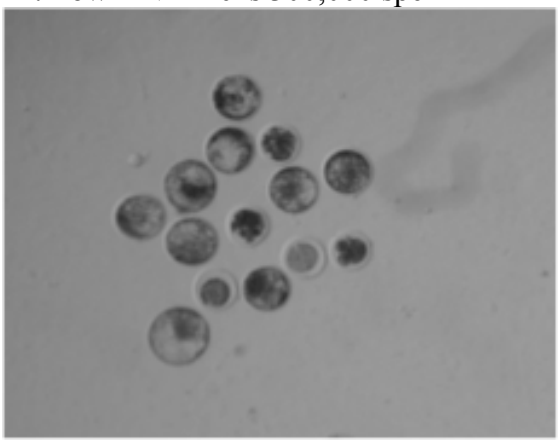
C. Supplementary information from IVF trial

Appendix C.1 Day 8 blastocyst development A)High DNA nick, 300,000 sperm per IVF droplet B) low DNA nick, 300,000 sperm C) high DNA nick, 30,000 sperm per IVF droplet D) low DNA nick, 30,000 sperm per IVF droplet.

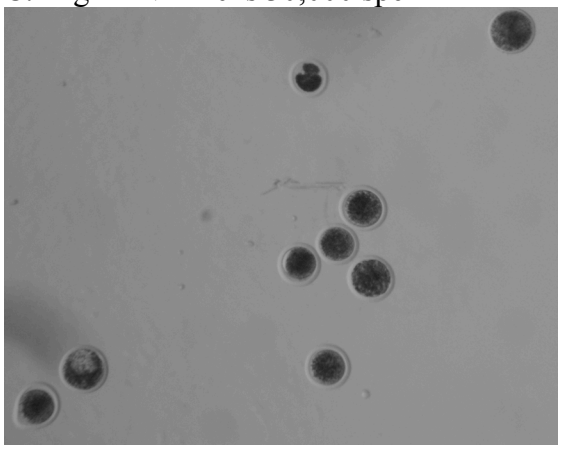
A. High DNA nicks 300,000 sperm



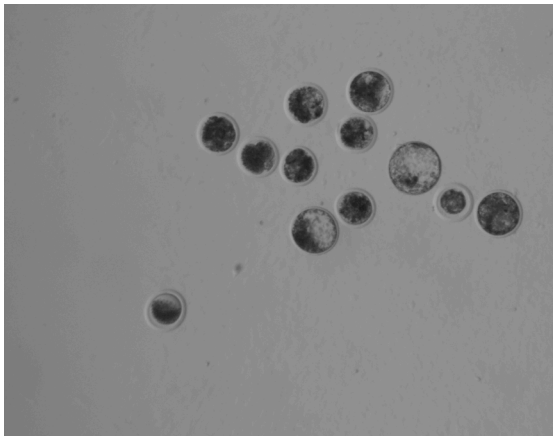
B. Low DNA nicks 300,000 sperm



C. High DNA nicks 30,000 sperm



C. Low DNA nicks 30,000 sperm



Data including hatched blastocyst rates not included in Experiment #3 data

Appendix C.2 Cleavage, blastocyst and hatched blastocyst rates in low and high DNA nick semen samples with two sperm concentrations (30,000 and 300,000 sperm per IVF droplet).

DNA nick/sperm concentration	COC's (n)	Cleavage Rate n (%)	P-Value	Blastocyst rate n (%)	P-Value	Hatched Blastocyst rate n (%)	P-Value
Low DNA nick 30,000	176	112 (64%)		57 (51%)		2 (1.8%)	
High DNA nick 30,000	177	60 (34%)	P<<0.001	19 (32%)	P<0.05	0 (0%)	NS
Low DNA nick 300,000	182	139 (76%)		67 (48%)		18 (13%)	
High DNA nick 300,000	169	112 (66%)	P<0.05	47 (42%)	NS	2 (1.8%)	P<0.01

D. Supplementary materials from Chapter 5

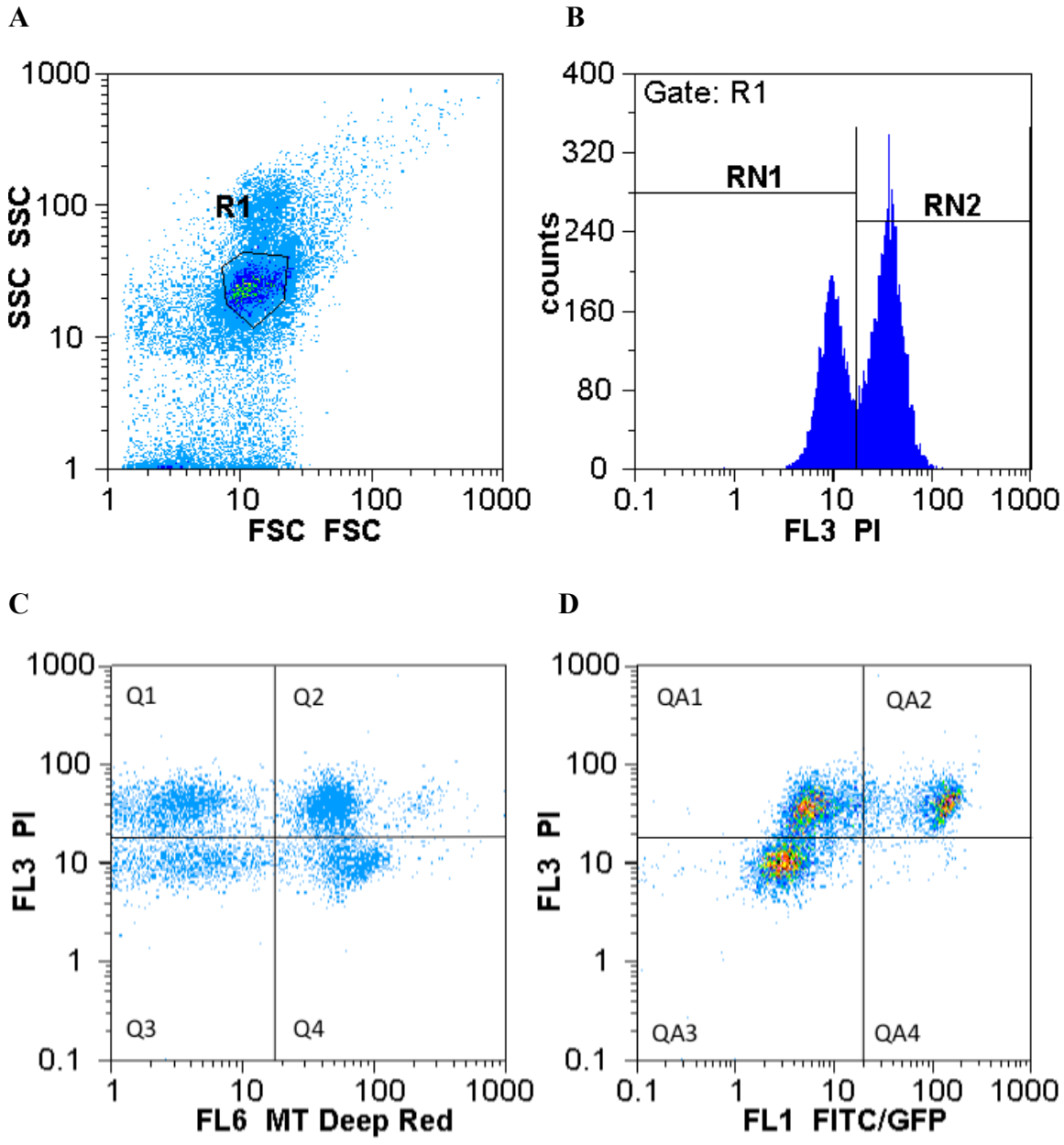
Appendix D.1 Tris-Citric Acid Buffer for sperm incubation

Product	g/100ml
Tris Base	2.42
Citric acid monohydrate	1.4
Fructose	1.0

Appendix D.2 Fluorescent Markers for Bull Sperm: Flow Cytometric Analysis

Fluorescent dyes	Stock Solution	Working Solution	Dilution in Semen	Final Concentration
Propidium Iodide	2.4mM in H_20	-	5 µl/ml (201x)	12 µM
Mito-tracker Deep Red	1mM (50 µg/vial in 92 µl DMSO)	2 µl + 18 µl DMSO [0.1 mM]	2 µl/ml (201x)	200 nM
FITC-PNA	1mg/ml in PBS	-	1 µl/ml (201x)	1 µg/ml

Appendix D.3 Flow cytometric figures. **A)** Isolation of sperm specific events, based on forward and side scatters (R1). **B)** Sperm populations with intact membranes (RN1) and sperm population with damaged membranes (RN2). **C)** MtDR/PI assay; Q1: dead sperm with low ΔΨm, Q2: dead sperm with high ΔΨm, Q3: live sperm with low ΔΨm, Q4: live sperm with high ΔΨm. **D)** FITC-PNA/PI assay; QA1: dead sperm with intact acrosomes, QA2: dead sperm with compromised acrosomes, QA3: live sperm with intact acrosomes, QA4: live sperm with compromised acrosomes.



Tables of raw data from Study 2

Appendix D.4 Effect of apoptosis inhibitors on sperm motility (%). Mean ± SEM.

	Time (h)			
Treatment	**0**	**3**	**6**	**12**
Untreated Control	57.7 ± 4.3	49.8 ± 5.8	25.9 ± 5.7	0.31 ± 0.3
Treated Control	53.7 ± 4.9	48.6 ± 2.8	39.4 ± 3.4	16.1 ± 5.4
Bax 5	49.9 ± 4.9	46.3 ± 2.5	34.3 ± 3.6	9.5 ± 4
Bax 10	52 ± 3.9	46.7 ± 4.1	36.1 ± 3.2	6.2 ± 2.1
VAD 5	52.8 ± 3.5	46.6 ± 2.7	32.3 ± 5.6	11.3 ± 4.9
VAD 10	55.5 ± 4.4	45.6 ± 2.8	32.3 ± 5.6	9.3 ± 4.1
Q10 5	49.3 ± 3.5	43 ± 2.3	33.9 ± 4.2	11.6 ± 4.4
Q10 50	46.4 ± 5	46.1 ± 2.7	36.5 ± 3.3	11.0 ± 4.7
XIAP 50	54.2 ± 3.2	48.6 ± 2.9	37.9 ± 4.2	16.3 ± 6.5
XIAP 100	54.4 ± 3.1	48.7 ± 2.8	41.4 ± 3.4	17.1 ± 6.6

Appendix D.5 Effect of apoptosis inhibitors on progressively motile sperm (%). Mean ± SEM.

	Time (h)			
Treatment	**0**	**3**	**6**	**12**
Untreated Control	49.3 ± 4.5	39.1 ± 6.2	15.3 ± 4.8	0.1 ± 0.1
Treated Control	46.1 ± 4.4	41.0 ± 3.5	30.6 ± 4.1	9.8 ± 4.3
Bax 5	45.0 ± 4.2	39.9 ± 2.7	25.0 ± 4.6	5.0 ± 2.9
Bax 10	47.7 ± 4.1	40.1 ± 4.4	26.2 ± 5.0	2.0 ± 0.7
VAD 5	46.5 ± 3.4	40.5 ± 2.4	25.6 ± 5.4	6.8 ± 4.1
VAD 10	47.3 ± 3.5	38.6 ± 2.7	23.6 ± 5.6	5.2 ± 3.3
Q10 5	43.2 ± 3.5	36.1 ± 2.5	26.9 ± 4.5	5.7 ± 2.8
Q10 50	39.7 ± 4.4	39.5 ± 2.2	29.2 ± 3.5	5.3 ± 3.4
XIAP 50	47.5 ± 2.4	40.7 ± 3.7	30.9 ± 4.8	10.9 ± 5.3
XIAP 100	48.0 ± 2.8	41.0 ± 3.4	33.69 ± 4.1	11.3 ± 5.1

Appendix D.6 Effect of apoptosis inhibitors on sperm curvilinear velocity (μ/sec). Mean ± SEM.

	Time (h)			
Treatment	**0**	**3**	**6**	**12**
Untreated Control	103.1 ± 5.7	83.9 ± 8.6	51.0 ± 4.6	3.6 ± 3.6
Treated Control	141.3 ± 6.7	121.1 ± 12.8	107.0 ± 14.0	69.1 ± 14.2
Bax 5	139.9 ± 4.5	129.9 ± 10.3	107.4 ± 9.1	51.6 ± 14.1
Bax 10	148.7 ± 6.1	130.1 ± 9.3	103.5 ± 11.8	59.0 ± 12.7
VAD 5	142.5 ± 5.3	129.8 ± 10.7	98.6 ± 12.9	57.1 ± 18.8
VAD 10	138.7 ± 6.1	123.4 ± 8.9	101.8 ± 11.6	50.3 ± 14.3
Q10 5	142.9 ± 8.3	122.4 ± 9.4	107.6 ± 12.4	69.6 ± 9.5
Q10 50	148.5 ± 6.1	124.9 ± 9.4	112.0 ± 11.8	52.3 ± 17.7
XIAP 50	138.3 ± 7.3	125.4 ± 10.2	105.5 ± 11.2	69.8 ± 15.2
XIAP 100	147.2 ± 6.9	125.8 ± 11.1	118.2 ± 10.2	78.1 ± 14.1

Appendix D.7 Effect of apoptosis inhibitors on sperm with higher mitochondrial membrane potential (%). Mean ± SEM.

	Time (h)			
Treatment	**0**	**3**	**6**	**12**
Untreated Control	4.6 ± 1.3	4 ± 1.3	1.9 ± 0.9	0.9 ± 0.4
Treated Control	43.5 ± 1.8	43.4 ± 1.7	44.2 ± 1.1	43.5 ± 1.2
Bax 5	42.2 ± 1.6	43.7 ± 1.6	42.7 ± 1.2	43.5 ± 1.3
Bax 10	43.4 ± 1.4	43.2 ± 1.6	42.8 ± 1.4	43.4 ± 1.4
VAD 5	43.9 ± 2.0	42.9 ± 1.6	42.6 ± 1.5	43.8 ± 1.4
VAD 10	44.1 ± 1.7	42.8 ± 1.5	42.2 ± 1.3	44.2 ± 1.3
Q10 5	43.7 ± 1.3	43.2 ± 1.7	43.0 ± 1.1	43.6 ± 1.5
Q10 50	43.9 ± 1.9	43.8 ± 2.2	44.0 ± 1.4	44.6 ± 1.4
XIAP 50	44.0 ± 1.6	43.8 ± 1.7	42.6 ± 1.4	43.9 ± 1.5
XIAP 100	44.3 ± 1.6	41.8 ± 2.3	43.8 ± 0.9	43.2 ± 1.4

Appendix D.8 Effect of apoptosis inhibitors on live sperm (%). Mean ± SEM.

	Time (h)			
Treatment	**0**	**3**	**6**	**12**
Untreated Control	47.9 ± 6.0	37.1 ± 6.2	31.9 ± 7.5	28.2 ± 5.3
Treated Control	44.5 ± 4.0	43.0 ± 4.3	31.9 ± 4.1	26.8 ± 4.2
Bax 5	39.1 ± 4.6	38.5 ± 3.4	26.9 ± 4.3	25.0 ± 4.7
Bax 10	41.2 ± 3.5	38.5 ± 3.0	29.9 ± 3.1	24.8 ± 4.1
VAD 5	41.6 ± 3.9	36.2 ± 2.9	27.8 ± 4.3	25.6 ± 4.2
VAD 10	39.8 ± 3.8	34.3 ± 3.5	30.4 ± 3.7	25.5 ± 4.36
Q10 5	40.6 ± 3.3	35.8 ± 3.4	31.6 ± 3.9	24.7 ± 4.6
Q10 50	40.2 ± 4.0	34.9 ± 4.4	28.2 ± 4.7	24.7 ± 5.4
XIAP 50	40.8 ± 3.8	37.8 ± 3.8	29.0 ± 4.5	25.2 ± 5.2