COMPARATIVE EVALUATION OF DIFFERENT EXTENDERS OF BULL SEMEN STORED UNDER DIFFERENT CONDITIONS

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DECLARATION

I, **Andrea Motswetla Raseona**, the undersigned, hereby declare that this dissertation submitted in fulfilment of the requirements for the Masters of Science in Animal Science degree at the University of Venda is my own work and has not been previously in part or in its entirely been submitted to any university for any other degree, and that all reference material contained therein has been duly acknowledged.

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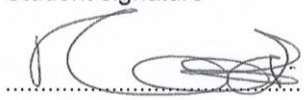

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A.M Raseona

DEDICATION

I dedicate this dissertation to my family. As no one in my family has had a chance to study this far besides myself, I really consider myself blessed and anointed. I thank them all for their constant support and challenge the young generation to surpass my endeavours.

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ABSTRACT

Preservation of semen is an important process to ensure that semen quality is sufficient for use in assisted reproductive technologies. This study evaluated the effectiveness of three different extenders to preserve bull semen stored under different conditions, as an alternative to frozen-thawed semen straws used for artificial insemination. Semen samples were collected from two Nguni bulls using an electro-ejaculator and transported to the laboratory at 37 °C for evaluation. Pooled semen was first aliquoted into three extenders namely Triladyl, Ham's F10 and M199 at a dilution ratio of 1:4 (semen:extender), and then stored at controlled room temperature 24 °C. Secondly pooled semen was aliquoted into four groups of Ham's F10 extender and diluted at a ratio of 1:4, then stored at 24 °C, 17 °C, 12 °C and 5 °C respectively. Sperm motility rates were analysed after 0, 24, 48 and 72 hours. Morphology, viability and sperm DNA fragmentation were analysed after 72 hours. The study was replicated four times and data was analysed by ANOVA. Triladyl had higher sperm viability rate and total motility rate for 72 hours ($P<0.01$). However, Ham's F10 had higher progressive motility rate as compared to the other extenders. There was no significant difference ($P<0.01$), in the viability rate between Ham's F10 and M199. No significant difference was also observed in total sperm abnormalities (absent tails, coiled tails and bent tails), except for reacted acrosomes ($P>0.05$), between the two Nguni bulls. Lower temperatures than 24 °C influenced sperm motility and viability in Ham's F10. There was no significant difference in sperm DNA fragmentation rates ($P<0.01$), between all the four storage temperatures which indicated that temperature did not have an influence on sperm DNA fragmentation. In conclusion, bull semen can be preserved in Triladyl or Ham's F10 and M199 culture media stored at 24 °C and stay alive for 72 hours. Triladyl proved to be the best suitable extender showing higher sperm viability and total motility rates as compared to Ham's F10 and M199. Lower temperatures than 24 °C noticeably decreased sperm motility and viability in Ham's F10 culture medium.

Key words: Bull semen, Triladyl, Ham's F10, M199, Sperm DNA fragmentation, Viability.

TABLE OF CONTENTS

CONTENTS **PAGE**

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS USED IN THIS DISSERTATION

AI : Artificial Insemination
ART : Assisted reproductive technology
ANOVA : Analysis of variance
ATP : Adenosine triphosphate
BCF : Beat cross frequency
BSA : Bovine serum albumin
BUSgp60 : Bulbourethral secretion glycoprotein-60
Ca : Calcium
CASA : Computer aided sperm analysis
CHO : Chinese hamster ovary
DNA : Deoxyribonucleic acid
EYCE : Egg yolk coagulating enzyme
FBS : Fetal bovine serum
FSH : Follicle Stimulating Hormone
IVF : In vitro fertilization
IVM : In vitro maturation
K : Potassium
LIN : Linearity
LN_2 :Liquid nitrogen
LPC : Lysophophatidycholine
Na : Sodium
NADH : Nicotinamide Adenine Dinucleotide
PC : Phosphatidycholine
PM : Progressive motility
RAP : Rapid motility
ROS : Reactive oxygen species
SCA : Sperm class analyser
SCD : Sperm chromatin decondensation
SCSA : Sperm chromatin structure assay
SDF : Sperm DNA fragmentation
SEM : Standard error mean
STR : Straightness

TCM-199 : Tissue culture medium 199

TM : Total motility

VAP : Average path velocity

VCL : Curvilinear velocity

VSL : Straight line velocity

WOB : Wobble

CHAPTER 1

1. INTRODUCTION

1.1. Background of the study

Semen is an ejaculate from the male animal's penis containing spermatozoa suspended in the fluid secretions of the three male accessory sex glands (Dean, 2005; Frandson *et al.,* 2009).The fluid portion of semen is called seminal plasma which functions primarily as a transport medium for spermatozoa. Seminal plasma contains a variety of substances that includes various electrolytes, fructose, citric acid and sorbitol. Fructose serves as the potential source of energy for the spermatozoa. Semen is collected and evaluated as part of the protocol to evaluate fertility of a male animal. Some characteristics of semen that are evaluated and appear to have some correlation with the fertility of a male animal when considered together are: sperm concentration per milliliter of semen, sperm motility parameters and morphology (Frandson *et al.,* 2009).

Spermatozoa motility is one of the most important characteristics measured during analysis of semen and generally is performed in a subjective way using a light microscopic evaluation. Computer aided sperm analysis (CASA) is an objective method that gives widespread information about the kinetic property of the male ejaculate based on measurements of the individual sperm cells (Januškauskas & Žilinskas, 2002). CASA is a system in which several parameters like concentration, motility and morphology can be analyzed by processing digital images of the spermatozoa (Kathiravan *et al.,* 2011).

After semen collection from mammalian species, the ejaculates are diluted in media called semen extenders that provide spermatozoa with nutrients as a source of energy, prevent microbial growth, and protect cells against damage from cold shock, and changes in pH and osmotic pressure. Semen collected from farm animals and used for artificial insemination is either extended to be used as fresh semen within several hours post collection or frozen to be used at a later date (Gadea, 2003). In order to use liquid semen for artificial insemination, diluted semen need to have a minimum shelf-life of 2 to 4 days to provide for easy transport and use in distant locations (Salisbury & Van Dermark, 1961b).

The primary purpose of extending semen is to maximize the number of females that can be inseminated with a single ejaculate. Cryopreservation of bovine semen is practiced worldwide for many reasons, but the use of liquid semen has its own advantages. The main benefit of using liquid semen is

that high conception rates are achieved with relatively lower number of sperms (Anzar *et al.,* 2003). The much higher sperm number required for frozen-thawed than for fresh semen is due to the detrimental effect of the freezing and thawing procedure on semen quality (Holt, 2000). The freezing and thawing of spermatozoa result in more reduced rates of sperm motility and fertilizing capacity when compared to liquid storage at 5 ^{o}C (Christensen *et al.,* 2000). Cooled bull sperm, however, suffers from a decrease in motility and membrane integrity, followed by a reduction in fertility for extending days of liquid storage (e.g. 12 days of storage) (Garner & Johnson, 1995; Bredbacka *et al.,* 1997).

Artificial insemination (AI) has proven to be the most effective tool for genetic improvement of animals of zootechnical importance, especially in the cattle industry (Bailey *et al*., 2000; Vishwanath & Shannon, 2000). As the most frequently used assisted reproductive technology (ART) today, AI had and still continues to have a remarkable impact on various animal breeding industries (Althouse, 2007). Through AI one ejaculate from a genetically superior male can be used to impregnate multiple females to maximize the distribution of mammalian favourable genes. AI also eliminates physical contact between animals, thus limiting the spread of sexually transmitted diseases (Bailey *et al*., 2000). AI also extends the usefulness of proven sires that for some physical reasons or age are unable to mate normally (Bale, 2003).

Successful liquid storage of spermatozoa is dependent on the reversible reduction of survival and metabolic activity of spermatozoa. This can be achieved by providing effective environmental conditions for the cooled semen and the use of beneficial extenders that maintain motility, DNA integrity and fertilizing ability of spermatozoa during liquid storage (Garner & Johnson, 1995; Fazeli *et al.,* 1997).
Livestock and crop farming are the major forms of agriculture in the Limpopo province, with communal cattle farming enterprises comprising approximately 50% of the farming in remote areas of the Vhembe district (Stroebel *et al.,* 2011). Production is primarily for subsistence purposes with very little marketable surplus. Furthermore, the livestock animals namely cattle, goats and sheep show clear signs of past inbreeding due to lack of selection of phenotypically superior bulls for breeding and poor management practices. This has resulted in lower reproduction rates which have led to poverty in this community.

Provision of improved livestock genes into a rural livestock production system can play an important role in changing the poor livestock farming in rural areas focused on household subsistence (Lebbi & Kagwini, 1996), into viable commercial enterprises that are open to the global market. Artificial insemination program can be the precise reproductive technology to improve genetic material of the

livestock animals in the community of Vhembe. It is however, a challenge to implement such a program in the community due to certain limitations such as the availability of liquid nitrogen, lack of storage of semen tanks, and the cost of those semen tanks. Thus, production of high quality extended bull semen stored at refrigerated temperatures for use in AI purposes will greatly benefit the rural community of Vhembe district.

Reproduction rates will increase leading to better production of livestock animals with superior genetic value. Animal genetic resources will be conserved and the indigenous livestock gene pool diversity will also be maintained. The economic value of the rural community of Vhembe will also improve as there would be an increase in livestock production with superior genetic value, for marketing. Ultimately poverty will be alleviated and the living conditions of the rural community of Vhembe will also improve.

1.2. Problem statement

Artificial insemination with frozen-thawed semen in the isolated rural areas in South Africa is not the optimal procedure, because of the not readily availability of liquid nitrogen (LN_2), safe storage of LN_2 tanks for genetic banking, and other factors such as the high cost of LN_2 tanks which are also vulnerable.

The livestock animals in Vhembe district, namely cattle, goats and sheep still shows clear signs of past inbreeding which results in low production. The indications include small body size, slow average daily weight gain, decreased fertility, extended calving intervals, and loss of natural resistance against diseases and parasites.

1.3. Justification

Deployment of extended freshly collected bull semen stored at a refrigeration temperature for artificial insemination programs in cows in the Vhembe district could be a valuable tool to maximize utilization of superior bulls between producers in fairly close proximity. In species where sperm survival and fertility rates after cryopreservation are poor (porcine and equine), the use of refrigerated spermatozoa is widely spread in genetic improvement practices. This alternative has been mostly overlooked in the bovine species.

1.4. Study objectives

1.4.1. Broad objective of the study:

i. The purpose of this study was to evaluate the effectiveness of different bull semen extenders stored under refrigerated temperatures as an alternative to frozen-thawed semen straws.

1.4.2. Specific objectives of the study:

i. To test different extenders and sperm protectors to keep the spermatozoa viable for more than 24 hours.
ii. To test different refrigerated storage temperatures for extended semen.

1.5. Hypothesis

i. Bull spermatozoa cannot be kept alive and viable for longer periods than 24 hours by extending the semen with M199, Ham's F10 culture media or Triladyl.

CHAPTER 2

2. LITERATURE REVIEW

2.1. Bull spermatozoa

Spermatozoa are unique specialised cells that are well designed to accomplish a single objective which is to fertilise an ovum (Hafez, 1993). Spermatozoa are formed within the seminiferous tubules of the testes. After production, they are released into the rete testis and enter the epididymis. During their transportation through the epididymis, spermatozoa obtain fertilizing capacity and are then stored at the cauda epididymis until ejaculation. The fully formed spermatozoa are elongated cells consisting of a flattened head containing the nucleus and a tail containing the apparatus necessary for cell motility.

2.2. Spermatozoa metabolism

The main function of spermatozoa is to fertilize oocytes, and to spread genetic material distant from where they are produced. Spermatozoa are one of the smallest cells in the body, and do not possess energy storage (Varner & Johnson, 2007). Spermatozoa use adenosine triphosphate (ATP) as the basic source of energy and require a constant supply for cell function and survival. Energy demands differs depending on spermatozoa activity, such as; hyperactivation, acrosome reaction, sperm-oocyte fusion, and motility (Miki, 2007). To meet energy supply demands, spermatozoa rely on an extracellular substrate. Spermatozoa primarily utilize carbohydrates to meet their energy requirements and the carbohydrates that are metabilizable cross the plasma membrane using protein transporters (ATP-dependent process). Spermatozoa are able to produce ATP through oxidative phosphorylation, however, glycolysis can be considered the central metabolic pathway for energy supply (Miki, 2007; Varner & Johnson, 2007). Glucose metabolized through glycolysis produces two molecules of pyruvate (ATP and NADH). Pyruvate is then oxidized by mitochondria to produce 36 ATP molecules (Miki, 2007). Bull and ram spermatozoa readily metabolize fructose using protein transporters other than the glucose protein transporter (Hiipakka & Hammerstedt, 1978).

2.3. Characteristics and quality of bull semen.

It is commonly accepted that there is a correlation between semen fertility and its measurable properties. The ultimate goal of semen evaluation is to find one or few parameters to predict the fertilising ability of the spermatozoa. Various methods have been evaluated, but only few methods have been adopted for practical work. Most of these studies have used light microscopic evaluations of classical spermatozoa parameters that include sperm concentration, motility, morphology and viability (Januškauskas & Žilinskas, 2002). The relationship of semen quality to fertility is the concept of compensable and uncompensable sperm quality traits and their interactions with numbers of sperm per dose (Salisbury & Van Demark, 1961a; Saacke, 1998).

If the number of sperm per AI dose is increased based on measurement of a compensable semen trait, there is an increase in fertility. However, the increase in fertility is not proportionate over the entire range of sperm dosages. Compensable semen quality traits are generally believed to be associated with measures of spermatozoa viability (i.e., motility, acrosomal integrity, cell membrane integrity, etc.). Conversely, if the number of sperm per AI dose is increased based on measurement of an uncompensable semen trait, there is no increase in fertility with additional sperm beyond that associated with a compensable trait that might be added at the same time (Januškauskas & Žilinskas, 2002).

2.4. The use of bull semen for artificial insemination

The primary use of bull semen is breeding either through natural or is collected and used in AI. Artificial insemination is the most frequently used assisted reproductive technology (ART) today and still continues to have a remarkable impact on various animal breeding industries (Althouse, 2007). AI has many advantages that include the prevention of sexually transmitted diseases, easy transport of genetic material and optimal use of superior sires that for some reasons or age are unable to mate normally (Bale, 2003).Through AI one ejaculate from a genetically superior male can be used to impregnate multiple females to maximize the distribution of mammalian favourable genes. Estrus synchronization of large groups of females can be inseminated at the same to simplify herd management before and after the offspring are born. The use of AI also reduces the cost of bulls required for breeding, maintenance expenditures and prevents overuse of males (Heise, 2012). AI semen is usually collected and evaluated before it is used either directly or stored at different lengths of time.

2.5. Evaluation of bull semen

Male fertility is an imperative factor in bovine reproduction as a single bull can be used to breed numerous cows, especially after the introduction of artificial insemination (AI) technology. Assessment of male animal fertility is primarily based on semen evaluation using parameters such as sperm motility, morphology, viability, biochemical estimations of enzyme release, membrane acrosome integrity (Kathiravan *et al.,* 2011) and DNA integrity (Januškauskas & Žilinskas, 2002).

2.5.1. Spermatozoa motility

Motility is considered to be the most important characteristic associated with the fertilizing ability of spermatozoa and is an expression of their viability and structural integrity (Kathiravan *et al.,* 2011). Motility can be divided into quantitative (percentage of sperm cells with a progressive motility) and qualitative motility. Qualitative motility involves several different parameters, some of which are the speed of the moving sperm cells, altitude of head displacement and movement pattern (circular vs. linear movement, total distance vs. progression etc.) (Januškauskas & Žilinskas, 2002). The traditional evaluation of spermatozoa motility using light microscopes has been very subjective because of the great variability of assessment that existed among observers. To overcome the subjective assessment, application of a computer aided sperm analysis (CASA) system offers an objective and rapid approach to assess spermatozoa motility (Malmgren, 1997; Januškauskas & Žilinskas, 2002).

In addition to the total and progressive motility, the CASA system measures other spermatozoa motion characteristics such as straight-line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), linearity (LIN), straightness (STR), wobble (WOB), and beat cross frequency (BCF). The CASA system consists of an objective microscope attached to a video camera, a video frame grabber card and a computer (Mortimer, 2000; Kathiravan *et al.,* 2011). The computer software is used to identify and follow all of the spermatozoa in the video images and to perform all of the data calculations. First, the image of the microscope field is sent from the camera and it is converted into a digital image (Mortimer, 2000).

To perceive spermatozoa easily, the CASA system uses a dark field or a negative high phase contrast image, which shows white sperm heads on a dark background. The brightness of the head image stays moderately consistent even when the spermatozoa move because the rotation of their heads does not

change the intensity of the white image (Mortimer, 2000; Kathiravan *et al.,* 2011).The image of each sperm head is then digitized, with the computer determining the number of picture elements (pixels) the sperm head covers (Mortimer, 2000). In fluorescent optics microscopy, the sperm head is identified by staining it with a fluorescent dye that binds to the sperm DNA (Kathiravan *et al.,* 2011).

2.5.2. Sperm morphology

Concentration and morphology assessment of sperm is based on the direct relation between the incidence of abnormal spermatozoa and the type of certain morphological defects with the *in vivo* fertility of the bull (Söderquist *et al.,* 1991). Morphological assessment of spermatozoa is accepted to be a more significant method of differentiating between semen of high and low fertilizing capacity (Ball & Poters, 2004). Before bulls are used in a progeny testing program or semen preservation, accurate morphological evaluation of the ejaculates is preformed to eliminate bulls with a low fertility potential, thus contributing to a major savings for AI enterprises. There is certainly a correlation between motility and fertility as well as for morphology and fertility, provided there is a wide range of variation in the quality of the parameters assessed as well as fertility obtained with that semen. However, these correlations are reduced concurrently with an increase in the lower limit set to accept an ejaculate for further processing. When the acceptable range for these parameters is narrow, motility and gross morphology only have limited value for separating ejaculates in respect to the expected fertility of the ejaculate (Januškauskas & Žilinskas, 2002).

Evaluation of sperm morphology using light microscopy by human eye is very subjective and different technicians often get different results on the same series of smears (Amann, 1981). The use of computerized methods focused on evaluating the measurements to quantify and classify sperm morphology correctly, offers repeatable and objective method of assessing bull sperm head morphometry within and between technicians. Measurements of sperm head variables such as area, length, width and perimeter have shown promise in the computerized analysis of bull sperm head morphology (Gravance *et al.,* 1999).

2.5.3. Sperm DNA Fragmentation

Sperm DNA fragmentation (SDF) is the physical breaking of one or both DNA strands in sperm chromosomes. It occurs when there is an alteration to the bases or a physical break in one or both of the DNA strands. If this occurs within a gene needed for embryo growth and is not repaired, the

consequence may be the death of the embryo. SDF has been advocated as a male fertility indicator (Bungum *et al.,* 2011). Evaluation of sperm DNA fragmentation is similar to the regular method of semen analysis. Tests used include the Halosperm kit based on the Sperm Chromatin Decondensation (SCD) technique in which approximately 100 sperm are characterized as to whether they have expanded nuclei, with five grades of expansion, or no expansion (good sperm); the Tunel assay; the Comet assay; and the Sperm Chromatin Structure Assay (SCSA) (Woodruff, 2012).

The SDF detected in the ejaculate is caused by different factors such as oxidative stress, apoptosis and failures in the histone-protamine replacement (Agarwal *et al.,* 2008; Sakkas *et al.,* 1999). These natural processes observed in semen samples are amplified when and after ejaculation, and when the semen samples are manipulated in the laboratory. It has also been proven that temperature fluctuations affect the rate of SDF (Lopez-Fernandez *et al.,* 2007; Toro *et al.,* 2009). Moreover, it has been reported that microbial contamination in the germ line can impact semen quality, also affecting the DNA molecule. Some authors have reported a number of factors that may affect SDF within species. Among environmental factors that show effects on SDF are presence of toxic agents (Spano *et al.,* 1996; Potts *et al.,* 1999), vaccination (Gosálvez *et al.,* 2008), biological contamination (González-Marín *et al.,* 2011), components of the extender in which semen is stored (Hammadeh *et al.,* 2001; Love *et al.,* 2005; Waterhouse *et al.,* 2010) or storage conditions (Love *et al.,* 2002; Waterhouse *et al.,* 2010), age and male genital infection (Ruiz-López *et al.,* 2010) or even the amounts of Zn in the dietary intake (García-Contreras *et al.,* 2011).

2.6. Diluents or extenders

The term diluent or extender refers to the aqueous solution used to increase the volume of the ejaculate until the required concentration is reached. This needs to be done while preserving the functional characteristics of the sperm. The spermatozoa are found in the seminal plasma which provides them with the required nutrients for the high metabolic activity demands of sperm transport through the female genital tract (Gadea, 2003), however, in the ejaculate this high metabolic activity can only be maintained for a limited period (Lewis, 1911). In order to preserve spermatozoa for extended periods, their metabolic activity needs to be reduced by diluting them in an appropriate medium and lowering the temperature. The medium should also supply the nutrients needed for the metabolic maintenance of the spermatozoa (Gadea, 2003).

The extenders used for semen preservation of domestic animals must have an adequate pH and buffering capacity suitable for osmolarity and should protect sperm cells against cold shock (Salamon &

Maxwell, 2000). To prevent microbial growth in stored semen, the diluents are supplemented with antibiotics. To perform its function, the extender should also provide an environment that inhibits the formation of harmful reactive oxygen species (ROS) or lipid peroxide (Orok *et al*., 2010). Reactive oxygen species damage the sperm membrane and DNA, which in turn reduces motility rate and the spermatozoan's capacity to fuse with the oocyte (Boonsorn *et al*., 2010).

2.7. Types of semen diluents

Diluents can be divided into two major groups: those designed for short-term preservation (semen stored in a liquid state) and extenders for long-term semen preservation (semen stored in a frozen state). Short term diluents are used for short distance semen insemination while long term diluents are generally used in programmes where the site of semen production is a long distance away from the site of insemination (Gadea, 2003).

Different synthetic (e.g. fructose-tris, glucose-citrate etc.) and non-synthetic-based extenders (e.g. skim milk) have been tested and recommended to be used for liquid storage of semen in several species (discovered by Maxwell & Salamon, 1993). The early diluents for storage of bovine semen at refrigerated temperatures were significantly influenced by the discovery of egg yolk as a useful additive, and the primary buffering component in this diluent is phosphate (Philips, 1939). Subsequently, citrate was found to have satisfactory buffering capacity with an improved period of survival of spermatozoa stored at 5 ^{o}C (Willett & Salisbury, 1942). Citrate then became the salt of choice as its chelating properties improved the solubility of protein fractions in egg yolk. Egg yolk addition to skim milk extender improves the quality of spermatozoa during chilled storage (Salamon & Maxwell, 2000). It has also been found that egg yolk can be a detrimental component in synthetic extenders (Colas, 1975) or it is only detrimental at high concentrations, thus suggesting moderate amounts of egg yolk to improve results (Colas *et al*., 1968).

Egg yolk and skim milk are the most common cryopreservation diluents for goat semen, but the dilution of goat semen into extenders with egg yolk or skim milk can be detrimental to spermatozoa. The harmful interactions between seminal plasma and egg yolk were first documented by Roy (1957) and with milk by Nunes *et al.* (1982). Nowadays, egg yolk or skim milk extenders are widely used for the frozen storage of small ruminant semen (Salamon & Maxwell, 2000). The presence of enzymes (bulbourethral secretion glycoprotein-60 and egg yolk coagulating enzyme) in the seminal plasma caused damaging interactions between seminal plasma and egg yolk or milk (Nunes *et al.,* 1982;

Leboeuf *et al.,* 2000). Bulbourethral secretion glycoprotein-60 (BUSgp60) has a triacylglycerol hydrolyse activity which reduces sperm motility and movement quality by disruption of the cell membranes (Pellicer-Rubio & Combarnous, 1998). Phospholipase A_2 activity of egg yolk coagulating enzyme (EYCE) catalyses the hydrolysis of egg yolk phosphatidycholine (PC) into fatty acids and lysophophatidycholine (LPC). LPC has toxic effect on buck spermatozoa by acting like a detergent on biomembrane, which results in loss of motility, membrane integrity and consequently low fertility rates (Upreti *et al.,* 1999).

Generally, egg yolk has been accepted to be an effective agent in semen extenders for sperm protection against cold shock and the lipid-phase transition effect (Aboagla & Terada, 2004). However, the use of chilled-stored semen diluted in egg-based extender is limited by relatively short-time fertilization capacity (Aurich *et al*., 1997), and the individual differences in egg yolk due to different period of egg storage. The removal of chicken egg yolk from a semen extender would improve uniformity in the components of semen extenders and eliminate hygienic risk. It is thus reasonable to develop synthetic semen extenders free of egg yolk. A number of commercially available semen extender containing a substitute for egg yolk like Andromeda® (Aires *et al*., 2003), Biociphos plus® (Gill *et al*., 2000) and Bioxcell® (Gil *et al*., 2003a,b; Stradaioli *et al*., 2007) have been used for the preservation of ovine, bovine and caprine semen, and maintained better semen quality. Bovine spermatozoa preservation (Hansen *et al*., 2005; Stradaioli *et al*., 2007) and ram spermatozoa preservation (Gil *et al*., 2003a,b) in soya lecithin-based extender Bioxcell® maintained the sperm quality and produced acceptable fertility rates (Gil *et al*., 2003b).

2.8. Temperature and dilution effect

The sperm plasma membrane separates the interior of the cell from the outside environment and it is selectively permeable to ions and organic molecules. The sperm membrane also controls the movement of substances in and out of the sperm cell (Alberts *et al.,* 2002). These membranes consist of phospholipids bilayer with randomly embedded protein complexes. When these complexes are uninterrupted, the membrane remains in a fluid state. As the temperature is lowered, restriction of lateral movement of membrane phospholipids could result in a transition from a fluid to a gel phase. Once in a gel phase lipids tend to aggregate, forming micro-domains in the remaining fluid areas of the membrane. The edges of these micro-domains turn into fragile areas which are prone to fusion or rupture and more permeable to ions (Hammersted *et al*., 1990).

Spermatozoa are diluted with seminal fluids from the accessory glands at ejaculation and their motility is retained for some few hours. To lengthen their survival *in vitro,* it is necessary to reduce the metabolic activity by chemical inhibitors or by lowering the temperature, which also requires dilution (Johnson *et al.,* 2000). The relationship between metabolic rate and temperature is often expressed as Q_{10}, which measures the rate increase for each 10 ^{o}C rise in temperature. For example, if the metabolic rate of an animal at 0 ^{o}C is x, then at 10 ^{o}C the rate would be 2x, at 20 ^{o}C 4x, etc. (ANON., 2012). The metabolic rate of a body cell (including spermatozoa) will thus decrease by a factor of 2x for every 10 ^{o}C drop in temperature. In addition to a prolonged lifespan, mammalian spermatozoa respond to dilution by an initial increase of activity, followed by a loss of motility and an increase in membrane damage.

With excessive dilution, there is considerable loss of cell viability, especially when using pure electrolyte media (Johnson *et al.,* 2000). A reduction in the concentration of important seminal plasma components may contribute to the effect, which can be eliminated by the addition of albumin. Addition of diluents in semen lowers the concentration of certain compounds in the seminal plasma such as K^{+} (Harrison *et al*., 1978) or the plasma proteins, changing sperm viability. These losses need to be compensated by adding the necessary ingredients in the diluents formulations. Also, minimal additions of K^{+} may assist to maintain the motility of spermatozoa, as a high concentration of K^{+} in the seminal fluid is essential for cell viability (Harrison *et al.,* 1978). Harrison *et al.* (1982) proposed that the dilution effect is due to the absence of proteinaceous motility stimulants from seminal plasma and showed that serum albumin could stimulate motility in a reversible manner. Again it was shown that bovine serum albumin (BSA) stimulated spermatozoan motility during a six day storage test (Waberski *et al.,* 1989).

Buhr (1990) used BSA in an attempt to overcome the fluidity of the membrane, and consequently the detrimental effect of lysophospholipids and free fatty acids, produced by cooling. The Na^{+} and Ca^{++} ions that enter the cell in spermatozoa are withdrawn by active transport. However, Ca^{++} permeability increases significantly at 5 ^{o}C accumulating within the cell and reaching toxic levels. Cold shock damage is partly determined by membrane cholesterol content and polyunsaturated fatty acid composition. Cholesterol/phospholipids ration has been revealed to have a key role in membrane fluidity. It was assumed that changes in membrane lipid fluidity could affect trans-membrane movement of Ca^{++}, which is essential in the capacitation process, but detrimental during storage. BSA induced a temporal decrease in membrane fluidity which was, however, difficult to interpret. BSA nonetheless improved fertility when semen was stored between 3 and 5 days (Waberski *et al.,* 1994).

2.9. Culture media

The earliest attempt to culture mammalian cell was in 1940s where the culture media contained human plasma or serum solely. In the1990s the well-defined albumin media additive was then introduced. Today, albumin is universally added in culture media for *in vitro* fertilization (IVF) and as a cryoprotectant for semen cryopreservation as it is well-known to be beneficial (Cabrita *et al.,* 2001; Blake *et al.,* 2002). Albumin makes up sixty percent of the total protein in the plasma. It has a large surface area and abundant binding sites. Albumin is also able to bind to a number of substances such as fatty acids, fat soluble vitamins (A, D, E, and K), cations (calcium, magnesium, zinc), and some toxins (Ghuman *et al.,* 2005). The binding of albumin to hydroperoxy fatty acid that can be detrimental to the sperm membranes, makes albumin as an effective lipid peroxidation inhibitor (Alvarez & Storey, 1995). Regardless of all the advantages, if the media is too concentrated with albumin, it will draw the water from the cell (osmotic effect) which may lead to shrinkage and crystallization inside the cell.

Embryo culture *in vitro* requires an artificial environment capable of sustaining the viability of the fertilized (*in vitro* or *in vivo*) ovum during one or more of the following phases: the initial oocyte-mediated cleavage stages, the period of genomic activation which varies according to species, and during the early differentiation events leading to formation of the visually distinct cell populations of the blastocyst-stage embryo. The artificial environment addresses empirical temperature, nutritional, and atmospheric components. This artificial environment is, however, immensely different from the dynamic spatial and temporal physiological environment found *in vivo* (Biggers & Summers, 2008).

Culture media for embryo growth was first described in 1912 for the growth of an embryo of a rabbit. Later on, in 1949, mouse embryos were grown in culture media from the 8-cells stage to blastocysts. These culture media, like Earle, Ham's F-10, Tyrode's T-6 and Whitten's WM1 were based on different salts and were constructed to support the development of somatic cells and cell lines in culture. These culture media, known as physiological salt solutions, were used by Robert Edwards for his first successful human IVF attempt and the birth of the first IVF baby, a girl named Louise Brown. These media were formulated for use with or without serum supplementation, depending on the cell type being cultured. The Ham's nutrient mixtures were originally developed to support growth of several clones of Chinese hamster ovary (CHO) cells, as well as clones of HeLa and mouse L-cells (Edwards, 1965).

The initial experimental conditions for the culture of immature bovine oocytes have survived 25 years of improvement. The use of TCM-199 (a somatic culture medium with 200 components) and the addition

of 10% fetal bovine serum (FBS) pyruvate, FSH, estradiol, and antibiotics, still remains the method used in most laboratories to culture mature bovine embryos (Sirard *et al.*, 1988). A reasonable blastocyst rate of 30% can be achieved reliably with this simple system either in open system or under oil. The same system was used by Chian for human IVM after using it for bovine oocytes for few years (Chian *et al.,* 2004). More recently defined systems were developed to remove serum and to have lesser components present. This system resulted in similar blastocyst rates with only Bovine Serum Albumin (BSA) as a biological supplement (Ali & Sirard, 2002). To our knowledge there is no study available up to this date, on the liquid preservation of bull semen using the M199 or Ham's F10 culture media. This study was therefore, conducted to evaluate the quality of bull spermatozoa preserved for 72 hours in Ham's F10 and M199 culture media or the commercial extender Triladyl stored at refrigerated temperatures as an alternative to frozen/thawed semen straws.

CHAPTER 3

3. METHODOLOGY

3.1. Design of the study

The study was conducted at the University of Venda situated in Thohoyandou, in the Limpopo Province of South Africa. Thohoyandou falls under Thulamela municipality in Vhembe district. Thohoyandou is located within a subtropical climate region with temperatures and humidity in summer and mild winters. It has hot summer months and cool winter months. Two Nguni bulls with good body condition scores and proven fertility records, kept at the University of Venda experimental farm under intensive care, at the ages of 2 to 3 years, were used for this study. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Animals under the guidelines of the University of Venda Animal Ethics Committee. Water and feed were given *ad libitum* throughout the duration of the study.

3.2. Bull semen collection and processing

Semen samples were collected at the experimental farm twice per week for 5 weeks from the two Nguni bulls. Ejaculates from the bulls were collected using the Pulsator IV-Auto Adjust™ electro-ejaculator (Lane manufacturing Inc, Denver Colorado, USA) into 15 mL graduated Cell-Star® tubes (Greiner Bio-One GmbH, Frickenhausen/Germany). The electro-ejaculator consists of a rectal probe that has a series of linear banded electrodes connected to a variable current and voltage source. The bull was restrained in a standing position inside the crush pen and the rectum emptied of faeces with an arm-length gloved covered hand before inserting the probe. The probe was lubricated with cooking oil and inserted into the rectum with a pushing, slightly turning motion, with the metal electrodes facing ventrally (downwards). The electro-ejaculator was then turned on and the voltage increased automatically in small increments until the bull maintains an erection and ejaculates. Immediately, after collection, the semen samples were placed into the thermo-flask with water at 37 ^{o}C and transported to the Biotechnology laboratory within 10 minutes. In the laboratory semen evaluations were performed within 1 hour.

3.3. Macroscopic evaluation of semen

3.3.1. Semen volume

Semen volume was measured by reading volume values on the 15 mL graduated Cell-Star® tubes and volumes were recorded.

3.3.2. Semen pH

The semen pH was determined using a pH meter SG2 (Mettler-Teledo AG, Switzerland) calibrated to measure semen pH for bulls. The electrode of the pH meter was rinsed with sterile water and wiped with clean paper to avoid any contamination and then dipped into the semen sample for the pH measurement. The reading on the pH meter was then recorded.

3.3.3. Spermatozoa concentration

Spermatozoa concentration was determined using a C30 portable spectrophotometer (PG instruments Alma Park, Wibtoft, UK) calibrated to measure spermatozoa concentration. Semen samples from the two bulls were diluted in Dulbecco's phosphate buffered saline (DPBS) at multiple ratios (1:10, 1:25, 1:100, 1:200) to generate the standard curve for each bull. Each diluted sample was placed into a microcuvette which was then placed in the spectrophotometer to measure the absorbance of each sample. To get the concentration of the diluted sample to set up the standard curve for each bull, 3 µL of each sample was placed inside one chamber of the eight chambered Leja® slide (Leja Products B.V, Nieuw-Vennep, Netherlands) and then evaluated using morphology and concentration programme of the CASA system. The formula (CASA reading / dilution rate) was used to determine the spermatozoa concentration of all samples. Spermatozoa concentration per sample was expressed as 10^9 spermatozoa/mL.

3.4. Experiment 01: Evaluation of different extenders and sperm protectors to keep the spermatozoa viable for more than 24 hours.

3.4.1. Treatments

Collected semen samples were examined macroscopically for any contamination like blood, pus and dirt. After the macroscopic evaluations, the two semen samples, one from each bull, were pooled to eliminate individual differences. The pooled semen sample was then divided into three aliquots using a pipette to withdraw equal amounts of semen. The three aliquots were diluted in two culture media M199 and Ham's F10 both supplemented with 0.025g/4mL Maltose monohydrate ($C_{12}H_{22}O_{11}.H_2O$),10% Bovine Serum Albumin (BSA), 10% HEPES buffer and 1% antibiotic cocktail (Penicillin, Streptomycin and Fungizone), and also in a commercial extender called Triladyl. The semen was diluted with an extender at a ratio of 1:4. The three extended samples were stored at controlled room temperature 24 °C for three days (72 hour).

3.4.2. Microscopic evaluations

3.4.2.1. Sperm motility evaluations

The sperm motility parameters (Table 6) were evaluated using the computer aided sperm analysis (CASA) system (Sperm Class Analyzer® [SCA] 5.3, Microptic, Barcelona, Spain) using the motility program at phase contrast Ph1 using 10x magnification after 0, 24, 48 and 72 hours (3 days). Three micro-litres of semen from the three extended samples was placed inside one chamber of the eight chambered Leja® slides on the warm glass stage at 37 °C of the CASA microscope for evaluation. For each analysis five fields were captured and the software automatically calculated the percentage of sperm in the sample by motility parameters.

3.4.2.2. Sperm morphology

Abnormal spermatozoa such as acrosome-reacted, coiled or bent tails, double head or tails, distal and proximal droplets were determined by staining with Spermac® (Stain Enterprises, P.O. Box 152, Wellington 7654, South Africa). For staining, sperm smears were first prepared at controlled room temperature 24 °C by pipetting a 15 µL drop of extended semen on a clear end of slide. The edge of the second slide was then placed on the drop at an angle of 20° on a horizontal plane and then pushed

forward to smear across the slide. The sperm smears were thereafter allowed to air dry at room temperature before staining. The dried smears were placed horizontally down on absorbent paper inside a staining tray and 1mL of spermac fixative was then dropped on the dried smear using a plastic disposable pipette. After 10 minutes the slides were placed vertically on their end on absorbent paper to drain off excess fixative. The slides were then washed by dipping seven times slowly (about 1 second for each dip) into distilled water. Excess water was drained off by touching the end of slide onto absorbent paper. One millilitre of stain A was then dropped on for 2 minutes and after that washed as above. The slides were washed for the second time in fresh distilled water to remove the excess of stain A. Stain B was introduced the same way as stain A, and then washed by dipping 7 times into fresh distilled water. The third stain, stain C was introduced for another 2 minutes and washed as above. The slides were then left in an upright position (at about 70° angle) to let fluid drain until air dry. Thereafter, the stained slides were placed on the CASA microscope stage at room temperature. A drop of immersion oil was poured on the stained slide and covered with a cover slip before evaluation of spermatozoa morphology. The CASA morphology program was used at 60x magnification to count 200 spermatozoa per each stained slide and the results were recorded. Under the microscope the post-acrosomal region and the nuclear portion of the head appears red, while the acrosome, midpiece and tail appears green (Figure 1A).

3.4.2.3. Viability (Live/dead) test

Spermatozoa were stained with nigrosin-eosin stain. For staining, two microscope slides were pre-warmed at 37 °C. Six micro-litres of the semen sample and 20 µL of eosin stain was poured on the end of the slide and mixed using a pipette tip on a warm glass stage at 37 °C. A drop of 20 µL nigrosin stain was then poured onto the mixture on the slide and mixed using the same pipette tip. The edge of the second slide also pre-warmed at 37 °C was thereafter placed on the mixture at an angle of 20° from the horizontal plane and pushed forward to smear across the slide. Immediately after smearing, the smeared slide was placed on a hot Buehler® slide warmer (Buehler Ltd., 41 Waukegan road, Lake Bluff, Illinois, USA) at 120 °C to allow drying. The dried stained slides were thereafter placed on the CASA microscope stage at room temperature. A drop of immersion oil was poured on the stained slide and covered with a cover slip before evaluation. The CASA vitality program was then used at 60x magnification to count the number of live and dead spermatozoa. A total of 200 spermatozoa were counted per each stained slide and the results were recorded. Under the microscope live sperm heads appears white, while dead sperm heads appears pinkish in colour (Figure 2).

3.5. Experiment 02: Comparison of different refrigeration storage temperatures for extended semen.

3.5.1. Treatments

For further experiment, only Ham's F10 was used to evaluate the effect of different refrigerated temperatures on spermatozoa motility, viability and sperm DNA fragmentation because it has not only proved to be the second best extender after Triladyl, but also showed a better decrease in progressive motility throughout the storage time, and again due to the fact that there is no information to date on the use of culture media as bull semen extenders. Semen samples were collected, macroscopically evaluated, pooled and extended with Ham's F10 as described in 3.4.1. The four extended samples were then stored at refrigerated temperatures 5 °C, 12 °C, 17 °C and controlled room temperature 24 °C for three days.

3.5.2. Microscopic evaluations

3.5.2.1. Sperm motility evaluations

The sperm motility parameters (total, progressive and rapid motility, curvilinear velocity, straight line velocity, average path velocity, linearity, straightness and wobble) were assessed using the CASA system as described in 3.4.2.1.

3.5.2.2. Viability (Live/dead) test

Spermatozoa were stained with nigrosin-eosin stain. Staining and evaluation of the stained slides was done in a similar manner as described in 3.4.2.3.

3.5.2.3. DNA Fragmentation

DNA sperm fragmentation was assessed using Sperm-halomax kit (Halotech DNA. Madrid, Spain). Lysis solution was set at room temperature. An eppendorf tube containing agarose in the float was incubated in water bath at 95 – 100 °C for 5 minutes until the agarose was fully melted. Thereafter, the agarose eppendorf tube was transferred in a water bath at 37 °C for 5 minutes until the temperature has equilibrated, meanwhile, 25 µL of each extended semen sample was transferred to an empty

eppendorf tube. Fifty micro litres of liquefied agarose was then transferred into the tubes with the semen samples and mixed gently at 37 °C. A drop of 2 µL from each of the prepared mixtures was then placed on a microscope slide placed on a horizontal plane then covered with a cover slip and pressed down gently without formation of bubbles. The slides were placed on a cold metal surface pre-cooled at 4 °C and then transferred into the refrigerator at 4 °C for 5 minutes for the agarose to solidify. Thereafter, the slides were removed from the refrigerator, placed horizontally on the float into a tray and the cover slips gently removed. Lysis solution was applied on the slides and then incubated at 37 °C for 5 minutes. After incubation the slides were drained by tilting and then placed horizontally on top of the float for washing by covering the slides for 5 minutes with distilled water using a disposable pipette and then drained by tilting. After draining, the slides were dehydrated by flooding first with 70% ethanol using a disposable pipette, incubated for 2 minutes, drained and then dehydrated for the second time with 100% ethanol which was applied for 2 minutes, drained by tilting and then allowed to air dry. After drying the processed slides were stained using Sperm-Blue and then evaluated for sperm DNA fragmentation. The CASA system was used at 60x magnification to count fragmented and non-fragmented spermatozoa. Two-hundred spermatozoa were counted per each stained slide and the results were recorded.

3.6. Statistical analysis

The study was conducted in four replicates and data was statistically analysed using General Linear Model (GLM) procedures of Minitab program (Minitab 2013), using 3 x 4 factorial in a completely randomized design. Analysis of variance (ANOVA) was used for analysis of the results. Means of different treatments were compared using Tukey's post hoc test. Significance was set at $P<0.05$.

Model used for Experiment 1:

$$y_{ijk} = \mu + D_i + T_j + (DT)_{ij} + \varepsilon_{ijk}$$

Where y_{ijk} was the observation

μ = overall mean.

D_i = effect of the i^{th} extender.

T_j = effect of j^{th} storage time

$(DT)_{ij}$ = interaction between the i^{th} extender and j^{th} storage time.

$\boldsymbol{\varepsilon_{ijk}}$= random error

Model used for Experiment 2 model:

$$\boldsymbol{y_{ijk} = \mu + T_i + P_j + (TP)_{ij} + \varepsilon_{ijk}}$$

Where $\boldsymbol{y_{ijk}}$ was the observation

$\boldsymbol{\mu}$ = overall mean.

$\boldsymbol{T_i}$ = effect of the ith storage temperature.

$\boldsymbol{P_j}$ = effect of jth storage time.

$(\boldsymbol{TP})_{ij}$ = interaction between the ith storage temperature and jth storage time.

$\boldsymbol{\varepsilon_{ijk}}$= random error.

CHAPTER 04

4. RESULTS

Table 1. Experiment 01: Motility rates of extended semen with Triladyl, Ham's F10 and M199 evaluated after 0, 24, 48 and 72 hours stored at controlled room temperature 24 °C.

Treatment	ST (h)	TM (%)	PM (%)	RAP (%)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)
Triladyl	0	98.8^{a}	27.5^{d}	48.8^{a}	146.3^{a}	56.8^{a}	83.5^{a}	46.0^{abc}	70.3^{ab}	61.5^{ab}
	24	98.3^{a}	23.5^{e}	50.3^{a}	146.8^{a}	46.5^{abc}	76.8^{a}	31.8^{bc}	60.3^{bc}	52.5^{b}
	48	96.5^{a}	22.3^{ef}	47.8^{a}	140.0^{a}	43.0^{abc}	75.0^{ab}	30.3^{bc}	56.3^{c}	53.8^{b}
	72	91.5^{a}	20.3^{f}	39.3^{cd}	119.5^{ab}	34.5^{bcd}	62.0^{abc}	28.5^{c}	55.3^{c}	52.0^{b}
Ham's F10	0	98.3^{a}	49.3^{a}	42.0^{bc}	105.8^{bc}	60.0^{a}	81.0^{a}	57.0^{a}	74.3^{a}	76.5^{a}
	24	90.8^{b}	43.0^{b}	48.3^{a}	120.5^{ab}	56.3^{ab}	77.0^{a}	46.3^{abc}	72.8^{ab}	64.0^{ab}
	48	86.3^{c}	30.8^{c}	35.5^{e}	116.0^{ab}	49.8^{abc}	70.0^{ab}	42.8^{abc}	71.0^{ab}	60.5^{cdef}
	72	72.0^{d}	28.0^{d}	27.5^{f}	78.8^{cd}	33^{cd}	45.0^{cd}	41.0^{abc}	72.3^{ab}	56.3^{b}
M199	0	98.3^{a}	48.5^{a}	36.5^{de}	102.0^{bc}	59.3^{a}	77.5^{a}	58.8^{ab}	76.3^{a}	76.3^{a}
	24	82.5^{c}	40.8^{b}	44.3^{b}	106.8^{bc}	52.5^{abc}	70.0^{ab}	49.0^{abc}	74.8^{a}	66.0^{ab}
	48	71.0^{d}	28.8^{cd}	20^{g}	79.5^{c}	39.8^{abc}	51.8^{bc}	50.8^{abcd}	77.8^{a}	65.0^{ab}
	72	55.0^{e}	8.8^{g}	4.3^{h}	47.8^{d}	17.0^{d}	24.3^{d}	34.0^{bc}	68.0^{abc}	49.8^{b}
SEM		0.9	0.5	0.7	6.4	4.4	5.0	4.3	2.8	3.5
Ext. Means										
Triladyl		96.3^{a}	23.4^{c}	46.5^{a}	138.1^{a}	45.2^{a}	74.3^{a}	34.1^{b}	60.5^{b}	54.9^{b}
Ham's F10		86.8^{b}	37.8^{a}	38.3^{b}	105.3^{b}	49.8^{a}	68.3^{a}	46.8^{a}	72.6^{a}	64.3^{a}
M199		76.7^{c}	31.7^{b}	26.5^{c}	84.0^{c}	42.1^{a}	55.9^{b}	48.0^{a}	74.2^{a}	64.3^{a}
SEM		0.4	0.2	0.3	3.1	2.2	2.5	2.1	1.4	1.8
ST means										
0		98.4^{a}	41.8^{a}	42.4^{b}	118.0^{a}	58.7^{a}	80.7^{a}	53.8^{a}	73.6^{a}	71.4^{a}
24		90.5^{b}	37.8^{b}	47.6^{a}	124.7^{a}	51.8^{ab}	74.6^{ab}	42.3^{b}	69.3^{ab}	60.8^{b}
48		84.6^{c}	27.3^{c}	34.4^{c}	111.8^{a}	44.2^{b}	65.6^{b}	41.3^{b}	68.3^{ab}	59.8^{bc}
72		72.8^{d}	19.0^{d}	23.7^{d}	82^{b}	28.2^{c}	43.8^{c}	34.5^{b}	65.2^{b}	52.7^{c}
SEM		0.5	0.3	0.4	3.8	2.6	2.9	2.5	1.6	2.0
Extender (E)		**	**	**	**	ns	**	**	**	**
ST		**	**	**	**	**	**	**	**	**
E x ST		**	**	**	ns	ns	*	ns	ns	ns

$^{a\,b\,c\,d}$ Mean values with different superscript letters in the same column differ significantly; SEM = Standard Error Mean; ST = Storage time; TM = total motility, PM = progressive motility, RAP = rapid motility, VCL = curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ns = Not significant; * = Significant (P<0.05); ** = Highly significant (P<0.01).

Table 1 shows the motility characteristics mean values of Nguni spermatozoa extended in three different extenders stored for three days at 24 ^{o}C of experiment 01. Significant difference in total, progressive and rapid motility was observed between all the three extenders and storage time intervals (P<0.01). No significant difference in TM (P>0.01), however, was observed with the commercial extender Triladyl. TM, PM and RAP was again affected by the interaction between extenders and storage time intervals (P<0.01). In terms of velocity parameters, no statistically significant difference (P>0.05) in VSL was found between all the three extenders, but a decrease in VCL, VSL, VAP, LIN, STR, and WOB was observed throughout the storage time (P<0.01). However, there was no decrease in VCL, VAP and WOB with Triladyl, and no decrease in WOB with M199 throughout the storage time. Again no significant difference was observed in STR velocity between Ham's F10 and M199. It was also observed that the extender x storage time interaction did not affect sperm cell velocity parameters (P>0.05), except for VAP velocity (P<0.05). The commercial extender Triladyl proved to be the best extender showing consistent motility throughout the storage time followed by Ham's F10 culture medium which also showed a better decrease in progressive motility amongst the three extenders used. Many studies have been done on extension and cryopreservation of bovine semen using Triladyl (Nabiev *et al.,* 2003; Herold *et al.,* 2003; Christensen *et al.,* 2005; etc); however, there is no available information about the use of culture media as bovine semen extenders. For that reason Ham's F10 was chosen for further experimentation on sperm motility, viability and DNA fragmentation.

Table 2. Experiment 01: Abnormal morphology rates of extended semen in Ham's F10 after 72 h of storage at 24 ^{o}C.

Treatment	Acrosome reacted (%)	Absent tails (%)	Coiled tails (%)	Bent tails (%)	Total abnormality (%)
Bull 01	15.0^{a}	7.0^{a}	1.0^{a}	2.0^{a}	25.8^{a}
Bull 02	17.3^{b}	7.8^{b}	0.8^{a}	1.5^{a}	27.3^{a}
SEM	0.4	0.2	0.2	0.4	0.8
Treatment (T)	*	*	ns	ns	ns

$^{a\,b}$ Mean values with different superscript letters in the same column differ significantly; SEM = Standard Error Mean; * = Significant (P<0.05); ns = Not significant.

The mean values of Nguni spermatozoa morphological abnormalities after 72 hours of storage at 24 ^{o}C are presented in Table 2 above. The most observed morphological abnormality in both the Nguni bulls was the acrosome reacted sperm heads which appear red in colour (Figure 1B), followed by absent tails (top arrow in Figure 1C). The other abnormalities observed were bent tails (second arrow in Figure 1C) and coiled tails (Figure 1 D). Figure 1A shows normal sperm cells with intact acrosome. No statistically significant difference in total morphological abnormality, bent tail and coiled tail was observed between the two Nguni bulls ($P>0.05$), but there was a significant difference in absent tails and acrosome reacted ($P<0.05$).

Table 3. Experiment 01: Bull spermatozoa viability (life/dead) percentages after 72 h of storage at 24 ^{o}C.

Treatment	Live cells (%)	Dead cells (%)
Triladyl	41.3^{a}	58.7^{a}
Ham's F 10	26.5^{b}	73.5^{b}
M199	25.0^{b}	75.0^{b}
SEM	2.6	2.6
Extender (E)	*	*

$^{a\ b}$ Mean values with different superscript letters in the same column differ significantly; SEM = Standard Error Mean; * = Significant ($P<0.05$).

Table 3 shows the live and dead mean values of bull semen diluted in different extenders after 72 hours of storage at 24 ^{o}C. Normal live sperm cells appear white in colour whilst the dead sperm cells appear pinkish in colour (Figure 2A and B). The highest viability value was obtained with Triladyl (41.3 ± 2.6). There was a significant difference between Triladyl and Ham's F10-M199 ($P<0.05$), but no significant difference was found between Ham's F10 and M199 ($P>0.05$).

Table 4. Experiment 02: Motility rates of extended bull semen in Ham's F10 evaluated after 0, 24, 48 and 72 hours stored at 24 °C, 17 °C, 12 °C and 5 °C.

Treatment	ST (h)	TM (%)	PM (%)	RAP (%)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)
24 °C	0	96.0^{a}	45.3^{a}	39.5^{a}	105.8^{ab}	60.0^{a}	81.0^{a}	57.0^{ab}	74.3^{a}	76.5^{a}
	24	90.8^{a}	39.0^{ab}	45.0^{a}	120.5^{a}	56.3^{a}	77.0^{a}	46.3^{abcd}	72.8^{ab}	64.0^{ab}
	48	87.3^{ab}	33.8^{bc}	39.8^{a}	116.0^{a}	49.8^{ab}	70.0^{ab}	42.8^{bcde}	71.0^{abc}	60.5^{bc}
	72	72.0^{bc}	22.8^{cde}	21.3^{bc}	78.8^{cd}	33.0^{c}	45.0^{cd}	41.0^{cde}	72.3^{ab}	56.3^{bcd}
17 °C	0	94.5^{a}	43.5^{ab}	38.8^{a}	108.5^{a}	62.8^{a}	83.3^{a}	57.8^{a}	75.5^{a}	76.5^{a}
	24	82.0^{ab}	25.5^{cd}	33.8^{ab}	106.0^{ab}	36.5^{bc}	54.8^{bc}	34.0^{def}	66.3^{abcd}	51.5^{bcde}
	48	60.25^{c}	13.5^{ef}	12.8^{cd}	80.5^{bcd}	25.3^{cd}	39.0^{cd}	31.3^{ef}	64.8^{abcd}	48.5^{cdef}
	72	42.5^{d}	6.0^{fg}	2.5^{d}	56.5^{de}	21.3^{cde}	30.5^{de}	36.8^{def}	68.0^{abcd}	53.3^{bcde}
12 °C	0	96.3^{a}	42.0^{ab}	37.3^{a}	107.8^{a}	59.5^{a}	81.3^{a}	55.3^{abc}	73.0^{a}	75.5^{a}
	24	57.5^{cd}	15.3^{def}	11.0^{cd}	77.5^{d}	27.8^{c}	39.5^{cd}	36.0^{def}	70.0^{abc}	51.3^{bcde}
	48	26.5^{e}	1.8^{g}	0.8^{d}	50.8^{e}	11.8^{def}	20.8^{ef}	22.3^{fg}	54.0^{cde}	41.0^{ef}
	72	21.0^{ef}	0.0^{g}	-0.0^{d}	32.3^{ef}	7.8^{ef}	13.5^{ef}	23.5^{fg}	55.5^{bcd}	42.5^{ef}
5 °C	0	97.0^{a}	42.8^{ab}	37.8^{a}	103.5^{abc}	56.3^{a}	78.0^{a}	54.5^{abc}	72.5^{ab}	75.5^{a}
	24	25.3^{e}	1.0^{g}	0.8^{d}	45.8^{ef}	12.0^{def}	20.0^{ef}	25.8^{fg}	58.8^{abcd}	43.8^{def}
	48	6.5^{f}	0.0^{g}	0.0^{d}	31.5^{ef}	4.5^{f}	11.8^{f}	13.8^{g}	37.0^{e}	37.5^{f}
	72	11.8^{ef}	0.0^{g}	0.0^{d}	31.0^{f}	5.5^{f}	9.8^{f}	23.0^{fg}	51.5^{de}	43.5^{def}
SEM		3.0	2.2	2.5	5	3.1	3.5	2.9	3.4	2.5
Temp. Means										
24 °C		86.5^{a}	34.2^{a}	36.4^{a}	105.3^{a}	49.8^{a}	68.3^{a}	46.8^{a}	72.6^{a}	64.3^{a}
17 °C		69.5^{b}	22.1^{b}	21.9^{b}	87.9^{b}	36.4^{b}	51.9^{b}	39.9^{b}	68.6^{ab}	57.4^{b}
12 °C		50.3^{c}	14.8^{c}	12.3^{c}	67.1^{c}	26.7^{c}	38.8^{c}	34.3^{c}	63.1^{b}	52.6^{c}
5 °C		35.1^{d}	10.9^{c}	9.6^{c}	50.4^{d}	19.6^{d}	29.9^{d}	29.3^{d}	54.9^{c}	50.1^{c}
SEM		1.5	1.1	1.3	2.5	1.5	1.8	1.4	1.7	1.3
ST means										
0		95.9^{a}	43.4^{a}	38.3^{a}	106.4^{a}	59.6^{a}	80.8^{a}	56.1^{a}	73.8^{a}	76.0^{a}
24		63.9^{b}	20.2^{b}	22.6^{b}	87.4^{b}	33.1^{b}	47.8^{b}	35.5^{b}	66.9^{b}	52.6^{b}
48		45.1^{c}	12.3^{c}	13.3^{c}	69.7^{c}	22.8^{c}	35.4^{c}	31.1^{bc}	56.7^{c}	46.9^{c}
72		36.8^{d}	7.2^{d}	5.9^{d}	47.1^{d}	16.9^{d}	24.7^{d}	27.5^{c}	61.8^{bc}	48.9^{bc}
SEM		1.6	1.0	1.4	2.7	1.3	1.6	1.3	1.6	1.1
Temperature (T)		**	**	**	**	**	**	**	**	**
ST		**	**	**	**	**	**	**	**	**
T x ST		**	**	**	**	**	**	**	**	**

$^{a\ b\ c\ d}$ Mean values with different superscript letters in the same column differ significantly; SEM = Standard Error Mean; ST = Storage time; TM = total motility, PM = progressive motility, RAP = rapid motility, VCL = curvilinear velocity, VSL = straight line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ** = Highly significant (P<0.01).

Table 4: Temperature, storage time and the interaction between temperature and storage time all had an effect on total, progressive and rapid motility (P<0.01). Spermatozoa samples stored at 24 °C, however, showed no significant decrease (P>0.05) in TM, PM and RAP from 0 h until 48 h of storage. No decrease (P>0.01) again in TM and RAP from 24 h until 72 h of storage was observed for 5 °C storage temperature. Velocity parameters were also affected by temperature, storage time and the interaction between extender and storage time (P<0.01). No significant difference, however, was observed for storage temperature 24 °C in VCL, VSL, VAP and LIN from 0 h until 48 h, and no decrease throughout the storage time in STR velocity. Storage temperature 17 °C and 12 °C also showed no significant difference (P<0.05) in WOB velocity. No significant difference was furthermore observed in VCL, VSL, VAP and LIN (P<0.05) for 5 °C storage temperature.

Table 5. Experiment 02: Bull spermatozoa viability (live/dead) and sperm DNA fragmentation percentages in Ham's F10 after 72 h stored at 24 °C, 17 °C, 12 °C and 5 °C.

Treatment	Viability (%)	SDF (%)
24 °C	26.5[a]	29.3[a]
17 °C	8.0[b]	29.8[a]
12 °C	2.5[c]	29.5[a]
5 °C	0.5[c]	29.5[a]
SEM	0.8	1.1
Temperature (T)	**	ns

[a b c] Mean values with different superscript letters in the same column differ significantly; SEM = Standard Error Mean; SDF = Sperm DNA fragmentation; ** = Highly significant (P<0.01); ns = not significant.

Table 5 shows viable/live and SDF mean values of bull spermatozoa after extension with Ham' F10 for three days stored at different temperatures. Spermatozoa samples stored at 24 °C gave higher viability value than the rest (26.5 ± 0.8) and the lowest viability value was obtained with spermatozoa samples at 5 °C (0.5 ± 0.8). There was no significant difference between spermatozoa samples stored at 5 °C and 12 °C (P>0.05), but they were significantly different to spermatozoa samples stored at 17 °C and 24 °C (P<0.05). Sperm heads with large expanded/dispersed nuclei contain fragmented DNA while heads without expanded/dispersed nuclei are without DNA fragmentation (arrows in Figure 3A and B). No significant difference was observed in any of the storage temperatures after 72 hours of storage (P>0.01).

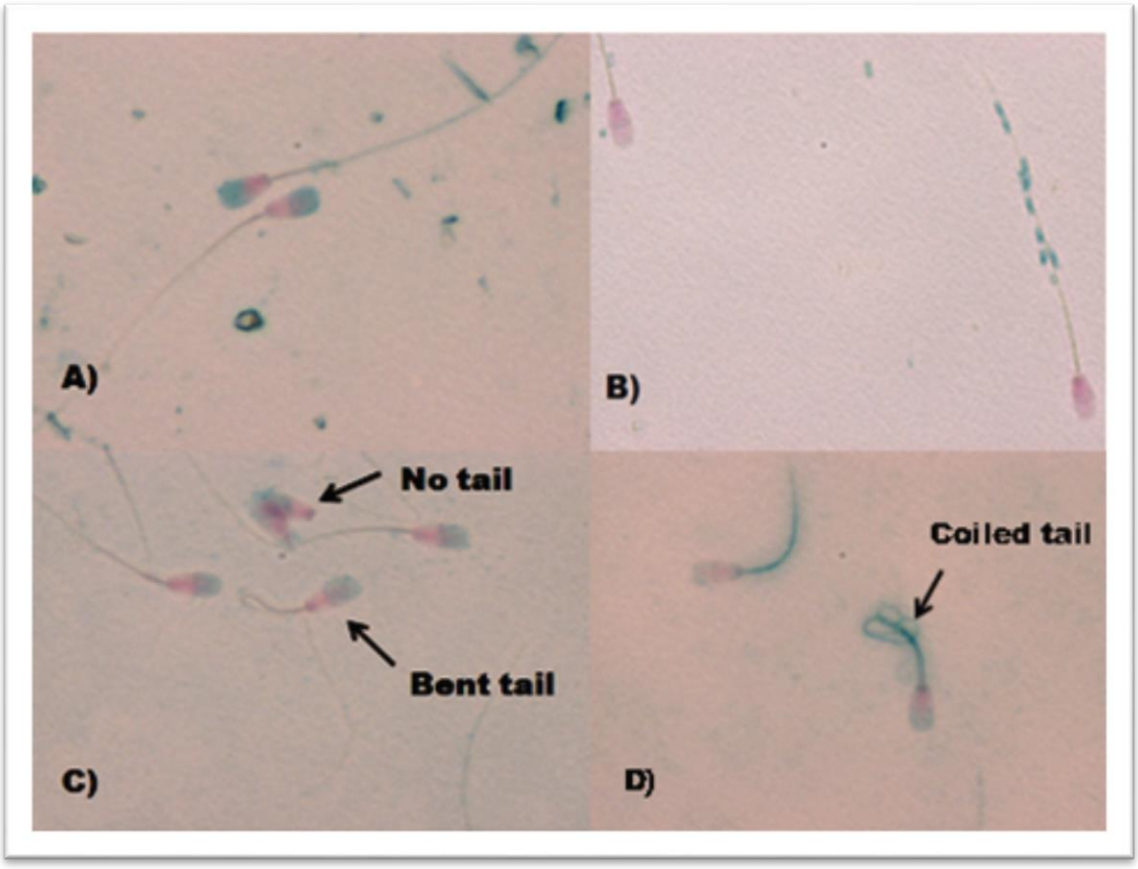

Figure 1. Images indicating bull spermatozoa morphology determined using spermac staining method.

A. Normal cells with intact acrosome.
B. Abnormal cell with reacted acrosome.
C. Abnormal cells: Top arrow above indicates absent tail and the arrow below indicates bent tail.
D. Abnormal cell: The arrow indicates coiled tail.

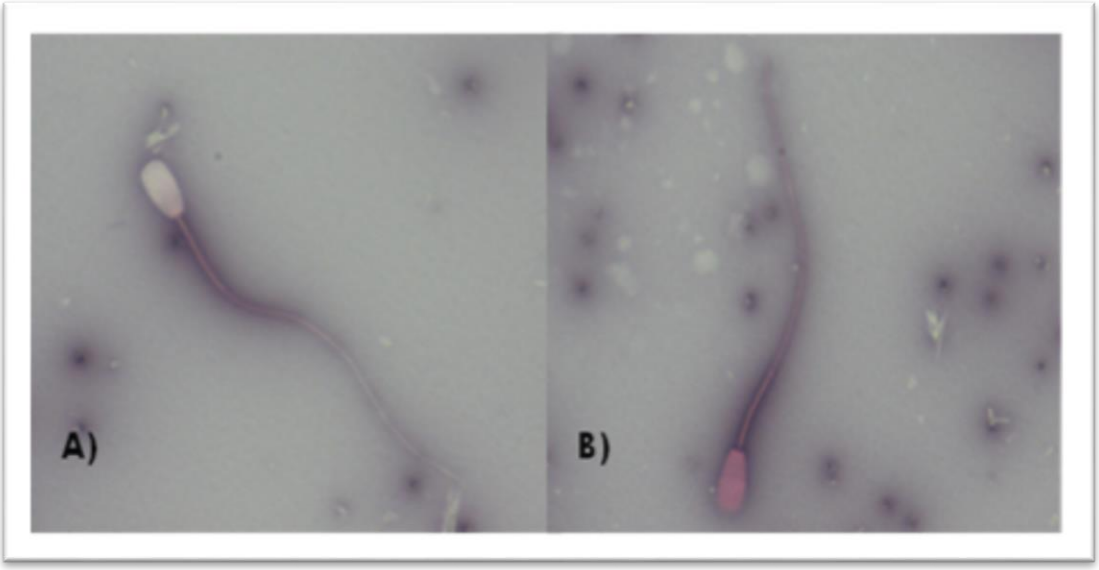

Figure 2. Images indicating bull spermatozoa viability determined using nigrosin-eosin staining method.

E. Live cell: head stained white.
F. Dead cell: head stained pink.

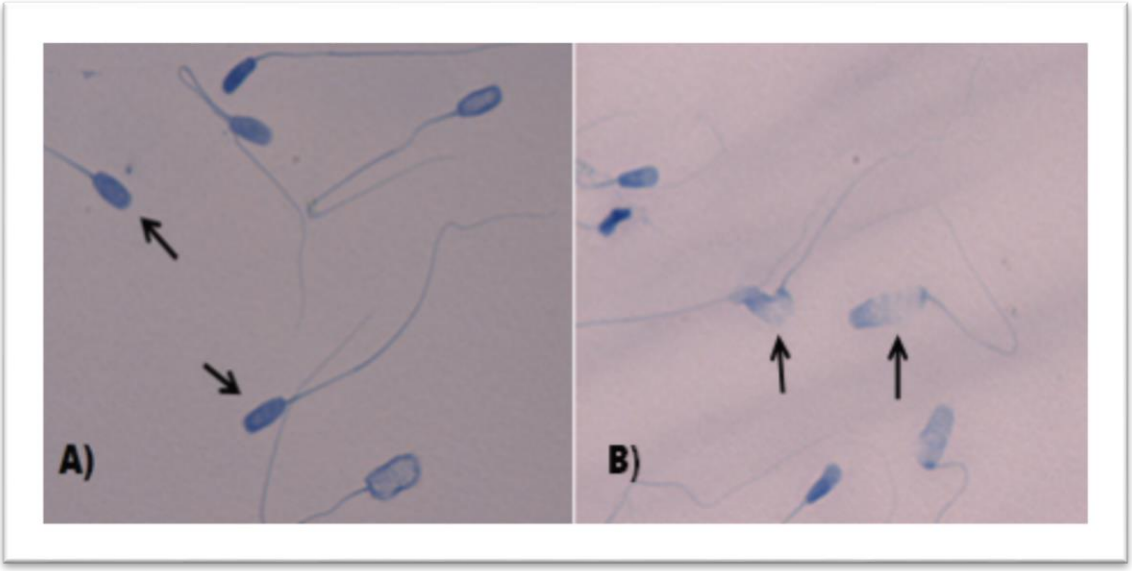

Figure 3. Images obtained when Sperm-blue staining was combined with the Sperm–Halomax protocol indicating sperm DNA fragmentation (SDF).

A. Arrows indicate sperm heads without DNA fragmentation.
B. Arrows indicate heads with fragmented DNA.

CHAPTER 05

5. DISCUSSION

Despite the effect of high storage temperature of 24 ^{o}C, the results from this study indicated that bull semen can be extended in Ham's F10 and M199 culture media, and the commercial extender Triladyl and stay viable for more than 24 hours. For the first time, bull semen has been extended in Ham's F10 and M199 culture media and survived for three days. Triladyl, however, showed consistent better results in TM, VCL, VAP and WOB throughout the storage time at 24 ^{o}C. Afroz *et al.,* (2008) also found consistency in buck spermatozoa motility rates extended in Triladyl at 5 ^{o}C. The earliest attempt to culture mammalian cell was in 1940s where the culture media contained human plasma or serum solely and in the 1990s the well-defined albumin media additive was introduced. Today, albumin is universally added in culture media such as Ham's F10 and M199 for *in vitro* fertilization (IVF) and as a cryoprotectant for semen cryopreservation as it is well-known to be beneficial (Cabrita *et al.,* 2001; Blake *et al.,* 2002). Albumin makes up sixty percent of the total protein in the plasma. The two culture media Ham's F10 and M199 were both supplemented with 10% BSA to serve as a protein supplement. The extender, storage time and the interaction between extender x storage time, however, affected spermatozoa motility and velocity throughout the storage time in both Ham's F10 and M199, but Ham's F10 showed a lower decrease than M199. Again Ham's F10 showed higher PM than both Triladyl and M199 throughout the storage time. Triladyl also showed high sperm viability (41%) than the two culture media with M199 showing the lowest viability percentage (26%) after 72 h of storage at 24 ^{o}C. This indicates that the extender had a major effect on viability of the sperm cells.

The percentages of spermatozoa with abnormal morphology between the two Nguni bulls were not significantly different and were within the physiological range. A detailed evaluation revealed that in both bulls spermatozoa with reacted acrosome made up the greatest portion of all the abnormal sperms, followed by spermatozoa with absent tails. The least observed abnormalities (less than 2%) were coiled and bent tails and there was no significant difference between the two bulls. Generally, the morphological quality of the two Nguni bull ejaculates under study was high.

Storage temperature, time and the interaction between the two factors influenced sperm motility and velocity. Storage temperature 24 ^{o}C decreased by 24% TM better throughout the storage time, while 17 ^{o}C, 12 ^{o}C and 5 ^{o}C storage temperatures decreased by 52%, 75%, and 85%, respectively. Again 24 ^{o}C storage temperature showed higher progressive and rapid motility throughout the storage time than the

rest and it also showed consistency in RAP for the first 48 h. Total, progressive and rapid motility decreased radically at 12 °C and 5 °C. At 5 °C storage temperature both PM and RAP were less than 2% from 24 h of storage. Moreover, at 72h of storage PM and RAP were 0% at both 17 °C and 5 °C storage temperatures. Spermatozoa velocity was also influenced by the decrease in temperature and more radical decrease was observed at 12 °C and 5 °C. At 5 °C VCL was less than 50% after 24 h and at 72 h it was 31%. The VCL is always the highest compared to the three velocity values (Mortimer, 2000), which is what is observed in this study. However, a slight decrease in STR and WOB was observed at 24 °C compared to the other storage temperatures.

Past studies indicated that spermatozoa motility highly depends on the available energy source in an extender or seminal plasma in the form of adenosine triphosphate (ATP) produced from metabolism and therefore requires a constant supply for cell function and survival (Wattimena *et al.,* 2009; Mikki, 2007). Exposing fresh ejaculated semen to room temperature conditions would likely deplete the nutrients of the seminal plasma as they would likely have been consumed by the spermatozoa. This depletion would eventually lead to ATP deficits. Overall, this condition would disturb the efficiency in sperm motility and affects its viability. The highest percentage of TM (91.5%) and viability (41%) at room temperature 24 °C after 72 hours of storage was obtained with spermatozoa samples extended with Triladyl (with egg yolk) and the lowest (55%) TM and (25%) viability was obtained with M199 (with BSA). This indicates that Triladyl extender was able to supply the spermatozoa with more nutrients as the source of energy than the two culture media Ham's F10 and M199. Furthermore, sperm viability was less than 10% for the three other storage temperatures 17 °C, 12 °C, and 5 °C. Our results therefore, indicates that motility and viability of bull spermatozoa decreased radically at lower temperatures than 24 °C due to the fact that the Ham's F10 culture medium which was used for semen extension did not contain any form of composition to prevent spermatozoa against damage from cold shock. Despite the fact that the two culture media were supplemented with 0.025g/4mL maltose monohydrate and 10% BSA, motility and viability still decreased greatly as compared to the commercial extender Triladyl which contained egg yolk as part of its composition to protect the sperm cells again cold shock. A study conducted by Wattimena *et al.* (2009) showed that maltose disaccharide sugar was able to give protection and also serve as the energy source substrate for spermatozoa during preservation at 5 °C for 5 days. Monosaccharide, disaccharide and polysaccharide sugars are energy resources for spermatozoa and could be utilized as extracellular cryoprotecant to protect spermatozoa during frozen process (Rizal *et al.,* 2003). However, Sarıözkan *et al.* (2012) obtained a lower cryoprotective effect for monosaccharides (fructose), when compared to di- (trehalose) and tri- (raffinose) saccharides. Our results indicate that both maltose monohydrate and BSA did not prevent

the sperm cells from cold shock damage, but only acted as energy source substrates for spermatozoa during preservation in Ham's F10 culture medium.

The transmission of genetic information to future generations depends on the DNA integrity of sperm. DNA integrity has become an important indicator of fertile sperm. Some authors suggested that sperm DNA integrity is a more objective marker of sperm function as opposed to the sperm parameters such as motility (Love *et al.,* 2005). Sperm cells are highly vulnerable to *in vitro* manipulations, which lead to the loss of sperm functions (Nakatsukasa *et al.,* 2001). Additionally, cold shock not only deteriorates the functional parameters of sperm, but also triggers DNA damages (Gandini *et al.,* 2006). Studies had shown that cell death releases reactive oxygen species (ROS) into their micro-environment as a mechanism to digest the dead cells. The released ROS will then accumulate and multiply in quantity due to the presence of large amount of unsaturated fatty acid (Lenzi *et al.,* 1996; Lenzi *et al.,* 2002). Additionally, ROS from other sources, including electron leakage from the spermatozoa mitochondria, would also aggravate this problem. Continuous accumulation of ROS would lead to sudden increase in lipid peroxidation in the surrounding spermatozoa. Since a sperm cell has a high content of polyunsaturated fatty acids and low amount of anti-oxidant, the spermatozoa tend to be more susceptible to lipid peroxidation process compared to other cells. When the production of ROS overwhelmed the limited antioxidant defence system, oxidative stress will then occur in the cell. The lipid peroxidation and dead sperm cells will further worsen the problem since lipid peroxidation is a cascade reaction. Past studies have also indicated that the excessive endogenous oxidative stress could also induce significant DNA damage in the spermatozoa. Since the deoxyribonucleic acid bases and phosphodiester backbones are linked by double bonds, these molecules become very susceptible to peroxidation (Teebor *et al.,* 1988). The loss of sperm's DNA integrity has been linked to a reduction of spermatozoa's viability (Uysal & Bucak, 2007) and this would likely be what was seen in this study. Our results indicate that the effect of different storage temperatures did not influence sperm DNA fragmentation as there was no significant difference between all the temperatures and viability was greatly reduced in all the four storage temperatures.

CHAPTER 6

6. CONCLUSIONS AND RECOMMENDATIONS

The results from this study shows that Nguni semen can be extended using the commercial extender Triladyl or Ham's F10 and M199 culture media and stay alive for 72 hours stored at controlled room temperature 24 ^{o}C. Triladyl showed consistency in total sperm motility rate for 72 h and thus proved to be the best extender. Ham's F10 proved to be the second best extenders showing higher sperm motility throughout the storage time. Lower preservation temperatures than 24 ^{o}C in Ham's F10, however, influenced a drastic decrease in spermatozoa motility and viability. Further research is necessary to evaluate fertility rate using Triladyl, Ham's F10 and M199 as semen extenders stored at 24 ^{o}C. Again, more work needs to be done on the use of antioxidants at appropriate optimal concentrations in order to mitigate the effects of ROS in extended bull semen.

Table 6. Spermatozoa motility and velocity parameters.

Parameter	Definition	Unit
Total Motility (TM)	Percentage of sperm showing any form of movement.	%
Progressive Motility (PM)	Percentage of sperm moving rapidly in a straight forward direction.	%
Rapid Motility (RAP)	Percentage of sperm travelling at a speed of 25 µm/sec or faster.	%
Curvilinear Velocity (VCL)	Time/average velocity of a sperm head along its actual curvilinear path.	µm/s
Straight Line Velocity (VSL)	Time/average velocity of a sperm head between its first detected position and its last position projected along the straight line.	µm/s
Average Path Velocity (VAP)	Time/average velocity of sperm head along its spatial average path.	µm/s
Linearity (LIN)	Measure of the curvilinear path. LIN = VSL/VCL.	%
Straightness (STR)	Measure of the spatial average velocity path. STR = VSL/VAP.	%
Wobble (WOB)	Measure of oscillation of the actual curvilinear trajectory about its spatial average path. WOB = VAP/VCL.	%

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