PRESERVATION OF BOER GOAT SEMEN IN LIQUID NITROGEN VAPOUR IN COMPARISON TO THE CONVENTIONAL FREEZING METHOD USING DIFFERENT EXTENDERS, FREEZING AND THAWING REGIMES

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DECLARATION

I, Kidinda Kalobo, the undersigned student, hereby declare that this dissertation, for the Master of Science in Animal Science (MSc. ANS) degree at the University of Venda, is my own work and has not been previously, in part or in its entirety, submitted to any university for any other degree.

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DEDICATION

I dedicate this work to my parents, the late Mr G.L. Kalobo and Mrs P.L. Mendaku. I love you both till eternity. I am indebted to my loving mother and friend whose love is like no other.

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ABSTRACT

The Boer goat *(Capra hircus*) is one of the most desirable goat breeds for meat production. The impact of cryopreservation on the viability of its semen depends on the extenders, freezing and thawing methods. This study evaluated the effects on sperm viability in Boer goat semen extended using Bioxcell, Biladyl and Ham's F10, and frozen in semen straws placed on a rack at 4, 5, 6 or 7 cm above the surface of liquid nitrogen. After storage in liquid nitrogen for 7 days, the frozen semen was thawed at 37 ^{o}C for 30 seconds or 90 ^{o}C for 5 seconds. Samples of sperm were also frozen to -196 ^{o}C in a programmable freezer, as the control regime for the freezing treatments. Sperm morphology, motility and viability were evaluated using the computer aided sperm analysis (CASA) system in a randomised design in which the treatments were in a 3 (extender) X 5 (freezing regime) and X 2 (thawing regime) factorial arrangement. The extenders Bioxcell and Biladyl were affected in the total motility, progressive motility and static ($P<0.01$), the motility was overall maintained only in straws placed at 5 cm above the liquid nitrogen level, with significant difference for the interaction extender X freezing regime in the total motility ($p<0.01$), non-progressive motility ($p<0.05$) and progressive motility ($p<0.01$), the 37 ^{o}C for 30 sec thawing regime had significantly more ($P<0.05$) in cut-head spermatozoa. Ham's F10 extender had significantly lower normal spermatozoa ($P<0.05$) compare to Biladyl and Bioxcell extenders. In conclusion, the extender type, freezing and thawing regime were important factors for consideration in goat semen preservation for artificial insemination purposes, with the nitrogen vapor method comparable to the conventional programmable freezer method only when semen straws are placed at 5 cm above the liquid nitrogen level.

Keywords: Conventional method of freezing, Cryopreservation, Different rates, Goat semen, liquid nitrogen vapour.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AI : Artificial insemination

ALH : Amplitude of Lateral Head Displacement

ANOVA : Analysis of variance

AO : Acridine Orange

ART : Assisted Reproductive Technology

ASMA : Automated Semen Morphology Analysis

ATP : Adenosine Tri-phosphate

AV : Artificial vagina

Bi : Biladyl

BCF : Beat Cross Frequency

Biox : Bioxcell

BSA : Bovine serum aluminium

Busgp 60 : Bulbourethral secretion glycoprotein 60

CASA : Computer Assisted Sperm Analyzer

CUE : Cornell University Extender

DNA : Deoxyribonucleic acid

E : Extender

EE : Electro ejaculator

EYCE : Egg yolk coagulating enzyme

Ca : Calcium

FAO : Food and Agricultural Organization

FAOSTAT : Food and Agricultural Organization Statistics

g : Grams

GLM : General Linear Model

Ham's : Ham's F10

HOST : Hypo-osmotic Swelling Test

HOP : Hamster Oocyte Penetration

IVT : Illini Variable Temperature

IVF *: In vitro* fertilisation

K : Potassium

LN_2 : Liquid nitrogen

LIN : Linearity of Cell Track

RNA : Ribonucleic acid

SCA : Sperm Class AnalyzerSCA

SCSA : Sperm Chromatin Structure Assay

T : Temperature

TCM : Tissue Culture Medium

Tr : Triladyl®

UV : Ultraviolet

VAP : Average Path Velocity

VSL : Straight Line Velocity

WHO : World Health Organization

CHAPTER 1: INTRODUCTION

1.1 Introduction

Boer goats *(Capra hircus*) are considered to be one of the most desirable breeds for meat production. They have gained worldwide recognition for excellent body conformation, a fast-growing rate and good carcass quality. Its popularity as a meat goat breed soared during the last decade due to its availability in Australia, New Zealand, and, later in North America and other parts of the world (S.A Boer goat Breeder's Association). It has been demonstrated that Boer goats can improve productive performance of many indigenous breeds through cross breeding because it has a strong impact on the goat meat industry globally. Although the exact origin of Boer goats is not clear, it is believed to be the result of a genetic pooling of African indigenous goats, Indian goats, and European dairy goats. The present-day Boer goats appeared in the 1900's when Eastern Cape Performance testing of Boer goats started in 1970 under the South African Mutton and Goat breeders (Casey & Van Niekerk, 1988). A combination of breeding standards and performance testing is likely to be the best approach for the effective selection and improvement of the Boer goat breed.

Goat spermatozoa require unique attention to maximise post-thaw viability, as the deleterious interaction between egg yolk and the bulbourethral gland secretions exists in goat semen but not in other species such as the bull, boar or ram (Purdy, 2006). Frozen-thawed goat semen can only be used when the spermatozoa are deposited into the uterus, for example, during laparoscopic and transcervical AI. However, if used for cervical AI, there would not be enough spermatozoa in a single inseminate that survive the freeze-thaw process to cause fertilization. The optimal freezing process for goat semen, therefore, has to be found. The rate of cryopreservation and thawing of goat semen are variables that can influence the survival of the spermatozoa.

The ability to cryopreserve spermatozoa from all the domestic species is challenging. Even though all the cells must endure similar physical stresses associated with the cryopreservation processes, spermatozoa from different species are different in size, shape and lipid composition, all of which affect cryosurvival. Thus, when a cryopreservation protocol has been optimised for sperm of one species, it may not be ideal for sperm of other species. Semen cryopreservation causes ultra-structural, biochemical and functional damage of spermatozoa, resulting in their decreased motility and viability. A specific problem limiting post-freeze properties of goat semen is the presence of the egg yolk coagulating enzyme (EYCE) in the seminal plasma (Leboeuf *et al.,* 2000). Therefore, a thorough understanding of the

specifics of spermatozoa freezing for a particular species will improve the cryosurvival of spermatozoa from that species (Saraswat *et al*., 2012). Due to the differences between the spermatozoa of the domestic species, this study was conducted to investigate the cryopreservation of Boer goat spermatozoa using the conventional method and programmable freezer. This study sought to compare the survival rate of goat semen frozen in liquid nitrogen vapour compared to the conventional freezing method after being stored in liquid nitrogen (-196^{o}C) for seven days.

1.2 Problem Statement

The productivity of goats in Vhembe District Municipality of Limpopo Province is poor. The constraining factors include both the reproductive management and the genetic potential. Improved reproductive management and genetic improvement through AI may be possible. However, conventional semen cryopreservation may not be optimal for goats in this production environment. Therefore, this study was designed to compare the survival rate of Boer goat semen extended using three different extenders and frozen by placement of the semen at different levels above liquid nitrogen vapour to the conventional freezing method, followed by comparison of different thawing regimes.

1.3 Justification

Goat semen processing for artificial insemination or preservation demands suitable specific protocol for a dilution to be developed or modified. Current extenders do not preserve goat semen 100% and there are several obstacles that need to be addressed. Improvement will help to facilitate the cervical and trans-cervical AI of the goat in this area. Consequently, the goat herd's quality will increase, and the genetic value of the goats will improve.

1.4 Main and Specific objectives

1.4.1 Main objective of the study

This study aimed to compare the survival rate of goat semen extended in different extenders and frozen at different levels of liquid nitrogen vapour to the conventional freezing method, followed by storage in liquid nitrogen (-196^{o}C) for seven days, and thawing at different rates.

1.4.2 Specific objectives

- To determine the effects of extending Boer goat semen with Bioxcell, Biladyl and Ham's F10 extenders on the quality after cryopreservation in liquid nitrogen for 7 days.
- To determine the effects of freezing method on quality of goat semen.
- To determine the effects of thawing method on the quality of Boer goat semen.

1.5 Research hypothesis

- There is no difference in the quality of semen extended using Bioxcell, Biladyl and Ham's F10 and cryopreserved in liquid nitrogen for 7 days.
- There is no difference in quality of Boer goat semen frozen in liquid nitrogen vapour and programmable freezer and stored in LN2 for 7 days.
- There is no difference in quality of Boer goat semen frozen and thawed at 37 ^{o}C for 30 seconds and 90^{o}C for 5 seconds.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

According to Lehloenya *et al.* (2004), indigenous goat breeds in South Africa, such as Nguni and Boer goats, have a moderate productive performance under natural conditions. These breeds are well-known for adaptability, hardiness, resistance to diseases, as well as good mothering ability. Regardless of their good characteristics older goats may have relatively low fertility due to their age, or where AI is applied, possibly because of prior multiple.

To determine the future outlook of goat populations and their productivity, accurate statistics are required. Before any improvement policies can be planned on a realistic basis, these policies are needed as this will help those implementing them to do so with confidence. Goats are part of the Caprinae family and are closely related to sheep as both are in the goat-antelope subfamily. Goats can be managed intensively, semi-intensively and extensively. It is difficult to sustain accurate data due to goat farming systems and communal practices under which goats are commonly kept. According to FAOSTAT (2008), the world's total number of goats and sheep were 861.8 and 1078.2 million respectively, meaning that there is about one goat to approximately 1.25 sheep in the world.

In Africa, Central America and Asia, the ratios of goats to sheep are equal, indicating the considerable importance of the goat population in these parts of the world, especially among the poor and landless peasants. Figure 2.1 shows the number of goats in the world between 1990 and 2008.

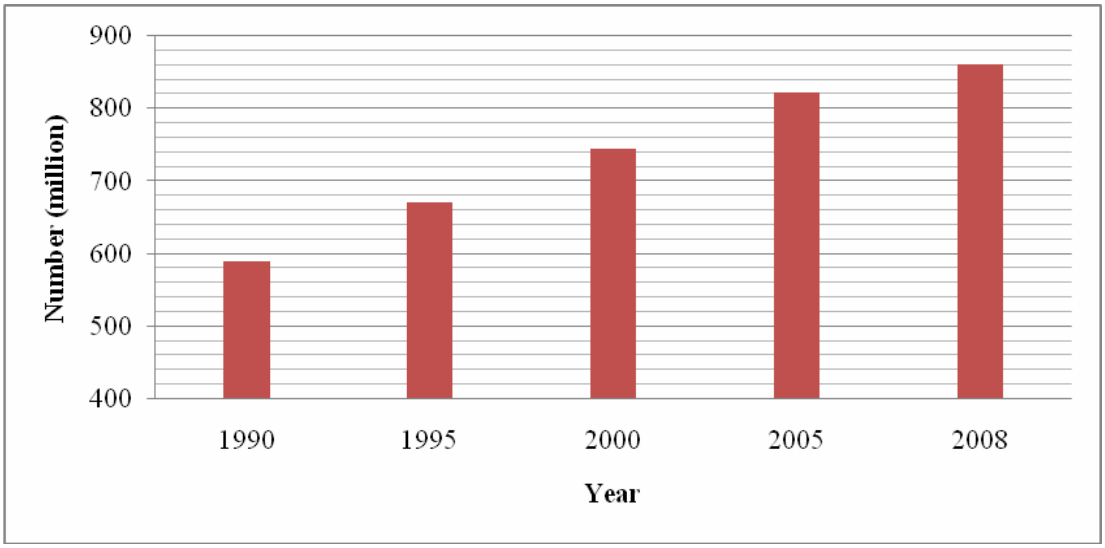

Figure 2.1: Number of goats in the world between 1990 and 2008 (FAOSTAT, 2008)

2.2 South African breeds of goats

South African indigenous goats are less susceptible to different parasites and diseases, such as blue tongue, are able to survive on the poorest vegetation and are well-known for being non-selective browsers (South African Boer Goat Breeders' Association, 2006). The majority of South African indigenous goats are kept in rural areas and they serve as a source of milk, hides and meat (Webb *et al.,* 2003). However, the indigenous goat breed is under threat of extinction and very little information on their reproductive status is known (Webb *et al.,* 2003). Fresh semen characteristics that were previously obtained from other South African indigenous goats included semen volume of more than 1.5 ml (Tuli & Holtz, 1994), sperm cell motility of 72% (Tuli & Holtz, 1995), sperm cell concentration of greater than 3 billion sperm cells and motility generally ranges from 80 to 90% (Gacitua & Arav, 2005).

2.2.1 The Boer goat

Boer goats are white with reddish-brown heads, ears and necks and white faces. They are large, long-legged goats with short soft hair and long lopped ears. The goat has a full and well-fleshed neck, which is moderately long, and a very powerful head with a compressed nose and strong horns with a gradual backward curve (Samurais, 1992). A mature Boer goat weighs between 90-100 kg, while a mature Boer buck weighs between 110-135 kg with well fleshed buttocks and thighs. The Boer goat is a meat rather than milk type (Malan, 2000). It is a famous subspecies which originated from South Africa, and is well-known for meat production. It has gained worldwide recognition for excellent body conformation, fast growth rate and good carcass quality (Lu, 2003). In addition, the Boer goat is highly adaptable to various environments and feeds; it grows fast and the meat is popular among consumers (Tao *et al.,* 2009). Globally, it has a strong impact on the goat meat industry.

Figure 2.2: Boer goat (White Forest, 2012)

2.3 Advantage of keeping goats

According to Devendra (1985), in many parts of the world where the geographical properties of the terrain are not suitable for other livestock species, goats seem to be the best choice. Based on the accumulated information on the characteristics of goats, it can be concluded that goats have a specific place in the animal agricultural economy of many countries. He further states that goats have played a key role in supplying food to humans. Dubeuf *et al*. (2004) opine that, as in many Western Countries, traditional and religious groups (e.g. Muslims and rural dwellers) are the predominant consumers of goat meat in South Africa. They also stated that, prior to 1994, the importance and size of this market, both locally and internationally, was ignored by marketers. This, in turn, resulted in a lack of marketing and research institutions even in many Western Countries. With the industrialisation of agriculture during the 20th century, cattle and other livestock developments progressed in the Northern Hemisphere, while less and less money was being invested in research to improve the genetic make-up of goats and the development of their production and management.

Today, goat meat and milk are considered healthier than mutton, beefor pork. In light of these new nutrition research results, as well as the advancement of information technology, the World markets, especially the opening of the east to trade in different commodities, goat farmers may see an increase in the demand for goat meat and its products. Traditional people are also migrating to different corners of the globe and many countries are seeing an increase

in the demand for goat meat, milk and goat products such as leather and cashmere). In the developing world, goats offer their owners a wide range of products to sustain them.

2.4 Characteristics of goats

According to Abdel-Aziz (2010), with the increasing frequency of drought and long-term environmental degradation, pastoralists are forced to change from raising cattle or sheep to camels or goats. This has compelled farmers and pastoralists to rely on goats as a means of survival and a way of boosting their income because goats can endure prolonged water deprivation because they have great adaptability to adverse climates where sheep and cattle cannot survive. A range of products and socio-economic services are provided by goats to their owners and they have played an important role in the social life of many people, for example, being used for rites of passage, dowry, religious purposes, rituals and as gifts. In addition, goats can efficiently utilize poor quality forage and cover long distances looking for food and their peculiar feeding habits make them easier for owners to choose diets to meet their requirements.

According to Restani (2004), goats are easily acquired by the poor as they require modest capital. They provide people with valuable nutrients and from past research, many people who cannot consume cow milk due to allergies are able to do so with goat milk. The increasing demand for goat meat presents an opportunity for increasing farmers' herds through animal assisted reproduction.

However, according to Celik and Boyazoglu (2010), there is a justified concern about the damaging effect of goats on forests, trees, woodlands and grazing. The severe damage that goats have caused in some regions is usually associated with high stocking density and mismanagement. Goats have a huge appetite for and the ability to effectively utilise many trees and shrubs not available or not palatable to sheep and cattle. Therefore, they can be more damaging to perennial vegetation and soil stability than cattle or sheep. This is greatly noticeable during a drought in arid zones, as goats have a reputation for being good survivors. It is clear that goats need careful management to prevent irreversible damage to the vegetation.

2.5 Goat spermatozoa

Spermatozoa continuously change and develop from their origins as somatic spermatogonia cells until their destination as highly specialized cells capable of fertilization.

Like other sperm cells, goat sperm has basically three functional regions, namely a head that contains the condensed nuclear material, amid piece serving as a powerhouse and a tail which is the propulsive region. Sperm development takes place in the seminiferous ducts of the testes, and subsequent maturation occurs within the epididymis, followed by further development induced first by contact with seminal plasma and then by the secretions of the female tract (Varner & Johnson, 2007). The final stages of spermatozoon development are induced by the immediate environment of the oocyte and its surrounding zona. In the process, most of the organelles are lost together with the cytoplasm, and the spermatozoa chromatin is remodelled. This specialisation, though, is achieved at a cost, reducing the spermatozoon's ability to repair itself, leading to a greater susceptibility to environmental change. Hence, even under ideal conditions, it is inevitable that some damage will occur to spermatozoa during the freezing process (Andrabi, 2007).

2.6 Cryopreservation of goat semen

Pegg (1972) states that cryopreservation is the use of very low temperatures to preserve structurally intact living cells and tissues. Unprotected freezing is normally lethal. He further opines that biological effects of cooling are dominated by the freezing of water, which results in the concentration of the solutes that are dissolved in the remaining liquid phase. Rival theories of freezing injury have envisaged either that ice crystals pierce or tease apart the cells, destroying them by direct mechanical action, or that damage is from secondary effects via changes in the composition of the liquid phase. By simply increasing the total concentration of all solutes in the system cryoprotectants, reduce the amount of ice formed at any given temperature. However, to be biologically acceptable, they must be able to penetrate into the cells and have low toxicity. Many compounds have such properties, for example, glycerol, dimethyl sulfoxide, ethylene glycose, and propylene glycol (Pegg, 1972).

2.7 Goat seminal plasma effect

Goat semen is different from that of other domestic species in its limited tolerance to the inclusion of egg yolk in the freezing medium. Tolerance depends on the presence of enzymes in the seminal plasma that reacts with egg yolk, thus producing toxic compounds to the spermatozoa. Moreover, the goat is a seasonal breeder that shows variations in semen quality throughout the year. Those variations may affect semen freezability, thus, in freezing protocols, for instance, removal of seminal plasma (washing) yields varying results.

2.8 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. The dilutions are done while preserving the functional characteristics of the sperm. Thus, in order to preserve spermatozoa for extended periods, their metabolic activity needs to be reduced by dilution in an appropriate medium and by lowering the temperature. The relationship between metabolic rate and temperature is often expressed as Q10, which measures the increase in metabolic rate 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 2 times, at 20^{o}C, 4 times, etc. (BIOG 1105–1106, 2012). The metabolic rate of a body cell (including spermatozoa) will thus decrease by a factor of 2 times for every 10^{o}C drop in temperature. The addition of diluents to the semen lowers the concentration of certain elements, ions and compounds in the seminal plasma (Harrison *et al.,* 1978) or the plasma proteins, altering sperm viability. These losses need to be compensated for by adding the necessary ingredients in the diluent formulations. Citrate is the salt of choice as its chelating properties improve 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).

Egg yolk and skim milk are the most common cryopreservation diluents for goat semen. However, the dilution of goat semen in extenders with egg yolk or skim milk can be detrimental to spermatozoa. Currently, 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 cause damaging interactions between seminal plasma and egg yolk or milk (Leboeuf *et al.,* 2000; Nunes *et al.,* 1982). 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).

Generally, egg yolk has been accepted as 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 yolk-based extender is limited by relatively short-time fertilisation capacity (Aurich *et al.,* 1997) and the individual differences in egg yolk due to different periods of egg storage. The removal of the chicken egg yolk from a semen extender would improve uniformity in the components of semen extenders and eliminate hygienic risks.

2.9 Temperature and dilution effect

The spermatozoa 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 the 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, as well as and more permeable to ions (Hammersted *et al.,* 1990).

Spermatozoa are naturally diluted with seminal fluids from the accessory glands at ejaculation and their motility is retained for some hours. To extend their survival *in vitro*, it is necessary to reduce the metabolic activity by chemical inhibitors or by lowering the temperature, which also requires dilution. 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 a pure electrolyte media (Johnson *et al.,* 2000). Harrison *et al.* (1982) argue that the dilution effect is due to the absence of proteinaceous motility stimulants from seminal plasma and show that serum albumin could stimulate motility in a reversible manner.

In a study by Buhr (1990) bovine serum albumin (BSA) was used 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 the trans-membrane movement of Ca^{++}, which is essential in the capacitating process, but detrimental during storage. However, BSA induced a temporal decrease in membrane fluidity and this was difficult to interpret. BSA nonetheless improved fertility when the semen was between 3 and 5 days (Waberski *et al.,* 1994).

The earliest attempt to culture mammalian cell was in the 1940s; the culture media then 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 (Blake *et al.,* 2002; Cabrita *et al.,* 2001). Albumin constitutes 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 an effective lipid peroxidation inhibitor (Alvarez &Storey, 1995). Regardless of all the advantages, if the media is too concentrated with albumin, it will pull the water out of the cell (osmotic effect) and this may lead to shrinkage and crystallisation in the cell.

2.10 Culture media and semen extension

Embryo culture *in vitro* requires an artificial environment capable of sustaining the viability of the fertilised *(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. However, this artificial environment is immensely different from the dynamic spatial and temporal physiological environment in *vivo* (Biggers & Summers, 2008).

Storing culture media is more advantageous than reproducing stock medium every two weeks. A number of batches can be made at the beginning of each IVF cycle, tested with the 1-cell mouse embryo bioassay and selected for those batches that perform the best. Those batches that perform best can, therefore, be properly stored in a refrigerator at 4^{o}C and subsequently, used for human embryo cultures during that cycle. This would also minimise variability among culture media and cut down on expenses and technician time. Storage would also enable gametes and embryos from a group of patients to be exposed to the same batch of culture medium in humans (Bernart*et al.,* 1990).

De Silva (1993) evaluated the effect of storage of Ham's F-10 medium on 1-cell mouse embryo development in *vitro*. The findings of this study indicated that Ham's F-10 medium can be stored in a refrigerator at 4^{o}C for longer than the recommended 2-week period without

compromising embryo development in *vitro*. These results were in agreement with two other studies by Naz *et al.* (1986) and Bernart *et al.* (1990), which showed that Ham's F-10 medium can be refrigerated at 4°C or frozen (-20°C) without affecting embryo development *in vitro*.

The objective of extending semen was to provide an appropriate volume of inseminate with a sufficient number of sperm to give high fertility without wasting sperm. Spermatozoa concentration of the ejaculate varies from 2 to 6.5 x 10^9 sperm/ml. Highest fertility in ewes was reported for sperm. The objective of extending semen is to provide an appropriate volume of inseminate with a sufficient number of sperm to give high fertility without wasting sperm. Spermatozoa concentration of the ejaculate varies from 2 to 6.5 x 10^9 sperm/ml. Highest fertility in ewes was reported for sperm suspension frozen at a concentration of 666 x 10^6 sperm/ml. Another worker inseminated goats with 1 x 10^9 sperm/ml and reported fertility. Semen of rams and bucks is routinely extended immediately after collection at 37 °C and cooled slowly to prevent cold shock, which would result in sperm damage. Lipoprotein and lecithins found in egg yolk and milk, provide protection from cold shock when added to the semen before cooling. The optimum cooling rate is one that prevents cold shock but is fast enough to maintain healthy, viable sperm cells. After extension, semen is cooled to 5 °C at approximately 0.5 °C/min. Glycerol is added to extenders to protect sperm against the detrimental effects of freezing. It has been suggested that the increase in intracellular salt concentration due to the removal of water from the cell during ice formation is a major cause of sperm damage during freezing. The protective action of cryo-protectants, such as glycerol, is largely attributed to their salt "buffering" capacity. Thus, electrolytic damage, as the water freezes is minimised.

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2.11 Freezing of semen

It is generally accepted that the cryopreservation process reduces more than 50% of sperm viability. During cryopreservation, the spermatozoa are subjected to biochemical, osmotic, thermal and mechanical stresses, which are conspicuous at dilution, cooling, equilibration, freezing and thawing stages. Diluted buck semen is cooled to 4 - 5°C over a period of 1.5 - 4.0 h and then frozen in either pellets or straws (Evans & Maxwell, 1987; Leboeuf *et al*., 2000). Higher sperm survival was observed when semen was cooled at 5°C within 2.0 - 2.5h. A fast cooling rate (less than 1.5h) has adverse effects on the freezing ability of buck semen (Purdy, 2006). Major changes in goat spermatozoa occurred during cooling from 15°C to 5°C and not below 0°C (Watson, 2000).

Freezing of sperm in straws is more expensive and laborious than the pellet freezing technique, but each sample can be accurately labeled for inventory management. In straw freezing, diluted and chilled semen samples are loaded to 0.25 or 0.5 ml straws, placed on a rack 3 to 4 cm above the liquid nitrogen (LN) for 7 to 8 min on LN vapour in a Styrofoam box or in programmable freezer; Straws are then plunged in LN for storage (Evans & Maxwell, 1987). In pellet freezing aliquots of 0.1 - 0.5ml of cooled semen sample is dispensed into indentations on a block of solid carbon dioxide −79°C and frozen for 2 - 4 min. Pellets are then plunged into liquid nitrogen for storage (Evans & Maxwell, 1987; Chemineau *et al*., 1991).

The pioneering work of Sahni and Roy (1972), on deep freezing of buck semen using the original Cambridge method (-79 °C), revealed 30 – 40 % post-thaw motility in citrate yolk and milk diluents containing 3 – 6 % glycerol as a cryoprotectant. Later, in 1972, survival of 35% was observed in buck semen frozen by straw method (-196 °C) (Maxwell *et al*., 1995). However, Chemineau *et al.* (1991) reported that the size or diameter of the straw should determine the freezing height above the liquid nitrogen. Ritar *et al* (1990 a, b) reported that sperm frozen in pellets yielded superior motility (39 %) following thawing compared to sperm frozen in straws. Differences in post-thaw motility, viability and fertility may be due to the different cooling rates produced by the pellet and straw methods. Programmable freezers are more convenient for the freezing of large quantities of semen straws and these are beneficial as freezing rate can be controlled and the freezing curve can be customized, for example 4 to −5 °C at 4 °C/min, −5 to −110 °C at 25 °C/min and −110 to −140 C at 35 °C/min, and then the semen straws can be plunged into liquid nitrogen (Sharma, 2004). However, the choice of the cryopreservation method should also take into account other factors like ease of handling, labour, insemination technique, inventory management and the cost of the bio-freezer. The kidding rate and prolificacy were significantly higher in goats inseminated with semen frozen

by the ultra-low freezing technique and it is a more suitable technique for the freezing of goat semen (Batista et al. 2009).

2.12 Thawing of frozen semen

Thawing of semen samples is based on the method used to freeze the semen sample. Sperm pellets should be thawed in a dry test tube at 37°C while the straws can be thawed using various time and temperature combinations (Evans & Maxwell, 1987). Thawing of straws by placing them in 37 °C water bath for 12 – 30 seconds was superior (36.1 % motility) to a slow thaw method (18.9 % motility), where the semen straws were placed in a 5 °C water bath for 2 minutes (Deka & Rao, 1987). Thawing of straws at 70°C for only 7 seconds resulted in significantly higher progressive motility (36.9 %) and plasma membrane integrity (39.8 %) compared to thawing straws at 37 °C for 2 minutes which resulted in 31.5%, 33.7% progressive motility and plasma membrane integrity, respectively or 40°C for 20 seconds resulting in 32.4% progressive motility and 33.5% plasma membrane integrity. Attention to temperature and timing becomes much more critical as the temperature goes higher than 37 °C since these high temperatures can result in tremendous sperm mortalities if performed incorrectly (Tuli *et al.,*1991).

2.13 Evaluation of semen

According to Partyka *et al.* (2012), light microscopy is usually used to analyse the quality and predict the fertility of the cryopreserved semen in a conventional way. The visual assessment requires such equipment as microscopes, heated stage and slides, as well as an experienced evaluator. However, the assessment is subjected to the evaluator's bias. Computer assisted sperm analysis has recently been introduced to veterinary andrology (Rijsselaere *et al.,* 2003; Verstegen *et al.,* 2002). It assures objective semen assessment, while the main disadvantage of conventional semen evaluation is the variability of obtained results. The subjectivity of traditional semen analysis is associated mainly with the experience and skill of the observer, the method of specimen preparation, staining technique and the number of cells evaluated. Variations in the results of conventional evaluation of the same semen samples by different observers and laboratories may be between 30 and 60% (Coetzee *et al.,* 1999; Davis & Katz, 1992).

The immediate measurement of spermatozoa concentration, the total number of spermatozoa in the ejaculate and the automated calculation of the number of insemination units which could be prepared from one ejaculate is one important advantage of computer aided sperm analysers. In addition, some machines are equipped with ultra-violet (UV) excitation module, which gives one the opportunity to analyse the percentage of live and dead spermatozoa after staining with vital fluorescent probes such as Hoechst 33258. Nevertheless, the CASA system needs standardisation and validation before it is used and image settings standardised (Davis & Katz, 1992; Iguer-Ouada & Verstegen, 2002; Rijsselaere *et al.,* 2003; Verstegen *et al.,* 2002). Other factors, such as the type and depth of the used chamber, number of fields analysed, the temperature during analysis and protocol of semen sample preparation, also affect results. Optimisation and validation of the technical settings would allow a comparison between intra and inter-laboratory results, regardless of the instruments used (Agarwal *et al.,* 1992).

2.13.1 Spermatozoa motility

Penaz-Martinez (2004) asserts that motility is an important attribute of spermatozoa because it is readily identifiable and reflects several structural and functional competence, as well as essential aspects of spermatozoa metabolism. It is expressed as the percentage of total motile or progressively motile spermatozoa. This parameter is usually assessed by the subjective visual examination under a phase contrast microscope at 37 °C using low objectives (x10 or 20x). Light microscopic evaluation of motile spermatozoa does not require expensive equipment and is a simple and rapid method for assessment of sperm quality. However, it is a highly subjective and not reliable assay for the prediction of fertility. Subsequently, correlations between spermatozoa characteristics and fertility trials in females are relatively low.

Computer aided sperm analysers allow for calculation of several motility parameters which characterise movement of individual sperm cells. They include VAP-average path velocity, VSL-straight line velocity, VCL-cell velocity, ALH-amplitude of lateral head displacement, BCF-beat cross frequency, STR-straightness of cell track, LIN-linearity of cell track, a subpopulation of rapid, medium and slow cells (Niżański *et al.,* 2009). Selected characteristics of spermatozoa motility parameters measured by CASA systems are shown below.

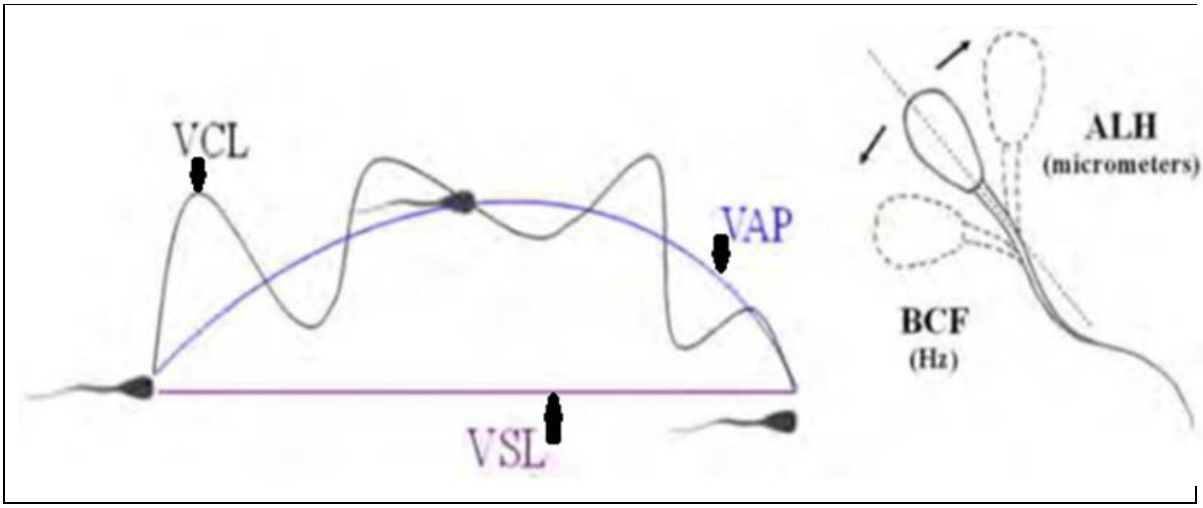

Figure 2.3: Schema of different velocities and parameters of sperm movement, measured by CASA systems (Partyka *et al.,* 2007)

2.13.2 Sperm morphology

Sperm morphology is one of the most important criteria for determining the quality of a semen sample for humans and animals assessment (Gravance *et al.,* 1998; WHO 1999; Henkel *et al.,* 2007; Van der Horst *et al.,* 2009). Many claims have been made about the relationship between spermatozoa morphology assessment and fertility (Enginsu *et al.,* 1991; Menkveld *et al.,* 2003). Accordingly, many stains and staining combinations, which have been employed in order to determine the percentage of spermatozoa with normal morphology as the accuracy of sperm morphology assessment, will depend on careful preparation, fixation, and staining of spermatozoa because these procedures can affect spermatozoa dimensions significantly (Garcia-Herreros *et al.,* 2006). Thus, one of the criteria for a staining method is that the process involved should cause as little change to spermatozoa morphology as possible (Meschede *et al.,* 1993; Gabo *et al.,* 1998, Hidalgo *et al.,* 2006; Lukaszewicz *et al.,* 2008).

Even though classification categories are different for the various species and that there is a need to adopt a uniform system within each species, many reports have shown common classification systems for the morphology of spermatozoa from different species. Mammalian spermatozoa abnormalities can be divided into primary and secondary abnormalities (Hafez, 2008), and in some classification systems, into major and minor abnormalities (Blom, 1968, 1983). Hafez (2008), argue that morphologic abnormalities of spermatozoa can be primary, secondary or tertiary. Primary abnormalities to the failure of spermatogenesis and secondary abnormalities occur during the passage of spermatozoa

through the epididymis, while the damage to sperm resulting during or after ejaculation or from the improper handling of the artificial insemination (AI) is designated as a tertiary abnormality.

The most common spermatozoa abnormalities are related to abnormal acrosomal regions heads, detached head, proximal droplets, distal droplets, abnormal mid-pieces, and bent coiled tails. Acrosome defects include knobbed, roughed, and detached acrosomes. Head defects include microcephalic, macrocephalic, pyriform, tapered, other shape defects, nuclear vacuoles and multiple heads. Mid-piece and principal piece (tail) abnormalities include simple bent, folded, fractured, thickened, swollen, roughed, disrupted sheet, duplicated and coiled. Various defects are typical of each species. For each slide, at least 100-300 spermatozoa should be counted at X400-1000 magnification; this would allow for accurate calculation of the percentage of different sperm defects (Morrell, 2011).

Analysis of spermatozoa morphology may be done using Diff-Quik stain recommended by the World Health Organization (WHO, 2010) or Sperm Blue®, which has been developed for the evaluation of human and animal spermatozoa morphology (Van der Horst & Maree, 2009; Maree *et al.,* 2010). According to Bjorndahl *et al.* (2003), eosin-nigrosin staining developed for ‘‘live-dead’’ staining of sperm has also been used to assess spermatozoa morphology for many animal species, but it does not clearly differentiate between the various components of the spermatozoa (Van der Horst *et al.,* 2009). Many other staining combinations (Jager *et al.,* 1984; Gravance *et al.,* 1998;Belletti & Melo 2004) have been used to access spermatozoa morphology for humans and several animal species. They have, however, not been shown to have wider application in a routine laboratory. Several studies have shown the need for Automated Semen Morphology Analysis (ASMA) in the routine clinical laboratory to improve accuracy and avoid bias when determining the percentage of normal spermatozoa (Gago *et al.* 1998; Coetzee *et al.,* 1999, 2001).

2.14 Semen extension

As mentioned earlier, seminal plasma plays an important role in activating spermatozoa in the female reproductive tract. However, it is detrimental to long-term sperm survival outside the body. Under physiological conditions, seminal plasma supports spermatozoa at ejaculation by creating a milieu beneficial for the spermatozoa in a vaginal surrounding that is normally acidic, activating and augmenting their motility, coating the sperm cells with capacitating inhibitors, and supplying nutrients for the sperm cells. The spermatozoa then swim away from the seminal plasma in the anterior part of the ewe’s vagina. It is only during

in vitro storage that spermatozoa become exposed to seminal plas50ma for a long time (Rodriguez-Martinez *et al.,* 2009).

It is customary to add a semen extender to the semen, in order to dilute toxic elements in seminal plasma, provide nutrients for the spermatozoa during in vitro storage and to buffer their metabolic by-products. The addition of the extender also permits the semen to be divided into several more doses, each containing a minimal number of spermatozoa that have been determined to be optimal for good fertility when used in inseminated females (Morrel *et al.,* 2010).

2.15 Components of semen extenders and their effects on spermatozoa

Currently, egg yolk is a common component of most semen cryopreservation extenders for domestic animals. It has been shown to have a beneficial effect on sperm cryopreservation, especially on the plasma membrane, and in association with the other components, it protects the acrosomal sac against temperature-related injury (Amirat *et al.,* 2004). There have also been numerous reports that egg yolk from avian species, such as the duck, quail, pigeon and chicken, has different combinations of fatty acids, phospholipids and cholesterol, which could result in different cryopreservation effects on the sperm (Trimeche *et al.,* 1997; Choi *et al.,* 2001; Andrabi *et al.,* 2007; Su *et al.,* 2008). However, there are a few reports comparing the effect of egg yolk of different species (domestic chicken, goose, turkey, duck, Japanese quail and chucker) in the extender on the efficiency of cryopreservation of ram or buck spermatozoa. The potential cause of decline in motility and fertility during the hypothermic storage of liquid semen is an oxidative damage of spermatozoa (Ball *et al.,* 2001). A wide variety of antioxidants, such as glutathione, oxidized glutathione, cysteine, taurine, hypotaurine, bovine serum albumin, trehalose or hyaluronan, have been tested to determine their abilities to minimise the damage caused by cooling and freeze-thawing of goat semen (Salvador *et al.*, 2006) and ram semen (Paulenzen *et al.,* 2002; Uysual *et al.,* 2005).

Pure substances and clean equipment have been used to exclude toxic materials and micro-organisms from the sperm environment. Extenders should be prepared aseptically and stored for less than a week unless frozen. Simple carbohydrates, such as glucose and/or fructose, are added as sources of energy for the sperm. Both egg yolk and milk are used to protect buck and ram sperm against cold shock as they are cooled from body temperature to 5^0C (Ax*et al.,* 2000). These substances also contain fructose, which is the principal seminal sugar for fructolytic activity in releasing energy in the form of adenosine tri-phosphate (ATP)

to the spermatozoa, thus, allowing them to survive under anaerobic conditions. This characteristic is important during the storage of spermatozoa for use in artificial insemination. A variety of buffers may be used to maintain a nearly neutral pH and an osmotic pressure of approximately 300 mMol, which is equivalent to that of semen, blood plasma and milk (Hafez, 2008).

To inhibit the growth of microorganisms in the semen, penicillin, streptomycin, polymyxin B, or other combinations of antibiotics are added (Hafez, 2008). Many extenders have been developed as a result of the discovery that spermatozoa in whole semen lived for only short periods of time and that cooling whole semen very slowly to 5^0C caused the death of spermatozoa due to lack of energy supply and protection. Therefore, apart from increasing the ejaculate volume, the extender protects spermatozoa during cooling, as well as extends the life of spermatozoa (Kalaba & Abdel-Khalek, 2001). Some known extenders are skim milk, glycerol, lactose, egg yolk glycerol, egg-yolk citrate, hydroxymethyl, amino ethane, citric acid, Illini Variable Temperature (IVT) extender, Cornell University Extender (CUE), Tris-coconut milk and coconut milk-citrate, to mention a few, has a different preservation power and fertilization rate. Some of these extenders are more expensive than others, and may sometimes not be readily available locally (Preciado *et al.,* 2011; Kaplan *et al.,* 2011).

According to Foote *et al.* (1959), the Cornell University Extender (CUE) is a self-gassing medium in which the carbon dioxide is derived from the reaction of citric acid and sodium bicarbonate. The Illini Variable Temperature (IVT) developed many decades back is another diluent which requires gassing with carbon dioxide (Salisbury & Van Demark, 1961). Both of these diluents have been modified for use with ram semen; they were saturated with carbon dioxide by gassing to pH 6.3 for about 10 minutes before use or were self-carbonating as a result of their carbonate or bicarbonate and acid content.

2.16 Summary

The success of artificial insemination depends on the management of semen collection, storage and use. The use of fresh or refrigerated semen is recommended when semen is stored for short periods of time or when it is used in small areas (Lebouf *et al.,* 2000). However, since frozen-thawed semen can be used for an indefinite period of time, and it can be used worldwide, it presents some advantages over fresh or refrigerated semen. Unfortunately, spermatozoa encounter damages during the cryopreservation process and, as a result, the fertilizing ability of frozen-thawed spermatozoa is lower than that of fresh or refrigerated

semen. A reduction in sperm motility and viability can be observed after cryopreservation (Salamon & Maxwell, 1995).

The damages caused by the cryopreservation process are due to several factors, such as the type of cryoprotectant used, the presence of seminal plasma, and the stage of the cryopreservation process (Leboeuf *et al.,* 2000; Watson, 2000; Purdy, 2006). Cryopreservation of goat semen serves diverse purposes, for example, cryopreserved spermatozoa can be used after a long period of time of storage (Martinez *et al.,* 2007). Cryopreservation of goat spermatozoa also extends the reproductive life of a buck after its death (Rahman *et al.,* 2008). According to Ritar *et al.* (1990), goat semen has been cryopreserved, using the Tris-based extender, which contains a safe margin of egg yolk to avoid coagulation and provide sperm cells with nutrients like protein. However, sperm cell motility rates following cryopreservation are usually lower than when fresh semen is used. Fresh goat sperm cell motility generally ranges from 80 to 90% (Gacitua & Arav, 2005).

However, Purdy (2006) argues that there are no or low recovery rates (0 to 18%) of motile sperm cells post-thawed in goats. However, the post/freezing sperm cell motility ranges from 23 to 65 % among different goat breeds (Tuli & Holtz, 1995; Gacitua & Arav, 2005). This clearly shows that sperm cell motility following freezing is relatively low, thus leading to low conception rates following artificial insemination (Lopez-Sebastian *et al.,* 2007; Houdeau *et al.,* 2008). Therefore, an increase in the recovery of good quality sperm cells following semen cryopreservation is necessary to achieve reasonable fertility rates (Gacitua & Arav, 2005).

CHAPTER 3: MATERIALS AND METHODS

3.1 Site description

This study was conducted at the School of Agriculture Experimental Farm. All semen samples were collected and analysed in the Biotechnology Laboratory of the Centre of Excellence in Animal Assisted Reproduction (CEAAR), University of Venda, Thohoyandou, in the Limpopo Province of South Africa. The daily temperatures at Thohoyandou vary from about 25˚C to 40˚C in summer and between approximately 12˚C and 26˚C in winter. Rainfall is highly seasonal with 95% occurring between October and March. The average rainfall is about 800 mm but varies (Mzezewa *et al*., 2010).

3.2 Animals and management

Three (n = 3) healthy adult Boer goat bucks aged 3.12 ± 0.55 years, with an average weight of 78.41 ± 5.29 kg and scrotal circumference of 28.75 ± 2.58 cm were used for this study. They were kept intensively at the feedlot of the School of Agriculture experimental farm, which located in the northern part of the University of Venda, Thohoyandou (22^0 57’ 0’’S and 30^0 29’ 0’’), Limpopo Province of the Republic of South Africa. The bucks were kept under natural light and maintained under a uniform nutritional regime as each buck were fed two percent of its body weight of pelleted concentrate each day with water provided ad *libitum*. A high standard of animal care was exercised at all times.

3.3 Extenders used for the study

3.3.1 Preparation of extenders used for the study

The extenders were prepared each day before semen collection and these were kept at 5 ^{0}C and reconstituted according to the manufacturer’s instructions. The quantity prepared for each extension was reduced proportionately, in order to avoid wastage due to prolonged cooling which could destabilize the media. Work tables were sterilized by wiping them with 100% methanol using clean paper towels. The two-step method of preparation was employed to prepare the Biladyl® concentrates. In order to avoid prolonged refrigeration of the final reconstituted Biladyl®, fractions A, B and AB were each prepared a day before semen collection.

3.3.2 Preparation of Biladyl

Fraction A

The stock solution containing fraction A was prepared by mixing 8.1 ml of the Biladyl® fraction A with 56.3 ml ultra-pure water in a graduated flask. Freshly laid eggs were sterilized by thoroughly wiping them with 70% methanol. The eggs were carefully opened, and the yolk was separated from the albumen by passing the three to four times from one half of the shell to the other, in order to get rid of the egg albumin as much as possible.

The yolk was transferred to a clean paper towel and rolled towards the edge of the towel until it was completely free of egg albumin. The yolk was completely wrapped in the paper towel, and slight pressure was applied on it to break open its membrane. The membrane-free yolk was collected in another graduated flask until it measured 16.7 ml. The stock solution was then carefully poured into the egg yolk and mixed well by gently stirring the mixture (to avoid foaming) using a sterile glass stirring rod. The mixture was filtered using a sterile filter funnel and 0.15g of Biladyl® fraction AB (antibiotics cocktail) was dissolved in 2 ml ultra-pure water and added to the stock solution. The final stock solution was kept in sterile 50 ml Cellstar tubes (Greiner Bio-one; Frickenhausen, Germany) (Zarazaga *et al*., 2009), and sealed with Parafilm “M”® (American National Can, Chicago, U. S. A.) to avoid contamination it was then stored in the refrigerator at 5 °C a day before semen collection.

Preparation of fraction B

Exactly 41.7 ml of Biladyl® fraction B was reconstituted from 25 ml ultra-pure water gently mixed into 16.7 ml fresh egg yolk in a separate beaker. The homogenous mixture was filtered using a sterile filter funnel and cooled to 5 °C. The final stock solution was kept in sterile 50 ml CellStar tubes, sealed with Parafilm “M”® (American National Can, Chicago, U. S. A.) and stored at 5 °C in the refrigerator.

Table 3.1 Constituents of Biladyl® extender

Fraction A Biladyl® (49 g)	Fraction B Biladyl® (250 g)	Fraction AB Biladyl® (250 g)
Double distilled water (56.3 ml)	Double Distilled Water (25 ml)	Gentamycin (300 g)
Citric acid (6.9 g)	Citric Acid (6.9 g)	Lincomycin (180 mg)
Fructose (5 g)	Fructose (5 g)	Spectinomycin (360mg)
TRIS (12.1 g)	TRIS (12.1 g)	Tylocin (60 mg)
Egg yolk(16,7ml)	Glycerol (86 g)	

The final restructured Biladyl® extender contains 20% egg yolk.

3.4 Semen collection

The three Boer goat bucks were trained to ejaculate using the artificial vagina (AV) once every four days to allow sexual rest. The semen samples for this experiment were collected using an artificial vagina (AV) (Ramsem, South Africa), with a doe in heat. The AV is an imitation of the vagina of the ewe and provides thermal (temperature) and mechanical (pressure) stimulation to the erect penis of the male to cause ejaculation (Steyn, 2010). The inner temperature ranges between 42 °C and 46 ^{0}C. The inner wall of the AV was lubricated with cooking oil. Just before semen samples were collected, preputial hairs of each buck were clipped, and the orifice thoroughly washed and rinsed and dried. The sheath was washed with phosphate buffer saline (PBS). PBS was injected into the sheath using a catheter syringe; the sheath was gently rocked between fingers as the PBS was allowed to run out of the sheath. The sheath washing was repeated 2 to 3 times and then a paper towel was rolled about 1.5 cm into the sheath for drying. The experimental bucks were taught to get used to the presence of the technician and the assistants in order for the bucks to easily mount while they are present. An assistant technician held the goat doe in heat by the horns and then allowed the male to mount. The technician collected the semen as quickly as possible by guiding the penis into AV.

Immediately after collecting the semen samples, the volume of each sample was measured because a low volume can be accompanied by low sperm concentration (Ajao, 2015). The colour of each semen sample was observed visually and then recorded. The samples were turned into their labeled, pre-warmed (37°C) 15 ml Cellstar tubes. The samples

were then transported to the biotechnology laboratory for processing in a vacuum flask with warm water at 37°C to prevent sperm death due to cold shock.

3.5 Semen preservation

3.5.1 Experimental design

The experiment was performed in a three extenders x five freezing regimes x two thawing regimes factorial arrangement of treatments in a randomised design (Table 3.2).

Table 3.2 Experimental design

Bucks	Extenders	Freezing	Storage	Thawing	Replicates
3	Bioxcell	Liquid N2 Vapour method; 4, 5, 6 and 7 cm above the LN2 level	7 days	37^0C for 30 sec 90^0C for 5 sec	3
		Conventional method; Freeze control at 1degree C/min		37^0C for 30 sec 90^0C for 5 sec	
	Biladyl	Liquid N2 Vapour method; 4, 5, 6 and 7 cm above the LN2 level	7 days	37^0C for 30 sec 90^0C for 5 sec	
		Conventional method; Freeze control at 1degree C/min		37^0C for 30 sec 90^0C for 5 sec	3
	Ham's F10	Liquid N2 Vapour method; 4, 5, 6 and 7 cm above the LN2 level	7 days	37^0C for 30 sec 90^0C for 5 sec	
		Conventional method; Freeze control at 1degree C/min		37^0C for 30 sec 90^0C for 5 sec	3

3.5.2 Semen extension, dilution, freezing and thawing method

After semen collection, the three semen samples were pooled together, and the pH was measured using a Metler Toledo (AG Analytical, Sonnenbergstrasse 74, Schwerzenbach) pH meter. Concentrations were measured using a Spectrophotometer and Sperm Class Analyzer® (SCA), version 5.4, (Microptic S.L., Barcelona, Spain) and recorded in millions per millilitre. Each buck was ejaculated six times, meaning a total of twenty-four semen samples were collected for the study. The collection was done every 4th day, in order to ensure sexual rest of the bucks and to allow enough time for evaluation of the collected aliquots. Immediately after reaching the laboratory, the concentration was calculated using a haemocytometer and the semen samples were transferred into a water bath at a temperature of 37 ^{0}C.Pooled semen samples were divided into three samples.

Semen samples were diluted into three groups at a ratio of 1:3 (semen to extender) with Bioxcell, Biladyl and Ham's F10. After the dilution, the samples were centrifuged at 300 G for 10 minutes at 37 ^{0}C in order to remove the seminal plasma. The supernatant was removed, and the pellets were re-diluted with the same extenders at the same ratio of 1:3 (semen to extender) to reach a concentration of 120 x 10^{6} per ml. Each sample was then divided into five sub-divisions (A, B, C, D and E) making a total of thirty samples, because it needed one sample for each thawing method.

The cryoprotectant 10% glycerol was then added to the Ham's F10 extender after cooling down the Ham's F10 sample to 4 ^{0}C.The sub-divisions were loaded in 0.25 ml semen straws by sucking the semen up and then sealed with the sealing powder. One straw per extender was placed in a programmable freezer for cooling at 1 ^{0}C per minute. The remaining four subdivisions per extender were arranged at the distances of 4 cm, 5 cm, 6 cm, and 7 cm above the level of liquid nitrogen (LN_2). These were then left for cryopreservation for 10 minutes.

The dilution procedure was repeated three times to generate the required 90 samples, that is in 3 extenders x 5(freezing regimes) x 2 (thawing regimes) 3 replicates. The difference in distance from the LN_2 level was considered descriptive of different cooling rates. The straws were plunged into LN_2 in which they were left for 1 week before thawing. The thawing was done at different temperatures of 37^{0}C for 30 seconds and 90^{0}C for 5 seconds.

The diluted sperm samples were equilibrated in the refrigerator at 5^{0}Cfor a period of 2 hours. After 2 hours extended semen samples were loaded into 0.25 ml labelled plastic straws. After filling, the semen straws were sealed using polyvinyl powder ofdifferent colours to differentiate the extenders, the levels above the liquid nitrogen and placed on a semen

freezing holding racks. For freezing, the Styrofoam box was filled with liquid nitrogen. The straws were placed on a freezing rack approximately 4 cm, 5 cm, 6 cm and 7 cm above the surface of the liquid nitrogen in a Styrofoam box for 10 minutes. After 10 minutes the straws containing semen were submerged in liquid nitrogen and loaded in to a LN_2 canister and then stored at −196 ^{0}C for a period of seven days.

The thawing of the frozen semen straws was done after seven days of storage at -196 ^{0}C. The semen straws were thawed at the temperature of 37 ^{0}Cfor 30seconds in the water bath. It was important to make the transfer of the semen straw from the tank where semen straws were stored to the flask containing water bath for thawing as quickly as possible, to avoid exposing the straw to the room temperature for not more than 3 to 5 seconds and not to handle the frozen straw to avoid contamination. The semen straw was plunged for thawing in the water bath for 37 ^{0}Cat 30 seconds, and waswiped with a clean tissue to dry the straw so as to get it ready for analyses. The other 50% of the straws of semen were thawed at a temperature of 90 ^{0}Cfor 5 seconds and were also dried and prepared for analysis.

3.6 Semen evaluation

3.6.1 Spermatozoa motility

The sperm motility was evaluated using the computer aided sperm analysis (CASA) system (Sperm Class Analyser), version 5.4(Microscopic SL, Barcelona, Spain), using the motility programme at phase contrast Ph1 using 10x magnification. Three micro-litres of semen from each extended samples were placed inside one chamber of the eight chambered Leja slides on the warm glass stage at 37 ^{0}C of CASA microscope for evaluation. Five fields were captured, and the software calculated automatically the motility parameters percentage of total motility of spermatozoa (TM), progressive motility (PM), Non progressive motility (NPM), static motility (STC), rapid motility (RAP), velocity curvilinear (VCL), straight-line velocity(VSL),average path velocity (VAP), linearity (LIN), straightness (STR) wobble (WOBB)of sperm in the sample.

3.6.2 Sperm morphology

Sperm morphology was evaluated. Abnormal spermatozoa such as bent tails or coiled, double head, distal and proximal cytoplasmic droplets were determined by staining with Spermac (Stain Entreprises, P.O.Box 152, Wellington 7654, South Africa). Sperm smears

were first prepared for staining in a controlled room, at a temperature of 24 ^{0}C by pipetting 15 µl drop of extender semen on a clear of a clean glass slide. The edge of the second slide was then placed on the drop at an angle of 20 ^{0}C on a horizontal plane and then pushed forward to smear the semen across the slide. The sperm smear was thereafter allowed to air dry at room temperature before staining. The dried smears were placed horizontally down on absorbent paper inside a staining stray. Spermac fixative was 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 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 the slide onto absorbent paper. One millilitre of stain A was then dropped onto the slide and left for 2 minutes and after that drained as above. The slides were washed for the second time in fresh distilled water to remove the excess of stain A, water was drained by touching the end of slide onto absorbent paper. Stain B was used in the same way as stain A, then washed by dipping seven times into the fresh distilled water. Stain C was added for two minutes and washed as above.

The slides were left in an upright position (about 70 ◦C angle) to let the fluid drain until air dry. 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.

3.6.3 Sperm vitality

Spermatozoa were stained with negrosin-eosin stain. Two slides were pre-warmed at 37 ^{0}C for staining. Ten µL of extended 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^{0}C. A drop of 20 µL nigrosine stain was then poured into the mixture on the slide and mixed using the same pipette tip. The edge of the second slide, also pre-warmed at 37^{0}C, was placed on the mixture at an angle of 20 ^{0}C from the horizontal plane and pushed forward to smear the mixture across the slide.

After smearing, the smeared slide was placed on a hot Buehler slide warmer (Buehler Ltd., 41 Waukegan road, Lake Bluff, Illinois, USA) at 120 ^{0}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 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.

3.7 Statistical analysis

Analysis of variance (ANOVA) of sperm quality parameters was performed in a 3 extenders x 5 freezing regimes x 2 thawing regimes factorial arrangement of treatments in a randomised design based on the model;

$$Y_{ijkl} = \mu + E_i + D_j + T_k + (ED)_{ij} + (ET)_{ik} + (DT)_{jk} + (EDT)_{ijk} + \sum_{ijkl}$$

Where;

Y_{ijk}	= observation
μ	= overall mean common to all observations;
E_i	= effect of the i^{th} extender; I=1, 2 or 3 extenders.
D_j	= effect of the j^{th}freezing regime; J= 1,2,3,4 or 5 levels.
T_k	= effect of the k^{th} time; K= 1 or 2 times.
$(ED)_{ij}$	= interaction of the i^{th} Extender and i^{th} distance
$(ET)_{ik}$	= interaction of the i^{th} Extender and k^{th} time
(DT)jk	= interaction of the i^{th} freezing regime and k^{th} times
$(EDT)_{ijk}$	= interaction of the i^{th} Extender, j^{th} freezing regime and kth time
∑ijkl	= random Error.

CHAPTER 4: RESULTS

TABLE 4.1 Shows semen parameters of the South African Boer bucks used in this study

Parameters	Values
Volume (ml)	1.73 ± 0.40
Colour	Creamy
pH	7.10 ± 0.7
Concentration (x10^6/ml)	610.5 ± 123.0

Table 4.2 Shows the effects of different extenders, freezing regime and thawing time on Boer goat sperm quality.

No significant differences (P>0.05) was observed in the total motility, progressive motility, static, rapid, velocity curvilinear, linearity and wobble, while non progressive, straight-line velocity and average path velocity were significantly different (P<0.05).Significant differences (P<0.05) were observed in freezing regime in total motility, progressive motility, non-progressive motility, static and wobble, while rapid, velocity, straight line velocity, average path velocity, linearity and straightness was showing non-significant difference(P>0.05) .Highly significant differences (P<0.01) were observed in rapid motility between all the treatments with the interactions among the treatments. A significant difference (P < 0.05) was observed in the total motility, progressive motility, non-progressive motility, static, velocity, straight line velocity, average path velocity, straightness and wobble for the interaction between extenders type and freezing regime, while non-significant differences(P>0.05) were observed in static and linearity. The interaction between extenders type, freezing regime and thawing time was non-significant for all the parameters. A non-significant difference (P>0.05) was also observed in the interaction between extenders and thawing time for all the parameters and the interaction between freezing regimes and thawing time for all the parameters

TABLE 4.2. Motility rates of extended semen with Biladyl, Bioxcell and Ham'sF10 evaluated after 7days of storage

Extr Means	FR means	Tt	TM (%)	PM (%)	NPM (%)	STC (%)	RAP (%)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)
Biladyl			17.8^{b}	6.7^{b}	11.1^{a}	82.2^{a}	6.7^{ab}	26.5^{ab}	11.7^{a}	16.6^{a}	40.8^{ab}	62.3^{ab}	58.2^{b}
Bioxcell			21.3^{ab}	8.2^{b}	12.7^{a}	79.1^{a}	3.2^{b}	23.9^{b}	12.2^{a}	16.8^{a}	48.7^{a}	66.2^{a}	67^{a}
Ham'sF10			26.6^{a}	12.3^{a}	14.3^{a}	70.5^{b}	10.3^{a}	30.3^{a}	12.5^{a}	18.3^{a}	36.7^{b}	57.2^{b}	55.4^{b}
SEM			2.1	1.7	1.3	2.5	2	1.5	1.2	1.2	2.7	2.2	1.9
	4cm		23.7^{a}	10^{a}	13.7^{a}	76.2^{a}	5.0^{b}	25.5^{b}	10.7^{a}	15.5^{ab}	38.8^{ab}	58.5^{b}	60.1^{a}
	5cm		19.2^{a}	7.9^{a}	11.4^{a}	80.7^{a}	4.0^{b}	25.2^{b}	9.5^{a}	15.1^{b}	36.5^{b}	59.4^{ab}	59.2^{a}
	6cm		20.7^{a}	7.9^{a}	12.8^{a}	75.4^{a}	2.9^{b}	22.2^{b}	10.9^{a}	15^{b}	39.2^{ab}	59.5^{ab}	58.1^{a}
	7cm		18^{a}	7.9^{a}	10.2^{a}	82.5^{a}	4.5^{b}	27.7^{ab}	15.3^{a}	18.9^{ab}	52.5^{a}	70.7^{a}	63.0^{a}
	PGF		27.9^{a}	12^{a}	15.4^{a}	71.3^{a}	17.1^{a}	33.5^{a}	14.1^{a}	21.6^{a}	43.2^{ab}	62.2^{ab}	60.5^{a}
SEM			2.7	1.4	1.6	3.2	2.6	1.9	1.5	1.6	3.5	2.9	2.4
		37 °C 30sec	23.2^{a}	9.9^{a}	13.1^{a}	74.7^{a}	5.8^{a}	26.2^{a}	11.3^{a}	16.1^{a}	40.2^{a}	61.1^{a}	59.1^{a}
		90 °C 5sec	20.7^{a}	8.3^{a}	12.3^{a}	79.8^{a}	17.1^{a}	27.6^{a}	12.9^{a}	18.3^{a}	43.9^{a}	63^{a}	61.3^{a}
SEM			1.7	0.9	1	3.2	2.6	1.2	0.9	1.0	2.2	1.8	1.5
P values													
E			0.01	0.00	0.21	0.00	0.00	0.01	0.90	0.60	0.01	0.02	0.00
FR			0.10	0.14	0.21	0.11	0.00	0.00	0.04	0.02	0.00	0.02	0.70
Tt			0.30	0.22	0.10	0.10	0.45	0.41	0.24	0.13	0.22	0.50	0.32
E*FR			0.00	0.00	0.01	0.00	0.44	0.00	0.00	0.00	0.20	0.01	0.03
E*Tt			0.50	0.20	0.71	0.21	0.71	0.20	0.50	0.12	0.20	0.50	0.32
FR*Tt			0.61	0.90	0.53	0.30	0.51	0.70	0.60	0.60	0.20	0.10	0.10
E*FR*Tt			0.10	0.20	0.50	0.23	0.63	0.70	0.70	0.50	0.20	0.22	0.10

For each set of means, different superscripts (a, b) within the same column indicate significant differences (P < 0.05. E = extender, FR= freezing regime, Tt = thawing time, E x FR = interaction between extender type and Freezing regime, E x Tt = interaction between extender type and thawing time, FR x Tt = interaction between temperature and storage time, E x FR x Tt = interactions between extender type, freezing regime and thawing time, TM = total motility of spermatozoa, PM= progressive motility, NPM= Non progressive motility, STC= static motility, RAP= rapid motility, VCL=velocity curvilinear, VSL= straight-line velocity, VAP= average path velocity, LIN= linearity, STR= straightness, WOB=wobble; ns= Not significant (P > 0.05) ; *=Significant(P<0.05); **=Highly significant(P<0.01).

Table 4.3 Shows the effects of different extenders in semen before freezing highly significant differences (P<0.01) were observed in the total motility, progressive motility and static, while non-significant differences(P>0.05) were observed in rapid motility, velocity, straight line velocity, average path velocity, linearity, straightness and wobble.

TABLE 4.3 Raw semen evaluated before storage

Extender Means	TM (%)	PM (%)	NPM (%)	STC (%)	RAP (%)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)
Biladyl	96	68.3	27.7	4	70.7	64.3	21.7	33.3	30	56.7	53.7
Bioxcell	93	69.7	23.3	7	88	85.7	80	82.7	79	83.7	87
Ham'sF10	95.3	64.7	30.7	4.7	67.3	56.7	19	30.3	31.3	2.9	56
SEM	1.8	6.3	4.8	1.8	1.4	3.6	3	1.3	2.9	1.4	2.7
Biladyl	96^{a}	68.3^{a}	27.7^{a}	4^{a}	70.7^{b}	64.3^{b}	21.7^{b}	33.3^{b}	30^{b}	56.7^{b}	53.7^{b}
Bioxcell	93^{a}	69.7^{a}	23.3^{a}	7^{a}	88^{a}	85.7^{a}	80^{a}	82.7^{a}	79^{a}	83.7^{a}	87^{a}
Ham'sF10	95.3^{a}	64.7^{a}	30.7^{a}	4.7^{a}	67.3^{b}	56.7^{b}	19^{b}	30.3^{b}	31.3^{b}	55.7^{b}	56^{b}
P values	0.50	0.84	0.60	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.0

TM= Total motility, PM= Progressive Motility, NPM= Non-progressive Motility, STC= Static, RAP= Rapid, VCL=Velocity Curvilinear, VSL= Straight-line Velocity, VAP= Average Path Velocity, LIN= Linearity, STR= Straightness, WOB= Wobble.

Table 4.4. Shows the effects of different extenders, freezing regime and thawing time on sperm morphology. There was no significant difference (P>0.05) that was observed for all the extenders in grouping for cut tail, bent tail, cut head sperms. Significant difference (P<0.05) was observed on normal sperm for Bioxcell and Ham's F10, Biladyl showed no significant difference (P>0.05). There was no significant difference (P>0.05) observed for all levels of straw above liquid Nitrogen. No significant (P>0.05) was observed for both 30seconds and 50seconds in cut tail, bent tail, and normal sperm. Significant difference (P < 0.05) for time was only observed from cut head sperms only in grouping.

TABLE 4.4 The effects of extenders, freezing regime and time on morphologic parameters (counts per 200 spermatozoa)

Extender means	Freezing regime	Thawing time	Cut tail	Bent tail	Cut head	Normal
Biladyl			10.8	11.8	76.4	101.5[ab]
Bioxcell			10.7	10.4	72.3	106.7[a]
Ham's F 10			13.0	12.5	75.6	98.6[b]
SEM			1.01	0.66	1.74	2.17
Freezing regime means						
	4cm		10.6	10.5	74.6	105.3
	5cm		11.8	12.0	79.0	97.1
	6cm		13.1	10.7	75.7	99.8
	7cm		12.0	12.2	73.7	101.8
	PGF		10.0	12.3	70.8	107.2
	SEM		1.3	0.85	2.25	2.80
Thawing time means						
		37ºC for 30sec	10.7	12.1	77.0[a]	100.1
		90 ºC for 5 sec	12.3	11.0	72.5[b]	104.4
SEM			0.83	0.54	1.42	1.77
P values						
Extender (E)			Ns	ns	ns	Ns
Freezing Regime (FR)			Ns	ns	ns	Ns
Thawing time (T)			Ns	ns	ns	Ns
E x FR			Ns	ns	ns	Ns
E x Tt			Ns	ns	ns	Ns
FR x Tt			Ns	ns	ns	Ns
E x FR x Tt			Ns	ns	ns	ns

For each set of means, different superscripts (a, b) within the same column indicate significant differences ($P < 0.05$) E = extender, FR= Freezing regime, Tt = thawing time, E x FR = interaction between extender type and Freezing regime, E x Tt = interaction between extender type and thawing time, FR x Tt = interaction between Freezing regime and thawing time, E x FR x Tt = interactions between extender type, Freezing regime and thawing time. C TAIL= cut tail, B.TAIL = bent tail, C. HEAD

= cut head, N. SPERM = normal sperm, * = significant (P < 0.05), **= highly significant (P < 0.01); ns = not significant (P >0.05).

CHAPTER 5: DISCUSSION

The freezing process negatively affected the spermatological parameters of goat spermatozoa. Freezing and thawing procedures (dilution, equilibration, and thawing) had negative effects on motility. These effects were also observed in the studies of Barbas and Mascarenhas (2009) and Dorado *et al.* (2009), which showed that sperm from small ruminants, such as goats, does not have high adaptability to temperature changes, which may contribute to sperm sensitivity. Generally, cold shock damage manifests as a decline in cell metabolism, altered membrane permeability, loss of intracellular components, irreversible loss of spermatozoa motility, and increase in the number of dead spermatozoa (Watson, 1990).

Optimal freezing and thawing rates are critical for developing successful semen cryopreservation protocols. Blanco et al. (2000) and Bittencourt *et al. (2007),* reported that freezing rates had no effect on post thaw motility or acrosome defects for poultry, goats, and rams, respectively.

The study suggested Bioxcell and Biladyl extenders are better than Ham'sF10. In a previous study, soy-based diluent (Bioxcell®) was superior to an egg yolk-based diluent in preserving the motility of cryopreserved goat sperm (Roof *et al.,* 2011). Jimenez-Rabadana *et al* (2012) studied the post-thaw quality of buck semen using Biladyl, Andromed and skim milk based diluents and found Biladyl and Andromed diluent superior to skim milk based diluent. Other studies (Raja *et al.,* 2006; Mishra *et al.,* 2010) suggested that Tris-citric acid may provide the most satisfactory buffering system and act as a better diluent for goat spermatozoa. In the current study, the observed difference in sperm motility in response to different freezing rates were in agreement with studies on Korean native buck reported by Choe *et al.,* (2006) that rapid freezing was found to be more effective than slow freezing for Korean buck sperm survival. Sperm motility differed between fast and slow freezing methods using Tris yolk glycerol extender. The extenders, freezing retime, thawing time and the interaction between extenders x freezing regime x thawing time, however, affected spermatozoa motility and morphology throughout the freezing regime and the thawing time in all the extenders, but Ham'sF10 showed a lower decrease than other two extenders. Past studies indicated that spermatozoa motility highly depends on the available source of energy in an extender or seminal plasma in the form of adenosine triphosphate (ATP) produced from metabolism and requires a constant supply for cell function and survival (Wattima *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 spermatozoa. This depletion would eventually lead to ATP deficits.

The results from this study on morphology showed no significant difference at all on freezing regimes for Biladyl, Bioxcell and Ham's F10 in the cut tail, bent tail and normal sperm. The effectiveness of extenders in preserving the longevity and fertilizing capacity of spermatozoa during liquid storage can be attributed to their constituents, so will their variations in the preservation depend on the differences in their constituents. The Biladyl and extenders examined in this study were based on fructose as the energy source. These extenders are commonly used for the liquid storage of bull (Vishwanath & Shannon, 2000), buck (Leboeuf *et al*., 2000) and ram s (Evans & Maxwell, 1987) semen. Similar extender have been used for liquid storage of alpaca (Vaughan *et al*., 2003, Morton *et al*., 2009), camel (Vyas *et al*. 1998; Deen *et al*. 2004; Wani *et al*. 2005; Niasari-Naslaji *et al*. 2006) and llama spermatozoa (Ratto *et al*. 1999; Giuliano *et al*. 2006). In a previous study (Udeh & Ogbenesode, 2011) the type of extender and storage conditions had significant negative effects on the motility of goat spermatozoa in fresh extended goat sperm.

CHAPTER 6: CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The extender type and thawing regime were important factors in preserving Boar goat spermatozoa. Compared to the programmable freezer, the quality of Boer buck spermatozoa was maintained when the sperm was placed at least 5cm above the liquid nitrogen. Overall, both extenders proved suitable for fresh goat semen preservation at the thawing times and freezing regimes tested in the present study.

6.2 Recommendation

Considering that spermatozoa motility is the preferred indicator of sperm viability, Biladyl and Bioxcell are better extender followed by Ham's F10. Where the nitrogen vapour method is applied in freezing semen, it is recommended semen straws be placed 5 cm above the liquid nitrogen level.

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APPENDIX: EDITORS REPORT

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