

**EFFECT OF BIOXCELL AND TRILADYL EXTENDERS AND REMOVAL OF SEMINAL
PLASMA ON EQUILIBRATED AND CRYOPRESERVED GOAT SEMEN**

By

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DECLARATION

I, Nethenzheni Livhuwani Pertunia, the undersigned, Student number 11590622, hereby declare that this dissertation for Master of Science in Animal Science (MscANS) at the University of Venda is my own work and has not been submitted previously to any university for any other degree.

Student Date

L.P. Nethenzheni

DEDICATION

A special dedication to my late grandparents, Mr. Wilson and Mrs. Rossinah Nethenzheni. My wonderful parents, Mr. Robert Siala and Mrs. Lucy Nethenzheni and my most precious son, Rotshidzwa.

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ABSTRACT

The objectives of the study were to evaluate the effect of two extenders (Triladyl[®] and Bioxcell[®]) and the removal of seminal plasma on goat buck semen. Six ejaculates were collected from six indigenous bucks by means of electro-ejaculator method, and semen was pooled, and replicated 10 times. Raw semen were randomly allocated into six groups as follows: (i) Raw non-washed, (ii) Raw washed, (iii) Triladyl[®]-washed, (iv) Triladyl[®]-non-washed, (v) Bioxcell[®]-washed and (vi) Bioxcell[®]-non-washed. All six groups were analysed for spermatozoa motility rates using computer-aided sperm analysis (CASA). The spermatozoa viability for all groups were assessed using Eosin-Nigrosin, acrosome integrity using Spermac, chromatin structure using Acridine Orange, and mitochondria using JC-1 staining solutions. Both the Triladyl[®] and Bioxcell[®] washed semen groups were diluted (1:4) with Phosphate Buffered Saline (PBS) then centrifuged at 1500 x g for ten min and seminal plasma was aspirated using 1 mL sterile plastic pipette. Semen samples were diluted (1:4) as follows: Triladyl[®] (washed and non-washed) or Bioxcell[®] (washed and non-washed) and then equilibrated at 5 °C for 2 hours. Following equilibration, semen parameters were analysed. Thereafter, the semen samples were loaded into straws and placed 5 cm above a liquid nitrogen vapour for 10 min, and then stored at -196 °C until use. Following one month of storage, frozen semen straws per treatment group were thawed at 37 °C for 30 seconds, then semen parameters were analysed again. Significant differences among the mean values of semen parameters were determined by Tukey's test using ANOVA, GLM procedure of SAS version 12.1 of 2010. Total Spermatozoa motility rate of Bioxcell[®] (92.5±4.6), (68.2±13.5) and Triladyl[®] (94.9±5.5), (63.1±15.1) were significantly reduced (P < 0.05) following equilibration and freeze-thawing process, respectively on washed semen groups. Live and normal spermatozoa percentages were drastically reduced in Bioxcell[®] (5.2±4.9) and Triladyl[®] (6.9±8.6) washed semen groups, following freeze-thawing. There was a significantly lower number of spermatozoa with high mitochondrial membrane potential in non-washed semen extended with Triladyl[®] (68.7±26.8) compared to non-washed semen extended with Bioxcell[®] (49.8±20.1) following the freeze-thawing process. In conclusion, the freezing-thawing process did reduce the indigenous buck semen parameters irrespective of removal or non-removal of seminal plasma. However, Bioxcell[®] extender was found to be more suitable for preserving spermatozoa during equilibration and freezing/thawing process of buck semen.

Keywords: Indigenous bucks, extenders, seminal plasma, motility, membrane integrity.

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LIST OF ABBREVIATIONS

µL	: Microliter
µm/sec	: Micrometres per second
AI	: Artificial insemination
ALH	: Amplitude of lateral head displacement
ART	: Assisted reproductive technologies
ATP	: Adenosine triphosphate
AV	: Artificial vagina
BCF	: Beat cross frequency
Biox NW 2 h	: Bioxcell® non-washed 2 hours
Biox NW FT	: Bioxcell® non-washed frozen-thawed
Biox W 2 h	: Bioxcell® washed 2 hours
Biox W FT	: Bioxcell® washed frozen-thawed
BUSgp60	: Bulbourethral secretion glycoprotein-60
Ca ²⁺	: Calcium ionophore
cAMP	: Cyclic adenosine monophosphate
CaNa ₂	: Calcium salt
CASA	: Computer assisted sperm analyser
CEAAR	: Centre of Excellence for Animal Assisted Reproduction
DHT	: Dihydrotestosterone
DMSO	: Dimethyl sulphoxide
DNA	: Deoxyribonucleic acid
E ₂	: Estradiol
EDTA	: Ethylene diamine triacetic acid
EE	: Electro-ejaculator
FSH	: Follicle stimulating hormone
FSHR	: Follicle stimulating hormone receptor
FT	: Frozen-thawed
g	: Grams
GnRH	: Gonadotropin releasing hormone
Hr	: Hour
Hz	: Hertz
ICSH	: Interstitial cell stimulating hormone
IVEP	: <i>In vitro</i> embryo production
IVF	: <i>In vitro</i> fertilization
LH	: Luteinizing hormone

LIN	: Linearity
LPC	: Lysophosphatidylcholine
MED	: Medium
Min	: Minutes
mL	: Millilitre
MOET	: Multiple ovulation and embryo transfer
NPM	: Non-progressive motility
NW	: Non-washed
PC	: Phosphatidylcholine
PM	: Progressive motility
RAP	: Rapid
Raw NW	: Raw non-washed
Raw W	: Raw washed
ROS	: Reactive oxygen species
SLW	: Slow
SSH	: Spermatogenesis stimulating hormone
STC	: Static
STR	: Straightness
TM	: Total motility
Tril NW 2 h	: Triladyl [®] non-washed 2 hours
Tril NW FT	: Triladyl [®] non-washed frozen-thawed
Tril W 2 h	: Triladyl [®] washed 2 hours
Tril W FT	: Triladyl [®] washed frozen-thawed
VAP	: Average path velocity
VCL	: Velocity on the curve line
VSL	: Velocity on the straight line
W	: Washed
WOB	: Wobble
$\Delta\psi_m^{\text{high}}$: High mitochondrial membrane potential
$\Delta\psi_m^{\text{low}}$: Low mitochondrial membrane potential

CHAPTER 1

INTRODUCTION

1.1 Background

Goats are prolific and require low inputs for a moderate level of production, and tend to reach maturity early and are profitable to keep (Webb & Mamabolo, 2004). The majority of South African indigenous goats are kept in rural areas and provide milk, hides, and meat (Webb & Mamabolo, 2004). These goats are tolerant to different parasites and diseases, non-selective browsers and able to survive on the poorest vegetation. However, there is limited information regarding their reproductive status (Ramukhithi, 2011), semen quality and their tolerance to cryopreservation (Matshaba, 2010). This is mainly because they have received little attention from researchers in the past. However, there is recent interest from commercial farmers and this may be due to their hardiness and adaptability to the local harsh environmental conditions as well as their exceptional capacity to produce and reproduce efficiently under poor nutritional conditions (Matshaba, 2010). Furthermore, there is a need to conserve their genetic materials. Genetic resource banks are normally utilised with reproductive technologies for the conservation of endangered species (Marco-jiménez *et al.*, 2005).

Semen collection and storage is essential in controlled breeding programmes of many endangered species (Okere *et al.*, 2011). The cryopreservation of gametes is vital, because it would allow researchers to support a genome resource bank for this breed for an indefinite period of time (Kulaksiz *et al.*, 2013). Cryopreservation of goat spermatozoa also extends the male reproductive life of a goat germline (Ajao, 2015). Frozen-thawed goat semen may be utilised for AI to enhance improvement of livestock as breeders mostly make use of genetically superior goats (Ramukhithi, 2011). However, condition to freeze goat semen remains a challenge.

The cryopreservation method includes temperature reduction, cellular dehydration, eventual freezing and subsequent thawing. Cryopreservation has been shown to stop all cellular activities, restarting its normal metabolic functions, following thawing. However, semen cryopreservation generally induces the formation of intracellular ice crystals, osmotic and chilling injury, which causes sperm cell damage, cytoplasmic fracture, or some effects on the cytoskeleton or the genome-related structures (Peña *et al.*, 2003; Farshad & Akhondzadeh, 2008; Munyai, 2012). The main

changes that occur through the freezing of gametes are primarily related to ultrastructural, biochemical and functional activities. These eventually impair spermatozoa transport and decrease the survival rate in the female reproductive tract, thus reducing fertility (Munyai, 2012).

Reduced spermatozoa motility rate may be due to the egg yolk-coagulating enzyme (phospholipase) that has harmful interactions with secretions of the bulbourethral gland, reducing the survival rates of the spermatozoa after cryopreservation. Among the factors affecting the freezing ability of spermatozoa, supplementation of the freezing medium with different types of cryoprotectant plays a vital role in minimising the physical and chemical stresses occurring during cryopreservation procedure (Anakkul *et al.*, 2013). Hence, it is of utmost importance to develop and modify protocols to improve cryopreservation protocols to preserve indigenous germplasm.

Seminal plasma plays an important role in spermatozoa survival during the cryopreservation process. This is because the causes of reduced spermatozoa motility are related to seminal plasma enzymes (Naing *et al.*, 2011). Goat semen is currently centrifuged (washed) to eliminate the seminal plasma from the spermatozoa prior to dilution with standards extenders containing egg yolk. Phospholipase A₂ activity of egg yolk coagulating enzyme (EYCE) catalyses the hydrolysis of egg yolk phosphatidylcholine (PC) into fatty acids and lysophosphatidylcholine (LPC). The LPC has a toxic effect on buck spermatozoa by acting like a detergent on biomembrane, resulting in a loss of motility, membrane integrity and consequently low fertility rate (Niang *et al.*, 2011). This harmful effect is observed in goat semen when it is extended with egg-yolk-based extenders (Daşkin *et al.*, 2011). Currently, soy-lecithin-based extenders are used to avoid problems encountered when egg-yolk-based extenders are used to extend goat semen (Vidal *et al.*, 2013). Goat semen has been reported to be centrifuged (washed) to eliminate the seminal plasma from the spermatozoa prior to dilution with standards extenders containing egg yolk (Ashmawy *et al.*, 2010). The present study focused on the effect of extenders (egg-yolk-based extender and soy-lecithin-based extender) and the removal of seminal plasma on South African indigenous goat semen parameters.

1.2 Problem statement

Frozen-thawed spermatozoa are subjected to chemical, osmotic, thermal and mechanical trauma that occurs during dilution, cooling, equilibration and thawing process. The cryopreservation of goat semen decreases spermatozoa motility rate; however, the extent of damage at consecutive stages of cryopreservation has not been extensively documented. The interaction between seminal plasma (phospholipase enzyme) and egg yolk have been reported to be toxic (Niang *et al.*, 2011). Consequently, egg yolk semen extenders have a detrimental effect on goat spermatozoa when interacting with seminal plasma, as the egg yolk coagulates with the phospholipase enzyme found in goat semen. This toxic effect is not observed when soy lecithin extenders are used to dilute goat semen during cryopreservation.

1.3 Justification

The effect of seminal plasma on fertility of goat semen as measured by semen parameters during cryopreservation needs to be investigated. There is a great need to further assess the damage on spermatozoa morphology (acrosomal intactness, chromatin structure, mitochondrial membrane potential), motility, and viability at consecutive stages of cryopreservation on indigenous goat semen. The assessment of quality of frozen-thawed goat semen extended with egg yolk or soy lecithin extender with or without seminal plasma will greatly help improve goat semen cryopreservation and semen fertility. This study will contribute to the improvement of cryopreservation protocol used to cryopreserve indigenous male goat genetic materials of South African goats. Furthermore, it will assist emerging farmers to use artificial insemination technology for future breeding programmes.

1.4 Objective of the study

The main purpose of this study was to improve the freezability and fertility of semen as measured by semen parameters following cryopreservation of South African unimproved indigenous bucks.

1.5 The specific objectives are to:

- i. Investigate the effect of two commercial extenders (Bioxcell® and Triladyl®) on semen parameters during the cryopreservation of buck semen.

- ii. Evaluate the effect of washing (removal) of seminal plasma on semen parameters in the two extenders during the cryopreservation of buck semen.

1.6 Hypotheses

- i. There will be no difference between the two commercial extenders in improving semen parameters' fertility of indigenous bucks prior and post-cryopreservation.
- ii. There will be no difference between removal and non-removal of seminal plasma in improving semen parameters' fertility of indigenous bucks prior and post cryopreservation.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Cryopreservation of goat semen facilitates the supply of male genetic material for AI in goats and storage for future use (Ahmad *et al.*, 2014). Semen cryopreservation is essential for the application of reproductive techniques such as AI and IVF, which contribute to the increase in the reproduction of goats and genetic selection breeding schemes or programmes. However, cryopreservation causes membrane integrity, ultrastructural, biochemical and functional damages on spermatozoa due to the temperature changes resulting in decreased motility and viability (Naing *et al.*, 2011; Ahmad *et al.*, 2014; Lee *et al.*, 2014). Seminal plasma plays an important role in spermatozoa survival during cryopreservation process, because the causes of reduced spermatozoa motility are related to seminal plasma enzymes (Naing *et al.*, 2011). The ability to cryopreserve spermatozoa of all domestic species is difficult (Yimer *et al.*, 2014). Even though cryopreservation protocols have been optimised for spermatozoa of specific species, it could not be good for spermatozoa of the other species (Ari & Daskin, 2010; Yimer *et al.*, 2014). Bovine and Caprine or ovine spermatozoa-freezing extenders include similar or the same ingredients. However, the interaction between goat seminal plasma and egg yolks (or skim milk) are deleterious to the spermatozoa, a condition not observed with bovine or ovine seminal and egg yolk (Ari & Daşkin, 2010). This is because the enzyme (phospholipase) present in seminal fluid secreted by the bulbourethral glands interacts with egg yolk lecithin and through hydrolysis producing toxic compounds (lysophosphatidylcholine) to the spermatozoa (Matshaba, 2010; Niang *et al.*, 2011; Yimer *et al.*, 2014; Ferreira *et al.*, 2014; Cabrera *et al.*, 2005).

Goat semen is currently centrifuged (washed) to eliminate the seminal plasma from the spermatozoa prior to dilution with standard extenders containing egg yolk (Ari & Daskin, 2010; Matshaba, 2010; Niang *et al.*, 2011; Yimer *et al.*, 2014). However, to minimise the harmful interactions, low concentrations of egg yolk are added to the extender and centrifugation (washing) is not required (Ashmawy *et al.*, 2010; Matshaba, 2010; Ramukhithi, 2011; Yimer *et al.*, 2014). Several factors such as semen collection methods, season and nutrition can affect semen characteristics; moreover, semen cryopreservation can be affected by factors such as semen diluents, cryoprotectants, and temperature (Ramukhithi, 2011). The process of fertilisation involves complex biochemical and physiological and morphological aspect of semen (Martins *et al.*, 2010).

The traditional evaluation of ejaculate quality has been based primarily on routine semen analyses (such as motility, morphology, and acrosome integrity). Routine semen analysis (such as motility, morphology, and acrosome integrity) have a limited capacity of predicting the potential fertility of the ejaculate. Thus, advanced techniques for semen evaluation (such as IVF, cervical mucus penetration, DNA, mitochondrial membrane and plasma membrane integrity) should be implemented to increase the accurate identification of high quality spermatozoa (Üstüner *et al.*, 2015).

2.2 Description of semen

Normal semen is a mixture of motile and non-motile spermatozoa and viscous fluid (Dehghani *et al.*, 2004). It is also described as the liquid cellular suspension containing spermatozoa and secretions (seminal plasma) from the accessory glands (seminal vesicles, bulbourethral and prostate gland) of the male reproductive tract (Ramukhithi, 2011; Munyai, 2012). The Semen parameters that are mostly assessed prior to semen dilution and cryopreservation are colour, volume, pH, concentration, motility, morphology, and viability. Semen colour and volume are measured in raw semen ejaculate and can indicate contaminations in a semen sample (Munyai, 2012). Semen pH is a measurement of the alkalinity and acidity of the semen ejaculate (Ramukhithi, 2011). On the other hand, concentration is the number of spermatozoa per millilitre of semen (Munyai, 2012). Spermatozoa motility and velocity are the motion parameters essential for normal fertilisation and male fertility. Spermatozoa morphology is the normal size and shape considered for the success of fertilisation (Rapuling, 2010). Viable spermatozoa are cells that possess an intact plasma membrane (Mocé & Graham, 2008).

2.3 Spermatogenesis

Spermatogenesis is a complex process that involves the development of undifferentiated germ cells into highly specialised spermatozoa capable of fertilising an oocyte (Sharma *et al.*, 2013). Spermatozoa are produced in the seminiferous tubules of the testis through a process called spermatogenesis. After formation in the seminiferous tubules, the spermatozoa are transported through the *rete testis* and *vasa efferentia* into the epididymis, where they are stored while undergoing maturation changes that make the spermatozoa capable of fertilisation (Munyai, 2012).

Spermatogenesis consists of two phases; namely, spermatocytogenesis and spermiogenesis. From puberty, spermatogenesis will continue as an on-going process throughout the life of the male. Spermatocytogenesis involves the mitotic cell division, which results in the production of stem cells and primary spermatocytes, while spermiogenesis is the maturation and formation of spermatozoa. Meiosis during spermiogenesis is a process involving two cell divisions, resulting in spermatids containing a haploid number of chromosomes. Each primary spermatocyte first undergoes a meiotic division, forming two secondary spermatocytes. In this division, half reduce the chromosome complement in the nucleus, so that the nuclei in secondary spermatocytes contain an unpaired (n) number of chromosomes.

Spermiogenesis is then the differentiation of spermatids, which are released as spermatozoa. Spermatids with spherical nuclei differentiate into spermatozoa and are released from the Sertoli cells into the lumen of the seminiferous tubules. Spermiogenesis is thus the process during which a haploid spermatid undergoes a metamorphosis (change in morphology) to form a mature elongated spermatid or spermatozoa (Pruslin *et al.*, 1985).

The number of Sertoli and Leydig cells is related to spermatozoa production, each Sertoli cell supporting a defined number of germ cells (Matshaba, 2010). The process of spermatozoa production and formation of germ cells to fully mature spermatozoa take about 70 to 80 days in goats. When the spermatozoa development is complete in the seminiferous tubules, the spermatozoa are released from the seminiferous tubules into the epididymis, where storage and maturation occurs. The spermatozoa are stored in the *epididymis* until ejaculation. Through the contractions of retractor muscles, the spermatozoa propelled to the *vasa deferentia* and prostate gland to the urethra. As spermatozoa travel down the urethra, secretions are added by the secondary sex glands (prostate and seminal vesicles) to produce semen (Ramukhithi, 2011).

2.4 Hormonal control of spermatogenesis

The functions of the testes are to produce spermatozoa and androgens (testosterone), regulated by specific hormones (follicle stimulating hormone (FSH) and luteinizing hormones (LH)). These hormones are called gonadotropins. The production of spermatozoa and androgens by the testes would cease without gonadotropins (interstitial cell stimulating hormone and spermatogenesis stimulating hormone) support.

The main gonadotropins maintaining and regulating spermatogenesis are FSH/SSH and LH/ICSH (Munyai, 2012). Follicle-stimulating hormone and luteinizing hormone are glycoprotein hormones secreted by the anterior pituitary that acts directly on the testes to stimulate somatic cell function in support of spermatogenesis (Holdcraft & Braun, 2004).

2.4.1 Follicle stimulating hormone (FSH) or spermatogenesis stimulating hormone (SSH)

The follicle stimulating hormone is one of the gonadotropins (GTHs) produced in the pituitary that are members of the pituitary glycoprotein family. The action of FSH is mediated by the FSH receptor (FSHR) expressed by Sertoli cells and promotes the production of various endocrine and growth factors. Therefore, FSH signalling on Sertoli cells plays an important role in the initiation of spermatogenesis (Takeshi *et al.*, 2007).

2.4.2 Luteinizing hormone (LH) or interstitial cell stimulating hormone (ICSH)

In the male, luteinizing hormone is also known as an interstitial cell stimulating hormone (ICSH). The LH stimulates the Leydig cells or interstitial cells of the testes (located outside the seminiferous tubules) to produce testosterone. The LH is then carried from the anterior pituitary by the blood to the interstitial cells (Matshaba, 2010).

2.4.3 Testosterone

Testosterone and its metabolites, dihydrotestosterone (DHT) and estradiol (E_2) are collectively referred to as sex hormones. This is because of their primary role in the regulation of gonadal and germ cell development in both males and females as well as in the sexual differentiation of males (Holdcraft & Braun, 2004). Testosterone is an androgenic steroid hormone produced by the interstitial cells or Leydig cells, richly supplied with nerves. Testosterone secretion is under endocrine control by a negative feedback mechanism involving the hypothalamus and anterior pituitary. Low levels of testosterone naturally stimulate the hypothalamus to release GnRH, which in turn is carried by the portal system to the anterior pituitary, where it stimulates the release of LH by the anterior pituitary. The LH stimulates the Leydig cells in the testes to produce testosterone and when testosterone levels get too high, the hypothalamus and anterior pituitary are inhibited and secretion of GnRH and LH suppressed (Matshaba, 2010).

2.5 Spermatozoa structure and function

Spermatozoa are a male reproductive cell that fertilizes the egg to form a zygote. Spermatozoa consists of several membrane compartments; namely, plasma membrane, head (nucleus and acrosome), midpiece (mitochondria) and the tail. Cell competency requires that each of these membrane compartments be intact (Graham *et al.*, 1990).

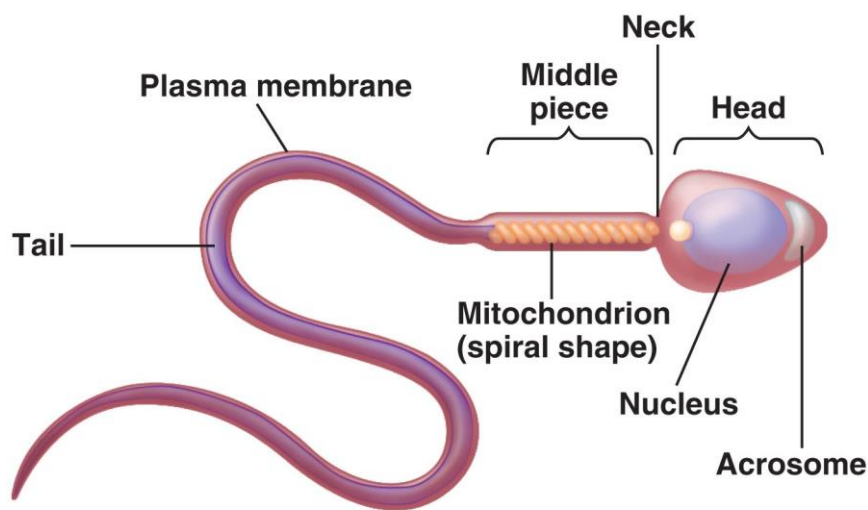


Figure 2.1: An illustration of a human spermatozoa structure (picture adapted from www.biology.lifeeasy.org)

2.5.1 Plasma membrane

The spermatozoa membrane is directly or indirectly related to many spermatozoa functions, warranting the capability of the cell to maintain homeostasis and depict motility and the capacity to interact with the environment, including the lining epithelium of the female genital tract or the oocytes-cumulus cell complex. Although the spermatozoa plasma membrane covers the entire cell, it consists of several distinct membrane compartments. Examples of these membrane compartments are the ones that covers the outer acrosome membrane, the one that covers the post acrosome portion of the spermatozoa head down to the annulus and lastly the one covering the principal piece and the rest of the tail (Hossain *et al.*, 2011).

Spermatozoa acquire the ability to move progressively during epididymal transit, but they are still fertilisation incompetent. Fertilisation capacity is gained after residing the spermatozoa in the female reproductive tract for a limited period. Capacitation is the physiological change that grants the spermatozoa the ability to fertilise. Capacitation is

an absolute prerequisite that spermatozoa must undergo in order to interact efficiently with the zona pellucida and accomplish one of the last steps leading to fertilisation; namely acrosome reaction. Capacitation includes several cellular changes in the spermatozoa particularly in the distribution and composition of certain glycoproteins, protein tyrosine phosphorylation, intracellular Ca^{2+} and cAMP concentrations (Nandi *et al.*, 2012).

2.5.2. Spermatozoa head

2.5.2.1 Spermatozoa acrosome

The acrosome is a membrane-enclosed structure located on the head of the spermatozoa (Lida *et al.*, 1999; Hossain *et al.*, 2011). It also contains hydrolytic enzymes that are exocytosed on acrosome reaction. The membrane fusion event that is required for spermatozoa penetration through zona pellucida and is crucial for subsequent spermatozoa-egg fusion (Lida *et al.*, 1999; Hossain *et al.*, 2011; Nandi *et al.*, 2012). It also contains a variety of proteins, including several protease zymogens, protease inhibitors, zona pellucida binding proteins and other ligand-binding proteins (Hossain *et al.*, 2011; Nandi *et al.*, 2012).

Acrosome integrity is a prerequisite for fertilisation, essential for spermatozoa penetration of the zona pellucida. Biologically, the plasma membrane and the outer acrosome membrane fuse and vesicate during the acrosome reaction when activated by spermatozoa binding to the zona pellucida (Hossain *et al.*, 2011). An acrosome reaction is an essential event to spermatozoa penetration into the zona pellucida and fertilisation. During the acrosome reaction, acrosomal enzymes are released and much of the anterior head membranes are lost as hybrid vesicles of the plasma membranes and the outer acrosomal membrane. Thus, the spermatozoa must maintain an intact acrosome up to the time it binds to the zona pellucida (Oliveira *et al.*, 2010).

2.5.2.2 Spermatozoa nucleus

The nucleus consists of the nucleus deoxyribonucleic acid (DNA) and chromatin structure. The integrity of DNA in the nucleus of mature ejaculated spermatozoa is one area of research that has been studied intensely during the past decade as a cause of male infertility. Normally, the spermatozoa chromatin is a well-organized, compact

structure consisting of DNA and heterogeneous nucleoproteins. It is condensed and insoluble in nature, features that protect the genetic integrity and facilitate transport of the paternal genome through the male and female reproductive tracts. For spermatozoa to be fertile, it must be capable of undergoing decondensation at a suitable time in the fertilisation process. It has been suggested that spermatozoa DNA integrity may be a more objective marker of spermatozoa function as opposed to the standard semen analysis (Agarwal & Said, 2003).

Evaluation of spermatozoa DNA integrity is of utmost importance owing to early embryo development, depending on its normality. Although spermatozoa DNA is packed in highly compacted and stable form, chromatin abnormalities and DNA damage exist, derived from either premeiotic testicular insults, during spermiogenesis, when DNA is packed or during further chromatin building (protamine dominance) during epididymal maturation. Alternatively, it could be the result of free radical-induced damage or consequence of apoptosis, in some species (Hossain *et al.*, 2011). Chromatin condensation begins during spermiogenesis when the histones are first replaced by transition proteins and finally by small, basic protamines. The final volume of the chromatin is highly reduced according to the volume of somatic cell metaphase chromosomes, with a corresponding reduction in DNA stainability and the nucleus adopts a species-specific morphology. During the passage of the spermatozoa through the epididymis, the protamines formulate inter- and intra-molecular disulphide bonds, and, as a result, spermatozoa chromatin is fully condensed (Garcia-Macias *et al.*, 2006).

2.5.3 Spermatozoa mitochondrion

Mitochondria are located in the spermatozoa mid-piece and are approximately 100 in number, depending on the species (Juhász *et al.*, 2000; Peña *et al.*, 2003; Hossain *et al.*, 2011). Mitochondria contain enzymes and co-factors required for the production of adenosine triphosphate (ATP) required for spermatozoa metabolism, membrane function, and motility, alongside with anaerobic glycolysis in the cytoplasm (Juhász *et al.*, 2000; Peña *et al.*, 2003; Hossain *et al.*, 2011). Further, mitochondria are the coordinators of apoptosis mechanisms in a number of cell systems, and they are involved in spermatozoa maturation and protection against damage induced by cryopreservation (Hossain *et al.*, 2011).

Mitochondria have a significant role in energy production, calcium homeostasis, and apoptosis and also provide motors for spermatozoa motility and relates to fertility

(Peña *et al.*, 2003; Fernández-pastor *et al.*, 2007; Sun & Yang, 2010). Both spermatozoa biosynthesis and motility require ATP. Two metabolic pathways provide ATP for normal spermatozoa function: oxidative phosphorylation in the mid-piece and glycolysis in the principal piece. Mitochondria are dynamic both in their morphological transitions and in distribution (Sun & Yang, 2010). Mitochondria provide maternal inheritance and ubiquitination of mitochondrial takes place to facilitate their degradation in the egg cytoplasm after fertilisation (Thompson *et al.*, 2003; Sun & Yang, 2010). Due to the indispensable role of mitochondria during fertilisation, a defect of spermatozoa mitochondria or associated proteins can lead to male infertility (Sun & Yang, 2010). Mitochondrial proteins are involved in the capacitation-dependent tyrosine phosphorylation in spermatozoa (Hossain *et al.*, 2011).

2.6 Seminal plasma

Seminal plasma is composed of secretions from the testis, epididymis and male accessory glands. It provides a favourable environment for spermatozoa and serves as a vehicle for spermatozoa as they travel to meet the oocytes (Teixeira *et al.*, 2006; Sharma *et al.*, 2013). Seminal fluid contains a variety of both inorganic and organic components, of which proteins are a major part of the high molecular mass substances. The protein composition of seminal plasma varies from species to species. However, studies on several mammalian species indicate that seminal plasma contains factors that influence the fertilising ability of spermatozoa and exerts important effects on female reproductive physiology. Boar spermatozoa adhesins are a group of proteins found in seminal plasma and they are major secretory products of the seminal vesicle epithelium (Teixeira *et al.*, 2006). These unique proteins are necessary for spermatozoa function and survival. Seminal plasma proteins play a variety of roles; they protect the spermatozoa by binding to the spermatozoa surface during ejaculation and play a key role in capacitation, acrosome reaction, and sperm-egg fusion. They can also modulate important response in male and female reproductive tracts, ensuring that the most competent spermatozoa meet the oocytes during fertilisation. Thus, seminal plasma proteins can serve as important biomarkers for male infertility (Sharma *et al.*, 2013). Seminal plasma plays an important role in spermatozoa survival during cryopreservation process (Peterson *et al.*, 2007; Niang *et al.*, 2011). The antioxidant presence in the seminal plasma is the most vital form of defence available to spermatozoa against ROS and they provide the defence mechanism through prevention, interception and repair level of protection. Nevertheless, the antioxidant capacity of spermatozoa may be insufficient in preventing

oxidative stress during centrifugation, cooling and freezing or thawing processes (Memon *et al.*, 2012).

Causes of reduced spermatozoa motility are related to seminal plasma enzymes. Today, egg yolk and milk extenders are widely used for the frozen storage of small ruminant semen (Niang *et al.*, 2011). Nevertheless, the deterioration and toxic effects of the seminal plasma were observed when goat semen was diluted in egg yolk extender. The presence of enzymes (bulbourethral secretion glycoprotein-60 and egg yolk coagulating enzyme) in the seminal plasma causes the harmful interactions between seminal plasma and egg yolk. Meanwhile, bulbourethral secretion glycoprotein-60 (BUSgp60) has a triacylglycerol hydrolase activity, which decreases spermatozoa motility and movement quality by disrupting the cell membrane. Phospholipase A₂ activity of egg yolk coagulating enzyme (EYCE) catalyse the hydrolysis of egg yolk phosphatidylcholine (PC) into fatty acids and lysophosphatidylcholine (LPC). The LPC has a toxic effect on buck spermatozoa by acting like a detergent on biomembrane, resulting in a loss of motility, membrane integrity and consequently low fertility rate (Peterson *et al.*, 2007; Amidi *et al.*, 2010; Miclea *et al.*, 2011; Niang *et al.*, 2011; Hussain *et al.*, 2012; Jimènez Rabadán, 2013; Yimer *et al.*, 2014). Furthermore, the strength of the detergent properties of LPC depends on the amount formed, temperature (enzyme activity and hence LPC formation is temperature dependent), dilution or degree of removal of seminal plasma, the season of semen production and breed of fowl providing the egg yolk (Miclea *et al.*, 2011).

During the washing process, not only is the seminal plasma separated but other beneficial components and compounds; such as inorganic compounds, organic compounds, and antioxidants, are also removed. These substances are essential for functionality and survival as well as preventing lipid peroxidation of spermatozoa after ejaculation (Memon *et al.*, 2014). During the storage of mammalian spermatozoa, phospholipids undergo peroxidation, the formation of toxic fatty acids peroxides causing structural damage to the spermatozoa accompanied by decreased motility (Mishra *et al.*, 2010).

2.7 Effect of cryopreservation on the spermatozoa structure and functions

The storage of frozen goat semen has been reported to cause ultrastructural, biochemical and functional damage to the spermatozoa, resulting in a reduction in

motility, viability, fertility, and impaired transport (Qureshi *et al.*, 2013). Spermatozoa parameters are associated with male fertility or infertility. Morphology evaluation is widely used for predicting fertility potential in farm animals and in humans (Rybar *et al.*, 2004; Mekasha *et al.*, 2007). Spermatozoa motility, viability, membrane integrity and acrosome integrity are therefore vital in the evaluation criterion of semen sample quality (Priyadharsini *et al.*, 2011).

The DNA integrity could be useful for assessing the potential fertility of a given spermatozoa sample when considered along with other semen quality assays. Spermatozoa DNA damage has been associated with poor semen quality (Agarwal & Said, 2003). Cryopreservation and freeze-thaw process alter or damage the chromatin structure and DNA (Hammadeh *et al.* 1999; Ekwall, 2007; Üstüner *et al.*, 2015). It has been speculated that damage to DNA integrity might be caused by environmental effects such as elevated temperature, toxic agents, components of semen storage extenders, storage conditions and the cold shock caused by freezing and thawing.

Cold shock increases the susceptibility of semen to oxidative damage due to an increase in reactive oxygen species (ROS) production (Memon *et al.*, 2012; Lee *et al.*, 2014). The ROS have been shown to change cellular functions through the disruption of the spermatozoa plasma membrane and damage to proteins and DNA (Hammadeh *et al.* 1999; Memon *et al.*, 2012; Lee *et al.*, 2014).

2.8 Factors affecting semen production and quality

Productivity in small ruminants is affected by the seasonality of reproduction. In rams and bucks, a decrease in quantitative and qualitative semen production and spermatozoa fertility during the non-breeding season has been reported (Munyai, 2012). Photoperiod or annual season has been suggested as the principal factor influencing seasonality of reproduction in bucks at high latitudes. However, other environmental stimuli, such as availability of food and social interactions, should not be disregarded as potential regulators of the seasonality of reproduction. Nutrition is considered an important factor affecting seasonality of reproductive functions in bucks. However, since changes in photoperiod also occur during times of scarce nutrition, it is possible that season and nutrition have complex effects on reproductive activity (Zarazaga *et al.*, 2009).

2.9 Semen collection methods

2.9.1 Semen collection using the artificial vagina

An artificial vagina (AV) is a device designed to simulate the female reproductive tract and it is easy to use and the semen collected is generally relatively clean and the ejaculate is similar to the natural ejaculate (Matshaba, 2010; Munyai, 2012). The AV briefly consists of a rigid cylinder of rubber or polyvinyl chloride (PVC) and a thin-walled rubber tube for the thin-walled rubber tube over the outer cylinder. The water jacket is filled with warm water (45-55 °C) to bring the inside temperature of the artificial vagina to a few degrees Celsius (°C) above normal body temperature. The temperature of the water provides the thermal stimulation, while the pressure in the AV provides the mechanical stimulation of the vagina over the glans penis. At one end of the AV, a graduated glass semen collection tube is fitted. A female that is in estrus is placed in a neck clamp and the male is allowed to mount. When the male mounts, the penis is deflected into the AV, where the male ejaculates naturally (Munyai, 2012). Prior to semen collection, all parts of the AV should be thoroughly cleaned and rinsed with sterile water, then sterilised with alcohol and finally with distilled water and allowed to air dry and be properly assembled (Matshaba, 2010; Munyai, 2012).

The prerequisites for using the AV method are that the male must be conscious, not significantly frightened by people and more interested in ejaculation than fighting with the people that are collecting semen and bucks need to be trained for this specific method (Ramukhithi, 2011; Matshaba, 2012). Bucks that are raised extensively could reject the training of AV method of collecting semen, due to infrequent contact with people (Ramukhithi, 2011). Semen collection with an AV offers the advantage of frequent sampling without the stress of chemical or physical restraint (Durrant, 1990).

2.9.2 Semen collection by electro-ejaculation

The ejaculation of semen is brought about by inserting a probe or electrode into the rectum of the male with the contacts facing down and stimulating the sacral plexus, hypogastric nerves, and parasympathetic outflow through pudendal nerve by gradually increasing the electrical current in rhythmic fashion, for a short period of time (Matshaba, 2010). By passing 5 to 10 seconds rhythmic electric stimuli through the electrodes, an ejaculation can be induced and the semen collected in a glass tube. During the

application of this electro-ejaculation method, the electric current produces strong contractions of all body muscles and a slight and temporary motor inability of the hindquarters and hind limbs, at the end of this treatment (Munyai, 2012). This method is easily adaptable, because it does not need training of males and can be the best choice to collect semen from valuable males, which are unable to perform natural services due to age or injury. Bucks are able to ejaculate when 3-5 volts of electrical stimulation is applied through the rectum to the accessory gland with a rest period of 3-5 seconds between stimulations (Ramukhithi, 2011).

2.10 Semen evaluation

Male fertility is a symphony of physiology, endocrinology, and behaviour. The ultimate test of male fertility is definitely conception. The fertilising capacity of spermatozoa can be evaluated by examining ova for evidence of fertilisation following natural breeding or AI. Traditional parameters of semen quality include volume and spermatozoa motility, concentration and morphology evaluations. When these parameters are combined, the accuracy of fertility assessment is enhanced but still incomplete (as none of these criteria can predict the ability of spermatozoa to perform effectively in the female reproductive tract). Truly comprehensive semen analysis involves the evaluation of the ability of spermatozoa to reach the site of fertilisation, undergo capacitation and the acrosome reaction, penetrate the zona pellucida, fuse with the ooplasm, and decondense (Durrant, 1990). The most important semen parameters that are subjectively or objectively evaluated immediately after semen collection include semen volume, colour, and pH, spermatozoa motility, morphology, viability and spermatozoa count/concentration (Ramukhithi, 2011).

2.10.1 Manual/visual spermatozoa analysis

Traditionally, the spermatozoa motility is assessed visually under contrast microscopy. Subjective motility evaluation performed by optical microscopic analysis presents variations from 30 to 60% for the same ejaculates (Konyali, 2009). Manual/visual microscopic spermatozoa analyses are conducted by placing a sample of the semen on a microscope slide and visually evaluating it, using specific criteria. These tests use fresh or fixed, stained or unstained semen and remain the mainstay of the assays conducted by most laboratories. The major limitations of manual analyses are that they can be influenced by human bias, as well as being a time-consuming process,

either in the semen sample preparation or during the analysis itself, resulting in relatively few spermatozoa being evaluated per ejaculate (Matshaba, 2010).

2.10.2 Automatic spermatozoa analysis procedures

Computer-assisted sperm analysis (CASA) is one of the systems that were developed in order to prevent human biases and is one of the utmost important. The CASA is a valuable tool for spermatozoa evaluation because it allows the evaluation of spermatozoa motion parameters objectively and more quickly if compared with manual methods. The assessment of spermatozoa motility parameters is important to evaluate the *in vivo* or IVF ability of semen samples and motility evaluated by CASA system provided repeatable estimates of many criteria of spermatozoa movements (Konyali, 2009).

To estimate more accurately the spermatozoa quality of a sample (fresh, cooled or frozen-thawed), several laboratory assays should be performed. Spermatozoa membrane integrity or spermatozoa viability is a parameter widely used to estimate spermatozoa quality. The sample is stained and then evaluated under bright field or fluorescence microscopy, depending on the stains used for the estimation of spermatozoa membrane integrity. Stains used for bright field evaluation are cheap and allow the estimation of this parameter with simple equipment and samples can be evaluated by flow cytometry. Flow cytometry measures and analyses simultaneously multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. Spermatozoa analysis by flow cytometry allows the objective, rapid and simultaneous analysis of a large number of spermatozoa and the results obtained with these analyses may allow the estimation of the fertility of a semen sample. Flow cytometry analysis by using one or more fluorescent stains can be applied for several spermatozoa analyses such as spermatozoa count, viability, apoptotic-like markers, motility, spermatozoa acrosome reaction or DNA content and integrity (Konyali, 2009).

The power of this technology is that approximately 50 000 sperm cell can be counted in a minute. The staining techniques are all very simple and rapid, air-dried sperm cell fixed in a fluorescent fixative or live sperm cell can be recorded. Fluorescent probes are currently available to evaluate nearly any cell attribute, which one would wish to measure using a fluorescent microscope (Matshaba, 2010).

2.11 Evaluation of semen parameters

2.11.1 Semen colour and volume

Buck raw (unaltered) semen appears as a thick whitish to slightly yellowish fluid (Bag et al., 2002; Munyai, 2012). Normal buck semen is grayish-white to yellow in colour and varies between bucks and ejaculates of the same buck. The dense colour (greyish white) may indicate a high spermatozoa concentration, while the less dense colour (yellow colour) may indicate low spermatozoa concentration (Hafez & Hafez, 2000; Matshaba, 2010; Munyai, 2012). The presence of blood in the semen is indicated by a pink colour of the semen (contamination) and can be due to injury or disease of the penis or reproductive tract (Matshaba, 2010; Munyai, 2012). Contaminated semen samples should be discarded (Matshaba, 2010).

Semen volume together with spermatozoa concentration play a major role during semen dilution, as the researchers will know how much extender is needed to dilute a certain semen volume. Dilution of goat semen containing a high spermatozoa concentration allows high volumes of extenders resulting in an increased number of semen straws that can be frozen; hence, more females could be served. In bucks, semen collected with electro-ejaculator method ranges from 0.7 mL to 2.2 mL and that collected with artificial vagina method ranges from 0.5 mL to 1 mL (Ramukhithi, 2011).

2.11.2 Semen pH

A pH of approximately 6.8 to 7.0 falls within the optimum activity ranges of most of the enzymes in the spermatozoa. A higher metabolic rate is expected when the pH of semen is maintained near neutrality (7.0). However, the pH of semen could deviate towards alkalinity or acidity and then the metabolic rates are increasing or reducing (Matshaba, 2010). The pH meter is used to measure semen pH. Changes in semen pH can be due to semen collection method and cryopreservation media. It is best to maintain the proper environment by controlling pH fluctuations in the cryopreservation media using buffers (Ramukhithi, 2011).

2.11.3 Semen concentration

Spermatozoa concentration of ejaculates determines the number of females that will be inseminated. Semen sample is diluted according to the concentration. Therefore,

high spermatozoa concentration results in high number of insemination doses or semen straws (Ramukhithi, 2011). Sperm cell concentration can be evaluated using spermacue, photometers and haemocytometers (Dombo, 2002).

2.11.4 Spermatozoa motility

Spermatozoa motility is an important, useful parameter of spermatozoa. It is a widely used criterion to assess the effects of freezing on spermatozoa of various species. The evaluation of spermatozoa motility is a crucial parameter in the assessment of semen quality and in the establishment of correlations between spermatozoa quality and fertility. Spermatozoa motility can be evaluated for both raw and frozen-thawed semen (Ramukhithi, 2011). The ability of spermatozoa to migrate through the female genital tract and penetrate or fertilise the oocytes thus depends on the hydrodynamic potential exerted by the flagella bending and the resistance exerted by the secretions present in the lumen of the genital tract. Different rates in the transport of spermatozoa are mainly based on the kinematic properties that define the propulsive strength. Spermatozoa motility is believed to be one of the most important characteristics used when evaluating the fertility potential of ejaculated spermatozoa. It has been stated that the spermatozoa motility parameters of goat semen can be useful in the selection and ranking of bucks regarding their potential fertility.

It is important to protect the ejaculated semen from harmful agents or conditions prior to evaluation because spermatozoa motility is susceptible to environmental changes (excessive warm or cold ambient temperatures). An experienced technician and a properly equipped laboratory are essential for a reliable estimation of the semen motility. At present, the objective assessment of spermatozoa motility is possible with CASA, which considers many motility properties, eliminating human errors. However, the equipment is expensive and is generally not used in routine semen evaluation procedures (Matshaba, 2012). The motility parameters evaluated by CASA are as follows; total motility (TM), progressive motility (PM), non-progressive motility, rapid (RAP) motility, and medium (MED), slow (SLW) and static (STC). The kinematic parameters evaluated by CASA are as follows; velocity on the curve line (VCL), velocity on the straight line (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), wobble (WOB), amplitude of lateral head displacement (ALH), beat cross frequency (BCF).

2.11.5 Spermatozoa morphology

Spermatozoa morphology seems to be one of the most important qualitative characteristics of semen and can serve as an indicator of some disorders in the process of spermatogenesis. Morphologic assessment of the spermatozoa is an integral component in the analysis of semen and is an important part of any breeding buck soundness examination (Mekasha *et al.*, 2007; Matshaba, 2010). Spermatozoa abnormalities have been found to negatively affect the motility, the survival and fertilisation rates in several species. Eosin is referred to as a differential stain, as it cannot pass through living cell membranes. A background stain such as Nigrosin, opal blue or fast blue provides a good contrast making the unstained spermatozoa heads more visible. The partial stained and totally stained spermatozoa represent dead cells, whereas unstained spermatozoa represent live cells (Matshaba, 2010). Abnormalities can occur in any region of the spermatozoa. The abnormalities' are classified into primary, secondary and tertiary abnormalities. Primary abnormalities occur during spermatogenesis in the testes (small, large, swollen and double heads, abnormal acrosome, elongated and abaxial mid-piece, double and short tail. Secondary abnormalities occur during maturation in the epididymis (detached, loose or damaged acrosomes, bent and protoplasmic droplets of the mid-piece, bent and shoe-hook tail). Tertiary abnormalities results from poor handling of the semen after collection (reacted acrosomes and coiled spermatozoa tails (Ramukhithi, 2011).

2.12 Cryopreservation of semen

Due to the development of semen collection, storage, and AI techniques, donor males have a great impact on reproductive success. This is because each of the ejaculates of the males can be used to inseminate many females. To increase storage period of the semen collected, it is necessary to develop an optimal cryopreservation protocol. Cryopreserved semen may have a great economic impact for breeders and animal production industries as cryopreservation permits the utilisation of semen outside the sexual season in seasonal species and it permits covering the demand of seminal doses in seasons where the seminal quality of the male decreases (Konyali, 2009).

2.13 Semen diluents

The purpose of diluents in semen cryopreservation is to provide the spermatozoa with energy, protection from temperature related damage/shock and maintain a

sustainable environment for the spermatozoa to survive temporarily. The volume of diluents to be added in semen is based on the specific volume of semen or by diluting semen to a specific spermatozoa concentration. A number of diluents have been evaluated in the past for the freezing of goat semen, e.g. reconstituted cow skimmed milk, sodium citrate-glucose yolk, lactose yolk, saccharose ethylene diamine triacetic acid (EDTA), CaNa₂ yolk, raffinose yolk, Spermasol yolk and Tris-yolk. All extenders used for semen preservation in domestic farm species must have the appropriate pH and buffering capacity, suitable osmolality and should protect the spermatozoa from any cryogenic injury (Matshaba, 2010). Most of the semen extenders have an egg yolk in their composition and different combinations of cryoprotectants at different concentrations. Some of the cryoprotectants that have been used are dimethyl sulphoxide (DMSO), glycerol, ethylene glycol, acetamide, trehalose, methylcellulose, sucrose and skimmed milk. The composition of the extenders assists in stabilising the cell during the freezing and thawing process (Soylu *et al.*, 2007). Egg-yolk-based extender is regarded as a non-permeable cryoprotectant. Extenders consist of one or more cryoprotectant, sugar, buffer and antibiotics (Chanapiwat *et al.*, 2012).

2.13.1 Cryoprotectants used in semen cryopreservation

Among factors affecting the freezing ability of spermatozoa, supplementation of the freezing medium with different types of cryoprotectant plays a vital role in minimising the physical and chemical stresses occurring during cryopreservation procedure (Anakkul *et al.*, 2013). The cryoprotectants and their modes of action have been focused on, with glycerol and DMSO being the most commonly used (Munyai, 2012). Glycerol is commonly added to semen extenders for freezing mammalian semen and has yielded successful results in the cryopreservation of sheep and goat semen (Soylu *et al.*, 2007; Sundararaman & Edwin, 2008; Farshad & Akhondzadeh, 2008; Chanapiwat *et al.*, 2012; Lee *et al.*, 2014). Although glycerol protects the spermatozoa from cryoinjury by removing the water found within the cell and by increasing extracellular osmolality (tonicity), the presence of glycerol in the semen extender causes certain structural damage at room temperature (Sundararaman & Edwin, 2008; Farshad & Akhondzadeh, 2008). For these reasons, many studies have been carried out on the amount of glycerol and the timing protocol for adding it to the extender and on the exclusion of the glycerol from the freezing media (Soylu *et al.*, 2007). Low molecular weight cryoprotectant, such as ethylene glycol may cause less damage to the spermatozoa than glycerol because its lower molecular weight allows it to cross the plasma membrane easier (Lee *et al.*, 2014). Dimethyl sulfoxide (DMSO) is the internal cryoprotectant that reduces the formation of

intracellular ice crystals, thus preserving the fertilising potential of spermatozoa (Varela Junior et al. 2015). DMSO can also increase the concentration of calcium ions in cytoplasm, causing a variety of metabolic responses such as depolymerisation and cytoskeleton assembly (Anil, 2013). Sugar is an energy source during incubation and also acts as a non-permeable cryoprotectant and minimises osmotic stress during the freezing and thawing processes. It protects the spermatozoa from dehydration and intracellular ice formation during the freezing process. Different types of sugars (trehalose, lactose, fructose, glucose, sucrose, sorbitol and raffinose) have been used in the freezing extender of semen (Farshad & Akhondzadeh, 2008; Chanapiwat *et al.*, 2012). The storage temperature, the molecular weight of the sugar and the type of buffer used in the extender affect the cryoprotective ability of sugars (Farshad & Akhondzadeh, 2008).

2.13.2 Antioxidants

Antioxidants such as glutathione, oxidised glutathione, cysteine, taurine, hypotaurine, bovine serum albumin, trehalose, and hyaluronan have been tested to determine their abilities to minimise the damage caused by cooling and freeze-thawing of goat semen and ram semen (Ajao, 2015).

2.13.3 Antibiotics

The antibiotics added to diluents allow for spermatozoa preservation without excessive bacterial growth (Van Staden, 2010). To prevent the growth of microorganisms in the semen, antibiotics such as penicillin, streptomycin, polymyxin B, sulfanilamide and other combinations of antibiotics are added in the diluents (Ajao, 2015).

2.13.4 Buffers

Buffers are solutions, which lessen the change of pH upon addition of small amounts of acids or base or upon dilution. A buffering solution should have a pH of 6.0 – 8.0 and should have minimal interactions with sodium citrate and citric acid and be minimally affected by temperature and ionic contents. Seminal plasma also functions as a buffer for spermatozoa against changes in pH in *in vivo* conditions (Ramukhithi, 2011).

CHAPTER 3

MATERIALS AND METHODS

3.1 Ethical approval

The present study was conducted with the approval of the Research Ethics Committee of the University of Venda (SARDF/15/ANS/08/0502).

3.2 Study site

This study was conducted at the experimental farm and the Biotechnology Laboratory of Centre of Excellence in Animal Assisted Reproduction (CEAAR) of the University of Venda located in Thohoyandou (22° 57' 0" South, 30° 29' 0" East), Limpopo Province, Republic of South Africa.

3.3 Experimental bucks

A total of six unimproved indigenous domesticated bucks aged 3 to 4 years were used as semen donors. The bucks were kept in pens at the experimental farm and maintained on ewe and lamb pellets with water provided *ad libitum* throughout the experiment.

3.4 Semen collection and processing

Semen samples were collected twice a week (Monday and Thursday) from October to December 2015 using an electro-ejaculator [(EE) Pulsator IV Complete, Lane Manufacturing incorporation, Denver, CO 80231, USA]. The collection was performed in the morning between 07:00 am and 10:00 am. Prior to semen collection, the hair around the sheath was clipped with a pair of scissors and the prepuce was washed with 70% ethanol and wiped with a sterile paper towel to prevent contamination by dirt and excess urine from the sheath. The buck was laid down on its side during semen collection. The probe was washed with clean tap water, wiped with a paper towel, and lubricated with KY Jelly®. The probe was inserted into the rectum of the buck, and the monitor was switched on and set for manual running. Semen samples were collected into pre-warmed (37 °C) 15 mL graduated plastic tubes and immediately placed in a thermo flask with water at 37 °C. The collected buck semen samples were then transported to the

laboratory for further analysis with an average interval of 15 minutes between each ejaculate and kept in a water bath at 37 °C.

The buck semen samples were pooled to eliminate individual differences between the samples. Pooled semen were randomly allocated into six aliquots, namely (i) Raw non-washed, (ii) Raw washed, (iii) Triladyl[®]-washed semen, (iv) Triladyl[®]-non-washed semen, (v) Bioxcell[®]-washed semen, and (vi) Bioxcell[®]-non-washed washed. Triladyl[®] and Bioxcell[®] washed semen groups were mixed with PBS at a ratio of 1:4. Semen samples were then centrifuged at 1500 x g for 10 min. Following centrifugation, seminal plasma was removed using 1 mL sterile plastic hand pipette, leaving only the spermatozoa pellets.

3.5 Semen evaluation before extension

The following semen parameters were evaluated: spermatozoa motility, viability, morphology, acrosome, and chromatin and mitochondria membrane potential

3.5.1 Evaluation of spermatozoa motility

Spermatozoa motility rate was analysed using the Sperm Class Analyzer[®] (SCA), version 5.4, (Microptic SL, Barcelona, Spain) system (Ajao, 2015) known as Computer-Aided Sperm Analyser (CASA). Five hundred microliters of phosphate buffered saline (PBS) (Sigma-Aldrich, St Louis, MO, USA) plus 20 µL of raw semen were mixed in an Eppendorf tube (Simport, Canada) using a hand pipette. A drop (2 µL) of semen sample was placed on a pre-warmed (37 °C) microscopic glass slide (MS labcon) and mounted with a cover of a microscopic glass slip and examined under a CASA microscope. Four fields per sample were captured under 10X magnification with a Ph1 phase contrast. The motility parameters recorded by CASA are as follows; total motility (TM), progressive motility (PM), non-progressive motility, rapid (RAP) motility, and medium (MED), slow (SLW) and static (STC). The kinematic parameters recorded by CASA are as follows; velocity on the curve line (VCL), velocity on the straight line (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), wobble (WOB), amplitude of lateral head displacement (ALH), beat cross frequency (BCF).

3.5.2 Evaluation of spermatozoa viability (live/dead)

Spermatozoa morphology/viability was analysed using an Eosin-Nigrosin (Merck Millipores Corporation) staining solution (Raseona, 2015). A drop of 20 μ L Eosin and a drop of 10 μ L Nigrosin were placed on the one end of the pre-warmed (37 °C) microscopic glass slide (MS labcon) using a hand pipette. Then 10 μ L of semen was placed on the stains and they were gently mixed together with the tip of the hand pipette and then a smear was made using a pipette tip (to avoid breakage of spermatozoa during the smear) at 37 °C. The slide was placed on the slide warmer (Buehler® LTD, Illinois, USA) at 120 °C to allow the smear to dry fast. Dried microscopic slides were analysed for viability using the CASA microscope at 60 X magnification with oil immersion (uniLab, Merck chemicals, (PTY) LTD) and 300 spermatozoa were counted/slide/replicate. The live spermatozoa are unstained and the dead spermatozoa are stained.

3.5.3 Evaluation of spermatozoa morphology and acrosome membrane damage

Spermatozoa morphology and acrosome membrane damage were analysed by staining spermatozoa with Spermac® stain (Stain enterprises, South Africa) (Raseona, 2015). A drop of 15 μ L of semen was placed on a microscopic glass slide using a hand pipette. A smear was made by gently pulling semen from one end to the other end using a hand pipette tip and was allowed to air dry for about 10 min on a warm plate at 37 °C. The absorbent paper was spread inside the staining tray to absorb excess stain during the staining procedure and the smeared slide was placed on the staining tray. The smeared slide was fixed with the Spermac® fixative by placing fixative drops on the smeared slide until it was covered with the fixative using a hand pipette. After 1 min, the microscopic slide was then placed vertically on absorbent paper to drain excess fixative into the staining tray. The slide was then washed gently by dipping it seven times in a glass beaker with distilled water to remove the stain. The fixed slide was placed on the staining tray with absorbent paper and stained with stain A by placing stain drops on the fixed slide until it was covered with stain A using the hand pipette for 1 min. The stained slide was then drained by holding the slide vertical on its side and washed by dipping the slide seven times in a glass beaker with distilled water. The slide was placed vertically on absorbent paper to drain excess water. The same procedures were repeated with stains B and C. The slides were allowed to air dry for 15 min and then observed under a light microscope (60 X magnification) using oil immersion and 300 spermatozoa per stained

slide were counted. The acrosome stained dark green and nucleus stained red and morphology were evaluated for the abnormal head, bent mid-piece and coiled tail.

3.5.4. Acridine orange (AO) staining procedure for evaluation of chromatin structure

Acridine orange staining solution was prepared as follows: 10 mL of 0.1% AO stock solution (Sigma-Aldrich, St Louis, MO, USA) in distilled water was added to a mixture of 40 mL of 0.1 M citric acid (MW= 192.12) and 2.5 mL of 0.3 M Na₂HPO₄·7H₂O (MW= 177.99) (Sadeghi *et al.*, 2009). The pH was adjusted to 2.5 (Sadeghi *et al.*, 2009). Acridine orange was used to analyse the chromatin membrane of the buck spermatozoa. The raw semen sample in a 15 mL tube was washed three times in PBS by centrifugation at 1500 x g for 10 min. Fifteen microliter drops of the spermatozoa pellets were smeared on a microscope glass slides using a hand pipette tip and was allowed to air-dry for 10 min. The smeared slides were fixed for 1 hour in ethanol-acetone (1:1) in a staining tray at 4 °C and were allowed to air-dry for 10 min. The microscope slides were then stained for 7 min with AO (0.19 mg/mL) at room temperature in the dark. The microscope slides were gently rinsed by dipping the slides in a glass beaker with distilled water and air-dried for 10 min, then viewed under a UV light inverted microscope at 40X magnification (Nikon eclipse TI, Narishige Co., Ltd. USA). Three hundred spermatozoa per stained slide were counted. Green spermatozoa have normal chromatin and red or yellow spermatozoa have denatured chromatin.

3.5.5 JC-1 staining procedure for evaluation of mitochondrial membrane potential

The JC-1 stain (Molecular probes[®], Eugene. Oregon. USA) was used to analyse the spermatozoa mitochondrial membrane potential (Kasai *et al.*, 2002). Five mg of JC-1 was dissolved in 5 mL of DMSO (Sigma-Aldrich, St Louis, MO, USA). All staining procedures were done in subdued light because the JC-1 stain is light sensitive. To avoid repeated freeze/thawing of the JC-1 stock solution small aliquots were made after the first thaw and stored at -20°C. Before staining the spermatozoa, the JC-1 stain was thawed. JC-1 stain and PBS were warmed to room temperature. Semen was diluted with PBS (1:4 v/v) and centrifuged at 1500 x g for 10 min. A hand pipette was used to suck the fluid carefully to avoid disturbing the spermatozoa pellets. Ten microliters of JC-1 staining solution supernatant was diluted in pre-warmed 100 µl of PBS in an Eppendorf tube (vortex during dilution to prevent the formation of precipitates) and placed in a water

bath at 37 °C. The JC-1 reagent was diluted immediately prior to use. The spermatozoa pellets were diluted with PBS and 5 µL of PBS with spermatozoa were mixed with 5 µl of the JC-1 staining solution in an Eppendorf tube. The Eppendorf tube was then placed in an incubator and the spermatozoa were incubated for 10 minutes at 37 °C. After incubation, washing was done twice by mixing 40 µL of PBS with incubated spermatozoa using a hand pipette. After washing, a 2 µl drop of PBS containing spermatozoa was placed on a slide and covered with a coverslip. Spermatozoa were observed immediately under a UV light inverted microscope at 40 X magnification (Nikon eclipse TI, Narishige Co., Ltd. USA). Three hundred spermatozoa per stained slide were counted. The spermatozoa mitochondria with high membrane potential appeared reddish and mitochondria with low membrane potential appeared greenish.

3.6 Preparation and composition of semen extenders

The extenders used in this study were Bioxcell® (IMV, L'Aigle, France) and Triladyl® (Minitüb, Germany). Bioxcell® is a soy lecithin extender and Triladyl® is an egg yolk extender. The following were the compositions and preparations of Bioxcell® and Triladyl® extenders used in the present study.

Table 3.1: Composition of Bioxcell[®] and Triladyl[®] semen extender

Ingredients	Quantity for Bioxcell[®]	Quantity for Triladyl[®] (250 g)
Tris	2.3 g	12.1 g
Fructose	1.2 g	5 g
Sodium citrate	6.2 g	-
Monohydrate lactose	0.8 g	-
Glycine	0.2 g	-
Anhydrous glucose	0.5 g	-
Taurine	0.005 g	-
Gentamicin sulphate	0.24 g	25 mg
Tylosin tartrate	0.33 g	5 mg
Linco-spectin, Lincomycin, Spectinomycin	0.383 g, (15 mg), (30 mg)	
Glycerol	40.2 g	86 g
Hydrate of calcium lactate	0.7 g	-
Soy lecithin	1.5 g	-
Monohydrate citric acid	2.5 g	6.9 g
Ultrapure water	1000 mL	140 g

One bottle of Bioxcell[®] (100 mL) was diluted with 400 mL of distilled water for a final extended, ready to use the volume of 500 mL. It was then aliquoted into 15 mL graduated tubes and stored at -20 °C. The egg yolk was separated from the albumen by passing the egg yolk from one-half of the shell to the other in order to get rid of the albumin (Ajao, 2015). The egg yolk was placed in a gauze swab (Johnson & Johnson (PTY) LTD, South Africa) and a 18-gauge needle (Sigma-Aldrich, St Louis, MO, USA) was used to punch the egg yolk in order to separate the egg yolk from its membrane and some contents of albumin to the 15 mL graduated tube (Cell Star[®] tubes, Greiner Bio-one GmbH, Germany). Triladyl[®] concentrate was added slowly to the water and the mixture was added to egg yolk. The mixture was stirred and filtered using a sterile filter funnel. The final mixture of Triladyl[®] extender was kept in 15 mL graduated tubes, sealed with Parafilm "M"[®] (American National Can, Chicago, USA) to avoid contamination and stored at 5 °C.

3.7 Semen extension, equilibration and evaluation of semen samples

Semen samples were extended with pre-warmed (37 °C) Bioxcell® or Triladyl® at a ratio of 1:4 (semen to extender). Rapid cooling was done by placing the tubes in a beaker with cool water (25 °C). After 10 min, the beaker was transferred into the refrigerator and cooled to 5 °C for two hours (Ajao, 2015). The equilibrated semen samples were removed from the 5 °C refrigerator for evaluation. The equilibrated semen samples were evaluated for spermatozoa motility, viability, morphology, chromatin and mitochondrial membrane potential.

3.8 Freezing of semen

After 2 hours of equilibration at 5 °C, the semen samples were loaded into 0.25 mL plastic straws per treatment group by sucking the semen into the straws. The loaded straws were sealed with polyvinyl powder by dipping the straws into a 50 mL graduated tube containing the polyvinyl powder and then placed horizontally on a semen freezing holding rack. The rack carrying the diluted cooled semen straws was suspended in liquid nitrogen vapour, 5 cm above a liquid nitrogen surface in a polystyrene box for 10 min. At the end of the 10 minutes, all semen straws were plunged directly into the LN₂ (-196 °C). The frozen semen straws were immediately transferred into LN₂ canisters that were contained in LN₂ tank and stored for a month before thawing.

3.9 Semen thawing procedure

Frozen semen straws were removed from the liquid nitrogen canister (-196°C) during the thawing process. The frozen semen straws were plunged into the water bath (37°C) for 30 seconds. The frozen-thawed semen was evaluated for spermatozoa motility, viability, morphology, chromatin and mitochondrial membrane potential.

3.10 Statistical analysis

The data were analysed by analysis of variance (ANOVA) for a 3 X 2 factorial design in a completely randomized design using the General Linear Model (GLM) procedure of SAS version 12.1 of 2010. A significant difference (P<0.05) among the mean values of semen parameters were determined by Tukey's test method.

The following statistical model was used;

$$Y_{ijk} = \mu + E_i + P_j + S_k + (EP)_{ij} + (ES)_{ik} + (EPS)_{ijk} + \Sigma_{ijk}$$

Where: Y_{ijk} is the observation

μ is the overall mean

E_i is the effect of the i^{th} extender

P_j is the effect of the j^{th} plasma status

S_k is the effect of the k^{th} semen status

$(EP)_{ij}$ is the interaction between the i^{th} extender and the j^{th} plasma status

$(ES)_{ik}$ is the interaction between the i^{th} extender and the k^{th} semen status

$(EPS)_{ijk}$ is the effect of the interaction of the i^{th} extender j^{th} plasma status k^{th} semen status

Σ_{ijk} is the random error

CHAPTER 4

RESULTS

4.1 The effect of extenders and removal of seminal plasma on semen parameters of South African indigenous goats following equilibration are presented in Table 4.1 to 4.4.

The spermatozoa total motility rate in non-washed semen extended with Triladyl® (90.5±7.5) was significantly lower ($P < 0.05$) compared to semen samples extended with Bioxcell® (97.8±2.3) extender (Table 4.1). Spermatozoa total motility rate in washed semen extended with Bioxcell® (92.5±4.6) was not significantly different ($P > 0.05$) from semen extended with Triladyl® (94.9±5.5). The spermatozoa progressive motility rate in non-washed semen extended with Triladyl® (77.0±10.8) was significantly ($P < 0.05$) lower than the semen extended with Bioxcell® (89.6±7.5). A higher ($P < 0.05$) percentage of non-progressive spermatozoa motility rate of semen extended with Triladyl® (13.5±5.5) extender was recorded compared to semen extended with Bioxcell® (8.2±5.6) extender in non-washed semen samples. The rapid spermatozoa motility rate of semen extended with Triladyl® (75.8±10.9) extender was significantly lower ($P < 0.05$) than the semen extended with Bioxcell® (87.8±8.4) of non-washed semen.

Table 4.1: Effect of extenders (Bioxcell® & Triladyl®) and seminal plasma (non-washed & washed) on equilibrated spermatozoa motility parameters (mean ± S.E.)

Treatment	TM (%)	PM (%)	NPM (%)	RAP (%)	MED (%)	SLW (%)	STC (%)
Raw NW	98.9±1.2 ^a	92.9±4.7 ^a	6.0±3.7 ^b	91.6±5.6 ^a	6.3±4.0 ^d	1.0±0.7 ^{bc}	1.1±1.1 ^b
Biox NW 2h	97.8±2.3 ^a	89.6±7.5 ^a	8.2±5.6 ^b	87.8±8.4 ^a	8.4±5.0 ^{dc}	1.7±1.6 ^b	2.2±2.3 ^b
Tril NW 2h	90.5±7.5 ^c	77.0±10.8 ^b	13.5±5.5 ^a	75.8±10.9 ^b	11.8±2.5 ^{bc}	3.8±1.9 ^a	8.7±7.7 ^a
Raw W	98.2±2.7 ^a	91.4±7.9 ^a	6.8±5.3 ^b	89.7±9.0 ^a	7.1±4.6 ^d	1.4±1.8 ^b	1.8±2.7 ^b
Biox W 2h	92.5±4.6 ^{bc}	78.8±6.4 ^b	13.7±2.1 ^a	75.8±7.7 ^b	12.9±4.0 ^{ab}	3.7±1.5 ^a	7.5±4.5 ^a
Tril W 2h	94.9±5.5 ^{ab}	78.6±10.8 ^b	16.3±5.7 ^a	75.1±9.6 ^b	16.1±3.4 ^a	3.7±2.7 ^a	5.1±5.5 ^{ab}

Different superscripts (a, b, c and d) within the same column indicate significant differences among the means ($P < 0.05$)

Raw NW = raw non-washed, Biox NW 2 h = Bioxcell® non-washed 2 hours, Tril NW 2 h = Triladyl® non-washed 2 hours, Raw W = raw washed, Biox W 2 h = Bioxcell® washed 2 hours, Tril W 2 h = Triladyl® washed 2 hours, TM = total motility, PM = progressive motility, NPM = non-progressive motility, RAP= rapid, MED = medium, SLW = slow, STC = static.

The spermatozoa velocity on the curve line (VCL) in washed semen extended with Triladyl® (79.0±7.9) was significantly lower ($P < 0.05$) than the semen extended with Bioxcell® (90.4±9.1) extender (Table 4.2). The percentage spermatozoa velocity on the straight line (VSL) was significantly higher ($P < 0.05$) in non-washed semen extended with Bioxcell® (53.0±9.7) extender than the semen extended with Triladyl® (42.5±4.5) extender. The percentage spermatozoa VSL was significantly higher ($P < 0.05$) in washed semen extended with Bioxcell® (58.5±5.6) extender than the semen extended with Triladyl® (45.9±3.9) extender. The spermatozoa average path velocity (VAP) was significantly higher ($P < 0.05$) in non-washed semen extended with Bioxcell® (72.3±11.3) extender than the semen extended with Triladyl® (61.2±6.3) extender. The spermatozoa VAP was significantly higher ($P < 0.05$) in washed semen extended with Bioxcell® (77.0±8.0) extender than the semen extended with Triladyl® (62.5±6.1) extender. The spermatozoa linearity (LIN) in non-washed semen extended with Bioxcell® (58.3±6.9) was significantly higher ($P < 0.05$) than the semen extended with Triladyl® (45.5±5.2) extender. The spermatozoa LIN in washed semen extended with Bioxcell® (64.8±1.9) was significantly higher ($P < 0.05$) than the semen extended with Triladyl® (58.2±3.2) extender.

Table 4.2 effect of extenders (Bioxcell® & Triladyl®) and seminal plasma (non-washed & washed) on equilibrated spermatozoa kinematic parameters (mean ± S.E.)

Treatment	VCL (µm/sec)	VSL (µm/sec)	VAP (µm/sec)	LIN (%)	STR (%)	WOB (%)	ALH (µm)	BCF (Hz)
Raw NW	96.2±7.8 ^a	47.2±6.5 ^{bc}	69.7±5.3 ^b	49.5±8.0 ^c	67.6±5.8 ^c	72.7±5.8 ^c	2.9±0.4 ^b	7.3±0.4 ^c
Biox NW 2h	90.8±12.7 ^a	53.0±9.7 ^{ab}	72.3±11.3 ^{ab}	58.3±6.9 ^b	73.1±4.6 ^{ab}	79.6±4.6 ^b	2.6±0.3 ^{cd}	7.4±0.4 ^c
Tril NW 2h	94.0±9.9 ^a	42.5±4.5 ^c	61.2±6.3 ^c	45.5±5.2 ^c	69.6±4.1 ^{bc}	65.3±4.0 ^d	3.5±0.4 ^a	10.1±0.8 ^a
Raw W	95.6±3.7 ^a	53.3±10.8 ^{ab}	73.8±7.8 ^{ab}	55.9±11.5 ^b	71.6±7.9 ^{bc}	77.3±8.0 ^b	2.7±0.4 ^{bc}	7.4±0.2 ^c
Biox W 2h	90.4±9.1 ^a	58.5±5.6 ^a	77.0±8.0 ^a	64.8±1.9 ^a	76.1±1.5 ^a	85.2±1.3 ^a	2.3±0.1 ^d	7.2±0.3 ^c
Tril W 2h	79.0±7.9 ^b	45.9±3.9 ^c	62.5±6.1 ^c	58.2±3.2 ^b	73.6±2.5 ^{ab}	79.0±2.2 ^b	2.4±0.2 ^{cd}	8.0±0.4 ^b

Different superscripts (a, b, c and d) within the same column indicate significant differences among means ($P < 0.05$)

Raw NW = raw non-washed, Biox NW 2 h = Bioxcell® non-washed 2 hours, Tril NW 2 h = Triladyl® non-washed 2 hours, Raw W = raw washed, Biox W 2 h = Bioxcell® washed 2 hours, Tril W 2 h = Triladyl® washed 2 hours, VCL = velocity on the curve line, VSL = velocity on the straight line, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency.

Live spermatozoa percentage in non-washed semen extended with Triladyl® (60.7±22.9) extender was not significantly different ($P > 0.05$) from the semen extended with Bioxcell® (60.7±22.8) extender (Table 4.3). Live spermatozoa percentage in washed semen extended with Triladyl® (27.7±17.1) extender was reduced significantly ($P < 0.05$) compared to semen extended with Bioxcell® (48.0±28.8). There was no significant difference ($P > 0.05$) in abnormal live spermatozoa morphology of semen samples extended with both Bioxcell® and Triladyl® extenders of non-washed and washed semen samples. A higher percentage of live spermatozoa with an abnormal tail was recorded, compared to head and mid-piece abnormalities.

Table 4.3 Effect of extenders (Bioxcell® & Triladyl®) and seminal plasma (non-washed & washed) on equilibrated spermatozoa viability and morphology parameters (mean ± S.E.)

Treatment	Viability		Head	Bent	Coiled
	Live & normal (%)	Dead (%)	Abnormal (%)	mid-piece (%)	tail (%)
Raw NW	71.7±12.3 ^a	28.3±12.3 ^c	14.1±16.4 ^a	23.6±11.9 ^a	62.3±23.2 ^a
Biox NW 2h	73.1±14.0 ^a	26.9±14.0 ^c	8.9±20.4 ^a	34.5±26.4 ^a	56.6±25.5 ^a
Tril NW 2h	60.7±22.8 ^{ab}	39.3±22.8 ^{bc}	10.1±8.5 ^a	34.2±28.6 ^a	55.7±26.6 ^a
Raw W	60.7±22.9 ^{ab}	39.3±22.9 ^{bc}	8.2±10.7 ^a	43.8±12.1 ^a	48.0±12.5 ^a
Biox W 2h	48.0±28.8 ^b	52.0±28.8 ^b	8.9±20.4 ^a	34.5±26.4 ^a	56.6±25.5 ^a
Tril W 2h	27.7±17.1 ^c	74.3±17.1 ^a	10.1±8.5 ^a	34.2±28.6 ^a	55.7±26.6 ^a

Different superscripts (a, b and c) within the same column indicate significant differences among means ($P < 0.05$)

Raw NW = raw non-washed, Biox NW 2 h = Bioxcell® non-washed 2 hours, Tril NW 2 h = Triladyl® non-washed 2 hours, Raw W = raw washed, Biox W 2 h = Bioxcell® washed 2 hours, Tril W 2 h = Triladyl® washed 2 hours.

The percentage of spermatozoa acrosome integrity in non-washed semen extended with Bioxcell® (71.1±13.8) extender was not significantly different ($P < 0.05$) from the semen extended with Triladyl® (60.1±15.9) extender (Table 4.4). The spermatozoa chromatin integrity was lower ($P > 0.05$) in washed semen extended with Triladyl® (46.3±9.4) than the semen extended with Bioxcell® (56.8±13.6). There was a lower ($P > 0.05$) percentage of spermatozoa with high mitochondrial membrane potential in non-washed semen extended with Bioxcell® (39.5±23.2) extender than the semen extended with Triladyl® (51.2±31.4) extender. There was a lower ($P > 0.05$) percentage of spermatozoa with high mitochondrial membrane potential in washed semen extended

with Bioxcell® (37.9±28.6) extender than the semen extended with Triladyl® (59.1±33.1) extender.

Table 4:4 Effect of extenders (Bioxcell® & Triladyl®) and seminal plasma (non-washed & washed) on equilibrated spermatozoa acrosome, chromatin and mitochondrial membrane potential (mean ± S.E.)

Treatment	Normal acrosome (%)	Reacted acrosome (%)	Normal chromatin (%)	Reacted chromatin (%)	$\Delta\psi_m^{\text{high}}$ (%)	$\Delta\psi_m^{\text{low}}$ (%)
Raw NW	62.8±13.7 ^a	37.2±13.7 ^a	82.2±16.5 ^a	17.8±16.5 ^d	83.4±14.2 ^a	16.6±14.2 ^c
Biox NW 2h	71.1±13.8 ^a	28.9±13.8 ^a	79.8±21.9 ^{ab}	20.2±21.9 ^{cd}	39.5±23.2 ^c	60.5±23.2 ^a
Tril NW 2h	60.1±15.9 ^a	40.3±16.0 ^a	67.5±24.4 ^{abc}	32.5±24.4 ^{bcd}	51.2±31.4 ^{bc}	48.8±31.4 ^{ab}
Raw W	68.4±19.0 ^a	31.6±19.0 ^a	65.1±17.5 ^{bc}	34.9±17.5 ^{bc}	65.0±20.8 ^{ab}	35.0±20.8 ^{bc}
Biox W 2h	71.3±22.7 ^a	28.7±22.7 ^a	56.8±13.6 ^{cd}	43.2±13.6 ^{ab}	37.9±28.6 ^c	62.1±28.6 ^a
Tril W 2h	70.1±13.4 ^a	29.9±13.4 ^a	46.3±9.4 ^d	53.7±9.4 ^a	59.1±33.1 ^{bc}	40.9±33.1 ^{ab}

Different superscripts (a, b, c and d) within the same column indicates significant differences among means ($P < 0.05$)

Raw NW = raw non-washed, Biox NW 2 h = Bioxcell® non-washed 2 hours, Tril NW 2 h = Triladyl® non-washed 2 hours, Raw W = raw washed, Biox W 2 h = Bioxcell® washed 2 hours, Tril W 2 h = Triladyl® washed 2 hours, $\Delta\psi_m^{\text{high}}$ = High mitochondrial membrane potential, $\Delta\psi_m^{\text{low}}$ = Low mitochondrial membrane potential.

4.2 The effect of extenders and removal of seminal plasma on semen of South African indigenous goats following freezing-thawing are presented in Table 4.5 to 4.8.

The spermatozoa total motility rate in non-washed semen extended with Bioxcell® (85.0±3.4) extender was significantly higher ($P < 0.05$) than the semen extended with Triladyl® (73.9±13.8) extender (Table 4.5). The spermatozoa progressive motility rate in non-washed semen extended with Bioxcell® (58.5±10.0) extender was significantly higher ($P < 0.05$) than the semen extended with Triladyl® (45.4±11.2) extender. The spermatozoa non-progressive motility rate in washed semen extended with Bioxcell® (21.1±5.8) extender was not significantly different ($P > 0.05$) from the semen extended with Triladyl® (23.2±3.2) extender. The spermatozoa rapid motility rate in washed semen extended with Bioxcell® (49.9±11.5) extender was not significantly different ($P > 0.05$) from the semen extended with Triladyl® (40.1±11.0).

Table 4.5: Effect of extenders (Bioxcell® & Triladyl®) and seminal plasma (non-washed & washed) on frozen-thawed spermatozoa motility parameters (mean ± S.E.)

Treatment	TM (%)	PM (%)	NPM (%)	RAP (%)	MED (%)	SLW (%)	STC (%)
Raw NW	98.9±1.2 ^a	92.9±4.7 ^a	6.0±3.7 ^d	91.6±5.6 ^a	6.3±4.0 ^c	1.0±0.7 ^c	1.1±1.1 ^d
Biox NW FT	85.0±3.4 ^b	58.5±10.0 ^b	26.6±8.5 ^{ab}	49.9±11.5 ^b	27.5±7.2 ^a	7.6±3.2 ^b	15.0±3.4 ^c
Tril NW FT	73.9±13.8 ^c	45.4±11.2 ^c	28.5±5.7 ^a	40.1±11.0 ^{bc}	23.9±6.1 ^a	9.9±1.4 ^a	26.1±13.8 ^b
Raw w	98.3±2.7 ^a	91.4±7.9 ^a	6.8±5.3 ^d	89.7±9.0 ^a	7.1±4.6 ^c	1.4±1.8 ^c	1.8±2.7 ^d
Biox W FT	68.2±13.5 ^{cd}	47.0±15.7 ^c	21.1±5.8 ^c	42.1±16.0 ^{bc}	17.8±5.3 ^b	8.2±2.7 ^{ab}	31.9±13.5 ^{ab}
Tril W FT	63.1±15.1 ^d	39.9±13.1 ^c	23.2±3.2 ^{bc}	36.1±12.4 ^c	18.1±4.3 ^b	8.9±1.4 ^{ab}	36.9±15.1 ^a

Different superscripts (a, b, c and d) within the same column indicate significant differences among means (P < 0.05)

Raw NW = raw non-washed, Biox NW FT = Bioxcell® non-washed frozen-thawed, Tril NW FT = Triladyl® non-washed frozen-thawed, Raw W = raw washed, Biox W FT = Bioxcell® washed frozen-thawed, Tril W FT = Triladyl® washed frozen-thawed, TM = total motility, PM = progressive motility, NPM = non-progressive motility, RAP= rapid, MED = medium, SLW = slow, STC = static.

The Spermatozoa VCL was not significantly different (P > 0.05) in non-washed semen extended with Bioxcell® (50.2±11.9) from the semen extended with Triladyl® (53.0±8.6) extender (Table 4.6). The Spermatozoa VSL was not significantly different (P > 0.05) in washed semen extended with Bioxcell® (33.7±12.7) from the semen extended with Triladyl® (25.8±5.0) extender. The Spermatozoa VAP was not significantly different (P > 0.05) in non-washed semen extended with Bioxcell® (33.3±12.7) from the semen extended with Triladyl® (32.8±7.2) extender. The Spermatozoa LIN was not significantly different (P > 0.05) in washed semen extended with Bioxcell® (57.7±16.5) from the semen extended with Triladyl® (48.1±8.6) extender.

Table 4.6: Effect of extenders (Bioxcell® & Triladyl®) and seminal plasma (non-washed & washed) on frozen-thawed spermatozoa kinematic parameters (mean ± S.E.)

Treatment	VCL (µm/sec)	VSL (µm/sec)	VAP (µm/sec)	LIN (%)	STR (%)	WOB (%)	ALH (µm)	BCF (Hz)
Raw NW	96.2±7.8 ^a	47.2±6.5 ^a	69.7±5.3 ^a	49.5±8.0 ^{abc}	67.6±5.8 ^b	72.7±5.8 ^{ab}	2.9±0.4 ^a	7.3±0.4 ^{ab}
Biox NW FT	50.2±11.9 ^b	24.1±12.1 ^c	33.3±12.7 ^c	46.1±13.8 ^{bc}	70.0±9.4 ^b	64.6±10.9 ^c	2.1±0.3 ^d	6.1±1.3 ^{bc}
Tril NW FT	53.0±8.6 ^b	23.3±6.7 ^c	32.8±7.2 ^c	41.2±6.2 ^c	66.9±6.1 ^b	61.3±3.9 ^c	2.5±0.1 ^{bc}	7.1±2.2 ^{ab}
Raw w	95.6±3.7 ^a	53.3±10.8 ^a	73.8±7.8 ^a	55.9±11.5 ^{ab}	71.6±7.9 ^{ab}	77.3±8.0 ^a	2.7±0.4 ^{ab}	7.4±0.2 ^{ab}
Biox W FT	57.7±11.9 ^b	33.7±12.7 ^b	43.8±12.2 ^b	57.7±16.5 ^a	75.3±11.3 ^a	75.1±12.2 ^{ab}	2.1±0.3 ^d	5.9±0.9 ^c
Tril W FT	53.8±4.8 ^b	25.8±5.0 ^{bc}	36.4±4.6 ^{bc}	48.1±8.6 ^{abc}	70.5±7.1 ^{ab}	67.6±6.6 ^{bc}	2.3±0.2 ^{cd}	7.6±2.3 ^a

Different superscripts (a, b, c and d) within the same column indicate significant differences among means (P < 0.05)

Raw NW = raw non-washed, Biox NW FT = Bioxcell® non-washed frozen-thawed, Tril NW FT = Triladyl® non-washed frozen-thawed, Raw W = raw washed, Biox W FT = Bioxcell® washed frozen-thawed, Tril W FT = Triladyl® washed frozen-thawed, VCL = velocity on the curve line, VSL = velocity on the straight line, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency.

There was a higher ($P < 0.05$) live and normal spermatozoa percentage in non-washed semen extended with Bioxcell® (45.7 ± 21.2) extender than the semen extended with Triladyl® (24.5 ± 22.2) extender (Table 4.7). There were a higher ($P < 0.05$) percentage of spermatozoa with head abnormalities in non-washed semen extended with Triladyl® (20.4 ± 10.2) extender than the semen extended with Bioxcell® (18.3 ± 12.4) extender. There were a higher ($P < 0.05$) percentage of spermatozoa with head abnormalities in washed semen samples extended with Triladyl® (34.0 ± 16.0) extender than the semen extended with Bioxcell® (10.1 ± 7.0) extender. The percentage of spermatozoa with coiled tail abnormalities were reduced significantly ($P < 0.05$) in washed semen extended with Triladyl® (35.9 ± 21.6) extender compared to the semen extended with Bioxcell® (65.4 ± 25.0) extender.

Table 4.7: Effect of extenders (Bioxcell® & Triladyl®) and seminal plasma (non-washed & washed) on frozen-thawed spermatozoa viability and morphology parameters (mean \pm S.E.)

Treatment	Viability		Head abnormal (%)	Bent mid-piece (%)	Coiled tail (%)
	Live & normal (%)	Dead (%)			
Raw NW	71.7 ± 12.3^a	28.3 ± 12.3^d	14.1 ± 16.4^{bc}	23.6 ± 11.9^c	62.3 ± 23.2^a
Biox NW FT	45.7 ± 21.2^b	54.3 ± 27.2^c	18.3 ± 12.4^{bc}	30.9 ± 15.5^{abc}	50.9 ± 15.5^{ab}
Tril NW FT	24.5 ± 22.2^c	74.5 ± 22.2^b	20.4 ± 10.2^b	41.1 ± 18.0^{ab}	38.5 ± 16.1^b
Raw w	60.7 ± 22.9^{ab}	39.3 ± 22.9^{cd}	8.2 ± 10.7^c	43.8 ± 12.1^a	48.0 ± 12.5^{ab}
Biox W FT	5.2 ± 4.9^d	94.8 ± 4.9^a	10.1 ± 7.0^{bc}	25.5 ± 20.4^{bc}	65.4 ± 25.0^a
Tril W FT	6.9 ± 8.6^d	93.1 ± 8.6^a	34.0 ± 16.0^a	28.0 ± 25.1^{abc}	35.9 ± 21.6^b

Different superscripts (a, b, c and d) within the same column indicate significant differences among means ($P < 0.05$)

Raw NW = raw non-washed, Biox NW FT = Bioxcell® non-washed frozen-thawed, Tril NW FT = Triladyl® non-washed frozen-thawed, Raw W = raw washed, Biox W FT = Bioxcell® washed frozen-thawed, Tril W FT = Triladyl® washed frozen-thawed.

The spermatozoa acrosome integrity was not significantly different ($P > 0.05$) in non-washed semen extended with Bioxcell® (50.4 ± 23.5) extender and semen extended with Triladyl® (55.6 ± 22.1) extender (Table 4.8). The spermatozoa chromatin integrity was

not significantly different ($P > 0.05$) in washed semen extended with Bioxcell® (48.7±21.3) extender and semen extended with Triladyl® (37.9±24.3) extender. There was a lower percentage ($P < 0.05$) of spermatozoa with high mitochondrial membrane potential in non-washed semen extended with Bioxcell® (49.8±20.1) extender than the semen extended with Triladyl® (68.7±26.8) extender.

Table 4.8: Effect of extenders (Bioxcell® & Triladyl®) and seminal plasma (non-washed & washed) on frozen-thawed spermatozoa acrosome, chromatin, and mitochondrial membrane integrity (mean ± S.E.)

Treatment	Normal acrosome (%)	Reacted acrosome (%)	Normal chromatin (%)	Reacted chromatin (%)	$\Delta\psi_m^{\text{high}}$ (%)	$\Delta\psi_m^{\text{low}}$ (%)
Raw NW	62.8±13.7 ^{ab}	37.2±13.7 ^{ab}	79.9±15.6 ^a	20.1±15.6 ^c	83.4±14.2 ^a	16.6±14.2 ^c
Biox NW FT	50.4±23.5 ^b	51.7±23.5 ^a	63.7±9.1 ^{ab}	36.3±9.1 ^{bc}	49.8±20.1 ^c	50.2±20.1 ^a
Tril NW FT	55.6±22.1 ^{ab}	44.4±22.1 ^{ab}	51.2±18.7 ^{bc}	48.8±18.7 ^{ab}	68.7±26.8 ^{ab}	31.3±26.8 ^{bc}
Raw w	68.4±19.0 ^a	31.6±19.0 ^b	65.1±17.5 ^{ab}	34.9±17.5 ^{cb}	65.0±20.8 ^{cb}	35.0±20.8 ^{ab}
Biox W FT	65.4±11.4 ^{ab}	34.6±11.4 ^b	48.7±21.3 ^{bc}	51.3±21.3 ^{ab}	65.3±14.9 ^{cb}	34.7±14.9 ^{ab}
Tril W FT	65.5±15.4 ^{ab}	34.5±15.4 ^b	37.9±24.3 ^c	62.1±24.3 ^a	78.4±16.8 ^{ab}	21.6±16.8 ^{bc}

Different superscripts (a, b and c) within the same column indicate significant differences among means ($P < 0.05$)

Raw NW = raw non-washed, Biox NW FT = Bioxcell® non-washed frozen-thawed, Tril NW FT = Triladyl® non-washed frozen-thawed, Raw W = raw washed, Biox W FT = Bioxcell® washed frozen-thawed, Tril W FT = Triladyl® washed frozen-thawed, $\Delta\psi_m^{\text{high}}$ = High mitochondrial membrane potential, $\Delta\psi_m^{\text{low}}$ = Low mitochondrial membrane potential.

CHAPTER 5

DISCUSSION

5.1. The effect of extenders and removal of seminal plasma on semen of South African indigenous goats following equilibration

The current study demonstrated that the removal of seminal plasma in indigenous goat semen maintained better results of motile spermatozoa in equilibrated and frozen-thawed samples in both Bioxcell® and Triladyl® extenders. The current results is similar to the previous report of seminal plasma removal with a physiological solution, which increased the percentage of motile spermatozoa before and after freezing of goats' semen (Leboeuf, Restall & Salamon, 2000; Ferrer *et al.*, 2004; Gacitua & Arav, 2005). However, contradictory results were reported by other authors (Jasko *et al.*, 1991; Ramukhithi, 2011). Centrifugation speed and time used in the current study might have influenced the decrease and increase of the percentage of motile spermatozoa. Ferrer *et al.* (2004), centrifuged extended semen at 900 X g for 10 minutes without causing a reduction in spermatozoa motility immediately after centrifugation. However, Ramukhithi (2011) recorded contradictory results that showed that centrifugation of extended semen up to 1500 X g for 10 minutes decreased the percentage of motile spermatozoa before and after freezing goat spermatozoa.

The results of spermatozoa total motility of non-washed (98.9%) and washed (98.3%) raw semen were better than the total motility of raw semen (89.3% and 69.4%) recorded by Ramukhithi (2011) and Roof *et al.* (2011). Progressive motility percentage of non-washed (92.9%) semen was similar to washed (91.4%) raw semen. The results of the current study were much better than (58.6% and 75%) spermatozoa progressive motility obtained by Ramukhithi (2011) and Vidal *et al.* (2013). These results show a significantly lower percentage on non-progressive (6.0% and 6.8%) and static (1.1% and 1.8%) spermatozoa of raw semen samples. The results were lower than the results obtained by Ramukhuthi (2011) who reported 30.7% and 10.7% of non-progressive and static spermatozoa. These differences were attributed to the individual buck difference, environment, semen collection facilities available and seasonal effect on spermatozoa quality (Câmara *et al.*, 2016; Van Staden. 2010).

The results for spermatozoa total motility rate of equilibrated non-washed semen samples extended with Bioxcell® (97.8%) were higher than those extended with Triladyl®

(90.5%). Roof *et al.* (2011) reported similar results where soy-lecithin-based (Bioxcell®) extended semen had higher results than the egg-yolk-based extended semen. However, Okukpe *et al.* (2012) reported spermatozoa total motility of 73.7% and 64.9% in semen extended with whole milk and soymilk extenders. However, these results contradict what was found in the present study. This may be due to the high concentration of unsaturated fatty acids present in the spermatozoa membrane, which in the presence of ROS are highly susceptible to lipid peroxidation that disrupts cells and causes a reduction in motility (Bustamante-filho *et al.*, 2009; Sariözkan *et al.*, 2010). The total motility of washed semen samples extended with Triladyl® was higher than that of Bioxcell® extended semen samples. However, the total motility of non-washed semen samples extended with Bioxcell® had a higher percentage than other semen sample groups. This shows that it is not necessary to wash semen before extending with a soy-lecithin-based extender (Bioxcell®) as it is shown to have higher spermatozoa total motility than the other groups. However, it was necessary to wash semen samples before extending with Triladyl® as it yields higher results than the other groups.

Spermatozoa VCL percentage of washed semen samples extended with Triladyl® were significantly lower than percentage spermatozoa VCL of other treatment groups. The percentage of spermatozoa VSL and spermatozoa VAP were lower in non-washed and washed semen samples extended with Triladyl® as compared to non-washed and washed semen samples extended with Bioxcell®. These results show that washing does not have an effect on semen samples but the extender has the effect on semen samples. These results were better than the results obtained by Ajao, (2015). This is because semen in the present study was equilibrated for two hour and that reported by Ajao, (2015) was of different equilibration time (12 hr, 24 hr, 36 hr, 48 hr and 60 hr). Equilibration time does also have an effect on preservation of semen.

The washing of semen samples had an effect on equilibrated spermatozoa live and normal percentages in semen samples that were extended with Bioxcell® and Triladyl®. However, the lowest spermatozoa live and normal percentage were observed in equilibrated semen samples that were extended with Triladyl®. There was no significant difference ($P > 0.05$) in spermatozoa, bent mid-piece and coiled tail abnormalities in non-washed and washed semen samples extended with both Bioxcell® and Triladyl®. However, the spermatozoa head abnormality percentages in non-washed semen samples were numerically lower than the raw semen samples, although there was no significant difference ($P > 0.05$). Moreover, spermatozoa head abnormality percentages in washed semen samples were significantly different and numerically

higher as than the raw semen samples. There were more abnormalities in live spermatozoa than normal live spermatozoa for semen samples that were washed and extended with Bioxcell® and Triladyl® in the present study. These are caused by the non-enzymatic antioxidants (ascorbic acid, α -tocopherol, taurine, and albumin) lost with the removal of seminal plasma (Bustamante-filho *et al.*, 2009). However, Bioxcell® extender seemed to compensate for the loss of non-enzymatic antioxidants because it has been supplemented with taurine and the results for washed semen samples extended with Bioxcell® was statistically better than the Triladyl® extended semen for live and normal spermatozoa percentages.

There is a relationship between the spermatozoa abnormalities and fertilisation ability of preserved semen. For example, normal acrosome is important for acrosome reaction, which is needed for fertilisation to take place (Akhter *et al.*, 2010). There was no significant difference ($P > 0.05$) in the percentage spermatozoa with normal acrosome in non-washed and washed semen samples extended with Bioxcell® and Triladyl®. Although there was no significant difference ($P > 0.05$) between the four treatment groups, there was a decrease in the percentage of spermatozoa with normal acrosome in washed semen samples that were extended with Triladyl®, compared to the other treatment groups. There was also a decrease in percentage spermatozoa with normal chromatin of washed semen samples extended with Triladyl®, compared to the other treatment groups. The percentage of spermatozoa with high mitochondrial membrane potential in non-washed and washed semen samples extended with Bioxcell® were significantly lower ($P < 0.05$) than the non-washed and washed semen samples extended with Triladyl®. There was a high percentage of abnormal spermatozoa in the current study, similar to what Al-Ghalban *et al.* (2004) and Kridli *et al.* (2007) reported during spring season.

The presence of glycerol is potentially toxic to fresh semen. Therefore, it is necessary to balance the equilibration periods with the glycerol in order for the protective properties of glycerol to take an effect without any unnecessary loss of spermatozoa prior to cryopreservation (Van Staden, 2010). Both extenders used in the present study had glycerol and the equilibration time used was 2 hours prior to cryopreservation. This maintained the spermatozoa' motility, kinematics parameters, viability, morphology, and membrane integrity. A short equilibration duration of 2 hours at 5 °C with 7% glycerol was beneficial to goat semen (Sundararaman & Edwin, 2008). Shahverdi *et al.* (2014) also reported that equilibration times of over 2 hours resulted in the optimum

preservation of sperm cell total and progressive motility, as well as the plasma and acrosomal membrane integrity during cryopreservation.

5.2. The effect of extenders and removal of seminal plasma on semen of South African indigenous goats following freezing and thawing

The diluents types, freezing rate, glycerol levels and glycerol equilibration time interact with the thawing procedure, which in turn affect the post-thaw fertilising ability (Jothipriya *et al.*, 2014). The results of frozen-thawed semen on spermatozoa total motility of washed semen samples extended with Bioxcell® were similar (68.2%) to Triladyl® (63.1%) extender. These results were higher than the results recorded by Ramukhithi (2011), who reported that frozen-thawed washed semen's recovery rate was 10.6% for South African unimproved indigenous goats and 16.9% for Boer goats. The results for washed semen samples extended with Bioxcell® extender (68.2%) were similar to the results (60.6%) obtained by Sariözkan *et al.* (2010). The results reported by Sariözkan *et al.* (2010) for semen that was centrifuged and extended with Triladyl® extender (31.5%) were lower than the results obtained in the current study.

Spermatozoa progressive motility of washed semen samples extended with Bioxcell® (47.0%) and Triladyl® (39.9%) were not significantly different ($P > 0.05$) from the spermatozoa progressive motility of non-washed semen samples extended with Triladyl® (45.4%). However, these results were significantly different from spermatozoa' progressive motility of non-washed semen extended with Bioxcell® (58.5%). Both extenders were found to maintain progressive motility of washed and non-washed semen. Frozen-thawed progressive motility of semen samples that were not washed, extended with Bioxcell® was significantly greater (58.5%) than other groups. These results concur with the results reported by Sariözkan *et al.* (2010).

The results of pre-treatment (removal and non-removal of seminal plasma) are not constant in different studies. In some studies, the removal of seminal plasma was essential for maximising spermatozoa motility of frozen-thawed goat semen (Üstener *et al.*, 2009). However, other authors reported that the presence of seminal plasma was beneficial for frozen-thawed spermatozoa motility of goat semen (Ramukhithi, 2011). In the present study, the removal and non-removal of seminal plasma maintained frozen-thawed motility in both extenders (Bioxcell® and Triladyl®). This concurred with what was reported by Dorji *et al.* (2014). Sariözkan *et al.* (2010) reported progressive motility of frozen-thawed semen samples extended with Bioxcell® to be 22.3% and with Triladyl® to

be 7.0%. Jiménez-Rabadán *et al.* (2012) and Kurien *et al.* (2012) also found similar results on frozen-thawed semen extended with Biladyl® (45.3%) and Triladyl® 32.5%, which were also higher. Daşkin *et al.* (2011) reported post-thaw motility of 38% in Angora buck semen when using Bioxcell®. Sundararaman and Edwin (2008) considered frozen-thawed percentage spermatozoa' progressive motility of 40% and above to be good and acceptable. Therefore, the results for frozen-thawed progressive spermatozoa motility in this study are acceptable.

For VSL, VAP, LIN, STR and WOB, the highest values (33.7 µm/sec, 43.8 µm/sec, 57.7%, 75.3%, 75.1%, respectively) were obtained from washed semen samples extended with Bioxcell® compared to other treatment groups. These results contradicts with the results reported by Sariözkan *et al.* (2010), who found that for VSL and LIN, the values were highest (103.2 µm/sec, 47.5%, respectively) in centrifuged semen samples extended with Bioxcell®. The results obtained by Sariözkan *et al.* (2010) for linearity were lower than the results obtained in the present study. This may be due to the loss of ability of spermatozoa to move in a straight line of sensitive spermatozoa that endure membrane and axonemal changes, which results in decreased kinematic traits such as linearity. The osmotic and structural damages induced in spermatozoa by glycerol can also reduce kinematic traits (Bezerra *et al.*, 2012). There was no significant difference for frozen-thawed non-washed and washed semen extended with Bioxcell® or Triladyl® for spermatozoa curvilinear velocity. These results show the beneficial effects of frozen-thawed semen sample pre-treated (washed and non-washed) and extended with Bioxcell® and Triladyl® on kinematic parameters.

The composition of the extender and suitable cryoprotectants are important factors for successful semen cryopreservation (Vidal *et al.*, 2013; Dorji *et al.*, 2014). The removal of the seminal plasma by washing is beneficial when the spermatozoa are frozen in a diluent containing egg yolk. The toxicity of egg yolk coagulating enzyme is influenced by season of the year (Ashmawy *et al.*, 2010). Bulbourethral glands increase their activity by high plasma concentrations of prolactin and produce more phospholipase A₂ enzyme during the non-breeding season (Çebi Şeni *et al.*, 2015). The current study was conducted during the spring months (September, October and November) and the beginning of summer (December) and the percentage of post-thawing spermatozoa motility was high during spring months. This concurred with Ashmawy *et al.* (2010), who reported a higher percentage of post-thaw spermatozoa motility as well as recovery rates during autumn months (September, October and November) than during the other months of the year. Although there was a significant effect on interactions in this study,

Bioxcell® extender did not show an advantage over Triladyl® extender as both similarly maintained spermatozoa motility. This may be due to the natural constituent of egg yolk (phospholipids, cholesterol and low-density lipoproteins) in Triladyl® extender which is equally effective in protecting spermatozoa against oxidative stress as the forfeited antioxidants found in Bioxcell® extender (Dorji *et al.*, 2014).

The live and normal spermatozoa percentages were greatly affected ($P < 0.05$) following freezing and thawing in both extenders compared to equilibrated semen in both extenders. The thermal stress and cryoinjury that occur on the spermatozoa membrane during cryopreservation processes and after thawing caused spermatozoa death in the current study. Asr *et al.* (2011) and Bezerra *et al.* (2012) also reported that freezing and thawing causes more damage than other stages of cryopreservation to the spermatozoa. Seminal plasma removal did not improve the live and normal spermatozoa percentages in both the extenders in this study. This may be because the protective effects of natural antioxidants present in the seminal plasma were available in low concentrations as seminal plasma was removed and spermatozoa become more susceptible during cryopreservation (Asr *et al.*, 2011).

Glycerol is a penetrating cryoprotectant used in the freezing of spermatozoa. However, it has chemical and osmotic toxicities on the spermatozoa membrane when it is in higher concentration. It was concluded that glycerol has the best anti-apoptotic effects when used in boar semen extenders in lower concentrations (2-3%). Nevertheless, the exact cryoprotective or anti-freeze mechanism of glycerol remains unknown (Yotov. 2015). The detrimental effects of cryopreservation on various spermatozoa organelles, including irreversible changes to the acrosomes, mitochondria and tails of spermatozoa, have been shown in ultrastructural studies (Watson, 1990; Üstüner *et al.*, 2015). Samardžija *et al.* (2008) and Bezerra *et al.* (2012) suggested that membrane integrity could be as important as spermatozoa motility, in predicting fertility rates. Extensive chemical and physical damage caused by cryopreservation to spermatozoa membranes increase lipid peroxidation of the membrane induced by reactive oxygen species (ROS). Furthermore, the osmotic stress and temperature changes cause cell membrane stress (Memon *et al.*, 2012).

Acrosome intactness is a pre-requisite for fertilisation, though the correlation between acrosome status and fertility vary (Rodríguez-Martínez, 2003). Spermatozoa' normal acrosome percentage was high in frozen-thawed non-washed and washed semen samples extended with Triladyl®. This was not evident in what Kubovičová *et al.*

(2010) and El-kon *et al.* (2010), reported about the effects the egg yolk added on extender has on acrosome integrity and the post-thaw viability of ejaculated spermatozoa in goats.

Normal spermatozoa chromatin structure and integrity are essential for the accurate transmission of paternal genetic information to the next generation. They are also an appropriate function of spermatozoa in the fertilisation process (Sadeghi *et al.*, 2009). Chromatin structure was reported to have been affected by the freeze-thawing process (Hammadeh *et al.*, 1999; Üstüner *et al.*, 2015). Hammadeh *et al.* (1999) reported that the mean percentage of human spermatozoa with damaged DNA content was higher in semen frozen in liquid nitrogen vapour than in a controlled biological freezer. Similarly, the buck semen in the present study was frozen in liquid nitrogen vapour and the freeze-thawing process affected the chromatin structure. From the results in the present study, there was lower spermatozoa membrane integrity and low chromatin integrity. Spermatozoa membrane integrity can be directly related to ROS production. However, it should not be a direct indicator of DNA integrity, as DNA fragmentation can be observed even in spermatozoa with normal morphology (Bassiri *et al.*, 2013). The antioxidants present in the seminal plasma were said to be an essential form of protection (prevention, interception, and repair) to spermatozoa against ROS (Memon *et al.*, 2012). In the present study removal of seminal plasma showed lowered membrane integrity and viability of frozen-thawed semen. However, it was not evident to all spermatozoa membranes. This might be because of the antioxidants present in the extenders (Bioxcell[®] and Trilady[®]) that provided protection in replacement to the antioxidant that is present in the removed seminal plasma.

In the present study, parts of the spermatozoa involved in locomotion (tail and mitochondria) were reduced to a lesser degree after freeze-thaw. The results also showed a higher percentage of high mitochondrial membrane potential of frozen-thawed non-washed and washed semen samples extended with Trilady[®]. In addition, there is a correlation between bent mid-piece and low mitochondrial membrane potential in the present study. This was because mitochondria are localised in the mid-piece area and are thought to provide the mid-piece and the spermatozoa head with the ATP required for maintenance processes of membranes (Silva & Gadella, 2006; Wusiman *et al.*, 2012). In the present study, motility and mitochondrial membrane potential were the least affected parameters. This is because the mitochondrial membrane potential is a good indicator of spermatozoa motility dysfunction (Mansour, 2009).

CHAPTER 6

CONCLUSIONS AND RECOMMENDATION

From the current study, the following conclusions were drawn:

1. The percentage of spermatozoa progressive motility rate was above 70% for all the groups after 2 hours of equilibration. Though there was a reduction in the percentage of progressive motility rate in non-washed and washed semen extended with Bioxcell® and Triladyl®, they still maintained acceptable motility (40%) post-thaw.
2. There was no difference in morphology of non-washed and washed semen extended with Triladyl® and Bioxcell® extender prior and post-cryopreservation.
3. The study also demonstrated that viability reduced drastically with or without washing of seminal plasma in both Triladyl® and Bioxcell® extended semen post-cryopreservation.
4. Triladyl® and Bioxcell® extender maintained acrosomal integrity with or without washing of seminal plasma during equilibration. However, only washing of seminal plasma maintained acrosomal integrity in semen extended with Triladyl® and Bioxcell® extenders post-cryopreservation.
5. Washed semen extended with Triladyl® did reduce the integrity of chromatin structure prior and post-cryopreservation.
6. Bioxcell® extender reduced the high mitochondrial membrane potential in non-washed and washed semen prior and post-cryopreservation.
7. The freezing-thawing process did reduce the indigenous buck semen parameters irrespective of removal or non-removal of seminal plasma. However, Bioxcell® extender was found to be more suitable for preserving spermatozoa during equilibration and freezing/thawing process of buck semen.

Therefore, the following recommendation was made:

1. Additional studies should be conducted following the success of the present study to determine spermatozoa' fertilising ability both *in vivo* and *in vitro* using South African unimproved indigenous bucks.

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APPENDIX



Figure 3.1: The South African indigenous bucks that were used as semen donors

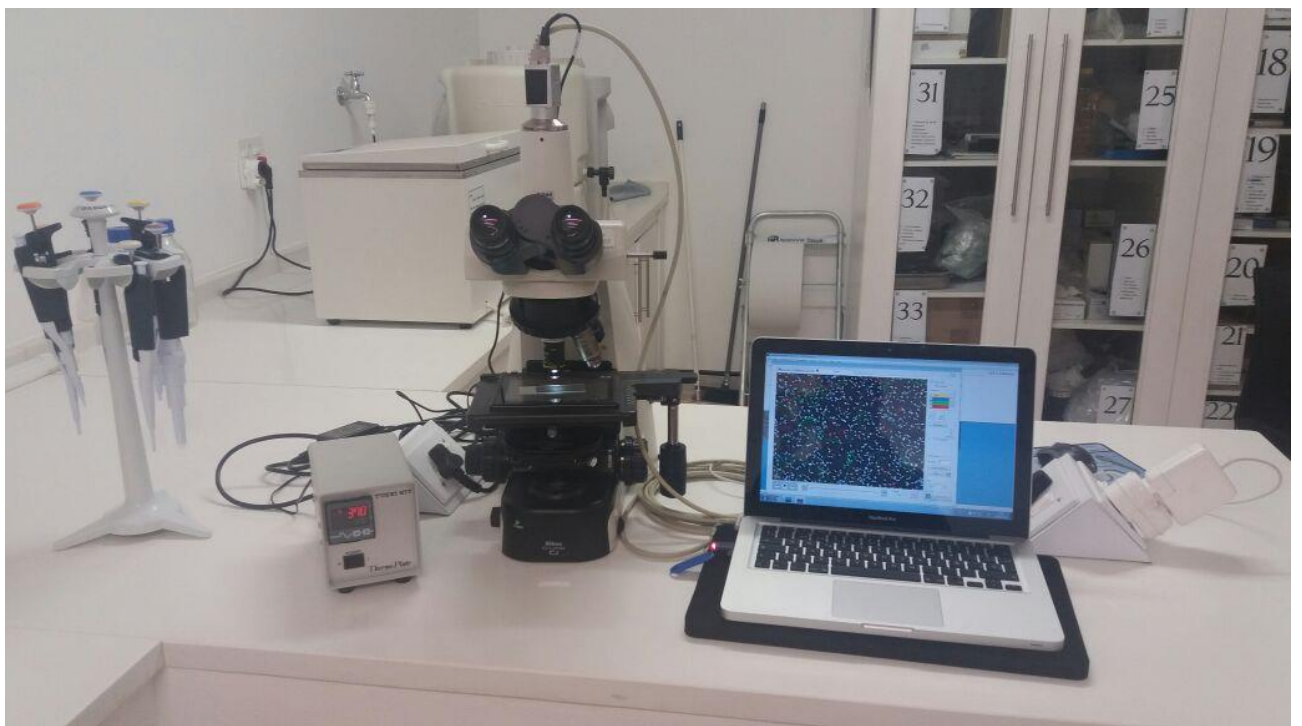


Figure 3.2: Computer-Aided Sperm Analysis (CASA) also known as the sperm Class Analyser[®] (SCA) system during semen analysis



Figure 3.3: Used UV light inverted microscope for evaluation of the spermatozoa chromatin and mitochondrial membrane potential



Figure 3.4: Polystyrene box, straw holding rack, and semen straws for semen cryopreservation