EFFECT OF DIFFERENT DISACCHARIDES AS ENERGY SUPPLEMENTS IN TRIS-EGG YOLK SEMEN EXTENDER ON THE QUALITY OF CRYOPRESERVED BOER GOAT SPERMATOZOA

BY

RAMMUTLA TSAKA LYZER

STUDENT NUMBER: 11615759

A dissertation submitted in requirements for the degree of Master of Science in Agriculture (Animal Science)

Department of Animal Science

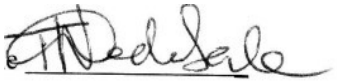
School of Agriculture

University of Venda

Thohoyandou

South Africa

June 2018

Signature  **Date** ...19/06/2018.....

Supervisor: Prof T.L Nedambale

Signature…….. **Date**……

Co-supervisor: Dr F.Fushai

Signature…….. **Date**……

Co-supervisor: Dr E. Bhebhe

DECLARATION

I, Rammutla Tsaka Lyzer student number 11615759, 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 previously, in part or in its entirety, been submitted to any other university for a degree.

Signature…….. **Date……**

Student: Rammutla T.L. (11615757)

ABSTRACT

The quality of cryopreserved Boer goat semen may be influenced by the source and concentration of energy supplements in the extender. The aim of the study was to improve the protocols for cryopreservation of Boer goat spermatozoa using different disaccharides concentrations as supplements in tris- egg yolk extender. Two experiments were carried out to investigate the effect of (a) addition of three disaccharides (maltose, sucrose and trehalose) and (b) disaccharides combination (maltose and trehalose) at different concentrations using tris-egg yolk extender. For experiment 1: the study was replicated six times and was conducted in a 3 x 2 x 2 factorial arrangement with three different sugars (sugars: maltose, sucrose and trehalose), two sugar concentrations (0.12g and 0.22g) and two evaluation times (0 hours before cryopreservation and 120 hours after cryopreservation). For experiment 2: the study was replicated six times and was conducted in a 2 x 2 factorial arrangement with two sugar concentrations (0.12g and 0.22g) and two evaluation times (0 hours before cryopreservation and 120 hours after cryopreservation). Semen ejaculates were collected at 7.00-9.00 am from three Boer goats twice per week. After collection, the semen samples were pooled and diluted with tris-egg yolk extender at the ratio of 1:7 (semen to extender). Sperm quality (progressive motility, non- progressive motility, kinetic motions, viability (live/dead) and morphology) were analyzed using computer aided sperm analyzer (CASA). For experiment 1: sucrose 0.12g had higher progressive motility (PM %) when compared to maltose, and trehalose at 0h but reduced after cryopreservation. Sucrose 0.12 showed high percentage of kinetic motions (straightness and average path velocity) when compared to other sugars at 0 hours. More morphological defects M (CH) were revealed by maltose 0.12 at 0 hours. Sugar type (ST) and evaluation time (ET) showed no significant difference (P>0.05) in progressive motility (PM %), sperm kinetic motion, sperm viability and morphology. For experiment 2: mixed/combined 0.12g (maltose and trehalose) revealed more progressive motility (PM %) at 0h and reduced after cryopreservation. Table 6 and 9: of experiment 1 and 2 showed an interaction caused by sugar concentration level and evaluation time (L X ET) on the percentage of cut head M (CH%) and coiled M(C%) morphological abnormalities. In conclusion addition of maltose 0.12g to the extender showed almost similar results with that of trehalose at 0h and 120h. Therefore addition of maltose and trehalose to the extender might improve the quality of Boer goat spermatozoa prior and post cryopreservation.

Key words: Boer goat, Spermatozoa, Disaccharides, Cryopreservation and CASA

TABLE OF CONTENT

ACKNOWLEDGEMENT

Firstly I would like to acknowledge God almighty for His faithfulness, grace and wisdom. He gave me strength when I was weak, and encouraged me when I was unable to stand. I wouldn't have completed my research if it was not for Him. I am extremely grateful to my mother for her love, prayers and sacrifices for educating and preparing me for my future. She always encourage me to be positive and do the best in every opportunity I get. I would like to appreciate Mr. Machaba, my former home language (Sepedi) teacher from High School for his everyday support and motivation. He always remind me that hard work bring success. Also not forgetting my friend, and roommate (Lindo) for her support and motivation.

I would like to express my sincere appreciation and gratitude to Prof D.M. Barry, for opening an entry for me to become a member of Centre of Excellence in Animal Assisted Reproduction (CEAAR). I appreciate his continua's support, his patience, inspiration, and immerse proficiency. His guidance helped me to develop love for my research project. It was a great privilege and honor to work and study under his guidance.

I would like to thank the following people for their assistance in my data collection and analysis: Raseona A (CEAAR Lab Technician and also Animal Science PhD student), Ajao O.A(Animal Science PhD student), Nethengwe L.D (Animal Science PhD student), Negota N.C (Animal Science MSc Student), Siphungu M.S (Animal Science MSc student), Monyelote V (Animal Science MSC Student), Kalonji M.P.V (Animal Science MSc Student), Kalobo K (Animal Science MSC Student), Serota N.R (Animal Science MSc Student), Makhuvha k (Animal Science MSC Student), Thaba L (Animal Science BScANN Student), Mahumele F.G (Animal Science BScANN Student), Mabena T (Animal Science BScANN Student).

I would like to express my inmost gratitude to Prof T.L. Nedambale, who agreed to supervise me despite his work load. In my darkest moment he showed up and made the light to shine in my research work. I would like to thank him for taking his time to revise my work, comment, for his corrections and also his suggestions on my research study. He worked overnight to ensure that there's progress in my study. His way of seeing things always motivate me. I am tremendously thankful for what he has done for me.

I would like to acknowledge Dr .F. Fushai and Dr. E. Bhebhe (My co- supervisors), for their contribution to my research work. Although they had lot of students to supervise, they were always available to assist me and ensured progress in my study. Their notions and advices made countless effect to my work. My special obligations are due to Prof J.J. Baloyi, Head of Department (HOD) of Animal Science for his moral support in hard times, prudent suggestions, and encouragement. Also not forgetting Mrs Mathaulula from the Institute of Rural Development (IRD) for her encouragement and motivation. Lastly, but not least my gratitude goes to the National Research Foundation (NRF) of the Republic of South Africa for funding this study under NRF scarce Skills Masters Scholarship for 2016, Grant UID: 105052, 2016. Much gratitude goes to the Research and Publication Committee of the University of Venda, South Africa for their post graduate funding support numbered SARDF/17/ANS/02, and also to the work study program of the University of Venda.

DEDICATION

I dedicate this research project to my late father (Thomas), to my mother (Reginah) who never had an opportunity to go to school but always encouraged me to be the best I could be in life. Her prayers and positive attitude toward my research work contributed a lot. Also not forgetting my two brothers (Phuti and Kapari) and also to my four sisters (Portia, Phylicia, Tracy, and Tinah) for their encouragement, love and support throughout my studies.

LIST OF ACRONYMS

%	: Percentage
^{0}C	: Degrees Celsius
AI	: Artificial Insemination
ANOVA	: Analysis Of Variance
ART	: Assisted Reproductive Techniques
AV	: Artificial Vagina
CASA	: Computer aided Sperm Analyzer
CEAAR	: Centre of Excellence in Animal Assisted Reproduction Biotechnology Laboratory
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic Acid
EE	: Electro-Ejaculator
G	: Gram
GLM	: General Linear Model
LDL	: Low Density Lipoprotein
LN_2	: Liquid Nitrogen
NRF	: National Research Fund
SCA	: Sperm Class Analyzer
STR	: Straightness of the Average Path
TRIS	: Tris (Hydroxymethyl) Amino Methane
VAP	: Average Path Velocity
VCL	: Curvilinear Velocity
VSL	: Straight Line Velocity

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: INTRODUCTION

1.1 Background of the study

Boer goat is a breed that was developed in South Africa in the early 1900s for meat production. According to Sambraus (1992) Boer goats are large, long-legged goats with short, soft hair, long lop ears, white with reddish-brown heads, ears and necks. They are now considered to be the most preferred goat breed for meat production because of their excellent body confirmation, fast growing rate, and good carcass quality (Naing *et al.,* 2010). Recently, loss of genetic diversity within indigenous livestock breeds has been a major concern. Drought is regarded as one of the major causes for loss in genetic diversity. Goto *et al.,* (1989) reported that sudden injury and or death of a valuable animal carrying genetically beneficial traits can be a marked loss to animal producers and industry. It is estimated that 35% of mammalian breeds and 63% of avian breeds are at risk of extinction. Cardellino (2004) also reported that due to loss of diversity, a large proportion of domestic animal breeds in the world is believed to be in danger of extinction. Cryopreservation is one of the techniques to be used for Boer goat semen preservation. (Kundu *et al.,* 2000; Dorado *et al.,* 2007 and Rahman *et al.,* 2008) described cryopreservation as the process whereby sperm cells are preserved in liquid nitrogen (LN_2). However Holt (1997), Andrabi and Maxwell (2007) describe it as a technique used for long-term conservation of genetic material (germplasm) of endangered species. One of the advantages of cryopreservation is that it extends the reproductive life of a buck after his death (Rahman *et al*., 2008). According to Celeghini *et al* (2008) the use of cryopreserved semen in animal breeding programmes frequently results in reduced fertility. Isachenko (2003) reported that sperm cryopreservation usually induces the formation of intracellular ice crystals, osmotic and chilling injury, which causes sperm cell damage, cytoplasmic fracture, or even effects on the cytoskeleton or the genome related structures. Hammerstedt *et al* (1990) reported that for successful semen cryopreservation, the composition of an extender and optimal freeze rate play important roles in minimizing extra- and intra-cellular stresses. Purdy (2006) supported this by saying that the choice of extender plays an important role in determining the viability of deep frozen semen. In order to get the best post-thaw semen characteristics which would lead to greater fertility, the influence of different sugars on Boer goat spermatozoa should be evaluated. Leibo and Songsasen (2002) reported that sugar maintains the osmotic pressure of the diluents by inducing cell dehydration and less ice crystal formation in the spermatozoa. Sugar is utilized by spermatozoa as an energy source through glycolysis and

mitochondrial oxidative phosphorylation to support sperm motility and movement. Naing *et al* (2010) also discovered that a combination of sugars supplemented to a freezing extender provided improvement to buck sperm function, viability and quality following cryopreservation. Many studies focused much on glucose and fructose as supplements in the extender for semen preservation but few on maltose, trehalose and their combination.

1.2 Problem statement

Genetic material from animals of economic interest can be lost anytime. This can be due to the effects of drought, sudden injury and or death of valuable animals carrying superior genetic traits. Although cryopreservation is a process of preserving sperm cells for long periods of time, in animal breeding programmes, it results in reduced fertility. This is mainly because of damage caused to cells during freezing and thawing. Many studies have been conducted on semen cryopreservation but still the ability to freeze Boer goat semen remains a challenge.

1.3. Justification

Due to the economic and meat value of the Boer goat breed, most local farmers are now trying to switch from unimproved indigenous goats to the Boer goat breed. Therefore cryopreservation of buck spermatozoa is necessary, in case bucks of high genetic value die unexpectedly, then their preserved semen may be used through assisted reproductive techniques (ART). Few studies on the use of different sugar (maltose, sucrose and trehalose) concentrations as supplements to the extender have been conducted and none have been done on mixed/combined (maltose and trehalose). Therefore, further research on the diluents and techniques that give protection of sperm ‘cells during cryopreservation is needed. This will allow for increased efficiency and better utilization of Boer goat spermatozoa in producing progeny of high genetic value that would otherwise be lost.

1.4 Study objectives

1.4.1 Broad objective

The aim of the study was to improve the protocols for cryopreservation of Boer goat spermatozoa using different disaccharides concentrations as energy supplements in extender (tris-egg yolk extender).

1.4.2 Specific objectives

i. To evaluate and characterize the effects of supplementing maltose, Sucrose and trehalose at two different concentrations (0.12g and 0.22g) to tris-egg yolk extender on the quality of Boer Goat prior and post cryopreservation.

ii. To determine the effect of combining maltose and trehalose as energy supplements at concentrations (0.12g and 0.22g) in the semen extender on the characteristics of Boer goat spermatozoa prior and post cryopreservation.

1.5 Research Hypothesis

i. The concentrations of (Maltose, sucrose and trehalose) does not affect the quality of Boer goat spermatozoa prior and post cryopreservation.

ii. Mixed/combined (maltose and trehalose) sugars in the semen extender does not affect the characteristics of Boer goat spermatozoa prior and post cryopreservation.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Semen preservation and artificial insemination is a powerful technology tools for genetic improvement and management of animal breeding programmes. This is because assisted reproductive techniques allow the storage of semen from genetically valuable animals and extend generation times. Cryopreservation is the technique of freezing cells and tissues at very low temperatures at which the biological material remains genetically stable and metabolically inert, while minimizing ice crystal formation. In general, when a tissue is subjected to low temperatures, ice crystals will eventually form. These crystals may disrupt the cell membrane leading to the death of the cell. One of the ways to solve problems related to cryopreservation is through the use of cryoprotective agents. Cryoproptective agents protect the cells from mechanical and physical stress and reduces the water content within the cells, thus minimizing the formation of cell-lysing ice crystals. Sugars are one of the cryoprotective agent reported to have beneficial effects on the post-thaw viability of mammalian sperm cells. Leibo and Songsasen (2002) reported that sugars provide an energy substrate for sperm cells during incubation, maintain the osmotic pressure of the diluent and act as cryoprotectants.

2.2 Origin of Boer goat

Boer goats were developed in South Africa in the early 1900s for meat production, and their name is derived from the Dutch "Boer," meaning “farmer” (Solaiman, 2010). Boer goats were probably bred from the indigenous goats of the Namaqua Bushmen and the Fooku tribes, possibly with some crossing of Indian and European bloodlines. Boer goats evolved in South Africa from the indigenous African and the introduced European stock (Epstein, 1971). These include Bantu and Nubian, Saanen, Toggenburg and probably Angora. Out of South Africa, they are also found in Botswana, Lesotho, Swaziland, Zimbabwe, Namibia, Kenya, Burundi, Mozambique, Australia, USA, New Zealand, Germany, Israel, France and China (Malan, 2000). Germany used to have a large goat production (used mostly for research), and nations like Mexico and Argentina are beginning to farm Boer goats, but on a smaller scale.

Figure 1: Boer goat (University of Venda)

2.2.1 Site of sperm production

Sperm cells are produced in the seminiferous tubules of the testis through a process called spermatogenesis. After formation in the seminiferous tubules of male goat, the sperm cells are forced through the rete testis and vasa efferentia into the epididymis, where they are stored while undergoing maturation changes that make the sperm capable of fertilization (Hafez and Hafez, 2000).

2.2.3 Spermatogenesis

Spermatogenesis is the process whereby spermatozoa containing half the number of chromosomes (haploid) are produced, compared to the somatic cells. This process takes place in the seminiferous tubules of the testis of a buck. In mammals, it occurs in the male testes and epididymis in a stepwise fashion and in humans it takes approximately 64 days. Spermatogenesis is highly dependent on optimal conditions (e.g. temperature) for the process to occur efficiently, and is critical in reproduction. Spermatogenesis starts at puberty and usually continues uninterrupted until death. A slight decrease in semen production is discerned with an increase in age of an animal (Knobil and Neil, 1999).

2.3 Reproduction performance of Boer goat

Reproductive fitness may be regarded as the most important criterion relating to adaptation. From the onset of its reproductive life span, the improved Boer goat has established itself as a most productive and prolific breeder. Mean data from 826 goat does, 1.5 ± 6.5 years of age, revealed that 7.6% of lambs were born as singles, 56.5% as twins, 33.2% as triplets, 2.4% as quadruplets and 0.4% as quintuplets (Erasmus *et al.,* 1985). Interestingly, the Boer goat appears to attain maximum fertility at the relatively early age of 3.5 years; all the quadruplets and quintuplets being born at this age. In another study, 15.2% of the kids were born as singles, 67.5% as twins, 16.3% as triplets and 0.9% as quadruplets (Els, 1995). On the other hand, it was reported that Boer goats exported to Re Âunion Island between 1976 and 1982 were less productive regarding reproductive performance when compared to the indigenous Creole goat. Kimmes (1992) found out that for Creole, Boer Creole and Boer goats, litter size averaged 2.28, 1.81 and 1.75 kids, respectively; the conception rate averaged 96, 94 and 90, respectively, while annual kid production per doe averaged 2.57, 1.79 and 1.6, respectively.

2.4 Method of semen collection

2.4.1 Semen collection using the artificial vagina

The artificial vagina as a means to collect semen is easy to use and the semen collected is generally relatively clean and the ejaculate is similar to the natural ejaculate (Salisbury *et al.,* 1978). Briefly the artificial vagina (AV) consists of a rigid cylinder of rubber and a thin walled rubber tube for the inner lining. A water-tight jacket is formed inside the cylinder by folding both

ends of 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 simulates the thermal, while the pressure in the AV provides the mechanical stimulation of the vagina over the glans penis (Donovan *et al.,* 2001). The major disadvantage of this method of semen collection is that the animals have to be trained beforehand to utilize this method (Mathews *et al.,* 2003).

2.4.2 Electro-ejaculator

Electro-ejaculation involves applying a series of short, low-voltage pulses of current to the pelvic nerves which are involved in the ejaculatory response. The technique is widely used for semen collection in domestic animals such as cattle, sheep and bucks (Wulster-Radcliffe *et al.,* 2001a). The buck has to be well restrained on the lateral position during EE technique for violent reaction arising from the electric shock (Ramukhithi *et al.,* 2011b).Three pieces of equipment are required for electro ejaculation. The electro ejaculator itself is a power supply with rheostats to control the amplitude of the delivered current and lots of circuitry to prevent accidental electrocution. Second, one needs a collection tube, usually attached to a latex rubber cone ("loving cup") in which to collect the semen. An electric probe is inserted into the rectum adjacent to the prostate gland. The probe delivers an AC voltage, usually 12-24 volts sine wave at a frequency of 60 Hz, with a current limited to usually 500 mA. The probe is activated for 1-2 seconds, referred to as a stimulus cycle. Ejaculation usually occurs after 2-3 stimulus cycles. Care must be taken when using currents greater than 500 mA, as tissue burns may result due to heating of the probe. The stimulus voltage stimulates nearby nerves, resulting in contraction of the pelvic muscles and ejaculation. The animals generally experience no harmful effects, no loss in body condition, no real change in disposition, and no special disinclination to further application of the treatment.

2.5 Evaluation of semen characteristics

2.5.1 Colour

The first measurement of raw or fresh semen to indicate quality is its overall appearance. Raw (unaltered) semen appears as a thick whitish to slightly yellowish fluid. The semen colour varies from milky- white to pale creamy in colour (Bag *et al.,* 2002). According to Hafez and Hafez (2000), there exists a correlation between the colour and the sperm concentration of the semen ejaculate. The viscosity of the semen sample is often a reflection of the number of sperm cells present.

Collected semen must not have a noticeable odor, and if so, poor sanitary procedures during collection probably occurred. The emitting odor is most likely reflective of prepuce fluids (urine) which are generally heavily laden with bacterial and foreign contaminants. It has been suggested that semen samples with abnormal colour and consistency should be examined for determining actual concentration of spermatozoa, as reduced concentration leads to low fertility (Roberts, 1971).

2.5.2 Sperm concentration

Sperm concentration refers to the number of sperm cells per milliliter of semen (Graffer *et al.,* 1988). He further reported that sperm concentration in the ejaculate serves as one of the criteria in semen characteristics, to qualify fertile males for breeding purposes. The most common means of determining sperm concentration is to simply count sperm under a microscope with the aid of a hemacytometer. A hemacytometer is a glass slide onto which a precision grid has been etched.

2.5.3 Sperm motility and motion

Factors that affect mass motion of the spermatozoa include concentration, percentage of progressively motile cells and the speed/vigor of sperm motion. If one or more of these factors is compromised, the swirling of mass motion will be suppressed. Sperm motility is the parameter assessed in semen analysis that consists of the ability of sperm to swim in a forward direction and is the most commonly evaluated trait for semen quality. To evaluate motility, a 5 mm diameter drop of the semen is placed on a warm glass-slide and mass motion is observed under bright field microscopy at X40 magnification with the field diaphragm closed (Karatzas *et al.,* 1997). There are several means for evaluating motility but alternatively to the subjective visual motility evaluation, photographic analysis or computer assisted semen analysis (CASA) can be used. Computer-assisted semen analysis allows the analysis of sperm concentration, sperm motility, sperm motion, and to a certain extent, sperm head morphology.

2.5.4 Sperm viability

Sperm viability is an important measure of fertility, doctors and scientists often test seminal fluid to measure the percentage of living and non-living sperm it contains. Sperm viability (vitality) should be assessed if a low percentage of sperm are progressively motile, e.g. 30-40%. Since motile cells are inherently viable, a viability assessment may not be necessary when motility is high. This test is important to determine if the non-motile spermatozoa are alive or dead. Viability

testing should be performed as soon as possible after liquefaction. To assess viability, a commonly used staining method is eosin-nigrosin (Matshaba, 2010). Vital staining of the spermatozoa allows quantification of the fraction of living cells independently of their motility. A modification of the staining procedure (Raseona, 2015) to distinguish live from dead sperm was applied by adding one drop of eosin stain to one drop of semen at room temperature and and drying off over one to two minutes depending on ambient temperature, followed by smearing the mixture on a microscopic slide. A 100 spermatozoa are classified as either colored orange-red, if the stain has passed through the membrane and therefore the cell is considered dead, or non-stained, the cell than being considered alive. This staining technique makes it possible to differentiate spermatozoa that are immotile but alive from those that are dead (Hafez and Hafez, 2000).

2.5.5 Sperm morphology

Sperm morphology is generally dependent on spermatogenesis. Poor handling techniques leads to primary abnormalities which occur during spermatogenesis in the testis and secondary abnormalities which occur during maturation in the epididymis and also tertiary abnormalities. The sperm is defined as a highly structured cell, designed to deliver (Deoxyribonucleic acid) DNA to the oocyte. Generally, sperm abnormalities associated with the head are classified as primary and those associated with the mid piece or sperm tail as secondary. Abnormalities of the sperm head include twin, tapering or pyriform, round, shrunken, large, narrow, elongated and diminutive heads. Abnormalities of the neck on the other hand include broken necks and loose necks (Evans and Maxwell, 1987). For morphological evaluation, microscope slide is prepared with a very thin coating of semen. The slide is stained to make the sperm clearly visible and several hundred sperm are then viewed under high magnification using differential interference phase contrast of fixed sperm or stained dried samples under oil immersion. The sperm are judged based on the percentage of normal cells and the nature of the defects as mentioned above (Evans & Maxwell, 1987).

2.6 Buck semen cryopreservation

Cryopreservation is a useful technique for storing semen in liquid nitrogen at (–196 ^{o}C) for long periods until needed for use. Successful cryopreservation of mammalian sperm cells was first reported in 1949 (Kundu *et al.,* 2000). The cryopreservation of mammalian semen is a combination process that involves balancing of many factors in order to obtain suitable results.

This process is based on proper diluents, semen dilution rates, cooling rates, freezing rates and thawing rates (Kundu *et al.,* 2000; Purdy, 2006; Medrano *et al.,* 2010). Good knowledge of the sperm cell physiology is important to maximize post-thaw recovery of sperm cells and the fertility (Purdy, 2006). Cryopreservation of goat semen has advantages, including advancement of reproductive techniques, such as artificial insemination and in vitro fertilization. The cryopreservation technique is also important for conduction of genetic research and for production of transgenic animals (Barbas and Mascarenhas, 2009). However, freezing and thawing of semen lead to sperm cells functional damage (Kundu *et al.,* 2000; Aboagla and Terada 2004a; Kozdrowski *et al.,* 2007). Other negative effects of freezing and thawing include reduction of motility, membrane integrity and fertilizing capability of the sperm cells (Dorado *et al.,* 2007). The problems that results from freezing and thawing are due to the intensive dehydration or formation of intracellular ice crystals (Kundu *et al.,* 2000; Aboagla and Terada 2004a; Kozdrowski *et al.,* 2007). In goats and other species, the fertility rates are mostly below 50 % following insemination with frozen-thawed semen (Kundu *et al.,* 2000; Martinez *et al.,* 2007).

2.7 Problems associated with cryopreservation of semen

Semen cryopreservation was made possible more than half a century ago upon the discovery of egg-yolk being protective during cooling. Since then, research on cryopreservation has introduced and developed several technical modifications of cryopreservation protocols for different species and to improve the existing process (Johnson *et al.,* 2000; Salamon and Maxwell, 2000). Although several developments have been made, post-thaw quality and fertility of cryopreserved sperm are reduced due to damage during cryopreservation (Medeiors *et al.,* 2002). Sperm have variable responses to freezing and thawing depending on individual males and species (Holt, 2000 and Thurston *et al.,* 2002). According to Hofmo and Grevle (1999) in most mammalian species, a substantial number of sperm lose their fertilizing ability at different stages of cryopreservation. For instance, porcine sperm are sensitive to cold temperatures and the fertility of post-thaw pig semen is remarkably low for commercial use. Although bovine semen is considered cryoresistant, it still suffers major damage during freezing, and about ten times more frozen-thawed sperm are required than fresh bull sperm to achieve equivalent in vivo fertility rates (Shannon and Vishwanath, 1995). During cryopreservation, a significant proportion of sperm which look normal otherwise in terms of motility and morphology, may have suffered sub-lethal damages compromising their fertilizing potential (Watson, 2000).

2.8 Effect of cryopreservation on spermatozoa

Spermatozoa continuously change and develop from their origins as somatic cells until their destination as highly specialized cells capable of fertilization. They have basically three functional regions comprising a head that contain the condensed nuclear material, a mid piece serving as a powerhouse and a tail which is the propulsive region. Subsequent maturation occur within the epididymis, followed by further development induced first by contact with seminal plasma and then by the secretions of the female tract (Varner and Johnson, 2007). The final stages of spermatozoon development are induced by the immediate environment of the oocyte and its zona. In the process, most of the organelles are lost together with the cytoplasm, and the spermatozoa chromatin is remodelled. This specialization, though, is achieved at a cost, reducing the spermatozoon's ability to repair itself leading to a greater susceptibility to environmental change. Hence, even under ideal conditions, it is inevitable that some damage will occur to spermatozoa during the freezing process (Andrabi, 2007).

2.9 Extenders used in freezing semen

The addition of a cryoprotectant to the semen sample is needed in order to protect spermatozoa from cold shock. A large variety of extenders combining various components (sugars, electrolytes, buffers, egg yolk, milk and milk products), have been proposed and used for extending sperm. Milk and milk-based extenders are known to be practical and efficient in protecting spermatozoa of various species (Batellier *et al.,* 2001; Varner *et al.,* 1989). Glycerol and egg yolk extenders are amongst the first to be used for freezing semen (Garner *et al.,* 1999; Curry, 2000; Holt, 2000; Medeiros *et al.,* 2002). Today many extenders use glycerol as a major cryoprotectant. According to Fahy (1986) glycerol is used at a relatively high concentration which can be detrimental to spermatozoan viability at higher temperatures hence it is added after the semen has been cooled. Examples of extenders for freezing include egg yolk and those based on skimmed milk with egg yolk (Pickett and Amann, 1993). Success has been reported with the use of trehalose as a cryoprotectant within a skimmed milk–egg yolk extender. It is suggested that trehalose has a stabilizing effect on the spermatozoon plasma membrane (Steinmann, 1996).

2.10 Cryoprotective agents

Cryoprotective agents or cryoprotectants are included in the cryopreservation medium to reduce the physical and chemical stresses derived from cooling, freezing and thawing on the sperm cells

(Gao *et al.,* 1997; Purdy, 2006). According to Karow (1981), Mazur (1984) and Brockbank (1995) cryoprotectants and their modes of action have been the subject of many reviews, with glycerol and DMSO being the most commonly used cryoprotective agents. The addition of cryoprotectants during the freezing process is necessary in order to increase post-thaw viability and survival. The most commonly used cryoprotectant agents are sugars and glycerol. "Sugars have shown the ability to lower the Van der Waals interactions at the membrane hydrocarbon chains that enhance trans-membrane transfer (especially phospholipids)" (Yildiz *et al.,* 2007)**.** The cryoprotectants can be classified as penetrating or non-penetrating agents (Aisen *et al.,* 2000).

2.11 Sugars in the diluent for freezing goat semen

Sugar is a class of edible crystalline substances which is divided into two categories, such as monosaccharide and disaccharides (McDonald *et al.,* 1995). Sugars are included in semen extenders for respiration, to provide osmotic balance, energy as well as for cryoprotection of the sperm cells (Singh, Sinha and Singh, 1995; Aboagla and Terada, 2003; Purdy, 2006). According to Aboagla and Terada (2003) the presence of sugars in the diluents, positively affects the pattern of ice crystallization which could possibly relieve or prevent fast-cooling damage to the sperm cells. Osmolalities of sugars in the semen extenders ranges from 6 mM–375 mM (Purdy, 2006). Monosaccharides are the most basic unit of carbohydrates; they are the simplest form of sugar, usually colourless, water-soluble and have a sweet taste. Monosaccharides examples include glucose, fructose, galactose, xylose and ribose (McDonald *et al.,* 1995). Glucose-fructose-raffinose yolk have been investigated in several studies and proven to have a potential in use for freezing goat sperm cells (Aboagla and Terada, 2003). Disaccharides are the carbohydrates which are formed when two monosaccharides are joined together. They are sometimes crystalline, watersoluble, sweet-tasting and have a sticky-feeling, depending on the monosaccharide constituents. Examples of disaccharides are sucrose, lactulose, lactose, maltose and trehalose (McDonald *et al.,* 1995).

2.12 Advantages of using the computer assisted sperm analysis (CASA) system

The CASA provides an accurate evaluation of semen parameters such as spermatozoa motility by avoiding errors that may arise as a result of subjective evaluation of different technicians and reduces the time spent on semen evaluation (Jane *et al.,* 1996). More objectivity and repeatability in assessing sperm motility can be achieved by the Computer Assisted Sperm Analysis (CASA)

(Davis and Siemers, 1995). The use of CASA offers a more reliable, unbiased and repeatable means of assessing sperm motility, compared to examination by the human eye (Colenbrander *et al.,* 2003). Individual spermatozoa can be analysed and video images of the sperm cells are captured and analysed by the software.

CHAPTER 3: MATERIALS AND METHODS

3.1 Experimental site

The experiment was conducted at the School of Agriculture in the CEAAR Biotechnology Laboratory, University of Venda, Thohoyandou which is about 70 km east of Louis Trichardt in the Limpopo Province of South Africa. Daily temperatures at Thohoyandou vary from about 25 ˚C to 40 ˚C in summer and between 12 ˚C and 26 ˚C in winter. Rainfall is highly seasonal with 95% occurring between October and March. The average annual rainfall is about 800 mm but varies from year to year.

3.2 Animals and management

During this study, semen was collected and replicated five times from three mature, healthy bucks housed at University of Venda experimental farm. The bucks were of sound conformation and of good body condition. Semen was collected twice per week. The experimental animals were housed in enclosed pens without roofing under natural ventilation. Every morning, the bucks were fed pellets and clean water was provided *ad-libitum*. All procedures for this study were approved by ethical committee of the University of Venda.

3.3 Experimental design: Experiment 1

The study was replicated six times. Boer goat semen was collected using electro-ejaculator. Three disaccharides (maltose, sucrose and trehalose) at two different concentrations (0.12g and 0.22g) were used as supplements in tris-egg yolk extender. The semen was evaluated at (0h and 120h) and stored in liquid nitrogen (LN_2) at -196 °C.

Table 1: Experimental design: Experiment 1

Method of collection	Sugars type	Concentration levels	Extender	Evaluation Time	Storage	Replication
Electro-ejaculator	Maltose	0.12g	Tris-egg yolk extender	0 before and 120 hours after freezing	Liquid N	6
		0.22g	Tris-egg yolk extender	0 before and 120 hours after freezing	Liquid N	6
Electro-ejaculator	Sucrose	0.12g	Tris-egg yolk extender	0 before and 120 hours after freezing	Liquid N	6
		0.22g	Tris-egg yolk extender	0 before and 120 hours after freezing	Liquid N	6
Electro-ejaculator	Trehalose	0.12g	Tris-egg yolk extender	0 before and 120 hours after freezing	Liquid N	6
		0.22g	Tris-egg yolk extender	0 before and 120 hours after freezing	Liquid N	6

3.4 Experimental design: Experiment 2

The study was replicated six times. Boer goat semen was collected using electro-ejaculator. Two mixed/combined disaccharide (maltose and trehalose) at two different concentrations (0.12g and

0.22g) were supplemented in tris-egg yolk extender and evaluated at (0h and 120h). The semen sample were stored in liquid nitrogen (LN_2) at -196 °C.

Table 2: Experimental design: Experiment 2

Method of collection	Sugars type	Concentration levels	Extender	Evaluation Time	Storage	Replication
Electro-ejaculator	(Maltose and Trehalose)	0.12g maltose and 0.12g trehalose	Tris-egg yolk extender	0h and 120h	Liquid N	6
Electro-ejaculator	(Maltose and Trehalose)	0.22g maltose and 0.22g trehalose	Tris-egg yolk extender	0h and 120h	Liquid N	6

3.5 Preparation of extenders

The egg yolk was prepared by modification of the procedure described by Alexandra (2013);

i. The eggs were obtained within one week of laying from University of Venda poultry house, were cleaned with 70% alcohol and dried with paper towel.
ii. The cleaned eggs were cracked in half and egg white discarded
iii. Egg yolk was placed on a paper towel and yolk rolled carefully on the paper towel to remove the remaining egg white.
iv. The yolk was then punctured with a thick sterile needle and internal egg yolk extracted.

The tris-egg yolk extender was prepared by modification of the procedure described by Chaveiro *et al*. (2006) (Table 3).

Table 3: The tris-egg yolk extender constituents

Constituents	A_1	A_2	B_1	B_2	C_1	C2	D_1	D_2
Tris (g)	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46
Citric acid (Monohydrate) (g)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Fructose(g)	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007
Maltose (g)	0.12	0.22	-	-	-	-	0.12	0.22
Trehalose(g)	-	-	0.12	0.22	-	-	0.12	0.22
Sucrose(g)	-	-	-	-	0.12	0.22	-	-
Mixed	-	-	-	-	-	-	-	-
Egg yolk(ml)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Distilled water(ml)	10	10	10	10	10	10	10	10
Glycerol (ml)	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25
Penicillin (g)	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Streptomycin(µl)	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06

Sugar supplements; Experiment 1: Semen extender was supplemented with three different sugars (Maltose, Sucrose and Trehalose) at two different concentrations (0.12 and 0.22g) each and replicated six times. *Experiment 2:* Semen extender was supplemented with mixed/combined sugars (maltose 0,12g and trehalose 0.12g; maltose 0.22g and trehalose 0.22g) and replicated six times.

3.6 Ejaculated spermatozoa collection:

The semen from three bucks was collected with the aid of an electro-ejaculator at the School of Agriculture Experimental Farm using modification of the procedure described by Raseona (2015). The bucks were ejaculated twice a week (7.00-10.00 am). Long hairs around the prepuce were removed by clipping with a clean scissors. The exterior of the sheath was washed using clean water and dried using paper towel. The probe was lubricated with cooking oil and inserted into rectum with metal electrodes facing ventrally (downwards). The control unit was then connected using the cable supplied. The control unit was switched on and the voltage increased automatically in small increments until the buck maintained erection and ejaculated. The semen ejaculated was collected directly into 15ml tubes, and immediately placed in a thermos flask at 37°C, without being exposed to any direct sunlight. Ejaculated spermatozoa was then transported to the Animal Science Biotechnology Laboratory within 20 minutes of collection for microscopic sperm evaluation. In the laboratory semen was put inside a water bath at 37 °C and the ejaculates were pooled and diluted at a ratio of 1:5 semen and extender.

3.7 Semen evaluations

3.7.1 Sperm motility rate evaluation

The sperm motility parameters was analyzed with the aid of computer aided sperm analysis (CASA) system (Sperm Class Analyzer® [SCA] 5.3, Microptic, Barcelona, Spain) (Figure 2). A total of 3 µl of semen solution was pipetted onto a pre-warmed bevel-edged, frosted-end microscope glass slide (Thermo Scientific Menzel-Gläser, Germany), gently covered with a microscope cover slip (Menzel-Gläser, Germany) and evaluated under 10X magnification. The percentage of motility, concentration and kinetic motion such as the straight line velocity (VSL), which represented the average velocity of the sperm head measured in a straight line from the beginning to end of its track (µm/s). The average path velocity (VAP) which accounts for the average point-to-point velocity of the sperm head along its average track (µm/s).The curvilinear velocity (VCL), that defines the average point-to-point velocity of the sperm head along its actual track (µm/s), and straightness (STR): measuring the departure of the cell path from a straight line were all assessed using CASA (Verstegen *et al.*, 2002, Mocé and Graham, 2008).

Figure 2: CASA

3.7.2 Viability (live/dead) spermatozoa

Viability was performed using a modification of the eosin-nigrosin stain procedure described by (Evans and Maxwell, 1987). Spermatozoa was stained with nigrosine-eosin stain. Microscope slides were labelled with black permanent marker and pre-warmed at 37 °C. Three micro-litres of the semen and ten micro-litres of eosin stain was then poured on the end of the slide and mixed using a pipette tip on a warm glass stage at 37 °C. A drop of ten micro-litres of nigrosine stain was poured on to the mixture and mixed using the same pipette tip. The edge of the second slide also pre-warmed at 37 °C was placed on the mixture at an angle of 20° from the horizontal plane and pushed forward to smear across the slide (Matshaba, 2010). The smeared slide was placed on a hot Buehler® slide warmer (Buehler Ltd., 41 Waukegan Road, Lake Bluff, Illinois, USA) at 120 °C to allow drying. The dried stained slides were then placed on the CASA microscope stage at room temperature. A drop of immersion oil was poured on the stained slide and covered with a cover slip before evaluation. The CASA vitality program was then used at 60x magnification to count the number of live and dead spermatozoa (Figure 3). A total of 200 spermatozoa was counted per each stained slide and the results were recorded.

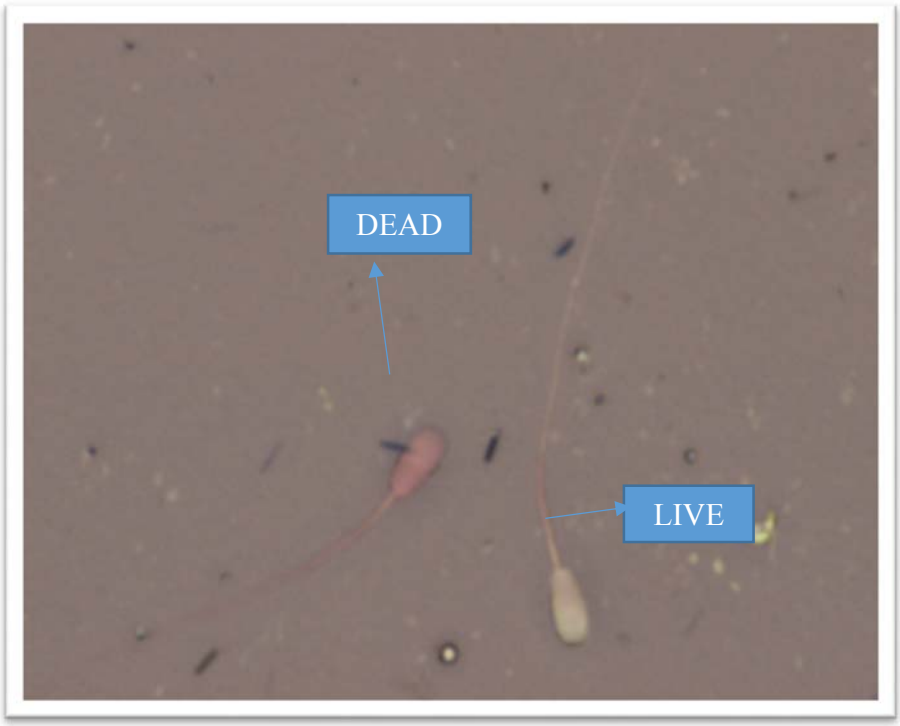


Figure 3: Live and dead semen

3.7.3 Evaluation of sperm morphology

Abnormal spermatozoa were determined by spamac stains using a modification of the procedure described by Matshaba (2010). For staining, a 5 micro-litres drop of extended semen was placed on the clear end of slide. The edge of a second slide was then placed on the drop of semen sample at an angle of 20° on a horizontal plane and pushed forward to smear the slide. The sperm smear was allowed to smear at room temperature (24°C). The dried smears were then placed horizontally down on paper towel inside a staining tray. Four staining procedures were used: for fix spermac stain: 1ml of spermac fixative was dropped on the dried smear using a plastic disposable pipette. After 5 minutes the slides were placed vertically on their end on absorbent paper for excess fixative to be drained off. The slides were washed slowly by dipping seven times in to distilled water. Excess water was dried off by placing the slides vertically on a paper towel inside the staining tray. For spermac A: 1ml of stain A was placed on the dried slide. After one minute, the slides were then washed twice seven times using fresh distilled water. For spermac stain B: stain B was introduced the same way as stain A, and then washed seven times using distilled water. For sperm stain C: stain C was introduced the same way as stain B and washed the same way. The slides were left in an upright position inside the staining tray to let the fluid

drain until air dry. The dried stained slides were then placed on the CASA microscope stage at room temperature. A drop of immersion oil was placed on the stained slide and covered with a cover slip before evaluation. The CASA morphology program was then used at 60x magnification to count the number of live and dead spermatozoa. A total of 200 spermatozoa was counted per each stained slide and the results of the normal sperm, headless sperm, and bent tail were recorded (Evans and Maxwell, 1987).

3.7.4 Cryopreservation and packaging

Cryopreservation was performed by modification of procedures described by Raseona (2015). The diluted sperm samples were equilibrated in the refrigerator at 5 °C for a period of 2 hours. After 2 hours, extended semen samples were loaded into 0.25 ml labelled plastic straws. After filling, the semen straws were sealed using polyvinyl powder and placed on a semen freezing holding racks. For freezing, the Styrofoam box was filled with liquid nitrogen. The straws were placed on a freezing rack approximately 4cm above the surface of the liquid nitrogen in a Styrofoam box for 10 minutes. After 10 minutes the straws containing semen were submerged inside liquid nitrogen and loaded into a liquid nitrogen canister and then stored at (−196 °C) for a period of five days.

Figure 4: Shows the process of semen cryopreservation

Figure 4: The process of semen cryopreservation

3.7.5 Post - thaw evaluation of sperm cells processes

Straws containing semen from the liquid nitrogen were thawed, by dipping them into a water bath at 37 ◦C for 40 seconds by modification of procedures described by Raseona (2015). The straws were then removed from the water bath, wiped dry using a paper towel and the tip cut open with a scissors. The contents was then emptied into a test tube and 3 µl sample of sperm from different straws was then placed onto a pre-warmed glass slide and evaluated by phase microscopy for sperm motility. For sperm viability and sperm morphology the same staining procedures was used and the results were recorded.

3.8 Statistical analysis for experiment 1

Data were analyzed by analysis of variance (ANOVA) for a 3x 2x 2 factorial in a completely randomized design using the GLM procedure of Minitab (Minitab, 2013).Three sugar types, two sugar concentrations and two evaluation times. Significant differences (P <0.05) among mean values of semen quality parameters resulting from treatments were subsequently determined by Tukey's procedure (Steel and Torrie, 1981).

3.8.1 Statistical model for the first experiment: 1

$$Y_{ijkl} = \mu + E_i + S_j + L_l + ES_{ij} + EL_{il} + SL_{jl} + ESL_{ijl} + \varepsilon_{ijkl}$$

Where Y_{ijk} is the observation
μ = Overall mean
E_i = the effect of i^{th} sugar type
S_j =the effect of j^{th} sugar concentration
L_l = the effect of l^{th} evaluation time
ES_{ij} is the interaction of i^{th} sugar type and j^{th} sugar concentration
EL_{il} is the interaction of i^{th} sugar type and l^{th} evaluation time
SL_{jl} is the interaction of j^{th} sugar concentration and l^{th} evaluation time
ESL_{ijl} = the interaction between i^{th} sugar type, j^{th} sugar concentrations and l^{th} evaluation time
ε_{ijkl}= Random error

3.9 Statistical analysis for experiment: 2

Data were analyzed by analysis of variance (ANOVA) for a 2 x 2 factorial in a completely randomized design using the GLM procedure of Minitab (Minitab, 2013). Combined sugar, two sugar concentrations and two evaluation times. Significant differences (P <0.05) among mean values of semen quality parameters resulting from treatments were subsequently determined by Tukey's procedure (Steel and Torrie, 1981).

3.9.1 Statistical model: experiment: 2

$$Y_{ijk} = \mu + E_i + S_j + ES_{ij} + \varepsilon_{ijk}$$

Where Y_{ijk} is the observation

μ = Overall mean

E_i = the effect of i^{th} sugar concentration

S_j =the effect of j^{th} evaluation time

ES_{ij} = the interaction of i^{th} sugar concentration and j^{th} evaluation time

ε_{ijk} = Random error

CHAPTER 4: RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 EXPERIMENT 1

Table 4: below shows the summarized results of sperm motility rate characteristics of semen exposed to extender supplemented with maltose, sucrose and trehalose at 0h and 120h. The percentage of sperm motility characteristics of maltose, trehalose and sucrose concentrations for progressive and static at 0hours revealed statistically significant difference (P<0.05). Progressive motility at 120hours showed no significant difference (P>0.05). NP (%) and Static (%) had no significant difference at 120hours. The concentration level (S) revealed interaction (P<0.05) for PM (%) values.

Table 4: Sperm motility rate characteristics of semen exposed to extender supplemented with maltose and trehalose at 0h and 120h.

Energy source	SL(g)	EVT(H)	PM (%)	NP (%)	Static (%)
Maltose	0.12	0	70.0^{abc}	24.5^{ab}	5.5^{de}
		120	10.8^{d}	15.7^{ab}	73.5^{abc}
Maltose	0.22	0	51.3^{bc}	32.8^{ab}	16.0^{de}
		120	6.5^{d}	24.5^{ab}	69.0^{abc}
Sucrose	0.12	0	84.0^{a}	13.3^{ab}	2.7^{e}
		120	5.5^{d}	5.8^{b}	88.7^{a}
Sucrose	0.22	0	46.5^{c}	32.7^{ab}	20.8^{de}
		120	4.2^{d}	15.0^{ab}	81.0^{ab}
Trehalose	0.12	0	72.0^{ab}	21.0^{ab}	7.0^{de}
		120	15.7^{d}	46.5^{a}	37.8^{cde}
Trehalose	0.22	0	59.8^{abc}	26.5^{ab}	13.8^{de}
		120	8.3^{d}	25.3^{ab}	66.3^{abc}
SEM±			5.0	7.7	8.4
Significance					
E			NS	*	*

S	**	NS	*
L	**	NS	**
ES	NS	NS	NS
EL	NS	NS	*
SL	*	NS	NS
ESL	*	NS	NS

[a-b-c] means within a column, that do not share a common superscript are significantly different (P<0.05); SL=Supplementary level, ST=Storage, PM=progressive motility, NP=Non-progressive motility and Static. NS= Non significant difference (P> 0.05),*= Significant difference (P< 0.05), **= Highly significant (P< 0.01).

Table 5: shows the summarized results of sperm velocity rate of semen exposed to extender supplemented with maltose, trehalose or sucrose at 0h and 120h. There was no significant (P>0.05) differences in VSL (µm/s), VAP (µm/s) and STR (%) at both 0h and 120h. VCL (µm/s) at 0h and 120h showed significant difference (P<0.05). Sucrose had increased VSL (µm/s), VAP (µm/s) and STR (%) at 0hours compared to all the other sugars at 0.12 concentration level (P<0.05). A significant difference was revealed by SL on VSL (µm/s) and VAP (µm/s) values on the other hand the interactions revealed non-significant difference on sperm velocity rate values.

Table 5: Sperm velocity rate of semen exposed to extender supplemented with maltose or sucrose at 0h and 120h.

Energy source	SL(g)	EVT(h)	VSL (µm/s)	VAP(µm/s)	VCL (µm/s)	STR (%)
Maltose	0.12	0	18.0^{a}	27.3^{a}	42.1^{a}	74.5^{a}
		120	9.4^{a}	12.5^{a}	16.9^{bcd}	73.1^{a}
Maltose	0.22	0	18.2^{a}	24.5^{a}	39.3^{abc}	74.4^{a}
		120	10.9^{a}	12.2^{a}	18.5^{bcd}	65.9^{a}
Sucrose	0.12	0	22.3^{a}	26.6^{a}	33.2^{abcd}	83.4^{a}
		120	6.0^{a}	9.3^{a}	15.8^{d}	60.7^{a}
Sucrose	0.22	0	15.0^{a}	19.7^{a}	27.4^{abcd}	75.9^{a}
		120	6.0^{a}	10.1^{a}	16.2^{cd}	57.5^{a}

Trehalose	0.12	0	20.5[a]	27.4[a]	42.6[a]	75.6[a]
		120	13.0[a]	16.9[a]	21.1[d]	73.6[a]
Trehalose	0.22	0	17.8[a]	24.5[a]	42.6[a]	72.8[a]
		120	7.8[a]	11.2[a]	15.3[d]	67.8[a]
SEM±			3.6	3.6	4.7	5.3
Significance						
E			NS	NS	NS	NS
S			*	*	NS	*
L			**	**	**	*
ES			NS	NS	NS	NS
EL			NS	NS	NS	NS
SL			NS	NS	NS	NS
ESL			NS	NS	NS	NS

[a-b-c] means within a column, that do not share a common superscript are significantly different (P<0.05); SL=Supplementary level, EVT=Evaluation Time, VCL = curvilinear velocity, VSL = straight-line velocity, VAP = average path velocity, STR = straightness. NS= Non significant difference (P> 0.05),*= Significant difference (P< 0.05), **= Highly significant (P< 0.01).

Table 6: shows the summarized morphological characteristics of sperm cells vitality (live and dead) and of semen exposed to extender supplemented with maltose, trehalose or sucrose at 0h and 120h.The results revealed no significant differences (P>0.05) on the morphological characteristics; M (CH), M (C) and M (BT) of Boer goat semen with the addition of different concentrations of maltose, sucrose and trehalose to the extender at 0h and 120h. The percentage of sperm vitality (live and dead) of maltose, trehalose and sucrose concentrations had statistically significant difference (P<0.05) at both 0h and 120h. All sugars had more live spermatozoa before freezing, after freezing the total live spermatozoa decreased. Further, sperm diluted in sucrose 0.12 and 0.22 experienced greater loss in vitality V (L) spermatozoa at 120hours when compared to other sugars. Trehalose had more live spermatozoa at both 0h and 120h when compared to all other sugars. There was significant (SL) interaction on M (CH %) and M (C %)

Table 6: Morphological characteristics of sperm cells vitality (live and dead) and of semen exposed to extender supplemented with maltose or sucrose at 0h and 120h.

Energy source	SL(g)	EVT(h)	M (CH %)	M(C %)	M (BT %)	V (L %)	V (D %)
Maltose	0.12	0	16.7^{a}	0.7^{a}	4.8^{a}	57.5^{a}	42.5^{d}
		120	11.2^{a}	2.0^{a}	3.2^{a}	7.8^{d}	92.2^{a}
Maltose	0.22	0	12.5^{a}	1.0^{a}	2.7^{a}	39.0^{ab}	61.0^{cd}
		120	11.2^{a}	0.7^{a}	2.7^{a}	4.3^{d}	95.7^{a}
Sucrose	0.12	0	14.7^{a}	1.3^{a}	0.7^{a}	54.7^{ab}	45.3^{cd}
		120	8.7^{a}	1.7^{a}	1.5^{a}	3.3^{d}	96.7^{a}
Sucrose	0.22	0	12.3^{a}	1.2^{a}	0.8^{a}	31.8^{bc}	68.2^{bc}
		120	6.3^{a}	1.2^{a}	2.7^{a}	2.2^{d}	97.8^{a}
Trehalose	0.12	0	15.3^{a}	0.5^{a}	2.8^{a}	58.3^{a}	41.7^{d}
		120	12.5^{a}	2.2^{a}	2.0^{a}	11.3^{cd}	88.7^{ab}
Trehalose	0.22	0	12.8^{a}	2.0^{a}	3.2^{a}	45.3^{ab}	54.7^{cd}
		120	15.0^{a}	1.0^{a}	2.7^{a}	6.3^{d}	93.3^{a}
SEM±			3.4	0.8	0.9	4.6	4.6
Significance							
E			NS	NS	NS	NS	*
S			*	*	NS	NS	NS
L			**	**	*	NS	NS
ES			NS	NS	NS	NS	NS
EL			NS	NS	NS	NS	NS
SL			*	*	NS	NS	NS
ESL			NS	NS	NS	NS	NS

$^{a\text{-}b\text{-}c}$ means within a column, that do not share a common superscript are significantly different ($P<0.05$); SL=Supplementary level, EVT=Evaluation Time, M (CH) =Morphology cut head, M(C) =Morphology coiled, M (BT) =Morphology cut tail. V (L) =Vitality Live, V (D) =Vitality Dead. NS= Non significant difference ($P> 0.05$),*= Significant difference ($P< 0.05$), **= Highly significant ($P< 0.01$).

4.1.2 EXPERIMENT 2

Table 7 shows the summarized results of Sperm motility rate characteristics of semen exposed to extender supplemented with mixed/combined sugar (maltose and trehalose) at 0h and 120h. Mixed/combined 0.12 sugar had higher progressive motility (56.17%) at 0h ($P<0.05$) when compared to mixed 0.22 and reduced after cryopreservation. NP (%) and S (%) showed no significant difference ($P>0.05$) at 0h but differed significantly after freezing ($P<0.05$). There was significant ($P<0.05$) (SL) interaction on PM (%) and also S revealed significant difference ($P<0.05$) on PM (%) values.

Table 7: Sperm motility rate characteristics of semen exposed to extender supplemented with mixed/ combined sugar (maltose and trehalose) at 0h and 120h.

Energy source	SL(g)	EVT(h)	PM (%)	NP (%)	STATIC (%)
Mixed	0.12	0	56.2^{bc}	33.5^{ab}	10.3^{de}
		120	8.3^{d}	24.3^{ab}	67.3^{abc}
Mixed	0.22	0	56.5^{bc}	33.0^{ab}	10.5^{de}
		120	7.5^{d}	47.0^{a}	45.5^{bcd}
SEM±			5.0	7.7	8.4
Significance					
S			**	NS	**
L			NS	NS	NS
SL			*	NS	NS

$^{a\text{-}b\text{-}c}$ means within a column, that do not share a common superscript are significantly different ($P<0.05$); L=levels, EVT=Evaluation Time, PM=progressive motility, NP=Non-progressive motility, Static. NS= Non significant difference ($P> 0.05$),*= Significant difference ($P< 0.05$), **= Highly significant ($P< 0.01$).

Table 8: shows the summarized results of sperm velocity rate of semen exposed to extender supplemented with mixed/combined sugar (maltose and trehalose) at 0h and 120h. The evaluation time revealed significant difference (P<0.05) on VSL (µm/s), VAP (µm/s), VCL (µm/s) and STR (%) values.

Table 8: Sperm velocity rate of semen exposed to extender supplemented with mixed/combined sugar (maltose and trehalose) at 0h and 120h.

Energy source	SL(g)	EVT(h)	VSL (µm/s)	VAP (µm/s)	VCL (µm/s)	STR (%)
Mixed	0.12	0	17.4^{a}	24.3^{a}	40.1^{ab}	72.7^{a}
		120	23.3^{a}	26.4^{a}	26.5^{abcd}	77.6^{a}
Mixed	0.22	0	18.5^{a}	25.9^{a}	43.0^{a}	71.5^{a}
		120	8.0^{a}	11.5^{a}	16.6^{cd}	62.3^{a}
SEM±			3.6	3.6	4.7	5.3
Significance						
S			*	*	NS	*
L			**	**	**	*
SL			NS	NS	NS	NS

$^{a\text{-}b\text{-}c}$ means within a column, that do not share a common superscript are significantly different (P<0.05); SL=Supplementary level, EVT= Evaluation Time, VCL = curvilinear velocity, VSL = straight-line velocity, VAP = average path velocity, STR = straightness. NS= Non significant difference (P> 0.05),*= Significant difference (P< 0.05), **= Highly significant (P< 0.01). VSL (µm/s), VAP (µm/s) and STR (%) had no significant difference (P>0.05) at both 0h and 120h.VCL (µm/s) at 0h and 120h showed significant difference (P<0.05). There was no interaction revealed by SL.

Table 9: shows the summarized results of morphological characteristics of sperm cells vitality (live and dead) and of semen exposed to extender supplemented with mixed/combined sugar (maltose and trehalose) at 0hand 120h. The S X L had interactions on M (CH), M (C) values and there was significant difference (P<0.05) revealed by S and L on M (CH %) and M (C %) values.

Table 9: Morphological characteristics of sperm cells vitality (live and dead) and of semen exposed to extender supplemented with mixed/combined sugar (maltose and trehalose) at 0h and 120h. There was interaction revealed by S X L on M (CH %) and M (C %) values.

Energy source	SL(g)	EVT(h)	M (CH %)	M (C %)	M (BT %)	V (L %)	V (D %)
Mixed	0.12	0	13.0^{a}	2.2^{a}	3.7^{a}	46.7^{ab}	53.3^{cd}
		120	9.7^{a}	1.3^{a}	2.0^{a}	5.7^{d}	94.3^{a}
Mixed	0.22	0	17.7^{a}	1.5^{a}	2.8^{a}	47.0^{ab}	53.0^{cd}
		120	10.0^{a}	1.0^{a}	2.5^{a}	4.0^{d}	96.0^{a}
SEM±			3.4	0.8	0.9	4.6	4.6
Significance							
S			*	*	NS	NS	NS
L			**	**	*	NS	NS
SL			*	*	NS	NS	NS

$^{a-b-c}$ means within a column, that do not share a common superscript are significantly different (P<0.05); S L=Supplementary level, EVT=Evaluation Time, M (CH) =Morphology cut head, M(C) =Morphology coiled, M (BT) =Morphology bent tail, V (L) =Vitality Live, V (D) =Vitality Dead. NS= Non significant difference (P> 0.05),*= Significant difference (P< 0.05), **= Highly significant (P< 0.01).

4.2 Discussion

According to Kathiravan *et al.* (2011) motility is considered the most important characteristic associated with the fertilizing ability of spermatozoa and as an expression of their viability and structural integrity. In the current study, sugar concentration levels (S) and evaluation time (L) showed significant difference ($P<0.05$) in sperm motility parameters (NP %) and (Static %) at 0h and 120h. Sugar concentration level (S %) was highly significant in (PM %) in experiment 1. This might be due to the fact that sugars vary according to types and functionality of their chemical and molecular weight (Purdy, 2006). Low molecular weight molecules can pass through the plasma membrane of spermatozoa and provide energy to function in metabolism. High molecular weight sugars are not capable of diffusing across a plasma membrane and create an osmotic pressure leading to eventually induced cell dehydration (Purdy, 2006). The percentage of progressive motile spermatozoa was significantly higher ($P<0.05$) in sucrose (84.0±5.0) followed by trehalose with (72.0±5.0) at 0 hours (Table 3). Addition of 0.12g sucrose to the extender improved sperm motility with higher percentage when compared to other sugars at 0hours. The results of the study revealed that addition of sucrose as a non-permeating cryoprotectant sugar in extender seems to provide more protection to the sperm cells.

Aisen, *et al.,* (2005) reported that sucrose promotes cell dehydration before freezing and prevents injury caused by intracellular ice formation. Although sucrose has been reported to exert a cryoprotective effect by direct interaction with the membranes preventing freeze-thaw bilayer destabilization, in the current study reduced motile spermatozoa after cryopreservation was observed when compared to the other sugars at 120h. This also agrees with the finding of Woelders *et al.* (1997) which revealed that addition of sucrose to bull semen extender exhibited deleterious effect on post-thaw sperm motility. From the results we can conclude that semen with sucrose can improve reproductive performance of goats only when used before freezing. This is due to high decrease in PM (%) revealed by sucrose after cryopreservation. Addition of 0.12g of maltose to the extender had less variation when compared to trehalose. Findings of this study clearly indicate that addition of 0.12 maltose to the extender showed almost similar results with that of trehalose at 0h and 120h. This might be due to the fact that trehalose and maltose have the same molar mass of 342.297 g/mol. They are formed from two glucose units but joined with different bonds. Therefore, addition of 0.12g maltose in the extenders could protect Boar goat spermatozoa against freeze damage and also brings positive results after artificial insemination. This is due to less damage revealed by maltose after cryopreservation when compared to sucrose. The results are in agreement with the findings of Abdelhakeam *et al.* (1991) which revealed that maltose yielded the best post-thaw motility in TEST extender when used in ram spermatozoa.

Maltose revealed lower PM (%) when compared to trehalose and sucrose at 0h, but had high motile spermatozoa at 120h when compared to sucrose (Table 4). Comparison between maltose, sucrose and trehalose revealed a higher progressive motile spermatozoa of (15.7%) after cryopreservation in 0.12g trehalose. Again when 0.22g of trehalose was added to the extender, an increase in PM (8.33±4.95) was observed when compared to addition of the other sugars. This clearly indicates that trehalose can be used in Boer goat semen extenders to improve reproductive fertility performance. These findings agree with the results obtained by (Malo *et al.* 2010). Trehalose is a non-reducing sugar formed from two glucose units joined by α 1-1 alpha bond. The bonding makes trehalose very resistant to acid hydrolysis, and therefore stable in solution at high temperatures even under acidic conditions. Trehalose has been extensively used to improve sperm quality parameters in semen cryopreservation and its protective effects significantly improved the freezability of goat spermatozoa (Aboagla and Terada, 2003). Furthermore Aboagla *et al.* (2004a) revealed that when trehalose was added to semen of buck, improved sperm motility, viability, and acrosomal intergrity was observed. In comparing two concentration levels of trehalose, the 0.12g concentration revealed better results than the 0.22g at both 0h and 120h. This is in agreement with the findings of Aisen *et al.* (2002), which revealed favourable effect of the lower trehalose concentrations on spermatozoa motility after thawing, and an unfavourable effect of higher trehalose concentrations, which was also proved by Hu *et al.* (2009). Addition of 0.12 maltose to tris egg yolk extender revealed more M (CUT H) and M (BT) abnormalities when compared to other sugars on the same treatment (table 5). On the contrary sucrose M (CUT H) and M (B T) showed less abnormalities when compared maltose and trehalose at 0h. For experiment 2: 0.12g of Mixed/combined had higher PM (%) at 0h ($P<0.05$) when compared to mixed 0.22. Reduction in PM (%) was observed after cryopreservation at 120h (Table 7). Mixed 0.22 showed more percentage of (CH) at 0h and 120h when compared to mixed 0.12. In comparison between sperm motility and morphological abnormalities, mixed 0.12g had high percentage of PM (%) and lower percentage of cut head at 0h and 120h when compared to mixed 0.22g. Mixed 0.22 revealed low PM (%) and high (CH %) experiment 2.

Table: 4 of experiment: 1 sugar concentration level and evaluation time revealed interaction on the percentage of progressive motility PM (%). All sugars at 0.12 concentration level had high percentage PM (%) at 0 hours and the reduction was observed after cryopreservation. On experiment: 2 (Table: 7), concentration level (S) and evaluation time (L) showed an interaction on percentage of progressive motility M (PM %), where the 0.12 concentration level revealed higher percentage of progressive motility at 0hours and reduction was observed after cryopreservation. This could be attributed to the

alteration of sperm structure during cryopreservation process and also the amount of energy supplemented. The fertilizing potential of the frozen thawed sperm is reduced because of alterations in the structure and physiology of the spermatozoa (Salamon and Maxwell, 2000; Barbas and Mascarenhas, 2009). Medeiros *et al.* (2002) reported that these alterations reduce the spermatozoon's life span and fertilizing ability. 0.22 sugar concentration level revealed less PM (%) at 0hours when compared to 0.12 concentration level. The results clearly indicate that the higher the sugar concentration, the more fertilizing potential of semen is reduced. Table 5 and 9: of experiment 1 and 2 showed an interaction caused by sugar concentration level and evaluation time (SL) on the percentage of cut head M (CH %) and coiled M(C %) morphological abnormalities. At 0.12 concentration level more cut head were revealed at 0 hours and reduced after cryopreservation. Sperm abnormalities may not affect motility but reduces life span, ability to interact with the female reproductive tract and sperm fertility. Spermatozoa morphological assessment is considered the most significant method of differentiating between semen of high and low fertilizing capacity (Ball and Poters, 2004). The sperm cell is divided into the head, neck, middle piece, plasma membrane and tail. When sperm have a double tail, no tail, or a head that is crooked, coiled, bent tail, it is considered to be abnormal, and therefore unable to successfully penetrate an egg. Abnormal characteristics of spermatozoa are analyzed to determine the percentage infertility of an animal. The abnormalities might have been caused during semen smearing and staining. When smearing the edge of a second slide is placed on the drop of semen sample at an angle of 20° on a horizontal plane and pushed forward to smear the slide. So sperm abnormalities might results during the process. The results of the current study clearly indicate that that 0.12g of sugar is the best concentration level to be used when compared to 0.22g. The results shows that the more the concentration level is added, the more the sperm parameters decreases. This is in agreement with the findings of Aisen *et al.* (2002) which says that higher concentrations of trehalose in the presence of glycerol have a detrimental effect on the acrosome integrity of frozen-thawed spermatozoa.

CHAPTER 5: CONCLUSION AND RECOMMENDATION

I. Results of this study revealed high percentages for sperm parameters when 0.12g concentration level of sugar is added to the semen extender (tris-egg yolk extender). Furthermore 0.12g concentration can assist in improving post-thawed sperm parameter characteristics of Boer goat spermatozoa when compared to 0.22g concentration. Decrease/increase in sperm motility parameters depends on the storage time and the concentration level of sugar. The higher the sugar concentration the less motile spermatozoa became.

II. Different sugars used revealed variable results and this may be due to their different functionality as offered by their chemical and molecular weight.

III. Trehalose and maltose can be used for cryopreservation of semen when storage is going to be for a long period of time, this observation does not apply to sucrose.

IV. All the sugars (trehalose, maltose and sucrose) are fit for AI (Artificial Insemination) purposes for a short period of time.

V. More research needs to be done on ways to preserve Boer goat spermatozoa for extended periods.

VI. Combined/mixed (maltose and trehalose) produced lower percentage for motility parameters when compared to when sugars were used individually.

VII. More studies need to be undertaken on the use of maltose and trehalose individually and when combined for Boer goat semen cryopreservation.

VIII. Although sperm motility parameters decrease with the storage time, the extender used can still be used for fresh semen preservation for artificial insemination purposes.

IX. Further studies need to be conducted on addition of different disaccharide (maltose, sucrose and trehalose) concentrations levels to the extender on Boer goat spermatozoa and also best ways to be used on improving cryopreservation using maltose and trehalose for Boer goat spermatozoa.

REFERENCES

Aboagla, E. M. E. and Terada, T., (2003). Trehalose-enhanced fluidity of the goat sperm membrane and its protection during freezing. Biology Reproduction. 69, 1245 – 1250.

Aboagla, E. M. E. and Terada, T., (2004a). Effects of the supplementation of trehalose extender containing egg yolk with sodium dodecyl sulphate on the freezability of goat spermatozoa. Theriogenology. 62, 809 – 818.

Aisen, E. G. Medina, V. H., Venturino, A., (2002). Cryopreservation and post thawed fertility of ram semen frozen in different trehalose concentrations. Theriogenology. 57, 1801-1808.

Aisen, E. G., Alvarez, H. L., Venturino, A. and Garde, J. J., (2000). Effect of trehalose and EDTA on cryoprotective action of ram semen diluents. Theriogenology. 53, 1053-1061.

Aisen, E. G., Quintana, M., Medina, V., Morello, H., Venturino, A., (2005). Ultramicroscopic and biochemical changes in ram spermatozoa cryopreserved with trehalose-based hypertonic extenders. Cryobiology. 50, 239-249.

Alexandra, R., 2013. Cryopreservation of bovine semen in egg yolk based extenders. Western College of Veterinary Medicine University of Saskatchewan Saskatoon, Saskatchewan S7N 5B4 Canada.

Andrabi, S. and Maxwell, W., (2007). A review on reproductive biotechnologies for conservation of endangered mammalian species. Animal Reproduction Science. 99, 223 -243.

Andrabi, S. M. H., (2007). Fundamental principles of cryopreservation of Bos taurus and Bos indicus bull spermatozoa. Mini review. International Journal of Agriculture and Biology. 9,367-369.

Bag, S., Joshi, A., Rawat, P. S. and Mittal, J. P., (2002). Effect of initial freezing temperature on the semen characteristics of frozen thawed ram spermatozoa in a semi-arid tropical environment. Small Ruminant Research. 43, 23–29.

Ball, P. J. and Peters, A. R., (2004). Reproduction in cattle. (3rd edition). John Wiley & Sons.

Barbas, J. P. and Mascarenhas, E.R.D., (2009). Cryopreservation of domestic animal sperm cells. Cell Tissue Bank. 10, 49-62.

Batellier, F., Vidament, M., Fauquant, J., Duchamp, G., Arnaud, G., Yvon, J. M., and Magistrini, M., (2001). Advances in cooled semen technology. Animal Reproduction Science. 68,181–190.

Brockbank, K. G. M., (1995). Essentials of cryobiology: In Principles of Autologous, Allogeneic, and Cryopreserved Venous Transplantation. (Ed. K. G. M. Brockbank), RG Landes Company, Austin, TX (Medical Intelligence Unit Series) and Springer- Verlag. 91-102.

Cardellino, R. A., (2004). Conservation of farm animal genetic resources – a global view. Farm Animal Genetic Research. 30, 1-14.

Celeghini ECC, Arruda RP, Andrade AFC, Nascimento J, Raphael CF, Rodrigues PHM (2008) Effects that bovine sperm cryopreservation using two different extenders has on sperm membranes and chromatin. *Anim Reprod Sci*, 104:119–131.

Chaveiro, A., Machado, L., Frijters, A., Engel, B., Woelders, H., (2006). Improvement of parameters of freezing medium and freezing protocol for bull sperm using two osmotic support.Theringenology.60, 1875-1890.

Colenbrander, B., Gadella, B.M. & Stout, T.A.E., (2003). The predictive value of semen analysis in the evaluation of stallion fertility. Reproduction of Domestic Animals. 38, 305-311.

Curry, M., (2000). "Cryopreservation of semen from domestic livestock." Reviews of Reproduction. 5, 46-52.

Davis, R.O. and Siemers, R.J., (1995). Derivation and reliability of kinematic measures of sperm motion. Reproduction Fertility. Dev. 7, 857-869.

Donovan, A., Hanrahan, J.P. and Lally, T., (2001). AI for sheep using frozen-thawed semen. End of project report. Teagase sheep research centre, Athenry, Co. Galway. 43.

Dorado, J., Rodriguez, I. and Hidalgo, M., (2007). Cryopreservation of goat spermatozoa: Comparison of two freezing extenders based on post-thaw sperm quality and fertility rates after artificial insemination. Theriogenology. 68, 168 – 177.

Els, J.F., (1995). Production analysis of a Boer goat flock in the Bush Savanna veld. M.Sc. (Agric.) Dissertation. Department of Animal and Wildlife Sciences, University of Pretoria, South Africa.

Epstein, H. (1971). The origin of the domestic animals of Africa Volume I. Africana Publishing Corporation. New York. London. Munich. 261-263.

Erasmus, J. A., Fourie, A. J. and Venter, J. J., (1985). Influence of age on reproductive performance of the Improved Boer goat doe. South African Journal of Animal Science. 15, 5-7.

Evans, G. and Maxwell C., (1987). Salamon's artificial insemination of sheep and goats. Sydney: Butterworth. 66-76, 158-161.

Fahy, G. M., (1986): The relevance of cryoprotectant "toxicity" to cryobiology. Cryobiology. 23, 1-13.

Gao, D. Y., Mazur, P., Critser, J. K., (1997). Fundamental cryobiology of mammalian spermatozoa. In: Karow, A.M. and Critser, J.K. (eds). Reproduction T Bank. Academic Press, San Diego, CA. 263–328.

Garner, D. L., Thomas, C. A. and Gravance, C. G., (1999). The effect of glycerol on the viability, mitochondrial function and acrosomal integrity of bovine spermatozoa. Reproduction of Domestic Animals. 34, 399-404.

Goto, K., Kajihara, Y., Koba, Y. M., Kosaka, S., Nakanishi, Y. and Ogawa, K., (1989). In vitro fertilization and development of in vitro matured bovine follicular oocytes. Journal of Animal Science.67, 2181-2185.

Graffer, T., Solbu, H. and Filseth, O., (1988). Semen production in artificial insemination bulls in Norway. Theriogenology. 30, 1011-1021.

Hafez, E. S. E and Hafez, B., (2000). Reproduction in Farm Animals. Seventh Edition. Edited by E.S.E Hafez and B. Hafez. Philadelphia: Lippincott Williams and Wilkens. 82-95.

Hammerstedt, R. H., Graham, J. K. and Nolan, J. P., (1990). Cryopreservation of mammalian sperm: what we ask them to survive. Journal of Andrology. 11, 73.

Hofmo, P. O. and Grevle, I. S., (1999). Boar Semen Preservation. Development and commercial utilization of frozen boar semen in Norway. In: Proceedings of the IV International Conference Beltsville, MD, 9.

Holt, W. V., (1997). Alternative strategies for the long-term preservation of spermatozoa. Reproduction Fertility. Dev. 9, 309–319.

Holt, W. V., (2000). Basic aspects of frozen storage of semen. Animal Reproduction Science 62, 3–22.

Hu, J. H., Li, Q. W., Jiang, Z. L., Yang, H., Zhang, S. S., Zhao, H. W., (2009). The cryoprotective effect of trehalose supplementation on boar spermatozoa quality. Reproduction of Domestic Animals .44, 571–575.

Isachenko, E., (2003). Vitrification of mammalian spermatozoa in the absence of cryoprotectants: from past practical difficulties to present success. Reproductive Biomedicine. 6(2), 191-200.

Jane, E. J., William, R. B. and Dawn, W. B., (1996). Manual versus Computer-automated semen analyses. Determination or the working range of a computer-automated semen analyzer. Fertility and Sterility. 65, 156-159.

Johnson, L. A., Weitze, K. F., Fiser, P. and Maxwell, W. M., (2000). Storage of boar semen. Animal Reproduction Science. 62, 143–172.

Karatzas, G., Karagiannidis, A., Varsakelil, S. and Brikas, P., (1997). Fertility of fresh and frozen-thawed goat semen during the nonbreeding season. Theriogenology. 48, 1049-1059.

Karow, A. M., (1981). Biophysical and chemical considerations in cryopreservation: In Organ Preservation for Transplantation. (Eds. A. M. Karow and D. E. Pegg). Dekker, New York 113.

Kathiravan, P., Kalatharan, J., Karthikeya, G., Rengarajan, K. and Kadirvel, G., (2011). Objective sperm motion analysis to assess dairy bull fertility using computer-aided system: a review. Reproduction of Domestic Animals. 46, 165-172.

Kimmes, A., (1992). On Re Âunion Island cross-bred goats provide a traditional product. Che Âvre Lepa, St. Joseph, Re Âunion. 189, 46-49

Knobil, E., and Neil, J.D., (1999). Encyclopedia of Reproduction. New York: Academic Press. 539–545

Kozdrowski, R., Dubiel, A., Bielas, W., Dzieciol, M., (2007). Two protocols of cryopreservation of goat semen with that use of computer assisted semen analysis system. Acta Veterinaria Brno.76, 601-604.

Kundu, C. N., Chakraborty, J., Dutta, P., Bhattacharyya, D. Ghosh, A. and Majumder G. C. (2000). Development of a simple sperm cryopreservation model using a chemically defined medium and goat cauda epididymal spermatozoa. Cryobiology. 40, 117 – 125.

Leibo, S. P., and Songsasen, H., (2002). Cryopreservation of gametes and embryos of non-domestic species. Theriogenology. 57, 303–326.

Malan S.W. (2000). The improved Boer goat. Small Ruminant Research. 36(2), 165-170.

Malo, C., Gil, L., Gonzalez, N., Cano, R., De Blas, I. and Espinosa, E., (2010). Comparing sugar type supplementation for cryopreservation of boar semen in egg yolk based extender. Cryobiology. 61(1), 17-21.

Martínez, R. R. D., Hernández, J., Reyna, L. and Santos, R., (2007). Intrauterine insemination with frozen-thawed semen in Creole goats, synchronized in oestrous during the non-breeding season. Research Journal of Animal Science. 1 (3), 76 − 80.

Matshaba, B., 2010. Characterization and cryopreservation of South African unimproved indigenous buck semen. MSc Dissertation, Bloemfontein, University of Free State.

Matthews, N., Bester, N. and Schwalbach, L. M. J., (2003). A comparison of ram semen collected by artificial vagina and electro-ejaculation. South African Journal of Animal Science. 4, 28-30.

Mazur, P., (1984). Freezing of living cells: mechanisms and implications. American Journal of Physiology. 247,125- 142.

Mcdonald, P., Edwards, R. A., Greenhalgh, J. F. D. and Morgan, C. A. (1995). Animal nutrition. 5th ed. Malaysia. Prentice Hall.

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

Medrano, A., Terrazas, A. and Soto, R., (2010). Principles and perspectives for the conservation of goat buck spermatozoa. Small Ruminant Research. 89, 140 – 143.

Mocé, E., and Graham, J.K., (2008). In vitro evaluation of sperm quality. Animal Reproduction Science. 105:10418.

Naing, S. W., Wahid, H., Mohd Azam, K., Rosnina Y, Zuki, A. B., Kazhal, S., Bukar, M. M., Thein M., Kyaw, T. and San, M. M., (2010). Effect of sugars on characteristics of Boer goat semen after cryopreservation. Animal Reproduction Science. 122(1-2), 23-28.

Pickett, B. W., and Amann, R. P., (19930. Cryopreservation of semen. In: McKinnon, AO and Voss, EL (eds) Equine Reproduction. Lea and Febiger, Philadelphia. 768–789.

Purdy, P. H., (2006). A review on goat sperm cryopreservation. Small Ruminant Research. 63, 215-225.

Rahman, A. N. M. A., Abdullah, R. B. and Khadijah, W. E. W., (2008). A review of reproductive biotechnologies and their application in goat. Biotechnology. 7 (2), 371 – 384.

Ramukhithi, F.V., (2011a). Characterisation and cryopreservation of South African unimproved indigenous and Boer buck semen. M-Tech Dissertation, Pretoria, Tshwane University of Technology, South Africa.

Raseona, A.M., (2005). Comparative evaluation of different extenders of bull semen stored under different condition. Masters dissertation, Thohoyandou. University of Venda, South Africa.

Roberts, S.J., (1971) Infertility in male animals. Veterinary obstetrics and genital diseases. Published by the author, Ithaca, N.Y, United State of America.

Salamon, S. and Maxwell, W., (2000a). Storage of ram semen. Animal Reproduction Science. 62(1), 77-111.

Salisbury, G. W., VanDemark, N. L. and Lodge, J. R., (1978). Semen evaluation: In "Physiology of Reproduction and Artificial Insemination of Cattle." 2nd edition. W.H. Freeman and Compagny, Sanfrancisco, USA. 400-427.

Sambraus, H. H., (1992). Goats. In: A colour atlas of livestock breeds. Eds. Sambraus, H. H., Wolfe Publishing, Germany 137-156.

Shannon, P. and Vishwanath, R., (1995). The effect of optimal and suboptimal concentrations of sperm on the fertility of fresh and frozen bovine semen and a theoretical model to explain the fertility differences. Animal Reproduction Science. 39, 1-10.

Solaiman, S. (2010). Goat Science and Production. Blackwell Publishing: Ames, IA.

Steel, G. and Torrie, M., (1981). Principles and Procedures of Statistics. A biometric approach, 2nd edition. McGraw-Hill Book Co. Inc., New York, United State of America.

Steinmann, H., (1996). Use of trehalose as a cryoprotectant in the freezing of stallion semen. A laboratory study. Thesis, Tierärztliche Hochschule Hannover, Germany.

Thurston, L. M., Siggins, K., Mileham, A. J., Watson, P. F. and Holt, W. V., (2002). Identification of amplified restriction fragment length polymorphism markers linked to genes controlling Boer sperm viability following cryopreservation. Biology of Reproduction.66, 545-554.

Varner, D. D., Blanchard, T.L., Meyers, P.J., Meyers, S.A., (1989). Fertilizing capacity of equine spermatozoa stored for 24 hours at 5 or 20 degrees C. Theriogenology. 32, 515–525.

Varner, D.D. and Johnson, L., (2007). From a sperm's eye view: revisiting our perception of this intriguing cell. Proceedings of the Annual Conference American Association. Equine Practitioners.104–177.

Verstegen J, Iguer-Ouada M, Onclin K., (2002). Computer assisted semen analyzers in andrology research and veterinary practice. Theriogenology. 57, 149-79.

Watson, P.F., 2000, 'The causes of reduced fertility with cryopreserved semen',Animal Reproduction Science 60–61, 481–492. http://dx.doi.org/10.1016/S0378-4320(00)00099-3

Woelders, H., Matthijs, A. and Engel, B., (1997). Effect of trehalose and sucrose, osmolality of the freezing medium and cooling rate on viability and intactness of bull sperm after freezing and thawing. Cryobiology.35, 93-105

Wulster-radcliffe M.C., Wereiams, M.A., Stellflug, J.N. & Lewis, G.S., (2001a). Technical note: AV vs. a vaginal collection vial for collecting semen from rams. South African Journal of Animal Science, 79, 2964-2967.

Yildiz, C., Ottaviani, P., Law, N., Ayearst, R., Liu, L., McKerlie, C., (2007). "Effects of cryopreservation on sperm quality, nuclear DNA integrity, in vitro fertilization, and in vitro embryo development in the mouse." Reproduction. 133, 585-595.

APPENDICES

Significant levels for the results of the analysis of variance of Boer goat spermatozoa motility parameters evaluated at 0h and 120h.

APPENDICES 1: Mean Squares and Degree of Freedom Obtained from the Analysis of Variance for Buck Motility Characteristics.

		SIGNIFICANT LEVELS							
Source of Variations	DF	P (%)	NP (%)	S	V (L %)	V (D %)	M (C H)	M (C)	M (C T)
E	3	NS	*	*	NS	NS	NS	NS	*
S	1	**	NS	NS	*	*	NS	NS	NS
L	1	**	NS	**	**	**	*	NS	NS
E X S	3	NS	NS	NS	NS	NS	NS	NS	NS
E X L	3	NS	NS	*	NS	NS	NS	NS	NS
S X L	1	*	NS	NS	*	*	NS	NS	NS
E X S X L	3	*	NS	NS	NS	NS	NS	NS	NS
ERROR	80								
TOTAL	95								

NS=Non Significant difference(P>0.05),* =Significance (P<0.05), ** =Highly significant(P<0.01), ST=Sugar Type, L=Levels, T=Time, DF=Degree of Freedom, P=progressive motility, NP=Non-

progressive motility, S=Static, V(Live)=Vitality Live, V(Dead)=Vitality Dead, M(CUT H)=Morphology cut head, M(COIL)=Morphology coiled, M(CUT T)=Morphology cut tail.

ST X L X T showed significant difference on progressive motility and non-significant difference NP (%) ST V (L %) V (D %) M (C H) M (C) M (C T). ST X L showed non-significant difference in all above mensioned sperm parameters: P (%) NP (%) ST V (L %) V (D %) M (C H) M (C) M (C T).ST showed significant difference in P (%) NP (%) and S.T had high significant rate in P (%), S (%), V (L %) and V (D %).

Significant levels for the results of the analysis of variance of Boer goat spermatozoa Kinematic features evaluated at 0h and 120h.

APPENDICE 2: Mean Squares and Degree of Freedom Obtained from the Analysis of Variance for Buck Motility Characteristics

		SIGNIFICANT LEVELS			
Source of Variations	DF	VSL	VAP	VCL	STR
E	3	NS	NS	NS	NS
S	1	*	*	NS	*
L	1	**	**	**	*
E X S	3	NS	NS	NS	NS
E X L	3	NS	NS	NS	NS
S X L	1	NS	NS	NS	NS
E X S X L	3	NS	NS	NS	NS
ERROR	80				
TOTAL	95				

NS=Non Significant difference (P>0.05),* =Significance (P<0.05), ** = Highly significant (P<0.01), ST=Sugar Type, L=Levels, T=Time, DF=Degree of Freedom, VCL = Curvilinear velocity, VSL = straight-line velocity, VAP = Average path velocity, STR = Straightness. ST, STXL, STXT, LXT,

STXLXT showed non-difference. T showed highly significant results in VSL, VAP and VCL.L showed significant results in VSL, VAP AND STR.

APPENDICES 3: SPERMATOZOA MOTILITY PARAMETERS

Parameter	Meaning
Level (L)	Level of sugar used
Evaluation Time (EVT)	Time used for evaluating Boer goat semen
Progressive Motility(PM)	Sperm swims forward in an essentially straight line.
Non Progressive motility (NP)	Sperm swims, but with an abnormal path, such as in tight circles.
Morphology cut head M(CH)	Spermatozoa without head
Morphology bent tail M(BT)	Spermatozoa with bent tail
Morphology coiled M(C)	Spermatozoa with coiled tail
Vitality live V(L)	Living spermatozoa
Vitality dead V(D)	Non-living spermatozoa
Straight line velocity(VSL)	Is the average velocity of the sperm head measured in a straight line from the beginning to end of its track (μm/s).
Curvilinear velocity(VCL)	The average velocity of the sperm head through its real path.
Avarage path velocity(VAP)	The average point-to-point velocity of the sperm head along its average track (μm/s).
Straightness(STR)	Measures the departure of the cell path from a straight line. This was assessed using CASA.