EVALUATION OF NGUNI BULL SEMEN EXTENDED IN TRIS EGG YOLK EXTENDER, SOYBEAN MILK AND COCONUT WATER BASED EXTENDERS AND STORED AT DIFFERENT TEMPERATURES

By

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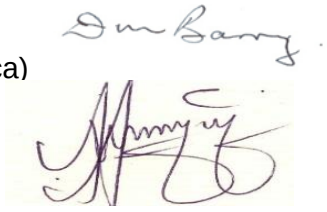

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DECLARATION

I, Pie Veillard Mayombo Kalonji, student number 15004155, the undersigned, hereby declare that the contents of this dissertation submitted for the degree of Master of Science in Animal Science (MScANS) at the University of Venda are my own original work and has not been previously in whole or in part been submitted to any university for the award of other degree.

Student **…….....** **Date** **…….**

M.P.V. Kalonji

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DEDICATION

This work is dedicated to my family.

Thanks for always believing in me and for encouraging me to follow my dreams, whatever it takes. Without your guidance, love and support, I would not have been able to achieve my goals.

To my wife for always being there with your support through the entire time of this study, and without your motivation and trust this would not have been possible.

To my parents for not giving up on me after so many years of education and not stopping to cheer me up, you are the best parents in the world, and I am honoured to be called your son.

To my brothers and sisters, thank you for always keeping me in touch with reality, you all mean a lot to me.

To my other brother, I really miss you but I know that you are happy and smiling down on me where you are now “Sit tibi terra lewis”.

All my love,

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ABSTRACT

In order to realize many of the potential advantages of AI, storage of semen is necessary. Semen storage is only possible using a system that decreases and/or halts the metabolic processes of the spermatozoa, allowing no significant loss of fertility. Numerous factors affect the success of spermatozoa storage. This study was designed to compare the effects of egg yolk, soybean milk and coconut water in Tris extender using different storage methods for Nguni bull spermatozoa storage. Bull semen was collected from two adult Nguni bulls approximately four years old and kept under similar managerial conditions. Using electro-ejaculator, semen was collected from each bull into a graduated semen collection tube. Macroscopically evaluation of the sample was performed immediately after collection. Only the semen free from contamination was processed. The kinetic properties namely: total spermatozoa motility, and progressive spermatozoa motility were analysed using CASA. Semen sample was stained and spermatozoa morphology and vitality also analysed using CASA. The extended semen was then split into three groups. The first group was stored at room temperature (25 °C). The second group was cooled to 4 °C and stored in the refrigerator. The third group was also cooled to 4 °C for 2 h in the refrigerator, then held in LN_2 vapour 5 cm above the surface of LN_2 at ~ -80 °C for 10 min and then plunged into LN_2 for storage at -196 °C. Different colours of straws and plugging powder were used for identifying each extender. After 3 days of storage at room temperature, in the refrigerator and in LN_2, the extended semen was split into three portions and assayed for kinetic properties using the first portion. The second portion was assayed for spermatozoa morphology and the third portion for spermatozoa vitality. The results from the fresh semen extended with all three extenders (TEYE, SBME and COWE), and analysed immediately after dilution at room temperature (25 ºC), showed no significant difference ($P > 0.05$) in the mean values of the kinetic and morphologic properties and viability, on spermatozoa TM, PM, AR, AT, CT; BT and LS. After three days of storage, there was no significant difference ($P > 0.05$) in the kinetic morphologic properties and viability of semen stored at room and refrigeration temperature regardless of the extender in use. There were, however, significant differences ($P < 0.05$) in the TM, PM, AR and DL of the frozen semen samples. For the short storage period of semen used for AI, from this study, it is recommended that semen should be kept at room or refrigeration temperature regardless of the three extenders used. However, for long storage of frozen semen TEYE is

recommended. The egg yolk-based extender provided greater preservation of motility and bull spermatozoa integrity during the freezing process than did SBME and COWE.

Keywords: Tris egg yolk, Soybean-milk, Coconut water, Spermatozoa, Nguni bull and storage methods.

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AI	: Artificial insemination
ATP	: Adenosine triphosphate
AV	: Artificial vagina
BCF	: Beat cross frequency
BIOX	: Bioxcell
CASA	: Computer aided sperm analysis
CE	: Conventional extender
CED	: Cauda epididymal dissection
^{o}C	: Degree Celsius
COWE	: Coconut water based extender
DMSO	: Dimethyl sulfoxide
EE	: Electro-ejaculator
ET	: Embryo transfer
EYC	: Egg-yolk citrate
g	: Gram
h	: Hour
IU	: International unit
IVF	: *In vitro* fertilisation
LN_2	: Liquid nitrogen
mg	: Milligram
min	: Minute
ml	: Millilitre
mm	: Millimetre
P	: Probability

PSM	: Progressive spermatozoa motility
SBME	: Soybean-milk based extender
sec	: Second
SM	: Spermatozoa motility
STR	: Straightness
TEYE	: Tris-citric egg-yolk
TM	: Total motility
Tris	: Tetra-hydroxymethylaminomethane
v/v	: Volume per volume

CHAPTER 1

RESEARCH THEME, HYPOTHESIS AND MOTIVATION

Artificial insemination (AI) had a major impact on animal breeding (Arzondo, *et al*. 2012). By means of AI, a single ejaculate of a bull is used for the insemination of various females, which could not have been possible without sperm extension and/or cryopreservation (Jones, 1976; Rauch, 2013).

Although there has been extensive research done on the subject of bovine semen cryopreservation, most of these studies were completed decades ago (Almquist, 1951; Dunn *et al*., 1953; Graham *et al*., 1956; Rauch, 2013). There has been significant progress in the components used for semen extenders, and also the preparation of these extenders which plays a role in determining the optimum equilibration periods for bovine semen cryopreservation (Almquist, 1954; Bean *et al*., 1963; Berndtson & Foote, 1976; Kumaresan *et al*., 2011). However, bull semen has been cryopreserved for AI for over half a century, many of the protocols used are still empirical, with the number of spermatozoa that do not survive the freezing process being considerable and those that do survive being affected structurally or functionally after thawing. In general, many of the strategies tested to achieve successful cryopreservation do not involve the spermatozoon itself, but the medium in which it is stored (Jones, 1976; El-Harairy *et al.,* 2011).

The aim of this study was to improve the cryopreservation and storage protocols of Nguni bull semen for artificial insemination purposes by determining the effect of egg yolk, soybean-milk and coconut water as extenders on kinetic, morphology properties and viability of spermatozoa during different storage methods. The null hypothesis for both experiments was that egg yolk, soybean milk and coconut water based extenders will not have a significant effect on protecting bull spermatozoa during different storage methods. The alternative hypothesis for both experiments was that there will be significant differences on kinetic and morphology properties of spermatozoa during different storage methods and between egg yolk, soybean milk and coconut water based extenders. The motivation of this study was to define and improve the cryopreservation and storage process of semen for cattle indigenous to Africa, in order to improve the pre-insemination motility of spermatozoa and in turn the conception rates of cows inseminated with the good quality extended semen. This in turn may

lead to farmers using artificial insemination in their herds, for accelerated genetic improvement of these indigenous cattle breeds.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Throughout history man and animals have always competed for nutritional resources. Due to the demands of growing and evolving human population, livestock producers are constantly under pressure to supply more products, from even more limited resources. Livestock producers are therefore continuously searching for ways to improve the production efficiency of their livestock. This includes selecting for animals with better feed conversion ratios, disease resistance and improved reproductive efficiency. These factors can all be improved by genetic selection, in other words selecting and breeding only superior animals. This is known as genetic improvement (Salisbury *et al.,* 1978; Bailey *et al.,* 2000; Vishwanath, 2003; Bozkurt *et al.,* 2011). For genetic improvement to take place in any population there has to be an increase in both the gene frequency and gene combinations of the desired genes. This is achieved by selecting individuals possessing the desired traits and then manipulating their breeding. Artificial insemination has contributed enormously to the genetic improvement of beef and dairy cattle in the last 50 years. Frozen semen in 0.5 ml or 0.25 ml straws has become the universally accepted unit of storage and transfer of bovine genetics to cattle producers (Baracaldo *et al*., 2007).

Cryopreservation of semen would be to minimize damage resulting from the freezing and thawing procedures and to maximize recovery of motile and viable spermatozoa used for AI. Many scientific studies have been carried out to improve the quality of frozen-thawed semen. For this purpose, different cryoprotective agents, freezing and thawing protocols, and various extenders have been used (England, 1993; Silva *et al*., 2002; 2003; Walters *et al*., 2009).

2.2. History of AI and semen cryopreservation in cattle

Research on artificial insemination (AI) dates back several centuries. The first scientific step in AI was the discovery by Leeuwenhoek in 1677 used a simple microscope to view the small motile cells which he named “animalcules”. This discovery was the cornerstone of any further research or discoveries that led to the development of current assisted reproductive

technologies. The next notable event was the first documented case of AI which occurred more than 100 years later in 1784, when Lazzaro Spallanzani performed the first successful AI. He inseminated a confined bitch in heat by depositing semen in the uterus with a syringe. This resulted in the birth of three pups 62 days later that resembled the dam as well as the semen donor. Spallanzani later also found that when semen is filtered, fertilization only occurred when the residue (sperm) left on the filter was used and not when the filtrate (seminal fluids etc.) was used. He also observed that when equine semen was cooled the sperm weren't killed, but only remained motionless until the temperature was increased. Another century passed before the use of AI was also reported by Walter Heape (1897) and several other researchers in independent studies on horses, dogs and rabbits. Walter Heape was also credited for establishing the basics for the relationship between reproduction and seasonality (Foote, 1999; 2002; Walters *et al*., 2009).

Later in 1899, Ivanoff was credited with initial attempted to establish AI as a breeding practice. By 1907 he had applied AI in the breeding management programmes for rabbits, dogs, foxes, poultry and domestic farm animals. As part of his research he trained technicians as inseminators and developed semen extenders to prolong the life of sperm. Ivanoff's work also stimulate interest in AI outside Russia. In 1912, Dr. Ishikawa, a scientist who studied with Ivanoff, started an AI programme in Japan with horses. Later similar programmes followed for poultry, swine, goats, sheep and cattle. Milovanov later succeeded Ivanoff and established projects for the breeding of cattle and sheep in Russia. Milovanov also designed and made practical versions of the artificial vagina, specifically for these species as well as other practical equipment for semen collection. Many apparatus currently used are still based on these designs (Foote, 2002). The artificial vagina used in AI today was first devised by Amantea in 1914. It was originally meant for the collection of dog semen, but was quickly adapted for use in bulls, stallions and rams. Gunn (1936) experimented with using the electro-ejaculator to collect semen from male animals. This is an alternative method used when animals are not trained for semen collection using the artificial vagina (Salisbury *et al.,* 1978; Foote, 1999; El-Sisy *et al*., 2016).

Although the discovery of AI had occurred much earlier, it was not until the mid-1930's that all the necessary ingredients for the successful commercial applications thereof, were available. There ingredients include an economic need to improve milk yield in dairy cows, as well as a measurement system for assessing the phenotypic expression of milk-producing capacity of cows and several other factors (Salisbury *et al*., 1978; Akhter *et al*., 2011). During

the period of the 1940's and 1950's, there were various revolutionary discoveries regarding the semen cryopreservation process, as well as the storage environment for cryopreserved semen. This, together with new methods of sire selection, resulted in the application of AI to herds in remote regions that previously did not have access to viable collected semen (Vishwanath, 2003). When the first calf emanating from AI with cryopreserved semen was born in 1951, the technique was already starting to be incorporated into commercial breeding management programmes, and it established itself rapidly thereafter (Curry, 2000). With time, the process of AI became more accessible, and by the next decade, the majority of replacement stock was the result of applying AI (Vishwanath, 2003; Walters *et al*., 2009).

Several discoveries and developments played crucial roles in establishing the basic principles for the freezing of bovine semen. Phillips developed a phosphate-buffered egg yolk based extender in 1940, and a year later Salisbury *et al*. (1941) improved on this by using a citrate buffer that resulted in a clearer medium. This allowed for more accurate and critical sperm evaluation (Foote, 1999). A few years later, two independent reports by Foote and Almquist (1948) described the remarkable results achieved when adding antibiotics to the semen extender. When Polge *et al*. (1949) discovered the cryopreservative properties of glycerol; it was the beginning of a new era in cryopreservation of semen. Semen could now be frozen and stored for long periods, while still yielding acceptable fertility results.

The discovery of protective agents within egg yolk and glycerol was a major milestone in sperm cryopreservation. These agents protected bovine sperm during cooling and freezing procedures and resulted in increased survival rates (Medeiros *et al*., 2002). Over the last few decades research has concentrated on the cryopreservation of bovine sperm, but this did very little to advance the preservation of any other body cells. This is partly due to the slow realization that bovine sperm cells are unique in its composition, and a lack of methods to measure damage caused by cryopreservation to sub-cellular compartment (Hammerstedt *et al.,* 1990; Walters *et al*., 2009).

2.3. Advantages and disadvantages of AI and the cryopreservation of semen

Some factors affecting the success rate of AI generally include herd management (including the nutritional status of the females), seasonal effects on reproduction and the quality of semen used (Kathiravan *et al*., 2011). These factors should be managed and controlled in such a way that the advantages of AI application within the herd will be fully utilised (Haugan *et al.,* 2005; Van Staden, 2010). The most obvious advantages of applying AI

in a herd include genetic improvement, increase of productivity, control of venereal diseases, as well as reduced cost (Vishwanath, 2003). When compared to other reproductive technologies, it becomes apparent that AI is very simple, yet successful and economical method that can be applied to introduce new genes in a population (Vishwanath, 2003; Kathiravan *et al*., 2011). The presence of lethal genes can also be reduced and genetic improvement of milk production traits by the selection of bulls used in AI programs can be seen as benefits on their own. However they also collectively lead to overall economic benefits for the dairy industry (Foote, 1999). Sperm cryopreservation is then also used to build fertility reserves for endangered species (Ehmcke & Schlatt, 2008).

The ability to control the transmission of venereal diseases possibly had a large effect on the initial decision to apply AI commercially than the genetic advantages. However careful and continuous monitoring of health status of donor sires, and regular disease testing of their semen is of critical importance. If this is not done efficiently, AI can become a very effective tool for transmitting diseases to herds worldwide (DeJarnette *et al.,* 2004). Much of the labour and money saved by not having to keep bulls on site may have to be spent in the detection of cows in oestrus and the restraint on these cows for Ai. As dairy cows are confined for milking each day, some of these costs are more easily justified in a dairy, compared to a beef farm (Salisbury *et al.,* 1978). Another limitation of semen cryopreservation is that only matured spermatozoa can be preserved, and thus sperm from pre-pubertal males cannot be preserved with any degree of success (Ehmcke & Schlatt, 2008).

2.4. Economic implication of AI in cattle

Effects resulting from the commercial implementation of AI, are most prominent in the dairy cattle industry. The overall result of implementing AI is accelerated genetic progress, resulting in improved production capabilities of the animals (Medeiros *et al*., 2002). Improving the production efficiency of dairy herds has the benefit of satisfying both producer and consumer. The increased demand for inexpensive product of acceptable quality is satisfied, and a higher profit potential for producers is generated. (DeJarnette *at al*., 2004; Kathiravan *et al*., 2011). Depending on the reason why AI or semen cryopreservation is implemented, the economic implications of these procedures will differ. The various reasons for preservation of male fertility may lead to different approaches, depending on the discipline of interest (Ehmcke & Schlatt, 2008).

2.5. Structure and functional characteristics of spermatozoon

Spermatozoa have certain characteristics that give them the distinction of being “terminal cells”. They are very susceptible to injury and are usually the preferred cells used in research into optimal cryopreservation procedures (Suarez, 2007). Other characteristics of spermatozoon include being haploid and containing very little cytoplasm and other organelles are the chromosomes within the nucleus are very condensed, which prevents protein transcription taking place (Hu, 2010). The minimal quantity of endoplasmic reticulum present on the Golgi apparatus is not sufficient to maintain membrane molecule to supply building blocks to maintain cell membranes and undergo maturational changes (Medeiros *et al.,* 2002).

2.5.1. Structure of spermatozoon

The fully formed spermatozoon is an elongated cell consisting of a flattened head containing the nucleus and a tail containing the apparatus necessary for cell motility (movement). The major feature of the sperm head is the oval, flattened nucleus consisting of highly compacted and condensed chromatin which contains DNA. The DNA is complexed to a special class of basic proteins called spermatozoa protamines (Garner & Hafez, 1993).

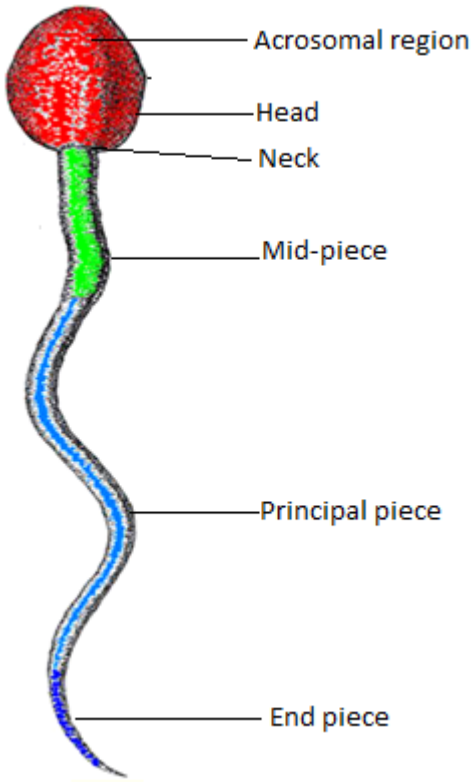

Figure 2.1. Structure of Spermatozoon (Ajao, 2015)

A spermatozoon is entirely covered by a plasma membrane, identical to somatic cells. The study of the spermatozoon membrane function status is of particular importance since an intact and functionally active membrane is required for metabolism, capacitation, acrosome reaction, attachment and penetration of the oocyte (Tardif *et al*., 1998). The spermatozoon can be divided in several membrane domains and subdomains, depending on the function. The domains of the spermatozoa head include the acrosomal and the postacrosomal region (Anzar *et al*., 2011). The plasma membrane can be divided into an acrosomal cap and an equatorial subdomain. The latter is separated by the posterior ring from the neck region of the midpiece. Besides the specialization in function of the different domains of plasma membranes, the lipids and proteins of the plasma membrane vary between different parts of the spermatozoon (Correa *et al*., 1997; Rauch, 2013).

2.5.2. Composition of spermatozoa plasma membrane

The spermatozoon plasma membrane consists of a phospholipid bilayer with cholesterol, complex carbohydrates and proteins, typical for plasma membranes. The carbohydrate structures are bound to proteins or specific lipids on the outside of the plasma membrane (glycocalyx) (Tiwari *et al.,* 2008). The phospholipids in the spermatozoa plasma membrane vary between mammalian species, but generally include phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), sphingomyelin, lysophosphatidylcholine and cardiolipin (Anzar *et al*., 2011). In contrast to other species, spermatozoa from bull have a high ratio of PC to PE. The proteins constitute about 50 % of the total membrane weight and can be either peripheral to or integrated in the plasma membrane (Gürler *et al*., 2015).

The cell membrane is described as a mosaic of different degrees of localized fluid areas that are also called lipid domains. These domains consist of certain lipids (mainly phospholipid and sterols) with certain functions. The lipids and proteins are mobile and able to move laterally in the plane of the membrane (Tiwari *et al.,* 2008). At room temperature, the membrane lipids are generally in a fluid (liquid crystalline) phase, but some domains contain lipids in the gel-phase. In the gel-phase, the lipids are more clustered and less mobile (Anzar & Graham, 1996). More recent reports claimed that the proteins in plasma membrane are predominant, cause less fluidity and therefore there is less lateral diffusion of the lipids (Gürler *et al*., 2015). Further, the degree of fluidity depends on the type and amount of the present lipids. For example, long chain polyunsaturated fatty acids in the phospholipids as well as a

smaller amount of cholesterol increase the fluidity of the membrane at room temperature (Rauch, 2013).

2.5.3. Role of plasma membrane in spermatozoa function

The assessment of spermatozoa membrane functional status appears to be a significant marker for the fertilizing capacity of spermatozoa (Correa *et al*., 1997). Concurrent with the acrosomal membrane, the spermatozoa plasma membrane has an impact on the spermatozoa shape and volume, motility, energy production, penetration, capacitation and acrosome reaction, and interaction with the oocyte. The spermatozoon membrane changes its lipid composition and location of the lipid domains during the physiological events before fertilization occurs (Correa *et al*., 1997; Kumaresan *et al.*, 2011).

2.5.3.1. Spermatozoa motility

Spermatozoa motility requires adenosine triphosphate (ATP) which is produced by mitochondria (10 %) and by anaerobic glycolysis in the spermatozoon tail (90 %). Thus, an intact transport mechanism of monosaccharides from the extracellular environment into the spermatozoon is essential for flagellar movement (Kumaresan *et al.*, 2011). Specific plasma membrane proteins enable the transport of glucose and fructose into the spermatozoa. These transporter systems require an intact plasma membrane and a specific chemical gradient of ions and soluble components to function properly (Singh *et al*., 2013).

2.5.3.2. Changes during capacitation

Ejaculated spermatozoa of mammalian species are not ready to fertilize an oocyte immediately, and have to be in the female reproductive tract for a period of time. This period is species-specific, and is regulated by female's hormone status in order to prevent gamete ageing (Medeiros *et al.*, 2002). In 1951, Austin and Chang independently discovered that spermatozoon has to undergo a process called capacitation within the female reproductive tract, before it could fertilize an oocyte. It was determined that the acrosome reaction could only take place if capacitation had already occurred, and it became standard practice to use the acrosomal reaction as an endpoint in determining whether spermatozoa were fully capacitated (Curry, 2000; Medeiros *et al.*, 2002; Jones *et al.,* 2007).

The process of capacitation is still not clearly defined, but it is accomplished by acquisition of the ability of the spermatozoon to fertilize an oocyte. The initial event of capacitation includes a rise in intracellular calcium, bicarbonate, and hydrogen peroxide (Medeiros *et al.*, 2002). This triggers the production of cyclic adenosine monophosphate (AMP; cAMP) which in turn causes the tyrosine phosphorylation of proteins in the plasma membrane and in the cytoplasm of the spermatozoa (Curry, 2000). Besides a change in conformation and dimerization of membrane proteins, tyrosine phosphorylation is linked to increased zona pelucida affinity, spermatozoa hyperactivity and the induction of acrosome reaction. Hyperactivation of the spermatozoa occurs *in vitro* at some point during capacitation, but the processes leading to hyperactivation and acrosomal responsiveness can happen independently (Kumaresan *et al*., 2011). In addition, so-called decapacitating factors (substances coating the spermatozoa surface) are removed from the plasma membrane during capacitation. The coating factors originating from the seminal plasma are also known as bovine seminal plasma proteins (BSPP) or phospholipid binding proteins, or binder of sperm proteins (Jones *et al.,* 2007). Their removal of BSPP is essential for fertilization. Another key event of capacitation is the efflux of cholesterol from the plasma membrane. This leads to reorganization of lipids within the plasma membrane and in an increase in membrane fluidity (Jones *et al.,* 2007; Kumaresan *et al*., 2011).

2.5.3.3. Changes during acrosome reaction and fertilization

The acrosome is located underneath the spermatozoa plasma membrane in the anterior head region and consists of a protein matrix that is enclosed by an inner and outer acrosomal membrane (Kumaresan *et al*., 2011). The conformational changes in tyrosine phosphorylated transmembrane proteins enable the binding of the spermatozoa to the zona pelucida (ZP3 proteins) of the oocyte. As a result of the phosphorylation, receptors aggregate in the spermatozoa plasma membrane and induce a calcium ion flux into the spermatozoon (Muin˜o *et al.,* 2008). The proteins involved form bridges between the outer acrosomal membrane and the apical spermatozoa plasma membrane (Muin˜o *et al*., 2008). The acrosomal matrix swells and both the plasma membrane and the outer plasma membrane are dissolved by vesiculation and fusion (Mogielnicka-Brzozowska & Kordan, 2011). This fusion is accomplished by the destabilization of the plasma membrane during capacitation. Subsequently, hydrolytic enzymes from the acrosomal matrix are released which dissolve the

cumulus cells and the zona pelucida, and enable the penetration of the sperm into the perivitelline space of the oocyte (Kumaresan *et al*., 2011).

2.6. Spermatozoa plasma membrane changes during cryopreservation

The spermatozoa plasma membrane is drastically altered by cryopreservation. There are several mechanisms proposed on how cryopreservation leads to spermatozoa damage. One mechanism involves lateral lipid rearrangement which destabilizes the membrane (known as phase transition during cold shock (Muin˜o *et al*., 2008). The lipid components (mainly phospholipids and sterols) of the plasma membrane undergo reorganization during the cooling process. In detail, the lipids in the bilayer start aggregating in different lipid domains, which results in new associations between proteins and lipids in the membrane (Jones *et al.,* 2007). Therefore, the fluidity of the plasma membrane decreases during cryopreservation which results in transition from liquid crystalline to gel phase. The temperature at which the phase transition occurs is specific for each particular lipid (Eidan, 2016). The shorter the fatty acyl chain and the more *cis*-unsaturated carbon bonds, the lower the phase transition temperature. Similarly, cholesterol decreases the phase transition temperature (Rauch, 2013; Eidan, 2016).

Freezing can further promote clustering of the lipids. Rewarming of the spermatozoa does not initially return lipids in their pre-cooling state. It was hypothesized that over time the original assembly could be achieved by lipid diffusion (Cheema *et al*., 2016). The reorganization of lipids also affects the proteins, whereas integral proteins become clustered and subsequently can lose their functionality. Another phenomenon related to cryopreservation is the change in membrane lipid composition. In boar sperm, sphingomyelin (a phospholipid) and the saturated fatty acids content of the phospholipids decreased during freezing, whereas the content of cholesterol did not change (Eidan, 2016).

Another mechanism that influences the plasma membrane constitution is the peroxidation of membrane lipids as a result of formation of reactive oxygen species. The high content of polyunsaturated fatty acids in the spermatozoa membrane increases the susceptibility of spermatozoa to peroxidation if oxygen is present (Divyaswetha *et al.,* 2008). The consequences of membrane peroxidation on spermatozoa include the irreversible loss of motility, impaired metabolism, damage to the plasma membrane, leakage of intracellular enzymes, and decrease in oocyte penetration and fertilizing capacity (Divyaswetha *et al.,* 2008). The consequences of the changes in lipid fluidity, lipid composition and lipid damage

lead to membrane destabilization and the spermatozoon becomes more susceptible to premature acrosome reactions (Curry, 2000). Further, functional proteins like ion channel proteins are affected by the changes during cooling, which is linked to a general leakiness of the spermatozoa membrane. Consequently, the decrease of spermatozoa motility and metabolism after cooling could be attributed to the loss of cations and enzymes (Singh *et al*., 2013).

Since calcium channels are affected, the intracellular calcium level increases and results in a decline in spermatozoa motility and spermatozoa necrosis. The increased calcium level and the reorganization of the plasma membrane during cooling also occur during the physiologic process of capacitation (Correa *et al*., 1997). Further, capacitation and changes during cooling and cryopreservation of spermatozoa have the production of reactive oxygen species and the phosphorylation of proteins in common. Due to these similarities, the damages during cryopreservation are often referred to as “capacitation-like changes” (Rauch, 2013). These induced alterations render the spermatozoa to a partially capacitated state and decrease their life span (Eidan, 2016).

2.7. Principles in semen cryopreservation

There are certain principal factors that should be kept in mind during the process of extending and cooling of semen. These factors will affect sperm independently, but interactions between these factors also affect the sperm. Many of these interactions have been studied, which have led to combining different procedures. The result was the development of “systems” for semen preservation (Saacke, 1983). Some of these factors involved ambient temperature, energy source, osmotic pressure and electrolyte balance, buffering and pH, proper gas phase, inhibition of microorganism growth, exclusion of toxic materials, the dilution effect and protection of against the cold (Perry *et al.,* 2017).

2.7.1. Semen temperature

As the metabolic rate of cells are generally proportional to the absolute temperature, semen is kept at lowered temperature (usually 5 ºC) during storage, as this slows down the semen to 5 ºC must be controlled to prevent the occurrence of cold shock (Bearden *et al*., 2004). The metabolic rate is not the only factor affected by the lower temperature, as the temperature decreases, but both the internal and external environments of cells undergo changes (Correa *et al*., 1997). So for instance gas solubility for environmental gases increase

as the temperatures decreases. As decreased temperatures as a large part of the total metabolic activities are due to the oxidative metabolism, because of the presence of the air and increased solubility of gases at these temperatures (Cheema *et al*., 2016).

2.7.2. Energy source

Spermatozoa do not only require energy for motility, but also for cell maintenance. Spermatozoa can utilize energy sources through both aerobic and anaerobic metabolism, as the energy source naturally available in seminal plasma is fructose, diluted greatly during semen extension. Thus a supplementary source of energy such as glucose or fructose should be added to the extender. It is known that egg yolk contains several compounds suitable for use as energy sources by bovine sperm (Bearden *et al*., 2004).

2.7.3. Osmotic pressure and electrolyte balance

Spermatozoa are capable of adapting in size according to the tonicity of different media. Cell permeability also differs for various substances. Although spermatozoa are tolerant towards the tonicity of media, if this deviates too much it could result in spermatozoa having bent tails, swimming in circles and ultimately dying (Salisbury *et al.,* 1978). Solutions with compositions similar to that of body fluids, like egg yolk and milk, seem to be the most compatible with spermatozoa (Salisbury *et al.,* 1978; Bearden *et al.,* 2004).

2.7.4. Buffering and pH

Spermatozoa need to be protected from auto-toxication from acids that build up as a product of the metabolism. This effect is more pronounced if semen is not kept at a lower temperature, which decreases the rate of metabolism (Mogielnicka-Brzozowska & Kordan, 2011). Extenders containing egg yolk or milk usually have a pH of approximately 7, but may even be beneficial to reduce the pH to 6.5 (Bearden *et al*., 2004).

2.7.5. Proper gas phase

As the cells' permeability for gases increase with decreased temperature, the proportion of gas liquid phase, as well as the composition of the gas phase should be controlled to maintain the desired gas conditions within the extender (Almquist, 1948). Care must also be taken to limit exposure to light, as such exposure in the presence of oxygen

damages the spermatozoa. Gassing with nitrogen will be beneficial to the cell (Salisbury *et al*., 1978; Mogielnicka-Brzozowska & Kordan, 2011).

2.7.6. Inhibition of microbial growth

The AI industry relies upon the use of antibiotics in semen extenders to control contaminating organisms in bull semen (Almquist, 1948; Contri *et al*., 2014). While there are many positive aspect for the use of natural products such as egg yolk and milk as additives, there are also downsides to their use, as part of the extension media. These products provide a fertile breeding ground for microorganisms, which produce many products harmful to spermatozoa, or that have the potential to infect the cows. This is one of the reason why it is standard procedure for antibiotics to be included in any medium used for the extension of bovine semen (Salisbury *et al.,* 1978). Although many of these organisms are not pathogenic, they compete with spermatozoa for nutrients and their by-products are toxic to spermatozoa. This reduces the livability of spermatozoa and reduces fertility (Foote & Berndtson, 1976; Contri *et al*., 2014).

2.7.7. Exclusion of toxic substances

Any extender should be prepared to be free of any and all harmful substances like heavy metals. These substances should also prevent the formation of any toxic products during storage (Urata *et al*., 2001). Even though care is taken to minimize exposure to toxic products, some products, such as glycerol or dead spermatozoa, cannot be totally eliminated. Glycerol, a cryoprotective agent that protects cells during freezing, can be toxic to sperm at high levels and at room temperature (Amann & DeJarnette, 2012). Dead spermatozoa also provide amino acid oxidase, which produces hydrogen peroxide (H_2O_2), a toxic substance to sperm (Salisbury *et al*., 1978; Amann & DeJarnette, 2012).

2.7.8. Semen extension and dilution effect

Semen should be extended in such a way that the initial spermatozoa concentration is reduced to a number that yields optimum result (Tirpak *et al*., 2015). The dilution (or extension) lowers the motility of spermatozoa, but that the addition of certain amino acids and macromolecules, such as egg albumin or casein, minimizes this effect (Vishwanath, 2003). Rottensten *et al.* (1960) found that if egg yolk or milk was included in the extender, the effect of spermatozoa motility was almost prevented.

2.7.9. Protection of spermatozoa against cold shock

Spermatozoa are subjected to cold shock when they are cooled down to 5 ºC, and this causes intracellular enzymes and other material of the cell to leak out, as well as decreased motility (Rego *et al*., 2015). Cold shock also causes the flagella of spermatozoa to bend. It is not known what the exact mechanisms occur at different rates both internally and on the surface of the cell, that results in both physical and chemical damage (Rego *et al*., 2015). Lecithin, proteins, lipoproteins, milk and egg yolk have proved to be the most effective agents in preventing damages of the spermatozoa caused by cold shock (Salisbury *et al.,* 1978; Rauch, 2013). The application of consistent, stringent quality control standards is essential for supply the best possible semen to the breeders, thus individual batches of semen should always be frozen using the best known cryopreservation procedures (Foote, 1975; Tirpak *et al*., 2015).

2.8. Extenders used in this study

2.8.1. Egg yolk and egg yolk extenders

The use of egg yolk in extenders dates back to 1939, when Phillips discovered its protective effect on cooled bovine semen (Rego *et al*., 2015). Van Denmark *et al*. (1957) and Foote (1975) contributed to modern egg yolk extender recipes by finding that 16%, 20% and 24% chicken egg yolk in the freezing extender is favorable. Dried chicken egg yolk consists of 63% lipids and 33% proteins. The fresh egg yolk can be fractionated into 78% plasma and 22% granules. The granules contain 16% high density lipoproteins (HDL), 4% phosvitin and 2% low-density lipoproteins (LDL) (Rego *et al*., 2015). In the yolk plasma, the main component is LDL (66%), followed by livetins (10%). Phosvitin is a highly phosphorylated protein with bactericidal and antioxidant properties. An egg yolk extender provides protection for cooled and cryopreserved bovine spermatozoa. It is now the most commonly used semen extender worldwide for bulls (Akhter *et al*., 2011).

It is widely accepted that the protective agent in the egg yolk is a phospholipid moiety of the low-density lipoprotein fraction (El-Sisy *et al.,* 2016). Thus, there are several mechanisms proposed on how the LDL fraction decreases damage to the spermatozoa during cooling and cryopreservation. One possibility is that the LDLs, particularly the phospholipids, associate with the spermatozoa membrane and thereby provide stabilization (Kathiravan *et al*., 2011). Another possibility is that the phospholipids lost during spermatozoa cryopreservation

are replaced by phospholipids of the egg yolk (El-Sisy *et al.,* 2016). However, Quinn *et al.* (1980) and Ricker *et al*., (2006) did not observe that the added phospholipids were integrated into the spermatozoa membrane. The most recent hypothesis is that the LDLs bind detrimental BSP proteins that are responsible for the efflux of cholesterol and phospholipids from the spermatozoa membrane (El-Sisy *et al.,* 2016).

2.8.2. Soybean milk based extenders

The use of vegetal extenders for preservation of live cells has been reported for a long time. Different types of vegetable products such as tylose, sodium alginate, agar-agar, semi-hydrolyzed starch, dextrin, flax and quince seed, as well as soybean milk were some of the first vegetable liquids used as semen extenders reported as good alternatives extenders for bovine semen (Vale *et al.,* 1997; El-Sisy *et al*., 2016). Fukui *et al*. (2008) found that egg-yolk can be replaced by soybean-milk in semen extenders, without any deleterious effects of spermatozoa during cryopreservation.

2.8.3. Coconut water based extenders

In 1987, coconut water was first used as semen extender during refrigerated storage of goat semen. During the last decade, researchers developed a technology to dehydrate coconut water and produce a stable and standardized powder (ACP®) (Viveiros *et al.,* 2008). Some vegetables derivate like coconut water and tomato juice seem to have some properties to protect bovine and caprine spermatozoa (Vale *et al.,* 1997). Coconut water has shown to be an excellent alternative for conservation of bovine and swine semen as well as caprine semen due the presence of phytohormones (Nunes *et al*., 1996). This substance protects the semen of this species against the deleterious effect of the enzyme phospholipase A, secreted by the Cowper's glands and present in the ejaculated semen (Nunes *et al*., 1996; Boonsorn *et al.,* 2010). Moreover, the *indole-3-acetic* appeared to be the main spermatozoa protective substance present in the coconut water (Vale *et al.,* 1997; Boonsorn *et al.,* 2010).

2.9. Fresh and frozen semen

Successful cryopreservation of animal semen have the potential to enhance the advantages of applying AI. However, in order to achieve this, the result have to be comparable to that of fresh semen (Bailey *et al*., 2000; England & Millar, 2008). In order to achieve success when applying AI with frozen semen, the damaging effects on the cells caused by

freezing procedures must be reduced to a minimum. This is generally a complex problem, as these effects can occur during any of the procedures of semen extension, equilibration and freezing (Fleisch *et al*., 2017). Therefore, all procedures should be kept as simple and straightforward as possible. Cryopreservation of bovine semen is good example of the potential success that can be achieved with cryopreserved semen (Curry, 2000; Amirat *et al*., 2005; Fleisch *et al*., 2017). Even though there has been considerable progress in semen cryopreservation; both the viability and fertility of semen still deteriorated during the cryopreservation process. This deterioration is a result of numerous cellular injuries occurring throughout the process. These injuries have to be minimised, in order to improve the survival rate of spermatozoa, which could thus result in higher fertility rates obtained (Medeiros *et al*., 2002; Fleisch *et al*., 2017).

Many researchers have reported a post thaw motility of 50 % of a fresh semen sample motility to be acceptable. This has been achieved in number of species. Regardless, the number of spermatozoa needed per successful insemination is still variable between species. In some species, the fertility rates of cryopreserved semen can be comparable to that of the fresh semen, if the number of spermatozoa per insemination is high enough (Curry, 2000; Medeiros *et al.,* 2002; Chaveiro *et al*., 2006). In 1995 Shannon and Vishwanath reported that to achieve fertilization rates in cattle comparable to that of fresh semen, the number of cryopreserved spermatozoa needed per insemination was 8 times more than the required number for fresh semen AI (Bailey *et al*., 2000; Akhter *et al*., 2016). When Shannon (1978) determined the number of fresh and frozen bovine spermatozoa needed per insemination to yield comparable fertility rates in the 1970's, it was clearly demonstrated that 10 times more frozen spermatozoa were required to yield the same result as a dose of fresh spermatozoa. Although the total number of spermatozoa required per fresh and frozen insemination dose has decreased from 2.5 million and 25 million in 1978 to 1 – 1.5 million and 10 – 15 million, respectively (Vishwanath, 2003). The fresh: frozen spermatozoa ratio still remained 1 to 10 (Holt, 1997). Matter *et al*. (1969) reported a poor *in vivo* survival rate of spermatozoa that were not irreversibly damaged by the cryopreservation process, as a possible reason for the vast difference in spermatozoa numbers required for fresh and frozen semen AI.

2.10. Effect of cryopreservation on spermatozoa

It is a well-known fact that freezing of spermatozoa causes structural damage. This is a major reason why progress in developing methods for the cryopreservation of sperm was slow

(Medeiros *et al*., 2002). Reduced fertility of frozen semen is attributed largely to altered membrane structure and function during cooling, freezing and thawing. The nature of this damage remains unclear, but it has been suggested that membranes are compromised due to reordering of membrane lipids during cooling and rewarming, thereby disturbing the lipid-lipid and lipid-protein associations required for normal membrane function (Poulos *et al*, 1973; Hammerstedt *et al*., 1990; Campanholi *et al*., 2017).

Cryopreservation of spermatozoa is associated with an oxidative stress (Mazur *et al*., 2000). Superoxide dismutase activity, a scavenger of the superoxide radical, is decreased in frozen/thawed spermatozoa (Bilodeau *et al*., 2000; Cheema *et al*., 2016). However, in 1979 Jones and Stewart found the cooling of spermatozoa to 5 ºC cause the acrosomal head to swell. The freezing and thawing of spermatozoa also caused the acrosome to rupture as well as causing damage to the middle piece in a large number of spermatozoa. Cryopreserved sperm display signs of being in an advanced stage of capacitation, prior to the freezing process (Medeiros *et al.,* 2002). Nishizono *et al.* (2004) reported that cryopreservation induces the morphological abnormalities including mitochondria with an increased relative area of the matrix, thickening of the outer membrane and swelling with loss of cristae.

The biggest problem encountered when freezing any cellular structure is the phase changes that all membranes and cell contents undergo. As the temperature lowers, eventually the lipid phase changes into solid phase and this causes structural changes (Januskauskas & Zilinskas, 2002). The lipids transform into solids, ice or crystals, which can cut through membrane. As the external solvents change a liquid to solid phase, the concentrations of other solutes increase dramatically in the remaining liquid. The entire cell must then respond to all these changes in the limited time allowed by the protocol used (Campanholi *et al*., 2017). During the thawing process, the cell again has to go through a reverse process, where the solid phase changes into a liquid phase. All of the above mentioned changes underline the importance of identifying a rate of temperature change that allows water and cryoprotectant movement without causing intracellular crystal formation or any irreversible membrane changes (Januskauskas & Zilinskas, 2002; Heise, 2012).

Many factors that seem to play a role in the success of cryopreservation of semen of different species have been studied. The species with lower cholesterol levels in the cell membranes (including bovine and ovine) were more susceptible to membrane damage during cryopreservation (Akhter *et al*., 2016). White (1993) also found that spermatozoa from species

with a higher ratio of unsaturated fatty acids (bull and ram), are more sensitive to lower temperatures. Another possible factor is the reactive oxygen species (ROS), which is thought to play role in the initiation of the capacitation process (Medeiros *et al*., 2002; Griffin, 2004; Fleisch *et al*., 2017).

As spermatozoa are not adapted to endure extreme temperatures, many of these spermatozoa are injured during the cryopreservation process. There are different mechanisms during each of the phases, resulting in different injuries to the cells. The spermatozoa injuries can be divided into two main groups: spermatozoa injuries occurring during cooling and due to cold shock, and cellular injuries inflicted during freezing (Hammerstedt *et al*., 1990). The whole practice of bovine semen processing can essentially be divided into 5 steps. Step 1 is the extension of semen and cooling to 5 ºC. Step 2 includes the addition and equilibration period of glycerol, as well as the packaging. Step 3 is the freezing. Step 4 the semen storage and step 5 the thawing process (Campanholi *et al*., 2017). Each of these steps has specific effects on the spermatozoon membrane and metabolism. Step 1 causes changes in the physical properties of the cell membrane due to temperature changes. Step 2 causes a large change in cell volume to which cells have to adapt, while step 3 causes further modifications to both the spermatozoa volume and membrane structures in a very limited period of time. Step 4 represents a dormancy period of the cell and step 5 requires the spermatozoa membranes to recover from changes caused by previous steps to expand back to normal (Hammerstedt *et al*., 1990; Medeiros *et al*., 2002).

Between the ejaculation of semen and fertilization of an oocyte there are however several phases where man can influence the ability of spermatozoon to successfully fertilize an oocyte. These phases include the evaluation of initial semen quality, extension, storage and thawing (Foote, 1975; Campanholi *et al*., 2017).

2.11. Protection of spermatozoa against cold shock

The cold shock phenomenal could be explained as the irreparable damage to several areas of an individual spermatozoon and it can be observed as a permanent loss of motility in the thawed spermatozoa. Cold shock usually takes place when the semen temperature rapidly decreases from approximately body temperature of the bull (37 ºC), to the freezing temperature of water (0 ºC) (Bailey *et al.*, 2000; Medeiros *et al*., 2002; Akhter *et al*., 2016). Cold shock injury is damage to cell structure and function resulting from a sudden reduction in

temperature. Spermatozoa incorrectly cooled to 5°C are subject to cold shock, which results in structural and biochemical damage. In particular, membranes lose their selective permeability with the result that many cellular components are released including lipids, proteins and ions (Salisbury *et al.,* 1978; Cheema *et al*., 2016). Additionally, sodium and calcium gain access to the interior of the cell and consequently, metabolic activities are diminished and further changes ensue. Cold shock can be prevented by cooling semen slowly in the presence of protective agents (Moussa *et al*., 2002; Pena *et al*., 2009).

2.12. Pre- and post-freezing evaluation of bovine semen

By improving AI and semen cryopreservation techniques, the minimum number of spermatozoa needed per dose can be decreased, while still yielding optimal fertility results. Methods of semen evaluation become essential in order to evaluate any potential improvements in these techniques (Saacke, 1983; Eidan, 2016). A successful commercial evaluation method must be fast, easy and relatively inexpensive (Januskauskas & Zilinskas, 2002; Fleisch *et a*l., 2017). Evaluating the viability of spermatozoa before, during and after cryopreservation and storage is the foundation of any research relating to semen preservation; because of this, accurate and dependable methods are critical. Many of these methods have been questioned during earlier research studies related to correlation between evaluated factors and fertility which seemed to be inconsistent (Pena *et al.,* 2009).

In 1961 Salisbury and VanDemark suggested an asymptotic model as explanation for these apparent inconsistencies. This model explained the relationship between fertility and semen viability to be as follows: as the number of spermatozoa increase towards a threshold value, which may differ between species, as well as individuals of the same species, fertility will also increase (Saacke, 1983). At this threshold value, maximum fertility is reached, which means that further increases in spermatozoon numbers will not result in increased fertility. This implies that if semen samples used in a study to observe the correlation between fertility and a specific trait surpass the threshold value for that specific quality trait, no correlation between fertility and the specific trait exists (Pena *et al.,* 2009). However, if the semen samples contained spermatozoa below the threshold value, the conclusion would be that a high correlation exists between the trait and fertility. Several studies done by researchers have supported this model (Saacke, 1983; Cheema *et al*., 2016).

2.12.1. Spermatozoon morphology

Spermatozoon morphology is generally dependent on spermatogenesis or events that occur after spermiation (Ahmad *et al*., 2014). Poor handling techniques or problems during cooling and freezing could also damage the acrosome or cause reflection of the spermatozoon tail (Fleisch *et al*., 2017). Disturbances during spermatogenesis in the testis or during epididymal transit that affect spermatozoa morphology can be classified in a variety of ways, including the spermiogram, a differential count of spermatozoa morphology (Ahmad *et al*., 2014). This system generates a frequency distribution of all defects. Frequent bovine defects affecting the spermatozoa head include knobbed acrosomes, nuclear vacuoles, pyriform and detached heads. Defects that affect the mid-piece such as the distal mid-piece reflexes, segmental aplasia of the mitochondrial sheath (gaps), fractures, proximal droplets and teratoids are common (Ahmad *et al*., 2014).

Another system classifies spermatozoon abnormalities based on their presumptive origin: primary defects occur during spermatogenesis and secondary defects are caused by abnormal function of the epididymis or from semen handling (Ahmad *et al*., 2014). A third classification system divides abnormal spermatozoa based on their relationship to male fertility: Major spermatozoon defects were considered to be more likely to affect male fertility and minor defects may have a minor effect on male fertility (Ahmad *et al*., 2014). A fourth system was based on whether the defect was compensable versus uncompensable. It distinguishes between the spermatozoa defects that can be compensated by inseminating high numbers of spermatozoa and spermatozoon defects that result in fertilization failure regardless of the sperm concentration inseminated (Fleisch *et a*l., 2017).

Morphological evaluation can be performed visually or with computer created images. A visual subjective microscopic evaluation of spermatozoon morphology can be conducted on unstained wet samples using differential interference phase contrast of fixed sperm or stained dried samples under oil immersion using eosin nigrosin (Ahmad *et al*., 2014). The spermatozoa are judged based on the percentage of normal cells and the nature of the defects as mentioned above. Generally, 70% morphologically normal spermatozoa and not more than 20% head defects are necessary to reach the highest possible fertility. Computer-aided spermatozoa head morphometric analysis uses the head length, width, and area to calculate spermatozoon specific permutations and perimeters. Digital images can also be analyzed

using Fourier functions which describe the shape of the spermatozoon head (Ahmad *et al*., 2014; Eidan, 2016).

2.12.2. Spermatozoon motility and motion

Spermatozoa motility is the most commonly evaluated trait for semen quality. A manual microscopic evaluation can be performed on an unstained semen sample which is evaluated for its percentage of total and/or progressively motile spermatozoa. It is an easy and fast method which does not need expensive equipment (Pena *et al*., 2009). However, the visual assessment is subjected to human bias and the repeatability within a laboratory and especially across laboratories may be of limited value. 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 spermatozoa concentration, spermatozoa motility, spermatozoa motion, and to certain extent spermatozoa head morphology (Akhter *et al*., 2016).

Besides the total and progressive motility, several spermatozoa motion characteristics can be determined (Ahmad *et al*., 2014). The use of CASA reduces human bias and allows for a more objective semen evaluation. Similar to the manual assessment of spermatozoa motility, there are limitations in comparing the spermatozoa parameters across laboratories (Fleisch *et a*l., 2017). There are differences in the optics and software between machines that are on the market. The settings and parameters entered for the spermatozoa vary along with the chamber type for the spermatozoa and the expertise and training have an effect on the reliability, accuracy and precision of CASA. In addition, the concentration, the extender used and the temperature at which the spermatozoa are examined has an impact on the motion parameters (Eidan, 2016).

CHAPTER 3

MATERIALS AND METHODS

3.1. Study Area

The study was conducted at the University of Venda located in Thohoyandou, in the Limpopo Province of South Africa. Thohoyandou 22° 57' 0" South, 30° 29' 0" East, falls under Thulamela municipality in Vhembe District. The Vhembe region extends northwards to the Limpopo River which forms the boundary between South Africa and Zimbabwe (Rix *et al.,* 1989). Thohoyandou is located within a subtropical climate region with high temperatures and humidity in summer and mild winters. This study was carried out in strict accordance with the recommendations in the guide for the care and use of animals under the guidelines of the University of Venda Animal Ethics Committee.

3.2. Nutrition and housing of the bulls

Both bulls were fed a standard daily diet consisting of 2 Kg of 12 % protein concentrate pellets and 10 Kg of dried Lucerne (*Medicago sativa*), Eragrostis (*Eragrostis curvula*) hay bales and minerals. Fresh water was available *ad libitum.* The bulls were housed at the feedlot of the experimental farm of the University of Venda and kept under similar managerial conditions.

3.3. Preparation of extenders

The different constituents of the extenders (tris, citric acid, fructose etc.) were dissolved in distilled water and split into the graduated tubes. Egg yolk, soybean milk and coconut water were added according to the formula given in Table 3.1.

Table 3.1. Illustration of the constituents of different extenders

Ingredients	**TEYE**	**SBME**	**COWE**
Tris (g)	2.442	2.442	2.442
Citric acid (g)	1.3	1.3	1.3
Monohydrate glucose (g)	1	1	1
Gentamycin sulphate (g)	0.1	0.1	0.1
Egg yolk (% v/v)	20	_	_
Soybean milk (% v/v)	_	2.5	_
Coconut water (% v/v)	_	_	35
Total volume (ml)	**100**	**100**	**100**

Distilled water was filed up to 100 ml

TEYE: Tris egg-yolk extender

SBME: Tris extender based on soybean-milk

COWE: Tris extender based on coconut water

These extenders were stored in the refrigerator and only used for a maximum of 7 days.

The pH was measured with pH meter (Metter-Toledo AG, Analytical, Sonnenbergstrasse 74, Schwerzenbach) and adjusted to 6.8 using acetic acid and sodium hydroxide.

3.3.1. Preparation of egg yolk

The chicken eggs used as a source of egg yolk were collected from the experimental farm of the University of Venda. Fresh eggs were collected the day before the extenders were prepared. All eggs were washed with clean water and wiped with a paper towel. Eggs were then sprayed with 70 % alcohol and again wiped with clean paper towel. This was done in order to ensure that no dirt or micro-organisms were present that could contaminate the extenders. The egg shell was broken by lightly tapping the egg against the side of an egg divider. Egg yolks were obtained by using a sterile egg divider and then transferred to a clean filter paper so that the remaining albumin could be absorbed. A sterile needle was used to carefully puncture the yolk membrane and aspirated into a 10 ml syringe. After it was aspirated, the egg yolk was then poured into a graduated tube.

3.3.2.. Preparation of soybean milk

Soybean grains were purchased from the local market. Ten grams of soybean were washed and soaked in 100 ml distilled water and boiled for 30 minutes. After boiling, the water was discarded and the whole grains were washed again and finally cooled down with 50 ml distilled water containing 0.25 % $NaHCO_3$. The grains were then grounded for 5 minutes and the slurry cooled. Soybean milk was extracted by filtration through a clean cotton cloth, centrifuged and then boiled again for 10 minutes. The slurry was then allowed to cool down. After that, the soybean milk (SBM) was ready for use (El-Keraby *et al,.* 2010).

3.3.3. Preparation of coconut water

The coconuts (*Cocos nucifera*) were purchased from the local market. Coconut was brushed during washing in clean water in order to thoroughly remove dirt and debris. Washed coconut was then cut horizontally at one end while the coconut was in a vertical position. The liquid was filtered to remove undesirable particles. The coconut water was then ready to be used.

3.4. Semen collection

Semen samples were collected from two adult Nguni bulls of approximately the same age (four years) using the Pulsator IV, Auto. Ajust^{TM}, 15 A. 125 V electro-ejaculator (Lane manufacturing INC Denver Colorado, USA) was used for semen collection. Semen samples were collected twice weekly for 6 weeks. The electro-ejaculation was performed in the morning, with the bull restrained in the crush-pen in a standing position. The prepuce hair were trimmed to about 0.5 mm. The prepuce was cleaned externally with 0.9 % normal saline and a sterilized paper towel to remove the dirt. The collection tubes were kept in thermos containing warm water at 35 °C, to avoid cold shock. The cone and graduated semen collection tubes were covered with an insulation jacket. Contamination of semen by water or other harmful substances was avoided.

The bulls were restrained in the crush-pen and the electro-ejaculator with the probe connected to the Pulsator IV with the battery charged was set for use. The rectum was emptied of faeces using an examination clove. The probe was lubricated with cooking oil and inserted into the rectum with the electrodes facing ventrally (downwards). The voltage was

increased (automatically) in small increments until semen was ejaculated. The clear pre-seminal fraction that started to flow from the protruded penis was not collected. As soon as the cloudy sperm rich fraction begains to flow from the penis, the collection cone with the graduated semen collection tube attached was placed over the penis and the sample was collected. After collecting a suitable sample, the stimulation was stopped and the rectal probe removed.

Figure 3.1. The electro-ejaculator used for semen collection: Carrying bag (1), Pulsator IV charger (2), Probe (3), Probe cord (4), Semen collection handle (5), Semen collection cone (6), Power supply cords (7), Scrotal tape (8).

3.5. Semen extension

The semen in the graduated semen collection tubes were placed in the thermos and transported to the laboratory to avoid semen temperature changes. Semen was evaluated macroscopically, and after pooling the semen from the two bulls, the semen sample was then split into three different tubes and each extended with equal fractions for the Tris Egg Yolk Extender (TEYE), SoyBean Milk based Extender (SBME) and Coconut Water based Extender (COWE) (Table 3.1. above). The spermatozoa concentration was determined by CASA [Sperm Class Analyzer® (SCA) 5.3.Microptic Barcelona, Spain] and semen was extended to approximately 120 x 10^6 /ml, a concentration that yields the optimal results.

3.6. Semen evaluation

3.6.1. Macroscopic evaluation of semen

Immediately after the semen was collected and taken to the lab, an initial evaluation of the sample was performed macroscopically. This included the assessment of general appearance, pH, volume and viscosity. Ejaculated semen volume was measured using the calibrations on the collection tubes and then recorded. The semen pH was determined using a pH meter (Mettler-Teledo AG, Switzerland) calibrated to measure semen pH for bulls. Before and after use, to avoid any contamination, the electrode of the pH meter was rinsed with sterile water and wiped with clean paper towel. Colour was determined by visual assessment. Bull semen sample should be uniformly near-white, but could be light yellow in colour due to higher riboflavin content in some bulls (Ahmad *et al*., 2014). The smell and the viscosity were also determined subjectively. This included the assessment of the presence of blood, pus and dirt. Only semen free from any contamination was further processed.

3.6.2. Microscopic evaluation

The semen samples were first diluted with the three extenders (TEYE, SBME and COWE) at 37 °C to a final concentration of 20 × 10^6 spermatozoa per ml. Immediately after dilution, microscopic evaluation was done in order to characterize the semen sample. Each extended semen sample was then split into three portions, the first kept at room temperature, the second kept in the refrigerator and the third frozen and stored in the Liquid Nitrogen (LN_2) for three days.

3.6.2.1. Spermatozoa kinetic properties evaluation

The characteristics relating to the kinetic (motion or movement) properties; namely total spermatozoa motility (TM), progressive spermatozoa motility (PM) were analysed through the computer aided sperm analysis (CASA) system (Sperm Class Analyzer® [SCA] 5.3, Microptic, Barcelona, Spain). Two micro-litres from each of the three extended semen samples were placed in one chamber of the eight chambered Leja® slides on the warm glass stage at 37 °C of the CASA microscope for evaluation. Five fields were captured for each analysis and the software automatically calculated and recorded the motility parameters of sperm in the sample.

3.6.2.2. Spermatozoa morphology properties evaluation

Spermac® stain (Stain Enterprises, Wellington 7654, South Africa) was used to determine spermatozoa acrosome integrity/acrosome reacted (AR), coiled (CT) or bent tail (BT) and absent tail (AT). Spermatozoa smears were prepared at room temperature (25 °C) by pipetting a 15 µL drop of extended semen on end of a clear glass slide. The edge of the second slide was then placed on the front side of the drop horizontally at an angle of 20º. The upper slide was then pushed forward to pull the semen sample across the slide to make a smear. The spermatozoa smears were then air dried at the room temperature before staining. The dried smears were placed horizontally down on absorbent paper inside a staining tray and 1 ml of Spermac® fixative was then dropped on the smear using a plastic disposable pipette. After 10 min the slides were placed vertically on their end on absorbent paper to drain off excess fixative. The slides were then washed by slowly dipping seven times (about 1 second each dip) into distilled water. Excess water was drained by touching the long end of slide onto absorbent paper.

One millilitre of stain A was then dropped onto the slide for 2 min and after that washed as above. The slides were washed for the second time in fresh distilled water to remove the excess of stain A. The smears were stained with B the same way as stain A, and then washed by dipping seven times into fresh distilled water. The stain C was used to stain the smears for another 2 min and washed as above. The slides were then left in an upright position (at about 70° angle) to let fluid drain until air dry. Thereafter, the stained slides were then placed on the CASA microscope stage at room temperature. A drop of immersion oil was placed on the stained slide and covered with the cover slip before evaluation of spermatozoa morphology. The CASA morphology program was used at × 60 magnification to count 100 spermatozoa per stained slide and the results were recorded. Under the microscope the post-acrosomal region and the nuclear portion of the head appears red, while the acrosome, midpiece and tail appears green.

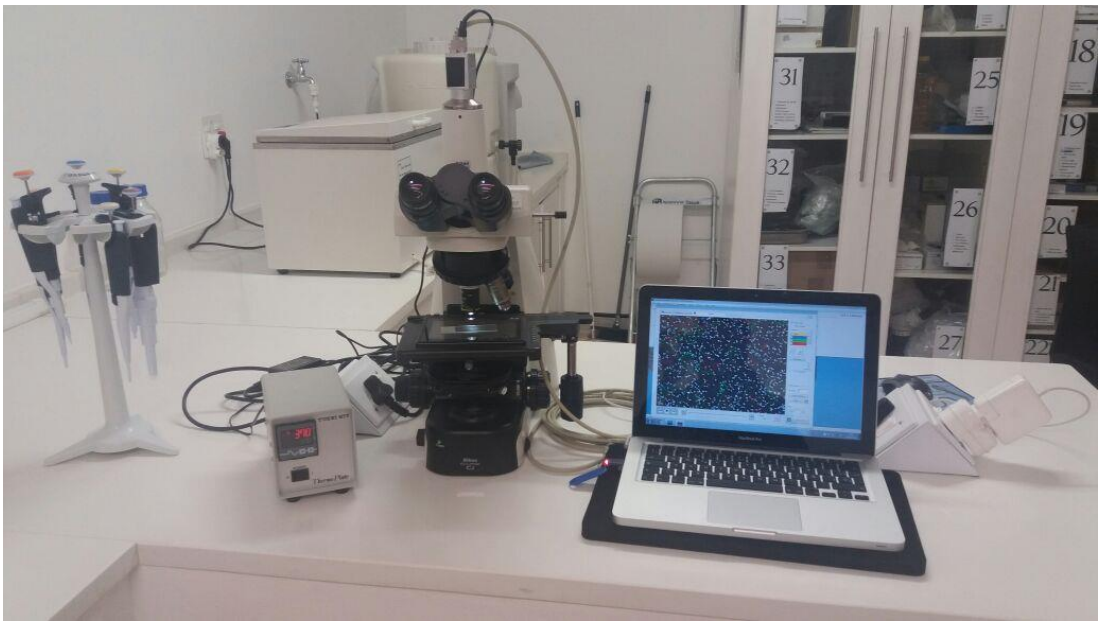

Figure 3.2. The Sperm Class Analyzer® (SCA) system

3.6.2.3. Viability (live/dead) test

Nigrosin-eosin stain was used to determine sperm viability. To stain, two clean microscope slides were warmed to 37 °C. Six micro-litres of a semen sample and 20 µL of eosin stain were placed on the end of the slide and mixed using a pipette tip on a warm glass stage at 37 °C. Twenty micro-litres of nigrosine stain was then added onto the mixture on the slide and mixed using the same pipette tip. The edge of the second slide pre-warmed at 37 °C was then placed on the mixture at an angle of 20° from the horizontal plane and pushed forward to pull the smear across the slide. The smeared slide was immediately placed on a hot Bluehler® slide warmer (Bluehler Ltd., 41 Waukegan road, lake Buff, 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 placed on the stained slide and covered with a cover slip before evaluation. The CASA vitality program was then used at × 60 magnification to count the number of live and dead spermatozoa. A total of 200 sperm were counted per stained slide and the results were recorded. Under the microscope, the live spermatozoa (LS) heads appeared white, while dead spermatozoa (DS) appeared pinkish in colour.

3.7. Experimental design, packing, freezing and storage of semen

The experiment was set up as 3 X 3 factorial arrangement in a completely randomized design. For each extender treatment (TEYE, SBME and COWE), three storage methods (room temperature, refrigeration and freezing) were used, each replicated 12 times. The packing of extended semen was done at room temperature into 0.25 ml straws by aspiration. The straws were then split into three groups of four straws each. The first group of four from each extender (a total of 12 straws), was kept at room temperature for three days then heated at 35 - 37 °C and analysed. The second group was cooled to 4 °C in the refrigerator for three days before warming and analysis. The third group of straws was refrigerated at the temperature of 4 °C for two hours. The straws were thereafter held in liquid nitrogen vapour at 5 cm above the surface of liquid nitrogen (approximately - 80 °C) for 10 minutes and then plunged into liquid nitrogen for storage at -196 °C in a semen storage tank for 3 days. Different colours of straws and plugging powder were used for identifying each extender.

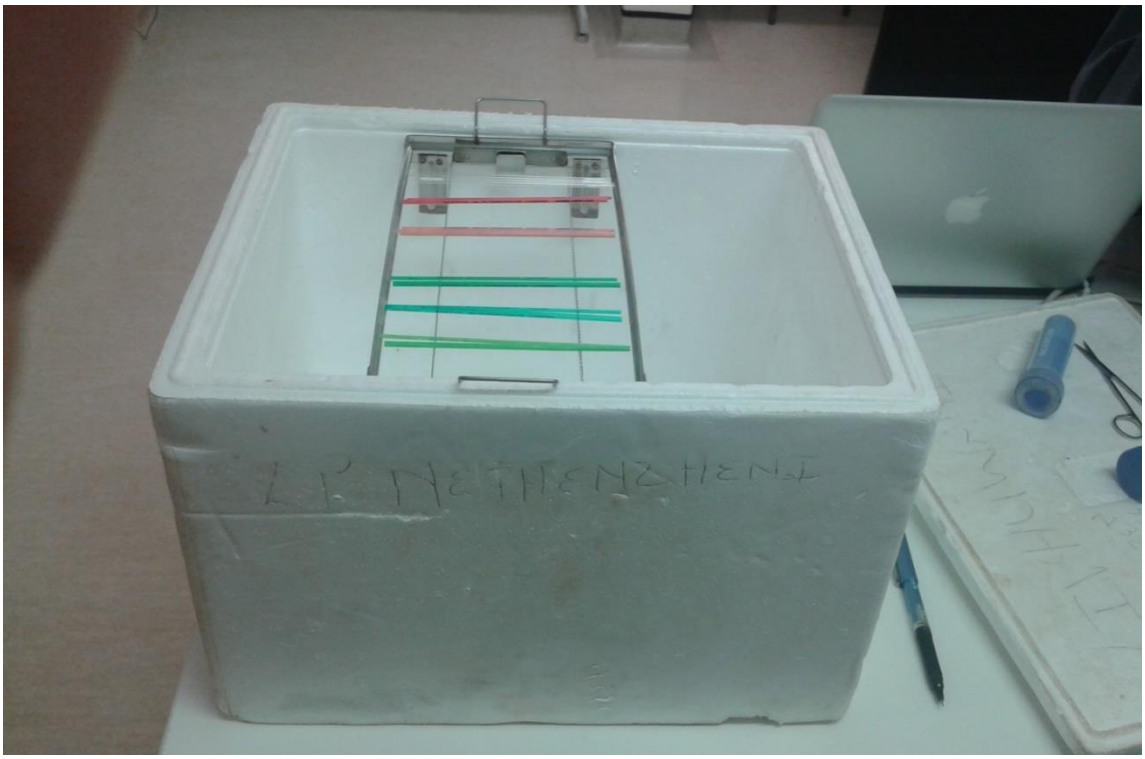

Figure 3.3. Styrofoam box, straw holding rack and semen straws

3.8. Thawing and post-freezing evaluation of semen

Refrigerated and frozen groups of semen straws were warmed for 30 seconds in a water bath at 35 - 37 °C. One semen straw from each group was wiped with the paper towel and split into three portions. The first portion was assayed for the characteristics relating to the kinetic properties. The second portion was evaluated for live-dead counts. The last portion was assayed for morphology properties.

3.7. Statistical analysis

Analysis of variance (ANOVA) on quality of fresh extended semen data (Model I) and extended semen stored with different methods data (Model II) was performed at P <0.01 and P <0.05 according to Steel and Torrie (1980) using general linear model procedures of Minitab Statistical package version 17 (Minitab, 2014). Where significant differences between the treatment groups was detected, means were separated using the Tukey's test (Tukey, 1953). The models used were as follows:

$$\mathbf{Y_{ij} = \mu + E_i + \varepsilon_{ij}} \qquad \text{Model I}$$

Where, Y_{ij} = the observation – TM, PM, AR, AT, CT, BT and DL;

μ = overall mean common to all observations;

E_i = effect of i^{th} extenders, i = 1, 2 or 3; and

ε_{ij} = random residual error.

$$\mathbf{Y_{ijk} = \mu + E_i + S_j + (ES)_{ij} + \varepsilon_{ijk}} \qquad \text{Model II}$$

Where, Y_{ijk} = the observation – TM, PM, AR, AT, CT, BT and DL;

μ = overall mean common to all observations;

E_i = effect of i^{th} extenders, i = 1, 2 or 3; and

S_j = effect of j^{th} storage temperatures, j = 1, 2 or 3;

$(ES)_{ij}$= interaction between i^{th} extenders and j^{th} storage methods; and

ε_{ijk} = random residual error.

CHAPTER 4

RESULT AND DISCUSSION

EXPERIMENT 1: Effect of different extenders on spermatozoa motility, morphology and viability

In the laboratory, before the semen was processed, an accurate macroscopic evaluation of the samples was performed. Detection of the quality of semen before use ensures achieving optimum reproductive efficiency. Therefore, physical properties, particularly volume of ejaculate, pH and semen color were investigated. The results are summarized below in Table 4.1.

Table 4.1. : Initial (macroscopic) characteristics of the collected bull semen

Parameters	Values
Volume (ml)	7.62 ± 0.12
pH	6.46 ± 0.08
Semen color	Ivory

The mean volume of Nguni bull semen was 7.62 ± 0.12 ml and the pH was 6.46 ± 0.08 cm. The colour of the semen was ivory which could be considered normal according to Hossain *et al* (2012).

The current study also investigated the behaviour of freshly collected Nguni bull semen following dilution with Tris Egg Yolk Extender (TEYE), Soy Bean Milk based Extender (SBME) and Coconut Water based Extender (COWE). The results are presented below.

Table 4.2. Effect of egg yolk, soybean milk and coconut water based extenders on kinetic and morphologic properties and viability on fresh bull semen

Extenders	TM (%)	PM (%)	AR (%)	AT (%)	CT (%)	BT (%)	LS (%)
TEYE	92.9	53	14.7	7.5	1.9	3	95.3
SBME	93.5	51	15.4	7.8	2.8	2.9	96.4
COWE	93.2	54.2	15.3	7.3	2.5	2.8	96.1
SEM	1.3	1.86	0.59	0.48	0.36	0.35	0.59
Significance	NS	NS	NS	NS	NS	NS	NS

SEM = Standard Error Means; TM = total motility; PM = progressive motility; AR = acrosome reaction; AT = absent tails; CT = coiled tails; BT = bent tails; LS = live spermatozoa; NS = not significant (P > 0.05); SEM = standard error mean.

Table 4.2. shows the mean values of the kinetic, morphologic properties and viability of fresh Nguni bull semen extended with three different extenders (TEYE, SBME and COWE), and analysed immediately after dilution at room temperature (25 °C). Results from Table 4.2. show no significant difference (P > 0.05) in spermatozoa TM, PM, AR, AT, CT; BT and LS. It was evident from the table that spermatozoa TM was numerically the highest in SBME (93.5 %), it had the lowest progressive spermatozoa (51 %) and lowest TM (92.9) in TEYE. The semen diluted in SBME and COWE showed higher percentages of reactive acrosomes (15.4 % and 15.3 % respectively) than in TEYE. Semen diluted in TEYE had the highest percentage of spermatozoa with bent tails. None of these were, however, significantly different (P > 0.05).

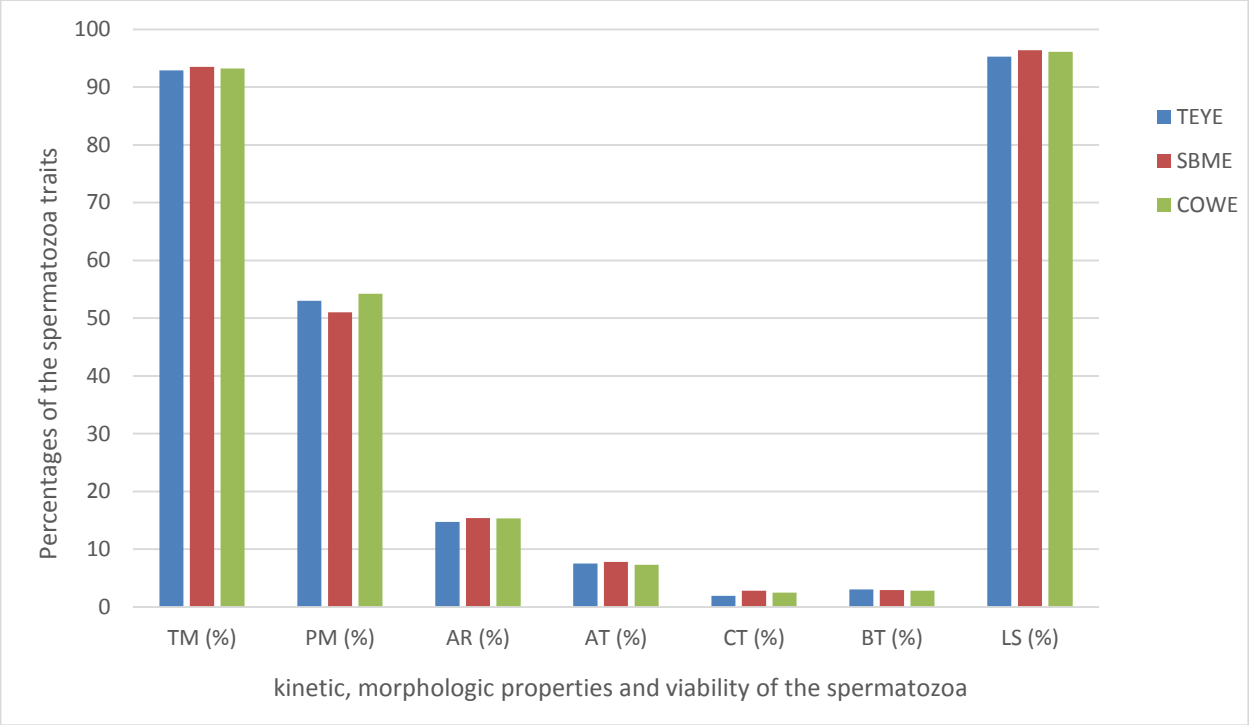

Figure 4.1. Fresh extended semen characterization immediately after collection and dilution

It was observed that indole-3-acetic acid (IAA) present in coconut water had a beneficial effect on boar sperm acrosomal integrity, but it is assumed that other molecules present in coconut water also play a protective role (Toniolli *et al.,* 1996). Azevêdo and Toniolli (1999) also reported that sperm motility and vigor increased with the addition of IAA to skim milk extender. Several studies have demonstrated that coconut water, after correcting osmolality and pH, is effective in the maintenance of *in vitro* and *in vivo* spermatic cell characteristics (Nunes, 1998). Therefore, we believe that the better results observed with the COWE (TM = 93.2 % and PM = 54.2 %) compared to the values observed with TEYE (TM = 92.9 % and PM = 53 %) and SBME (TM = 93.5 % and PM = 51 %) can be attributed to the characteristics mentioned above, as well as the presence of sodium citrate that exerts a buffering power preventing rapid changes in the pH of the medium (Holt, 2000).

Experiment 2: Effect of extenders on bull spermatozoa kinetic, morphologic properties and viability after three days of storage

Nguni bull semen samples were diluted with TEYE, SBME and COWE extenders and were stored for 72 hours at room temperature (25 °C), refrigerator temperature (4 °C) and cryopreserved in LN_2 (-196 °C). The results of analyses are presented in Table 4.3. below.

Table 4.3. Effect of egg yolk, soybean milk and coconut water based extenders on bull spermatozoa kinetic; morphologic properties and viability after three days of storage

Treatment	SM	TM (%)	PM (%)	AR (%)	AT (%)	CT (%)	BT (%)	LS (%)
TEYE	RT	72.6^{a}	32.2^{a}	23.5^{c}	7.7^{ab}	4.5^{b}	5.1^{bc}	59.1^{a}
	RF	72.7^{a}	32.4^{a}	22.3^{c}	7.1^{ab}	4.7^{b}	6.0^{bc}	61.2^{a}
	FR	32.7^{b}	14.8^{b}	35.2^{b}	8.3^{ab}	6.3^{ab}	6.9^{b}	36.0^{b}
SBME	RT	73.6^{a}	31.2^{a}	21.3^{c}	7.6^{ab}	4.5^{b}	4.2^{c}	59.1^{a}
	RF	71.1^{a}	32.9^{a}	22.8^{c}	6.5	5.3^{b}	5.1^{bc}	61.0^{a}
	FR	28.8^{b}	13.1^{b}	37.8^{b}	9.1^{a}	6.3^{ab}	6.9^{b}	25.0^{c}
COWE	RT	73.9^{a}	34.6^{a}	21.6^{c}	7.9^{ab}	4.8^{b}	5.3^{bc}	57.3^{a}
	RF	73.6^{a}	33.1^{a}	23.1^{c}	6.8^{ab}	4.7^{b}	4.8^{c}	60.3^{a}
	FR	16.1^{c}	6.7^{c}	54.8^{a}	8.3^{ab}	8.0^{a}	9.9^{a}	13.7^{d}
SEM		1.05	1.15	0.88	0.5	0.41	0.44	1.1
EXT Means								
TEYE		59.3^{a}	26.4	27.0^{b}	7.7	5.2	6.0^{ab}	52.1^{a}
SBME		57.8^{a}	25.7	27.3^{b}	7.7	5.4	5.4^{b}	48.4^{b}
COWE		54.5^{b}	24.8	33.1^{a}	7.7	5.8	6.7^{a}	43.8^{c}
SEM		0.61	0.67	0.51	0.29	0.24	0.25	0.65
SM Means								
RT		73.4^{a}	32.7^{a}	22.1^{b}	7.7^{ab}	4.6^{b}	4.8^{b}	58.5^{b}
RF		72.4^{a}	32.8^{a}	22.7^{b}	6.8^{b}	4.9^{b}	5.3^{b}	60.8^{a}
FR		25.9^{b}	11.5^{b}	42.6^{a}	8.6^{a}	9.6^{a}	7.9^{a}	24.9^{c}
SEM		0.61	0.67	0.51	0.29	0.24	0.25	0.65
Significance								
EXT (E)		**	NS	**	NS	NS	*	**
SM (S)		**	**	**	**	**	**	**
E × S		**	**	**	NS	NS	**	**

Mean values that do not share superscript letters in the same column, are significantly different. SEM = Standard Error Means; TM = total motility; PM = progressive motility; AR = acrosome reacted; AT = absence tails; CT = coiled tails; BT = bent tails; LS = live spermatozoa; RT = room temperature; RF = refrigeration; FR = freezing; NS = not significant ($P > 0.05$); * = significant ($P < 0.05$); ** = highly significant ($P < 0.01$); SM = storage methods; EXT = extenders.

Results from Table 4.3. above show that although, there is no significant difference ($P > 0.05$) in the kinetic morphologic properties and viability of semen stored at room temperature and refrigerator temperature regardless of the extender in use, they were significantly difference ($P < 0.05$) in the TM, PM, AR and DL to that of frozen semen. While the percentage of sperm with reactive acrosome was highest ($P < 0.01$) in the cryopreserved sample diluted with COWE, there were no significant difference ($P > 0.05$) between the percentages of total acrosomes that reacted as shown with Spermac® stain.

The percentage of absent tails showed no significant difference ($P > 0.05$) in any of the storage methods in TEYE and COWE, but it was significantly different ($P < 0.05$) in the means of all the tested storage methods. Table 4.3. indicated that there was no significant difference ($P > 0.05$) between the percentages of sperm with coiled tails in semen samples diluted with all three extenders and stored at room temperatures and, they were significantly different ($P < 0.05$) from the cryopreserved sample in each case of the extenders. The semen sample diluted with COWE and cryopreserved had the highest percentage ($P < 0.01$) of spermatozoa with bent tails; while the sample diluted with TEYE and SBME and stored at room and refrigerator temperature were not significantly different ($P > 0.05$). The percentages of live spermatozoa stored at room and refrigeration temperature (25 °C and 4 °C respectively) in all three extenders showed no significant difference ($P > 0.05$) and they are significantly different ($P < 0.05$) from cryopreserved samples in all extenders.

In this study it was observed that better post thaw MT, MP, AR, AT, CT, BT, and LS values were associated with the use of TEYE, followed by SBME and COWE in the frozen semen. However, at room and refrigeration temperature, the values were similar regardless of the extenders. This result is in line with the observation of Celeghini *et al*. (2008) that greater post-thawing TM, PM, AR, AT, CT, BT and LS values were associated with the use of Botu-Bov® (BB) containing 20 % egg yolk compared to Bioxcell® medium composed of soy lecithin. It has been verified that BB gives greater sperm protection than does Tris medium when used for refrigeration, as was previously observed for bull semen cryopreservation.

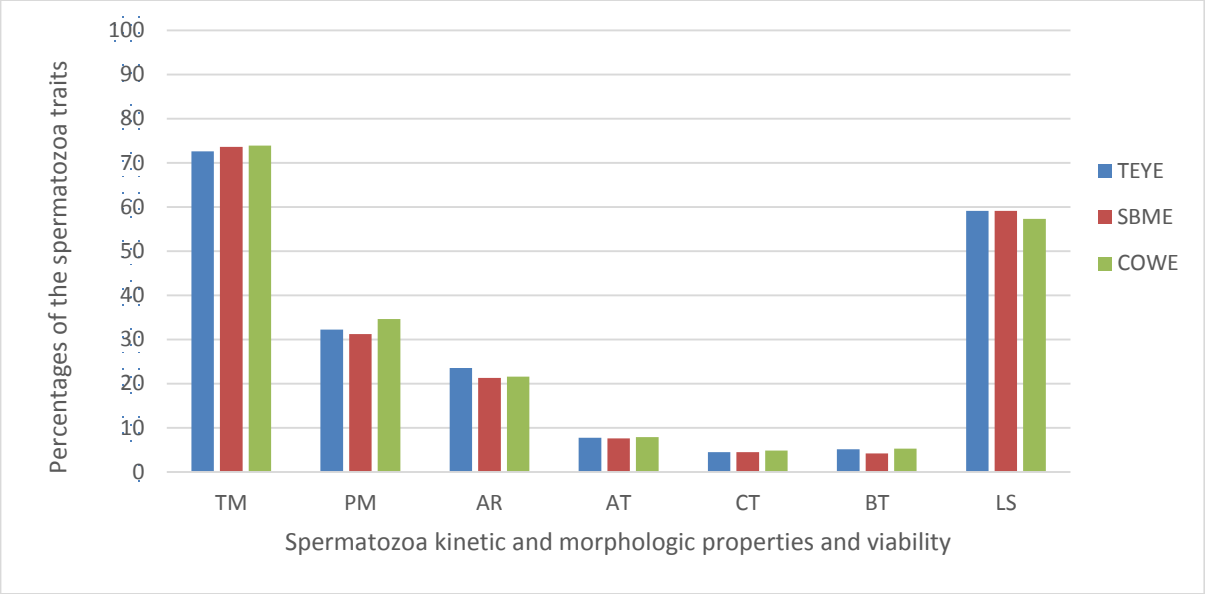

Figure 4.2. Semen characterization after three days of storage at room temperature (25 ˚C)

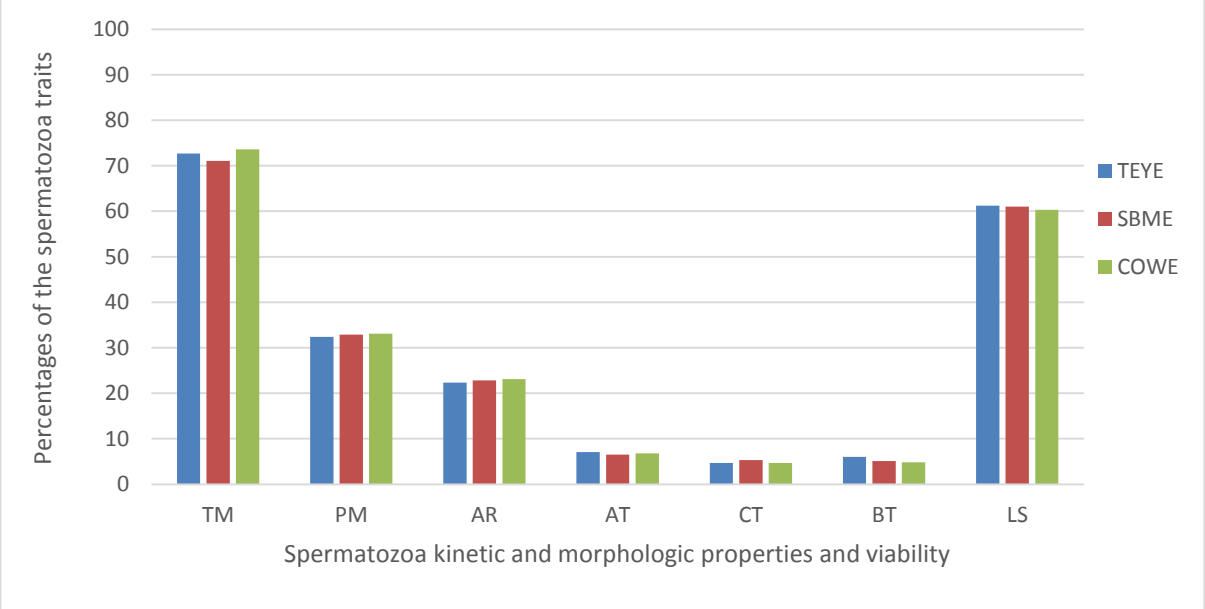

Figure 4.3. Semen characterization after three days of refrigeration (4 ˚C)

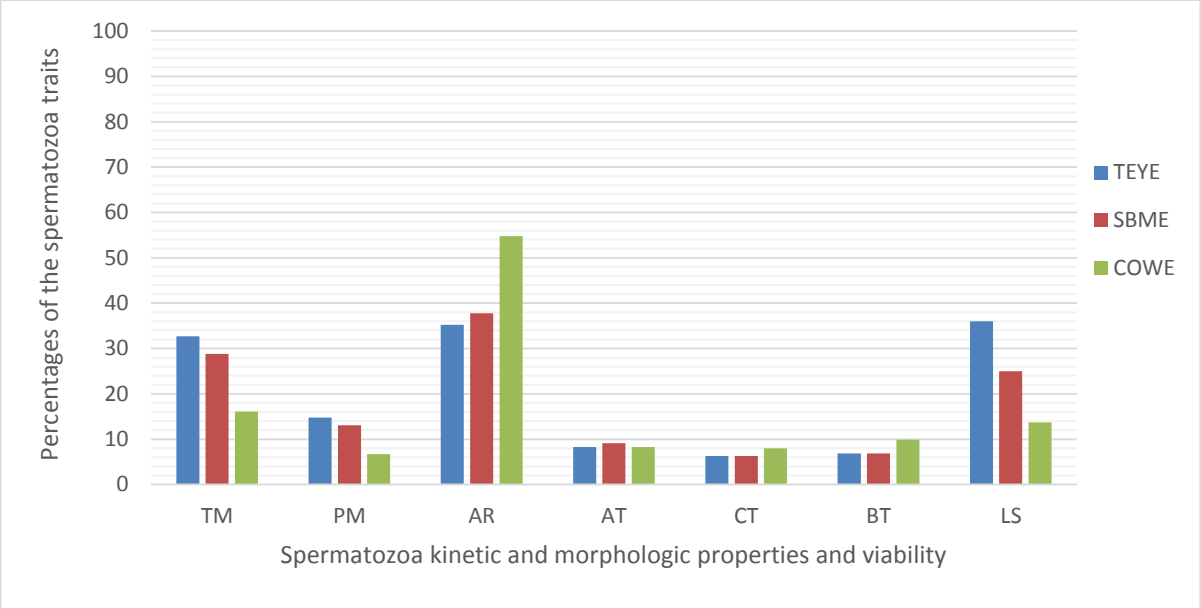

Figure 4.4. Semen characterization after three days of storage in the LN_2 (-196 °C)

It can be argued that storage of semen at room temperature would provoke degenerative changes leading to cell death (Aalseth & Saacke, 1985). Barros and Toniolli (2011) reviewed the potential effects of coconut water on semen technology and reported that its utilization as an extender is important, because it is an easily prepared and cheap alternative for semen transport over small distances and for use in artificial insemination programs in several species. Initially, it was reported that isotonic sodium chloride solution was the first extender used to dilute semen from domestic animals to evaluate spermatozoa metabolic activity (Salisbury and Nakabayashi, 1957).

In the present study, superior numeric results have also been observed with COWE (TM = 73.9 % and PM = 34.6 %) compared to TEYE (TM = 72.6 % and PM = 32.2 %) and SBME (TM = 73.6 % and PM = 31.2 %). The temperature exerts a significant influence on spermatozoa survival throughout the refrigeration period (Batellier *et al*., 2001). All variables related to bull semen motility (total and progressive motility) decreased significantly over the 72 h of refrigeration period regardless of the extender in use, consistent with the results obtained by Verberckmoes *et al*. (2005). Even though the decrease in temperature causes a significant decrease in sperm metabolism, reducing the rates of fructolysis and oxygen consumption (Blackshawet *et al*., 1957), spermatozoa quality decreases throughout the refrigeration period regardless of the extender, dilution rate or storage conditions (O'Hara *et al.,* 2010).

The present study shows that COWE can be used to refrigerate spermatozoa, since similar results in terms of TM and PM were observed compared to TEYE and SBME. The differences observed in the TM and PM variables among the different extenders for the freezing storage method were not similar to those observed in previous studies evaluating the extenders containing 20 % of egg yolk [Tris (Tris-R) and Botu-Bov® (BB)] and another composed of 1 % soy lecithin [Botu-Bov® Lecithin (BB-L)] for semen cryopreservation.

Crespilho *et al*. (2012) noted more TM and PM for cryopreserved bull spermatozoa stored in Botu-Bov® compared to spermatozoa processed in Tris egg yolk fructose extender. In the previous study it was concluded that the lipid particles found on egg yolk based extenders could also have a deleterious role on spermatozoa motility, acting as a physical barrier for spermatozoa. For this reason, the lesser viscosity of the Botu-Bov® medium (clarified by centrifugation) allowed greater preservation of post-thaw spermatozoa movement. However, in the present study, better results from frozen semen, were observed with TEYE (TM = 32.7 % and PM = 14.8 %) as compared to SBME (TM = 28.8 % and 13.1 %) and COWE (TM = 16.1 % and PM = 6.7 %).

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1. Conclusions

The success of spermatozoa used for AI and its ability to fertilize, is affected at different level during its course from its origin until it reaches the ovum.

Cryopreservation continues to be the most frequently employed technique for use in modern animal production. Commercial AI will inevitably use this technique for long storage and transport semen over a wider area around the world. However, even with the most up to date procedures, cryopreservation still causes severe detrimental effect to spermatozoa and their function. To ensure that semen used for AI is of a relatively high quality, artificial breeding organizations should also consider the different storage methods of semen based on the period between the collection and the AI.

5.2. Recommendations

For the short storage period of semen used for AI, it is recommended that semen should be kept at room or refrigeration temperature regardless of the three extenders used. However, for long storage of frozen semen TEYE is recommended. The egg yolk-based extender provided greater preservation of motility and bull spermatozoa integrity during the freezing process than did SBME and COWE.

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APPENDICES

APPENDIX 1.

Table 6.1. ANOVA for kinetic and morphologic properties and viability of fresh bull semen on egg yolk, soybean milk and coconut water based extenders

Source	df	TM	PM	AR	AT	CT	BT	LS
Ext	2	1.03	31.04	2.028	0 .75	2.194	0.083	4.333
Error	33	20.33	41.54	4.129	2.773	1.581	1.472	4.245

df = degree of freedom; TM = total motility; PM = progressive motility; AR = acrosome reacted; AT = absence tails; CT = coiled tails; BT = bent tails; LS = live spermatozoa; EXT = extenders.

APPENDIX 2.

Table 6.2. ANOVA for kinetic and morphologic properties and viability of fresh extended semen. Effect of egg yolk, soybean milk and coconut water based extenders on bull spermatozoa kinetic; morphologic properties and viability after three days of storage

source	df	TM	PM	AR	AT	CT	BT	LS
Ext	2	220.4**	25.2 NS	433**	0.009 NS	4.231 NS	14.704 **	627.4**
SM	2	26561.2 **	5402.6 **	4863.2 **	28.454 NS	54.176 **	99.287 **	14552.5 **
EXT	4	355.5**	115.7**	472.2**	1.815 NS	4.843 NS	14.954 **	442.4**
Error	99	13.3	16	9.3	3.013	2.046	2.305	15

df = degree of freedom; TM = total motility; PM = progressive motility; AR = acrosome reacted; AT = absence tails; CT = coiled tails; BT = bent tails; LS = live spermatozoa; NS = not significant (P > 0.05); ** = highly significant (P < 0.01); SM = storage methods; EXT = extenders.