Dietary supplementation of selenium and addition of vitamin C and E in extender to enhance semen cryopreservation and reproductive performance of Saanen goats

By

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Then God said, "Let Us make man in Our image, according to Our likeness; let them have dominion over the fish of the sea, over the birds of the air, and over the cattle, over all the earth and over every creeping thing that creeps on the earth." Genesis 1: 26

Declaration

I, Kambulu Lukusa, declare that by submitting this thesis for the degree of Doctor of philosophy at the University of Pretoria, the entirety of the work contained therein is my own, original work, and has not been previously in its entirety or in part submitted by me for obtaining any qualification at this or any other tertiary institution.

Preface

This dissertation argues that supplementation of animals or semen extender with antioxidants and the use of appropriate cooling rate as well as equilibration period might prevent detrimental effects of oxidative stress (OS), intracellular ice crystals formation and development of regions of high solute concentrations during the semen freezing process. This might offer a great opportunity for the improvement of post-thaw sperm quality and capturing of superior genetics from proven sires; therefore, promoting the use of cryopreserved Saanen buck semen. The general objective of this work was to determine the antioxidative capability of dietary selenium (Se) supplementation, addition to extenders of vitamin C, E and their combination (C+E) on post-thaw sperm quality in relation to cooling rates (slow and fast) and equilibration times (2, 4 and 6). The specific objectives were addressed in the following chapters, which have been published or are to be submitted for publication in peer review journals.

1. **K. Lukusa** and **K. C. Lehloenya**. 2017. Selenium supplementation improves testicular characteristics and semen quality of Saanen bucks. Small Rumin. Res. 151: 52-58.
2. **K. Lukusa., A. Hassen** and **K. C. Lehloenya**. 2018. Dietary selenium supplementation and slow cooling improve freezability of Saanen buck sperm extended in clarified egg yolk medium (submitted for publication in cryobiology)
3. **K. Lukusa., A. Hassen., F. V. Ramukhithi., M. B. Matabane** and **K. C. Lehloenya.** 2018. Antioxidants and equilibration times improve post-thaw sperm kinematic parameters of Saanen buck semen (to be submitted for publication in cryobiology).

The first chapter of this thesis introduces the study with emphasis on a brief background, justification for the study and highlights of the entire thesis. In the second chapter, the different factors affecting buck semen freezability and fertility are reviewed in detail. In the third chapter, the effects of dietary selenium supplementation on reproductive performance and semen characteristics of Saanen buck were investigated. In the forth chapter, the effects of cooling rates and selenium supplementation on post-thaw sperm characteristics diluted in different extenders were studied. In the fifth chapter of this thesis, the antioxidative capability of Se, vitamin C, vitamin E and their combination (C+E) as well as equilibration times on post-thaw sperm motility and kinematic parameters were studied. Finally, general conclusion, recommendations and critical analysis based on this research work were discussed in the sixth chapter.

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- And last but not least, a special thanks to my wife Esther Lukusa and son Eldad Lukusa for not giving up on me after so many years of education and not stopping to cheer me up.
- Eben ezer: "thus far the Lord has helped me despite my weakness and mistakes". 1 Samuel 7: 12

TABLE OF CONTENTS

List of tables

List of figures

List of abbreviations

µL:	Microliter
ADP:	Adenosine diphosphate
AI:	Artificial insemination
ALH:	Amplitude of lateral head movement
ANOVA:	Analysis of variance
ARC:	Agricultural Research Council
ART:	Assisted reproductive technologies
ATP:	Adenosine triphosphate
AV:	Artificial vagina
BCF:	Beat cross frequency
BW:	Body weight
CASA:	Computer-assisted semen analysis
CEY:	Clarified egg yolk
CG:	Control group
CPA:	Cryoprotective agent
Cm:	Centimetre
DD:	Distal droplet
DH:	Detached head
DLD:	Drag-like defects
DMR:	Distal mid-piece reflex
DSS:	Defects in size and shape
EE:	Electro-ejaculation
EYC:	Egg yolk citrate
FSH:	Follicle stimulating hormone
GLM:	General linear model
GnRH:	Gonadotropin releasing hormone
GSH-Px:	Glutathione peroxidases
H:	Hour
IU:	International unit
IVF:	In Vitro Fertilization
LH:	Luteinizing hormone
LIN:	Linearity
LN_2:	Liquid nitrogen
LPO:	Lipid peroxidation
Kg:	Kilogram
min:	Minute

mg:	Milligram
mL:	Millilitre
mM:	Millimolar
^{o}C:	degrees Celsius
s:	Second
TG:	Treatment group
TL:	Testicular length
TTh:	Testicular thickness
Tris:	Tris (hydroxymethyl) aminomethane
TV:	Testicular volume
TWEY:	Tris without egg yolk
TW:	Testicular width
OD:	Optical density
OS:	Oxidative stress
PD:	Proximal droplet
PVC:	Polyvinyl chloride
ROS:	Reactive Oxygen Species
SC:	Scrotal circumference
Se:	Selenium
SEM:	Standard error of means
SL:	Scrotal length
STR:	Straightness
v/v:	Volume by volume
VAP:	Average path velocity
VCL:	Curvilinear velocity
VSL:	Straight-line velocity
WEY:	Whole egg yolk
WOB:	Wobble coefficient

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Summary

The present study investigated the effects of antioxidants supplementation, freezing extenders, cooling rates and equilibration times on Saanen buck reproductive performance and post-thaw sperm quality. For this reason, selenium was supplemented orally to evaluate its antioxidative potential on reproductive performance as well as on cooled and frozen-thawed semen. The effects of cooling rates, and different extenders were also investigated on post-thaw sperm quality. This was followed by the addition of vitamin C, E and their combination vitamis (C+E) to freezing extender to evaluate their effects on the cooled and post-thaw sperm motility and kinematic parameters post-thaw in relation to equilibration time.

Selenium is an integral part of glutathione peroxidase (GSH-PX), an enzyme which protects cell internal structures against free radicals and is an antioxidant for cellular membrane lipids. Its deficiency has been reported to cause impaired motility, reduced fertility and sperm production. In the present study dietary Se supplementation increased testes measures, semen attributes and hormone concentrations of Saanen buck. Therefore, Se supplementation is recommendable, especially for animals depending on Se deficient Lucerne diets or pastures, to boost their natural antioxidants and enhance the ability of the seminal plasma to fight oxidative stress (OS).

Although Saanen buck reproductive performance and fresh semen characteristics were improved with supplementation of Se, semen cooling and freezing is still a big concern due to excessive production of reactive oxygen species (ROS), leading to structural damage of sperm membranes. The suitable extenders, proper cooling rate and equilibration time are key elements to develop suitable protocol of semen cryopreservation. In this regard, a study was conducted to investigate the interactions between cooling rates (slow and fast) and freezing extenders: clarified egg yolk (CEY), whole egg yolk (WEY) and tris without egg yolk (TWEY) on post-thaw sperm quality in relation to dietary Se supplementation. The study revealed that the combination of clarified egg yolk (CEY) and slow cooling yielded higher percentages of cooled and frozen-thawed sperm characteristics of semen from Se supplemented bucks. This indicted that, supplementing animals with Se prior to the semen freezing process and using clarified egg yolk (CEY) in combination with slow cooling could be recommended for buck semen cryopreservation.

Further to these results another study was conducted to determine the effects of both dietary selenium supplementation and addition of vitamin C (5 mM), E (4.8 mM) and their combination vitamins (C+E) to freezing extender as well as equilibration times (2, 4 and 6) on post-thaw sperm motility and kinematic parameters. Antioxidants supplementation prior to semen freezing led to higher post-thaw sperm motility and kinematic parameters when 2 or 4 h equilibration time was used. It can be concluded that the use of dietary selenium or the addition to extender of the combination of vitamins (C+E) as antioxidants agents, associated with optimum equilibration time, may protect better spermatozoa against free radicals during semen cryopreservation.

In general, the different experiments conducted in this thesis revealed that supplementing bucks with selenium, and the addition of the combination of vitamins (C+E) to clarified egg yolk (CEY) extender together with slow cooling and 2 or 4 h equilibration period, can be an alternative option to enhance reproductive performance and post-thaw sperm motility and velocity parameters.

CHAPTER 1

General introduction

Breeding buck selection is the most critical decision for improvement of a herd. Artificial Insemination (AI) is one of the most important techniques for increasing the rate of genetic improvement and breeding efficiency in livestock production (Anand and Yadav 2016). Male fertility is highly influenced by semen quantity and quality (Mittal *et al*., 2014). The quality of frozen-thawed semen is very important for success of AI. Therefore, understanding the sources of variation in semen quality and identification of highly fertile bucks with good quality of frozen-thawed semen would be of great interest to all livestock breeders (Arredondo *et al*., 2015). Although progress in this area of AI with reference to its application in the caprine species has not progressed as fast as with cattle, the need is still there for improving the cryopreservation process, as about 40 to 50% of the viable sperm are damaged during freezing and thawing processes (Watson, 2000). With the major populations of goats residing in the developing countries, there is obvious demand to accelerate the understanding in this field with the goal to boost the productivity for these countries, including South Africa.

In that context, the Saanen goat breed has been considered in this study because it is well adapted to the South African climate and can better survive tick borne diseases better have been considered in the study (Malan, 2000; Erasmus, 2000). The choice of this breed has been motivated by their popularity in the South African goat farming industry. The implementation and popularity of frozen semen has opened the way for massive use of higher quality bucks. Furthermore, Genome Resource Banking through semen cryopreservation is a fundamental conservation strategy for any potential genetic resource.

Artificial insemination (AI) has gained widespread acceptance in other animals such as dairy cattle, horse and swine industries of most developing countries in general and in particular South Africa. However, its application in the goat industry has been much more limited. The AI in goats with cryopreserved semen is still not as developed as it is with other animal breeding. The poor ability of goat sperm to resist the freezing-thawing process and its much reduced performance resulted from its use are the reasons for limited application of this technique in the breeding programs of various goat breeds.

The major setback in semen cryopreservation technology is that the freezing-thawing process of mammalian sperm generally leads to a decrease in motility and viability of sperm cells as a result of damage to membrane integrity and ultrastructure (Watson, 2000). Frozen-

thawed sperm are subjected to chemical, thermal, osmotic and mechanical stresses that occur during dilution, cooling, equilibration and thawing as well as semen collection method, which may result in reduced sperm motility and viability. These deteriorative changes are more profound in buck than cattle spermatozoa due to unique physiology of the buck spermatozoa and higher polyunsaturated phospholipids levels in its plasma membrane (Aitken *et al*., 1993).

The most probable reason for poor post-thaw characteristics of buck semen is oxidative stress (OS) (Bucak *et al*., 2010). Spermatozoa are subjected to OS resulting from lipid peroxidation, which can lead to membrane damage, reduced sperm viability and lower fertility (Donghue and Donoghue, 1997). All sperm components including lipids, proteins, nucleic acids, and sugars are potential targets of OS (Agarwal *et al*., 2003). Although seminal plasma contains antioxidants that equilibrate lipid peroxidation and prevent excessive peroxide formation (Lewis *et al*., 1997), the seminal plasma antioxidants are not sufficient during semen storage (Maxwell and Salamon, 1993). In addition, the levels of antioxidant decreased during the cryopreservation process due to dilution of semen with extender and excessive generation of ROS (Andrabi, 2009; Kumar *et al*., 2011). The production of ROS at low concentration is a normal physiological event in various organs including the testis. Overproduction of ROS during cryopreservation has been associated with reduced post-thaw motility, viability, membrane and acrosome integrity, antioxidant status and fertility (Akiyama, 1999).

Spermatozoa are protected by non-enzymatic and enzymatic antioxidants in the seminal plasma or in spermatozoa itself to prevent oxidative damage (Kim and Parthasarathy, 1998). An antioxidant that reduces OS and improves sperm motility and viability could be of great importance in the management of cryopreserved semen. Therefore, animal and/or diluted semen need to be supplemented externally with natural antioxidants for improved post-thaw semen quality (Ahmad, 1994).

Selenium (Se) is an essential trace element in mammalian diet and its importance has been well established in goat (Ganabadi *et al*., 2010; Kumar *et al*., 2013; Lukusa and Lehloenya, 2017). Se antioxidant function is mediated through glutathione peroxidase enzyme activity. Se is an essential element in spermatogenesis and male fertility (Chavarro *et al*., 2010) and its antioxidant. Several studies on the effect of Se supplementation on semen quality and reproductive performance have been conducted in cattle (Ceballos *et al*., 2009) and rams (Mahmoud *et al*., 2013).

There are several other antioxidants in semen that are known to improve sperm quality such as vitamin E and C, which are components of antioxidant systems. Vitamin C and E are the major antioxidants naturally present in mammalian semen that regulates ROS, protect the sperm from lipid peroxidation and provides higher integrity to plasma membranes and mitochondria, as well as improves kinematics for sperm post-cryopreservation (Akhter *et al*., 2011; Silva *et al*., 2013; Mittal *et al*., 2014).

Recent studies demonstrate that supplementation of cryopreservation extenders with antioxidants has been tested successfully to provide a cryoprotective effect on bull, ram, goat, boar and canine sperm quality, thus improving sperm parameters such as motility and membrane integrity after thawing (Bucak *et al*., 2010; Mittal *et al*., 2014). In spite of these encouraging results, the use of antioxidants in freezing extenders is not common and there is lack of data with respect to its use in Saanen buck semen cryopreservation. Supplementation with these antioxidants prior to the cryopreservation process may be recommended to facilitate the enhancement of sperm cryopreservation technique for the goat breeding industry (Bucak *et al*., 2010). This is because goats present a wider range of fertility when frozen-thawed semen is used, varying from 3% up to 70% (Corteel, 1973). Modification in cryopreservation protocol, and experimental methodology and antioxidant concentration may influence the effectiveness of the antioxidant capacity.

The wider range fertility and the highly individual semen variability have resulted in a growing interest in the characterization of the sperm fertility and freezability of buck semen. Assessment methods of semen quality will help to improve early fertility prediction of semen following preservation. These methods will contribute significantly to improve animal reproductive techniques, and will aid in the selection of high-quality fertile animals. In the present study, traditional and CASA evaluation methods will be used to monitor changes that occur during cryopreservation. Understanding of the causes of phenotypic variation will offer some knowledge for the phenomenon of good and bad freezable buck semen, and provide the basis for improving the quality of cryopreserved semen through development of protocols of sperm cryopreservation with wide use.

CHAPTER 2

Literature review

2.1. Introduction

Fertility is one of the most important economical traits in goat production, affecting both animal productivity and genetic progress (Bauersachs *et al*., 2010). Knowledge of reproductive physiology and use of artificial insemination (AI) technology are powerful tools to achieve acceptable fertility. The AI is one of the most important reproductive techniques to accelerate the genetic improvement of animals. However, fertilizing capability of sperm is one of the most important factors in achieving a successful AI program. Moreover, the successful utilisation of AI depends mainly on the use of cryopreserved semen and on the techniques that could produce a higher fertility rates. However, the poor fertility obtained when frozen-thawed buck semen is used has attracted more research interest in the goat industry.

The insignificant result obtained when performing AI with cryopreserved semen in bucks has restricted its practical use in goat breeding, demanding for an improvement of AI technique itself and/or of the survival rate of the cryopreserved sperm. To accomplish the selection of highly fertile buck based on reproductive traits is of great importance. The body condition score, body growth, scrotal growth and fertilizing ability of sperm are the most important traits to consider prior to an AI program. Implementation of AI in goat production could allow for the improvement of buck selection based on production traits, but at the same time, it stresses the importance of the individual buck's reproductive performance. Dietary energy profoundly affects spermatogenesis and overall animal growth. Dietary intake of certain vitamins and antioxidants agents can improve both reproductive performance (Abecia *et al*., 1993) and fertility (Kassa and Tegene, 1998).

Buck reproductive performance is highly affected by semen quality such as sperm motility, acrosome integrity, morphology and viability. The quality of sperm after freezing is very important for success of AI. However, variations in sperm quality have been reported (Correa *et al*., 1997). Therefore, understanding the sources of variation in semen quality would be of great interest in goat the industry (Arredondo *et al*., 2015). AI with cryopreserved semen in goats is not common, and mostly fresh or cooled semen is utilised. In vitro survival of cooled semen decreases as the semen storage time is prolonged (Roca *et al*., 1997; Leboeuf and Restall Salamon, 2000). In addition, reports concerning fresh semen indicated that the sperm have a shorter fertile life span outside the body (Morrier *et al.,* 2002) and this is due to increased cellular metabolism at higher temperatures. Even though Brinsko *et al.*

(2000) suggested that, the freezing of semen would increase its longevity, the biggest challenge is fertility of the sperm cell after thawing which prevents their widespread use. Therefore, knowledge of factors that could affect fertility after thawing is of great importance in goat breeding programs.

A number of factors have been implicated in affecting the success and effectiveness of AI such as nutrition, environmental conditions, breeding season, parity, farm, breed, depth of semen deposition and extender composition (Nunes and Salgueiro, 2011). Despite all these factors affecting AI, failure to implement it in goat breeding program will result in a massive loss of valuable genetics. Therefore, proper knowledge and management of all these factors, including variations that could compromise the success of AI are vital.

2.2. Factors affecting buck semen production and fertility

The reproductive performance of goat breeds depends on age of the buck, environment, management system and genetic makeup of the animal. The combination of these factors is what determines the adaptability of goats and their reproductive performance (Robertshaw, 1982). Photoperiod and temperature are main factors that affect animals with seasonal reproductive parameters in temperate regions. In tropical regions, the rain is the environmental factor-affecting animals and has an influence on the quality of forage (Rege *et al*., 2000). However, additional factors related to climate including humidity and temperature fluctuations can induce thermal uneasiness, resulting in reduced feed intake and eventually affecting spermatogenesis and semen quality (Kunavongkrit *et al*., 2005). Therefore, the AI industry has to optimize the factors that alter semen production and sperm quality. Both genetic and non-genetic factors affect semen production (Chauhan *et al*., 2010). A number of these factors are discussed in this section.

2.2.1. Environmental factors

Sheep and goat are seasonal breeders (Rosa and Bryand, 2003). Seasonality has been demonstrated to affect semen quality in bucks (Thongtip *et al*., 2008). Ghalban *et al*. (2004) indicated that buck performance is most favorable during the period of increased daylight length. They further demonstrated that both semen quantity and quality were higher in spring and summer compared to winter or autumn in bucks. Semen production of goats living in temperate environments is influenced by the season, with photoperiod being the main determining factor (Loubser and van Niekerk, 1983; Ritar, 1993). Concentrations of many proteins molecules are under seasonal control and are associated with sperm function during breeding and non-breeding seasons (La-Folic *et al*., 2002). The total protein concentration of the seminal plasma was reported to be affected by seasonal variations in

rams, with a higher concentration recorded in autumn compared to summer and winter (Gundogan, 2006). The concentration of fructose and total protein in the goat seminal plasma was consistently lower in the dry season compared to the rainy season (Anguiar *et al*., 2013). Juma *et al*. (2009) reported decreased total cholesterol concentration in ram semen during the summer season. Habeeb *et al.* (2008) reported that the hot conditions were accompanied by significantly increased cortisol level. The biochemical parameters like protein and cholesterol increased during the summer season, which may be responsible for poor motility and libido.

A study conducted by Arrebola *et al*. (2010) in the Mediterranean demonstrated that photoperiod treatment allowed adequate sperm production in winter. Ansari *et al*. (2017) reported a significant seasonal variation in buck semen characteristics. They recorded better quality semen during winter as compared to summer season. Schwab *et al*. (1987) recorded the highest sperm concentration, semen volume and sperm number per ejaculate during winter. South African indigenous bucks have been reported to produce higher semen volumes and sperm motility in the hot summer months of December and January (Webb *et al*., 2004). The semen wave motion and sperm motility were found to be significantly lower during winter (June to August) period (Malejane *et al*., 2014). Zamiri *et al*. (2010) also added that high summer temperatures affected sperm motility to a lower degree. Gallego-Calvo *et al*. (2015a) reported that, in goats the values of sperm linearity coefficient (LIN), straightness coefficient (STR), curvilinear velocity (VSL), wobble (WOB), as well as motile, rapid and progressive sperm percentages, fluctuated between seasons. They observed lowest values and percentages during winter. They further reported seasonal effect on plasma testosterone content and body weight (BW). The plasma testosterone content increased in summer and autumn, and BW was higher during spring.

Photoperiod is however, the main environmental factor that affects sheep and goat reproduction. Photoperiodic variations caused by season have a significant effect on the reproductive performance due to modification in the concentrations of the hormones released by the hypothalamus, pituitary, epiphysis and gonads (Pérez and Mateos, 1996). Seasonal fluctuations of fertility in bucks are mostly a result of day length change during the year (Chemineau *et al.,* 1992; Talebi *et al.,* 2009; La Falci *et al*., 2002). Short days stimulate the release of luteinizing hormone (LH). In turn, LH stimulates testicular growth and the secretion of testosterone, resulting in improvements of semen quality and production as well as increased sexual behavior. Perversely, long days decrease LH release and testicular growth. This result in a reduction in the plasma testosterone content, decreased sperm quality, and eventually reduced sexual behaviour (Delgadillo and Chemineau, 1992;

Zarazaga *et al*., 2009). The hormone responsible for spermatogenesis and sexual behaviour is testosterone. However, the seasonal variation of testosterone production could influence the buck reproductive performance during some seasons of the year (Chemineau and Delgadillo, 1994). The study conducted on bucks by Gallego-Calvo *et al.* (2015a) recorded high testosterone contents in summer and autumn. They also recorded less testosterone contents in winter and spring. They further reported lowest total numbers, concentrations and decreased motility of sperm in winter when plasma testosterone was low. In winter, values of sperm velocity parameters and percentages of rapid, progressive as well as motile sperm were lower than in summer. In contrast, no differences were observed concerning other seasons.

2.2.2. Age of buck

The age of the buck at semen collection has been reported to affect ejaculates characteristics such as volume of the ejaculate, the sperm concentration, and sperm motility (Fuerst-Waltl *et al*., 2006). Numerous reports have suggested that when the age of the male animal increases, there is a decline in some semen parameters (Centola and Eberly, 1999). In rodents, age seems to induce certain histological modifications such as degeneration of testicular tissue, leading to the decrease in number of spermatogenic cell layers as well as degeneration of seminiferous tubules (Sarma and Devi, 2016). This may lead to the decrease of sperm quality (Mahal *et al*., 2013).

Osinowo *et al*. (1988) and Toe *et al*. (2000) reported that the scrotal circumference and semen volume normally increase when the ram age increases up to 5 years. Suggesting that, the genital system of the ram may undergoe certain maturational modifications during this period. The study of Mahal *et al*. (2013) reported that the age of buck affects all semen parameters except mass motility. Semen volume and sperm concentration were reported to increase with age, while live sperm percentages decreased significantly with age. The percentage of normal sperm was also reported to increase significantly with age. In addition, analysis of sperm motility parameters using CASA technique have also demonstrated an age-related decrease in values of LIN, VSL and average path velocity (VAP) (Sloter *et al*., 2006). Osinowo *et al*. (1988) also indicated that older rams produce higher volumes of semen, sperm concentrations and total sperm per ejaculate compared to younger rams. In another study, Langford (1987) found sperm production to increase proportionately with scrotal circumference.

The age of the buck is an indispensable physiological factor that changes the semen characteristics. Rajuana *et al.* (2008) reported a positive correlation of age with semen

volume in Black Bengal bucks. This implies that as the age of bucks increases, sperm production is expected to increase proportionately. In a research paper by Bitto and Egbunike (2012) that compares semen characteristics of pubertal and adult WAD bucks, they found that adult bucks were significantly superior to pubertal bucks in all the semen traits measured except semen colour. Incidences of abnormal and dead spermatozoa were higher in pubertal compared to adult bucks. In addition, Amann *et al*. (2000) found the percentages of abnormal sperm head shape to increase at younger and older age; while a gradual decrease was observed after sexual maturity. Abd-Allah *et al*. (2007) had earlier foundthat the optimal age of bucks for either natural service or AI was 2 years of age.

2.2.3. Management

The production and fertility of semen in buck depends largely on proper management of the animal, semen collection, preservation, storage and their application. However, handling the buck and how the semen is collected is also responsible for semen production and quality. Fuerst-Waltl *et al.* (2006) suggested that the handler must ensure appropriate sexual preparation of the animal before semen collection. Mathevon *et al*. (1998) reported a considerable variable effect of a group of people responsible for semen collection on the volume of ejaculates and total number of sperm. On the other hand, no significant effect was observed on sperm concentration and motility. The interval and frequency of semen collection largely affected the semen quality in the study conducted by Everett and Bean (1982) and Mathevon *et al*. (1998). Everett *et al.* (1978) and Everett and Bean (1982) found that, first ejaculates produced higher semen volumes, sperm concentration and total number of sperm. It was also further reported that if the intervals separating semen collections were shorter the number of sperm produced per ejaculate would decrease (Everett and Bean, 1982; Schwab *et al*., 1987), while the volume of semen ejaculated increases (Mathevon *et al*., 1998).

2.2.4. Genetics

Buck fertility is an indispensable economic trait, controlled by genetic factors. Generally, genetic factor is considered narrow because the heritability of fertility is normally low (Rollinson, 1955; Foote, 1970). Numerous investigations done in different species demonstrated the importance of various genes in the process of male reproduction and fertilization. However, reports on genetic control of fertility in bucks are still in their early stage and need detailed investigation to meet the future needs. The application of AI in modern breeding programs allows breeders to use small number of healthy males for improving the livestock genetics of economically important traits. The widespread utilization of AI associated with proper genetic evaluation can speed up the spread of genetic material

of commercial importance. The genetic factor has been observed in some goat breeds such as the Alpina goat, which produces good semen quality at a younger age, while the Damascus and Murciano-Grandina breeds produce good semen quality at an older age (Ghalban *et al*., 2004)**.**

In South Africa goat genetic improvement programs are mainly done using a conventional method (Campbell, 2003; Casey and Webb, 2010). The method has resulted in a considerable number of genetic improvements mainly in the meat goat sector (Mohlatlole *et al*., 2015). Despite success accomplished by South Africa to develop meat goat breeds, the need to develop goats that meet smallholder production systems persist. The smallholder production system is generally characterized by elevated diseases prevalence and poor quality nutrition (Mohlatlole *et al*., 2015).

2.3. Factors affecting the survival of cooled and frozen-thawed buck spermatozoa

AI with frozen-thawed semen could be utilized favorably for preservation of goat breeds, *ex situ* conservation and dissemination of germplasm (Kharche *et al*., 2013). However, successful AI depends on good quality of frozen-thawed sperm. Semen freezing involves an adjustment of sperm to the thermal and osmotic stresses that happen throughout dilution, cooling, freezing and thawing procedures (Watson *et al*., 1992; Holt, 2000). After dilution, sperm is kept at approximately 4 or 5^{o}C prior to cryopreservation. This process is responsible for the reduction of sperm cells metabolism and their adaptation to low temperatures, and cryoprotectants present in extenders (Dong *et al*., 2008; Gao and Zhou, 2012). The cryoprotectants eventually penetrate the sperm cells and set up equilibrium between intra- and extracellular concentrations (Salamon and Maxwell, 1995). During this time thermal shock may occur resulting in the loss of the sperm's potential to fertilize properly (Watson, 1995).

It is therefore important to develop a freezing protocol, which can preserve a considerable amount of functionally and structurally normal sperm (Sundararaman and Edwin, 2008). Therefore, acceptable cryopreservation of sperm would help in the provision of prolonged preservation of goat germplasm. This would also allow the preservation of genetic materials that could increase meat and milk production, and their related commercial benefits. The starting point in semen freezing is the use of an appropriate semen collection method (Marco-Jiménez *et al*., 2005; Jiménez-Rabadán *et al*., 2012). The method of collection may influence post-thaw sperm quality due to differences in their seminal plasma compositions. In addition, the survival of sperm in cryopreserved semen is also altered by other factors (

including individual effect, seminal plasma composition and semen processing) that need to be well understood before any cryopreservation procedure can be initiated.

2.3.1. Semen collection methods

The starting point to establish a sperm cryo-bank is the utilisation of a suitable method for the collection of the semen. Normally the semen collection method should be appropriate and should not affect sperm quality (Morrell *et al*., 1996). For bucks, collection of the ejaculates can be done by artificial vagina (AV) and/or electro-ejaculation (EE) (Marco-Jiménez *et al*., 2005; Jiménez-Rabadán *et al*., 2012; Lukusa and Lehloenya, 2017). The AV method is the most used procedure (Leboeuf*et al*., 2000), but this method needs a training period prior to semen collection (Wulster-Radcliffe *et al*., 2001). Sometimes this technique can be impractical and time wasting if the buck is difficult to handle or becomes excessively sensitive.

The EE technique is regarded as an alternative collection method where it is impossible to use an AV, as is the case for wild animals or when males are not trained. This technique is also a preferred method for collecting repeated ejaculates from animals without inducing any harm (Santiago-Moreno *et al*., 2009). However, differences between AV and EE methods in regard to sperm characteristics have been reported, with AV performing better than EE (Greyling and Gobbelaar, 1983). In addition, in certain species, sperm quality after thawing may present some variations inherent from semen collection methods. In a study conducted by Marco-Jiménez *et al*. (2005) ram sperm obtained by EE were found to be more resistant to cryo-damage than the one obtained using AV. In contrast, Jiménez-Rabadán *et al*. (2012) indicated that semen samples obtained by AV produced better post-thaw sperm quality than EE method.

Semen collection using EE technique may modify the secretory function of some accessory sex glands, therefore changing the entire biochemical composition of the seminal plasma (Marco-Jiménez *et al*., 2008). Seminal plasma plays a great role as a vehicle for ejaculated sperm. It is composed with various biochemical components such proteins, enzymes, lipids, organic acids and minerals released by the accessory sex glands (Manjunath and Thérien, 2002; Boisvert *et al*., 2004). These components are combined together with sperm during ejaculation; contributing to the volume and composition of semen. (Moura *et al*., 2006). Each biochemical component has an indispensable role in sperm function (Chacur, 2012; Sarsaifi *et al*., 2015; Marco-Jiménez *et al*., 2008). Their variation in the composition and content in seminal plasma is dependent on the collection method. It has been reported that proteins, sodium and potassium concentrations increased significantly when semen was collected

using EE (Marco-Jiménez *et al.,* 2008). In a study conducted in bulls, total protein concentration was found to increase significantly when semen was collected by an EE method (Sarsaifi *et al*., 2015). However, these proteins and electrolytes may play indispensable roles in protecting sperm from the detrimental effects cold-shock (Barrios *et al*., 2005), and maintaining stability of the membrane before capacitation happens in the genital tract of the female animal (Manjunath *et al*., 2002). Therefore, modification in their relative balance in seminal plasma may lead to poor cryo-resistance and fertility of spermatozoa.

2.3.2. Individual effect

One major factor affecting the survival of buck sperm during cryopreservation is the buck itself. The buck may be categorised as freezable or unfreezable depending on suitability of their semen to be used for freezing. This does not only rely on the quality of fresh semen, but also on the make-up of their seminal plasma as well as sperm plasma membranes (Aurich, 2005). Semen from individual buck can be more sensitive to cooling and freezing to the point that semen freezing is impossible. Sperm from males of the same species may present physiological variations.

Curry (2000) reported that physiological disparities among sperm from males of the same species might exist. In addition, Sultana *et al*. (2018) reported a significant individual variation on semen volume of five bucks of the same breed, similar age, nutritional status and general health condition. However, such disparities could be relatively genetic or non-genetic. This demonstrates inter-buck variability in sperm freezeability as a source of variation during the cryopreservation procedure (Soler *et al*., 2003; Ramón *et al*., 2013). Several reports have demonstrated individual differences in goats concerning sperm freezeability and fertility (Furtoss *et al*., 2010; Medrano *et al*., 2010). Variability at the level of ejaculates within the same buck has also been reported (Furtoss *et al*., 2010).

2.3.3. Seminal plasma

Seminal plasma is produced firstly from secretions of the epididymis and accessory sex glands. It is recognised to contain proteins, enzymes, lipids, electrolytes and other different components which play a major role in the regulation of sperm metabolism. There are different types and amount of seminal plasma proteins between individuals. Some proteins prevent sperm from damage caused by cold shock (Pérez-Pe *et al*., 2002). These proteins could be affected by some environmental factors including season, stress, feeding,

temperature (Pérez-Pe *et al*., 2001) and semen collection method (Marco-Jiménez *et al*., 2008).

The seminal plasma-induced detrimental effects on sperm during the process of cooling and storage may be related to the action of specific enzymes. The seminal plasma enzyme phospholipase A2 has been reported to play a role during acrosome reaction and spermatozoa-oocyte fusion (Yuan *et al*., 2003). In goats, coagulating egg yolk enzyme (EYCE) and bulbourethral III secretion (SBUIII) are secreted by bulbourethral glands and have phospholipase activity. The EYCE hydrolyses egg yolk lecithin (contained in freezing extenders) into fatty acids and lysolecithin, which is toxic to sperm (Sias *et al*., 2005). This hydrolysis causes acrosome reaction (Upreti *et al.*, 1999) and chromatin decondensation (Sawyer and Brown, 1995). Similarly, SBUIII hydrolyses residual triglycerides in the skim milk from freezing extenders leading to production of fatty acids, which are toxic to spermatozoa (Pellicer-Rubio and Combarnous, 1998). This causes a decrease in the percentage of motile sperm, breakage of acrosome and cellular death. In order to avoid the detrimental effect of these enzymes (EYCE and SBUIII), it has been suggested that the removal of seminal plasma by means of centrifugation may increase the sperm motility, membrane integrity and fertility after the freeze/thawing procedure (Machado and Simplicio, 1995; Kozdrowski *et al*., 2007). Other reports have found no effect of seminal plasma removal on sperm quality after thawing (Daskin and Tekin, 1996; Cabrera *et al*., 2005). Jiménez-Rabadán *et al*. (2013) indicated that the removal or not of seminal plasma before freezing by centrifugation did not affect sperm quality at thawing regardless of collection method and extender used. However, they observed a beneficial effect of seminal plasma removal when the semen collection was performed during the non-breeding season.

2.3.4. Semen processing

The success of cryopreservation depends upon many factors such as freezing extenders, cooling rate, equilibration time packaging and thawing (Cooter *et al*., 2005; Curry, 2007). During all of these processes, spermatozoa are exposed to damaging stresses such as temperature and osmotic changes. These stresses are produced by exposure to cryoprotectants, formation and dissolution of ice crystals in the extra-cellular space (Watson, 2000). Therefore, understanding of all steps involved in semen processing prior to the cryopreservation program is of great importance.

2.3.4.1. Extenders for freezing semen

Extenders are used to dilute and protect sperm during liquid storage or freezing. The successful storage of buck semen in liquid and frozen forms depends on the composition of

the extender used to ensure survival and fertility of the sperm for long period (Purdy, 2006; Mara *et al*., 2007). The major role of semen diluents is to provide nutrients as energy source and ensure appropriate physiologic at osmotic pressure as well as protection from cold shock during cooling and freezing procedures (Concannon *et al*., 1989).

In general, the diluents used for cooling or freezing of semen include egg yolk, skimmed milk, glycerol or their combination (Maxwell and Watson, 1996; Sharafi *et al.,* 2009; Kulaksiz *et al.,* 2013). Several extender compositions containing different concentrations of these compounds have been studied for cryopreservation of goat semen (Bittencourt *et al*., 2007). Regardless of the good fertility rate after using egg yolk, skimmed milk or glycerol as extenders, some limitations following their utilisation have been reported in goats (Leboeuf *et al.,* 2000). Goat semen contains lipases secreted from bulbourethral glands that interact with egg yolk and skimmed milk eventually producing harmful substances that have detrimental effects on sperm during storage. However, glycerol and egg yolk are the most popular cryoprotectants utilised. There is a need to stop utilization of egg yolk in freezing media, because it may contain microbial contaminants that can compromise fertilization ability of sperm (Bousseau *et al.,* 1998; Bittencourt *et al.,* 2007). To avoid the deleterious effects caused by egg yolk, Soybean lecithin-based extenders free of animal protein were proposed as alternatives (Bousseau *et al*., 1998). The soybean lecithin is naturally composed of phosphatidylcholine and some fatty acids (stearic, oleic and palmitic). Papa *et al*. (2010) had demonstrated the importance of soybean lecithin as the main source of lipoproteins in freezing extenders. It been also been indicated that addition of soybean lecithin to freezing extender increased the percentages of sperm motility, viability and acrosome integrity after thawing in ram and goat semen (Sharafi *et al.,* 2009; Forouzanfar *et al*., 2010(Jiménez-Rabadán *et al*., 2012; Salmani *et al*., 2014; Yodmingkwan *et al.*, 2016). In addition, some researchers that compared egg yolk-based extender and soybean lecithin published contradictory reports (Aires *et al*., 2003; Gil *et al*., 2000; Thun *et al*., 2002; Vidal *et al*., 2013). They suggested that this might be attributed to different breeds of goats used and geographic regions. The seasons in which the semen was collected may also influence the semen quality (Leboeuf *et al*., 2000). The well-planned processes of storage of semen with appropriate extenders, suitable cooling rate and equilibration period are main key components to develop an acceptable and species-specific cryopreservation cycle.

2.3.4.2. Cooling rates

Cooling semen from temperature of 37^{o}C to 4^{o}C, defines the success of cryopreservation process concerning sperm viability (Holt *et al*., 2005). This process requires suitable cooling rate, physiologically equilibrated homeostasis and metabolism (Martorana *et al*., 2014). The

cooling process decreases metabolic function and increases the life of the sperm cells. However, inappropriate cooling rate initiates thermal discomfort and induces membrane damage due to proteins structural disturbance, disruption of ion channels and production of ROS as well as reduced potential of the mitochondrial membrane (Watson, 2000; Holt *et al*., 2005; Martorana *et al.*, 2014).

Various cooling rates (rapid and slow) have been studied in different species. Even with slow cooling, sperm cells are subjected to thermal stresses and become compromised. Excessively slow or fast rates of cooling may cause irreparable damage to the spermatozoa integrity due to osmotic and oxidative changes (Koshimoto and Mazur, 2002; Ahmad *et al*., 2015). The appropriate rate of cooling must be sufficiently slow in order to allow water to come out of sperm cells to avoid intra-cellular ice formation, and sufficiently fast to avoid excessive cell dehydration and damage due to the medium effect (Mazur, 1970). Apart from goat where no appropriate cooling rate has not been established, other species have been reported to have appropriate cooling rates, such as between 76 and 140°C/min in bull (Woelders *et al*., 1997), 30°C /min in boar (Fiser and Fairfull, 1990), 27°C to 130 °C / min in mouse (Koshimoto and Mazur, 2002) and 10°C/min in human (Henry *et al.,* 1993).

Even with an appropriately slow cooling rate, sperm cells face temperature stresses that lead to dysfunction. The intensity of damage is related to the rate of lowering temperature (Watson, 2000). Rapid cooling on the other hand, causes a lethal condition for sperm (Dhami *et al.*, 1992), known as ‘‘cold shock’’ (Fiser and Fairfull, 1986), which induces osmotic and oxidative damages. As a result there is a disruption in cell physio-chemical functions, inducing disturbed homeostasis and over-regulation of ROS (Ahmad *et al*., 2015). The magnitude of these negative changes is relatively associated to the sperm cell membrane composition. This is because the low ratio of poly-unsaturated fatty acids allows the sperm to be more resistant to cold shock (Bouchard *et al*., 1990), and have been explored in various species (Devireddy *et al.*, 2002; Leboeuf *et al*., 2000). However, the effects of different rates of cooling on buck sperm have not yet been explored in detail (Ahmad *et al*., 2015). In addition to cooling, sperm must retain their viability at thawing. It is believed that suitable cooling rate associated with appropriate equilibration time can help sperm to cope with detrimental effects caused by cold stresses during freezing (Holt *et al*., 2005).

2.3.4.3. Equilibration time

Prior to freezing, diluted semen in extender is kept at a temperature of 4 or 5^{o}C; this decreases the sperm cells metabolism, allowing them to adjust to low temperatures (Dong *et*

al., 2008). This is followed by the acclimatisation of the sperm to osmotic variations once in contact with the cryoprotectant (Gao and Zhou, 2012). The period during which the sperm cells stay mixed with the cryoprotectant in freezing extender, prior to freezing is generally named equilibration period. This period allows cryoprotectants (e.g. glycerol) to penetrate the sperm cell to establish an equal intracellular and extra-cellular concentration. The equilibration time comes after the cooling stage during the freezing process (Leite *et al*., 2010). This process affects the stabilization of sperm in extended medium to keep homeostasis, osmotic and cryo-tolerance (Herold *et al*., 2006). Therefore, various equilibration periods have been investigated in different species including goats (Ahmad *et al.*, 2015), cattle (Shahverdi *et al*., 2014; Shah *et al*., 2016) and sheep (Câmara *et al*., 2016). It is noteworthy that the reports of several fertility studies developed to define suitable equilibration period for buck semen, proposed a longer equilibration time (2-8 h) at 5˚C prior to freezing to reach maximal fertility (Jiménez-Rabadán *et al*., 2013; Ahmad *et al*., 2015).

Yi *et al*. (2002) reported the most suitable equilibration time for the cryoprotectant to penetrate sperm cells is to be between 2-4 h in boar. Ahmad *et al*. (2015) reported that equilibration of 2-8 h maintained better sperm quality of buck sperm. It has also been claimed that 2 h of equilibration time is suitable for buck semen (Sundararaman and Edwin, 2008). However, all these studies used whole egg yolk based extenders. The equilibration time depends upon type and concentration of cryoprotectant used (Iaffaldano *et al*., 2012).

2.3.4.4. Packaging, storage, freezing and thawing rates

Following the dilution of buck semen with freezing extender, it is cooled to 4-5^{o}C and packaged in either straws or pellets (Ritar, 1993; Gravance *et al*., 1997; Leboeuf *et al*., 2000). Both straws and pellets are the most popular packaging system utilized since the inception of cryopreservation.

The use of straws in sperm freezing is more expensive than using the pellets freezing technique, but allows labelling and precise identification of samples. It also decreases the risk of contamination between samples during storage and cryopreservation. In straw freezing, diluted and chilled semen samples are loaded to 0.25 or 0.5 ml straws, placed on a rack 3 to 4 cm above the liquid nitrogen (LN_2) for 7 to 8 min on LN_2 vapour in a Styrofoam box or in programmable freezer and straws are then plunged in LN_2 for preservation (Evans and Maxwell, 1987). In pellet freezing, aliquots of 0.1-0.5 ml of cooled semen sample are indented on a block of solid carbon dioxide (−79°C). The pellets are then frozen for a period of 2-4 min followed by plunging them into LN_2 for preservation (Evans and Maxwell, 1987; Chemineau *et al*., 1991).

Packaging of semen has great significance seeing that it allows the identification of each dose and its preservation in the LN_2 container (Maxwell *et al*., 1995). However, the size and type of package may affect the semen cooling and freeze/thawing rates (Maxwell *et al*., 1995). It has been reported that the use of 0.5 ml straws produced better preservation of goat sperm than 0.25 ml straw concerning sperm progressive motility and acrosome integrity after cryopreservation process (Bezerra *et al.,* 2012). Ritar *et al.* (1990) reported increased motility of sperm frozen in pellets after thawing compared to sperm frozen in straws. However, no difference was recorded in terms of sperm motility between sperm frozen in 0.25 and 0.5 ml straws.

The detrimental effect caused by thawing occurs when the sperm passes through the critical zone between −50^{o}C and −15^{o}C or −5^{o}C (Lahnsteiner, 2000). In addition, sperm undergoes osmotic stress when the period of thawing is insufficient to efflux the excess of cryoprotectants from inside the cells (Penitente-Filho *et al*. (2014). Sperm becomes turgid and smooth due to the abrupt dilution of the medium caused by thawing of extra-cellular ice (Andrabi, 2007).

The entire processing of cryopreservation including dilution, cooling, packaging method, and freezing semen in straws or pellets determines the optimal rate of thaw. Sperm cells frozen by pellets method are thawed using the dry test tube method at 37^{o}C while the straws may be thawed using different techniques (Evans and Maxwell, 1987). A straw is normally thawed by submerging it in a 37^{o}C water bath for about 12–30 s (Deka and Rao, 1987). This technique yielded higher sperm motility compared to a slow thawing technique, where the straw containing semen is placed in a 4-5^{o}C water bath for approximately 2 min (Deka and Rao, 1987). In another study, when temperature of thawing was increased to 70^{o}C and thawing rate for 7 s. The percentages of sperm progressive motility and plasma membrane integrity were increased significantly more than straws thawed at 37^{o}C for 2 min or 40^{o}C for 20 s, respectively (Gangwar *et al*., 2016). Penitente-Filho *et al*. (2014) tested two different thawing rates in goats, 38^{o}C for 60 seconds or 60^{o}C for 7 s followed by 60 seconds at 38^{o}C, and found no difference in terms of sperm motility and vigor. Nevertheless, thawing at 60^{o}C for 7 seconds increased functional integrity of the sperm membrane.

2.4. Oxidative stress (SO)

The OS constitutes one of the most contributing factors to poor sperm quality during semen cryopreservation (Bucak *et al*., 2010). This phenomenon is related to higher rate of sperm damage caused by oxygen and ROS (Sikka *et al*., 1995). Excessive ROS production in

seminal plasma results in OS, which is detrimental to sperm cells (Desai *et al*., 2010). All cellular properties including lipids, proteins, nucleic acids, and sugars are potential targets of OS (Agarwal *et al*., 2003).

2.4.1. Free radicals

Free radicals are short-lived reactive chemical bonds which possess unpaired electrons that are highly unstable (Sanocka and Kurpisz, 2004; Kefer *et al*., 2009). To become stable, these electrons react immediately with other free radicals or non-radicals in their surroundings. Therefore, free radicals have the ability to capture the electrons from nucleic acids, lipids and proteins and eventually induce damages to sperm cells (Flora, 2009). They contribute to sperm cell destruction when they go through this unpaired electron onto surrounding cellular structures, thus leading to oxidation of cellular membrane lipids, amino acids in proteins or within nucleic acids (Ochsendorf, 1999). Membrane leakiness, DNA fragmentations and mitochondrial damage are the possible outcomes of free radical damage (Saraswat *et al*., 2016). Free radicals are unavoidable for intracellular signaling involved in the normal process of cell proliferation, differentiation, and migration (Ford, 2001; Agarwal *et al*., 2005).

2.4.2. Reactive oxygen species (ROS)

The term reactive oxygen species (ROS) is a diversity of reactive molecules derivative of oxygen (O_2) or highly reactive oxidizing agents related to the group of free radicals (Bolisetty and Jaimes, 2013). ROS are a result of the excitation of O_2 to form singlet (O_2^1). They can also result from the transfer of one, two or three electrons to form a superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) or a hydroxyl radical (OH.) (Saraswat *et al*., 2014). The production of ROS is a normal physiological event but a discrepancy between ROS production and scavenging activity is harmful to the sperm and has been linked to male infertility (Sharma and Agarwal, 1996). ROS produced by spermatozoa play an indispensable role in normal physiological processes such as sperm capacitation, acrosome reaction and maintenance of fertilizing ability. The mitochondrial capsule in the sperm mid-piece is stabilized by ROS (Agarwal *et al*., 2008; Gonçalves *et al*., 2010). ROS are also involved in sperm motility initiation by enhancing the cAMP synthesis and protein phosphorylation during ejaculation (Baumber *et al*., 2000).

Preserving normal physiological ROS concentration is, therefore, indispensable for efficient sperm functionality. The concentration of ROS present in the sperm cell and in the medium affects both sperm capacitation and acrosome reaction processes. Excessive concentration of ROS may have a negative effect in the male genital tract by altering antioxidant capability

to prevent sperm from undergoing capacitation (saraswat *et al*., 2014). The main targets for ROS have been reported to be in the plasma membrane of sperm and DNA integrity and physiological functions, therefore, altering the sperm quality. In the plasma membrane, ROS destroy mainly polyunsaturated fatty acids resulting in sperm damage leading to infertility (Saraswat *et al*., 2014). The DNA damage induced by ROS may also alter sperm function such as motility (Saraswat *et al*., 2012) by reducing the ATP needed for the sperm motion.

Procedure of cryopreservation increases premature capacitation of spermatozoa because of the generation of excessive production of ROS. The over-production of ROS has been responsible for decreased sperm motility, viability and membrane integrity. The antioxidant activity, sperm fertility and functions are also altered by ROS over-production after thawing (Uysal and Bucak, 2007). Furthermore, antioxidant concentration and the period of storage have a considerable effect on sperm quality parameters such as motility, viability, acrosomal integrity and membrane integrity (Upreti *et al.,* 1994).

2.4.3. Physiology and mode of action of reactive oxygen species

The ROS, at low concentrations, are involved in the maintenance of male fertility. As mentioned earlier, evidence suggests that ROS are involved in the activation of intracellular activities responsible for sperm maturation, capacitation and acrosomal reaction in order to ensure appropriate fertilization (Saleh and Agarwal, 2002). Excessive ROS may negatively affect the sperm plasma membrane. The vulnerability of goat sperm to ROS is a result of the higher concentration of polyunsaturated fatty acids found in sperm's plasma membrane. The polyunsaturated fatty acids are responsible for the membranes fluidity and flexibility, which assist the sperm to take part in the membrane fusion processes related to the fertilization. However, the presence of double bonds in polyunsaturated fatty acids makes them susceptible to lipid peroxidation (LPO). LPO is a consequence of the detrimental effect induced by oxidation on the sperm membrane lipids (Sharma and Agarwal, 1996). The LPO reaction causes alterations on fluidity of the sperm membrane, damage of membrane integrity and non-remediable decrease of sperm motility (Storey, 1997). The functions of some enzymes (cytochrome oxidase, lactase dehydrogenase and glucose-phosphate dehydrogenase) are also altered by LPO (Ferrandi *et al*., 1992). In addition, mitochondria constitute a potential target for ROS by disrupting its functions. The synthesis of DNA, RNA and proteins is also inhibited. The DNA fragmentation has been reported to increase due to excessive ROS (De Lamirande and Gagnon, 1992).

The mode of action of ROS consists of several physiological processes in sperm cells. The processes are organised in three different stages known as initiation, propagation and

termination (Nogushi and Niki, 1999). During the initiation stage, the ROS produce lipid radicals. The propagation stage is characterized by alterations of other unsaturated fatty acids by lipid radicals from the initiation stage. In this stage, lipid radicals may catch electrons from oxygen (O_2) and transform them into hydrogen peroxide (H_2O_2). In turn, (H_2O_2) destroys other polyunsaturated fatty acids on the cell membrane. The final stage is known as termination stage of the LPO. During this stage, free radicals are combined to form paired stable electrons. The termination stage can be prevented earlier by using antioxidant agents that can destroy free radicals (Silva, 2006; Saraswat *et al*., 2012). Due to limited reports in goats, information from other species was used in the present study. The production of superoxide radicals ($O_2^{\bullet}-$) has been reported during cryopreservation processes in Human, bovine and dog (Tselkas *et al.,* 2000; Chatterjee and Gagnon, 2001; Michael *et al.,* 2008). Furthermore, the low concentration in semen of antioxidants glutathione (GSH) or superoxide dimutase (SOD) was found to be related to freezing-thawing processes in the bovine (Bilodeau *et al.,* 2000) and human (Alvarez and Storey, 1992). Therefore, it can be suggested that, sperm damage induced by ROS is more prevalent during cryopreservation processes.

The ROS have double role in sperm functions which are both beneficial and detrimental. During normal concentrations, ROS play an indispensable role in various biochemical processes. At excessive concentrations, they cause damage to sperm cells. The excessive production of ROS may be responsible for their elevated concentration in the cells. This can be attributed to inadequate antioxidants of the cell to prevent over-production of the ROS or pathological conditions. In case where natural defences are unable to keep a normal physiological balance between ROS and antioxidants, there are numerous ways to combat over-production of ROS, such as dietary supplementation and addition of antioxidants to freezing extenders. Therefore, antioxidant supplementation may be required to boost the natural antioxidants and enhance the ability of the seminal plasma to fight OS (Agarwal *et al*., 2005). The following section discusses the different antioxidants, including non-enzymatic and enzymatic, which can be used to fight OS.

2.5. Supplementation of antioxidants in freezing extenders

Sperm cells are protected by enzymatic and non-enzymes antioxidants present in the seminal plasma or in the sperm itself to reduce or prevent oxidative damage (Kim and Parthasarathy, 2008). However, it may happen that these natural antioxidants fail to maintain the physiological (normal) balance between ROS and antioxidants leading to OS. In such cases, strategies can be applied to suppress OS, such as supplementation of antioxidants

(both enzymatic and non-enzymatic). Recently, many researchers reported the beneficial effects of antioxidant addition to freezing extenders of goat semen (Bucak *et al*., 2010; Daramola and Adekunle, 2015), and other animal species bull and ram (Mittal *et al*., 2014; Kirilova *et al*., 2015; Vichas *et al*., 2017). Vitamin C and E have been suggested as effective antioxidants. Daramolaand Adekunle (2015) reported higher spermatozoa motility, and acrosome and membrane integrities in extenders supplemented with vitamin C. While vitamin E is responsible for sperm structural integrity and kinematics after cryopreservation in rams (Silva *et al*., 2013). In contrast, in a study conducted by Vichas *et al*. (2017), the supplementation of vitamin E did not provide significant protection to sperm based on evaluated parameters. Câmara *et al*. (2011) reported that addition of three different antioxidants (glutathione, superoxide dismutase or catalase) in freezing extender failed to neither increase the total antioxidant potential of semen nor improve the quality of the ram sperm after cryopreservation. However, controversy surrounding the effect of antioxidant on sperm freezing still persists (Bucak *et al*., 2009; Vichas *et al*., 2017).

2.5.1. Addition of non-enzymatic antioxidants in freezing extenders

Various non-enzymatic antioxidants in seminal plasma prevent oxidative attack against sperm cells (Khan *et al*., 2012). However, in the event where there is excessive production of ROS above natural antioxidant defence, sperm DNA, proteins, and lipids will be subjected to damages. ROS can also have detrimental effect on mitochondrial ATP generation in sperm and induce LPO (Sikka, 2001). Thus, for better post-thaw sperm quality, supplementation of freezing extender with natural antioxidants has been shown to improve the quality of sperm against ROS damage (Memon *et al*., 2011).

Vitamin C, E and reduced glutathione (GSH) are the most important natural and non-enzymatic antioxidants present in mammalian semen. These antioxidants control the concentration of ROS, protect the sperm from LPO damage and maintain higher plasma membrane and mitochondria integrity as well as increase sperm kinematics parameters post-thaw (Akhter *et al*., 2010; Silva *et al*., 2013). Excessive ROS generation and reduced antioxidant levels are a consequence of sperm cryopreservation. Therefore, semen freezing extenders require supplementation with natural antioxidants to enhance post-thaw semen quality (Maia *et al*., 2010). A study conducted by Azawi and Hussein (2013) and Aminipour *et al.* (2013) in rams investigated the efficiency of both vitamin C and E on sperm quality and showed that there were significant beneficial effects of vitamins C and E addition to semen freezing extenders on sperm quality.

2.5.1.1. Supplementation of freezing extender with vitamin C (ascorbic acid)

Vitamin C is among the most important antioxidants in seminal fluid released from the seminal vesicles. It has been reported to be a major antioxidant present in seminal plasma of fertile males (Lewis *et al*., 1995). Vitamin C represents the major water-soluble antioxidant in blood plasma and seminal plasma (Carr and Frei, 2002). The water solubility and low toxicity of vitamin C are claimed to be responsible for the protective effect from ROS damage on sperm (Asadpour *et al*., 2011; Daramola and Adekunle, 2015). It has the capability of alleviating OS (Min *et al*., 2016) and neutralizing hydroxyl, superoxide, and hydrogen peroxide radicals produced in a hydrophibic environment (Azawi and Hussein, 2013).

The addition of vitamin C in freezing extender can have beneficial effect on sperm capability by preventing cell alterations through its unlimited radical-scavenging mechanism during sperm cryopreservation process. The addition of vitamin C to freezing extender of ram semen decreased sperm abnormalities and acrosomal defects values compared to the control group (Azawi and Hussein, 2013). Daramolaand Adekunle, (2015) indicated that the percentages of sperm quality parameters evaluated were increased when extenders were supplemented with vitamin C during freezing of buck semen. The increase in sperm motility, acrosome and membrane integrity was recorded in extenders supplemented with 8 mM of vitamin C. Memon *et al.* (2013) demonstrated that cooled and post-thaw sperm quality of Boer goat were improved when a Tris-based extender added with vitamin C was used. The antioxidant capability of vitamin C has been suggested extensively in other species such as bovine where it has been demonstrated that addition of vitamin C in semen extender improved sperm motility, as well as acrosome and membrane integrity (Hu *et al*., 2010; Reza *et al*., 2011; Asadpour *et al*., 2011; Mittal *et al*., 2014). Min *et al*. (2016) demonstrated that supplementation of vitamin C can suppress the negative effect of DEX-induced OS on sperm viability and motility of breeder roosters. Azawi and Hussein (2013) showed that vitamin C was more efficient in protecting ram sperm viability and acrosomal integrity.

2.5.1.2. Supplementation of freeing extenders with vitamin E (α-Tocopherol)

Vitamin E is believed to be the primary components of the antioxidant system of the spermatozoa, and is one of the major membrane protectors against ROS and LPO (Surai *et al*., 1998; Akiyama, 1999). It is a lipophilic antioxidant located in the sperm cell membrane known to act as chain-breaker. It acts by breaking and neutralizing the covalent bonds that ROS have established between fatty acid chains (Jeong *et al*., 2009). Vitamin E can also halt LPO reactions by removing peroxyl, alkoxyl, and other lipid-related radicals, therefore protecting the membrane against detrimental effect caused by ROS (Silva, 2006).

The efficiency of vitamin E was evidenced after dietary supplementation in cattle and rams, where the semen production and sperm concentration were increased considerably (Liu *et al*., 2005; Bansal and Bilaspur, 2009). The mammalian cells do not synthesize vitamin E. Once membrane tocopherol is reduced during period of OS, cell lipids are subjected to peroxidation, which can result in detrimental effect to spermatozoa (Zhang *et al*., 2001). The ascorbate and thiols are external agents responsible for the recycling as well as enabling vitamin E to maintain a stable and low concentration of peroxyl radical in the plasma membrane (Maia *et al*., 2009; Maia *et al*., 2010).

AminiPour *et al*. (2013) reported that addition of vitamin E in semen extender improved sperm progressive motility and viability before and after cryopreservation. In another studies, it was demonstrated that the addition of vitamin E in freezing medium increased rams sperm survival, longevity, and decreased free radicals concentrations after cooled storage (Azawi and Hussein, 2013; Zeitoun and Al-Damegh, 2014). The experiments reported in other species, revealed the efficiency of vitamin E in protecting the sperm plasma membrane integrity after deep freezing of bull sperm (O'Flaherty *et al.,* 1997). Cerolini *et al*. (2000) reported similar results with boar semen stored at 19 °C in liquid form. A study conducted in mice revealed that male mice injected with vitamin E have a lower quantity of sperm with defective heads than non-injected mice (Raza *et al*., 2011).

Addition of vitamin E to the semen extenders may prevent or reduce the production of free radicals that can damage the plasma membrane, therefore improving hypo osmotic swelling scores and sperm motility post-thaw. It has also been reported to reduce LPO, thus efficiently protecting the sperm against OS. The anti-oxidative property of vitamin E prevents the expression of apoptosis genes by lowering DNA fragmentations (Jeong *et al*., 2009).

2.5.1.3. Supplementation of freezing extenders with reduced glutathione (GSH)

Reduced glutathione (GSH) is a tripeptide that occurs naturally in semen and plays an important role in the intra-cellular defence mechanism against OS during sperm cryopreservation. It maintains the active forms of externally supplemented vitamin C and E (Gadea *et al*., 2004). The GSH is considered as catalyser of the reduction of H_2O_2 into water and hydro peroxides. It also involved in the maintenance of a normal level of free radicals (Bilodeau *et al*., 2001). However, dilution of seminal plasma with semen freezing extender is responsible for the decrease in GSH concentration, probably because of OS and cell death (Bilodeau *et al*., 2000; Gadea *et al*., 2004).

Glutathione supplementation in semen extender is reported to prevent detrimental effects caused by free radicals (Bilodeau *et al*., 2001). The supplementation of glutathione to semen extender protected sperm motility by inhibition of lipid peroxidation caused by ROS in cryopreserved buck semen (Sinha *et al*., 1996). Moreover, Saranji *et al*. (2017) reported that addition of glutathione in tris extender helped to preserve diluted buck semen up to 72 h at 4°C in refrigerator. They also reported higher percentages of sperm progressive motility, liveability, and acrosome integrity. The decrease in lipid peroxidation followed by increase in antioxidant enzymes has also been reported.

2.5.1.4. Selenium as an antioxidant

Selenium (Se) is one of the most indispensable trace nutrient found in human andanimal cells. The most important role of Se in biological cell is prevention and/or removal of free radicals through glutathione peroxidise. Glutathione peroxide is the main enzyme activated by Se to combat OS. Selenium also acts as a cofactor of glutathione synthetase. Zhang *et al*. (2006) added Se in form of sodium selenite in a cell culture medium to protect cells against detrimental effects caused by oxidation. Se has been reported to improve semen quality by increasing SOD, GSH-Px level, total antioxidant capacity and decreased hydroxyl free radical in seminal plasma in goat (Shi *et al*., 2010; Li-guang *et al*., 2010).

The reproductive performance of male goats can be improved through dietary Se supplementation. Dietary supplementation of Se has been found to improve semen quality by increasing the antioxidative defence of seminal plasma in buck (Shi *et al*., 2010), Boer goats (Li-guang *et al*., 2010) and ram (Kendall *et al*., 2000). Kumar *et al*. (2011) have also reported improved post-thaw sperm quality of Barbari bucks supplemented with Se. In another study, Elsheikh *et al*. (2014) reported a significant increase of Se and glutathione concentrations in serum of Aaradi goats after dietary Se supplementation.

In sheep, inadequate supplementation of Se was linked to reproductive complications and reduced sperm quality (Baiomy *et al*., 2009). A deficiency of Se induces changes in the sperm's mid-piece leading to the breakage of the head and tail of sperm and impaired sperm motility (Maiorino *et al*., 2006). Furthermore, male hypogonadism, reduced production and semen quality have been reported to be prevalent in males kept on a low Se diet (Kleene, 1993).

2.5.2. Addition of enzymatic antioxidants in freezing extenders

Mammalian spermatozoa have evolved defence mechanisms through an enzymatic antioxidant system to protect themselves against OS. The best known enzymatic antioxidant

system is super oxide dismutases (SOD), catalases (CAT) and glutathione reductase. The concentration of these antioxidative enzymes differs among species in both seminal plasma and spermatozoa (Asadpou, 2012). Under normal conditions, there is a balance in spermatozoa between ROS production and antioxidant enzyme system for sperm stability and function (Cassani *et al*., 2005; Kotheri *et al*., 2010; Guthrie and Welch, 2012).

2.5.2.1. Super oxide dismutase (SOD)

Superoxide dismutase (SOD) is one of the enzymes present in seminal plasma and naturally combats ROS (Weir and Robaire, 2007). The SOD converts O^{2-} to H_2O_2, diminishing ROS activity and plays an indispensable role in decreasing LPO (Shiva *et al*., 2011). Its supplementation to freezing medium prevented ROS generation, leading to the increase of ram sperm survival and in vitro fertility of sperm stored in liquid form (Maxwell and Stojanov, 1996). Shaflei *et al*. (2015) reported that the addition of SOD to freezing extender, such as Andromed, improved the viability and motility of goat semen samples after thawing. In another study conducted in boar, Roca *et al*. (2003) indicated that supplementation of SOD in sperm freezing extender reduced ROS production after thawing. They further reported an increase in sperm motility, viability and in vitro fertilizing capability of thawed sperm.

2.5.2.2. Catalase (CAT)

Among enzymatic antioxidants contained in both sperm cytoplasm and seminal plasma, catalase (CAT) plays a major role protecting sperm against ROS damage. Catalase also plays a vital role in detoxification of both intracellular and extracellular hydrogen peroxide (H_2O_2) by breaking it down into water (H_2O) and oxygen (O_2) (Foote, 1962). Besides maintaining low concentration of LPO and enhancing the quality of post-thaw sperm; It is a major antioxidant of ROS (Shiva *et al*., 2011). The existence of CAT in sperm cells has been confirmed in ram and bull and it plays an indispensable role in the ageing process and management of OS in cells (Saraswat *et al*., 2016). Shaflei *et al*. (2015) indicated that goat semen cryopreservation extenders supplemented with CAT increased sperm motility and viability post-thaw by destroying excessive ROS.

Prior to sperm cryopreservation semen must be diluted with suitable freezing extender to provide nutrient and other cryogenic components. However, this process is responsible for the reduction of antioxidants content in seminal plasma exposing sperm to OS damage (Martinez-Páramo *et al*., 2012). It has been evidenced that controlled levels of ROS is beneficial to maintain normal physiological functions of sperm including acrosome reactions, capacitation and hyper-activation for acceptable fertilization. Therefore, ROS must be kept at low concentrations to avoid underlying detrimental effects to sperm.

2.6. Research problem statement

The preservation of spermatozoa through cryopreservation process facilitates the dissemination of improved genetics across breeds and different geographical regions. However, there are some challenges during goat semen cryopreservation including its intolerance when egg yolk is used in the freezing extender. The components of egg yolk are shown to be the primary cause of damage in sperm. The egg yolk (lysolecithin) interacts with the seminal plasma lipase, a content of the bulbourethral secretion, and this interaction is known to be harmful for the sperm (Purdy, 2006). In addition, whole egg-yolk (WEY) has been reported to interfere with microscopic observations or biochemical assays as it contains granular material of the same size and shape as spermatozoa; it also reduces respiration and motility of sperm cells (Wall and Foote, 1999; Moussa *et al*., 2002). By centrifugation, egg yolk can be separated into its two main fractions (plasma and granules) (Pillet *et al*., 2011). Therefore, it is essential to remove large particles in WEY by centrifugation and allowing only plasma (clarified) to be used to obtain better sperm quality post-thaw. This clarified egg yolk (CEY) extender has been successfully used for stallion and bull semen cryopreservation (Vidament *et al*., 2000; Moussa *et al*., 2002). Therefore, it can be assumed that CEY extender in combination with antioxidants may better protect Saanen buck sperm against detrimental effects associated with egg-yolk components. In addition, the stages of semen freezing such as cooling and equilibration play important role for the survival of sperm after cryopreservation. However, cooling is a highly stressful process which leads to irreparable damages to the spermatozoa membrane (Garner *et al*., 2001). These detrimental effects can be reduced by optimizing cooling rates before freezing. The optimal cooling rates have been established in several species such as bull (Woelders *et al*., 1997), boar (Fiser and Fairfull, 1990), mouse (Koshimoto and Mazur, 2002) and human (Henry *et al*., 1993). However, goat sperm, temperatures over which goat semen must be cooled are quite diverse (Memon *et al*., 2013; Ahmad *et al*., 2015), making it difficult to establish optimal cooling rates, therefore necessitating further investigation to find an appropriate cooling rate for goat semen cryopreservation. On the other hand, equilibration affects the sperm survival after freezing-thawing. While progress has been made in various species to establish suitable equilibration times only few studies have investigated the effect of equilibration time for goat semen (Sundararaman and Edwin, 2008; Ahmad *et al*., 2015; Ranjan *et al*., 2015). They reported the equilibration time of several hours varying from 2 to 8 h. Therefore, the minimum period of equilibration with acceptable fertility after freezing remains controversial (Dhami *et al*., 1992; Dhami and Sahni, 1993). Antioxidants supplementation in combination with equilibration time has been reported to increase post-thaw sperm motility and kinematic parameters (Câmara *et al.,* 2011; 2016). Therefore, the dietary supplementation and addition of antioxidants in freezing extender in combination with

equilibration period to reduce damage of cold shock and free radicals still needs to be investigated in the goat semen cryopreservation process. Although semen contains antioxidants that control LPO and prevent excessive peroxide formation, these antioxidants are decreased by dilution and during storage (Kumar *et al*., 2011). The dietary supplementation of antioxidants and its addition to extenders has been demonstrated to be useful for the improvement of post-thaw sperm quality (Bucak *et al*. 2010; Kalthur *et al*., 2011; Saraswat *et al*., 2014; Mittal *et al*., 2014; Lukusa and Lehleonya, 2017). Among them, Selenium (Se) has a biological function, which is present in various selenoproteins to protect membranes from oxidative damage due to free radicals (Burk and Hill, 2000). Vitamin C is a ROS scavenger and sperm membrane protector (Asadpour *et al*., 2011; Daramola and Adekunle, 2015) and Vitamin E provides greater structural integrity and sperm kinematics after cryopreservation (Silva *et al*. 2013). Despite this, the use of antioxidants both in diet and freezing extenders is not common with respect to its use in goat semen cryopreservation.

2.7. Hypothesis of the experiment

In the present study, we tested the following hypothesis:

- Oral supplementation of selenium could increase testes measures, semen attributes and hormone concentrations
- Se supplementation could reduce sperm damage in cooled and frozen-thawed Saannen buck semen
- The use of CEY extender will preserve better the quality of buck sperm before and after cryopreservation.
- Slow cooling rate for cooled and frozen semen from Se supplemented bucks will preserve better then sperm quality.
- The addition of combination of vitamin (C+E) to freezing extender will provide better motility and kinematics parameters of cooled and frozen-thawed buck sperm than vitamin C and E alone.
- Equilibration times for frozen semen after dietary Se supplementation or addition to extender of the combination of vitamin (C+E) will improve post-thaw sperm quality.

2.8. Aims and objectives of the project

The overall aims of the current project were to investigate the effects of dietary supplementation of sodium selenite on reproductive performance of Saanen bucks and addition of vitamin C and E in freezing extender during cooling or freezing-thawing of buck semen.

In order to achieve these aims, the following objectives were set:

- To determine the effect of orally supplemented sodium selenite on testes measures, semen attributes and hormone concentrations of Saanen bucks.
- To evaluate the effects of cooling rates and dietary Se supplementation on post-thaw sperm characteristics of Saanen bucks.
- To establish the most appropriate extender to cryopreserve buck sperm through comparison between clarified egg yolk, whole egg yolk and tris without egg yolk extenders.
- To evaluate the effect of different types of extenders and cooling rates on cooled and frozen-thawed sperm of Se supplemented Saanen bucks.
- To determine the effects of vitamin C, E, their combination (C+E) and selenium as well as different equilibration times on post-thaw sperm motility and kinematic parameters of Saanen buck semen.

CHAPTER 3

Effects of dietary selenium supplementation on reproductive performance and semen characteristics of Saanen buck

Abstract

In the resent years, interest in goat sperm cryopreservation as a potential source of indispensable genes has escalated to improve reproductive performance and productivity in livestock. Based on this, the present study was conducted to determine the effect of orally supplemented sodium selenite on testes measures, semen attributes and hormone concentrations of Saanen bucks. Twenty mature healthy Saanen bucks were divided into two equal groups (10 bucks). The treatment bucks received sodium selenite at 10-day intervals for ninety days. Testicular measurements were recorded at 10-day intervals and fresh semen analysis was performed weekly. Testes and scrotal parameters of bucks were significantly ($p<0.001$) different between the groups. The testes length and left testis thickness were not affected by treatment. The semen pH was significantly ($p<0.001$) different between the groups. Moreover, the ejaculate volume, sperm mass activity, progressive motility and normal morphology were increased ($p<0.001$) in the selenium (Se) supplemented group compared to the control. The plasma glutathione peroxidase (GSH-Px), luteinizing hormone (LH), testosterone and selenium concentrations were significantly ($p<0.001$) higher in the Se supplemented group. No trace of plasma Se was found in the control group, therefore its supplementation proved to be beneficial. It can be concluded that supplementation with sodium selenite improved testicular parameters and semen quality of Saanen bucks. In addition, Se supplementation increases the concentration of GSH-Px, LH and testosterone in Saanen bucks.

Keywords: Sodium selenite, Testes measures, Semen attributes, Testosterone, LH Glutathione peroxidase

3.1. Introduction

Selenium (Se) is currently acknowledged to be an essential dietary trace element required for various body functions such as growth, reproduction, immune system and protection of tissue integrity. It is an essential component which is found in all cells particularly in the kidneys, liver, and pancreas (Pilarczyk *et al*., 2013). A biological function associated with Se, which is present in various selenoproteins, is to protect membranes from oxidative damage due to free radicals. The most important antioxidants related to Se are glutathione peroxidases, iodothyronine deiodinases, selenoproteins P, W, R. T, N and thioredoxine

reductase (Birringer *et al*., 2002**).** The principal constituent of plasma Se is selenoprotein-P, which contributes to Se transportation from blood to testis (Kehr *et al*., 2009). Selenoprotein-P also has a redox function and may protect cell membranes (Burk and Hill, 2000**).** Both testis and epididymis require dietary Se intake in order to synthesize a variety of known selenoproteins, whose precise role in spermiogenesis and post testicular sperm maturation are not clearly defined (Ali *et al*., 2009). Therefore, it is clear that Se deficiency in the testis can compromise its antioxidant role (Kryukov *et al*., 2003).

Any Se deficiency in the soil leads to low Se concentrations in plants growing in that soil. In many parts of the world plants do not provide adequate Se to meet dietary requirements (Hogan *et al*., 1993). In South Africa, Cloete *et al*. (1994**)** and van Ryssen *et al*. (1992) reported subclinical Se deficiencies and production responses to supplementation in South African Southern Cape coastal area and the Kwa-Zulu Natal midlands, respectively. Selenium is not distributed evenly across the planet; rather concentrations differ markedly depending on local conditions. Climate also exerts a very significant effect on the incidence of Se deficiency, mainly during winter in South Africa Se supplementation might be necessary, especially for animals depending on lucerne diet or pastures (Harthoom and Turkstra, 1976). This is because the lucerne may be produced from Se-deficient environments, since not all Se-deficient areas in South Africa have been mapped.

Supplementing ruminants with Se can be done in different ways as the use of trace element-amended fertiliser, intraruminal Se pellets (Langlands *et al*., 1991) or glass boluses. Although they serve as a readily available source of Se, but these products are not available in South Africa. Oral supplementation of Se is widely used in other countries due to its fast response and has been demonstrated to be important for normal semen quality and reproductive function in different studies of sheep (El-Mokadem *et al*., 2012; Pilarczyk *et al*., 2013). In South Africa, under an intensive production system, animals are given a balanced diet including Se and therefore, oral Se supplementation is not necessary. However, sheep, goats and beef cattle are produced under extensive production system in South Africa, in a way making oral Se supplementation a more applicable method. This method has been proposed as a preferred way to improve male reproductive performance by reducing the extent of oxidative damage (Barragry, 1994**).** Several studies on the effect of Se supplementation on semen quality and reproductive performance have been conducted in cattle (Ceballos *et al*., 2009) and rams (Mahmoud *et al*., 2013) and little has been done in goats. No studies have been done so far to ascertain the effects of oral Se supplementation on the reproductive performance of Saanen goats.

The use of artificial insemination (AI) with cryopreserved semen in goats is not a common practise as compared to cattle due to poor freezability of buck spermatozoa (Kumaresan *et al*., 2005). The poor freezability is mainly attributed to elevated amount of phospholipid and unsaturated fatty acid production in buck sperm, which makes it more vulnerable OS (Aitken *et al*., 1993). Furthermore, semen collection also plays a vital role in post-thaw sperm quality. Buck semen is commonly collected by an artificial vagina (AV) and electro-ejaculation (EE).

The collection of semen by AV is more preferable as it resembles natural mating, it is however more time consuming, as it requires training of animals for more than four weeks prior to semen collection (Matthews *et al*., 2003). The EE on the other hand, can be an alternative method in cases where males are not or impossible to train (Santiago-Moreno *et al*., 2009). However, differences in seminal plasma composition between semen collected with AV and EE have been reported in goats (Greyling and Gobbelaar, 1983; Marco-Jiménez *et al*., 2008). These differences may have substantial effect on the response of sperm to different steps of cryopreservation.

Cooling rate prior to sperm cryopreservation is an important step to minimise damages during the freezing process (Kumar *et al*., 2003). The cooling period decreases the effect of temperature changes and allows equilibration of the spermatozoa with the cryoprotectants before freezing (Foote *et al*., 2002). However, cooling is a highly stressful process, which leads to irreparable damages to the spermatozoa membrane that result in either cell death or premature capacitation-like changes (Garner *et al*., 2001).

These detrimental effects can be avoided by using an appropriate cooling rate prior to semen freezing (White, 1993). According to the limited number of studies on comparison of cooling rates in which the cooled and post-thawing sperm quality and fertility were the criteria for evaluation in bucks (Memon *et al*., 2013; Ahmad *et al*., 2015), no clear preference can be established among the cooling rates examined. Furthermore, the response of sperm to cooling rates of bucks semen obtained by AV or EE has not been explored yet in detail. The rates of cooling affecting freezability of goat semen in both collection methods are little understood especially in Se supplemented bucks. The specific aim of the present investigation is to study the preservability of sperm in relation to cooling rates in semen obtained by both AV and EE methods from Se supplemented bucks. This would help to establish appropriate freezing protocol with acceptable sperm quality post-thaw.

The processes of semen cryopreservation are responsible for certain negative effects in

terms of sperm structure, biochemical and functional damage. These detrimental effects induce reduction of sperm motility, membrane integrity and fertilizing ability (Salamon and Maxwell, 2000). This can occur during dilution in an extender, as well as at any step, during or after the freezing process. Therefore, there is an urgent need to improve the reproductive potential of breeding with cryopreserved semen. This may involve increasing the post-thaw quality of sperm through improvements in cryopreservation extenders.The composition of extender and suitable cryoprotectants are important factors for successful semen cryopreservation (Hammerstedt *et al.*, 1990; Curry *et al.*, 1994).

Currently, egg yolk is an important constituent of extenders for cryopreservation of semen of domestic animals. Their use has been encouraged because it provides protection for spermatozoa (Polge and Rowson 1952). However, this protection is affected to some extent by some limitations. This led to increasing needs to replace whole egg yolk in semen extenders because it presents microbiological contamination risks. It also contains particulate debris and greater viscosity in yolk that decreases the sperm motility and interferes in microscopic examination (Watson and Martin, 1975; Vishwanath and Shannon, 2000). For these reasons, substitutes with defined compositions to limit the risks cited above and maintain a high protection of the cells need to be investigated. Jiménez-Rabadán *et al*. (2012) reported less detrimental effect of egg yolk based extender when clarified egg yolk was used. This clarified extender has been successfully used in stallion and bull (Wall and Foote, 1999; Vidament *et al*., 2000), rams (Watson and Martin, 1975), boars (Jiang *et al*., 2007), and dogs (Bencharif *et al*., 2008) semen cryopreservation. However, the nature of the protection is still a matter for debate since clarified egg yolk is a main component of the commonly used whole egg yolk and because is easy to extract. It has been used in the present study to acquire a better knowledge of the events occurring during sperm preservation. Based on this, there is a necessity to investigate the cryoprotective effects of clarified egg yolk extender on post-thaw sperm quality of Se supplemented Saanen bucks following two cooling rates and different semen collection methods.

Therefore, the aim of the present study was: (1) to determine the effect of orally supplemented sodium selenite on testes measures, semen attributes and hormone concentrations of Saanen bucks. (2) to evaluate the effects of cooling rates and dietary Se supplementation on post-thaw sperm characteristics of Saanen bucks. In this context, clarified egg-yolk, whole egg-yolk and tris without egg-yolk extenders were also compared to establish the most appropriate extender of sperm cryopreservation.

3.2. Materials and methods

3.2.1. Study area

The study was conducted at the University of Pretoria, Hatfield Experimental Farm in Pretoria. The mean annual average air temperatures range from 11.8^{o}C in June to 23.0^{o}C in January. The average annual rainfall is 674 mm (Mengistu *et al*., 2016).

3.2.2. Animals and their management

All animal care and procedures used were performed in accordance with the Animal Ethics Committee of the University of Pretoria (Project no: EC079-14). The present study was carried out over a period of three months during autumn (February to May 2015). Figure 3.1 shows the recap of the experimental design for chapter 3. A total number of 20 Saanen bucks aged between 16 to 17 months and weighing 50.7±2.27 kg were used in this study. Bucks were grouped according to body weight and age, thereafter, allocated randomly into two groups comprising of 10 animals per group. Each group was further subdivided into semen collection methods; artificial vagina (AV) and electro-ejaculator (EE). The animals were raised only on locally available milled lucerne from weaning with no access to fresh growing forages or other feed for four months before the start of the experiment. Fresh water was provided *ad libitum* during the experimental period. Lucerne hay was milled and tested for Se concentration prior to supplementation and no Se traces were detected using the spectrophotometer (Perkin-Elmer 2380 Atomic Absorption Spectrophotometer; Varian, Australia).

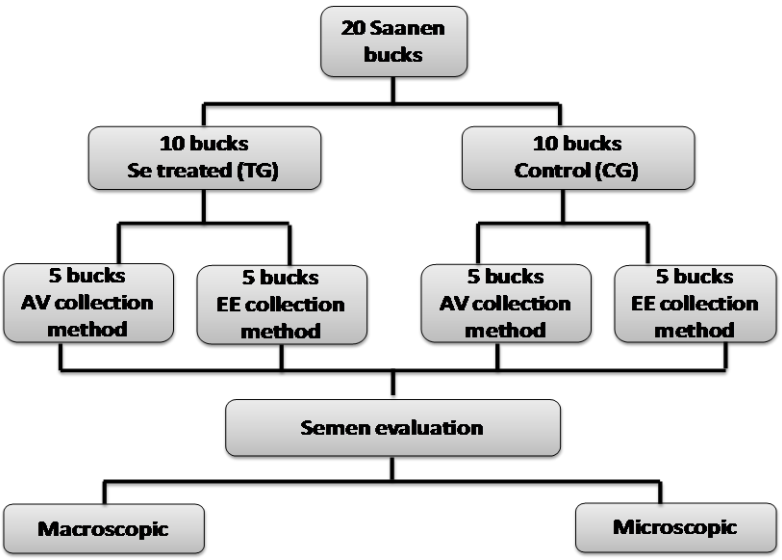

Fig. 3. 1. Recap of the experimental design for chapter 1

3.2.3. Animal treatment

The treatment animals received sodium selenite (ACECHEM, South Africa) at a dose rate of 0.34 mg/kg body weight and were adjusted according to Mahmoud *et al*. (2013). It was administered orally at 10-day intervals for three months, except for the third treatment on day 30 that was skipped due to fear of Se toxicity as advised by the veterinarian.

3.2.4. Testicular and scrotal measurement

Scrotal circumference (SC) and testicular parameters were measured at 10-day interval. Scrotal length (SL) was measured with a digital caliper (KTV150, MAJOR tech) as the distance between the tip of the scrotal sack and its neck. The scrotal circumference was measured with a flexible tape at the point of maximum circumference of the paired testes. Testicular length (TL) was measured by placing the fixed arm of the calliper at the proximal end and the sliding arm at the distal end of the testes avoiding as much as possible the caudal aspects of the epididymis (Schinkel *et al*., 1983). Testicular thickness (TTh) was measured by placing the fixed arm of the caliper at the anterior part and the sliding arm at the posterior part of each testis, at the point of maximum depth (Perumal, 2014). Testicular width (TW) of each testis was measured by placing one arm of the caliper at the medial part and the other at the lateral part, at the point of maximum width of testis Mahmoud *et al*., 2013).

3.2.5. Semen collection

The collection of ejaculates was performed using artificial vagina (AV) and electro–ejaculator (EE). Half of control and treated groups were either collected with the EE or AV. Semen collection started 20 days following Se supplementation. Ejaculates were collected once weekly for a period of 10 weeks (Nur *et al*., 2010).

For the artificial vagina (AV) collection method bucks were trained for more than six weeks prior to semen collection using a doe on heat. The AV consisted of an outer casing of rubber or plastic 15-20 cm x 5-6 cm and an inner liner made of latex (Ramsem, South Africa). The liner was folded back and secured over the end of the casing. On the day of semen collection the AV was prepared by filling the space between the casing and liner with approximately 55^{o}C warm water (Matthews *et al*., 2003). The lubricant (K-Y* Lubricating Jelly, Johnson & Johnson Medical, South Africa) was applied to the inner liner at the open end of the AV before collection. At the other end of the AV, a graduated glass tube (Ramsem, South Africa) was inserted for semen collection. A doe on oestrus was used and the semen was collected as described by Moore (1985). After ejaculation, the graduated

glass tube was separated from the AV, and then semen volume was recorded and transferred into the conical tube (Minitube, South Africa).

For the electro-ejaculator collection method, Semen was collected using an electro-ejaculator (Ramsem, South Africa) with a standardized rectal probe (28 cm long and 2 cm diameter) for small ruminants. Briefly, the animal was physically restrained in a lateral position on the floor and the rectum was cleaned of faeces. Then the prepuce area was shaved using a clean pair of scissors, cleaned with distilled water and dried with a paper towel (Kimberly-Clark, South Africa). The collection tube was placed over the penis in order to collect semen and a lubricated (K-Y* Lubricating Jelly) rectal probe was inserted into the rectum. Thereafter, the electro-stimulation of a maximum of 5 voltages was applied for 4 to 5 times at the 4–6 s intervals between stimuli until the semen was collected in a conical tube. When the electro-stimulation was stopped briefly, further massage was applied with the probe. If the animal did not give semen after 5 repetitions, it was then released without semen collection.

3.2.6. Semen evaluations

The collected semen sample was evaluated macroscopically (volume, pH and colour), as well as microscopically (sperm mass motility, progressive motility, sperm concentration, morphologically normal and abnormal sperm and viability).

3.2.6.1. Macroscopic evaluation

Collected semen was placed immediately in a warm water bath (33°C) and evaluated for normal semen attributes. The Ejaculate Volume collected by AV was determined after ejaculation, the graduated glass tube was separated from the AV, and then semen volume was recorded and transferred into the conical tube. The semen volume collected by EE was determined immediately after collection by reading the volume on graduated conical tubes. The ejaculate pH was determined using a litmus pH paper (colour-fixed indicator strips: (MACHEREY-NAGEL, Düran, Germany). The litmus paper was dipped into the semen and colour change was evaluated after 5 minutes by comparing colour between litmus pH paper and colour indicators suggested. The colour of semen was determined by visual observation for indication of the sperm density and the possibility of semen contamination (Greyling and Grobbelaar, 1982). The scoring system for sperm density was 1 (watery-cloudy) – 5 (creamy) according to Shamsuddin *et al*. (2000).

3.2.6.2. Microscopic evaluation

Mass sperm motility and progressive motility were determined subjectively by evaluating the degree of movement observed using a drop of semen under a microscope. The ejaculate mass motility was evaluated by placing a drop (10 µl) of semen on a pre-warmed slide without cover slip and examined under a phase contrast microscope (OLYMPUS, CX21FS1, Tokyo, Japan) at 40x magnification. The mass motility score of 1 (no motion) - 5 (dense, very rapidly moving waves) was used (Avdi *et al*., 2004). For sperm progressive motility, a drop (5 µl) of fresh semen diluted at 1: 50 with saline solution was placed on a pre-warmed slide and covered with a coverslip. The motility was estimated according to Biswas *et al*. (2002) using a phase contrast microscope at 200x magnification. At least 200 spermatozoa selected randomly from a minimum of four microscopic fields were examined. The sperm motility was scored as 0 (no sperm movement) – 5 (very rapid progression in which cells are difficult to follow visually (100%) (Martin *et al*., 2013).

The sperm concentration was determined by means of a haemocytometer (Hausser, Horsham, PA USA) where the semen samples were diluted with water 1: 200 to kill the spermatozoa (Salisbury *et al*., 1978; Mitchell and Doak, 2004. A drop of 10 µl diluted semen was then loaded on each side of the counting chambers using a micropipette (Socorex ISBA SA, Switzerland) and covered with a coverslip. The content was then placed on the phase contrast microscope under a (x10) magnification and allowed to settle for 5 min. The concentration of spermatozoa per mL of semen was calculated as: Concentration/mL = (Dilution Factor) (Count in 5 squares)($0.05x10^6$).

The morphologically normal and viable spermatozoa were assessed using nigrosin/eosin-stain smears (Evans and Maxwell, 1987; Mamuad *et al*., 2004). After mixing 5 µL of semen and 20 µL of nigrosin/eosin into a 1 mL eppendorf tube, 5 µL of the mixture was placed on a warmed microscope slide and smeared using another slide. The smeared slide was then allowed to dry for 5 min. A drop of immersion oil was placed on top of the slide followed by a coverslip and examined using a microscope to determine the percentages of live and dead sperm by counting a total of 100 sperm across the slide. The sperm that did not absorb stain appeared as white or clear and were considered as live while those that absorbed stain and appeared pinkish were considered dead (Malejane *et al*., 2014).

The live spermatozoa were classified into normal and abnormal sperm. Sperm abnormalities were evaluated for the abnormalities that occurred at a specific location by observing the head, mid-piece and tail of the sperm cell (Bearden *et al*., 2004). The head sperm abnormalities included detached head, and defects in size and shape. The mid-piece sperm

abnormalities observed were distal mid-piece reflex and proximal droplet. The tail sperm abnormalities included distal droplet, dag-like defects, bent tail and coiled. The slide was examined using a phase contrast microscope at 1000x magnification under oil immersion objective and bright fields (Yildiz *et al*., 2000).

3.2.7. Blood collection and analysis

Blood samples from each buck were collected using BD vacutainer® needle 18G x 1.5" (1.2 x 38 mm) (BD, Plymouth PL67BP, UK) and heparinised BD vacutainer® tubes (4.0 mL 13 x 75 mm) (BD, Plymouth PL67BP, UK). The blood was collected from the jugular vein at 10 day intervals throughout the experimental period. Immediately after collection, the blood samples (4 mL) were centrifuged at 3000 rpm for 20 min. Plasma aliquots of about 2 mL were harvested and then stored at -20^{o}C until subsequent analysis for Se, glutathione peroxidase (GSH-Px), luteinizing hormone (LH) and testosterone concentrations were done.

Plasma samples were analysed to determine Se concentration using the continuous hydride generation atomic absorption method (AOAC, 2000**)** and samples were read using a Perkin-Elmer 2380 Atomic Absorption Spectrophotometer (Varian, Australia) with the absorbency of 196 nm. Bovine pancreas powder was used as standard reference material and included in each batch of analyses to verify the accuracy of the Se assays.

Plasma GSH-Px, LH and testosterone analyses were performed using a spectrophotometer (Multiskan^{TM}Go Microplate, Thermo scientific). Plasma GSH-Px concentration was determined using GSH-Px assay kit (ab102530) (Abcam, England). 100 µL standard from the kit was mixed with 50 µL blood plasma samples in the wells. After preparing reaction mix, 40 µL was added to the blood plasma samples. Positive controls and reagent control wells and contents were incubated for 15 min. Following incubation 10 µL were then analysed to determine the concentration of GSH-Px using a microplate reader at optical density (OD) of 340 nm.

The LH concentration was determined using sheep LH ELISA kits (Elabscience Biotechnology Co. Ltd, E-ELS0783, Beijing). Briefly, 25 µL blood plasma samples together with kits standard were mixed into the wells containing antibody. After the addition of the enzyme conjugate the samples were incubated at room temperature for 30 min. After washing with the provided buffer, the substrate solution was added to the wells followed by 10 min incubation period at room temperature. After that, the top solution was added. The concentration of the LH was determined using microplate reader set at an absorbency of 450±10 nm.

The testosterone concentration was determined using ELISA kits (Demeditec Diagnostics GmbH, D-24145 Kiel, Germany). To accomplish this, 25 µL blood plasma sample and standards from the kits were added into the wells containing antibody of testosterone hormone. The enzyme conjugate was added to the wells followed by a 60 min incubation at room temperature. After washing with the provided buffer, a substrate solution was added to the wells and eventually incubated for 15 min at room temperature followed by the addition of the top solution. The concentration of the testosterone was then determined using a microplate reader set at an absorbency of 450±10 nm.

3.2.8. Statistical analysis

Statistical analysis was performed with the General Linear Model using statistical software SPSS (Version 23) (2015). The results were expressed as mean±SEM. Data of sperm characteristics and testicular measures were compared using one-way ANOVA for repeated measures. When ANOVA revealed a significant effect, values were compared using the Duncan's multiple range tests (Duncan, 1955). A probability of $p<0.05$ was considered to be statistically significant. Pearson's correlation coefficients were also calculated to verify the relationships among the different traits when the variances of the pairs of observations were independent.

3.3. Results

The testicular parameters of bucks are illustrated in Table 3.1. The overall average scrotal length, scrotal circumference, right and left testes were significantly ($p<0.001$) larger for the treated group, compared to the control. The right testes thickness was significantly ($p<0.001$) larger respectively for the treated group than the control.

Table 3.1**.** Overall (mean ± SEM) testicular measurements and plasma selenium concentration of Saanen bucks supplemented with selenium over a period 90 days

Items	**Selenium supplemented**	**Control**
Scrotal length (cm)	12.8±0.08[a]	11.9±0.13[b]
Scrotal circumference (cm)	29.7±0.29[a]	27.4±0.15[b]
Right testes width (cm)	6.2±0.07[a]	5.6±0.04[b]
Left testes width (cm)	5.7±0.07[a]	5.3±0.04[b]
Right testes length cm)	9.1±0.05[a]	8.9±0.10[a]
Left testes length (cm)	8.7±0.05[a]	8.5±0.09[a]
Right testes thickness (cm)	6.4±0.08[a]	5.8±0.05[b]
Left testes thickness (cm)	5.8±0.09[a]	5.7±0.06[a]
Plasma Se concentration (ng/ml)	0.2±0.00[a]	0.000[b]

Means with different superscripts in a row differ significantly at P<0.001

Table 3.2 and 3.3 present the effect of Se supplementation, semen collection technique and their interaction on quality and quantity of goat semen. Semen quality and quantity were significantly affected ($p<0.001$) by Se supplementation and methods of semen collection. Regardless of the method of semen collection, Se supplementation had a significant ($p<0.001$) effect on semen characteristics. The volume of semen was significantly higher ($p<0.001$) in the supplemented group, compared to the control. The percentages of all sperm attributes were significantly higher ($p<0.001$) in the supplemented group compared to the control group. The ejaculate volume and sperm viability were significantly ($p<0.001$) higher in semen collected with AV than EE.

The interaction between treatment and semen collection methods were significant ($p<0.001$). Treatment group resulted in higher ($p<0.001$) sperm concentration and acrosome integrity when AV was used but when EE was used the defference between TG and CG was not observed. Similarly, TG yielded higher ($p<0.001$) sperm motility when EE was used but when AV was used no difference was observed between TG and CG.

Table 3.2**.** Overall (mean±SEM) semen characteristics collected with AV or EE of Saanen bucks supplemented with selenium over a period of 90 days

Items	**Ejaculate volume (ml)**	**Sperm progressive motility (%)**	**Sperm viability (%)**	**Normal morphology (%)**
Seleniumsupplementation				
TG	1.5±0.05[a]	88.5±1.41[a]	82.3±0.64[a]	83.6±0.38[a]
CG	1.3±0.04[b]	80.7±1.30[b]	79.6±0.38[b]	80.8±0.32[b]
Semen collection methods				
AV	1.5±0.04[a]	83.2±1.48[a]	83.8±0.52[a]	82.4±0.45[a]
EE	1.3±0.04[b]	86.0±1.35[a]	78.8±0.05[b]	82.1±0.31[a]

For each factor, Means with different superscripts in a column differ significantly at ($p<0.001$).TG: treatment group, CG: control group, AV: artificial vagina, EE: electro ejaculator.

Table 3.3. Interaction effect between Se treatment and collection methods in terms of overall semen characteristics (mean±SEM)

Parameter	Se treatment	Semen collection method	
		Artificial vagaina	**Electro ejaculator**
Semen pH	Se suppelmented	6.6±0.11^{aA}	6.5±0.11^{aA}
	Control	6.4±0.07^{aA}	5.8±0.11^{bB}
Mass motility (score 1-5)	Se suppelmented	4.4±0.10^{aA}	4.5±0.08^{aA}
	Control	4.2±0.00^{aA}	4.1±0.10^{bA}
Sperm concentration(x109/ml)	Se suppelmented	2.9±0.10^{aA}	2.4±0.12^{aB}
	Control	2.4±0.11^{bB}	2.7±0.13^{aB}
Acrosome integrity (%)	Se suppelmented	77.3±0.70^{aA}	75.9±1.15^{aA}
	Control	72.4±0.79^{bA}	73.9±1.05^{aA}

For each factor, within a column means followed by small letter differ significantly at $p<0.05$. Within a row means followed by different capital letter differ significantly at $p<0.001$.

Table 3.4 present the effect of Se supplementation on sperm morphological abnormalities of Saanen bucks. The mean percentage of abnormalities such as detached head (DH), defects in size and shape (DSS), distal mid-piece reflex (DMR), distal droplet (DD) and proximal droplets were significantly lower ($p<0.001$) in TG compared CG. Bent and Coiled abnormalities were significantly higher ($p<0.001$) in TG compared to CG group. Dag-like defects (DLD) did not differ significantly ($p<0.001$) among treatments.

Table 3.4. Effect of selenium on sperm morphological abnormalities of Saanen bucks (mean±SEM)

Parameters		Selenium supplemented	Control
Head defects	Detached head (%)	3.4±0.08^{b}	4.7±0.10^{a}
	Defects in size and shape (%)	0.5±0.03^{b}	0.8±0.04^{a}
Mid-piece defects	Distal mid-piece reflex (%)	3.5±0.09^{b}	3.7±0.10^{a}
	Proximal droplet (%)	3.2±0.12^{b}	3.6±0.10^{a}
Tail defects	Distal droplet (%)	1.2±0.06^{b}	1.5±0.07^{a}
	Dag-like defects (%)	0.7±0.04^{a}	0.7±0.05^{a}
	Bent tail (%)	1.4±0.06^{a}	1.0±0.07^{b}
	Coiled tail (%)	1.6±0.08^{a}	1.0±0.06^{b}

Means with different superscripts in a row differ significantly at $p<0.001$

Table 3.5 presents the effect of semen collection methods and Se supplementation on sperm morphological abnormalities of Saanen bucks. The mean percentage of abnormalities such as detached head (DH), defects in size and shape (DSS), distal mid-piece reflex (DMR) and bent tail, were significantly ($p<0.001$) lower in Se supplemented samples when semen was collected using AV method. Proximal droplet (PD) defects were significantly ($p<0.001$) lower in the Se supplemented group when semen was collected by EE. Coiled tail defects were significantly ($p<0.001$) higher in the Se supplemented group when semen was collected by AV. No significant ($p<0.001$) difference was observed regarding distal droplets and dag-like defects in both AV and EE group.

Table 3.5. Effects of semen collection methods and selenium supplementation on sperm morphological abnormalities of Saanen bucks (mean±SEM)

	Artificial vagina		**Electro-ejaculator**	
Sperm Defects (%)	**Selenium supplemented**	**Control**	**Selenium supplemented**	**Control**
Head defects				
Detached head (DH)	2.9 ± 0.15^{c}	3.8 ± 0.13^{b}	3.8 ± 0.07^{b}	5.5 ± 0.11^{a}
Defects in size and shape (DSS)	0.5 ± 0.05^{c}	0.7 ± 0.05^{b}	0.5 ± 0.05^{cb}	0.9 ± 0.06^{a}
Mid-piece defects				
Distal mid-piece reflex (DMR)	2.8 ± 0.13^{c}	3.3 ± 0.14^{b}	3.6 ± 0.11^{b}	4.2 ± 0.12^{a}
Proximal droplet (PD)	3.5 ± 0.15^{b}	4.4 ± 0.15^{b}	2.7 ± 0.11^{c}	1.23 ± 0.07^{b}
Tail defects				
Distal droplet (DD)	1.2 ± 0.11^{b}	1.7 ± 0.13^{a}	1.2 ± 0.06^{b}	1.2 ± 0.07^{b}
Dag-like defects (DLD)	0.5 ± 0.06^{b}	0.7 ± 0.07^{ba}	0.8 ± 0.06^{b}	0.6 ± 0.05^{a}
Bent tail	1.1 ± 0.08^{c}	1.5 ± 0.09^{ba}	1.7 ± 0.08^{b}	0.9 ± 0.08^{c}
Coiled tail	1.8 ± 0.14^{a}	1.2 ± 0.11^{b}	1.3 ± 0.07^{b}	0.9 ± 0.08^{c}
Total abnormalities	15.5 ± 0.87^{c}	16.4 ± 0.87^{b}	15.6 ± 0.61^{c}	17.5 ± 0.71^{a}

Means with different superscripts in a row differ significantly at $p<0.001$

Table 3.6 shows the correlation analysis between scrotal and testicular measurements of Saanen bucks. The results showed that scrotal length and scrotal circumference were positively correlated ($p<0.001$) with right and left testicular length. Right testicular thickness was positively ($p<0.001$) correlated with left testicular thickness. Sperm mass motility was positively ($p<0.001$) correlated with sperm progressive motility. Sperm concentration had a positive ($p<0.001$) correlation with sperm viability and acrosome integrity.

Table 3.6. Pearson correlation coefficients (r) among scrotal measures, testicular measures, and semen characteristics in Saanen bucks over a period of 90 days of selenium supplementation

Parameters	R	Parameters	r
Scrotal length and right testicular length	0.590**	Right testicular length and left testicular length	0.901**
Scrotal length and scrotal circumference	0.590**	Right testicular thickness and left testicular thickness	0.940**
Scrotal length and right testicular width	0.571**	Mass motility and progressive motility	0.731**
Scrotal length and left testicular width	0.527**	Sperm concentration and sperm viability	0.533**
Scrotal circumference and selenium concentration	0.598**	Sperm concentration and sperm acrosome integrity	0.523**

**. Correlation coefficient is significant at p<0.001.

The effect of Se supplementation on plasma concentration of glutathione peroxidases (GSH-Px), luteinizing hormone (LH), testosterone and Se is presented in Fig.3. 2, 3.3, 3.4 and 3.5. The GSH-Px concentration in blood plasma increased significantly (p<0.001) in treatment group from day 30 (8.55±0.21 U/mL) to 80 (10.47±0.19 U/mL) of the experiment, whereas no changes in GSH-Px were recorded in the control group. The increase in plasma LH was significant (p<0.001) and started rising from day 30 to 80 of Se supplementation in treatment compared to control group for day 30 and 80 respectively when the animals reached 19 and 20 months of age. The concentration of blood plasma testosterone increased significantly (p<0.001) in the treated group from day 40 to 80, compared to the control group. There were no traces of Se picked up in the control group throughout the experimental period. The sharp increase in plasma Se occurred from day 10 (37.2±4.35 ng/mL) to 30 (128.6±6.86 ng/mL) of supplementation. The decrease in day 40 (61.4±2.04 ng/mL) occurred due to skipping of Se supplementation on day 30. Blood plasma GSH-Px, LH and testosterone concentrations increased with the age of animal and reached its peak at day 80 of the trial.

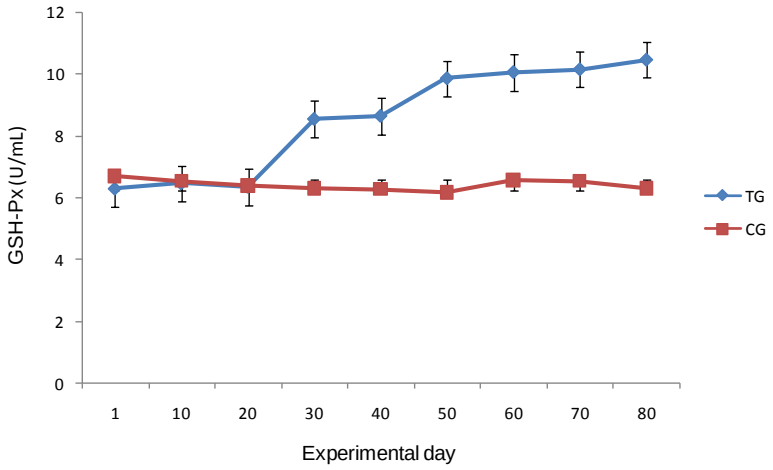

Fig. 3.2. Gluthathione peroxidase concentration of Saanen bucks supplemented with selenium for 90 days. TG: Selenium supplemented group. CG: control group.

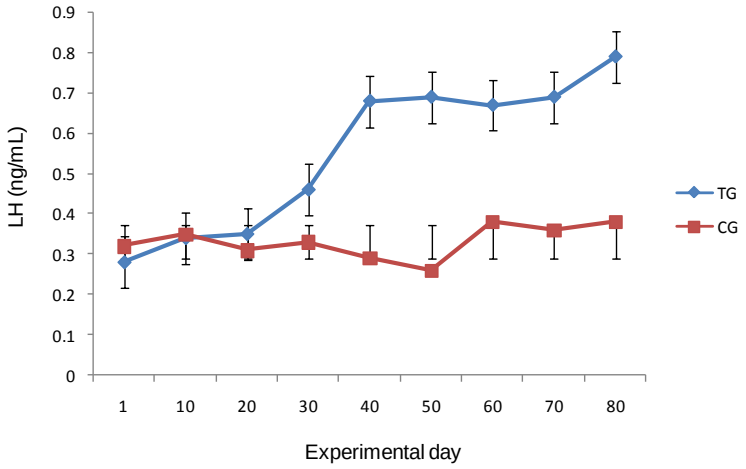

Fig. 3.3. Luteinizing hormone concentration of Saanen bucks supplemented with selenium over a period of 90 days. TG: Selenium supplemented group. CG: control group.

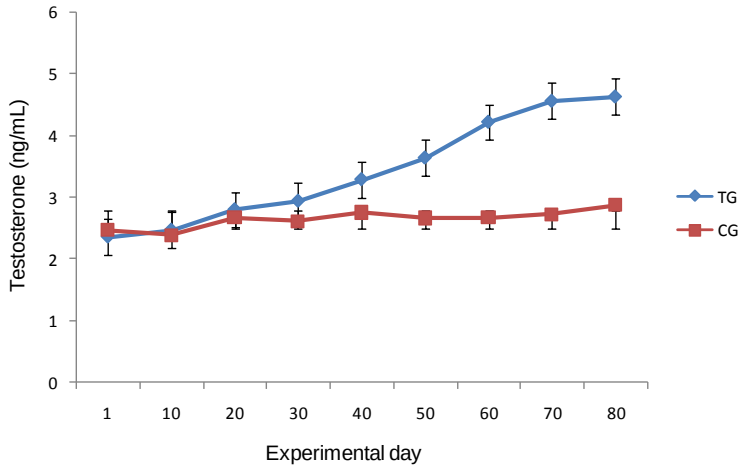

Fig. 3.4. Testosterone concentration of Saanen bucks supplemented with selenium over a period of 90 days. TG: Selenium supplemented group. CG: control group.

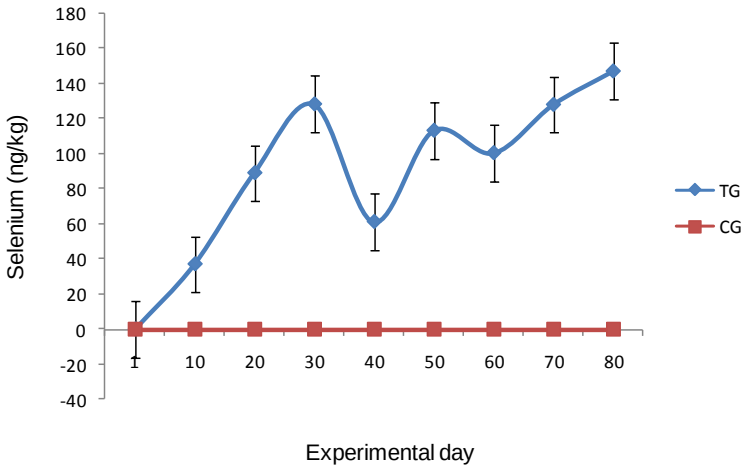

Fig. 3.5. Selenium concentration of Saanen bucks supplemented with selenium over a period of 90 days. TG: Selenium supplemented group. CG: control group.

3.4. Discussion

Plasma Se concentration at the start of the experiment revealed that all of the experimental bucks did not have any trace of Se in their blood plasma. This lack of Se confirms that the lucerne fed to the animals was Se deficient, as they had no access to pastures. This observation emphasizes the need for Se supplementation in regions such as Midlands and mountainous areas of the KwaZulu-Natal province and southern coastal region of the Western Cape where Se is deficient (van Ryssen, 2001). Moreover, during winter times when animals are fed with Lucerne that may be produced from these areas and other areas that are not yet marked as Se deficient in South Africa. Three animals died from the control group just after the experiment ended, although they did not show subclinical symptoms related to Se deficiency. It might be necessary to supplement the animals with Se even if they do not show subclinical signs of deficiency.

There was a rapid increase of Se concentration in the supplemented group at 30 days following the onset of treatment, with the highest level reaching 128.6 ng/ml. This might have been due to rapid absorption of orally supplemented Se as sodium selenite, especially when the animals are deficient in Se as was the case in this study. The sharp increase observed at day 20 to 30 followed by rapid decline of plasma Se concentration at day 40 after skipping day 30 supplementation, may also suggest that plasma Se in goat may be a sensitive indicator of Se intake. Therefore, when animals are deficient, excessive supplementation might lead to Se toxicity (Mehdi and Dufrasne, 2016). Selenium has a very narrow margin of safety (Ramirez-Bribiesca *et al*., 2005). Blood plasma Se concentration of greater than 3000 ng/ml is considered to be in the toxic range (Hodges *et al*., 1986). The highest peak of Se

concentration recorded in blood plasma at the end of experiment was 170 ng/ml. This falls within the range of 80 ng/mL-400 ng/mL considered as adequate for blood plasma Se concentration in sheep (Puls, 1994; Grace, 1995).

From the results it was observed that Se has more physiological effects on testicular measures, hormones and enzyme activities than semen parameters. The increase in glutathione peroxidase enzyme confirms that Se acts as cofactor of glutathione peroxidase enzyme. Selenium is also a component of some hormones and therefore is directly regulating the endocrine system and metabolism of animal body (Dumont *et al*., 2006).

In the present study, the results observed in terms of semen characteristics, scrotal and testes measures are in agreement with the report of Marai *et al*. (2009). This can be explained by the fact that Se plays a major role in metabolism of thyroid hormones known to be involved in growth mechanisms (Chadio *et al.*, 2006).

The significant increase in scrotal circumference and scrotal length in Se the supplemented group confirms the involvement of Se as an antioxidant, on the stability of cell membranes, processes of cell growth and testicular morphology (Behne *et al*., 1996). The increases in testes measurements including the right and left testes width and right testes thickness, could be ascribed to the role of Se for development of germ and Sertoli cells, hence, increasing the size and volume of the developing testes (Griswold, 1998).

The increased quality and quantity of seminal attributes such as ejaculate volume, sperm mass and progressive motility, sperm concentration, morphologically normal sperm, sperm viability, and acrosome integrity in Se supplemented group is in agreement with earlier published findings (Mahmoud *et al*., 2013; Kumar *et al*., 2014).

The increase in ejaculate volume supports the findings of Kendall *et al*. (2000) and Shi *et al*. (2010) in bucks and rams, respectively. The increased ejaculate volume may be linked to the involvement of Se in the development of primary and secondary sex glands, spermatogenesis, and prostate function. Therefore, the ejaculate volume increased due to the increase in secretion of seminal plasma from the secondary sex glands and spermatogenesis (Gabryszuk and Klewiec, 2002; Kolodziej and Jacyno, 2005).

The increased sperm motility in the supplemented group was also noted in bucks and rams (Xu *et al*., 2003; Mahmoud *et al*., 2013). The increase in sperm motility was expected, as Se has been reported to facilitate the transport of glucose across cell membranes required to

support aerobic and anaerobic glycolysis (Furnsinn *et al*., 1996). Selenium may also act in the maintenance of mitochondrial structural integrity, which is reflected by increase in ATP of spermatozoon, thus causing an increase in sperm motility (Liang *et al*., 2007). It also shows that Se supplementation played a major role to provide a sufficient protection to the sperm membrane against lipid oxidation (Mahmoud *et al*., 2013).

The increase in sperm concentration of supplemented group supports the report of Marin-Guzman *et al*. (2000). This implies that Se supplementation can increase the Se concentration in testes and epididymides and therefore supports the process of sperm production and maturation as well as metabolism and conformation of spermatozoa (Marin-Guzman *et al*., 1997). Se is also indispensable for the development of sperm and plays an important role in increasing the number of the germ cells (Liu *et al*., 1982). Furthermore, Se is also responsible for the proliferation of Sertoli cells in the developing testes, which in turn participate in the health and nourishment of the germ cells which in turn leads to an increase in the number of sperm cells, thereby increasingsperm concentration (Marin-Guzman *et al*., 2000).

The observed increase in sperm acrosome integrity and of percentages of morphologically normal sperm is in agreement with the reports of Ball *et al.* (2001) and Speight *et al*. (2012), respectively. The increase in sperm acrosome integrity may be due to Se protection of lipid component of the plasma membrane over the entire sperm acrosome (Kumar *et al*., 2014). Marai *et al*. (2009) reported a decline in acrosome damage when rams were supplemented with sodium selenite.

The increase in morphologically normal sperm may be linked to the activity of selenoprotein P, which supplies Se for spermatogenesis (Kehr *et al*., 2009). It has been observed in the present study that the concentration of GSH-Px increased when bucks were supplemented with Se. An increase in GSH-Px concentration demonstrates that there was higher Se concentration in bucks since Se is an integral component of GSH-Px enzyme (Rotruck *et al*., 1973). It can be suggested that an increase in GSH-Px concentration might have contributed to the increase of morphologically normal sperm. This enzyme has been reported to provide protection for the sperm membrane against oxidative damage, which causes sperm dysfunction, especially in terms of the loss of membrane fluidity or sperm membrane damage (Agwaral *et al*., 2003).

The increase in viable sperm in Se supplemented bucks supports the findings of Shi *et al*. (2010) who reported similar results. It believed that Se acts as a powerful antioxidant which

protects the sperm from structural damage by altering free radicals from damaging sperm cells (Marin-Guzman *et al*., 2000). Chen *et al*. (2012) suggested that Se plays an indispensable role during the maturation process of spermiogenesis for the formation of the structural normal and viable sperm.

The slightly basic semen pH in the Se supplemented group clearly demonstrates the involvement of Se in semen pH. Our results show for the first time that Se can maintain or balance semen pH depending on the semen collection method used. The EE method led to more acidic semen pH compared to semen pH of ejaculates obtained when using the AV semen collection method. Patel (1967) reported that the average semen pH range of buck semen is between 6.5 and 7.4. This supports the present study where semen pH was within the suggested range of goat semen when the animals were supplemented with Se and semen was collected using AV method. When semen was collected with an EE, semen was below the suggested range and more acidic in the control group. Ramukhithi (2011) also reported acidic pH (6.1±0.2), suggesting that the EE method leads to more acidic semen pH possibly by the modification of seminal plasma biochemical composition caused by excessive accessory glands secretion due to electrical stimulation (Ortiz-de-Montellano *et al*., 2007; Ramukhithi *et al*., 2011).

The more acidic semen pH 6.1 obtained by EE may still indicate good quality semen since highly active sperm samples produce lactic acid as a metabolic waste product (Aghangari, 1992)**.** However, if the acidity is not neutralized during a certain time period, and the metabolism of sperm continue to produce waste products, lactic acid will accumulate and that may lead to sperm damage. This can be noticed through the decrease in spermatozoa motility and viability. The altered pH of the semen above or below the normal range will affect the sperm ability to move and finally cause sperm death (Wahjuningsih *et al.,* 2012).

Greater semen volume (1.5±0.04 mL) obtained when using the AV method compared to the EE method (1.3±0.04 mL) confirms the report of Martin *et al*. (2013) and Malejane *et al*. (2014). The authors obtained higher semen volume with the AV method in summer, autumn and winter. However, another study is contradictory to the present study, where higher semen volume was obtained when using the EE method (Marco-Jimenez *et al.*, 2008). The main difference in these studies is the season of semen collection, therefore, suggesting season of semen collection to be a more determining factor than the method of semen collection. This suggestion is supported by the report that the AV method may give a higher semen volume than the EE method during the breeding season as is the case for the present study this is because the sexual glands are more active (Loubser and van Niekerk,

1983).

Semen ejaculates obtained by AV was also reported to be of higher sperm concentration than those obtained by EE (Memon *et al*., 1986; Marco-Jiménez *et al*., 2005; Jiménez-Rabadán *et al*., 2012; Jiménez-Rabadán *et al*., 2016). The lower semen concentration obtained by EE might be attributed to the intense stimulation of the accessory sex glands by electric pulses that led to the secretion of higher amount of seminal fluid (Marco-Jiménez *et al*., 2008). These results suggest that if semen is collected regularly for AI, AV is a suitable method as it produces higher sperm concentration as recommended by Matthews *et al*. (2003). The higher sperm concentration presents the advantage of getting more insemination doses in AI.

The result obtained for sperm viability is in agreement with Greyling and Grobbelaar (1983) and Matthews *et al*. (2003) who reported the higher percentage of viable sperm when using the AV method compared to the EE method in bucks and rams respectively. In addition, Carter *et al.* (1990) indicated that the percentage of normal sperm is also improved by using the AV technique, supporting the present results. The results in the present study, suggest that samples collected by AV should be preferred as they result in a higher percentage of live sperm cells, indicating better semen quality. Matthews *et al*. (2003) is also of the same opinion.

Our results presented for the first time to our knowledge, the interaction between Se supplementation and method of semen collection for ejaculate volume, sperm concentration, and sperm viability. Thus, values for these sperm parameters were increased when semen samples were collected using the AV method on the Se supplemented group compared to both AV and EE methods on the control group. In the present study, Se supplementation improved the semen quality in terms of high ejaculate volume, sperm concentration and viability when collected with the AV method compared to the EE method. For the results of this experiment, it can be said if semen is collected frequently for the purposes of AI, it seems that Se should be supplemented as it result in higher ejaculate volume (more production), higher concentration (more insemination doses) and higher percentage of live sperm cells (better semen quality).

In the present study, it was observed that Se supplementation reduced head and mid-piece defects such as detached head (DH), defects in size and shape (DSS), distal mid-piece reflex (DMR), distal droplet (DD) and proximal droplet (PD) compared to the control group. These results are in agreement with Wanatabe and Endo (1990) who reported similar

findings in rats. This was expected since these kinds of defects usually occur during spermatogenesis (Memon *et al*., 2012) and maturation phase in the epididymis (Salisbury *et al*., 1978). So, the reduced defects caused by Se supplementation may be attributed to the increased concentration of GSH-Px recorded in the present study. The higher concentration of GSH-Px is indispensable for the protection of germ cells and sperm membranes against OS during spermatogenesis (Shi *et al*., 2010). The reduced percentages of proximal and distal droplets indicate that Se was sufficient in the epididymis to enhance proper sperm maturation as evidence by semen from the control group that produced high proximal and distal droplet defects. Se supplementation did not have any effect on bent tail, coiled tail and dag-like defects (DLD). These defects are mainly caused by mishandling after ejaculation (Chenoweth and Lorton, 2014).

In the present study, it was observed that when bucks were supplemented with Se and semen was obtained using the AV method, the sperm defects affecting sperm head and mid-piece were reduced. These reduced sperm abnormalities may be attributed to Se supplementation and not the AV method because these abnormalities are usually related to disruptions during spermatogenesis and sperm maturation in the epididymis (Roca *et al*., 1992) and not during ejaculation. The higher percentages of head and mid-piece abnormalities in Se treated bucks with the EE method may be due to the stress induced during semen collection (Ortiz-de-Montellano *et al*., 2007), therefore leading to lower GSH-Px activity. The lower GSH-Px has been reported to be associated with damage of the chromatin structure of the sperm in the epididymis thereby leading to increased number of abnormal sperm morphology (Hansen, 2009). This observation suggests that Se should be supplemented when semen is collected using EE method. As evidenced in the present study, when bucks were supplemented with Se and semen was collected using the EE method buck produced better semen quality compared to control group. On the other hand, the AV method produced higher percentages of coiled tail defects. This was expected because the method has been reported to produce higher percentages of tail abnormalities compared to the EE method (Adenji *et al*., 2010).

The positive correlation between paired testicular volume with scrotal lengths and scrotal circumferences has been reported in bulls (Mahmood *et al*., 2014; Perumal, 2014). As would be expected the different measurements of testicular size were positively correlated with each other supporting the study of Perumal (2014) who reported positive correlation between right and left testicular length, and right and left testicular thickness. Sperm mass motility was also positively correlated with sperm progressive motility. This was in agreement with another study in bulls where a significant positive correlation was reported between

sperm mass activity score and the percentage of progressively motile spermatozoa (Ray and Ghosh, 2013).

Selenium has antioxidant activity and one of the mechanisms it uses is through GSH-Px activity. In this study, this enzyme activity was increased in Se supplemented bucks. The increase of plasma GSH-Px concentration in Se supplemented group has been reported previously in bucks (Kumar *et al*., 2014) and rams (Mahmoud *et al*., 2013). Confirming that, Se induces the biosynthesis of GSH-Px, a seleno-dependent enzyme, which regulates cellular peroxidases (Bengoumi *et al*., 1998). Furthermore, the GSH-Px concentration has been considered as an indicator of Se status (Ganther *et al*., 1976).

The present study demonstrates that Se supplementation increased testosterone concentrations in blood plasma of Saanen bucks. Similar observations were reported in the blood serum of bucks (EI-Sisy *et al*., 2008). It may be possible that the metabolic pathway of testosterone biosynthesis requires protection against peroxidation since glutathione peroxidase (Se-dependant) has been found in the Leydig cells (Murakoshi *et al*., 1983). In this study glutathione peroxidase was increased by Se supplementation. Therefore, it is assumed that it protected the testes from ROS (EI-Sisy *et al.*, 2008).

The results also revealed increase in plasma concentrations of LH in the group supplemented with Se. Similar results have been reported by Hezarjaribi *et al*. (2016) in male broiler breeders. It has been reported that testicular function is controlled by gonadotropin releasing hormones (GnRH) secretion which is responsible for stimulating the gonadotrophes of the pituitary gland to secrete LH (Griswold, 1998). As Se is known to accumulate in the anterior pituitary (Thorlacius-Ussing and Jensen, 1988), it is possible that increase in plasma Se concentration may have activated GnRH receptors in the anterior pituitary gonadotrophes, leading to increased LH production in the Se supplemented group (Ottinger *et al*., 2004).

3.5. Conclusion

In conclusion, oral supplementation with sodium selenite for ninety days significantly improved testis measures and semen characteristics of Saanen bucks. Better quality semen can be obtained by supplementing goats with Se and collecting the semen using the artificial vagina method, whereas the electro-ejaculator method of semen collection did not improve semen quality, but did improve mass and progressive sperm motility. Selenium supplementation increased plasma concentration of luteinizing hormone (LH), and testosterone as well as glutathione peroxidase (GSH-Px) activity.

CHAPTER 4

Effects of cooling rates and selenium supplementation on semen characteristics extended in different extenders

Abstract

Two experiments were conducted to evaluate the effect of three extenders, cooling rates and selenium (Se) supplementation on cooled and frozen-thawed Saanen buck sperm. Twenty bucks were divided into two groups: Se-treated (TG) and control (CG). Ejaculates were collected once weekly by artificial vagina (AV). In the first experiment, pooled semen was diluted with triladyl extender and split into two aliquots (slow and fast cooling). The second experiment was conducted in two steps. The first step analyzed the effect of extenders and cooling rates on post-thaw sperm quality. The mixed ejaculates regardless of Se supplementation were divided into three aliquots and diluted with clarified egg yolk (CEY), whole egg yolk (WEY) and tris without egg-yolk (TWEY) extenders. The diluted samples were further subdivided into two aliquots (slow and fast cooling). The second step analyzed the effect of selenium supplementation and extenders on post-thaw sperm quality. Ejaculates from each treatment samples (Se and control) were divided into three aliquots and diluted with the three extenders (CEY, WEY, TWEY). All samples were cooled for 2 h at 4^{o}C and frozen at −196^{o}C. Slow cooling resulted in higher ($p<0.001$) percentages of all sperm parameters compared to fast cooling. All sperm parameters were significantly ($p<0.001$) higher in Se treated samples when slow cooling was used. The post-thaw sperm motility and viability were significantly ($p<0.001$) higher in CEY with slow cooling. All post-thaw sperm parameters diluted with CEY were significantly ($p<0.001$) higher in Se treated bucks. In conclusion, clarified egg yolk combined with slow cooling yielded higher percentages of cooled and frozen-thawed sperm parameters in Se treated bucks. In addition, tris without egg yolk did not present any advantage over whole egg yolk extender.

Keywords: extenders, sperm characteristics, cooled semen, selenium

4.1. Introduction

Artificial insemination (AI) in goats is mostly done using freshly collected semen due to low fertility rates obtained with cryopreserved sperm. However, sperm freezing is an indispensable tool for genetic improvement or conservation programs in various species, including goats. For this reason, numerous investigations have been conducted recently with the objective of optimizing sperm cryopreservation protocols in goats. Irrespective of the protocol, viability of spermatozoa deteriorates at low temperatures during the storage. During

the freezing process, sperm cells are subjected to OS and ROS that can lead to harmful effects on sperm (Serviddio *et al*., 2013).

Antioxidants have the ability to prevent or reduce oxidative process by scavenging released free radicals. There are several antioxidants in semen that are known to improve sperm quality such as vitamin E and C, as well as Se and Zn which are components of antioxidant systems. These antioxidants may be insufficient in seminal plasma to protect spermatozoa against OS and ROS during the freezing-thawing process.

Se can be added to freezing extender to combat ROS in case animals are Se deficient, but the addition of Se to the medium sometimes results in contradictory results. Some authors have reported a protective effect of Se on sperm against free radical-induced damage (Dorostkar *et al*., 2012), while deleterious effect on sperm quality has been reported by others (Seremak *et al*., 1999). Oral supplementation of Se has been proposed as a potential way to reduce the oxidative damage due its fast response (El-Mokadem *et al*., 2012; Pilarczyk *et al*., 2013). There are several studies on the effect of oral Se supplementation to improve reproductive performance in sheep and goats (Mahmoud *et al*., 2013; Lukusa and Lehloenya, 2017). Consequently, it was hypothesized that Se supplementation could reduce sperm damage in cooled and frozen-thawed Saanen buck semen.

To cryopreserve semen, dilution with a protective extender is important in order to maintain fertilizing capacity of sperm during *in vitro* storage at low temperatures. The most widely used extenders for freezing goat semen are tris-egg yolk and skim milk extenders and their components are shown to be the primary cause of damage in semen. Egg yolk (lysolecithin) and milk-based diluents (SBUIII) interact with the seminal plasma lipase, a content of the bulbourethral secretion, and this interaction is known to be harmful for the sperm (Purdy, 2006). In addition, whole egg yolk (WEY) has been reported to interfere with microscopic observations or biochemical assays as it contains granular material of the same size and shape as spermatozoa; it also reduces respiration and motility of sperm cells (Wall and Foote, 1999; Moussa *et al*., 2002). By centrifugation, egg yolk can be separated into its two main fractions, plasma and granules (Pillet *et al*., 2011). Therefore, it is essential to remove large particles in WEY by centrifugation and only plasma (clarified) is used to obtain better sperm quality post-thaw. This clarified egg yolk (CEY) extender has been successfully used for stallion and bull semen cryopreservation (Vidament *et al*., 2000; Moussa *et al*., 2002). The advantage of CEY is that the concentrations of egg yolk or egg yolk constituents are reduced without decreasing sperm survival (Wall and Foote, 1999). Therefore, it can be

assumed that the use of CEY extender will preserve better the quality of buck sperm before and after cryopreservation.

Cooling of semen prior to sperm cryopreservation is an important step to minimize damages during the freezing process. The cooling step is important to minimize the effect of temperature variations and to allow equilibration of the spermatozoa with the extenders prior to freezing. However, cooling is a highly disturbing process, which induces irreparable damages to the spermatozoa membrane that result in either cell death or premature capacitation-like changes (Garner *et al*., 2001).These detrimental effects can be reduced by optimizing cooling rates before freezing. The optimal cooling rates have been established in several species such as 76 and 140°C/min in bulls (Woelders *et al*., 1997), 30°C /min in boars (Fiser and Fairfull, 1990), 27°C to 130 °C /min in mice (Koshimoto and Mazur, 2002) and 10°C/min in human (Henry *et al*., 1993). But for goat sperm, temperatures over which goat semen must be cooled are quite diverse for frozen sperm; ranging from -0.3^{o}C/min to 0.55^{o}C/min (Memon *et al*., 2013; Ahmad *et al*., 2015). These cooling rates lead to different results, making it difficult to establish optimal cooling rates. It was then hypothesized that an appropriate cooling rate in combination with oral Se supplementation will preserve buck sperm quality better following cryopreservation. Therefore, the objectives of the present study are to evaluate the effect of different types of extenders and cooling rates on cooled and frozen-thawed sperm of Se supplemented Saanen bucks.

4.2. Materials and methods

4.2.1. Animals

All animal care and procedures used were performed in accordance with the Animal Ethics Committee of the University of Pretoria (Project no: EC079-14). The present study was carried out over a period of 10 months from autumn to summer (from April to January). A total number of 20 Saanen bucks aged between 18 to 19 months and weighing 55.13±0.75 kg average body weight were used. The same animals used in chapter 3 section (3.2.2) were utilized for this experiment under the same management.

4.2.2. Semen collection

The semen collection was performed using an artificial vagina (AV). Ejaculates were collected twice weekly at 3 day intervals throughout the experiment. The bucks were trained for more than six weeks prior to successful semen collection using a doe on heat. On the day of semen collection, the AV was prepared according to Matthews *et al*. (2003). A doe on oestrus was used and the semen was collected as described by Moore (1985).

4.2.3. Evaluation of semen

All semen samples were evaluated macroscopically for ejaculate volume and pH, and microscopically for sperm mass motility, progressive motility, concentration, normal morphology, acrosome integrity and viability as described previously in chapter 3 section (3.3.6).

4.2.4. Extenders preparation

Three different freezing extenders namely the ready-to-use triladyl, clarified Egg yolk (CEY), whole egg yolk (WEY) and tris without egg yolk (TWEY) extenders were used. Triladyl extender was purchased from University of Pretoria, faculty of veterinary sciences. The CEY was prepared as described by Wall and Foote (1999). The WEY-based extender consisted of Tris 2.422 g, Citric acid monohydrate 1.36 g, Glucose 1 g, Gentamycin 1000µg/mL, Kanamycin 1000µg/mL, Egg yolk (v/v) 20%, Glycerol (v/v) 16%, and Distilled H_2O to final volume (mL) (Liu *et al*., 1982). The composition of TWEY-based extender comprised of Tris 4.54 g, Citric acid monohydrate 2.61 g, Glucose 0.82 g, Gentamycin 1000µg/mL, Kanamycin 1000µg/mL and Distilled H_2O to final volume (mL). The pH was then adjusted to approximately 7.2.

4.2.5. Cooling and freezing procedures

In the Fig 4.1 showed the experimental design for cooling and freezing procedures. The study was subdivided into two sets of experiments. Experiment 1 assessed the effects of cooling rates (slow and fast) and dietary Se supplementation on semen characteristics. In the experiment 2, the cryoprotective effects of CEY, WEY and TWEY extenders on cooled and frozen/thawed sperm of Se supplemented bucks was determined

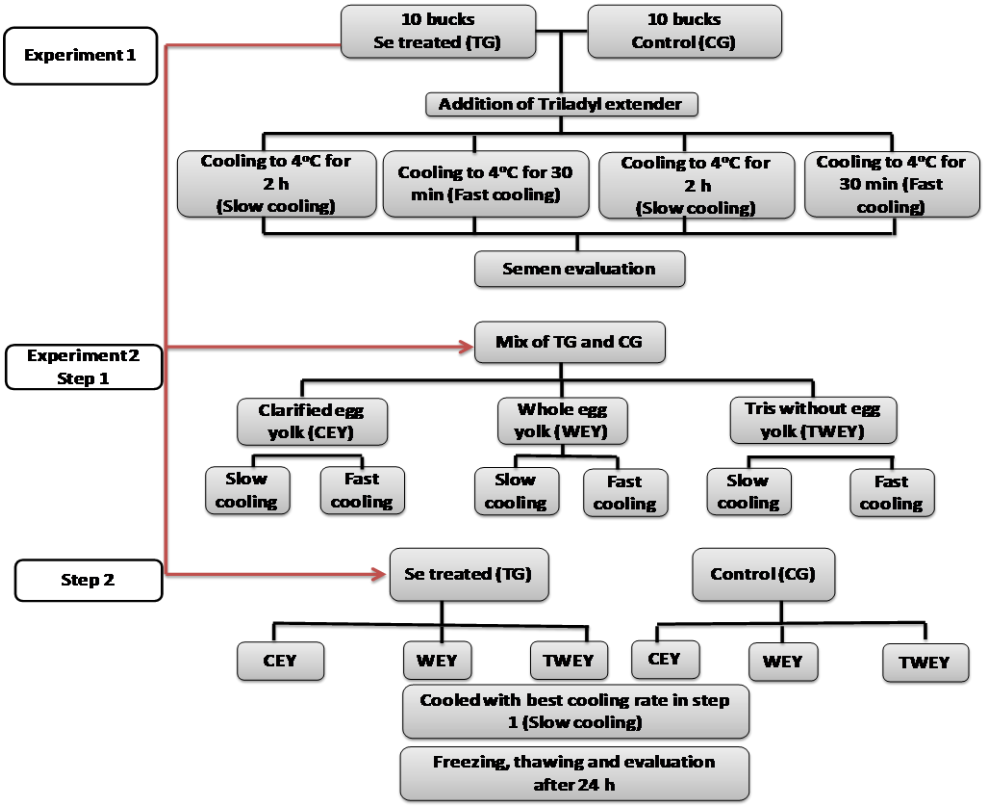

Fig 4.1. Experimental design for cooling and freezing procedures

Experiment 1: Effects of cooling rates and selenium supplementation on semen characteristics of Saanen bucks.

The animals were divided into two equal groups: treatment (TG) and control (CG) groups containing 10 animals in each group. The semen samples for both group (TG and CG) were diluted with no glycerolated fraction of freezing extender (fraction A) and further subdivided into two equal aliquots each. One aliquot was cooled using a slow cooling rate and the other used a fast cooling rate for 24 h at 4^{o}C. The samples were evaluated for both slow and fast cooling at 0 h and 24 h following storage. The collection of ejaculates started three months after first administration of Se. For each male, twelve collections of ejaculates were performed once weekly for three months.

Semen was considered for cooling using standard criteria established by Hidalgo *et al.* (2007). The semen samples used for cooling were those presenting the following characteristics: ejaculate volume of 1 to 2 mL, sperm concentration >1.5x10^{9} sperm/mL, >70% progressive motility and >70% morphologically normal sperm. The ready-to-use triladyl extender (Veterinary Sciences, University of Pretoria) was used as a cooling extender. Individual ejaculates were diluted at a ratio of 1:2 (semen: extender) and mixed

gently to ensure homogeneity of the mixture. The extended semen was transferred into two 15 mL conical tubes for either slow or fast cooling.

For slow cooling a 15 mL conical tube containing extended semen was placed inside a 50 mL conical tube, and the space between the tubes was filled with warm water at 33^{o}C. The combined tubes were transferred into a 500 mL beaker containing water at 33^{o}C. The beaker was placed directly in a refrigerator (4^{o}C) and kept for 2 h. This allowed a gradual cooling of the semen from 33^{o}C to 4^{o}C in 2 h at 0.22^{o}C/min (Memon *et al*., 2013). For fast cooling a 15 mL conical tube containing extended semen was placed inside a 50 mL conical tube and the space between the tubes was filled with warm water at 33^{o}C. The combined tubes were placed inside a 500 mL beaker containing tap water and ice blocks. The beaker was immediately placed in a refrigerator (4^{o}C) and kept for 30 min. This allowed a faster cooling rate from 33^{o}C to reaching 4^{o}C after 30 min at 0.55^{o}C/min (Memon *et al*., 2013). The sperm characteristics were evaluated immediately after cooling at 0 h and at 24 h following storage at 4^{o}C. For evaluation, the samples were removed from the refrigerator and placed in a water bath at 33^{o}C for 5 min prior to semen evaluation.

Experiment 2: Effects of freezing extenders on cryosurvival of Saanen buck spermatozoa.
The experiment was conducted in two steps the first step analyzed the effect of extenders and cooling rates, and the second step analyzed the effect of selenium supplementation and extenders. Sixteen semen collections were performed once weekly for seven months with 14 collections in each step.

First step: The ejaculates from Se supplemented and control groups were mixed together and divided into three equal aliquots. Each aliquot was diluted at 33°C to a final concentration of 150×10^{9}/mL with different type of extenders CEY, WEY and TWEY. The diluted samples for each extender were further subdivided into two equal aliquots. One aliquot was cooled using the slow cooling rate and the other aliquot used fast cooling.

Second step: Since the slow cooling rate produced better results at thawing in the first step, it was used in the second step to evaluate the effect of Se supplementation and extenders on cooled and frozen-thawed sperm. The animals remained in their main two groups of treatment and control groups as described in experiment I. The semen samples from treatment and control groups were divided into three equal aliquots that were diluted with CEY, WEY and TWEY using a 2-step dilution method. The samples were diluted to a ratio of 1:2 (semen: extender) with extender A (no cryoprotectant) and cooled to 4 °C for 2 h. The cooled semen was further diluted to a ratio of 1:1 (semen: extender) with extender B (8%

glycerol). After equilibration time at 4°C for 2 h, semen samples were aspirated into 0.25 mL French straws. Thereafter, the straws were sealed with polyvinyl alcohol powder followed by their suspension in liquid nitrogen vapour inside a cooler box container at a height of 4 cm above liquid nitrogen for 10 min. Then they were subsequently submerged into liquid nitrogen at –196 °C where they were stored for 24 h. A minimum of 3 straws from each treatment (CEY, WEY and TWEY) were thawed at 37 °C for 30 s in a water bath 24 h after freezing to evaluate post-thaw semen characteristics.

4.3. Statistical analysis

Statistical analysis was performed with the General Linear Model using statistical software SPSS (Version 23) (2015). The results were expressed as mean±SEM. Data of sperm characteristics were compared using one-way ANOVA for repeated measures. When ANOVA revealed a significant effect, mean±SEM were compared using the Duncan's multiple range tests (Duncan, 1955). A probability of $p<0.05$ was considered to be statistically significant.

4.4. Results

Experiment 1: Effects of cooling rates and selenium supplementation on semen characteristics of Saanen bucks.

Table 4.1 presents the effect of cooling rates and Se supplementation on quality of goat sperm. Sperm quality were significantly affected ($p<0.001$) by cooling rates and Se supplementation. Slow cooling provided higher percentages of sperm progressive motility, normal morphology, acrosome integrity and viability when semen was collected from Se supplemented bucks compared to the control group. Irrespective of Se supplementation, slow cooling resulted in significantly ($p<0.001$) higher percentages of sperm progressive motility and viability in comparison with fast cooling. No difference was observed for sperm acrosome integrity and normal morphology between Se-treated and control group. The interaction between treatment and cooling rates were significant ($p<0.001$). The sperm progressive motility, acrosome integrity, normal morphology and viability in supplemented samples with slow cooling rate were significantly ($p<0.001$) higher compared to sperm cooled with both slow and fast cooling in the control group.

Table 4.1 and 4.2 presents the effect of cooling rates and Se supplementation on quality of goat sperm. Sperm quality was significantly affected ($p<0.001$) by cooling rates and Se supplementation. Slow cooling provided higher percentages of sperm progressive motility, normal morphology, acrosome integrity and viability when semen was collected from Se

supplemented bucks compared to the control group. Irrespective of Se supplementation, slow cooling resulted in significantly ($p<0.001$) higher percentages of sperm progressive motility and viability in comparison with fast cooling. No difference was observed for sperm acrosome integrity and normal morphology between Se treated and control group. The interaction between treatment and cooling rates were significant ($p<0.001$). The sperm progressive motility, acrosome integrity, normal morphology and viability of semen cooled using slow cooling rate in Se supplemented samples were significantly ($p<0.001$) higher compared to sperm cooled with both slow and fast cooling in the control group.

Table 4.1. Overall (mean±SEM) sperm fertility parameters of slow and fast cooled semen of of Saanen bucks supplemented with selenium over a period of three months

Treatment	Sperm motility (%)	Acrosome integrity (%)	Normal Morphology (%)	Viability (%)
Overall cooling rates				
Slow cooling	70.8±1.30^{a}	68.2±0.60^{a}	79.3±0.70^{a}	74.6±0.61^{a}
Fast cooling	59.6±1.50^{b}	68.5±0.41^{a}	78.5±0.62^{a}	67.5±1.10^{b}
Overall selenium supplementation				
TG	71.8±1.40^{a}	69.5±2.02^{a}	73.1±1.83^{a}	72.1±1.03^{a}
CG	65.1±2.80^{b}	65.2±2.03^{b}	67.8±0.70^{b}	59.4±0.05^{b}

Means with different superscripts in a column differ significantly at $p<0.001$.

Table 4.2. Interaction effect between Se treatment and cooling rates in terms of overall semen characteristics (mean±SEM)

Parameter	Se treatment	Cooling rates	
		Slow cooling	**Fast cooling**
Sperm motility (%)	Selenium supplemented	74.2±0.98^{aA}	69.5±1.02^{aB}
	Control	67.7±2.01^{bA}	62.5±1.60^{bB}
Acrosome integrity (%)	Selenium supplemented	70.3±1.70^{aA}	68.7±1.15^{aA}
	Control	66.8±1.79^{bA}	63.6±1.15^{bB}
Normal Morphology (%)	Selenium supplemented	76.2±1.60^{aB}	70.0±0.50^{aA}
	Control	71.4±0.48^{bA}	64.2±0.44^{bB}
Viability (%)	Selenium supplemented	75.1±1.80^{aA}	69.1±0.80^{aB}
	Control	67.7±0.26^{bA}	51.2±0.64^{bB}

For each factor, within a column means followed by small letter differ significantly at $p<0.001$. Within a row means followed by different capital letter differ significantly at $p<0.001$.

Figure 4.2 shows the overall mean percentages of progressive motility, acrosome integrity, normal morphology and viability of Saanen bucks sperm subjected to different cooling rates. Slow cooling resulted in higher ($p<0.001$) percentages of sperm progressive motility, acrosome integrity, morphologically normal sperm and viability compared to fast cooling.

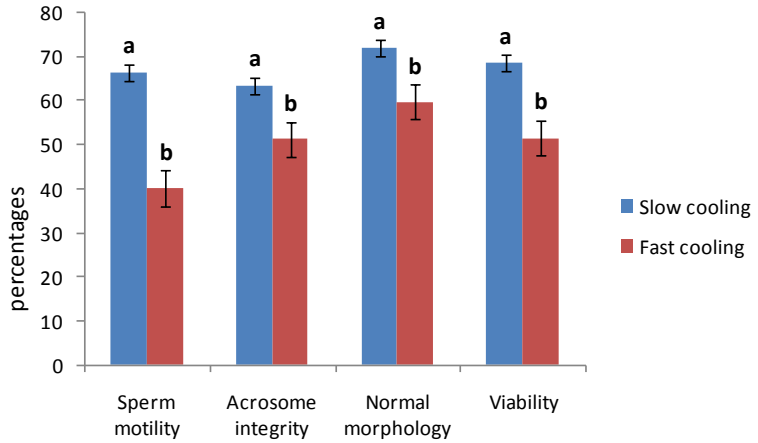

Fig. 4. 2. Mean values (±SEM) for sperm progressive motility, viability, normal morphology and acrosome integrity of slow and fast cooling rates. SC: slow cooling, FC: fast cooling. Error bar = SEM and ^{ab} bars with different letters differ significantly at p<0.001.

Table 4.3 Shows Overall (Mean±SEM) of Saanen goat sperm characteristics cooled using slow and fast cooling rates for 24 h. Slow cooled semen had significantly (p<0.001) higher sperm progressive motility immediately after cooling (0 h). However, there was a significant (p<0.001) decrease on sperm progressive motility at 24 h following storage, although still >50 %. Progressive motility following fast cooling immediately (0 h) and 24 h following storage was significantly (p<0.001) lower compared to slow cooling. The progressive motility, morphologically normal sperm and viability was significantly (p<0.001) higher following slow cooling immediately (0 h) and 24 h following storage compared to fast cooling. On the other hand, the percentages of acrosome integrity after 24 h of storage using slow cooling rate and 0 h storage using fast cooling rate did not differ significantly (p<0.001).

Table 4.3. Overall (Mean±SEM) of Saanen goat sperm characteristics cooled using slow and fast cooling rates at different times

Treatment		Sperm motility (%)	Acrosome integrity (%)	Normal morphology (%)	Viability (%)
Slow Cooling	0 h	72.9±1.03^{a}	66.7±0.06^{a}	76.3±0.42^{a}	71.9±0.14^{a}
	24 h	54.6±1.05^{b}	58.0±1.02^{b}	67.6±1.30^{b}	63.2±1.13^{b}
Fast Cooling	0 h	47.6±1.13^{c}	56.0±0.61^{b}	64.0±0.45^{c}	56.4±0.09^{c}
	24 h	32.7±1.20^{d}	46.6±1.22^{c}	55.8±1.21^{d}	46.8±1.04^{d}

Means followed by different superscripts in a column differ significantly at p<0.001.

Experiment 2: Effects of freezing extenders on cryosurvival of Saanen buck spermatozoa.

The overall (mean±SEM) sperm characteristics of cooled and frozen-thawed semen extended in clarified egg yolk (CEY), whole egg yolk (WEY) and tris without egg yolk (TWEY) freezing extenders are presented in Table 4.4. The overall percentages of sperm motility, acrosome integrity, normal morphology and viability were affected by extender type

in cooled semen. The CEY extender yielded higher ($p<0.001$) values of acrosome integrity and morphologically normal sperm than both WEY and TWEY extenders. The values of sperm motility and viability did not differ significantly ($p<0.001$) between CEY and TWEY extenders, however, there were lower in the WEY extender. The post-thaw sperm quality resulted in significantly ($p<0.001$) higher percentages of all studied sperm parameters in the CEY extender compared to both WEY and TWEY extenders.

Table 4.4. Overall (mean±SEM) sperm characteristics of cooled and frozen-thawed Saanen goat semen extended in different types of freezing extenders

Extenders	**Sperm motility (%)**	**Acrosome Integrity (%)**	**Normal morphology (%)**	**Viability (%)**
Cooled semen				
Clarified egg yolk	69.1±1.30[a]	70.3±0.81[a]	71.9±0.80[a]	70.1±1.10[a]
Whole egg yolk	59.6±1.91[b]	62.8±1.30[c]	61.2±1.81[b]	60.8±1.40[b]
Tris without egg yolk	67.8±2.21[a]	66.1±1.20[b]	64.8±1.52[b]	67.9±1.22[a]
Frozen-thawed semen				
Clarified egg yolk	52.6±1.71[a]	53.5±0.91[a]	62.3±1.31[a]	54.4±1.03[a]
Whole egg yolk	37.7±1.26[b]	47.4±1.21[b]	55.1±1.60[b]	46.2±1.71[b]
Tris without egg yolk	34.7±1.70[b]	40.3±1.22[c]	42.4±1.73[c]	42.2±1.61[c]

Means with different superscripts in a column differ significantly at $p<0.001$.

Figure 4.3 presents the effect of CEY, WEY and TWEY extenders on cooled sperm characteristics of Saanen buck sperm using slow cooling rate. The percentages of sperm normal morphology and viability were significantly ($P<0.001$) higher in the CEY when slow cooling was used, whereas no significant differences were observed between CEY and TWEY extenders in terms of sperm motility and acrosome integrity.

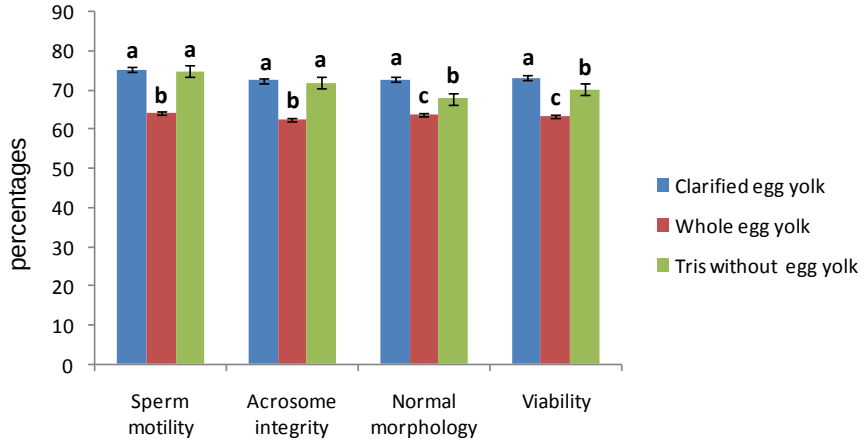

Fig. 4.3.Mean values (±SEM) for cooled sperm characteristics of Clarified egg yolk and Tris without egg yolk extenders in slow cooling rate. Error bar = SEM and[abc] bars with different letters differ significantly at p<0.001.

Figure 4.4 presents the effect of CEY, WEY and TWEY extenders on sperm characteristics of Saanen buck semen frozen-thawed using slow cooling rate. The sperm characteristics were significantly (p<0.001) affected by extenders. Higher percentages of sperm motility and viability were found for frozen-thawed spermatozoa from CEY extender when slow cooling was used compared to WEY and TWEY extenders. However, no significant (p<0.001) differences were observed in term of acrosome integrity and normal morphology in slow cooled samples.

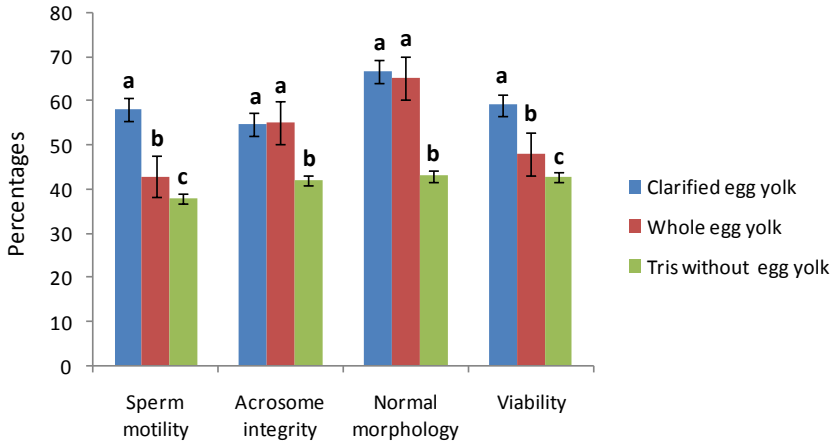

Fig. 4.4.Mean values (±SEM) for post-thaw Saanen buck sperm characteristics of slow cooled in clarified and whole egg yolk extenders. Error bar = SEM and[abc] bars with different letters differ significantly at p<0.001.

Figure 4.5 presents the effect of slow and fast cooling rates on post-thaw sperm characteristics of Saanen buck semen in clarified egg yolk extender. The results revealed significantly (P<0.001) higher percentages of sperm motility and viability when slow cooling rate was used in combination with clarified egg yolk extender. However no significant differences were observed in term of acrosome integrity and normal morphology.

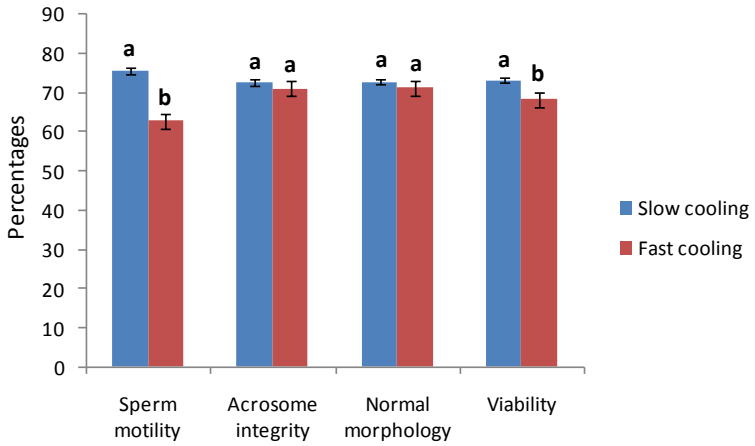

Fig. 4.5. Mean values (±SEM) for post-thaw sperm characteristics of slow and fast cooling rates in Clarified egg extender. Error bar = SEM and ^{ab} bars with different letters differ significantly at p<0.001.

Table 4.5 presents the effects of Se supplementation on sperm characteristics of cooled and frozen-thawed Saanen goat semen cryopreserved in different extenders. In cooled semen, sperm motility, normal morphology and viability were significantly (p<0.001) higher in both CEY and TWEY extenders in Se supplemented samples compared to WEY extender. After freezing-thawing, CEY Se supplemented samples, sperm parameters were all significantly (p<0.001) higher compared TWEY extenders. However, in whole egg yolk Se supplemented samples, normal morphology and acrosome integrity were similar (p<0.001) to CEY extender.

Table 4.5. Effects of selenium supplementation on sperm of Saanen bucks in cooled and frozen-thawed semen stored in different extenders (mean±SEM)

Extenders	Se treatment	Sperm motility (%)	Acrosome integrity (%)	Normal morphology (%)	Viability (%)
Cooled semen					
Clarified egg yolk	TG	72.1±0.11^{a}	74.1±0.21^{a}	74.1±0.61^{a}	73.1±0.52^{a}
	CG	67.1±0.32^{b}	70.1±0.12^{b}	73.5±0.42^{a}	70.0±0.42^{b}
Whole egg yolk	TG	66.5±0.44^{b}	69.5±0.53^{b}	67.0±1.23^{b}	70.5±0.13^{b}
	CG	64.7±0.21^{bc}	66.7±1.53^{c}	64.0±0.33^{cb}	62.0±0.14^{c}
Tris without egg yolk	TG	70.5±0.32^{a}	70.3±1.14^{b}	73.1±1.24^{a}	72.5±0.35^{a}
	CG	67.5±0.43^{b}	68.8±0.15^{bc}	66.1±0.44^{b}	71.1±0.36^{b}
Frozen-thawed semen					
Clarified egg yolk	TG	51.2±0.21^{a}	56.2±0.32^{a}	64.3±0.44^{a}	60.5±0.11^{a}
	CG	46.3±0.11^{b}	53.8±1.13^{b}	60.1±2.34^{b}	58.5±2.81^{ba}
Whole egg yolk	TG	45.4±2.13^{cb}	55.6±0.41^{a}	63.2±1.23^{a}	52.0±2.93^{c}
	CG	45.0±2.62^{cb}	44.6±1.23^{c}	54.5±2.82^{c}	50.5±2.92^{dc}
Tris without egg yolk	TG	38.0±2.71^{d}	33.5±1.52^{d}	40.1±1.40^{d}	49.5±2.41^{dc}
	CG	37.5±2.72^{d}	35.3±0.32^{d}	39.5±2.10^{d}	39.5±2.41^{e}

Means with different superscripts on a column differ significantly at p<0.001.

4.5. Discussion

Experiment 1: Effects of cooling rates and Se supplementation on semen characteristics of Saanen bucks.

The increased percentages of Saanen buck sperm characteristics observed with slow cooling rate in Se supplemented samples demonstrated clearly that Se had a significant protective effect against LPO on slow cooled sperm. This Indicates that Se is more effective in protecting different sperm parameters of slow cooled sperm against OS. It is evident that Se might have enhanced the protective effects during slow cooling rate by reducing the production of ROS and maintaining better sperm quality during cooling (Dorostkar *et al*., 2012). However, it can be suggested that in order to start the freezing process with acceptable sperm quality, supplementing the animals with Se to boost their antioxidant status is necessary. Then use slow cooling for better results.

The increase in sperm motility in slow cooled semen indicated that stabilization of sperm cells with slow cooling rate coupled with Se supplementation enables the sperm to cope with detrimental effects of physical, osmotic and cold stresses during cooling. This may be

attributed to the combined role of Se and slow cooling in the protection of sperm membrane integrity and lowering enzyme leakages during the cooling process (Memon *et al*., 2013). In addition, Se may also act in the maintenance of mitochondrial structural integrity, leading to the increase in ATP of spermatozoon, therefore causing an increase in sperm motility (Liang *et al*., 2007). In the present study, it was also observed that fast cooling could maintain sperm acrosome integrity equal to slow cooling in Se supplemented bucks. Similar to our results, Zhao *et al*. (2009) also reported also no differences between slow and fast cooling rates in terms of acrosome integrity.

The higher percentages of morphologically normal sperm in slow cooled semen from Se supplemented bucks in the current study may be attributed to higher level of GSH-Px activity. Kehr *et al*. (2009) documented that higher GSH-Px activity plays an indispensable role in chromatin structure protection of the sperm in the epididymis thereby leading to increased population of morphologically normal sperm. Similar results were observed by Rezaeian *et al*. (2016) who indicated that the addition of 5 mg/mL of Se to human sperm before freeze-thawing procedures caused an increase in spermatozoa with normal morphology.

In the present study, improvement was observed in viability of spermatozoa with slow cooling in Se supplemented bucks. Indicating that slow cooling rate in combination with Se have significant protective effects on sperm viability, and there is a strong interaction between cooling rate and Se supplementation. Similar results were reported by Memon *et al*. (2013) with slow cooling in Boer goat semen. Salazar *et al*. (2011) also reported increased percentages of sperm characteristics when slow cooling rate was used on stallion sperm compared to fast cooling. The present results confirm that slow cooling rate is required to reduce damage to Saanen buck sperm cells during the freezing process. The temperature of $4^{o}C$ must be attained within 2 h for desirable viability to result after thawing. On the otherr hand, the beneficial effects of Se can be attributed to the fact that Se is a very efficient antioxidant and a scavenger of oxygen free radicals that are toxic to metabolic activity and cellular viability of cryopreserved spermatozoa.

Cooling processes are known to damage sperm membranes and reduce sperm viability and their fertilising ability. The cooling rate of semen from $37^{o}C$ to $4^{o}C$ determines the success of freezing protocols in terms of sperm quality. The results of the present study regarding cooling rates are similar to the report of Memon *et al*. (2013) who observed that slow cooling increased percentages of sperm motility, acrosome integrity, normal morphology and viability. However, it is obvious that all sperm characteristics are low in fast cooled semen as

evidenced in the decline of the percentages of sperm parameters during storage at 4^{o}C from 0 h to 24 h. This confirm the report which stated that fast cooling induces damaging effect in sperm structure and function as well as intracellular ice crystal formation and loss of osmotic tolerance (Mazur, 1984). The results also showed that slow cooled sperm could be stored at 4^{o}C for more than 24 h; and will still maintain its fertilising ability while fast cooled sperm should be stored for less than 24 h at 4^{o}C. This Indicates that short-term storage of buck sperm is possible when AI cannot be performed immediately after semen collection or in the case where a selected buck is geographically distant from the doe requiring that fresh semen be shipped overnight. This is advantageous, as it reduces the expense and stress of transporting female or animals.

Experiment 2: Effects of freezing extenders on cryosurvival of Saanen buck spermatozoa

In cooled semen, the extender containing WEY was more deleterious for buck sperm than with CEY or TWEY. Better survival of spermatozoa was observed when CEY or TWEY extender was used. The CEY provided better sperm quality in terms of sperm acrosome integrity and normal morphology while no differences were observed between CEY and TWEY in terms of sperm motility and viability. The present results contradict the findings of Tabarez *et al*. (2017) who did not find any superiority of CEY extender in buck semen over WEY. However, our result is in agreement with Wall and Foote (1999) who indicated that CEY preserved better motility and fertility of cryopreserved bull sperm. Differences in results may be due to the different protocols used. For example, Tabarez *et al*. (2017) removed seminal plasma by centrifugation before sperm cryopreservation; this may affect the quality of sperm. In the present study we did not remove seminal plasma.

Our results with TWEY extender in cooled samples did not differ significantly from CEY extender in term of sperm motility and viability, showing that it is possible to preserve buck semen at low temperatures (4^{o}C) for short-term storage in the absence of egg yolk. The present results disagree with the report of Yodmingkwan *et al*. (2016) who indicated higher sperm motility and viability with WEY extender, while TWEY did not affect these parameters in the cooled stored sperm of Boer buck. This may be the result of the differences in goat breeds or extender composition which may affect the quality of semen (Leboeuf *et al*., 2000). They used low concentration of tris (2.4 g) and low percentages (1.4%) of glycerol in the freezing extender. In this study we used high concentration of tris and glycerol percentages. However, Silva *et al*. (2002) observed similar results to our study with canine semen. This can be explained by the fact that tris buffer removes the hydrogen ions originating from sperm metabolism during the thermal shock caused by the freezing process. Tris plays a major role in preserving sperm energy by reducing fructolysis (Silva *et al*., 2002).

In frozen semen, the current study demonstrated clearly that the type of semen extender used had an effect on sperm characteristics of Saanen buck semen during the freezing process. Overall sperm quality was improved when frozen-thawed semen was extended in CEY extender. Higher percentages of sperm motility, acrosome integrity, normal morphology and viability obtained after thawing with CEY extender confirm the results of earlier studies (Vidament, 2000; Nouri *et al*., 2013), where similar observations were reported with frozen-thawed semen. Also, Fernández-Santos *et al*. (2006) observed higher sperm quality using CEY during the cryopreservation of Iberian red deer spermatozoa. This may be explained by the fact that CEY extender contains less particles than WEY extender. Watson and Martin (1975) observed that some substances or particles in WEY extender inhibit respiration of spermatozoa or decrease their motility and acrosome integrity. The procedure of centrifugation used for obtaining CEY might have removed substances harmful to spermatozoa. Another possibility is that during the cryopreservation process, phospholipids of CEY were not hydrolyzed by enzymes of seminal plasma (phospholipase A2 and lysophospholipase), as has been shown by Chauhan and Anand (1990).

Application of a slow cooling rate combined with CEY revealed that buck sperm can maintain better motility, acrosome integrity, normal morphology and viability. Confirming the reports which indicated that a slow cooling rate is optimal and necessary to maintain membrane integrity and motility, and limit oxidative damage to sperm (Salazar *et al*., 2011; Martorana *et al*., 2014). This suggests that damages of spermatozoa are generally reduced if the cooling rate is slower than fast due the absence of intracellular ice crystal formation.

Despite the fact that fast cooling induces cold shock to sperm, the rapidly cooled samples can maintain acrosome integrity and normal morphology post-thaw equal to slow cooled samples when extended with CEY extender. This confirms the previous report which stated that slow and fast cooling resulted in similar membrane integrity after thawing in buck semen (Fernández-Santos *et al*., 2006; Memon *et al*., 2013). Utilisation of fast cooling associated with CEY extender may have lead to resistance of the outer acrosomal membrane and overlying plasma membrane to cooling and cryo-injury, indicating that reducing the period of contact between spermatozoa and seminal plasma during the cooling step could minimize detrimental effects for sperm extended in CEY post-thaw (Pradiee *et al*., 2016).

The results of post-thaw analysis of both slow and fast cooling methods combined with three extenders (CEY, WEY and TWEY) have produced interesting results in the present study. Before freezing sperm motility and viability must be at least 60% to obtain 30% progressive motility post-thaw for acceptable fertility (Ahmed *et al*., 2014). Considering this the current

study demonstrated that most of the sperm attributes regardless of cooling method and extender used reached above this limit (30% and 60%) before and after freezing. Slow cooling rate combined with CEY extender yields better results than fast cooling rate based on post-thaw sperm attributes. The post-thaw values of sperm acrosome integrity and normal morphology were similar in both CEY and WEY extenders when slow cooling was applied. This observation suggested that the acrosome was more resistant to membranous damage following cold shock when slow cooling was applied (Salazar *et al*., 2011).

Our results showed clearly that, supplementing Se as a component of the antioxidant system increased percentages of cooled sperm parameters in both CEY and TWEY extenders. The present results are in agreement with the findings of earlier researchers who reported that Se supplementation led to significant increases of sperm viability as well as motility before and after freezing (Dorostkar *et al*., 2012). The protective effect of Se supplementation on cooled sperm motility, acrosome integrity, normal morphology and also viability in both CEY and TWEY extenders observed in the current study may be explained by the increase of GSH-Px in Se-treated bucks as reported by Lukusa and Lehloenya (2017). These findings suggest that Se supplementation could increase antioxidative status of seminal plasma and spermatozoa to reduce excessive production of ROS during the cooling process. Regardless of Se supplementation, these results differ from those observed by Tabarez *et al.* (2017) and Yodmingkwan *et al*. (2016) who reported no superiority in the use of CEY and TWEY over the effectiveness of WEY. However, the results from this study are in agreement with the report of El-sheshtawy *et al*. (2016) who stated that sperm motility and semen characteristics improved with CEY extender as compared to WEY in bull cooled-stored semen. In our study, the improved sperm quality observed with CEY and TWEY extenders are mainly due to the absence of large particles and some components of egg yolk toxic to sperm (Wall and Foote, 1999).

In the present study, all frozen sperm parameters analyzed post-thawing were higher on Se supplemented samples preserved in CEY extender. These results support the report of Wall and Foote (1999) who indicated that the concentration of egg yolk or egg yolk constituents could be reduced by centrifugation without decreasing sperm cryosurvival of bull semen. Fernandez-Santos *et al*. (2006) added that centrifuged egg yolk provided a higher protection than whole egg yolk during the freeze-thawing of Iberian red deer epididymal spermatozoa. Similarly, El-Sheshtawy *et al*. (2016) revealed that bull sperm motility and semen characteristics improved with CEY extender supplemented with strawberry juice as antioxidant compared to WEY. It is clear that buck semen frozen using CEY extender supplemented with antioxidant such as Se can produce acceptable sperm quality post-thaw

that can be used an AI program. The removal of some detrimental components from egg yolk by centrifugation in CEY also played a greater role on the effectiveness of Se to provide the best cryoprotective effect on post-thaw sperm quality during the freeze-thawing process. Therefore, antioxidant such as Se may be necessary to protect sperm against ROS in both CEY and WEY for cryopreservation of Saanen buck semen.

4.6. Conclusion

The fast cooling rate did not improve semen quality. However, it can maintain acrosome integrity and normal morphology post-thaw equal to slow cooling with clarified egg yolk extender. The use of tris without egg-yolk extender did not present any advantage to the whole egg-yolk extender. However, cooled and frozen-thawed sperm parameters are higher when clarified egg yolk extender in combination with slow cooling rate is used in semen from Se supplemented bucks. This suggestes that before starting freezing process, supplementing animals with Se to boost their antioxidant status followed by the use of clarified egg yolk extender with slow cooling rate may be beneficial to yield acceptable sperm quality post-thaw.The cooled and post-thaw percentages of sperm acrosome integrity and normal morphology were similar in both clarified egg yolk and whole egg yolk extenders in Se supplemented bucks.

CHAPTER 5

Antioxidative capability of selenium, vitamin C, vitamin E and equilibration times on post-thaw sperm and kinematic parameters of Saanen buck semen

Abstract

Three experiments were conducted to determine the effects of the combination of vitamins (C+E) and dietary selenium supplementation in relation to equilibration times on post-thaw sperm quality of Saanen buck. Ejaculates were collected once weekly by artificial vagina (AV) from 16 bucks for eight months. In the first experiment, pooled ejaculates from eight bucks (no Se treated) were divided into five aliquots and diluted with extender containing different concentrations of vitamin C (3 and 4 mM), vitamin E (2.4 and 4.8 mM) and control. Cooled and post-thaw sperm characteristics were analyzed subjectively. The second experiment was conducted in two steps: the first step determined the antioxidative capacity of the best concentrations of vitamin C and E obtained in the first experiment, and their combination (C+E) on post-thaw sperm kinematics. The pooled ejaculates (no Se treated) were divided into four aliquots and diluted with extender containing vitamin C (4 mM), vitamin E (4.8 mM), vitamin (C: 4 mM+E: 4.8 mM) and control. The second step, since vitamin (C+E) produced better results in the first step; it was used to determine optimal equilibration time. The pooled ejaculates were divided into two parts: vitamin (C: 4 mM+E: 4.8 mM) and control. In the third experiment, bucks were grouped into two groups comprising eight animals per group (Se treated and control). The pooled ejaculates were diluted with no antioxidant added extender and divided into three aliquots for each group: with three aliquots corresponding to equilibration times (2, 4 and 6 h) and other three as their controls. Frozen-thawed spermatozoa were evaluated for kinematic parameters. The concentration of vitamin C (4 mM) and vitamin E (4.8 mM) resulted in significantly ($p<0.001$) higher percentages of cooled and frozen-thawed sperm characteristics. The post-thaw percentages of sperm total motility, rapid and medium-speed, curvilinear velocity (VCL), straight-line velocity (VSL) and beat cross frequency (BCF) were significantly ($p<0.001$) higher with the combination of vitamin (C+E) after 2 or 4 h of equilibration time. The VCL, VSL, average path velocity (VAP), BCF and total motility, rapid, medium-speed, and progressive motility were significantly ($p<0.001$) higher when semen was equilibrated for 2 or 4 h in Se supplemented bucks. In conclusion, to improve and maintain the quality of frozen-thawed buck spermatozoa, 2 and 4 h equilibration time in combination with vitamin (C+E) or selenium supplementation may be the best option.

Keywords: Antioxidant, cryopreservation, equilibration time, buck, spermatozoa

5.1. Introduction

Modern livestock breeding depends on artificial insemination (AI) to accelerate genetic improvement, although its application in the goat breeding sector is not common. This is due to inconsistent and often low fertility rates especially when frozen-thawed semen is used. Indicating that buck semen cryopreservation still needs improvements (Mata-Campuzano *et al*., 2015). The success of AI resides in fertilizing capability of the diluted, cooled or frozen-thawed semen, as well as the suitability of the extender to maintain the motility of spermatozoa. However, the cryopreservation process induces sperm cryo-damage due to excessive production of ROS. Therefore, leading to the loss of sperm and mitochondrial membrane integrity, inhibition of sperm adenosine triphosphate (ATP) production decreased motility and fertilizing capability (Bucak *et al*., 2007). These detrimental effects are more profound in goat spermatozoa due to high content of polyunsaturated fatty acids in their membranes (Foote *et al*., 2002), making sperm vulnerable to lipid perooxidation (Perumal *et al*., 2011). Although semen contains antioxidants that control LPO and prevent excessive perooxide formation, these antioxidants are decreased by dilution and during storage (Kumar *et al*., 2011). However, dietary or additions of antioxidants to extenders have been reported to protect sperm against LPO (Azawi *et al*., 2013; Kowalczyk *et al*., 2017). Antioxidants such as vitamin C and E are naturally occurring free radical scavengers that protect the sperm from LPO and provide higher integrity to the plasma membrane and mitochondria as well as better kinematics for sperm post-cryopreservation (Azawi *et al*., 2013; Sarangi *et al*., 2017).

Although both vitamin C and E are free radical scavengers, vitamin C represents the major water-soluble antioxidant in plasma and may reduce ROS induced DNA fragmentation and recycle inactive vitamin E (Keshtgar *et al*., 2012). Beconi *et al*. (1993) reported that the addition of 5mM vitamin C in the freezing diluents exerted an antioxidant effect during freezing and thawing of bovine sperm. On other hand, Vitamin E as a lipid soluble antioxidant defends the sperm against OS. Vitamin E is one of the major membrane protectants against ROS and has been reported to improve sperm motility and kinematic parameters in bull semen (Motemani *et al*., 2017). Sarangi *et al*. (2017) reported that 3 mM vitamin E helped in maintaining the buck seminal parameters at 4^{o}C up to 72 h and protected the spermatozoa form oxidative damage. However, the ability of vitamin E to maintain a steady-state rate of ROS reduction in the plasma membrane depends on its recycling by vitamin C (Maia *et al*., 2009; 2010). Therefore, it was hypothesized that addition of combination of vitamin C and E to freezing extender will effectively provide better motility and kinematics parameters of cooled and frozen/thawed buck sperm than vitamin C and E alone.

Another antioxidant substance is Se. It is known that Se serves as a component of the enzyme GSH-Px that protects cellular membranes and lipid containing organelles from peroxidative damage. Dietary Se supplementation has been reported to increase the percentages of motile sperm and reproductive performance in buck (Lukusa and Lehloenya, 2017). However, as an antioxidant its interaction with equilibration times on sperm motility and kinematics has not yet been proven.

Equilibration time is the period where cryoprotectants (glycerol) penetrate within sperm cell to establish a balanced intracellular and extracellular concentration. The results of several studies designed to determine the optimal equilibration time for buck semen established the beneficial effect of a period between 2 to 8 h of equilibration to obtain acceptable fertility (Sundararaman and Edwin, 2008; Ahmad *et al*., 2015). All these studies did not add antioxidant compound to freezing extenders. It is believed that equilibration period can interact with other osmotically active extender components such egg yolk, buffers and antioxidants (Muiño *et al*., 2007; Ranjan *et al*., 2015**;**Câmara *et al*., 2016). Câmara *et al., (*2011; 2016) reported that the equilibration time in combination with antioxidants may results in increased post-thaw sperm motility and velocity parameters. Suggesting that, antioxidants and equilibration times play a major role on the protection of sperm against ROS. This relationship of antioxidants and equilibration times is indispensable in ordeer to establish a suitable extender and optimal duration of equilibration. This interaction effect is still needed to be investigated during the buck semen cryopreservation process. Consequently, it can be hypothesized that equilibration times for frozen semen after dietary Se supplementation or addition to extender of the combination of vitamin (C+E) may improve post-thaw sperm quality due to their great effect on decreasing the ROS production.

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Therefore, the objective of the present study was to determine the effects of vitamin C, E, their combination (C+E) and selenium as well as different equilibration times on post-thaw sperm motility and kinematic parameters of Saanen buck semen.

5.2. Materials and methods

5.2.1. Animals and their management

The study was carried out in spring (September to November). The same animals used in chapter 3 were used for this study under the same management and treatment conditions. The animals were fed only locally available milled lucerne and they had no access to fresh growing forages or other feed. Fresh water was provided *ad libitum* during the experimental period as described in section (3.2.2). The experimental animals were 8 no Se

supplemented bucks and 4 Se supplemented Saanen bucks, ranging between 30 to 31 months of age and weighing 80.3±2.14 to 120.6±3.23 kg at the start of the experiment. The Se treated animals received sodium selenite at dose rate of 0.34 mg/kg body weight as described in chapter 3 section (3.2.3). The animals were kept on the experimental farm in Hatfield, University of Pretoria.

5.2.2. Semen collection and evaluation

The collection of ejaculates was performed using an artificial vagina (AV) (55^{o}C) (lukusa *et al*., 2017). Ejaculates were collected once, weekly for a period of 12 weeks and evaluated subjectively for semen attributes as described previously in chapter 3 section (3.2.6). Sperm kinematics were evaluated using the CASA system (Sperm Class Analyzer, (SCA, Microptic SL., Barcelona, Spain). The semen samples used for freezing were those presenting the following characteristics: ejaculate volume of 1 to 2 mL, sperm concentration >1.5 x10^{9} sperm/mL, >70% progressive motility and >70% morphologically normal sperm (Hidalgo *et al.,* 2007).

5.2.3. Sperm motility and kinematic assessment

Sperm motility, VAP, VCL, and VSL parameters were assessed with a CASA, using existing species-specific evaluation parameters for bucks. Preset values for the instrument were as follows: for the Basler camera, which can take 60 frames per second, image brightness of 60, contrast of 750, and light of 1000 were adjusted. The minimum average path at 50 µm/s and >50% progressive motility were accepted. Motility parameters of static, slow (>40 µm/s), medium (>70 µm/s), and rapid (>100 µm/s) were set; and kinematic parameters of VCL (>80 µm/s), VSL (>50 µm/s), and VAP (>25 µm/s) were set. 5 µL of each sample was evaluated on microscopic slides covered with a coverslip.

For each sample, 200 to 300 spermatozoa in three different areas were analyzed to evaluate the motility. Total motility was taken as the sum of progressive and non-progressive motility. The curvilinear velocity (VCL: µm/s) is the time-averaged velocity of a sperm head along its actual curvilinear path as perceived in two dimensions with the microscope. The VSL is a measure of the time-average velocity (µm/s) of the centroid of the sperm head along the straight-line trajectory between its first and last points. It is computed by finding the total distance travelled along the linear path divided by the acquisition time. The VAP is a measure of the time-average velocity (µm/s) of the centroid of the sperm head along the smoothed trajectory, which is constructed by averaging several points on the actual curvilinear path. It is computed by dividing the length of the smoothed track by the acquisition time.

The LIN (%) refers to the linearity of the curvilinear path and is calculated as (VSL/VCL) X 100, it was previously described as a progressiveness ration. Straightness STR (%) measures the linearity of the average path and is calculated as (VSL/VAP) X 100, the STR was formerly known as linear index (Stephens *et al*., 1988). The Wobble WOB (%) measures the magnitude of the oscillation of the actual path around the average path, formerly called curvilinear progressiveness ratio and is calculated as (VAP/VCL) X 100 (Stephens *et al*., 1988).

The amplitude of lateral head displacement (ALH) measures the degree of lateral displacement of the sperm head's centroid around its average path (μm). The ALH can be determined mathematically by measuring the length of the risers, which are straight lines extending between each point on the average path and its corresponding point on the actual curvilinear path. The ALH value can be calculated from the maximum riser value, which is then doubled to give the track-maximum measurement (ALHmax), or from the averaged riser values to be doubled and expressed as the track-average measurement (ALHmean) (Mortimer, 1994).

Beat/cross frequency (BCF) indicates the frequency (hertz [Hz]) with which the curvilinear path crosses the average path; such crossovers occur two times within each flagellar beat cycle. Therefore, BCF is considered a measure of the flagellar beating frequency as a new flagellar beat is initiated once the actual sperm trajectory crosses the average path. BCF provides further indication of the frequency of the rotational movement of the sperm head around its longitudinal axis of progression, providing the sperm head rotates by 180 degrees at the peak of each lateral displacement with each beat initiation (Mortimer, 1997).

Sperm were classified as medium-speed spermatozoa when their velocity was between 45 and 75 μm/s, and as rapid spermatozoa when their velocity was >75 μm/s. Spermatozoa were considered progressively motile when they travelled straight over at least 80% of their trajectory.

5.2.4. Semen cryopreservation

In the Fig. 5.1 is showed the experimental design of semen cryopreservation process for experiment 1 and 2. For all experiments, clarified egg yolk extender supplemented with vitamin C, E and their combination was used except for the extender considered as control and Se experiment where extender was not supplemented with antioxidant. The clarified egg-yolk extender was prepared as described by Wall and Foote (1999).

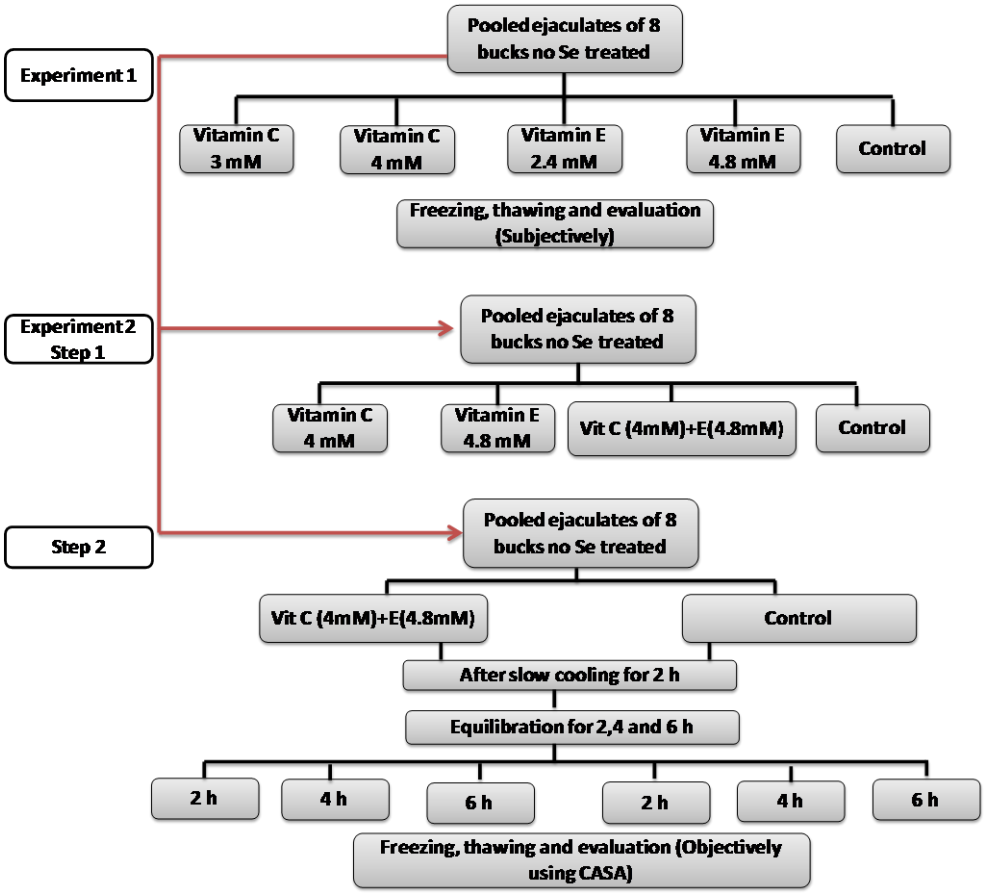

Fig. 5.1. The experimental design of semen cryopreservation process for experiment 1 and 2.

Experiment 1: Effects of different concentrations of vitamin C and E added in freezing extender on cooled and post-thaw sperm quality of Saanen buck semen.

Ejaculates from eight bucks (no Se supplemented) collected on the same day were pooled to eliminate individual differences. Each pool of semen was divided into 5 aliquots and diluted with five extenders containing different vitamin concentrations of vitamin C (3 and 4 mM), vitamin E (2.4 and 4.8 mM), and control (no-supplemented). The extenders were added in two steps: solution A (without glycerol) was added in the first step at 33^{o}C, to obtain a sperm concentration of 150x10^{9} sperm/ml. Diluted semen was cooled for 2 h in a refrigerator at 4^{o}C, followed by the addition of an equal volume of solution B (containing 16% glycerol) at 4^{o}C to obtain a final sperm concentration of 75×10^{9} sperm/ml and equilibrated for 2 h. After equilibration time, the semen samples were aspirated into 0.25 mL French straws and sealed with polyvinyl alcohol powder and suspended in liquid nitrogen vapor inside a Styrofoam box container at height 4 cm above liquid nitrogen for 10 min. They were subsequently submerged into liquid nitrogen at –196 °C, where they were stored at –196 °C for 24 h before analysis (Naing *et al*., 2010). Immediately after cooling, semen samples were

evaluated subjectively for pre-freezing sperm parameters, while post-thaw evaluation was performed 24 h after freezing using a phase contrast microscope (OLYMPUS, CX21FS1; Tokyo, Japan).

Experiment 2: Effects of equilibration times and different antioxidants on frozen-thawed Saanen buck sperm

Since 4 mM of vitamin C and 4.8 mM of vitamin E produced better results in experiment 1 at thawing, these concentrations were used in this experiment. The experiment was conducted in two steps: the first step determined the antioxidative capacity of vitamin C, E and their combination (C+E) on post-thaw sperm motility and kinematic parameters of Saanen buck semen. In the second step, since the combination of vitamin (C+E) produced higher sperm quality at thawing in first step it was used to determine optimal equilibration time.

In the first step: The pooled ejaculates from no Se supplemented animals were divided into four aliquots. The first aliquot was diluted with extender containing 4 mM of vitamin C, the second aliquot with extender containing 4.8 mM of vitamin E, the third aliquot with extender containing combination of vitamin C (4 mM) and E (4.8 mM) and the fourth aliquot was considered as a control (without antioxidant).

In the second step: The pooled ejaculates were divided into two parts. The first part was diluted with extender containing the combination of vitamin (C+E) and the second part was considered as the control. After cooling at 4^{o}C for 2 h, each part (treatment and control) was diluted with Fraction B containing 16% glycerol at 4^{o}C and subdivided into three aliquots each and maintained at 4^{o}C for 2, 4 and 6 h (equilibration time) respectively. After equilibration, samples were frozen as described in experiment 1. Post-thaw sperm quality evaluation was carried out 24 h after freezing using CASA system as described in previous section.

Experiment 3: Effect of selenium supplementation on sperm motility and kinematic parameters of cooled and post-thaw based on different equilibration times

Figure 5.2 illistrates experimental the design for experiment 3 on the effect of selenium supplementation based on different equilibration times. Bucks were grouped into two groups comprising of 4 animals per group (Se-supplemented and control group). The Se supplemented animals were from a group that was continuously fed Se from chapter 3 section (3.2.3). Semen was collected using the AV method, diluted and evaluated as described in chapter 3 sections (3.2.5) and (3.2.6). The pooled ejaculates from main groups (Se-treated and control) were diluted with no antioxidant added freezing extender as

described in chapter 4 section (4.2.5). The diluted samples were further subdivided into three aliquots corresponding to equilibration times (2, 4 and 6 h) respectively and frozen as in experiment 1 paragraph (5.2.4).

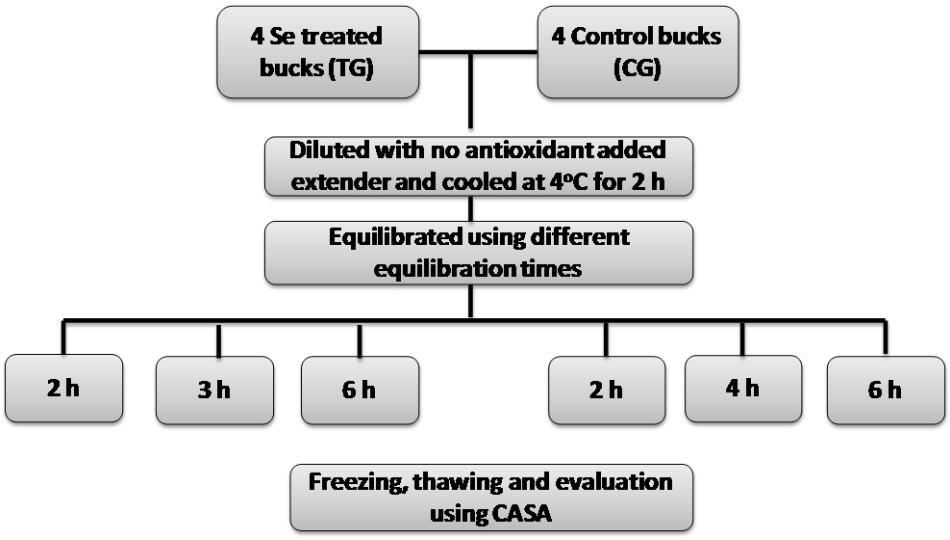

Fig. 5.2. Experimental design for experiment 3 on effect of selenium supplementation based on different equilibration times

5.2.5. Post-thawing sperm analysis

Sperm quality was evaluated after thawing. A minimum of 3 straws from each treatment were thawed at 37 °C for 30 s in a water bath 24 h after freezing for quality evaluation. A sample of semen was transferred to a warm slide and the motility variables were measured as described in section (5.2.3) using CASA system.

5.3. Statistical analysis

The effects of treatment (vitamin C, E or their combination) and equilibration times were analyzed using the repeated measures option of the General Linear Model procedure of SPSS (Version 13) computer program (2015). In this model, the different treatments and equilibration time were considered as intra-subject factors. ANOVA for repeated measures with the treatments (vitamin C. E or their combination C+E) as intra-subject factors was also performed to determine their effect on the quality of frozen-thawed semen. When ANOVA revealed a significant effect the values were compared using the Duncan's multiple range tests. A probability of $p<0.05$ was considered to be statistically significant. Pearson's correlation coefficient was also calculated to verify the relationships among the different variables when the variance of the pairs of observations was independent.

5.4. Results

Experiment 1: Effects of different concentrations of vitamin C and E added in freezing extender on cooled and post-thaw sperm quality of Saanen buck semen.

Table 5.1 and 5.2 show the effect of different concentrations of vitamin C and vitamin E on cooled and frozen-thawed buck sperm evaluated subjectively. In cooled semen, the percentages of sperm motility and acrosome integrity increased significantly ($p<0.001$) in the extender supplemented with 4 mM of vitamin C compared to other concentrations and the control group. No differences were observed between extenders supplemented with 4 mM vitamin C and 4.8 mM vitamin E in terms of sperm normal morphology and viability which were lower in other concentrations and the control group. The percentages of abnormal sperm decreased significantly ($p<0.001$) in the control group compared to supplemented groups.

No differences were observed in frozen-thawed semen between extenders supplemented with 4 mM of vitamin C and 4.8 mM of vitamin E regarding percentages of sperm motility and viability which were significantly ($p<0.001$) lower when other concentrations (3 mM of vitamin C and 2.5 mM of vitamin E) and control group were used. The percentages of sperm normal morphology increased significantly ($p<0.001$) in extender supplemented with 4.8 mM of vitamin E compared to other vitamin concentrations and control group. The percentages of sperm acrosome integrity were significantly ($p<0.001$) higher in extender supplemented with 4 mM of vitamin C compared to other vitamin concentrations (3 mM of vitamin C and 2.5 mM of vitamin E) and control groups. The percentages of abnormal sperm decreased significantly ($p<0.001$) in extender supplemented with 4.8 mM of vitamin E compared to other concentrations and control groups.

Table 5.1**.** Effects of different concentrations of vitamin C and E in buck semen extender on overall cooled sperm characteristics (Mean±SEM)

Parameter	Vitamin concentration (mM)	Sperm motility (%)	Normal morphology (%)	Acrosome integrity (%)	Viability (%)	Abnormal sperm (%)
Vitamin C	4	69.9 ± 0.79^{a}	77.5 ± 0.21^{a}	76.7 ± 0.02^{a}	76.0 ± 0.02^{a}	22.4 ± 0.24^{d}
	3	63.7 ± 0.71^{b}	74.7 ± 0.23^{b}	73.9 ± 0.23^{c}	70.7 ± 0.21^{c}	25.1 ± 0.28^{c}
Vitamin E	2.4	61.3 ± 0.70^{c}	72.9 ± 0.50^{c}	72.7 ± 0.32^{d}	73.1 ± 0.31^{b}	26.8 ± 0.56^{b}
	4.8	64.4 ± 0.71^{b}	76.4 ± 0.51^{a}	74.9 ± 0.31^{b}	76.8 ± 0.32^{a}	23.5 ± 0.61^{d}
Control	0	60.3 ± 0.74^{c}	70.5 ± 0.30^{d}	70.6 ± 0.44^{e}	69.7 ± 0.03^{c}	29.4 ± 0.38^{a}

Means with different superscripts in a column differ significantly at P<0.001

Table 5.2. Effects of different concentrations of vitamin C and E in buck semen extender on overall frozen-thawed sperm characteristics (Mean±SEM)

Parameter	Vitamin concentration (mM)	Sperm motility (%)	Normal morphology (%)	Acrosome integrity (%)	Viability (%)	Abnormal sperm (%)
Vitamin C	4	57.1±0.8^{a}	68.9±1.00^{b}	71.2±0.50^{a}	72.6±0.51^{a}	31.3±0.08^{a}
	3	52.5±0.8^{cb}	66.7±1.21^{b}	69.1±0.61^{bc}	68.4±0.42^{b}	33.1±0.28^{a}
Vitamin E	2.4	53.5±0.1^{b}	67.2±0.25^{b}	68.1±0.50^{c}	68.2±0.34^{b}	33.0±0.19^{a}
	4.8	57.6±0.6^{a}	72.7±0.56^{a}	70.1±0.32^{ba}	71.9±0.33^{a}	27.2±0.23^{b}
Control	0	50.8±0.5^{c}	68.6±0.17^{b}	69.2±0.41^{bc}	67.6±0.40^{b}	32.3±0.14^{a}

Means with different superscripts in a column differ significantly at P<0.001

Experiment 2: Effects of equilibration times and different antioxidants on frozen-thawed Saanen buck sperm

Overall motility patterns exhibited by spermatozoa during the cooling and freezing-thawing process as determined by CASA method are presented in Figure 5.3 and 5.4. In cooled semen, significantly (p<0.001) higher percentages of total motility, rapid-speed sperm and progressive motility were observed in extender supplemented with the combination of vitamins (C+E) compared to vitamin C and E alone. In frozen-thawed semen, the proportions of total motility, rapid and medium-speed sperm in extender supplemented with the combination of vitamin C+E were significantly (P<0.001) higher compared to vitamin C and E alone.

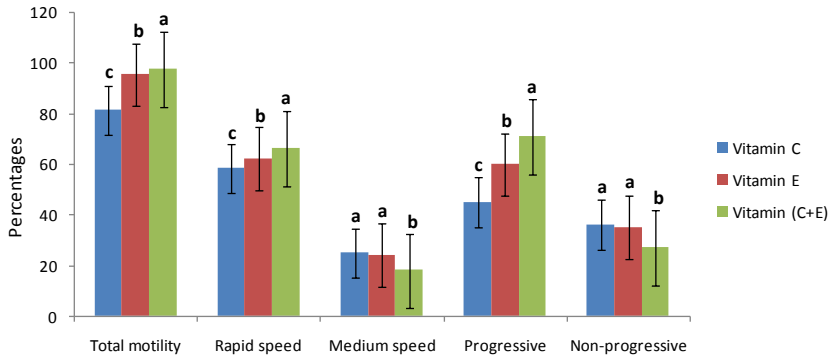

Fig. 5.3. Overall (Mean±SEM) for sperm motility parameters of cooled Saanen buck semen stored in extenders supplemented with vitamin C, E and their combination (C+E). Error bar = SEM and ^{ab} bars with different letters differ significantly at p<0.001.

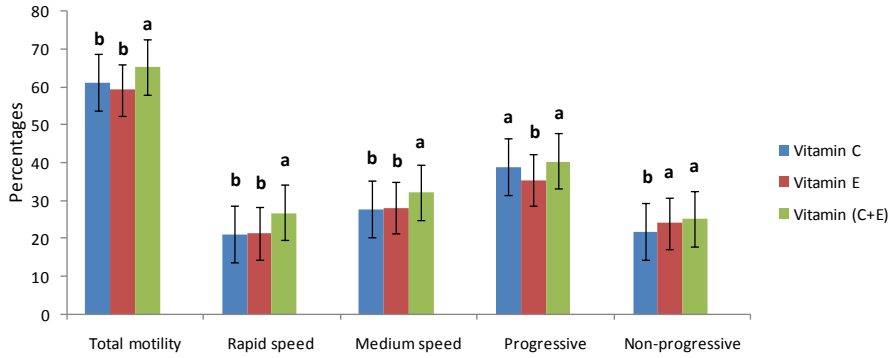

Fig. 5.4. Overall (Mean±SEM) for sperm motility parameters of frozen-thawed Saanen buck semen stored in extenders supplemented with vitamin C, E and their combination (C+E). Error bar = SEM and ^{ab} bars with different letters differ significantly at p<0.001.

Overall sperm kinematic parameters exhibited by spermatozoa during the cooling and freezing-thawing process as determined by CASA method are presented in Table 5. 3 and 5.4. In cooled semen, significantly (p<0.001) higher values were recorded for sperm kinematic parameters such as VCL, VSL and LIN in the extender supplemented with the combination of (C+E) compared to vitamin C and E alone. Similarly, the values of WOB were significantly (p<0.001) higher in extender supplemented with vitamin C alone. In frozen-thawed semen, sperm kinematic parameters such as VCL, VSL and BCF in extender supplemented with the combination of vitamin C and E were significantly (p<0.001) higher compared to semen suspended in vitamin C and E alone. The parameter LIN in vitamin C supplemented semen was significantly higher compared to semen extender with vitamin E and their combination vitamins (C+E).

Table 5.3. Overall (Mean±SEM) of Saanen buck sperm kinematic parameters of cooled semen diluted in extenders supplemented with vitamin C, E and their combination (C+E)

	VCL(µm/s)	VSL(µm/s)	VAP(µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm)	BCF (Hz)
Vitamin C	100.6±0.63^{c}	54.1±0.11^{b}	78.2±1.16^{a}	67.8±1.23^{b}	85.6±0.93^{a}	86.0±0.81^{a}	3.1±0.36^{b}	17.1±1.42^{a}
Vitamin E	105.1±0.33^{b}	53.1±1.13^{b}	75.6±1.31^{b}	66.2±1.32^{b}	86.6±1.00^{a}	70.1±0.61^{c}	4.6±0.38^{a}	14.1±1.26^{b}
Vitamin (C+E)	111.1±0.11^{a}	57.2±1.20^{a}	79.2±0.03^{a}	71.1±1.24^{a}	86.3±0.66^{a}	82.1±1.09^{b}	4.1±1.73^{a}	17.3±0.42^{a}
Control	98.3±1.11^{d}	48.2±1.11^{d}	73.1±0.11^{cb}	63.1±0.32^{c}	85.1±1.20^{a}	71.2±1.71^{c}	2.6±1.38^{c}	14.4±0.43^{b}

Means with different superscripts in a column differ significantly at p<0.001.

Table 5.4. Overall (Mean±SEM) of Saanen buck sperm kinematic parameters of frozen-thawed semen stored in extenders supplemented with vitamin C, E and their combination (C+E)

	VCL(µm/s)	VSL(µm/s)	VAP(µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm)	BCF (Hz)
Vitamin C	61.8±1.53^{bc}	37.2±1.21^{b}	53.2±1.16^{a}	64.8±1.23^{a}	78.6±0.93^{a}	83.1±0.71^{a}	1.8±0.36^{b}	14.3±4.32^{b}
Vitamin E	62.3±1.43^{b}	38.8±1.13^{b}	50.2±1.31^{b}	60.2±1.32^{b}	77.6±1.00^{a}	79.2±0.61^{b}	2.6±0.38^{a}	13.9±0.46^{b}
Vitamin (C+E)	64.1±1.10^{a}	41.1±1.01^{a}	52.2±1.52^{a}	61.0±1.00^{b}	77.1±1.15^{a}	84.5±0.12^{a}	2.6±0.55^{a}	18.1±3.02^{a}
Control	59.4±0.13^{c}	36.8±1.15^{b}	49.1±0.11^{b}	57.1±0.32^{c}	78.1±1.20^{a}	78.2±1.71^{b}	1.6±1.38^{c}	12.±1.43^{b}

Means with different superscripts in a column differ significantly at p<0.001

Significant differences were recorded between the vitamins (C+E) supplemented extender and control with respect to the kinematic parameters of the rapid (Fig. 5.5) and medium-speed spermatozoa (Fig. 5.6). In these treatments, the VCL, VSL, VAP and LIN values recorded after addition to freezing extender of the combination of vitamins (C+E) were significantly (P<0.001) higher for the rapid spermatozoa compared to control samples. No differences were observed in term of STR, WOB, ALH and BCF (Fig. 5.5). For the medium-speed spermatozoa, the VCL, VSL, VAP, STR and BCF values were significantly (P<0.00) higher in vitamins (C+E) supplemented samples than the control. No differences were observed in term of LIN, WOB and ALH (Fig. 5.6).

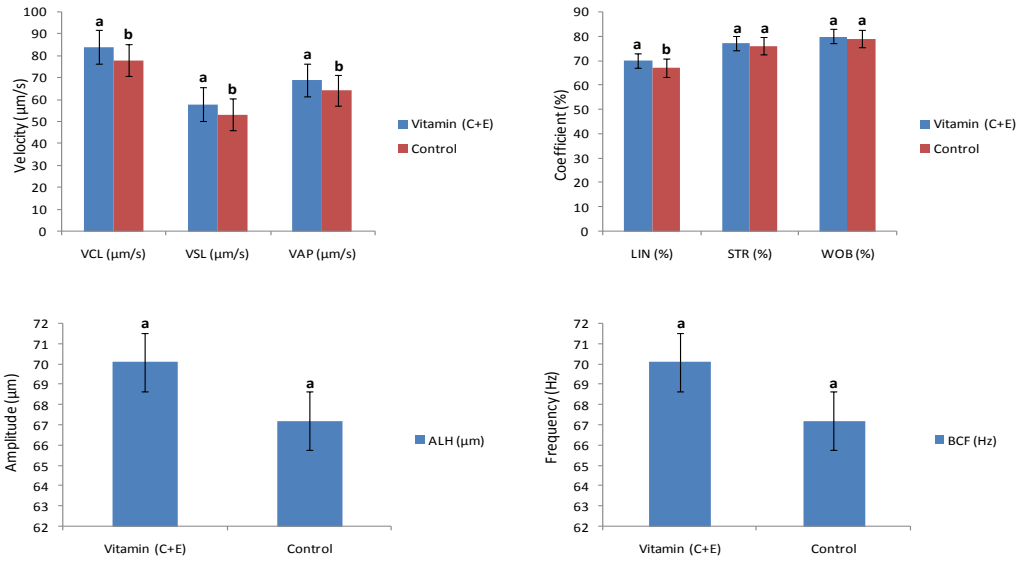

Fig. 5.5. Effects of the combination of vitamins (C+E) on kinematic parameters for rapid-speed spermatozoa in frozen-thawed sperm (mean±SEM).Error bar = SEM and ^{ab} bars with different letters differ significantly at p<0.001.

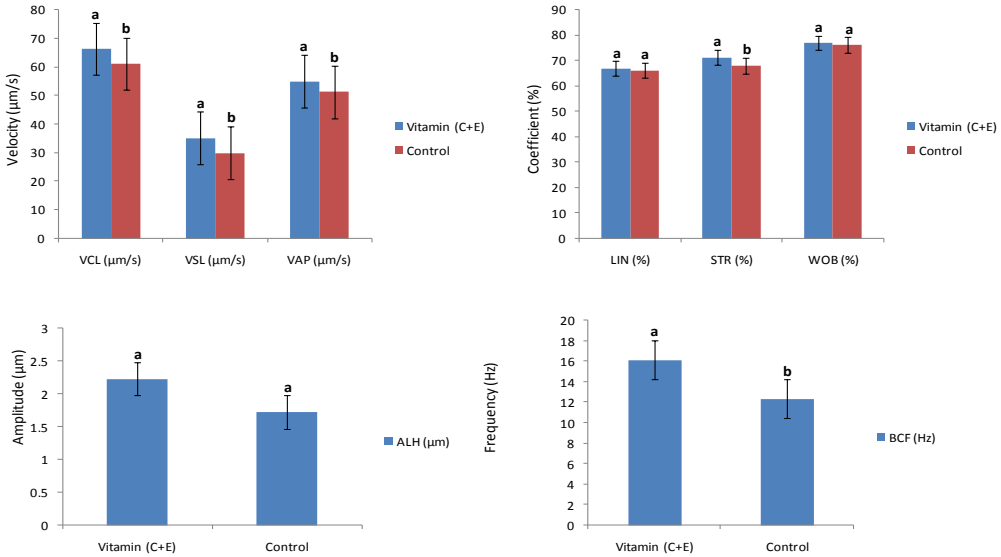

Fig. 5.6. Effects of the combination of vitamins (C+E) on kinematic parameters for medium-speed spermatozoa in frozen-thawed sperm (mean±SEM). Error bar = SEM and ^{ab} bars with different letters differ significantly at p<0.001.

Table 5.5 shows sperm motility and velocity parameters in frozen-thawed semen stored in extender supplemented with the combination of vitamins (C+E) and different equilibration times. Rapid and medium speed spermatozoa were significantly (p<0.001) higher during 2 h of equilibration time compared to 4 and 6 h. Sperm total and progressive motility were similar for both 2 and 4 h of equilibration times. Equilibration time did not have any effect on non-progressive motility.

Table 5.5. Effect of the combination of vitamins (E+C) on post-thaw sperm motility parameters of Saanen buck semen equilibrated at 2, 4 and 6 h (mean±SEM)

Equilibration times		Total motility (%)	Rapid speed (%)	Medium speed (%)	Progressive motility (%)	Non-progressive motility (%)
2 h	Vitamin (C+E)	66.4±1.23^{a}	26.9±0.01^{a}	31.2±1.10^{a}	42.6±1.81^{a}	24.1±2.01^{a}
	Control	58.4±1.12^{b}	22.4±1.11^{cb}	27.9±1.45^{b}	38.0±1.63^{b}	20.2±0.15^{b}
4 h	Vitamin (C+E)	65.7±1.24^{a}	24.7±1.11^{b}	28.1±1.04^{b}	42.2±0.63^{a}	23.3±0.45^{a}
	Control	57.3±0.12^{c}	21.1±1.11^{c}	26.3±1.45^{bc}	37.0±0.63^{b}	20.2±1.15^{b}
6 h	Vitamin (C+E)	60.2±2.12^{b}	23.1±1.01^{b}	28.1±1.03^{b}	36.5±1.34^{bc}	24.3±1.65^{a}
	Control	54.4±0.12^{d}	20.2±1.11^{c}	25.0±1.45^{c}	34.0±0.63^{c}	19.1±1.15^{b}

Means with different superscripts on a column differ significantly at P<0.001.

Table 5.6 shows sperm kinematic parameters in frozen-thawed semen stored in extender supplemented with the combination of vitamins (C+E) and different equilibration times. Kinematic parameters VCL, VSL and VAP were significantly (p<0.001) higher during 2 h of equilibration compared to 4 and 6 h, while LIN and WOB were significantly (p<0.001) higher

during 4 h of equilibration time. The BCF was significantly ($p<0.001$) higher during 6 h of equilibration compared to 2 and 4 h.

Table 5.6. Effect of the combination of vitamins (C+E) on post-thaw sperm kinematic parameters of Saanen buck sperm equilibrated at 2, 4 and 6 h (mean±SEM)

Equilibration times	Selenium treatment	VCL(μm/s)	VSL(μm/s)	VAP(μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)
2 h	Vitamin (C+E)	67.3±1.53^{a}	42.1±1.13^{a}	56.3±2.16^{a}	61.0±1.11^{b}	79.5±0.93^{a}	77.2±0.67^{b}	2.8±0.36^{a}	14.3±4.32^{b}
	Control	63.1±0.12^{b}	38.1±0.23^{b}	53.5±0.13^{b}	59.9±1.15^{b}	63.1±1.17^{b}	76.1±0.09^{b}	1.3±1.16^{b}	13.2±1.82^{b}
4 h	Vitamin (C+E)	62.8±1.43^{b}	39.5±0.21^{b}	52.3±1.31^{b}	65.7±0.23^{a}	77.8±1.10^{a}	82.3±1.71^{a}	2.6±0.38^{a}	14.9±0.46^{b}
	Control	60.1±1.12^{c}	37.1±1.23^{b}	51.5±1.53^{b}	55.9±1.15^{c}	62.2±1.97^{b}	68.0±0.09^{c}	1.02±1.14^{b}	13.2±0.82^{b}
6 h	Vitamin (C+E)	63.0±1.10^{b}	38.2±1.01^{b}	51.2±1.42^{b}	60.3±1.01^{b}	78.2±0.15^{a}	78.5±0.12^{b}	2.6±0.55^{a}	18.1±3.02^{a}
	Control	60.1±0.12^{c}	34.1±1.23^{c}	48.1±1.53^{c}	54.9±1.15^{c}	63.1±1.97^{b}	68.1±0.09^{c}	1.2±1.15^{b}	14.2±0.82^{b}

Means with different superscripts on a column differ significantly at $p<0.001$.

Experiment 3: Effect of selenium supplementation on sperm motility and kinematic parameters of cooled and post-thaw based on different equilibration times

Fresh, cooled and frozen-thawed sperm motility parameters from Se supplemented and control bucks are shown in Figure 5.7, 5.8 and 5.9. In fresh semen total, rapid, medium and progressive motility were significantly ($p<0.001$) higher from Se treated bucks compared to the control group while the percentage of non-progressive spermatozoa were higher in control groups.

In cooled semen the rapid, medium and progressive motile spermatozoa were significantly ($p<0.001$) increased in the Se treatement compared to the control group. No differences were observed between semen from the treated and control groups in terms of medium speed spermatozoa while non-progressive sperm motility was higher from the control group semen. In frozen-thawed semen total, rapid, medium and progressive motile spermatozoa were significantly ($p<0.001$) higher from Se treated bucks compared to control groups while no differences were observed between the Se treated and control group semen concerning non-progressive sperm motility.

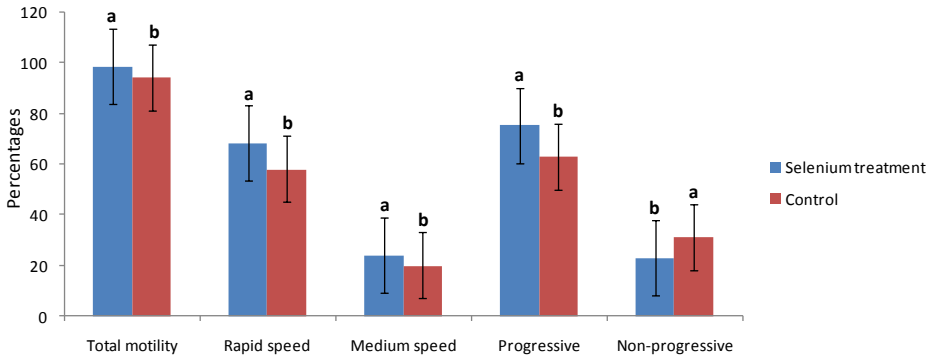

Fig. 5.7.Mean values (±SEM) for sperm motility parameters of fresh semen from selenium supplemented Saanen buck determined by CASA. Error bar = SEM and ^{ab} bars with different letters differ significantly at $p<0.001$.

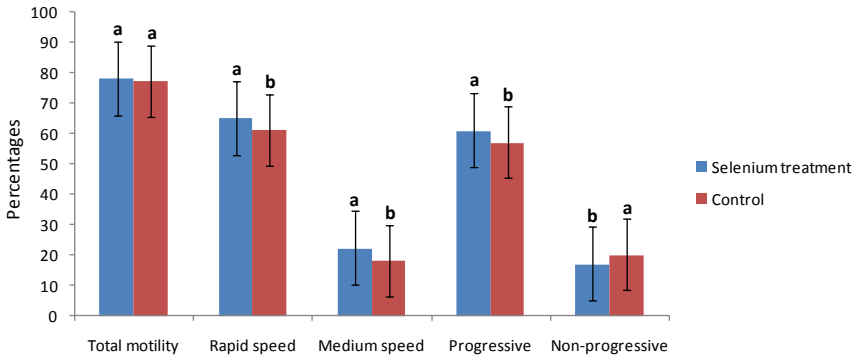

Fig.5. 8. Mean values (±SEM) for sperm motility parameters of cooled semen from selenium supplemented Saanen buck determined by CASA. Error bar = SEM and ^{ab} bars with different letters differ significantly at p<0.001.

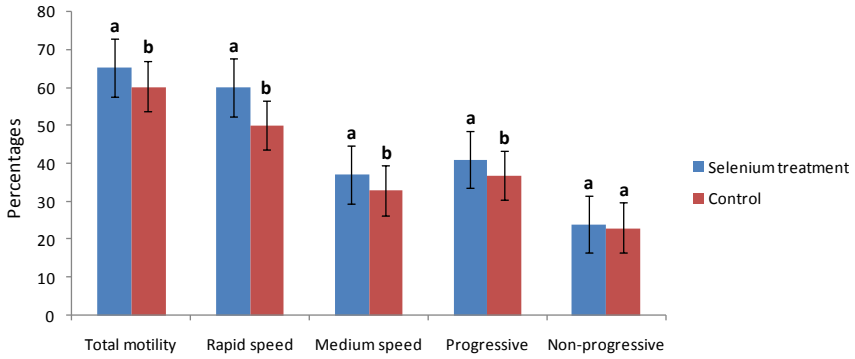

Fig. 5.9. Mean values (±SEM) for sperm motility parameters of frozen-thawed semen from selenium supplemented Saanen buck determined by CASA. Error bar = SEM and ^{ab} bars with different letters differ significantly at p<0.001.

Fresh, cooled and frozen-thawed sperm kinematic parameters from Se supplemented and control bucks are shown in Table 5.7 and 5.8. In fresh semen, the spermatozoa VCL, VSL, VAP, LIN and STR were significantly (p<0.001) higher in semen from Se treated bucks compared to the control group. No differences were observed between the Se treated and control group with respect to the spermatozoa with the WOB, ALH and BCF. In cooled semen the spermatozoa VCL, VSL, VAP and BCF were significantly (p<0.001) increased in semen from Se treated compared to the control group. No differences were observed in semen from the treated and control group in terms of LIN, STR, WOB and ALH spermatozoa. In frozen-thawed semen the spermatozoa VCL, VSL, VAP, STR and BCF were significantly (p<0.001) higher in semen from Se treated bucks compared to control

group while no differences were observed between semen from the Se treated and control group concerning LIN, WOB and ALH spermatozoa.

Table 5.7. Overall (means±SEM) sperm kinematic parameters in fresh semen from selenium supplemented goats

Selenium treatment	VCL(µm/s)	VSL(µm/s)	VAP(µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm)	BCF (Hz)
TG	136.1±1.23[a]	92.3±0.16[a]	111.5±1.02[a]	67.4±0.72[a]	69.5±1.52[a]	83.1±1.26[a]	4.2±1.02[a]	14.8±1.43[a]
CG	123.2±0.32[b]	69.2±1.15[b]	95.2±1.02[b]	60.2±0.42[b]	64.3±1.42[b]	84.2±1.11[a]	4.4±1.01[a]	13.2±0.11[a]

Means with different superscripts in a column differ significantly at p<0.001. TG: treatment group, CG: control group.

Table 5.8. Overall (means±SEM) sperm kinematic parameters in cooled and frozen-thawed semen from selenium supplemented goats

Selenium treatment	VCL(µm/s)	VSL(µm/s)	VAP(µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm)	BCF (Hz)
Cooled semen								
TG	108.2±0.02[a]	56.2±1.20[a]	79.2±0.03[a]	57.1±1.24[a]	63.3±1.66[a]	74.1±1.09[a]	4.1±1.73[a]	16.2±0.92[a]
CG	95.1±0.13[b]	51.3±1.32[b]	73.1±2.23[b]	58.0±0.12[a]	62.1±0.01[a]	73.2±1.05[a]	3.7±0.13[a]	17.1±0.47[b]
Frozen-thawed semen								
TG	72.5±1.71[a]	54.4±0.42[a]	70.1±1.52[a]	55.2±0.34[a]	68.4±1.11[a]	71.2±0.32[a]	2.2±1.74[a]	16.2±1.12[a]
CG	65.3±1.12[b]	49.1±1.23[b]	59.2±1.53[b]	54.9±1.15[a]	64.1±1.97[b]	67.1±0.09[b]	2.3±1.16[a]	15.1±0.82[b]

Means with different superscripts between row, within cooled and frozen semen differ significantly at p<0.001. TG: treatment group, CG: control group.

Significant differences were recorded between the Se treated and control with respect to the kinematic parameters of the rapid (Fig. 5.10) and medium-speed spermatozoa (Fig. 5.11). In the present study, the spermatozoa VCL, VSL, VAP, STR and ALH values observed in Se treated bucks were significantly (P<0.001) higher for the rapid spermatozoa compared to control bucks. No differences were observed for LIN, WOB and BCF spermatozoa between both Se treated and control (Fig. 5.10). For the medium-speed spermatozoa, the spermatozoa VCL, VSL, VAP, STR and BCF values were significantly (P<0.001) higher in Se treated bucks than in the control. No differences were observed in term of LIN, WOB and ALH spermatozoa (Fig. 5.11).

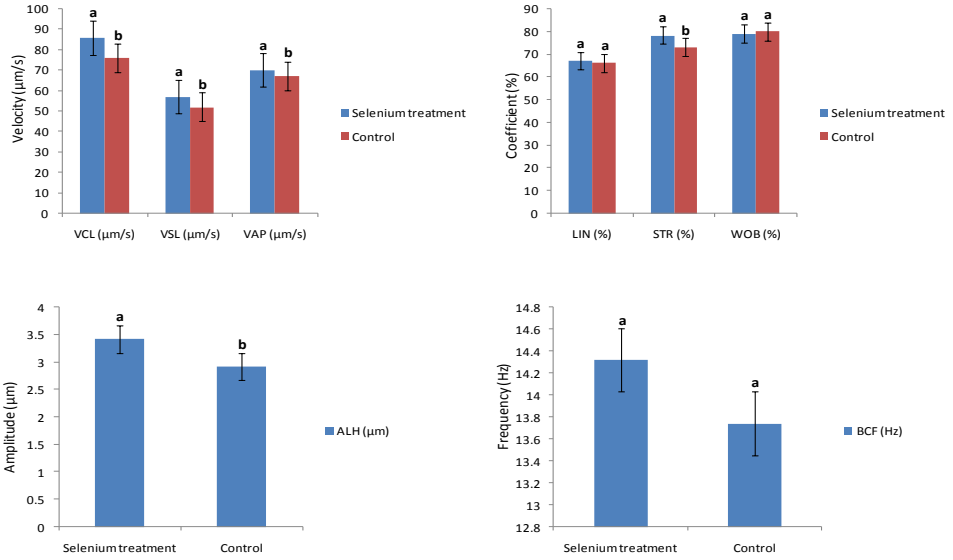

Fig 5.10. Effects of selenium supplementation on kinematic parameters for rapid-speed spermatozoa in frozen-thawed sperm (mean±SEM).Error bar = SEM and ^{ab} bars with different letters differ significantly at p<0.001.

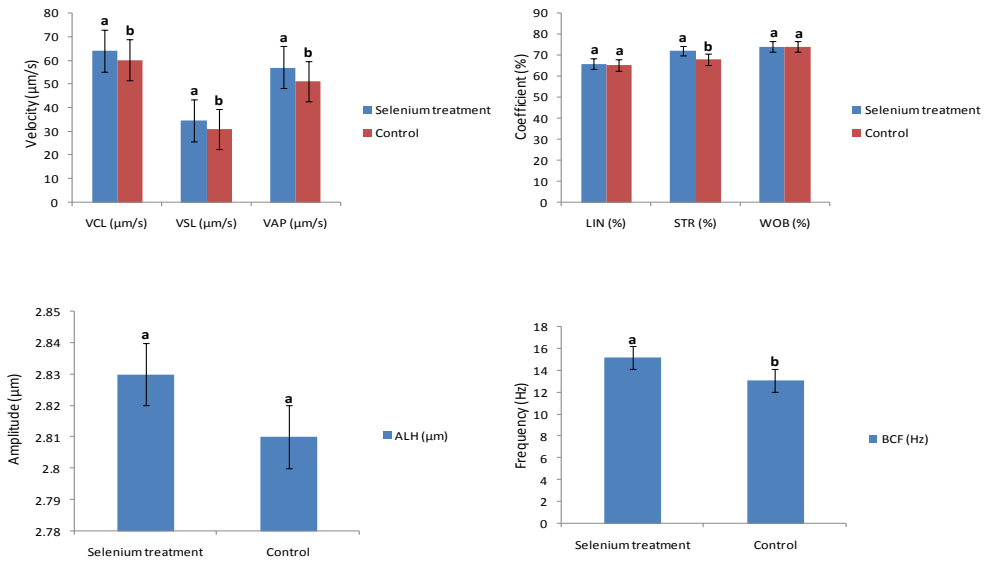

Fig. 5.11.Effects of selenium supplementation on kinematic parameters for medium-speed spermatozoa in frozen-thawed sperm (mean±SEM).Error bar = SEM and ^{ab} bars with different letters differ significantly at p<0.001.

Table 5.9 shows the correlation analysis between post-thaw sperm motility and kinematic parameters of semen from Se supplemented Saanen bucks. The results showed that sperm total motility, rapid, medium-speed and progressive motility were positively correlated (p<0.001) with spermatozoa VCL, VSL, VAP, LIN, STR, ALH and BCF. Sperm total motility was positively correlated (p<0.001) with rapid, medium-speed and progressive motility. Rapid-speed sperm were positively correlated (p<0.001) with medium-speed and progressive motility spermatozoa. Medium-speed sperm was positively correlated (p<0.001) with progressive motility spermatozoa.

Table 5.9. Pearson correlation coefficients (r) between post-thaw sperm motility and kinematic parameters of selenium supplemented Saanen bucks ejaculates

Parameters	**r**	**Parameters**	**R**
Total motility and rapid-speed sperm	0.817**	Rapid-speed sperm and VSL	0.952**
Total motility and medium-speed sperm	0.684**	Rapid-speed sperm and VAP	0.783**
Total motility and progressive motility	0.935**	Medium-speed sperm and progressive motility	0.591**
Total motility and VCL	0.892**	Medium-speed sperm and VCL	0.784**
Total motility and VSL	0.956**	Medium-speed sperm and VSL	0.852**
Total motility and VAP	0.794**	Medium-speed sperm and VAP	0.563**
Rapid-sperm and medium-speed sperm	0.583**	Progressive motility and VCL	0.792**
Rapid-speed sperm and progressive motility	0.924**	Progressive motility and VSL	0.874**
Rapid-speed sperm and VCL	0.894**	Progressive motility and VAP	0.713**

**. Correlation coefficient is significant at p<0.001.

Table 5.10 shows the correlation analysis among sperm kinematic parameters of Saanen bucks. The results showed that sperm VCL was positively correlated ($p<0.001$) with sperm VAP and BCF. Sperm VSL was positively correlated ($p<0.001$) with spermatozoa VCL, VAP, LIN, STR and WOB. Sperm LIN was positively correlated ($p<0.001$) with sperm STR and WOB. A positive correlation ($p<0.001$) was observed between STR and WOB spermatozoa, and between ALH and BCF spermatozoa.

Table 5.10. Pearson correlation coefficients (r) among sperm kinematic parameters of selenium supplemented Saanen bucks ejaculates

Parameters	r	Parameters	R
VCL and VCL	0.891**	VAP and LIN	0.782**
VCL and VAP	0.912**	VAP and STR	0.712**
VCL and BCF	0.711**	VAP and WOB	0.732**
VSL and VAP	0.952**	LIN and STR	0.901**
VSL and LIN	0.801**	LIN and WOB	0.981**
VSL and STR	0.743**	STR and WOB	0.843**
VSL and WOB	0.812**	ALH and BCF	0.666**

**. Correlation coefficient is significant at $p<0.001$.

The mean values of sperm motility parameters in frozen-thawed semen of Se supplemented and control group equilibrated using different equilibration times are shown in Table 5.11. Sperm parameters such as total motility and progressive motility increased significantly ($p<0.001$) when semen was equilibrated for 2 h in Se supplemented bucks compared to 4 and 6 h of equilibration, and control group. However, rapid and medium spermatozoa were similar in both 2 and 4 h of equilibration.

Table 5.11. Sperm motility parameters in frozen-thawed semen of Saanen buck supplemented with selenium and equilibrated at different times (mean±SEM)

Equilibration times	Selenium treatment	Total motility (%)	Rapid speed (%)	Medium speed (%)	Progressive motility (%)	Non-progressive motility (%)
2 h	TG	66.1 ± 0.13^{a}	28.1 ± 0.01^{a}	36.8 ± 1.13^{a}	41.0 ± 0.61^{a}	25.1 ± 1.01^{a}
	CG	61.3 ± 0.32^{cb}	23.3 ± 1.11^{b}	33.0 ± 1.45^{b}	37.1 ± 1.63^{b}	24.2 ± 0.15^{a}
4 h	TG	63.8 ± 1.02^{b}	26.8 ± 0.33^{a}	37.1 ± 1.03^{a}	38.4 ± 1.04^{b}	25.4 ± 2.65^{a}
	CG	60.4 ± 0.23^{c}	23.3 ± 1.23^{b}	30.7 ± 1.23^{cb}	37.1 ± 1.41^{b}	23.0 ± 1.21^{b}
6 h	TG	59.1 ± 1.22^{c}	20.1 ± 1.61^{c}	28.9 ± 1.75^{c}	33.3 ± 0.53^{c}	26.1 ± 0.45^{a}
	CG	60.2 ± 0.12^{c}	21.4 ± 0.41^{c}	27.4 ± 1.43^{c}	34.1 ± 1.44^{c}	26.2 ± 0.35^{a}

Means with different superscripts on a column differ significantly at $P<0.001$.

The mean values of sperm kinematic parameters in frozen-thawed semen of Se treated and control group equilibrated using different equilibration times are shown in Table 5.12. The sperm kinematic parameters such as VCL, STR, WOB and ALH increased significantly ($p<0.001$) when semen was equilibrated for 2 h in semen from Se treated bucks compared to the control 4 and 6 h of equilibration, while, sperm VSL, VAP and BCF were similar in both 2 and 4 h of equilibration of semen from Se treated bucks.

Table 5.12. Sperm kinematic parameters of frozen-thawed semen from Saanen bucks supplemented with selenium and equilibrated at different times (mean±SEM)

Equilibration times	Selenium treatment	VCL(µm/s)	VSL(µm/s)	VAP(µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm)	BCF (Hz)
2 h	TG	69.4±0.51^{a}	58.1±0.42^{a}	63.2±1.52^{a}	66.6±0.34^{a}	86.4±0.11^{a}	76.2±0.42^{b}	3.1±0.74^{a}	18.2±1.12^{a}
	CG	66.1±0.12^{b}	53.2±1.23^{b}	57.5±0.53^{b}	65.9±1.15^{a}	82.1±1.97^{b}	75.1±0.09^{b}	2.4±1.16^{b}	15.1±0.82^{b}
4 h	TG	65.3±0.24^{b}	57.9±2.06^{a}	59.2±1.65^{b}	65.7±7.11^{a}	87.5±0.40^{a}	79.8±1.37^{a}	2.0±0.72^{b}	17.2±0.57^{a}
	CG	62.6±0.02^{cd}	48.8±1.22^{b}	55.8±0.58^{bc}	64.9±1.44^{a}	86.8±2.11^{a}	72.4±0.42^{c}	1.3±1.02^{c}	13.6±0.60^{dc}
6 h	TG	54.9±0.85^{e}	47.6±1.41^{b}	53.2±0.36^{c}	65.4±0.52^{a}	87.1±0.71^{a}	76.1±0.51^{b}	2.2±0.07^{a}	11.3±0.06^{d}
	CG	60.0±0.68^{e}	48.1±1.45^{b}	54.1±1.31^{c}	66.9±1.30^{a}	86.0±0.15^{a}	73.4±1.12^{c}	1.3±1.17^{c}	14.1±0.17^{bc}

Means with different superscripts on a column differ significantly at $p<0.001$.

5.5. Discussion

Experiment 1: Effects of the different concentrations of vitamin C and E added in freezing extender on cooled and post-thaw sperm quality of Saanen buck semen

The results in the present experiment showed increased percentages of all sperm parameters in cooled and frozen-thawed sperm when freezing extender was supplemented with 4 mM of vitamin C or 4.8 mM of vitamin E. This suggests that to obtain acceptable sperm quality after thawing these vitamins concentrations may be recommended.

The results observed in cooled semen are in agreement with that of Asadpour *et al*. (2011) and Silva *et al*. (2013), in which they reported increased sperm motility with the addition of vitamin C in extender. It seems that vitamin C added to freezing extender protects sperm cells better against ROS and consequently improves sperm motility.

The increased percentages of sperm acrosome integrity, normal morphology and viability when vitamin C or E was added to freezing extender in cooled semen agrees with the findings of Azawi and Hussein (2013) and Memon *et al*. (2013).The authors reported an increase in percentages of sperm acrosome integrity, normal morphology and viability following addition to extender of vitamin C or E in ram and Boer goat semen respectively. This can be likely related to an inhibition of lipid peroxidation by these antioxidants in the sperm plasma membrane as was revealed by Barati *et al*. (2011).

On frozen-thawed sperm, it was observed that, 4 mM of vitamin C and 4.8 mM of vitamin E showed a suitable protective effect against freezing damages. This indicates that addition of vitamin C or E in freezing extenders is necessary due to its ability to quench ROS accumulation during cryopreservation. The present results support several studies in bull that reported protective effects of vitamin C and E at thawing (Asadpour *et al*., 2011;Motemani *et al*., 2017). The increased sperm motility and viability obtained with extender supplemented with vitamin C or E post-thaw is in agreement with other researchers who reported increased recovery rate of sperm motility and viability when vitamin C or E was added to freezing extender in ram (Silva *et al*., 2013), buck (Memon *et al*.,2013) and rooster (Min *et al*., 2016). Memon *et al*. (2013) also observed an increase in the percentages of sperm normal morphology and acrosome integrity post-thaw with the addition of vitamin C or E to extender, confirming the findings of the present study. These beneficial effects of adding vitamin C to freezing extenders can be attributed to its ability as a water soluble antioxidant to react with ROS and thereby protecting sperm cell components such as proteins, lipids and nucleic acids against oxidative damages (Donnelly *et al*., 1999). On the other hand, vitamin E, because of its solubility in lipids, might have played a major protective role against OS

and prevents the production of lipid peroxides by scavenging free radicals which are toxic byproducts in biological membranes (Takanami *et al*., 2000; Verma and Nair, 2001). On the basis of the present results, it is clear that vitamin C and E are very efficient antioxidants and their addition to an extender can reduce the OS induced during sperm cryopreservation process.

Experiment 2: Effects of equilibration times and different antioxidants on frozen-thawed Saanen buck sperm

Sperm motility and velocity are the most affected parameters during the semen freezing process and thus are the main cause for the reduced fertility after freezing-thawing processes. In the present study, their increased percentages and values in cooled and frozen-thawed sperm suggested that the addition of the combination of vitamins (C+E) better protected the sperm membranes against ROS and lipid peroxidation attack than the addition of vitamin C or E alone.

Several reports have indicated that increased levels of ROS were significantly correlated with decreased motility parameters, including total motility, progressive motility and rapid motility (Agarwal *et al*., 1994; Khosravi *et al*., 2014). The higher percentages of these parameters in cooled semen demonstrate the possible role of the combination of vitamins (C+E) in the protection of sperm cells against ROS and lipid peroxidation damage. This study also revealed higher values of sperm kinematic parameters such as VCL, VSL and LIN when vitamins (C+E) were added to freezing extender in cooled semen. These observations suggest that the combined actions of vitamin C and E as water and lipids soluble antioxidants may have played a vital role in reducing the oxidation process that accompanied sperm metabolism (Bansal and Bilaspurl, 2009). In this way, the sperm membrane phospholipids are being maintained in a stable condition and their sensitivity to peroxidation is being decreased. Similar to our study, Mittal *et al*. (2014) reported increased sperm motility with the combination of vitamins (C+E), although the authors did not use CASA evaluation method.

From frozen-thawed semen, our study shows that the combination of vitamins (C+E) produce higher total motility, rapid and medium speed spermatozoa, and also higher sperm kinematic parameters such as sperm VCL, VSL and BCF. These results are similar to that reported by Mittal *et al*. (2014) in bull and Min *et al*. (2016) in roosters sperm evaluated, subjectively. They found that supplementing freezing extender with the combination of vitamins (C+E) better protected sperm cells against ROS production and increases sperm motility in frozen-thawed semen. This indicates that vitamin C and E have considerable

synergistic effect and their addition to freezing extender is essential for the maintenance and improvement of Saanen buck sperm fertilizing ability post-thaw. It has been reported that progressive motility and kinematic parameters such as sperm VCL, VSL and VAP could be used to predict the fertilizing capability of the bull spermatozoa (Kathiravan *et al*., 2011).

This can be explained by the fact that vitamin C can penetrate into mitochondria through facilitated glucose transporter and as water-soluble antioxidant with ability to scavenge aqueous peroxyl radicals (Wainer *et al*., 1986). Daramola and Adekunle (2015) reported that vitamin C supplementation in stored buck semen improved motility of spermatozoa. On the other hand, vitamin E is a major chain-breaking antioxidant in the sperm membrane. Vitamin E can inhibit lipid peroxidation reaction in the membrane by eliminating peroxyl (ROO-), alkoxyl (RO-), and other lipid-derived radicals (Silva, 2006). The combination of the two vitamins (C+E) is superior to individual supplementation due to the ability of vitamin C to recycle inactive vitamin E and recover its antioxidant effect (Keshtgar *et al*., 2012).

In our study, the addition of the combination of the two vitamins (C+E) improved the VCL, VSL, VAP and LIN in rapid-speed spermatozoa. The post-thaw sperm velocity (VCL, VSL and VAP) parameters also showed the same trend as well as medium-speed spermatozoa, including STR and BCF. These findings are in agreement with the findings of Casao *et al*. (2010) and Gallego-Calvo *et al*. (2015b) in ram and buck semen, respectively. Although the authors did not add vitamins (C+E) to freezing extender, they reported an increase in all motility parameters except for WOB in the rapid-speed spermatozoa, and all motility parameters except for VCL and VAP in the medium-speed spermatozoa of cooled semen in the melatonin-treatment period. Therefore, it possible that the combination of vitamins (C+E) in the present study played a vital role in protecting and maintaining rapid and medium-speed spermatozoa motility variables after cryopreservation. The reason for this protective effect can be attributed to the ability of the combination of the two vitamins (C+E) to induce neutralization of some detrimental metabolic substances, as evidenced by the improvement and stabilization of velocity parameters such as VCL, VSL and VAP post-thaw both in rapid and medium-speed spermatozoa. It can be assumed that that the combination of vitamins (C+E) exerts its influence by moderating or inhibiting the oxidation process that accompanies sperm metabolism. In this way, the sperm membrane phospholipids are being maintained in a stable condition and their sensitivity to peroxidation is being decreased (Kirilova *et al*., 2015).

The higher post-thaw rapid and medium-speed sperm motility, and kinematic parameters such VCL, VSL and VAP when the combination of vitamins (C+E) and 2 h of equilibration

were used, suggest that addition of antioxidant to freezing extender is very important during equilibration time to protect sperm cells against LPO induced damages. Contrary to our results Almeida *et al*. (2017) found no beneficial effects of the combination of catalase or superoxide dismutase and 0, 4 or 6 h of equilibration on sperm kinetics, plasma and acrosomal membrane integrity of frozen-thawed bull epididymis spermatozoa. Mehdipour *et al*. (2016) also observed no differences between equilibration times (0 and 4 h) for sperm samples frozen with or without antioxidants in ram. This discrepancy can be attributed mainly to differences in antioxidants and animal species used.

The higher post-thaw sperm total motility and progressive motility when the combination of vitamins (C+E) and 2 or 4 h equilibration time were used confirm the results of Sundararaman and Edwin (2008) and Ranjan *et al*. (2015) who reported an optimal equilibration time of 2 and 4 h when freezing buck semen. This suggestes that the equilibration time of 2 or 4 h is appropriate to allow glycerol in the freezing extender to work optimally to prevent the formation of ice crystal, thereby inhibiting the death of spermatozoa (Eriani *et al*., 2017). It has also been reported that 2 to 4 h is the most suitable duration for sperm cryopreservation (Yi *et al*., 2002).

The higher post-thaw sperm LIN and WOB when the combination of vitamins (C+E) and 4 h of equilibration were used implies that the addition of antioxidant to freezing extender allegedly affects equilibration period. Regardless of the antioxidant addition to freezing extender, similar to our results Shah *et al*. (2016) observed maximum post-thaw recovery with 4 h of equilibration than other equilibration periods. This equilibration period is considered as a long equilibration time in buck cryopreservation. Anzara *et al*. (2011) stated that in the long equilibration time, lipoproteins in egg yolk interact with the plasma membrane of spermatozoa to be prepared for low temperature.

The improved post-thaw sperm BCF when the combination of vitamins (C+E) and 6 h equilibration were used support the findings of Belala *etal*. (2016) who reported increased post-thaw percentages of motile and progressive spermatozoa when freezing canine semen. The higher sperm BCF indicated that longer equilibration time was more effective at preserving some sperm kinematic parameters. Câmara *et al.* (2016) also observed higher sperm total motility at thawing with the combination of catalase and prolonged equilibration time of 12 h. This can be attributed to the modifications occurring in the spermatozoa membrane enabling adaptation to low temperature and increasing of cryo-tolerance of spermatozoa (Okano *et al*., 2004). These modifications may be associated with the duration of equilibration time. It is, therefore, suggested that if the equilibration time is within the

range of 2 and 6 h it will help spermatozoa to stabilize in extended environment to maintain homeostasis, osmotic and cryo-tolerance (Herold *et al*., 2006).

Experiment 3: Effect of selenium supplementation on sperm motility and kinematic parameters of cooled and post-thaw based on different equilibration times

The observed higher values of sperm kinematic parameters such as VCL, VSL, VAP, STR and BCF in fresh, cooled and frozen-thawed semen in Se supplemented bucks agrees with other studies in human (Rezaeian *et al*., 2016) and ram (Baiomy *et al*., 2009). The authors reported that the use of Se as a component of antioxidant system optimized sperm parameters such motility after freeze-thawing procedures. The parameters VCL, VSL and VAP are measures of sperm progressive velocity and are revealed to play a vital role in sperm competition (Malo *et al*., 2005). They have also been suggested as potential reliable indicators of male fertility (Farooq *et al*., 2017; Santolaria *et al*., 2015). BCF is one of the useful parameters that contribute substantially to the overall sperm linear progression. It indicates the rate at which the curvilinear path crosses the average path. The sensitivity of these parameters to the deleterious effects of LPO has been reported to be higher than that of the total motility (Ayad, 2018). Therefore, the increases of these parameters in the current study indicate the importance of Se in the alleviation of ROS-induced oxidative damage, thus reducing the cytotoxicity to spermatozoa. This suggestes that Se was more effective at preserving flagellar structures and stimulate ATP production, by affecting sperm mitochondrial oxidative phosphorylation. Therefore, enhancing enzymatic rates of adenosine triphosphate (ATP)-utilizing and ATP-regenerating pathways of the sperm, which are assessed by motility and oxygen consumption of the sperm (Marin-Guzman *et al*., 2000).

The higher values of sperm kinematic parameters such as VCL, VSL, VA P and STR in both rapid and medium-speed spermatozoa, in Se supplemented bucks indicates that Se played an indispensable role to protect sperm cells against ROS, thus increasing sperm motility after cryopreservation. The same increase was observed for ALH in rapid-speed and BCF in medium-speed spermatozoa. However, there is no study about the protective effect of Se on the buck's rapid and medium-speed spermatozoa for comparison with our study. Other authors also examined separately post-thaw rapid and medium-speed spermatozoa in bucks. They found no differences in terms of kinematic parameters in both sperm subpopulations (Gallego-Calvo *et al*., 2015a; Gallego-Calvo *et al*., 2015b). This can be explained by the fact that, authors did not supplement animals with Se or any other antioxidant prior to semen cryopreservation. Their studies focused on seasonal changes in sperm variables. It is known that during the cryopreservation the OS levels and ROS production are increased. The supplementation of animal with a suitable antioxidant ensures

a good protection of the spermatozoa. In the present study, Se supplementation may have contributed to the preservation of the energy potential of the rapid and medium-speed sperm, which is important for the fertilization ability (Kirilova *et al*., 2015).

As would be expected the positive correlation between spermatozoa motility and kinematic parameters (VCL, VSL, VAP, LIN, STR, ALH and BCF) in Se supplemented bucks support the findings of Perumal *et al*. (2014) who reported positive correlation between total motility, progressive motility and kinematic parameters in freezable Mithun Semen. Some studies also reported also in bull and rabbit a strong correlation between sperm progressive motility and velocity parameters (Januskauskas *et al*., 2003; Lavara *et al*., 2005), indicating that spermatozoa with forward motility and a straight linear path may cover more distance in a short period of time so that they can reach the oocyte and be able to penetrate the zona pellucida (Conell *et al*., 2002). Sperm motility and some kinematic parameters are essential for the sperm to achieve fertilization. Farrell *et al*. (1996) and Perumal *et al*. (2011) found that sperm motility and kinematic parameters such as progressive motility, VSL, VCL, ALH, and LIN were correlated with bull fertility.

The rapid and medium-speed sperm were also positively correlated with sperm progressive motility and kinematic parameters (VCL, VSL, VAP, LIN, STR, ALH and BCF). This indicates that there is similarity of sperm velocity pattern between both subpoputation of buck's ejaculates. However, there is no study describing correlations between rapid and medium-speed with sperm kinematic parameters of post-thawed buck semen assessment by CASA.
The positive correlation between various spermatozoa kinematic parameters detected in the present study has also been reported previously by Inanç *et al*. (2018) in bull. The authors detected the highest positive correlationbetween the VCL and VAP followed by WOB and LIN. They also reported a positive correlation between VSL with LIN and STR. The same trend was observed between VAP with LIN, STR and WOB. In a study conducted on Mithun semen, Perumal *et al*. (2014) reported positive correlations between VCL, VSL, VAP, and BCF, between VSL and VCL, and between ALH and BCF in accordance with the present study. In other studies, a strong and highly significant correlation was also found between STR and LIN in bulls (Januskauskas *et al*., 2003). These observations suggested that sperm kinematic parameters are correlated and interrelated among themselves.

The combination of equilibration time plus the beneficial effects of Se supplementation significantly increased the post-thaw percentages of sperm total and progressive motility as well as the values of sperm kinematic parameters such as VCL, VAP, STR and ALH when semen was equilibrated for 2 h. These results are similar to those reported in ram by

Câmara *et al*. (2016), who tested other antioxidants (catalase) besides Se, although they used a longer equilibration time of 12 h. Anzar *et al*. (2011) reported the beneficial effects of equilibration on plasma and acrosomal membranes of sperm.

The higher percentages of rapid and medium speed spermatozoa as well as the values of sperm kinematic parameters such as VSL, WOB and BCF in Se supplemented samples when 2 or 4 h of equilibration were used agrees with previous study which reported increased sperm motility and viability post-thaw when semen samples were equilibrated for 2 or 4 h (Ahmad *et al*., 2015), although the authors did not use CASA technique to evaluate sperm quality. It can be suggested that the combination of Se supplementation and equilibration time of 2 and 4 h used in the present study enabled the sperm to resist cold shock stresses during cryopreservation of buck semen, as it yielded some improvements in motility and kinematic parameters of frozen-thawed buck sperm. It is believed that this duration of equilibration may help sperm reach an osmotic equilibrium following supplementation of Se, probably due to biochemical balance between ROS generation and scavenging and supporting physiological aspects of sperm cell metabolism. We inferred that the interaction between equilibration time and Se supplementation was necessary to preserve sperm motility and velocity parameters during cryopreservation.

5.6. Conclusion

In summary, according to subjective evaluation, our findings suggested that vitamin C and E at the concentrations of 4 and 4.8 mM respectively, can be efficient for preservation of buck spermatozoa in cooled and frozen-thawed semen. Based on objective analysis, there were some significant interactions between antioxidants and equilibration time which lead to an increasing trend of sperm survival.The combination (4 mM vitamin C and 4.8 mM vitamin E) added to freezing extender yielded an increased tendency of post-thaw sperm motility and velocity parameters, and when combined with 2 or 4 h equilibration, resulted in increased quality of frozen-thawed semen. Furthermore, dietary Se supplementation and 2 or 4 h equilibration during cryopreservation was essential for maintaining motility and velocity parameters as evidenced by higher sperm survival in cooled and frozen-thawed semen.

CHAPTER 6

General conclusion, recommendations and critical evaluation

6.1. General conclusion and recommendations

Maintaining the highest fertilizing potential of semen through the freezing-thawing process requires the best available conditions for cryopreservation. Despite many attempts to optimize the freezing-thawing conditions to improve post-thaw sperm quality, spermatozoa still fail to survive the freezing and thawing process. Specific problems limiting post-freezing properties of goat semen are the LPO, formation of ice crystals when fast cooling rates are used and development of regions of high solute concentrations when slow rates of cooling are employed. The quality of cryopreserved semen depends on the interactions between extender, cooling rate and equilibration time. In this regard, dietary antioxidant supplementation or its inclusion to well defined extender prior to the freezing process is essential to combat excessive production of ROS. This includes optimization of cooling rate to prevent sperm cell damage due to formation of intracellular ice and hypertonic solutions.

The experiment, which was conducted to determine the antioxidative effect of orally supplemented sodium selenite on reproductive performance, showed that oral supplementation with selenium in the form of sodium selenite significantly improved testis measures and semen characteristics of Saanen bucks. In addition, better quality semen can be obtained by supplementing bucks with Se and collecting the semen using AV method. Selenium supplementation also increased plasma concentration of LH and testosterone as well as GSH-Px activity. The oral supplementation of sodium selenite could possibly be used as supplementary diet, since in many parts of the world plants do not provide adequate Se to meet dietary requirements. In South Africa goats and sheep are produced under an extensive production system. Therefore, oral selenium supplementation may be a preferred method .In addition, Se is not distributed evenly across the planet; rather concentrations differ markedly depending on local conditions. Climate also exerts a very significant effect on the incidence of Se deficiency, mainly during winter in South Africa. Based on this, oral supplementation of Se might be necessary and a more applicable method, especially for animals depending on Lucerne diet or pastures. This is because the Lucerne or pastures may be produced from Se-deficient environments, since not all Se-deficient areas in South Africa have been mapped.

It can be noted that, the oral selenium supplementation may not be applicable to commercial semi-intensive producers, since these farmers supplement their animals with diets containing vitamins, minerals and trace elements such as selenium (in the form of a premix).

The use of various premixes is necessary as they have positive effects on maintenance, growth, health and milk production as well as reproductive performance.

The current work demonstrated that buck sperm total motility decreased with every step of cryopreservation (cooling, equilibration and frozen storage). However, the outcome of the cryopreservation process depends on the protocol used in diluting semen with different extenders and additives as well as the use of different cooling, freezing and thawing rates. The influence of freezing medium composition is of great importance for sperm survival during cryopreservation. The experiment conducted to study the effects of dietary Se supplementation, cooling rates and different extenders on buck semen cryopreservation demonstrated that, spermatozoa survived the freezing-thawing cycle better in clarified egg yolk extender under conditions where slow cooling was used. This indicates that clarified egg yolk can be used to replace whole egg yolk thus ensuring less viscosity due to removal of granules through centrifugation process and a more homogeneous composition of the extender. In addition, all sperm parameters studied showed an increasing trend when bucks were supplemented with Se prior to sperm cryopreservation. This suggests that supplementing animals with Se, then using clarified egg yolk extender followed by slow cooling; may be a useful combination to improve sperm quality post-thaw. It improves sperm quality by preventing the production and propagation of reactive oxygen species in goat semen. Furthermore, some sperm parameters were similar in both clarified egg yolk and whole egg yolk extenders in Se supplemented bucks. This suggests that, regardless of freezing extender type, spermatozoa were more resistant to cryo-injury when bucks were supplemented with Se before starting cryopreservation process. Sodium selenite seems to be more effective in enhancing the antioxidant enzyme capacity of the sperm cells by increasing the activities of plasma glutathione peroxidase (GSH-Px) content as evidenced in the previous chapter. However, the fertilizing ability of sperm preserved using this combination still need more detailed investigation.

The semen cryopreservation process causes oxidative stress on the sperm membrane, leading to irreversible sperm cells damage and changes in enzymatic activity associated with a reduction in sperm motility, functional membrane integrity and fertilizing ability. Our preliminary experiments on the addition of vitamin C at the concentration of 4 mM and vitamin E at 4.8 mM to freezing extender provided superior results of cooled and frozen-thawed sperm compared to other concentrations. These preliminary experiments were the basis for the current study where it was proposed that addition of the combination of vitamins (C+E) to semen extender could improve spermatozoa motility and velocity parameters in cooled and frozen-thawed semen. In the context of this proposition, clarified egg yolk

extender was added with the combination of vitamins (C+E) and compared to vitamin C and E alone to establish the extender most suitable for buck semen cryopreservation. It was observed that the combination of vitamins (C+E) yielded an increased post-thaw sperm quality with substantial improvement of sperm motility and velocity parameters. Furthermore, addition of the combination of vitamins (C+E) to freezing extender before freezing, combined with 2 or 4 h equilibration time also increased the resistance of the outer acrosomal membrane and overlying plasma membrane to cryo-injury. This resulted in an increased tendency of sperm motility and velocity parameters in cooled and frozen-thawed semen.Thus, it is possible to use the combination of vitamins (C+E), especially when animals are not supplemented with an antioxidant prior to semen cryopreservation, to maintain an acceptable percentage of motile sperm and values for most kinematic parameters related to sperm progressive motility.

The antioxidant capacity of the sperm cell is limited due to a small cytoplasmic component, which contains these antioxidants to scavenge the oxidants. In addition, the concentration of these antioxidants may decrease considerably by the dilution of the semen. Sperm may however be incapable in preventing LPO during the freezing–thawing process. Based on this, utilization of dietary Se supplementation prior to semen cryopreservation to boost natural antioxidants of animals could be recommended. In the present study, dietary Se supplementation reduced the impact of ROS induced damage due to LPO as evidenced by the increased sperm motility and velocity parameters in fresh, cooled and frozen-thawed semen. In an attempt to establish the relationship between Se supplementation and equilibration times for buck semen in the current study, the effect of the dietary supplementation of Se and different equilibration times on frozen-thawed sperm motility and velocity parameters was investigated. The results show that the combination of Se supplementation and 2 or 4 h equilibration period yielded an increasing trend of sperm motility and velocity parameters.This implies that Se supplementation in combination with short period of equilibration proved to be better than longer period.

Thus, this improvement in sperm motility and kinematic parameters might indicate that, if animals are supplemented with dietary Se or freezing extender is supplemented with the combination of vitamin (C+E), there is more chance of reducing ROS responsible for reduced sperm quality and fertility during cryopreservation. However, the extent of vitamin C and E addition to freezing extender and oral Se supplementation need further attention in order to clarify their optimal concentration levels for sperm protection when used in combination.

6.2. Critical evaluation

Although the present study has reached its aims and objectives, there are always avenues to explore for improvement and a number of shortcomings and limitations were identified. Firstly, this study highlights the effect of different antioxidants on buck spermatozoa during semen cryopreservation. Further, the study addresses many aspects of sperm protection, sperm motility, viability and membrane stabilization of sperm cells during storage. Various enzymatic and non-enzymatic antioxidants are available that play an important role in protecting sperm from free radical species during cryopreservation. Therefore, the current need is to improve existing techniques for semen processing, cooling, packaging and freezing with the use of those antioxidants.

Due to to unexpected damage to the pH at the start of the trail we were forced to use a litmus pH paper to monitor the pH of the semen. While litmus pH paper is great for quick qualitative work, it will not provide accurate quantitative data. Therefore, it can be noted that, the pH obtained in the present study may not be exact pH values but can be used as an indication, since the litmus pH paper only allow us to know whether the solution is basic or acidic but will not tell the degree of acidity or alkalinity. The pH meter is far more accurate compared to a pH paper. In a pH meter results are obtained and analysed within the set ranges according to the set standards.

The Se concentration adjustment studies should be conducted to determine the precise dosage with maximal beneficial and minimal detrimental effects on buck health and sperm quality post-thaw. A major limitation of the current study was its design based on single administration route. However, various administration routes such injection and addition to extender can provide a better understanding of the blood plasma Se concentration and its effects on male hormone. Such determinations should be covered in further studies.

With the aim of defining an adequate extender composition, we tested whether the addition of vitamin C, E and their combination to the sperm freezing extender was able to protect buck spermatozoa during the cryopreservation process. However, only one extender was tested. It may be important to study different extenders, especially whole egg yolk and tris without egg-yolk extender. The dose and duration of these antioxidants should also be determined and standardized. With the increase in the use of assisted reproductive technology procedure, there should be an effort to develop optimum combinations of antioxidants to supplement sperm preparation media.

The findings of the present work may have been more defined with an expended animal sample pool, but because of time and other practical restrictions, the amount of samples had to be limited. Therefore, the study provides a great opportunity for future research to determine the optimum cooling rates and equilibration periods for goat breeds. It will be beneficial if other indigenous goat breeds are included in future study on this topic to develop new techniques that can be used as standard for buck semen cryopreservation.

Critical studies to establish the minimum number of frozen-thawed sperm per inseminating dose for acceptable fertility (about 60%) and strict quality control of the frozen semen at various stages of production, processing, storage and final use are necessary.

The findings in this dissertation are obtained through laboratory experiments that might not reflect the true fertilizing capability of cooled and frozen-thawed spermatozoa. Further *in vitro* or *in vivo* fertilization studies are needed in order to determine their success rates at insemination.

There is a need to identify the seminal characteristics which directly affects the freezing ability of spermatozoa. Critical studies to establish the fertility marker-based selection of the bucks for use in semen cryopreservation might be of great importance in years to come.

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