CHARACTERISATION AND CRYOPRESERVATION OF BAPEDI RAM SEMEN IN TRIS EGG YOLK EXTENDER SUPPLEMENTED WITH PHOSPHATIDYLCHOLINE

MASTER OF SCIENCE IN AGRICULTURE (ANIMAL PRODUCTION)

K.S MAFOLO

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CHARACTERISATION AND CRYOPRESERVATION OF BAPEDI RAM SEMEN IN TRIS EGG YOLK EXTENDER SUPPLEMENTED WITH PHOSPHATIDYLCHOLINE

KGAOGELO STIMELA MAFOLO

BSc in Agriculture (Animal Production), University of Limpopo

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SUPERVISOR: DR T. CHITURA (UL)

CO-SUPERVISOR: DR C.M. PILANE (ARC)

CO-SUPERVISOR: PROF T.L. NEDAMBALE (TUT)

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DECLARATION

I declare that this mini-dissertation submitted to the University of Limpopo for the degree of Master of Science in Agriculture (Animal Production) is my independent work and research, and that it has not previously been presented as a study for this university or elsewhere. I further declare that all sources have been duly acknowledged.

Name: Mafolo Kgaogelo Stimela

Signature………………………… Date…………………………

DEDICATION

This mini-dissertation is dedicated to my academic father, Dr M Malatje and my son, Pholosho Mafolo for their everlasting support and inspiration.

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ABSTRACT

The study was conducted to determine the macroscopic and microscopic raw semen characteristics of Bapedi rams, to evaluate the effect of different egg yolk (EY) concentrations in Tris-based extenders on cryopreservation of Bapedi ram semen and to determine the effect of supplementing different phosphatidylcholine (PC) concentrations in Tris-based extenders with or without egg yolk on cryopreservation of Bapedi ram semen. Semen ejaculates were collected from four matured Bapedi rams aged 2-4 years using artificial vagina (AV) and pooled to eliminate individual differences. The first experiment was performed to characterise Bapedi ram semen parameters immediately after semen collection. The macroscopic semen parameters such as volume, pH and concentration and microscopic semen parameters such as motility, viability and morphology, membrane integrity and acrosome integrity were evaluated. The experiment was replicated 8 times and the data was subjected to descriptive statistics. The second experiment evaluated the effect of Tris-based extenders with five different EY concentrations (0, 5, 10, 15 and 20 %) on the microscopic quality of cryopreserved Bapedi ram semen. The treatments were subjected to a Completely Randomized Design (CRD) and replicated 4 times. The third experiment evaluated the effects of different PC concentrations supplemented to Tris-based extenders with or without 10% EY and the PC was added as liposomes. The experiment was a 2 x 4 factorial design in a CRD with two concentrations of EY: 0 and 10 %, and four concentrations of PC: 0, 0.25, 0.50, 0.75 mg/ml in Tris-based extenders. Pooled semen samples were divided into 5 and 8 aliquots to comply with objective 2 and objective 3, respectively. The semen aliquots were diluted with Tris-based extenders and equilibrated in a refrigerator at 5°C for another 4 hours. The semen was frozen using a programmable freezer and plunged into liquid nitrogen tank (-196°C).

The volume, sperm concentration and pH of Bapedi ram semen ranged between 0.4-1.5 ml, 0.52-8.84 × 10^9 sperm/ml, and 5-7, respectively. The average total motility (TM), progressive motility (PM) and rapid motility (RM) characteristics were 85.95±2.58 %, 29.33±2.11 % and 39.47±4.99 %, respectively. The results for the mean percentage live spermatozoa, abnormalities, intact membrane and intact acrosome were 70.19±2.29 %, 2.50±1.34 %, 72.39±1.71 % and 75.37±5.39 %, respectively. There was a general decrease trends in frozen-thawed motility

characteristics such as TM, PM and RM as compared to raw semen ($p<0.05$). The frozen-thawed semen in Tris-based extenders with 10, 15 and 20% EY concentrations resulted in significantly ($p<0.05$) higher TM, PM and RM motility characteristics compared to 0 and 5%. The percentage of live spermatozoa, membrane and acrosome integrities were found higher in raw semen than in frozen–thawed semen of respective extenders ($p<0.05$). The supplementation of PC in extenders either with or without EY did not improve the TM, PM and RM parameters ($p>0.05$). The membrane integrity in extenders either with or without EY were not influenced by the supplementation PC after freezing and thawing ($p>0.05$). The supplementation of PC in treatments with EY did not improve the acrosome integrity ($p>0.05$). Interestingly, the supplementation 0.75 mg/ml PC resulted in acrosome integrity that was not significantly different ($P>0.05$) to treatments with EY. In conclusion, the macroscopic and microscopic semen parameters of raw Bapedi ram semen were characterized. The use of 10% EY concentration resulted in higher motility parameters and membrane integrity of frozen-thawed Bapedi ram semen. However, 20% EY resulted in higher acrosome integrity of frozen-thawed Bapedi ram semen. The supplementation of PC in extenders in extenders with or without EY did not improve the motility parameters, percentage live spermatozoa and membrane integrity. However, the acrosome integrity was improved in extenders without EY supplemented with 0.75 mg/ml PC.

TABLE OF CONTENTS

Contents **Page**

DECLARATION i

DEDICATION ii

ACKNOWLEDGEMENTS iii

ABSTRACT iv

TABLE OF CONTENTS vi

LIST OF TABLES ix

LIST OF FIGURES x

CHAPTER ONE

1.0 GENERAL INTRODUCTION 1

1.1 Background 2

1.2 Problem statement 3

1.3 Motivation 3

1.4 Purpose of the study 4

1.4.1 Aim 4

1.4.2 Objectives 4

CHAPTER TWO

2.0 LITERATURE REVIEW 5

2.1 Introduction 6

2.2 Characteristics of semen 6

2.3 Ram semen characteristics and quality evaluations 7

2.3.1 Macroscopic semen characteristics 7

2.3.2. Microscopic semen characteristics 9

2.3.2.1. Sperm motility 9

2.3.2.2. Viability and morphology 10

2.3.2.3. Membrane integrity 11

2.3.2.4 Acrosome Integrity 12

2.4 Factors affecting ram semen quality 12

2.4.1 Nutrition 12

2.4.2 Ram age 13

2.4.3 Season of collection 13

2.4.4 Method of semen collection 14

2.5 Ram semen cryopreservation 15

2.6 Characteristics of ram semen extenders 17

2.7 Characteristics of egg yolk on semen cryopreservation 18

2.8 Effect of phosphatidylcholine on semen cryopreservation 19

CHAPTER THREE

3.0 MATERIALS AND METHODS 21

3.1 Study location 22

3.2. Experimental animals and semen collection 22

3.2.1 Semen collection 22

3.3 Preparation of extenders 23

3.4 Experimental design 23

3.4.1 Preparation of liposomes 24

3.5 Semen dilution, freezing and thawing 26

3.6 Semen evaluation 26

3.6.1 Macroscopic semen evaluation 27

3.6.2 Microscopic semen evaluation 27

3.6.2.1 Sperm motility 27

3.6.2.2 Viability and morphology 30

3.6.2.3 Membrane integrity 30

3.6.2.4 Acrosome integrity 30

3.7 Data analysis 31

CHAPTER FOUR

4.0 RESULTS 32

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS 43

5.1 Discussion 44

5.2 Conclusion 51

5.3 Recommendations 51

CHAPTER SIX

6.0 REFERENCES 53

LIST OF TABLES

Table	Title	Page
2.1	Macroscopic characteristics of ram semen and collection methods from different breeds	9
3.1	The composition of Tris-based extenders	25
3.2	Description of experimental treatments and codes	25
3.3	Sperm Class Analyser® V.5.3.Settings used to analyse the ram spermatozoa motility and velocity characteristics	28
3.4	The definition and descriptions of spermatozoa motility descriptors for CASA system	29
4.1	Macroscopic semen characteristics of Bapedi rams	35
4.2	Sperm motility characteristics of Bapedi ram semen determined by CASA	36
4.3	Viability, morphology, membrane integrity and acrosome integrity characteristics of Bapedi ram semen	37
4.4	Mean±SEM of raw and frozen-thawed CASA motility characteristics of Bapedi ram semen extended with different concentrations of egg yolk in Tris-based extenders.	38
4.5	Mean±SEM of raw and frozen-thawed viability, membrane and acrosome integrities of Bapedi ram semen extended with different concentrations of egg yolk in Tris-based extenders	39
4.6	The effect of different concentrations of phosphatidylcholine supplemented in extenders with or without egg yolk on CASA motility characteristics following cryopreservation of Bapedi ram semen	40
4.7	The effect of different concentrations of phosphatidylcholine supplemented in extenders with or without egg yolk on viability, membrane and acrosome integrities following cryopreserved Bapedi ram semen	41

LIST OF FIGURES

Figure	Title	Page
4.1	Viability and morphology of the frozen-thawed Bapedi ram spermatozoa stained with eosin-nigrosin.	42
4.2	Membrane integrity of the frozen-thawed Bapedi ram spermatozoa evaluated with HOST.	42
4.3	Acrosome integrity of frozen-thawed Bapedi ram spermatozoa evaluated with PSA-FITC.	42

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background

The fat-tailed Bapedi sheep has been reported to have arrived in South Africa between 200 and 400 AD with the Bapedi people who migrated southwards into the Northern Province of South Africa and settled in the area south of Soutpansberg (Ramsey *et al.*, 2001). The indigenous breeds such as Bapedi sheep are important assets to the country because they possess unique combinations of adaptive traits to survive harsh local environments (Baduram, 2004). The Bapedi sheep are characterised by long straight fat tail with colours varying from brown to red head and to a variety of black and white patterns on the body (Snyman, 2014). Their extreme hardiness triggers their adaptability to survive in different climatic conditions and tolerance to ticks and diseases (Soma *et al.*, 2012). However, a decline in their numbers and loss of genetic variations has been reported, which would lead to their extinction (Baduram, 2004; Soma *et al.*, 2012). These are as a result of cross-breeding and the replacement with exotic breeds (Kunene, 2010). South Africa is currently experiencing climatic changes such as rising temperatures, lack of rainfall, change in vegetation, and increase in animal diseases and parasites which threatens food security. Therefore, Bapedi sheep has a potential to contribute significantly to food security.

The use of reproductive technologies such as semen cryopreservation would allow the conservation and sustainable utilisation of indigenous livestock breeds like Bapedi sheep (Kunene, 2010). Semen cryopreservation has been predominantly used to establish genetic resource banks for endangered species (Day and Stacey, 2007). However, semen cryopreservation is a detrimental procedure to the spermatozoa, which affects the semen quality after freezing and thawing (Watson, 1995). Consequently, cryoprotectants were developed in this procedure to play a central role in resisting sudden temperatures changes, protecting spermatozoa against cold and warm shock as well as preventing ice formation during freezing and dissolution during the thawing process (Salamon and Maxwell, 2000; Purdy, 2006). Egg yolk (EY) is the predominantly used cryoprotective component of semen extenders which protects the spermatozoa during cryopreservation (Salamon and Maxwell, 2000; Kulaksiz *et al.*, 2010). Egg yolk is composed of the biochemical components such as phospholipids, cholesterol and low density lipoprotein (LDL) which protect the spermatozoa against cold shock (Kulaksiz *et al.*, 2010; Azizunnesa *et al.*, 2014). According to Bergeron and Manjunath (2006), phospholipids in EY protect spermatozoa by forming a protective

film on the spermatozoa surface or by replacing spermatozoa membrane phospholipids that are lost or damaged during the cryopreservation process. In addition, phosphatidylcholine (PC) as a major phospholipid of EY was reported to be the major protective component of spermatozoa against cold shock (Quinn *et al.*, 1980).

1.2 Problem statement

South Africa has a distinctive group of indigenous livestock, however, the erosion of their gene pool is a serious concern because they significantly contribute to the rich biodiversity of South Africa (Nedambale *et al.*, 2008). Bapedi sheep breed is under threat of extinction, therefore, conservation strategies such as cryopreservation of their genetic materials are required for their sustainability (Baduram, 2004; Soma *et al.*, 2012). Cryopreservation of ram semen has several disadvantages caused by its negative impact on the spermatozoa, which result in reduced survival rate after freezing-thawing process (Hamedani *et al.*, 2013). Spermatozoa are affected by cold shock, osmotic stress, ice crystal formation and oxidative damage which causes cryoinjuries that results in loss of viability and fertility (Alvarez and Storey, 1992; Amirat *et al.*, 2004). Spermatozoa membranes are considered to be the primary site of cryoinjury resulting from their disruption during cold shock (Situmorang, 2002; Ropke *et al.*, 2011). Furthermore, the phospholipids of the spermatozoa membrane, especially the phosphatidylcholine are lost during semen cryopreservation (Hinkovska-Galcheva *et al.*, 1989; Situmorang, 2002; Ropke *et al.*, 2011). Consequently, characterization and finding suitable protocols for cryopreservation of Bapedi ram semen such as the optimization of egg yolk concentration and supplementation with phosphatidylcholine in extender are required.

1.3 Motivation

Indigenous breeds such as Bapedi sheep have a potential role of sustaining livestock production in South Africa. Hence, the use of reproductive technologies has the potential to improve, utilise and conserve Bapedi sheep (Baduram, 2004). Semen cryopreservation as a conservation strategy has a potential to support the genetic resource gene-bank of a breed for an indefinite period of time (Kulaksiz *et al.*, 2013).

Consequently, knowledge of the semen characteristics prior to their cryopreservation is essential.

During cryopreservation, egg yolk plays a major role in protecting spermatozoa against cold shock and to maintain the quality of cryopreserved semen (Sharafi *et al*., 2009; Kulaksiz *et al*., 2010). The cryoprotective effect of egg yolk is attributed to its biochemical components such as phospholipids, cholesterol and low density lipoprotein (LDL) which protect the spermatozoa against cold shock (Kulaksiz *et al*. 2010). According to Watson (1981), PC as a phospholipid is the major component that protects spermatozoa membrane against cold shock damage. Hen egg yolk has a lower concentration of PC compared to that of other avian, hence supplementation is necessary (Trimeche *et al*., 1997). In addition, the introduction and supplementation of PC in extenders proved to have the potential to enhance the success of semen storage and cryopreservation (He *et al*., 2001; Ropke *et al*., 2011; Pillet *et al*., 2012; Kumar *et al*., 2015; Belala *et al*., 2016)

1.4 Purpose of the study

1.4.1 Aim

The aim of the study was to characterise and improve the quality of cryopreserved Bapedi ram semen using suitable egg yolk concentration in Tris-based extender supplemented with phosphatidylcholine.

1.4.2 Objectives

The objectives of this study were:

i. To determine the macroscopic and microscopic raw semen characteristics of Bapedi rams.
ii. To evaluate the effect of different egg yolk concentrations in Tris-based extenders on cryopreservation of Bapedi ram semen parameters.
iii. To determine the effect of supplementing different concentrations of phosphatidylcholine in Tris-based extenders with or without egg yolk on cryopreservation of Bapedi ram semen parameters.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

This chapter will review the characteristics of semen, ram semen characteristics and quality evaluations, factors affecting ram semen quality, ram semen cryopreservation, characteristics of ram semen extenders, characteristics of egg yolk on semen cryopreservation and the effect of phosphatidylcholine on semen cryopreservation.

2.2 Characteristics of semen

Semen is a secretion from male reproductive tract which is composed of the spermatozoa and seminal plasma fluid (Hafez and Hafez, 2000). The seminal plasma is a complex fluid portion that facilitates the chemical functions of the semen (Juyena and Stelleta, 2013). The components of the seminal plasma are secreted from the compartments of the male reproductive tract such as rete testis, epididymis and accessory glands (seminal vesicle, prostate and bulbo-urethral glands) (Mann and Lutwak-Mann, 1981). The seminal plasma also contains lipids, proteins, enzymes, sugars and electrolytes that influence the metabolism, motility and surface properties of the spermatozoa (Fernandez-Juan *et al.*, 2006). Furthermore, the seminal plasma lipids particularly phospholipids and cholesterol have special reference in the structure and function of the plasma membrane of spermatozoa and might play significant roles in the structure, metabolism, capacitation and gamete fertilization (Hafez, 1987; Juyena and Stelleta, 2013). Juyena and Stelleta (2013) reported a PC concentration of 24.5 to 30.0 %, 21.7 to 34.1% and 15.2 to 22.0% of total phospholipids in bulls, buffalo and goat seminal plasma, respectively. However, there was no corresponding information reported on ram seminal plasma.

The spermatozoa are haploid cells produced in the seminiferous tubules of the testis through the process called spermatogenesis and are morphologically characterized by the head, midpiece and tail (Hafez and Hafez, 2000). The head consists of the nucleus and surrounded by an acrosome which contains enzymes required to penetrate an oocyte during fertilization. The acrosome covers the first two thirds of the sperm head, and forms a cap-like structure around it (Taloni *et al.,* 2017). The midpiece houses the mitochondria that provide energy for the tail required for the sperm motility (Trout, 2012). The spermatozoa are covered by a plasma membrane which acts as a physical barrier against external environment (Trout, 2012). Cholesterol and phospholipids are the integral components of the plasma membrane.

Plasma membrane of different mammalian species contains approximately 70% phospholipids, 25% neutral lipids and 5% glycolipids (Mann and Lutwak-Mann, 1981; Flesch and Gadella, 2000). Major phospholipids of the plasma membrane include PC, phosphatidylethanolamine and phosphatidylserine (Mann and Lutwak-Mann, 1981; Sitimurang, 2002). In addition, phosphatidylcholine is a major phospholipid of the plasma membranes (Gerv, 1993).

2.3 Ram semen characteristics and quality evaluations

Semen evaluations were initially used to predict the fertilizing ability of the spermatozoa and also to evaluate the potentiality of the male as a breeder (Juyena, 2011). However, recently they are utilized to evaluate the freezeability of the spermatozoa during cryopreservation. According to Patel and Siddiquee (2013), there is a correlation between semen characteristics and cryopreservation stages. Furthermore, the assessment of semen quality is also associated with the need for predicting fertility or to enable maximum number of offspring from a valuable sire (Amann and Hammerstedt, 1993). Semen characteristics are classified as macroscopic which can be evaluated physically and microscopic which can be evaluated conventionally or with specialized equipment (Boshoff, 2014). Furthermore, the use of only a single test is not reliable to evaluate the fertility of the semen (Salisbury *et al.,* 1978). Therefore, the use of multiple laboratory evaluations of semen quality would provide better prediction of the male fertilizing ability after cryopreservation.

2.3.1 Macroscopic semen characteristics

The macroscopic semen evaluations are predominantly used to determine the appearance of the sample especially the semen volume, concentration and pH. They are termed macroscopic, because evaluation can be carried out with the naked eye and without a microscope (Evans and Maxwell, 1987). Macroscopic semen characteristics from different breeds around the world were reported in the literature (Table 2.1). The ejaculate volume generally ranges between 0.5 and 2ml in mature rams and 0.5 and 0.7ml in young rams (Hafez and Hafez, 2010). Consequently, semen volume was reported to be as low as 0.59 and 0.61 ml in Arrabi and Karradi rams, respectively (Al-Samarrae, 2009). Table 2.1 is showing that ram semen volume ranges

between 0.59 and 3 ml as previously reported in the literature. These differences may be due to the effect of age, environment, health status, semen collection procedure, season of the year, frequency of collection, as well as breed and individual animal differences (Hafez and Hafez, 2008). The spermatozoa concentration generally refers to the number of sperm cells per milliliter of semen (Hafez, 1993). The sperm cell concentration of the ejaculate serves as one of the criteria in semen characteristics that qualify male fertility for breeding purposes (Graffer *et al.,* 1988). The concentration of semen is essential to determine how much to dilute the semen, to ensure adequate number of sperm cells in each insemination dose. The average spermatozoa concentration of ram ejaculates range from 1.21 $\times 10^9$ to 6.2 $\times 10^9$ sperm/ml (Table 2.1). In addition, the spermatozoa concentration is also correlated to semen volume (Marx *et al.,* 1975). The concentration of the spermatozoa can be measured using a hemocytometer or a spectrophotometer (Hafez and Hafez, 2008). The spectrophotometers that are calibrated with the hemocytometer are most preferred because they do not consume time (Evans and Maxwell, 1987; Hafez and Hafez, 2008; Boshoff, 2014). Semen pH plays a significant role in maintaining the viability and quality, ensuring successful fertilization by the spermatozoa (Zhou *et al*., 2015). Ejaculated ram semen samples usually have a pH either near neutrality or slightly alkaline (7.0-7.2) (Bar-Sagie *et al*., 1981). However, the occurrence of a slightly acidic pH was observed in ram and bull ejaculated semen (Juyena and Stelleta, 2013). According to Bar-Sagie *et al*. (1981) and Latif *et al*. (2005), an acidic pH environment affects both the sperm metabolism and motility, resulting in reduced fertility of the spermatozoa.

Table 2.1 Macroscopic characteristics of ram semen and collection methods from different breeds

Ram Breed	Method of collection	Volume (ml)	pH	Sperm concentration (x 10^9 cells/ml)	Reference
Ghezel-Merinose and Merino-Moghani	AV		6.8		Soltanpour and Moghaddam 2013
Dorper	AV	1.1	6.8	3.4	Malejane *et al.,* 2014
	EE	0.7	6.9	2.2	
Dallagh	EE	1.2		3.5	Hamedani *et al*., 2013
Nondescript indigenous	AV	1.4		4.6	Azizunnesa *et al*., 2014
Zel	EE	1.34		3.6	Hamedani *et al*., 2016
Karradi	EE	0.61	6.89	1.22	Al-Samarrae, 2009
Arrabi	EE	0.59	6.9	1.21	
Indigenous	AV	0.8		3.9	Mahmuda *et al*., 2015
Guirra	AV	1.2		6.2	Marco-Jimenez *et al.,* 2005
	EE	1.0		5.2	
NariSuvarna	AV	0.71	6.76	5.08	Tejaswi *et al.,* 2016
Awassi	EE	3.0	7.4	6.07	Nameer *et al* 2016
Moghani	AV	1.42		4.44	Abd-Benemar *et al*., 2015

AV: Artificial vagina, EE: Electro Ejaculation

2.3.2. Microscopic semen characteristics

2.3.2.1. Sperm motility

Generally, the sperm motility describe the proportion of the sperm cells that are moving and the proportion of sperm with productive movements such as rapid, linear and progressive motility. The sperm progressive motility is considered important for the spermatozoa to achieve fertilization (Perumal *et al*., 2014). The motility of the spermatozoa is affected by different factors such as the exposure to heat or cold, pH, type of extenders, urine in the semen sample and infertility (O'Hara *et al*., 2010). Sperm motility is an important characteristic of the spermatozoa that is associated with the fertilizing capacity of the semen (Verstegen *et al*., 2002). Therefore, the spermatozoa must run across the cervical mucus of female genital tract to approach the oviduct and fertilize the oocytes (Tsakmakidis, 2016). The evaluation of sperm motility was previously performed subjectively under phase contrast microscope and

the results were dependent on the experience of the technicians (Rodriguez-Martinez, 2003; Kumar *et al.*, 2016). Amann (1989) demonstrated that the evaluator's skills contribute about 30-60% variability when the sperm motility is evaluated subjectively using light microscopy. In addition, the evaluation of subjective sperm motility using light microscope lacks the ability to measure the kinematics of the spermatozoa (Tsakmakidis, 2016). In order to decrease this variation, computer-assisted semen analysis (CASA) instruments were developed with software to analyze and record every spermatozoa characteristic that improved the semen evaluation (Kumar *et al.*, 2016). A high accuracy and repeatability using the CASA systems have been previously reported (Davis *et al.*, 1992; Farrell *et al.*, 1995).

The CASA system provides objective and detailed information on various motility characteristics that cannot be evaluated subjectively under light microscopic semen analysis (Rijsselaere *et al.*, 2005). In brief, CASA system consists of a microscope to visualize the sample, a digital camera to capture images and a computer with specialized software to analyze the movement of the spermatozoa (Vincent *et al.*, 2012). The CASA individual motion parameters such as straight line velocity (VSL) and linearity (LIN), curvilinear velocity (VCL) and average path velocity (VAP) kinematic parameters were correlated to fertility in different species (Amann *et al.*, 2000; Olmo *et al.*, 2013; Tsakmakidis, 2016). The use of CASA system is associated with constrains that affects the results such as the type of specimen chamber used, temperature, concentration at which semen is analyzed, sampling conditions and the type of extender used (Vincent *et al.*, 2012). In addition, the components of semen extenders such as egg yolk and milk based extenders poses debris of similar size to sperm head, ultimately causing the CASA software to include them in the analysis (Vincent *et al.*, 2012).

2.3.2.2. Viability and morphology

One of the important semen qualities to evaluate is the spermatozoa viability, which is an indicative of the percentage live spermatozoa. Numerous techniques were applied to differentiate between viable and non-viable spermatozoa such as eosin-nigrosin (Hafez and Hafez, 2008). When eosin-nigrosin is combined with aliquots of

spermatozoa and smeared for evaluation, live spermatozoa will not absorb the stain, while dead ones will stain red against the dark nigrosin (Boshoff, 2014). Although the use of eosin-nigrosin stain is cheap, it is regarded as unreliable because sometimes spermatozoa show partial staining (Hancock, 1957). Eosin-nigrosin is also used to determine the structure or morphology of the spermatozoa especially the abnormalities. Although samples of the spermatozoa are expected to have abnormalities, they are also associated with infertility and sterility of males (Saacke, 2008). Spermatozoa abnormalities are characterized as primary, secondary and tertiary. Those with spermatozoa head and acrosome abnormalities are primary, while secondary abnormalities have a droplet on the midpiece of the tail and tertiary abnormalities have other tail defects such as coiling (Hafez and Hafez, 2008).

2.3.2.3. Membrane integrity

The physiological processes during fertilization such capacitation, acrosome reaction and fusion of spermatozoa and ovum requires an active spermatozoa membrane. Hence, there are difficulties for fertilization to occur with physically inactive membranes (Jeyendran *et al.*, 1984). Furthermore, the spermatozoa with an intact plasma membrane have the ability to undergo a series of complex changes in the female reproductive tract and acquire the ability to fertilize an oocyte (Yanagimachi, 1994). Plasma membrane functional activity is important for the viability and fertilizing ability of spermatozoa, which makes it very important to assess the structural and functional activity of the spermatozoa membrane (Hafez, 1993). Jeyendran *et al.* (1984) developed hypo-osmotic swelling test (HOST) to evaluate spermatozoa membrane functional integrity. The test is based on the semi-permeability of the intact cell membrane, which allows the spermatozoa to swell under hypo-osmotic conditions and indicates the functional integrity of the spermatozoa membrane (Mandal *et al.*, 2003). The exposure of spermatozoa with intact plasma membrane to HOST solution results in tail swelling, which is indicative of normal transportation of water across the spermatozoa membranes (Ahmadi and Soon-Chye, 1992). In addition, the cell size and shape of the spermatozoon changes and can be seen as swelling by a phase contrast microscope (Cabrita *et al.*, 1999). Other researchers reported a positive correlation between spermatozoa membrane integrity and motility characteristics (Neild *et al.*, 1999; Mandal *et al.*, 2003).

2.3.2.4 Acrosome Integrity

The acrosome is an acidic secretory organelle on the spermatozoa head filled with hydrolytic enzymes (Hafez and Hafez, 2000). An acrosome must remain normal to ensure the occurrence of acrosome reaction at a suitable time to facilitate fertilization (Partyka *et al.*, 2012). Furthermore, an acrosome must remain intact before and during the transit of the spermatozoa to the isthmus until the occurrence of zona binding and hence, an early acrosome reaction renders spermatozoa infertile (Juyena, 2011). The evaluation of the acrosomal status of a spermatozoa is an important factor to consider when evaluating semen quality, since some instances of male infertility could be the result of a lack of spermatozoa with functional intact acrosomes at the time of ejaculation (Odhiambo *et al.*, 2011).The acrosomal integrity of the spermatozoa can be evaluated using fluorescently labeled plant lectins, which are proteins that recognize and bind glucosidic residues in different parts of the acrosomal membrane (Graham, 2001). The commonly used components are the *Pivum sativum* agglutinin (PSA) and *Arachis hypogaea* (peanut) agglutinin (PNA), which are usually labeled with FITC fluorochromes, allowing them to be used by all cytometers. The PSA are derived from the pea plant and while the PNA are derived from the peanut plant and they are the most commonly used due to their specificity (Graham, 2001).

2.4 Factors affecting ram semen quality

The macroscopic and microscopic characteristics of ram semen are affected by different factors that have an impact on their reproductive potential and fertility. These include factors such as the nutritional status of the ram, age of the ram, season of collection and methods of semen collection.

2.4.1 Nutrition

Generally over-nutrition and under-nutrition have adverse effects on the reproductive capacity of rams. Several studies on nutrition in rams have demonstrated that diet may have an effect on testicular size and spermatozoa production (Brown, 1994). Kheradmand *et al.* (2006) studied the effect of improved dietary intake above maintenance requirements and found a significance increase in scrotal size and

spermatozoa concentration. However, semen volume and spermatozoa viability percentage were not significantly affected. It was also demonstrated that specific components of the diet, such as vitamin E have a positive effect of increasing semen quality and quantity (Yue *et al.*, 2010). This is supported by Cheah and Yang (2011) who reviewed the effect of essential nutritional elements such as zinc, selenium, and vtamins on spermatogenesis. These elements serve as antioxidants that protect spermatozoa from oxidative damage during the entire spermatozoa production (Hidiroglou and Knipfel., 1984; Beckett and Arthur., 2005). Mohamed and Abdelatif (2010) found a correlation between nutrition and thermal temperature on their effect on quality of ram spermatozoa.

2.4.2 Ram age

The age of a ram is an important factor that affects semen quality, as sexual maturity is a prerequisite for the production of viable spermatozoa (Boshoff, 2014). This is initiated by the gonadotrophin releasing hormone (GnRH) from the hypothalamus that triggers the increase of gonadotrophin hormones in order to initiate the process of spermatogenesis during puberty (Boshoff, 2014). According to Salhab *et al*. (2003), good quality semen can be collected from Awassi rams at the age of 11 months with both semen volume and spermatozoa concentration increasing with age. Hassan *et al*. (2009) also reported that the increase in age improves semen quality and remains constant between 3 to 4 years. The increase in age of the ram is associated with the body weight, body condition scores (BCS) and scrotal circumferences (SC) resulting in improved semen characteristics (Salhab *et al*., 2003; Hassan *et al*., 2009). These findings were supported by by Toe *et al.* (2000) who regarded scrotal circumference as an indicator of spermatozoa quality in rams.

2.4.3 Season of collection

Most species in temperate climatic region, including sheep, have an annual cycle of reproduction which can be divided into breeding and non-breeding seasons (Langford *et al.,* 1987). Generally, sheep are short-day breeders that breed mostly during days with decreasing length (Dorostghoal *et al*., 2009).The breeding season of a ram is

determined by the photoperiod which synchronizes their breeding activity (Moghaddam *et al.*, 2012). Furthermore, the breeding season of rams is determined by the seasonal changes when the days become shorter especially in autumn. Briefly, the light is transmitted through the eyes to the pineal gland which synthesizes and secretes melatonin in the blood plasma when there is no light, and stops secretion when light is present (Boshoff, 2014). The secretion of melatonin, determines the activity of the hypothalamic neurons which control luteinizing hormone (LH) and follicle stimulating hormone (FSH) resulting in the secretion of testosterone (Boshoff, 2014).

The changes in testicular volume, hormonal profiles, sexual behaviour and semen quality that affect the reproductive performance of rams have been reported (Casao *et al.*, 2010). Furthermore, semen production and freezeability are better in the autumn due to the seasonal fluctuation in seminal plasma protein components (Dacheux *et al.* 1981; D'Alessandro and Martemucci, 2003). Monthly variations in semen quality and quantity are due to differences in length of daylight throughout the year (Chemineau *et al.*, 1992). Hence, rams will most likely have the highest quality semen in autumn and summer than in winter and spring (Karagiannidis *et al.*, 2000). Tabbaa *et al.* (2006) found that good quality semen can be collected from Awassi rams throughout the year. However, Malejane *at al.* (2014) studied the seasonal variation in semen quality of Dorper rams using different semen collection methods, and reported high semen quality in summer, autumn and spring.

2.4.4 Method of semen collection

The quality of semen is affected by the collection methods, collection interval and semen handling. There are two commonly used methods for successful collection of ram semen, namely the artificial vagina (AV) and electro-ejaculation (EE) (Moore, 1985). The AV method produces thermal and mechanical stimulations that induce ejaculation, making it ethically acceptable to collect ram semen compared to EE (Munyai, 2012). The AV method is a simple semen collection method, which mimic natural mating and ejaculation (Salisbury *et al.*, 1978). However, AV semen collection requires training of ram initially in the presence of ewes on heat (Tiwary, 1990). Furthermore, rams with good libido and also the skill and experience of the collector are the prerequisite for the AV method (Gordon, 2007). The EE method is performed

by inserting a bipolar electrical probe into the rectum, with low electrical voltage used to stimulate ejaculation within few seconds (Boshoff, 2014). The EE semen collection method is regarded as stressful to the rams, rendering the method to be ethically unacceptable (Hafez and Hafez, 2008).

The use of AV collection method is associated with uncontaminated semen samples compared to EE that sometimes produces semen contaminated with urine (Watson, 1990). Both semen collection methods may vary in terms of semen quality and hence, different breeds of rams exhibited differences in terms of quality versus the collection method (Table 2.1). However, the total spermatozoa and fertility levels were found to be comparable between the two collection methods (Bearden and Fuquay, 1980). In addition, the spermatozoa collected with an AV were more resistant to cold shock compared to when collected with the EE (Quinn *et al*., 1968). Furthermore, semen should be collected at regular intervals to ensure the recovery of good quality semen. The collection of semen at regular intervals would ensure the spermatozoa maturity during spermatogenesis (Mahmuda, 2014). Jennings and Mcweeney (1976) reported that the semen volume, spermatozoa concentration and motility characteristics were reduced following consecutive ejaculates in rams. Ari *et al*. (2011) found improved freeze-ability with higher motility, viability and membrane integrity, and lower abnormality and abnormal acrosome percentages from semen collected at one day interval compared with at four days interval in Tushin ram. Furthermore, the proper handling and processing of semen during and after collection are crucial to ensure the survival of spermatozoa during cryopreservation. Semen should not be exposed to light, direct sun shine, fumes of volatile chemicals or disinfectants as these would affect their quality (Hunter, 1985). Light can affect fertilizing capacity of spermatozoa by photo oxidation. Lastly, both semen and extender needs to be maintained at the same temperature during dilution and usually at 30 ºC (Foote, 1974).

2.5 Ram semen cryopreservation

Cryopreservation is a procedure that stabilizes cells at cryogenic temperature (-196 ºC), with the main aim of restraining the movement and metabolic activities of the spermatozoa (Bashawat *et al*., 2016). Currently, semen cryopreservation is a very useful tool in establishment of genetic resource banks for endangered species (Day

and Stacey, 2007). Also, it has a potential to be applied in assisted reproduction technologies such as AI and IVF, which contributes to increased production and genetic selection (Ahmed *et al*., 2016). Although semen cryopreservation has numerous advantages, it is also associated with drawbacks such as partial irreversible damage to the sperm cells (Amann and Pickett, 1987; Purdy, 2006). There are numerous factors that causes spermatozoa injuries during cryopreservation such as cold shock, oxidative stress, spermatozoa membrane modification, cryoprotectant toxicity, intracellular ice crystal formation, and fluctuations in osmotic pressure (Watson, 1995; Isachenko, 2003). The sperm cell injuries causes a decline in motility, leading to poor fertility following AI (Matsuoka *et al*., 2006; Rodriguez-Martinez and Barth, 2007). According to Watson (2000), cryopreservation damages about 40-50 % of the spermatozoa. This is caused by the exposure of the spermatozoa to different stages such as dilution, incubation, cooling, freezing or thawing, resulting in ultrastructural, biochemical and functional alterations of spermatozoa during cryopreservation (Holt, 2000).

The plasma membrane of the spermatozoa is the primary site of cryoinjuries due to membrane alterations induced by phase transitions, occurring when membranes undergo cooling, mechanical stress caused by osmotic stress and temperature changes caused by freezing and thawing and intracellular ice crystallization during cryopreservation (Watson, 1995; Salamon and Maxwell, 2000; Ropke *et al*., 2011). Furthermore, the spermatozoa membrane cryoinjuries depend on the species due to several elements such as cholesterol/phospholipids ratio, lipid bilayers content, hydrocarbon saturation degree and protein/phospholipid ratio (Juyena and Stelleta, 2013). Ram spermatozoa are more sensitive to cold shock than other species (Ollero *et al*., 1998). The spermatozoa membrane of rams are characterised by high ratio of unsaturated to saturated fatty acids and low phospholipid/cholesterol ratio which makes them to be susceptible to cold shock (Darin-Bennett and White, 1977). According to Joshi *et al*. (2006), previous research focused on ram semen extenders, semen processing, freezing and thawing methods to improve the quality of frozen thawed spermatozoa. The methods of freezing and thawing contribute significantly to the most damages during cryopreservation (Salamon and Maxwell, 2000). Therefore, the use of suitable extenders and cryoprotectant additives has a potential to prevent damages caused during cryopreservation (Emamverdi *et al*., 2013).

2.6 Characteristics of ram semen extenders

Semen freezing extenders are used to dilute semen for preservation and also to create sufficient semen for multiple insemination doses from a single ejaculate (Kuster and Althouse, 1999). The composition of ram semen freezing extenders should have similar properties to extenders for raw use of semen (Soltanpour and Moghaddam, 2013). Semen extenders are characterised by the ability to supply the spermatozoa with energy, buffering against pH changes and protection from cryoinjuries (Juyena, 2011). The composition of extenders has major effects on the freezeability and fertilizing ability of spermatozoa during cryopreservation (Salamon and Maxwell, 1995). Tris-based extenders are mostly used in ram semen cryopreservation, with fructose or glucose as source of energy, antibiotics to combat microbial infections as well as lipids and cryoprotectants for protection of spermatozoa against freezing (Purdy, 2006; Juyena, 2011). The cryoprotectants used for freezing provides the spermatozoa with protection against cold shock and other damages during freezing and glycerol is the extensively used cryoprotectant (Salamon and Maxwell, 2000; Purdy, 2006). Glycerol is an intracellular cryoprotectant that is characterized by a low molecular weight, and induces membrane lipid and protein rearrangement, resulting in increased membrane fluidity, greater dehydration at lower temperatures, reduced intracellular ice formation, and an increased survival rate of spermatozoa following cryopreservation (Holt, 2000). Moreover, glycerol has the ability to dissolve sugars and salts in semen freezing extenders (Purdy, 2006). Glycerol is successfully added to the semen in a separate diluent fraction (two-step dilution) and it is predominantly added in the second fraction extenders (Salamon and Maxwell, 1995). Consequently, five to eight percentages of glycerol in extenders has shown to yield better spermatozoa motility during cryopreservation of ram semen (First *et al*., 1961; Ali *et al*., 1994). In addition, lipid sources such as EY are commonly used for protection of spermatozoa membrane during temperature changes (Salamon and Maxwell, 2000; Bergeron and Manjunath, 2006).

2.7 Characteristics of egg yolk on semen cryopreservation

Cryopreservation of semen has extensively relied on EY as cryoprotective component of ram semen freezing extenders (Salamon and Maxwell, 2000; Kulaksiz *et al*., 2010). Egg yolk as a component of semen freezing extenders was first recommended by Phillips and Lardy (1940). Although EY has disadvantages such as variability in composition and risk of microbial contaminations, it provides good protection to the spermatozoa against cold shock and also during freezing and thawing (Salamon and Maxwell, 2000; Emamverdi *et al*., 2013). The phospholipids, cholesterol and low density lipoprotein of the EY are the components that protect the spermatozoa membrane integrity and also prevent cold shock during cooling and freezing (Kulaksiz *et al*., 2010). The mechanisms of EY on the protection of spermatozoa from cryoinjury were reviewed by Bergeron and Manjunath (2006). The phospholipids of the EY protect the spermatozoa by forming a protective film on the spermatozoa surface or by replacing spermatozoa membrane phospholipids that are lost or damaged during the cryopreservation process (Quinn *et al*., 1980; Bergeron and Manjunath, 2006). Furthermore, it was reported that when EY phospholipids are included in extenders interact with the spermatozoa membrane and provide protection to the spermatozoa similar to extenders with EY (Quinn *et al*., 1980).

The hen (*Gallus gallus domesticus*) EY has been extensively used in freezing extenders due to its reliability and availability (Bathgate *et al*., 2006). Due to variations in the biochemical components of the EY, there were attempts to replace hen EY with that of other avian species (Trimeche *et al*., 1997; Andrabi *et al*., 2008). However, hen EY proved its competence as a protective component of ram semen during cryopreservation (Moreno *et al*., 2008; Kulaksiz *et al*., 2010; Gholami *et al*., 2012). Biochemically, EY contains 31.8-35.5% of lipids with phospholipids having 30% of total lipids and 66-76% PC (Wang, 2007). In addition, hen EY was reported to have a lower concentration of PC compared to yolks from other avian species (Trimeche *et al*., 1997). Currently, there are uncertainties with regards to the correct EY concentration to use in ram semen freezing extenders. Previously, Evans and Setchell (1978) regarded 20% as a standard EY concentration while other researchers used as much as 30-50% (Salamon and Maxwell, 2000). It was shown that the increase in EY concentration reduces the quality of cryopreserved semen. According to Amirat *et al*. (2004), this is attributed to the granules of the EY which prevents the metabolic

exchange of the spermatozoa, reduces their motility and inhibit their respiration. Furthermore, the fat globules of the EY also make sperm evaluation a challenge (Singh *et al.*, 2012). Consequently, Graham *et al.* (1978) reported that low concentrations (1.5-3.75%) of EY were ineffective on semen cryopreservation. Currently, researchers use EY concentrations with a range of 5-20% (Moreno *et al.*, 2008; Kulaksiz *et al.*, 2010; Gholami *et al.*, 2012; Emamverdi *et al.*, 2013; Alcay *et al.*, 2015). Therefore, these uncertainties elucidate the need for optimal EY concentration for cryopreservation of Bapedi ram semen to be determined.

2.8 Effect of phosphatidylcholine on semen cryopreservation

Cryopreservation of semen has a detrimental effect on the composition and organization of the spermatozoa plasma membrane (Hinkovska-Galcheva *et al.*, 1989). This results in a substantial loss of total phospholipids and membrane disruption during cold shock and freezing (Sitimurang, 2002). The major phospholipids of the spermatozoa were reported to be PC, phosphatidylethanolamine and phosphatidylserine (Sitimurang, 2002). Quinn *et al.* (1980) first studied the effect of PC on ram semen cryopreservation in extenders without EY and discovered that PC forms a protective film at the surface of the spermatozoa membrane. In addition, Graham and Foote (1987) reported that phospholipids are able to merge with spermatozoa membrane and replace lost phospholipids. By definition, phosphatidylcholines are waxy solids that form colloidal suspensions in water and are prevalent in cell membranes (Stoker, 2016). The PC of the EY is biochemically made up of the saturated fatty acids, namely palmitic and stearic (41-46%), oleic acid (35-38%), linoleic acid (15-18%) and alpha-linoleic (0-1%) (Muss, 2011). Furthermore, PC also proved to be the major protective component of the spermatozoa during cryopreservation of ram semen when other sources of PC such soybean lecithin were compared with EY (Emamvardi *et al.*, 2013; Khalifa and Abdel-Hafez, 2014). According to Voet and Voet (1995) lecithin (a form of PC) is distributed widely in plants and it plays an important role in the regulation of the physiological function of animal cells bio-membrane. Currently, PC are considered as liposomes because they are converted to liposomes prior to being used in semen cryopreservation (Ropke *et al.*, 2011). The introduction and supplementation of PC in extenders as liposomes

exhibited the potential to enhance the success of semen cryopreservation in different species such as boar (He *et al*., 2001), bulls (Ropke *et al*., 2011), stallion (Pillet *et al*., 2012), buffalo (Kumar *et al*., 2015) and Canine (Belala *et al*., 2016). Liposomes are characterized as vesicular structures composed of phospholipids that enclose aqueous solutions and are built from concentrically arranged lipid bilayers similar to the arrangement of biological membranes (Grad, 2010). The effect of liposomes in cryopreservation of ram semen was previously studied (Quinn *et al*., 1980, Simpson *et al*., 1986; Ollero *et al*. 1998. However, the current information on ram semen cryopreservation using PC liposomes in extenders is still questionable. In addition, previous studies did not prove the effectiveness of supplementing PC liposomes in EY extenders for cryopreservation of ram semen. Therefore, the supplementation of PC liposomes in extenders with reduced concentration EY would be advantageous, considering the latest reports of sanitary issues possessed by EY as a component of extenders. According to Kumar *et al*. (2015), lipids used for preparation of liposomes are not vectors of any infective agent compared to EY and therefore, they can comply with the biosafety requirements.

CHAPTER THREE

MATERIALS AND METHODS

All the procedures performed during the study were approved by both the University of Limpopo Research and Ethics Committee and Agricultural Research Council Animal Ethics Committee (APIEC15/048).

3.1 Study location

The study was conducted at the Germplasm Conservation and Reproductive Biotechnologies Laboratory of the Agricultural Research Council (Irene) (25º 55' S; 28º 12' E) in Pretoria, South Africa. The area is situated on the Highveld at an altitude of 1525 m above sea level. The weather conditions range from hot days and cool nights in summer (17.5 ºC to 32 ºC) to moderate winter days with very cold nights (1 ºC to 17 ºC) (Webb *et al.*, 2004).

3.2. Experimental animals and semen collection

Due to the scarcity of the Bapedi sheep, four healthy and sexually matured Bapedi rams of good body conformation, well developed testes and good vigor within the age group of 2 to 4 years were selected from eight rams. The rams that had good libido and adaptability to artificial vagina semen collection technique were selected. The rams were kept in a separate camp and maintained with *Panicum maximum* hay, supplemented with concentrate and water provided *ad libitum*. The initial average body weight and scrotal circumference of the rams were 54.5±2.25 kg and 31.8±0.85 cm, respectively. Sixty four (n=64) ejaculates were collected twice per week during breeding season from April to May 2016. The experimental rams were trained to mount ewes on estrus as teasers for collection of semen by artificial vagina (AV) in the early morning at 7.30 to 9.00 A.M.

3.2.1 Semen collection

Semen was collected using AV methods adopted from Mahmuda (2014) and Bopape (2015). All apparatus used for semen collection were sterilized a day before collection using an autoclave machine. The rams were prepared before collection by wiping their prepuce with 70% alcohol to avoid semen contamination. The rams were allowed to have at least 1-2 false mounts before collection of each ejaculate. The AV was also prepared before semen collection by placing the inner rubber sleeve into the outer hard cylinder of the AV and both ends of inner sleeve were deflected over the cylinder to form a water tight space. The space between the sleeve and cylinder of AV was

filled with distilled water at a temperature of 42- 45°C and closed with a rubber stopper. The open side of AV was lubricated with a small amount of gel and a graduated semen collection tube was connected to the other open side of the AV. During collection, the AV was held with the right hand along the ram's flank. The open end was facing towards the penis and downwards at an angle of 45°. During mounting, the erected penis of ram was directed into the open end of the AV to permit a vigorous upward and forward thrust which signifies the occurrence of ejaculation. The ram was allowed to withdraw its penis immediately after ejaculation in the AV. Semen volume was measured by reading the measurements on the graduated semen collecting tube and the semen samples were transferred to a 15 ml graduated Falcon tube, labelled and then placed in a thermoflask at 37 °C.

3.3 Preparation of extenders

All chemicals were purchased from Sigma-Aldrich® (USA), unless otherwise stated. The Tris-based extenders were prepared according to the ingredients of Mahmuda *et al*. (2015) on Table 3.1 below. A day before semen collection, raw hen EY was used to prepare 100ml Tris-based extenders. The extenders were separated into two fractions, namely fraction A and fraction B for each treatment, which were differentiated by the presence or absence of glycerol. Fraction A contained EY without glycerol, whilst fraction B had EY and 7% glycerol (Munyai, 2012). In fraction A, distilled water was added instead of glycerol. Also, fraction A without EY was used as Tris-buffer for dilutions and analysis throughout the study.

3.4 Experimental design

Experiment one was performed to characterise Bapedi ram semen parameters immediately after semen collection. The macroscopic semen parameters such as volume, pH and concentration were evaluated. The microscopic semen parameters such as motility, viability and morphology, membrane integrity and acrosome integrity were evaluated. The experiment was replicated 8 times and the data was subjected to descriptive statistics.

Experiment two evaluated the effect of Tris-based extenders with five different EY concentrations (0, 5, 10, 15 and 20 %) on the microscopic quality of cryopreserved Bapedi ram semen. The EY concentrations were prepared according to the formula: EY Concentration= (Final volume of extender) X (%Concentration). The treatments

were subjected to a Completely Randomized Design (CRD) and the experiment was replicated 4 times.

Experiment three evaluated the effects of different PC concentrations supplemented to Tris-based extenders with or without 10% EY. Commercial PC (Sigma-Aldrich, P3556) derived from EY were added to Tris-based extenders as liposomes, which were prepared according to the protocol below. The experiment was a 2 x 4 factorial design in a CRD with two concentrations of EY: 0 and 10, and four concentrations of PC: 0, 0.25, 0.50, 0.75 mg/ml in Tris-based extender. The experiment had 8 treatments (table 3.2 below) and it was replicated 4 times.

3.4.1 Preparation of liposomes

The liposomes were prepared with modifications according to the procedures of Ropke *et al*. (2011) and Long and Conn (2012). Egg yolk PC in lyophilized form was dissolved and mixed in chloroform to ensure a clear homogeneous mixture of lipids. The PC and chloroform were dried together in a pear-shaped flask on a rotary evaporator for 30 minutes, followed by 30 minutes under airflow hood. The dried lipid films were rehydrated in Tris-buffer for 30 minutes. The suspension was vortexed for 10 minutes to form multilamellar vesicles (MLV). The resulting solution was frozen and thawed using liquid nitrogen(LN) (-196 °C) and hot water (60 °C) five times to yield highly homogenous MLV suspension. The MLV suspensions were passed 20 times through a 100 nm polycarbonate pore size filters using a mini-extruder to form small unilamellar vesicles (SUV). The liposomes were stored under liquid nitrogen until used.

Table 3.1 The composition of Tris-based extenders

Ingredients	Fraction A	Fraction B
Tris (Hydroxyethyl) amino methane (g)	3.63	3.63
Citric acid (g)	1.99	1.99
Fructose (g)	0.5	0.5
Gentamycin sulphate (g)	0.05	0.05
Glycerol (ml)	0	7
Egg Yolk (ml)	0,5,10,15,20	0,5,10,15,20
Phosphatidylcholine (mg/ml)	0, 0.25, 0.50, 0.75	0, 0.25, 0.50, 0.75
Distilled water (up to 100 ml)	100	100

Table 3.2 Description of experimental treatments and codes

Code	**Description**
E_0P_0	Tris-based extender without EY and PC
E_0P_1	Tris-based extender without EY supplemented with 0.25 mg/ml PC
E_0P_2	Tris-based extender without EY supplemented with 0.50 mg/ml PC
E_0P_3	Tris-based extender without EY supplemented with 0.75 mg/ml PC
E_1P_0	Tris-based extender with EY without PC
E_1P_1	Tris-based extender with EY supplemented with 0.25 mg/ml PC
E_1P_2	Tris-based extender with EY supplemented with 0.50 mg/ml PC
E_1P_3	Tris-based extender with EY supplemented with 0.75 mg/ml PC

Egg Yolk (EY); Phosphatidylcholine (PC)

3.5 Semen dilution, freezing and thawing

Semen processing for cryopreservation was carried out with minor modifications according to Munyai (2012). Only samples containing more than 75% motile spermatozoa were used for further processing and treatment, otherwise the rest were discarded. Semen samples from 4 rams were pooled in order to obtain sufficient semen for replication and also to eliminate individual variations. Pooled semen samples were divided into 5 and 8 aliquots to comply with objective 2 and objective 3, respectively. Semen aliquots were diluted with fraction A extender (37°C) at a ratio (1:2) and transferred to a refrigerator at 5°C together with fraction B. After 2 hours, fraction B was slowly added into semen previously extended with fraction A at same ratio (1:2). The extended semen samples were loaded into 0.25 ml plastic straws and sealed with dry polyvinyl powder. The sealed straws were equilibrated in a refrigerator at 5°C for another 2 hours and the straws were frozen in a programmable freezer using the rates: 3 ºC/min from 5 to -8 ºC and at 15 ºC/min from -8 to -120 ºC (Alcay *et al.*, 2015). The frozen semen straws were plunged into liquid nitrogen tank (-196°C) and stored for a month before thawing and evaluation.

The frozen semen straws were thawed by dipping in water bath at 37°C for 40 seconds before evaluations (Munyai, 2012). Briefly, the straws were thawed individually by letting the straw directly from the liquid nitrogen on air for 10 seconds and then dipped into prepared water bath at 37°C for 40 seconds. Each straw was dried with a paper towel, cut open with clean scissors, and the contents of the straw emptied into a 1.5 ml Eppendorf tube. The semen in the tubes were allowed to stabilize for a minute at 37°C before evaluated for motility, viability and morphology, membrane integrity and acrosome integrity (Boshoff, 2014).

3.6 Semen evaluation

All microscopic semen characteristics were evaluated after semen collection and after thawing. All semen samples were frozen by the same person, and each of the studied semen parameters were measured by the same person on each occasion throughout the study.

3.6.1 Macroscopic semen evaluation

The semen volume was measured by recording the value on graduate semen collecting tube before being transferred into the 15 ml graduated Falcon tubes. Semen pH was measured using a litmus paper due to low semen volume. Briefly, a litmus paper was dipped inside a 15 ml graduated tube and the numbers matching colours were recorded as the pH value. The spermatozoa concentrations were determined using a spectrophotometer (Jenway 6310, Bibby Scientific, England) and calculated according to the formula: Spermatozoa concentration (x 10^9/ml) = (Dilution factor) x [25.97x (Absorbance) – 0.30] for ram semen. A total amount of 1.5 ml (2.9% of Sodium citrate) was placed in a microcuvette and calibrated using a spectrophotometer for 30 seconds. After calibration, sodium citrate solution was removed from the spectrophotometer. Thereafter, 7.5 µl of raw ram semen was pipetted into a microcuvette with sodium citrate and returned in the spectrophotometer. The absorbance reading was recorded within 30 seconds and it was substituted on the above formula to determine the spermatozoa concentration for all the samples.

3.6.2 Microscopic semen evaluation

3.6.2.1 Sperm motility

The sperm motility characteristics were evaluated using Computer Assisted Sperm Analysis (CASA) system of Sperm Class Analyser®-SCA® (V.5.3 Animal/Veterinary Microptic S.L, Barcelona, Spain) (see table 3.3 for settings). Briefly, 5µl of semen was diluted with 100 µl of Tris-buffer in a microcuvette at 37°C to prepare swim-up and 5 µl of this diluted semen was pipetted onto microscope glass slide, gently covered with a microscope cover slip and evaluated under X10 magnification with the SCA® microscope projecting an image on a monitor. The results were saved on a Microsoft excel sheet. The motility parameters evaluated were expressed as the percentage total motility (TM), progressively motility (PM) and rapid motility (RM). The kinetic parameters evaluated were classified as curvilinear (VCL), straight-line (VSL), average path (VAP), beat cross frequency (BCF), amplitude of lateral (ALH), linearity (LIN), straightness (STR) and wobble (WOB) (see table 3.4 for description).

Table 3.3 Sperm Class Analyser® V.5.3.Settings used to analyse ram spermatozoa motility and velocity characteristics

Parameter	Setting
Brightness	166
Chamber	Cover slide
Circular	50% of Linearity
Connectivity	12
Contrast	450
Optics	Ph-
Number of images	50
Images per second	50
Particle area	15 – 70 μm2
Progressivity	80% of STR
Scale	10X
Slow	VAP of 0 - 30 μm/s
Medium	VAP of 30 - 80 μm/s
Rapid	VAP of 80 μm/s and above
Velocity on the average path points	7

Table 3.4 The definition and descriptions of spermatozoa motility descriptors for CASA system

Descriptors	Abbreviation	Unit	Description
Curvilinear velocity	VCL	μm/s	The sum of the incremental distance moved in each frame along the sampled path divided by the total time of the track.
Average path velocity	VAP	μm/s	A derived path based on an average number of points and divided by the time of track.
Straight line velocity	VSL	μm/s	The straight line distance between the start and end points of the track divided by the time of track.
Beat cross frequency	BCF	Hz	Cycles per second of the spermatozoa head across the mean path.
Amplitude of lateral	ALH	μm	The average deviation from the smoothed path, based on difference in linearity between the smooth and real paths
Linearity of track	LIN	%	The ratio of net distance moved to total path distance (VSL/VCL×100)
Straightness of track	STR	%	The ratio of net distance moved to total path distance (VSL/VAP×100)
Wobble	WOB	%	VAP/VCL×100

Source: Kime *et al.* (1996)

3.6.2.2 Viability and morphology

The percentage of live spermatozoa and abnormalities were evaluated using eosin-nigrosin staining according to the protocol of Munyai (2012). Briefly, 5 µl of semen was diluted in 20 µl of eosin-nigrosin staining and 7 µl of the dilution was pipetted and smeared on a glass slide and evaluated within an hour of drying at room temperature. The slides were placed on a microscope table and a drop of immersion oil was placed on the slide to evaluate live, dead and abnormal spermatozoa using a fluorescent microscope (Olympus Corporation BX 51FT, Tokyo, Japan) under 100x magnification. A total of 200 spermatozoa per slide were evaluated and counted for each sample using a laboratory counter and the total spermatozoa abnormalities were recorded. Under the microscope, the live spermatozoa fluoresced and dead spermatozoa were brown or dark in colour showing that they absorbed the eosin-nigrosin stain (figure 4.2). All abnormalities such as proximal, distal, bent or coiled tails were recorded under one category.

3.6.2.3 Membrane integrity

The spermatozoa membrane integrity was evaluated using hypo-osmotic swelling test (HOST) solution prepared using 9 g fructose and 4.9 g sodium citrate per liter of distilled water. Briefly, 10 µl of semen was diluted with 100 µl HOST solution and incubated at 37°C for 60 minutes. After incubation, 20 µl of diluted semen was spread on a warm slide (37°C) and covered with a cover slip. For evaluation, 200 spermatozoa were counted under 1000x magnification using phase-contrast microscope and spermatozoa with swollen or coiled tails were recorded as intact and those with curled tails as damaged (figure 4.2).

3.6.2.4 Acrosome integrity

The spermatozoa acrosome integrity was evaluated using *Pisum sativum* agglutinin fluorescein conjugate (PSA-FITC) according to the protocols of Alcay *et al*. (2015). Five micro liters (µl) of ram semen were diluted with 200 µl of Tris-buffer and 20 µl of diluted semen was re-suspended in 500 µl of phosphate buffered saline (PBS) and then centrifuged at 2000 rpm for 20 minutes. The supernatant was discarded and the spermatozoa pellet was re-suspended in 250 µl PBS solution. One drop of re-

suspended spermatozoa was smeared on a glass microscope slide and air dried. Air dried slides were fixed with acetone at 4ºC for 10 minutes and covered with PSA-FITC solution (50 µg/ml in PBS solution) in dark room for 30 minutes. The slides were rinsed with PBS solution, covered with immersion oil and 200 spermatozoa were evaluated for intact and damaged acrosome under a fluorescence microscope (figure 4.3).

3.7 Data analysis

All data were analyzed using version 9.4 of Statistical Analysis System (SAS, 2013). Data on semen characterization was analyzed as descriptive statistics and expressed as mean, standard error of the mean and range (minimum and maximum). The effect of different EY concentrations on Bapedi ram semen cryopreservation were analyzed using one-way analysis of variance (ANOVA) for motility, viability and morphology, membrane integrity and acrosome integrity. The supplemental effect of phosphatidylcholine was analyzed using ANOVA in General Linear Model (GLM) procedure, also for motility, viability, membrane integrity and acrosome integrity. Duncan's Multiple Range Test was used to compare means for significant differences ($p<0.05$).

CHAPTER FOUR

RESULTS

The raw semen macroscopic characteristics of Bapedi rams are outlined in Table 4.1. The volume of Bapedi ram semen recorded ranged between 0.4 to 1.5 ml, while the concentration of sperm cells in semen varied widely from 0.52 to 8.84 billion sperm cells per ml, with the pH ranging from 5 to 7. Tables 4.2 and 4.3 show the microscopic semen parameters of Bapedi rams. Figure 4.1, 4.2 and 4.3 represent the sperm viability and morphology, membrane integrity and acrosome integrity of raw semen. The average TM, PM and RM characteristics determined by CASA were 85.95±2.58 %, 29.33±2.11 % and 39.47±4.99 %, respectively. Furthermore, the average means for kinetic parameters were 102.34±5.75 µm/s, 61.39±4.31 µm/s, 80.89±5.12 µm/s, 60.14±2.25 %, 75.76±1.53 %, 78.66±1.65 %, 3.21±0.15 µm and 8.0±0.20 Hz for VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF, respectively. The results further showed mean percentages of live spermatozoa, abnormalities, intact membrane and intact acrosome for 70.19±2.29 %, 2.50±1.34 %, 72.39±1.71 % and 75.37±5.39 %, respectively.

Table 4.4 depicts the Mean±SEM of raw and frozen-thawed CASA motility characteristics of Bapedi ram semen extended with different concentrations of EY in Tris-based extenders. There was a general decrease of frozen-thawed semen parameters evaluated by CASA on motility rates such as TM, PM, RM, VCL, VSL and VAP as compared to raw semen ($p<0.05$). The frozen-thawed semen in Tris-based extenders with 10, 15 and 20% EY concentrations resulted in significantly higher ($p<0.05$) TM, PM, RM, VCL, VSL and VAP motility rate compared to 0 and 5%. Frozen-thawed semen extended with different concentrations of EY did not differ significantly ($p>0.05$) in comparison with raw semen on LIN, STR, WOB, ALH and BCF motility rate. Cryopreservation was detrimental to the percentage of live spermatozoa, membrane and acrosome integrities (Table 4.5). The percentage of live spermatozoa, membrane and acrosome integrities were found higher in raw semen than in frozen–thawed semen of respective extenders ($p<0.05$). The treatments with 0% EY concentration resulted in significantly ($p<0.05$) lower percentage of live spermatozoa, membrane and acrosome integrities compared to 5, 10, 15 and 20% following cryopreservation. Interestingly, frozen-thawed semen extenders with 5, 10, 15, 20% EY concentrations were not significantly different ($p>0.05$) with regards to the percentage of live spermatozoa, membrane and acrosome integrities. The

spermatozoa abnormalities of raw and frozen-thawed semen were not significantly different ($p>0.05$) irrespective of the EY concentration used in extenders.

The effects of PC supplementation in Tris-based extenders with or without EY on cryopreserved Bapedi ram semen are presented in Tables 4.6 and 4.7. A decline in CASA motility parameters such as TM, PM, RM, VCL, VSL and VAP was observed in frozen-thawed semen compared to raw semen ($p<0.05$). The TM, PM, RM, VCL, VSL and VAP parameters in frozen-thawed semen treatments were significantly different ($p<0.05$). However, the extenders with EY were significantly higher ($p<0.05$) than those without EY on the TM, PM, RM, VCL, VSL and VAP parameters. The supplementation of PC in extenders either with or without EY did not improve the TM, PM, RM, VCL, VSL and VAP parameters ($p>0.05$). The STR and BCF motility parameters were not affected during freezing and thawing ($p>0.05$). The LIN, WOB and ALH motility parameters were significantly different ($p<0.05$) on the treatment extenders and raw semen. In the present study, freezing and thawing reduced the percentage of live spermatozoa, membrane and acrosome integrities ($p<0.05$). The frozen-thawed treatments did not differ significantly ($p>0.05$) on the percentage of live spermatozoa. A significant difference ($p<0.05$) was observed in extenders with EY compared to those without EY on the membrane integrity. The membrane integrity in extenders either with or without EY were not influenced by the supplementation of PC after freezing and thawing ($p>0.05$). The supplementation of PC in treatments with EY did not improve the acrosome integrity ($p>0.05$). Interestingly, the supplementation of 0.75 mg/ml PC resulted in acrosome integrity that was not significantly different ($P>0.05$) to treatments with EY.

Table 4.1 Macroscopic characteristics of Bapedi ram raw semen

Characteristics	Mean±SEM	Range	
		Minimum	Maximum
Volume (ml)	0.62±0.03	0.4	1.1
pH	6.0±0.36	5.0	7.0
Concentration [x 10^9 cells/ml]	3.62±2.39	0.52	8.84

SEM: Standard error of the mean

Table 4.2 Sperm motility characteristics of Bapedi ram raw semen evaluated by CASA

Characteristics	Mean±SEM	Range	
		Minimum	Maximum
TM (%)	85.95±2.58	57.42	99.93
PM (%)	29.33±2.11	11.70	52.55
RM (%)	39.47±4.99	5.46	81.60
VCL (µm/s)	102.34±5.75	43.42	148.46
VSL (µm/s)	61.39±4.31	25.67	114.81
VAP (µm/s)	80.89±5.12	32.76	134.70
LIN (%)	60.14±2.25	32.70	82.03
STR (%)	75.76±1.53	55.68	89.16
WOB (%)	78.66±1.65	58.73	92.0
ALH (µm)	3.21±0.15	1.82	5.15
BCF (Hz)	8.0±0.20	5.85	10.63

Total motility (TM), progressively motility (PM) and rapid motility (RM), curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), Beat cross frequency (BCF), Amplitude of lateral (ALH), linearity (LIN), straightness (STR) and wobble (WOB).

SEM: Standard error of the mean

Table 4.3 Viability, morphology, membrane integrity and acrosome integrity characteristics of Bapedi ram raw semen

Characteristic	Mean±SEM	Range	
		Minimum	Maximum
LIVE (%)	70.19±2.29	46.50	93.27
DEAD (%)	27.22±2.13	5.74	47.5
ABN (%)	2.50±1.34	0	29.5
IM (%)	72.39±1.71	55.5	85.0
DM (%)	27.6±1.71	15.0	43.0
IA (%)	75.37±5.39	18.91	95.0
DA (%)	24.63±5.39	5.0	81.09

Abnormalities (ABN); intact membrane (IM); damaged membrane (DM); intact acrosome (IA); damaged acrosome (DA)

SEM: Standard error of the mean

Table 4.4 Mean±SEM of CASA motility characteristics of raw and frozen-thawed Bapedi ram semen extended with different concentrations of egg yolk in Tris-based extenders.

Characteristic	Raw semen	Egg yolk concentration (%)				
		0	5	10	15	20
TM (%)	91.97±2.61^{a}	21.55±6.32^{d}	56.52±5.47^{c}	82.05±5.47^{b}	65.77±5.47^{bc}	73.51±5.47^{bc}
PM (%)	27.47±2.87^{a}	1.19±1.30^{c}	10.78±2.86^{b}	15.97±2.86^{b}	14.81±2.86^{b}	11.58±2.86^{b}
RM (%)	42.87±7.38^{a}	0.13±3.52^{c}	5.88±3.05^{bc}	12.6±3.058^{b}	6.96±3.05^{bc}	7.19±3.05^{bc}
VCL (μm/s)	105.75±9.41^{a}	31.15±7.22^{c}	46.77±6.25^{bc}	59.94±6.25^{b}	50.67±6.25^{bc}	45.89±6.25^{bc}
VSL (μm/s)	56.78±6.69^{a}	14.53±5.14^{c}	26.41±4.45^{bc}	30.22±4.45^{b}	31.51±4.45^{b}	26.12±4.45^{bc}
VAP (μm/s)	79.21±7.84^{a}	19.98±6.45^{c}	34.02±5.59^{bc}	42.46±5.59^{b}	40.39±5.59^{b}	34.63±5.59^{bc}
LIN (%)	54.69±3.80^{ab}	45.43±4.66^{b}	55.97±4.03^{ab}	48.87±4.03^{ab}	62.21±4.03^{a}	56.90±4.03^{ab}
STR (%)	71.61±2.76	70.22±4.26	77.56±3.69	69.57±3.69	78.68±3.69	75.43±3.69
WOB (%)	75.36±2.75^{a}	63.89±3.29^{a}	72.11±2.84^{ab}	69.57±2.84^{ab}	79.13±2.84^{a}	75.30±2.84^{a}
ALH (μm)	3.49±0.25	3.32±0.21	2.92±0.18	3.16±0.18	2.87±0.18	2.91±0.18
BCF (Hz)	7.70±0.23	10.08±0.82	8.37±0.71	7.73±0.71	7.90±0.71	8.51±0.71

Total motility (TM), progressively motility (PM) and rapid motility (RM), curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), beat cross frequency (BCF), amplitude of lateral (ALH), linearity (LIN), straightness (STR) and wobble (WOB).

$^{a\text{-}d}$: Means in the same row not sharing a common superscript are significantly different (P<0.05).

SEM: Standard error of the mean

Table 4.5 Mean±SEM of raw and frozen-thawed viability, membrane and acrosome integrities of Bapedi ram semen extended with different concentrations of egg yolk in Tris-based extenders.

Characteristic	Raw semen	Egg yolk concentration (%)				
		0	5	10	15	20
LIVE (%)	86.76±2.26^{a}	38.61±6.07^{c}	49.39±6.07^{bc}	59.30±6.07^{b}	55.34±6.07^{bc}	56.52±6.07^{bc}
DEAD (%)	12.86±2.29^{c}	57.65±5.30^{a}	48.86±5.30^{ab}	39.80±5.30^{b}	43.22±5.30^{ab}	39.86±5.30^{b}
ABN (%)	0.39±0.4	3.74±1.70	1.76±1.70	0.90±1.70	1.44±1.70	3.62±1.70
IM (%)	90.23±3.17^{a}	38.0±6.42^{c}	51.33±5.24^{bc}	61.23±4.54^{b}	59.25±4.54^{b}	55.09±5.24^{b}
DM (%)	9.77±3.17^{c}	62.91±6.42^{b}	48.67±5.24^{ab}	38.77±4.54^{b}	40.75±4.54^{b}	44.91±5.24^{b}
IA (%)	91.65±2.0^{a}	19.25±7.80^{c}	47.17±6.37^{b}	51.38±5.51^{b}	64.13±5.51^{b}	65.67±6.37^{b}
DA (%)	8.35±2.0^{c}	80.75±7.80^{a}	52.38±6.37^{b}	48.62±5.51^{a}	35.88±5.511^{b}	34.33±6.37^{b}

Abnormalities (ABN); intact membrane (IM); damaged membrane (DM); intact acrosome (IA); damaged acrosome (DA)

$^{a-c}$: Means in the same row not sharing a common superscript are significantly different (P<0.05).

SEM: Standard error of the mean

Table 4.6 The effect of different concentrations of phosphatidylcholine supplemented in Tris-based extenders with or without egg yolk on CASA motility characteristics following cryopreservation of Bapedi ram semen (Mean±SEM).

Characteristic	Raw semen	Treatments							
		E_0P_0	E_0P_1	E_0P_2	E_0P_3	E_1P_0	E_1P_1	E_1P_2	E_1P_3
TM (%)	91.29 ± 2.86^{a}	30.6 ± 7.92^{cd}	14.73 ± 7.92^{d}	22.60 ± 7.92^{d}	19.90 ± 9.70^{d}	60.51 ± 9.70^{b}	60.94 ± 7.92^{b}	60.71 ± 7.92^{b}	52.96 ± 7.92^{bc}
PM (%)	35.89 ± 3.12^{a}	4.78 ± 3.17^{cd}	1.43 ± 3.17^{d}	2.13 ± 3.17^{d}	2.79 ± 3.88^{d}	17.57 ± 3.88^{b}	23.42 ± 3.17^{b}	16.75 ± 3.17^{b}	13.51 ± 3.17^{bc}
RM (%)	49.65 ± 7.92^{a}	3.90 ± 3.66^{cd}	1.37 ± 3.66^{d}	0.61 ± 3.66^{d}	1.17 ± 4.48^{d}	19.19 ± 4.48^{b}	20.48 ± 3.66^{b}	14.18 ± 3.66^{bc}	12.90 ± 3.66^{bc}
VCL (µm/s)	115.76 ± 9.92^{a}	48.87 ± 8.85^{bc}	43.51 ± 8.85^{c}	34.59 ± 8.85^{c}	41.40 ± 10.84^{c}	77.03 ± 10.84^{b}	76.43 ± 8.85^{b}	65.30 ± 8.85^{bc}	63.78 ± 8.85^{bc}
VSL (µm/s)	71.15 ± 7.07^{a}	25.30 ± 7.26^{cde}	19.34 ± 7.26^{de}	15.84 ± 7.26^{e}	21.23 ± 8.89^{cde}	45.64 ± 8.89^{bc}	56.73 ± 7.26^{b}	44.41 ± 7.26^{bcd}	36.86 ± 7.26^{bcde}
VAP (µm/s)	93.09 ± 8.0^{a}	34.41 ± 8.02^{cde}	26.04 ± 8.02^{e}	22.77 ± 8.02^{e}	28.22 ± 9.82^{de}	61.31 ± 9.82^{bc}	67.38 ± 8.02^{b}	54.78 ± 8.02^{bcd}	48.28 ± 8.02^{bcde}
LIN (%)	61.0 ± 4.19^{ab}	50.32 ± 6.83^{abc}	40.77 ± 6.83^{c}	45.83 ± 6.83^{bc}	48.94 ± 8.36^{bc}	58.26 ± 8.36^{abc}	74.13 ± 6.83^{a}	67.18 ± 6.83^{ab}	59.27 ± 6.83^{abc}
STR (%)	75.80±8.0	72.56±4.88	70.49±4.88	69.58±4.88	71.28±5.98	73.38±5.98	84.18±4.88	80.18±4.88	76.80±4.88
WOB (%)	80.01 ± 3.96^{ab}	68.79 ± 4.91^{bcd}	56.80 ± 4.91^{d}	65.83 ± 4.91^{cd}	66.47 ± 6.01^{bcd}	78.96 ± 6.01^{abc}	88.06 ± 4.91^{a}	83.57 ± 4.91^{ab}	76.60 ± 4.91^{abc}
ALH (µm)	3.23 ± 0.27^{ab}	3.19 ± 0.32^{ab}	3.53 ± 0.32^{a}	2.81 ± 0.32^{ab}	2.90 ± 0.39^{ab}	2.84 ± 0.39^{ab}	2.32 ± 0.32^{b}	2.51 ± 0.32^{ab}	3.10 ± 0.32^{ab}
BCF (Hz)	8.55±0.24	7.29±0.56	7.71±0.56	7.16±0.56	7.89±0.66	8.24±0.66	7.78±0.56	7.67±0.56	9.16±0.56

Total motility (TM), progressively motility (PM) and rapid motility (RM), curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), beat cross frequency (BCF), amplitude of lateral (ALH), linearity (LIN), straightness (STR) and wobble (WOB), Tris-based extender without EY and PC (E_0P_0), Tris-based extender without EY supplemented with 0.25 mg/ml PC (E_0P_1), Tris-based extender without EY supplemented with 0.50 mg/ml PC (E_0P_2), Tris-based extender without EY supplemented with 0.75 mg/ml PC (E_0P_3), Tris-based extender with EY without PC (E_1P0), Tris-based extender with EY supplemented with 0.25 mg/ml PC (E_1P_1), Tris-based extender with EY supplemented with 0.50 mg/ml PC (E_1P_2), Tris-based extender with EY supplemented with 0.75 mg/ml PC (E_1P_3).

SEM: Standard error of the mean

$^{a-e}$: Means in the same row not sharing a common superscript are significantly different ($P<0.05$).

Table 4.7 The effect of different concentrations of phosphatidylcholine supplemented in Tris-based extenders with or without egg yolk on viability, membrane and acrosome integrities following cryopreservation of Bapedi ram semen (Mean±SEM).

Characteristic	Raw semen	Treatments							
		E_0P_0	E_0P_1	E_0P_2	E_0P_3	E_1P_0	E_1P_1	E_1P_2	E_1P_3
LIVE (%)	85.75±3.90^{a}	46.0±2.97^{b}	44.49±3.43^{b}	43.75±2.97^{b}	44.50±4.20^{b}	45.33±3.43^{b}	44.17±3.43^{b}	43.82±2.97^{b}	47.69±3.43^{b}
DEAD (%	14.17±3.85^{b}	51.8±3.09^{a}	53.0±3.57^{a}	53.5±3.09^{a}	51.0±4.38^{a}	53.17±3.57^{a}	51.67±3.57^{a}	53.06±3.09^{a}	50.63±3.57^{a}
ABN (%)	0.20±0.15^{b}	2.13±1.37^{a}	3.77±2.37^{a}	2.63±1.37^{a}	4.50±1.93^{a}	1.5±1.56^{a}	4.17±1.56^{a}	4.17±1.56^{a}	5.05±2.73^{a}
IM (%)	91.10±2.62^{a}	31.88±3.93^{d}	32.65±4.53^{d}	36.3±3.93^{cd}	32.76±5.55^{d}	55.76±4.53^{b}	47.08±4.53^{bc}	58.35±3.93^{b}	53.65±4.53^{b}
DM (%)	8.90±2.62^{d}	68.12±3.93^{a}	67.35±4.53^{a}	63.67±3.93^{ab}	67.24±5.55^{a}	44.24±4.53^{c}	52.92±4.53^{bc}	41.75±3.93^{c}	46.35±4.53^{c}
IA (%)	90.59±2.25^{a}	38.43±4.61^{d}	47.83±5.32^{cd}	46.35±4.61^{cd}	62.5±6.52^{bc}	75.98±5.32^{b}	70.83±5.32^{b}	76.13±4.61^{b}	62.67±5.32^{bc}
DA (%)	9.41±2.25^{d}	61.57±4.61^{a}	52.18±5.32^{ab}	53.65±4.61^{ab}	37.50±6.52^{bc}	24.02±5.32^{c}	29.17±5.32^{c}	23.88±4.61^{c}	37.33±5.32^{bc}

Abnormalities (ABN), intact membrane (IM), damaged membrane (DM), intact acrosome (IA), damaged acrosome (DA), Tris-based extender without EY and PC (E_0P_0), Tris-based extender without EY supplemented with 0.25 mg/ml PC (E_0P_1), Tris-based extender without EY supplemented with 0.50 mg/ml PC (E_0P_2), Tris-based extender without EY supplemented with 0.75 mg/ml PC (E_0P_3), Tris-based extender with EY without PC (E_1P0), Tris-based extender with EY supplemented with 0.25 mg/ml PC (E_1P_1), Tris-based extender with EY supplemented with 0.50 mg/ml PC (E_1P_2), Tris-based extender with EY supplemented with 0.75 mg/ml PC (E_1P_3).

SEM: Standard error of the mean

$^{a-d}$: Means in the same row not sharing a common superscript are significantly different ($P<0.05$).

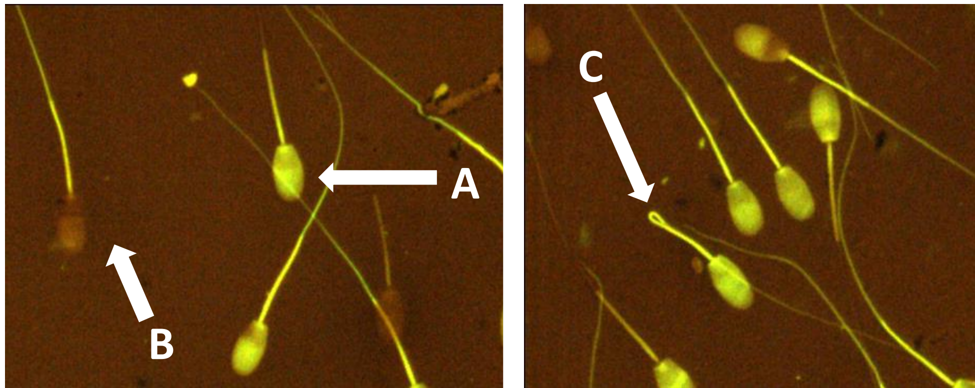

Figure 4.1 Viability and morphology of the raw Bapedi ram spermatozoa stained with eosin-nigrosin. (A): live spermatozoa, (B): dead spermatozoa and (C): live spermatozoa with abnormalities.

Figure 4.2 Membrane integrity of the raw Bapedi ram spermatozoa evaluated with HOST. (A): spermatozoa with intact membrane and (B): spermatozoa with damaged membrane.

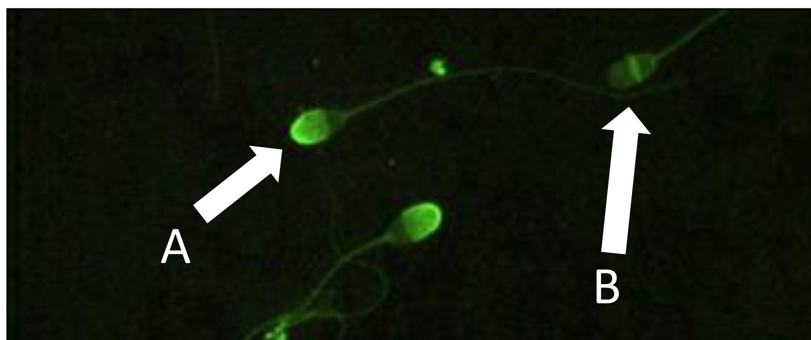

Figure 4.3 Acrosome integrity of raw Bapedi ram spermatozoa evaluated with PSA-FITC. (A): intact acrosome and (B): damaged acrosome.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Characterization in the present study was done to observe the semen parameters of Bapedi rams before diluting with extenders. The macroscopic and microscopic semen parameters are the important indicators of the reproductive potential of the male animals. In this study, Bapedi rams had similar semen volume and spermatozoa concentrations compared to previous breeds studied (Al-Samarrae, 2009; Hamedani *et al.*, 2013; Mahmuda *et al.*, 2015; Hamedani *et al.*, 2016; Tejaswi *et al.,* 2016). Although Prins (1999) reported an average semen pH that ranges from 7.2-7.8 in ram semen, the present study had an acidic semen pH ranging from 5-7. Other researchers found ram semen pH that is between 5 and 7 similar to the current study (Al-Samarrae, 2009; Soltanpour and Moghaddam 2013; Tejaswi *et al.,* 2016). The low semen pH of Bapedi rams is an interesting finding that warrants further research, since low semen pH has been documented to be detrimental and affecting the spermatozoa by reducing both the cellular activities and the quality of the spermatozoa (Latif *et al.*, 2005; Rehman *et al.*, 2013). However, the current study has shown that Bapedi ram semen has good freezeability based on the observations made after freezing and thawing. In addition, the semen pH in rams and bulls was reported to be slightly acidic (Juyena and Stelleta, 2013). According to Hafez and Hafez (2000), ram semen volume ranges between 0.5 to 2 ml and 0.5 to 0.7 ml in matured and young rams, respectively, while a spermatozoa concentration of 2.5×10^9 sperm/ml is regarded as normal and acceptable for ram semen (Gil *et al.*, 2003). Both the semen volume and spermatozoa concentration are essential to determine how much to dilute the semen and also to ensure adequate number of sperm cells during insemination doses.

The computer aided sperm analysis (CASA) was used to determine the sperm motility parameters in the present study. The CASA system is an objective method of analyzing semen motility and gives extensive information about the kinematic properties of the ejaculate based on measurements of individual spermatozoa (Awad, 2011). The total motility (TM) and rapid motility (RM) of raw Bapedi ram semen were similar to the reported findings by Boshoff (2014). However, the observed average PM of raw Bapedi ram semen was very low compared to other breeds (Sundararaman *et al.*, 2012; Boshoff, 2014). The occurrence of low PM of Bapedi ram semen in the present study is a serious concern, particularly that PM is very important for fertilization

(Perumal *et al*., 2014). The low PM of Bapedi ram semen might have been influenced by the acidic semen pH finding reported in the present study. A decrease in pH in semen has previously shown to reduce the PM of ram semen, which possibly affected the results of the entire study (Soltanpour and Moghaddam, 2013). Similar to the PM, the CASA motility parameters such as VSL, VCL, VAP, ALH and LIN were correlated to fertility in different species (Olmo *et al*., 2013; Perumal *et al*., 2014). The VSL, VAP, STR, ALH and LIN parameters of raw Bapedi ram semen were similar to other studies reported (Hamidi *et al*., 2012; Boshoff, 2014). However, the LIN and WOB parameters in the current study were higher, while the VCL and BCF parameters were extremely lower compared to that reported previously (Boshoff, 2014). According to Tsakmakidis (2016), the individual motion characteristics of spermatozoa such as VSL and LIN, VCL and VAP characteristics were reported to be correlated to fertility in pigs and sheep. In addition, a decrease in VSL, VAP and STR characteristics reduce the distance that the spermatozoa will travel to fertilize an egg, resulting in infertility (Kime *et al*., 1996; Makinta *et al*., 2005).

The microscopic semen characteristics such as percentage live spermatozoa, membrane and acrosome integrities are also very important for fertility (Mehmood *et al*., 2009; Odhiambo *et al*., 2011; Dogan *et al*., 2013). In the present study, the percentage live spermatozoa of raw Bapedi ram semen were low in comparison to previous reports (Hamidi *et al*., 2012; Hernandez *et al*., 2012; Hamedani *et al*., 2013; Mahmuda *et al*., 2015; Hamedani *et al*., 2016). On the contrary, the semen resulted in higher percentage live spermatozoa compared to other indigenous breeds (Al-Samarrae, 2009; Saltanpour and Moghaddam, 2013). Furthermore, the raw semen collected from Bapedi rams resulted in lower spermatozoa abnormalities, which are in agreement with previous studies (Hamedani *et al*., 2013; Hamedani *et al*., 2016). The observed lower abnormalities of Bapedi ram spermatozoa signifies good semen quality, because abnormalities are associated with infertility of male animals (Saacke, 2008). The maximum range and average of spermatozoa with intact membrane and acrosomes were similar to that reported in the literature (Aisen *et al*., 2002; Wusiman *et al*., 2012; Alcay *et al*., 2015; Hamedani *et al*., 2016). These results emphasize the competency of raw Bapedi ram semen to achieve fertility (Odhiambo *et al*., 2011; Dogan *et al*., 2013).

Sperm motility remains an important characteristic associated with the fertilizing ability of sperm (Saacke and White, 1972). In the present study, a decrease in motility characteristics was observed in frozen-thawed semen, reflecting a stressful situation due to the detrimental effects of cryopreservation (Salamon and Maxwell, 2000). The observations of the current study were able to distinguish between the extenders with or without EY based on the comparison of extenders with 0% and 5-20% EY. The beneficial effects of EY in ram semen freezing extenders were previously reported (Salamon and Maxwell, 2000; Kulaksiz *et al*. 2010). Mahmuda *et al*. (2015) found improved subjective motility using 10% EY in extenders with similar Tris-based ingredients. There is limited information regarding the use of CASA system to evaluate the effect of different EY concentrations in extenders for ram semen cryopreservation. According to Verstegen *et al*. (2002), the use of CASA facilitates the comparison of results, making it possible to find subtle differences between treatments. Egg yolk does not have any specific protective effect on the motility of the spermatozoa (Anel *et al*., 2003). The TM of the present study was similar to that of Maia *et al*. (2009) and higher than that reported by Emamverdi *et al*. (2013) in Tris-based extenders with 20% EY in frozen-thawed ram semen. Although the present study and Maia *et al*. (2009) found higher TM rate, a weak correlation (r^2=0.34) was observed between TM and field fertility (Farrell *et al*., 1998). The LIN motility parameter observed in the current study was higher compared to that reported previously (Maia *et al*., 2009; Emamverdi *et al*., 2013). According to Silva *et al*. (2013), spermatozoa with higher percentage of STR and LIN parameters would be able to migrate through the cervical mucus of the female reproductive tract, which is good for fertility. Although cryopreservation in the current study did not affect the LIN, STR, WOB, ALH and BCF motility characteristics, the observed PM, VAP, VCL, VSL, ALH and BCF parameters were lower compared to that reported previously (Maia *et al*., 2009; Emamverdi *et al*., 2013). Olmo *et al*. (2013) previously found a relationship between VAP, VCL and VSL parameters with fertility of ram semen. On contrary, the VAP, VSL and LIN parameters did not appear as good predictors of ram fertility (Anel *et al*., 2003). The occurrence of higher TM and low PM in extenders with different EY concentrations signifies that more spermatozoa were non-progressive similarly to the study of Olmo *et al*. (2013). A reduction in PM has an effect on the fertility, because PM and other velocity parameters such as VCL, VCL, ALH and LIN are necessary for the spermatozoa to achieve fertilization (Perumal *et al*., 2014). The observed low PM possibly influenced the other CASA motility

parameters due to the acidic semen pH of Bapedi rams. The acidic pH of the semen decrease the Na^+/K^+-ATPase activity which is associated with the energy and movement of the spermatozoa (Zhou *et al*., 2015). This ultimately affects the hyperactivation of the spermatozoa, which is the high energy state responsible for the penetration of sperm through the cervical mucus, zona pellucida, fusion with oocyte and successful fertilization (Aitken *et al*., 1985).

Motility is preserved better than the morphological integrity of the spermatozoa and an increase in motility is associated with the physiological functions of the spermatozoa (Anel *et al*., 2003; Olmo *et al*., 2013). Similar to the observations of the current study, the mammalian spermatozoa undergoes the biochemical, ultrastructural and functional damage during semen cryopreservation (Holt, 2000). In the current study, a decrease in sperm quality parameters such as the percentage of live spermatozoa, membrane and acrosome integrities indicates the detrimental effects of cryopreservation when raw semen is subjected to freezing and thawing. However, the inclusion of different EY concentrations in the extenders proved to counter the effect of cryopreservation based on the results showing that semen cryopreserved with 0% EY resulted in lower percentage of live spermatozoa, membrane and acrosome integrities. It was observed that concentrations of EY between 5-20% were sufficient to protect the percentage of live spermatozoa, membrane and acrosome integrities of the frozen-thawed semen. The acrosome integrity results were similar to those reported by Emamverdi *et al.* (2013) and Alcay *et al.* (2015) using 20% EY in extenders. On contrary, Hernandez *et al.* (2012) observed low acrosome integrity using 25% EY in Tris-based extenders following cryopreservation of ram semen. The acrosome integrity of ram spermatozoa is very sensitive during cryopreservation (Alcay *et al*., 2016). Therefore, its evaluation is important to predict the fertilizing ability of cryopreserved spermatozoa (Odhiambo *et al*., 2011). Similar to the acrosome integrity, sperm membranes are also the sensitive parts of the spermatozoa (Anel *et al*., 2003). The concentrations of EY between 5-20% protected the spermatozoa membrane similar to that obsereved by Alcay *et al.* (2015) and higher than that reported by Emamverdi *et al.* (2013) using 20% EY. The percentage of live spermatozoa in the present study was similar to that reported by Mahmuda *et al.* (2015) using 10% EY in Tris-based extenders on cryopreservation ram spermatozoa. Percentage live spermatozoa and membrane integrity were positively correlated

during ram semen cryopreservation (Bohlooli *et al.*, 2012). The integrity of the membrane is essential for survival of the spermatozoa in the female reproductive tract and its fertilizing ability (Mehmood *et al.*, 2009; Emamverdi *et al.*, 2013). Similarly, the importance of the percentage live spermatozoa is related to the ability of the sperm to fertilize and activate the ovum in supporting early embryogenesis (Dogan *et al.*, 2013). Interestingly, the percentage live spermatozoa of all the EY concentrations including the treatment without EY (0%) in Tris-based extenders were higher compared to that reported by Maia *et al.* (2009) using 20% EY. The results of the current study may have been influenced by the effectiveness of the protocol used to cryopreserve Bapedi ram semen considering the combination of the basic extender, glycerol level, freezing and thawing rates which were adopted from previous studies (Munyai, 2012; Alcay *et al.*, 2015; Mahmuda *et al.*, 2015). This is supported by Curry *et al.* (2000) who stated that the success of cryopreservation dependents on the basic extender used and the concentration of cryoprotectant as they interact with the freezing and thawing rates. In addition, freezing and thawing did not affect the abnormalities of the spermatozoa irrespective of EY concentrations used. The observed lack of effect on spermatozoa abnormalities after freezing and thawing is probably a good indication of sperm freezeability, particularly that abnormalities are associated with infertility (Saacke, 2008).

The improvement of semen extenders and supplementation with different ingredients under optimized conditions has the potential to improve the semen quality during cryopreservation (Rehman *et al.*, 2013). In the present study, cryopreservation remained detrimental to the spermatozoa regardless of the extenders with or without EY supplemented with different PC concentrations. However, the treatments extended with EY had higher CASA motility parameters such as TM, PM, RM, VCL, VSL and VAP compared to those without EY. Egg yolk has extensively been proven as an excellent cryoprotective component of semen extenders during cryopreservation (Salamon and Maxwell, 2000; Kulaksiz *et al.*, 2010). The cryoprotective effect of EY has been attributed to the biochemical components such as phospholipids, cholesterol and LDL which protect the spermatozoa against cold shock (Kulaksiz *et al.*, 2010). The supplementation of PC in extenders with or without EY did not improve the TM, PM, RM, VCL, VSL and VAP parameters in the present study. It was expected that the use of PC alone in extenders would improve the quality of cryopreserved ram

semen similar to other species such as bull, stallion, buffalo and boar semen (He *et al.*, 2001; Ropke *et al.*, 2011; Pillet *et al.*, 2012; Kumar *et al.*, 2015). Although the procedure for semen cryopreservation is similar in different species, the success of this process is a species dependent. Therefore, different species will react differently to cryopreservation due to different size, shape and lipid composition of the spermatozoa (Saraswat *et al.*, 2013). Similarly, cryopreservation did not affect the STR, LIN, WOB, ALH and BCF motility parameters. The reason for this is unknown and this may provide the basis for future research.

Although frozen-thawed semen reduced the percentage live spermatozoa, the observed results for all the treatment extenders with or without EY supplemented with different PC concentrations were similar. This means that EY and PC had no effect on the percentage live spermatozoa similar to the results of different EY concentrations. On the contrary, Sitimurang (2012) observed significant differences on the percentage live spermatozoa when 0.5 mg/ml PC was supplemented in extenders with or without EY for freezing of bull semen. The frozen-thawed semen in the current study resulted in increased number of spermatozoa with abnormalities. However, the treatment extenders with or without EY and PC were similar. The increase in spermatozoa abnormalities might have been influenced by the sperm cell water exchange during the early stages of the freezing which causes swellings and shrinkages on the majority of sperm organelles (Medeiros *et al.*, 2002).

Phosphatidylcholine was reported as a major protective component of the sperm membrane during cryopreservation (Bergeron and Manjunath, 2006). However, the supplementation of PC in extenders without EY did not improve the membrane integrity of frozen-thawed semen in the present study. Similar results were observed by Ollero *et al.* (1998) in which PC in extenders without EY did not improve the sperm membrane integrity of frozen-thawed ram semen. Although there are no clear reasons, the assumptions are on the phospholipids composition of Bapedi ram spermatozoa membrane and the incubation period. Ropke *et al.* (2011) studied the effect of incubation period of PC on cryopreservation of bull semen and found that a longer incubation period (24 hours) prior to freezing allow more time for PC and spermatozoa membrane interaction, resulting in improved semen quality following cryopreservation. Furthermore, the components of the spermatozoa membrane vary with species and

breeds of animals. Previously, researchers assumed PC as the major phospholipid of spermatozoa membrane and as a major protective component during cryopreservation (Quinn *et al.*, 1980; Watson, 1981). However, Fang *et al.* (2016) determined the effect of different plasma membrane phospholipids on spermatozoa during ram semen cryopreservation. The composition of PC on spermatozoa membrane did not exhibit significant difference when different freezing rates were studied compared to other phospholipids such as phosphatidylethanolamine (PE) and phosphatidylserine (PS). From their study, it can be postulated that cryopreservation does not destabilize the sperm membrane PC. Therefore, this could be one of the reasons that made PC supplementation to be less effective on protecting the spermatozoa against cold shock and other factors of cryopreservation.

Although the supplementation of PC in extenders without EY did not improve the CASA motility parameters, percentage live spermatozoa and membrane integrity, different results were observed in acrosome integrity. Interestingly, the supplementation of 0.75 mg/ml PC in Tris-based extenders without EY resulted in acrosome integrity similar to Tris-based extenders with EY. This imply that sorely using PC in extenders has the ability to maintain integrity of the acrosome of frozen-thawed ram semen. During buffalo semen cryopreservation, PC liposomes maintained the acrosome integrity similar to EY extenders (Kumar *et al.*, 2015). According to Simpson *et al.* (1986), the PC is able to prevent the premature acrosome reaction. A spermatozoa with premature reacted acrosome may not fertilize an oocyte. Acrosome reaction is an exocytotic process occurring after the binding of the spermatozoa with the zona pellucida of the oocyte (Ickowics *et al.*, 2012). In addition, only the spermatozoa with reacted-acrosome can penetrate the zona pellucida and fertilize (Marti *et al.*, 2000).

The supplementation of PC in extenders with EY improved none of the semen quality parameters including CASA motility, percentage of live spermatozoa, membrane and acrosome integrities. Although there are no previous records of PC supplementation to EY in ram semen freezing extenders, Sitimurang (2012) observed no significant differences when freezing bull semen using Tris-based extenders with 10% EY supplemented with 0 and 0.5 mg/ml PC. This imply that supplementing PC in extenders with EY does not improve the quality of frozen-thawed semen. This

phenomenon can be related to the hypothesis of Purdy and Graham (2004) that cholesterol supplemented in EY extenders is attracted to the EY lipids instead of the sperm membrane. Probably the PC was attracted to the EY lipids instead of the sperm membrane, resulting in less benefit to the spermatozoa during cryopreservation. This imply that the supplementation of PC in Tris-based extenders with EY is not necessary for cryopreservation of Bapedi ram semen.

5.2 Conclusion

The macroscopic and microscopic semen parameters of raw Bapedi ram semen were characterized. The study observed an acidic pH of the semen that affected the progressive motility (PM) of Bapedi ram semen in the entire study. Cryopreservation affected the motility parameters, percentage live spermatozoa, membrane and acrosome integrities of frozen-thawed Bapedi ram semen. The use of Tris-based extenders with 10% EY concentration resulted in higher motility parameters and membrane integrity of frozen-thawed Bapedi ram semen. However, Tris-based extenders with 20% EY resulted in higher acrosome integrity of frozen-thawed Bapedi ram semen. The freezing and thawing did not affect the abnormalities of the spermatozoa irrespective of EY concentrations used. The supplementation of PC in Tris-based extenders in extenders with or without EY did not improve the motility parameters, percentage live spermatozoa and membrane integrity. However, the acrosome integrity was improved in Tris-based extenders without EY supplemented with 0.75 mg/ml PC.

5.3 Recommendations

The study has established a freezing protocol for successful cryopreservation of Bapedi ram semen. The use of 10% EY in Tris-based extenders is recommended for cryopreservation of Bapedi ram semen. Therefore, the practices of AI and IVF with cryopreserved Bapedi ram semen are recommended as part of breed conservation programs. Future studies should also investigate the possible causes of acidic pH in Bapedi ram semen and its possible effect on progressive motility up to the cellular level. Further studies should examine the composition of Bapedi ram spermatozoa membrane phospholipids and their interactions with EY phospholipids. Moreover,

more studies are recommended on the incubation periods of the PC in extenders prior to freezing to allow more time for their interaction with the spermatozoa. In addition, higher concentrations of PC should be tested and other phospholipids such as PE and PS should be studied in extenders without EY.

CHAPTER SIX

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