CRYOPRESERVATION OF SOUTH AFRICAN INDIGENOUS RAM SEMEN

by

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Dedication

To my father (Freddy) and mother (Tshinakaho)

My wife (Lutendo), son (Mulweliwashu) and daughter (Londani)

My late brother (Mafanedza) and Emmanuel

My sisters (Ntshimbidzeni and Mashudu)

Thank you for your encouragement, love and support throughout my studies.

Declaration

I hereby declare that the work in this dissertation submitted to the University of the Free State for the degree**, Magister Scientiae Agriculturae,** is my own independent work and has never been previously submitted to any other university. I cede the copyright of this dissertation to the University of the Free State.

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Pfananani Hendrick Munyai
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List of Abbreviations

ADP	Adenosine diphosphate
AI	Artificial insemination
ALH	Amplitude of lateral head movement
ANOVA	Analysis of variance
ARC	Agricultural Research Council
ART	Assisted reproductive technologies
ATP	Adenosine triphosphate
AV	Artificial vagina
BCF	Beat cross frequency
BO	Bracket and Oliphant
BSA	Bovine serum albumin
CASA	Computer assisted sperm analysis
CPA	Cryoprotective agent
DMSO	Dimethylsulfoxide
EE	Electro-ejaculation
EDTA	Ethylenediaminetetraacetic acid
EYC	Egg yolk citrate
FBS	Fetal Bovine Serum
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
ICSH	Interstitial cell stimulating hormone
IU	International unit
IVEP	In Vitro Embryo Production

IVF	In Vitro Fertilization
LH	Luteinizing hormone
LIN	Linearity
LN_2	Liquid nitrogen
LSD	Least significant difference
MOET	Multiple Ovulation and Embryo Transfer
PVC	Polyvinyl chloride
ROS	Reactive Oxygen Species
SEM	Standard error of means
SSH	Spermatogenic stimulating hormone
STR	Straightness
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight line velocity
WOB	Wobble

Chapter 1

General Introduction

The South African sheep population is consistently being improved as result of local and international trade of superior genetic material. The two major systems that are used for this purpose are the transport of live animals and export of frozen ram semen. For decades there has been speculation regarding the exploitation of sheep breeds indigenous to Southern Africa regarding food security. It has been alleged that these indigenous sheep breeds (including the Damara, Namaqua Afrikaner, Pedi and Zulu breeds) are specially adapted to the South African arid environmental conditions and possess certain favourable traits (excellent mothering ability, natural tolerance to external parasites and diseases, high fertility, etc.), which could be incorporated into a viable and profitable crossbreeding programme (Ramsay *et al*., 2001). In order to exploit the productive traits of the Damara, Namaqua Afrikaner, Pedi and Zulu sheep, it is however essential that the genetic material (in this case the male) firstly be characterized and gametes collected and preserved (*in-situ* and *ex-situ*), for future use and incorporation in sheep breeding programmes.

For improving reproductive performance, several assisted reproductive technologies (ART's), such as artificial insemination, multiple ovulation and embryo transfer (MOET), *in vitro* embryo production (IVEP) and semen cryopreservation can be used. Artificial insemination is the most widely used ART and has made the most significant contribution to genetic improvement worldwide (Evans & Maxwell, 1987; Leboeuf, 2000). For successful artificial insemination, ram semen specific cryopreservation protocols should be developed. The cryopreservation technique includes temperature reduction, cellular dehydration, eventual freezing and subsequent thawing (Medeiros *et al.*, 2002). The lowering from room temperature to 4°C reduces cellular metabolic activity and increases the life span of the sperm cells. Cryopreservation has been shown to stop all cellular activities, restarting its normal metabolic functions, after thawing (Mazur, 1984).

Sperm cryopreservation usually induces the formation of intracellular ice crystals, osmotic and chilling injury, which causes sperm cell damage, cytoplasmic fracture, or even effects on the cytoskeleton or the genome related structures (Isachenko, 2003). The main changes that occur during the freezing of gametes are mainly related to ultra-structural, biochemical and

functional activities, which may ultimately impair sperm transport and decrease the survival rate in the female reproductive tract, thereby reducing fertility. Ultra-structural sperm damage is generally greater in the ram than in the bull and thus seems to be species-related (Salamon & Maxwell, 2000).

Sperm preservation protocols differ between animal species, due to their inherent abilities to accommodate variations in semen extenders used in the cooling and freezing processes (Barbas & Mascarenhas, 2009). These differences between species regarding the sensitivity of their sperm to cooling are then largely attributed to the compositional variation of the sperm plasma membranes (Bailey *et al*., 2000). Differences in fatty acid composition and sterol levels of the cell membrane have also been associated with the tolerance of sperm to cold shock and cryopreservation. Thus, the observed variation between species in sperm survival rate, after freezing and thawing, has been attributed to these differences. There may then also be considerable differences between breeds and between individual males, regarding the 'freezability' of their semen (Hiemstra *et al*., 2005).

A thorough knowledge of the sperm physiology for a specific species is thus essential to maximize post-thaw sperm survival and subsequent fertility (Purdy, 2006). Protocols for different species, including the ram have been developed and tested over time on various exotic breeds. There is however a need to study and characterize the quality of indigenous (in this case, South African) ram semen, as it ultimately determines the fertility rate achieved. It is deemed necessary to cryopreserve indigenous ram semen and to develop extenders that may optimise the sperm cryosurvival and guarantee their survival and viability. Sperm quality and its relationship to male fertility are of utmost importance in animal breeding. Moreover, standard sperm analyses are routinely implemented to determine the acceptability of processed semen for breeding purposes. In this study, the Computer Assisted Sperm Analysis (CASA) system has been used to accurately measure the motility characteristics of the indigenous ram sperm cells, as it gives reliable and repeatable results.

Semen evaluation and cryopreservation studies have been done in the past, using different cryoprotectants and ram breeds of different ages, on different nutritional regimes and at various time of the year (season) – all factors that could affect the semen quality and quantity. To date no study has been conducted on the semen quality of certain indigenous South African ram breeds (in this case the Damara, Namaqua Afrikaner, Pedi and Zulu) and the

potential of their gametes (sperm) to be preserved. Due to practical reasons, the semen collected from the different breeds was done with the aid of the electro-ejaculator, which is not the preferred method. It is generally accepted that the quality of semen obtained when using the electro-ejaculator is inferior to that obtained when using the artificial vagina (Greyling & Grobbelaar, 1983).

The objectives of this study were thus to characterise indigenous South African ram semen macroscopically (volume, pH and sperm concentration) and microscopically (sperm cell viability and motility rate), determine a suitable storage temperature (5°C vs. 15°C) and storage time for diluted ram semen prior to AI. Also to determine the effect of storage temperature and period on ram sperm motility and velocity characteristics of semen diluted with an extender containing glycerol, prior to cryopreservation and artificial insemination. It was further to determine the optimal glycerol inclusion level in a standard cryopreservation diluent for South African indigenous ram semen, and compare programmable slow cooling rates with the use of liquid nitrogen vapour in the cryopreservation of indigenous ram semen.

Chapter 2

Literature review

Factors affecting cryopreservation and post thaw viability of ram semen

2.1 Description of semen

Semen is the liquid cellular suspension containing sperm cells and secretions from the accessory organs of the male reproductive tract. The fluid portion of the ejaculate is known as seminal plasma (Hafez & Hafez, 2000). The medical dictionary describes semen as the penile ejaculate; a thick, yellowish-white, viscous fluid containing sperm cells.

2.2 Production of semen

2.2.1 Site of production

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

2.2.2 Hormones involved in the control of spermatogenesis

The functions of the testes, are namely the production of sperm and androgens (testosterone), regulated by specific hormones. These hormones are called the gonadotropins, and are released into the bloodstream (endocrine hormones) by the pituitary gland located in the base of the brain. The production of sperm cells and androgens by the testes cease without gonadotropin (interstitial cell stimulating hormone and spermatogenesis stimulating hormone) support. Production and release of these gonadotropins by the pituitary are in turn controlled by other centres in the brain (hypothalamus), which also respond to environmental stimuli. The main gonadotropins maintaining and regulating spermatogenesis are FSH (SSH) and LH (ICSH) (Evans & Maxwell, 1987).

2.2.2.1 Follicle stimulating hormone (FSH)

The Sertoli cells of all mammals have FSH receptors and are known to regulate the differentiation and transformation of germ cells to spermatozoa. However, there appear to be species and age differences in the way in which FSH regulates spermatogenesis. FSH has a critical role in regulating spermiogenesis, the process that controls the formation of normal mature sperm with fertilising ability (Moudgal & Sairam, 1998).

2.2.2.2 Luteinizing hormone (LH)

In the male, LH is known as interstitial cell stimulating hormone (ICSH) (Hafez & Hafez, 2000). It acts on the Leydig cells of the testes to stimulate testosterone production. The testosterone in turn acts on the seminiferous tubules to promote spermatogenesis (Evans & Maxwell, 1987). Foster *et al.* (1978) stated an increase in both volume and activity of the Leydig cells to be caused by the secretory pattern of LH.

2.2.2.3 The male sex hormone, testosterone

Testosterone is an anabolic androgenic steroid occurring naturally in both males and females (secreted by the adrenal cortex and ovaries in small quantities). It is the principal male sex hormone, which belongs to the class known as androgens. Testosterone is produced by the interstitial (Leydig) cells of the testis, and acts locally to stimulate the development of sperm, and via the circulating blood to promote the secondary male characteristics.

Testosterone levels in the body are controlled by a negative feedback mechanism that involves the hypothalamus, the anterior pituitary gland, and the testes. Briefly, the hypothalamus releases gonadotropin-releasing hormone (GnRH) that is transported to the anterior pituitary via the portal system (that lies between the two areas of the brain). The anterior pituitary then releases follicle stimulating hormone (FSH) and luteinizing hormone (LH), which target the testes. FSH induces the seminiferous tubules to produce sperm and a feedback hormone called inhibin. LH on the other hand promotes the production of testosterone by the interstitial cells of the testes. Inhibin and testosterone initiate a feedback on the anterior pituitary to inhibit the production of FSH and LH and, on the hypothalamus to inhibit the production of GnRH. When inhibin and testosterone levels drop GnRH, FSH, and LH production increases once again (Evans & Maxwell, 1987).

2.2.3 Spermatogenesis

Spermatogenesis is the process whereby spermatozoa containing half the number of chromosomes (haploid) are produced, compared to the somatic cells. This process takes place in the seminiferous tubules of the testis. The germ cells progress from the diploid to haploid state and then change shape to become fully developed sperm cells. Spermatozoa are the matured male gamete in many sexually reproducing organisms. Thus, spermatogenesis is the male version of gametogenesis. In mammals it occurs in the male testes and epididymis in a stepwise fashion and in humans it takes approximately 64 days. Spermatogenesis is highly dependent on optimal conditions (e.g. temperature) for the process to occur efficiently, and is critical in reproduction. Spermatogenesis starts at puberty and usually continues uninterrupted until death. A slight decrease in semen production is discerned with an increase in age. The entire process of spermatogenesis can be sub-divided into several distinct stages, each corresponding to a particular type of cell or stage of maturation (Hafez & Hafez, 2000).

The spermatogenic process in mammals is composed of three functionally and morphologically distinct phases: the spermatogonial (proliferative or mitotic), spermatocytary (meiotic) and spermiogenic (differentiation) phases, which are under the control of specific regulatory mechanisms (Russell *et al*., 1990; Sharpe, 1994). The meiotic and spermiogenic phases are very similar in all mammals. Spermatogenesis is divided into three phases (Figure 2.1). The first being spermatocytogenesis, entailing a series of mitotic divisions during which spermatogonia form the primary spermatocytes. The second phase is meiosis, when the primary spermatocytes undergo reduction division forming rounded spermatids with haploid nuclei. The third phase is spermiogenesis, a phase during which spermatids undergo a metamorphosis, forming sperm cells. The entire process will be completed within 46 to 49 days in rams. Time estimates reported are shorter in the boar (36 to 40 days) and longer in bulls (56 to 63 days). As spermatogenesis progresses, the developing gametes migrate from the basement membrane of the seminiferous tubules toward the lumen and then, towards the rete testis (Bearden *et al*., 2004).

2.2.3.1 Spermatocytogenesis

There are two types of cells located along the basement membrane of the seminiferous tubules. The first are the Sertoli cells, which are larger, less numerous and are somatic cells which play a supporting role during both spermatocytogenesis and spermiogenesis. Second are the spermatogonia, small, rounded and more numerous cells which are the potential

gametes. After migrating to the embryonic testes, the primordial germ cells undergo a number of mitotic divisions before forming the gonocytes. Before puberty these gonocytes differentiate into A_0 (stem cells), A_1 (dormant) and A_2 (dormant) spermatogonia, located along the basement membrane of the seminiferous tubules. The A_2 spermatogonium will divide, forming either dormant (A_1) spermatogonium or an active (A_3) spermatogonium (Figure 2.1), starting a new generation of developing germ cells. The active spermatogonia will undergo four mitotic divisions in bulls and rams, eventually forming 16 primary spermatocytes. In rams, these mitotic divisions are completed by day 15 to 17 (Bester, 2006).

2.2.3.2 Meiosis

Meiosis is a two-step process. Each primary spermatocyte will undergo a first meiotic division to form two secondary spermatocytes. With this division, the chromosome number in the nucleus is reduced by half so that nuclei in the secondary spermatocytes contain an unpaired (n) or haploid number of chromosomes. This step requires approximately 15 days in the ram. Within a few hours after their formation, each secondary spermatocyte will again divide, forming two spermatids. Thus, four spermatids form from each primary spermatocyte, or 64 from each active (A_3) spermatogonium, in bulls and rams. As the A_1 spermatogonia divide by mitosis to form A_2 spermatogonia, the potential yield of spermatids is higher than is actually realized. Degeneration of the spermatogonia during mitotic division could account for this loss in efficiency. The Sertoli cells then remove the degenerating germ cells by phagocytosis.

Following a resting or dormant state of several weeks, the dormant (A1) spermatogonium will divide, forming A2 spermatogonia which will divide, forming new active (A3) and new dormant (A1) spermatogonia. Even though A0 spermatogonia (reserve stem cells) will occasionally divide, forming new A0 and A1 spermatogonia, the formation of dormant spermatogonia from A2 spermatogonia is the key process to maintaining the continuity of spermatogenesis and thereby not diminishing the supply of potential gametes within the testes (Bearden et al., 2004).

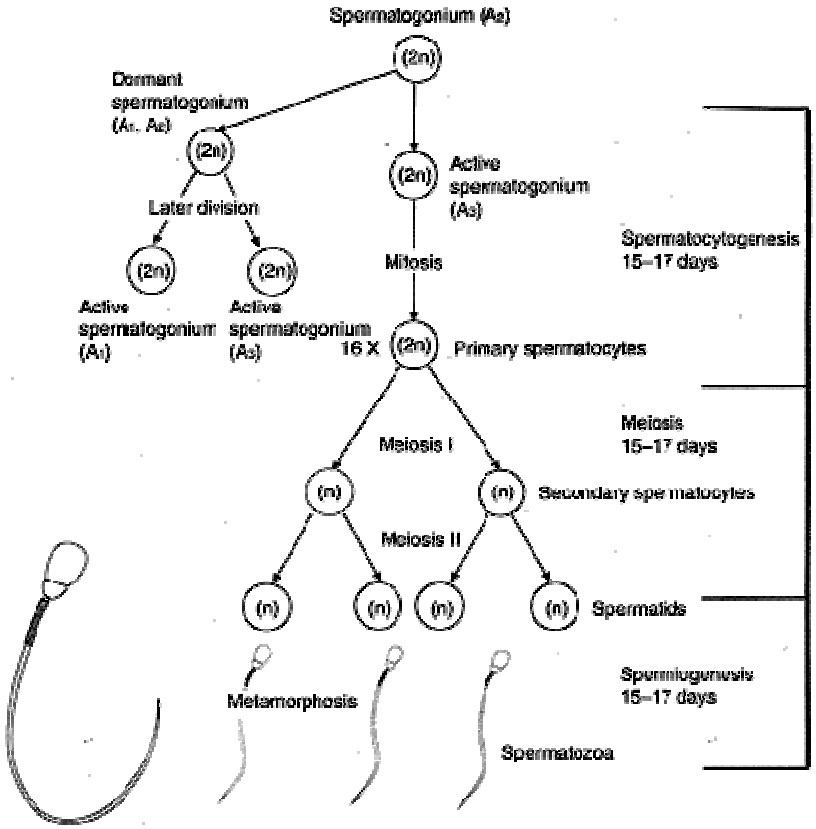

Figure 2.1 Spermatogenesis indicating the sequence of events and time involved in spermatogenesis in the ram
Source: http://nongae.gsnu.ac.kr/~cspark/teaching/chap6.html

2.2.3.3 Spermiogenesis

During spermatogenesis the spermatids are attached to the Sertoli cells. Each spermatid then undergoes a metamorphosis, forming a spermatozoon. During this metamorphosis the nuclear material will compact in a certain area of the cell, forming the head of the sperm, while the rest of the cell elongates, forming the tail. The acrosome, a cap around the head of the sperm, will then form from the Golgi apparatus of the spermatids. As the cytoplasm from the spermatid is cast off during formation of the tail, a cytoplasmic droplet will form on the neck of the sperm. The mitochondria from the spermatid will form in a spiral around the upper one-sixth of the tail, forming the mitochondrial sheath. Newly formed sperm cells will then be released from the Sertoli cell and forced out through the lumen of the seminiferous tubules into the rete testis. Sperm cells are unique cells in that they have no cytoplasm, and after maturation possess the ability to be progressively motile. The process of spermiogenesis is completed after 15 to 17 days in rams (Bester, 2006).

2.3 Seminal plasma

The seminal plasma is the extracellular fluid that provides the medium for sperm cells. It is a composite mixture of secretions that originate from the male accessory organs of reproduction (Gundogan, 2006). Seminal plasma as such is synthesized and secreted by the testes and accessory sex glands in males and plays a significant role in the development of sperm motility and hence its freezing ability. Mammalian seminal plasma is thus composed of secretions from several glands in the reproductive tract (Mann & Lutwak-Mann, 1976) that are mixed with the sperm at ejaculation and contribute to the majority semen volume and components (Moura *et al*., 2006). The seminal plasma is known to contain proteins, enzymes, lipids, electrolytes, sugars and various other factors, which may play significant roles in the metabolic regulation of sperm cells. In ejaculated semen, fructose is a major saccharide contained in the seminal plasma of most farm animals. This fructose in the seminal plasma plays an important role in sperm metabolism, and the sperm cells utilize it to produce adenosine triphosphate (ATP) (Maxwell *et al*., 1999). Certain accessory sex gland proteins are also known to bind and be absorbed into the sperm cell membrane, affecting its functions and properties (Mentz *et al*., 1990; Desnoyers & Manjunath, 1992). It is known that the seminal plasma proteins coat and protect sperm cells during ejaculation. Many studies have shown that the low content of seminal plasma proteins is associated with poor semen quality (White *et al*., 1987; Ashworth *et al*., 1994). The seminal plasma proteins are mainly composed of albumin and globulin, in addition to small quantities of non-protein nitrogen, amino acids and peptides (Zedda *et al*., 1996). These compounds make up the amphoteric property of the seminal plasma proteins, while the low protein content in seminal plasma reduces its buffering capacity and in turn the semen quality (Paz *et al*., 1992).

Seminal lipids play significant roles in the membrane structure of the sperm cell, sperm metabolism, sperm capacitation and fertilization of the female gamete (Hafez, 1993). In addition, some researchers have reported that reductions in sperm concentration and motility are associated with a decrease in seminal plasma lipid content (Kelso *et al*., 1997; Taha *et al*., 2000). Seminal plasma has also been reported to maintain sperm motility and viability in many species (Baas *et al*., 1983; Graham, 1994; Maxwell *et al*., 1996) and increase the sperm resistance to cold shock injury (Berger *et al*., 1985; Barrios *et al*., 2000), by providing specific components that stabilize the membrane of the frozen-thawed sperm cells (Maxwell & Watson, 1996; Ollero *et al*., 1997). Maxwell *et al*. (1999) evaluated the effects of resuspending ram sperm cells in 20% seminal plasma post-thawing and reported the

penetration of sperm cells through the cervical mucus to be improved, and fertility after cervical insemination of ewes significantly increased. Mortimer and Maxwell (2004) subsequently reported frozen-thawed sperm, resuspended in artificial seminal plasma or ram seminal plasma to have improved motility and increased plasma membrane stability, compared to those resuspended in PBS. It was suggested that this was due to the components of the medium.

The detrimental effects of seminal plasma on sperm motility (Iwamoto *et al*., 1993; De Lamirande *et al*., 1984), viability (Dott, 1979) and post thaw survival rate (Ritar & Salamon, 1982, Kawano *et al*., 2004) have also been reported. Seminal plasma has been shown to suppress sperm capacitation and to decapacitate previously capacitated sperm (Cross, 1993).

2.4 Semen collection methods

2.4.1 Semen collection using the artificial vagina

The artificial vagina as a means to collect semen is easy to use and the semen collected is generally relatively clean and the ejaculate is similar to the natural ejaculate (Salisbury *et al*., 1978). The artificial vagina (AV) briefly consists of a rigid cylinder of rubber or PVC and a thin walled rubber tube for the inner lining. A water-tight jacket is formed inside the cylinder by folding both ends of the thin walled rubber tube over the outer cylinder. The water jacket is filled with water, warm enough (45-55℃) to bring the inside temperature of the artificial vagina to a few degrees Celsius (℃) above normal body temperature. The temperature of the water simulates the thermal, while the pressure in the AV provides the mechanical stimulation of the vagina over the glans penis (Donovan *et al*., 2001). At one end of the artificial vagina, a graduated, glass semen collection tube is fitted. A female, preferably in oestrus, is placed in a neck clamp and the male is allowed to mount. When the male mounts, the penis is deflected into the AV, where the male ejaculates naturally. The major disadvantage of this method of semen collection is that the rams have to be trained beforehand to utilize this method (Mathews *et al*., 2003).To avoid contamination of the semen sample and prevent the transmission of venereal diseases from one ram to another, all rubber parts should be thoroughly cleaned and rinsed with water, then with alcohol, and finally with distilled water and allowed to dry.

2.4.2 Semen collection using an electro-ejaculator

The electro-ejaculation (EE) method of semen collection was first used by Gunn (1936), in Australia. The technique was based on the principle of stimulating the spinal cord, between the 4th lumbar and the 1st sacral vertebrae by placing one electrode in the rectum and the other in the back muscle. By passing a few 5 to 10 second rhythmic electric stimuli through the electrodes, an ejaculation can be induced and the semen collected in a glass tube. The animals generally experienced no harmful effects, no loss in body condition, no real change in disposition, and no special disinclination to further application of the treatment. However, during the application of this electro-ejaculation method, the electric current produces general strong contractions of all body muscles, and a slight and temporary motor inability of the hindquarters and hind limbs, at the end of this treatment. Later a bipolar rectal electrode in contact with the floor of the rectum was introduced to facilitate the process (Salisbury *et al*., 1978). Carter *et al.* (1990) described the EE as a two phase process. The first entailed an emission phase, involving stimulation of the lumbar sympathetic nerves which form the hypogastric nerve and which supply the ampullae and vasa deferentia. The second is an ejaculatory phase involving the contraction of the urethral muscles, which are serviced by the sacral parasympathetic nerves, forming the pelvic and pudendal nerves. Electro-ejaculators are basically electrical generators, which deliver an oscillating current which serves as a stimulus to the nerve controlling the emission and ejaculation of semen. Researchers have claimed that EE generally produce ejaculates with larger volumes, but a lower sperm concentration than that obtained using the AV. Bearden and Fuquay. (1980) indicated that the total number of sperm cells obtained using EE is comparable, and fertility levels also seem to be comparable to that of ejaculates collected from the same rams when using the AV. According to Matthews *et al*. (2003), semen collected with the aid of an AV produce a higher sperm concentration, but with a similar volume and sperm morphology, when compared to that of semen collected by an EE. Carter *et al*. (1990) also compared EE and AV semen collection methods in rams and found the repeatability of the volume of the ejaculate obtained, sperm concentration, total sperm number, percentage of normal sperm, and wave motion were slightly higher when using the artificial vagina technique.

The advantages of the EE are that no prior training is needed for rams, more ejaculates can be collected within a short period of time, and semen can be collected from superior sires that are incapable of mounting, possibly as a result of injury or ageing (Sundararaman *et al*., 2007).

2.5 Semen evaluation

2.5.1 The importance of semen evaluation

Semen quality and its relationship to fertility are aspects of major concern in the animal production industry. The average ejaculate volume of ram semen is 1.1mL (Seremak *et al*., 1999). Semen however needs to be evaluated using a light microscope to estimate the sperm viability and the percentage motile (and progressively motile) sperm cells, prior to its use in AI (Rowe *et al*., 1993).

2.5.2 Subjective assessment of semen

2.5.2.1 Colour and volume of the ejaculate

The first measurement of raw or fresh semen to indicate quality is its overall appearance. Raw (unaltered) semen appears as a thick whitish to slightly yellowish fluid. The colour of ram semen varies from milky- white to pale creamy in colour (Bag *et al*., 2002). According to Hafez and Hafez (2000), there exists a correlation between the colour and the sperm concentration of the semen ejaculate. The viscosity of the semen sample is often a reflection of the number of sperm cells present. In practice the semen sample should be free of any odour, as this is indicative of an infection or the presence of urine, which could be detrimental to the fertilizing ability of the semen sample. Other contaminations considered to be detrimental to the ejaculate can be detected in the colour of the semen e.g. blood, urine, and faeces, which may cause the semen to be pink to brownish in colour. White clumps or flakes in the ejaculate indicate pus and the presence of an infection in the reproductive tract of the male.

Hafez and Hafez (2000) further reported age of the ram and body condition, season of the year, skill of the technician involved and the frequency of collection to affect the ejaculate volume. The ejaculate volume generally ranges between 0.5 and 2mL in mature rams, and 0.5 and 0.7mL in young rams. The ejaculate volume will generally decrease if a ram is collected three or more times per day, or for a lengthy period of time. Gil *et al*. (2003) using the AV to collect semen from rams, regarded a volume of between 0.75 and 2mL to be normal.

2.5.2.2 Sperm concentration

Sperm concentration generally refers to the number of sperm cells per millilitre of semen (Hafez, 1993). Sperm concentration in the ejaculate serves as one of the criteria in semen characteristics, to qualify fertile males for breeding purposes (Graffer *et al*., 1988). The concentration of semen is essential to determine how much to dilute the semen, while providing adequate number of sperm cells in each insemination dose. The sperm concentration in the ejaculate is physically measured with the aid of a haemocytometer or a spectrophotometer. The haemocytometer is composed of a microscope slide with a precisely calibrated chambers generally used for counting red blood cells (Evans & Maxwell, 1987).

According to Hafez and Hafez (2000) there exists a correlation between the colour of the semen sample and the concentration of the ejaculate. The density of the semen sample is then a reflection of the number of spermatozoa present. Table 2.1 demonstrates how sperm concentration varies, based on the semen sample colour.

Table 2.1 Subjective assessment of semen concentration using colour variation

Semen score	**Ejaculate colour**	**Number of sperm(10^9/mL)**	
		Mean	Range
5	Thick creamy	5.0	4.5-6.0
4	Creamy	4.0	3.5-4.5
3	Thin creamy	3.0	2.5-3.5
2	Milky	2.0	1.0-2.5
1	Cloudy	0.7	0.3-1.0
0	Clear (watery)	Insignificant	Insignificant

Source: Hafez and Hafez, 2000

2.5.2.3 Sperm motility

Sperm motility is the simplest trait to evaluate the quality of a semen sample. Hafez and Hafez (2000) reported sperm motility assessment to involve the subjective microscopic estimation of the viability of the sperm cells and the quality of the sperm motility.

Sperm motility in raw and extended semen at various steps of the freezing process can be assessed microscopically by examining a uniform drop of semen on a slide with a coverslip, under a phase contrast microscope, fitted to a warm stage at 37°C. This motility assessment is

generally made on the basis of an arbitrary scale from 0 to 5 (0 = no motility, 1 = 20%, 2 = 40%, 3 = 60%, 4 = 80% and 5 = 100% motility) (Karatzas *et al*., 1997). Although it is important to look for progressively motile sperm (sperm moving in a straight line), it may be just as relevant to evaluate the viability – to determine if sperm are alive and motile (total motility or sperm being able to propel themselves forward with a beating tail). For people evaluating semen for the first time, the process of assessment seems difficult, inaccurate and not very repeatable.

Although useful, these sperm motility evaluations are not completely reliable or repeatable, because of the small number of sperm evaluated, the lack of objectivity and human bias (Graham *et al*., 1980).

2.5.2.4 Sperm morphology

The structure or morphology, of the sperm cell has been studied using light and electron microscopy techniques. The sperm as such has been defined as a highly structured cell, streamlined to deliver DNA to the oocyte. Primary abnormalities may occur during spermatogenesis in the testis, while secondary abnormalities may occur during maturation in the epididymis and tertiary abnormalities result from poor handling of the semen following ejaculation. Generally sperm abnormalities associated with the head are classified as primary and those associated with the mid piece or sperm tail as secondary. Abnormalities of the sperm head include twin, tapering or pyriform, round, shrunken, large, narrow, elongated and diminutive heads. Abnormalities of the neck on the other hand include broken necks and loose necks (Evans & Maxwell, 1987).

The most common abnormalities of the sperm mid-piece include bent, broken, and short, enlarged or thickened, double, filiform, vestigial mid-piece or abaxial attachment of the mid-piece to the head of the sperm cell. The principal abnormalities of the tail include coiled, twin, broken, crooked, kinky, or truncated tails. Salisbury *et al.* (1978) reported the ageing of the sperm cells to result in morphological changes, even in semen kept under controlled temperatures. Periods of high ambient temperatures, together with high humidity may render a male infertile for up to 6 weeks and many abnormal sperm cells may appear in the ejaculates collected during the recovery period. The ram's fertility is often questionable when 20% or more cells are abnormal in a semen sample. Gil *et al.* (2003) regarded semen with less than 10% abnormalities, to be normal for sheep. Seasonal variations may influence the

percentage of abnormal sperm, with the number of abnormalities being highest in spring, and declining as the natural breeding season advances.

2.5.3 Objective semen evaluation

2.5.3.1 Introduction

Semen quality and its relationship to fertility are of major concern in animal production. The accurate evaluation of semen quality is thus of utmost importance. Conventionally, the laboratory tests for standard semen evaluation at most AI centres use light microscopy to estimate sperm survival and the percentage of motile sperm (Rowe *et al*., 1993). Although useful, these tests are not completely reliable or repeatable because of the small numbers of sperm eventually evaluated, lack of objectivity, and human bias (Graham *et al*., 1980). More objectivity and repeatability in the assessment of sperm motility can be achieved with the aid of the Computer Assisted Sperm Analysis (CASA) (Davis & Siemers, 1995).

2.5.3.2 Semen evaluation with the aid of the computer assisted sperm analyser (CASA)

Computer Assisted Sperm Analysis (CASA) has been introduced in the laboratory as a routine method to improve the accuracy and repeatability of sperm quality data collection, to avoid technician error resulting from the subjective evaluation of different technicians and to reduce the time spent in semen evaluation (Jane *et al*., 1996).

The use of this Computer Assisted Sperm Analysis allows for the objective measurement of several sperm parameters, for example motility, which offers a more reliable, unbiased and repeatable method of assessing sperm motility, than the subjective evaluation by the human eye (Colenbrander *et al*., 2003). Several CASA systems are available commercially, and may differ in their mode of functioning and in their ability to detect and measure the motility of sperm in different species. The majority of CASA systems (e.g. the ISASTM by Proiser, the Hobson Sperm Tracker using Sound and Vision or CEROSTM system by Hamilton Thorne), record the path and type of movement of a group of sperm in a wet preparation under a cover slip using a video camera. The signal received by the camera is digitized and the information is processed by a computer which reconstructs each individual sperm path trajectory for a certain number of frames. Subsequently, these sperm trajectories are mathematically processed, permitting them to be defined in a numerical form (Quintero-Moreno *et al*., 2003). The CASA system is able to determine a series of semen variables, including the number of

moving sperm, curvilinear velocity (VCL), linear velocity (VSL), linear coefficient (LIN), straightness coefficient (STR), frequency of sperm head displacement (BCF), etc. The kinematic parameters obtained from the CASA system are thus useful for research purposes, making the identification of sperm sub-populations co-existing in an ejaculate possible (Quintero-Moreno *et al*., 2003).

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

The CASA provides an accurate evaluation of semen parameters such as spermatozoa motility by avoiding errors that may arise as a result of subjective evaluation of different technicians and reduces the time spent on semen evaluation (Jane *et al*., 1996). More objectivity and repeatability in assessing sperm motility can be achieved by the Computer Assisted Sperm Analysis (CASA) (Davis & Siemers, 1995). The use of CASA offers a more reliable, unbiased and repeatable means of assessing sperm motility, compared to examination by the human eye (Colenbrander *et al*., 2003). Individual spermatozoa can be analysed and video images of the sperm cells are captured and analysed by the software.

2.5.3.2.2 Disadvantages of using the computer assisted sperm analysis (CASA)

The major problems with CASA are centered on the high cost of the instruments, which suggests its use only in sophisticated laboratories that perform a high number of routine semen evaluations (Verstegen *et al*., 2002). The instrument settings are relatively subjective and different CASA instruments use different mathematical algorithms (Rabinovitch, 2006). The degree of comparability of measurements across all instruments is not quite clear.

There are problems encountered regarding the accurate counting of high and low sperm concentrations (Rabinovitch, 2006). The measurements obtained following sperm counting, include a statistical counting error, while CASA requires extensive training and cross validation regarding technician competencies (Verstegen *et al*., 2002). The clinical significance of the kinematical variables is however severely limited. The analyses are not standardized due to the different instrument settings and algorithms (Rabinovitch, 2006).

2.6 Effect of environmental factors on sperm production and quality

The fertilization rate generally depends on the availability of a sufficient number of fertile sperm in the vicinity of the fertile ovum. In turn, the quality of these sperm depends on a number of biological and environmental factors. Certain factors, such as inadequate nutrition,

high ambient temperatures, and aging of the animal have negative effects on the overall semen production. On the other hand, an extended photoperiod in small stock, frequent semen collection, and certain genetic factors could positively stimulate sperm production (Flowers *et al.*, 1997). A thorough knowledge of factors affecting sperm quality and ultimately semen production is important in all AI centres (Soderquist *et al*., 1996).

2.6.1 Age of the ram

Much attention in the past has been paid to the effect of age of the individual on semen production and the season of collection, regarding the sperm morphology in bulls (Almquist & Amann, 1976; Almquist, 1982). The age of the bull at semen collection generally affects the volume of the ejaculate, the sperm concentration, and sperm motility. Several studies have suggested that an increase in age of the male is associated with a decline in certain semen parameters (Centola & Eberly, 1999). Ageing in rodents appears to cause certain histological changes in the testes, which in turn results in the decline of sperm quality (Tanemura *et al.*, 1993; Centola & Eberly, 1999). The scrotal circumference and ejaculate volume normally increase with increasing ram age, up to 5 years of age. These findings seem to indicate that the genital system of the ram undergoes maturational changes during this period (Osinowo *et al*., 1988; Toe *et al*., 2000). In men, semen volume, sperm concentration, total sperm count, sperm motility, progressive motility, and normal morphology have been found to decrease as age increases (Tanemura *et al*., 1993; Pasqualotto *et al*., 2005). Similarly, quantitative analysis of sperm motility characteristics using CASA has indicated an age-related decline in linearity (LIN), straight line velocity (VSL), and average path velocity (VAP) (Sloter *et al*., 2006).

Shannon and Vishwanath (1995) and Garner *et al*. (1996) have reported the morphology of sperm, semen concentration, semen motility and the volume of the ejaculate to improve with an increase in the age of the bulls. This supports the findings of Osinowo *et al*. (1988) who reported mature rams to generally have higher ejaculate volumes, sperm concentrations and total sperm per ejaculate than younger rams. Langford (1987) also found sperm output to increase with an increase in scrotal circumference. Generally, scrotal circumference can be used as an indicator of sperm production in sheep (Toe *et al*., 2000)

2.6.2 Season of the year

Seasonality has been shown to affect semen quality in bulls, boars, bucks, stallions, and rams (Thongtip *et al*., 2008). Seasonal variation in the thyroid activity and seminal characteristics has also been observed in Iranian fat-tailed rams (Zamiri *et al*., 2005). It was specifically shown that the highest values for thyroid stimulating hormone (TSH), T4, free T4 index, testosterone, total sperm number, percentage normal sperm, percentage live sperm, sperm concentration, semen volume and scrotal circumference were recorded from early summer to winter with the lowest values being detected at the end of spring. It has also been suggested that the thyroid gland may be involved in seasonal transition of reproductive activity in the ram (Thongtip *et al*., 2008).

Low semen quality with a decreased sperm concentration and motility and increased percentage abnormal sperm has also been found in thyroidectomized rams (Brookes *et al*., 1965). Most studies found evidence that season of collection significantly affects semen production (Graffer *et al*., 1988; Stalhammar *et al*., 1988). According to Schwab *et al*. (1987) the highest volume of semen, sperm concentration, and number of sperm per ejaculate are produced during winter. Menendez-Buxadera *et al*. (1984) also reported semen quality to be higher in winter. These results are contrary to the findings of Fuente *et al*. (1984), who obtained the lowest semen quality during winter. Seasonal effects on semen quality are caused by several factors, such as ambient temperature or humidity, day length, and available feed quality. Seasonal variations in total protein content of the seminal plasma were found in rams, being higher in autumn than in summer and winter (Gundogan, 2006).

2.6.3 Daylight length (Photoperiod)

Sexual behaviour in the ram can be influenced by many factors, including season of the year, genetics, breed differences, hormonal effects, post-weaning management, ambient temperature and nutrition (Mickelsen *et al*., 1982). Photoperiod is however the main environmental cue affecting sheep reproduction (Chemineau *et al*., 1992). Variation in the sexual response of sheep breeds, to photoperiodic stimuli appear to be affected by the latitudes where the animals are raised. Sheep and goats exhibit great seasonal variation in semen quality (Lebouef *et al*., 2000). Animals in the temperate zones are highly affected by photoperiod, while those in the tropical zones are less sensitive. D'Alessandro and Martemucci (2003) reported an improvement in the percentage of motile sperm to occur during decreasing photoperiod.

Reproductive responses to photoperiod are determined to a large extent by the degree of photo responsiveness, the nature of the photoperiodic signal, nutritional and social environment (Wildeus, 1995; Walkden-Brown & Restall, 1996). The nature of the photoperiodic signal is however important in determining the reproductive activity in seasonal breeders (Walkden- Brown & Restall, 1996). Photoperiodic signals are translated into effects on the reproduction system by changes in the pattern of secretion of melatonin from the pineal gland (Shelton, 1980; Wildeus, 1995; Walkden- Brown & Restall, 1996). This results in changes in the pulsatile release of GnRH, from the hypothalamus (Mori, 1992). In sheep and all mammals, the circulating levels of melatonin are generally low during the day and high at night. This profile of melatonin secretion is an endocrine signal, which relays the photoperiodic information to the reproductive axis (Karsch *et al*., 1985). As short days are characterized by a longer duration of melatonin secretion compared to long days, attempts have been made to mimic the duration and amplitude of the presence of melatonin in the blood. Continuous melatonin administration via nutrition, subcutaneous or intravaginal implants can stimulate an early onset of breeding activity by mimicking the onset of short photoperiodic environments (Poulton *et al*., 1987). In a study conducted by David *et al.* (2007), melatonin implants were found to produce a shorter response than photoperiodic treatment as such, which was less repeatable. Accelerated production of sperm by induction has been performed using different methods. These include the administration of Clomid® (Herbert *et al.*, 2002), testosterone implants (Adamopoulous *et al*., 1990). However these methods implicate certain problems regarding animal health, embryo mortality, fertility, immunology and environmental contamination. As blood testosterone levels and therefore the sexual activity are affected by photoperiod, rams need to be treated by other means that are less expensive and easier to apply.

2.6.4 Ambient temperature and testicular thermoregulation

2.6.4.1 Ambient temperature

For normal semen production to occur the testes have to be at a temperature several degrees below that of normal body temperature, otherwise sperm production may be affected. To provide the necessary thermoregulation for spermatogenesis, the ram has large sweat glands in the skin of the scrotum, as well as a system of muscles that raise or lower the testes nearer to the body for the purpose of temperature regulation. Blood flow to the testes also helps to

regulate temperature through a heat exchange mechanism. Heat is transferred from the testes to the blood and is then transported to other parts of the body for dissipation.

If the temperature in the testes cannot be kept low enough, as can happen in warm weather (e.g. ambient temperatures over 32°C for long periods or short spells of very high temperatures (38 °C or more)), the production of normal, viable sperm will be affected. Fully developed stores of sperm are less affected than those sperm still in the developing stages.

High body temperatures produced in rams by high summer temperatures or with fever is generally a cause of poor quality semen. This also affects semen formation or spermatogenesis and ultimately induces temporary sterility. These high temperatures can also affect mating, with subsequent reduced sexual activity. Elevated body temperature during periods of high ambient temperature leads to testicular degeneration and reduction in the percentage of normal and fertile spermatozoa in the ejaculate (Marai *et al.*, 2008).

2.6.4.2 Testicular thermoregulation

Several physiological mechanisms play a significant role in testicular thermoregulation. These include the regulation of blood flow, the control of the testis position, relative to the body by scrotal musculature, sweating, counter-current heat exchange in the vascular cone, and overall radiation of heat from the scrotal surface. The counter-current exchange of heat in the neck of the scrotum has been identified as the primary mechanism of regulating the temperature in the testes. It has been shown that the scrotum and testes have complimentary temperature gradients that contribute to testicular thermoregulation (Kastelic *et al*., 1996).

The testicular vascular cone is made up of a complex venous network that surrounds the highly coiled testicular artery. The counter-current, heat exchange within the vascular cone functions by allowing, the transfer of heat from the warm blood flowing down the testicular artery towards the testis, to the cooler blood returning from the testis through the testicular venous system (Cook *et al*., 1994). Waites and Moule (1961) reported the counter-current exchange to only cool the testis if a temperature gradient exists between the venous and arterial blood. The extent of this heat exchange then depends solely on the magnitude of the temperature gradient. The vascular cone also plays an important role in the radiation of heat from the scrotum, as the scrotal skin overlying the vascular cone is usually the warmest area on the scrotum (Acevedo, 2001)

2.6.5 The effect of nutrition on semen quality and fertility

Nutrition has a direct and dramatic effect on testicular size, which again has a corresponding effect on sperm production. Rams grazing on pastures of fluctuating quality may have testes which double (or halve) in size during the year due to the seasonal variation in quality of the pasture. Research has shown that an improvement in nutritional intake of both protein and energy during the two-month period prior to mating may increase the testicular size and subsequent sperm production by as much as 100%. Nutritional changes also affect testicle size much more rapidly than is reflected in the live weight or general body condition. This highlights the importance of checking the rams' reproductive soundness prior to the mating season. On the other hand, rams should not be allowed to become over-fat (body condition score more than 4), as obese rams tend to be less sexually active and are more prone to heat stress (Hafez, 1993). It is well documented that adequate nutritional management is crucial for successful mating in sheep flocks (Smith & Akinbamijo, 2000; Fernandez *et al*., 2004).

Carbohydrates, protein and nucleic acid metabolism and their deficiency may impair spermatogenesis and libido in males, with resultant lower fertility rates, embryonic development and survival, post-partum recovery activities, milk production, later development and lower survival rates in the offspring (Smith & Akinbamijo, 2000).

Vitamin A is essential for sperm production. Rams deficient in Vitamin A often have soft testicles and produce poor quality semen. Where rams have spent six months or more without access to any green feed, supplements which contain Vitamin A may be required (e.g. green hay, vitamin supplements).

A number of studies have demonstrated that spermatogenesis in rams is sensitive to an increase in protein intake. This effect has been related to an increase in testicular size, due to an increase in the volume of the seminiferous epithelium and the diameter of the seminiferous tubules (Oldham *et al*., 1978; Hotzel *et al*., 1998). The improvement of testicular efficiency with nutrition has also been reported by Oldham *et al.* (1978). It has been shown that rams maintained on a high plane of nutrition produce more sperm than those raised on a low plane of nutrition.

Masters and Fels (1984) demonstrated testicular size to be controlled by nutrition, even to the extent that well-fed rams in spring may have larger testes, compared to poorly-fed rams in autumn. Nutrition appears to mediate its effect by increasing the frequency of pulses of LH and probably FSH (Lindsay *et al*., 1984; Boukhliq *et al*., 1997; Hotzel *et al*., 2003). However, the energy components of the diet, particularly the fatty acids, appear to play a key role in reproductive responses following changes in nutrition. Fatty acids for example can stimulate the GnRH-dependent pathways that initiate changes in testicular function (Boukhliq & Martin, 1997; Blache *et al*., 2002).

2.7 Factors affecting the viability of sperm after semen collection

2.7.1 Temperature

The most important physical condition that sperm are extremely sensitive to is temperature. An excessive, fast decrease or increase in temperature causes sperm mortality (temperature shock). Such a change normally involves damage to the plasma membrane of the sperm cell, which contain temperature sensitive, unsaturated fatty acids. These lipids are sensitive to oxidization and excessive peroxidization disrupts the cell membrane, rendering the cell incapable of fertilization (Bester, 2006).

2.7.2 Semen pH

Stored semen following collection produce hydrogen ions, and as a result the pH decreases. Therefore, buffers are usually required to maintain semen at acceptable pH levels. If extended semen is maintained at body or room temperature, the sperm will be metabolically active, secreting acids, increasing the pH and will soon die, if not introduced into the female reproductive tract. Latif *et al.* (2005) also reported that in an acidic pH environment, the motility of sperm is affected, probably due to a change in the metabolic activity and a disturbance in the cellular respiration of the sperm cell.

2.7.3 Osmotic pressure

Semen and diluents must be isotonic as sperm maintain their maximum metabolic activity when semen is diluted with an isotonic extender. Swanson (1949) observed that bovine sperm are more sensitive to hypertonic solutions of sodium citrate than to hypotonic solutions. It was suggested that, as a result of glycolytic metabolism, an increase in the osmotic pressure of semen occurred during storage. However, both hypotonic and hypertonic extenders reduce

the metabolic activity and disrupt the membrane integrity, which leads to clumping and finally death of the sperm (Latif *et al*., 2005).

2.7.4 Concentration of sperm per ejaculate

A too concentrated semen sample decreases sperm metabolic activity, due to an increase in potassium content of the sperm cell. Dilution does not normally change the metabolic activity of the sperm, but will increase its lifespan. Excessive dilution (1 to 1000) on the other hand will depress the motility and metabolism. So when semen is prepared for artificial insemination (AI), the semen is diluted in an extender and the number of sperm per AI dose is standardized. This reduces the direct advantage of high sperm output in the ejaculate on the fertilizing potential. However, when a sample is diluted to standard sperm cell numbers, the seminal plasma is also diluted. The final dilution ratio of seminal plasma to the extender used in the AI straws generally depends on the sperm concentration at semen collection (Karoliina, 2009)

2.7.5 Gas environment

CO_2 stimulates aerobic metabolism, if kept below 5 to 10%.Too much oxygen however also decreases the sperm cell metabolism. The gaseous environment under which semen is stored can thus influence the motility via a change in intracellular ATP. The possible explanation for the CO_2-based inhibition of sperm motility is that the presence of CO_2 in the semen storage environment leads to the depletion of intracellular ATP. The CO_2 reacts with water to generate carbonic acid, a reaction catalyzed by intracellular carbonic anhydrase. An increase in CO_2 concentration in an aqueous environment, results in a decrease of the pH in that environment. The extent of this change in pH is a function of the magnitude of the CO_2 increase, and the buffering capacity of the solution. (Bencic *et al*., 2000)

2.7.6 Light exposure

Exposure to light can depress the sperm metabolic rate, motility, and fertilizing capacity that only occurs aerobically. It is recommended to never expose semen to direct sunlight as ultraviolet light can be lethal to sperm. That is why it is so important to transport the semen following collection inside a box, to protect it from direct sunlight (Bester, 2006).

2.8 Semen extenders or diluents

Cryopreservation as a technique for long term storage of semen has many advantages, but the freezing and thawing processes induce detrimental effects in terms of sperm ultrastructural, biochemical and functional damage (Watson, 2000), resulting in a decrease of sperm motility, membrane integrity and fertilizing ability (Purdy, 2006). The detrimental effects induced by cryopreservation may however be compensated for by using higher sperm numbers in the insemination dose (Watson, 1995). Related to this, various techniques of processing and freezing of sperm have been developed over the years, to reduce the cryogenic injury to sperm (Salamon & Maxwell, 1995**)**.

The purpose of a semen cryopreservation extender is generally to supply the sperm cells with a source of energy, to protect the cells from temperature related damage, while maintaining a suitable environment for the sperm to survive temporarily. Diluents are used in the semen cryopreservation process as these media increase the ejaculate volume without affecting semen quality and preserve the fertilizing capacity of the sperm for the longest period of time possible. Egg yolk is a general component of semen cryopreservation extenders used for domestic animals. This yolk has been shown to have a beneficial effect on sperm cryopreservation as a protector of the sperm plasma membrane and acrosome against temperature related injury – in association with other components, because of the lipids that it contains (Purdy, 2006). The semen extender for ram semen cryopreservation should also contain buffers for controlling the pH (6.7 to 7.0), antibiotics to prevent bacterial growth and cryoprotectants to prevent the crystallization of water within the sperm cells. This will ultimately allow sperm cells to be cryopreserved effectively (Salamon & Maxwell, 2000).

2.8.1 Components of ram semen extenders

Buffers are essential to control the pH between 6.7 and 7.0. Sodium citrate, egg yolk and Tris buffers are commonly used for this purpose. Lipids generally provide protection of the sperm membranes from temperature changes. Skim milk and egg yolk are generally good sources of lipids. Nutrients are also essential to provide energy for sperm cell. Fructose and glucose are typically used in the extenders. Antibiotics are included to prevent bacterial growth, while glycerol serves as a cryoprotective agent in the semen freezing process. It prevents the crystallization of water within the sperm cells, which ultimately allows the sperm cells to be frozen rapidly (Holt, 2000)

2.8.1.1 Example of a semen extender

1) Egg yolk- citrate (Fraction A)
20% (v/v) egg yolk
80% (v/v) of a 2.9% (weight/vol) sodium citrate dehydrate
1000 IU Penicillin/mL
1000 μg Streptomycin/mL

2) Egg yolk- citrate + glycerol (Fraction B)
20% (v/v) Egg yolk
66% (v/v) of a 2.9% (weight/vol) sodium citrate dehydrate
14% (v/v) glycerol- provides final concentration of 7% glycerol

Glycerol is added after the semen has been cooled to 5°C. This prevents morphological damage to sperm, compared with glycerol added at room temperature. The glycerol fraction is generally added in three equally timed steps and volumes. The final volume of the extender should contain equal quantities of Fraction A and B.

2.8.2 Cryoprotective agents

Cryoprotective agents or cryoprotectants are included in the cryopreservation medium to reduce the physical and chemical stresses derived from cooling, freezing and thawing on the sperm cells (Gao *et al*., 1997; Purdy, 2006). Cryoprotectants and their modes of action has been the subject of many reviews, with glycerol and DMSO being the most commonly used cryoprotective agents (Karow, 1981; Mazur, 1984; Brockbank, 1995). Fetal bovine serum (FBS) is also often used in mammalian cryopreservation solutions, but it is not a real cryoprotective agent. However dextrans, glycols, starches, sugars, and polyvinylpyrrolidone provide considerable cryoprotection in a variety of biologic systems (Mazur, 1981).

The cryoprotectants can be classified as penetrating or non- penetrating agents.

The penetrating cryoprotectants or intracellular cryoprotectants (glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol) have low molecular weights, and induce membrane lipid and protein rearrangement, resulting in increased membrane fluidity, greater dehydration at lower temperatures, reduced intracellular ice formation, and an increased survival rate to

cryopreservation (Holt, 2000). Additionally, the penetrating cryoprotectants are solvents that normally dissolve sugars and salts in the cryopreservation medium (Purdy, 2006).

Non-penetrating cryoprotectants or extracellular cryoprotectants (egg yolk, non-fat skimmed milk, trehalose, amino acids, dextrans, and sucrose) on the other hand have relatively high molecular weights, and do not cross the plasma membrane and only act extracellularly (Aisen *et al.*, 2000). Therefore, the non-penetrating cryoprotectant may alter the plasma membrane, or act as a solute, lowering the freezing temperature of the medium and decreasing the extracellular ice formation (Amman, 1999; Kundu *et al*., 2002).

Cryoprotectants generally protect frozen sperm cells by one or more of the following mechanisms: Suppressing the high salt concentrations; reducing cell shrinkage at a given temperature; reducing the fraction of the solution frozen at a given temperature and minimizing intracellular ice formation. Combinations of cryoprotectants may also result in the additive or synergistic enhancement of cell survival following cryopreservation (Brockbank, 1992; Brockbank & Smith, 1993).

2.9 Semen cryopreservation techniques

Two methods are currently being used for gamete cryopreservation. These entail the slow freezing and vitrification techniques. The slow freezing method uses a low concentration of cryoprotectants, which may be associated with chemical toxicity and osmotic shock to the semen sample. Vitrification as such is a rapid freezing method that decreases the occurrences of cold shock, but is usually not performed as heat transfer in sperm cells is too slow to permit vitrification without the risks of solution effects or ice crystal formation (Arav *et al*., 2002). Vitrification also does not require expensive freezing equipment and the method (vitrification/warming) only takes a few seconds (Isachenko, 2003). Classical vitrification generally requires a high proportion of permeable cryoprotectants in the medium (30–50% compared to 5–7% for slow freezing) and seems to be detrimental for the sperm cells, due to lethal osmotic effects and possible chemical alterations. The failure to successfully vitrify sperm can be further explained by the extreme sensitivity of spermatozoa to high concentrations of cryoprotectants and the low osmotic tolerance of most mammalian sperm (Gao *et al*., 1995). The cryopreservation technique thus includes temperature reduction, cellular dehydration, eventual freezing and subsequent thawing (Medeiros *et al*., 2002). The lowering from room temperature to 4°C reduces the cellular metabolic activity and increases

the life span of the sperm cells. Cryopreservation generally stops all cellular activities, restarting its normal metabolic functions, after thawing (Mazur, 1984).

In domestic animal species, fast cooling between 30 and 0°C causes cell damage in certain sperm cells, the so called ‘‘cold shock’’, which is dependent on the cooling rate and temperature interval (Gilmore *et al*., 1998; Watson, 2000). The cooling or freezing rate must be slow enough to allow water to leave the cells by osmosis, preventing intracellular ice formation, which causes irreversible damage to the sperm cells (Fiser & Fairfull, 1986). Sperm cells are usually frozen at fast rates (15–60°C/ min), which gives rise to best post thawing results (Byrne *et al*., 2000; Anel *et al*., 2003).

Semen cryopreservation induces the formation of intracellular ice crystals, osmotic and chilling injury that gives rise to sperm damage e.g. cytoplasmic fracture, effects on the cytoskeleton and genome related structures (Isachenko, 2003). The membrane permeability is increased after cooling, and may be a consequence of increased membrane leakiness, and specific protein channels. Calcium regulation is again affected by cooling and this has severe consequences on cellular function, including cell death. The uptake of calcium during the cooling process then influences capacitation changes and fusion events between plasma membrane and acrosomal membrane. As known, the sperm membrane is a structure that undergoes vast reorganization during the capacitation process. Cold shock reduces membrane permeability to water and solutes, while damaging the acrosomal membrane (Purdy, 2006).

The main changes that occur during semen freezing are mainly ultrastructural, biochemical and functional. These impair the sperm transport and survival in the female reproductive tract and reduce the resultant fertility in domestic animal species. The ultrastructural damage has been found to be greater in the ram than in bull sperm. Greater damage has also been detected in the plasma and acrosomal membranes, mitochondrial sheath and axoneme (Salamon & Maxwell, 2000). In frozen-thawed semen, the sperm motility is generally better preserved than the morphological integrity. The plasma and the outer acrosome membranes of the sperm cell are the most cryosensitive. Biochemical changes have been detected, including the release of glutamic-oxaloacetic transaminase (GOT), losses of lipoproteins and amino acids, a decrease in phosphatase activity, a decrease in loosely bound cholesterol protein, an increase in sodium and a decrease in the potassium content, inactivation of hyaluronidase and

acrosin enzyme, the loss of prostaglandins, the reduction of ATP and ADP synthesis and a decrease in the acrosomal proteolytic activity (Salamon & Maxwell, 1995).

The cryopreservation protocol as such causes several injuries to the sperm cell by way of several factors e.g. the dramatic changes in temperature, submission to osmotic and toxic stresses derived from exposure to molar concentrations of cryoprotectants and finally the formation and dissolution of ice in the intracellular and extracellular environment. These damaging effects of cooling and freezing on the sperm membrane differ among domestic species and is influenced by several components, namely cholesterol/ phospholipids ratio, content of lipids in the bilayer, the degree of hydrocarbon chain saturation and protein/ phospholipid ratio (Medeiros *et al*., 2002). Boar sperm is generally the most sensitive. Bull, ram and stallion sperm are also very sensitive; while dog and cat sperm are somewhat sensitive; rabbit, human, and rooster sperm are the least sensitive to cold shock (Parks, 1997).

Cryoprotectants are generally included in cryopreservation medium to reduce physical and chemical stresses derived from cooling, freezing and thawing of sperm cells (Gao *et al*., 1997; Purdy, 2006). These cryoprotectants, as mentioned previously, are classified as either penetrating or non-penetrating (See 2.8). Glycerol is frequently used as a cryoprotectant for the freezing of ram semen. Glycerol or dimethyl sulfoxide (DMSO) can however induce osmotic stress and toxic effects on the sperm, but the extent of the damage varies according to the species and depends on the concentration of the cryoprotectant in the extender solution (Purdy, 2006). Egg yolk is a normal component of semen extenders, protecting the sperm cell against cold shock and supporting the cell membrane during freezing and thawing. The protective mechanisms are determined by the phospholipids (lecithin) and the low density lipoproteins (Medeiros *et al*., 2002; Purdy, 2006). Egg yolk thus acts on the cell membrane, having a greater effect on bull than ram sperm. For the freezing of ram semen in ampoules, 3 to 6% egg yolk has been used, but for straws and pellet freezing higher concentrations are required (15-17%), although the effect is dependent on the extender composition (Salamon & Maxwell, 2000).

It would seem as if the increased concentrations of egg yolk in the semen extender may reduce the glycerol levels. In the formulation of semen extenders, glycerol may be added initially or later in a separate fraction (glycerolated fraction), after semen refrigeration. In the first situation, the complete extender is added after semen collection (one step method). In the

second situation a fraction of the extender (without glycerol) is added after semen collection, and the remaining portion (with glycerol) is added after refrigeration, prior to semen freezing (two step method) (Evans & Maxwell, 1987). Effective cryoprotection after a short (5-10 seconds) contact with glycerol, has been demonstrated for bull, boar and ram semen (0-5 minutes) – which proves that the penetration of glycerol into the cell is not essential for sperm protection. This still remains a controversial subject (Barbas & Mascarenhas, 2009).

The freeze-thaw process increases the maturation of sperm membranes and induces capacitated acrosome reaction in sperm. These modifications may not affect the initial sperm motility, but can reduce the lifespan, the ability to interact with the female reproductive tract and ultimately affect the sperm fertility (Medeiros *et al*., 2002). Cooled sperm have displayed, an increase in the intracellular free Ca^{2+}, typical of capacitated sperm following chlortetracycline staining. Cryopreservation of semen also induces the formation of reactive oxygen species (ROS), which impair good fertilization (Alvarez & Storey, 1993; O'Flaherty *et al*., 1997).

In ram and buck semen freezing methods, there are many similarities, e.g. in the type of extenders, cryoprotectants, and cooling rates utilized (Salamon & Maxwell, 2000). The cryopreservation extenders used for goat and ram semen generally include either egg yolk or non-fat dried skimmed milk. Sanchez-Partida *et al*. (1992) showed that low concentrations (50 mM) of proline and glycine-betaine improved the post thaw motility of ram sperm. In rams and bucks, semen may be diluted using either one or two step method. Normally, diluted semen is cooled to 4-5°C during a 1.5-3h period, and thereafter aspirated into mini straws (0.25mL). Freezing may then be performed over liquid nitrogen vapour or in a programmable biofreezer. In the first method, filled straws are arranged horizontally at a height of 4 to 5cm over the liquid nitrogen vapour for a variable time (10-20 min), with good post thawing results (Byrne *et al*., 2000; Leboeuf *et al*., 2000).

Programmable freezers are frequently used at AI centres when freezing large quantities of semen straws. The freezing rates vary according to the research laboratories, so for example, the freezer may be at the following freezing rates: from 4 to -5°C at 20°C/min, -5 to -110°C at 55°C/min and -110 to -140°C at 35°C/min, followed by the immersion of the straws into liquid nitrogen (Byrne *et al*., 2000; Leboeuf *et al*., 2000).

2.10 Thawing of cryopreserved semen

Before the thawing of semen straws is attempted, the liquid nitrogen tank must be filled to avoid an increase in temperature**.** A liquid nitrogen container temperature above -120℃ has been shown to lead to irreversible damage to the sperm. For the thawing of semen, the canisters containing the semen straws must be raised up to the neck of the nitrogen tank and then lowered to the bottom. These manipulations may cause temperature fluctuations in the straws remaining in the canister (Nur *et al*., 2006).

Thus during the freeze-thawing of semen, the warming phase is critical for the survival of the sperm as, well as the cooling phase (Fiser *et al*., 1987). During thawing, frozen semen will cross the critical temperature range between -15 and -60°C. The thawing rate is dependent on whether the cooling rate has been sufficiently high to induce intracellular freezing, or low enough to produce cell dehydration. In the first instance, fast thawing is required to prevent recrystallization of any intracellular ice present in the sperm cell. Sperm thawed at a fast rate are also exposed for a short period of time to the concentrated solute and cryoprotectant, and the restoration of the intracellular and extracellular equilibrium is more rapid, than with slower thawing (Fiser *et al*., 1987). Ram and buck semen is generally thawed at 38 to 42℃ for 30s, but thawing at higher temperatures (60-75°C), may produce similar post-thaw sperm motility, acrosome integrity and fertility of the sperm (Evans & Maxwell, 1987).

Chapter 3

Materials and methods

3.1 Study location

This study was conducted at the Agricultural Research Centre (ARC)-Irene campus (25° 55' S; 28° 12' E), South Africa (S.A). The centre is located in the Highveld region of South Africa, at an altitude of 1525m above sea level. The climatic conditions and ambient temperatures range from hot days to cool nights in summer, to moderate winter days with cool nights.

3.2 Experimental animals

Eight indigenous rams, between 2 and 4 years of age and weighing between 40 and 56kg were used in the trials. Hundred and twenty eight (128) ejaculates were collected during the entire study. Semen was collected twice a week (every Monday and Tuesday) from Damara, Namaqua Afrikaner, Pedi, and Zulu rams, using the electro-ejaculator. The animals were maintained on natural grazing, supplemented with 300 to 350g concentrate per day, with water being available *ad lib*.

Plate 3.1 Damara ram used as a semen donor

Plate 3.2 Namaqua Afrikaner ram used as a semen donor

Plate 3.3 Pedi ram used as a semen donor

Plate 3.4 Zulu ram used as a semen donor

3.3 Preparation of Diluents

Most of the chemicals were supplied by Sigma except for the ultrapure water which was obtained from TRANSFARM, South Africa. Glycerol was purchased from Pal Chemicals. All semen extenders were prepared on the day before semen collection, and freshly laid eggs (egg yolk) were always used. The egg yolk- citrate extender was derived from two different portions, the one without glycerol (diluent A) and the other containing glycerol (diluent B). Different glycerol inclusion levels were used and the two step dilution procedure was always used.

3.3.1 Preparation of the egg yolk- citrate extender

The composition of the fractions (A and B) of the ram semen extenders used is set out in Table 3.1. Following the preparation, the semen extenders were stored at 5°C, until utilized. The procedure for the preparation and composition of the sperm washing medium is set out in Table 3.2. This solution was stored and used within 30 days, thereafter a new batch was prepared.

Table 3.1 Preparation of egg yolk extender (g/100mL)

Fraction A

Na.Citrate. $2H_20$: 1.856g

Glucose: 1.0g

Water: 80mL

CPA: 0mL

Egg yolk: 20mL

pH: 7.0

Gentamycin Sulphate (optional): 0.1g

Fraction B

Na.Citrate. $2H_20$: 1.856g

Glucose: 1.0g

Water: 66mL

CPA%: 14mL

Egg yolk: 20mL

pH: 7.0

Gentamycin Sulphate (optional): 0.1g

3.3.2 Protocol for preparing the sperm washing solution (BO-W) (Brackett & Oliphant, 1975)

Table 3.2 Preparation of 10xBO stock solution A (effective for 30 days)

Component	Molecular Wt.	mM	g/100mL
NaCl	58.44	112.00	6.5453
KCl	74.56	4.02	0.2997
$NaH_2PO_4.H_2O$	137.99	0.83	0.1145
$MgCl_2.6H_2O$	203.30	0.52	0.1057
$CaCl_2.2H_2O$*	147.02	2.25	0.3308
Glucose	181.16	13.90	2.5181
Antibiotics (penicillin) or			5mL or 10mL
Streptomycin (0.02g/ mL)		50mg/mL	0.68 or 2.5mL
0.2% Phenol Red			4mL

Addition of ultrapure water (Sabax) to a volume of 100mL

The BO-W solution was filtered using a craft suction unit (Rocket) and a 250mL filter system and prepared by firstly dissolving the components separately in approximately 5mL of ultrapure water (Sabax). Thereafter water was added to a volume of approximately 80mL, after which the pH was checked and adjusted to 7.4. This sperm washing solution was then stored at 4°C, until utilized.

Table 3.3 Preparation of 1xBO working solution B (effective for 2 weeks)

Component	Molecular Wt.	mM	g/100mL
$NaHCO_3$	84.01	37.0	0.3108
Na-Pyruvate	110.04	1.25	0.0138
Take 10mL of BO stock (solution A)			(10mL)

Solution B was prepared as set out in Table 3.3. After adding all the ingredients listed in Table 3.3, ultrapure water (Sabax) was added to a volume of 100mL. The pH was checked and adjusted to 7.4 after adding caffeine and then filtered.

3.3.3 Preparation of the sperm washing solution (BO-W)

80 mL of BO working solution B was taken to prepare the sperm washing solution, to which 3.0mg/mL BSA (Fraction V, A-9418) was added and the solution filtered. The remaining 20mL was used for preparing IVF maturation medium.

3.4 Semen collection and quality evaluation

3.4.1 Semen collection

The semen of all individual rams was collected with the aid of an electro-ejaculator (Ramsem, South Africa). Two ejaculates were collected per week/ram at an interval of two days. The semen was collected directly into 15mL tubes, and immediately placed in a thermo flask at 37°C. The collected ram semen was then transported to the laboratory for microscopic sperm evaluation within an hour. The raw semen samples were firstly evaluated macroscopically for the ejaculate volume, pH and sperm concentration. Spermatozoa parameters (motility and velocity) were microscopically evaluated using the computerized Sperm Class Analyser® (CASA system).

Plate 3.5 Electro-ejaculator used for semen collection

Plate 3.6 Thermo flask used for temporary semen storage after collection

3.4.2 Semen evaluation

3.4.2.1 Semen concentration

Semen concentration (sperm/mL) was determined with the aid of a spectrophotometer calibrated for ram semen (SpermaCue®, Minitüb, Germany). Briefly, 20µl of undiluted raw

semen sample was pipetted into a microcuvette (HemoCue AB, Ängelholm, Sweden). This sample was then inserted into the spectrophotometer to give an automated sperm concentration reading in terms of the number of sperm/mL ($X10^6$).

Plate 3.7 SpermaCue® used for the determination of sperm concentration

Plate 3.8 Semen pH meter used in this study

3.4.2.2 Semen pH

The semen pH was measured using a microprocessor pH/mV/°C meter fitted with a glass probe (Hanna HI 931401, Portugal). The probe was rinsed in ultrapure water and wiped dry with a paper towel before and after each semen sample measurement.

3.4.2.3 Sperm motility evaluation using the CASA system

The Computer Assisted Sperm Analysis (CASA) system was used to analyse the sperm motility with the aid of a Sperm Class Analyzer®-SCA® (V.4.0.0.1 Animal/Veterinary Microptic S.L, Barcelona, Spain). The sperm swim-up technique was used, where 10µl of the semen sample was diluted with 500µl of the Bracket and Oliphant (BO) medium, developed by Bracket and Oliphant (1975) and stored for 5 minutes in an MCO-20 AIC Sanyo CO_2 incubator (Sanyo Electric Biomedical Co., Ltd, Japan), at 37°C. Following the storage period, 5µl of this semen solution was pipetted onto a pre-warmed bevel-edged, frosted-end microscope glass slide (Thermo Scientific Menzel-Gläser, Germany), gently covered with a microscope cover slip (Menzel-Gläser, Germany) and evaluated under 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 progressively motile sperm (sperm with forward movement), percentage non-progressively motile sperm and percentage static (immotile) sperm). Sperm velocity parameters evaluated included the static, slow, medium, rapid, curvilinear (VCL), straight-line (VSL), average path (VAP), linearity (LIN), straightness (STR) and wobble (WOB) velocities.

Table 3.4 The definitions of sperm motility descriptors when using the CASA system

Descriptors	Abbreviation	Unit	Description
Curvilinear velocity	VCL	µm/s	Velocity of progression along the entire trajectory
Average path velocity	VAP	µm/s	Velocity of progression along the smoothed trajectory
Straight line velocity	VSL	µm/s	Velocity of progression from first to last coordinates
Beat cross frequency	BCF	Hz	Frequency that the sperm head crosses the smoothed trajectory
Amplitude of lateral	ALH	µm	Mean lateral sperm head displacement along the smoothed head displacement trajectory
Linearity of track	LIN	%	VSL/VCL×100
Straightness of track	STR	%	VSL/VAP×100
Wobble	WOB	%	VAP/VCL×100

Source: Holt *et al*. (2007)

Table 3.5 Sperm Class Analyser® V.4.0.0 settings used to analyse the ram sperm cell 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 - 70µm^2
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

Plate 3.9 Incubator used for semen incubation prior to sperm motility evaluation

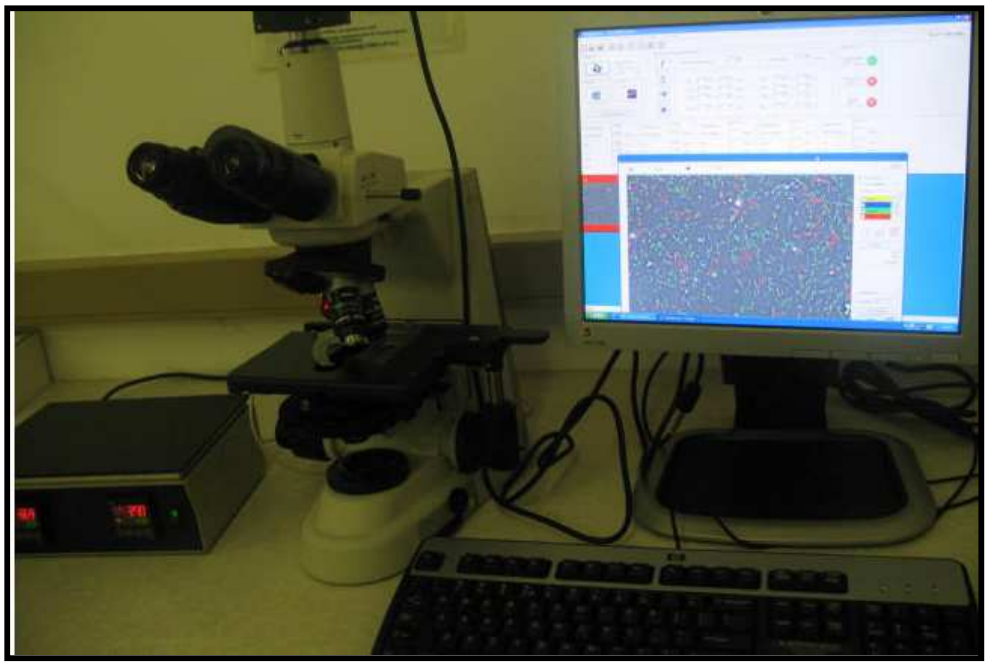

Plate 3.10 Sperm Class Analyzer® used for sperm motility evaluation

3.4.2.4 Sperm morphology and viability

Sperm viability was determined with the aid of an eosin/nigrosin stain (pH=8.39; osmolarity=411), manufactured by the Onderstepoort Faculty of Veterinary Sciences' pharmacy (60µl of eosin/nigrosin stain plus 6µl of semen). This staining method indicates the percentage live or dead sperm cells, while allowing a good evaluation of the morphology of the sperm cell (Bjoerndahl *et al.*, 2004).

The sperm smears were prepared on a clean, warmed glass slide to avoid temperature shock and evaluated on the same day of semen collection with the aid of a fluorescent microscope (Olympus BX 51TF) – using an oil immersion objective (X100 magnification). A total of 100 sperm/slide were evaluated and counted for each animal per collection, using a DBC.6 Model laboratory counter (Han Lien International Corp) and the gross structural sperm abnormalities recorded (Hidalgo *et al.*, 2007).

Under the microscope the live sperm fluoresced white as no stain was absorbed by the cell, while the dead sperm on the other hand fluoresced red, as the cells absorbed the stain (Bearden *et al.*, 2004). The live spermatozoa were further categorized into morphologically normal or abnormal cells. Abnormalities were recorded using two different sets of criteria. The first set of criteria used the location of the abnormality i.e. head (e.g. bulb, small,

enlarged, looped, etc.), mid-piece and tail (e.g. swelling, looping, partial or totally lacking, etc.) as described by Łukaszewicz *et al*. (2008).

Sperm abnormalities were further classified as either being primary or secondary, according to the degree of the lesion. These abnormalities were thus set out, using the following criteria (Loskutoff & Crichton, 2001):

The primary sperm abnormalities included the following:
Sperm head: microcephalic (small heads), macrocephalic (large/swollen heads), double heads and abnormal acromosomes. Mid-piece of the sperm cell: swollen, elongated and abaxial bodies. Tail of the sperm: double and short tails.

The secondary sperm abnormalities included the following:
Sperm head: detached, loose and damaged acrosome. Mid-piece of the sperm cell: bent and containing protoplasmic droplets. Tail of the sperm cell: bent, shoe-hook and protoplasmic droplets on the tail.

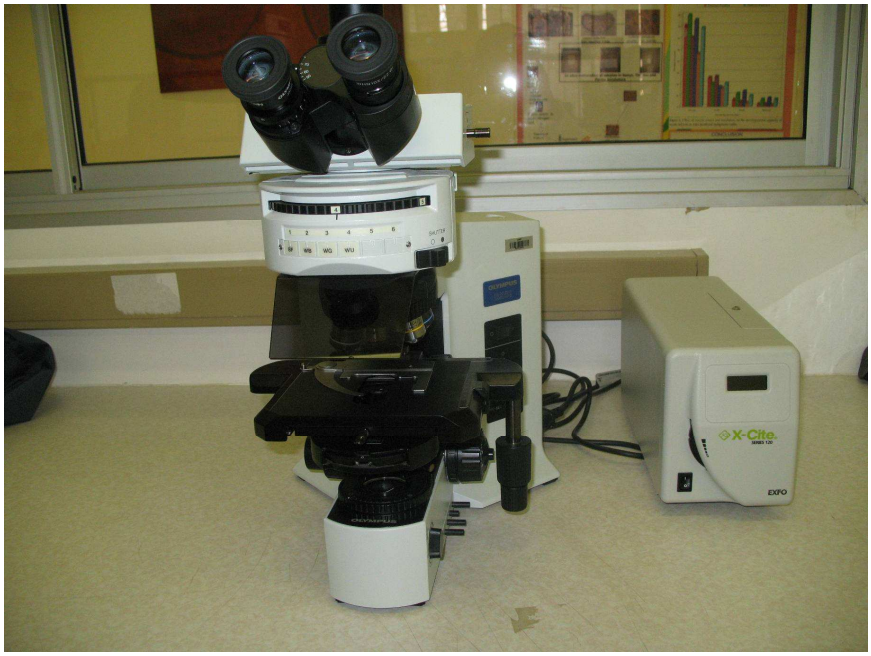

Plate 3.11 Fluorescent microscope (BX 51TF) used for sperm morphology and viability

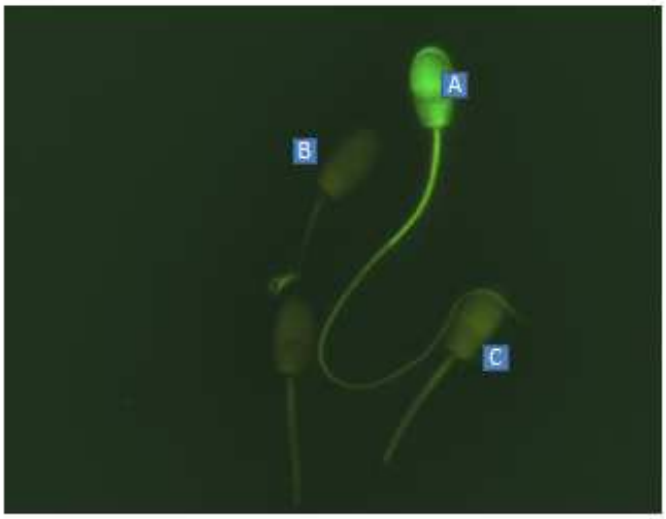

Plate 3.12 Eosin/nigrosin stained ram sperm cells. A is a live and normal ram sperm; B is a dead sperm with a mid-piece abnormality and C is a dead, normal sperm.

3.5 Liquid storage of ram semen

Two experiments were conducted where a comparison was made between semen stored at two different temperatures (5 and 15°C) for various storage periods. In the first experiment only fraction A (without glycerol) of the egg yolk-citrate diluent was used and in the second experiment both fractions A and B were used. For the experiment where only fraction A of the egg yolk-citrate extender was used, semen samples were diluted 1:1. For the experiment where egg yolk-citrate diluent containing 14% glycerol was used, the samples were first diluted with 1:1, v/v of fraction A, and later diluted 2:1 v/v with fraction B (with glycerol) – resulting in a final glycerol inclusion level of 4.7%. After semen evaluations, the ejaculates were divided into two aliquots. The one aliquot being kept at 5°C and the other at 15°C in a Defy VT60 cooler**.** Sperm motility and velocity evaluations were performed following 3, 6, 9 and 24h of storage.

Plate 3.13 Walk-in refrigerator used during semen storage and processing

Plate 3.14 Defy VT60 cooler used during liquid semen storage

3.6 Semen cryopreservation

After macroscopic semen evaluation in the laboratory, semen samples were diluted 1:1 with the egg yolk citrate (EYC) fraction A (without glycerol, at a ratio of 1:1, v/v), at 37°C and maintained in a cold room (Recam international, South Africa) at 5°C for 2h. Two hours

after the addition of the first extender (Fraction A), fraction B (with glycerol) of the egg yolk citrate extender was added at a ratio of 2:1, (v/v) and the semen samples were further cooled for another 2h. The cooled semen samples were then loaded into 0.25mL straws. The semen straws were put into the straw holder and frozen in a programmable freezer (CBS freezer 2100 series, Custom Biogenic Systems, SA), using a customized freezing curve as set out in Table 3.4. The straws were then plunged into a liquid nitrogen tank (-196ºC) for later sperm analysis (Hammadeh *et al*., 2001).

Table 3.6 The freezing rates used to cool indigenous ram semen during cryopreservation

From(°C**)**	**To(**°C**)**	**Rate of cooling(**°C/min**)**
5	-5	4
-5	-110	25
-110	-140	35

Ram semen was pooled because of the low ejaculate volumes obtained from the indigenous rams, to test the three different glycerol concentrations. The pooled semen sample was diluted 1:1 v/v with an egg yolk citrate fraction A (without glycerol) and was then divided into 4 aliquots. The semen samples were then maintained in a cold room (Recam international, South Africa) at 5°C for 2h. Two hours after the addition of the first extender, one group from the four aliquots was diluted with EYC fraction A, which served as a control. The other three aliquots were diluted with EYC fraction B containing 7, 10 and 14% GLY in a ratio of 2:1(v/v) resulting in the final glycerol inclusion levels of 2.3, 3.3 or 4.7% respectively. The samples were further cooled for another 2h, then loaded into 0.25mL semen straws and frozen, by placing the straws 5cm above the liquid nitrogen (LN_2) for 10 minutes, before being plunged into LN_2 and stored in LN_2 storage tank (-196°C) for future evaluation.

Plate 3.15 The programmable freezer used for semen freezing

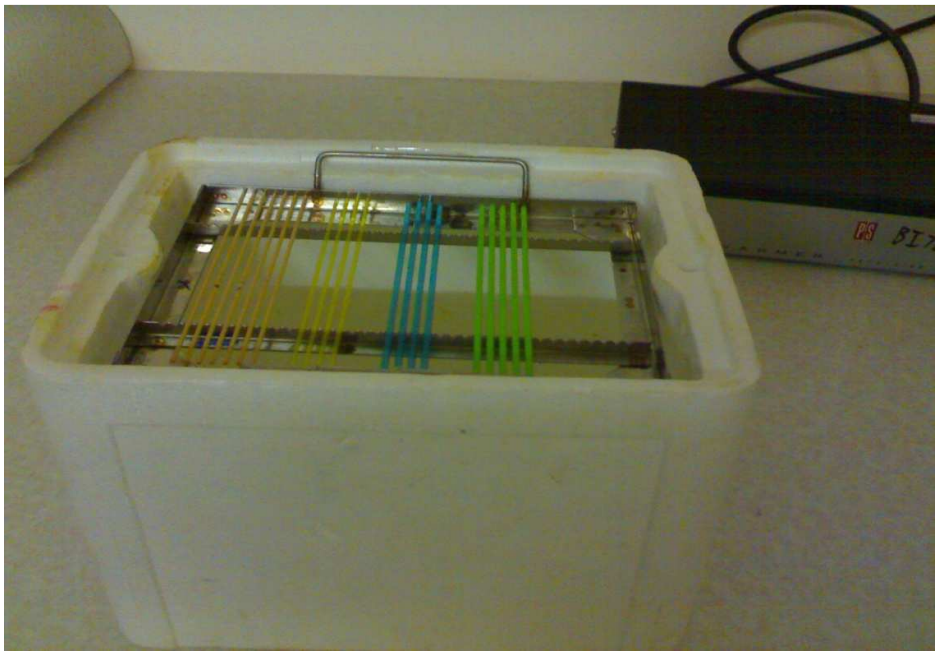

Plate 3.16 Freezing of semen in liquid nitrogen vapour

Plate 3.17 Liquid nitrogen tanks used for semen storage

3.7 Thawing of semen for the post-thaw semen analyses

Semen straws were thawed 7 days after cryopreservation by placing them in a water bath [Julabo P (Julabortechnik GMBH) West Germany] at 37°C, for 30 seconds (Purdy, 2006). Both ends of the sealed straws were cut with a pair of scissors and the semen poured into 15mL test tubes. 10µL of semen was diluted with 500µL BO sperm washing solution and stored at 37°C for 5 minutes in an MCO-20 AIC Sanyo 5%CO_2 incubator (Sanyo Electric Biomedical Co., Ltd, Japan). The semen samples were then evaluated for sperm motility and velocity parameters using the same method as for raw semen.

Plate 3.18 Water bath used during thawing of the semen straws

3.8 Statistical analyses

Data were analysed using the statistical program GenStat®. Analysis of variance (ANOVA) was used to test for significant differences in semen volume, semen concentration, semen pH, sperm morphology, sperm motility and in the treatments (temperature, storage periods, glycerol, different glycerol levels and freezing methods). Treatment means were separated using the Fishers protected t-test least significance difference (LSD), at the 5% level of significance (Snedecor & Cochran, 1980).

Chapter 4

Characterization of South African indigenous ram semen

4.1 Introduction

Several adapted indigenous sheep breeds with superior genetic traits are available in South Africa. These include the Damara, Namaqua, Pedi and Zulu sheep breeds, each with unique traits that make them adapted to South African conditions. Therefore, for preservation of these valuable indigenous genetic resources, it is essential to maintain the animals with certain unique production qualities, using the techniques of semen cryopreservation and AI (Ehling, 2006). Most of the South African indigenous sheep breeds are currently under threat of extinction mainly due to crossbreeding practices, and thus their genetic properties need to be preserved – in order to be used in genetic sheep improvement programmes of the specie and genetic resource banking (Holt, 1997). Cryopreservation is then one of the technologies which can be utilized to preserve these genetic resources e.g. the oocytes, sperm, somatic cells and embryos (Bester, 2006).

Generally, the technique of semen cryopreservation involves temperature reduction, cellular dehydration, freezing and thawing processes (Medeiros *et al.*, 2002). The lowering of the environmental temperature, from room temperature (20°C) to 4°C generally reduces the cellular metabolic activity, and thus increases the life span of the sperm cells. Cryopreservation as such, stops the cellular activity, restarting its normal metabolic functions after thawing (Mazur, 1984).

Sperm cryopreservation usually induces the formation of intracellular ice crystals, osmotic and chilling injury that may cause sperm cell damage, cytoplasmic fracture, and even has effects on the cytoskeleton or the genome related structures (Isachenko, 2003). The main changes that occur during the freezing of gametes are mostly related to ultra-structural, biochemical and functional activities, which may ultimately impair sperm transport and decrease the survival rate in the female reproductive tract, post thawing – thereby reducing fertilization rate. Ultra-structural sperm damage is generally greater in the ram than in the bull and seems to be species related (Salamon & Maxwell, 2000).

Sperm preservation protocols differ between animal species, due to their inherent abilities to accommodate variations in semen extenders used in the cooling and freezing processes (Barbas & Mascarenhas, 2009). These differences between species regarding the sensitivity of their sperm to cooling are then largely attributed to the compositional variation in the sperm plasma membranes (Bailey *et al*., 2000). Differences in fatty acid composition and sterol levels of the cell membrane have also been associated with the tolerance of sperm to cold shock and cryopreservation. Thus, the observed variation among species in sperm survival rate, after freezing and thawing, has been attributed to these differences. A higher ratio of unsaturated/saturated membrane fatty acids, and lower levels of cholesterol in the bull and ram sperm cell membranes, compared to the human and dog, have been suggested to be a reason for the differences encountered in cold shock and cryopreservation tolerance recorded between these species (White, 1993). There may then also be considerable differences between breeds and between individual males, regarding the 'freezability' of their semen (Hiemstra *et al*., 2005).

A thorough knowledge of the sperm physiology for a specific species or even breed is thus essential to maximize post-thaw sperm survival and subsequent fertility (Purdy, 2006). Protocols for different species, including the ram have been developed and tested over time, on various exotic breeds. There is however a need to study and characterize the quality of indigenous (in this case, South African) ram semen, as it will ultimately determine the fertility rate achieved. It is deemed necessary to cryopreserve indigenous ram semen and to develop extenders that may optimise the sperm cryosurvival and guarantee their survival. Semen quality and its relationship with male fertility are of utmost importance in animal production. Moreover, standard semen analyses are routinely implemented to determine the acceptability of processed semen for breeding purposes. In this study, the Computer Assisted Sperm Analysis (CASA) system has been used to accurately measure the motility characteristics of the indigenous ram sperm cells. The aim of this study was thus to characterise indigenous South African ram semen macroscopically (volume, pH and sperm concentration) and microscopically (sperm motility rates).

4.2 Materials and Methods

Semen was collected from rams of different South African indigenous breeds (Damara, Namaqua Afrikaner, Pedi and Zulu), during May (end of autumn – i.e. during the natural breeding season), 2009. Two rams per breed were available and used due the scarcity of these

breeds of rams. The ages of the rams ranged between 2 and 4 years and all animals were maintained on natural pastures, supplemented with a commercial pelleted diet. Water was available *ad libitum*. A total of four ejaculates were collected from each ram. Semen was collected twice a week, at an interval of two days using the electro-ejaculator. Samples were collected in graduated test tubes and placed in a thermo flask at a temperature of 37°C. Semen was then transported to the laboratory for evaluation within a period of 1h. The raw or fresh semen was macroscopically and microscopically evaluated for sperm concentration, sperm cell motility rate, and semen pH. The sperm concentration was determined with the aid of a spectrophotometer (Spermacue®) and the semen pH using a pH meter (Microprocessor pH/mV/°C Meter Hanna HI 931401). A Computer Assisted Sperm Analysis (CASA) system was used to evaluate the sperm cell motility rates.

The sperm viability (percentage live/dead) was determined using an eosin/nigrosin stain (60µl eosin/nigrosin and 6µl semen in a thin smear). This staining method indicates the live or dead status of the sperm cells and also allows a good evaluation of the sperm cell morphology (normal or abnormal) (Bjoerndahl *et al*., 2003). The semen smears were prepared on a clean, warmed microscope slide, to avoid temperature shock to the sperm cells, and evaluated on the same day of collection with the aid of a fluorescent microscope (BX 51TF), using an oil immersion objective (X100 magnification). A total of 100 sperm cells per slide were evaluated and recorded for each ram per ejaculate, with the aid of a cell counter, with the gross structural normal/abnormalities being recorded (Hidalgo *et al*., 2007). The live sperm cells fluoresced green, as these sperm do not absorb the stain, while the dead cells coloured red, as they absorbed the stain (Bearden *et al*., 2004). The live sperm cells were further categorized as morphologically normal or abnormal. Abnormalities were recorded according to the location of the abnormality e.g. head (i.e. bulb, small, enlarged, looped, etc.), mid-piece and tail (i.e. swelling, looping, partial or totally lacking, etc.), as described by Lukaszewicz *et al*. (2008).

All data were analysed using the statistical GenStat® program. The analysis of variance (ANOVA) was used to test for significant differences between the treatments. Treatment means were compared using the Fishers protected t-test for the least significant differences (LSD), at the 5% level of significance (Snedecor & Cochran, 1980).

4.3 Results and Discussion

In Table 4.1, the mean live-weight, scrotal circumference, semen volume, pH and sperm concentration of the indigenous rams are set out. The weight and scrotal circumference of the rams ranged between 41.3 and 57.4kg and 28 and 31.3cm respectively. There were no significant ($P<0.05$) differences between the rams with regards to scrotal circumferences. The volume of the indigenous ram ejaculates ranged from 0.4 to 0.9mL. The volume recorded in this study was generally lower to that reported by Gil *et al*. (2003), who reported an ejaculate volume of 0.75 to 2mL as being normal for rams, when using the artificial vagina (AV) to collect semen from rams. Hafez and Hafez (2000) also reported the semen ejaculate volume in rams to range from 0.5 to 2mL in mature rams, and from 0.5 to 0.7mL in young yearling rams. Furthermore, the sperm concentration recorded in the current study ranged from 0.9 to $1.3x10^9$sperm/mL, which was also lower, when compared to other studies. According to Evans and Maxwell (1987) the sperm concentration of an adult ram ejaculate was found to vary from 3.5 to $6.0x10^9$ sperm/mL. This was also supported by Hafez and Hafez (2000). Gil *et al.* (2003) however considered a sperm concentration of $2.5x10^9$ sperm/mL for rams to be normal and acceptable. The mean sperm cell concentration recorded in the current study was however higher to the reported results by Fourie *et al.* (2004), on intensively managed Dorper rams ($731.6x10^6$ sperm/mL), also using the electro-ejaculator as the method of semen collection. This difference could thus generally be attributed to the method of semen collection, breed of the rams or age of the individuals. The semen pH recorded in this study ranged from 6.5 to 7.3. Greyling and Grobbelaar (1983) recorded a similar semen pH for Boer goats (ranging from 6.40 to 7.02). Semen pH is considered to be normal when it ranges between 7.2 and 7.8 (Prins, 1999). It is generally accepted that ejaculates obtained following the use of the electro-ejaculator, tend to induce semen with a higher pH, due to excessive stimulation of the accessory sex glands with their alkaline secretions (Greyling & Grobbelaar, 1983). The reason for the significantly ($P< 0.05$) lower semen pH in the Pedi rams is unclear. This could possibly be attributed to less accessory gland fluid being produced.

In Table 4.2 Pearson correlations between the bodyweight, scrotal circumference, semen volume, sperm concentration, semen pH and total sperm motility are set out. Body weight was positively correlated with total sperm motility ($r = 0.228$). However negative correlations were found to exist between body weight and scrotal circumference ($r = -0.003$), semen volume ($r = - 0.773$), sperm concentration ($r = - 0.730$), semen pH ($r = - 0.783$). Semen volume was positively correlated with sperm concentration ($r = 0.997$) and pH ($r = 0.566$).

The scrotal circumference was positively correlated with semen volume (r = 0.197), total sperm motility (r = 0.537) and sperm concentration (r = 0.172). Sarder (2005) recorded the increases in semen volume and total sperm/ejaculate to be associated with an increase in scrotal circumference. Langford (1987), Devkota *et al.* (2008), Hassan *et al.* (2009) and Okere *et al.* (2011) also found scrotal circumference to be positively correlated with semen volume and sperm concentration. Scrotal circumference was found to be positively correlated with sperm motility, and these traits were closely correlated with the fertility in bulls (Okere *et al*., 2011. The sperm concentration was positively correlated with semen pH (r = 0.556). Negative correlations were also recorded in this study, between total sperm motility and semen volume (r = - 0.562), sperm concentration (r = - 0.613), semen pH (r = - 0.613).

Table 4.1 Mean (±SD) semen volume, pH and sperm concentration of different South African indigenous ram breeds

Breed	Body weight(kg)	Scrotal circumference(cm)	Ejaculate volume (mL)	Sperm concentration (10^9/ mL)	Semen pH
Damara	41.3±0.8^{b}	30.8±0.4^{a}	0.4±0.1^{b}	1.3±48.5^{a}	7.3±0.3^{a}
Namaqua Afrikaner	47.4±0.5^{ab}	29.8±0.9^{a}	0.9±0.2^{a}	1.2±30.5^{ab}	7.3±0.3^{a}
Pedi	57.4±0.4^{a}	31.3±0.8^{a}	0.5±0.1^{b}	0.9±84.2^{b}	6.5±0.4^{b}
Zulu	51.6±0.2^{ab}	28.0±0.7^{a}	0.5±0.3^{b}	0.9±177.2^{b}	7.3±0.4^{a}

^{a,b} Values with different superscripts within a column differ significantly (P< 0.05)

In Table 4.3 the sperm morphology of the indigenous ram semen determined using the eosin/nigrosin stain, is set out. Sperm morphology is generally considered to be a good predictor of successful fertilizing capacity (Lukaszewicz, 1988). The proportion of live sperm in the current study ranged between 32 and 64.3%. The Namaqua Afrikaner ram (32%) demonstrated a significantly lower proportion of live sperm cells, compared to the Damara (58.8%), Pedi (59.3%) or Zulu (64.3%) rams. The reason for the lower % live sperm recorded in the Namaqua rams is not clear. It is speculated that the percentage live sperm in the raw ejaculates should have been high – due to the semen being collected in the breeding season. The Namaqua Afrikaner rams then resulted in the highest occurrence of abnormal sperm. These morphological abnormalities of the sperm cells have been generally associated with a decrease in fertility rate of the rams (Mithat *et al*., 2001). In the present trial the sperm abnormalities ranged between 5.2% and 8.2%, which are regarded as acceptable values for a high fertility rate. The proportion of abnormal sperm cells recorded in this study were generally lower than those cited by Perez *et al.* (1997) (9.4%) and Taha *et al.* (2000) (14.2%). This may indicate the semen collection and staining procedures used in this trial, to be acceptable.

Table 4.2 Pearson correlations between bodyweight, scrotal circumference, semen volume, sperm concentration, semen pH and total sperm motility in South African indigenous rams

Variables	Body weight	Scrotal circumference	Semen volume	Sperm concentration	Semen pH
Body weight	—				
Scrotal circumference	-0.003	—			
Semen volume	-0.773	0.197	—		
Sperm concentration	-0.730	0.172	0.997	—	
Semen pH	-0.783	-0.606	0.566	0.556	—
Total sperm motility	0.228	0.537	-0.562	-0.613	-0.613

Table 4.3 Sperm morphology evaluation of raw semen from South African Indigenous rams of different breeds

Breed	%_Sperm cells		%_Abnormal sperm		
	Live	**Dead**	Head	Midpiece	Tail
Damara	58.8±4.8[a]	36.0 ± 6.0[b]	1.5 ± 0.7[b]	1.5 ± 1.3[a]	2.2 ± 0.9[a]
Namaqua Afrikaner	32.0±9.9[b]	59.0 ± 10.6[a]	4.5 ± 0.8[a]	2.5 ± 0.6[a]	2.2 ± 0.9[a]
Pedi	59.3±4.5[a]	35.2 ± 5.3[b]	1.2 ± 0.5[b]	1.5 ± 1.0[a]	2.7 ± 0.9[a]
Zulu	64.3±6.8[a]	29.5 ± 7.8[b]	1.2 ± 0.5[b]	2.0 ± 0.8[a]	3.0 ± 0.8[a]

[a,b] values with different superscripts within a column differ significantly (P< 0.05)

Table 4.4 Mean (±SD) sperm motility and velocity rates of South African indigenous ram breeds, as recorded by CASA

Characteristics	Breed			
	Damara	Namaqua Afrikaner	Pedi	Zulu
Total motility (%)	69.6±16.5[a]	37.1±19.9[a]	74.9±11.0[a]	56.0±22.6[a]
Progressive motility (%)	36.4±15.1[b]	17.4±14.7[c]	52.7±13.3[a]	32.6±15.4[b]
Non-progressive motility (%)	32.2±13.7[a]	19.7±12.9[a]	22.2±19.3[a]	23.4±17.5[a]
Rapid (%)	59.75±13.6[a]	23.4±17.8[b]	60.1±19.5[a]	40.4±19.1[ab]
Medium (%)	4.9±33.0[a]	5.0±2.0[a]	3.9±4.4[a]	7.0±3.0[a]
Static (%)	30.3±16.4[a]	62.9±19.9[a]	25.1±11.0[a]	44.0±22.6[a]
Slow (%)	5.1±3.1[a]	8.8±4.8[a]	11.0±18.6[a]	8.6±11.0[a]
VCL(µm/s)	213.3±39.3[a]	143.4±20.3[b]	201.7±63.3[a]	193.0±47.0[a]
VSL(µm/s)	128.5±39.7[a]	94.0±24.8[a]	143.7±58.1[a]	123.4±52.6[a]
VAP(µm/s)	177.8±30.3[a]	117.8±21.3[a]	164.5±62.1[a]	148.0±49.5[a]
LIN (%)	59.2±9.8[a]	65.1±11.0[a]	69.0±10.1[a]	61.8±12.2[a]
STR (%)	71.5±16.0[a]	79.4±12.1[a]	86.5±6.7[a]	81.5±8.1[a]
WOB (%)	83.8±6.6[a]	81.8±3.4[a]	79.7±10.2[a]	75.3±7.5[a]

VCL = curvilinear velocity, VSL = straight-line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency

[a,b] Values with different superscripts within a row differ significantly (P< 0.05)

In Table 4.4 the sperm motility and sperm velocity rates of the indigenous rams, as measured by the CASA system, are set out. In this analysis the proportion of total motile sperm ranged from 37.1 to 74.9%. Ram as such had no effect on the total motile sperm, although the Namaqua

tended to record lower sperm motility rates than the Damara or Pedi. The Pedi (52.7%) recorded a significantly ($P<0.05$) higher proportion of progressive motile sperm cells, compared to the Damara (36.4%), Namaqua Afrikaner (17.4%) and the Zulu (32.6%) rams. The total sperm motility and progressive sperm motility in the Namaqua (Table 4.4) was significantly ($P<0.05$) lower, than the other breeds, when using the CASA system for sperm evaluation. This lower motility could possibly be related to the lower percentage of live sperm as set out in Table 4.3. Furthermore the breeds did not differ in the percentage of non-progressive motile, static, medium motile or slow motile sperm. Breed also recorded no effect on the straight line sperm velocity, average path velocity (VAP), linearity (LIN), straightness (STR) and proportion of wobbling (WOB) sperm cells, as evaluated by the CASA system.

4.4 Conclusions

In this study, the volume of the indigenous ram ejaculates ranged from 0.4 to 0.9mL. Furthermore, the sperm concentration recorded ranged from 0.9 to $1.3x10^9$sperm/mL, which is lower when compared to other studies. The semen pH recorded ranged from 6.5 to 7.3. The Pedi ram semen recorded the highest total motile (74.9%) and most progressive (52.7%) sperm cells, compared to the other breeds. The Namaqua rams resulted in lowest total motile (37.1%) and progressive (17.4%) sperm cells, compared to other breeds, as measured by CASA. The relatively small standard deviation in the semen volume is indicative of a satisfactory and repeatable semen collection technique being used, although EE is not the most acceptable technique used. Body weight was positively correlated with total sperm motility. However negative correlations were found between body weight and scrotal circumference, semen volume, sperm concentration, semen pH. Semen volume was positively correlated with sperm concentration and semen pH. Scrotal circumference was positively correlated with semen volume, total sperm motility and sperm concentration and these traits are closely correlated with fertility especially in bulls. The sperm concentration was positively correlated with semen pH. Negative correlations were also recorded in this study, between total sperm motility and semen volume, sperm concentration, semen pH.

This study thus gave an overall knowledge regarding the characterization of the indigenous ram semen and semen quality in the breeds. The use of the CASA system offered a more reliable,

unbiased and repeatable means of microscopically assessing sperm motility traits, compared to traditional visual assessment. The low percentage of sperm abnormalities demonstrated the technique used to collect and evaluate sperm abnormalities to be acceptable for obtaining normal, viable sperm.

Chapter 5

Effect of storage temperature on the viability of diluted ram semen stored for different periods of time

5.1 Introduction

The three South African indigenous sheep breeds (Damara, Namaqua Afrikaner and Zulu) from which semen was collected, are adapted to the harsh arid South African conditions. They are generally more tolerant to ticks and resistant to certain diseases, while they are also said to be highly fertile and the dams are said to have an excellent mothering ability, being very protective and able to defend their young against smaller predators (Ramsay *et al*., 2001). The characterization of these indigenous breeds have however been neglected in the past, especially regarding their production potential although they have been crossbred with other exotic breeds – with no records regarding their production performances. The need has however long been recognized to preserve the genetics of these breeds for future use. Semen storage (long or short term) is a method of preserving the genetic potential of these indigenous breeds.

As the cryopreservation and thawing process of semen induce serious damage to the sperm cells especially in rams, this may result in impairing the subsequent fertility rate following AI (Maxwell & Watson, 1996; Soderquist *et al*., 1997). The use of raw, diluted and cooled semen shortly following semen collection may be an alternative method to the cryopreservation of semen for use in AI programs. Compared to raw (fresh) semen, cooled ram semen however exhibits a decrease in sperm motility and sperm morphological integrity over time – accompanied with a decline in the survival rate of the sperm in the female reproductive tract, with a reduction in fertilizing ability and increased embryonic losses. The injury to the sperm cells are usually less pronounced in diluted and chilled semen, compared to frozen/thawed ram semen (Maxwell & Salamon, 1993). Irrespective of the semen diluent used, the dilution rate, temperature, or conditions of storage, the quality of the sperm deteriorates as the duration of storage increases (O'Hara *et al*., 2010). It is however important to know when the deterioration in sperm quality occurs, and therefore there is a need to study the effect of semen storage on sperm survival rate for different time intervals. The temperature at which semen is stored plays a

critical role in acceptable sperm motility rates recorded. Temporary storage of ram semen at 5°C or 15°C would be better in terms of fertilization rate, following the transportation of semen, while also being more affordable to small scale farmers. The main method of long term semen storage (cryopreservation) is generally in liquid nitrogen at a temperature of -196°C. This temperature then lowers the metabolic rate of the sperm, and contributes to enhanced sperm survival (Vishwanath & Shannon, 2000). Vishwanath and Shannon (2000) also reported the storage of semen at room temperature to be superior to the storage at a temperature of 5°C, provided the medium with which sperm is diluted, inhibits those pathways that are detrimental to their survival at higher temperatures. So for example Grasa *et al*. (2004) successfully used 15°C as the storage temperature for liquid ram semen, before artificial insemination being implemented.

It is critical to inseminate at an optimal time during the oestrous period, relative to ovulation, to achieve an acceptable fertility rate. It is then also vital to preserve and store the semen used for AI under optimal environmental conditions. The recommended maximum storage period for raw ram semen has been set as short as 6 to 12h. Depending on the time required for the transportation of semen from the AI stations to the farms, a short-term storage time will make it possible to inseminate the ewes at an optimal time during oestrus (Paulenz *et al*., 2002).

The processing and storage has been shown to promote destabilisation of the cell membrane, hampering capacitation and acrosome integrity of the sperm (Watson, 1981; Guillan *et al*., 1997). Semen extenders are thus generally added to semen to supply the sperm with a source of energy, protect the cells from temperature related injury, and maintain a suitable environment for the sperm to survive temporarily (Purdy, 2006).

The aim of this study was to determine an acceptable storage temperature (5°C vs. 15°C) and different storage times for raw ram semen, prior to AI.

5.2 Materials and Methods

Semen was collected during the winter (June, 2009), from 6 healthy mature rams of different South African indigenous breeds i.e. the Damara, Namaqua Afrikaner and the Zulu breed (2

rams per breed being used). The ages of the rams ranged between 2 and 4 years and all animals were maintained on natural pastures, supplemented with a commercial pelleted diet. Water was available *ad libitum.* Semen was collected using an electro-ejaculator from all the rams, twice a week for a period of 2 weeks, with 4 ejaculates being collected in total from each ram. Semen was collected directly into graduated test tubes, which were then placed into a thermo flask, with the water being maintained at a temperature of 37°C. All collected semen was then transported to the laboratory for macroscopic and microscopic evaluation within 1h of collection. The fresh undiluted semen was evaluated for sperm concentration, semen pH, and sperm motility (refer to Chapter 3 for more details). The sperm concentration was determined with the aid of a spectrophotometer (Spermacue®), the semen pH with the aid of a pH meter and the Computer Assisted Sperm Analysis (CASA) system used for measuring the sperm motility attributes. After the initial evaluation, all semen samples were pooled and diluted equally in an egg yolk citrate extender in the ratio of 1:1(v/v). The pooled semen sample was then divided into two; one sample being stored at 5ºC, and the other at 15°C, for periods of 3, 6, 9, and 24h respectively. Sperm characteristics were then recorded for each interval of storage.

Data were analysed using the statistical program, GenStat®. The analysis of variance (ANOVA) was used to test for significant differences between the treatments. Treatment means were compared using Fishers protected t-test least significant difference (LSD), at the 5% level of significance (Snedecor & Cochran, 1980).

5.3 Results and Discussion

Raw or fresh ram semen generally has a short fertile lifespan outside the body (*in vitro*) (Morrier *et al*., 2002). However, a decreased metabolic rate could extend the lifespan of the sperm cells. Subsequently a low storage temperature generally extends the fertile lifespan of the sperm – by reducing the metabolic rate. Sperm cells from fresh ejaculates are generally more fertile for a few hours after collection and their metabolic rates are high at higher temperatures. However, as the metabolic rate increases, the life span of the sperm cells decreases (Hafez and Hafez, 2000). At normal body temperature (37°C) the sperm cell only survives for a few hours, because of this increased cellular metabolism. It is therefore imperative to lower and stabilize the environmental temperature in which the semen is to be stored, thereby decreasing the metabolic rate of the

sperm cells and thus increasing its longevity (Brinsko *et al*., 2000). The ejaculates from the three indigenous rams were pooled to eliminate the individual seminal differences and compare the effect of two storage temperatures (5°C and 15°C). In Table 5.1 the sperm motility characteristics of the diluted semen of the indigenous rams stored at 5°C or 15°C, as measured by the CASA system, are set out.

Storage period (3h)

After 3h of semen storage, no effect of temperature (5°C or 15°C) was recorded regarding all the sperm cell motility characteristics evaluated. Values for the different sperm characteristics of semen stored at a temperature of 15°C were generally superior to those for semen stored at 5°C – except for the proportion of static (immotile) sperm cells (although these differences were not significant). Semen stored at 15°C also exhibited a significantly higher proportion of motile sperm (9.6%) at the medium rate of motility, compared to semen stored at 5°C (5.3%).

Storage period (6h)

After a 6h period of semen storage, the proportion of total motile sperm, progressively motile, non-progressively motile and rapid motile sperm cells recorded were generally higher in semen stored at 15°C, compared to that at 5°C. Similarly, the proportion of immotile sperm cells was higher in semen stored at 5°C. No temperature effect was recorded regarding the proportion of sperm cells for the medium or slow motile, the VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF characteristics. For 15°C, all values recorded for the sperm characteristics (CASA) tended to be higher than at 5°C. It was evident that all the parameters measured were decreasing with time and were not as metabolically active as following a 3h interval.

Storage period (9h)

After 9h of semen storage at 5°C (15.1%), a significantly lower progressive motility rate was recorded compared to 15°C (22.3%) group. Although no significant differences were recorded, the percentage total motile, non-progressive motile, rapid motile, medium motile, slow motile, STR, WOB and ALH sperm were higher when stored at 15°C. Semen stored at 5°C recorded higher values for the immotile sperm, VCL, VSL, VAP, LIN and the BCF characteristics.

Following this period of time, irrespective of the storage temperature, it was evident that the sperm motility characteristics had drastically decreased.

Storage period (24h)

After 24h of semen storage, the proportion of total motile, progressive motile, non-progressive motile and rapid motile sperm cells were significantly($P<0.05$) higher at 15°C, compared to 5°C. The proportion of static (immotile) sperm cells was significantly ($P<0.05$) lower at 15°C. No significant differences were recorded for the proportion of medium motile, slow motile, VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF characteristics. In general values recorded tended to be higher for the semen that had been stored at 15°C, compared to 5°C. This is because the sperm cells kept at 5°C are believed to lose their motility, while maintaining their viability (Appell & Evans, 1977). The proportion of total motile sperm recorded after 24h of storage at 15°C was higher (61.2%), compared to that at 3h (51.4%), 6h (50.1%) and 9h (50.6%). The percentage rapid motile sperm recorded following 24h of semen storage at 15°C was higher (48.6%) than following 3h (30.7%), 6h (37.0%) and 9h (34.3%) of storage. The trend followed by the sperm motility rates over time was not consistent, as a linear decrease in semen quality is generally expected with time.

The cooling of semen to 4°C has been shown to have an adverse effect on the sperm motility rates. In a study conducted by Appell and Evans (1977), where semen samples stored at 3 temperatures (4°C, 20°C or 37°C) were compared, sperm viability followed a motility pattern very close to that in the semen samples kept at room temperature (20°C) and body temperature (37°C). However at 4°C the sperm viability was well preserved, despite the loss in motility of the sperm. At 20°C and 37°C all static sperm were dead, however this was not the case at 4°C.

Semen stored at 15°C recorded a value for the total motile sperm of 51.4%, following a 3h storage period. This however increased to 61.2% after 24h. This increase in the total sperm motility may be attributed to a high proportion of immotile sperm that possibly regained their motility after a long time exposure to 15°C. The overall percentage of total motile sperm however decreased from 39% (at 3h storage) to 27% (at 24h storage) in semen stored at 5°C. This decrease in recorded sperm motility of the semen stored at 5°C after 24h period may be

attributed to the immobilisation of sperm cells that generally occurs at lower storage temperatures. Generally semen stored at 15℃ resulted in better preservation of the sperm motility characteristics, when compared to semen stored at 5℃. Langford and Fiser (1980) reported the storage of ram semen in skim milk at 15℃ to be more satisfactory in terms of sperm survival, than at 4℃ – which is in agreement with the current study. The results recorded in this study however differ with the observations of O'Hara *et al*. (2010), who found the storage of semen at 5℃ to be superior to 15℃ in terms of both sperm motility and viability of the semen stored for a period of 72h. Paulenz *et al*. (2002) also reported the motility of sperm stored at 5℃ to be superior to storage at 15℃. The difference recorded in the current study may be ascribed to the semen diluent used. Morton *et al*. (2009) clarified the variation in the effectiveness of the semen diluents in preserving the longevity and potential fertilising capacity of sperm - during liquid storage, using different constituents. This was in a comparative study using reconstituted skim milk and CUE (Cornell University Extender) for ram semen storage at 4 or 15℃. Interactions between the type of diluent, dilution rate and the temperature of storage have been reported by Maxwell and Salamon (1993).

5.4 Conclusions

Semen stored at 15℃ recorded higher sperm motility, compared to semen stored at 5℃, over the entire time period. It would thus seem, according to the results in this trial that ram semen diluted and stored at 15℃ for a period of up to 24h was more satisfactory in retaining sperm motility. However, the fertilizing capacity of the sperm still has to be evaluated. It can also be seen as practical means of storing semen (15℃) for the application of artificial insemination in a short-term semen storage programme, incorporated with AI. Semen stored at 15℃ also resulted in the better preservation of sperm characteristics as recorded by CASA, compared to the 5℃ storage group. CASA can be seen as a very reliable test regarding the sperm characteristics. More studies need to be conducted to investigate the longer term effect (more than 24h) of semen storage at different temperatures on sperm viability and the relationship to actual fertilizing capacity. The ultimate test regarding sperm viability would however be in the fertilizing ability of the sperm cell – an aspect not investigated in this study.

Table 5.1 The mean (±SE) sperm motility characteristics of pooled diluted South African indigenous ram semen stored at 5°C or 15°C following evaluation using the CASA system

Pooled semen characteristics	Temperatures at the different storage periods							
	3h		6h		9h		24h	
	5°C	15°C	5°C	15°C	5°C	15°C	5°C	15°C
Total Motility (%)	39.0±8.5^{abc}	51.4±8.1^{ab}	24.4±5.7^{c}	50.1±9.6^{ab}	31.3±8.8^{bc}	50.6±10.0^{ab}	27.0±7.1^{c}	61.2±10.0^{a}
Progressive Motility (%)	20.3±5.5^{ab}	30.4±6.8a	14.2±3.4c	24.8±5.4ab	15.1±4.3c	22.3±6.4^{ab}	13.6±3.8^{c}	28.8±5.6^{a}
Non- progressive Motility (%)	18.7 ± 3.9^{bcd}	21.1±4.1^{abcd}	10.2 ± 2.5^{d}	24.9 ± 5.2^{abc}	16.2 ± 5.0^{bcd}	28.3 ± 5.0^{ab}	13.4 ± 3.5^{cd}	32.4 ± 5.3^{a}
Rapid (%)	25.9±6.5^{bc}	30.7±6.2^{bc}	17.7±4.3^{c}	37.0±7.7^{ab}	21.3±6.3^{bc}	34.3±7.5^{abc}	18.3±5.1^{c}	48.6±9.3^{a}
Medium (%)	5.3±1.4^{b}	9.6±2.5^{a}	3.1±1.1^{b}	5.0±1.2^{b}	3.2±1.2^{b}	6.1±1.5^{ab}	2.6±0.8^{b}	4.7±0.9^{b}
Static (%)	61.0±8.5^{abc}	48.5±8.1^{bc}	75.6±5.7^{a}	49.9±9.6^{bc}	68.7±8.8^{ab}	49.4±10.0^{bc}	73.0±7.1^{a}	38.8±10.0^{c}
Slow (%)	7.8±1.6^{ab}	11.2±3.8^{a}	3.6±1.0^{b}	8.1±1.8^{ab}	6.8±2.2^{ab}	10.2±1.7^{a}	6.2±1.8^{ab}	7.9±1.4^{ab}
VCL (µm/s)	180.1±16.4^{a}	184.3±16.4^{a}	168.5±24.3^{a}	186.5±10.9^{a}	164.0±25.4^{a}	163.4±13.2^{a}	165.5±25.3^{a}	203.6±17.4^{a}
VSL (µm/s)	112.3±14.9^{ab}	130.8±14.7^{a}	113.7±16.8^{ab}	115.3±9.7^{ab}	101.8±20.3^{ab}	88.0±11.4^{b}	100.9±17.5^{ab}	122.6±14.3^{ab}
VAP (µm/s)	145.2±16.1^{a}	153.2±14.7^{a}	150.4±23.2^{a}	148.1±8.7^{a}	130.8±26.1^{a}	121.4±13.8^{a}	131.5±21.9^{a}	163.2±16.6^{a}
LIN (%)	59.4±5.0^{ab}	70.8±4.3^{a}	59.8±7.6^{ab}	62.04±4.2^{ab}	54.6±7.4^{b}	53.3±4.8^{b}	53.5±7.3^{b}	59.2±3.4^{ab}
STR (%)	75.1±4.3^{a}	84.7±3.3^{a}	70.4±8.9^{a}	77.1±2.6^{a}	69.5±8.8^{a}	73.1±4.5^{a}	68.0±8.7^{a}	74.6±2.3^{a}
WOB (%)	78.4±4.0^{a}	83.1±2.5^{a}	75.6±9.6^{a}	79.9±2.9^{a}	69.9±9.5^{a}	72.8±4.4^{a}	69.6±9.1^{a}	79.0±2.6^{a}
ALH(µm)	3.3±0.5^{a}	3.5±0.2^{a}	2.9±0.4^{a}	3.3±0.1^{a}	2.9±0.4^{a}	3.2±0.2^{a}	3.3±0.2^{a}	3.6±0.2^{a}
BCF(Hz)	13.6±1.9^{b}	14.8±0.7^{ab}	14.2±2.3^{ab}	16.4±0.8^{ab}	14.8±2.0^{ab}	18.0±1.2^{a}	15.2±0.7^{ab}	17.5±0.9^{ab}

VCL = curvilinear velocity, VSL = straight-line velocity, VAP = average path velocity, LIN = linearity, STR = straightness WOB = wobble ALH = amplitude of lateral head displacement and BCF = beat cross frequency. ^{a,b} Values with different superscripts within a row differ significantly (P< 0.05)

Chapter 6

The effect of temperature and different storage times, on sperm motility of ram semen diluted with an extender containing glycerol

6.1 Introduction

When utilising males with outstanding traits in a controlled breeding program, it is necessary to store the semen for a period of time. This storage could be in liquid form at lower temperatures or by cryopreservation of semen. As long term cryopreservation and thawing of ram semen induces serious structural damage to the sperm cell and impairs fertility, raw diluted and cooled semen is generally considered as an alternative to frozen semen, when used for AI within a short period after semen collection (Maxwell & Watson, 1996; Soderquist *et al*., 1997). Compared to raw or fresh semen, diluted cooled ram semen is however also exposed to a decrease in sperm motility and morphological integrity, accompanied with a subsequent decline in sperm survival rate in the female reproductive tract, a reduction in fertility and even increased embryonic losses (Maxwell & Salamon, 1993).

Regarding fertilization as such, it is essential to inseminate, in this case the ewe at an optimal time during the oestrous period, to achieve acceptable fertility results. It is also important to preserve and store ram semen under optimal conditions, for maximal AI success. The recommended maximum storage period of raw semen has been said to be as short as 6 to12h (Paulenz *et al*., 2002). Depending on the time needed to transport semen from the AI station to the farm, such a short storage time often makes it difficult to inseminate the ewes at the prescribed time during oestrus. The length of the natural oestrous period in sheep has been reported to vary between 24 and 36h, with ovulation generally occurring some 24 to 30h after the onset of oestrus. Timing of insemination should thus be 12 to18h after the onset of oestrus (Hunter *et al*., 1980). Semen extenders are generally added to the semen to supply the sperm cells with a source of energy, protect the cells from temperature related injury and maintain a suitable environment for the sperm to survive temporarily (Purdy, 2006). Cryoprotectants in the case of semen freezing are then added as components to the semen extenders to protect the sperm from these temperature related injuries. In this trial glycerol was included, as a cryoprotectant as

it is currently the most extensively used agent in mammalian semen cryopreservation. Glycerol as such is a penetrating cryoprotectant that causes membrane lipid and protein rearrangement, which results in increased membrane fluidity and permeability for ions and an increase in ATP consumption. Glycerol thus generally causes greater sperm cell dehydration at lower temperatures and an increased ability for sperm cells to survive cryopreservation (Holt, 2000). Certain artificial insemination centres even include glycerol in the semen extender for the conservation of raw ram semen at higher temperatures (Hackett & Wolynetz, 1982). In liquid semen diluents, glycerol has been shown to reduce the decline in fertility associated with the aging of sperm (Shannon, 1964). Purdy (2006) also indicated the presence of glycerol to induce osmotic damage to sperm, while Morrier *et al*. (2002) only recommended glycerol for use in semen cryopreservation.

The aim of this study was thus to determine the effect of temperature and different storage times, on sperm motility of ram semen diluted with an extender, containing glycerol.

6.2 Materials and Methods

Semen was collected during the winter (June, 2009, outside the natural breeding season) from 6 healthy mature rams of different indigenous breeds i.e. the Damara, Namaqua Afrikaner and the Zulu breed, with 2 rams per breed being used. The ages of the rams ranged from 2 to 4 years and all animals were maintained on natural pastures and supplemented with a commercial diet. Water was available *ad libitum*. Semen was collected twice weekly for a period of 2 weeks and 4 ejaculates were collected in total from each ram, with the aid of an electro-ejaculator. Semen was collected directly into graduated test tubes, which were then placed into a thermo flask, containing water at a temperature of 37°C. All collected semen was transported to the laboratory for evaluation within 1h of collection. The raw, undiluted ejaculate was evaluated for sperm concentration, semen pH, and sperm motility (for more details, see Chapter 3). The sperm concentration of each ejaculate was determined with the aid of a spectrophotometer (Spermacue®), the semen pH with the aid of a pH meter and the Computer Assisted Sperm Analysis (CASA) system used for measuring the sperm motility. After initial evaluation, all semen samples were pooled to eliminate individual ram differences and diluted equally with an egg yolk citrate extender, containing 14% glycerol in the ratio of 1:1 (v/v), making a final

glycerol concentration of 7%. The pooled semen sample was then divided into two, one sample being stored at 5ºC and the other at 15°C, for storage periods of 3, 6, 9, and 24h. Sperm motility characteristics were then recorded at each interval of semen storage.

All data were analysed using the statistical program, GenStat®. The analysis of variance (ANOVA) was used to test for significant differences between the treatments. Treatment means were compared using Fishers protected t-test for the least significant difference (LSD), at the 5% level of significance (Snedecor & Cochran, 1980).

6.3 Results and Discussion

Raw extended ram semen generally has a very short fertile lifespan and the reduced metabolic rate of sperm at a lower storage temperature should extend the storage life of the semen (Maxwell & Salamon, 1993). Thus low temperatures tend to extend the fertile life of sperm by decreasing the cell metabolism (Morrier *et al.*, 2002). The sperm cells of fresh ejaculates are generally fertile for a few hours at a high rate of metabolism. A high temperature will increase the metabolic rate, and subsequently decrease the life span of a sperm cell. At normal body temperature (37°C) the sperm cell lives for a few hours only, due to this increased cellular metabolism (Brinsko *et al*., 2000). The semen of the three South African indigenous breeds of rams were diluted in an egg yolk citrate diluent, containing 14% glycerol and stored at 5°C and 15°C, were compared in this study.

In Table 6.1 the sperm motility characteristics of the diluted indigenous ram semen exposed to glycerol as a cryoprotectant and stored at the two storage temperatures for the different periods of time, as measured by the CASA system, are set out.

Storage period (3h)

In the current trial, the effect of storage temperature (5°C vs. 15°C) was recorded with the aid of the CASA system following 3h of storage – regarding VAP (average path velocity) and VSL (straight-line velocity). Sperm stored at 15°C recorded significantly ($P<0.05$) higher values for the VAP and VSL, when compared to that of semen stored at 5°C. The percentage of total motile sperm, progressive motile, non-progressive motile, rapid motile, slow motile, VCL (curvilinear

velocity), LIN (linearity), STR (straightness), WOB (wobble), ALH (amplitude of lateral head displacement), and the BCF (beat cross frequency) characteristics were recorded to be numerically higher at an storage temperature of 15°C, compared to semen stored at 5°C. The semen stored at 15°C resulted in numerically (not statistically) lower static (immotile) sperm percentage and a medium motile sperm percentage than that of semen stored at 5°C (Table 6.1).

Storage period (6h)

Following 6h of semen storage, no temperature effect (5°C vs. 15°C) was recorded regarding all the sperm motility and velocity characteristics. Values for the percentage of total motile sperm and percentage static sperm were similar for both semen storage temperatures (5°C and 15°C).

Storage period (9h)

Following 9h of semen storage, no temperature effect (5°C vs. 15°C) was recorded for the semen characteristics measured. The percentage of total motile sperm, progressive motile sperm and non-progressive motile sperm, rapid motile sperm, medium motile, slow motile, the VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF characteristics were once again only numerically higher for semen stored at 15°C, than at 5°C, although these differences were not significant. The percentage of immotile sperm was higher in semen stored at 5°C than at 15°C.

Storage period (24h)

A significant effect of storage temperature for this temperature was only recorded for the percentage wobbling sperm, where semen stored at 15°C recorded a significantly ($P<0.05$) higher WOB than semen stored at 5°C. Semen stored at 15°C recorded 48.3% total motile sperm after 3h of storage but this increased to 50.4% following 24h of storage. Similar percentages of total motile sperm were recorded after 3h and 24h (40% and 40.8%, respectively) in the semen stored at 5°C (Table 6.1). This may be due to the reduced metabolism induced by the low temperature of 5°C. The results of the present study were thus similar for the two temperatures. However it was found that most of the seminal characteristics (sperm motility characteristics) were higher in semen stored at 15°C, compared to those stored at 5°C, although these differences were not significant.

Table 6.1 The mean (±SE) sperm motility characteristics of indigenous ram semen diluted with glycerol, stored at two temperatures for different periods of time as measured by the CASA system

Characteristics	Temperatures at different storage periods							
	3h		6h		9h		24h	
	5°C	15°C	5°C	15°C	5°C	15°C	5°C	15°C
Total Motility (%)	40.0 ± 7.4^{a}	48.3 ± 9.0^{a}	46.9 ± 8.3^{a}	46.7 ± 8.8^{a}	31.8 ± 6.4^{a}	47.1 ± 7.9^{a}	40.8 ± 7.7^{a}	50.4 ± 10.6^{a}
Progressive Motility (%)	26.1 ± 5.2^{a}	32.9 ± 6.4^{a}	25.6 ± 4.9^{a}	29.5 ± 6.3^{a}	22.1 ± 4.4^{a}	34.9 ± 6.2^{a}	21.7 ± 4.9^{a}	33.9 ± 7.7^{a}
Non-progressive Motility (%)	13.9 ± 2.9^{abc}	15.4 ± 2.9^{abc}	21.3 ± 4.0^{a}	17.3 ± 3.4^{ab}	9.7 ± 2.6^{c}	12.2 ± 2.6^{bc}	19.1 ± 3.8^{ab}	16.4 ± 3.3^{abc}
Rapid (%)	28.7 ± 5.8^{a}	35.7 ± 6.8^{a}	33.3 ± 6.1^{a}	33.3 ± 7.6^{a}	25.2 ± 5.6^{a}	37.8 ± 7.1^{a}	31.6 ± 6.1^{a}	40.3 ± 8.9^{a}
Medium (%)	5.9 ± 1.3^{ab}	5.8 ± 1.7^{ab}	6.7 ± 1.6^{a}	5.5 ± 1.4^{ab}	2.9 ± 0.9^{b}	3.8 ± 1.1^{ab}	3.4 ± 0.6^{b}	4.1 ± 1.1^{ab}
Static (%)	60.0 ± 7.4^{a}	51.8 ± 9.0^{a}	53.1 ± 8.3^{a}	53.3 ± 8.8^{a}	68.2 ± 6.5^{a}	53.0 ± 7.9^{a}	59.2 ± 7.7^{a}	49.7 ± 10.6^{a}
Slow (%)	5.4 ± 1.5^{a}	6.8 ± 1.5^{a}	6.9 ± 1.8^{a}	7.9 ± 1.3^{a}	3.7 ± 0.9^{a}	5.5 ± 1.1^{a}	5.9 ± 1.2^{a}	6.0 ± 1.3^{a}
VCL (µm/s)	157.5 ± 9.2^{a}	169.5 ± 6.6^{a}	164.2 ± 9.8^{a}	147.5 ± 12.6^{a}	165.8 ± 11.4^{a}	180.3 ± 8.8^{a}	175.4 ± 8.3^{a}	163.8 ± 9.0^{a}
VSL (µm/s)	102.3 ± 8.4^{b}	125.5 ± 6.4^{a}	101.9 ± 6.9^{b}	96.6 ± 9.0^{b}	110.1 ± 8.0^{ab}	126.4 ± 6.9^{a}	92.7 ± 6.6^{b}	103.1 ± 9.6^{b}
VAP (µm/s)	121.6 ± 6.9^{b}	140.6 ± 6.2^{a}	120.8 ± 6.9^{b}	111.0 ± 10^{b}	126.8 ± 7.8^{ab}	141.1 ± 7.0^{a}	118.1 ± 5.7^{b}	125.7 ± 7.2^{ab}
LIN (%)	65.2 ± 4.7^{ab}	74.2 ± 3.2^{a}	63.4 ± 3.8^{b}	69.5 ± 4.5^{ab}	66.5 ± 1.9^{ab}	70.2 ± 1.9^{ab}	53.2 ± 4.1^{c}	62.3 ± 5.2^{bc}
STR (%)	84.0 ± 4.5^{ab}	88.9 ± 1.8^{a}	84.2 ± 1.9^{ab}	83.8 ± 2.5^{ab}	86.3 ± 1.8^{ab}	89.4 ± 1.2^{a}	77.9 ± 3.1^{b}	79.9 ± 5.4^{b}
WOB (%)	77.7 ± 2.7^{ab}	83.0 ± 2.2^{a}	74.7 ± 3.0^{bc}	77.0 ± 2.1^{ab}	77.1 ± 1.9^{ab}	78.5 ± 1.7^{ab}	68.4 ± 3.7^{c}	77.1 ± 2.4^{ab}
ALH(µm)	2.6 ± 0.3^{a}	2.8 ± 0.1^{a}	3.1 ± 0.2^{a}	2.7 ± 0.2^{a}	3.0 ± 0.2^{a}	3.1 ± 0.1^{a}	3.2 ± 0.4^{a}	2.7 ± 0.3^{a}
BCF(Hz)	20.3 ± 2.1^{a}	21.9 ± 1.2^{a}	23.8 ± 1.0^{a}	23.1 ± 1.2^{a}	23.5 ± 1.1^{a}	25.0 ± 1.0^{a}	23.0 ± 2.3^{a}	22.1± 2.3^{a}

VCL = curvilinear velocity, VSL = straight-line velocity, VAP = average path velocity, LIN = linearity, STR = straightness WOB = wobble ALH = amplitude of lateral head displacement and BCF = beat cross frequency. ^{a,b} Values with different superscripts within a row differ significantly ($P< 0.05$)

Storage temperature and period had no significant ($P<0.05$) effect on total sperm motility as the total sperm motility of semen stored at 5°C for 3h (40%) was not significantly different to the sample that was stored for 24h (40.8%). The same results were recorded for semen that was stored at 15°C, where 3h and 24h, recorded (48.3%) and (50.4%) total sperm motility respectively. Progressive sperm motility was also not affected by either storage temperature or time (Table 6.1).

Storage temperature and the period employed had no significant ($P<0.05$) effect on total sperm motility, progressive motility, rapid motility, slow motility, static, VCL, ALH and BCF, as the sperm motility and velocity characteristics recorded did not show significant differences for semen stored at both temperatures for different periods of time. For semen stored at 5°C, a 9h storage period recorded significantly lower non-progressive sperm motility, compared to 6h and 24h storage periods – with 9h recording a significantly lower proportion of medium motile sperm, compared to the 6h storage. Storage period as such had no effect on the VSL of semen that was stored at 5°C, but significantly($P<0.05$) lower VSL values were recorded at 6h and 24h compared to 3 and 9h in semen stored at 15°C.

6.4 Conclusions

Storage temperature and period had no significant ($P<0.05$) effect on total sperm motility, progressive motility, rapid motility, slow motility, static, VCL, ALH and BCF, as the motility and velocity characteristics recorded did not show significant differences for semen stored at both temperatures for different time periods. However it was found that most of the seminal characteristics (sperm motility characteristics) were higher in semen stored at 15°C, compared to those stored at 5°C, although these differences were not significant.

The addition of glycerol as a cryoprotectant, demonstrated a protective effect on the sperm motility characteristics of sperm cells that were stored at both 5°C and 15°C, as the total sperm motility was maintained (40 to 50%) for up to 24h of storage. More studies are however needed to further investigate amongst others, the viability of indigenous ram sperm following storage at these lower temperatures for more than a 24h storage period – and the effect on fertilising ability.

Chapter 7

The effect of different glycerol inclusion levels in the semen diluent on the sperm motility characteristics, following cryopreservation in indigenous South African rams

7.1 Introduction

The three indigenous sheep breeds (Damara, Pedi and Zulu), from which semen was collected in this study, are adapted to the harsh South African conditions and have superior traits which make them to be in demand. In the past these breeds have been overlooked and have generally been crossbred with exotic breeds to incorporate some of the desirable adaptation traits. There is thus a need to preserve this genetic material (semen, oocytes, embryos and somatic cells), because of their superior characteristics for future use (Ramsay *et al*., 2001).

Polge *et al*. (1949) first reported glycerol to have a protective action on the survival of frozen/thawed sperm in chickens and humans. Generally a cryoprotectant (in this case glycerol) provides a protective action, serving as a source of energy for the sperm cell, while maintaining the osmotic pressure by forming hydrogen bonds with the membrane phospholipids and sugars (Watson, 1979). Thus reducing sperm cell membrane damage and minimizing membrane destabilization during the freezing and thawing processes (Strauss *et al*., 1986). Glycerol is also known to cause greater cell dehydration at lower temperatures, and thus induce an increased ability of the sperm cells to survive cryopreservation – due to reduced ice crystal formation (Holt, 2000). However, the inclusion levels of glycerol to diluents for the cryopreservation of ram semen may be limited by its toxicity towards the sperm cell (Watson, 2000).

In general raw or fresh extended ram semen has a relatively short fertile lifespan and the reduction in the metabolic rate of the sperm cell could extend the storage life. Low temperatures generally extend the fertile life of the sperm, by decreasing the cell metabolic rate (Morrier *et al*., 2002). Processing and the storage of semen have however been shown to promote destabilisation of the cell membranes, hampering capacitation and acrosome integrity of the sperm (Watson, 1981; Guillan *et al*., 1997). However, when a semen extender is added to the semen, it will supply the sperm with

a source of energy, and protect the cells from temperature-related damage, while also maintaining a suitable environment for the sperm to survive in, temporarily (Purdy, 2006).

Most semen extenders for cryopreservation contain egg yolk in their composition, with different combinations of cryoprotectants, at different concentrations (Fiser *et al*., 1987). For the cryopreservation of mammalian semen, glycerol is generally added to the semen extenders to protect the sperm (Fiser & Fairfull, 1984). When semen is frozen by the slow conventional method and hypertonic extenders are used, glycerol is frequently used within the range of 6 to 8%. The glycerol levels higher than these may cause damage to the sperm cell, thus lowering the post thawing survival of the sperm (Barbas & Mascarenhas, 2009). The present study investigated the freezability and sperm characteristics of ram semen diluted with egg yolk citrate diluents, with different glycerol inclusion levels.

The aim of this study was thus to determine the optimal glycerol inclusion level in a standard cryopreservation diluent for South African indigenous ram semen.

7.2 Materials and Methods

Semen was collected during winter (July, 2009 – outside the natural breeding season) from 6 healthy South African indigenous rams (Damara, Pedi and Zulu) with 2 rams per breed being used. The ages of the rams ranged between 2 to 4 years, with all animals being maintained on natural pastures, supplemented with a commercial diet, while water was available *ad libitum*. The ram semen was collected using an electro ejaculator twice a week for 2 weeks – with 4 ejaculates in total being collected from each ram. The semen was collected directly into a graduated test tube, which was then placed into a thermo flask with water (37°C) and transported to the laboratory for microscopic evaluation within a period of 1h. The raw, undiluted semen was evaluated for sperm concentration, semen pH, and sperm motility. The sperm concentration was determined with the aid of a spectrophotometer (Spermacue®) – calibrated for ram semen, the semen pH measured with the aid of a pH meter and a Computer Assisted Sperm Analysis (CASA) system used for the monitoring the sperm motility characteristics. After the initial evaluation of the ejaculates, the semen samples were diluted with an egg yolk citrate extender (EYC) fraction A (without

glycerol), in the ratio of 1:1 (v/v) and cooled over a period of 2h to 5°C. All ram ejaculates were pooled and then divided into 4 parts or treatment groups. The first group was diluted with EYC (fraction A), and served as a control while the other 3 groups were diluted with EYC (fraction B), containing 7, 10 or 14% glycerol (GLY) in the ratio of 2:1 (v/v); resulting in the final glycerol inclusion levels of 2.3, 3.3 or 4.7%, respectively . The semen samples were equilibrated for 2h and then loaded into 0.25mL semen straws. The straws were frozen in liquid nitrogen (LN_2) vapour, by placing the straws 5cm above the LN_2 surface for 10 minutes. Thereafter the semen straws were then plunged into the LN_2 (-196°C). The semen straws were thawed 7 days later, in a water bath (37°C) for 30 seconds. The sperm characteristics (motility and velocity) were microscopically evaluated using the Sperm Class Analyzer® (CASA) system. Data were analysed using the statistical program GenStat®. The analysis of variance (ANOVA) was used to test for significant differences between the treatments. Treatment means were compared using Fishers protected t-test for the least significant difference (LSD), at the 5% level of significance (Snedecor & Cochran, 1980).

7.3 Results and Discussion

In Table 7.1 the sperm motility and velocity characteristics of the raw indigenous ram semen are set out, prior to pooling. Breed (although not considered due to the limited numbers) had no effect on the sperm progressive motility, total motility, slow, medium, rapid and static (immotile) sperm. No breed effect was also recorded regarding the VCL (curvilinear velocity), VSL (straight-line velocity), VAP (average path velocity), LIN (linearity), STR (straightness) and BCF (beat cross frequency) sperm characteristics. The percentage of non-progressive sperm cells in the fresh ejaculates was generally significantly ($P<0.05$) lower in the Zulu (15.9%), compared to the Damara (34.6%) and Pedi (32.3%) semen. The Pedi breed (88.2%) recorded a significantly ($P<0.05$) higher percentage of WOB (wobbling) sperm cells, compared to the Damara (74.7%) and the Zulu (76.4%) breeds. The sperm cells from the Pedi breed (2.6µm) demonstrated significantly ($P<0.05$) shorter amplitude of lateral sperm head displacement (ALH) than the sperm cells of the Damara (3.4µm) and Zulu (3.4 µm) breeds (Table 7.1).

Table 7.1 The mean (±SE) sperm motility and velocity characteristics for different S.A. indigenous rams, as measured by CASA following dilution, prior to cryopreservation

Characteristics	Breed			Pooled
	Damara	Pedi	Zulu	
Total motility (%)	75.9±9.1[a]	78.8±7.4[a]	62.6±14.6[a]	72.4±10.4[a]
Progressive motility (%)	41.3±8.3[a]	46.5±6.8[a]	46.7±12.3[a]	44.8±9.1[a]
Non-progressive motility (%)	34.6±2.5[a]	32.3±6.4[a]	15.9±3.0[b]	27.6±4.0[a]
Rapid (%)	60.0±8.8[a]	66.3±5.6[a]	52.7±14.5[a]	59.7±10.4[a]
Medium (%)	8.3±1.2[a]	8.3±2.7[a]	4.4±1.2[a]	7.0±1.7[a]
Static (%)	24.1±9.1[a]	21.2±7.4[a]	37.4±14.6[a]	27.6±7.0[a]
Slow (%) %)	7.6±3.2[a]	4.2±1.4[a]	5.5±1.5[a]	5.8±2.0[a]
VCL(µm/s)	162.8±10.2[a]	156.6±15.1[a]	171.4±17.7[a]	163.6±14.3[a]
VSL(µm/s)	93.9±8.7[a]	107.6±6.9[a]	113.9±16.4[a]	105.1±10.7[a]
VAP(µm/s)	121.1±6.4[a]	137.7±12.8[a]	131.8±18.3[a]	130.2±12.5[a]
LIN (%)	57.5±2.6[a]	69.6±3.0[a]	65.9±3.7[a]	64.3±3.1[a]
STR (%)	77.4±5.0[a]	79.0±3.6[a]	86.2±0.7[a]	80.9±3.1[a]
WOB (%)	74.7±2.9[b]	88.2±2.0[a]	76.4±3.7[b]	79.8±2.9[b]
ALH(µm)	3.4±0.4[a]	2.6±0.3[b]	3.4±0.3[a]	3.1±0.3[a]
BCF(Hz)	17.5±1.5[a]	14.6±1.9[a]	19.4±3.0[a]	17.2±2.1[a]

VCL = curvilinear velocity, VSL = straight-line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency

[a, b] Values with different superscripts within a row differ significantly (P< 0.05)

In Table 7.2 the sperm motility and velocity characteristics of the pooled indigenous ram semen stored with glycerol as a cryoprotectant, prior to freezing, as measured by the CASA system, are set out. The glycerol inclusion level had no effect on all the sperm characteristics recorded, except for the percentage of total motile, progressive motile, immotile and rapid motile sperm cells. A 14% glycerol inclusion level resulted in a significantly (P<0.05) lower percentage total motile sperm cells (22.3%) being recorded, compared to the 0% glycerol or control (50.6%), 7% glycerol (57.1%) and 10% glycerol (49.9%) levels of inclusion. The percentage of progressive motile sperm cells was significantly (P<0.05) lower in the 14% glycerol (18.4%), compared to the

0% glycerol (35.8%), 7% glycerol (42.7%) and 10% glycerol (35.7%) inclusion levels. An inclusion level of 14% glycerol also resulted in a significantly ($P<0.05$) lower percentage rapid motile sperm (19.2%) being recorded, compared to the 0% glycerol (40.4%), 7% glycerol (49.8%) and 10% glycerol (41.1%) inclusion levels. The reduced sperm motility rates recorded in the semen equilibrated with 14% glycerol, indicates the toxicity of cryoprotectants at high levels. The sperm motility decreased with the increase in glycerol inclusion levels. The percentage static (immotile) sperm cells was significantly ($P<0.05$) higher in the 14% glycerol (75.2%) treated group, compared to the 0% glycerol (49.4%), 7% glycerol (43.0%) and 10% glycerol (50.4%) inclusion levels.

In general it can be said that cryopreservation causes a reduction in sperm motility and viability following injury even after glycerol addition (irrespective of the inclusion level) and equilibration. This has been indicated by the low sperm motility and the high percentage of immotile sperm recorded prior to freezing. A high proportion of sperm cells lost their motility during cooling and equilibration; with 0%, 7, 10 or 14% glycerol resulting in sperm motility losses of 31%, 21%, 31% or 71%, respectively. High glycerol inclusion levels resulted in higher pre freezing sperm motility losses compared to low inclusion levels, which confirm the toxicity of a cryoprotectant at a high concentration. One of the major causes of the reduced sperm motility after equilibration could be the negative effect of glycerol supplementation. The addition of glycerol to diluted ram semen has been said to cause changes in the permeability of the sperm cell membranes which ultimately affect the viability (Maxwell & Watson, 1996).

In Table 7.3 the sperm motility and velocity characteristics of the indigenous ram semen after freezing, as measured by the CASA system, are set out. The percentage glycerol inclusion had no effect on the percentage of non-progressive motile sperm, the medium motile sperm, the slow motile sperm and the percentage of STR.

Table 7.2 The mean (±SE) effect of different glycerol inclusion rates on the pre-freezing sperm motility and velocity characteristics of indigenous ram semen, as measured by CASA

Characteristics	Glycerol inclusion rate (%)			
	0	7	10	14
Total motility (%)	50.6±3.7[a]	57.1±4.9[a]	49.9±5.5[a]	22.3±5.8[b]
Progressive motility (%)	35.8±4.0[a]	42.7±3.6[a]	35.7±5.3[a]	18.4±4.5[b]
Non-progressive motility (%)	14.8±1.0[a]	14.3±2.4[a]	13.9±2.4[a]	6.4±0.7[a]
Rapid (%)	40.4±5.4[a]	49.8±3.9[a]	41.0±5.2[a]	19.2±5.8[b]
Medium (%)	6.0±1.3[a]	4.9±1.1[a]	5.2±1.3[a]	2.6±0.9[a]
Static (%)	49.4±3.7[b]	43.0±4.9[b]	50.4±5.8[b]	75.2±4.0[a]
Slow (%)	4.2±1.6[a]	2.3±1.1[a]	3.5±0.8[a]	3.0±1.6[a]
VCL(µm/s)	171.8±10.2[a]	197.7±6.1[a]	178.6±8.9[a]	155.8±14.2[a]
VSL(µm/s)	104.0±7.8[a]	117.4±2.9[a]	103.0±8.4[a]	100.1±12.4[a]
VAP(µm/s)	121.0±6.6[a]	136.2±4.2[a]	118.8±8.3[a]	111.6±11.7[a]
LIN (%)	60.4±1.6[a]	59.7±3.2[a]	57.7±3.3[a]	63.7±2.1[a]
STR (%)	85.7±2.0[a]	86.3±1.6[a]	86.7±2.5[a]	89.2±1.7[a]
WOB (%)	70.5±1.3[a]	69.1±3.4[a]	66.4±2.6[a]	71.4±1.2[a]
ALH(µm)	3.5±0.1[a]	3.6±0.2[a]	3.4±0.2[a]	3.7±1.9[a]
BCF(Hz)	22.4±1.8[a]	25.2±1.8[a]	25.9±1.2[a]	48.5±12.7[a]

VCL = curvilinear velocity, VSL = straight-line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency

[a, b] Values with different superscripts within a row differ significantly (P< 0.05)

Most researchers focus their analyses on sperm VCL, VSL, VAP, ALH and STR as these are indicators of potential fertility (Dorado *et al*., 2007). ALH indicates the vigour of flagellar beating, which affects the outcome of IVF, and is strongly correlated with sperm-oocyte fusion (Verstegen, 2002).

Sperm frozen without a cryoprotectant (0% glycerol or control) recorded a significantly (P<0.05) lower VSL, VAP, LIN, WOB (wobble), ALH (amplitude of lateral head displacement) and BCF, when compared to that of semen frozen with 7%, 10% or 14% glycerol. So for example the 10% glycerol inclusion level recorded a

higher percentage total motile sperm (15.6%), compared to the 7% glycerol (12.8%) and 14% glycerol (8.5%) inclusion levels, although all these differences were not significant. The same trend was observed for the percentage progressive motile sperm cells. The low percentage total motile sperm recorded when semen was cryopreserved without a cryoprotectant as illustrated in Table 7.3 indicates the importance of its inclusion in the semen extender. The low values of motility and velocity characteristics recorded when no glycerol was used to cryopreserve semen, may render the sperm cells incapable of fertilizing the oocytes. The low sperm motility results again recorded when semen was cryopreserved in an extender with 14% glycerol inclusion level indicated the possible toxicity of glycerol at high inclusion levels in a semen extender.

Abdelhakem *et al*. (1991) suggested that it is possible to freeze ram semen in the absence of glycerol with resultant good post-thaw sperm motility. However, in this study, a significant decrease was obtained regarding the post-thaw sperm motility and percentage live sperm in a diluent without glycerol, when compared to the other group of diluents containing glycerol. Thus, indicating the necessity of glycerol as a cryoprotectant. Glycerol protects the sperm by causing greater cell dehydration at lower temperatures, inducing an increased ability of the sperm cells to survive cryopreservation – due to reduced ice crystal formation (Holt, 2000).

It is thus essential to use an optimal cryoprotectant (glycerol) level in ram semen diluents, when cryopreserving semen, in order to improve sperm cryosurvival. This is emphasized in Table 7.3, where semen cryopreserved in an extender without glycerol recorded the lowest sperm cryosurvival. The results obtained in this study are lower, when compared to the study conducted by Aisen *et al.* (2002), who recorded 66% post thawing motility. Anel *et al*. (2003) also recorded a total sperm motility of 63% in Churra rams, which was higher than the current results. The low percentage of total motile sperm post-thaw was however comparable to that obtained by D'Alessandro and Martemucci (2003), who obtained a sperm motility rate of 19.6% during winter, 26.3% during summer, 27.3% during spring and 29.4% during autumn in Lecesse ram semen. Kozdrowski *et al*. (2007) also recorded 19.3% sperm motility in French Alpine goats, using the electro ejaculator as the method of semen collection.

Table 7.3 The mean (±SE) effect of different glycerol inclusion levels on the post thaw sperm motility and velocity characteristics of pooled indigenous ram semen, as measured by CASA

Characteristics	Glycerol inclusion level (%)			
	0	7	10	14
Total motility (%)	0.6±0.4[b]	12.8±2.4[a]	15.6±6.0[a]	8.5±1.0[ab]
Progressive motility (%)	0.0±0.0[b]	7.3±1.5[a]	9.5±4.4[a]	4.4±0.7[ab]
Non-progressive motility (%)	0.7±0.4[a]	5.5±2.5[a]	6.1±2.2[a]	4.2±0.5[a]
Rapid (%)	0.7±0.4[c]	9.5±1.7[ab]	12.8±4.8[a]	5.6±1.0[bc]
Medium (%)	0.0±0.0[a]	2.2±1.0[a]	2.1±1.0[a]	2.6±0.4[a]
Static (%)	99.3±0.4[a]	87.2±2.4[b]	84.4±6.0[b]	91.4±1.0[ab]
Slow (%)	0.0±0.0[a]	1.1±0.4[a]	0.7±0.4[a]	0.5±0.5[a]
VCL(µm/s)	82.0±49.5[b]	175.0±10.5[a]	207.0±30.6[a]	155.0±23.8[ab]
VSL(µm/s)	24.2±14.4[b]	98.7±16.5[a]	92.5±10.5[a]	83.6±10.7[a]
VAP(µm/s)	37.7±22.1[b]	128.3±8.4[a]	142.4±10.9[a]	105.6±15.7[a]
LIN (%)	15.0±8.7[b]	57.0±9.4[a]	50.0±12.4[a]	54.8±4.2[a]
STR (%)	31.9±18.5[b]	75.8±8.9[a]	67.6±12.3[a]	79.9±2.9[a]
WOB (%)	23.5±13.7[b]	73.7±4.5[a]	71.5±6.8[a]	68.4±3.0[a]
ALH(µm)	0.0±0.0[b]	2.7±0.3[a]	3.3±0.4[a]	2.7±0.3[a]
BCF(Hz)	0.0±0.0[b]	22.2±0.7[a]	24.9±2.5[a]	21.9±1.9[a]

VCL = curvilinear velocity, VSL = straight-line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency

[a,b] Values with different superscripts within a row differ significantly (P< 0.05)

7.4 Conclusions

This study demonstrated that the egg yolk-citrate extender containing 10% glycerol can be used to cryopreserve indigenous ram semen effectively, based on the sperm motility characteristics recorded. This study also confirmed the need for the inclusion of a cryoprotectant in the semen cryopreservation extender, as semen that was cryopreserved in an extender without glycerol (control), recorded the lowest cryosurvival of the sperm.

The low sperm motility results recorded in this study, when semen was cryopreserved in an extender containing 14% glycerol on the other hand indicated the toxicity of glycerol at high inclusion levels in the semen extender. Further studies are however needed to improve the entire sperm cryopreservation process and this has, ultimately to be evaluated regarding the fertilizing ability of the ram semen. Other protocols on ram semen cryopreservation, diluents and thawing procedures could also improve the efficiency of semen cryopreservation.

Chapter 8

Comparison of the slow cooling and liquid nitrogen vapour method on ram sperm motility rate following cryopreservation

8.1 Introduction

In general raw extended ram semen has a relatively short fertile lifespan and by reducing the metabolic rate of the sperm cell, the storage life of ram semen may be extended. The lowering of the storage temperature is one of the ways in which the fertile lifespan of sperm can be prolonged i.e. by decreasing the cellular metabolism (Morrier *et al*., 2002).

Vapour freezing is a simple and standard method of semen freezing, but different computer-controlled freezing programmes have been suggested because of their ability to control cooling and freezing rates in a more reliable way (Stanic *et al*., 2000). McLaughlin *et al*. (1990) have shown the computer-controlled freezing methods to preserve sperm quality better than vapour freezing, but others have found no beneficial effects. Whatever freezing procedure is used, there is always damage to the sperm cells, resulting in either structural or functional changes (Check *et al*., 1991).

Successful cryopreservation of sperm cells is affected by the rate of freezing and composition of the solution in which the cells are frozen (Holt, 2000). Therefore, optimum cooling rates must be used in the cryopreservation of many cell types as it influences the extent and rate of cell dehydration. Slow cooling may dramatically reduce the sperm cryodamage in many species (Hammadeh *et al*., 2001). Effective cooling rates for sperm of the horse have been reported to be around 5°C/min or less (Varner *et al*., 1988). However, quicker rates of cooling are required for some other species, with human sperm being about 10°C/min (McLaughlin *et al*., 1990), and optimal rates of cooling of 20°C/min or more for sperm of ram (Fiser *et al*., 1986), boar (Fiser *et al*., 1993) and mice (Stacy *et al*., 2006). When cooled to about -5°C, cells and their surrounding medium remain unfrozen and supercooled. Between -5 and -10°C, ice forms in the external medium, but the cell contents remain unfrozen and

supercooled. The supercooled water in the cells has a higher chemical potential than that of water in the partially frozen extracellular solution, and thus water flows out of the cells osmotically and freezes externally (Mazur, 1990).

The cooling rate affects the subsequent physical events within the sperm cells and determines the outcome of the freezing process. If sperm cells are cooled rapidly, water is not lost fast enough to maintain equilibrium. The cells become increasingly supercooled, eventually attaining equilibrium by freezing intracellulary (Mazur, 1990). Cells that undergo intracellular ice formation during cryopreservation are mostly killed (Muldrew & McGann, 1994). If cooling is slow, the sperm cells will lose water rapidly enough to concentrate the intracellular solutes sufficiently to eliminate supercooling. The sperm cells will then dehydrate and the intracellular freezing will not occur. If sperm cells are cooled too slowly, they experience a severe volume shrinkage and long-time exposure to high solute concentrations. Both factors could cause cell injury (Mazur, 1990).

The aim of this study was thus to compare two methods of cryopreserving indigenous ram semen i.e. with the aid of a programmable freezer versus the freezing of semen in the liquid nitrogen vapour – with respect to post-thawing sperm motility characteristics.

8.2 Materials and Methods

Ram semen was collected during August, 2009 (end of winter, outside the natural breeding season) from 6 healthy South African indigenous rams (Damara, Pedi and Zulu), with two rams per breed being used. The age of the rams varied between 2 and 4 years. All animals were maintained on natural pastures, supplemented with a commercial pelleted diet. Water was freely available throughout the trial. Semen was collected with the aid of an electro ejaculator, twice weekly, for a period of 2 weeks, with a total of 4 ejaculates being collected from each ram. Semen was collected directly into graduated test tubes, which were then placed and stored in a thermo flask (with water at a temperature of 37°C). The collected semen was then transported to the laboratory for sperm microscopic evaluation and semen processing within 1h.

The raw undiluted semen sample of each ram was evaluated for sperm concentration, semen pH, and sperm motility. Sperm concentration being measured with the aid of a

spectrophotometer (Spermacue®), the semen pH using a pH meter and the Computer Assisted Sperm Analysis (CASA) system was used for the sperm motility analysis (Verstegen *et al.*, 2002). After the initial evaluation of the raw semen samples, all ejaculates were pooled and then diluted with an egg yolk citrate extender (EYC) fraction A (without glycerol), in the ratio of 1:1(v/v) and cooled over a 2h period at 5°C. After the 2h period of equilibration, the pooled semen sample was further diluted with EYC fraction B, containing 14% glycerol in a ratio of 2:1(v/v), resulting in a final glycerol concentration of 4.7%. The pooled semen sample was then further equilibrated for a further 2h period and then loaded into 0.25mL semen straws. Half of the straws (n=10) were frozen in liquid nitrogen (LN_2) vapour by placing the straws 5cm above the LN_2 surface, for 10 minutes and then plunging the semen straws directly into the LN_2. The other half of the semen straws (n=10) were frozen with the aid of a programmable freezer, using the following freezing curve: The semen sample was cooled from 5°C to -5°C, at a rate of 4°C/min; then from -5°C to -110°C, at a rate of 25°C/min and from -110°C to -140°C at a rate of 35°C/min. Thereafter the frozen semen straws were transferred and stored in a LN_2 tank until further use (Anel *et al.*, 2003).

After 7 days, the semen straws were removed from the liquid nitrogen and thawed in a water bath at 37°C, for 30 seconds. The sperm characteristics (sperm motility and velocity) were microscopically evaluated using the Sperm Class Analyzer® (CASA) system. All sperm motility data were analysed using the statistical program GenStat® and the analysis of variance (ANOVA) was used to test for differences between the two treatments. Treatment means were compared using the Fishers' protected t-test least significant difference (LSD), at the 5% level of significance (Snedecor & Cochran, 1980).

8.3 Results and Discussion

In Table 8.1 the comparison of sperm motility and velocity characteristics following the two freezing methods, as measured by CASA are set out. In general the freezing technique had no effect on all the sperm motility characteristics. The values of all the other sperm motility and velocity characteristics, except for the percentage immotile sperm were higher in the semen frozen with the aid of the programmable freezer, compared to the semen frozen in the liquid nitrogen vapour. The results of this study

tended to indicate that sperm frozen with the aid of a programmable freezer recorded a better quality sperm motility following thawing compared to semen frozen in the liquid nitrogen vapour. This may have been due to the overall faster decline in temperature in the Styrofoam® box than in the programmable freezer. Moreover, the Styrofoam® box may have provided a more variable freezing rate than the programmable freezer as the level of liquid nitrogen in the box was subjectively estimated and subject to evaporation, and difficult to standardise for each freezing run. This is in agreement with a study conducted by Hammadeh *et al.* (2001), who also recorded better sperm motility results when the programmable freezer was used. Similarly, Clulow *et al.* (2008) found the motility of the sperm cryopreserved in the programmable freezer to be higher than when sperm were frozen in liquid nitrogen vapour in a Styrofoam® box. Petyim and Choavaratana (2006) also demonstrated that freezing semen with a computerized freezer causes less cryodamage to the sperm cell affecting sperm motility and cryosurvival rate, compared to freezing with liquid nitrogen vapour. Programmable freezers have the ability to control cooling and freezing rates in a more reliable way, and they give results that are more repeatable than LN_2 vapour method (Stanic *et al.*, 2000).The advantages of the LN_2 vapour method are that it is generally cheaper, easy to use, has a low liquid nitrogen requirement, it is portable and thus more practical. In contrast, the programmable freezer is an expensive apparatus, uses a large volume of liquid nitrogen and is generally fixed within the laboratory (Clulow *et al.*, 2008).

Table 8.1 Comparison of ram sperm motility and velocity characteristics following cryopreservation by two freezing methods as analysed by the CASA system

Characteristics	Freezing method (mean ± SE)	
	Programmable freezer	Vapour freezing
Total motility (%)	15.3±3.0	8.8±0.9
Progressive motility (%)	8.9±1.6	4.4±0.7
Non-progressive motility (%)	6.5±1.5	4.2±0.5
Rapid (%)	11.7±2.0	5.6±1.0
Medium (%)	1.3±0.4	2.6±0.4
Static (%)	85.1±2.8	91.5±1.0
Slow (%)	2.2±0.8	0.5±0.5
VCL(µm/s)	199.1±14.0	155.4±23.8
VSL(µm/s)	121.0±8.9	83.6±10.7
VAP(µm/s)	146.4±8.9	105.6±15.7
LIN (%)	60.7±4.3	54.9±4.2
STR (%)	82.0±2.5	79.9±2.9
WOB (%)	73.7±3.9	68.4±3.0

VCL = curvilinear velocity, VSL = straight-line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BCF = beat cross frequency

8.4 Conclusions

It can be concluded that a controlled rate of cooling semen gives better sperm motility results in rams, compared to semen frozen in LN_2 vapour, but significantly lower than the acceptable sperm motility rate. It should however be noted that controlled rate (programmable) freezers are costly, when compared to the liquid nitrogen vapour technique. As the sperm motility differences recorded were not significant, it is suggested that the freezing of semen on a small scale be done in LN_2 vapour, without any significant decrease in sperm motility and possible fertility. The microscopic sperm characteristics only give an indication of the potential fertility and the actual test for the two techniques would lie in the conception rates (*in vivo*).

Chapter 9

General Conclusions and Recommendations

9.1 General Conclusions

This study gave an overall insight into the characterization of indigenous ram semen and the semen quality in the breeds. The CASA system gave objective, reliable and repeatable sperm motility results. Semen volume and sperm concentration of the indigenous ram ejaculates were lower when compared to other breeds in other studies. The semen pH recorded in this study was consistent with that reported for other mammalian studies. The Pedi ram semen recorded the highest total sperm motility and the highest progressive sperm movement, compared to the other indigenous breeds.

Body weight was found to be positively correlated with total sperm motility. However negative correlations were recorded between body weight and scrotal circumference, semen volume, sperm concentration and semen pH. Semen volume was positively correlated with sperm concentration and semen pH. In the literature scrotal circumference was positively correlated with semen volume, total sperm motility and sperm concentration and these traits have been shown to be closely correlated with fertility in bulls. The sperm concentration was positively correlated with semen pH, with negative correlations also recorded in this study, between total sperm motility and semen volume, sperm concentration, semen pH. The low percentage of sperm abnormalities demonstrated in the current study that the technique used to collect and evaluate sperm abnormalities were acceptable for obtaining normal, viable sperm.

Semen stored at 15°C recorded the higher sperm motility, compared to semen stored at 5°C, over the entire storage period. It would thus seem, according to the results in this trial that ram semen diluted and stored at 15°C for a period of up to 24h was more satisfactory in retaining sperm motility. It can thus be seen as a practical means of storing semen (15°C) for the application of artificial insemination in a short-term semen storage programme, incorporated with AI – for possible acceptable fertilisation rates.

In semen that was diluted with a glycerol extender, the storage temperature and period had no significant effect on most of the sperm motility and velocity characteristics. The sperm motility and velocity characteristics recorded did not show any significant differences for semen stored at both temperatures (5°C and15°C) for the different storage periods. However it was found that most of the seminal characteristics (sperm motility characteristics) were better in semen stored at 15°C, compared to those stored at 5°C, although these differences were not significant.

In the trial where different glycerol inclusion levels were compared, it was demonstrated that the egg yolk-citrate extender containing 10% glycerol can be used to cryopreserve indigenous ram semen effectively – based on the sperm motility characteristics recorded. This study also confirmed the need for the inclusion of a cryoprotectant in the semen cryopreservation extender, as semen that was cryopreserved in an extender without glycerol (control), recorded the lowest cryosurvival of the sperm. The low sperm motility results recorded in this study, when semen was cryopreserved in an extender containing 14% glycerol, on the other hand indicated the toxicity of glycerol at high inclusion levels in the semen extender.

The controlled rate of cooling semen recorded better sperm motility results, compared to semen frozen in liquid nitrogen vapour, but significantly lower than the acceptable sperm motility rate. It should however be noted that programmable freezers are costly, when compared to the liquid nitrogen vapour technique. As the sperm motility differences recorded were not significant, it is suggested that the freezing of semen on a small scale be performed in liquid nitrogen vapour.

9.2 Recommendations

It is recommended that further studies be conducted using more rams and a different semen collection technique (artificial vagina). The longer term effect (more than 24h) of semen storage at different temperatures on sperm viability, acrosome integrity and the relationship to actual fertilizing capacity should be investigated. The ultimate test regarding sperm viability would however be in the fertilizing ability of the sperm cell – an aspect not investigated in this study.

Storage of semen at 15°C can be seen as practical means of storing semen for the application of artificial insemination in a short-term semen storage programme, as it resulted in the better preservation of sperm motility characteristics as recorded by CASA, compared to the 5°C storage group.

Further studies are however needed to improve the entire sperm cryopreservation process where other protocols on ram semen cryopreservation, diluents and thawing procedures could also improve the efficiency of semen cryopreservation. When the two cryopreservation techniques were compared, sperm motility differences recorded were not significant, and it is recommended that freezing of semen on a small scale be performed in LN_2 vapour, as it is cheaper and easier, when compared to costly programmable freezer.

ABSTRACT

CRYOPRESERVATION OF SOUTH AFRICAN INDIGENOUS RAM SEMEN

by

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Semen was collected from the indigenous Damara, Namaqua Afrikaner, Pedi and Zulu rams. Hundred and twenty eight (128) ejaculates were collected throughout the entire study, with semen being collected twice a week (every Monday and Tuesday) from each ram, using the electro-ejaculator. Ejaculates were collected in graduated test tubes, placed in a thermo flask at 37°C, and transported to the laboratory for evaluation within 1h interval. The raw or fresh undiluted semen was then microscopically evaluated for volume, concentration, pH and sperm motility. The sperm concentration was determined with the aid of a spectrophotometer (Spermacue®) and the semen pH using a pH meter (Microprocessor pH/mV/°C Meter Hanna HI 931401). A Computer Assisted Sperm Analysis (CASA) system was used to evaluate the different sperm motility characteristics. All data were analysed using the statistical GenStat® program. The analysis of variance (ANOVA) was used to test for significant differences between treatments.

Characterization of the South African indigenous ram sperm viability (percentage live/dead) of the semen samples was determined, using an eosin/nigrosin stain (60µl

eosin/nigrosin and 6µl semen), in a thin smear. All sperm cells were evaluated on the same day of semen collection with the aid of a fluorescent microscope (BX 51TF), using an oil immersion objective (X100 magnification). The live sperm fluoresced green, while the dead cells stained red. The live sperm cells were further categorized as morphologically normal or abnormal. The volume of the indigenous ram ejaculates ranged between 0.4 and 0.9mL. The sperm concentration recorded in this study ranged between 0.9 and $1.3x10^9$ sperm/mL, which are much lower when compared to other studies. The semen pH recorded in this study ranged between 6.5 and 7.3 and the sperm abnormalities ranged between 5.2% and 8.2% – which is regarded as acceptable for fertilization.

To test the effect of storage temperatures on the viability of the diluted ram semen stored for different periods of time, the same procedure of semen collection and semen evaluation was followed. After the initial semen evaluation, all semen samples were pooled and diluted equally in an egg yolk citrate extender in the ratio of 1:1(v/v). The pooled semen sample was then divided into two portions, one sample being stored at 5ºC, and the other at 15ºC, following storage periods of 3, 6, 9, and 24h respectively. Sperm characteristics were then recorded for each interval of storage. In general the percentage total motile sperm recorded after a 24h period of storage at 15ºC was higher (61.2%), compared to that at 3h (51.4%), 6h (50.1%) and 9h (50.6%). From the results of this study it was concluded that diluted ram semen can be successfully stored for 24h at 15ºC, retaining sperm motility for the application of AI.

When evaluating the effect of glycerol as a cryoprotectant, in the diluted ram semen stored at two temperatures for different periods of time**,** the same procedure for semen collection and evaluation was followed. After initial evaluation, all semen samples were pooled and diluted equally with an egg yolk citrate extender containing 14% glycerol in the ratio of 1:1 (v/v), resulting in a final glycerol concentration of 7%. The pooled semen sample was then divided into two portions, one sample being stored at 5ºC and the other at 15°C, for periods of 3, 6, 9, and 24h. Sperm characteristics were recorded at each interval of semen storage. Semen stored at 15°C recorded a 48.3% total motile sperm after 3h of storage, but this increased to 50.4% following 24h of storage. The percentage of total motile sperm remained relatively constant at 40%

after 3h of storage and 40.8% after 24h in the semen stored at 5°C. The addition of glycerol as a cryoprotectant demonstrated a protective effect on the sperm motility characteristics of sperm stored at both 5°C and 15°C for up to 24h of storage. The effect of different glycerol inclusion levels in the diluent, on the indigenous ram semen characteristics following cryopreservation were evaluated. The same procedure for semen collection was followed and semen was subjected to the initial evaluation comprising sperm concentration, semen pH and sperm motility. After initial evaluation of the ejaculates, the semen samples were diluted with an egg yolk citrate extender (EYC) fraction A (without glycerol), in the ratio of 1:1 (v/v) and cooled over a period of 2h to 5°C. All ram ejaculates were pooled and then divided into 4 portions treatment (groups). The first group was diluted with EYC (fraction A), which served as a control and the other 3 groups with EYC (fraction B) contained 7, 10 or 14% glycerol (GLY) in the ratio of 2:1 (v/v), making final glycerol concentrations of 2.3, 3.3 or 4.7% respectively. The semen samples were equilibrated for 2h and then loaded into 0.25mL semen straws. The straws were frozen in liquid nitrogen (LN_2) vapour, whereafter semen straws were plunged into the LN_2 (-196°C). The semen straws were thawed 7 days later, in a water bath (37°C) for 30 seconds. The sperm characteristics (motility and velocity) were microscopically evaluated using the Sperm Class Analyzer® (CASA) system. A 10% glycerol inclusion rate recorded a higher percentage of total motile sperm (15.6%), compared to the 7% glycerol (12.8%) and 14% glycerol (8.5%) inclusion levels, although all these differences were not significant. This study demonstrated that an egg yolk- citrate extender containing 10% glycerol can be used to cryopreserve indigenous ram semen effectively, based on the sperm motility characteristics. The low sperm motility results recorded when semen was cryopreserved in an extender containing 14% glycerol also indicated a degree of toxicity of glycerol at high inclusion levels in the semen extender.

Regarding the conventional slow cryopreservation (programmable freezer) of ram semen versus semen cryopreservation in liquid nitrogen vapour, the same procedure for semen collection and evaluation was followed. After the initial evaluation of the raw semen samples, all ejaculates were pooled and then diluted using an egg yolk - citrate extender (EYC) fraction A (without glycerol), in the ratio of 1:1(v/v) and cooled over a 2h period at 5°C. After equilibration, the pooled semen sample was further diluted with EYC fraction B, containing 14% glycerol, in a ratio of 2:1(v/v)

resulting in a final glycerol concentration of 4.7%. The pooled semen sample was then further equilibrated and loaded into 0.25mL semen straws. Half of the straws were frozen in liquid nitrogen (LN_2) vapour and then plunged into the LN_2. The other half of the semen straws were frozen with the aid of a programmable freezer. After 7 days, the semen straws were thawed in a water bath at 37°C, for 30 seconds. The sperm characteristics (sperm motility and velocity) were microscopically evaluated using the CASA system. From the findings in this study, it can be concluded that a controlled rate of semen cooling gave superior sperm motility results (15.3±3.0%), compared to semen frozen in LN_2 vapour (8.8±0.9%). It should be noted that programmable freezers are costly, when compared to the liquid nitrogen vapour technique. Due to the fact that sperm motility differences recorded were not significant, it is suggested that the freezing of semen on a small scale be done using the LN_2 vapour technique, without any significant decrease in sperm motility or possible fertility.

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