IN VITRO EMBRYO PRODUCTION AND SEMEN CRYOPRESERVATION IN SHEEP

by

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NTS'EMELO MAHOETE

Dedication

To my mother, Sisinyane Mahoete and my late sister Baleseng Mahoete for their support and inspiration.

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Declaration

I hereby declare that this dissertation submitted by me for the degree, **Magister Scientiae Agricuturae**, at the University of the Free State has never been previously submitted to any other university. I furthermore cede copyright of the dissertation in favour of the University of the Free State.

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List of abbreviations

ANOVA	Analysis of variance
ART	Assisted reproductive technologies
AV	Artificial vagina
BO	Brackett and Oliphant medium
BSA	Bovine Serum Albumin
CO_2	Carbon dioxide
COC's	Cumulus Oocyte Complexes
CR1	Charles Rosenkrans medium
DMSO	Dimethyl sulphoxide
DPBS	Dulbecco's phosphate buffered saline
E_2	Estradiol
EDTA	Ethylenediaminetetraacetic acid
EE	Electro-ejaculation
EG	Ethylene glycol
EY	Egg yolk
ESS	Estrous Sheep Serum
FBS	Foetal Bovine Serum
FSH	Follicle stimulating hormone
GPx	Glutathione peroxidase
ICSI	Intracytoplasmic sperm injection
IVEP	In Vitro Embryo Production
IVC	In Vitro Culture
IVF	In Vitro Fertilization
IVM	In Vitro Maturation
KSOM	Potassium Simplex Optimization Medium
LH	Luteinizing hormone

LN_2	Liquid nitrogen
MEM	Minimum Essential Medium
mg	milligram
ml	milliliter
MOET	Multiple Ovulation and Embryo Transfer
mPBS	Modified phosphate buffered saline
O_2	Oxygen
OPU	Ovum Pick-Up
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SOF	Synthetic oviduct fluid
TCM 199	Tissue Culture Medium 199
VCV	Vaginal collection vial
µl	microliter

CHAPTER 1

GENERAL INTRODUCTION

Sheep are said to have been one of the first species to be domesticated and hence have been closely associated with man from early times (Shelton, 1995). These livestock are important around the world, but more important in the less developed countries, which are known to have limited land and other natural resources. Apart from meat production sheep are also valuable regarding their production of milk, manure, fibre, and employment. Besides these conventional usages, sheep, in recent years, have become an important research tool for new and advanced technologies. The animal's size and physiology provides an appropriate sculpt to study a variety of mammalian biological functions, which are important - such as reproduction, embryology and fetal development, with regard to increasing the performance efficiency. Increasing production of sheep offers an opportunity for improving the livelihood of people, especially in the less developed countries. Sheep numbers (surplus) can be increased through improved reproductive efficiency of the flock and this reproductive efficiency can be attained through the manipulation of the reproductive activities and technologies (Devendra, 1980; Zhu et al., 2001; Mahammadpour, 2007).

Sheep are thus suitable and more adapted to small, low-capital input farms in the rural areas of developing countries. Certain sheep breeds are also adapted to the semi-arid environments (Terrill, 1985; Turner, 2002). For the achievement of improved reproductive performance, several assisted reproductive technologies (ART's), such as multiple ovulation and embryo transfer (MOET), *in vitro* embryo production (IVEP) and semen and embryo cryopreservation are available. IVEP and cryopreservation are also some of the most powerful tools in controlling and manipulating mammalian reproduction (Cognie et al., 2003, Martinez et al., 2006). *In vitro* embryo production is rendered important in sheep as it has shown the potential of producing sheep embryos, even during the non-breeding season,

through *in vitro* maturation and *in vitro* fertilization techniques and shortening of the generation interval (Pugh et al., 1991). Lambs can thus be produced continuously using IVEP, even though sheep are seasonal breeders.

The improved IVEP technologies of *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) have further led to another generation of reproductive techniques, such as intracytoplasmic sperm injection (ICSI), the production of transgenic animals and even cloning. With ICSI, only one sperm is needed to fertilize an oocyte and the motility of that sperm is not of great importance for the fertilizing ability. Ironically, when cloning techniques are used, sperm are no longer needed (Cibelli et al., 2002).

The cryopreservation of gametes is rendered an important technique in ART, especially when the distance between donors leads to male and female gametes not being readily available. However, the cryopreservation process exposes sperm to physical and chemical stress and less than 50% of the sperm can survive with the fertilizing ability being maintained (Waterhouse et al., 2006). Long term conservation of sperm is essential when IVF or artificial insemination is to be performed at a future date (Merlo et al., 2008). Embryos as such can also be stored if there is a limitation in the number of recipients, until the required numbers of recipients are available. If the production of desired offspring needs to be postponed to a later date, efficient cryopreservation techniques are essential (Leoni et al., 2001; Cognie et al., 2003). Cryopreserved gametes are easier and less expensive to transport from one location to the next and allow for long-term storage. This creates the opportunity to maintain superior genetic material at low costs and also conserve endangered species or breeds (Fogarty et al., 2000, Begin et al., 2003; Gonzalez-Bulnes et al., 2004, Mapletoft & Hasler, 2005). While cryopreservation of bovine semen and embryos has made great progress in recent years, little progress has, however, been obtained in the sheep industry (Zhu et al., 2001)

For gamete cryopreservation, two techniques are currently being used, namely the conventional slow freezing and the vitrification techniques. Slow freezing, however, has been reported to be the most preferred technique, particularly in embryos, both *in vivo* and *in vitro* (Martinez & Matkovic, 1998). The slow freezing technique has been reported to have several limitations, such as being costly - because of the slow freezing equipment required, physical damage to the embryos due to crystal formation and the process being time consuming (Kasai, 1996; Vajta, 2000; Okada et al., 2002; Naik et al., 2005). Vitrification on the other hand is said to be a cheaper method than the slow freezing technique. This technique leads to minimum cell injury through crystal formation, although damage may still occur due to cryoprotectant toxicity (Okada et al., 2002; Garcia-Garcia et al., 2005; Naik et al., 2005; Sharma et al., 2006). Cryoprotectant toxicity is one of the most important barriers to be overcome for successful vitreous preservation of these complex, spatially extended bio systems. Less toxic vitrification solutions are currently still being researched (Fahy et al., 2004).

Semen cryopreservation involves sperm dilution, cooling, freezing and thawing. Each of the steps may cause sperm damage which may impair the normal sperm functioning and fertilizing potential. In sheep, the mostly used semen preservation technique is slow cooling than vitrification (Salamon & Maxwell, 1995a; Thuwanut, 2007). However, there is still a lot of work that needs to be carried out, in order to improve semen cryopreservation efficiency and further identify factors affecting sperm survival (Bester, 2006).

The objectives of this study were as follows:

i) Compare two different oocyte harvesting techniques in ovine IVEP
ii) Evaluate the effect of different culture media on the development of *in vitro* produced ovine embryos.
iii) Evaluate the effect of breed on the efficiency of cryopreservation of ram semen.

iv) Test the fertilizing ability of frozen-thawed ram semen following incubation.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Artificial insemination (AI) has for many years been the predominant method used for controlled breeding and to increase the reproductive rate of males in animal production. It has been practiced in many farm animal species, but has been particularly useful and more successful when applied in cattle, where the cost benefit over natural mating is more substantial (Elder & Dale, 2000; Mitchell & Doak, 2004). Sheep on the other hand have been targeted regarding controlled breeding for years, due to the species seasonality in breeding, with the aim of increasing and concentrating the lamb crop per year. As a result a number of ART's have also been tried in sheep, including *in vivo* and *in vitro* embryo production (IVEP) (Shelton, 1995).

Embryo production either *in vivo* or *in vitro* is a well-established practice to spread or conserve desirable genes of valuable individuals, also in small ruminants. IVEP has been considerably refined in recent years due to initial fertilisation failures and embryo degeneration experienced, that affect the yields obtained when using traditional MOET (Gibbons et al., 2007). A cheap and abundant source of oocytes is usually available from slaughterhouse material. However these oocytes are highly variable in their developmental competence and genetic make-up (Bilodeau-Goeseels & Panich, 2002). This technique of IVEP also has a greater potential of increasing the number of embryos produced for conducting basic research (uniform offspring) and the stud application of emerging biotechnologies, such as embryo sexing, sperm injection, nuclear transfer and transgenesis (Baldassare et al., 2002; O'Brien et al., 2004). This technique has successfully been used in sheep and goats. IVEP has several advantages, including the production of offspring from sub-fertile males and females, an increase in the number of progeny from selected mature or juvenile

females and saving oocytes or sperm from valuable dead or dying animals, which would otherwise have been naturally lost (Nedambale, 1999; Cognie et al., 2004).

In sheep, less progress has been attained with IVEP over the past years due to several shortfalls in the technique, such as the *in vitro* maturation, fertilisation and culturing procedures. Apart from factors coupled to the basic *in vitro* procedure itself, oocytes from small follicles (2-3mm) have been shown to have a reduced developmental competence *in vitro*. This occurs due to the lack of prematuration events that should occur during the final follicular growth phase (Cognie et al., 2004). This also shows that there is a direct relationship between the ovarian follicle size and oocyte diameter and this affects the oocyte developmental capacity (Arlotto et al., 1996). *In vitro* produced ovine embryos have been found to be sensitive to cryopreservation, hence showing a reduced capacity to establish pregnancy after transfer into recipients (Leoni et al., 2007). These factors leading to less progress of IVEP in sheep, thus, warrants the conduction of more intensive studies in order to improve the levels of producing transferable embryos.

The cryopreservation of genetic material is another important extension in the conservation of genetic resources, whether produced *in vivo* or *in vitro* (Sharkey et al., 2002; Andrabi & Maxwell, 2007). This technique involves the preservation of sperm, oocytes, embryos or somatic cells. Semen cryopreservation and AI could also offer many advantages to the livestock industry for optimization of animal production. Cryopreservation of embryos could preserve important genes for future use, provide insurance against the loss of a particular superior sire, permit long distance transport of semen and allow the insemination of large numbers of females over extended periods of time or at different times of a year (Gillan et al., 2004; Peris et al., 2004) and could also even be used to solve human infertility problems (Barbas & Mascarenhas, 2009).

2.1.1 *In vitro* embryo production

The *in vitro* embryo production (IVEP) system includes three major steps, namely *in vitro* maturation (IVM) of the primary oocytes, *in vitro* fertilisation (IVF) of matured oocytes and *in vitro* culture (IVC) of presumptive embryos, until transferred or cryopreserved for future use (Cognie et al., 2003; Gandolfi et al., 2005; Zhu et al., 2007). The availability of enough oocytes is a prerequisite for IVEP, as it determines the number of embryos produced. Primary oocytes for IVEP are, however, mostly obtained from the ovaries of slaughtered animals (Wani, 2002). These oocytes from slaughterhouse material have the limitation that their genetic potential or origin are unknown and make no contribution to genetic progress (Cunningham, 1999). Oocytes can also be recovered from live donor animals. Although a large number of oocytes are present in mammalian ovaries, only a restricted number of them are chosen to develop to their mature size under hormonal control. These oocytes become competent to develop, be fertilized, and contribute to embryonic development. Generally most of the immature oocytes collected from mammalian ovaries fail to develop up to the pre-implantation stage following IVM, IVF and IVC. This failure has been attributed to factors such as the quality of oocyte and the embryo culture conditions (Kane, 2003; Merton et al., 2003; Russo et al., 2007; Morton et al., 2008).

2.1.2 Oocyte collection techniques

The method of oocyte recovery could affect the efficiency of IVEP (Katska-Ksiazkiewicz et al., 2007). There are different methods of oocyte recovery used from slaughtered animals. Oocytes can, however, also be recovered from live animals. In slaughtered sheep, slicing of the ovary or aspiration has generally been used for oocyte recovery (Wani et al., 2000). In live animals on the other hand, the oocytes are recovered through transvaginal ovum pick-up (OPU) or with the aid of the laparoscope.

2.1.2.1 Ovarian slicing

Slicing refers to a method whereby ovaries are placed in a petri dish containing a harvesting medium. Incisions are then made with the aid of a surgical blade over the entire ovarian surface (Pawshe et al., 1994; Wani et al., 2000; Wang et al., 2007). The oocytes are subsequently released from the follicles into the culture medium in the dish. In sheep, there are contradicting findings regarding slicing of the ovaries yielding a higher number of oocytes per ovary. However, resultant debris interferes with the microscopic search of the oocytes. This technique can also lead to a loss of harvested oocytes (Wani et al., 2000; Wang et al., 2007).

2.1.2.2 Oocyte aspiration

This method of oocyte recovery entails that all visible ovarian follicles are aspirated, using a hypodermic needle attached to a disposable syringe. Aspiration of follicular oocytes has been found to be difficult in the small ovaries as e.g. in sheep and goats. Only about 2 cumulus oocyte complexes (COC's) of acceptable quality per sheep or goat ovary are generally attained by aspiration (Pawshe et al., 1994; Cognie, 1999). The aspirated follicular fluid is transferred to a search Petri dish for microscopic recovery of the oocytes. Aspiration pressure above 50mmHg has been found to decrease the oocyte recovery rate and recovery of good quality oocytes, and thus increases the number of denuded oocytes from the bovine ovary (Pfeifer et al., 2008). The problem of denuded oocytes in sheep and goats can be overcome by using an 18-G needle attached to silicon tubing of 1mm internal diameter under 25mmHg aspiration vacuum. This will maintain the adhesion of cumulus cells to the oocytes. However, the oocyte recovery rate is generally lower (50-60%), when compared to the recovery rate (85-90%) obtained when using 50mmHg aspiration vacuum in sheep (Baldassarre et al., 1996; Cognie et al., 2004).

2.1.2.3 Oocytes recovered via ovum pick-up (OPU)

Immature oocytes can be collected by using either transvaginal OPU or by laparoscopic aspiration from live animals. Oocytes collected through these

techniques are usually of acceptable quality. The pedigrees of animals used are generally known and, therefore, superior quality genetic material can be produced. Animals with superior traits can be aspirated repeatedly, even when pregnant, without causing any harm to the animal or the fetus (Cunningham, 1999). Transvaginal OPU can also be performed with, or without ultrasound. The latter is the most commonly used method. This is because of the low risk of infection and injury and it can be performed approximately twice a week with approximately 4 to 8 oocytes being harvested per collection in small stock. Thus OPU may be an alternative to superovulation procedures in future. Oocytes following OPU can be harvested from adult or prepubertal animals, although a large number of oocytes can also be collected from ewes superovulated with the aid of hormones, which ultimately leads to increased embryo production. The production of embryos from young donors may greatly reduce the generation interval and hence accelerate the genetic gain. This increase in the number of offspring that a female animal can produce will be acceptable under normal circumstances, when an animal is not artificially manipulated in any way. This technique can, therefore, have a significant effect on a sheep or goat breeding programme (Hafez & Hafez, 2000; Valasi et al., 2007; Chen et al., 2008; Morton et al., 2008).

In sheep and goats laparoscopic OPU is generally performed and it provides an efficient and relatively non-invasive method for oocyte collection from the small ruminants, where other techniques may not have been feasible or desirable. This technique is mostly used in animal species or age groups where it is not possible or easy, to manipulate the reproductive tract via the rectum (as in cattle) during oocyte retrieval (Tervit, 1996; Koeman et al., 2003). The time required for the recovery of oocytes through laparoscopic OPU is approximately 20 minutes in smallstock, which reduces stress and the technique may be repeated several times without ovarian damage or a decrease in the donors' fertility (Kuhholzer et al., 1997; Stangl et al., 1999). Sheep and goats that are not hormonally treated to induce multi ovulations naturally tend to produce 4-6 oocytes per female, per

flushing session. However, in FSH-treated sheep, oocyte yields may be increased to between 9-16 oocytes per female. If ovarian stimulations are continuous, follicular development decreases with age, the decrease can be reversed by performing OPU at earlier stages (Baldassarre et al., 1996; Alberio et al., 2001; Koeman et al., 2003; Valasi et al., 2007). Oocytes retrieved from large follicles at the preovulatory stage may be mature, but the majority of oocytes aspirated via an ovum pick-up session are usually from the smaller ovarian follicles. These oocytes require a 24 hour period of maturation in the laboratory, using a culture medium. It is, therefore, very important to make sure the oocytes are mature before *in vitro* fertilisation is considered (Elder & Dale, 2000).

In other findings it has been reported that approximately 8.1 oocytes per ovary were recovered by aspiration, compared to 6.3 per ovary by slicing in sheep. Of the total number of oocytes recovered per ovary using the slicing technique, 1.7 of the oocytes were of acceptable quality for maturation, as opposed to 0.82 by aspiration (Shirazi et al., 2005). In goats, more oocytes are reported to be recovered by the aspiration method than the slicing method of oocyte recovery, but poorer quality oocytes being harvested (Pawshe et al., 1994). This is similar to the report by Shirazi et al. (2005). The slicing method of oocyte recovery is, therefore, an acceptable method to harvest sheep oocytes, compared to the oocyte aspiration technique.

2.1.3 Factors affecting oocyte quality

The goal of an oocyte recovery method is to maximize the total number of good quality oocytes obtained per ovary. The oocytes recovered must be utilised for the production of viable embryos, without reducing their developmental competence (Wani et al., 2000; Shirazi et al., 2005; Morton et al., 2008). Naturally the oocyte quality is determined by the oocyte's ability to mature, be fertilised and give rise to normal offspring (Duranthon & Renard, 2001; Hussein et al., 2006; Sirard et al., 2006). The quality of the oocyte is also related to the

oocytes' follicular environment (Camargo et al., 2006), as well as several factors, such as age of the donor animal, stage of follicular development and the media used for maturing the oocytes (Keskintepe et al., 1994).

2.1.3.1 Donor age

Age of a ewe or doe from which oocytes are to be recovered determines the developmental competence of the oocytes. In the case of prepubertal lambs, collection of oocytes can start as early as 4 weeks of age. It has been reported that most ovarian follicular responsiveness occurs between 4 to 6 weeks of age (Armstrong et al., 1997). In goats, more oocytes are generally recovered from prepubertal animals than adult does. This is possible as more follicles can be stimulated to develop than in the adult doe. Follicular development in the lamb, however, lacks ovarian follicle atresia with the maximum number of antral follicles being reached at 4 to 8 weeks of age. This number then gradually declines to a stable low number, when the ewe lambs reach puberty. The effect of lamb superovulation is best when the number of follicles on the ovarian surface have reached their maximum (Koeman et al., 2003; Chen et al., 2008). However, oocytes from prepubertal animals show a reduced developmental competence, when compared with oocytes derived from their adult ruminant counterparts (Khatir et al., 1996; Morton, 2008). So for instance, under *in vitro* conditions, 29% of the oocytes developed to the blastocyst stage when oocytes were derived from prepubertal lambs, compared to a 39.3% developmental rate for oocytes collected from adult ewes (Morton et al., 2005). In agreement with this, for *in vivo* embryo production, fertilised ova were flushed from the oviducts of inseminated prepubertal lambs and adult ewes, and then transferred to adult recipients. It was reported that 33% live lambs were born from prepubertal donors, compared to 73% from adult ewes (Armstrong, 2001). In goats, about 19% of cleaved prepubertal oocytes developed to the blastocyst stage, compared to 65% in the adult counterparts (Baldassarre et al., 2002). However, in some instances similar developmental capacity of oocytes has been reported from prepubertal and adult goats (Koeman et al., 2003). Apart from reduced oocyte developmental

competence, there is also the occurrence of polyspermy in sheep, especially after IVF of juvenile oocytes. This polyspermy may be due to the defective dispersal of cortical granules around the cortex (Cognie et al., 2003). Ignoring the possibility of polyspermy, blastocysts from prepubertal oocytes tend to develop a day later, than those from adult oocytes (O'Brien et al., 1997).

2.1.3.2 Ovarian follicular size

The stage of development of the follicle and growth of the oocyte go hand in hand. It has been reported that follicular size profoundly influences the quality of the oocyte obtained during ovulation and the quality of embryo obtained (Sirard et al., 2006). The growth of oocytes inside a follicle is generally a slow process, in which the oocytes must acquire a competence for meiotic maturation by the interaction with the theca and granulosa cells (Carmago et al., 2006; Krisher, 2004). This occurs during the developmental stages that precede ovulation, through a process normally referred to as 'oocyte capacitation' (Hyttel et al., 1997). During this time, the oocyte undergoes maturation changes (Elder & Dale, 2000). Follicle size thus affects the oocyte quality, potentially involving mRNA or protein reserves as factors involved in determining the oocyte competence (Krisher, 2004). This is a common problem that is associated with the use of non-ovulated immature oocytes collected from the ovary. This not only involves the degree of oocyte maturation, but also the fact that many oocytes in the ovary are undergoing a process of apoptosis (Kane, 2003). In sheep and goats, the oocyte developmental ability is generally associated with cumulus expansion, increasing with follicle size and decreasing with increasing granulosa atresia. Follicles ranging from 2 to 6 mm in size in sheep generally contain fully grown oocytes showing good competence for *in vitro* nuclear maturation, as they have variable diameters.

Furthermore, it has been found that bovine follicles greater than 6 mm in diameter yield significantly more oocytes with many layers of granulosa cells. These oocytes tend to yield a higher proportion of in vitro produced blastocysts,

suggesting that larger follicles may contain growth factors enhancing morphological and functional status of the COC's and embryo yields (Lonergan et al., 1994; Shirazi & Sadeghi, 2007; Abdullah et al., 2008). In cattle for example, greater rates of embryonic development result when follicles greater than 2 to 3 mm in diameter are used for IVEP (Carmago et al., 2006). In sheep and goats, the oocyte developmental competence is clearly related to oocyte diameter, as an increase in developmental competence is achieved when follicles greater than 8 mm in diameter are used (Crozet et al., 1995; Hyttel et al., 1997; Hendriksen et al., 2000; Lonergan et al., 2003).

During the growth phase, oocytes increase in diameter to more than 120μm (Hyttel et al., 1997). Studies have shown that oocytes with a diameter of less than 110 μm may still be in the growth phase (Fair et al., 1995). These oocytes are less capable of developing after fertilisation and results with lower rates of blastocyst formation. Such small oocytes are also prone to undergo certain chromosome alterations during maturation, which impairs further development (Armstrong, 2001; Lechniak et al., 2002). The oocyte diameter is directly proportional to the follicle diameter, and oocytes continue to grow, even in follicles with a diameter of > 10 mm (Arlotto et al., 1996). In sheep, this relationship of ovarian follicular size and oocyte diameter exists and has been shown to be influential in meiotic progression (Shirazi & Sadeghi, 2007). Not only is follicle size important in embryonic development, but the number of follicles on the ovarian surface also plays an important role. Sheep ovaries with 8 or more follicles on the surface have been shown to yield higher percentages of cleavage and blastocyst rates (94% and 52.4%, respectively), compared to 57% and 30.2% attained from ovaries having 4 or less follicles on the ovarian surface (Mossa et al., 2000).

2.1.4 *In vitro* maturation

The morphological features of oocytes are visually assessed during the selection of immature oocytes for *in vitro* maturation in mammals (Katska-Ksiazkiewicz et al., 2007). Oocytes for IVM are generally selected using the following criteria:

follicle size, cytoplasmic appearance, the appearance and number of cumulus cells around the oocytes (COC's). Cumulus expansion can importantly be used to microscopically assess the *in vitro* maturation rate of oocytes (Gupta et al., 2005). The thickness of the cumulus cell layer around the oocyte determines the meiotic competence of the oocyte. The thicker cumulus cell layers indicate that the corona radiata cells are sufficient for the oocyte to complete nuclear maturation. These cells are then used for selecting oocyte quality, as they secrete factors that assist the oocyte to progress through to meiosis (Schoevers et al., 2007), and act as a 'go-between' between the oocyte and the follicular or culture environment. An important indication of the attainment of both nuclear and cytoplasmic maturation of oocytes is thus the layers of cumulus cells surrounding the oocytes (Kidson, 2005). Hence, the cumulus expansion can importantly be used to assess the *in vitro* maturation rate of oocytes (Gupta et al., 2005). The cumulus cells support the penetrability of the oocyte by the sperm by preventing the zona from hardening, caused by the premature exocytosis of the cortical granules (Schoevers et al., 2007). The cumulus expansion assists the sperm capacitation, fertilisation and embryo development *in vivo* (Chen et al., 1990).

Although morphological criteria are reasonable for indicating oocyte quality and suitability for IVM, it is still insufficient in identifying oocytes that are competent for *in vitro* development to the blastocyst stage. *In vitro* matured oocytes, whether collected from live animals or from slaughterhouse material, usually show a high percentage of cleavage after IVF with a lower percentage of oocytes developing to the blastocyst stage. This is less of a problem when oocytes are surrounded by four or more cumulus cell layers, suggesting that the problem is mainly for follicle maturity (Kane, 2003). There is, however, still 60% failure of IVM/IVF oocytes reaching the blastocyst stage following *in vitro* embryo production (Katska-Ksiazkiewicz et al., 2007).

Oocyte maturation, whether *in vivo* or *in vitro*, is the most important stage of oocyte development, even in IVEP (Nedambale, 1999). Oocyte maturation is the climax of a prolonged period of oocyte growth and development within the growing follicle, and the short interval of meiotic maturation at ovulation (Sutton et al., 2003). *In vitro* oocyte maturation briefly involves the artificial removal of COC's from follicles and the culturing to reach the metaphase II stage. However, only a small proportion of *in vitro* matured oocytes show full developmental potential to term (Gilchrist & Thompson, 2007).

Oocyte maturation entails several aspects, including nuclear and cytoplasmic maturation. Nuclear maturation refers to the resumption of meiosis and progress to the metaphase II stage, whereas cytoplasmic maturation encompasses other poorly understood, maturational events related to the cytoplasmic capacitation of the oocyte (Kidson, 2005). However, there are still many inadequacies in the IVM of oocytes in domestic species, especially small ruminants (Shi et al., 2009).

2.1.4.1 Media used for IVM

The technique of IVM has been standardized in animal species, and currently the efforts are aimed at reducing the cost of technology by substituting expensive inputs of the IVM process with less expensive and chemically defined inputs (Gupta et al., 2005). Generally culture media used for oocyte IVM are almost universally complex formulations, originally designed for the culture of somatic cells and tissues. This is the case for most widely employed oocyte IVM reagents, tissue culture medium (TCM 199) and minimum essential medium (MEM). The media formulations are designed to meet the metabolic needs of the somatic cells, particularly for long-term requirements of cell lines, and not for the complex and dynamic requirements of maturing COC's. There is an urgent need for media formulations to be designed, specifically for oocyte IVM. Substantial improvements in embryo culture media have been made over the past two decades, by bypassing media formulations on the major cation and anion concentrations and metabolic substrates of reproductive tract fluids as well as

bypassing the metabolic needs of growing pre-implantation embryos. This type of approach is currently being applied to the design of new IVM media. Clearly the composition of the follicular fluid varies from the commonly used IVM media and most notably in the concentration of glucose, which is a major energy substrate for the COC's (Gilchrist & Thompson, 2007).

During IVM, the oocyte and the surrounding cumulus cells form a functional unit. Therefore, it is important to take into consideration the nutrient requirements of the COC in order to improve the *in vitro* maturation culture media (Sutton et al., 2003). In most of the mammalian IVM, the basic medium is supplemented with serum and hormones. Selection of protein supplements and hormones such as follicle stimulating hormone (FSH) and luteinizing hormone (LH) for IVM medium is important even in the subsequent IVF and embryonic development (Pawshe et al., 1996; Wang et al., 1998). FSH is generally used in *in vitro* maturation protocols as it has been shown to improve fertilisation, early embryo development and cumulus expansion. Although FSH and LH are by no means necessary for spontaneous oocyte maturation, it is generally believed that these hormones improve oocyte cytoplasmic maturation by significantly altering a range of cumulus cell activities. It is unclear, however, if this beneficial effect of gonadotrophins is mediated by changes in cumulus cell metabolic activity (Izadyar et al., 1998; Sutton et al., 2003; Cecconi et al., 2008). Previously, a maturation rate of 70.1% in medium containing FSH and LH has been obtained, compared to a 50.3% maturation rate in medium containing human chorionic gonadotrophin (hCG). Moreover, blastocyst formation is also improved in goats by media containing FSH - a 19.4% to 22.6% was reported, compared to a 12% blastocyst formation rate in media without FSH (Wang et al., 1998; Wang et al., 2007). FSH generally upholds the developmental competence differently during folliculogenesis and *in vitro* oocyte maturation. It also maintains follicular growth and is vital for LH receptor appearance during the final stages of follicular development (Sirard et al., 2007).

2.1.4.2 Type of albumin or serum used

Other essential components of the *in vitro* culture medium are the serum and albumin fraction used (Ocana Quero et al., 1994). Albumin should be included in the culture media as it is the most commonly found protein in the mammalian reproductive tract. Serum provides nutrients to an oocyte and apart from this, tends to nurture the cells surrounding the oocyte, rather than the oocyte itself. It also lessens the possibility of the Zona Pellucida hardening when an oocyte is liberated from its follicular environment (Thompson, 2000; Wani, 2002). The addition of serum to the culture medium during *in vitro* maturation of oocytes is partly responsible for the induction of maturation. So for instance, in sheep, a 69 to 72% maturation rate was obtained with medium supplemented with mare serum, compared to 50% maturation rate in media without. This is in agreement with results obtained in goats, where a 61 to 78% maturation success rate with oestrous goat serum was obtained, compared to a 28% maturation rate with medium without serum (Tajik & Shams Esfandabadi, 2003; Kharche et al., 2006; Motlagh et al., 2008).

There are several sera utilized in the maturation of oocytes. These include oestrous cow serum, foetal bovine serum (FBS), homologous and heterologous oestrous serum. FBS has mostly been used for sheep and goat oocyte maturation, where a significant difference between the maturation rate in medium supplemented with FBS and the maturation rate in medium without FBS has been recorded. Approximately 4% of the oocytes reached the metaphase II stage of development in medium without serum (FBS) supplementation, compared to 79 to 84% of the oocytes in FBS supplemented medium. Oestrous sheep serum (ESS) has also been used for sheep oocyte IVM as it stimulates the maturation process and subsequent embryonic development in immature prepubertal sheep oocytes. When comparing ESS with FBS, previous trials have shown FBS to display a slightly lower maturation rate than ESS (70% vs. 82%) (Walker et al., 1996; Ghasemzadeh-Nava & Tajik, 2000; Tibary et al., 2005). During the maturation stage problems emanating, affect the fertilisation rate and yield quality

of pre-implantation embryos. The exposure duration to maturation medium is said to have little effect on the cleavage rate, but markedly influences the rate of development to the blastocyst stage (Nedambale, 1999).

2.1.5 *In vitro* fertilisation (IVF)

IVF procedures are similar in both naturally ovulated and matured aspirated oocytes (Tajik & Shams Esfandabadi, 2003). The procedure is generally carried out in a media specifically formulated to mimic both biochemical constituents of the uterine environment and to promote sperm capacitation. The oviduct simulates the environment needed for oocyte maturation and acquisition of developmental competence for fertilisation (Elder & Dale, 2000). However, it is often difficult to recreate the physiological conditions occurring in the closely regulated environment of the oviduct and uterus (De La Torre-Sanchez et al., 2006).

Sperm cells do not attain their full capacity for fertilisation until after they are transported in the female reproductive tract. These cells have to undergo a crucial process with further physiologic changes before penetrating the Zona Pellucida and fusion with the vitellus of the ova (Katska-Ksiazkiewicz et al., 2004; Camargo et al., 2006). These changes are referred to as sperm capacitation. Sperm capacitation is the first stage of membrane destabilisation that involves intracellular ionic modifications. These modifications are then regulated by transient association of molecules to the sperm surface, such as an efflux of cholesterol and redistribution of the intrinsic membrane proteins and lipids (Gillan et al., 2004; Dominguez et al., 2008). Capacitation as such allows the sperm cells to undergo a normal acrosome reaction before fertilisation. Some early experiments with IVF have been unsuccessful as the sperm cells were not capacitated (Hafez & Hafez, 2000; Graham & Moce, 2005; Camargo et al., 2006). However, with semen cryopreservation, capacitation is said to be induced earlier as the sperm cells bypass certain normal processes (Morrier et al., 2002).

The culture media used for IVF must be beneficial in providing sperm motility and capacitation, providing for the fusion of two gametes and then the start of embryonic development. This is not always the case as the needs and metabolic activities of the two gametes are not identical. Therefore, it is important to use suitable medium for sperm preparation and the other medium suitable for oocyte insemination (Izquierdo et al., 1998). It has become evident from other studies that media which support sperm capacitation and fertilisation in cattle can also support the capacitation in goats and do the same with ram sperm (Cox & Alfaro, 2007).

In most of the fertilisation media, heparin is included, as it induces and is responsible for sperm capacitation. Heparin binds to the sperm and induces changes in the intracellular environment of the sperm, thus resulting in Ca^{2+} uptake and an increase in intracellular free calcium and intracellular pH. Another change associated with heparin-induced capacitation in sperm is an increase in protein phosphorylation (Lane et al., 1999). Fertilisation rates in sheep are thus stimulated, as heparin improves the efficiency of sperm capacitation. However, increased concentrations of heparin tend to reduce the cleavage rate and could affect the percentage of blastocysts formed. So for example, a medium not supplemented with heparin yielded an 86.7% cleavage rate, compared to media supplemented with 5IU heparin (85.8%) and 10IU heparin (75.5%). Cleavage rate thus decreased with an increase in the concentration of heparin (Wani, 2002; Li et al., 2006; Cox & Alfaro, 2007). Other agents such as caffeine and a mixture of penicillamine, hypotaurine and epinephrine have also been included in fertilisation medium to stimulate and prolong sperm motility. Bovine serum albumin (BSA) is another supplement in the IVF media which can be used to improve the *in vitro* capacitation of sperm. However, the effect of heparin together with BSA on fertilisation rate in sheep is sparsely documented. In cattle, sperm capacitation is enhanced by use of heparin which acts synergistically with caffeine. However, caffeine depresses the fertilisation rate obtained with fresh sperm in sheep (Izquierdo et al., 1998; Wani, 2002).

2.1.5.1 Semen used for IVF

The fertilisation rates obtained fluctuate, depending on the semen used. In IVF, the semen utilised can either be fresh, frozen or sexed semen. The quality of semen normally deteriorates with the degree of processing (dilution and cryopreservation). So for instance, semen which is considered acceptable immediately after collection may have decreased sperm motility after dilution (Sugulle et al., 2006). Frozen semen is also said to have impaired fertility, when compared with fresh semen due to lower viability post-thawing and dysfunction in the surviving sperm population. Fresh sperm cells have also demonstrated a higher oocyte binding ability, when compared to frozen-thawed sperm cells (Watson, 2000; Niu et al., 2006).

Offspring have been produced in several farm animal species e.g. cattle, pigs and sheep following the use of sex-sorted sperm, in conjunction with *in vitro* fertilisation and embryo transfer (Catt et al., 1996; Johnson et al., 2000; Fry et al., 2004). However, lower pregnancy rates after fertilisation with sex-sorted semen, compared to non-sorted sperm, have been reported in a number of studies (Hollinshead et al., 2002; Seidel & Garner, 2002; Wheeler et al., 2006). So for instance, reduced developmental potential of embryos produced from sorted-frozen boar sperm have been reported, although pregnancies were established (Johnson et al., 2000). On the contrary, sex-sorted ram sperm was reported to exhibit a higher fertility rate than non-sorted sperm when inseminated into the uterus at lower semen dose levels. With laparoscopic insemination in the uterus, semen is normally deposited close to the fertilization site. This success which led to improved fertility of sexed sperm can also be attributed to the refinement of the sorting procedure and accompanying semen processing (Salamon & Maxwell, 2000; Beilby et al., 2009). The DNA stains used for the sexing of semen have certain cytotoxic effects on fertility, embryonic development and the normality of the offspring. So for example, in cattle, the blastocysts development is lower, compared to blastocyst development in unsorted or unsexed semen (Cran &

Johnson, 1996). In sheep, it has been reported that there was no statistical difference on cleavage rates between the use of sorted semen, compared to the use of non-sorted semen (66.7% and 76.8%, respectively) (Hollinshead et al., 2004).

2.1.5.2 Albumin or serum used for IVF

Heat-inactivated oestrous serum is mostly used for supplementing the oocyte culture media. Such media are capable of capacitating sperm in sheep and goats. Different concentrations of serum have been used, depending on the type of semen used. So for example, 20% serum is used with fresh semen, while with frozen-thawed semen only 2% serum is used in sheep and goats (Cognie et al., 2003). The addition of serum to IVF media improves the cleavage rate. However, the concentration of the serum also plays an important role. Where serum is not added to fertilising media, no cleavage was obtained, compared to 78%, 72.6% and 73.9% recorded when 20% serum was added to the fertilising medium. These cleavage rates were higher when compared to cleavage rates obtained when 10% or 5% serum concentrations were used (Cognie et al., 2003; Li et al 2006). When albumin was involved, the total protein content of *in vitro* derived embryos, demonstrated a decrease during early cleavage. During compaction and blastulation, there is also an increase in protein content. It would thus seem that protein degradation exceeds protein synthesis during early cleavage (Thompson, 2000). However, serum has been observed to inhibit the first cleavage division in bovine embryos but, at the same time promote blastocyst development (Van Langendonckt et al., 1997). In sheep, serum results in larger lambs, if added during *in vitro* culture of sheep embryos (Totey et al., 1993; Thompson et al., 1995).

2.1.6 *In vitro* culture

The environment that can sustain embryo development could be achieved through the utilisation of several culture conditions, media supplements and gaseous atmospheres (Hafez & Hafez, 2000). Embryo yield and survival usually

differ between the different culture systems and culture media (Dobrinsky, 2002; Camargo et al., 2006).

2.1.6.1 Culture media used

Media used do not only influence embryonic development, but also have an effect on embryo survival following cryopreservation (Nedambale et al., 2004). Culture media also sometimes tend to be species specific. So for example, embryos of other ruminants are difficult to develop in systems developed for cattle and sheep i.e. it is difficult to culture red deer embryos to the blastocyst stage *in vitro* (Thompson, 2000).

There are different culture systems available for *in vitro* fertilised oocytes. In the culture systems different base media are used. The most commonly used media in sheep which supports embryonic development *in vitro*, is synthetic oviduct fluid (SOF) (Walker et al., 1996). This medium, when supplemented with amino acids, supports embryonic development in ruminants. This SOF has been formulated to mimic the oviductal fluid, which is a complex medium emanating from the blood and active secretion from cells of the epithelium. Ovine embryos immersed in this fluid for a period of 72h post fertilisation, develop to the 8-16-cell stage. During this period embryonic development is regulated by slight changes in composition of the fluid. The importance of this environment to early embryo development is clearly displayed on foetal development and the well-being of the subsequent offspring. Apart from SOF there are other media which can be used, e.g. TCM 199, Hams-F10 and Tyrodes medium (Walker et al., 1996; Wani, 2002; Carmago et al 2006; Cox & Alfaro, 2007). Other culture media have been reported to be successful for bovine embryo culture. Among them are potassium simplex optimization medium (KSOM) and Charles Rosenkrans medium (CR1). There have been several reports regarding CR1 for use in ovine embryo culture (Rosenkrans et al., 1993; Wan et al., 2009).

2.2 CRYOPRESERVATION OF RAM SEMEN

Cryopreservation of gametes is rendered an important tool in ART, especially when distance between donors results in the non-simultaneous availability of male and female gametes. Long term conservation of semen is also important when IVF and, or artificial insemination is to be performed at a future date (Merlo et al., 2008; Barbas & Mascarenhas, 2009). However, the quality of semen following cryopreservation is always a limitation. There are a number of processes involved in cryopreservation and thawing that potentially could damage the sperm cell. The cryopreservation of semen often results in reduced fertility, compared to the fertility of fresh semen. This then arises from the loss of sperm viability and the impairment of function in the population of sperm that survived the cryopreservation process (Watson, 2000). Semen cryopreservation also affects the sperm attributes such as motility and plasma membrane integrity, consequently reducing sperm survivability.

Ram sperm cell membranes seem to have a particular composition that makes them more sensitive to cryopreservation. It is, therefore, more difficult to cryopreserve ram semen than other farm animal species. This may be due to ram sperm having a higher saturated fatty acids ratio and a lower phospholipids molar ratio than the other species. This can then be responsible for all membrane disarrangement in the first place, with subsequent sperm damage. This damage is then more severe in ram than in bull semen (Abdelhakeam et al., 1991; Salamon & Maxwell, 1995b; Ollero et al., 1998; Byrne et al., 2000). So for example the post-thawing semen quality is generally better in cattle, ranging between 50 to 70% motile sperm, while in goats sperm motility can be reduced from 70% (fresh semen) to 30% (frozen-thawed semen). Survivability of the sperm cell is also affected by the cryopreservation procedures and following thawing. This survivability can be reduced from 85.6 to 34.3% (Hiemstra et al., 2005; Marco-Jimenez et al., 2006). In sheep, although 40 to 60% of the sperm cells can preserve their motility after freezing and thawing, only 20 to 30% of the

sperm population remain biologically undamaged (Salamon & Maxwell, 2000; Watson, 2000).

Cryopreservation as such involves a sequence of events, with important effects between the different steps. Cooling from 37˚C to 5˚C causes a specific type of alteration that is related to membrane lipid phase transitions. This alteration is different from those caused by freezing and thawing processes, and includes mechanical and osmotic changes (Ollero et al., 1998). These two processes also damage ram semen and impair fertility (Molinia et al., 1996; Gillan et al., 2004; Marco-Jimenez et al., 2005; Kasimanickam et al., 2007).

The site of semen deposition during insemination and the female genital morphology are also contributing factors regarding the fertility obtained with the frozen semen. In sheep, very poor results have been obtained when using frozen semen, compared to fresh semen, especially if it is cervically deposited (Molinia et al., 1996). However, fertility can be increased with increasing depth of deposition in cervical insemination. Cervical penetration is generally a problem in smallstock and can be overcome by surgically bypassing the cervix, via laparoscopic inseminations in order to obtain acceptable conception rates (Gillan et al., 2004; Hiemstra et al., 2005; Sabev et al., 2006). Satisfactory fertility results have been achieved in sheep and goats using intrauterine insemination with frozen semen (Salmon & Maxwell, 1995a; King et al., 2004).

2.2.1 Methods of semen cryopreservation

The methods of gamete cryopreservation reported to be extensively used in sheep are controlled slow freezing and vitrification (Papadopoulos et al., 2002).

2.2.1.1 Slow freezing

In the slow freezing technique, biological material is cooled fast enough to prevent cooling damage, yet at the same time slow enough to allow dehydration of the cells, without intracellular ice formation. The cell dehydration

accompanying this slow freezing technique is potentially beneficial to sperm cell survival, while rapid freezing rates are considered more likely to cause cellular death. Slow freezing is also typified by a more stable thermodynamic equilibrium. It uses low concentrations of cryoprotectants, which are generally associated with chemical toxicity and osmotic shock (Byrne, 2000; Arav et al., 2002; Hiemstra et al., 2005; Barbas & Mascarenhas, 2009). The rate of both cooling and thawing have been shown to have an effect on the plasma membrane and thus on the survival of the sperm cell.

2.2.1.2 Vitrification

Vitrification is a rapid cryopreservation method referred to as solidification of a solution at low temperatures without the formation of ice crystals. It involves rapid cooling rates and high concentrations of cryoprotectants which depress ice crystal formation in the cell and minimise cold shock (Vatja, 2000; Barbas & Mascarenhas, 2009). Vitrification as such requires 30 to 50% cryoprotectants in the medium, compared to 5 to 10% for the slow freezing technique (Dinnyes et al., 2007). Although vitrification seems to give better post-thawing results, most mammalian sperm cells are extremely sensitive to these high concentrations of cryoprotectants and have a low osmotic tolerance. Heat transfer in cells, tissues or organs, which have larger volumes, are, however, too slow to permit vitrification without the risk of crystallization. It is, therefore, preferable to freeze large volumes such as semen and tissue by means of the slow cooling rate procedures. Thus, vitrification is generally not popular for semen cryopreservation (Arav et al., 2002; Isachenko, 2003; Hiemstra et al., 2005).

2.2.2 Semen collection techniques

Semen collection can be performed in a variety of ways, depending on the species. In sheep and goats, the most commonly used techniques are the artificial vagina (AV) and electrical stimulation (use of electro-ejaculator). There is also another technique using a device called the vaginal collection vial (VCV), developed by Wulster-Radcliffe et al. (2001b). The VCV unlike the AV, does not

require the training of rams, which is often time consuming and could last for about 3 weeks, depending on the individual ram (Wulster-Radcliffe et al 2001a, 2001b; Ortiz-de-Montellano et al., 2007). In the VCV technique a glass vial of approximately 9cm long, bent at a 10˚ angle, is inserted into the vagina of the ewe about 10 minutes before mating. The bend assists in securing the vial being in place during mating (Wulster-Radcliffe et al., 2001b).

Collection of semen using the AV mimics the natural ejaculate and tends to give semen of a high concentration, than when collected by electro-ejaculation (EE). The numbers of sperm in ejaculates collected through EE generally tend to be lower than those found in ejaculates collected by the AV. (Wulster-Radcliffe et al., 2001b; Marco-Jimenez et al., 2008). However, the AV requires males with adequate libido and which are able to mount females after the necessary training. The apparatus used for electro-ejaculation involves a power source, transformer and a rectal probe. The size of the probe is normally determined by the species involved. The EE also allows the collection of semen from males that are incapacitated and unable to mount females. It can, therefore, be used as an alternative method to the AV (Barker, 1958; Sundararaman et al., 2007; Guiliano et al., 2008). So for example, EE has been used in male goats that were raised under extensive conditions and that rejected AV training for semen collection (Ortiz-de-Montellano et al., 2007). In bulls, the EE was compared with transrectal massage which it outperformed. Semen can thus successfully be collected by EE from all bulls in which transrectal massage failed. The collected semen by EE has generally displayed a higher sperm concentration (724 x 10^6sperm/ml), motile sperm (60%) and live sperm (78%), than semen collected by transrectal massage (320 x 10^6 sperm/ml, 50% and 67%, respectively) (Palmer et al., 2005). The EE in sheep, however, is faster, more convenient with a higher semen volume, but lower sperm concentration and is stressful to the animal (Mattner & Voglmayr, 1962; Salamon & Morrant, 1963; Wulster-Radcliffe et al., 2001b; Marco-Jimenez et al., 2005).

2.2.3 Semen processing

Immediately after semen collection, semen can be macroscopically evaluated for attributes such as colour and volume, before being taken to the laboratory for microscopic evaluation of sperm motility, semen pH and semen concentration. The visual estimation of the percentage motile sperm in a semen sample is probably the most common laboratory analysis performed. This method can be very useful, although it evaluates a single important sperm attribute, and is subject to human bias (Moce & Graham, 2008).

In all animal species, semen is collected at body temperature and as a result it has to be kept warm before extension, to avoid cold shock to the sperm. Then processing, which involves semen cooling to 5˚C, is similar, whether it is going to be used in the frozen or unfrozen state (Hafez, 1987). The cooling of semen should be a task which is carefully performed, as it is known that by cooling semen at too rapid rate, between 30˚C and 0˚C induces lethal stress to the sperm cell. The cooling of semen below freezing leads to the formation of ice crystals, nucleating and pure water crystallising out as ice. The remaining liquid water fraction dissolves the solutes, and osmotic strength of the solution increases (Watson, 2000). The osmotic pressure of the remaining solution and proportion of water crystallizing out as ice are dependent on temperature. If the temperature is lower, the unfrozen fraction will be smaller, hence the higher the osmotic pressure of the solution. It is, therefore, recognised that the cooling rate should be rapid in order to minimize the duration of exposure to cold, for optimal cell survival (Watson, 2000). Sperm cells have been frozen at rapid rates in the range 15-60˚C/min and reported to give acceptable survival rates. The cooling rate however must be slow enough to allow water to leave the cells by osmosis, preventing intracellular ice formation which is lethal (Mazur, 1984). The ram sperm mid-piece and tail have been shown to be particularly vulnerable, if cooled at slower rates (Kumar et al., 2003).

2.2.4 Factors affecting the quality of cryopreserved semen

There are several important factors involved in the cryopreservation of semen, which ultimately affect the quality and viability of the end product. The quality of ram sperm deteriorates as a consequence of cooling, freezing, thawing and the addition of cryoprotectants (Fernandez-Santos et al., 2006). Ram sperm cells are very sensitive to the extreme temperature changes during the freezing process. Procedures used to cryopreserve sperm cells have been shown to also induce damage to the sperm plasma membrane (Marco-Jimenez et al., 2005).

2.2.4.1 Age and breed

Age of an animal can affect its fertility. So for instance, ejaculates collected from rams at puberty tend to contain sperm with a high percentage of abnormalities and low percentages of motile cells, compared to adult rams (Lymberopoulos et al., 2008). This is in agreement with what has been previously reported by Al Ghalban et al. (2004), that in goats, the percentage of abnormal sperm was lower in mature bucks, compared to yearlings. Sperm motility also decreases with increasing age. Fresh semen from rams of more than 6 years of age have fewer motile and more abnormal sperm than younger rams of less than a year and a half old (Wiemer & Ruttle, 1987). The frozen-thawed semen for mature rams was reported to display a lower proportion of sperm motility compared to that of young rams (37% vs. 45%) (Lymberopoulos et al., 2008).

Breeds of sheep show differences in semen quantity (semen volume, concentration and ejaculate) and quality (semen motility, percentage alive and percentage of abnormal sperm), during and after the breeding seasons. This difference between breeds and individual rams in semen characteristics makes it necessary to perform semen evaluation, in order to select the best rams for breeding and thus optimizing reproductive performance. The breeds of sheep differ significantly in terms of semen volume and concentration, and these differences are mostly observed in different seasons of the year. Semen quality and quantity deteriorate after the breeding season e.g. low semen motility and

high percentage of abnormal sperm. A significant difference was observed between two breeds of sheep, namely Hamari and Kabashi rams. It was observed that poor semen ejaculates were obtained outside the breeding season, and a high percentage of semen samples were rejected after freeze-thawing, due to poor freezability. Mostly the semen samples of the Hamari rams were the ones rejected, as opposed to the semen samples of Kabashi rams. However better results were obtained during the breeding season (autumn and winter) for both breeds, with Kabashi rams having the high percentages comparatively (Karagiannidis et al., 2000, Purdy, 2006, Makawi et al., 2007).

The freezability of semen generally also differs between breeds and between males of the same breed. Consequently, frozen semen of certain genetically important breeds or males may not be suitable for gene bank resource storage and can only be used with limited efficiency (Hiemstra et al., 2005). The freezability of semen can also be affected by the season of collection. Semen collected during the breeding season freezes better than semen collected during the non-breeding season (Hafez & Hafez, 2000).

2.2.4.2 Semen collection frequency

Semen collection intensity is an important aspect relating to semen quality in domestic animals. In the ram, semen attributes such as ejaculate volume, sperm concentration and motility are highly correlated with the frequency of ejaculation. The mentioned sperm attributes gradually decline with an increase in the frequency of ejaculation. Normally, the first ejaculate tends to be more than the volume in the consecutive ejaculates. In a case where semen was collected twice per day, the average semen volume from the first ejaculate was recorded as 1.62ml, compared to 1.06ml from the second ejaculate. This is in agreement with the fact that, where collection of semen was performed once a day, 1.1ml was obtained compared to 0.8ml from 3 collections per day. However, post-thaw sperm motility improved with an increase in the frequency of ejaculation. A post-thaw motility of 44.1% was obtained from the second ejaculate, compared to

34.1% from the first ejaculate (Kaya et al., 2002; Bester, 2006; Nel-Themaat et al., 2006).

2.2.4.3 Extenders used

Semen extension or dilution is performed in specific ratios so that the volume of semen for insemination will contain sufficient sperm per dose to give high fertility, without wasting cells. So for instance, in sheep, the dilution of semen more than 10-fold significantly reduced fertility, although sperm motility was not affected for up to 40-fold (Hafez, 1987).

Extenders or diluents are dilution media with a protective ability, used to maintain sperm for longer periods of time (Hafez, 1987; Royere et al., 1996). The extenders are usually used for the purpose of supplying the sperm cells with a source of energy, protecting the cells from temperature-related damage and maintaining a suitable environment for sperm to temporarily survive. The sperm maintenance for prolonged periods can only be achieved by using extenders usually designed on an empirical basis to do so. In sheep, reports state that cooling non-extended semen has a detrimental effect on sperm viability. Sperm viability is generally a semen parameter closely related to the sperm functionality. A decrease in viability was found to be 14%, while motility was not significantly affected with 60% of the cells motile, after cooling (at 5°C). In contrast, when an extender is used, the decreases related to the seminal parameters after cooling are slight, with the extended semen showing a viability of 51% and a motility of 62%. Freezing and thawing, however, induce more serious modifications that lead to a total loss of motility. Sperm motility and sperm viability were severely affected by freezing and thawing; only 18% of the initial sperm viability and 33% motility in sheep and goats were maintained after freeze-thawing (Ollero et al., 1998; Paulenz et al., 2002; Dorado et al., 2007). As a result, extenders which maintain motility of the concentrated suspension of sperm during cooling to sub-ambient temperatures are a prerequisite for a successful AI program in sheep (Watson, 2000).

The extender composition must assist in stabilising the sperm cell during the freezing and thawing process (Soylu et al., 2007). Extenders generally include permeating cryoprotectants, non-permeating cryoprotectants, a buffer, salts, sugars and antibiotics. Antibiotics control the proliferation of any microbial contaminants, while the sugars on the other hand are said to have several functions in an extender. This includes providing an energy substrate for the sperm, maintaining osmotic pressure of the diluents and acting as a cryoprotectant (Hafez, 1987; Berlinguer et al., 2007). Buffers are also added to the extenders to maintain the pH of the semen. The addition of lipids to an extender overcomes the initial detrimental effect of cold shock and egg yolk (EY) is one of the sources of lipids with its protective effect primarily ascribed to its phospholipid components. So for example, semen extenders containing egg yolk have been extensively used in the preservation of bovine sperm (Maxwell & Salamon, 1993; Marti, 2003). Unfortunately, egg yolk may be a prospective source of viral infections or allergic reactions. As a result the extender composition has a major effect on post-thawed semen viability. In goat semen, egg yolk has been found to reduce sperm motility, viability and increase acrosomal damage. Egg yolk in sheep has led to many inseminate doses being discarded after thawing (Hafez, 1987; D'Alessandro et al., 2001; Dorado et al., 2007). Frozen-thawed ram semen extended with 15% EY recorded an increase in motility (46.5%), when compared to the one extended with 4.5% EY, together with trehalose (36.9%). However, sperm viability has been proven to be better in 4.5% EY together with trehalose extender, compared to 15% EY extender (66.5 vs. 58.9%). Fertility in ewes after AI was found to be higher with 15% EY extended frozen-thawed semen (20.5 vs. 3.8%) (Valente et al., 2010). The addition of trehalose to an extender thus provides acceptable thermo-protection for ram semen. About 57.6% sperm motility can be obtained from semen frozen with an extender containing trehalose, compared to 24.8% motility in semen frozen with an extender not supplemented with trehalose. However, high concentrations of trehalose have been shown to be deleterious on ram sperm

during the cooling process (Aisen et al., 2000; Aisen et al., 2002). The expectation is for the semen extenders to have pH values ranging from 6.75 to 7. These components are normally grouped according to their chronological use such as citrate-sugar based, milk diluents, lactose based, tris based diluents, etc (Salamon & Maxwell, 2000).

Although several studies have been conducted to develop different extenders and protocols for ram semen freezing, the fertility results are still lower than those obtained with fresh semen and natural mating (Woelders et al., 1997; Stanic et al., 2000). Fresh extended ram semen generally has a short fertile lifespan, whereas acceptable fertility with cryopreserved semen is achieved only by laparoscopy. This limits the widespread use of AI in sheep husbandry using frozen semen (Soylu et al., 2007).

Seminal plasma alone limits the survival of ejaculated sperm to a few hours. This seminal plasma is a complex mixture of organic and inorganic substances, with properties inducing the activation of sperm motility and protection of sperm from the effects of reactive oxygen species (ROS). An excess of ROS can cause cell damage and thus lead to a reduction in sperm motility and fertility. ROS are induced by cryopreservation, especially exposure to light during semen manipulation before storage. These reactive oxygen species are detrimental to the subsequent performance of semen (O'Flaherty et al., 1997; Aisen et al., 2005; Love et al., 2005; Mara et al., 2007). The ROS are produced by dead sperm in the ram and bovine semen (Upreti et al., 1995; Pitt, 2007) and play an important role in sperm survival. In this respect, ram sperm motility can be prolonged with the use of agents that protect sperm against the harmful effects of ROS, such as anti-oxidants e.g. superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase. These anti-oxidants are cytoplasmic substances common to every cell. Sperm cells lose most of their cytoplasm during cryopreservation, hence losing these substances. The cells then become prone

to the peroxidase processes. However, use of anti-oxidants in diluents is not a common practice (Watson, 2000; Marti et al., 2003; Mara et al*.,* 2005).

2.2.4.4 Cryoprotectants used

In order for semen cryopreservation to be successful, cryoprotective additives known as cryoprotectants should be present (Cunningham, 1999). Cryoprotectants are additional chemicals used in the cryopreservation process to avoid crystallization of the cell water and shock effects. These reagents are also referred to as the primary components in the freezing and vitrification solutions (Moore & Bonilla, 2006; Pereira & Marques, 2008). Although cryoprotectants are essential for the protection against cryo-injury, they could also be harmful, even at relatively low concentrations (Fernandez-Santos et al., 2006). The type of cryoprotectants used, vary widely between species (Viveiros et al., 2000).

Before their use, cryoprotectants are evaluated regarding their permeating property. Rapidly permeating agents are favoured if the exposure time prior to rapid cooling can be reduced, and they are likely to diffuse rapidly out of the cell, thus preventing osmotic injury (Kasai, 1996). The addition and removal of cryoprotectants also play an important role in the cryopreservation of semen. So for instance, the addition and removal of cryoprotectants in molar proportions could apply a substantial, but transient osmotic stress to the plasma membrane of the sperm cell, depending on the relative permeability of the cryoprotectants. The sperm stress could be reduced to tolerable limits by the stepwise addition and removal of cryoprotectants, and this substantially improves the proportion of surviving sperm, unlike the single step cryoprotectant addition (Watson, 2000).

Since the discovery of the cryoprotective properties of glycerol, its choice as a penetrating cryoprotectant has been confirmed as being most effective in lowering the intracellular water freezing point (Royere et al., 1996). Consequently glycerol is used extensively, although it still induces osmotic stress (Watson, 2000; Morrier et al., 2002). Furthermore, glycerol has been termed the most

suitable cryoprotectant in bird, fish and mammalian species (Hiemstra et al., 2005). When semen is frozen in diluents without glycerol, intracellular crystals that can damage the sperm structure during thawing are formed. However, its addition to diluents is limited by its toxicity. The addition of glycerol to cooled ram semen generally reduces the sperm motility and acrosomal integrity, which leads to reduced fertility following AI. So for example, sperm motility rates of 79% and 69% were reported in semen extended with diluents containing 0% and 7% glycerol, respectively after equilibration. Due to these properties, lower glycerol levels are suggested in order to maintain acrosomal integrity (Anel et al., 2003; Kumar et al., 2003; Sonmez & Demirci, 2004). Apart from using glycerol, other cryoprotectants such as dimethyl sulphoxide (DMSO) and ethylene glycol (EG) have also been used. The success of a particular cryoprotectant seems to be specie-specific. In the hamster, DMSO has been shown to be less efficient, while EG was shown to be the alternative for human sperm cryoprotection (Serafini & Marrs, 1986; Royere et al., 1996). However, among the cryoprotectants used, glycerol seems to be the most widely used cryoprotectant in sheep semen cryopreservation. It has been found that glycerol has no effect on quality of frozen-thawed sperm, as assessed by motility and viability (Salamon & Maxwell, 2000; Morrier et al, 2002).

2.2.4.5 Thawing procedure

Semen thawing may be performed in variable thawing solutions or physiological serum. The thawing solution has been reported to have a relationship with the freezing extender. Apart from the solutions, temperature and time as such should also be considered when thawing semen in order to minimize cellular damage. Thawing can either be performed rapidly or slowly. So for example it has been found that fast thawing (70˚C for 5 sec) results in a higher post-thawing sperm motility and membrane integrity (67% and 50%, respectively), than slow thawing (35˚C for 12 sec) of 63% and 41% (Soderquist et al., 1997).

2.3 CONCLUSIONS

An increase in animal numbers is essential to help feed an escalating human population. Several assisted reproductive technologies (ART's) have been employed to try and increase the number of embryos produced, e.g. in superovulation and embryo transfer. Currently, *in vitro* embryo production (IVEP) is also commonly practiced for the same reason and it could be an efficient tool to produce animals of higher genetic merit. Yet there are several factors that can influence the success of IVEP. The factors can either increase or decrease the subsequent embryo yields.

The oocytes used for IVEP can be obtained either from live or slaughtered animals. The inexpensive readily available oocytes are generally obtained from the slaughtered animals. Cumulus oocyte complexes (COC's) used for IVEP form a heterogeneous population, as they differ in quality and size of the follicles from which they originate and the method of collection. In sheep, the most common method of oocyte collection used is slicing of the ovaries, which seem to generate acceptable quality oocytes. However, the other methods of oocyte collection e.g. aspiration is still being used. Proper production of embryos may be inhibited if poor quality oocytes, which may not mature, are used. A deficiency of compact cumulus cells is said to negatively affect the IVM procedure, which leads to a decreased production of *in vitro* produced embryos.

In vitro maturation is yet another important factor contributing to successful IVEP. From the onset, the oocytes must be matured in a proper maturation medium. Most maturation media are in a way, species-specific. However, some of the maturation media used in ovine IVEP can also be used in bovine IVEP. When the oocytes are mature, *in vitro* fertilisation then follows. The semen used for *in vitro* fertilisation is also one of the factors contributing to the success of the IVEP programme. Fresh or frozen-thawed semen and sex-sorted or unsorted semen can all be used for IVEP. The use of fresh semen for IVEP seems to be a better

option, as it tends to increase the fertilisation rates. However, frozen-thawed semen is still used in cases where fresh semen is not available.

The *in vitro* culture environment is another factor greatly influencing *in vitro* embryo production. Different culture systems and culture media are available for IVEP and could lead to varying embryo yields. Presumptive zygotes are then cultured *in vitro* in a suitable media and environment to stimulate cleavage, and finally enhance development to the blastocyst stage. The culture media which is commonly used for IVEP in sheep is the synthetic oviductal fluid (SOF). Embryos can be cryopreserved, if suitable recipients are not immediately available for embryo transfer. Yet during cryopreservation most of the embryos tend to die due to certain agents, e.g. the cryoprotectant used. In some instances, embryos fail to grow after transfer due to the physiological status of the recipient. The recipient may, for example, not be properly prepared (hormonally) for embryo transfer (stage of the uterine cycle).

Semen cryopreservation in small ruminants is an important part of *in vitro* production of embryos and artificial insemination. When cryopreserving semen, the method of semen collection, number of ejaculates collected and environmental differences contribute to the quality of the cryopreserved semen. The artificial vagina tends to be the best technique of semen collection in ovine. However, the electro-ejaculation technique is still used, even though it tends to result in semen with a lower sperm concentration.

Extenders are also used in order to protect and maintain the sperm against cold shock. The extenders used tend to eventually affect the viability of cryopreserved semen. Egg-yolk based extenders are mostly used in the cryopreservation of ram semen. It has been found that egg yolk plays an important role in protecting the sperm against cold shock damage. This cold shock causes damage to the sperm during cooling (from body temperature to lower temperatures -5^{o}C). Thus extenders should include cryoprotectants. These agents help to dehydrate sperm

and prevent the formation of ice crystals in the cell. These cryoprotectants are designed in such a way that they help to maintain survivability and motility of the sperm, even after freezing and thawing. However, the cryoprotectants highly affect the viability of sperm, as they tend to dehydrate the sperm cells during cryopreservation. Glycerol as such, has been found to be the most used cryoprotectant in ram semen cryopreservation. Therefore, if the percentage live sperm and motility is maintained, there is a high possibility of successful fertility.

CHAPTER 3

MATERIALS AND METHODS

3.1 LOCATION

This study was carried out between March and October (autumn to mid-spring) 2008, at the ARC Germplasm Conservation and Reproduction Biotechnologies' (ARC-GCRB) laboratory at Irene, Pretoria in the Gauteng province. The ARC is situated at 25˚ 55′ south latitude and 28˚ 12′ east longitude, at an altitude of 1525m above sea level. This location experiences hot summer days with cool nights and cool moderate winter days with cool nights (Webb et al., 2004).

3.2 EXPERIMENT 1: EFFECT OF TWO DIFFERENT HARVESTING TECHNIQUES ON OVINE OOCYTES

3.2.1 Method of oocyte collection

Ovaries were collected during autumn (March – April) and the onset of winter at the Boekenhout abattoir (Pretoria) from unknown, untreated sheep (Plate 3.3), and transported to the laboratory in sterile saline water (37°C), in a thermos flask, within 3h of slaughter. Prior to oocyte recovery, the temperature of the saline water was checked and recorded. Ovaries were then removed from the saline water and lightly sprayed with 70% alcohol (70% of ethanol + 30% ultra pure water [Adcock Ingram, South Africa]) to remove any contaminants present. The ovaries were washed 3 times in fresh warm saline before processing. All ovaries were freed of the surrounding tissues, using sterile scalpel blades and forceps. The oocytes were then harvested from the ovaries by the slicing and aspiration techniques (Wani et al., 1999).

Cumulus oocytes complexes (COC's) were recovered from the ovarian follicles by aspiration of the follicular fluid using an 18-gauge needle attached to a 10 ml syringe, containing 1ml modified PBS (mPBS), at 22°C. The mPBS was made up

of warm Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco, BRL, Grand Island, New York), modified by the addition of 0.4% Bovine Serum Albumin (BSA) (Sigma, St. Louis, MO, USA) and a 10% antibiotic/antimycotic (Gibco, BRL, Grand Island, New York). The follicular fluid containing the oocytes was emptied into 50ml falcon tubes containing warm mPBS. Later the fluid was transferred into a Falcon tissue culture search dish. For the slicing technique, each ovary's cystic follicle (>10 mm) was aspirated to prevent gelling of the follicular contents. An ovary was placed in a search culture dish containing mPBS, enough to cover the ovary. A sharp sterile blade was used to dissect the ovary so that the follicular contents could be released into the dish, containing mPBS. Using a stereo microscope (Olympus SZ61, Japan), COC's were microscopically scrutinized, oocytes identified and classified based on their morphological appearance. The COC's with complete, compact cumulus layers and an evenly granulated cytoplasm were selected for maturation. Denuded oocytes with irregular granular cytoplasm were not considered mature and were discarded. Oocytes were classified as good (COC's with two or more complete, compact cumulus layers and an evenly granulated cytoplasm) or poor (denuded or with incomplete layers of cumulus cells) oocytes. Acceptable quality oocytes recovered by both methods of recovery were matured *in vitro.*

3.2.2 Oocyte maturation

The selected COC's were washed six times. The first 3 times in mPBS solution, before being further washed 3 times in a pre-incubated tissue culture medium 199 (M 199)(Gibco, BRL, Grand Island, New York) plus 10% fetal bovine serum (FBS). The latter medium was referred to as the washing medium. Four-well Petri dishes containing 500µl maturation medium, covered with 250µl mineral oil in each well were pre-incubated for at least 2h, before being used to mature the oocytes. The maturation medium was made up of the washing medium + 0.5µl/ml FSH + 5µl/ml LH + 2µl/ml estradiol (E_2) + 1µl/ml sodium pyruvate (all 4 reagents from Sigma, St. Louis, MO, USA) and 10µl/ml antibiotic/antimycotic (Wang et al., 1998). The COC's (50 oocytes/well) were then transferred to a four-well dish and

incubated (Plate 3.4) for 24h at 38°C (5% carbon dioxide and 90% relative humidity). Following maturation, the oocytes were again microscopically evaluated under a stereo microscope. Mature oocytes (Plate 3.5) were selected for fertilization, based on full cumulus all expansion involving all layers, as well as corona radiata cells.

3.3 EXPERIMENT 2: EFFECT OF CULTURE MEDIA ON EMBRYONIC DEVELOPMENT

3.3.1 Oocyte fertilization

Oocytes were harvested during winter using the slicing technique, as described in paragraph 3.2.1 and matured as in paragraph 3.2.2. A total of 1405 mature oocytes were used in the entire experiment. The mature oocytes were washed three times in 100µl drops of Brackett and Oliphant (BO)-IVF medium, covered with 3ml mineral oil. The BO stock solution was made up of 65mg/ml sodium chloride ($NaCl_2$), 2.99mg/ml potassium chloride (KCl), 1.15mg/ml sodium phosphate ($NaH_2PO_4 \cdot H_2O$), 25.2mg/ml glucose, 3.31mg/ml calcium chloride ($CaCl_2 \cdot 2H_2O$), 1.05mg/ml magnesium chloride ($MgCl_2 \cdot 6H_2O$), 0.015ml/ml phenol red (Sigma, St. Louis, MO, USA) and 10% antibiotic (Gibco, BRL, Grand Island, New York), suspended in 100ml ultra pure water. The BO-IVF medium working solution was then prepared by adding 10ml of the BO stock solution, 3.1mg/ml sodium bicarbonate ($NaHCO_3$) and 0.138mg/ml sodium pyruvate in 100ml ultra pure water. The IVF medium was made up of 20ml BO working solution, supplemented with 0.006g/ml essential fatty acid-free BSA and 10µl/ml heparin (Sigma, St. Louis, MO, USA). Similarly, the sperm wash medium used was made up of 80ml BO working solution, 0.1553g caffeine (Sigma, St. Louis, MO, USA) and 0.003g/ml essential fatty acid-free BSA. Both the aliquots (IVF and wash aliquots) were pre-incubated for 24h at 38°C (5% carbon dioxide and 90% relative humidity) for 2h, prior to use. From the wash aliquots, mature oocytes were placed in the BO-IVF medium aliquots. About 15-20 mature oocytes were

placed in each 50µl aliquot of IVF medium and covered with 3ml of mineral oil (Natarajan et al., 2010).

3.3.1.1 IVF using fresh ram semen

Fresh semen (Zulu ram), which was used to fertilize the oocytes was collected at least 2h prior to fertilization with the aid of an electro-ejaculator (Ramsem, South Africa). The probe of an electro-ejaculator which was connected to the power supply was lubricated before being inserted into a rectum of the restrained ram. After insertion of the probe, short low-voltage pulses of electrical current were applied to the pelvic nerves. Once the nerves were electrically stimulated, ejaculation resulted. When the animal failed to ejaculate initially, it was allowed to rest for 10 minutes. All the ejaculates were collected in a 15 ml, pre-warmed falcon tube, and temporarily stored in a thermos flask (37˚C). This was done to avoid cold shock to the semen. Within 1h of collection, semen was transported to the laboratory for microscopic quality evaluation. Sperm traits such as sperm motility and the percentage live sperm, volume of the ejaculate (ml) and pH of semen sample were recorded.

The semen volume was recorded by taking readings from the numbered falcon tube used for collection. A 5µl aliquot of semen was placed on a microscopic slide and covered with a cover slip for microscopic evaluation of sperm motility and percentage live sperm (x 40 magnification), with the aid of a phase-contrast microscope (Olympus BH-2, Japan) mounted to a warm stage (Plate 3.8). The evaluation was subjective. Semen pH was also measured by immersing a pH meter (Plate 3.9) (Laboratory Chemical, Switzerland) probe into the semen sample and leaving it for 1 - 2 min. in the sample, in order to allow the pH meter to stabilize the reading, before being recorded. The pH probe was then removed from the sample, and immersed into ultra pure water container and wiped clean with soft tissue paper, before immersing it into another semen sample.

After evaluation, semen was extended with an egg yolk citrate diluent and equilibrated for an hour. After 1h, 1ml semen was placed into a 15ml falcon tube, the tube then filled up to 6ml mark with warm BO sperm wash solution. Afterwards the semen sample was centrifuged (Plate 3.1) twice at 1500rpm for 8 minutes at 38°C. The supernatant was then carefully aspirated immediately after centrifugation without disturbing semen pellet, using a sterile serological pipette. The semen pellet was then diluted with pre-incubated BO-IVF medium to reach a final concentration of approximately 200 x 10^6 sperm/ml. Then 50µl of the prepared semen was placed in the IVF aliquot containing the mature oocytes.

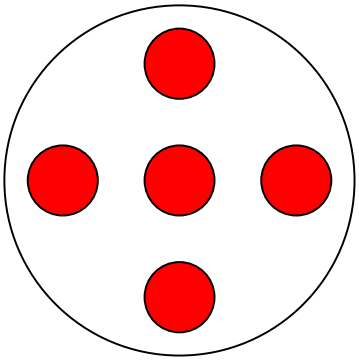
Plate 3.1 Centrifuge (Hermle, Germany) for washing of the semen

3.3.1.2 IVF using frozen ram semen

A frozen semen straw was taken from the liquid nitrogen (LN_2) and thawed by dipping the straw into a water bath of 37°C, for 30sec. The contents of the straw was then emptied into a 15ml falcon tube (by cutting both ends of the straw) and the tube filled up to 6ml with warm (37°C) BO sperm washing solution. The frozen-thawed semen suspension was then centrifuged, similarly to that of fresh semen and the semen pellets for both fresh and frozen-thawed semen were then

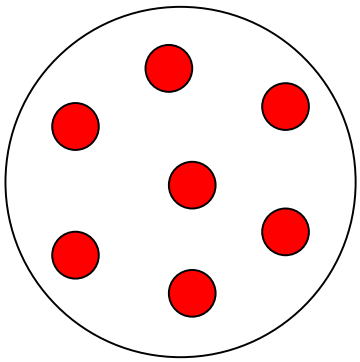
diluted with the BO sperm wash solution. A 5µl aliquot of semen after dilution was placed on a microscopic slide and covered with a cover slip for microscopic evaluation of sperm motility as is described in paragraph 3.3.1.1. Sperm motility was thus evaluated and recorded before semen was placed in the IVF aliquot containing the mature oocytes, and incubated for 18h at 38°C (5% carbon dioxide and 90% relative humidity).

Plate 3.2 A vortexing device for stripping off of cumulus cells



The 5 wash aliquots of 100µl

(a)



The 7 culture aliquots of 50µl

(b)

Figure 3.1 Two dishes prepared to wash (a) and culture (b) presumptive ovine zygotes respectively

3.3.2 *In vitro* embryo culture (IVC)

After 18h of oocyte-sperm incubation, presumptive zygotes were removed from the IVF aliquots into a 1.5ml Eppendorf tube containing 100µl of M199 + 10% FBS and vortexed (Plate 3.2) for 1.5 min. in order to strip off the cumulus cells. After vortexing, zygotes were washed 3 times in both M199 + 10% FBS and culture media (Fig. 3.1). The zygotes were then allocated to groups of 20 per aliquot and three different pre-incubated IVC media covered with 3ml mineral oil: KSOM (potassium simplex optimization medium), SOF (synthetic oviductal fluid) and CR1 (Charles Rosenkrans medium) (Fig. 3.1). Twenty to 25 zygotes were placed in each 50µl aliquot of the culture medium.

The IVC media used were made up as follows:

1. **KSOM** – A KSOM stock solution was composed of 55.5mg/ml sodium chloride ($NaCl_2$), 1.86mg/ml potassium chloride (KCl), 0.5mg/ml potassium phosphate (KH_2PO_4), 0.3mg/ml glucose, 1.46mg/ml L-glutamine, 0.04mg/ml EDTA, 2.51mg/ml calcium chloride ($CaCl_2 \cdot H_2O$), 0.49mg/ml magnesium sulphate ($MgSO_4 \cdot 7H_2O$), 0.016ml/ml of 0.5% phenol red (Sigma, St. Louis, MO, USA), 5.96mg/ml hepes and 10% antibiotics (Gibco, BRL, Grand Island, New York) mixed in 100ml ultra pure water (Adcock Ingram, South Africa). The KSOM working solution was then made up by using 10ml of the KSOM stock solution, 0.21g sodium bicarbonate ($NaHCO_3$), 0.005g taurine, 0.0022g sodium pyruvate, 0.2% sodium lactate, 1% minimum essential medium (MEM) and 1% Earle's basal medium (BME), all suspended in ultra pure water, to make up a total volume of 100ml. The KSOM working solution was then divided into two (50ml) equal parts. The first 50ml portion was supplemented with 0.01% BSA and called KSOM (step 1) while the second 50ml portion was supplemented with 0.1% BSA, called the KSOM (step 2).

2. The **SOF** IVC medium was prepared by first making up 4 stock solutions, namely, stock solutions A, B, C and D. Stock solution A was composed of 3.15g $NaCl_2$, 0.27g KCl, 0.08g KH_2PO_4, 0.3ml lactic acid ($C_3H_5O_3$), 0.09g $MgSO_4 \cdot 7H_2O$

(Sigma, St. Louis, MO, USA) and 0.5ml antibiotic dissolved in 50ml ultra pure water.

Stock solution B consisted of 1.05g $NaHCO_3$, 50µl 2% phenol red (Sigma, St. Louis, MO, USA) and antibiotic mixed and dissolved in 50ml ultra pure water.
Stock solution C consisted of 0,04g pyruvic acid (Sigma, St. Louis, MO, USA), dissolved in 5ml ultra pure water.

Stock solution D consisted of 0.26g $CaCl_2 \cdot H_2O$, dissolved in 10ml ultra pure water.

A complete SOF stock solution was then prepared by using 2ml of the stock solution A, 2ml stock solution B, 200µl stock solution C, 200µl stock solution D, 0.002g citric acid, 0.01g myo-inositol, 600µl BME, 200µl MEM, 0.003g L-Glutamine, 100µl antibiotics, dissolved using ultra pure water to make up a volume of 20ml. The 20ml complete SOF stock solution was divided into two parts, the first 10ml being supplemented with 0.1% BSA (SOF-BSA) and the other 10ml supplemented with 5% FBS (SOF-FBS).

3. **CR1** was prepared using 6.7mg/ml $NaCl_2$, 0.05ml/ml KCl stock solution, 2.2mg/ml $NaHCO_3$, 0.044mg/ml sodium pyruvate, 0.55mg/ml calcium lactate hydrate, 2µl/ml phenol red, 1% antibiotics, 0.5mg/ml myo-inositol, 0.01ml/ml magnesium chloride ($MgCl_2 \cdot 6H_2O$) stock solution, 1% MEM and 2% BME dissolved in ultra pure water. This CR1 solution was also divided into two portions, where 64ml was supplemented with 0.384g BSA (CR1-BSA) and the other 36ml supplemented with 4ml FBS (CR1-FBS) (Wan et al., 2009).

Presumptive zygotes were cultured for 6 days at 38°C, in humidified incubator (5% oxygen, 5% carbon dioxide and 90% nitrogen). For the first 48h of culture, the presumptive zygotes were cultured in either KSOM (step 1) or SOF-BSA and CR1-BSA. After 48h, the IVC media were changed from KSOM (step 1) to KSOM

(step 2), SOF-BSA to SOF-FBS and CR1-BSA to CR1-FBS. The media were changed by aspirating 50µl medium from aliquots containing the presumptive zygotes, and replacing the medium with the fresh 50µl pre-incubated medium, with the aid of a glass pipette. During the changing of the culture medium cleavage rate was also evaluated under a stereomicroscope and noted. The cleaved zygotes were also separated from the dead oocytes during the changing of the medium. After another 48h the media were changed again. On the 7th day of culture the embryos were fixed (Plate 3.6). For fixation, the embryos were removed from the IVC media and placed into a 1.5ml Eppendorf tube, containing 200µl fixing medium before being stored at 4°C, until stained. The fixing medium of the embryos was made up of DPBS and 3% paraformaldehyde (Sigma, St. Louis, MO, USA). The blastocysts were stained (Plate 3.7) with Bisbenzamide-Hoechst 33342 (H33342, Sigma) (Pawshe et al, 1996) to allow for the counting of the blastomeres.

3.4 EXPERIMENT 3: QUALITY OF CRYOPRESERVED RAM SEMEN

3.4.1 Semen cryopreservation

Animals used for semen collection in this study were kept at the Small Stock Section of the ARC – Irene, Pretoria. Three rams used were a Merino and 2 Nguni (Pedi and Zulu) rams. The ages of rams were estimated by identifying the number of permanent teeth. The Merino and Pedi rams were 2 years of age, while the Zulu ram was 3 years of age, with the body weights being 61.5kg, 58kg and 51.5kg for the Merino, Pedi and Zulu rams, respectively. The experiment was conducted during spring (August – September).

In this experiment, semen was collected from the 3 rams twice per week (every Monday and Thursday). The semen was collected with the aid of an electro-ejaculator, in a pre-warmed 15ml Falcon tube, maintained in a thermos flask (37°C). A 5µl aliquot of semen from each ejaculate was evaluated for sperm motility and the percentage live sperm, with the aid of a phase-contrast (Plate

3.8) microscope (x 40 magnification), attached to a warm stage. Another aliquot of 10µl from the same semen sample was used to determine the semen concentration with the aid of a Spermacue spectrophotometer (Plate 3.9) (Minitub, Germany). Semen pH was also measured as described in paragraph 3.3.1.1.

After microscopic semen evaluation, the semen was further processed for cryopreservation. The semen was extended at a ratio of 1:1 with an egg yolk-based extender (Egg-Yolk Citrate). The extender consisted of two parts, which were referred to as fraction A and B. Fraction A contained 1mg/ml gentamycin sulphate, 10mg/ml glucose, 18.6mg/ml sodium citrate (Sigma, St. Louis, MO, USA), ultra pure water and 10ml egg yolk. Fraction B was similar to fraction A, however, it contained 14% glycerol (Sigma, St. Louis, MO, USA). Both fractions were stored at 5°C. The semen was first extended with fraction A (which was warmed to approximately 37°C), and then the extended semen was equilibrated for 2h at 5°C. Fraction B was added and equilibrated for another 2h at 5°C. The semen sample was then evaluated for the percentage live sperm and motility, before being loaded into 0.25ml straws (5°C) and placed into a programmable freezer (Plate 3.10), for cryopreservation. Only semen with the sperm motility and percentage live sperm of 60% or more was further processed and frozen. The freezing procedure briefly entailed the following: In the programmable freezer the semen was cooled gradually at the rate of -0.08°C/min from 5°C to 4°C, thereafter at a rate of -6.20°C/min from 4°C to -130°C. This cooling process took 35 minutes. In addition to the 35 min of cooling, another 5 minute holding period was incorporated, while the straws were still in the programmable freezer. When the freezing process was completed, the straws were immediately removed from the programmable freezer and plunged into liquid nitrogen (-196°C). A single straw was then thawed to evaluate percentage live sperm and the motility rates of the batch. After that, the rest of the straws containing viable semen were loaded into hangers, and placed into liquid nitrogen tank for storage, until used for IVF.

3.5 EXPERIMENT 4: EFFECT OF FROZEN-THAWED RAM SEMEN ON *IN VITRO* FERTILIZATION (IVF) AND EMBRYONIC DEVELOPMENT

Frozen semen from the previous experiment (Experiment 3, discussed in 3.4.1) was used to fertilize *in vitro* matured oocytes (IVF). Fresh semen was used as a control in this experiment. The oocytes were collected during spring using the slicing method, as discussed in 3.2.1 and the oocyte maturation, semen preparation for IVF, fertilization of the oocytes and SOF for IVC were similar to those described in 3.2.2, 3.3.1 (3.3.1.1 & 3.3.1.2) and 3.3.2. The SOF IVC medium was the only medium used for this experiment. A total of 1593 mature oocytes were used for this trial, of which 791 oocytes were fertilized with the use of fresh semen, while 802 oocytes were fertilized using frozen-thawed semen. The straws of frozen semen were taken from the liquid nitrogen tank using pre-cooled forceps. The straws were then plunged into a water bath of 37°C, for 30sec.

3.6 DATA COLLECTION

The data collected included the number of ovaries, number of oocytes aspirated, cleavage and blastocyst rate formation for Experiments 1, 2 and 4. For Experiment 3, which dealt with semen cryopreservation, data on the following parameters – ejaculate volume, percentage live sperm, motility, semen concentration and semen pH was recorded. The parameters were recorded during both the pre-freezing and post thawing stages.

3.7 DATA ANALYSES

All collected data (total number of oocytes, good oocytes, poor oocytes, oocytes per ovary, all embryo developmental stages and semen parameters) were analyzed using the one way analysis of variance (ANOVA) procedures of SAS (2004). Differences between treatments were determined using the Tukey's Studentized Range (HSD) Test (SAS, 2004). Differences with a confidence level of $P<0.05$ were considered to be significantly different.

Plate 3.3 Collection of ovine ovaries from abattoir material

Plate 3.4 Incubators used for the maturation and culture of oocytes and embryos

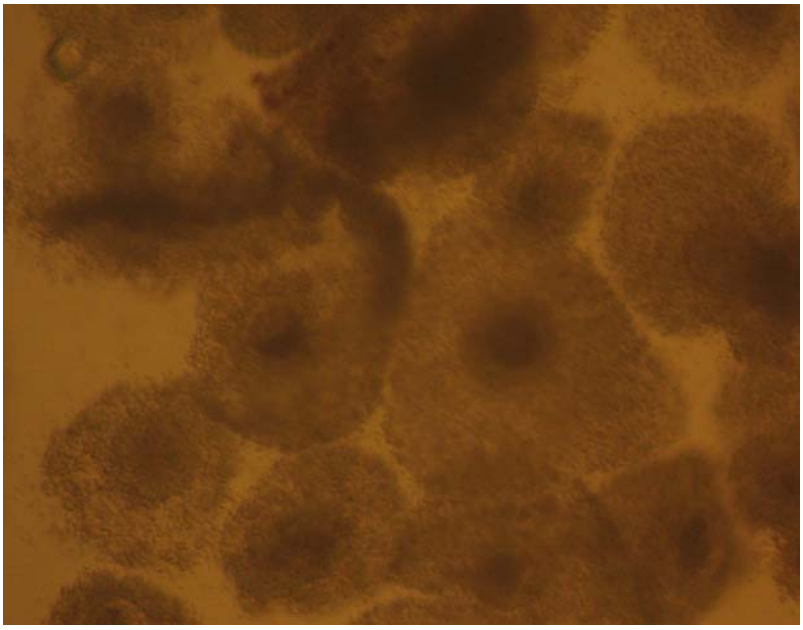


Plate 3.5 An example of mature ovine oocytes

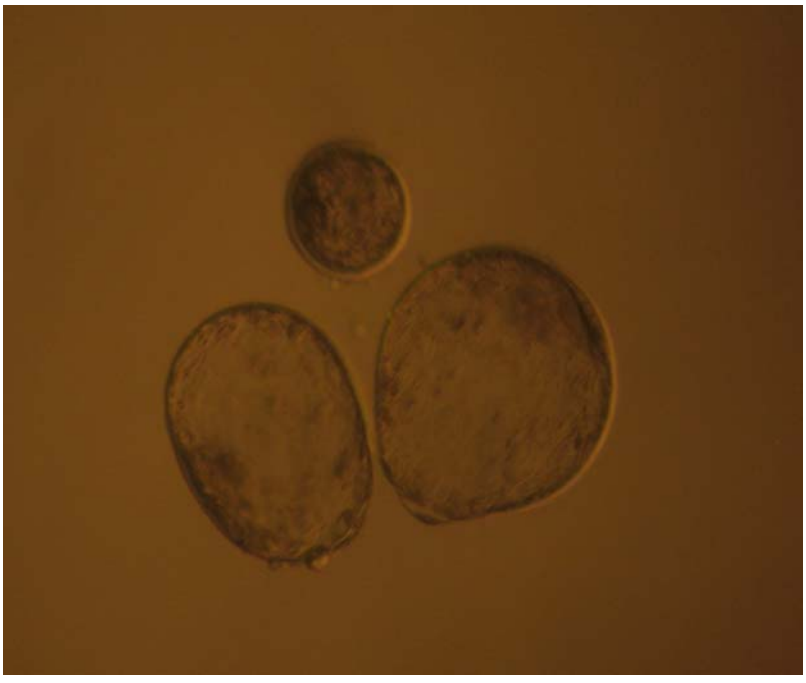


Plate 3.6 An example of day 7 ovine blastocysts

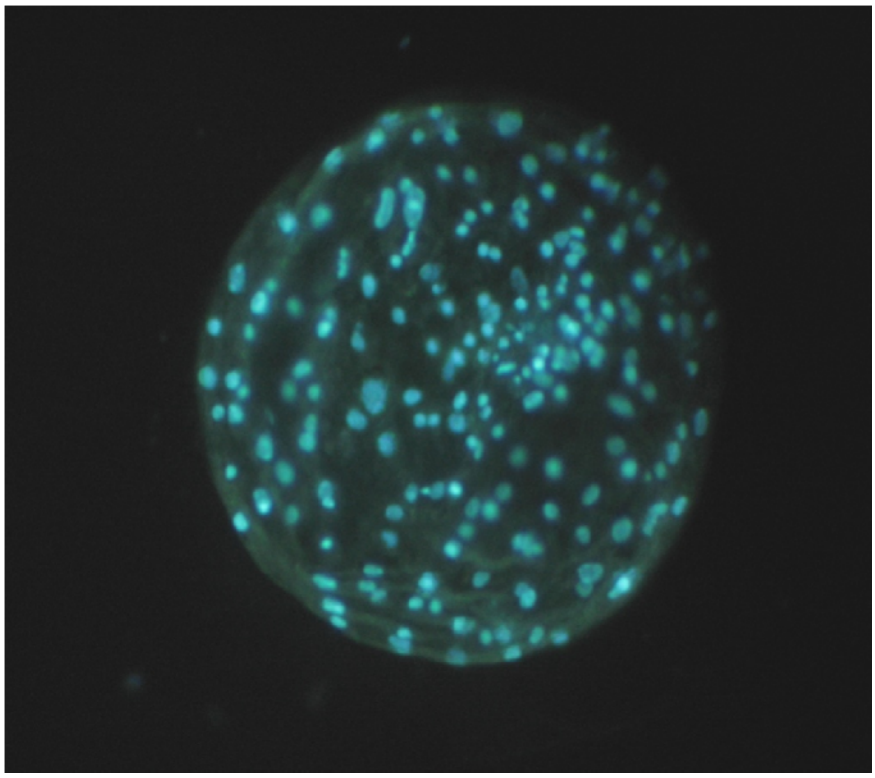


Plate 3.7 A stained ovine blastocyst exhibiting 202 blastomeres

Plate 3.8 The subjective evaluation of semen for percentage live sperm and motility

Plate 3.9 A spectrophotometer and the pH meter used in semen evaluation

Plate 3.10 Placing of loaded straws into a programmable freezer

CHAPTER 4

RESULTS

4.1 EFFECT OF OOCYTE HARVESTING TECHNIQUE ON THE RECOVERY RATE IN SHEEP

The effect of the oocyte harvesting technique on the ovine ovary oocyte recovery rate is set out in Table 4.1. The overall number of oocytes, acceptable quality oocytes and mean oocytes recovered per ovary using the slicing technique was higher ($P<0.05$) for the entire period of the experiment, compared to the aspiration technique. There was, however, no difference ($P>0.05$) between the two oocytes harvesting techniques with regard to the total number of inferior (poor) oocytes recovered.

Table 4.1 Effect of the aspiration and slicing techniques on the quantity and quality of ovine oocytes recovered

Treat.	Ovaries n	Oocytes (mean ±SE)			
		Tot. oocytes	Good oocytes	Poor oocytes	Oocytes/Ovary
Overall	307	57.8±8.7	28.5±6.6	29.3±4.2	3.9±0.4
Aspiration	167	40.3±9.1[b]	10.3±2.0[b]	30.0±7.5[a]	2.6±0.5[b]
Slicing	140	75.3±12.9[a]	46.7±10.3[a]	28.5±4.2[a]	5.2±0.4[a]

[ab] Mean values within same column with different superscripts differ significantly ($P<0.05$).

4.2 EFFECT OF CULTURE MEDIA ON *IN VITRO* EMBRYO DEVELOPMENT

Figure 4.1 demonstrates the developmental stages of ovine embryos cultured in the 3 different culture media (KSOM, SOF and CR1). The overall percentage cleavages to the 1-cell, 2-4-cell, 8-cell embryo, morula, and blastocyst stage were 17.9±2.0%, 46.7±2.5%, 14.5±1.6%, 3.4±0.7%, 2.1±0.5% and 0.5±0.2%, respectively. The cleavage rates recorded and embryonic developmental stages following culture using the 3 different media showed no statistical difference. The highest percentage was obtained for the 1-cell stage, followed by cleavage to the 2-4-cell, 8-cell, and morula and lastly blastocyst stages, respectively (although

not statistically different). For all the culture media, there was a similar tendency of decreasing rates in embryonic development with advancing incubation period of the embryos, with the lowest percentage occurrence being recorded for the blastocyst stage.

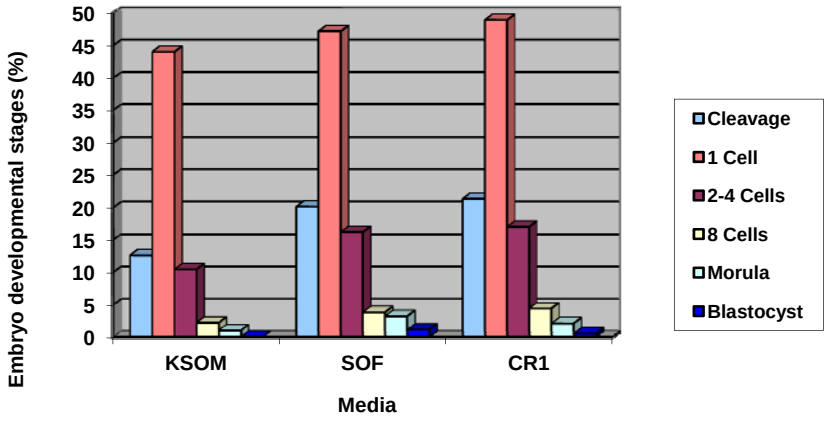


Figure 4.1 Effect of different culture media (KSOM, SOF and CR1) on *in vitro* produced ovine embryonic development

4.3 EFFECT OF BREED ON SEMEN PARAMETERS BEFORE AND AFTER FREEZING

Tables 4.2 and 4.4 demonstrate the semen parameters for the different sheep breeds before and after cryopreservation, respectively. The Merino breed produced a significantly ($P<0.05$) higher mean ejaculate volume, compared to the Zulu ram (0.6±0.09 vs. 0.3±0.01ml). The mean percentage live sperm for fresh semen 0h, 2h (after the first dilution) and 4h (after the second dilution) were similar ($P>0.05$) for all 3 breeds. Similarly sperm motility and semen pH of the fresh semen did not differ ($P>0.05$) between the breeds. The Zulu breed, however, recorded a significantly ($P<0.05$) lower semen concentration.

Regarding cryopreservation, the mean sperm survival rate (percentage live sperm) of the Pedi ram (26.7±2.8%) post-thawing was, significantly lower

(P<0.05), than in the Zulu and Merino rams (45.8±1.9% and 38.3±4.2%, respectively). The percentage live sperm for all breeds declined with equilibration time (2h - 4h) and a sharp decline was recorded post-thawing. The sperm motility between the 3 breeds did not differ (P>0.05) for the entire trial. The semen of the Zulu ram displayed a lower (P<0.05) sperm concentration, than that of Merino and Pedi rams at 2h and 4h of equilibration and for the entire observation period. As could be expected the fresh semen (prior to dilution) exhibited the highest sperm concentration and this declined with dilution and time (2h and 4h).

Table 4.2 Effect (mean ±SE) of breed on the fresh semen parameters in 3 sheep breeds

Breed	Semen parameters				
	Vol. (ml)*	Live Sperm (%)	Sperm motility (%)	Sperm concentration ($x10^6$/ml)	Semen pH
Merino	0.6±0.1[a]	75.8±1.5[a]	77.5±1.8[a]	742.1±18.4[a]	6.4±0.1[a]
Pedi	0.5±0.1[ab]	76.7±1.9[a]	80.0±1.2[a]	690.2±41.7[a]	6.8±0.2[a]
Zulu	0.3±0.0[b]	75.0±2.3[a]	75.0±2.0[a]	463.0±36.2[b]	6.5±0.2[a]
Overall	0.5±0.0	75.8±1.1	77.5±1.0	631.8±30.2	6.6±0.1

[ab] Values within same column with different superscripts differ significantly (P < 0.05)

4.4 EFFECT OF FROZEN-THAWED RAM SEMEN ON *IN VITRO* FERTILIZATION (IVF) AND EMBRYO DEVELOPMENT

In Table 4.3, the effect of breed following frozen-thawing of semen used for IVF, is set out. The rates recorded were as follows: for the overall cleavage rate (31.4±1.7%), 2-4-cell (24.3±1.4%), 8-cell (7.0±0.7%), morula (5.4±0.6%) and blastocyst formation (1.4±0.3%). No significant differences (P>0.05) were recorded between the fresh and frozen-thawed semen, regarding all the embryo developmental stages. The performance of both fresh and frozen-thawed semen followed the same trend, with the cleavage rate gradually declining with the progression in time and the embryonic developmental stage. The lowest developmental stage recorded was the occurrence of blastocyst formation, ranging from 0.4±0.4% to 2.6±1.0%. For the 3 breeds, no significant difference

was observed from the cleavage to the 2-4-cell stages. The use of fresh and frozen-thawed Zulu semen resulted in a higher (P<0.05) percentage of 8-cell embryos, compared to the Pedi semen. However, the 8-cell stage percentages recorded with the use of the Zulu ram semen (fresh and frozen-thawed), did not differ (P>0.05) from those of the Merino breed. There were no significant differences (P>0.05) between the 3 breeds regarding blastocyst formation rates, although the Zulu ram had a tendency to exhibit more 8-cell and morula embryos.

Table 4.3 Effect (mean ±SE) of breed, fresh and frozen-thawed (F/T) ram semen on *in vitro* fertilization and embryo development

Breed	Semen	Total oocytes	Overall cleavage (%)	2-4-Cell (%)	8-Cell (%)	Morula (%)	Blastocyst (%)
Overall		1593	31.4±1.7^{a}	24.3±1.4^{a}	7.0±0.7	5.4±0.6	1.4±0.3^{a}
Merino	Fresh	189	36.0±3.8^{a}	28.9±2.6^{a}	7.2±1.9^{ab}	4.2±1.6^{b}	1.4±1.0^{a}
	F/T	226	32.7±3.7^{a}	26.0±2.9^{a}	6.7±1.8^{ab}	4.7±1.5^{b}	2.0±1.1^{a}
Pedi	Fresh	281	28.2±4.7^{a}	22.9±4.1^{a}	4.8±1.2^{b}	3.2±1.4^{b}	0.4±0.4^{a}
	F/T	281	26.8±4.0^{a}	23.1±3.6^{a}	3.7±1.2^{b}	2.5±1.1^{b}	1.0±0.7^{a}
Zulu	Fresh	321	32.1±3.5^{a}	24.4±2.9^{a}	7.6±1.4^{a}	8.3±1.1^{a}	2.6±1.0^{a}
	F/T	295	34.2±5.0^{a}	22.2±3.5^{a}	12.0±2.3^{a}	8.6±1.4^{a}	1.4±0.6^{a}

^{ab} Values within same column with different superscripts differ significantly (P < 0.05).

Table 4.4 Effect (mean ±SE) of breed on ram semen parameters before and after cryopreservation

Breed	After 1st dilution (2 h equilibration)				After 2nd dilution (4 h equilibration)				Post-thawing	
	Live sperm (%)	Sperm motility (%)	Sperm concentration ($x10^6$/ml)	Semen pH	Live sperm (%)	Sperm motility (%)	Sperm concentration ($x10^6$/ml)	Semen pH	Live sperm (%)	Sperm motility (%)
Merino	70.8±2.3[a]	77.5±1.3[a]	577.8±17.8[a]	6.5±0.1[a]	66.7±1.9[a]	71.7±2.4[a]	366.1±19.9[a]	6.6±0.1[a]	38.3±4.2[a]	58.3±3.2[a]
Pedi	63.3±2.6[a]	80.0±1.7[a]	485.6±56.8[a]	6.7±0.1[a]	60.8±2.9[a]	75.0±1.5[a]	329.1±31.5[a]	6.6±0.1[a]	26.7±2.8[b]	59.2±2.3[a]
Zulu	66.7±2.6[a]	76.7±1.4[a]	311.6±40.8[b]	6.6±0.1[a]	64.2±2.6[a]	76.7±1.4[a]	182.9±25.0[b]	6.6±0.1[a]	45.8±1.9[a]	66.7±1.9[a]
Overall Mean	66.9±1.5	78.1±0.9	458.0±31.5	6.6±0.1	63.9±1.5	74.4±1.1	292.7±21.1	6.6±0.3	36.9±2.2	61.4±1.6

[ab] Values within same column with different superscripts differ significantly ($P < 0.05$).

CHAPTER 5

DISCUSSION

5.1 EFFECT OF THE OOCYTE HARVESTING TECHNIQUE ON THE OOCYTE RECOVERY RATE

The quality and quantity of oocytes harvested from sheep ovaries at the abattoir are important factors to be borne in mind during IVEP. In this study, two methods of oocyte recovery were used. The slicing method yielded higher numbers of better quality (morphologically intact) oocytes and more oocytes per ovary for the entire trial period. Similar results have been previously reported in sheep, goats (Pawshe et al., 1994; Wani et al., 1999; Wang et al., 2007) and cattle (Yoo et al., 1993). The slicing method generally led to higher recovery rates, as the ovarian surface and the core was dissected and oocytes microscopically retrieved ensuring the retrieval of most of the oocytes. The lower number of oocytes obtained per ovary (2.6±0.5) using the aspiration method in sheep has been confirmed by other researchers (3.17±1.4 oocytes/ovary) (Marques et al., 2006). This observation may be due to, amongst others, the small size of the ovine ovary and the follicles also being small and deeply implanted in the matrix of the ovary. Follicles are, therefore, easily missed by the needle during the aspiration process. This phenomenon is common also in other animal species when the aspiration technique is utilized to harvest oocytes (Wani et al., 2000; Nowshari, 2005). However, the number of inferior oocytes harvested using both techniques are relatively similar. Slicing would, therefore, seem to be the better option in ovine oocyte recovery procedures.

5.2 EFFECT OF CULTURE MEDIA ON *IN VITRO* EMBRYONIC DEVELOPMENT

No statistical difference regarding embryonic developmental stages between the 3 culture media used, following fresh ram semen fertilization, was recorded. The rates of cleavage for 1-cell, 2-4 cell, 8-cell, morula and the blastocyst stages did

not differ between culture media (KSOM, SOF and CR1). The occurrence of 1-cell oocytes (unfertilized) was high; while those induced to the blastocyst stage was very low. Cleavage rate from the present study was lower, when compared to other studies (Natarajan et al., 2010). So for example, high cleavage rates in sheep have been reported using CR1 and SOF media (72.5% to 84.3%) (Li et al., 2006; Wan et al., 2009). Higher percentages of blastocyst formation have also been reported (Natarajan et al., 2010). The percentages may be high due to the fact that the maturation medium was supplemented with sheep serum, and prepared on a weekly basis. From the present experiment IVM medium was supplemented with fetal bovine serum and prepared bi-weekly (Walker et al., 1996).

It was observed that a lower percentage of cleaved oocytes cultured in SOF and CR1 *in vitro* developed to lower percentages of blastocysts. No cleaved oocytes cultured in KSOM developed to blastocyst stage. This indicates that SOF and CR1 culture media are more suitable for ovine *in vitro* culture, compared to the KSOM medium. Failure of blastocyst development may be ascribed to the fact that the KSOM medium was initially formulated for bovine embryo culture *in vitro* hence more suited to bovine oocytes and sperm than to those of sheep. The KSOM medium also contains ethylenediaminetetraacetic acid (EDTA), hepes and taurine, which are absent in the CR1 and SOF media. Therefore, this slight difference in formulation of the media could have induced a prominent difference in embryonic development. In cattle, for example, Nedambale et al. (2006) reported more bovine blastocysts to form in sequential KSOM-SOF and SOF media, than in CR1 medium. Although the CR1 medium was initially formulated for bovine *in vitro* embryo production (IVEP), the current trial shows that this culture system can also be used for *in vitro* production of ovine embryos (Rosenkrans et al., 1993). The cleavage rates generally decreased with advancing stages of development. This is consistent with similar reports in bovine embryos (Sagirkaya et al., 2006).

5.3 EFFECT OF BREED ON THE QUALITY OF FRESH AND CRYO-PRESERVED RAM SEMEN

Small ruminants tend to produce ejaculates with small volumes. In this study, a marked variation between rams of 3 different breeds was recorded only in the ejaculate volume. The Zulu ram produced ejaculates with a lower semen volume, compared to the Merino ram and a low sperm concentration throughout the entire experiment. These volumes are similar to those obtained in goats (0.55±7.6ml) by Sundararaman et al. (2007). However, the mean ejaculate volume reported by Batisda et al. (2009) in goats was more (1.1±0.1ml to 1.7±0.2ml) than that obtained in the present study (0.3±0.01ml to 0.6±0.09ml). There are, however, many external factors that could play a role in the different volumes recorded e.g. breed, age, nutritional status and environmental factors. The difference in ejaculate volume may also be due to the method of semen collection. In this study the method of electro-ejaculation was used, and the semen was collected twice per week (every Monday and Thursday). In the other trials where semen volume was high, semen was generally collected by artificial vagina, also twice a week. These results, however, contradict previous reports that the electro-ejaculation method of semen collection results in larger ejaculate volumes than with the use of the artificial vagina in sheep and camels (Marco-Jimenez et al., 2005; Guiliano et al., 2008).

A reduction in the percentage live sperm within breeds was recorded post-thawing. In all the breeds, the post-thawing percentage live sperm ranged between 26.7 and 45.8% compared to a percentage live sperm for fresh semen, ranging between 75 and 76.7%. The Pedi ram exhibited the lowest percentage live sperm, post-thawing. A percentage live sperm variation between rams was also observed by Garcia-Alvarez et al. (2009). In addition to this, variation in quality of preserved semen between different breeds of sheep was reported. A decline in sperm motility parameters was observed with increasing time of storage (Kasimanickam et al., 2007). A significant difference was recorded regarding the sperm concentration between breeds. The Zulu breed displayed a

lower sperm concentration with fresh and diluted semen, compared to the Pedi and Merino sheep breeds. The semen for this trial was collected during the non-breeding season. Although the sperm concentrations are relatively lower in this study, these sperm concentrations were still more concentrated than those reported in goats of different breeds which ranged from 126.5±73.2 x 10^6 sperm/ml to 161.3±83.6 x 10^6 sperm/ml. This difference may be ascribed to the season of semen collection, as season also has an effect on semen quality (Makawi et al., 2007). Generally the natural breeding season results in higher sperm concentrations per ejaculate compared to the non-breeding season (Webb et al., 2004). The pH observed in this study was similar between all the breeds from the fresh to the diluted semen samples. It was also similar to that reported by Sabev et al. (2006).

5.4 EFFECT OF BREED AND SEMEN CRYOPRESERVATION ON *IN VITRO* PRODUCED OVINE EMBRYOS

The results obtained in this study showed no statistical difference in cleavage rates for fresh or frozen-thawed semen. For fresh semen the cleavage varied between 28.2±4.7% and 36.0±3.8%, while for the frozen-thawed semen the cleavage rate was between 26.8±4.0% and 34.2±5.0%. The overall mean cleavage rate recorded was 31.4±1.7%, compared to the 40.9±16.5% reported by Marques et al. (2006), which was also low. The reduced cleavage rates recorded in this study may be ascribed to the quality of the oocytes used. The trial was performed out of natural breeding season, that is, September and October. Generally the oocytes collected from different sheep breeds during the season of short-day length (natural breeding season) demonstrate a better ability to mature *in vitro* (Marques et al., 2006).

Individual ram differences were recorded. The Zulu breed performed better than Pedi and Merino rams, with both the fresh and frozen-thawed semen. This was especially evident in the 8-cell and morula stages of embryonic development. This is in agreement with Morris et al. (2003), although this variation was

reported throughout the entire *in vitro* culture period. In the current study variation was only observed at the 8-cell and morula stages of embryonic development.

CHAPTER 6

GENERAL CONCLUSIONS AND RECOMMENDATIONS

The oocyte harvesting technique affects the recovery rate of oocytes in sheep. The slicing technique in this trial yielded more oocytes of acceptable quality per ovary compared to the aspiration technique. Therefore, it is concluded that the slicing of the ovaries for oocyte retrieval is more warranted. This was brought by the fact that the slicing technique yielded better results in this study. Oocyte quality ultimately affects the embryonic survival and development rates, and it is, therefore, important to ensure that good quality oocytes are recovered for IVEP.

The *in vitro* maturation of oocytes is a prerequisite for *in vitro* fertilization. After fertilization presumptive zygotes have to be cultured to further assist them to develop. The differences recorded were not statistically significant between 3 culture media used. However, embryonic development decreased with advancement of the developmental stages, that is, the percentages obtained in all developmental stages decreased as developmental stages advanced. It could, therefore, be concluded that of the 3 used culture media, SOF and CR1 culture media can support embryonic development in sheep.

Breed and the equilibration period play major roles in the semen cryopreservation process. The breeds used in this study differed regarding ejaculate volume and sperm concentration. Regarding ejaculate volume, the Merino ram tended to have a higher volume than the Zulu ram, which recorded the lowest volume. The Merino ram again produced an almost similar ejaculate volume to that of the Pedi ram. The Zulu ram had the lowest fresh semen sperm concentration and the diluted semen, compared to the 2 other rams. As a result, the indigenous fresh semen with its relatively low sperm concentration, compared to the exotic (Merino) breed, was further reduced following dilution. The other semen parameters, such as percentage live sperm, sperm motility and semen pH

were not affected by breed or semen dilution. The percentage live sperm and motility displayed drastic deterioration with cryopreservation. However, the change in the percentage of live sperm in the less concentrated indigenous semen (Zulu ram) post-thawing was less evident, compared to the changes displayed by the more concentrated semen of the Pedi rams.

The overall cleavage, 2-4cell and blastocyst embryonic developmental stages (31.4±1.7%, 24.3±1.4% and 1.4±0.3%, respectively) following the use of fresh and frozen-thawed semen from the different rams were generally lower than those recorded by other researchers. The percentage of blastocyst formation was extremely low and not satisfactory. Regarding the 8-cell embryo development stage obtained following the use of both fresh and frozen-thawed semen, the Zulu ram semen recorded highest occurrences, yet equal to that recorded with the Merino ram semen. The Pedi ram recorded the lowest percentages which were also similar to those of the Merino ram. The Zulu ram displayed a higher morula rate of development than both the Merino and Pedi rams. However, no difference was recorded regarding the type of semen used at similar embryonic developmental stages. These results, thus, show that the performance of both the fresh and frozen semen was generally poor. Ovine semen fertility may be improved by investigating several external factors, such as the season of semen collection, technique used during semen collection and the method of semen cryopreservation. In conclusion, the low blastocyst rates obtained warrant more research in order to improve ovine blastocyst rate formation.

Recommendations

Factors that lead to the recovery of poor quality oocytes while using the aspiration technique should be investigated e.g. type of needle used and its size, the vacuum pressure for aspiration, together with the syringe may have contributed to the quality of the oocytes recovered.

For semen cryopreservation it is recommended that the extenders, cryoprotectants and freezing protocols should continually be evaluated and improved. The evaluations should be done until a diluent and protocol that suits ram semen freezing and records at least an 80% post-thawing sperm motility and percentage live sperm, with acceptable fertility rates can be identified.

ABSTRACT

IN VITRO EMBRYO PRODUCTION AND SEMEN CRYOPRESERVATION IN SHEEP

by

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Two trials were conducted at Agricultural Research Council (Irene – Pretoria), between March (autumn) and October (spring), 2008. The first trial evaluated the effect of the oocyte harvesting technique on the quantity and quality of ovine oocytes recovered, as well as the effect of the culture media used on embryonic development. The second trial evaluated the effect of breed and semen cryopreservation on the embryonic development following *in vitro* fertilization (IVF).

For the first trial, ovine ovaries were collected from slaughtered animals at the Boekenhout abattoir near Pretoria. All ovaries were immediately transported to the laboratory for further processing and use in the trials. On reaching the

laboratory, ovaries were processed, and oocytes were harvested, either by slicing (140 ovaries), or follicular aspiration (167 ovaries). The oocytes collected were matured, fertilized with fresh ram semen and cultured in order to produce embryos. The average total number of ovine oocytes recovered per ovary, and the total number of oocytes collected were higher ($P<0.05$) when using the slicing (5.2±0.4 oocytes/ovary and 75.3±12.9 total oocytes) technique, compared to the aspiration technique (2.6±0.5 oocytes/ovary and total of 40.3±9.1 oocytes). The number of acceptable quality (intact cumulus layers) oocytes was also higher ($P<0.05$) following the slicing (46.7±10.3 oocytes), compared to aspiration (10.3±2.0 oocytes) technique. However, the number of poor quality oocytes did not differ between the 2 oocyte harvesting techniques. The acceptable oocytes were then matured *in vitro* for 24h and no difference ($P > 0.05$) in oocyte maturation rate between the oocytes recovered using the slicing or aspiration technique was recorded.

A comparison of the 3 culture media (Potassium simplex optimization medium - KSOM, Synthetic oviductal fluid - SOF and Charles Rosenkrans medium - CR1) used for maintaining subsequent embryonic development was then evaluated. All oocytes were further matured, using the maturation medium - TCM 199 containing FSH, LH and E_2, supplemented with 10% FBS. After maturation, the oocytes were fertilized (using fresh ram semen) and incubated for a period of 18h. At the end of 18h fertilization period, oocytes were vortexed in an Eppendorf tube containing 100µl M199 + 10% FBS for 1.5min. The vortexing was performed to remove the cumulus cells surrounding the zygote. A total of 1405 presumptive zygotes were randomly allocated to the 3 different culture media (481, 461 and 463 zygotes in KSOM, SOF and CR1, respectively). The zygotes were then cultured for a period of 7 days. No significant difference between all 3 culture media was recorded regarding cleavage rates, showing that culture media had no effect on the subsequent cleavage. However, the percentages of embryos decreased with an advancement of the embryonic developmental stages.

Regarding the semen cryopreservation, 3 rams (Merino, Pedi and Zulu) were used. Semen from each ram was collected twice per week, using the electro-ejaculation method. The semen was first macroscopically evaluated for ejaculate volume and then microscopically for the percentage live sperm, motility, semen concentration and also semen pH immediately after collection. Thereafter, the semen samples were diluted with an egg-yolk citrate extender. Semen samples were diluted in a ratio of 1:1, before being equilibrated for a 2h period at 5˚C. Following equilibration, the semen samples were further diluted, with an extender containing a cryoprotectant and equilibrated further for another 2h, also at 5˚C. The semen samples were then loaded into plastic mini straws (0.25ml) and frozen in a programmable freezer. A straw from each ram was thawed in a water bath at 38˚C for 30sec. Post-thaw motility and the percentage live sperm were subjectively assessed using a phase contrast microscope. All straws were transferred and stored in liquid nitrogen (LN_2) for a minimum period of 2 weeks before being evaluated. The Merino breed produced a significantly ($P<0.05$) higher ejaculate volume, compared to the Zulu ram (0.6±0.09 vs. 0.3±0.01ml). The percentage live sperm for fresh semen sample after the first dilution and after the second dilution were not different between the 3 breeds. However, the percentage live sperm in the Pedi ram (26.7±2.8%) post-thawing was lower than that of the Zulu and Merino rams (45.8±1.9% and 38.3±4.2%, respectively). The sperm motility and semen pH of the fresh semen did not differ significantly between the breeds. The Zulu breed, however, recorded a lower semen concentration, compared to the other 2 breeds.

The embryonic developmental stages were monitored and recorded. The overall cleavage rate recorded was 31.4±1.7%, with no significant differences for cleavage at the 2-4 cell embryonic development stage. For the 8-cell developmental stage, the Zulu ram performed similar to the Merino ram, but better than Pedi. Regarding the morula stage of embryonic development, the Zulu performed better than the other 2 breeds for both fresh and frozen-thawed semen. Generally, a low rate of blastocyst formation was recorded for all the

breeds following the use of both fresh and frozen-thawed semen. The Zulu ram produced a higher percentage of morula stage embryos for both fresh (8.3±1.1%) and frozen-thawed (8.6±1.4%) semen, compared to the other 2 breeds (Merino and Pedi).

In conclusion, it can be said that both harvesting techniques can be used for ovine oocyte collection, as they do not have a real effect on oocyte maturation. However, the slicing technique resulted in a higher number of good quality oocytes, compared to the aspiration method. Therefore, the slicing technique proved to be the best for oocyte recovery in sheep. During *in vitro* embryo production (IVEP) 2 of the 3 culture media resulted in similar embryonic development showing that they can be used in ovine IVEP. The semen cryopreservation protocols, however, still need to be improved. The preliminary results show that frozen-thawed semen can successfully be used, like fresh semen, in *in vitro* ovine embryo production. Very little difference in the performances of both semen treatments was recorded. The Zulu ram recorded a lower ejaculate volume and sperm concentration, but led to a high 8-cell and morula formation (for fresh and frozen-thawed semen) which proved to be better than the other breeds (Merino and Pedi). However, unacceptable low percentages of blastocyst formation were recorded in all three breeds. Therefore, more research is needed on *in vitro* culture in order to improve blastocysts formation in sheep.

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