EFFECT OF DIFFERENT CRYODILUENTS ON NGUNI BULL SEMEN VIABILITY AND IN VITRO FERTILIZING CAPACITY

by

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DEDICATION TO MY FAMILY

- To my parents Thabiso and Mathapelo Sebotsa for their love, encouragement and guidance in my life which have contributed to what I am today.

- To my husband Tlelima for his endless support and encouragement especially during the tough times of my studies.

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DECLARATION

I hereby declare that the dissertation hereby submitted by me for the **Magister Scientiae Agriculturae** degree to the University of the Free State is my own independent work and has not previously been submitted by me to another University. I furthermore cede copyright of the dissertation in favour of the University of the Free State.

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LIST OF ABBREVIATIONS

AI	Artificial Insemination
ANOVA	Analysis of variance
ARC	Agricultural Research Council
ART	Assisted reproductive technology
BO	Brackett Oliphant
BSA	Bovine serum albumin
BSP	Bull seminal plasma
cm	Centimetre
CO_2	Carbon dioxide
DMSO	Dimethyl sulfoxide
DPBS	Dulbeccos phosphate buffered saline
EAA	Essential amino acid
ET	Embryo transfer
FAF	Fatty acid free
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
g	Gram
h	Hours
GCRB	Germplasm, Conservation and Reproductive Biotechnology
GV	Germinal vesicle
ICM	Inner cell mass
IMO	Isolated mouse oviduct
IVC	In vitro culture
IVEP	In vitro embryo production
IVF	In vitro fertilisation
IVM	In vitro maturation
KSOM	Potassium simplex optimizing medium
LDL	Low density lipoprotein
LH	Luteinizing hormone
m	Metre

Min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
MOET	Multiple Ovulation and Embryo Transfer
mPBS	Modified phosphate buffered saline
NEAA	Non-essential amino acid
OPU	Ovum pick up
PBS	Phosphate buffered saline
PDP	Professional development programme
PVA	Polyvinyl alcohol
RH	Relative humidity
ROS	Reactive oxygen species
rpm	Revolutions per minute
SAS	Statistical analysis system
SE	Standard error
SOF	Synthetic oviductal fluid
TALP	Tyrode's medium supplemented with albumin, sodium lactate and sodium pyruvate
TCM	Tissue culture media
USA	United States of America
µg	Microgram
µl	Microlitre
Vs	Versus
%	Percentage
^{0}C	Degrees Celcius

CHAPTER 1

GENERAL INTRODUCTION

Cattle play a major role in the well-being of people, which include the provision of meat, hides, milk, skins and manure, while also serving as a source of income. Over 70% of the poor farmers in South Africa are located in the rural harsh agro-ecological zones, where cropping is not possible and farmers therefore rely on livestock production for their livelihoods (Bester et al., 2003). Most of the small scale rural farmers in South Africa depend on cattle for milk and meat production, draught power, manure, cash and other socio-cultural uses (Bayer et al., 2004). However, in the rural areas especially of Southern Africa, the cattle production rate is generally low and unable to meet the animal protein demands of the rapidly increasing human population. This need thus calls for an increase in offspring numbers (production efficiency), which relies on efficient reproduction. Reproductive efficiency (whether measured as calving or weaning percentage) is an important factor that influences the sustainability of any livestock enterprise (Gordon, 1983; Wiltbank, 1994).

Several assisted reproductive technologies (ART's) are currently being used to improve the reproductive efficiency in cattle, including the manipulation of the female reproductive behaviour by using oestrous synchronization, artificial insemination (AI), in vitro fertilization (IVF) and ultimately in vitro embryo production (IVEP). AI as such could involve the use of either fresh or frozen-thawed semen, depending on the availability of semen. The use of fresh semen generally requires the availability of suitable recipient cows at the time of semen collection, in order to utilize all of the semen collected almost immediately. Semen used in either AI or IVF is generally collected from bulls possessing superior genetic traits, with the aim being to accelerate genetic progress. However, due to limitations regarding recipient cows and the quantity of semen ejaculated, it is necessary and feasible to freeze and store (long term) semen for future use, or even transporting the semen over vast distances.

Cryopreservation of gametes and embryos, as well as the development of genetic resource banks could allow for the availability of genetic material for an indefinite period of time (Watson & Holt, 2001). Within the aspects of the genetic material in a cryobank, the question of storage and subsequent use of sperm cells has found widespread consideration and application in cattle breeding programs. Semen cryopreservation has thus also become a valuable tool for the preservation of genetic material of endangered species or sires with superior breeding traits (Schafer-Somi et al., 2006). This technology of semen cryopreservation also enables cattle breeders to obtain sperm of genetically superior bulls without the expenses of buying, raising or maintaining such bulls. Semen cryopreservation overcomes problems of increased expenses related to the transport, labour and quarantine costs of the bull. Additionally, semen cryopreservation limits the transmission of sexual diseases from one herd to the next.

Although semen cryopreservation as such has a great potential and is an important technique, it still has certain limitations. Fertility results in cattle after AI with frozen-thawed semen have been reported to generally be low, compared to the use of fresh semen (Watson, 2000; Celeghini et al., 2008). Several trials have been performed to improve the protocols for the freezing of bovine semen, which include the use of better semen extenders, cryoprotectants, as well as improved cryopreservation and thawing methods. No matter which of these techniques are used in semen cryopreservation, sperm cryopreservation is still detrimental to sperm function and the technique eliminates or injures a considerable number of sperm (approximately 50%) (Gravance et al., 1998; Watson, 2000). In bovine, semen cryopreservation has led to reduced sperm viability and fertility, when compared to fresh semen (Bilodeau et al., 2000). Thus, fertility results following AI with frozen-thawed semen in cattle remain low and warrant further research.

The low conception rates obtained are often attributed to the depletion of seminal plasma and cellular antioxidant systems, as seminal plasma is either removed or highly diluted during freezing, while some cellular antioxidants are lost during the freezing and thawing processes (Alvarez and Storey, 1992;

Bilodeau et al., 2000). Intracellular ice crystallization during cryopreservation is one of the main reasons responsible for mortalities in the sperm cell (Mazur, 1984). This may be attributed to several factors including the extender used, the cryoprotectant used or the thawing rate and procedure (Platz & Seager, 1977; Linde-Forsberg, 1991; Rijsselaere et al., 2002). Thus it can be seen that the composition of the semen extender, a suitable cryoprotectant and an optimal freezing and thawing rate are important factors to consider in semen cryopreservation (Hammerstedt et al., 1990; Curry et al., 1994). The viability of cryopreserved bovine semen can be evaluated either following AI or after IVF. Although the use of AI for assessing bull fertility is the most accurate and practical method, it is a time consuming and an expensive exercise, compared to IVF if this technology is available.

Although cryopreserved bull semen has been used successfully for many years for AI purposes, there is still a shortcoming in research on the performance of cryopreserved semen in the different South African indigenous cattle breeds, such as the Nguni, following IVF. Research is also lacking on the quality of semen from indigenous African breeds of cattle such as the Nguni, prior and post freezing and thawing processes. This is due to the fact that most of the studies have focused on the use of frozen-thawed semen from exotic bovine breeds. This condition has led to a situation whereby the indigenous breeds of various adapted species, including indigenous cattle are currently facing a problem of extinction There is thus a need to carry out research and state recommendations concerning the use of frozen-thawed semen of the local South African cattle breeds - as they are well adapted to the local conditions and are easier to raise extensively, compared to the exotic breeds. There could be great advantage in finding an effective protocol for the freezing of semen from this indigenous South African cattle breed (Nguni). Cryopreservation of Nguni bull semen could enable cattle breeders to store genetic material in gene banks for use at a later stage in either AI programmes, or for IVF purposes.

South African indigenous cattle breeds such as the Nguni possess a wide range of gene pool diversity and have developed over generations to adapt to the local agro-climatic and socio-economic needs of the people. Global diversity in indigenous species of domestic animals is considered to be under threat. A large number of the indigenous cattle breeds are endangered worldwide, thus cryopreservation of genetic material (e.g. semen) from livestock, especially cattle through cryopreservation, is an important technique to conserve genetic diversity in those breeds. Due to a potential high meat production, (higher calving percentage) and adaptability to local climatic conditions, the Nguni breed is in high demand, especially in the Southern part of Africa. Thus special efforts are needed to propagate superior males and females of this breed. The performance of frozen-thawed Nguni bull semen has to be tested regarding its in vitro fertilizing ability of the bovine oocytes. This may thus be a potential technique to increase the reproductive efficiency of the Nguni breed.

There are currently few reports in the literature regarding the tolerance of Nguni bull sperm to cryopreservation. There is thus a need to perfect the technique of freezing semen of this local breed - in an attempt to use it as a tool for upgrading the genetic make-up of this local breed, and also possibly improving the overall reproductive efficiency. This study therefore aims at evaluating the fertilizing ability of frozen-thawed Nguni bull semen, following the use in IVF to improve the reproductive efficiency and genetic traits. The technique of IVF is used as a tool to test the fertilizing ability of frozen-thawed bull semen. This technique could also help in increasing the animal numbers, thus meeting the animal protein requirements of rapidly growing human populations in the third world countries. In the view of the foregoing, the production of beef cattle must be drastically increased, as an alternative to help feed the undernourished, poor third world communities.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

In the past number of years artificial breeding technologies have been extensively used throughout the world. The aim of the cryopreservation process has been to keep the cellular metabolism in an inactive state for an indefinite period of time (Squires et al., 2004). Cryopreservation of sperm cells in general involves the cooling and storage of cells in a frozen state at extremely low temperatures - which gives the sperm cells a greater chance of survival following thawing. The cryopreservation of bovine semen has been reported to lead to reduced sperm viability and fertility, when compared to fresh semen (Bilodeau et al., 2000). Membrane damage of the sperm cells includes changes in the lipid composition, fluidity and permeability of the plasma and outer acrosomal membranes (Januskauskas et al., 2003).

Other findings have indicated that 60-70% of the bull sperm are said to be killed by the current methodologies employed in semen cryopreservation (Watson, 2000). Intracellular ice crystallization during sperm cryopreservation being one of the main causes that leads to damaged cells (Mazur, 1984). During the freezing and thawing processes, there are also several other factors that could also reduce the fertility of semen. These include sudden temperature changes, ice formation and dissolution of the cell contents during deep freezing and thawing (Watson, 1999; Thun et al., 2002). Cell damage during cryopreservation is said mainly to affect the sperm membranes, the cytoskeleton, the motile apparatus as well as the nucleus of the sperm cell (Thun et al., 2002).

Frozen-thawed sperm produce reactive oxygen species (ROS), of which excessive formation is associated with a decrease in the quality of frozen-thawed sperm (Alvarez & Storey, 1992; Stradaioli et al., 2007). The cold shock induced in sperm cells during freeze-thaw process, is associated with oxidative stress imposed by these free radicals (Sanocka & Kurpisz, 2004;

Salvador et al., 2006). The cryopreservation process induces physical and chemical stress on the sperm membrane, which in turn reduces sperm viability and fertilizing ability. Antioxidants play a major role in expelling free radicals, which may cause lipid peroxidation of sperm plasma membranes during cryopreservation (Baumber et al., 2000). Although the process of cryopreservation generally impairs sperm cell function and thereby reducing the motility and fertility rates achieved, it is still extensively used by cattle breeders with a great degree of success - as it improves the genetic progress when using bulls of superior breeding value (Cotter, 2005). Some findings have reported a motility rate of 63% following semen cryopreservation (Samardzija et al., 2006). Nevertheless the low motility rate in cattle sperm is considered to be a 'compensative semen trait', as a large dose could compensate for a low percentage of motile sperm cells (Den Daas et al., 1998).

2.2 Cryopreservation techniques

The objective of any cryopreservation technique is to preserve living cells without decreasing their survival rate following long periods of storage in the liquid nitrogen (-196^{0}C) in a frozen state. The preservation of bull semen in a liquid form allows storage for a few days only, while the deep frozen form permits storage for many years without any significant decrease in semen quality. Various methods of cryopreservation of bovine semen have been used successfully e.g. conventional slow freezing, ultra-rapid cooling and vitrification (Vincent & Johnson, 1992; Leibo et al., 1996).

The containers in which semen is generally stored, e.g. straws, ampoules or pellets can also have an influence on the quality of sperm cells following the freezing and thawing process. The advantage of using semen straws for storing bovine semen compared to the ampoule is that more units can be stored in bulk at AI centres. The straw also allows a more complete deposition of the semen during insemination, compared to an ampoule. The straw also permits a more uniform control of the freezing and thawing procedures, which

ultimately leads to improved recovery of viable sperm cells (O'Conner, 1999). Bull sperm stored in straws has been reported to exhibit higher percentages of viable cells following thawing (47%), compared to those frozen in the form of a pellet (31%) (Award & Graham, 2004). This implies that bull semen generally survives cryopreservation more effectively in straws than in pellets. Semen stored in straws recorded a higher percentage of motile sperm, compared to those stored in pellets (Award & Graham, 2004).

2.2.1 Conventional slow freezing of semen

The conventional slow freezing method involves the gradual cooling of the semen over a period of about 2 to 4h in two or three steps, either manually (Thachil and Jewett, 1981), or by using a programmable freezer (Serafini and Marrs, 1986). This technique involves the use of a cryoprotectant to protect the sperm against the lethal effects of cooling. The initial cooling rates of a semen sample from room temperature to 5°C, has been shown to be optimal at approximately 0.5 to 1°C per min (Mahadevan and Trounson, 1984). The sample is then frozen at a rate of 1 to 10°C per min from 5°C to –80°C, after which the semen straw is plunged into liquid nitrogen (Thachil and Jewett, 1981; Mahadevan and Trounson, 1984; Serafini and Marrs, 1986).

2.2.2 Vitrification of semen

This method also involves the direct plunging of the semen straws into liquid nitrogen. The post-thaw sperm motility however is still low after vitrification. It has been reported that 11.6% sperm are motile after vitrifying using the swim-up technique, prepared bull sperm (Nawroth et al., 2002; Isachenko et al., 2003).

Ultra-rapid freezing and the vitrification methods do not generally involve the use of the classic permeable membrane mechanism, and therefore by-pass

the lethal effects of osmotic shock on the sperm (Isachenko et al., 2003). Although some research has shown the conventional slow freezing method to be superior (Mahadevan and Trounson, 1984), others have published data favouring more rapid cooling rates for semen freezing (Sherman, 1963). Despite these findings, the more rapid cooling method of semen cryopreservation has not been universally accepted. This may be due to the unavailability of suitable ultra-rapid cryopreservation vials.

The conventional slow freezing method of bovine semen cryopreservation is currently the most commonly used technique, as it involves the use of a cryoprotectant, which affords protection to the sperm cells. In this method, the sperm cells are cooled gradually to minimize the effects of the sudden cold shock. Semen straws are commonly used for the freezing of bull semen as higher percentages of viable and motile sperm are obtained with the use of straws.

2.3 Factors affecting quality of frozen-thawed semen

In the process of cryopreservation of bovine semen, the viability and the fertilizing capacity of the sperm cells must be maintained during storage. Although the cryopreservation of sperm cells is feasible, the survival rate (as measured by motile sperm) after thawing can vary widely. Factors influencing the survival rate of the cells include the rate of freezing and thawing, as well as choice and concentration of the cryodiluents used (Royere et al., 1996)**.**

2.3.1 Effect of extenders/diluents

The use of a suitable semen extender also plays a vital role in the successful preservation of bull semen. An extender is generally a dilution medium which is added to the semen preservation medium. Several semen diluents are currently being used for both short-term and long-term storage of bovine semen. However, the extenders used for bull semen preservation must have

the optimum pH and buffering capacity, a suitable osmolality, an antibiotic (e.g. penicillin, gentamycin, etc.) to inhibit microbial contamination, and also a cryoprotectant to afford protection to the sperm cells against cryogenic injury (Salamon & Maxwell, 2000). The main purpose for diluting semen is generally to increase the number of females inseminated by one ejaculation. However, a good extender does not only increase the volume of the ejaculate, but also provides a favorable environment with the necessary nutrients for maintaining sperm survival (Webb, 1992). The survival rate of ejaculated sperm in seminal plasma alone is only limited to a few hours. Thus in order to maintain the live of the sperm for longer periods and to cryopreserve semen, the addition of diluents is essential (Hafez, 1987). Sperm metabolism can be sustained more effectively in diluents containing degradable sugars e.g. glucose or fructose, which provide a source of energy to the sperm cells (Mann & Lutwak-Mann, 1981; Amirat et al., 2005).

The principal ingredients frequently used in bovine semen extenders contain egg yolk, skimmed milk or a combination of the two (Amann, 1989). There are also certain commercial extenders in which egg yolk is replaced by soybean. Extenders of different chemical compositions protect the different cellular structures to a varying degree during the cryopreservation process (Celeghini et al., 2008). Various substances have been used as extenders for bull semen, most of which are variations of a few basic formulae. The most commonly used bovine semen extenders include egg yolk Tris, egg yolk citrate, and commercial extenders, such as low density lipoprotein (LDL), Optidyl, Tryladyl, Biociphos Plus and Bioxcell, to mention but a few (Hafez, 1987).

Egg yolk is beneficial for the cryopreservation of sperm, as a result its use for this purpose is widespread and it is routinely included in cryopreservation protocols of semen in domestic and exotic mammalian species (Holt, 2000). Egg yolk is considered to protect sperm function by preventing the binding of the major proteins of bull seminal plasma (BSP) to the sperm (Drobnis et al., 1993; Holt, 2000). Despite its protective effect on the sperm cell, egg yolk still has some short-comings. Its preparation is time-consuming and can also be a

source of virus infection or allergic reactions (Hafez, 1987; Thun et al., 2002). As a consequence, post-thaw sperm motility has been reported to be significantly reduced when semen is extended in an egg yolk based diluent, compared to fresh semen (Aires et al., 2003). It has been reported that cryopreserved bull semen extended with a Tris egg yolk extender, containing glycerol, exhibited lower progressive sperm motility, compared to fresh semen (43.3% vs. 76.6%). The percentage of live sperm and sperm with intact acrosomes were also reduced in cryopreserved bovine semen, compared to fresh semen samples (54.0% & 64.6% vs. 79.3% & 85.3% respectively). Morphological sperm abnormalities were also higher in cryopreserved semen samples, than in fresh semen samples (15.46 vs. 3.85%) (Dhali et al., 2008).

When comparing two egg yolk-based extenders, it was found that bovine sperm cryopreserved in Tris egg yolk extender containing glycerol, had only 15% of their plasma membranes intact after thawing (Arruda et al., 2005). However, the use of an egg yolk citrate extender for bull semen resulted in higher percentage of progressively motile sperm as determined microscopically following thawing, compared to the use of an egg yolk Tris extender. It has also been found that the semen extended in egg yolk citrate had 18% lower activity in bound amidase, than that extended in an egg yolk Tris extender (Schenk et al., 1987).

So for example when comparing Botu-Bov with Bioxcell commercial extenders, which are generally used to extend bovine semen, both were found to induce reduced sperm motility by damaging the plasma and acrosomal membranes as well as decreasing the mitochondrial function. However, the former extender was found to be more effective in maintaining higher sperm motility and membrane integrity, than the latter (Celeghini et al., 2008). In the same study, losses of total and progressive sperm motility following cryopreservation was 55.8% and 49.6% for Bioxcell, compared to the 39.8% and 39.5% respectively for the Botu-Bov extender (Celeghini et al., 2008). Thun et al. (2002) observed that the utilisation of an extender at different temperatures also affects the sperm motility rates. Sperm motility rates of 25% and 32% were recorded when the semen sample was diluted with egg yolk

Tris extender at 4^0C and room temperature (18-22^0C), respectively. In another trial it was observed that the use of the egg yolk Tris extender resulted in low sperm motility compared to fresh semen (43.3% vs 76.6%). In the same study, the use of the egg yolk Tris extender resulted in low percentage of live sperm, compared to the fresh semen (54% vs 79.3%) (Dhali et al., 2008).

Nevertheless, the freezing and thawing of bull semen leads to damage or death of about 30% of the sperm, thus reducing the percentage of motile sperm to approximately 50-60% (Woelders et al., 1997). Since the use of fresh semen is not practical in a number of situations, the improvement of cryopreservation methods is necessary in an attempt to increase the percentage of motile bull sperm and also reduce mortality rate of the sperm cells.

2.3.2 Role of cryoprotectants

A cryoprotectant is defined as an agent added to protect living biological material (e.g. oocyte, embryo, sperm) to be cryopreserved in a viable state. The cryoprotectant protects the cells or tissues from the lethal effects of freezing, mainly by preventing large ice crystals from forming (Watson, 1995). The use of cryoprotectants is beneficial for the viability of sperm after thawing as these cryoprotective agents thus minimize intracellular ice formation and restrict the solution effect of dehydrating the sperm cell during the freeze and thaw process (Medeiros et al., 2002). The ability of a compound to become an effective cryoprotectant is based on its ability to protect cells against cryopreservation damage and to be non-toxic to the cells (Squires et al., 2004).

There are generally two groups of cryoprotectants generally used for cryopreservation of sperm. These are classified as permeating and non-permeating cryoprotectants. Examples of permeating cryoprotectants include; glycerol, ethylene glycol, propylene glycol, dimethyl sulfoxide. The non-permeating cryoprotectants on the other hand include sucrose, glucose,

fructose and raffinose, to mention but a few. Both groups of cryoprotectants operate by causing a shift in the isotonic state between the intracellular and extracellular spaces of the cell, thus causing water to flow out of the cell (Mazur & Schneider, 1986). The most commonly used cryoprotective agent to freeze and preserve bovine semen is glycerol (McGonagle et al., 2002; Senger, 2003), but ethylene glycol has started to replace glycerol in many species. Some research has recorded better post-thaw results with ethylene glycol e.g. in the human (Gilmore et al., 2000) and bovine semen (Guthrie et al., 2002). These better post-thawing results may be attributed to the low molecular weight of ethylene glycol, which enables it to penetrate the cell more easier and rapidly, compared to glycerol (Guthrie et al., 2002).

So for example, the molecular weight of glycerol, dimethyl sulfoxide and ethylene glycol are 92.10, 78.13 and 62.07 respectively. Dimethyl sulfoxide provides some protection during freezing and thawing of bovine semen, although less than the protection afforded by glycerol (Snedeker & Gaunya, 1970). These authors observed low sperm motility rate and sperm survival rate with the use of dimethyl sulfoxide, compared to the high sperm motility rate and sperm survival rate observed with the use of glycerol. In the same study it was also observed that combination of 1% dimethyl sulfoxide with 6% glycerol in an egg yolk Tris extender provided above 40% sperm motility, which quite higher than the use of each cryoprotectant alone. The penetration of dimethyl sulfoxide into the cells thus being more rapid because it has a lower molecular weight, compared to glycerol, thus resulting in a poor reproductive performance (Lovelock & Bishop, 1959). Other cryoprotectants with low molecular weights are formamide with 45 and dimethyl formamide with 73. These cryoprotectants are able to penetrate the sperm plasma membrane more readily, thus decreasing the osmotic toxicity, compared to glycerol (Squires et al., 2004).

In a study done elsewhere in cattle, it was observed that the motility of bovine sperm following a one-step addition and removal of 1M glycerol, dimethyl sulfoxide or ethylene glycol was reduced by 31%, 90% and 6% respectively (Guthrie et al., 2002). In another study bull sperm where semen had been

frozen utilizing several amides as cryoprotectants, lactamide provided a greater post-thaw sperm motility than glycerol, acetamide and formamide (Nagase et al., 1972). The cryopreservation of bovine semen with 6% glycerol has been reported to result in higher sperm motility than when using 3% glycerol (Razul et al., 2007). In another trial again conducted on equine, it was observed that 3% glycerol resulted in higher percentage of progressively motile sperm than 3% ethylene glycol (36.2% vs. 30%) (Mantovani et al., 2002).

Glycerol is used worldwide as a cryoprotective agent for the freezing of bull semen and protecting the sperm against the lethal effects of freezing (Sherman, 1964). The cryoprotection of glycerol is mainly due to its ability to buffer the salt at low temperatures, bind with metallic ions, dehydrate the cell and reduce the ice expansion during water solidification (Gao et al., 1995). Glycerol as a cryoprotectant is able to penetrate the sperm cell and dehydrate the cell thus reducing the risk of water crystallization (Bearden et al., 2004). Although glycerol is the most commonly used cryoprotectant for sperm cryopreservation on different animal species, including cattle, it is reported to have some toxic effects on the sperm cells (Fahy, 1986; Sherman, 1987; Hammerstedt & Graham, 1992). Glycerol as such becomes toxic to bull sperm in the egg yolk citrate diluents when added at room temperature, but is not toxic to bull sperm if added to a warm Tris based egg yolk extender (McGonagle et al., 2002). The detrimental effects of glycerol are lessened as the temperature and the concentration used are decreased (Sherman, 1987). This thus implies that the percentage of glycerol added to the diluents is very important to the survival of the sperm and its motility following freezing and thawing processes.

Nevertheless, addition of cryoprotectant before freezing and removal after thawing result in osmotic volume changes. While glycerol offers cryoprotection to spermatozoa, it may also cause structural damage during the pre- freezing process. Consequently, it was suggested that glycerol should be added not earlier than 20 to 30 minutes before the freezing of the semen. Effective cryoprotection after short (5 to 10 seconds) periods of contact with glycerol

has been demonstrated for bull and boar semen, and also for ram semen (0 to 5 min) – which supports the earlier view that the penetration of glycerol into the cell is not essential for cryoprotection (Hammerstedt & Graham, 1992).

2.3.3 Effect of the thawing technique on sperm survival

The thawing technique is equally dangerous to the plasma membrane of the sperm cell, following freezing. There are different methods used for thawing frozen semen. Some of these methods may be harmful to the plasma membrane and it is necessary to ensure that an optimal thawing temperature and time is implemented, in order to minimize the damage to the plasma membranes of the sperm (Borg et al., 1997). However, the resistance of sperm cells to the thawing process depends on the semen extender used and concentration of the cryoprotectant, as these interact during the freezing and thawing processes (Curry, 2000).

The rate of thawing frozen semen depends on a number of factors. These include the size, shape and composition of the semen straw, ampoule or pellet, the thawing medium and the temperatures used. Methods used for the thawing of frozen bull semen for AI include, ice water thawing for semen packaged in ampoules, warm-water thawing for semen packaged in straws or pellets, (Salisbury et al., 1978; Kaproth et al., 2005), pocket thawing (Kaproth et al., 2005) and air-thawing methods (DeJarnette & Marshall, 2005).

For warm-water thawing, the straw taken from the liquid nitrogen tank, is immediately dipped in a water bath at 33-37^{0}C, for a minimum period of 40 to 45 seconds (Pace & Edwards, 1981; Herman et al., 1994; Kaproth et al., 2005). The success of warm-water thawing is based on the fact that sperm cells are exposed to high temperatures rapidly in order to minimize sperm damage. However, the disadvantage of this method is the danger of cold shock introduced by the incorrect handling of the straw following thawing (O'Conner, 1999). On the other hand, for the pocket-thawing method, the semen straw is removed from the liquid nitrogen tank and immediately placed

in a folded paper towel for protection and then placed into a thermally regulated pocket for 2 to 3 minutes to thaw - before preparing the inseminating gun. This method has the advantage in that it minimizes the thermal stress risk under routine field conditions. It also helps to avoid the risk that water quality or inaccurate temperatures in the thawing vessel could impair fertility (Kaproth et al., 2005). In the air-thawing method, a straw is removed from the liquid nitrogen tank, wrapped in a paper towel and then placed directly into the inseminating gun (DeJarnette & Marshall, 2005).

Cryopreserved bovine semen extended in egg yolk citrate has been recommended to be thawed in a 33-37^{0}C water bath for 45 seconds, as it results in the higher survival of sperm, in terms of motility and acrosome integrity, than thawing at 5^{0}C for 1 to 4 minutes (Pace & Edwards, 1981; Herman et al., 1994; Nur et al., 2005). In a study, the conception rate in cows (60%) inseminated with frozen semen after thawing at 37^{0}C for 45 seconds, was higher than that of cows inseminated with fresh semen incubated in ice water for 30 to 60 minutes (47.7%) (Kaproth et al., 2005; Anwar-Mohamed et al., 2008).

In another study conducted with cattle, warm-water thawing resulted in a higher percentage of motile sperm (75%), than the air-thawed method (71%) at 0 hour. Also 3 hours post thawing, a similar trend was still observed, whereby warm-water thawing still exhibited a higher percentage motile sperm (29%), compared to the air-thawing method (16%). In this study semen thawed by warm-water thawing exhibited a higher conception rate than that for semen thawed in air (35% vs. 27%) (DeJarnette & Marshall, 2005). In another trial it was observed that thawing of bull semen at 70^{0}C for 5 seconds resulted in higher sperm motility, compared to thawing at 50^{0}C for 15 seconds and 37^{0}C for 30 seconds (60% vs 56.7% and 56.6%) (Nur et al., 2003). It has been shown that thawing temperatures above 35^{0}C result in higher sperm motility, but it must be noted that the duration of the thaw must be shortened and carefully timed (Senger, 1980). This author further stated that if the semen samples are exposed to very high temperatures, protein denaturation occurs, which in turn results in the death of the spermatozoa.

The thawing method most commonly used in practice for bull semen is at 37^0C for 1 minute, as it is easier to work with than higher temperatures. The higher temperatures also require special equipment to heat the water and need careful timing to avoid damage to the sperm cell (Borg et al., 1997).

2.3.4 Effect of storage time and temperature pre and post thawing on sperm survival

The time interval during which semen is incubated, either prior to or post freezing and thawing has an effect on the quality of the sperm. As semen is stored for longer periods of time, the sperm quality is reduced. It is thus recommended that fresh bovine semen be stored at 5^0C to maintain better sperm motility and survival rates. Storage of semen at 5^0C extends the lifespan of the sperm and also decreases the risk of growth of contaminants. Several suppliers of bovine semen recommend keeping sperm no longer than 15 minutes post-thawing, in order to avoid a reduction in fertility and sperm motility rate (Yang & Chou, 2000).

Changes in sperm motility over time have been reported to differ largely for individual bulls, within the same breed (Holstein). In this trial it was observed that semen from one bull recorded 68% sperm motility immediately post-thawing and 71% after being stored for 15h in a water bath at 34.4^0C. In contrary, another bull exhibited 68% immediately post-thawing and 17% after 15 hours of incubation in the same water bath as the first bull (Miller & Edwards, 1999). In this study the fertilization of oocytes with sperm stored for 14h at 34.4^0C resulted in a reduced proportion of presumptive zygotes that cleaved, but this did not alter the ability of embryos to develop to the 8-16 cell stage or blastocyst stage.

2.4 In vitro embryo production (IVEP)

IVEP refers to artificial production of embryos in the laboratory. Given that favourable conditions are provided, the embryo will develop up to the blastocyst stage. The IVEP procedures are used worldwide with different goals for a variety of livestock species (exotic, wild and endangered animals). Over the last decade IVEP in cattle has improved remarkably. The IVEP process involves four steps; namely, oocyte harvesting, in vitro maturation (IVM), in vitro fertilization (IVF) and in vitro culture (IVC) of the presumptive zygotes. These processes are then followed by either embryo transfer (ET) or cryopreservation of embryos for later use when suitable recipients are available. A broader knowledge has been developed in the fields of IVM, IVF and IVC of cattle embryos over a number of past years. These techniques have thus been applied in IVEP of cattle (Earl & Kotaras, 1997). The advantages of IVEP include amongst others, obtaining the embryos, even after the death of a donor cow or bull. This makes it relatively less expensive since an embryo is obtained without having to raise and maintain the cow or the bull. It is also important in the case where a cow has an infection that renders her infertile, but her ovaries are still functional (Gordon, 1994).

IVEP was first performed in order to produce relatively cheap embryos on a large scale for various experimental procedures. Besides for experimental procedures, IVEP can be seen as a possible method to produce embryos in abundance in order to improve the reproductive efficiency of livestock (Rust & Visser, 2001). The major advantage of IVEP is that the donor female is no longer involved in early embryo development. This thus implies that the donor female is only required to donate oocytes in the same manner as the male donates sperm cells (Gordon, 1994). Despite the progress achieved in IVEP over the past couple of years, the production of high quality transferable cattle embryos is still very low (Pivato, 2001). These poor results may be attributed to the in vitro maturation and fertilization conditions or maybe the developmental competence of the oocytes obtained from small follicles (2-6mm diameter) (Blondin & Sirard, 1995). The collection of good quality oocytes is the first step in the process of IVEP. The importance of oocyte

quality is a factor in the developmental competence of the embryo, which is more apparent and is determined by the oocyte's nuclear and cytoplasmic maturation which are attained during its growth in the follicle (Sirard, 2001). A competent oocyte is generally described as the one which is able to sustain embryonic development to term (Brevini-Gandolfi & Gandolfi, 2001). Some important factors to consider in IVEP include sources of oocytes, age of the donor, IVM, IVF as well as IVC of the presumptive zygotes.

2.4.1 Source of bovine oocytes

Bovine oocytes used in IVEP programmes can be obtained from various sources. The most common and cheapest source is from ovaries collected from animals, following slaughter at the abattoir. Another source may be from live animals, where the follicles are aspirated using the ultrasound-guided system through laparoscopy (Earl and Kotaras, 1997; Tibary et al., 2005) The most commonly used source of oocytes for IVEP purposes in the bovine is abattoir material, because as already said it is relatively cheap and the ovaries are readily available. However, the limitation of this source is that it is not easy to select superior genetic material and the genetic make-up and age of the donors are unknown (Rust & Visser, 2001).

Available evidence clearly states that an unfertilized cow oocyte has a significantly short viable life span of about 6 to 12h after being released from the ruptured follicle. This condition is brought about by the fact that the microtubules of the meiotic spindle of the ovulated cow oocyte become disorganised within a few hours, with pairs of microtubules escaping from the spindle and the subsequent loss of chromosomes from the metaphase plate. This thus implies that the age of the oocyte is inversely proportional to the estimated fertilization rate (Hunter & Greve, 1997). Some of the factors that influence the oocyte collection rate include experience of the operator, type of needle used, as well as suction pressure employed (Lansbergen et al., 1995).

2.4.1.1 Abattoir material as a source of oocytes

Bovine oocytes can be collected from ovaries obtained from slaughtered animals (abattoir), using either the slicing or aspiration techniques (Tibary et al., 2005). Early work on IVEP has involved the aspiration of oocytes from ovaries obtained from the abattoir, thus abattoir oocytes play a major role in the development of IVEP technology (Gordon, 1994). However, this source of oocytes has some limitations in that some inherent disease risks have been reported elsewhere and also each ovary is harvested only once (Ball & Peters, 2004). These authors also reported that in cattle, using abattoir material, it has been reported to lead to the recovery of a significantly higher number of oocytes per ovary when using aspiration method (15.1), than that recovered when using the slicing method (6.7).

Using an endoscopic method of oocyte retrieval, a blunt trocar is positioned dorsolateral to the fornix of the vagina, thus opening the abdominal cavity using a sharp-edged trocar. A forward viewing 0^0 angle endoscope attached to a xenon light source is inserted through the trocar. The puncturing process is controlled via microvideo television camera on a TV-monitor and the follicular fluid is then collected through a Teflon tube into plastic tubes. The benefit of this method is that it allows the exact positioning of the aspiration needle, thus becoming more effective when working with small follicles (2-3mm diameter). The disadvantages of this method are that random insertion of the trocar through vaginal fornix, especially if it is performed repeatedly, can lead to injuries to the abdominal organs and there is also a high risk of peritoneal infections in this method, compared to ultrasonography (Becker et al., 1994).

It was observed that the cleavage rates and the development to the blastocyst stage of oocytes collected by aspiration were higher. However, the number of transferable embryos obtained following the slicing of the ovaries was found to be higher than those obtained following aspiration (Korean Medical Database, 2001). Aspiration is the commonly used method for collecting oocytes in bovine. The reason for popularity of this method is that it is inexpensive, time

saving and convenient for collecting a large number of oocytes, resulting in a moderate number of oocytes per ovary (Hafez and Hafez, 2000). The yield of oocytes can be improved by slicing the tissue to reach deep cortical follicles. However, slicing is time-consuming and results in a lot of debris that may interfere with the recovery of oocytes (Arav, 2001).

2.4.1.2 Live animal oocyte retrieval

The use of live animals as a source of oocytes is another alternative for IVEP. In live animals the oocytes can be retrieved by transvaginal ovum pick-up (OPU), ultrasonography and endoscopy. OPU involves the insertion of a needle into the ovary of a live cow, and the aspiration of the oocyte containing fluid (follicular fluid). It can be done at least twice a week as this technique is considered to be the least traumatic method for the repeated collection of oocytes. OPU is generally used in large animals such as cattle or horses, even when follicles are aspirated from juveniles or pregnant animals (Ball & Peters, 2004; Tibary et al., 2005). The main advantage of OPU is that oocytes can be collected repeatedly within a short period of time. However, the major problem with this technique is the increased loss of cellular layers during aspiration, and the passage through the tubing (Kuhholzer et al., 1997). OPU thus appears to be the only means available for obtaining cattle oocytes from live animals on a large scale. However, the oocyte yield in OPU depends upon the number of follicles available for puncture, which is in turn influenced by breed, nutritional status and the climatic conditions to which the animal is exposed (Boland et al., 2001).

Ultrasonography is also an OPU method, which involves utilization of ultrasound to visualize the ovary. This method makes use of an ultrasound probe inserted in the vagina of the cow to guide the aspiration needle to the follicles in the ovary. The more recent development in the field of oocyte aspiration and IVEP has been the recovery of oocytes from pregnant cows. This observation has made the utilization of ultrasonography more popular. It has further been indicated that it is possible to recover the same number of

oocytes from a pregnant cow twice a week, as when using a non-pregnant cow. The only limitation to this technique is the fetus becoming too big after about 4 months of pregnancy, which makes it very difficult to manipulate the ovary for ultrasound scanning. It is even possible to hormonally stimulate a cow during pregnancy to produce even more follicles for aspiration (Rust and Visser, 2001). It has been further indicated that ultrasound guided OPU can be performed on live donors at weekly intervals from adult and prepubertal cattle (Rodrigues & Rodrigues, 2006). The mean total number of oocytes collected in adult cows using ultrasonography had been reported to be 7.4 (Lansbergen et al., 1995).

Heifers subjected to two aspirations per week were reported to yield more follicles (17.2) per session, than those subjected to a single OPU (12.4). A similar trend was also observed with the development of the cumulus oocyte complexes where heifers subjected to two aspirations exhibited more cumulus oocyte complexes (7.7) per session, compared to those subjected to a single OPU (5.4). OPU appears to induce and synchronize the follicular waves and when done twice a week, it is associated with a higher number of harvestable follicles and more oocytes being recovered than when performed once a week (Garcia & Salaheddine, 1998). For heifers studied in another trial, it was reported that a higher denudation rate of cumulus oocyte complexes occurred when using the endoscopy aspiration, than when using ultrasonography (62% vs. 6.6%). However, the number of aspirated oocytes was similar between these two techniques. However, the ultrasonographic method is less traumatic to the vagina and the abdominal organs, while endoscopy is less traumatic to the ovary (Becker et al., 1994).

2.4.2 Age of the donor

The quality of cattle oocytes is influenced by the age and the pubertal status of the donor (Silva et al., 2002). A common practice in IVEP is to work with younger animals with the aim of decreasing the generation gap and with the added bonus of even faster genetic improvement in herds. This has led to the

collection of oocytes and embryos cultured from heifers as young as 6 months of age. The quality of the embryos cultured from the oocytes of young heifers was however low (Rust and Visser, 2001).

The potential to produce viable embryos from fetal and pre-pubertal calf oocytes has received much attention in cattle breeding programmes, as it reduces the generation interval of genetically superior cows, thus accelerating genetic gain (Betteridge et al., 1989). The establishment of pregnancy and the birth of live calves following the IVF of oocytes from pre-pubertal and pubertal calves have been reported, but their developmental ability for in vitro cleavage and blastocyst formation is still very low compared to that of adult cow oocytes (Revel et al., 1995). IVM of oocytes from the ovaries of cow fetuses has been reported to be 80.1%, while that of adult cows has been reported to be 92%. In this study the cow fetuses recorded a lower fertilization and cleavage rate (69.3% and 36.7%, respectively), than observed in adult cows (79.9% and 49.9%, respectively). It was further indicated that poor IVM, IVF and embryonic development of the fetal oocytes may be attributed to a higher incidence of blockage of the germinal vesicle (GV). Although IVF results with fetal oocytes were lower than with adult cow oocytes, they were still high enough to be considered for use in research, particularly in the case of premature or sudden death of the dam or fetus (Chohan & Hunter, 2004).

Although the establishment of pregnancies and birth of live calves have been reported after IVF following the utilization of oocytes from pre-pubertal and pubertal calves, their developmental ability for in vitro cleavage to blastocyst formation is still lower than in the adult cow oocyte (Chohan and Hunter, 2004). The reasons for the lower embryonic development of pre-pubertal calf oocytes have been attributed to insufficient, delayed or abnormal nuclear and cytoplasmic maturation (Khatir et al., 1996). In another trial the oocytes obtained from 4 to 7 months old heifers were found to be less likely to develop to blastocysts after IVF, than those collected from adult cows (44% vs. 54%, respectively) (Camargo et al., 2005).

Adult cows are commonly used for the retrieval of oocytes either alive or after slaughter, and their oocytes are more competent and viable than those from the fetuses or pre-pubertal calves. Oocytes from pre-pubertal calves thus have less developmental competence because of impaired cytoplasmic maturation, which further impairs the embryo development. Nevertheless the use of oocytes from pre-pubertal calves in IVEP is an option mainly useful for genetic improvement in cattle breeds, which attain puberty later such as the Bos Indicus (Salamone et al., 2001).

2.5 In vitro maturation (IVM)

In vitro maturation is a process whereby oocytes are further matured outside the body of an animal, before they can be fertilised. Maturation of mammalian oocytes is defined as a sequence of events occurring from the germinal vesicle stage to completion of the second meiotic division with formation of the second polar body (McGaughey, 1983). At birth, the oocytes formed in the ovaries are not fully functional and therefore must undergo further maturation processes before they can take part in the fertilization events. Following collection, cattle oocytes with an evenly granulated cytoplasm and 2 to 4 layers of cumulus oocyte complexes are selected and matured in vitro, by incubating at 39°C in 5% CO_2, and 95% air with a high humidity for 24h (Hafez and Hafez, 2000). Oocyte maturation either in vivo or in vitro is an important stage of oocyte development and is monitored by the appearance of the first polar body and several layers of cumulus oocyte complexes (Hafez & Hafez 2000). Cattle oocytes are generally matured in vitro in tissue culture media (TCM) 199, supplemented with pyruvate, heat-treated serum and hormones (FSH, LH, estradiol) (Birler et al., 2002). The use of this maturation media in cattle has been reported to result in a high maturation rate reaching the metaphase II stage (Samake et al., 2000; Bornmann et al., 2003).Maturation media are generally supplemented with a protein source such as bovine serum albumin (BSA) and fetal bovine serum (FBS) to enhance the maturation process (Hafez & Hafez, 2000).

Oocyte in vitro maturation is a reproductive technology which enables mature oocytes to be generated in vivo, without the use of ovarian gonadotrophin treatment (Gilchrist & Thompson, 2007). Unlike sperm, the oocyte requires no exposure to the reproductive tract after release from the gonad (ovulation) in order to be fertile (Hafez and Hafez, 2000). For successful IVM, oocytes must undergo nuclear and cytoplasmic maturation in vitro (Tibary et al., 2005). However, IVM differs from in vivo oocyte maturation with the cumulus oocyte complexes being harvested from mid-sized antral follicles which have not completed oocyte capacitation. Thus the cumulus oocyte complexes do not have the full molecular and cellular machinery needed to support early embryogenesis (Gilchrist & Thompson, 2007).

2.6 In vitro fertilization (IVF)

IVF superficially involves the fusion of the male gamete (sperm) and the female gamete (oocyte) to produce an embryo outside the female reproductive tract. This process follows successful IVM of oocytes and it can be performed using either fresh or frozen-thawed semen. The medium used in the IVF process must be able to provide secondary oocytes and capacitated sperm, with a favourable environment which will allow the fertilization process to occur (Gordon, 1994). IVF is actually a signal of the beginning of transition from the oocyte to the embryo. This is thus regarded as a dual process during which the oocyte is activated and acquires the hereditary material from the sperm introduced to it. In that case, activation can be regarded as an important event for the start of the oocyte's developmental process, after fertilization (Gordon, 2003).

Nonetheless, successful IVF requires appropriate preparation of both gametes (oocyte and spermatozoa) as well as favourable culture conditions. In setting up an appropriate system for cattle IVF, it is important to ensure that the medium utilized is capable of providing the mature oocyte and the capacitated sperm with the favourable environment, in which sperm penetration into the oocyte can readily occur (Hafez & Hafez, 2000).

2.6.1 Semen used for in vitro fertilization (IVF)

The quality of spermatozoa is probably an important factor in stimulating fertilization. Most IVF studies use freshly ejaculated sperm (Izquierdo et al., 1998; Birler et al., 2002). Semen used for IVF, whether fresh or frozen-thawed should be prepared appropriately before it can be used. Preparation of semen includes washing and separation of the highly motile and normal sperm from the rest of the population. However, the sperm separation technique is necessary when semen quality and sperm motility are poor (Gordon, 2003). Washing of the bull sperm involves centrifugal sedimentation to remove seminal plasma proteins rapidly and effectively prior to the use in IVF (Gordon, 2003). A few IVF trials have however been carried out using frozen–thawed sperm (Bornmann et al., 2003; Berlinguer et al., 2004). Frozen bull semen can be used immediately after sexing or re-frozen for later use, with a high rate of correct predetermined sex (86.7%). However fertilization rate when using sorted frozen–thawed sperm is low, but the developmental capacity of the fertilized oocytes was similar to when using fresh sorted sperm (Hollinshead et al., 2004). This implies that more thawed sperm is needed to obtain the same fertilization rate following AI than when using fresh semen (Thundathil et al., 1999).

However, the use of fresh semen is limited in cattle. Frozen-thawed bull semen is widely used in commercial cattle farming and also in research (Gordon, 2003).

It is much easier for an IVF laboratory to obtain a supply of frozen semen, rather than having to keep a bull for the production of fresh semen (Gordon, 2001). However, prolonged storage of bovine sperm at ambient temperatures resulted in the reduced integrity of the sperm cell membrane, motility and fertilizing ability (Gordon, 2001). Lonergan et al. (2000) reported no significant difference in the cleavage rate when in vivo matured bovine oocytes were fertilized either in vivo (92.8%) or in vitro (87.3%). However, these authors further reported that in vitro fertilized oocytes generally resulted in a higher

blastocyst yield (73.9%), compared to in vivo fertilized oocytes (52.8%) when using frozen-thawed bull semen.

2.7 In vitro culture (IVC)

In vitro culture is a stage that refers to the process of growing the in vitro produced embryos in specific salt solutions. Pre-implantation embryos produced in vitro are generally very sensitive to their environment, and the conditions of culture can affect the embryonic developmental potential. Following IVF, the presumptive zygotes must be cultured in vitro for further development before being transferred into the uterus or cryopreserved (Hafez & Hafez, 2000). There are several methods of culturing the in vitro produced embryos. These include the use of a ligated oviduct of a temporary recipient such as a sheep or a rabbit (in vivo culture) or in vitro culture. The improvement of in vitro culture systems is essential for the production of embryos with high developmental competence that can be used in agricultural and biomedical research, and accelerated animal biotechnology techniques (Hansen & Block, 2004). Many regulatory molecules, cytokines, growth factors, enzymes and inhibitors which influence cell growth and differentiation have been identified in the oviduct. Such agents may act either in an autocrine or a paracrine way to regulate processes in the oviduct, including progress of early cleavage-stage embryonic development (Gordon, 2003). This therefore implies that the embryo culture media should mimic the environmental conditions in the oviduct.

The embryos are incubated in the IVC medium for a longer period of time than the oocytes in the IVM or IVF media. This therefore implies that IVC medium is more likely to have a greater effect on embryo development following fertilization, on the timing of development, blastocyst quality, hatchability sex ratio and total blastomere numbers. These factors may then contribute to the low pregnancy rates or a greater sensitivity to cryopreservation (Lonergan et al., 1999). The IVC of mammalian embryos needs a favourable environment

in order to stimulate cleavage, which will ultimately result in the formation of blastocysts. The essential environmental factors for embryo survival rate include temperature, pH, and osmolarity of the medium, energy sources, serum components, gas and water (Petter, 1992). The use of essential and non-essential amino acids is also important in order to support the function of the feeder cells, and result in increased blastocyst development. However, degradation of the amino acids can result in ammonia toxicity and therefore the embryos should be transferred to fresh media every 48h (Hafez and Hafez, 2000). Major important events occur during the development of embryos from post fertilization to the blastocyst stage. These include zygote formation, first cleavage division, embryonic genome activation (EGA), compaction of the morulae and blastocyst formation (Lonergan et al., 2003; Rizos et al., 2003).

Preimplantation bovine embryos can develop in different media whose compositions range from simple balanced salt solutions and carbohydrates such as Charles Rosenkrans 1 (CR 1), synthetic oviductal fluid (SOF) and potassium simplex optimizing media (KSOM) - to very complex constituents such as TCM 199 with further supplementation of serum or a feeder layer of somatic cells (Niemann & Wrenzycki, 2000; Summers & Biggers, 2003). Medium used for IVC does not only influence the development of the embryo, but also assists in the embryo survival rate following the process of cryopreservation (Nedambale et al., 2004).

In a study that compared tyrode's medium supplemented with albumin, sodium lactate and sodium pyruvate (TALP) medium with SOF, it has been observed that the quality of the embryos developed on day 7 was significantly higher when IVF was done with SOF medium supplemented with essential amino acids (EAA), non essential amino acids (NEAA), glutamine and glycine (Lazzari et al., 1999). These authors further concluded that SOF devoid of glucose proved to be a suitable medium for cattle IVF, and also that supplementation of the medium improved the quality of the developing embryos.

Cattle embryos cultured in SOF, supplemented with FBS have been reported to result in lower embryo survival rates following freezing, but higher numbers of hatched blastocysts, than those cultured in the same media supplemented with BSA (Yoshioka et al., 1997, Enright et al., 2000).The effect of the serum in the culture media has been associated with early blastocyst formation (Carolan et al., 1995, Holm et al., 1999). Thus the serum in the culture media has been reported to stimulate the hatching process, by providing a pool of plasminogen, which is converted into plasmin by the bovine embryo. Plasmin then proteolytically degrades the zona pellucida and facilitates hatching of the embryo. The SOF medium contains factors such as citrate, pyruvate and lactate, which help to protect the embryo against free radical injury (Holm et al., 1999). To the contrary, embryos cultured in SOF, without non-essential amino acids, have been reported to have more lipids in their cytoplasm than those of in vivo derived embryos. The lipids in the cytoplasm are considered to be detrimental to embryo survival, following cryopreservation (Tominaga et al., 2000).

Sodium pyruvate, sodium lactate and glucose are common sources of energy in the culture media (Earl & Kotaras, 1997). However, bovine embryos do not use significant amounts, until the 16-cell stage (Gardener & Batt, 1991; Rieger, 1992). Sodium pyruvate and sodium lactate are beneficial in embryonic development to the morula stage, but the addition of high concentrations of glucose have resulted in detrimental effects during the first 4 to 5 cleavage stages (Thompson et al., 1992).

In vitro mammalian embryonic development is negatively affected by the increased oxidative stress occurring under culture conditions. Culture conditions are known to stimulate the production of reactive oxygen species, which may alter certain functions of the embryonic cell (Guerin et al., 2001). In vitro embryo culture conditions have been shown to generally influence metabolic, endocrine and hematological parameters in calves (Rerat et al., 2005).The oxidative damage to the cell components passing through the reactive oxygen species interferes with the normal sperm cell function. Most

mammalian cells possess efficient antioxidant systems, such as catalase or superoxide dismutase, and thiol compounds that act as metabolic buffers which scavenge the reactive oxygen species (Del Corso et al., 1994).Cattle embryos cultured in SOF supplemented with fetal bovine serum (FBS) have resulted in higher cell numbers than those cultured in TCM 199 with BSA and SOF-BSA (92.1 vs. 74.8 and 71.6, respectively) (Gandhi et al., 2000). Sequential KSOM-SOF culture system had been reported to have improved Day-7 blastocysts (36%), cell numbers and total hatching rate (79%), compared to KSOM alone in bovine. On the other hand the embryos cultured in KSOM + BSA had been found to develop slower and most hatched late on Day 9 (Nedambale et al., 2004). Other researchers recorded blastocyst formation rates (Day 7) to be 15.8% (KSOM) and 15.9% (SOF), which were similar (Lima et al., 2004). The culture of embryos in isolated mouse oviducts (IMO) in KSOM, resulted in an increased number of the inner cell mass (ICM) nuclei (Rizos et al., 2007).

When SOF is used to culture cattle zygotes, the cleavage rate of in vitro cultured zygotes was 82.5%, while no cleavage for in vivo cultured zygotes was obtained, due to the degeneration of non-developing embryos in the oviduct. However, there was no difference in proportion of oocytes developing to the blastocyst stage between the two culture systems (34.1 vs. 34.5%) respectively (Lonergan et al., 2001). In another study in cattle, the culturing of the presumptive zygotes in SOF-BSA, resulted in 63.1% day 2 cleavage, 20.4% day 7 blastocysts and 10.6% day 10 hatched blastocysts (Samardzija et al., 2006). It was observed that the culturing of bovine embryos in SOF and in SOF + BSA, using 5% oxygen compared to 20% oxygen increased the day 8 blastocyst yield (Lonergan et al., 1999).

In another trial, bovine embryos cultured in KSOM resulted in heavier calves than their counterparts cultured in SOF. In this study, a higher percentage of embryonic disc formation in heifers was observed in embryos cultured in KSOM than those cultured in SOF (72% vs. 46%) (Fischer-Brown et al., 2000). It was also observed that culturing bovine embryos in KSOM medium

until day 3 after IVF with addition of BSA followed by SOF with BSA increased the blastocyst formation rate and cell numbers. The use of the SOF culture media supplemented with BSA resulted in 78% cleavage rate and 28% day 8 expanded blasocyst yield (Boni et al., 1999). In vitro produced embryos are held in culture medium for a longer period than in either maturation, or fertilization media. As a result, in vitro culture (IVC) medium is more likely to have a greater effect on the quality of cultured embryos, especially on the timing of development, blastocyst quality, hatchability, and total cell numbers (Lonergan et al., 1999).

In another trial conducted elsewhere, it was observed that the use of KSOM culture media resulted in a higher percentage of embryos at the 8-cell stage compared to the use of SOF media (70% vs 63%). However, the percentage of day 7 blastocysts was found to be low with the use of KSOM, compared to the use of SOF media (34% vs 41%) (Gardener et al., 1999). The culturing of bovine zygotes in the presence of EDTA for the first 72 h of development stimulates cleavage, but its presence at later stages of development inhibits blastocyst formation and thus reduces blastocyst quality and cell numbers. This explains the discrepancy observed in KSOM media (Gardener et al., 1999). These authors assumed that the presence of sodium citrate and myo-inositol (in SOF) may be time-dependent in promoting competent blastocyst development, or those factors may be effective for post 8-celled embryonic development. This implies that early addition of these embryotrophic factors may not be functional, at least not sufficient for competent development. In another trial it was observed that the use of SOF media resulted in a higher proportion of day 7 blasocysts, compared to the use of KSOM media (31% vs 28%). To the contrary, the percentage ob blastocysts obtained with the use of KSOM media at days 8-9 were higher than those obtained from the use of SOF media (43% vs 38%) (Nedambale et al., 2004). The use of SOF media and KSOM media in bovine resulted in similar cleavage rates, but SOF media resulted in higher day 7 blasocyst rate than KSOM media (Sagirkaya et al., 2006). This effect was associated with the fact that KSOM media contains

glutamine, while SOF media does not contain glutamine, which may affect the development to the blastocyst stage in KSOM media.

The optimization of embryonic development in vitro is not only dependent on the composition of the culture medium or media used, but is also affected by physical parameters, such as the incubation environment and gas phase. The embryo culture environment plays an important role in the degree of embryonic development. The developing embryo interacts actively with its environment and responds to culture conditions by altering its metabolism as well as the expression of relevant genes (Rizos et al., 2002). The culture environment includes culture medium, oxygen tension, as well as the culture method employed (Lonergan et al., 2006; Lane et al., 2008). The gaseous environment is therefore an important component of a culture system, and generally 5% CO_2, with 95% RH is used (Wright & Bondioli, 1981; Yang et al., 1994). The oxygen concentration of the oviduct is about one-third that of the atmosphere. The bovine embryos cultured in high oxygen tension may produce more free radicals which are detrimental to the embryonic development (Fischer & Bavister, 1993).

All the culture media mentioned above can be used successfully for culturing bovine embryos due to the fact that they produce more or less the same cleavage and blastocyst formation rates. In addition even the media that results in less cell or blastomere numbers, cleavage and blastocyst rates is still acceptable for use in IVEP (Nedambale et al., 2006).

2.8 Summary

Cryopreservation of semen has become an important tool in ART's. Cryopreserved bull semen can be used for a prolonged period of time following storage, while also facilitating the shipping of cryopreserved semen throughout the world. The dilution of semen with an extender prolongs the life span of the sperm, while also increasing the number of females that can be

inseminated from one ejaculation. Extenders containing egg yolk, such as egg yolk Tris or egg yolk citrate are generally used in the freezing of bull semen - as the egg yolk protects the sperm cell against cold shock and provides nutrients for the survival of the sperm. Other commercial extenders that can be used to dilute semen include low density lipoprotein, biociphos plus, bioxcell, tryladyl, as well as optidyl. Different cryoprotectants such as glycerol, ethylene glycol, dimethyl sulfoxide, formamide and dimethyl formamide can be used in cryopreservation of bovine sperm. The major role of a cryoprotectant being to dehydrate semen, in order to prevent formation of ice crystals and cryoinjury to the sperm cell during the cryopreservation process. This helps to maintain the viability and motility of the sperm following the freezing and thawing process.

The high motile sperm, exhibiting greater than 60% motility following thawing can be used either for AI or IVEP programmes. The method used to thaw semen following cryopreservation also plays a major role in maintaining viability of the sperm. If the semen is thawed at very high temperatures for a prolonged period of time sperm motility and survival rates can be impaired thus reducing the fertilizing ability of such sperm. Nonetheless, the time period and the temperature at which semen is stored either before, or after freezing also contribute towards the quality of semen. This implies that if semen whether fresh or frozen-thawed can be stored for a longer period of time before being used, the sperm quality can be greatly impaired or reduced. Also if semen can be stored at temperatures below 5^0C, sperm motility and survival rates can be affected negatively.

The major aim of IVEP in cattle is to improve the reproductive efficiency and productivity of cattle. IVEP includes several stages namely, IVM, IVF and IVC. The oocytes are harvested from the ovaries of either live or slaughtered animals. Transvaginal OPU, ultrasonography and endoscopy are the techniques that are commonly used to collect oocytes from live and even pregnant donors. Methods used to harvest the oocytes from the ovaries of slaughtered animals include aspiration and slicing. There are however certain

factors that can affect the yield and quality of oocytes harvested. These include the source of oocytes, pubertal status of the animal, age of the donor, as well as method of oocyte recovery. Good quality oocytes are generally those that are surrounded by several layers of cumulus cells and having an evenly granulated cytoplasm.

Following recovery, then the oocytes can be matured in vitro using TCM 199 medium. After about 24 h of incubation, the oocytes are evaluated under a phase contrast microscope to determine whether they have matured or not. The appearance of the first polar body, the expansion of the cumulus oocyte complexes or metaphase II of the cumulus oocyte complexes are used as an indication of maturation. Following maturation, then the oocytes can be inseminated in vitro using either fresh, sex-sorted or frozen-thawed semen, in a favourable IVF media. After successful IVF, the presumptive zygotes are cultured in vitro under a suitable culture media and an environment created to stimulate cleavage and finally development to the blastocyst stage. Various IVC media can be used for further successful development of the embryos. These include CR 1, TCM 199, TALP, SOF and KSOM to mention but a few. However, in some cases the embryos can be cultured in vivo in the uterus of a well prepared recipient. Following IVC the embryo is then transferred to a previously synchronized recipient where it can develop to term or the in vitro produced embryo can be cryopreserved for later use.

However, IVEP is not quite efficient, as there are several limiting factors affecting the end product at the end of each stage involved in the process. These include the recovery of poor quality oocytes, the failure of the oocyte to mature, transmission of diseases from the donor to the offspring, failure to develop to morula stage after fertilization. Also the death of the embryo during the cryopreservation process, or the failure of the embryo to implant in a suitable recipient can also impair the process of IVEP.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study area

All experiments were conducted on the Research Institute and laboratory of the Agricultural Research Council (ARC), Irene, ($25^{0}55´$ South, $28^{0}12´$ East), South Africa. The ARC is situated close to Pretoria, in the Gauteng Province at an altitude of 1525m above sea level. The average rainfall in this area is 640mm per annum, predominantly precipitating in the summer (October to January). The dry season generally lasts from March to August, with winter from June to August (Vilakazi & Webb, 2004). These trials were carried out from April to October, 2008 (autumn to spring).

3.2 Experimental animals and design

3.2.1 Experimental animals

Three Nguni bulls (average age of 5 years) were used to evaluate the effect of different cryodiluents on semen cryopreservation, sperm viability and in vitro fertilizing capacity. The bulls were maintained on natural pastures with strategic lick supplementation.

3.2.2 Experimental outline

This study consisted of two trials. In the first experiment, semen from three Nguni bulls was collected, evaluated and cryopreserved using different cryodiluents. In addition, the quality of semen was again evaluated post-thawing. The semen quality parameters evaluated included sperm motility and survival rates, semen pH and sperm concentration. In the second experiment only frozen-thawed semen with sperm motility and survival rates of more than 60% was further processed by in vitro fertilization In this experiment two different in vitro culture media were utilised.

3.3 Experiment 1: Semen cryopreservation

3.3.1 Semen collection method

Semen was collected from each bull (Plate 3.1) using an electro-ejaculator (CGS electrojector; model Pty Ltd, Australia). The collection of semen was performed twice every week throughout a 28 week evaluation period. The semen collection location was close to the laboratory, in order to facilitate immediate evaluation of the semen samples following collection. This method of semen collection (electro-ejaculation) involves the stimulation of the spinal cord between the 4th lumbar and the first sacral vertebrae, by inserting the electrode in the rectum. During each collection session the contents of the rectum i.e. the dung, were removed before insertion of the probe in order to ensure a good contact with the rectal mucosae. This thus enables easy stimulation of the sympathetic and the parasympathetic nerves of the pelvic plexus. These nerves are then responsible for erection of the penis, and ultimately ejaculation of the animal (Hafez & Hafez, 2000).

For semen collection the bulls were kept in a standing position, in a standard cattle crush pen, fitted with a neck clamp to restrain the animals with minimal stress. Prior to use the probe of the electro-ejaculator was cleaned using a dry towel with a disinfectant. A small amount of paraffin (lubricant) was applied to the probe before insertion into the rectum for ease of insertion, to minimize trauma to the rectum of the animal and also for maximum effectiveness (conductivity). The genital area was also cleaned and disinfected to reduce the risk of contamination during semen collection. For inducing ejaculation a few rhythmic electric stimuli were passed through the electrodes and semen was collected in an insulated sterile glass test-tube (to prevent cold shock). A bipolar rectal electrode in contact with the rectal floor is commonly used, although it has been observed that the use of the electro-ejaculator usually produces ejaculates with larger volumes but a lower sperm concentration, compared to the use of the artificial vagina (Matthews et al., 2003).

Nevertheless the main advantage of using an electro-ejaculation method is that no training is needed for the bull used for semen collection.

Plate 3.1 Nguni bulls used for semen collection

3.3.2 Semen evaluation techniques

After collection, the semen samples were maintained at 37^0C in an insulated flask and immediately transported to the laboratory for evaluation. Samples were evaluated for ejaculate volume, semen pH, sperm motility and the percentage live sperm. The volume (ml) of the ejaculate was recorded by reading the value directly from the calibrated collection tube used for

collecting the semen samples. The semen pH was measured with the aid of a pH meter (Plate 3.2 - Right), and the value recorded.

The sperm motility and percentage live sperm were assessed microscopically (X 40 magnification) and recorded immediately after collection. The values for motility and percentage live sperm were allocated a score on a subjective scale of 0 to 5 (Elmore, 1985).

The scale for sperm motility and percentage live sperm used was as follows:

5 — Very strong progressive dark semen waves (90% plus live cells).
4 — Strong progressive semen undulations (70 to 85% live cells).
3 — Weak semen undulations (50 to 65% live cells).
2 — Very few, weak, non-progressive semen undulations (30 to 45% live cells).
1 — No semen undulations (5 to 25% live cells).
0 — No movement (all cells dead).

Sperm concentration (X 10^6 sperm/ml) determinations were performed by using a spermacue photometer (Minitube) (Plate 3.2 - Left). The slides for determining the percentage live and motile sperm were prepared and screened (X 40 magnification) under a phase contrast microscope system, equipped with a warm stage (37^0C) (Plate 3.3). The thin semen smears were made on the pre-warmed slides by placing a small drop of semen on a slide using a pasteur pipette and then covering the semen drop with a cover slide. All these semen quality parameters were evaluated and recorded at 3 h intervals from 0 to 9 h. Only semen samples with acceptable sperm motility (>60%) and percentage live sperm (>60%) were used for further processing and freezing (Lu & Seidel Jr., 2004).

Plate 3.2 Left) Spermacue Photometer for determining sperm concentration in bulls
Right) pH meter for measuring pH of the bovine semen samples

Plate 3.3 Microscopic evaluation of Nguni bull semen

3.3.3 Semen dilution and equilibration

Two different semen extenders were prepared, namely; an egg yolk citrate and an egg yolk Tris solution. The extenders were prepared at the onset of

the trial and were both prepared using the ingredients as set out in Table 3.1 and Table 3.2.

Table 3.1 Composition of egg yolk citrate extender solution

Component	Quantity / 100ml
Sodium citrate (Buffer)	1.856g
Glucose (Source of energy)	1g
Sabax water (Solvent)	80ml
Gentamycin sulphate (Antibiotic)	0.1g
Egg yolk (reduces cold shock)	20ml

All these ingredients, except egg yolk were obtained from Sigma St. Louis, Mo, USA.

Table 3.2 Composition of egg yolk Tris extender solution

Component	Amount/ 100ml
Tris	2.422g
Citric acid	1.36g
Glucose	1g
Sabax water	80ml
Gentamycin sulphate	0.1g
Egg yolk	20ml

All these ingredients, except egg yolk were obtained from Sigma St. Louis, Mo, USA.

Briefly, for both extenders this mixture was prepared by weighing and adding the first three ingredients (Table 3.1 and Table 3.2) separately to Sabax water, followed by the addition of egg yolk and the antibiotic gentamycin sulphate. The semen extender was maintained at 37^0C on a warm stage, prior to use.

Semen with acceptable sperm motility and percentage live sperm (3 or more out of score of 5) as indicated by Elmore (1985), was diluted with the two different extenders in a ratio of 1:1, at 37^0C. At each semen collection session the semen sample was divided into 3 equal parts in a 15 ml plastic screw top tube, to apply different treatments. One portion or part was diluted with egg yolk citrate, while the other one was diluted with egg yolk Tris, and the last portion was left undiluted and used as a control. Each semen extender was then slowly added to the semen sample in small aliquots, while the sample was gently swirled. All samples were then gently agitated, tightly closed and placed in a cold room (5^0C). This procedure then slows down the rate of cooling and thus protects the sperm cells from cold shock. After that, the extended semen samples as well as the undiluted semen sample were then evaluated for sperm concentration, sperm motility rate and the percentage of live sperm in 3h intervals for a period of 9h, so as to determine the effect of the extender. Furthermore, the semen samples extended with the egg yolk citrate diluents were incubated at different temperature regimes (5^0C and 25^0C) for a period of 12h. These semen samples were also evaluated on the sperm quality parameters mentioned above at 3h intervals in order to determine the effect of temperature.

3.3.4 Semen freezing procedure

The semen samples extended with egg yolk citrate recorded a better sperm survival in terms of motility rate and percentage live sperm, compared to the semen samples extended with the egg yolk Tris extender. Therefore, the semen samples extended with the egg yolk citrate, were further divided into three portions in 15 ml plastic screw top tubes, for further evaluation of the effect of the three different cryoprotectants (glycerol, ethylene glycol and dimethyl sulfoxide) following cryopresevation. Three 100ml solutions of egg yolk citrate were prepared as indicated above, and in each solution 5 % glycerol, ethylene glycol or dimethyl sulfoxide were added.

Each semen sample was further diluted with a solution containing the different cryoprotectants, at the rate of 1:1 of the original semen volume,and equilibrated again for 2h in a cold room (at 5^0C). Thereafter microscopic sperm evaluation was performed to determine the effect of the cryoprotectants on the quality of semen, prior to freezing following 2 h of incubation. The semen samples exhibiting >60% sperm motility and >60% live after equilibration with cryoprotectants were then loaded into 0.5ml plastic straws and maintained at 5^0C. The straws were then sealed with polyvinyl alcohol, placed on the straw freezing racks, and transferred to a programmable freezer for freezing in step wise manner (Plate 3.4). Semen was initially cooled gradually at a rate of -0.08^0C/min from 5^0C to 4^0C; -6.20^0C/min from 4^0C to -130^0C. This cooling process took 35 minutes. There was an initial holding temperature of 5 minutes in the programmable freezer, which made the processing period 40 minutes.

After completion of the cryopreservation process, the semen straws were removed from the programmable freezer and placed above the liquid nitrogen vapour, in a styro-foam box for 5 minutes. The straws were then plunged directly into the liquid nitrogen (-196^0C) to cool the semen straws from -130^0C to -196^0C. Two straws from each treatment were thawed in order to evaluate sperm motility and survival rates before storage. Only semen samples exhibiting >60 % sperm motility and survival rates were stored in liquid nitrogen tanks (Plate 3.5) for further evaluation. A total of 228 semen straws (0.5ml) were stored in the liquid nitrogen tanks (-196^0C) during this experiment.

Plate 3.4 Programmable freezer for the cryopreservation of bull semen

Plate 3.5 Liquid nitrogen tanks for the storage of cryopreserved bovine semen

3.3.5 Semen thawing procedure

Two weeks after freezing, one straw from each treatment, was thawed by holding the straw in air (10 seconds) and then in water (38^0C) for 1 minute. A thin smear was then prepared by placing a small drop of semen on the pre-warmed slide, covered with a cover slip and evaluated under a phase contrast microscope (X 40 magnification) to determine the sperm motility and survival rate (percentage live sperm). All sperm quality parameters were evaluated

and recorded at 30 minutes intervals from 0 to 90 minutes post-thawing and semen was stored at 5^0C.

3.4 Experiment 2: Bovine in vitro embryo production

3.4.1 Ovary collection

Ovaries were collected at a local abattoir (Strydfontein) in Pretoria from cows immediately after slaughter and transported to the laboratory in sterile saline solution at 35°C, in a thermos flask within 4 to 6h post-mortem. Upon arrival, the ovaries were rinsed in 70% alcohol, washed three times in a saline solution and transferred to a fresh saline solution, maintained at 35°C in a water bath. Each ovary was dissected and freed of the surrounding tissue and the overlying bursa. This was performed using a pair of scissors to remove the tissue surrounding the ovaries, before processing.

3.4.2 Oocyte harvesting

Visible ovarian follicles were aspirated using a 18G x 1.5” hypodermic needle, attached to a 5ml sterile disposable syringe. The collected oocytes, together with the follicular fluid, were then emptied into a 50ml falcon tube, containing warm modified phosphate buffered saline (mPBS) in a water bath at 38^0C. The solution of mPBS consisted of 500ml dulbecco’s phosphate buffered saline (DPBS), 10ml antibiotics (Gibco, Grand Island, New York), 0.5g polyvinyl alcohol (PVA), and 200 µl phenol red (Sigma, St. Louis, Mo, USA).

After aspiration the mixture of mPBS and the follicular fluid were left for about 10 minutes to allow the oocytes to settle at the bottom, as a supernatant. The supernatant was then carefully drained twice, with a Pasteur pipette. The pellet of the supernatant was then transferred to a 100mm Petri dish and the oocytes surrounded by a compact cumulus were recovered, removed with a micro-pipette under a stereo microscope, and placed into a Petri dish containing 3ml of mPBS. Then the oocytes were finally washed three times in

TCM 199 (Gibco, Grand Island, New York), supplemented with 10% FBS (Sigma, St. Louis, Mo, USA), before in vitro maturation.

3.4.3 In vitro maturation (IVM)

For IVM, the bovine oocytes were placed into a pre-warmed four-well dish containing oocyte maturation media, (TCM199), follicle stimulating hormone (FSH), luteinizing hormone (LH) and estradiol (Sigma, St. Louis, Mo, USA). A 500 µl drop of maturation media was placed in each well of the dish and covered with 250 µl of mineral oil. Fifty oocytes were placed in each well, and left for 24h in a thermo water-jacketed incubator at 39^0C, 5% CO_2, and 90% relative humidity (RH) (Plate 3.6). After 24 h all mature oocytes were selected under a stereo microscope, as indicated by the compact cumulus oocyte complexes (Plate 3.7).

Plate 3.6 Thermo incubator used for in vitro maturation (IVM) and in vitro culture (IVC) of bovine oocytes and embryos respectively

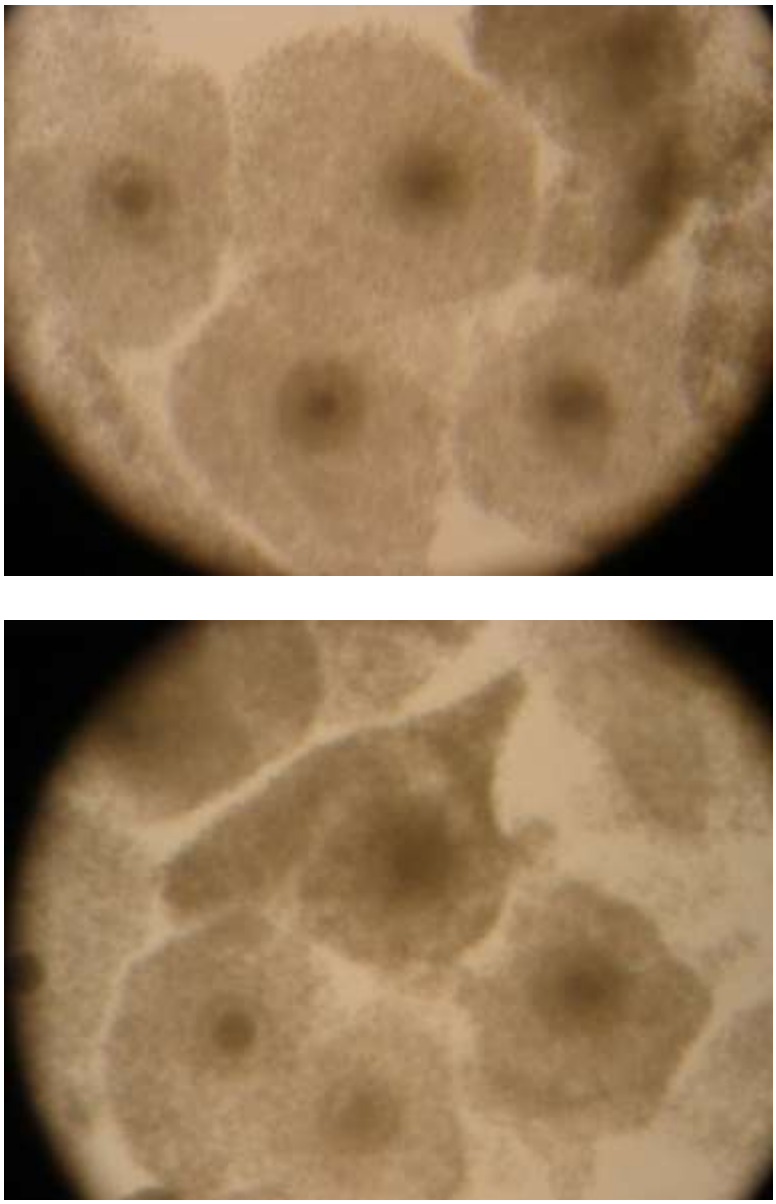

Plate 3.7 Examples of matured bovine oocytes with compact cumulus

3.4.4 In vitro fertilization (IVF)

Following IVM, a total of 1283 matured oocytes were fertilized in vitro, using the frozen-thawed semen from Experiment 1, while 1280 oocytes were fertilized with fresh semen (control). Briefly, the oocytes were washed three times in fertilization medium. The fertilizing medium consisted of Brackett and Oliphant (BO) fertilization medium supplemented with 6 mg/ml essential fatty

acid-free (FAF)-BSA and 10 µg/ml of heparin, as originally described by Brackett and Oliphant (1975). For fertilization, about 15 to 20 cumulus oocyte complexes were placed in pre-warmed 50µl droplets of fertilizing medium, covered with mineral oil.

Frozen semen was thawed by holding the straw in air (10 seconds) and then in water (38^0C), for 1 minute and thoroughly washed by centrifuging two times for 8 minutes at 1500rpm. The fresh semen, which was used as the control was also washed in the same way, as the frozen-thawed semen before insemination. For fertilization, 50 µl of diluted bull semen solution, with a sperm concentration of approximately 2×10^6 / ml was used to inseminate each 50µl droplet containing the oocytes (Wani et al., 2000). After this the oocytes and sperm were co-incubated for a period of 18h.

3.4.5 In vitro culture (IVC)

Following IVF, presumptive zygotes were washed by vortexing for 90 seconds, in order to remove the cumulus oocyte complexes. Following vortexing, the presumptive zygotes were washed three times in TCM199 + 10%FBS and transferred to two different pre-warmed IVC media droplets. The presumptive zygotes were allowed to develop (incubated) for 7 days, until reaching the blastocyst stage. The embryo culturing media used were SOF and KSOM.

The SOF culturing media was prepared by using the components as indicated in the following tables:

Table 3.3 SOF stock solution A (10X Sodium solution)

Component	Quantity/50ml
NaCl	3.145g
KCl	0.267g
K_2PO_4	0.081g
$MgSO_4.7H_2O$	0.091g
Ultrapure water	49.2ml
$C_3H_5O_3$ Lactic acid (60%)	0.3ml
Antibiotic-Antimyotic	0.5ml

Table 3.4 SOF stock solution B (Bicarbonate solution)

Component	Quantity/50ml
$NaHCO_3$	1.050g
0.5% Phenol red	50µl
Antibiotic-Antimyotic	0.5ml
Ultrapure water	50ml

Table 3.5 SOF stock solution C (Pyruvic acid)

Component	Quantity/5ml
Sodium pyruvate	0.04g
Ultrapure water	5ml

Table 3.6 SOF stock solution D (Calcium chloride solution)

Component	Quantity/10ml
$CaCl_2.2H_2O$	0.262g
Ultrapure water	10ml

Table 3.7 SOF medium solution

Component	Quantity/20ml
Citric acid	0.002g
Myo-inositol	0.01g
Ultrapure water	15.6ml
SOF stock A	2ml
SOF stock B	2ml
SOF stock C	0.2ml
SOF stock D	0.2ml
BME (50X)	0.6ml
MEM (100X)	0.2ml
L-Glutamine	0.003g
Antibiotic-Antimyotic	0.1ml

For step 1 of culture, SOF-BSA was prepared with 10ml SOF medium solution (Table 3.7) supplemented with 1mg BSA. For step 2 of culture, 9.5ml SOF medium solution (Table 3.7) was supplemented with 10mg BSA.

Table 3.8 Composition of KSOM culture media

Component	Quantity/100ml
NaCl	95.00mM
KCl	2.50mM
K_2PO_4	0.35mM
Glucose	0.35mM
L-Glutamine	1.00mM
EDTA	0.01mM
Hepes	2.50mM
$CaCl_2.2H_2O$	1.71mM
$MgSO_4.7H_2O$	0.20mM
$C_3H_5O_3$ Lactic acid (60%)	25.00mM
Sodium pyruvate	0.40mM
$NaHCO_3$	10 µl/ml
Taurine	10 µl/ml
Antibiotic-Antimyotic (100X)	10 µl/ml
EAA (50X)	10 µl/ml
NEAA (100X)	10 µl/ml
Phenol red	10 µl/ml

After adding all the components, all were dissolved in Sabax water, which was added to make up 100ml.

For step 1 of culture, KSOM media (Table 3.8) was supplemented with BSA at the rate of 1mg/ml. For step 2 of culture, KSOM media (Table 3.8) was supplemented with BSA at the rate of 10mg/ml.

The presumptive zygotes fertilized with fresh semen (n=481) were randomly allocated to the two culture media; KSOM (n=242) and SOF (n=239), while the presumptive zygotes fertilized with frozen-thawed semen (n=559) were also randomly allocated to the two culture media; KSOM (n=280) and SOF (n=279). Approximately 15 to 20 presumptive zygotes were placed in each of the IVC droplets, and incubated in 5% oxygen, 5% carbon dioxide, 90% nitrogen, and 90% relative humidity, at 38^0C for 7 days. Thereafter, on days 2 and 5 of culture the culture media were changed. KSOM-step 1 was changed to KSOM-step 2, while SOF-step 1 was changed SOF-step2.

Following 48h of IVC, the cleavage rate, proportion of the presumptive zygotes with 2 to 4 cells and 8 cells were evaluated, using a contrast microscope and development recorded. The stained day 7 blastocysts (Plate 3.8) were also evaluated and recorded (day 7 of culture).

3.5 Data collection

Data was collected during each semen collection by recording the sperm quality parameters, namely; sperm motility, percentage live sperm, semen pH, semen volume and sperm concentration before freezing semen and the same parameters were evaluated again after thawing to evaluate cryotolerance of the semen samples. Following IVF, parameters measured were cleavage rate, presumptive embryos with 2-4 cells and 8 cells and the number of blastocysts formed. Cleavage rate was evaluated 48 hours following IVC and the number of embryos reaching the expanded day 7 blastocyst stage was recorded at 7-8 days (onset of IVC = day 0).

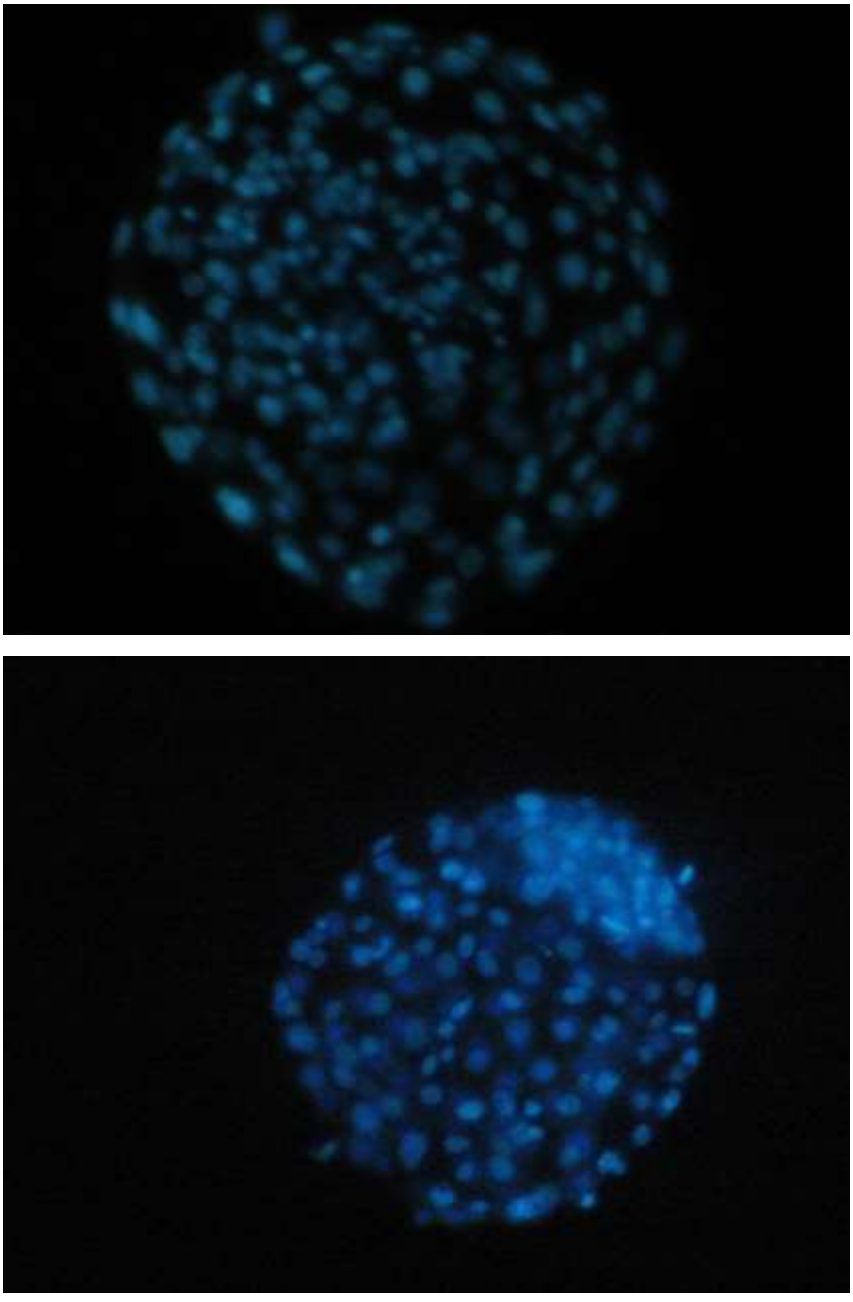

Plate 3.8 Examples of day 7 stained expanded bovine blastocysts

3.6 Data analysis

The one-way analysis of variance (ANOVA) (SAS, 2004) procedure was used to test the effect of extender and cryoprotectant on the sperm motility rate and

survival rate of the frozen-thawed semen. The same procedure was used again to test the in vitro fertilizing capacity of the frozen-thawed semen and the embryos were cultured in different culture media and significance was declared at 5% probability level.

CHAPTER 4

RESULTS

4.1 Effect of extender and incubation time on the quality of Nguni bull semen

The results on the effect of egg yolk citrate and egg yolk Tris extenders on the quality of Nguni bull semen at 3h intervals (for a period of up to 9 h) are illustrated in Table 4.1. The mean concentration of undiluted semen (control group) (302±30 x 10^6sperm/ml) was significantly ($P<0.05$) higher than that of semen extended with egg yolk citrate, or egg yolk Tris extender (186±38 x 10^6sperm/ml and 161±17 x 10^6sperm/ml, respectively). At 0 h the percentage live sperm was similar in all the different treatment groups. After 3h of incubation the semen extended with egg yolk citrate or egg yolk Tris extender (77±4.9% and 79±5.1 respectively) exhibited a significantly ($P<0.05$) higher percentage live sperm, compared to the undiluted semen (53±24%). After 6 h of incubation, the semen sample extended with egg yolk citrate or egg yolk Tris (74±6.7% and 62±9.4% respectively), exhibited a significantly ($P<0.05$) higher percentage of live sperm, compared to the undiluted semen (38±23%). At 9h of incubation, the semen extended with egg yolk citrate (69±10%) recorded a significantly ($P<0.05$) higher percentage live sperm, compared to that of the semen extended with egg yolk Tris (54±15%). These values were also much higher ($P<0.05$) than for the undiluted semen or the control group (29±18%).

Regarding the sperm motility, no significant differences were recorded between the different treatment groups at 0 h (onset). Following 3h of incubation, no significant difference between the semen sample extended with egg yolk citrate (73±11%) and that extended with egg yolk Tris extender (59±15%) were recorded. Similarly, no significant difference between the semen sample extended with egg yolk Tris (59±15%) and the undiluted semen sample (51±24%) were recorded. However, the egg yolk citrate diluent resulted in a significantly ($P<0.05$) higher sperm motility rate, when compared to the undiluted semen (control). After a period of 6 h of incubation, the semen

extended with egg yolk citrate (70±11%), recorded a significantly (P<0.05) higher sperm motility rate, than that of semen extended with egg yolk Tris (52±12%), or the undiluted semen (control group) (39±23%). At 9h of incubation, all the treatments exhibited significantly (P<0.05) different sperm motility rates, with the egg yolk citrate diluents exhibiting the highest sperm motility rate, followed by the egg yolk Tris diluents and lastly the undiluted semen (63±13%, 45±15% and 19±16% respectively).

Table 4.1 Mean (±SE) of semen parameters following dilution with egg yolk citrate or egg yolk Tris and incubated for a period of 9 h

Time of incubation (h)	Treatments			
	Egg yolk citrate	Egg yolk Tris	Undiluted semen (Control)	Overall mean
	Concentration(10^6/ml)			
0 h	186±38^{b}	161±17^{b}	302±30^{a}	217±68
	Percentage live (%)			
0 h	79±2.9^{a}	76±5.1^{a}	78±4.5^{a}	78±4.4
3 h	77±4.9^{a}	76±5.1^{a}	53±24^{b}	65±18
6 h	74±6.7^{a}	62±9.4^{a}	38±23^{b}	58±21
9 h	69±10^{a}	45±15^{b}	29±18^{c}	51±22
	Motility rate (%)			
0 h	77±9.8^{a}	70±11^{a}	74±14^{a}	74±12
3 h	73±11^{a}	59±15^{ab}	51±24^{b}	61±19
6 h	70±11^{a}	52±12^{b}	39±23^{b}	53±21
9 h	63±13^{a}	45±15^{b}	19±16^{c}	42±23
	pH			
0 h	6.7±0.3^{a}	6.4±0.3^{b}	6.9±0.6^{a}	6.7±0.4
3 h	6.8±0.3^{b}	6.5±0.2^{c}	7.1±0.6^{a}	6.8±0.5
6 h	6.9±0.3^{a}	6.5±0.3^{a}	5.9±2.8^{a}	6.5±1.7
9 h	7.0±0.2^{a}	6.5±0.3^{a}	6.0±2.9^{a}	6.5±1.7

^{abc} Values in the same row with different superscripts differ significantly (P < 0.05)

At 0h (immediately following dilution), the semen sample extended with the egg yolk citrate diluent and the undiluted semen (6.7±0.3 and 6.9±0.6) recorded a significantly ($P<0.05$) higher mean pH, compared to the semen sample extended with egg yolk Tris diluents (6.4±0.3). Following 3h of incubation, the mean pH of the undiluted semen (7.1±0.6) was significantly ($P<0.05$) higher than that of the semen sample extended with the egg yolk citrate (6.8±0.3), and also significantly ($P<0.05$) higher than that of the semen sample extended with egg yolk Tris (6.5±0.2). Following a period of 6 and 9h there were no significant differences between the different treatment groups with regard to the mean semen pH recorded.

4.2 Effect of the cryoprotectant on the quality of the frozen-thawed Nguni bull semen

The results on the performance of glycerol, dimethyl sulfoxide and ethylene glycol on the quality of Nguni bull semen prior to and post freezing and thawing, are illustrated in Table 4.2. The mean sperm concentration was not affected by the different treatments prior to cryopreservation. There was also no significant difference in sperm motility between the different treatment groups before semen freezing. Howwever, the mean percentage live sperm and mean pH differed significantly ($P<0.05$) between the different treatment groups following the addition of the cryoprotectants. The use of glycerol as a cryoprotectant (75±5.3%) resulted in a significantly ($P<0.05$) higher percentage live sperm, compared to the use of ethylene glycol (55±8.5%). Similarly, the use of glycerol (6.6±0.2) exhibited a significantly ($P<0.05$) higher pH, than that of dimethyl sulfoxide (6.3±0.3).

The sperm survival and motility rates declined drastically following the freezing and thawing processes. The post-thawing results indicated glycerol to demonstrate a significantly ($P<0.05$) higher sperm survival and motility rate, compared to the other cryoprotectants (dimethyl sulfoxide or ethylene glycol) used. The mean sperm survival rate obtained when using glycerol as a

cryoprotectant (58±7.9%) post thawing was significantly (P<0.05) higher, compared to that obtained following the use of dimethyl sulfoxide and ethylene glycol (14±5.2% and 7.5±2.6%, respectively). The mean sperm motility rate did not differ between the different cryoprotectant (treatment) groups prior to freezing. However, following cryopreservation and thawing, sperm motility rate differed significantly (P<0.05) between the different treatment groups, with glycerol (52±6.3%) exhibiting a significantly (P<0.05) higher sperm motility rate, compared to dimethyl sulfoxide or ethylene glycol (12±4.2% and 8.0±2.6%, respectively).

Table 4.2 The mean (±SE) effect of different cryoprotectants on the quality of frozen-thawed Nguni bull semen

Treatments	Semen parameters before cryopreservation				Post-thaw semen parameters	
	Semen concentration (10^6/ml)	Live sperm (%)	Sperm motility (%)	Semen pH	Live sperm (%)	Sperm motility (%)
Glycerol	171±60.1^{a}	75±5.3^{a}	71±7.4^{a}	6.6±0.2^{a}	58±7.9^{a}	52±6.3^{a}
Dimethyl sulfoxide	142±57^{a}	65±15^{ab}	56±22^{a}	6.3±0.3^{b}	14±5.2^{b}	12±4.2^{b}
Ethylene glycol	118±14^{a}	55±8.5^{b}	56±22^{a}	6.4±0.3^{ab}	7.5±2.6^{c}	8.0±2.6^{b}
Overall	143±48.6	65±10.5	61±18.6	6.5±0.3	26.5±5.2	24±4.4

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

4.3 Nguni bull sperm quality of diluted semen following incubation at different temperatures

The results of the semen quality parameters obtained when semen was incubated at 5^0C and 25^0C, are set out in Table 4.3. The mean concentration of semen sample incubated at 5^0C (227±65 x 106 sperm/ml) was significantly (P<0.05) higher than that of the semen sample incubated at 25^0C (206±72 x 106 sperm/ml) at 0h of incubation. Semen incubation resulted in reduced

percentage of the live sperm over time. However, there was no significant ($P<0.05$) difference between the treatment groups up to 9h of incubation. The sperm maintained at 5^0C (46±21%) exhibited a significantly ($P<0.05$) higher percentage live sperm, compared to that maintained at 25^0C (35±31%) after 12h of incubation.

The mean sperm motility rate of the semen sample incubated at different temperatures declined drastically over time, while immediately after incubation the mean sperm motility rate was similar for both treatment groups (5^0C and 25^0C). The mean sperm motility rate of the semen sample maintained at 5^0C (67±10%), was significantly ($P<0.05$) higher, compared to that of the semen sample incubated at 25^0C (55±24%) following 3h of incubation. After a period of 6h of incubation, the semen maintained at 5^0C (60±12%) exhibited a significantly ($P<0.05$) higher sperm motility rate than that of the semen incubated at 25^0C (47±25%). At 9h of incubation, semen sample maintained at 5^0C (47±20%) exhibited a significantly ($P<0.05$) higher sperm motility rate, compared to the semen sample incubated at 25^0C (38±25%). Similarly, following 12h of incubation, the semen sample incubated at 5^0C (40±20%) recorded significantly ($P<0.05$) higher motility rates, compared to that of the semen sample incubated at 25^0C (28±27%).

The mean pH of the semen sample incubated at 25^0C increased over time, and reached a maximum after 3h of incubation and then declined drastically. On the other hand, the semen sample incubated at 5^0C resulted in mean pH values, increasing with a prolonged incubation time, thus becoming less acidic. The mean pH of the semen maintained at 25^0C (7.0±0.5) was significantly ($P<0.05$) higher, compared to that of the semen incubated at 5^0C (6.4±0.1) at 0h. A similar trend was also observed after 3 h of incubation, where the semen sample incubated at 25^0C (7.1±0.5) exhibited a significantly higher pH than the semen sample incubated at 5^0C (6.5±0.1). After 6h of incubation the mean pH of the semen sample incubated at 25^0C (7.3±2.4) was significantly ($P<0.05$) higher, compared to that of the semen sample incubated at 5^0C (6.6±0.1). At 9h of incubation, the semen sample incubated at 5^0C (6.7±0.1) exhibited a significantly (($P<0.05$)) higher pH than that

incubated at 25^0C (6.1±2.3). After 12h of incubation, the mean pH of the semen maintained at 5^0C (6.9±0.1), was significantly (P<0.05) higher than that incubated at 25^0C (4.7±3.5), which tended to be more acidic.

Table 4.3 The mean (±SE) of the semen parameters extended with egg yolk citrate diluent following incubation at different temperature regimes

	Treatments (Mean±SE)		
Time of incubation (h)	25^0C	5^0C	Overall
	Concentration(10^6/ml)		
0 h	206±72[b]	227±65[a]	217±68
	Percentage live (%)		
0 h	78±4.3[a]	77±4.6[a]	78±4.4
3 h	63±24[a]	67±8[a]	65±18
6 h	56±27[a]	60±13[a]	58±21
9 h	47±26[a]	55±17[a]	51±22
12 h	35±31[b]	46±21[a]	40±27
	Motility rate (%)		
0 h	71±16[a]	76±5[a]	74±12
3 h	55±24[b]	67±10[a]	61±19
6 h	47±25[b]	60±12[a]	53±21
9 h	38±25[b]	47±20[a]	42±23
12 h	28±27[b]	40±20[a]	34±25
	pH		
0 h	7.0±0.5[a]	6.4±0.1[b]	6.7±0.4
3 h	7.1±0.5[a]	6.5±0.1[b]	6.8±0.5
6 h	7.3±2.4[a]	6.6±0.1[b]	6.5±1.7
9 h	6.1±2.3[b]	6.7±0.1[a]	6.5±1.7
12 h	4.7±3.5[b]	6.9±0.1[a]	5.8±2.7

[ab] Values in the same row with different superscripts differ significantly at P<0.05

The interaction between the extender and the incubation temperature did not affect the quality parameters of the semen (sperm concentration, percentage of live sperm, sperm motility and semen pH) at the various time intervals, during which the semen samples were incubated.

4.4 The sperm survival (percentage live) and motility rates of frozen-thawed Nguni bull semen stored at 5^0C, at different time intervals

The results of the mean (±SE) sperm survival (percentage live) and motility rates of frozen-thawed Nguni bull semen evaluated following periods of 0, 30, 60 and 90 minutes, post thaw are set out in Table 4.4. The mean sperm survival rate of the frozen-thawed semen stored for 0, 30 and 60 minutes (56±9.7%, 53±4.8% and 51±13.7%, respectively) were significantly (P<0.05) higher, compared to the semen sample stored for a period of 90 minutes (33±10.6%). Similarly, the mean sperm motility rate of the frozen-thawed semen sample incubated for 0, 30 and 60 minutes (51±5.7%, 49±3.2% and 48±7.9%, respectively) were significantly (P<0.05) higher, compared to that of the semen sample incubated for a period of 90 minutes (29±8.8%).

Table 4.4 The mean (±SE) percentage live sperm and motility rate of frozen-thawed Nguni bull semen incubated at 5^0C, over different time intervals

Treatment	Observations (n)	Semen parameters	
		Percentage live (%)	Sperm motility (%)
0	10	56±9.7[a]	51±5.7[a]
30	10	53±4.8[a]	49±3.2[a]
60	10	51±13.7[a]	48±7.9[a]
90	10	33±10.6[b]	29±8.8[b]
Overall	40	48.3±13.4	44.3±11.1

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

4.5 Bovine embryonic development following IVF, using frozen-thawed Nguni bull semen

The results regarding the mean cleavage rates (%) at different stages of embryonic development from cleavage, to the day 7 blastocyst stage following IVF using frozen-thawed semen and fresh semen are set out in Table 4.5. Regarding the cleavage rate no significant differences between the two treatment groups (fresh or frozen-thawed semen) were recorded. A significant ($P<0.05$) difference in the percentage of the presumptive zygotes at the 2-4 cell stage were obtained when using frozen-thawed semen (32.1±13%). This was higher than that recorded when fresh semen (24.3±12.8%) was used following IVF. There was also no significant difference between the two treatment groups with regard to the development of the zygotes to the 8 cell stage and containing dead cells. The use of the fresh semen (23.2±16.5%) resulted in a significantly ($P<0.05$) higher percentage of day 7 blastocysts, compared to the use of the frozen-thawed semen (14.2±11.9%).

Table 4.5 Embryonic development following IVF with frozen-thawed Nguni bull semen

Treatments	**Parameters (Mean±SE)**				
	Cleavage (%)	**2-4 cell stage (%)**	**8 cell stage (%)**	**Dead cells (%)**	**Blastocysts day 7 (%)**
Fresh semen	46.1±22.0[a]	24.3±12.8[b]	22.5±14.6[a]	26.0±14.4[a]	23.2±16.5[a]
Frozen-Thawed semen	49.6±18.0[a]	32.1±13.0[a]	17.4±16.1[a]	20.6±12.0[a]	14.2±11.9[b]
Overall	47.9±19.9	28.2±13.3	19.9±15.4	23.3±13.4	18.7±14.9

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

4.6 Effect of IVC media on bovine embryonic development following IVF with frozen-thawed Nguni bull semen

The results regarding the mean cleavage rates obtained at various stages of embryonic development from cleavage to day 7 blastocyst stage following in vitro culture with KSOM and SOF media are set out in Table 4.6. No significant differences were recorded between treatments (KSOM and SOF), with regard to the cleavage rate, presumptive zygotes at cell stage, as well as those containing dead cells recorded between the different treatments. The use of KSOM culture media (23.2±17.5%) exhibited a significantly ($P<0.05$) higher mean percentage of day 7 blastocysts than the use of SOF culture media (14.2±10.4%).

Table 4.6 Effect of different IVC media on bovine embryonic development following IVF with frozen-thawed Nguni bull semen

Treatments	Embryonic Development (Mean±SE)				
	Cleavage (%)	**2-4 cells (%)**	**8 cells (%)**	**Dead cells (%)**	**Blastocysts day 7 (%)**
KSOM	49.1±21.6[a]	27.8±12.3[a]	22.0±16.5[a]	22.8±13.0[a]	23.2±17.5[a]
SOF	46.6±18.5[a]	28.7±14.6[a]	17.9±14.3[a]	23.8±14.1[a]	14.2±10.4[b]
Overall	47.9±19.9	28.2±13.3	19.9±15.4	23.3±13.4	18.7±14.9

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

CHAPTER 5

DISCUSSION

5.1 Effect of extender on the quality of Nguni bull semen

The semen quality evaluation is an important factor to consider, as it is responsible for determining the fertilization rate. The use of semen extenders, namely egg yolk citrate and egg yolk Tris, greatly reduced the sperm concentration. This reduction in concentration occurred during the dilution, before freezing of the semen. This effect may be attributable to the fact that semen dilution increases the volume of semen, thus reducing concentration (Webb, 1992). Thus semen should be diluted in a suitable extender to increase its volume so as to increase the number of the females that can be inseminated with one ejaculation, and also to improve the viability of the sperm cells by more exposure to the extender.

Higher percentage live sperm and sperm motility rates were recorded when egg yolk citrate and egg yolk Tris extenders were used. This implies that semen dilution increases the number of females that can be serviced from one ejaculation, and also sperm cells in diluted semen can remain viable for a long period of time after ejaculation (Hafez, 1987; Webb, 1992). Both extenders seemed to be non-toxic to the sperm cells during incubation, for a period of up to 6 h. However, after 9 h of incubation egg yolk citrate extender seemed to result in a better percentage live sperm and motility rate than egg yolk Tris extender. This indicates that egg yolk citrate extender affords better protection, in terms of the percentage of live sperm, as well as the sperm motility rate for Nguni bull sperm cells, compared to an egg yolk Tris extender. This therefore suggests that egg yolk citrate is an excellent extender for the incubation of Nguni bull semen and can therefore probably be used for the preservation of fresh semen for short periods of time, before use.

Similar results have been observed where egg yolk citrate was compared with egg yolk Tris extender. It was reported that dilution of bull semen with egg yolk citrate resulted in a higher percentage of progressively motile sperm

cells, compared to dilution with an egg yolk Tris extender (Schenk et al., 1987). This implies that egg yolk citrate is the most acceptable extender that can be used to dilute Nguni bull semen, before being used for insemination without reducing motility and viability of the sperm cells. The percentage of live sperm and the sperm motility rate were found to be higher in the semen sample extended with the egg yolk citrate diluents, after 9 h of incubation at 5^0C, when the semen pH was approximately neutral. This therefore suggests that the semen sample should be buffered to maintain a nearly neutral pH in order to maintain a better percentage live sperm and sperm motility rate (Hafez & Hafez, 2000).

In experiment 1, the use of the egg yolk Tris diluent resulted in poor results in terms of the percentage live sperm and the sperm motility rate, compared to the use of the egg yolk citrate extender. Thus it was not studied any further in experiment 2.

5.2 Effect of cryoprotectant on the quality of frozen-thawed Nguni bull semen

A high percentage live sperm was recorded in semen diluted with egg yolk citrate, with glycerol and dymethyl sulfoxide used as the cryoprotectants. Performance of dymethyl sulfoxide was also similar to that of ethylene glycol. These observations were made before the freezing and thawing processes. With regard to the sperm motility before freezing, there was no significant difference between the treatments. Following freezing and thawing processes, the percentage survivability and the sperm motility rate obtained were low, compared to those obtained before freezing. The low percentage sperm survivability and the sperm motility rate recorded following semen cryopreservation in the present study, have also been reported by Guthrie et al., (2002).

However, following the freezing and thawing processes, glycerol resulted in higher percentages of survival and the sperm motility rate, compared to the

use of other cryoprotectants, e.g. dymethyl sulfoxide and ethylene glycol. This implies that the survival rate and sperm motility rate improved significantly with the addition of glycerol. Thus glycerol seems to be an effective cryoprotectant for cryopreservation of bull sperm cells. This effect may be attributable to the fact that glycerol has a higher molecular weight, than the other two cryoprotectants. This feature of glycerol does not allow it to move rapidly into and out of the sperm cell membrane, thus enabling it to afford better protection (percentage survival and sperm motility rate) to the bovine sperm cells (Lovelock & Bishop, 1959). This implies that glycerol can be used to cryopreserve Nguni bull semen with less cryoinjury to the sperm cell. As it resulted in a high percentage survival and sperm motility rate following the freezing and thawing processes. The results of the current study agree with the work of Snedeker & Gaunya (1970), who found dimethyl solfoxide to be inferior to glycerol for the freezing of bovine semen.

Nevertheless, these results are also contradictory to what was observed earlier where the use of glycerol as a cryoprotectant provided a lower post-thaw sperm motility, compared to the use of lactamide in bovine (Nagase et al., 1972). This discrepancy may be attributed to the fact that lactamide has a lower molecular weight, compared to glycerol. This property therefore enhances rapid movement of lactamide through the sperm cell membrane, thus making it result in a better performance. Also this discrepancy with other studies may be attributed to the use of different concentrations of a cryoprotectant in the method of freezing used (programmable freezer versus liquid nitrogen vapour), as well as the use of different thawing methods (38^0C versus room temperature) (Mahadevan & Trounson, 1984).

5.3 Nguni bull sperm quality of diluted semen following incubation at different temperatures

The sperm concentration of semen samples incubated at 5^0C was found to be higher than that of the samples incubated at 25^0C. With regard to the percentage live sperm, there were no significant differences between the

treatment groups for 0 to 9h of incubation. At 12h of incubation, the percentage live sperm in the semen sample incubated at 5^0C, was higher than that of the sample incubated at 25^0C. In terms of sperm motility rate, there was no difference between the treatment groups at 0h. However, from 3h up to 12h of incubation, the semen samples incubated at 5^0C exhibited higher motility rates - compared to the samples incubated at 25^0C. The results of this study have also been obtained elsewhere, where it has been recommended that semen be stored at 5^0C in order to maintain better sperm motility and a sperm survival rate (Yang & Chou, 2000). These authors further stated that incubating semen at 5^0C extends the life span of the sperm, and also reduces the risk of microbial contamination. This thus explains why it results in better sperm survival and motility rates. However, these authors further recommended that semen should be kept no longer than 15 minutes post-thaw, so as to avoid a reduction in fertility and sperm motility rates.

The pH values recorded for the semen samples incubated at 25^0C were higher than those obtained from the samples incubated at 5^0C up to 6h of incubation. A different trend was obtained with the semen samples incubated for 9 to 12h. The semen samples incubated at 5^0C resulted in higher pH values, compared to the samples incubated at 25^0C. The results of the current study indicate a high percentage live sperm and motility rate within 12h of incubation at 5^0C - whereby the semen pH value was 6.9. To the contrary, within the same time frame at 25^0C, the percentage live sperm and sperm motility rate recorded were lower at a pH value of 4.7. This therefore implies that a high temperature decreases the semen pH (becomes more acidic). Thus leading to an impaired percentage live sperm, as well as sperm motility rate. This may be attributable to the fact that if the semen sample is more acidic (low pH), and is stored for a longer period of time with the quality becoming badly affected (Yang & Chou, 2000). The results obtained in the current study thus imply that fresh Nguni bull semen (diluted) can be successfully utilised within 9 h following collection, if incubated at a temperature of 5^0C.

5.4 Effect of incubation time on the quality of frozen-thawed Nguni bull semen

Sperm cells incubated for a period of up to 60 minutes post-thaw, exhibited significantly higher sperm survival and motility rates than those incubated for 90 minutes. This trend found in this study is similar to the one recorded in another trial - where bovine sperm motility declined from 68% to 17% after being incubated for 15h (Miller & Edwards, 1999). In the current study, the percentage survival and the sperm motility rate of the cryopreserved sperm cells declined drastically after incubating semen for 90 minutes. The results of this study are contradictory to what was observed elsewhere, where it was indicated that bull semen has to be kept no longer than 15 minutes post-thaw, to avoid a reduction in fertility and sperm motility (Yang & Chou, 2000). However, the results obtained in the current study are all within the acceptable range, especially for IVF processes.

The high motility and survival rates of sperm cells obtained within 60 minutes post-thaw in the current study may be attributed to the good preservation of the sperm cells prior to freezing. The good preservation of the sperm cells involve the use of a suitable extender, as well as the incubation of the sperm cells at a suitable temperature. This implies that frozen-thawed Nguni bull semen can be used successfully for insemination, within 60 minutes after thawing. On the other hand, if frozen-thawed bull semen is to be used after being incubated for 90 minutes, its fertilizing capacity will be low, due to a reduced sperm survival and motility rate.

5.5 Fertilizing capacity of frozen-thawed Nguni bull semen

Fertilization of bovine oocytes with fresh and frozen-thawed Nguni bull semen resulted in similar cleavage rates, as well as the proportions of the presumptive zygotes at 1 cell stage and 8 cell stage. However, a higher proportion of the presumptive zygotes at the 2-4 cell stage was obtained, when frozen-thawed semen was used, compared to the use of fresh semen.

This effect may be attributable to the good preservation of sperm cells, prior to freezing. This thus implies that frozen-thawed Nguni bull semen can be successfully used for the in vitro fertilization of oocytes.

The oocytes fertilized with fresh semen resulted in a higher proportion of embryos that reached day 7 blastocyst stage, than those fertilized with frozen-thawed semen. This observation is similar to that recorded in another trial, where in vivo fertilization was compared with in vitro fertilization. In that trial there was a higher blastocyst yield (73.9%), when using fresh semen for the in vitro fertilization of oocytes, compared to the in vivo fertilization of oocytes (52.8%) (Lonergan et al., 2000).

The results obtained in the current study may be attributable to the fact that a high sperm (frozen-thawed semen) dose is needed to obtain the same fertilization rate following in vitro fertilization, than when using fresh semen (Thundathil et al., 1999). In the current study similar insemination doses were used for both fresh and frozen-thawed semen, which may be a reason why the use of fresh semen resulted in a higher proportion of embryos reaching the day 7 expanded blastocyst stage. This therefore implies that the impaired sperm quality, following freezing and thawing processes, can be compensated by using increased sperm concentration during inseminations. Thus frozen-thawed Nguni bull semen can probably be used for in vitro fertilization of oocytes with an increased insemination dose in order to compensate for the semen quality traits impaired during the freezing and thawing processes. Maybe additional studies are needed using higher insemination doses for frozen-thawed Nguni bull semen, compared to using fresh semen from the same breed.

5.6 Effect of IVC media on bovine embryonic development following in vitro fertilization with frozen-thawed Nguni bull semen

The use of KSOM and SOF media to culture the presumptive zygotes resulted in the same cleavage rate and also similar proportions of the presumptive

zygotes at 2-4 cell stage and 8 cell stage. These results are similar to another study where SOF media and resulted in similar cleavage rates (Nedambale et al., 2006). This confirms that both KSOM and SOF media can be used to culture bovine embryos.

These results are however contradictory to where SOF resulted in a higher cleavage rate of cattle embryos (82.5%) (Rizos, et al., 2007). The differences in the experimental design may partly have accounted for the discrepancies in the results. However, the use of KSOM media resulted in significantly higher proportions of day 7 expanded blastocysts than the use of SOF media. It could be assumed that glutamine present in KSOM media had a positive effect on the day 7 blastocyst formation. This observation is contradictory to what was observed by other researchers where the use of KSOM (15.8%) and SOF (15.9%) did not show any significant difference on the production of day 7 expanded blastocysts (Lima et al., 2004). In another trial the use of SOF resulted in a higher percentage of day 7 blastocysts, compared to the use of KSOM media (Nedambale et al., 2006). This effect could be associated with the fact that KSOM media contains glutamine while SOF media does not contain glutamine, which might affect development to the blastocyst stage in KSOM media.

Possible explanations for the discrepancy with other studies may include the use of a different IVF media, that is, BO as compared to the use of TCM 199 in other studies - BO has been found to promote bovine blastocyst formation (Nedambale et al., 2006). The results of the present study also indicate that although KSOM and SOF can be used to culture bovine embryos, KSOM media display a better performance, as it produces more day 7 blastocysts than the SOF media. The results of the current study also contradict another trial where it was observed that sequential KSOM-SOF culture system improved day 7 blastocyst cell numbers and the total hatching rate, compared to the use of KSOM alone, in bovine embryos (Nedambale et al., 2004). Certainly, KSOM media was proven to be the media of choice for culturing bovine embryos. This thus implies that KSOM media can probably be used for

culturing the presumptive zygotes in cattle, as it has resulted in a higher proportion of day 7 expanded blastocysts than in the SOF media.

CHAPTER 6

GENERAL CONCLUSIONS

When Nguni bull semen quality parameters were evaluated following dilution with different extenders, all the sperm quality parameters that were evaluated deteriorated over time - regardless of the semen extender used within 9 h of incubation. Sperm concentration decreased after dilution, with both the egg yolk citrate and egg yolk Tris extenders. However, semen dilution seemed to lessen sperm quality deterioration, compared to undiluted semen following a 3 h period of incubation following dilution. Semen extended with egg yolk citrate recorded a better percentage live sperm and sperm motility rate, than semen extended with an egg yolk Tris extender. Both egg yolk citrate and the egg yolk Tris extenders can be used for diluting bovine semen, as the extenders recorded a better sperm survival and motility rate, compared to the undiluted semen, within a period of 9h. However, the best extender which can be recommended for the dilution of bull semen is egg yolk citrate, as it proved to be superior in terms of maintaining a higher percentage live sperm and sperm motility (>60%) - which is acceptable for field work. It is also important to consider the temperature at which semen is stored in order to minimize a decline in the sperm quality. Storing Nguni bull semen at 5^0C tended to provide better motility results, compared to storage at 25^0C with regard to percentage live sperm and sperm motility rate. Thus the former may be recommended for incubating fresh bull semen for a period of 12h before use, without much reduction in sperm quality.

The use of glycerol as a cryoprotectant resulted in a better percentage of live sperm and sperm motility rate, while the dimethyl sulfoxide and ethylene glycol cryoprotectants resulted in reduced sperm quality following the freezing and thawing processes. Therefore glycerol was proved to maintain better sperm survival and motility rates (>70%) before freezing. Dimethyl sulfoxide and ethylene glycol reduced the sperm survival and motility rates, as well as the semen pH. The same trend was observed following freezing and thawing, where the use of glycerol as a cryoprotectant resulted in acceptable sperm

survival and motility rates (>50%). These semen quality parameters declined drastically with the use of dimethyl sulfoxide and ethylene glycol - therefore glycerol proved to be a suitable cryoprotectant for freezing Nguni bull semen.

Sperm motility and survival rates declined with incubation time following the freezing and thawing processes. Nguni semen had acceptable sperm quality up to 60 minutes of incubation. Thereafter the sperm quality with regard to sperm survival, and motility rate declined drastically. This therefore implies that frozen-thawed Nguni bull semen can be used successfully or kept for at least 60 minutes after thawing, without much sperm quality impairement.

The fertilization of oocytes with frozen-thawed Nguni bull semen resulted in a higher proportion of the presumptive zygotes developing to the 2-4 cell stage, compared to the use of fresh semen. However a smaller proportion of the presumptive zygotes managed to develop to the day 7 expanded blastocyst stage, compared to those fertilised with fresh semen. There was a high blastocyst rate obtained following fertilization with the fresh semen, compared to fertilization with the frozen-thawed semen. Frozen-thawed Nguni bull semen can presumably be used for in vitro fertilization of cow oocytes, as it resulted in similar proportions of oocytes that cleaved. A higher proportion of the presumptive zygotes were recorded with 2 to 4 cells, compared to the use of fresh semen. Apart from that a promising proportion of the presumptive zygotes obtained from its use still managed to reach day 7 expanded blastocyst stage (14.2%).

The culturing of the presumptive zygotes with potassium simplex optimizing medium resulted in a higher proportion of the presumptive zygotes produced that managed to reach the day 7 expanded blastocyst stage – compared to culturing with synthetic oviductal fluid (SOF). However, both media resulted in similar proportions in terms of the oocytes that cleaved, the presumptive zygotes with 2 to 4 cells, as well as those zygotes with 8 cells.

In conclusion Nguni bull semen can be best extended with an egg yolk citrate extender incubated at 5^0C and cryopreserved with glycerol in order to achieve acceptable results, with regard to sperm quality. Nguni bull semen can be

successfully used within 60 minutes post-thaw as it still has an acceptable sperm survival and motility rate within this time frame. Frozen-thawed Nguni bull semen also gave acceptable results in terms of the proportion of the oocytes that cleaved, thus it can be successfully used for fertilization purposes.

Cattle oocytes cultured in potassium simplex optimizing medium resulted in a high proportion of day 7 expanded blastocysts, compared to those cultured in synthetic oviductal fluid medium. The results obtained in this study thus show that semen from this indigenous cattle breed (Nguni), can be successfully cryopreserved. In turn cryopreservation gave an acceptable cleavage rate, and further development to the blastocyst stage, post-thaw. However, further studies have to be conducted with more Nguni bulls in South Africa in order to verify the results obtained in this study, as this was only seen as a preliminary study.

ABSTRACT

EFFECT OF DIFFERENT CRYODILUENTS ON NGUNI BULL SEMEN VIABILITY AND IN VITRO FERTILIZING CAPACITY

by

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Degree:	M. Sc. (Agric)

This study was aimed at evaluating the effects of different extenders and cryoprotectants on the quality of Nguni bull semen after cryopreservation, and to evaluate the performance of frozen-thawed Nguni bull semen in IVF. The study was conducted at ARC-Animal Improvement Institute in Pretoria, in conjunction with the University of the Free State from April to October 2008.

Three Nguni bulls (average age of 5 years) were used, and the semen collected from each bull, twice a week, using an electroejaculator. The semen quality parameters were evaluated prior and post freezing. The parameters evaluated included sperm motility rate and percentage live sperm, semen pH and semen concentration. The semen samples collected were divided into three equal portions following every collection and allocated to three groups - based on the semen extender used. One portion was extended with egg yolk citrate, the other extended with egg yolk Tris, while the other sample was left

undiluted and served as a control. Following the addition of the extenders, the semen samples were incubated for a period of 9h. Evaluation of semen quality parameters was done at 3h intervals, within that incubation period of 9 h. The egg yolk Tris extender exhibited a reduction in performance in terms of the sperm motility rate and the percentage live sperm, compared to the egg yolk citrate extender after 6 and 9h of incubation respectively. Thus the diluted semen was not further used in the second experiment. The semen samples extended with the egg yolk citrate diluents were incubated at different temperature regimes (5^0C and 25^0C) for a period of 12h, to evaluate the effect of temperature on the sperm quality of the diluted semen.

In the second trial, the semen sample that was diluted with egg yolk citrate was further divided into three portions - in order to add three different cryoprotectants, namely glycerol, dimethyl sulfoxide and ethylene glycol. The percentage live sperm and the sperm motility rate of the semen sample following addition of the cryoprotectants were also evaluated after 2h of incubation but prior to freezing. The semen samples were then loaded into 0.5ml semen straws, which were sealed with polyvinyl alcohol. The semen straws were then placed in a programmable freezer for 40 minutes for semen cooling from an initial temperature of 5^0C, to a temperature of -130^0C. After freezing the straws were removed from the programmable freezer and placed in liquid nitrogen vapour in a styro-foam box, for 5 minutes to cool the semen straws from -130^0C to -196^0C, after which the straws were plunged directly into liquid nitrogen (-196^0C) tank, for storage until thawing.

A total of 1560 bovine oocytes were retrieved by aspiration from 127 ovaries collected from Strydfontein abattoir in Pretoria. The oocytes were then matured *in vitro* in bovine maturation media (consisting of TCM 199, FSH, LH and estradiol), for a period of 24h. After 24h of incubation, the matured bovine oocytes with expanded layers of cumulus cells were washed in a BO-IVF solution and fertilized *in vitro* using frozen-thawed Nguni bull semen from the first trial, while the others were fertilized with fresh Nguni bull semen, used as a control.

For IVF, mature oocytes were incubated with semen for 18h. Thereafter, the presumptive zygotes were vortexed in TCM 199 for 90 seconds in order to remove the cumulus cells. After that, the presumptive zygotes from each treatment were randomly allocated into two different culture media namely, KSOM and SOF. The control group, that is the fresh semen group (n=481), 242 zygotes were allocated to KSOM culture media, while 239 zygotes were allocated to SOF culture media containing BSA. The treatment group, that is the frozen-thawed semen (n=559), 280 zygotes were allocated to the KSOM culture media, while 279 zygotes were allocated to SOF- BSA culture media. The presumptive zygotes were then allowed to develop (incubated) for 7 days until reaching the blastocyst stage. On day 2 following IVC (onset of IVC = day 0), cleavage rate was evaluated, the presumptive zygotes at 2-4 cell stage and those at 8 cell stage were evaluated under a contrast microscope and development recorded. Thereafter, on days 2 and 5 of culture the culture media were changed. KSOM-step 1 was changed to KSOM-step 2, while SOF-BSA was changed SOF-FBS. On the 7th day the expanded blastocysts were evaluated and recorded.

Extended semen exhibited a significantly ($P<0.05$) lower sperm concentration, than undiluted semen. The semen pH values were significantly ($P<0.05$) different at 0 to 3h of incubation between the treatment groups. After a period of 6h of incubation, no significant differences were recorded between the treatment groups, in terms of the semen pH. The semen pH was found to be acidic, however it became neutral after 9h of incubation in the semen sample that was diluted with egg yolk citrate extender and incubated at 5^0C. The percentage live sperm was similar for semen extended with egg yolk citrate and egg yolk Tris extenders incubated at 5^0C up to a period of 6h of incubation. Thereafter the percentage live sperm decreased in the semen sample extended with egg yolk Tris diluents, after a period of 9h of incubation (5^0C). The sperm motility rate was similar between the treatment groups up to 3h of incubation at 5^0C. After 6 and 9h of incubation (5^0C), there was a drastic decline in the sperm motility rate of the semen samples extended with an egg yolk Tris extender. The percentage live sperm and pH values differed

significantly (P<0.05) following addition of a cryoprotectant. The semen sample in which glycerol was used (75±5.3%) exhibited a significantly (P<0.05) higher sperm survival rate, compared to the semen sample in which ethylene glycol was used (55±8.5%). Semen sample in which glycerol was used as a cryoprotectant (6.6±0.2) exhibited a significantly (P<0.05) higher pH, compared to the semen sample in which dimethyl sulfoxide was used as a cryoprotectant (6.3±0.3).

The semen samples diluted at 5^0C exhibited a significantly (P<0.05) higher sperm concentration immediately following dilution, compared to samples diluted at 25^0C. The sperm motility rate immediately following dilution was similar between the treatment groups. However, the sperm motility rates at 3, 6, 9 and 12h of incubation were significantly (P<0.05) different (67±10% vs. 55±24%; 60±12% vs. 47±25%; 47±20% vs. 38±25% and 40±20% vs. 28±27%) at 5^0C and 25^0C respectively. The percentage live sperm was found to be similar between the treatment groups, up to 9h of incubation. However, after 12h incubation the semen sample incubated at 5^0C exhibited a significantly (P<0.05) higher percentage live sperm, compared to the sample incubated at 25^0C (46±21% vs 35±31%). The interaction between incubation temperature and the semen extender used did not affect all the measured sperm quality parameters.

In vitro fertilization of cow oocytes with the frozen-thawed bull semen exhibited a significantly (P<0.05) higher percentage of presumptive zygotes at the 2-4 cell stage, compared to the use of fresh semen (32.1±13.0% vs. 24.3±12.8%). IVF with fresh semen (23.2±16.5%) resulted in a significantly (P<0.05) higher percentage of day 7 blastocysts, compared to the use of frozen-thawed semen (14.2±11.9%). Culturing of the presumptive zygotes with KSOM media (23.2±17.5%) exhibited a significantly (P<0.05) higher percentage of day 7 blastocysts, than culturing with SOF media (14.2±10.4%) *in vitro*.

In conclusion, egg yolk citrate proved to be the best extender for diluting Nguni bull semen. Fresh Nguni bull semen diluted with egg yolk citrate can

probably be incubated up to a period of 9h at 5^0C, without any detrimental effect on the percentage live sperm and the sperm motility rate. Nguni bull semen can be best frozen using glycerol as a cryoprotectant. Frozen-thawed Nguni bull semen can be successfully used in IVEP since it resulted in higher percentage of the presumptive zygotes at the 2-4 cell stage and also attained day 7 blastocysts. Frozen-thawed Nguni bull semen can also be used successfully within 60 minutes following thawing incubated at 5^0C. Nevertheless, fresh Nguni bull semen can still be used successfully for IVF purposes since it resulted in a higher percentage of day 7 blastocysts, compared to frozen-thawed semen. KSOM medium proved to be a better IVC medium for bovine semen than SOF medium in terms of the percentage of day 7 blastocysts obtained.

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