IMPROVEMENT OF CATTLE OOCYTE RETRIEVAL TECHNIQUES AND HORMONAL INFLUENCE ON *IN VITRO* EMBRYONIC DEVELOPMENT

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BY

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A MINI-DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN AGRICULTURE (ANIMAL PRODUCTION), DEPARTMENT OF AGRICULTURAL ECONOMICS AND ANIMAL PRODUCTION, FACULTY OF SCIENCE AND AGRICULTURE, UNIVERSITY OF LIMPOPO, SOUTH AFRICA

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DECLARATION

I declare that the dissertation hereby submitted to the University of Limpopo for the degree of Master of Science in Agriculture (Animal Production) has not previously

been submitted by me for a degree at this or any other university, that it is my own work in design, execution and that all material contained herein has been duly acknowledged.

Signature…… Date……..

Lekola Khomotso Podile Molvia

DEDICATION

This study is dedicated to my mom (Mosobane Lekola), my sister (Mavis Kekana) and my brother in law (Jim Kekana) for their endless support and never giving up on me. To my late father Modiba Lekola, thank you for the agricultural knowledge that you shared with us. It was the first teaching we received, through that knowledge I have managed to pursue a degree in agriculture, your spirit will always be with us. Special thanks to my fiancé Ambrose Bosoga for all the love and support through the sleepless nights. To my son Bokang Lekola, I love you so much.

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ABSTRACT

The objectives of this study were: 1) To determine the effect of oocyte retrieval techniques (slicing and aspiration) on the quality and quantity of cattle oocytes, 2) To evaluate the effect of different concentrations of hormones on the maturational rate of cattle oocytes selected by brilliant cresyl blue staining, 3) To evaluate fertilization rate and cleavage/embryonic development of oocytes with or without cumulus cells, and 4) To compare the effect of fresh and frozen thawed semen on the fertilization rate of cattle oocytes. In Experiment 1: oocytes were recovered from abattoir derived ovaries using slicing and aspiration. The recovered oocytes were exposed for 90 minutes to 26µM of brilliant cresyl blue (BCB) stain and classified according to the colour of their cytoplasm: BCB+ (oocytes with blue cytoplasm) and BCB- (unstained oocytes). There was no difference ($P>0.05$) in the quality of oocytes recovered using slicing (60.7 %) or aspiration (53.7 %) techniques. In experiment 2: The BCB selected and the non-selected immature oocytes were randomly allocated into medium 199 + 10 % fetal bovine serum (FBS) maturation media. The media was supplemented with three different concentrations of hormones as treatments (T). The T1 (0.5 µg/ml of follicle stimulating hormone (FSH), 5mg/ml of luteinizing hormone (LH) and 2 µg/ml of estradiol (E_2) as the control group. Then, T2 (1 µg/ml of FSH, 6 mg/ml of LH and 2.5 µg/ml of E_2) and T3 (1.5 µg/ml of FSH, 7 mg/ml of LH and 4.5 µg/ml of E_2). Maturation rate of oocytes was determined by the protrusion of the first polar bodies 24 hours following *in vitro* maturation. Treatment 2 yielded higher ($P<0.05$) maturation rate for both BCB+ (65.6 %) and without BCB (60.3 %) oocytes with T1 giving lower ($P<0.05$) maturation rate for BCB+ (22 %) and without BCB (16 %) oocytes. However, BCB- oocytes had lower ($P<0.05$) polar body extrusion (3.03 %, 8.1 % and 2.2 %) for T1, T2 and T3, respectively. In Experiment 3: one group of the presumptive zygotes was denuded of cumulus cells and the other group was cultured with cumulus cells. The presumptive zygotes were *in vitro* cultured in SOF-BSA and changed to SOF-FBS after 48 hours. High fertilization/cleavage rate was observed in oocytes cultured with cumulus cells (29.0 %) compared to the denuded oocytes (20.0 %) for 2-4 cells stage. Day 7 blastocysts were more ($P<0.05$) on oocytes cultured with cumulus cells (32 %) compared to denuded oocytes (13 %). In experiment 4: The matured oocytes were fertilized using fresh and frozen thawed semen. The oocytes fertilized with frozen thawed semen obtained a better number of

2-4 cell cleavage (23 %) when compared to fresh semen (19 %). Oocytes that were fertilized with frozen thawed semen also obtained higher morula (13 %) and blastocyst (8 %) compared to fresh semen with morula (3.4 %) and blastocyst (2 %). In conclusion, immature oocytes that were exposed to BCB+ and cultured in M199 supplemented with 10 % FBS, 0.5 µg/ml of FSH, 5 mg/ml of LH and 2 µg/ml of E_2 had a higher ($P<0.05$) number of matured oocytes (extrusion of first polar body) compared to those that were not exposed to BCB (no BCB). Oocytes that were cultured with cumulus cells yielded a higher ($P<0.05$) number of cleaved embryos compared to the denuded oocytes. Slicing yielded a higher ($P<0.05$) number of oocytes, however the quality of oocytes recovered was similar compared to those recovered by the aspiration technique ($P>0.05$). Oocytes fertilized with frozen thawed semen yielded higher ($P<0.05$) number of 2-4 cell, morula and blastocyst when compared with oocytes that were fertilized using fresh semen.

Keywords: ovaries, oocytes, slicing, aspiration, COCs, BCB, polar body and cattle

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LIST OF ABBREVIATIONS

Abbreviations	Full names
AI	Artificial insemination
ART	Assisted reproductive technologies
ANOVA	Analysis of varience
BSA	Bovine serum albumin
BSPs	Bovine seminal plasma proteins
BCB	Brilliant cresyl blue
BO	Bracket and Oliphant
CCs	Cumulus cells
COCs	Cumulus oophurus complexes
cAMP	Cyclic adenosine monophosphate
CASA	Computer aided sperm analysis
CR1	Charles and Rosenkrans 1
E_2	Estradiol
ET	Embryo transfer
FBS	Fetal bovine serum
FCS	Fetal calf serum
FF	Follicular fluid
FSH	Follicle stimulating hormone
GHRH	Growth hormone releasing hormone
GVBD	Germinal vesicle break down
GV	Germinal vesicle
G6DPH	Glucose 6 phosphate dehydrogenase enzyme
IVC	In vitro culture

IVEP	In vitro embryo prodduction
IVF	In vitro fertilization
IVM	In vitro maturation
LH	Luteinizing hormone
MOET	Multiple ovulation transfer
mRNA	Messenger ribonucleic acid
mDPBS	Modified dulbeco’s phosphate buffered saline
RNAs	Ribonucleic acids
SOF	Synthetic oviduct fluid
TCM 199	Tissue culture medium 199
ZP	Zona-pellucida
SOF	Synthetic oviduct fluid
TCM199	Tissue culture medium 199

CHAPTER 1

INTRODUCTION

1.1 Background

Livestock plays a vital role in the agricultural and rural economics of the developing countries (Rosengrant *et al*., 2002). Cattle, together with small ruminants, account for the largest part of the economy in developing countries in terms of milk, meat, hides and wool production (Francis, 1988). However, cattle productivity is low, leading to economic losses in the livestock industry. Poor productivity can be observed in cases where a cow does not become pregnant during a defined breeding season (Chakravarthi and Balaji, 2010). Such matters can be resolved by the use of assisted reproduction technologies such as oestrus synchronization, embryo transfer, artificial insemination and *in vitro* embryo production which are important in maximising production (Rodriguez-Martinez, 2012).

The *in vitro* embryo production (IVEP) technique is one of the assisted reproduction technologies (ART) that have the potential for speeding up genetic improvement in cattle (Baldassarre and Karatzas, 2004). The IVEP process encompasses three steps: recovery of oocytes from cattle ovaries and maturing them *in vitro* (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of the obtained zygotes to the blastocyst stage. The IVEP is currently one of the most important biotechnologies in cattle breeding and husbandry (Camargo *et al*., 2006). It has become a repetitive method of producing embryos from abattoir-derived ovaries with minimal cost (Hoque *et al*., 2011).

The quality of oocytes is important for successful IVEP. The cumulus cells around the oocytes play a vital role in oocyte maturation by supplying the oocyte with nutrients and serve as a communication channel between the oocyte and the outside world (Hoque *et al*., 2011). Cumulus cell concentration is dependent on the efficiency of harvesting oocytes. Aspiration and slicing are among the techniques used to harvest oocytes (Ferdous, 2006). The quality of oocytes can be verified by the use of the brilliant cresyl blue stain. The stain is able to detect the activity of the glucose-6-phosphate dehydrogenase (G6PDH). This enzyme is active in young incompetent oocytes that are still in the growing phase, and it is decreased in oocytes that have finished the growth phase and are more competent (Wang *et al*., 2012). Such tests are known to improve the viability of embryos produced *in vitro* (Alm *et al*., 2005).

Farahavar *et al.* (2010) observed that *in vitro* handling and culture conditions may cause oxidative stress to oocytes and embryos. Thus, the overall objective of this study is to generate a suitable protocol for *in vitro* embryo production in cattle for the South African laboratories. There are a number of protocols that have been established for cattle IVEP, but due to various reasons such as environmental differences, type of breeds used and laboratory to laboratory differences, these protocols may not be efficient for South African IVEP laboratories.

1.2 Problem statement

It is important that oocytes of good quality are selected for IVEP. However, the production of embryos *in vitro* is low and oocytes experience an embryonic arrest after 48 hours of culture (Zhang *et al*., 2010). *In vitro* embryonic development in cattle is low compared to *in vivo* counter parts; this is attributed to *in vitro* maturation medium or conditions (Ferre *et al*., 2002). It is not clear if the growth of oocytes from the small antral follicles is possible under the basal level of luteinizing hormone (LH), follicle stimulating hormone (FSH) and estradiol (E_2). It is, however difficult to produce higher number of embryos in the laboratory due to various reasons (e.g. environmental differences, breeds used, laboratory to laboratory differences, etc.). Protocols differ from one laboratory to the next which entails that a protocol that is successful in a certain laboratory might not necessarily be efficient locally in RSA or another laboratory.

1.3 Motivation of the study

This study will generate information on the suitable concentration of hormones used for *in vitro* maturation, specifically for the Germplasm, Conservation Reproduction and Biotechnology Laboratories (GCRB) since protocol differ from one laboratory to the next. The study will generate a suitable IVEP protocols to be used in South African indigenous cattle oocytes. The information will be useful for research purposes on how to produce and conserve embryos from animals with high genetic value.

1.4 Objectives

The objectives of this study were to:

i. compare oocytes retrieval techniques (slicing and aspiration) on cattle oocytes quality and quantity;
ii. to determine the maturation rate of cattle oocytes matured in m199+10% FBS medium supplemented with different concentrations of hormones [luteinizing hormone (LH), follicle stimulating hormone (FSH), Estradiol(EST)];
iii. the effect of cumulus cells on cleavage and embryonic development of cattle oocytes and
iv. to compare fertilization rates between oocytes fertilized with fresh or frozen thawed semen.

CHAPTER 2

LITERATURE REVIEW

2.1 *In vitro* embryo production in cattle

In vitro embryo production (IVEP) is the process whereby female gametes are recovered from the ovaries, fertilized by sperm cells and cultured in order to produce viable embryos under the *in vitro* conditions (Ferre, 2002). The IVEP has advantages: it can be used to produce embryos from cows in different physiological conditions and semen from different bulls can be used to fertilize oocytes recovered from cattle ovaries resulting in embryos with different sires being produced at the same time (Vigneault *et al*., 2009). The use of IVEP by commercial embryo companies has increased especially in South America. Currently cattle IVEP represents a considerable percentage of the total number of cattle embryos produced in the whole world (Camargo *et al.,* 2006).

According to Nedambale *et al.* (2004), IVEP involves three major processes: to recover immature oocytes and to complete *in vitro* maturation, then insemination *in vitro* and to develop the resulting zygotes to blastocyst stage in a culture medium. The IVEP is strongly influenced by the results of these three major processes (Suthar and Shah, 2009). The resulting blastocysts after culturing can be cryopreserved for future use or directly transferred into the recipient cow. Oocytes used for IVEP can be obtained from the ovaries of live donor cows by using the transvaginal oocytes recovery or from ovaries of slaughtered cows (Heleil *et al*., 2010). There is abundant evidence showing that differences between *in vivo* and *in vitro* produced embryos still exist, which involve morphological and molecular aspects that prejudice IVEP efficiency (Ferre *et al*., 2002; Nedambale *et al*., 2004). These differences are probably influenced by several factors such as breed, oocyte quality, follicular environment, fertilization and embryo culture environment (Camargo *et al*., 2006). Considerable progress has been made in the development of techniques for IVEP of cattle for research and commercial purposes, but the success rates in terms of blastocyst yield remain modest and range between 30 and 40% which is still lower than that obtained from embryos produced *in vivo* (Blanco, *et al*., 2011). The use of IVEP technologies has the potential of maximum utilization of ova,

which would have been naturally lost in a process called follicular atresia (Nedambale *et al*., 2004).

2.2 Collection of oocytes

The number of good quality oocytes harvested from the ovary is an important consideration in the IVEP. Oocytes for IVF are collected from one of the following sources: the oviducts soon after ovulation, mature follicles shortly before ovulation or from immature and atretic follicles from abattoir derived material (Baldarssarre *et al*., 1996). Oocytes are recovered from ovaries of slaughtered cows by using aspiration, slicing or puncturing as methods of recovering oocytes. Oocytes can also be recovered from live cows using the ultra-sound guided transvaginal oocytes retrieval-ovum pick up. This recovery permits the production of more embryos than might be possible by the standard embryo transfer (ET) practice following IVEP. *In vivo* mature oocytes can be recovered by using multiple ovulations. Multiple ovulation and embryo transfer (MOET) may be of popular benefit to beef and genetic improvement schemes where only a short period of time is available for creation of embryos from selected donors if the theoretical contribution of a reduced generation interval to the improvement in the rate of genetic gain is to be achieved (Goodhand *et al*., 2000). Follicle size affects oocyte quality, communication between the oocyte and its surrounding cumulus cells is important for the development of a competent oocyte.

2.3 Oocyte *in vitro* maturation in cattle

In vitro maturation (IVM) is the first and the most critical step towards successful IVEP. Briefly, oocytes are formed within the ovary during gestation when a foetus is 18 weeks old. At that time the oocytes are known as primordial germ cells, primordial cells are found as a cluster of cells before the formation of follicles. The primordial germ cells known as oogonia proliferate and undergo the mitosis to form the primary oocytes. The first meiotic division occurs once the animal is born with the formation of granulosa and theca cells derived from the germinal epithelium, they are collectively known as the primordial follicles, this development is not dependent on

hormones (Eggan *et al*., 2006). The primordial follicle will then start to develop an antrum which is the space between the oocyte and the theca interna cells which will later be filled with follicular fluid, after the formation of an antrum, the oocyte will then be known as an antral follicle. The oocytes contain a nucleus which is referred to as a germinal vesicle. At the end of the first meiosis the germinal vesicle will break resulting in the formation of the first polar body (Jamil *et al*., 2010). Maturation of oocytes includes both the nuclear and cytoplasmic maturation. An oocyte is considered to be morphologically mature when the first polar body is extruded and the oocyte is arrested at the metaphase of the second meiotic division. Cytoplasmic maturation encompasses a variety of cellular processes that must be completed in order for oocytes to be fertilized and develop into normal embryos and offspring (Heleil, 2010). During the course of gametogenesis, fully grown oocytes generally arrest at prophase I (germinal vesicle stage) of meiosis. Mostly meiosis is reinitiated upon hormonal stimulation and results firstly in germinal vesicle breakdown (GVBD) and extrusion of polar bodies with a secondary arrest usually occurring at either metaphase I (MI) or metaphase II (MII) of meiotic maturation which is later released by fertilization. In well-known mammals, progression from prophase I to MII is initially promoted by a surge in progesterone or luteinising hormone (LH) levels (Jahn and Sudhof, 1999).

According to Rodrigues *et al*. (2010) full oocytes maturation does not only involve the acquisition of meiotic competency, but also cytoplasmic maturation as evidenced by the synthesis and storage of ribonucleic acids (RNAs) and proteins. Oocytes maturation is linked to adequate regulation of many molecular pathways, which are possibly those associated with genes that are only expressed by maternal transcriptome. Expression of pro- and anti-apoptotic genes may shift the developmental potential of the oocyte towards either cell death or cell survival. From distinguishable ovarian population, only fully-grown oocytes are able to resume meiosis and progress to maturation.

Messenger RNA (mRNA) transcription is first down regulated in fully-grown immature oocytes and ceases after GVBD. Certain genes are specifically up or down regulated in the MII stage oocytes relative to germinal vesicle stage (Fair *et al*., 2007). The first designed strategy used to provide information on oocytes development capacity is the identification of a healthy gamete, because oocytes intrinsic viability is considered the most critical factor influencing IVM success (Van Soom *et al*., 2007). The ability of oocytes to undergo IVM and subsequent embryo development is influenced by maturation media and supplements there in. A chemically defined medium is considered important to address the exact effects of any supplement of interest in the medium on oocytes maturation or embryo development (Motlagh *et al*., 2008).

2.4 Function of hormones in IVM

The strategic way of increasing the developmental ability of *in vitro* matured oocytes is by supplementing the maturation with gonadotropic hormones and growth hormones (De Lima *et al*., 2010). Kojima *et al*. (2003) discovered that the common hormones that are used in IVM are luteinizing hormone (LH), follicle stimulating hormone (FSH) and estradiol (EST). The continuation of oocyte maturation in the ovary of a live animal is initiated by the preovulatory surge of the gonadotrophins (LH and FSH) in the follicle. The LH hormone is regarded as the key hormone regulating oocyte maturation and ovulation (Albuz, 2009). Nuttinck *et al*. (2004) discovered that LH does not act directly on oocytes or cumulus oocytes complexes (COCs) to promote maturation but it stimulates the paracrine factors which are released by the granulosa cells and they promote the process of oocytes maturation. The preovulatory LH surge was discovered by Yamashita *et al*. (2011), that is vital for cumulus cell expansion which is often used as an indicator of oocyte maturation. It has been discovered that LH binds to receptors on mural granulose cells and stimulate cyclic Adenosine Mono Phosphate (cAMP) signalling (park *et al*., 2004). cAMP plays an crucial role in oocyte maturation not only by controlling meiotic arrest but also in meiotic resumption (Eppig, 1991). Lin *et al*. (2011) suggests that FSH as

another gonadotropic hormone induces the cumulus cells to produce cAMP, which is then transferred to the oocytes by gap junctions.

According to Izadyar *et al*. (1997), the presence of growth hormones (GH) during IVM accelerates the kinetics of meiosis, increases cumulus cell expansion and improves cytoplasmic maturation. GH can also stimulate nuclear maturation *in vitro* in rat (Apa *et al*., 1994) and pig (Hagen and graboski, 1990). Growth hormone releasing hormone (GHRH) as it was postulated by (Apa *et al*., 1996), it stimulates *in vitro* maturation via GH-mediated pathway of cattle oocytes. E_2 as a steroid hormone alters the LH secretion by influencing the secretion of LHRH (Kojima *et al*., 2003). The stimulation of gonadotropins by LHRH contributes to the production of mRNA for gonadotropin subunits (Moenter *et al*., 1990).

2.5 *In vitro* fertilization of cattle oocytes

In animals, sexual reproduction encompasses the fusion of two distinct gametes to form a zygote. Gametes are produced by a type of cell division called meiosis. Gametes are haploid (containing only one set of chromosomes) while the zygote is diploid (containing two sets of chromosomes). During fertilization the head of sperm, the acrosome releases enzymes that begin to break down the outer layer of the egg's membrane, for a sperm cell to penetrate the egg. Once a single sperm has successfully penetrated the cell membrane of the egg, it changes its electrical characteristics ensuring that only one sperm cell fertilizes the egg in the process called cortical reaction (Wright and Bondioli, 1981). Once the egg is fertilized by the sperm, it is called a zygote. The embryo starts as a single cell, the single cell splits into two cells, with time the two cell splits into four and then into 8 cells. On day 8 of gestation the embryo is expected to be at the blastocyst stage which contains about a 100 cells (Wasserman *et al.,* 2001).

The IVF processes take place in the laboratory, inside a dish containing growth hormones and medium necessary for fertilization. Historically, IVF of mammalian ova

has been possible for over 20 years, however only recently has IVF demonstrated and verified by the birth of at least one live calf after transfer of resulting embryos to recipients (Baibakov *et al*., 2007). The initial purpose of commercial IVF was to obtain viable embryos from females that may not be able to produce progeny through conventional techniques. At present, IVF is a complement to an embryo transfer (ET) program. Its application could be for females that will not respond to super stimulatory treatments and fail to produce transferable embryos or possess abnormalities in their reproductive tracts (Faber *et al*., 2003).

The IVF allows an improvement in efficiency of sperm utilization and provides opportunities to use relatively low number of sperm cells to produce viable embryos. This allows also the utilization of high value semen and may provide significant opportunities when coupled with gender separated semen (Ferre *et al*., 2002). Fertilization encompasses a series of different steps which have to be performed to create a new individual. The steps include sperm capacitation, binding and penetration of zonapellucida (ZP), transversing the perivitelline space, binding and fusion with oolemma, activation of the oocyte and decondensation of the sperm head to form the male pronucleus (Van Soom *et al.,* 2002).

2.5.1 Sperm capacitation

Mammalian sperm cells are not able to fertilize eggs immediately after ejaculation. Sperm acquire fertilization capacity after residing in the female tract for a predetermined period of time (Salicioni *et al*., 2007). Freshly ejaculated sperm cells must first undergo a series of changes known collectively as capacitation. Capacitation is associated with the removal of adherent seminal plasma proteins, reorganization of plasma membrane lipids and proteins. It also involves an inflow of extracellular pH. The molecular details of capacitation appear to diverge somewhat among species (Bailey, 2010).

Capacitation occurs while sperm resides in the female reproductive tract for a period of time, as they normally do during gamete transport. The length of time required varies among species. The sperm of many mammals including human beings can alternatively be capacitated *in vitro* by centrifugation and incubation in certain fertilization media by mimicking the processes taking place in the female reproductive tract (Beiley, 2010). Sperm cells that have undergone capacitation are said to become hyperactivated and display hyper activated motility. Capacitation performs to disrupt the membrane of the sperm cell to prepare it for the acrosome reaction (Talbot *et al*., 2003). Capacitation can be divided into (a) fast events such as the initiation of sperm motility, occurring as soon as the sperm cells are released from epididymis and (b) slow events such as changes in the motility pattern and the acquisition of the sperm capacity to undergo agonist-stimulated acrosome reaction, which are activated only after a certain period of time in conditions that support the sperm's ability to fertilise the egg. Capacitation is also the result of simultaneous processes involving changes at the molecular level occurring both in the head and tail (Harrison, 2004).

2.5.2 Acrosome reaction of sperm

The acrosome is a membrane-limited organelle which overlies the sperm nucleus. In response to either physiological or pharmacological stimuli, the outer acrosomal membrane and the overlying plasma membrane undergo fusion and vesiculation leading to the exposure of the acrosomal contents to the extracellular environment. This process is known as the acrosomal reaction and its completion is an absolute prerequisite for fertilization (Salicioni *et al*., 2007). Only acrosome-reacted sperm is able to penetrate through the zona pellucida (ZP), bind to the oocyte plasma membrane and fuse with the oocyte (Patrat *et al*., 2000).

According to Yanagimachi (1994) the acrosome reaction leads to the discharge of a range of hydrolytic and proteolytic enzymes, mainly acrosin and hyaluronidase, which are essential for sperm penetration through the oocyte envelopes. The acrosome reaction also results in the modification of some plasma membrane

proteins at the acrosomal equatorial segment and post acrosomal level necessary for the fusion with the oocyte membrane. Patrat *et al*. (2000) reported that, the electron microscopic analysis of spermatozoa identified different morphological stages of the acrosome reaction. The acrosome matrix become swollen and diffused and the volume of the acrosomal cap content between the outer and inner membrane expands dramatically. This first step was followed by multiple fusion points between outer acrosomal and plasma membranes with subsequent membrane vesiculation and exposure of the inner acrosomal membrane (Yanagimachi, 1994).

During *in vivo*, the acrosome reaction is induced by a ZP glycoprotein (ZP_3) which may act as a ligand for one or more sperm plasma membrane receptor(s). Although zonapellucida is the main physiological inducer of acrosome reaction, many arguments indicate that progesterone, secreted by cumulus cells is present in this exocytotic process (Breitbart and Sungin, 1997). The acrosome reaction presents differences with other known exocytotic events, the differences are (1) the acrosome is a single secretory vesicle, (2) there are multiple fusion points between the outer acrosomal membrane and the plasma membrane, (3) both these membranes form mixed vesicles that are shed during the acrosome reaction resulting in membrane loss and (4) the acrosome reaction is a one shot fusion event; thus there is no membrane recycling (Jahn and Sudhof, 1999). The acrosome reaction results from a cascade of intracellular signals which have been largely documented although the exact sequence of events is not yet completely elucidated. The evaluation of the acrosome reaction can be used to predict fertilization success and can be good in choosing the most appropriate technique of assisted reproductive technology (Patrat *et al*., 2000).

2.5.3 Sperm-zona pellucida binding

For mammalian sperm to exhibit the ability to bind the (ZP) they must undergo three distinct phases of maturation, namely, spermatogenesis in the testis, epididymal maturation inside the epididymis and capacitation which takes place within the female reproductive tract. An impressive array of sperm surface remodelling events

accompany these phases of maturation and appear critical for recognition and adhesion of the outer vestments of the oocyte, a structure known as the ZP (Reid *et al*., 2011).

Sperm penetration through the cumulus matrix is facilitated by a membrane-bound hyaluronidase. Eventually the sperm encounters the ZP, the extracellular coat of the egg where species-specific gamete recognition is believed to occur. In the mouse, the zona is composed of three glycoproteins, referred to as ZP1, ZP2 and ZP3, with the sperm-binding activity residing on ser/thr-linked oligosaccharide chains of the ZP3 glycoprotein (Salicioni *et al*., 2007). The ZP is regarded as a porous extracellular matrix that surrounds mammalian oocytes as well as ovulated eggs and embryos until the blastocyst stage of development. It is composed of a small number of glycoproteins that are held together through non-covalent bonds forming long, interconnected fibrils (Reid *et al*., 2011).

2.5.4 Polyspermy

Polyspermy is an abnormal fertilization whereby an oocyte is penetrated by more than one sperm cell. In most animals, a mechanical barrier against sperm and microorganisms is established in the egg's extracellular matrix by action of the egg's secretory vesicles known as the cortical granules, which contain structural proteins and/or enzymes (Coy *et al*., 2008). The cortical granules undergo a cortical reaction which is initiated by calcium oscillation during sperm penetration, whereby the intracellular granules induces the membrane of the granules to fuse with ooplasma and the cortical granule contents are then exposed to the perivitelline space (Nedambale *et al*., 2004). The ZP has a role in the blockage of polyspermy during fertilization. After one sperm has penetrated the oocyte, the ZP develops a resistance to proteolytic digestion as a consequence of cortical granule exocytosis that occurs soon after fertilization by masking the sites of attack to proteolytic enzymes or cross-linking the ZP to prevent unfolding of proteins (Wong & Wessel, 2006). It has been observed by Dean, (2004) that the ZP proteins remain inter- and intramolecularly bound by means of disulphide bonds Coy *et al*. (2008) discovered

that the zona pellucida of freshly ovulated pig and cow oocytes harden considerably before fertilization. This pre-fertilization ZP could be involved in the control of polyspermy. High incidences of polyspermy are observed when *in vitro* fertilization is performed than in natural mating in the oviducts (Talpot and Danderkar, 2003). Oocytes are mostly penetrated by the sperm within 4 to 6 hours of incubation with pronuclei forming between 6 and 10 hours after sperm addition (Eid *et al*., 1995). Incubation of oocytes with sperm for more than 6 hours can lead to high rates of polyspermy. High sperm concentration increases chances of polyspermy *in vitro* (Galli and Lazzari, 2008).

2.5.5 Evaluation of sperm fertilizing capability

The purpose of semen evaluation procedures is to ensure that only good quality and highly fertile semen is used for IVF purposes (Giritharan *et al*., 2004). The prediction of sperm fertilizing ability has great economic importance for breeding herds when artificial insemination is used and also good sperm is needed to fertilize oocytes in vitro (Gadea, 2005). To be able to know the fertilizing ability of sperm cells, there methods or ways used to evaluate for sperm motility. However there are conditions that needs to be adhered to before semen evaluation, whereby semen has to be diluted with a suitable extender or other medium so that individual sperm cells can be observed easily (about 10 x 10^6 sperm/ml work well). A contrast microscope is ideal, although for sperm extended with milk extenders, fluorescence microscope can be used (Seidel, 2012). The opinion of Seidel, (2012) stipulates that a progressive motility is when sperm cells are moving in a forward progression at a reasonable speed, however a non-progressive motility is when a circular movement is observed and when tails are moving but with little or no forward movement. Non-progressive motility is generally not a good measure of potential fertility (Brito, 2010). A method which is widely used in IVF is the subjective assessment of sperm motility which is done usually by visual assessment on a microscope fixed with phase-contrast optic (Nedambale *et al*., 2004). However the subjective assessment is considered to be unreliable (Schenk *et al*., 2009). Another method used to evaluate motility and morphology is computer aided sperm analysis (CASA), it is an objective

method used for semen evaluation. Flow cytometry has also been used to evaluate mammalian spermatozoa (Rodriquez-Martinez, 2001). Flow cytometry is used to analyse many structures and functional characteristics of sperm cells, such as cell membrane integrity, mitochondrial activity, changes in spermatozoa surface during capacitation (Katska-Ksiazkiewicz *et al*., 2005).

2.5.6 Bull effect in IVF

According to Jamil *et al*. (2007), bull differ in their ability to fertilize the oocyte and produce offsprings in both the natural and the artificial breeding systems (AI, IVF). Sperm from different bulls also differ in their ability to fertilize oocytes *in vitro*, thus the cleavage rate at the early stages can be used to differentiate bulls of low and high fertilizing ability. Leahy *et al*. (2011) stipulated that different ejaculates of the same bulls can as well have an effect on the fertilization of cattle oocytes. The differences between bulls appear to be dependent on their individual characteristics, such as the mechanism by which the sperm of an individual bull activates the oocyte and triggers synthesis of compounds needed for the cell cycle (Ward *et al*., 2002). However Tsuzuki *et al*. (2010) suggested that there are reagents that can be used such as: caffeine, heparin to overcome variation of bull effect during IVF. Heparin has the ability to stimulate both the acrosome reaction and induce capacitation of cattle sperm (Parrish *et al*., 2001). For these to be possible heparin must first bind to bull sperm with the aid of cattle seminal plasma proteins (BSPs) that binds to the epididymal sperm at ejaculation (Manjunath *et al*., 2002)

2.6 *In vitro* culture

Oocyte or embryo IVC is an assisted reproductive technology (ART) that enables embryo development *in vivo*. This involves artificial culture of fertilized oocytes or embryos in essentially standard cell culture conditions until they are ready to be transferred to the recipient animals. Embryos obtained from ruminant species have been cultured in a number of defined and undefined media. A defined medium is a medium which is prepared using identifiable components prior to embryo culture and

that the components must address the biochemical needs of the embryo as it develops and the physical environment (Nasar *et al*., 2012). Embryo yield and survival usually differ between the different culture systems and culture media (Camargo *et al*., 2006).

2.7 *In vitro* culture systems

To date there are different types of culture systems that available for culturing zygotes that resulted from in vitro fertilized oocytes for further embryonic development. These culture systems can be classified as: undefined; semi-defined and fully defined (Vanrose *et al*., 2001). The most common media used in these culture systems are SOF (synthetic oviductal fluid), KSOM, and tissue culture medium (TCM199). Rizos *et al*. (2002) observed that the culture system and the composition of the medium can affect embryo quality while the quality of the oocyte is regarded as the major determinant of the blastocyst yield. However Pereira *et al*. (2005) emphasized that the culture environment which the embryos are exposed to after fertilization is the key in determining blastocyst quality.

2.8 Defined and semi-defined culture media

Mammalian embryos are cultured in a variety of chemically defined and undefined media. A chemically defined medium has been described as a liquid prepared from a couple of chemicals, the concentration of components in a defined media are all known, which enables the evaluation of the effects of changing concentration on embryonic develop (Bavister, 1992). The contents that are usually found in a defined culture media at known concentrations include: inorganic salts, amino acids, vitamins and energy source (Thompson, 2000). The semi-defined or defined systems are characterised by a culture media without somatic cells and serum (Abe *et al*., 2002). Chemically defined media such as C. Rosenkrans (CR1), synthetic oviduct fluid (SOF), and KSOM, have been developed in an attempt to satisfy the metabolic requirements for embryonic development *in vitro* (Sung *et al*., 2004). In the undefined culture medium is a liquid containing any biological fluid where the

composition and the components can vary considerably. Serum is one of the main components in an undefined culture system. It can provide many beneficial factors to the embryo such as amino acids, vitamins, growth factors and energy substrates; however, Bavister (1992) discovered that these can contaminate the culture media with embryotoxic factors.

2.9 Culture media

The IVC protocols have since been further improved and allowed the development of superior cell free media. Media used do not only influence embryonic development, but also have an effect on embryo survival following cryopreservation (Nedambale *et al*., 2004).Culture media tend to be species specific. There are different culture systems available for IVF oocytes. The most commonly used media in cattle which supports embryonic development *in vitro*, is synthetic oviduct fluid (SOF). This medium, when supplemented with amino acids, has the ability to support embryonic development in ruminants. The SOF has been formulated to mimic the oviductal fluid, which is a complex medium emanating from the blood and active secretion from cells of the epithelium (Mahoete *et al*., 2010). Apart from SOF, other culture media can be used such as tissue culture medium 199 (TCM), Hams-F10 and tyrodes medium (Camargo *et al*., 2006). Some culture media have been reported to be successful for cattle embryo culture, which include potassium simplex optimization medium (KSOM) and Charles Rosenkrans medium (CR1) (Mahoete *et al*., 2010). Nedambale et al. (2004) discovered that KSOM-BSA in combination with SOF-FCS resulted in higher blastocyst formation as well as higher hatching rate.

2.10 Function of cumulus cells during fertilization

Cumulus cells communicate to the oocyte across ZP through corona radiate cells which penetrate the ZP and form gap junctions with oolemma. These intercellular communications allow metabolic transfer as molecules of small molecular weight and help in nutrition of oocyte and oocyte growth and maturation (Kakkassery *et al*., 2010). Cumulus oophorus is unique to the egg of eutherian mammals. It consists of

a mass of granulosa cells surrounding the oocyte. The cumulus oophorus expands after ovulation due to the deposition of a proteoglycan matrix. Cumulus cells are involved in oocyte growth and maturation. There are two specific populations of granulosa cells which can be distinguished by the following: (1) cumulus granulosa cells, which enclose the oocyte with the corona cells as the innermost layers and (2) mural granulosa cells which are lining the follicular wall (Van Soom *et al*., 2002). During follicular development, oocyte quality is affected by the communication between the oocyte and surrounding cells. The removal of cumulus cells before IVM decreases the quality of oocytes during maturation (Zhang *et al*., 2010). Cumulus and mural granulosa cells together with the oocyte form a gap-junction-mediated syncytium, which is essential for oocyte growth to proceed. The granulosa cells supply the oocytes with nutrients and connect them to external world.

During a natural fertilization process, i.e. bringing oocytes and sperm together as it happens in a petri dish, the cumulus cells definitely improve the fertilization rates in most mammalian species, the removal of cumulus cells before IVF has decreased sperm penetration in cattle and in pigs (Van Soom *et al*., 2002). However species dependence is obvious since it was found that in different mouse strains cumulus removal did not affect fertilization rates (Van Soom *et al*., 2007). Although cumulus cells are not critical for IVF, they do in some species and definitely improve IVF rates. The increased volume of the cumulus cells after maturation is important, especially in species with a wide ampulla and a relatively low number of sperm in the oviduct since it makes it more likely that a sperm will encounter the oocyte. The radial orientation of the cumulus cells themselves could guide the sperm to the oocyte and that secretions of the cumulus cells closest to the oocyte create an attractant gradient for sperm within the cumulus oophurus (Eisenbach, 1999).

2.11 Factors affecting oocyte quality

The oocytes have a unique ability to combine its own world components with those of the incoming sperm, followed by the creation of an embryo (Mtango *et al*., 2008).

The definitive test of the oocytes quality is its ability to be fertilized and develop to the blastocyst stage, to establish pregnancy and produce a live calf (Lonergan *et al*., 2001). An oocyte is a highly differentiated, molecular complex product developed through the process of gametogenesis (Mtango *et al*., 2008). The oocyte is expected to combine the two haploid gametes into a single embryonic genome.

2.12 Grading oocytes

According to Torner *et al*. (2008), oocytes quality can be assessed morphologically based on the compactness of the cumulus cells surrounding the oocyte and the intactness of the cytoplasm. Khandoker *et al*. (2001), has classed the oocytes into four groups/grades: Grade A: oocytes completely surrounded by cumulus cells; Grade B: oocytes partially surrounded by cumulus cells; Grade C: oocytes not surrounded by cumulus cells and Grade D: degeneration observed both in oocytes and their cumulus cells. Grade A and B are considered as good acceptable good quality oocytes, while grade C and D are considered to be abnormal. However morphological evaluation alone is insufficient to distinguish competent oocytes that have the ability to cleave, develop into blastocyst and bring about full-term pregnancy (Lonergan *et al*., 2003).

The Brilliant cresyl blue (BCB) staining was used previously to select good quality oocytes for IVM-IVF in pigs (Ericsson *et al*., 2000) and cattle (Pujol *et al*., 2000). During the course of their growth, immature oocytes are known to synthesise a variety of proteins, one of these proteins is the glucose-6-phosphate dehydrogenase (G6PDH) (Wasserman, 1988). The activity of this enzyme is reduced once the growth phase of the oocytes is complete and oocytes are likely to have achieved a developmental competency. The BCB is a stain which is degraded by the G6PDH enzyme which is found in access amounts in growing oocytes, this results in the growing oocytes appearing to be colourless whereas the grown oocytes with reduced activity of G6PDH having a blue cytoplasm (Torner *et al*., 2008).

2.13 Factors affecting IVEP

2.13.1 Heat stress

Heat stress has been shown to be harmful to cattle oocytes and embryos. Wolfenson *et al*. (2000) reported that Holstein cows have lower reproductive performance in autumn than in winter, which is likely a late effect of high temperature during the summer. Oocytes obtained at the beginning of autumn are of low quality, and quality improves as the winter season approaches. The effect of heat stress may also interfere with follicular development and with the secretion of hormones such as LH and progestone (Rensis and Petters, 2003).

2.13.2 Oocyte diameter

Oocytes from larger follicle of >5mm mature better compared to oocytes from smaller follicles of 2-5mm (Crozet *et al*., 2000). Oocytes from larger follicles were also observed to produce higher rates of morula and blastocysts (Arlotto *et al*., 1996). Lechniak *et al*. (2002) observed that oocytes from smaller follicles tend to follow an abnormal path of meiotic maturation resulting in the disturbance of the maturation process *in vitro* while they can also die through the process of atresia.

2.13.3 Follicular fluid

Recommencement of meiosis is triggered either by hormonal induction *in situ* or spontaneously after the release of a cumulus-enclosed oocyte from the follicles. Follicular fluid (FF) is a fluid which fills the cavity known as the antrum inside the follicle. The FF is produced naturally and accumulates proteins and transcripts that will guide the process of maturation, fertilization and initiate embryonic development (kruip *et al*., 2000). However it has been stipulated by Dostal and Pavla, (1996); Ayoub and Hunter, (1993), that follicular fluid from larger follicles appeared to have less inhibitory effect on oocyte maturation as compared to FF from small and medium follicles. Maturation of oocytes in FF at 100 % levels has been found to

inhibit maturation. Elmileik *et al*. (1995) reported that the addition of follicular fluid at the concentrations of 10-20 % has enhanced maturation and fertilization rates and subsequent embryonic development in cattle.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study site

This study was conducted at the Germplasm Conservation and Reproductive Biotechnologies (GCRB) laboratories of the Agricultural Research Council in Irene (Pretoria), Animal Production Institute. The study was done during the months of September to December, 2013. The area is located at 25º 55' south latitude and 28º 12' east longitudes in Pretoria, South Africa and it is situated on the Highveld with altitude of 1525 m above sea level (Webb *et al*., 2004).

3.2 Experimental design, treatment and procedures

All the required materials for the experiment were purchased in advance, prior to the commencement of the study. All chemicals were purchased from sigma-aldrich® (USA), unless indicated.

Experiment 1: comparison of aspiration and slicing methods on the recovery of oocytes from cow ovaries

Ovary collection

Ovaries were collected from freshly slaughtered local cow carcasses (Nguni, Bonsmara and Afrikaneer) at the local abattoir (Morgan abattoir in springs, South Africa). The ovaries were immediately transported after collection to the GCRB laboratory in 0.9 % saline water in a thermo flask at 37 °C. They were transported within two hours following collection. Upon arrival at the laboratory, the ovaries (Figure 1) were washed with 0.9 % saline water to remove excess blood. Then, the ovaries were transferred into a dish containing saline water and placed in a water bath at 37 °C.

Slicing of ovaries

The ovaries, as shown in Figure 1, were trimmed free of any tissues on their surfaces and they were placed in a searching petri dish containing modified dulbecco phosphate buffered saline (mDPBS), which was modified by adding 1 % polyvinyl alcohol and 10 % antibiotic. The ovaries (Figure 1) were sliced with a sharp sterile blade. The ovaries were held by the forceps over the dish to ensure easy slicing. The recovered oocytes were searched under the microscope. They were washed three times in mDPBS.

Figure 1: cow ovaries collected from the abattoir.

Aspiration of ovaries

Aspiration was carried out using a 10 ml syringe and an 18-gauge needle. The needle was pushed inside the ovaries and suck out the follicular fluid of visible follicles. The recovered follicular fluid was then mixed together with modified Dulbecco phosphate buffered saline (mDPBS). The oocytes were allowed to settle for about 10 minutes. Once the pellet was formed, the supernatant which was the top part was removed without disturbing the pellet. The pellet was washed again with mDPBS and the remaining fluid was transferred into a petri dish containing mDPBS for searching of oocytes. The recovered oocytes were washed three times in mDPBS and three times in pre-warmed tissue culture medium (m199) + 10 % foetus cattle serum (FBS). The oocytes were searched under a microscope and under the lamina floor to ensure sterility.

Grading oocytes

The oocytes which were retrieved by aspiration and slicing method, then divided into three groups according to the level of cumulus cells surrounding the oocytes. The three groups were labelled as follows: (1) oocytes completely covered with more than two layers of cumulus cells, (2) oocytes partially surrounded by cumulus cells and (3) denuded oocytes. After washing the oocytes, they were exposed for 90 min in 26 µM of brilliant cresyl blue (BCB) stain at 39 °C with 5 % CO_2. Following the 90 min of exposure to BCB, the oocytes were washed three times in mDPBS and classified into two groups depending on the colour of their cytoplasm. The oocytes with the blue cytoplasm were recorded as BCB+ and the oocytes with a colourless cytoplasm were recorded as BCB-.

Experiment 2: Effect of different concentrations of LH, FSH and estradiol on the *in vitro* maturational rate of cow oocytes selected by brilliant cresyl blue staining

Three different concentrations of hormones were used to supplement the maturation media for IVM. The aspiration technique was used to recover oocytes from cow ovaries. Before maturation, oocytes were washed six times, whereby they were washed three times in mDPBS and three times in M199 + 10 % FBS. Before maturation the oocytes were exposed to BCB. A group of oocytes which was not exposed to BCB was used as a control. The medium used was supplemented with different concentrations of Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and Estradiol (E_2). The numbers of BCB+, BCB- and no BCB oocytes were equally distributed randomly into the three medium. The first group of oocytes was matured in the medium supplemented with 0.5 µg/ml of FSH, 5 mg/ml of LH and 2 µg/ml of E_2. The second group was matured in a medium supplemented with 1 µg/ml of FSH, 6 mg/ml of LH and 2.5 µg/ml of E_2. The third group was matured in a medium supplemented with 1.5 µg/ml of FSH, 7 mg/ml of LH and 4.5 µg/ml of E_2. A

four well dish was used for maturation and each well contained 500 µl of maturation medium coved with 250 µl of oil to prevent evaporation. After the insertion of COCs in the maturation media, the dish containing oocytes was incubated at 39 °C with 5 % CO_2 in humidified air for 24 hours.

Following incubation for 24 hours in maturation medium, the oocytes were checked for the expansion of cumulus cells as a sign of maturation, maturation was also assessed by the extrusion of the first polar body on the oocytes. The extrusion of polar body was examined under the micro-manipulation microscope, USA, using the Oosight Imaging system. The number of oocytes with the presence and absence of polar bodies were recorded.

Table 3.1 Treatment codes for experiment 1

Treatment codes	Description
COCs	Cumulus oophurus complex (oocytes that are completely covered with cumulus cells
SCOCs	Oocytes recovered using slicing and are completely covered with cumulus cells
SDO	Oocytes recovered using slicing and do not have cumulus cells (denuded oocytes)
SPC	Oocytes recovered using slicing and partially covered with cumulus cells
ACOCs	Oocytes recovered using aspiration and are completely covered with cumulus cells
ADO	Oocytes recovered using aspiration and do not have cumulus cells (denuded oocytes)
APC	Oocytes recovered using aspiration and partially covered with cumulus cells

Table 3.2 Treatment codes for Experiment 2

Treatment code	Description
BCB	Brilliant cresyl blue stain
BCB+T_1	Oocytes with blue cytoplasm and treated with 0.5 µg/ml of FSH, 5 mg/ml of LH and 2 µg/ml of E_2
BCB+T_2	Oocytes with blue cytoplasm and treated with 1 µg/ml of FSH, 6 mg/ml of LH and 2.5 µg/ml of E_2
BCB+T_3	Oocytes with blue cytoplasm and treated with 1.5 µg/ml of FSH, 7 mg/ml of LH and 4.5 µg/ml of E_2
BCB-T_1	Unstained oocytes that were treated with 0.5 µg/ml of FSH, 5 mg/ml of LH and 2 µg/ml of E_2
BCB-T_2	Unstained oocytes that were treated with 1 µg/ml of FSH, 6 mg/ml of LH and 2.5 µg/ml of E_2
BCB-T_3	Unstained oocytes that were treated with 1.5 µg/ml of FSH, 7 mg/ml of LH and 4.5 µg/ml of E_2
No BCBT_1	Oocytes not exposed to the brilliant cresyl blue (BCB) and treated with 0.5 µg/ml of FSH, 5 mg/ml of LH and 2 µg/ml of E_2
No BCBT_2	Oocytes not exposed to the BCB stain and treated with 1 µg/ml of FSH, 6 mg/ml of LH and 2.5 µg/ml of E_2
No BCBT_3	Oocytes not exposed to the stain and treated with 1.5 µg/ml of FSH, 7 mg/ml of LH and 4.5 µg/ml of E_2

Experiment 3 Effect of cleavage/embryonic development of oocytes cultured with or without cumulus cells

Thawing process

Stored bull semen (at -196°C) was used for IVF. The frozen straws of semen from the liquid nitrogen tanks were thawed for ten seconds in the air and placed for one minute in the water at 37 °C. The straw was dried and cut on both of the sealed ends and the contents inside the straw were collected into 15 ml Falcon ® tube. Five µl of the semen was used to check for motility using the computer aided sperm analysis (CASA) before fertilization. An average motility of 60 % was used for IVF.

Capacitation process

The frozen-thawed semen was mixed together with 6 ml pre-warmed Bracket and Oliphant (BO) wash medium containing caffeine. The mixture was centrifuged at 1500 rpm for 8 minutes at 37 °C. Following centrifusion a pellet was formed at the bottom of the tube, and the top part was removed carefully using the serological pipette without disturbing the sperm pellet, the same level of BO medium was added to the pellet and the mixture was centrifuged for the second time at the same speed. After centrifusion the supernatant was removed leaving the pellet at the bottom of the tube. The sperm pellet was diluted with BO IVF medium depending on the number of drops having oocytes and the concentration of the sperm cells.

IVF process

Only Cumulus Oophurus Complexes (COCs) were used for *in vitro* fertilization. Before fertilization, sperm motility of the capacitated semen was evaluated using computer aided sperm analysis (CASA). The total sperm motility of 60 % was considered as acceptable for IVF. Prior to IVF, five wash drops of 100 µl were made in 1008 falconi ® petri dishes and covered with 3 ml of mineral oil and 7 drops of 50 µl of the same medium were also created in similar dishes, these drops were used as *in vitro* fertilization drops. The oocytes were first washed in pre-warmed 100 µl of

Brackett and Oliphant's (BO) fertilization medium containing 6mg/ml essential fatty acid-free (FAF) Bovine Serum albumin (BSA) and 10 µg/ml of heparin. Up to 20 oocytes were placed in 50 µl of BO medium covered with mineral oil. Fifty µl of capacitated diluted sperm from an Nguni bull with good fertility history was used to fertilize the oocytes. The oocytes were incubated together with the sperm cells for a period of 6 hours in 5 % CO_2 in humidified air at 39 °C. Following fertilization, the oocytes were divided into two groups; one group of oocytes was denuded by vortexing for 1 min and 30 seconds before culture while the other group of oocytes was cultured with cumulus cells. The oocytes were washed three times in pre-incubated M199 + 10 % FBS solution and again washed in 100 µl of SOF-BSA. The presumptive zygotes were cultured in synthetic oviductal fluid (SOF) containing 1mg/ml BSA in 50 µl droplets for further embryonic development. The dish containing the presumptive zygotes was placed in a modular chamber which was filled with three mixed gases (oxygen at 5 %, CO_2 at 5 % and 90 % humid air which was controlled by placing water in the modular chamber) and they were incubated in the CO_2 incubator. After 48 hours of culture cleavage rate was recorded as a sign of fertilization and SOF-BSA was changed to SOF-FBS (5 %). The process of determining fertilization was performed under the micro manipulation microscope using the Oosight Imaging system.

Experiment 4 Effect of fresh and frozen thawed semen on the cleavage/embryonic development of cow oocytes

Semen was collected from the Nguni bull on the same day of IVM; fresh semen was diluted with egg yolk citrate extender. Semen was mixed with BO sperm wash medium in a 14 ml test tube and centrifuged twice at 1500 rpm speed for 8 min at 37 °C. After centrifusion the top liquid part was removed and the supernatant was left in the tube. The BO IVF medium was added to the supernatant. A 50 µl of medium containing sperm cells was added to the 50 µl of fertilization drops each containing 20-25 oocytes. The oocytes and sperm cells were incubated for 6 hours at 39 °C in humidified air in 5 % CO_2. Following the 6 hours of IVF, the presumptive zygotes were washed three times in M199 + 10 %FBS, again washed in the 100 µl drops of

SOF-BSA. The oocytes were further cultured in SOF-BSA for two days. On the second day of culture fertilization rate was recorded based on the cleavage of the oocytes.

3.3 Data analysis

Experiment 1:

Data on comparison of aspiration and slicing methods on the recovery of oocytes from cow ovaries was analysed using general linear model procedure of SAS with 4 replications per treatment. Differences considered to be significant ($P<0.05$) were compared by fisher's least significance difference (LSD) test. A completely randomised design was used.

Experiment 2:

Data on the effect of different concentrations of LH, FSH and estradiol on the *in vitro* maturational rate of cow oocytes selected by brilliant cresyl blue staining was analysed using general linear model procedure of SAS with 4 replications per treatment. Differences considered to be significant ($P<0.05$) were compared by fisher's least significance difference (LSD) test. A randomised complete block was used.

Experiment 3:

Data on the effect of cleavage/embryonic development of oocytes cultured with or without cumulus cells were analysed using general linear model procedure of SAS with 5 replications per treatment. Differences considered to be significant ($P<0.05$) were compared by fisher's least significance difference (LSD) test. A completely randomised design was used.

Experiment 4:

Data on the effect of fresh and frozen thawed semen on the cleavage/embryonic development of cow oocytes were analysed using general linear model procedure of SAS with 5 replications per treatment. Differences considered to be significant ($P<0.05$) were compared by fisher's least significance difference (LSD) test. A completely randomised design was used.

CHAPTER 4

RESULTS

4.1 Comparison of slicing and aspiration methods of oocytes recovery from cow ovaries

The results for comparing the effects of slicing and aspiration methods on oocytes recovery from cattle ovaries are presented in Tables 4.1 and 4.2. The Slicing technique resulted in higher (P<0.05) number of oocytes compared to the aspiration technique. There was no significant difference (P>0.05) on the quality of oocytes recovered using slicing and aspiration. However slicing recovered a higher (P<0.05) number of denuded oocytes (DO). The denuded oocytes resulted in a higher (P<0.05) number of unstained oocytes compared to cumulus oophurus complexes and the oocytes that were partially covered with cumulus cells.

Table 4.1 Effect of slicing and aspiration technique on the quantity of recovered oocytes from cattle ovaries (Mean ± SD).

Treatment	Ovaries	Oocytes
Slicing	31	121.25 ± 39.54[a]
Aspiration	28	93.75 ± 7.41[b]

a,b Values with different superscripts within the column differ significantly at P<0.05.

Table 4.2 The quality of recovered oocytes selected by the brilliant cresyl blue stain (mean ± SD)

Treatment code	Oocytes	BCB+ (%)	BCB- (%)
SCOCs	249	60.75 ± 34.56[a]	1.50 ± 1.73[c]
SDO	201	1.50 ± 2.38[b]	48.75 ± 2.22[a]
SPC	35	4.50 ± 3.69[b]	4.25 ± 2.63[c]
ACOCs	218	53.75 ± 13.15[a]	0.75 ± 0.96[c]
ADO	70	1.25 ± 1.50[b]	16.25 ± 8.54[b]
APC	52	9.50 ± 7.59[b]	3.50 ± 3.69[c]

[a,b,c] Values with different superscripts within columns differ significantly at P<0.05.

SCOCs= cumulus oophorus complexes recovered by slicing

SDO = denuded oocytes recovered by slicing

SPCO = partially covered oocytes recovered by slicing

ACOCs= cumulus oophorus complexes recovered by aspiration

ADO = denuded oocytes recovered by aspiration

APCO = partially covered oocytes recovered by aspiration

BCB+ = brilliant cresyl blue plus

BCB- = brilliant cresyl blue negative

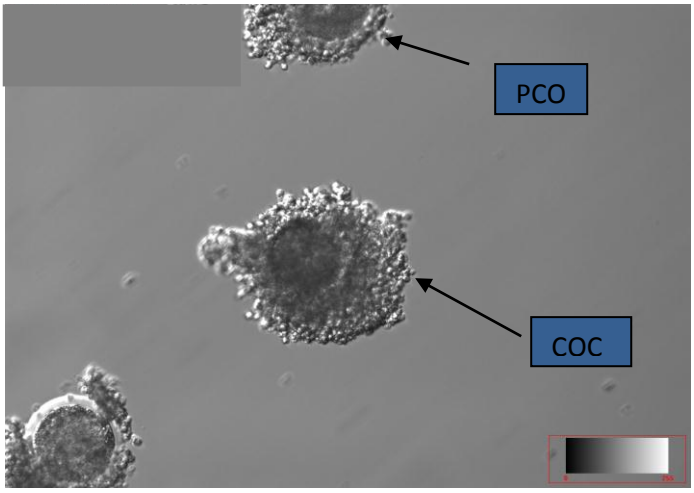

Figure 2 Immature oocytes recovered from cow ovaries at 10X/0.25 RC1 magnification

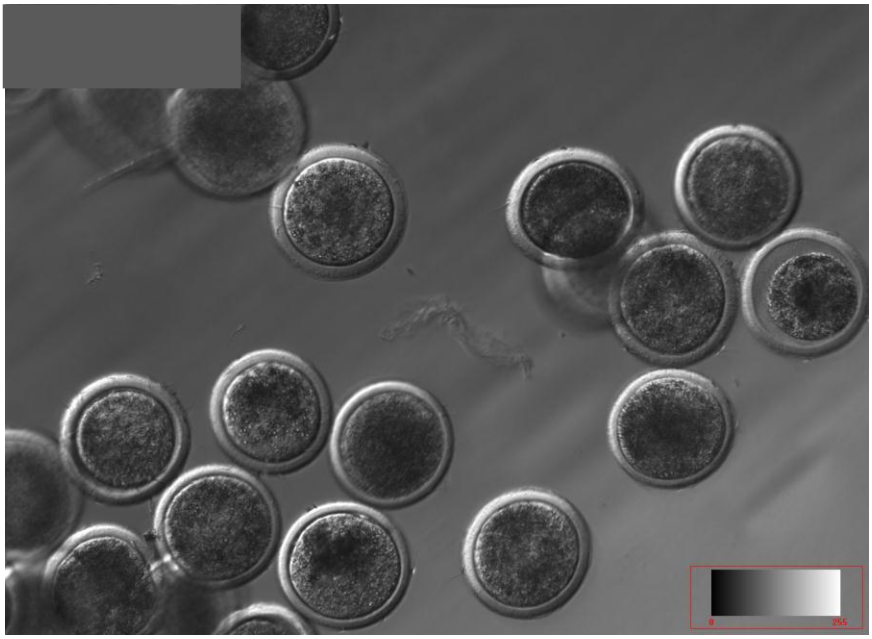

Figure 3 Denuded oocytes at 10X/0.25 RC1

4.2 Effect of different concentrations of LH, FSH and estradiol on the *in vitro* maturational rate of cow oocytes selected by brilliant cresyl blue staining

The results of the effect of different hormonal supplementation on the maturation of cow oocytes are presented in Table 4.3. The means of polar body extrusions of oocytes that were matured in the m199 medium supplemented with 1 µg/ml of FSH, 6 mg/ml of LH and 2.5 µg/ml of E_2 were found to be significantly higher ($P<0.05$) than the medium supplemented with 1.5 µg/ml of FSH, 7 mg/ml of LH and 4.5 µg/ml of E_2 and 0.5 µg/ml of FSH, 5 mg/ml of LH and 2 µg/ml of E_2. The BCB+ oocytes obtained higher ($P<0.05$) polar body extrusions in all the treatments than the BCB- oocytes. The oocytes that were not exposed to the BCB stain and the BCB+ oocytes obtained similar ($P>0.05$) number of polar body (Figure 5) extrusions. The BCB- group of oocytes recorded higher ($P<0.05$) number of oocytes without the extrusion of polar compared to the BCB+ group, the BCB- group has also exhibited a significantly lower number of polar body extrusions in comparison with the BCB+ group.

Table 4.3 Effect of different hormonal concentration in maturation media for the maturation of BCB selected cow oocytes (%) (Mean ± SD)

Oocyte classification	Treatments	Oocytes (n)	Polar body extrusions (%)	No polar body (%)
No BCB	T1	234	16.6 ± 4.11^{cd}	83.3 ± 4.78^{a}
	T2	232	60.3 ± 10.39^{a}	39.5 ± 4.69^{b}
	T3	198	48.0 ± 6.24^{bc}	52.0 ± 1.90^{b}
BCB-	T1	132	3.03 ± 1.41^{d}	96.9 ± 1.83^{ab}
	T2	136	8.1 ± 2.22^{d}	91.9 ± 2.02^{ab}
	T3	135	2.2 ± 0.95^{d}	97.8 ± 3.62^{b}
BCB+	T1	190	22.6 ± 5.73^{cd}	77.4 ± 4.54^{ab}
	T2	186	65.6 ± 1.68^{ab}	34.4 ± 3.64^{b}
	T3	187	48.1 ± 7.14^{bc}	51.8 ± 1.50^{b}

^{a,b,c,d} : Values with different superscripts within column differ significantly at $P<0.05$

T1= 0.5 µg/ml of FSH, 5 mg/ml of LH and 2 µg/ml of E_2

T2= 1 µg/ml of FSH, 6 mg/ml of LH and 2.5 µg/ml of E_2,

T3= 1.5 µg/ml of FSH, 7 mg/ml of LH and 4.5 µg/ml of E_2 and

No BCB = oocytes without Brilliant cresyl blue stain

BCB+ = oocytes with blue cytoplasm after exposure to BCB

BCB- = oocytes with a colourless cytoplasm after exposure to BCB

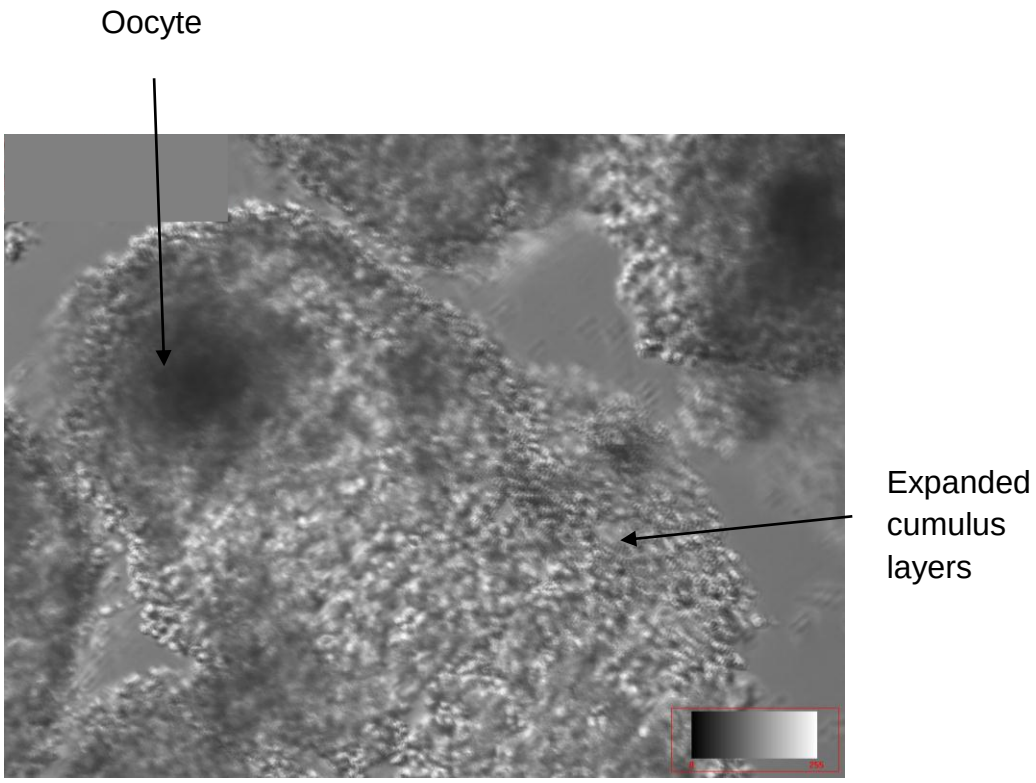

Figure 4 A mature oocytes with cumulus cells at 10X/0.25 RC1 magnification

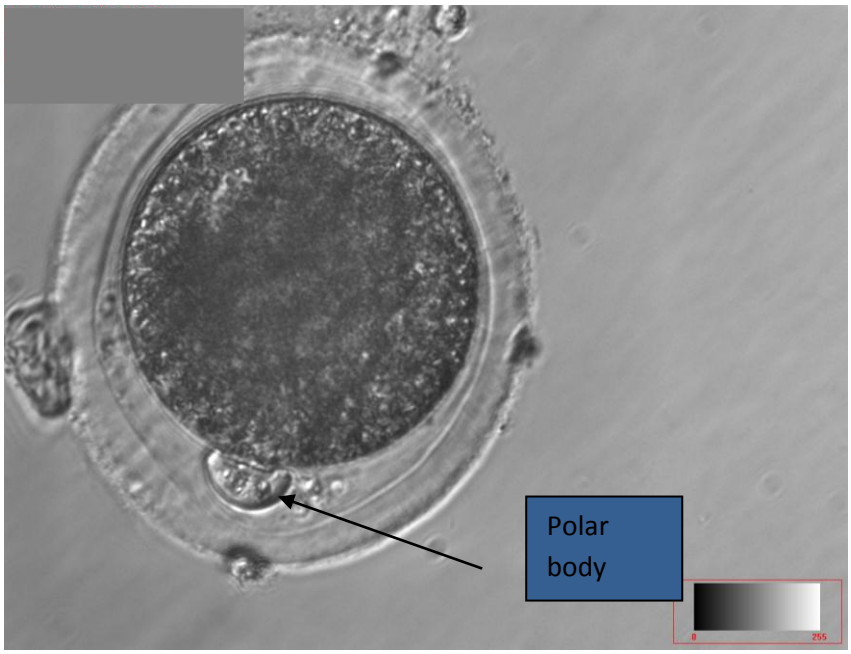

Figure 5 A mature oocyte with a protruded polar body following removal of cumulus layer at 40X/0.60 RC3 magnification

4.3 Comparison of cleavage/embryonic development of oocytes in the presence and absence of cumulus cells

The effect of cumulus cells on the cleavage/ embryonic development of cow oocytes *in vitro* are presented in Table 4.4. The cumulus oophurus complexes exhibited a higher rate ($P<0.05$) of 2-4 cell embryos (Figure 7) as compared to the denuded oocytes. There was no difference ($P>0.05$) of the harvested 8 cell embryos between cultured COCs and denuded oocytes. Similarly, the oocytes that were cultured with cumulus cells and the denuded oocytes had the same ($P>0.05$) morula (Figure 8) development. There was, however a difference ($P<0.05$) in blastocyst (Figure 6) development between the oocytes that were cultured with cumulus cells and those that were denuded of cumulus cells before culturing.

Table 4.4 Fertilization rate (%) and cleavage/embryonic development of oocytes in the presence and absence of cumulus cells (Mean ± SD).

Treatments	Oocytes	2-4 cells (%)	8 cells (%)	Morula (%)	Blastocyst (%)
COCS	340	29.0 ± 15.12[a]	5.0 ± 1.14[a]	32 ± 1.30[a]	32 ± 1.39[a]
Denuded oocytes	340	20.0 ± 6.04[b]	2.0 ± 1.92[b]	27 ± 0.84[a]	13 ± 0.49[b]

[a,b] : Values with different superscripts within rows differ significantly at *P*<0.05.

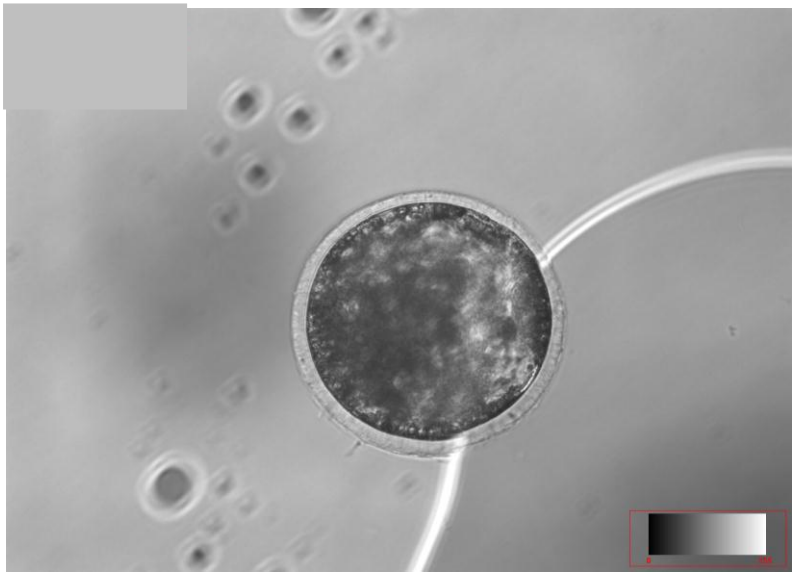

Figure 6 A blastocyst aged 7 days at 20X/0.45 RC2 magnification

4.4 Effect of fresh and frozen thawed semen on the fertilization rate of cow oocytes

The effect of frozen thawed and fresh semen on the fertilization rate of cow oocytes is represented in the Table 4.5. The 2-4 cell embryos differed (P<0.05) between the oocytes that were fertilized using fresh semen to those fertilized with frozen thawed semen. However, 8 cell embryo development for oocytes fertilized with frozen thawed and fertilized with fresh semen was the same (P>0.05). Number of embryos

that reached the morula stage was higher (P<0.05) in oocytes which were fertilized with fresh semen as compared to that from those fertilized with frozen thawed semen. The was a difference (p<0.05) in the blastocyst development of oocytes fertilized with fresh semen compared to those oocytes fertilized with frozen thawed semen.

Table 4.5 Effect of fresh and frozen thawed semen on the fertilization rate (%) of cow oocytes (Mean ± SD).

Treatment	Oocytes (n)	2-4 cells (%)	8 cells (%)	Morula (%)	Blastocyst (%)
Fresh semen	336	19 ± 1.1^{b}	6.6 ± 7.50^{a}	3.4 ± 0.55^{b}	2.3 ± 0.55^{b}
Frozen thawed semen	336	23 ± 10.29^{a}	6.0 ± 2.55^{a}	13.0 ± 1.82^{a}	8.0 ± 1.34^{a}

[a,b] : Values with different superscripts within the columns differ significantly at P<0.05

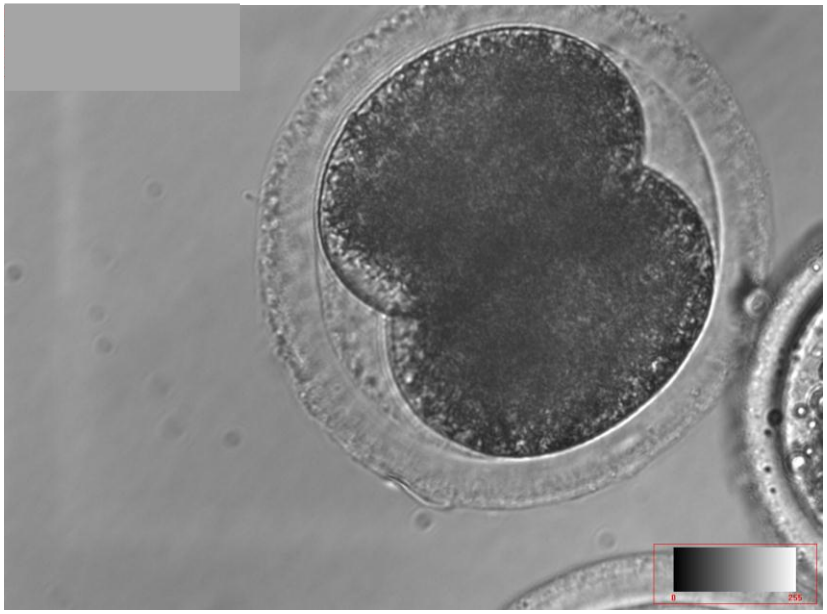

Figure 7 Day 2 cleaved 2 cell embryo at 40X/0.60 RC3 magnification

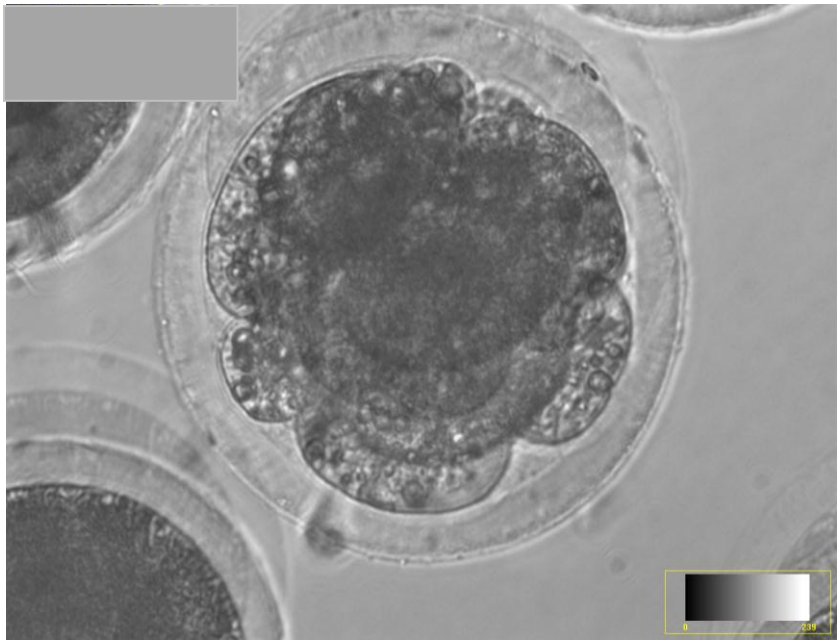

Figure 8 Morula embryo at day 5 of culture at 40X/0.60 RC3 magnification

CHAPTER 5

DISCUSSION

5.1 Comparison of slicing and aspiration methods of oocytes recovery from cow ovaries

In this study, two methods (slicing and aspiration) of recovering oocytes in order to achieve good quality oocytes and quantity were used. The slicing method yielded a higher number of oocytes (121.25 ± 39.54) as compared to aspiration (93.75 ± 7.41). Thus, the lower yield of oocytes when using aspiration may be attributed to the presence of oocytes which are imbedded deep within the cortex and could not be reached by a needle while with slicing the ovary is cut by a blade deeply into the cortex which makes it possible to reach the deeply embedded follicles (Lonergal *et al*., 2003). In this study it was observed that the slicing method produced a lot of debris which obstructed the search of oocytes, this was also accentuated by Ferdous. (2006), that the slicing technique is time consuming and it produces a lot of debris which makes it hard to search for oocytes under the microscope. The results of this study are comparable with the observations of Hogue *et al*. (2011), whereby the author yielded a higher number of oocytes from using the slicing technique but the good quality COCs were obtained when using the aspiration technique. However, morphologically there was no significant difference (60.75 and 53.75%) between the two methods; this was determined by the brilliant cresyl blue stain. Heleil *et al*. (2010) discovered that the brilliant cresyl blue stain has the ability to detect the activity of the glucose-6-phosphate dehydrogenase enzyme which is synthesized more in the growing oocytes while its activity is decreased in oocytes that have finished growth phase. Due to the high activity of the enzyme in the growing up oocytes they are able to convert the blue colour of the staining into colourless. The colourless oocytes are known as the BCB-. The grown oocytes absorb the blue colour of the stain, these types of oocytes are known as the BCB+ oocytes. The denuded oocytes recovered by using both of the methods appeared to be colourless after the exposure of the oocytes to the stain. The slicing technique yielded a higher number of denuded oocytes. The COCs from both of the recovering techniques were regarded as good quality oocytes; this was attributed to their ability to absorb the blue colour of the stain. The good quality COCs recovered by both of the retrieval techniques may be endorsed by the surrounding cumulus cells which play an important role in providing the oocyte with nutrients and energy substrates

that are necessary for the development of oocytes (Sutton *et al*., 2003). In this study the larger group of oocytes which showed the blue colour of the stain where the oocytes which were completely surrounded by cumulus cells followed by those which were partially surrounded by cumulus cells, this study is supported by the study of Torner *et al*. (2008) who observed that the good quality oocytes are the oocytes which are completely covered with cumulus cells and with an intact cytoplasm. This study emphasizes that cumulus cells are important and are involved in oocyte growth and maturation and that cumulus cell concentration is dependent of the efficiency of oocyte harvesting.

5.2 Effect of different hormonal supplementation on the maturation of BCB selected cattle oocytes

The IVM medium with 1 µg/ml of FSH, 6 mg/ml of LH and 2.5 µg/ml of E_2 yielded a higher number of oocytes which extruded polar bodies for both not exposed to BCB (60.3 ± 10.395%) and for BCB+ (65.6 ± 1.68%) oocytes. Some of the growing oocytes which did not absorb the stain under the same medium managed to protrude polar bodies (8.1 ± 2.22%). This clearly indicates that IVM medium supplemented with 1 µg/ml of FSH, 6 mg/ml of LH and 2.5 µg/ml of E_2 is suitable for maturing cattle oocytes of South African origin, and even the oocytes from the atretic follicles can be able to mature *in vitro*. The supplementation of the combination of hormones improves maturation in cattle oocytes (Farag *et al*., 2009). This study is comparable with the study of Younis *et al*. (1991) which reported that the addition of the gonadotropic hormones (LH and FSH) to the maturation medium enhanced cumulus cell expansion of goat oocytes. Chen *et al*. (1994) also suggested that FSH has beneficial effect in the presence of FBS and enhances cumulus expansion of cattle oocytes. The IVM medium with 0.5 µg/ml of FSH, 5 mg/ml of LH and 2 µg/ml of E_2 obtained lower numbers of mature oocytes under the BCB selected oocytes as well as the oocytes which were not exposed to BCB (22.6 ± 5.73, 16.6 ± 4.11 and 3.03 ± 1.41; respectively). The hormonal concentration of 0.5 µg/ml of FSH, 5 mg/ml of LH and 2 µg/ml of E_2 is a standard concentration which is widely used in IVM. This medium has, however, showed that it is unsuitable to mature South African cattle

oocytes. The results of this study showed that the BCB+ had more maturity than the BCB- oocytes in all the three maturation medium whereby many oocytes under the BCB+ (22.6 ± 5.73; 65.6 ± 1.68 and 48.1 ± 7.14) oocytes obtained higher polar body extrusion as compared to the BCB-. Shourbagy (2006) reported that BCB+ oocytes have a larger cytoplasmic volume, and they are more likely to get fertilized and contain far higher numbers of mtDNA copy which have been linked to fertilization outcome in the buffalo and humans. This study is comparable to the study of Wang *et al*. (2012), whereby it was observed that the BCB+ oocytes had higher maturation rates than the BCB- oocytes. Therefore, the BCB+ oocytes have a better developmental competency as compared to the BCB- oocytes. The BCB stain has proven to select the good quality oocytes which have better survival chances and embryonic developmental competency. This study has generated a suitable protocol which can be used for IVM of cattle oocytes of the African origin.

5.3 Comparison of cleavage/embryonic development of oocytes in the presence and absence of cumulus cells

Fertilization is determined by the number of oocytes that cleave during the second day of culture after they have been fertilized using sperm cells (Krisher. 2004). In this study fertilization rate of oocytes cultured with and without cumulus cells was determined by the cleavage rate of the oocytes. Fertilized oocytes cultured with cumulus cells had obtained a higher 2-4 cell (29 %) embryonic development as compared to the oocytes denuded (20 %) of cumulus cells before culture. However, there was no statistical difference between the 8 cell embryonic development and the morula for COCs (5 %) cultured oocytes as well as the denuded oocytes (2 %). The COCs cultured oocytes obtained a higher blastocyst rate (9.2 %) compared to the denuded oocytes (3.7 %), although the oocytes cultured with cumulus cells tended to attach to the culture plates which makes it harder to change them from one medium (SOF-BSA) to another (SOF-FBS) at the second day of culture. The low embryonic development in the denuded oocytes may be ascribed to the damaged cytoplasm due to the removal of cumulus cells by vortexing after fertilization. The role of cumulus cells during embryonic development should further be studied. Thus, the study encourages that oocytes should be cultured with cumulus cells to avoid

damage to the cytoplasm which is caused by vortexing. This study is in line with the study of Taveres *et al*. (2011) which reported that oocytes which are cultured with cumulus cells secrete a series of factors that act in a paracrine manner while stimulating development to the blastocyst stage. However, in this study, the cleavage and blastocyst yield was found to be low when compared with other studies (Nedambale *et al*., 2004; Machatkova *et al.*, 2006; Walters *et al*., 2004). However Nedambale *et al*. (2004) observed that regardless of the method used to obtain oocytes whether from live or slaughtered cows, there is still a problem with *in vitro* matured oocytes, where by a higher number of oocytes may cleave but a lower percentage will reach the blastocyst stage. This may be attributed to unsuitable culture conditions and toxic substances secreted during culture such as ammonia (Khurana *et al.*, 2000). Earl and Kotaras (1997), suggested that the improvement in IVEP technologies could enable more elementary research in the control of early blastocyst development. More studies on *in vitro* culture conditions as well as culture medium should to be conducted in order to find suitable protocols for IVC of South African indigenous cattle oocytes to increase the blastocyst yield.

5.4 Effect of fresh and frozen thawed semen on the fertilization rate of cattle oocytes

Sperm fertility is the main important factor to be considered when conducting the IVEP experiments (Camargo *et al*., 2002). In this study fresh and frozen thawed semen were used for fertilization of cattle oocytes. The oocytes fertilized with frozen thawed semen obtained a better number of 2-4 cell cleavages (23 %) when compared to fresh semen (19 %). Oocytes that were fertilized with frozen thawed semen also obtained higher morula (13 %) and blastocyst (8 %) compared to fresh semen with morula (3.4 %) and blastocyst (2 %). These results are supported by the findings of Parrish (2014), who states that when frozen thawed semen is used, capacitation and acrozomal reaction time is shortened due to extenders, cryoprotectants and cryo-environment. Parrish *et al*. (2001) states that these additions to the sperm cells weaken the ZP membrane which will in turn shorten capacitation time. It was, however, suggested by Parrish (2014) that since many straws can be frozen from an ejaculate of a single bull, it is then advisable to fertilize

oocytes with sperm cells from the same bull to eliminate the bull-bull or ejaculate-ejaculate variability. There was no statistical difference of the 8 cell embryonic development of both the frozen thawed (6.0 ± 2.55) and the fresh (6.6 ± 7.50) semen. This entails that frozen thawed semen promote the same production of cow embryos as compared to fresh semen. However, the blastocyst yield was higher in frozen semen (8.0 ± 1.34) compared to fresh semen (2.3 ± 0.55). The cleavage rates from this study differ with the findings of other studies (Nedambale *et al*., 2004) whereby they got higher cleavage and blastocyst rates. This study is comparable with the findings of Lane (2001), which states that mammalian embryos show low quality and viability when they are under *in vitro* environments or culture. The cleavage rate in this study is an indication that the oocytes were penetrated by sperm cells but, however, a small number developed to the blastocyst stage. This may be due to unfavourable conditions during culture which is supported by Pereira *et al*. (2005) who states that there are indications that the culture system and the composition of the medium can affect embryo quality. This low embryonic development may be influenced by the effect of breed. According Camargo *et al*. (2006), the developmental competence of oocytes and embryos in other stains of mice was low. Embryos developed until the blastocyst stage while others developed until between the 2.4 and 8 cell stages (Goddard and Pratt, 1983). Hence the culture medium used (SOF) was widely tested with the Holstein oocytes rather than the breeds found in South Africa (Nguni, Bonsmara and Afrikaneer). More studies need to be made on the factors affecting embryonic development and how to overcome those factors. Camargo *et al*. (2006) accentuated that there are different culture systems and media which are available for *in vitro* fertilized cattle embryos, and embryonic development differs amongst them. It has been shown in this study that the sperm cells used whether from frozen or fresh semen were able to penetrate through the ZP and fertilize the oocytes, since there was cleavage which was observed and it was not significantly different in both the oocytes fertilized with fresh semen and those fertilized with frozen thawed semen. The effectiveness of the culture system may also differ among laboratories which make it difficult to compare data from different laboratories (Wasserman, *et al*., 2001). It is, therefore, important to test the best culture system or medium which will provide better results for a certain laboratory. Generally, there was no significant difference in embryonic

develop when using fresh semen in comparison with frozen thawed semen. The effectiveness of the frozen thawed semen is as good as the fresh semen which is supported by several authors (Parrish, 2002; Parrish, 2014; Camargo *et al*., 2006) who reported that the fertilization rate of oocytes with either fresh or frozen thawed semen is the same.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

The recovering techniques of cattle oocytes affect their quality and subsequent *in vitro* embryonic development. In this study, slicing and aspiration were used as recovering techniques of oocytes from ovaries of slaughtered cattle. The quality of oocytes in this study was determined by using the BCB stain, whereby the number of BCB+ oocytes was not statistically different between the techniques. These led to the conclusion that both slicing and aspiration obtained good quality oocytes. However, the aspiration technique was a desired method as it is less time consuming compared to slicing.

Immature oocytes recovered from ovaries of slaughtered cattle are heterogeneous and at different stages of growth, *in vitro* maturation has to be carried out so that the oocytes are mature and ready for fertilization. Hormones that are essential for growth were used at different concentrations. In this study the media supplemented with 1µg/ml of FSH, 6mg/ml of LH and 2.5µg/ml of E_2 generated high maturation rates (polar body extrusion) compared to other concentrations which were used. The BCB+ oocytes generated higher polar body extrusions when compared to the BCB- oocytes. This proves that the BCB+ oocytes can be regarded as good quality oocytes since they were able to mature better in all the three different concentrations supplemented in the maturation media. The BCB stain has been proved to be a suitable tool used for the selection of good quality oocytes which can have better chances of survival and subsequently develop into embryos.

In this study cleavage rate was used to measure fertilization rate and performance of oocytes cultured with or without cumulus cells. There was no difference in embryonic development of oocytes fertilized with fresh or frozen thawed semen. Therefore, frozen thawed semen can work as good as the fresh semen.

Further studies should be conducted on *in vitro* culture to find a suitable protocol which will optimise embryo production and blastocyst yield. The IVEP performer must always work aseptically to minimize contamination of the cells and the medium which are being used in the laboratory.

CHAPTER 7

REFERENCES

Abe, H., Yamashita, S., Satoh, T. and H. Hoshi. 2002. Accumulation of cytoplasmic lipid droplets in cattle embryos and cryotolerance of embryos developed in different culture systems using serum-free containing media. *Molecular of Reproduction Development*. 61: 57-66.

Albuz, F. 2009. Induced IVM: A New Approach to Oocyte Maturation *In Vitro*. Doctor of philosophy thesis. The Research Centre for Reproductive Health. University of Adelaide. Australia.

Alm, H., Torner, H., L'ohrke, B., Vergutz, T., Ghoneim, I.M. and W. Kanitz. 2005. Cattle blastocyst development rate in vitro is influenced by selection of oocytes by brilliant cresyl blue staining before IM as indicator for glucose-6-phosphate dehydrogenase activity. *Theriogenology* 63: 2192-2205.

Ayoub, M. and A. Hunter. 1993. Inhibitory effect of cattle follicular fluid on *in vitro* maturation on cattle oocytes. *Journal of Dairy Science* 76: 95-100.

Arlotto, T., Schwartz, J.L., First, N.L. and M.L. Leibfried-Rutledge. 1996. Aspects of follicle and oocyte stage that affect *in vitro* maturation and development of cattle oocytes. *Theriogenology* 45: 943-956.

Apa, R., Lanzone, A., Miceli, F., Mastrandrea, M., Caruso, A., Mancuso, S. and R. Canipari. 1994. Growth hormone induces *in vitro* maturation of follicle and cumulus enclosed rat oocytes. *Molecular and Cellular Endocrinology* 106: 207-212.

Apa, R., Caruso, A., Andreani, G., Miceli, F., Lazzarin, N., Mastandrea, M., Ronsisville, E., Mancuso, S. and A. Lanzone. 1996. Growth hormone stimulates androsterone synthesis by rat theca-interstitial cells. *Molecular and Cellular Endocrinology* 118: 95-101.

Baibakov, B., Gauthier, L., Talbot, P., Rankin, L.T. and J. Dean. 2007. Sperm binding to the zona pellucida is not sufficient to induce acrosome exocytosis. *Development* 134: 933-943.

Baldassarre, H. and C.N. Karatzas. 2004. Advanced assisted reproduction technologies. ART in goats. *Animal Reproductive Science*, 82(83): 255-266.

Baldassarre, H., Furnus, C., Matos, D., Pessi, H. and D. De Matos. 1996. *In vitro* production of sheep embryos using laparoscopic folliculocentesis. *Theriogenology* 45: 707-717.

Bavister, B.D., Rose-Hellenkant, T.A. and T. Pinyopummintr. 1992. Development of *in vitro* matured/in vitro fertilized cattle embryos into morulae and blastocysts in defined culture media. *Theriogenology* 37: 127-146.

Bailey, J.L. 2010. Factors regulating sperm capacitation. *Systems Biology in Reproductive Medicine* 56: 48-338.

Blanco, M.R., Demyda, S., Moreno, M.M. and E. Genero. 2011. Developmental competence of *in vivo* and *in vitro* matured oocytes: Review. *Biotechnology and Molecular Biology Review* 6: 155-165.

Breibart, H. and B. Spungin. 1997. The biochemistry of the acrosome reaction. *Molecular of Human Reproduction* 3: 195-202.

Brito, L.F.C. 2010. Defining the requirements for cattle embryo culture. *Theriogenology*. 45: 27-40.

Camargo, L.S.A., Viana, J.H.M., SA, W.F., Ferreira, A.M., Ramos, A.A. and V.R. Vale Filho. 2006. Factors influencing *in vitro* embryo production. *Animal Reproduction* 3: 19-28.

Camargo, L.S.A., SA, W.F., Ferreira, A.M., Viana J.H.M. and M.C.C. Araujo. 2002. Taurina no desenvolvimento de embrioes cattles fecundados *in vitro*. *Brazil Medical Veterinary* 54: 396-404.

Chakravarthi, V.P. and N. Sri Balaji. 2010. Use of assisted reproductive technologies for livestock development. *Veterinary World* 3: 238-240.

Chen, L., Russell, P.T. and W.J. Larsen. 1994. *Sequencial* effects of follicle-stimulating hormone and luteinizing hormone on mouse cumulus expansion *in vitro*. *Biology of Reproduction* 51: 290-295.

Coy, P., Grullo, L., Canovas, S., Romar, R., Matas, C. and M. Aviles. 2008. Hardening of the zona pellucida of unfertilized eggs can produce polyspemic fertilization in the pig and cow. *Reproduction* 135: 19-27.

Crozet, N., Dahirel, M. and L. Gall. 2000. Meiotic competence of in vitro grown goat oocytes. *Journal of reproduction and Fertility* 118: 367-373.

Dean, J. 2004. Reassessing the molecular biology of sperm-egg recognition with mouse genetics. *Bio Essays* 26: 29-38.

De Lima, A.S., Marques, G.M., Nascimento, A.B., Ortiz, M.E., Assumpcao, D. and J.A. Visitin. 2010. Effect of hormonal supplementation periods and *in vitro* maturation media on developmental competence of pig oocytes. *Acta Scientiae Veterinariae* 38: 407-413.

Dostal, J. and A. Pavolok. 1996. Isolation and characterization of maturation inhibiting compound in cattle follicular fluid*. Reproduction of Nutrition Development* 36: 6810-90.

Earl, C.R. and P.J. Kotaras. 1997. Oocyte and *in vitro* embryo production in sheep and cattle. *Australian Agricultural Research* 48: 125-135.

Eggan, K., Jurga, S., Gosden, R., Min, I.M. and A.J., Wagers. 2006. Ovulated oocytes in adult mice derive from non-circulating germ cells. *Nature* 441: 1109-1114.

Eid, L.N., Lorton, S.P. and J.J. Parrish. 1995. Partenal influence of a-phase in the first cell cycle of cattle embryo. *Biology of Reproduction* 51: 1232-7.

Eisenbach, M. 1999. Sperm chemotaxis. *Biology of Reproduction* 4: 56-66.

Elmileik, A., t., Maeda. and T. Terada. 1995. Higher rates of development into blastocyst following the *in vitro* fertilization of cattle oocytes matured in a medium supplemented with the fluid from large cattle follicles. *Animal Reproduction* 38: 85-96.

Ericsson, S.A., Boyce, M.L., Funahashi, H. and B.N. Day. 2000. Assessment of porcine oocytes using brilliant cresyl blue. *Theriogenology* 39: 214.

Eppig, J.J. 1991. Maintenance of meiotic arrest and the induction of oocyte maturation in mouse oocyte granulosa cell complexes developed *in vitro* from preantral follicles. *Biology Reproduction* 45: 824-830.

Faber, D.C., Molina, J.A., Ohlrichs, C.L., Zavaag, D.B.V. and L.D. Ferre. 2003. Commercialization of animal biotechnology. *Theriogenology* 39: 125-138.

Fair, T., Carter, F., Park, S., Evans, A.C.O. and P. Lonergan. 2007. Global gene expression analysis during cattle oocyte *in vitro* maturation. *Theriogenology* 68: 91-97.

Farag, I.M., Girgis, S.M., Khalil, W.K.B., Hassan. N.H.A., Sakr, A.A.M., Allah S.M.A. and N.I. Ali. 2009. Effect of hormones, culture media and oocyte quality on *in vitro* maturation of Egyptian sheep oocytes. *Journal of Applied Biosciences* 24: 1520-1534.

Farahavar, A., Shahne1, A.Z., Hamid kohram1, H. and V. Vahedi. 2010. Effect of melatonin on *in vitro* maturation of cattle oocytes. *African Journal of Biotechnology* 9: 2579-2583.

Ferdous, J. 2006. Collection, grading and evaluation of goat cumulus oocyte complexes (COCs) in view of *in vitro* maturation, fertilization and culture. Master of Science thesis, Department of Animal Breeding and Genetics. Bangladesh agricultural university. Mymensigngh.

Ferre, L.B., Dalla, L.M., Medina, M. and G. Brogliatti. 2002. *In vitro* embryo production and pregnancy rates from problem, pregnant and cyclic cows by tranvaginal ovum pick-up. *Theriogenology* 57: 664.

Francis, P.A. 1988. Ox draught power and agricultural transformation. *Northern Zambia Agricultural Systems* 27: 35-49.

Galli, C. and G. Lazzari. 2008. The manipulation of gametes and embryos in farm animals. *Reproduction In Domestic Animals* 43: 1-7.

Gadea, J. 2005. Sperm factors related to *in vitro* and *in vivo* porcine fertility. *Theriogenology* 63: 431-444.

Gritharan, G., Ramakrishnappa, N., Balendran, A., Cheng, K.M. and R. Rajamahendran. 2004. Development of *in vitro* tests to predict fertility of bulls. *Canadian Journal of Animal Science* 85: 47-52.

Goddard, M.J. and H.P. Pratt. 1983. Control of events during early cleavage of the mouse embryo. *Journal Embryology and Morphology* 73: 111-133.

Goodhand, K.L., Staines, M.E., Hutchinson, J.S. and P.J. Broadbent. 2000. *In vivo* oocyte recovery and *in vitro* embryo production from cattle donors treated with progesagen, estradiol and FSH. *Animal Reproduction Science* 63: 145-158.

Hagen, D.R. and R.A. Graboski. 1990. Effect of porcine pituitary growth (pGH) on cytoplasmic maturation of porcine oocytes *in vitro*. *Journal of Animal Science* 68: 446.

Harrison, R.A. 2004. Rapid PKA-catalysed phosphorylation of boar sperm proteins induced by capacitation agent bicarbonate. *Molecular Production and Development* 67: 337-352.

Heleil, B., Kuzmina, T., Novikova, N., Torner, H. and H. Alm. 2010. Effect of prolactin on development competence of cattle oocytes selected by brilliant cresyl blue staining. *Journal of Reproduction and Infertility* 1: 1-7.

Hoque, S.A.M., Kabiraj, S.K., Khandoker, M.A.M., Mondal, A. and K.M.A. Tareq. 2011. Effect of collection techniques on cumulus oocyte complexes (COCs) recovery, *in vitro* maturation and fertilization of goat oocytes. *African Journal of Biotechnology* 10: 9177-9181.

Izadyar, F., Colenbrander, B. and M.M. Bevers. 1997. Stimulatory effect of growth hormone on *in vitro* maturation of cattle oocytes is exerted through the cyclic adenosine 3′,5′-monophosphate signalling pathway. *Biology of Reproduction* 57: 1484-1489.

Jahn, R. and T.C. Sudhof. 1999. Membrane fusion and exocytosis. *Annual Reviews of Biochemistry* 68: 863-911.

Jamil, H., Samad, H.A., Rehman, N., Qureshi. and L.A. Lodhi. 2010. Effect of somatic cell types and culture medium on *in vitro* maturation, fertilization and early development capability of buffalo oocytes. *Pakistan Veterinary Journal*.

Jamil, H., Samad, H.A., Qureshi, Z.I., Rehman, N. and L.A. Lodhi. 2007. Effect of bull and sperm preparation method on *in vitro* fertilization of buffalo oocytes. *Pakistan Veterinary Journal* 27: 29-34.

Kakkassery, M.P., Anand, L.F., Rijaryakumaran, V. and T. Sreekumaran. 2010. *In vitro* maturation of Bos indicus oocytes- effect of cumulus oocyte complex morphology. *Tamilnaduj. Veterinary & Animal sciences* 6: 247-249.

Katska-Ksiazkiewicz, L., Bochenek, M. and B. Rynska. 2005. Effect of quality of sperm chromatin structure on in vitro production of cattle embryos. *Dummerstorf* 48: 32-39.

Khandoker, M., Imai, K., Takahashi, T. and K. Hashizume. 2001. Role of gelatinase on follicular atresia in the cattle ovary. *Biology of Reproduction* 65: 720-732.

Kojima, F.N., Bergfeld, E.G.M., Wehrman, M.E., Cupp, A.S., Fike, K.E., Mariscal-Aguayo, D.V., Sanchez-Torres, T., Garcia-Winder, M., Clopton, D.T., Roberts, A.J. and J.K. Kinder. 2003. Frequency of luteinizing hormone pulses in cattle influences duration of persistence of dominat ovarian follicles, follicular fluid concentrations of steroids, and activity of insulin-like growth factor binding proteins. *Animal Production Science* 77: 187-211.

Khurana, N.K. and H. Niemann. 2000. Effects of oocyte quality, oxygen tension, embryo density, cumulus cells and energy substrates on cleavage and morula/blastocyst formation of cattle embryos. *Theriogenology* 54: 741-756.

Krisher, R.L. 2004. The effect of oocyte quality on development. *Journal of Animal Science* 82: 14-23.

Kruip, A.M., Bevers, M.M. and B. Kemp. 2000. Environment of oocyte and embryo determines health of IVP offspring. *Theriogenology* 53: 611-618.

Lane, M. 2001. Mechanisms for managing cellular and homeostatic stress *in vitro*. *Theriogenology* 55: 225-236.

Leahy, T. and B.M. Gadella. 2011. Sperm surface changes and physiological consequences induced by sperm handling and storage. *Reproduction* 42: 759-78.

Lechniak, D., Kaczmarek, D., stanisawski, D. and T. Adamowicz. 2002. The ploidy of in vitro matured cattle oocytes. *Theriogenelogy* 57: 1303-1308.

Lin, Y.H., Hwang, J.L., Seow, K.M., Huang, L.W., Hsieh, B.C., Chen, H.J., Tzeng, C.H. and C.H. Bai. 2011. Effect of incubation with different concentrations and durations of FSH for *in vitro* maturation of murine oocytes. *Reproductive Biomedicine* 23: 111-117.

Lonergan, P., Rizos, D., Gutierrez-Adan, A., Fair, T. and M.P. Boland. 2003. Oocyte and embryo quality: effect of origin, culture conditions and gene expression patterns. *Reproduction in Domestic Animals* 38: 259-267.

Lonergan, P., Rizos, D., Ward, F. and M.P. Boland. 2001. Factors influencing oocyte and embryo quality in cattle. *Reproduction Nutrition and Development* 41: 427/437.

Machatkova, M., Hanzalova, K., Horakova, J., Reckova, Z. and P. Hulinska. 2006. Collection of oocytes from donors in the growth phase of follicular development can enhance the production of cattle embryos for cryopreservation. *Veterinarni Medicina* 5: 232-238.

Mahoete, N., Lehloenya K.C., Greyling J.P.C. and T.L. Nedambale. 2010. *In vitro* embryo production and semen cryopreservation in sheep. Faculty of Natural and Agricultural sciences. University of Free State. Bloemfontein.

Manjunath, P. and I. Therien. 2002. Role of seminal plasma phospholipid binding proteins in sperm membrane lipid modification that occurs during capacitation. *Journal of Reproduction Immunology* 53: 19-109.

Motlagh, M.K., Shahneh, A.Z., Daliri, M., Kohram, H. and F. Ghargozion. 2008. *In vitro* maturation of sheep oocytes in different concentration of mare serum. *African Journal of Biotechnology* 7: 3380-3382.

Moenter, S.M., Caraty, A. and F.J. Karsch. 1990. The estradiol-induced surge of gonadotropin releasing hormone in the ewe. *Endocrinology* 127: 1375-1384.

Mtango, N.R., Potireddy, S. and K.E. Latham. 2008. Oocyte quality and maternal control of development. *International Review of Cell and Molecular Biology* 268: 1937-6448.

Nasar, Md. and A. Rahman. 2012. Factors influencing *in vitro* culture of goat embryos with special reference to ICSI-derived oocytes. *Biotechnology* 11: 1-9.

Nedambale, T.L., Dinnyes, A., Groen, W., Dobrinsky, J.R., Tian, X.C. and X. Yang. 2004. Comparison on *in vitro* fertilized cattle embryos cultured in KSOM or SOF and cryopreserved by slow freezing or vitrification. *Theriogenology* 63: 437-449.

Nuttinck, F., Charpigny, G., Mermillod, P., Loosfelt, H., Meduri, G., Freret, S., Grimard, B. and Y. Heyman. 2004. Expression of components of insulin-like growth factor system and gonadotropin receptors in cattle cumulus complexes during oocyte maturation. *Domesticated Animal Endocrinology* 27: 179-195.

Park, J.Y., Su, Y.Q., Ariga, M., Law, E., Jin, S.L. and M. Conti. 2004. EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science* 303: 7-682

Parrish, J.J. 2014. Cattle *in vitro* fertilization: in vitro oocyte maturation and sperm capcitation with heparin. *Theriogenology* 81: 67-73.

Parrish, J.J. and J.L. Susko-Parrish. 2001. Calcium increases in the anterior head of cattle sperm during capacitation. *Biology of Reproduction* 64: 112.

Patrat, C., Serres, C. and P. Jouannet. 2000. The acrosome in human spermatozoa. *Biology of the Cell* Review 92: 255-266.

Pereira, D.C., Dode, M.A.N. and R. Rumpf. 2005. Evaluation of different culture systems on the in vitro production of cattle embryos. Theriogenology 63: 1131-1141.

Pfeifer, L.F.M., Schneider, A. and M. Correa. 2008. Factors that affect *in vitro* production of cattle embryos. *Revista Colombiana de Ciencias Pecuarias* 21: 109-120.

Pujol, M., Lopez-Bejar, M., Mertens, M.J., Rodriguez-Gonzalez, E., Velilla, E. and P. Paramio. 2000. Selection of immature heifer oocytes using the brilliant cresyl blue test. *Theriogenology* 53: 466.

Reid, A.T., Redgrove, K., Aitken, R.J. and B. Nixon. 2011. Cellular mechanisms regulating sperm-Zona pellucida interaction. *Asian Journal of Andrology* 13: 88-96.

Rensis F.D. and R.J. Petters. 2003. Heat stress and seasonal effects on reproduction in the dairy cow: review. *Theriogenology* 60: 1139-1151.

Rizos, D., Ward, F., Duffy, P., Boland, M.P. and P. Lonergan. 2002. Consequences of cattle oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*. *Molecular Reproduction and Development* 61: 48-234.

Rodrigues, B.A. and J.J. Rodrigues. 2010. *In vitro* maturation of canine oocytes; unique conundrum. *Animal Reproduction* 7: 3-5.

Rodriguez-MartineZ, H. 2012. Assisted reproductive techniques for cattle breeding in developing countries: a critical appraisal of their value and limitations. *Reproduction in Domestic Animals* 47: 21-26.

Rodriguez-Martinez, H. 2001. Sperm function in cattle and pigs: morphological and functional aspects. *Dummerstorf* 44: 118-120.

Rosegrant, M.W., Cai, X. and S.A. Cline. 2002. Global water outlook to 2025, averting crisis. A 2025 vision for food, agriculture, and environment initiative. Washington DC. IFPRI and IWMI.

Salicioni, A.M., Platt, M.D., Wertheimer, E.V., Arcelat, E., Allaire, A., Sosnik, J. and P.E. Visconti. 2007. Signalling pathways involved in sperm capacitation. *Spermatology* 65.

Schenk, J.L., Cran, D.G., Everett, R.W. and G.E. Seidel Jr. 2009. Effects of sperm numbers per inseminate, sorting pressure, and sperm storage before sorting. *Theriogenology* 71: 717-728.

Seidel Jr, G.E. 2012. Several insights on evaluation of semen. *Animal Reproduction* 9: 329-332.

Shourbagy, S.H., Spikings, E.C., Freitas, M. and J.C. St John. 2006. Mitochondria directly influence fertilization outcome in the pig. *Reproduction* 131: 233-245.

Sung, L., Du, F., Xu, J., Chang, W., Nedambale, T.L., Zhang, J., Jiang, S., Tian, X.C. and X. Yang. 2004. The differential requirement of albumin and sodium citrate on the development of in vitro produced cattle embryos. *Reproduction Nutrition and Development* 44: 551-564.

Suthar, V.S. and R.G. Shah. 2009. Cattle in vitro embryo production. *Veterinary World* 12: 478-479.

Sutton, M.L., Gilchrist, R.B. and J.G. Thompson. 2003. Effects of *in vivo* and *in vitro* environments on the metabolism of the cumulus oocyte complex and its influence on the oocyte developmental competence. *Reproduction* 9: 35-48.

Talpot, P. and P. Dandekar. 2003. Perivitelline space: does it play a role in blocking polyspermy in mammals. *Microscopy Research Technique* 61: 349-357.

Taveres, L.M.T., Feitosa, W.B., de Oliveira, V.P., Nichi, M., Ortiz, M.E. and J.A. Visintin. 2011. *In vitro* development of cattle embryos culture under different fetal calf serum concentration and cell types. *Journal of Animal Science* 48: 38-45.

Thompson, J.G. 2000. *In vitro* culture and embryo metabolism of cattle and sheep embryos. *Journal of Animal Science* 61: 263-275.

Torner, H., Ghanem, N., Ambros, C., Holker, M., Tomek, W., Phatsara, C., Alm, H., Sirard, M., Kanitz, W., Schellander, K. and D. Tesfaye. 2008. Molecular and subcellular characterisation of oocytes screened for their developmental competence based on glucose-6-phosphate dehydrogenase activity. *Reproduction* 135: 197-212.

Tsuzuki, Y., Toyama, H., Nabenishi, H., Mrita, T. and K. Ashizawa. 2010. The effect of various concentrations of taurine during *in vitro* fertilization on the development of cattle embryos fertilized with spermatozoa from three different bulls. *Asian-Australian Journal of Animal Science* 7: 873-879.

Vanrose, G., Van Soom, A. and A. De Kruif. 2001. From co-culture to defined medium. *Reproduction of domestic Animal* 36:25-28.

Van Soom, A., Tanghe, S., De Pauw, I., Maes, D. and A. De Kruif. 2002. Function of cumulus oophorus before and during mammalian fertilization. *Reproduction of Domestic Animals* 37: 144-151.

Van Soom, A., Vandaele, L., Goosens, K., De Kruif, A. and L. Peelman. 2007. Gamete origin in relation to early embryo development. *Theriogenology* 68: 131-137.

Vigneault, C., Gravel, C., Vallée, M., McGraw, S. and M. Sirard. 2009. Unveiling the cattle embryo transcriptome during the martenal-to-embryonic transition. *Reproduction* 137: 245-257.

Walters, A.H., Eyestone, W.E., Saacke, R.G., Pearson, R.E. and F.C. Gwazdauskas. 2004. Sperm mopholorgy and preparation method affect cattle embryonic development. *Journal of Andrology* 25: 4.

Wang, L., Lin, J., Huang, J., wang, J., Zhao, Y. and T. Chen. 2012. Selection of ovine oocytes by brilliant cresyl blue staining. *Journal of Biomedicine and Biotechnology.* 7: 1155.

Wassarman, P.M., Jovine, L. and E.S. Litscher. 2001. A profile of fertilization in mammals. *Nature Cell Biology Review* 3.

Wasserman, M. 1988. The mammalian ovum. *The physiology of reproduction* 3: 69-102.

Webb, E.C., Dombo, M.H. and M. Roets. 2004. Seasonal variation in semen quality of Gorno Altai cashmere goats and South African indigenous goats. *South African Journal of Animal Science* 34: 240-243.

Wolfenson, D., Roth, Z. and R. Meidan. 2000. Impaired reproduction in heat-stressed cattle. *Animal Reproduction Science* 61: 535-547.

Wong, J.L. and G.M. Wessel. 2006. Defending the zygote: search for the ancestral animal block to polyspermy. *Developmental Biology* 72: 1-151.

Wright, R.J. and K.R. Bondioli. 1981. Aspects of *in vitro* fertilization and embryo culture in domestic animals. *Journal of Animal Science* 53: 702-729.

Ward, F., Brian, E., Dimitrios, R., Maurce, B., and L. Patrick. 2002. Optimization of *in vitro* cattle embryo production: effect of duration of moderation, length of gamete co-incubation, sperm concentration and sire. *Theriogenology* 57: 2105-2117.

Yamashita, Y., Okamoto, M., Kawashima, I., Okazaki, T., Nishimura, R., Gunji, Y., Hishinuma, M. and M. Shimada. 2011. Positive feedback loop between prostaglandin E_2 and EGF-like factors is essential for sustainable activation of $MAPK_{3/1}$ in cumulus cells during in vitro maturation of porcine cumulus oocyte complexes. *Biology of Reproduction* 6: 105-114.

Yanagimachi, R. 1994. Mammalian Fertilization. *The physiology of reproduction*, vol. 2. Raven press, New York.

Younis, A.I., Zuelke, K.A., Harper, K.M, Oliveira, M.A.L. and B.G. Brackett. 1991. *In vitro* fertilization of goat oocytes. *Biology of Reproduction* 44: 1177-1182.

Zhang, X., Miao, Y., Zhao, J.G., Spate, L., Bennett, M.W., Murphy, C.N., Schatten. and R.S. Prather. 2010. Porcine oocytes denuded before maturation can develop to the blastocyst stage if provided a cumulus cell-derived coculture system. *Journal of Animal Science* 88: 2604-2610.