

**EFFECT OF DIFFERENT CULTURE MEDIA AND INCUBATION METHODS ON  
CULTURING MURINE EMBRYOS *IN VITRO* USING A SEMEN STRAW AS AN  
ALTERNATIVE RECEPTACLE**

BY

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Science in Agriculture degree (Animal Science)**

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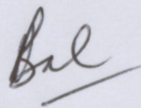
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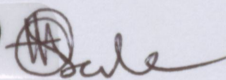


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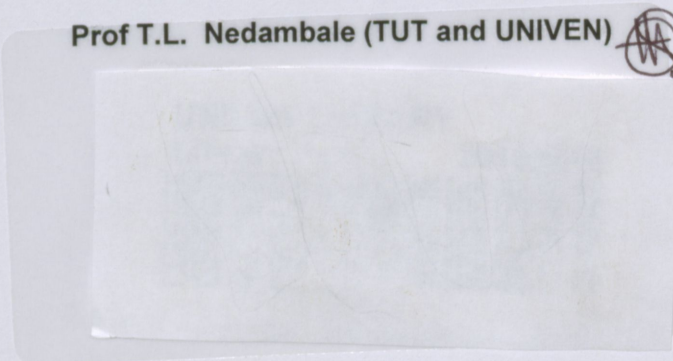


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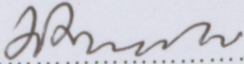


**Date: May 2016**



## DECLARATION

I, the undersigned, hereby declare that this dissertation submitted by me for the degree of Master of Science in Agriculture in the Department of Animal Science of the University of Venda, is my own work and has never been submitted previously by myself or any other student at any other university.



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Date



## DEDICATION

I would like to dedicate this dissertation to my wonderful family for their endless love, support and encouragement, and the new generation to come.

I owe a deep debt of gratitude to my university for giving me an opportunity to complete this work.

My sincere gratitude goes to my supervisor Prof. Daniel Nkomo Barry, and co-supervisor Prof. Tshenengadzo Lucky Nedembale, for their thoughtful advice and guidance, and also to Dr. Paul Barry who assisted me with the statistical analysis of my data and the rest of the animal science department staff, who assisted me.

I would like to dedicate this work to my friends: Ratsimilaho Lufano, Nanyudzhof Khuliso, George Kwenani, Masindi Mphahathi and Ndou Mchaho and CEAAE team (Andrew Kweneng, Gwende Ajae, Pie Vellard Kaloa), Dikalo Nathengwe, Patsima Ntshenzheni and Khato (Ntsheni) who have been supporting me.

I would also like to thank my spiritual father Dr. Shengor Heshengolwe from E-tshadai Christian Center who showed me love, corrected me when I was wrong as a father correcting his son, encouraged and believed in me so that I could be successful.

I would like to thank my parents Mr. Madzhe Phisoa and Mr. Madzhe Gernard, as well as my siblings Mr. Madzhe Dikalo and my brother Mr. Madzhe Shonizani, for their encouragement.

## ACKNOWLEDGEMENT

Firstly I would like to thank the almighty God, who made it possible for me to write this dissertation and to be where I am today.

I owe a deep debt of gratitude to my university for giving me an opportunity to complete this work.

My sincere gratitude goes to my supervisor Prof. Daniel Malan Barry, and co-supervisor Prof. Tshimangadzo Lucky Nedambale, for their thoughtful, advice and guidance, and also to Dr. Kow Benyi who assisted me with the statistical analysis of my data and the rest of the animal science department staff, who assisted me.

I would like to dedicate this work to my friends: Radzilani Lufuno, Nevhudzholi Khuliso, Makoya Nonhlanhla, Masindi Mphaphathi and Ndou Ntevheli and CEAAR team (Andrea Raseona, Olumide Ajao, Pie Veillard Kalonji, Dakalo Nethengwe, Pertunia Nethenzheni and Kidinda Kalobo), who have been supporting me.

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Lastly, I would like to thank my parents Mrs Madzhie Priscilla and Mr Madzhie Bernard, as well as my sister Ms Madzhie Dakalo and my brother Mr Madzhie Shonisani, for their unwavering support.



The aim of the study was to fertilize mouse oocytes and culture the resulting zygotes in bi-gas incubator and in a goat vagina and compare the *in vitro* embryo development in TCM-199 and Ham's F10 culture media until the blastocyst formation. The spermatozoa and oocytes of an F<sub>1</sub> generation (Balb C x C57) were fertilized in Ham's F10 and TCM-199 in semen straws and in micro-drops in petri dishes. The embryos were cultured in TCM-199 and Ham's F10 in semen straws and micro-drops in petri dishes. The semen straws were incubated in a bi-gas incubator and goat vagina. The blastocyst-stage embryos were stained using Hoechst 33528 solution and blastomeres count. The results were analyzed by 2 X 3 factorial design and the student t-test was used to separate the mean and they showed that there was no statistical difference ( $p > 0.05$ ) between the media, receptacles and incubators. The overall fertilization rate was 94 % to 99 %. The semen straw with Ham's F10 incubated in the bi-gas incubator had the highest rate (80.5 %), reaching the blastocyst-stage of the embryos. The lowest rate of murine embryos reaching the blastocyst-stage were those cultured in semen straws incubated in the goat vagina, with its highest rate of 60 %. The embryos blocking at the 8 - cell and morula stage recorded the lowest rate on both culture media and incubation methods, compared to the other embryo developmental stages. The overall mean number of blastomeres in the blastocyst-stage of the embryos ranged from 85±9 – 90±9 cells in all receptacles and incubators. It was concluded that the fertilization and culturing of murine embryos are possible in French semen straws incubated in a bi-gas incubator and in the goat vagina as an alternative method of fertilizing oocytes and culturing murine embryos in addition TCM-199 and Ham's F10 can both be used to fertilize oocytes and culture murine embryos until blastocyst formation embryo *in vitro*, incubated in a bi – gas incubator or in the vagina.

**Keywords:** *in vitro* fertilization, semen straw, TCM-199, Ham's F10, bi-gas incubator, goat vagina.



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## LIST OF ABBREVIATIONS

|                 |                                                  |
|-----------------|--------------------------------------------------|
| Am              | Ante meridian (morning)                          |
| ARC             | Agricultural Research Council                    |
| BSA             | Bovine serum albumin                             |
| CO <sub>2</sub> | Carbon dioxide                                   |
| COCs            | Cumulus oocytes complexes                        |
| DNA             | Deoxyribonucleic acid                            |
| D-PBS           | Dulbecco's Phosphate Buffered Saline             |
| Ecg             | Equine chorionic gonadotropin (Folligon 1000)    |
| EDTA            | Ethylendiaminetetraacetic acid                   |
| F1              | First filial generation                          |
| FBS             | Fetal bovine serum                               |
| hCG             | Human chorionic gonadotropin (Chorulon 1500)     |
| IU              | International unit                               |
| IV              | <i>In vitro</i>                                  |
| IVC             | <i>In vitro</i> culture                          |
| IVF             | <i>In vitro</i> fertilization                    |
| IVMFC           | <i>In vitro</i> maturation fertilization culture |
| IVM             | <i>In vitro</i> maturation                       |
| Mg              | Milligram                                        |
| ml              | Milliliter                                       |
| Mw              | Molecular weight                                 |
| N               | Nitrogen                                         |
| PHE             | Penicillamine Hyotaurine Epinephrine             |
| Pm              | Post meridian (afternoon)                        |
| R               | Rand                                             |
| TCM -199        | Tissue culture medium 199                        |



|        |                                  |
|--------|----------------------------------|
| TUT    | Tshwane University of Technology |
| UFO    | Unfertilized oocytes             |
| μl     | Micro liter                      |
| UNIVEN | University of Venda              |
| UV     | Ultraviolet                      |
| WOW    | Well of the well                 |
| ZP     | Zona pellucida                   |

# CHAPTER 1

## LIST OF EQUATIONS

### INTRODUCTION

| Equation number | Equation                                                 | Page |
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The mouse is a popular model system because it is a mammal with sophisticated genetic tools and significant genetic resources. Mice are less sensitive to and recover more easily from changes in pH than either human or bovine embryos. Laboratory mice are unique in that there are large numbers of inbred strains. Inbred strains are a strain of mice in which every individual is essentially genetically identical and homozygous at all loci. The advantage of using mice is that it is easy to handle and hence cheaper to house and feed as compared to other species. They also breed rapidly, which saves a considerable period of time. Mice are thought to share around 80 % of the genes of the humans and most of that of livestock (Ronard, 1984). The gestation period for laboratory mice varies among the strains from 16 to 21 days and they mature sexually by 5 weeks of age (Grazzian, 2007).

A bi-gas or bi-gas incubator provides precise temperature, CO<sub>2</sub> and O<sub>2</sub> control as well as humidity. This is why they are widely-used in scientific research to grow and maintain cell cultures. Also, the bi-gas incubator is available in two models: high supply (with 22 % - 25 %) O<sub>2</sub> and low (with 3 % - 20 %) O<sub>2</sub>, depending on the user's needs. However, the incubator's availability varies, based on countries, and their cost is very high. In addition, the incubator is often considered as a major source of biological contamination and can also be a source of chemical contamination (John, 2000).

The 0.25 ml French straw is the most popular semen straw package available today, but the 0.5 ml straws may be available in some areas. Each package system has a different surface-volume ratio, which requires unique handling procedures. Although, semen straws cost much less than petri dishes (Michael et al. 2013), their disadvantage is that they can cause environmental contamination if not properly monitored (Zemplier et al. 2013).

In vitro fertilization (IVF) involves fertilization of mature oocytes with capacitated sperm in a tissue culture dish in a laboratory incubator. This technique can generate large numbers of cleavage stage embryos without using a significant number of single oiled stud males for mating. Sperm penetration is synchronous during incubation with mature oocytes, leading to synchronous development unlike fertilization in vivo, when natural ovulation is usually spread over time. Another use of IVF is to generate offspring from cryopreserved sperm from mice, which for one reason or another, will not mate. IVF has become an important way to recover cryopreserved mouse strains. The success of mouse IVF is critically dependent on medium and manner in which both oocytes and sperm are handled (Hoppe & Pike, 1973).



## CHAPTER 1

### INTRODUCTION

#### 1.1. Background

The mouse is a popular model system because it is a mammal with sophisticated genetic tools and significant genetic resources. Mouse embryos are less sensitive to and recover more easily from changes in pH than either human or bovine embryos. Laboratory mice are unique in that there are large numbers of inbred strains. Inbred strains are a strain of mice in which every individual is essentially, genetically identical and homozygous at all loci. The advantage of using mice is that it is easy to handle and hence cheaper to house and feed as compared to other species. They also breed rapidly, which saves a considerable period of time. Mice are thought to share around 80 % of the genes of the humans and most of that of livestock (Ronard, 1984). The gestation period for laboratory mice varies among the strains from 18 to 21 days and they mature sexually by 6 weeks of age (Dressman, 2007).

A tri-gas or bi-gas incubator provides precise temperature, CO<sub>2</sub> and O<sub>2</sub> control as well as humidity. This is why they are widely-used in scientific research to grow and maintain cell cultures. Also, the tri-gas incubator is available in two models: high supply (with 22 % - 85 %) O<sub>2</sub> and low (with 3 % - 20 %) O<sub>2</sub>, depending on the user's needs. However, the incubator's availability varies, based on countries, and their cost is very high. In addition, the incubator is often considered as a major source of biological contamination and can also be a source of chemical contamination (John, 2000).

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In many laboratories, bovine morula and blastocyst embryos are routinely obtained from *in vitro* matured (IVM) and IVF oocytes. When media supplemented only with serum are used, embryos mostly cease development before or at the 8 – 16 cell stage (Eyestone & first, 1989). At the 8 – 16 cell stage the embryos enter the uterine environment, developing into a blastocyst in which the first events of cellular differentiation are observed. During this stage the specific cells are induced to change their morphological and functional phenotype to a polarized form. At the blastocyst-stage the embryos hatch from the surrounding zona pellucida and subsequently implant in the uterus. The block to development at the 8 – 16 cell stage can be overcome by culturing embryos in a medium containing cumulus cells or oviduct cell monolayers (Rexroad, 1989). Alternatively, a cell-free medium, typically tissue culture medium TCM-199, plus serum in which oviductal tissue has been cultured for 24 - 48 hours, is capable of supporting early bovine embryo development equally as well as oviduct cell monolayers (Wang *et al.* 1990).

*In vitro* culture systems may result in embryos of reduced morphological quality compared to those produced *in vivo* (Iwasaki *et al.* 1990). Pregnancy rates following transfer of bovine blastocysts produced *in vitro* are lower than pregnancy rates following transfer of blastocysts produced *in vivo* (Hasler *et al.* 1995). This may be due to the lack of agreement among evaluators when they determine the quality grade of embryos produced *in vitro* (Farin & Farin, 1995). Furthermore, bovine blastocysts produced *in vitro* have fewer total cells as well as fewer inner mass cells. *In vitro* conditions for the development of bovine embryos vary from culture in the presence of serum to culture under serum-free conditions. Bovine blastocyst produced in serum-free medium has morphology, compared with that of blastocysts produced *in vivo* (Keskintepe *et al.* 1995). However, *in vitro* production of embryos in serum-free culture resulted in fewer blastocyst-stage embryos than culture in serum-supplemented medium (Pinyopummintr & Bavister, 1994).

## 1.2. Problem statement

Mammalian embryos are very sensitive to the culture environment. They require a well-equipped laboratory with tri-gas or bi-gas incubators and ultra-hygienic conditions. It is important to maintain a stable culture condition. Bi-gas incubators are widely used to maintain an optimal culture environment in terms of gas phase, temperature and humidity. A new concept for an incubator suitable for mammalian embryo culture has been developed in recent years. However, these incubators are still large and require considerable installation space. The semen straw may be the possible solution to fertilize oocytes and culture embryos, where laboratory conditions are not available. This is because semen straws are permeable to



carbon dioxide gas that can maintain the pH of the bicarbonate buffer culture medium in the vagina. In addition, the vagina might serve as an incubator, as it has the same temperature and possibly the same carbon dioxide concentration.

## 2.1. Introduction

### 1.3. Justification

The proposed alternative method (semen straw) was used to fertilize oocytes and culture murine embryos outside a well-equipped laboratory. The cost and maintenance of a well-equipped laboratory is high. The French semen straw was used outside the laboratory and inside the vagina of a goat doe to fertilize and culture murine embryos. The gas phase, temperature and humidity are at constant levels in the vagina of the goat doe.

### 1.4 Study objectives:

#### 1.4.1. Main objective

The purpose of this study was to evaluate the potential of using a French semen straw to fertilize mouse oocytes and culture the resulting embryos in a semen straw inserted in the vagina of a doe and also in a bi-gas incubator as a control.

#### 1.4.2. Specific objectives

- i. To compare the *in vitro* fertilization and development of murine embryos until the blastocyst-stage in TCM-199 and Ham's F10 culture media.
- ii. To determine the number of blastomeres for each developmental stage of the embryos incubated in a bi-gas incubator and the vagina of a goat doe as an incubator.

### 1.5. Hypothesis

A semen straw cannot be used to fertilize and culture early stage murine embryos in an incubator or in the vagina of a doe.



## CHAPTER 2

### LITERATURE REVIEW

#### 2.1. Introduction

The absence of rigorous selection of gametes *in vitro* and suboptimal culture conditions both decrease embryo quality, but there is no consensus on which factor is more important in the incidence of apoptosis during early *in vitro* embryo development. The importance of oocyte quality, and the second “intrinsic factor” in the developmental competence of embryos, might be more apparent and are determined by the oocytes nuclear and cytoplasmic maturation which are attained during its growth in the follicle (Sirard, 2001). A competent oocyte is by definition one that is able to sustain embryonic development to term (Brevini-Gandolfi & Gandolfi, 2001). Nuclear maturation is characterized by the ability of the oocytes to resume meiotic division up to the metaphase II stage during IVM. Nuclear maturation can be visualized by the extrusion of the first polar body and the appearance of the metaphase plate. The efficiency of the processes of IVM, IVF of oocytes and development of embryos is not optimal (Sirard *et al.* 2006).

The number of pregnancies obtained under the same conditions is lower when the transferred embryos are produced *in vitro* than when they are produced *in vivo*. Despite efforts to improve bovine *in vitro* embryo production (IVP), its efficiency is lower, since only 30 % to 40 % blastocyst development is obtained from oocytes after IVMFC (Sirard *et al.* 2006). In both *in vivo* and *in vitro* conditions, only the fully grown oocytes can resume meiosis and acquire the ability to be fertilized. Meiotic events are sensitive to various perturbations, such as oxidative stress, toxins and *in vitro* culture conditions that may have profound effects on the genetic competence of IVM oocytes (Carrell *et al.* 2005). Embryo quality assessment is most commonly performed by non-invasive morphological evaluation of embryos, based on embryo colour, blastomere numbers, symmetry, cytoplasmic granulation and fragmentation (Merton, 2002).

A semen straw is a long narrow tube with an inside volume of about 0.25 ml, which generally has one of its ends semi-closed. Depending on the application, the tube is filled with animal semen or with some other biological liquid such as viruses or an embryo kept in a liquid medium. The straws can be closed through ultrasound welding or other technique (Andrabi & Ullah, 2008).

In the past few decades, culture media have been developed that finally allowed for complete *in vitro* development of preimplantation embryos, including human embryos, from fertilized oocytes through to the blastocyst stage (Biggers, 2002). The newer generations of culture



media were designed in part by trying to satisfy the physiological needs of embryos and in part by trying to empirically optimize *in vitro* outcomes and subsequent developmental potential. This has led to divergent systems for embryo culture, particularly for human embryos in the clinic, where both single-step and sequential culture systems are available for culture from fertilized oocytes to blastocysts (Lane & Gardner, 2007; Biggers & Summers, 2008). Despite such differences, successful culture media for pre-compaction mammalian embryos have a number of common characteristics, directly related to their ability to adequately support the physiological needs of the early embryo. The study of cell volume homeostasis in embryos was inspired by the results of empirical optimization of culture media, which included a lowering of osmolarity and the inclusion of several amino acids involved in volume regulation. Perturbations of the early embryo's ability to control its volume, including suboptimal *in vitro* conditions, it leads to blocked embryo development *in vitro*. The cell volume homeostasis is also a key factor in successful early embryo development (Lane & Gardner, 2007; Biggers & Summers, 2008).

## 2.2. Mouse embryo development

Embryonic development of the mouse begins with fertilization of the oocytes by the sperm. The important feature of the mouse embryogenesis is that early development is much slower than in such models as sea urchin, fruit fly (*Drosophila*) and African clawed frog (*Xenopus*) (Ginsburg *et al.* 1990). After 24 hours of fertilization the embryo of these species (*Drosophila* and *Xenopus*) are well on their way to become free living, feeding larvae and they contain more than 60 000 cells, organised into many different tissue layers. In contrast, the mouse embryo is still at the two cell stage and will continue to divide slowly without any increase in mass as it comes along the oviduct into the uterus for the implantation 4.5 days after fertilization. This slow development allows the uterine tissue time to prepare for receiving the embryo. The embryo in turn, generates the first two lines (the trophectoderm and the primitive endoderm) that make major contributions to the placenta and the extra-embryonic yolk sacs required for the successful interaction with the mother (Ginsburg *et al.* 1990).

Once implantation has been achieved, a dramatic increase in the growth rate of the embryo occurs, particularly in the small group of pluripotent cells known as the epiblast or primitive ectoderm from which the fetus will develop. Between the fifth and tenth day after fertilization, the three primary germ layers, the ectoderm, mesoderm and definitive endoderm are formed through gastrulation and the basic body plan and organ primordial of the future mouse are established (Ginsburg *et al.* 1990). The gestation period of the mouse embryo is 18 - 21 days,



depending on the strain (Dressman, 2007). Generally, mouse development depends on the number of day's postcoitum (dpc) (Ginsburg *et al.* 1990).

### 2.3. Oocyte development

The formation of the secondary oocytes is accomplished by the first stages of meiosis, and the nuclear division process that reduces the diploid state of the gamete precursor cells to the haploid condition of the completed gamete nucleus. At the extreme distal tip of the gonad, where oogenesis begins, the nuclei are predominantly in mitotic interphase (King, 1970). Each oocyte is contained within the follicle, composed of multiple layers of follicle or granulosa cells, which are of the same embryonic origin as Sertoli cells of the testis and have various roles in oocyte growth and differentiation (Richards *et al.* 1987; Matzuk *et al.* 2002).

The follicular granulosa cells surrounding the oocytes have numerous projections that form specialized junctions with the oocytes. This junctional complex involves gap junctions and allows metabolite transfer. They are maintained even when the follicle cells and oocytes are gradually separated through the deposition of the zona pellucida, a layer of extracellular material synthesized and deposited by the growing oocyte (Bleil & Wassarman, 1980). The zona is composed of three major acidic, O-linked sulphated glycoproteins (ZP1, ZP2 and ZP3) and reaches a thickness of about 7  $\mu\text{m}$  in the mouse. The ZP3 functions as a sperm receptor and initiates the acrosome reaction which occurs if a sperm is to fertilize the oocytes (Rosiere & Wassarman, 1992).

Molecular analysis has shown that the mouse ZP3 gene encodes a polypeptide of 402 amino acids, which is extensively glycosylated with both N-linked and O-linked complex oligosaccharides. Sperms do not attach to the polypeptide backbone of ZP3, but to oligosaccharides associated with a glycopeptide fragment from the carboxy-terminal region of the intact protein (Rosiere & Wassarman, 1992). Presumably apoptosis, programmed cell death, begins in the follicle and then affects the oocyte. There is some evidence that even good quality cumulus-oocyte-complexes (COCs) isolated from bovine follicles with a high degree of apoptosis in the follicle wall lose their developmental capacity (Jewgenow *et al.* 1999). On the other hand, it has been suggested recently that a limited degree of atresia or apoptosis in the cumulus cells surrounding the oocyte may be associated with improved oocyte competence (Hendriksen *et al.* 2000).

### 2.4. Sperm- oocyte fusion during fertilization

The process of membrane fusion between gametes is influenced by temperature, pH and  $\text{Ca}^{2+}$  dependent and the two membranes must be in close approximation. Fusion appears to be



mediated by membrane associated proteins, but terminal saccharides of glycoproteins are not directly involved in the process. Spermatozoa penetrate the zona in 2 – 15 minutes and sperm binding to the mouse zona is mediated by the ZP3 glycoproteins. During penetration of the zona, the spermatozoon loses its acrosomal contents and only the inner acrosomal membrane is in direct contact with the zona. While passing through the zona the spermatozoon beats its tail strongly leaving a sharp pathway behind. In eutherian mammals, the post-acrosomal region of the sperm head plasma membrane only attains fusibility after the acrosome reaction. However, this area apparently fuses with the oocyte plasma membrane and the two membranes become continuous. Following gamete fusion, the sperm plasma membranes remain in the oocyte plasma membrane and indicate the point of fusion (Elder & Dale, 2011).

## **2.5. Embryonic growth**

It is often assumed that growth is an essential aspect of embryonic development and that the cell number will always increase. However, cell divisions in early embryonic development are typically cleavage divisions which produce daughter cells half the size of the mother cell (Slack, 1983). Growth factors play a key role in growth and differentiation from the time of the morula or blastocyst transition. However, defining their precise role and potential for improving *in vitro* preimplantation development is completed by factors such as gene expression in both of the factors and their receptors (Elder & Dale, 2011).

## **2.6. Stages of embryonic development**

After fertilization, the zygote divides through mitosis into the number of smaller cells called blastomeres. This process of division is known as cleavage. The blastomere becomes organized in layers or groups, each group having a characteristic rate of a cleavage. Cleavage increases the number of nuclei, which amplifies the number of templates to facilitate the production of specialized protein added for the processes of compaction and differentiation to the blastocyst-stage embryo. The early-stage embryo has very little metabolic activity during its first few cleavages (Elder & Dale, 2000) and involves intense DNA replication and cell division in the absence of growth. The overall size of the embryo does not change as a result of the early cleavage divisions. At about the third cleavage division, the embryos undergoes the process of compaction to form the morula stage. At this stage, there is a significant increase in RNA and protein synthesis and the synthetic patterns of phospholipids. At the 32 cell stage, a second morphological change occurs which gives rise to the blastocyst stage embryo. The forms of the blastocyst-stage vary between species, but essentially are made up of an outer layer of trophectoderm cells and an essentially placed inner mass layer.



Throughout these blastocyst stages, the embryo is enclosed in the zona pellucida (ZP). During this stage, the trophectoderm cells produce proteolytic enzymes which digest a passage through the ZP, as the blastocyst hatches free of the zona (Elder & Dale, 2011).

The mouse embryo reaches the blastocyst-stage at 3.5 days following five rounds of cell divisions; cleavage, 2-cell, 4-cell, 8-cell and morula stage (Fujimori, 2010). There are variations in cell division patterns (Fujimori *et al.* 2009), and the timing differences in cell divisions depend on the condition of the embryo and size differences between the blastomeres. This suggests that the characteristics of the cells are not always the same between sister blastomeres (Plusa *et al.* 2005).

## **2.7. Embryo developmental block stage**

In many mammalian species, the development of one-cell embryo is usually blocked at various early stages *in vitro*. This is probably due to the switch in mitochondrial DNA control of the cleavages to nuclear DNA control (Fatehi *et al.* 2000). One-cell mouse embryos, with the exception of mouse inbred strains and F<sub>1</sub> hybrids of those strains, exhibit a block at the two-cell stage. Hamster embryos at both the two and four-cell stage *in vitro* embryo exhibit similar arrest (Minami *et al.* 2001). In porcine embryos, the developmental block was observed to occur at the four cell stage and in bovine, ovine and caprine between the eight to sixteen-cell stage embryos. In humans a similar block occurs between the four to eight-cell stages. Developmental block, however, is due to lack of some components in the culture medium or is caused by an inappropriate environment. Various co-culture systems have been applied to improve *in vitro* culture conditions (Naomi & Marcela, 2010).

## **2.8. In vitro cell culture**

When mammalian cells are placed in culture they grow rapidly, often at a rate substantially exceeding that in the intact animal. However, this growth does not continue indefinitely. Most frequently, after a variable interval, the cells die (Haff & Swim, 1961). In some cases, usually thought to be uncommon, changes in the cell population are observed to occur, culminating in the development of an established line having a variety of properties which distinguish it from the strain of origin (Haff & Swim, 1956). One of these properties is the ability to produce tumors when injected into suitable hosts (Earlm & Nettleship, 1961), so that the mechanism by which the normal cell is converted into an established cell lines that may have a bearing on the problem of carcinogenesis, can be reached. In experiments the growth properties of mouse embryonic fibroblasts were closely studied from the time they were placed in culture,



and especially during their conversion to established lines. The changes in growth properties were related to chromosomal and morphological changes, and certain criteria were set up for the established condition (Earlm & Nettleship, 1961).

## **2.9. Effect of culture conditions on viability of mouse embryonic development *in vitro***

There are many factors affecting the efficiency of *in vitro* development of embryos in various mammalian species. One of the most important is embryo density (number of embryos per volume of medium) during culture, influencing the embryo's interaction with soluble factors. The culture medium may dilute some important autocrine/paracrine factors, affecting the viability and embryo developmental performance *in vitro* (Paria & Dey, 1990). Preimplantation embryos are particularly susceptible to *in vitro* developmental blocks. These could be alleviated by lowering culture medium osmolarity, because mammalian cells regulate their volumes by adjusting intracellular osmotic pressure, as cell volume regulation could be critical to early embryos. Low osmolarity *in vitro* simply replicates the normal environment of the developing embryo (Borland *et al.* 1977).

## **2.10. *In vitro* development of mouse embryos under 5 % or 20 % oxygen**

Several studies have shown that mammalian embryos develop better at reduced oxygen tension (Bavister, 2004). A higher concentration of oxygen was found to be toxic, probably due to the formation of oxygen radicals which induces detrimental affect not only on *in vitro* development, but also on embryo metabolism (Orsi & Leese, 2001) and gene expression (Rinaudo *et al.* 2006). However, little is known about possible species-specific differences in sensitivity to oxygen tension of mouse and rat embryos. In a mouse embryo culture, oxygen concentration as low as 1 % was sufficient to support embryo development to the blastocyst stage and a transient exposure for 1 hour to 20 % oxygen reduced mouse development *in vitro* (Trouson & Gardner, 2000).

## **2.11. *In vitro* development of mouse embryos in different culture systems**

*In vitro* cultivation of mammalian ova is a very important tool for studying early embryogenesis and environments in which the early embryo can undergo a number of divisions to form a blastocyst. The greatest success and considerable information concerning *in vitro* culture requirements for embryos of preimplantation stages have been reported for mouse embryos (Brinster, 1965). Later different *in vitro* culture systems have been developed for embryos for a variety of agricultural mammals, including rabbit, sheep, cows and pigs (Dattena *et al.* 2007). Impressive results were also obtained with the *in vitro* manipulation of human embryos. The



main finding was that there are great species-specific and stage-dependent differences in the sensitivity of embryos removed from the female reproductive tract to different *in vitro* culture compounds and conditions. In spite of this, mouse preimplantation embryos are still the most popular model which is commonly used for the investigation of mammalian early development *in vitro* (Dattena *et al.* 2007).

Nunc 4-well dishes with 0.7 mL culture medium per well and 10 to 20 embryos per well is one of the most popular and conventional *in vitro* culture system for embryos of different mammalian species. Glass capillaries with 10 – 50  $\mu$ L of culture medium and 10 embryos per capillaries have been described for *in vitro* culture of mammalian embryos by Brinster many years ago (Brinster, 1968). This culture system was optimized and used to study the development of mouse embryos *in vitro* in a protein-free medium, depending on the number of embryos in a small volume of medium (Krivokharchenko *et al.* 1993). The Well-of-the-Well (WOW) culture system is another approach in which embryos are cultured in small indentations or micro-wells formed on the bottom of a plastic culture dish. This system was first developed for the individual culture of zona-free embryos (Peura, 2003). Moreover, it was also shown to be efficient for culturing of zona-intact cattle embryos (Vajta *et al.* 2000).

## 2.12. Straw *in vitro* fertilization system

The use of straw-IVF system allows a gradual exposure of oocytes to a suitable number of capacitated spermatozoa, avoiding the massive presence of capacitated spermatozoa in the immediate vicinity of oocytes as occurs with use of the micro-drops IVF system (Li *et al.* 2003). The straw IVF system increases the efficiency of fertilization and the quality of blastocyst in terms of cell number/ as compared with the micro-drops-IVF systems (Alminana *et al.* 2008).



## CHAPTER 3

### MATERIALS AND METHODS

#### 3.1. Study area

The study was conducted in the Biotechnology laboratory of the Centre of Excellence in Animal Assisted Reproduction (CEAAR) of the University of Venda situated in Thohoyandou, Limpopo Province, South Africa. Its geographical coordinates are 22° 57' 0" South, 30° 29' 0" East. Thohoyandou is the administrative centre of the Thulamela Local Municipality and Vhembe District.

#### 3.2. Experimental procedure

Table 3.1 The experimental procedure design

| Receptacles         | Medium    | Incubation       | Number of replicates | Total time of embryo evaluation |
|---------------------|-----------|------------------|----------------------|---------------------------------|
| Petri dish          | TCM-199   | Bi-gas incubator | 10                   | 120 hours                       |
|                     | Ham's F10 |                  |                      |                                 |
| 0.25 ml semen straw | TCM- 199  | Bi-gas incubator | 10                   | 120 hours                       |
|                     | Ham's F10 |                  |                      |                                 |
| 0.25 ml semen straw | TCM-199   | Goat vagina      | 10                   | 120 hours                       |
|                     | Ham's F10 |                  |                      |                                 |

#### 3.3. Experimental animal and animal care

Balb C and C57 strains of mice were kept in plastic cages with sawdust as bedding (one female in a cage and one male to a cage) in a room at ambient temperature (25 °C). The light in the room was on for 14 hours (5 am - 7 pm) and off for 10 hours (7 pm - 5 am). They were fed daily with mice pellets and water *ad libitum*. Sawdust inside the cages was changed every 5 days and cages were cleaned by washing with a disinfectant soap and tap water and then sterilized with 70 % alcohol.

Figure 3.1: Digital display of culture conditions (Bi-gas incubator)

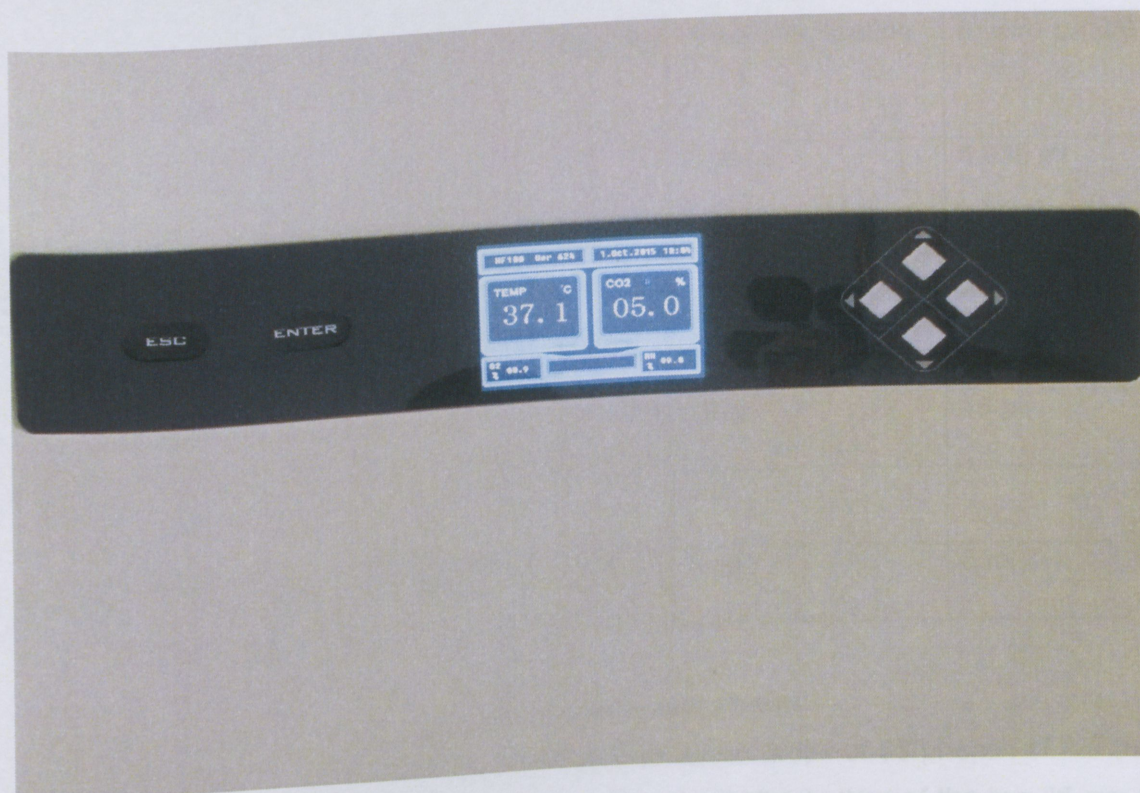


### 3.4. Mice first filial (F1) generation

The F1 generations were produced by mating white females (Balb C) and black males (C57), and black females (C57) and white males (Balb C) in the cages (one male and one female per cage). The male was placed in the cage with a female mouse for 7 days. This produced the first hybrid generations that were used for the study at 6 weeks of age.

### 3.5. *In vitro* fertilization (IVF) and *In vitro* culture media (IVC) preparation

Tissue culture media 199 (TCM-199) (Highveld Biological, South Africa) and Ham's F10 (Highveld Biological, South Africa) were used to wash oocytes, to produce drops for fertilization and for embryo culture. The media were prepared 1 day before IVF and placed in the bi-gas incubator (HF 100, Shanghai Lishen Scientific Equipment Co., Ltd) and incubated in the vagina of a goat doe to equilibrate the gas and temperature. The air in the incubator is atmospheric with extra CO<sub>2</sub> and consists of ~20 % oxygen, 5 % CO<sub>2</sub>, ~ 75 % nitrogen. The temperature of the incubator was maintained constant at 37 ° C and humidity maintained at 93 %.



**Figure 3.1:** Digital display of culture conditions (Bi-gas incubator)



### 3.5.1. Ham's F10 medium

The media (Ham's F10 and TCM-199) used for sperm and oocytes handling, *in vitro* fertilization and *in vitro* culture are described below in table 3.2 to table 3.7

**Table 3.2** Ham's media preparation

|                                                    | Sperm and oocytes handling | <i>In vitro</i> fertilization | <i>In vitro</i> culture |
|----------------------------------------------------|----------------------------|-------------------------------|-------------------------|
| Ham's F10                                          | 8.9 ml                     | 4.93 ml                       | 4.935 ml                |
| Anti-anti cocktail<br>(Antibiotic-antimycotic) 1 % | 100 µl (0.01 ml)           | 50 µl                         | 50 µl                   |
| Fetal bovine Serum                                 | 1 ml                       | 15 µl                         | 15 µl                   |
| Heparin                                            |                            | 5 µl                          |                         |
|                                                    | 10 ml                      | 5 ml                          | 5 ml                    |

**Table 3.3** TCM-199 medium preparation

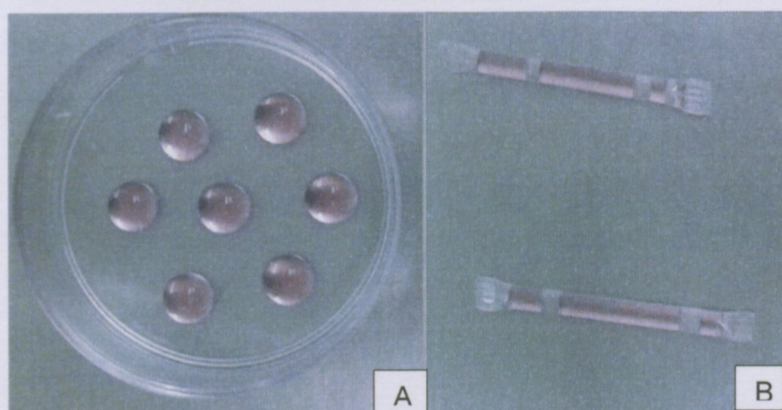
|                                                    | Sperm and oocytes handling | <i>In vitro</i> fertilization | <i>In vitro</i> culture |
|----------------------------------------------------|----------------------------|-------------------------------|-------------------------|
| TCM-199                                            | 8.9 ml                     | 4.93 ml                       | 4.935 ml                |
| Anti-anti cocktail<br>(Antibiotic-antimycotic) 1 % | 100 µl (0.01 ml)           | 50 µl                         | 50 µl                   |
| Fetal bovine Serum                                 | 1 ml                       | 15 µl                         | 15 µl                   |
| Heparin                                            |                            | 5 µl                          |                         |
| Total                                              | 10 ml                      | 5 ml                          | 5 ml                    |

### 3.6. Preparation of *In vitro* fertilization drops and straws

A pipette (10 µl – 100 µl) was used to dispense seven drops of 50 µl each of IVF medium (TCM-199 and Ham's F10 in separate petri dishes), to the bottom of the two 35 mm sterile plastic tissue culture dish. The dish was flooded with light mineral oil immediately after dispensing the drops and the other dish was not flooded with oil. Thereafter, the dish flooded



with oil was placed in the incubator and the other dish with 50  $\mu$ l IVF drops, without oil, were aspirated into 40 mm length of semen straws using a pipette (2  $\mu$ l – 5  $\mu$ l pipette tip tightly fitted to the semi-closed end of the straw). The partially filled semen straws were sealed using heated mosquito forceps, leaving an air space on both ends of the fluid column in French semen straws and the remaining empty section of the straws were removed. The sealed straws were divided into two groups: the first group of straws was covered with a lubricated (sunflower oil) sponge and the second group was not covered with a sponge. The first group was placed in the vagina of a goat doe as an incubator. The vagina was opened with a vaginal speculum and then the lubricated sponge was pushed in with a forceps. The second group was placed in the bi-gas incubator 1 day before the experiment to equilibrate the temperature and gas. This step was also the same for 50  $\mu$ l drops each of IVC medium (TCM-199 and Ham's F10).



**Figure 3.2** Petri dish (A) and semen straws (B) with IVF medium

### 3.7. Superovulation of mice

The frozen (-20 °C) injections of hCG and eCG, as prepared in appendix (Table 6.1), was thawed for 5 minutes at room temperature before each injection. The mouse oocytes donors were picked up from the cage by the scruff of its neck as close to the ears and the tail was twisted around the little finger. In order to stimulate multiple follicular growth (super-ovulation), the embryo donor was injected intraperitoneally with 5 IU equine chorionic gonadotropin (eCG; Folligon, Intervet SA, MSD Animal Health) using a short (0.5 x 16 mm) 25 gauge needle by pushing the needle through the abdominal wall on the midline at a 45 degree angle. After 48 hours of injecting eCG, the mouse oocytes donors were injected intraperitoneally with frozen-thawed 5 IU human chorionic gonadotropins (hCG; Chorulon 1500 IU , Intervet SA MSD Animal Health), for the induction of ovulation.



### 3.8. Spermatozoa collection and capacitation

Twelve hours after injecting oocytes donors with hCG, the male was sacrificed humanely by cervical dislocation. The mouse was placed on its back to expose the abdomen; the four limbs were fixed to the board using (0.90 x 38 mm) 20 gauge needles. The abdomen of the mouse was sprayed with ethanol for skin sterilization. The skin was then cut with fine scissors making a 1.5 cm transverse incision at a point level of the back of the scrotum of the back legs, and a similar incision made on the body wall. Forceps were used to grasp and pull out the fat pad with the testis, in order to bring the vas deferens and epididymis out and to easily detach it from the testicles using a pair of scissors. The detached vas deferens and epididymis (from one testis) were placed in a chamber of a 24-well plate containing 1 ml of TCM-199 (sperm handling medium from the incubator). The other detached vas deferens and epididymis (from the second testis) were also placed in another chamber of a 24-well plate containing 1 ml of Ham's F10 (sperm handling medium from the incubator). The same procedure was done on the other chamber containing medium from the vagina of a goat doe as an incubator. Using a pair of forceps, the spermatozoa were squeezed out of the vas deferens and the cauda epididymis in both petri dishes. The dish with the fresh spermatozoa suspension was shaken for 30 seconds and then capacitated by incubating the suspension at 37 °C for 1 hour before fertilization. The medium from the incubator was placed in the bi-gas incubator and the medium from the vagina of a goat with spermatozoa was aspirated into the semen straws (100 µl in each straw) and inserted in the goat vagina.

### 3.10. The IVF process

### 3.9. Oocytes collection

The mouse oocyte donors were sacrificed humanely by cervical dislocation, 12 hours after the hCG injection. The mouse was placed on its back to expose its abdomen on the board. The four limbs were fixed to the board using 20 gauge needles. The spray bottle was used to spray alcohol on the abdomen of the mouse to sterilize the skin. The small lateral incision was made at the midline, holding the skin above and below the incision with forceps. The skin was pulled towards the head and the tail until the abdominal muscles were completely exposed. Using the pair of forceps to lift the body wall, a pair of scissors was used to cut the body wall (muscles and peritoneum) in order to locate the two horns of the uterus, oviduct and ovaries.

The oviduct was dissected and placed in a 35 mm petri dish containing Dulbecco's Phosphate Buffered Saline (D-PBS). The petri dish was placed on the IVF workstation (IVF workstation, K-system workstation L125, Klinehøj Nænge 3-5, Dk – 3400 Bikerød, Denmark) at a surface temperature of 37 °C.



Two 4-well dishes were placed under the laminar flow hood and filled with (1) D-PBS at ambient temperature into the first well, (2) TCM-199 or Ham's F10 oocytes handling medium (from the 37 °C water bath that was prepared 2 hours before the experiment) into the second well and (3) the third well with TCM-199 or Ham's F10 IVF medium from the incubators (bi – gas incubator and the vagina of a goat doe as an incubator).

The ampullae of the oviduct were opened by making a small incision with a 25 gauge needle to release the oocytes with cumulus cell complexus (COCs). The oocytes were aspirated from the 35 mm petri dish by aspirating them with a pipette and placed in the first well of the 4-well dishes (above). These were washed three times: once in D-PBS, once in oocytes handling medium and once in IVF medium. After oocytes washing, IVF drops for semen straw filling were prepared in a similar manner as described in 3.6, without oil immersion and incubation.

Immediately after washing, up to 10 oocytes were distributed among each four IVF drops (50 µl) under the oil in a 35 mm petri dish from the incubator and the four IVF drops in a petri dish without oil using a pipette. Oocytes washed in TCM-199 IVF medium were distributed in TCM-199 IVF drops and the same procedure was used for those washed in Ham's F10 IVF medium.

The 500 µl eppendorf vial (covered with aluminum foil) and containing PHE (see appendix 4) at –20 °C was thawed at room temperature for 20 minutes before IVF.

### **3.10. The IVF process**

The IVF protocol described below was carried out following the procedures described by Fraser & Drury (1975) & Sztein *et al.* (2000).

#### **3.10.1. *In vitro* fertilization**

The capacitated sperm were taken from the incubators and placed on the laminar flow hood at 37 °C capacitated semen from the straws inserted in the goat vagina. These were opened and pooled together in a chamber on the lamina flow to make 1 ml. The sperm concentration of  $\sim 8 \times 10^6$  per ml was determined using the Computer Aided Sperm Analyser microscope (CASA, Sperm Class Analyser®, [SCA], 5.3, Microptic, Barcelona, Spain). Two microliters of the medium containing capacitated sperm were placed inside one chamber of the four chambered Leja® slide (Leja Product B.V, Luzernestraat 10, 2153 GN Nieuw Vennepe, Netherland ) on the warm glass stage at 37 °C of the CASA microscope and evaluated using sperm motility and concentration programme.



The volume of 2 - 4  $\mu$ l of Ham's F10 containing capacitated sperm were placed into Ham's F10 fertilization drops under the oil, containing ten oocytes and PHE (Penicillamine, Hypotaurine and Epinephrine) for enhancing sperm motility and penetration of oocytes. The same procedure was used with the TCM-199 medium and IVF drops without oil (both TCM-199 and Ham's F10) for straw filling.

The 50  $\mu$ l IVF drops, without oil, were aspirated into semen straws using a 2  $\mu$ l – 5  $\mu$ l pipette tip tightly fitted to the semi-closed end of the straw. About a 4 cm length of the straws were filled with the mixture of oocytes and sperm. The partially filled 0.25 ml semen straws were sealed using a mosquito forceps heated in a flame, leaving air space on both ends of semen straw and the remaining empty section of the straw removed. The sealed straws were divided into two groups: the first group were covered with a lubricated sponge and placed in the goat vagina which was used as an incubator. The vagina was opened with a vaginal speculum and then the sponge pushed in with a forceps. The second group was placed in the bi-gas incubator with the petri dishes.



**Figure 3.3.** Opening of the goat vagina with a speculum for insertion of sponge and straws

After 6 hours semen straws from the incubator and petri dishes from the bi-gas incubator were withdrawn and taken to the IVF workstation and pronucleus were assessed on the embryo to



check if fertilization took place. The sponge containing the straws was pulled from the goat vagina using tissue forceps, while a vaginal speculum was used to open the vagina of the goat doe. The vaginal straws were cleaned on the outside with methanol and taken to the IVF workstation. The straws were opened by cutting both ends on the air space and the medium with presumptive embryos was flushed into a 35 mm petri dish.

A 4-well dish filled with pre-incubated IVC medium (TCM-199 and Ham's F10) was used to wash the presumptive embryos from both group of straws and the petri dishes. The 4-well plate contained oocyte handling medium in well one and *in vitro* culture media from the incubators (vagina and the bi-gas incubator) in well two and three. The presumptive embryos were washed three times; once in oocytes handling medium and twice *in vitro* culture medium, by aspirating them with a pipette from one well to another.

Figure 3.4. The two 4-well 25 mm cell culture plates (one TCM-199 and one Ham's F10) made for oocytes and presumptive embryos washing.

### 3.10.2. *In vitro* culture

After washing the presumptive embryo, IVC drops for the semen straws were prepared as described in 3.6 without oil immersion and incubation. Immediately after, the 10 washed presumptive embryos were distributed among IVC drops (50  $\mu$ l) under the oil in a 35 mm petri dish from the incubator and the IVC drops in a petri dish without oil. Presumptive embryos washed in TCM-199 IVC medium were distributed in TCM-199 IVC drops and taken back to the incubator for culturing. The same procedure was performed for those washed in Ham's F10 IVC medium. The semen straws were filled with drops containing ten presumptive embryos and sealed as described above. Presumptive embryos from the straws and petri dishes from the incubator were placed back into the incubator and those from the goat vagina were placed back into the vagina as described above.

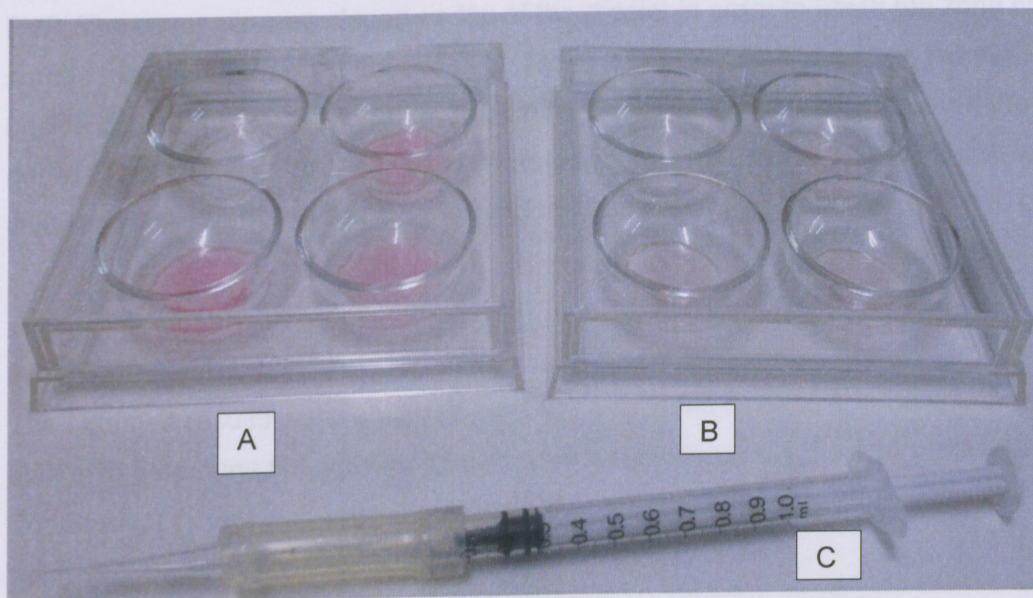
stage.

### 3.11. Embryo staining

The blastocyst-stage embryos were stained using Hoechst 33258 stain solution. A 4-well plate was used for embryo staining. Wells one and two were filled with 0.5 ml phosphate buffered saline (PBS) and polyvinyl pyrrolidone (PVP-PVA) each three with 0.5 ml fixative (paraformaldehyde in PBS, 40 mg/ml) and well four with Hoechst 33258.

The blastocyst-stage embryos from the IVC petri dish and the semen straws were washed in well one and then fixed in well 3 for 30 – 40 minutes. After fixing, the blastocysts were transferred to well four to stain for 10 minutes and then to well two for washing once again. The stained embryos were transferred to a clean microscope slide with 10  $\mu$ l drop of PBS.





**Figure 3.4.** The two 4-well dishes with (A) TCM-199 , (B) Ham's F10 media for oocytes and embryos washing and (C) pipette used to aspirate zygotes

### 3.10.3. Evaluation of *in vitro* development of the embryos

After every 24 hours, embryos from both the petri dishes and the two groups of straws (one straw per group) were evaluated. A straw from each group was opened every day and the embryos were flushed into a 35 mm petri dish under the lamina flow. After evaluation, the embryos from the straws were aspirated back into the straws and placed in their respective incubators. The petri dishes were also placed back into the incubator. After 3 days of *in vitro* culture all the straws and petri dishes were evaluated and the embryos of the blastocyst stage were stained. The other embryos at different cell stages were recorded according to their cell stage.

### 3.11. Embryo staining

The blastocyst-stage embryos were stained using Hoechst 33258 stain solution. A 4-well plate was used for embryo staining. Wells one and two were filled with 0.5 ml phosphate buffered saline (PBS) and polyvinyl pyrrolidon medium (PBS-PVP); well three with 0.5 ml fixative (paraformaldehyde in PBS, 40 mg/ml) and well four with Hoechst 33258.

The blastocyst-stage embryos from the IVC petri dish and the semen straws were washed in well one and then fixed in well 3 for 30 – 60 minutes. After fixing, the blastocysts were transferred to well four to stain for 10 minutes and then to well two for washing once again. The stained embryos were transferred to a clean microscope slide with 10 µl drop of PBS-



PVP. The cover slip was placed on top of the droplet in order to break the zona pellucida and spread the blastomeres as described by Owiny et al. (2009). Thereafter, the slide was turned upside down and examined under the inverted microscope in order to count the blastomere nuclei. The stained nuclei of the blastomere were then counted on a fluorescent UV light inverted microscope at 400x magnification (Nikon eclipse TI, Narishige co., Ltd. USA).

### 3.12. Statistical analysis

The obtained results were analyzed by a 2 x 3 factorial designs in a completely randomized design to determine the significance difference and comparing the TCM-199 with Ham's F10. The Student t - test was used to separate the mean and the following model was used:

$$Y_{ijk} = \mu + S_i + M_j + (SM)_{ij} + \epsilon_{ijk}$$

$Y_{ijk}$  = the observation of the embryo development

$\mu$  = The overall mean

$S_i$  = The effect of the  $i^{\text{th}}$  receptacles

$M_j$  = The effect of the  $j^{\text{th}}$  medium

$(SM)_{ij}$  = The interaction of the  $i^{\text{th}}$  receptacles and  $j^{\text{th}}$  medium

$\epsilon_{ijk}$  = The random error

### 3.13. Ethics

The clearance (SARDF / 15 / ANS / 02 / 0110) was obtained from the University of Venda Ethics Committee for permission to use the animals.



## CHAPTER 4

### RESULTS

Table 4.1 shows the number of receptacles (micro-drops, incubator straws and vaginal straws) and media (TCM-199 and Ham's F10) used and the total number of oocytes collected and fertilized.

**Table 4.1** The *in vitro* fertilization rates in two receptacles and media

| Fertilization rate |           |                         |                                 |                     |
|--------------------|-----------|-------------------------|---------------------------------|---------------------|
| Receptacles        | Media     | Total number of oocytes | Total number of cleaved embryos | Total number of UFO |
| Micro-drops        | TCM       | 74                      | 70                              | 4                   |
| Micro-drops        | Ham's F10 | 74                      | 73                              | 1                   |
| Incubator straws   | TCM       | 74                      | 72                              | 2                   |
| Incubator straws   | Ham's F10 | 77                      | 76                              | 1                   |
| Vaginal straws     | TCM       | 71                      | 67                              | 4                   |
| vaginal straws     | Ham's F10 | 80                      | 75                              | 5                   |

TCM = Tissue culture medium, UFO = Unfertilized oocytes

Different types of media (TCM-199 and Ham's F10) and incubators (bi-gas and vagina of a goat doe) were used for the assessment of IVF and IVC of murine embryos. The receptacles that were used were micro-drops in a petri dish in the bi-gas incubator (micro-drops), a semen straw in the incubator (incubator straw) and a semen straw in the vagina of a goat doe (vaginal straw).



The results on Table 4.1 show that during *in vitro* fertilization more embryos cleaved in the incubator straw (98 %) and micro-drops (97 %) than in the vaginal straws (94 %), although there was no significant difference ( $p > 0.05$ ) between the receptacles.

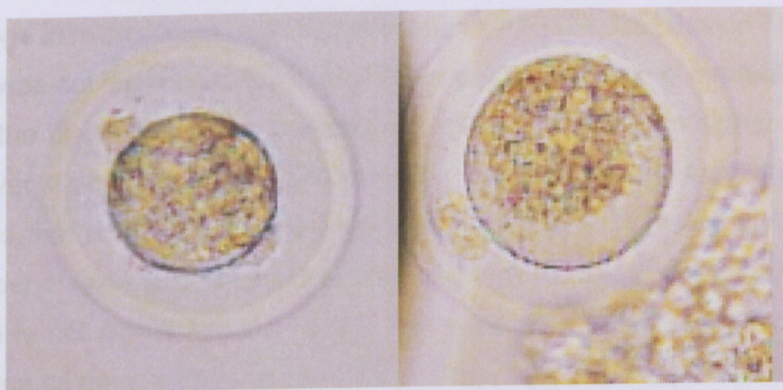


Figure 4.1 Matured murine oocytes

Table 4.2: Embryo developmental rates in the different receptacles and media (M±SE)

|                     |       | Embryo development |                    |                   |                   |                    |                    |
|---------------------|-------|--------------------|--------------------|-------------------|-------------------|--------------------|--------------------|
| Receptacles         | Media | UFO<br>(%)         | 2-cell<br>(%)      | 4- cell<br>(%)    | 8- cell<br>(%)    | Morula<br>(%)      | Blastocyst<br>(%)  |
| Micro-drops         | TCM   | 4/74 <sup>a</sup>  | 9/74 <sup>a</sup>  | 6/74 <sup>a</sup> | 1/74 <sup>a</sup> | 2/74 <sup>a</sup>  | 52/74 <sup>a</sup> |
|                     |       | 5.4                | (12.2±0.3)         | (8.1±0.3)         | (1.4±0.2)         | (2.7±0.3)          | (70.3±1.0)         |
| Micro-drops         | Ham's | 1/74 <sup>a</sup>  | 5/74 <sup>a</sup>  | 2/74 <sup>a</sup> | 1/74 <sup>a</sup> | 10/74 <sup>a</sup> | 55/74 <sup>a</sup> |
|                     | F10   | 1.35               | (6.8±0.3)          | (2.7±0.3)         | (1.4±0.2)         | (13.5±0.3)         | (74.3±1.0)         |
| incubator<br>straws | TCM   | 2/74 <sup>a</sup>  | 2/74 <sup>a</sup>  | 6/74 <sup>a</sup> | 1/74 <sup>a</sup> | 6/74 <sup>a</sup>  | 57/74 <sup>a</sup> |
|                     |       | (2.7)              | (2.7±0.3)          | (8.1±0.3)         | (1.4±0.2)         | (8.1±0.4)          | (77.0±1.1)         |
| incubator<br>straws | Ham's | 1/77 <sup>a</sup>  | 8/77 <sup>a</sup>  | 4/77 <sup>a</sup> | 1/77 <sup>a</sup> | 1/77 <sup>a</sup>  | 62/77 <sup>a</sup> |
|                     | F10   | (1.3)              | (10.4±0.3)         | (5.2±0.3)         | (1.3±0.2)         | (1.3±0.40)         | (80.5±1.0)         |
| vaginal<br>straws   | TCM   | 4/71 <sup>a</sup>  | 8/71 <sup>a</sup>  | 2/71 <sup>a</sup> | 3/71 <sup>a</sup> | 8/71 <sup>a</sup>  | 46/71 <sup>a</sup> |
|                     |       | (5.6)              | (11.3±0.3)         | (2.8±0.3)         | (4.2±0.2)         | (11.3±0.3)         | (64.8±1.0)         |
| vaginal<br>straws   | Ham's | 5/80 <sup>a</sup>  | 12/80 <sup>a</sup> | 5/80 <sup>a</sup> | 3/80 <sup>a</sup> | 9/80 <sup>a</sup>  | 46/80 <sup>a</sup> |
|                     | F10   | (6.3)              | (15.0±0.3)         | (6.3±0.3)         | (3.8±0.2)         | (11.3±0.3)         | (60.0±1.0)         |

TCM = Tissue culture medium 199 and UFO = unfertilized oocytes

<sup>a</sup> = The superscript shows there was no difference in the fertilization rates  $p > 0.05$



the mean percentage in the vaginal straws (62.4 %). The difference was, however, not significant ( $p > 0.05$ ).

#### 4.1. The percentage of embryos that reached the blastocyst-stage of the embryo

##### 4.1.1 Embryo development in Ham's F10 and TCM-199 in all receptacles

The results of this study also show that the mean percentage of embryos that reached the blastocyst-stage in micro-drops, was higher in Ham's F10 (74.3 %) than in TCM-199 (70.7 %), although this was not significant ( $p > 0.05$ ). The mean percentage of incubator straw embryos that reached the blastocyst-stage in Ham's F10 (80.5 %), was also higher than in TCM-199 (75 %), however, it does not differ significantly ( $p > 0.05$ ). The mean percentage of the vaginal straw embryos that reached the blastocyst-stage embryo in TCM-199 (64.8 %) was higher than the number of embryos that reached the blastocyst-stage in Ham's F10 (60 %), although the difference is not significant ( $p > 0.05$ ). The mean percentage of embryos reaching the blastocyst-stage of development in Ham's F10 was higher (71.6 %) than the percentage in TCM-199 (70.7 %). The difference was, however, not significantly different ( $p > 0.05$ ).



**Figure 4.2.** The blastocyst-stage murine embryo (84 hours post IVF)

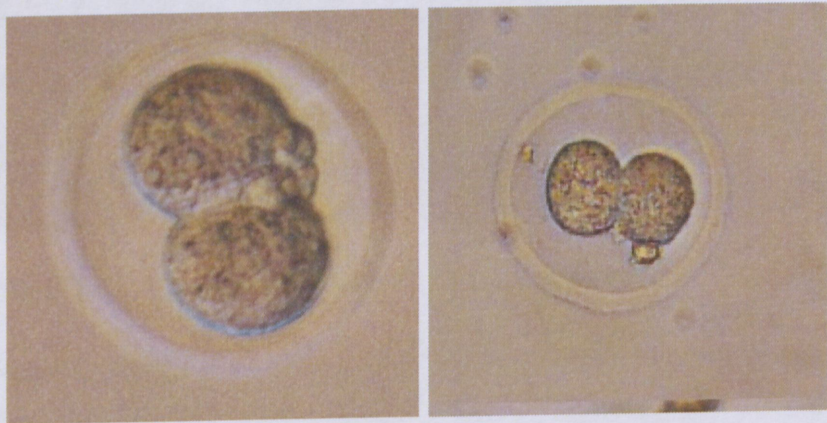
The mean percentage of embryos that reached the blastocyst-stage of the embryo in incubator straws was higher (78.8 %), than in the micro-drops (72.3 %). The difference was, however, not significant ( $p > 0.05$ ). The mean percentage of the embryos that reached the blastocyst-stage of the embryo in incubator straws (78.8 %), was higher than that in the vaginal straws (62.4 %), although the difference was not significant ( $p > 0.05$ ). The mean percentage of the embryos that reached the blastocyst-stage of the embryo in micro-drops was higher (72.3 %), than that in the vaginal straws (62.4 %). The difference was, however, not significant ( $p > 0.05$ ). The mean percentage of embryos reaching the blastocyst-stage in straws was higher (70.6 %), than that of micro-drops (72.3 %). The difference was, however, not significant ( $p > 0.05$ ). The mean percentage of embryos reaching the blastocyst-stage development in the incubator straws (77.8 %) was highest, followed by micro-drops (72.3 %) and the least was



the mean percentage in the vaginal straws (62.4 %). The difference was, however, not significant ( $p > 0.05$ ).

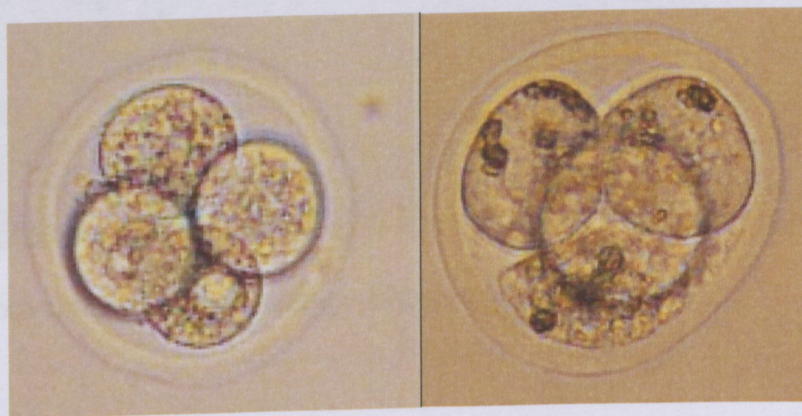
#### 4.2. The percentage of the embryo that blocked at the 2-cell to morula stage in different media and receptacles.

The current study shows that more embryos are blocked at the 2-cell stage, with mean percentages of 15 % in Ham's F10 and 11.3 % in TCM-199 in the vaginal straw embryos, followed by the micro-drop with 12.2 % in TCM-199 and 6.8 % in Ham's F10. The lowest numbers of embryos that were blocked at the 2-cell stage were observed in the incubator straw with 2.7 % in TCM-199. This difference was, however, not significant ( $p > 0.05$ ).



**Figure 4.3.** The normal 2-cell stage murine embryos (24 hours post IVF)

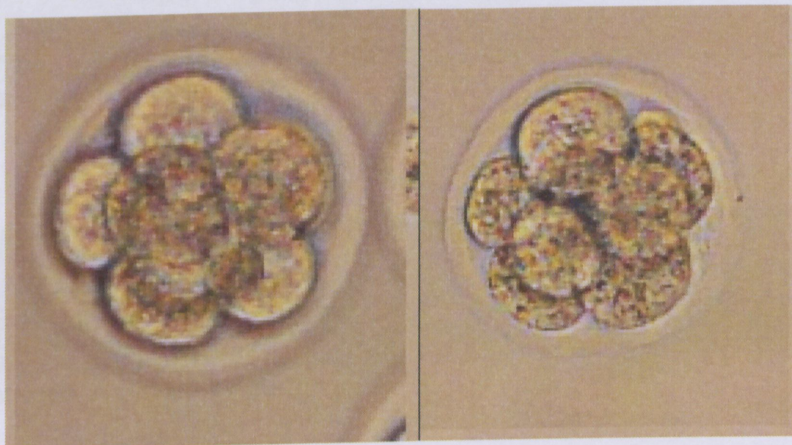
The mean percentage of the embryos that were blocked at the 4-cell stage in micro-drops in Ham's F10 was the least (2.7 %), followed by the vaginal straw in TCM-99 (2.8 %) and the incubator straw in Ham's F10 (5.2 %). Although, the difference is not significant ( $p > 0.05$ ) between the different media and the receptacles, the micro-drops and incubator straws had the highest mean percentage of embryos blocking at the 4-cell stage (8.1 %) in TCM-199, followed by the vaginal straw in Ham's F10 (6.3 %).





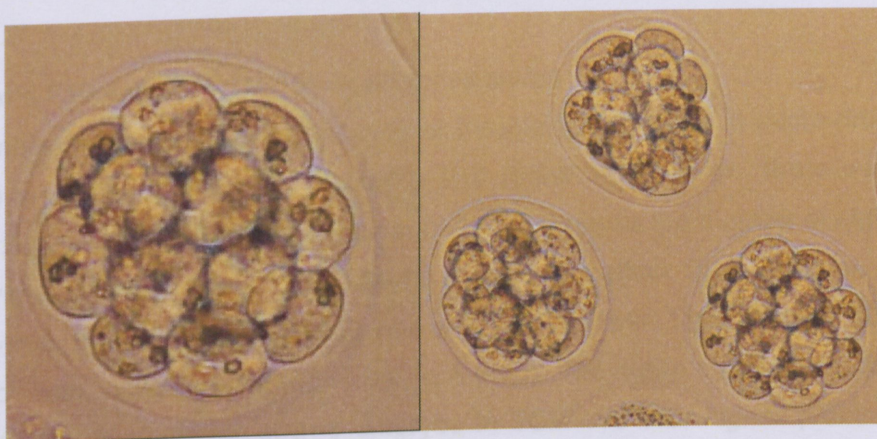
**Figure 4.4.** The normal 4-cell stage murine embryos (24 hours post IVF)

The embryos blocking at the 8-cell stage had an average percentage of 1.4 % in TCM-199, Ham's F10 in micro-drops, TCM-199 in incubator straws and 1.3 % in Ham's F10 in the incubator straws. The percentage of vaginal straw embryos that blocked at the 8-cell stage was 4.2 % in TCM-199 and 3.8 % in Ham's F10. There was no significant difference ( $p > 0.05$ ) between incubators for embryos that blocked at the 8-cell stage (Table 4.2).



**Figure 4.5.** The normal 8-cell stage murine embryos (48 hours post IVF)

The mean percentage of embryos that blocked at the morula stage in micro-drops in Ham's F10 was the highest (13.5 %), followed by vaginal straws in TCM-199 (11.3 %) and Ham's F10 (11.3 %). The mean percentage of embryos that blocked at the morula stage in the incubator straws was the lowest percentage (8.1 %), followed by the micro-drops in TCM-199 (2.7 %) and incubator straw in Ham's F10 (1.3 %). This difference was not significant ( $p > 0.05$ ).



**Figure 4.6.** The pre-compacted morula stage embryos (48 hours post IVF)



**Table 4.3: The blastomere counts for the two receptacles and media**

| Receptacles      | Media     | Total number of cleaved embryos | Blastocyst (%)                   | Mean No of blastomere at the blastocyst-stage (M±SD) |
|------------------|-----------|---------------------------------|----------------------------------|------------------------------------------------------|
| Micro-drops      | TCM       | 70                              | 52/74 <sup>a</sup><br>(70.3±1.0) | 87±9.8                                               |
| Micro-drops      | Ham's F10 | 73                              | 55/74 <sup>a</sup><br>(74.3±1.0) | 85±9.0                                               |
| Incubator straws | TCM       | 72                              | 57/76 <sup>a</sup><br>(77.0±1.0) | 90±9.2                                               |
| Incubator straws | Ham's F10 | 76                              | 62/77 <sup>a</sup><br>(80.5±1.0) | 88±6.9                                               |
| Vaginal straws   | TCM       | 67                              | 46/71 <sup>a</sup><br>(64.8±1.0) | 90±9.4                                               |
| Vaginal straws   | Ham's F10 | 76                              | 46/80 <sup>a</sup><br>(60.0±1.0) | 88±10.9                                              |

TCM = Tissue culture medium and SD = standard deviation

<sup>a</sup> = The superscript shows that there was no deference in the fertilization rates ( $p > 0.05$ )

#### 4.3. Ham's F10 versus TCM-199 in all receptacles

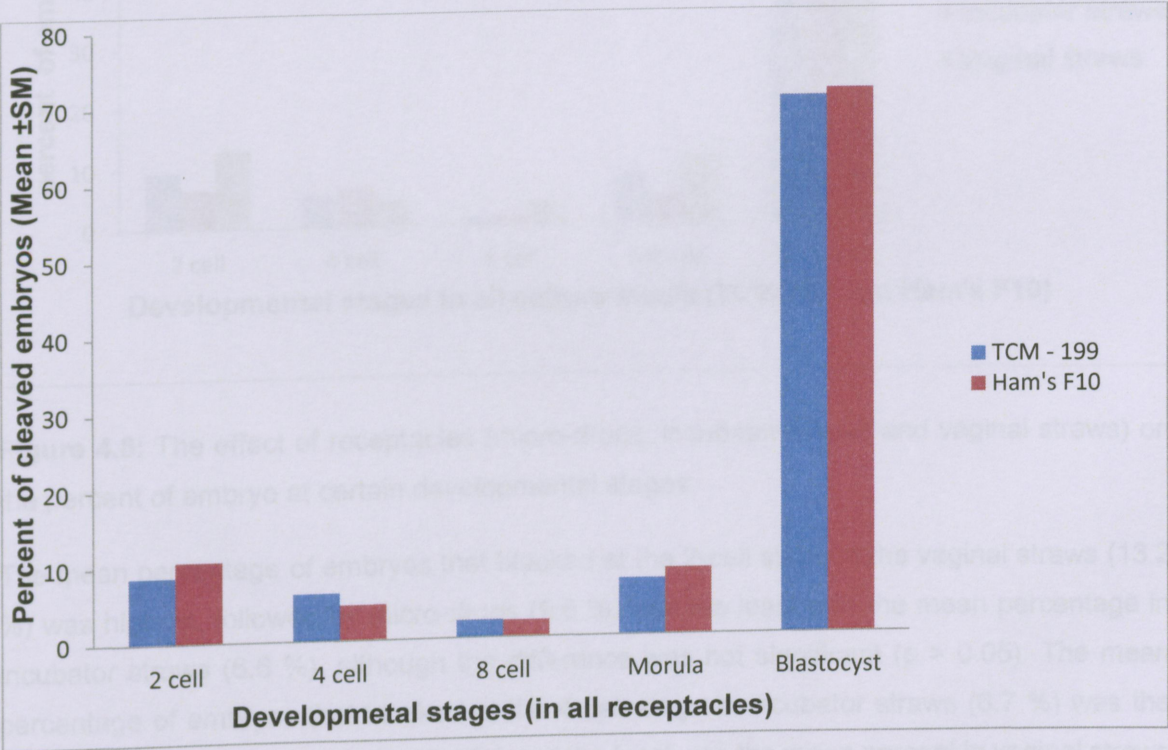
The number of blastomeres of embryos at the blastocyst-stage in micro-drops in TCM-199 (87±9.8) was higher than that in Ham's F10 (85±9.0). Although, there was no statistical difference ( $p > 0.05$ ) between the media. In different media the number of blastomeres at the blastocyst-stage in the incubator straws showed no effect ( $p > 0.05$ ) on the number of blastomeres, but TCM-199 (90±9.2) had a higher number, than Ham's F10 (88±6.9). This difference was, however, not significant ( $p > 0.05$ ). The mean number of blastomeres at the blastocyst-stage of vaginal straws embryos in TCM-199 was higher (90±9.4), than that in Ham's F10 (88±10.9). This difference was, however, not significant ( $p > 0.05$ ). The number of



blastomeres of embryos at the blastocyst-stage in TCM-199 was higher ( $89\pm9.5$ ), than that in Ham's F10 ( $87\pm8.9$ ) (figure 4.3). This difference was, however, not significant ( $p > 0.05$ ).

**4.4. The total mean number of blastomeres of embryo at the blastocyst-stage in both media (TCM-199 and Ham's F10)**

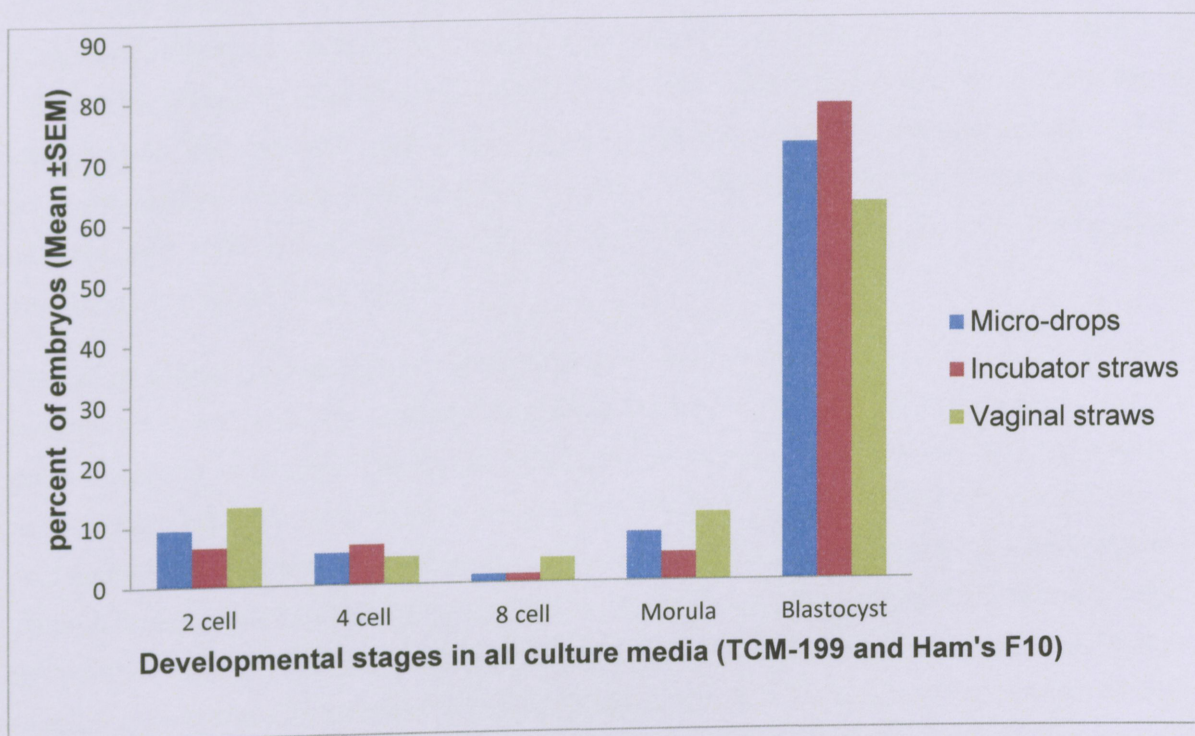
The number of embryo blastomeres at the blastocyst-stage in the incubator straws was higher  $89\pm8.1$ , than that in micro-drops ( $86\pm9.4$ ). This difference was, however, not significant ( $p > 0.05$ ). The number of embryos blastomeres at the blastocyst-stage in incubator straws  $89\pm8.1$  was equal to that in vaginal straws ( $89\pm10.2$ ). This difference was, however, not significant ( $p > 0.05$ ). The number of embryo blastomeres at the blastocyst-stage in vaginal straws was higher  $89\pm10.2$ , than the micro-drops ( $86\pm9.4$ ). This difference was, however, not significant ( $p > 0.05$ ). The number of blastomeres of embryos at the blastocyst-stage in straws was the highest ( $89\pm9.1$ ), than in micro-drops ( $86\pm9.4$ ), although the difference is not statistical ( $p > 0.05$ )



**Figure 4.7:** Effect of the culture media on the murine developmental stages from 2-cell stage to blastocyst-stage.



The mean percentage of embryos that blocked at the 2-cell stage in Ham's F10 was higher (10.7 %), than that in TCM-199 (8.7 %). This difference was, however not significant ( $p > 0.05$ ). The mean percentage of embryos that blocked at the 4-cell stage in TCM-199 was higher (6.3 %), than in Ham's F10 (4.7 %). This difference was, however, not significant ( $p > 0.05$ ). The mean percentage of embryos that blocked at the 8-cell stage in TCM-199 was higher (2.3 %), than in Ham's F10 (2.2 %). This difference was, however, not significant ( $p > 0.05$ ). The mean percentage of embryos that blocked at the morula stage in Ham's F10 (8.7 %) was higher, than that in TCM-199 (7.4 %). This difference was, however, not significant ( $p > 0.05$ ).



**Figure 4.8:** The effect of receptacles (micro-drops, incubator straws and vaginal straws) on the percent of embryo at certain developmental stages.

The mean percentage of embryos that blocked at the 2-cell stage in the vaginal straws (13.2 %) was highest, followed by micro-drops (9.5 %) and the least was the mean percentage in incubator straws (6.6 %), although the difference was not significant ( $p > 0.05$ ). The mean percentage of embryos that blocked at the 4-cell stage in incubator straws (6.7 %) was the highest, followed by micro-drops (5.4 %) and the least was the mean percent in vaginal straws (4.6 %), although the difference was not significant ( $p > 0.05$ ). The mean percentage of embryos that blocked at the 8-cell stage in the vaginal straws (4 %) was the highest, followed by the micro-drops and incubator straws with (1.4 %), although the difference was not significant ( $p > 0.05$ ). The mean percentages of embryos that blocked at the morula stage in







## CHAPTER 5

### DISCUSSION

The overall fertilization rate in the current study ranged from 94 % - 99 %. During *in vitro* fertilization 98 % and 97 % of oocytes cleaved in the incubator straws and micro-drops, respectively, which were higher than 94 % of cleaved embryos in the vaginal straw, although the difference was not significant ( $p > 0.05$ ) between the two different incubation methods. These results were similar to those reported by Cross and Brinster (1970) which were 95 % - 99% fertilization rate. However, it was higher than the fertilization rate of 63.7 % reported by Mohammed *et al.* (2010). The higher fertilization rate obtained in the present study is similar to the fertilization rate obtained by Cross and Brinster (1970) when the same 0.3 % BSA but different mice breeds were used. The Balb C and C57 mice breed was used in the present study, while the random bred Swiss mice breed was used in previous studies. The supplementation of the culture media with different exogenous protein sources (e.g. serum albumin and fetal calf serum) yields variable effects on the rate of IVF and embryo development (Robert *et al.* 1991).

The albumin that was added to most of human IVF culture media implies that it is widely considered to be beneficial. Indeed, the roles of albumin in culture media include: pH buffer, colloid osmotic regulation, membrane stabilization, carrier of growth promoting substances (amino acids, vitamins, fatty acids, hormones, growth factors, etc.), surfactant (anti-adhesion) and nutrient (breakdown to amino acids). It is, therefore, used universally as a supplement for embryo culture. Albumin is the major protein constituent of the environment of the embryo and in human blood, with various physiological roles, as stated above. It is also the most abundant macromolecule that the gametes come into contact with in the human oviduct (Leese, 1988).

The current study showed that semen straws can be a possible solution for fertilizing oocytes and culturing murine embryos *in vitro*. A fertilization rate of 94 % was obtained in the vaginal straw. In addition, 65 % of the cultured presumptive embryos reached the blastocyst-stage using the vaginal straws. These results concur with the findings of Barry (2005), where it was shown that semen straws were permeable to CO<sub>2</sub> gas and therefore, could be used as a receptacle material for culturing embryos.

The overall cleavage rates and embryonic developmental stages recorded from the current study following culture using the different media (TCM-99 and Ham's F10) and incubators (bi-gas and the goat vagina) showed no significant differences ( $p > 0.05$ ). In ten replicates, 72.3 % of embryos in the micro drops, 77.8 % in the incubator straws and 62.4 % in the vaginal straws developed to the blastocyst-stage of the embryo. The rest arrested at the lower stage



of development or degenerated. However, the murine embryo developmental stages were not affected ( $p > 0.05$ ) by either media or receptacle. The percentage of embryos that reached the blastocyst-stage of the embryo during this study was lower than that reported by Summers *et al.* (2005), who reported that 79.9 % of embryos reached the blastocyst-stage. The murine embryos reached the blastocyst-stage of the embryo after 3 days which was similar to the findings reported by Lorthongparic (2008), but contrasted with the results reported by Zhu *et al.* (2004), where embryos developed to the blastocyst stage in 120 hours of culture (5 days).

The current study demonstrated that the blastocyst-stage embryos cultured in the incubator straws had a mean percentage of blastomeres  $90 \pm 9.2$  in TCM – 199 and  $88 \pm 6.9$  in Ham's F10. Those cultured in the doe's vagina had a mean of  $90 \pm 9.4$  in TCM-199 and  $88 \pm 10.9$  in Ham's F10. Those embryos cultured in micro-drops had blastomeres with a mean of  $85 \pm 9$  in Ham's F10 and  $87 \pm 9.8$  in TCM-199. These differences were, however, not significant ( $p > 0.05$ ).

From the current study, the number of blastomeres of the blastocyst-stage embryos ranged from  $85 \pm 9$  –  $90 \pm 9.2$  in all receptacles (micro-drops, incubator straw and vaginal straw). The number of blastomeres per blastocyst embryo obtained from this study was higher than that obtained by Evisikon and Solomon (1996), Zhu *et al.* (2004) who reported  $60 \pm 1.4$  blastomeres per blastocyst (after 120 h of *in vitro* culture). Zhu *et al.* (2004) also concluded that *in vitro* culture condition significantly delays the development and reduces the number of blastocyst cells, compared to *in vivo* produced murine blastocyst, especially when cultured in a medium without EDTA. *In vitro* cultured blastocysts can reach a blastomeres number of up to 110 cells after 190 hours of culture. Fabian *et al.* (2009) reported that the average number of blastomeres of early blastocysts increased to 85.80 and the hatched blastocysts contained an average of 115.05 cells and showed the ability to grow *in vitro*, which concurs with the results of the current study. Zhu *et al.* (2004) concluded that the number of blastomeres at the blastocyst-stage embryo could affect the ability of embryonic development beyond the blastocyst stage embryo.

Successful animal and human IVF depend on using suitable culture conditions for gametes and early embryo development. Various investigators have tried a number of different media and culture conditions. Media used for IVF have ranged from simple buffered balanced salt solutions to more complex media, supplemented with amino acids and various co-factors (Olson & Seidel, 2000).

Ham's F10 medium shows the best performance on the developmental stages of the embryo compared to the TCM-199, although there was no statistical significant difference ( $p > 0.05$ ). More embryos were blocked at the 2 - cell stage with mean percentages of  $9.6 \pm 0.2$  and



10.7±0.2 in TCM-199 and Ham's F10, respectively, in all receptacles. Ham's F10 produced higher mean blastocyst-stage embryos of 71.6±0.6 than TCM-199 (70.0±0.6). The current study indicated that Ham's F10 is better suited for the *in vitro* fertilization and *in vitro* culture of the murine embryos than TCM-199. There is, however, no significant difference ( $p > 0.05$ ) observed between the two media, TCM-199 and Ham's F10 used for *in vitro* fertilization and *in vitro* culture in the present study. These results concur with the findings of Mohammed *et al.* (2010), who used different types of media (TCM-199, medicult-IVF and RPMI-1640) to assess *in vitro* fertilization and culture. These authors concluded that there was no significant difference ( $p > 0.05$ ) between the media (TCM-199, medicult-IVF and RPMI-1640). These contradict with Erbach *et al.* (1994) who reported that there was a significant difference ( $p < 0.05$ ) between different media (SOM, mSOM and KSOM).



## CHAPTER 6

### CONCLUSION AND RECOMMENDATION

It was concluded that *in vitro* fertilization and culturing of the murine embryos are found to be effective in a short length of a French semen straw incubated in a bi-gas incubator and in the goat vagina. The vagina of a goat doe can therefore be used as an alternative incubation method for fertilizing oocytes and culturing murine embryos while in a semen straw. TCM-199 and Ham's F10 can both be used to fertilize oocytes and culture murine embryos *in vitro* until the blastocyst-stage, while incubated in the bi-gas incubator or in the goat vagina.

It is therefore recommended that more research should be conducted to test oocyte fertilization from different animal species, while using the vaginas of different species as alternative incubators for *in vitro* fertilization and the culturing of embryos.



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## Appendix 1. Preparation of superovulation hormone and BSA

**Table 6.1** Solution preparations as described by Heide Schatten, (2005)

| Component   | mg/ml of saline | Vol (ml) of saline | Concentration | Storage |
|-------------|-----------------|--------------------|---------------|---------|
| eCG         | 1000 iu         | 20                 | 5 iu / 0.1 ml | -20 °C  |
| hCG         | 1500 iu         | 30                 | 5 iu / 0.1 ml | -20 °C  |
| BSA (0.3 %) | 1200            | 10                 | 0.3 mg/ml     | -20 °C  |

### 1. eCG (equine chorionic gonadotropin) preparation

The 1000 iu eCG powder was dissolved with 20 ml of saline. 1 ml syringes were used to load 0.1 ml (5 iu) and stored in the frozen form in a freezer at -20 °C.

### 2. hCG (human chorionic gonadotropin) preparation

The 1 500 iu hCG powder was dissolved in 30 ml of normal saline. 1 ml syringes were used to store 0.1 ml (5 iu) of the hCG at -20 °C.

### 3. BSA (0.3 %) preparation

The BSA powder (1.2 g) was dissolved in 10 ml of normal saline and stored in 0.5 ml eppendorf vials at -20 °C.

## Appendix 2. Preparation of PHE

### Preparation of stock solutions.

#### 4.1 Lactate metabisulphite

100 mg of sodium metabisulphite ( $\text{Na}_2\text{S}_2\text{O}_5$ ) and 328.1 mg of sodium lactate (60 % Na-Lactate) were dissolved in 100 ml of saline. The pH was adjusted to 4.004 with Sulfuric acid/ Sodium hydroxide at room temperature.

#### 4.2 Preparation of penicillamine stock

15 g of penicillamine (mw: 149.21) was dissolved in 50 ml of saline.



### 4.3 Hypotaurine stock

5 mg of hypotaurine (mw: 109.15) was dissolved in 50 ml of saline.

### 4.4 Epinephrine stock

18.2 mg of epinephrine (183.20), 5 ml of lactate metabisulphite, 19.5 ml of sodium metabisulphite, and 0.5 ml of hypotaurine stock were added in a 50 ml tube and the solution was called epinephrine stock.

PHE solution preparation

**Table :** preparation of stock solutions

|                                                             | Lactate metabisulphite stock | penicillamine stock | Hypotaurine stock | Epinephrine stock |
|-------------------------------------------------------------|------------------------------|---------------------|-------------------|-------------------|
| Component                                                   |                              |                     |                   |                   |
| Sodium metabisulphite ( $\text{Na}_2\text{S}_2\text{O}_5$ ) | 100 mg                       | -                   | -                 | -                 |
| Sodium lactate (60 % Na-Lactate)                            | 328.1 mg                     | -                   | -                 | -                 |
| Penicillamine                                               | -                            | 15 mg               | -                 | -                 |
| Hypotaurine                                                 | -                            | -                   | 5 mg              | -                 |
| Epinephrine                                                 | -                            | -                   | -                 | 18.2 mg           |
| Lactate metabisulphite                                      | -                            | -                   | -                 | 5 ml              |
| Sodium metabisulphite                                       | -                            | -                   | -                 | 19.5 ml           |
| Hypotaurine stock                                           | -                            | -                   | -                 | 0.5 ml            |
| pH                                                          | 4.004                        | 7.4                 | 7.4               | 7.4               |
| <b>Final volume (ml)</b>                                    | <b>50</b>                    | <b>50</b>           | <b>50</b>         | <b>25 ml</b>      |

The stock solutions were prepared in a low light intensity room and the tubes were covered with aluminium foil (to prevent vitamins to break when exposed to light).



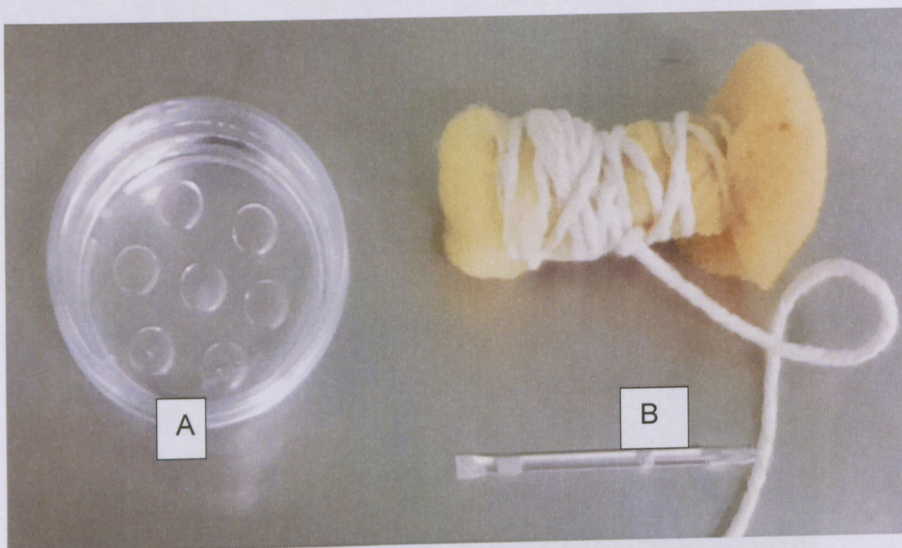
**Table 6.3.** PHE final solution

| Component           | Vol (ml)     | Storage      |
|---------------------|--------------|--------------|
| Normal saline       | 16 ml        |              |
| Penicillamine stock | 10 ml        |              |
| Hypotaurine stock   | 10 ml        |              |
| Epinephrine stock   | 8 ml         |              |
| <b>Total</b>        | <b>44 ml</b> | <b>-20°C</b> |

The PHE final solution was aliquoted to 500 µl eppendorf vials and covered with aluminium foil before storage to prevent the solution to be exposed to light.

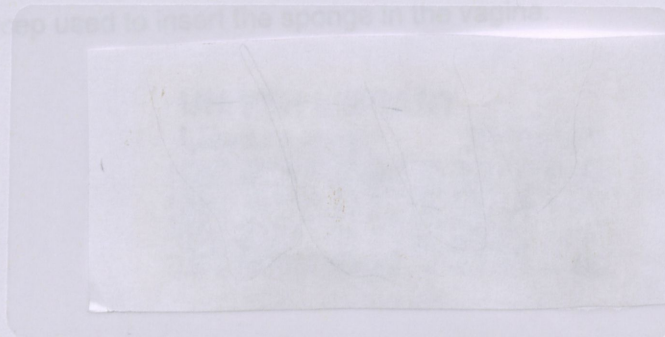
Figure 5.2 The larvae removed

### Appendix 3. Receptacles used for IVF and IVC



**Figure 6.1** Petri dish (A) immersed with oil and semen straw (B) covered with a sponge.

Figure 6.2 (A) Semen straw used to as a receptacles, (B) speculum used to open the vagina of the goat and (C) forcep used to insert the sponge in the vagina

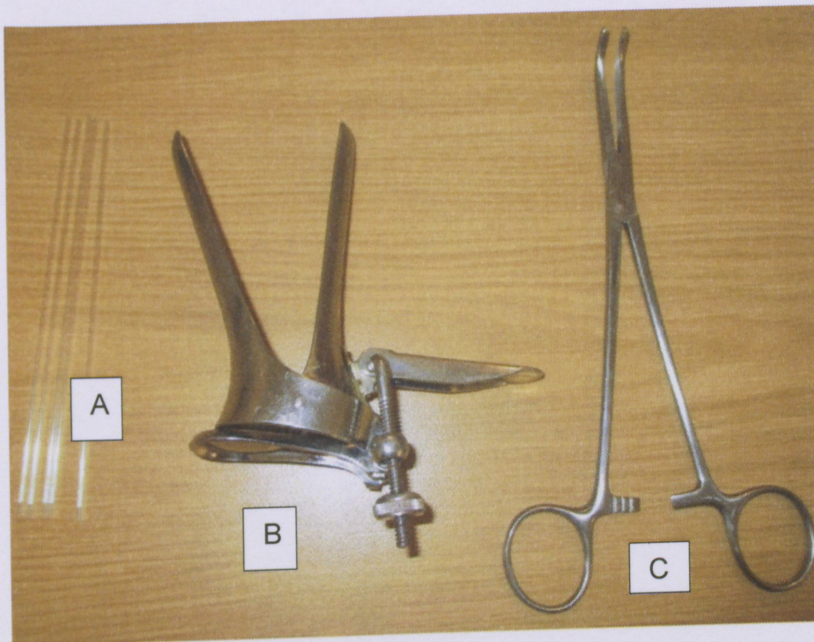




#### Appendix 4. Equipment used in the study



**Figure 6.2** The laminar flowhood



**Figure 6.3** (A) Semen straws used as a receptacles, (B) speculum used to open the vagina of the goat and (C) forcep used to insert the sponge in the vagina.