EFFECT OF *IN VITRO* CULTURE MEDIA AND ASSISTED HATCHING TECHNIQUES ON MICE EMBRYO SURVIVAL RATE FOLLOWING CRYOPRESERVATION

By

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DECLARATION

I, Serota Nthabiseng Ruth, student number.11618263, hereby declare that this dissertation for the Masters of Science in Agriculture (Animal science) hereby submitted by me to the Department of Animal Science at the University of Venda, has not previously been submitted for a Masters degree at this or any other University and is my own work in design, execution and that all reference material contained herein has been duly acknowledged.

…….. ……..

Student Signature Date

Serota N.R

DEDICATION

This dissertation is dedicated to my family, my parents: Mr Serota Nthale Darius and Mrs Serota Kgadi Elizabeth, my siblings: Mr Serota Matsobane, Ms Serota Shirley, Mr Serota Daniel and Ms Serota Mosotho and my friend Ms Tshwana Lerato.

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ABSTRACT

This study determined the effects of *in vitro* culture media (Ham's F10 and TCM199) and assisted hatching techniques (laser or mechanical) on mice embryo survival following cryopreservation. Pure strain C57BL/6 (B6) female (50) and strain BALB /c (C) Male (25) mice were crossed to produce F_1 generation of females which were injected for follicular growth and super ovulation at 6 weeks of age and from which embryos were produced 21 h later through *in vivo* fertilization. Embryos were randomly divided into Petri dishes with different culture media, and the development of embryos was assessed until the morula stage. At the morula stage, selected embryos were assisted to hatch using different techniques, and then cryopreserved in liquid nitrogen using the slow freezing method for a period of 1 week. After 1 week of cryopreservation, the embryos were thawed and cultured in the two different *in vitro* culture media for 72 hours. Thereafter, the numbers of embryos hatched or survived were recorded after 24 h, 48 h and 72 h. Data was analyzed using ANOVA in Minitab Software Version 16 (2010). Significant difference in embryo quality development was observed between *in vitro* culture media and stage of embryo development ($P<0.05$). In the TCM-199 *in vitro* culture medium, embryo quality development yielded 72, 69 and 69% from day 1 to day 3, while in Ham's F10 embryo quality development yielded 68, 63 and 60% respectively. Relative to the control (18.1%) assisted hatching improved hatchability significantly ($P<0.05$) in the order laser (23.6%)>, mechanical (20.8%). There was significant ($P<0.01$) interaction between assisted hatching techniques and evaluation time, whereby laser assisted hatching was most successful at 48 h (42.0%) while mechanical assisted hatching was most successful at 72 h (36.8%). Cryopreservation reduced the embryo survival compared to fresh embryos. In conclusion laser was the best assisted hatching technique, while TCM-199 was the better medium for *in vitro* culture of embryo.

Key words: Cryopreservation, assisted hatching, culture media, embryo

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ABBREVIATIONS

AH	Assisted hatching
ART	Assisted reproductive technology
D-PBS	Dulbecco's Phosphate Buffered Saline
eCG	Equine chorionic gonadotropin
hCG	Human chorionic gonadotropin
IVF	*In vitro* fertilization
mm	millimeter

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CHAPTER ONE

INTRODUCTION

1.1 Background to the research

The ability of an embryo to develop and implant primarily depends on the quality of the gametes which in turn depends on their source (Ziebe *et al.,* 1997). However, a proportion of euploid embryos with full developmental potential may fail to implant because of hatching difficulties. The zona hatching process is of importance for the implantation of the embryo in the uterus. The main factors affecting the implantation include quality of the embryo (Ewards *et al., 1984*) and factors linked to impairment of the zona hatching process (*Cohen et al.,* 1992). Artificially breaching the zona pellucida facilitates the process of hatching (*Cohen et al., 1992*). Assisted hatching can be achieved by mechanical (Cohen *et al*., 1990), chemical (Lanzendorf *et al., 1998*) or laser manipulation (Antinori *et al.,* 1996).

Tissue culture was established in the early 1900s, accompanied by the development of serum free media which can be reproduced in different laboratories (Bigger *et al.,* 1971). There is now a variety of different *in vitro* culture systems available ranging from simple solutions to more complex culture media, to maintain the physiological needs of embryos. These *in vitro* culture media are primarily used to maintain gametes and embryonic growth. The design of chemically defined media which are entirely free from animal-derived components accelerated in the 1940s, as different media were customised to support the growth of plant and animal cells. These developments enabled the culture of mammalian pre-implantation embryos in the late 1940s. Defined media can be modified in a controlled manner, and are free of enzyme activities that may interfere with the responses being studied.

Successful mice embryo freezing and thawing was firstly reported by Whittingham *et al.* (1972). Since then, embryos of many other mammalian species have also been successfully cryopreserved (Cohen & Li 1991). Cryopreservation protects the strain from genetic contamination, mutation, or change in genetic trait, natural disaster, and loss due to pathogenic infections. It also helps in storage, as well as improvement of transportation of genetic material across the globe. It is challenging to obtain a high survival rate and a high implantation after transferring post thaw embryos. The improvement of the survival rate after cryopreservation depends on several variables: effectiveness of the cryoprotectants, the

correct embryonic stages for freezing, quality embryo selection for cryopreservation and advanced freezing/thawing methods (Li *et al*,. 1991). However, improvements in cryopreservation and cryoprotective additives have made slow progress is the last 10 years (Camus *et al.,* 2004).

1.2 Problem statement

In multiple ovulation embryo transfer (MOET) programs, different media are currently used in the culture of embryos. Failure of the zona pellucida to rupture impairs the hatching of the blastocyst thereby preventing the implantation of the embryo. Assisted hatching of embryos improves their implantation. The hatching process can be facilitated by rupturing the zona pellucida using chemical, mechanical or laser methods, thus allowing earlier embryo–endometrial contact. After fertilization, fresh embryos are not easy to preserve for future use. Therefore, improved cryopreservation protocols are required. Therefore, the study evaluated different culture media, methods of assisted hatching and a slow freezing embryo cryopreservation method.

1.3 Justification

The use of genetically modified mice in research as models is essential to the development of new and more effective methods for diagnosing and treating diseases that affect both humans and animals. There is low implantation rate of embryos used for *in vitro* fertilization or following cryopreservation due to the failure of zona pellucida to extrude. Cryopreservation of embryos is an important technique in the whole multiple ovulation and embryo transfer (MOET) program, which could assist in cryo-conservation, as well as helping in the improvement of transportation of genetic material across the globe. According to the Food and Agriculture Organization 2011 world population is estimated to grow rapidly and by the year 2050 will reach nine billion. These estimates impose a great challenge on the current agricultural systems as food supply will need to increase by more than 100% in the next 30 years. Hence, technologies such MOET maybe is used to enhance the production efficiency of livestock.

1.4 Objectives of the research

1.4.1 Broad objective

The main objective of this study is to find suitable *in vitro* culture medium, cryopreservation protocol and assisted hatching techniques on mice embryo development.

1.4.2 Specific objectives

1. To compare the efficacy of two *in vitro* culture media (Ham's F10 and TCM199) on the quality development of mice embryos

2. To compare the efficacy of two assisted hatching techniques (Laser and Partial mechanical) on the hatchability of mice embryos cultured in Ham's F10 and TCM199.

3. To determine the effect of cryopreservation on mice embryos survival.

1.5 Hypotheses

1. Both *in vitro* culture media will yield similar quality of mice embryos.

2. Both assisted hatching techniques (Laser and Partial mechanical) will improve hatching of mice embryo cultured in Ham's F10 and TCM199 similarly.

3. Cryopreservation will damage *in vitro* produced mice embryos following thawing

CHAPTER TWO

LITERATURE REVIEW

1.1. Introduction

In vitro cultivation of mammalian ova is critical tool to study early embryogenesis and the suitable environments for an embryo to develop. The greatest success and considerable information on *in vitro* culture requirements for pre-implantation embryo stages have been reported for mouse embryos (Brinster *et al*., 1968). 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 from the *in vitro* manipulation of human embryos. Mice pre-implantation embryos are still the most popular model which is commonly used for investigation of mammalian early development *in vitro.* However, to understand the fundamental mechanisms of pre-implantation development other models have to be studied in addition (Dattena *et al*., 2007).

In assisted reproductive technology, *in vitro* culture media have the potential to affect embryo viability before or after transfer, and potentially the health of offspring. Researchers found that culture media conditions can result in delayed cell division (Bowman & McLaren 1970*)* and increased cell death (Brison & Schultz, *1997*). Other studies have shown that the environment for pre-implantation development can affect the expression and imprinting of key genes (Doherty *et al.,* 2000; Fauque *et al.,* 2007), which can result in chromosomal defects and abnormal embryos, and may act as markers of abnormal health in offspring. Embryo culture can induce changes to epigenetic marks1 in mammal embryos (Katari *et al*., 2009; Zhang et al. 2010) and such changes have been shown to affect the development and future disease risk (Kelsey *et al*., 2007). The suboptimal *in vitro* culture media and 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 (Sirard *et al.*, 2006).

A key event for successful embryo implantation is the expansion and thinning of the zona pellucida and embryonic hatching. One of the suggested explanations for impaired hatching, which may result in failure to implant and achieve pregnancy, is thickening or hardening of the zona pellucida. Hormonal factors including basal follicle stimulating hormone (FSH) and pre-ovulatory oestrogen levels are associated with thick zona pellucida, and some studies have reported a direct relationship between the number of mammalian gonadotropin ampoules given for ovarian stimulation and ZP thickness.

According to Gordon & Dapunt (1993), several techniques for assisted hatching have been introduced over the years which can be performed chemically, mechanically, or by using a laser beam. Taken together, these findings indicated that assisted hatching can exert both facilitating and deleterious effects on subsequent embryonic development depending on several factors, such as zona thickness, the area of thinning treatment, the size of the hole created, mechanical damage to the embryo by manipulation, chemical damage by acid solution, and the technical skill of the operator. For example, an inappropriately small hole may cause the embryo to become trapped during hatching, creation of trophectodermal vesicles, or strangling of the intracellular matrix, which was suggested as the possible reason for the higher rate monozygotic twinning after assisted hatching.

Whittingham (1972) stated that cryopreservation of embryos defines the long-term preservation of the pre-implantation embryos of any species which suspends its metabolism and development by freezing them at very low temperature. Whenever need arises, the cryopreserved embryos are thawed and implanted back into pseudo pregnant females which subsequently gives progeny and further expands the colony. Scientists have adopted/ invented different protocols for cryopreservation of mice and rat embryos at different laboratory conditions and are still engaged in refining the entire procedure of cryopreservation.

Cryopreservation protects the strain from genetic contamination, mutation, or change in genetic trait, natural disaster, and loss due to pathogenic infections. Due to cryopreservation, there is simplicity in transportation of embryos than the actual live animals. It also helps in planned animal production or a material for making of genetically engineered mouse. The first successful cryopreservation of a mouse embryo was reported by Whittingham *et al.* (1972) by using slow freezing and thawing method. This method reports high survival rates and excellent reproducibility. In slow freezing, if the rate of cooling is controlled, then chances of cryo-injury by forming intracellular ice are less. Use of appropriate cryoprotectant also limits the formation of intracellular ice crystals formation. In Whittingham *et al.,* (1972) study, 1, 2 Propanediol as a cryoprotectant was used because it permeates rapidly into the blastomeres and diffuse rapidly after thawing. We have developed an embryo bank at Laboratory Animal Facility of Advanced Centre for Treatment, Research and Education in Cancer (ACTREC) for various mice strains by “Slow freezing and Fast revival method”. This is expected to preserve our original genotype to re-establish our foundation stock whenever required.

2.2 In *vitro* culture media

The culture media used for the early years of human embryo were those designed for tissue culture and were either simple salt solutions or complex tissue culture media. ‘Simple’ media

such as Tryode's or Earle's T6 medium consisted of balanced salt solutions with added carbohydrates glucose, pyruvate and lactate and were commonly supplemented with patient's serum. These media lacked many components that we now know are important for maintaining embryo physiology and health such as amino acids. Alternatively, embryos were grown in tissue culture media that were designed to support immortal cell lines in culture and were more complex containing salts supplemented with carbohydrates, amino acids, vitamins, nucleic acids and metal ions and include media such as Ham's F-10, MEM or TCM-199 (Menezo *et al.,* 1984; Lane *et al.,* 2001). None of these media were designed to support embryo development and they contained many components that have subsequently been shown to be detrimental to embryo development in vitro such as high levels of glucose, metal ions and hormones Brown & Whittingham *et al.,* 1991; Bowden *et al.,* 1993; Quinn *et al.,* 1995, Pinsino *et al.* 2010).

2.2.1 Culture media (components and systems)

The fact that many different culture media, with different formulations, have been used in the past generally led to the view that the choice of culture media for cleavage stage embryos is not important. However, outcomes of recent studies and increased knowledge about the biochemistry, genetics and epigenetic control of the mammal embryos have led to this view being challenged. As outlined in a review by Lane & Gardner (2007), historically mammal embryos were cultured in either simple salt solutions (commonly supplemented with the patient's serum) or in complex tissue culture media. The simple salt solutions lacked many components, such as amino acids, that are now known to be important for the maintenance of embryo physiology and health.

The tissue culture media was designed to support immortal cell lines in culture and were far more complex, being supplemented with carbohydrates, amino acids, vitamins, nucleotides and metal ions. Many of these components, such as high levels of glucose, divalent metal ions, nucleotides and certain hormones, have subsequently been shown to be detrimental to embryo development *in vitro*. The majority of centres no longer prepare their own culture media in house and now obtain commercially produced media, manufactured under good manufacturing procedures, specifically for use in clinical IVF applications. This has resulted in a greater standardisation of practice between centres. There are two main systems for culturing embryos: monoculture systems or sequential culture systems. Monoculture systems are 'a single medium formulation that is used to support zygote development to the blastocyst stage (Lane & Gardner *et al*., 2007).

A monoculture system does not reflect any changes in the physiology of the embryo. The majority of commercial culture systems are sequential culture systems. These systems are designed to address the changing requirements of the developing embryo – optimum conditions to support the development of a zygote will not be the optimum conditions to support the development of a blastocyst. The majority of centres culture embryos for 2 or 3 days, but some centres culture to the blastocyst stage (5-6 days) before they are transferred. A number of media have recently been designed specifically to support development to blastocyst stage, such as DM1/2/3, G1/G2 media, KSOM and Quinns**3** Advantage (Lonergan *et al.,* 2003).

The following are possible components of embryo culture media:

- **Glucose**

Glucose, when used together with amino acids, vitamins or EDTA (ethylene diaminetetra acetic acid) and phosphate, is necessary for the development to blastocyst stage and to prevent loss of viability. However, if a high level of glucose is used in a media containing phosphate and lacking amino acids, then there is a risk of retardation or developmental arrest of cleavage-stage embryos (Chatot *et al.,* 1989)

- **Amino acids**

Amino acids have proven to be the most important components of culture media for all mammalian embryos. Amino acids enhance embryo development to the blastocyst stage and increase subsequent viability15. They have now been shown to have many important roles in the culture media, including Chelators, osmolytes, pH, buffers, and antioxidants, regulators of energy metabolism, biosynthetic precursors and energy substrates (Lane & Gardner, 2007).

- **Ammonium**

Amino acids in the culture media break down in the culture medium to produce ammonium. Ammonium does not always alter blastocyst development; but it does significantly affect the cellular health of the blastomeres. Studies on animal models have shown that the presence of ammonium results in a significant reduction in the ability of embryos to implant and significant foetal loss after pregnancy was established. Moderate levels of ammonium have been shown to affect foetal growth rates so that foetuses were smaller than controls. One study has found that the development of human blastocyst is inhibited by the ammonium levels produced whilst culturing embryos in medium containing glutamine (Virant-Klun *et al.,* 2006).

- **Chelators: EDTA**

It is well established that EDTA has an important role in the development of cleavage-stage embryos, but it has a negative effect on post-compaction stage embryos. The presence of EDTA inhibits the development of the inner cell mass (Abramczuk *et al.,* 1977).

- **Macromolecules**

Supplementation of culture media with a patient's serum used to be common practice. However, more recently, there is evidence that serum can be detrimental to the developing mammalian pre-implantation embryo and may be responsible for alterations in metabolism, ultra structure, methylation of imprinted genes and foetal overgrowth. Therefore, instead of using patient's serum, culture media are now supplemented with serum albumin. Hyaluronan is a macromolecule present in elevated levels in the reproductive tract when an embryo is present. Subsequently, hyaluronan has an important role in embryo culture media and can substitute albumin and its use in a culture media containing albumin has been shown to significantly increase development and improve survival of thawed embryos. A recent randomised controlled trial in clinical IVF confirmed that elevating the levels of hyaluronan increased pregnancy and implantation rates, which was particularly evident in selected patient groups (Valojerdi *et al.,* 2006).

2.2.3 Effect of culture conditions on viability of mouse developed *in vitro*

There are a lot of 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 culturing, influencing the embryo's interaction with soluble factors. The culture medium may dilute some important autocrine/paracrine factors effecting the viability and embryo developmental performance *in vitro* (Paria *et al.,* 1990). Pre-implantation 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 replicate the normal environment of the developing embryo (Borland *et al.,* 1977).

2.4 Assisted Hatching

Assisted Hatching was performed mechanically by partial zona dissection (PZD) just before embryo transfer to the uterine cavity. The cryopreserved embryos underwent the procedure after thawing and prior to the thawed embryo transfer. AH was carried out on the Nikon microscope using Nomarski modulation optics with three-dimensional manipulators

(Narishige, Tokyo, Japan). The procedure was performed as described by (Feng *et al.,* 2011). The embryo was placed in 5 µl of cleavage medium supplemented with 10% SSS under mineral oil (Sage, USA), held by a holding pipette. The PZD pipette (Cook, Australia) was advanced through the perivitelline space to impale the ZP. Then suction was discontinued from the holding pipette, and the micro needle impaling the ZP was brought below the holding pipette, which was rubbed over the impaled section of the ZP to create an incision.

2.4.1 Mechanical technique

The technique of assisted hatching using partial zona dissection (PZD) to create an artificial opening of the zona pellucida of early cleaved embryos was first described by Cohen *et al*., 1990, while the embryo is stabilised by holding pipette, the zona pellucida is pierced with a micro needle that is pushed tangentially through the space between the zona pellucida and blastomeres until it pierces through the zona pellucida again. The embryo is released from the holding pipette. The small part of the zona trapped against the micro needle is then rubbed against the holding pipette, thus opening the area between the two sides pierced by the micro needle.

The mechanism of PZD is quick to perform, but it produces holes of variable sizes that may not always be optimal. A refinement of PZD has been described where a second cut is made in the zona pellucida under the first cut at a right angle, leaving a cross-shaped hole on the surface of the zona pellucida (three-dimensional PZD). This method allows the creation of larger openings while permitting the protection of the embryo by the zona pellucida flaps during embryo transfer. Nijs *(*1993) described a zona rubbing technique to assisted hatching, by reducing the thickness of the matrix through gentle rubbing with a micro needle. No gap is made in the zona pellucida, so the risk of blastomeres loss and invasion of micro-organisms or immune cells is minimized.

2.4.2 Laser-assisted hatching

The technique of laser-assisted zona drilling was first reported by Hellebaut, & Kort (1996). Laser technique presents an ideal tool for microsurgical procedures, as the energy is easily focused on the targeted area to produce a controlled and precise hole to allow smooth hatching of the embryo. The laser produces beams which may either be directed to the zona outer cover using an optical lens tangential to the embryo through the zona pellucida in a non-contact mode, or the laser may be guided through an optical fiber touching the embryo in a contact mode.

Laser assisted micro dissection of the ZP can be done with high precision and repeatability with no negative impact on embryo development. The technique is easy to perform and very

effective with regard to the overall time requirement and can be performed in a sterile environment without any additional micromanipulations. Using the infrared 1.48 µm diode laser it is feasible to open the zona pellucida even in largely expanded blastocysts without visible blastomere damage. The safety of the 1.48 µm diode laser beam has been evaluated in mouse and human oocytes and zygotes (Khalifa et al., 1992).

2.5 Cryopreservation

2.5.1 Slow freezing

In slow-freezing, cells in a medium are cooled to below freezing point. At some stage, ice masses containing pure crystalline water will form. What remains between the growing ice masses is the so-called unfrozen fraction, in which all cells and all solutes are confined. The concentrations of sugars, salts and cryoprotectant (e.g. glycerol) increase, while the volume of the unfrozen fraction decreases. The increase in osmotic strength causes an efflux of water from the cells. Slow cooling is needed in order to allow sufficient efflux of water to minimize the chance of intracellular ice formation. As cooling continues, the viscosity of the unfrozen fraction ultimately becomes too high for any further crystallization. The remaining unfrozen fraction turns into an amorphous solid that contains no ice crystals (Bank *et al.*, 1973).

2.5.2 Chilling injury and cold shock

The first challenge in cryopreserving cells from homeotherm (warm-blooded) animals is in cooling the cells below body temperature. Cells may be damaged by very rapid cooling (cold shock) or be damaged by low temperature *per se* (chilling injury). Behaviour and function of membrane lipids and proteins may be affected by temperature. For example, membrane lipids that are normally in a liquid crystalline state may solidify at non-physiological temperatures, which can change their function and begin processes such as cryocapacitation of the production of reactive oxygen species that increase damage to membranes. Decreasing the temperature may cause an imbalance in cellular processes because the rate of one process may be affected more strongly than that of another. One example is the disintegration of the metaphase spindle of oocytes caused by a change in the dynamic equilibrium of the association/dissociation of the tubulin filaments (Woelders & Malva, 1998).

2.5.3 Super cooling

In slow-freezing methods, cells are brought into a suitable freezing medium and cooling is continued below the freezing point of the medium. Ice formation does not necessarily start at the freezing point. Small ice crystals have a lower melting/freezing point than “bulk” ice, due to their large surface tension. Spontaneous ice nucleation will in most cases occur after the

solution is super-cooled to a temperature between 5 and 15°C. Thereafter, ice will grow rapidly in all directions, and the release of the latent heat of fusion will cause the sample to warm up abruptly until the freezing/melting temperature of the solution (i.e. of the remaining unfrozen fraction) is reached. At this point, the ice formation will stop, or will proceed at a rate governed by the rate at which the heat of fusion is transported from the sample. Finally, the sample can "catch up" again with the lower temperature in the freezing apparatus. When the initial freezing medium contains only salts (electrolytes), salt concentrations in the unfrozen fraction will reach extremely high levels as the temperature decreases. In contrast, in a medium that contains a large proportion of non-electrolytes, the total solute concentration at each subzero temperature will be the same as that found at the equivalent temperature in a medium containing only salts; however, the salt concentration will be much lower (Fiser *et al.*, 1991; Henry *et al.,* 1993).

2.5.4 Effect of cryoprotectants

Sugars can be used as non-electrolyte solutes, but they will only affect the extracellular salt concentration. Moreover, high concentrations of impermeable solutes impose osmotic stress on the cells already before freezing. This is much less the case when a membrane permeable solute, such as glycerol, is used rather than a non-permeable solute. When cells are brought into a hypertonic glycerol medium, water will leave the cells because of the osmotic pressure difference. However, at the same time, glycerol will enter the cells. After a short period of equilibration, the cells will have regained their original volume (Henry *et al.,* 1993).

The osmotic stress imposed by a hypertonic glycerol solution is therefore much smaller than that imposed by a hypertonic sugar solution. Hence, glycerol can be used at greater concentrations than sugars without damaging the cells. A substantial initial glycerol concentration in the medium means that part of the extracellular and intracellular water is replaced by the glycerol. Hence, the amount of ice formed is lower, the unfrozen fraction remains larger, the degree of shrinkage of the cells is limited, and the electrolyte concentration in the unfrozen solution and in the cells will be relatively small.

The mechanisms through which other membrane permeable substances, such as ethylene glycol and dimethyl-sulfoxide (DMSO), provide cryoprotection are similar to those involving glycerol. There are additional mechanisms through which polyols, such as glycerol and several sugars provide cryoprotection. These substances can stabilize lipid membranes by hydrogen bonding with the polar head groups of membrane lipids (Crowe and Crowe 1984; Crowe *et al.,* 1985), which is especially important under severely dehydrated conditions. In addition, these substances may affect the mechanical properties of the unfrozen fraction, especially its viscosity and glass-forming tendency (Chaveiro *et al.,* 2006).

The degree to which cells shrink and re-swell after addition of a membrane-permeable cryoprotectant depends on the concentration of the cryoprotectant and the relative permeability of the membrane to water and to the cryoprotectant (Kleinhans, 1998). For instance, bull sperm shrink very little when brought into a freezing medium with glycerol (Chaveiro *et al.,* 2006), whereas bovine embryos react much more strongly. Upon thawing, removal of the cryoprotectant has the opposite effect on cells: they first swell and then they shrink again. This may lead to damage if the cells expand too much. Damage due to over-swelling of cells can be prevented by stepwise removal of the cryoprotectant.

2.5.5 Interactions of cooling rate with thawing rate and cryoprotectant concentration

The optimal cooling rate may depend on various other factors, such as the cryoprotectant concentration and the thawing rate. It has been observed in semen from a number of species that the combination of fast cooling and slow thawing is particularly damaging to the cells. (Rodriguez *et al., 1*975). If intracellular ice nucleation occurs at a low temperature and cooling proceeds rapidly, it may be that the cytoplasm turns into glass before the intracellular ice crystals grow to a significant size, thus causing only sublethal, or no, damage. During slow thawing, the small crystals can grow and subsequently damage the cells (Rall *et al.,* 1984). In addition, cells may be damaged by extracellular restructuring of ice masses, a process known as “recrystallization” (Bank *et al.,* 1973).

CHAPTER THREE

THE EFFECTS OF DIFFERENT CULTURE MEDIA ON THE DEVELOPMENT OF MICE EMBRYOS

3.1 Introduction

The aims of embryo culture in assisted reproductive technology (ART) programmes are to improve the quality of embryos which are developing *in vitro* as well as the chances of successful implantation. Tissue culture was established in the early 1900s, along with the development of serum free and chemically defined media which can be reproduced in different laboratories (Bigger *et al.,* 1971). *In vitro* culture media were designed to maintain gametes and embryonic growth. This encouraged different mammalian embryo culture systems in many laboratories, where in both single-step and sequential culture systems are available for culture from fertilized oocytes to blastocysts (Lane & Gardner, 2007; Biggers & Summers, 2008). Regardless of such differences, successful culture media for pre-compaction mammalian embryos have a number of common characteristics, which is related to their ability to effectively support the physiological needs of the embryo. The study investigated the effect of two *in vitro* culture media (Ham's F10 and TCM199) on the quality development of mice embryos.

3.2 Materials and methods

3.2.1 Study site description

The study was conducted at the Center of Excellence in Assisted Animal Reproduction, Biotechnology Laboratory, School of Agriculture, University of Venda, Thohoyandou, Limpopo, South Africa (22 ° 58 ' S, 30 ° 26 ' E. Daily temperatures at Thohoyandou vary from about 25˚C to 40˚C in summer and between approximately 12˚C and 26˚C in winter. Rainfall is highly seasonal with 95% occurring between October and March. The average rainfall is about 800 mm but varies (Mzezewa et al., 2010).

3.2.2 Experimental procedure

3.2.2.1 Ethical clearance

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

3.2.2.2 Management of animals

Embryos were collected from the mice at the University mice colony. The ambient temperature range was 16 – 18˚C. Mice were kept in barred cages and provided with feed and water *ad libitum*.

3.2.2.3 Breeding animals

Pure C57BL / 6 (B6) and BALB / c (C) mice were mated to produce the F_1 generation. At the age of 3 weeks the offspring were weaned and separated into different barred cages according to their gender avoid reproduction. At the age of 6 weeks, the animals had matured for the experiment.

3.2.2.4 Ovarian Stimulation and Ovulation Induction

3.2.2.4.1 Preparations of chorionic hormones

Folligon

Folligon (Intervet SA, MSD Animal Health), 1000 international units (IU) of Folligon was diluted to 5 IU/ 0.1 ml using 1.0 ml syringe. The solution was stored in syringes and kept frozen at -30° C.

Chorulon

Chorulon (Intervet SA, MSD Animal Health). This was dissolved in the saline solvent. The Folligon was brought to a concentration of 50 IU /ml= 5 IU/ 0.1 ml. The solution was stored as 5 IU in 1.0 ml syringes and kept frozen at -30° C.

3.2.2.5 Administration of injections

The F_1 female mice were injected peritoneally with 0.1 ml (5IU) of eCG using a 1 ml syringe 0.5 x 16 mm needle, and 46-48 h later, 0.1 ml (5 IU) of hCG was injected. After the injections, the male and female mice were allowed to mate and embryos were collected at 21 h later.

3.2.2.6 Preparations of the media

Tissue culture media 199 and Ham's F10 (Highveld Biological, South Africa) were used for embryo culture. The media were prepared 1 day before use (Table 3.1 & 3.2), in the Bi - gas incubator (HF 100, Shanghai Lishen Scientific Equipment Co., Ltd) at 37ºC and 93% humidity in atmospheric air enriched in CO_2 (20% oxygen, 5% CO_2, 75% Nitrogen). Antibiotic-antimycotic was used to target microorganisms such as bacteria to avoid contaminations.

Bovine serum albumin was used as protein stabilizer and to act as a small molecule carrier (Heide *et al.,* 2005).

Table 1 Shows; the preparation of Ham's F10 *in vitro* culture medium.

Ham's F10	4.935Ml
Anti-anti cocktail (Antibiotic-antimycotic) 1%	0.05mL
Bovine serum albumin	0.015mL
Heparin	
	5mL

Table 2 Shows; the preparation of TCM-199 *in vitro* culture medium.

TCM-199	4.935mL
Anti-anti cocktail (Antibiotic-antimycotic) 1%	0.05mL
Bovine serum albumin	0.015mL
Heparin	
	5mL

3.2.2.7 Collection of embryos

After 21 h, mice were sacrificed by cervical dislocation. Then laid on its back on the absorbent paper and soaked with ethanol 70% to reduce contamination from the fur. Small lateral incision was made at the midline using surgical scissor and the skin was pulled anterior. Using a fine forceps, the uterus, oviduct, ovary and fat pad taut were gently pulled and cut between the oviduct and ovary. The oviduct and attached segment of uterus were then transferred in to a 35 mm petri dish containing holding medium (Whittingham et al.1993). The petri dish was placed on the laminar flow (IVF workstation, K-system workstation L125, Klinehøj Nænge 3-5, Dk – 3400 Bikerød, Denmark) at 37°C. The embryos were then collected from the oviduct by flushing using 1 ml syringe 26-gauge needle filled with holding medium (phosphate buffered saline). Thereafter, embryos were allowed to sit in Hyaluronidase for one min to strip off the cumuli.

3.2.2.8 Washing Embryos

Embryos were moved in a 1 ml volume of embryo holding media into the first well of embryo washing medium (TCM-199 and Ham's F10). Embryos were moved in a minimal volume of

medium into each successive wash, using sterile embryo handling tips that are changed between each successive wash. Embryos were moved through four more washes as described.

3.2.2.9 Embryo culturing

After washing the embryos, a pipette (10 µl – 100 µl) was used to dispense seven drops of 50 µl *in vitro* culture medium (TCM - 199 and Ham's F10 separately), on the bottom of a 35 mm sterile plastic tissue culture dishes. Immediately after, 10 washed embryos were distributed among the *in vitro* culture media drops. Embryos washed in TCM-199 medium were distributed in TCM-199 drops, and same procedure was performed for those washed in Ham's F10 medium. Immediately after distributing the embryos, the dishes were flooded with light mineral oil and placed in the incubator for culture.

3.2.2.10 Embryo quality (grading)

After every 24 h of culture, the embryos were taken out of the incubator and graded (Steer *et al*., 1992) under 400X inverted microscope.

Table 3 Shows; the characteristics of embryo grading

Stage (Days)	Characteristics		
	Good	Fair	Poor
1	2-4 even cells, ≤ 10% fragments	2- 4 even cells, 10 - 20% fragments	Few blastomeres, ≥ 20% fragments
2	6-8 even cells, ≤ 10% fragments	6- 8 even cells, 10 - 20% fragments	Few blastomeres ≥ 10% fragments
3	Different cell size, Fair compaction	Different cell size, Poor compaction	Large cell size Poor compaction

3.3 Experimental design

The experiment evaluated two *in vitro* culture media at three stages of embryo development in a randomized factorial design with three replications.

3.4 Statistical analysis

Data were analyzed using the Minitab Software Version 16 (2010) in a completely randomized design with two x three factorial.

$$Y_{ijK} = \mu + M_i + S_j + (MS)_{ij} + £_{ij}$$

Where:

- Y_{ijk}= the observation (quality development)
- μ = The overall mean
- M_i = The effect of i^{th} *in vitro* culture media
- S_j = The effect of j^{th} stages of embryo development
- MA_{ij} = The interaction of the i^{th} media and j^{th} stage of embryo development
- $£_{ijk}$=The random error

3.5 Results

Table 4 Shows; the effect of *in vitro* culture media on day 2 and 3 embryo development

STAGE	CM	LESS NC	NORM NC	MORE NC	NONE F	NORM F	MORE F	TGQ
Day1	TCM	5.5^{b}	80.7^{a}	10.5^{a}	18.3^{ab}	61.5^{a}	22.0^{b}	79.5^{a}
	Hams	15.3^{a}	74.5^{b}	10.5^{a}	22.0^{a}	59.0^{a}	28.0^{a}	73.5^{b}
Day2	TCM	8.5^{b}	78.5^{a}	12.0^{a}	15.3^{b}	50.0^{b}	23.5^{b}	78.0^{a}
	Hams	15.8^{a}	71.5^{b}	13.7^{a}	19.0^{ab}	49.0^{b}	30.0^{a}	70.3^{c}
SEM		1.4	0.7	1.3	1.0	0.5	0.8	0.6
MEANS								
Day1		10.6^{b}	77.6^{a}	12.0^{a}	20.1^{a}	54.0^{b}	25.1^{b}	76.5^{a}
Day2		11.8^{a}	75.5^{b}	12.9^{a}	17.2^{b}	55.7^{a}	27.0^{a}	74.1^{b}
SEM		0.9	0.5	0.9	0.7	0.4	0.5	0.4
	TCM	7.0^{b}	80.1^{a}	12.8^{a}	16.7^{b}	60.3^{a}	22.0^{b}	78.7^{a}

	Hams	15.5[a]	73.0[b]	11.0[a]	20.5[a]	50.2[b]	29.2[a]	71.7[b]
SEM		0.9	0.5	0.9	0.7	0.3	0.5	0.4
P Values								
CM		0.00	0.00	0.48	0.00	0.00	0.00	0.00
Stage		0.37	0.01	0.72	0.01	0.08	0.03	0.03
CM x Stage		0.21	0.25	0.13	1.00	0.37	0.43	0.18

[a b] Means with different superscript letters in the same column differ significantly, CM= *in vitro* culture media, TCM= TCM-199, Hams= Ham's F10, For day1: less NC= less number of cell (<2 cells), NORM NC= normal number of cell (2-4 cell), MORE NC= more number of cell (>4 cells),For day 2: less NC= less number of cell (<6 cells), NORM NC= normal number of cell (6-8 cell), MORE NC= more number of cell (>8 cells), NO F= no fragments, NORM F= normal fragments (10-20%), MORE F= more fragments (>20%), stage= stage of embryo development, TGQ= Total good quality embryo, P<0.05= Significant, P<0.01= Highly significant, P>0.05= Not significant, SEM: standard error mean.

Stage of embryo development was significantly different (P<0.05) on the degree of fragments and not significantly different (P>0.05) on less number of embryos. *In vitro* culture media significantly influenced all the embryo quality parameters, with high significant difference (P<0.01) on the degree of fragments and more number of cells. While the interaction between *in vitro* culture media and stage of development did not (P>0.05) affect the number of cells and degree of fragments.

Table 5 Shows; the effect of *in vitro* culture on the total quality development from day 1 to day 3

Stage	**CM**	**TGQ**
Day1	TCM	79.5[a]
	Hams	73.5[b]
Day2	TCM	78.0[a]
	Hams	70.0[b]
Day3	TCM	73.5[b]
	Hams	63.3[c]
SEM		0.8
MEANS		76.5[a]
Day1		74.1[b]
Day2		68.3[c]
Day3		0.5

SEM		
	TCM	77.0[a]
	Hams	69.0[b]
SEM		0.4
P Values		
Stage		0.00
CM		0.00
Stage X CM		0.05

[a bc] Means with different superscript letters in the same column differ significantly, CM= culture media, TCM= TCM-199, Hams= Ham's F10, TGQ= Total good quality embryo, P<0.05= Significant, P<0.01= Highly significant, Not significant P>0.05.

Both stage of embryo development and *In vitro* culture media significantly influenced (P<0.01) total good quality embryos, and the interaction between stage of embryo development and *In vitro* culture media was also significantly different (P=0.05).

3.6 Discussion

In the present study, the effect of culture media on embryo quality development was quantitatively analyzed. The overall embryo quality development ranged 73.5% - 79.5% in TCM-199 culture medium, compared to 63.3% - 73.5% in Ham's F10 culture medium. Therefore, the test on the efficiency of culture media on embryo quality development indicated that embryos can develop better in TCM-199. The results obtained from the current study were related to findings on sheep embryos by Abolfazl *et al.* (2012), where they obtained good quality embryos from cleaving cells 89% to 60% morula in TCM-199. The difference percentages between Abolfazl *et al.,* 2012 results and the results obtained from the current study could be due to species differences. Bavister *et al.* (1995) reported that superior quality of embryos grown in TCM-199 compared to Ham's F10 may be due to the absence or lower concentration of glucose, phosphates, sulfates and transition metal ions such as cu^{+}, Zn^{+} and Fe^{+}. Some of these chemicals have been shown to inhibit or decrease embryo quality development in rats, mouse, cow and human (Bavister *et al.* 1995).

The current study yielded high percentage of fragment in Ham's F10 *in vitro* culture medium than in TCM-199. The fragmentation likely delayed the embryo development to morulae and later disrupted the blastulation. Bastias *et al.* (1993) reported that Ham's F10 contains a high concentration of hypoxanthine which is associated with more fragmentation than TCM-199 which contains less hypoxanthine concentration. Matsumoto *et al.* (2003) found that high

hypoxanthine concentrations and changeover of metal ions can increase the relative oxygen radicals in embryos and slow down embryo quality development.

In the present study, the *in vitro* culture media were supplemented with antibiotic and bovine serum albumin. According to Leese,*et al.,* (1988), antibiotic and bovine serum albumin encourage better embryo development. Indeed better embryonic development was obtained, more especially in TCM-199 *in vitro* culture medium from day 1 to day 3. Sagirkaya *et al*., (2007) findings showed higher oocyte maturation and subsequent embryonic development by using serum supplemented TCM-199 compared to serum supplemented synthetic oviductal fluid medium. Other researchers like Edward E *et al*., (1990), who also used different *in vitro* culture media (TCM-199,Ham's F10 and CZB + glucose) found that there were large differences in the degree to which the three media tested supported embryo development, where TCM-199 was clearly superior over both Ham's F10 and CZB + glucose media. Edward found that TCM-199 differs from Ham's F10 in that it has a lower concentration of hypoxanthine and higher concentrations of amino acids which results in improved embryo development.

3.8 Conclusions

The TCM-199 *in vitro* culture medium yielded better embryo quality. This was attributed to enough antioxidant elements formulated to ensure correct development. Hence, TCM-199 *in vitro* culture medium is a better option for future applications for *in vitro* culture of embryos.

CHAPTER FOUR

EFFECT OF ASSISTED HATCHING TECHNIQUES ON HATCHABILITY AND SURVIVAL RATE OF MICE EMBYROS CRYOPRESERVED USING SLOW-FREEZING METHOD

4.1 Introduction

Several assisted hatching techniques have been introduced over the years including; drilling a hole in the zona pellucida, partial zona dissection, thinning the zona pellucida or total removal of the zona (Gordon & Dapunt, 1993). These techniques can be performed chemically (using acid Tyrode's solution), mechanically (using special tapered micropipettes, or by using a laser beam. Researches indicated that assisted hatching can have both helpful and harmful effects on the development of an embryo depending on many factors, such as zona thickness, the area of thinning treatment, the size of the hole created, mechanical damage to the embryo by manipulation, chemical damage by acid solution, and the technical skill of the operator. The quality of gametes plays an important role in embryo development and implantation (Ziebe *et al.,* 1997). However, embryos with full developmental potential may fail to implant because of hatching difficulties.

Embryo cryopreservation can be defined as a long-term conservation of the pre-implantation embryos of any species. It hangs up its metabolism and development by freezing them at very low temperature (Whittingham *et al.,* 1972). Cryopreservation could be in use for the protection of strain from genetic contamination, mutation, or change in genetic trait, natural disaster, and loss due to pathogenic infections. It could also be helpful in planned animal production or a material for making of genetically engineered mice. Thus, the improved embryo cryopreservation procedures should be utilised to achieve optimal results. However, improvements in cryopreservation and cryoprotective additives have made slow progress is the last 10 years (Camus *et al.,* 2004). The study investigated the efficacy of two assisted hatching techniques (Laser and Partial mechanical) on the hatchability of mice embryos cultured in Ham’s F10 and TCM199, and determined the effect of cryopreservation on mice embryos survival.

4.2 Materials and methods

4.2.1 Management of animals

The animals used in this study were managed as described in chapter 3. Good quality embryos obtained during chapter 3 from TCM-199 and Ham's F10 *in vitro* culture medium were selected and used in this study.

4.2.2 Experimental procedure

4.2.2.1 Embryos equilibration prior to cryopreservation

Embryos were transferred from the *in vitro* culture media (TCM and Ham's F10) into the freezing medium (1.5 molar ethylene glycol), and allowed to sit for 5 minutes to equilibrate. Thereafter, the embryos were loaded into semen straws by firstly aspirating a small volume of ethylene glycol using embryo loading device. A tiny air bubble was created within the straw and aspirated five embryos with ethylene glycol. A second tiny air bubble was created within the straw (air bubbles served to physically isolate the embryo within the straw), then ethylene glycol was aspirated again to completely fill the straw. The straw was sealed with polyvinylchloride powder (Bank *et al.*, 1973).

4.2.2.2 Cryopreservation

The freezing machine was cooled from ambient temperature to -6°C. The straws were then load into the freezing machine and allowed to sit for 2 minutes before proceeding. Once embryos have cooled to -6°C, a pair of tongs super cooled in liquid nitrogen was used to touch the straw just below the embryos to induce ice crystal formation in the ethylene glycol. The embryos were held at seeding temperature for 10 minutes before further cooling. Thereafter, the embryos were cooled at a rate of 0.5°C/min down to a temperature of -34°C to ensure continued dehydration. The embryos were held at -34°C for 10 minutes before being transferred into liquid nitrogen (-196°C). Cryopreserved embryos were placed into the canister of a liquid nitrogen dewar for seven days.

4.2.2.3 Thawing of cryopreserved straw with embryos

Immediately after seven days, the straws were taken out of the liquid nitrogen and held in air for 5 seconds, and then dipped into a 37°C water bath for an additional 30 seconds. The embryos were then transferred into a petri dish containing thawing solution (1.0 Molar of sucrose). The embryos were allowed to remain in sucrose solution for 10 minutes (Pegg *et* al., 2002).

4.2.2.4 Laser assisted hatching

Immediately after 10 minutes in thawing solution, the dish was placed on the stage of the inverted microscope and the embryos were located under focus. The laser lens was rotated to the position of the drops and was focused on the first embryo. The laser target was placed over the ZP in a place that had no blastomeres directly underneath. The laser beam was guided by safety circles which help the operator to choose the area of interest and the target was positioned on the ZP and ensure that the safety circle did not overlap the blastomeres. Beaming of the ZP was done with a single medium pulse on the ZP of the embryo. Embryo was fired by one laser beam following the other until all embryos were fired by the laser beam, without breaking the inner layer. The same procedure was repeated for all the embryos in TCM-199 or Ham's F10 medium.

4.2.2.5 Mechanical assisted hatching

The dish containing embryo was taken and placed on the micromanipulator microscope. The embryo was held by a holding pipette at 30° from the left and the partial zona dissection needle was inserted from the right of the embryo. The zona pellucida of the embryo was pierced with the zona dissection needle. The needle was removed after making a hole in the zona pellucida. The same procedure was repeated on all the remaining embryos in the two *in vitro* culture media.

4.2.2.6 Embryo culturing

After assisted hatching, a pipette (10 µl – 100 µl) was used to dispense seven drops of 50 µl *in vitro* culture medium (TCM - 199 and Ham's F10 separately), on the bottom of a 35 mm sterile plastic tissue culture dishes. Immediately after, 10 washed embryos were distributed among the *in vitro* culture media drops. The dishes were flooded with light mineral oil and placed in the incubator for culture. The survival and number of embryos hatched were recorded after every 24 h for 3 days.

4.3 Experimental design

The experiment evaluated two (culture media) X three (assisted hatching methods & control) X three (evaluation time) factorial in a randomized factorial design with three replications (Table 6).

Table 6 Shows; the experimental procedure design.

Types of media	AH techniques	Evaluation time	Replicates
Hama's	Laser Mechanical Control	24, 48 and 72 h	3
TCM	Laser Mechanical Control	24, 48 and 72 h	3

4.4 Statistical analysis

Data were analyzed using the Minitab Software Version 16 (2010), ANOVA General Liner Model in a completely randomized design with 2 x 3 x 3 factorial.

$$Y_{ijK} = \mu + M_i + A_j + T_k + (MA)_{ij} + (MT)_{ik} + (AT)_{jk} + (MAT)_{ijjk} + £_{ijk}$$

Where:

- Y_{ijk}= the observation (hatchability/survival)
- μ = The overall mean
- M_i = The effect of i^{th} media
- A_j = The effect of j^{th} Assisted hatching technique
- T_k = the effect of k^{th} evaluation time
- MA_{ij} = The interaction of the i^{th} media and j^{th} assisted hatching technique
- MT_{ik} = The interaction of the i^{th} media and k^{th} evaluation time
- AT_{jk} = The interaction of the j^{th} assisted hatching technique and k^{th} evaluation time
- MAT_{ijk} = The interaction of the i^{th} media and j^{th} assisted hatching technique and k^{th} evaluation time.
- $£_{ijk}$=The random error

4.5 Results

Table 7 Shows; hatchability and survival rate of embryos in two different *in vitro* culture media and assisted hatching techniques evaluated at 24 h, 48 h and 72 h

CM	AH	ET	HR (%)	LIVE (%)	DEAD (%)
TCM	Laser	24 h	14.5^{ef}	72.0^{ab}	15.5^{b}
		48 h	47.5^{a}	60.8^{abcd}	5.0^{def}
		72 h	15.3^{def}	36.0^{e}	1.5^{f}
	Mechanical	24 h	11.0^{f}	71.0^{abc}	16.0^{b}
		48 h	17.5^{cdef}	58.5^{cd}	8.5^{bcdef}
		72 h	38.5^{ab}	16.5^{f}	3.8^{ef}
	Control	24 h	10.0^{f}	73.5^{a}	11.3^{bcde}
		48 h	16.3^{def}	62.5^{abcd}	5.8^{cdef}
		72 h	32.3^{abcd}	40.0^{e}	1.3^{f}
Hams	Laser	24 h	12.3^{ef}	67.5^{abcd}	27.5^{a}
		48 h	36.5^{ab}	59.5^{bcd}	13.0^{bcd}
		72 h	3.5^{f}	40.0^{f}	3.3^{ef}
	Mechanical	24 h	7.5^{f}	66.3^{abcd}	29.5^{a}
		48 h	15.5^{def}	57.0^{d}	5.5^{edf}
		72 h	35.0^{abc}	32.5^{e}	5.5^{edf}
	Control	24 h	6.8^{f}	72.3^{ab}	14.4^{bc}
		48 h	14.0^{ef}	60.0^{bcd}	9.0^{bcdef}
		72 h	29.0^{bcde}	40.0^{e}	0.5^{f}
SEM			3.4	2.5	1.7
TCM	Laser		22.3^{a}	52.9^{ab}	7.0^{b}
	Mechanical		22.0^{a}	49.3^{bc}	6.1^{b}
	Control		19.6^{a}	58.6^{a}	9.4^{b}
Hams	Laser		21.1^{a}	49.0^{bc}	10.3^{a}
	Mechanical		19.3^{a}	44.4^{c}	6.8^{b}
	Control		16.5^{a}	56.0^{a}	15.0^{a}
SEM			1.9	1.4	0.9
TCM		24 h	12.0^{b}	72.5^{a}	23.8^{b}
		48 h	28.7^{a}	60.8^{b}	14.3^{a}
		72 h	23.4^{a}	30.8^{c}	2.1^{e}
Hams		24 h	8.8^{b}	68.6^{a}	23.8^{a}
		48 h	22.5^{a}	58.8^{b}	9.2^{c}
		72 h	25.7^{a}	25.6^{c}	3.1^{de}
SEM			1.9	1.4	0.9
	Laser	24 h	13.4^{C}	70.5^{a}	12.9^{b}
		48 h	42.0^{a}	61.3^{bc}	5.6^{cde}
		72 h	9.4^{c}	34.3^{d}	0.9^{e}
	Mechanical	24 h	9.3^{c}	60.1^{c}	21.5^{a}
		48 h	16.5^{c}	57.8^{c}	7.0^{cd}
		72 h	36.8^{ab}	10.3^{e}	3.5^{de}
	Control	24 h	8.6^{c}	72.6^{a}	22.8^{a}
		48 h	15.1^{c}	68.6^{ab}	10.8^{bc}
		72 h	30.6^{b}	40.3^{d}	3.5^{de}
SEM			2.4	1.7	1.2
AH MEANS					
	Laser		23.6^{a}	54.3^{a}	10.3^{a}
	Mechanical		20.8^{a}	46.9^{b}	6.5^{b}
	Control		18.1^{b}	57.3^{a}	12.6^{a}

SEM		1.4	1.0	0.7
CM MEANS				
TCM		21.5[a]	56.6[a]	7.6[b]
Hams		19.0[a]	51.1[b]	12.0[a]
SEM		1.1	0.8	0.6
ET MEANS				
	24 h	10.4[b]	70.6[a]	19.0[a]
	48 h	24.5[a]	59.7[b]	7.8[b]
	72 h	25.6[a]	28.3[c]	2.6[c]
SEM		1.4	1.0	0.7
P VALLUES				
CM		0.149	0.03	0.02
AH		0.193	0.03	0.02
ET		0.000	0.00	0.00
CM X AH		0.873	0.71	0.01
CM X ET		0.107	0.04	0.01
AH X ET		0.000	0.00	0.00
CM X AH X ET		0.0170	0.011	0.032

[a b c d e f] Means with different superscript letters in the same column differ significantly, CM=*in vitro* culture media AH=assisted hatching, ET= Evaluation time, HR= Hatchability rate, Live=live embryos, Dead=dead embryos, ±SEM = Standard error mean, P<0.05 = Significant, P<0.01 = Highly significant, P>0.05= Not significant.

In vitro culture media, showed a significant difference (P<0.05) on live and dead embryos and no significant difference (P>0.05) on embryo hatchability. However, TCM-199 yield better results on all parameters. There was a significant difference (P<0.05) between two assisted hatching techniques on live and dead embryos, but no significant difference (P>0.05) on hatchability. Evaluation time had a highly significant difference (P<0.01) on all parameters. The highest hatching percentage was observed at 48 h, and the survival rate of embryos was decreasing as time increases. The interaction of main factors (culture media, assisted hatching techniques and evaluation time) had significant difference on all parameters. TThe highest hatching percentage was obtained from laser in TCM-199 at 48 h. Highest live embryo percentage was observed from control group in TCM-199 at 24 h. Interaction between *in vitro* culture media and assisted hatching techniques had a significant difference (P<0.05) on dead embryos and no significant different (P>0.05) on embryo hatchability and live embryos.

4.6 Discussion

Laser assisted hatching technique was simpler and precise, hence considered as a good technique in the current study. Embryo hatchability is mostly determined by the thickness of the zona pellucida as well as the age of the animal (Sung *et al.,* 2014). The hatchability percentages obtained from the present study are in agreement with the finding of Hsiesh *et al.,* (2002), where they reported laser (31.8%) to have a high hatchability percentage than

mechanical (16.1%). The difference in these results could be due to the fact that Hsieh performed assisted hatching on two cell embryos whilst morulae were used in this current study. Many recent studies indicated that laser assisted hatching technique appears to have significant advantages over other methods of assisted hatching, because it prevents mechanical, thermal, or chemical injury to the embryo (Schopper *et al.,* 1999).

In the current study, laser assisted hatching technique had the potential to promote rapid embryo hatching whereby 42.6% was obtained at 48 h compared to mechanical assisted hatching technique (36.8%) at 72 h. Laser assisted hatching technique differs from mechanical assisted hatching technique in that, it involved zona thinning whilst mechanical assisted hatching method created a small zona opening, which likely delayed the hatching process. During mechanical assisted hatching, the internal pressure that is generated by expanding blastocyst is released through the zona opening, of which was too small to slit the zona opening to a large size. Mantoudis *et al*., (2001) reported that, higher Implantation rates were mostly obtained by zona thinning rather than zona opens. The results obtained from this present study were related to the findings of Kim *et al.* (2015) whereby high embryo hatchability was in laser (49%) at 48 h and mechanical (40%) at 72 h.

Focusing on the influence of *in vitro* culture media, there was no significant difference (P>0.05) on embryo hatchability. However, the comparison of *in vitro* culture media between TCM-199 and Ham's F10 yielded 21.5% and 19.0% hatching, respectively, without interactions factors. The superior numeric results observed in TCM-199 was attributed to enough amounts of nutrients available in the culture medium, in addition to the lysine which was initially generated from cleaved embryos prior blastocyst. Letterie *et al*., (1997) reported that, upon reaching the blastocyst stage, a combination of lysine 'proteases' produced by the cleaved embryo (trophectoderm) and/or the uterus, all assist in zona dissolution. Furthermore, Nedambale et al., (2004) reported that addition of serum in culture media has the potential to encourage hatching of embryos by providing plasminogen that thins the zona pellucida, hence facilitating hatching.The results from this present study attested that laser assisted hatching is most successful at 48 h in TCM-199 *in vitro* culture medium.

With regard to the embryo survival rate (live & dead embryos) in the present study, the results showed that fresh embryos (control) can survival better than cryopreserved embryo (using slow freezing technique). The low viability rate in cryopreserved embryos could be as a result of damages caused by the liquid nitrogen low temperatures. However, cryopreserved embryos life span can be prolonged for the future use more especially when the appropriate method is used. The results obtained from the current study are in agreement with the findings of Park

et al. (2009) who reported that the survival rate of control group (fresh embryos) and vetrification groups were significantly higher than those of slow freezing group. According to Pegg *et* al. (2002) the slow freeze–thaw procedure makes various cryo damages (osmotic shock and ice crystal formation) in embryos. The structure of zona pellucida is composed of glycoproteins (with low amount of water) and could be more tolerable to cryo damages, while blastomeres of embryos contain high amount of water, which are vulnerable in freeze–thaw procedure (Pegg *et* al,. 2002) .

4.7 Conclusions

Laser is the better embryo assisted hatching technique. However, investment in these technologies could be constrained by cost given the small improvement in hatchability with assisted hatching. Slow-freezing cryopreservation is not the best method to cryopreserve compared to fresh embryos (control) that had better live embryo percentage.

CHAPTER FIVE
GENERAL DISCUSSION AND CONCLUSIONS

5.1 Overall discussion

The present study had three objectives; the first objective was to compare the efficacy of two *in vitro* culture media (Ham's F10 and TCM-199) on the quality development of mice embryos. The results showed that TCM-199 *in vitro* culture medium can sustain the quality development of embryo from the early stage until morulae compared to Ham's F10 *in vitro* culture medium. However, the embryo quality gradually decreases as the embryo develops. The results obtained from this study are closely in line with the findings of Abolfazl *et al.,* (2012).

The second objective was comparing the efficacy of two assisted hatching techniques (Laser and Partial mechanical) on the hatchability of mice embryos in Ham's F10 and TCM-199. The study showed that laser assisted hatching technique could be more effective in TCM-199 *in vitro* culture medium at 48 h, and mechanical assisted hatching technique at 72 h. Therefore, laser assisted hatching technique has the ability to promote rapid embryo hatching. These results were similar to the findings of Kim *et al.,* (2015).

The last objective was to determine the effect of cryopreservation on the survival of mice embryos. Slow freezing method yielded low percentage of viable (live) embryo as compared to control (fresh embryo). This was supported by the findings of Park *et al,.* (2009). The embryo survival rate percentage was decreasing as time increases. The high survival rate percentage was obtained in TCM-199 *in vitro* culture medium. During the thawing process following cryopreservation, embryos experienced various damages such as osmotic shock, ice crystal formation and hardening of the zona pellucida which negatively affected its viability and hatchability (Pegg *et* al., 2002).

5.2 Conclusions and recommendations

5.2.1 Conclusions

The comparison of the effect of assisted hatching techniques and *in vitro* culture media on mice embryo development, hatchability and viability following cryopreservation were carried out in this study thus, the following conclusions were made:

1. Even though the quality of embryos decreased as it develops, the *in vitro* culture media used in the present study yielded favourable results. However, TCM-199 medium yielded better embryo development results compared to Ham's F10 medium.
2. Laser assisted hatching technique has a high hatchability over mechanical assisted hatching and control. However, investment in these technologies could be constrained by cost given the small improvement in hatchability with assisted hatching.

3. Laser assisted hatching technique has the ability to promote rapid embryo hatching in TCM-199 *in vitro* culture medium.

4. The low embryo viability post thawing obtained from this study, showed that slow-freezing was not the best method to cryopreserve compared to fresh embryos (control) that had better survival rate (live embryo percentages).

5.2.2 Recommendations

It is recommended that embryos should be cultured in TCM-199 *in vitro* culture medium. More studies should be done to test assisted hatching techniques after cryopreservation on different culture times. Slow freeze method is not highly recommended to cryopreserve embryos.

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APPENDIX

Appendix 1. Mean squares of analysis of variance for less, normal and more number of cells, none, normal and more fragmentation and total good quality embryos.

Source of variation	DF	LESS NC	NORM NC	MORE NC	NONE F	NORM F	MORE F	TGQ
Stage	2	6.25	18.06	1.00	36.00	4.00	14.00	139.62
CM	1	289.00	203.06	4.00	56.25	400.00	162.00	384.00
Stage X CM	2	12.25	3.06	20.50	0.00	1.00	1.57	9.13
Error	18	7.29	2.15	7.71	4.12	1.17	2.39	2.58

DF= degree of freedom, CM= *in vitro* culture media, less NC= less number of cells, NORM NC= normal number of cells, MORE NC= more number of cells, NONE F= no fragments, NORM F= normal fragments, MORE F= more fragments, stage= stage of embryo development, TGQ= Total good quality embryo

Appendix 2. Mean squares of analysis of variance for hatchability rate and survival rate (live and dead embryos)

Source of variation	DF	HR	LIVE	DEAD
CM	1	100.35	231.10	351.13
AH	2	79.43	696.00	220.01
ET	2	1722.51	11600.00	1691.06
CM X AH	2	8.35	8.40	61.54
CM X ET	2	109.85	17.80	125.17
AH X ET	4	1728.85	688.80	42.97
CM X AH X ET	4	77.31	48.10	13.83
Error	54	46.83	24.4	11.53

DF=degree of freedom, , CM=*in vitro* culture media AH=assisted hatching, ET= Evaluation time, HR= Hatchability rate, Live=live embryos, Dead=dead embryos.

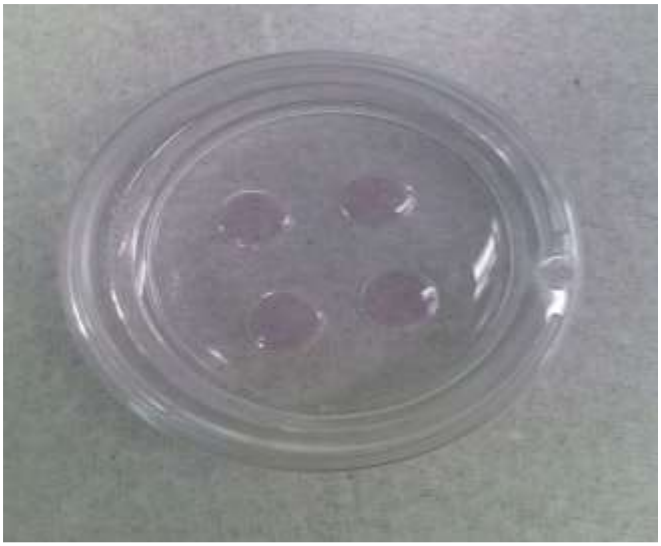

Figure 1: TCM-199 culture medium droplets used for culturing embryos

Figure 2: Ham's F10 culture medium used for culturing embryos

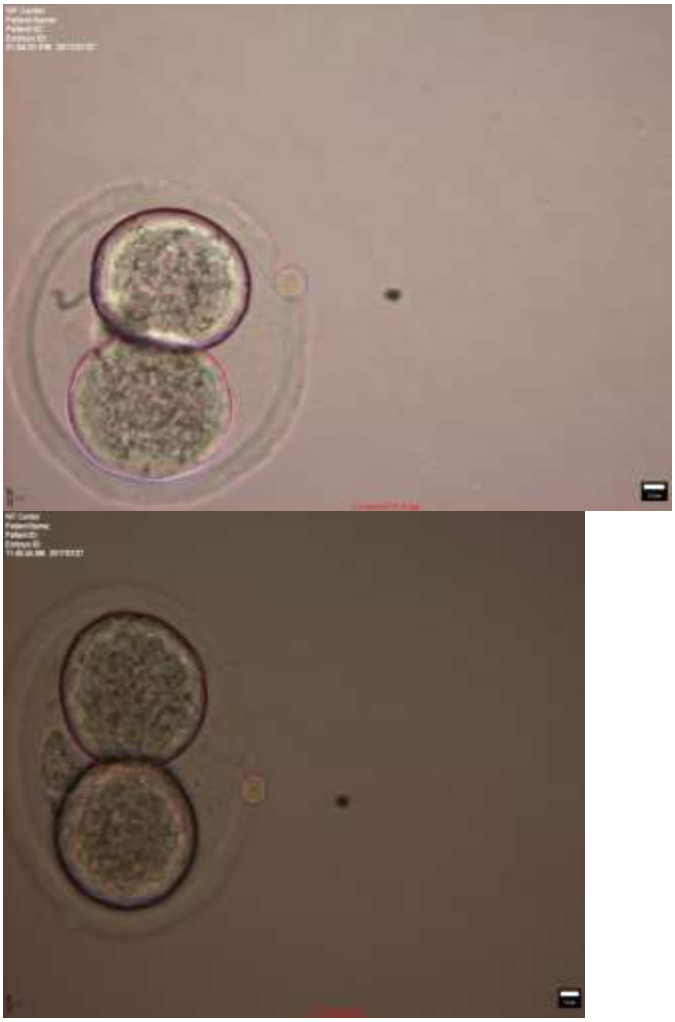

Figure 3: two cell embryos with no fragments (good quality embryos)

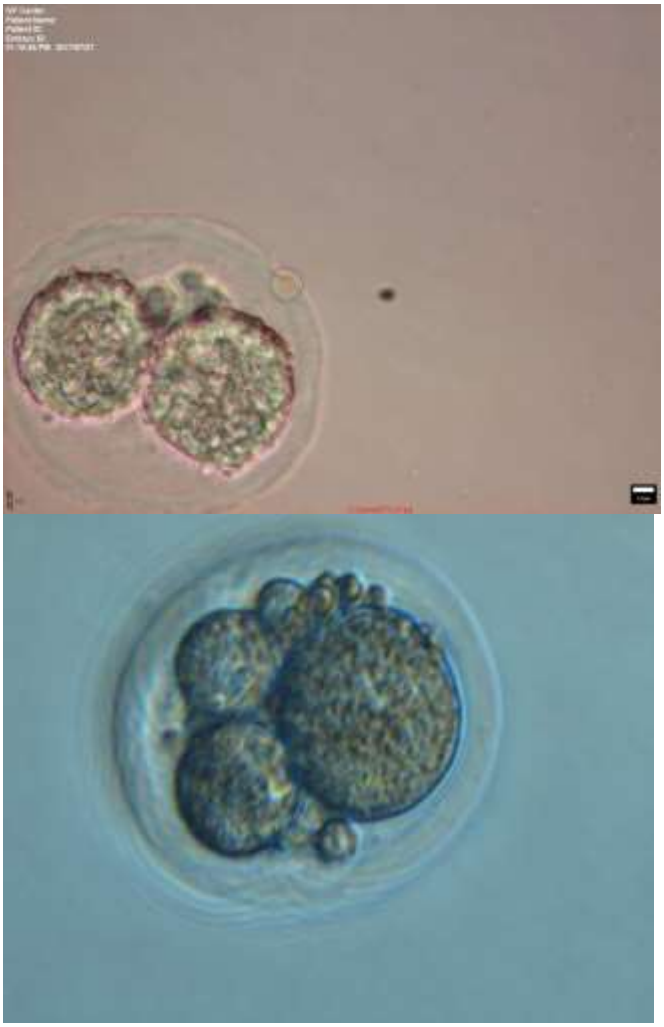

Figure 4: two & three cell embryos with fragments

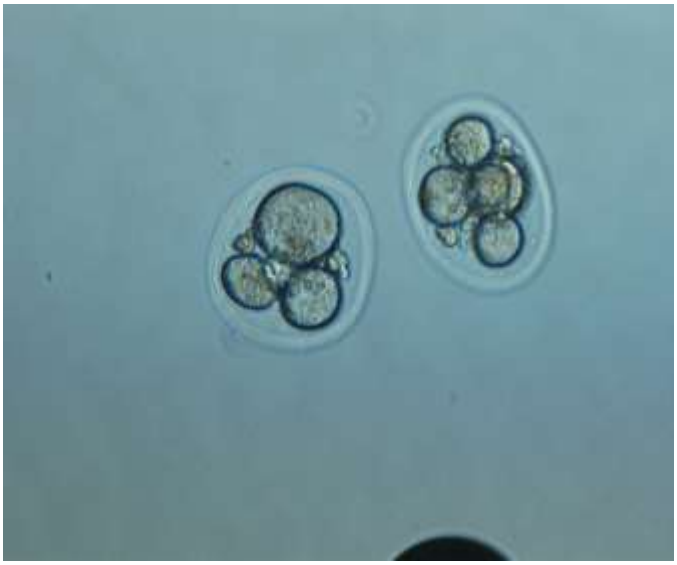

Figure 5: three & five cell embryos with fragments

Figure 6: six cell embryo with no fragments (good quality embryo)

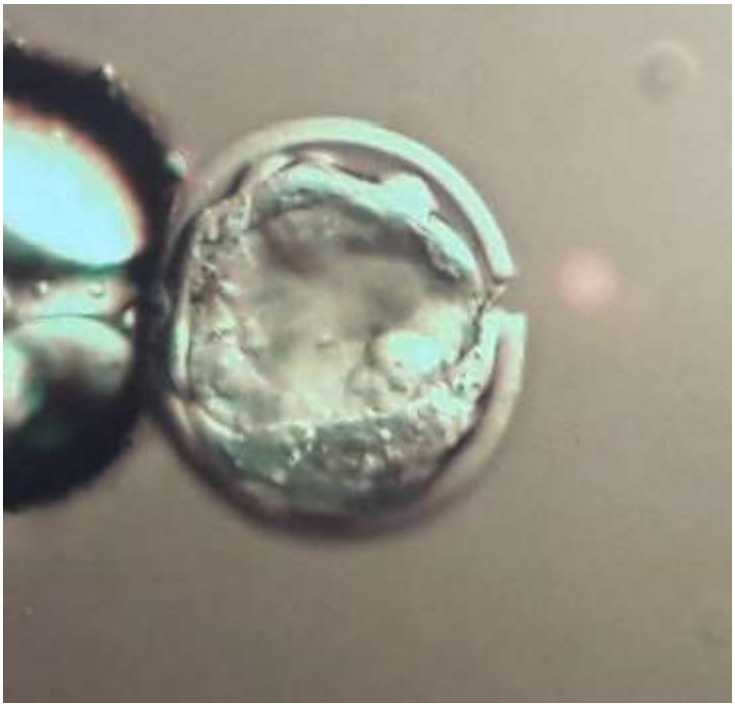

Figure 7: laser assisted hatching

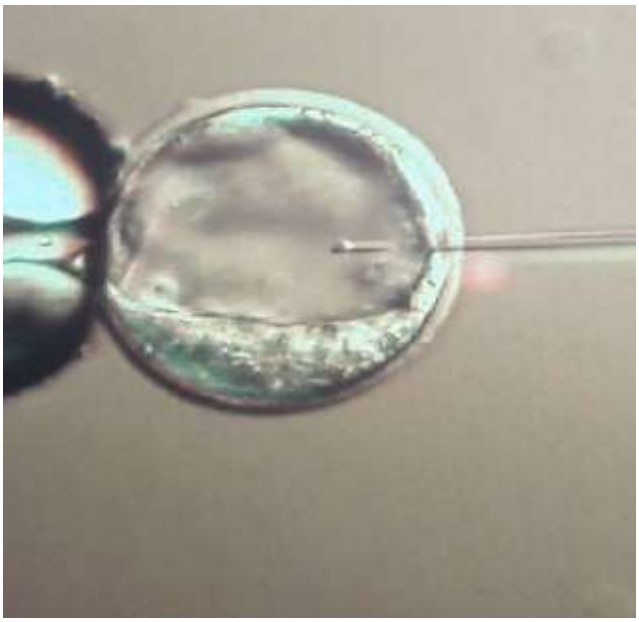

Figure 8: mechanical assisted hatching

Figure 9: C57BL/6 (B6) & BALB/C (C) micePure C57BL / 6 (B6) and BALB / c (C) mice

Figure 10: programmable freezer and liquid nitrogen tank