COMPARISON OF TWO DIFFERENT MEDIA AND ASSISTED HATCHING TECHNIQUES ON THE EMBRYO HATCHING RATE USING THE MOUSE AS A MODEL

BY

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DECLARATION

I, **Nkhumeleni Cathbert Negota**, the undersigned, student number: 11618288, hereby declare that this dissertation for the Master of Science in Agriculture (Animal Science) submitted to the University of Venda, has not previously been submitted to any other University for any other degree and it is my own work in design and execution and that all reference materials contained herein have been duly acknowledged.

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ABSTRACT

The use of *in vitro* culture media and assisted hatching techniques remain a challenging obstacle to hatching of blastocyst-stage embryos. Mechanical, chemical, enzymatic thinning and laser assisted techniques have been used previously, but there is still a lack of information on its application and implication in livestock. The aim of this study was to compare the effect of two *in vitro* culture media ((Ham's F10 and Tissue Culture Medium 199 (TCM-199)) and four assisted hatching techniques (mechanical, chemical, enzymatic and laser) on blastocyst formation and hatching rate using murine embryos as a model. The C57BL/6 and BALB/c mouse breeds were bred and raised until they reach maturity and then bred naturally to produce a hybrid F1 generation. The light in the breeder house was controlled at 14 hours light and 10 hours darkness. Feed and water were provided *ad libitum* for the mice. Mature female mice were super-ovulated using equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG). A total of 400 blastocysts were collected from the F1 generation and these were allocated equally for the four assisted hatching techniques (laser, mechanical, chemical and enzymatic) as well as a non-treated control group. The blastocysts were paired into a group of 10 and replicated 4-four times for each assisted hatching techniques and control group. The embryos were then cultured for 24 hours and the hatching of the embryos were observed. Hatched embryos were stained for blastomere counting. The general linear model (GLM) of statistical analysis software (SAS) version 9.4 was used to analyze the data. Assisted hatching techniques (laser, mechanical, enzymatic and chemical) yielded 46.86±37.12; 51.07±40.19; 39.05±35.83 and 33.32±37.50% of hatching, respectively under *in vitro* culture in Ham's F10. There was a significant difference ($p<0.05$) observed between assisted hatching techniques using Ham's F10 as culture medium. In the TCM-199, laser, mechanical, enzymatic and chemical assisted hatching techniques yielded 56.25±43.30; 52.55±35.50; 49.16±37.50 and 33.85±35.50%, respectively, with significant differences ($p<0.05$). However, the hatching rate of embryos for all techniques was higher when *in vitro* cultured in TCM-199 compared to those cultured in Ham's F10, and statistically higher than the control group. In conclusion, laser assisted hatching technique is the best of the techniques to use to assist the hatching of murine embryos and TCM-199 is the best of the two *in vitro* culture media for the hatching percentage.

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

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DEDICATION

Herewith this dissertation dedicated to the Negota family and to God who made all things possible.

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LIST OF ABBREVIATIONS

AH	Assisted hatching
ARC	Agricultural Research Council
ART	Assisted Reproductive Technology
Bl	Blastocyst
CAH	Chemical assisted hatching
CO_2	Carbon dioxide
CEAAR	Centre of Excellence in Animal Assisted Reproduction
CRD	Completely Randomized Design
D-PBS	Dulbecco`s Phosphate Buffered Saline
EDTA	Ethylene-diamine-tetra-acetic acid
et al	And others
F1	First filial generation
h	Hour
ICM	Inner cell mass
In vivo	Inside the body
IU	International unit
IVF	*In vitro* fertilization
LAH	Laser assisted hatching
MAH	Mechanical assisted hatching
Ml	Milliliter
PZD	Partial zona dissection
TCM	Tissue culture medium
TUT	Tshwane University of Technology
UNIVEN	University of Venda
ZP	Zona pellucida

CHAPTER 1

INTRODUCTION

1.1 Background

Assisted hatching (AH) is a method under assisted reproductive technology (ART) that aims to positively improve the implantation rate of embryos in both animals and humans (Debrock *et al*., 2009). It uses different techniques to create a hole or weaken the zona pellucida (ZP) at the blastocyst stage of the embryo. This allows the easy hatching of the blastocyst-stage embryo from the zonal cover. Biologically, after an embryo has reached the blastocyst stage, it must hatch from the ZP before it can implant into the uterine wall (Hammadeh *et al*., 2011). The hatching of the blastocyst is the crucial step that must take place before implantation and failure to hatch will result in failure of the embryo to implant, thus limiting the reproductive efficiency (Gabrielsen et al., 2004; Park *et al*., 2014).

The ZP is made of glycoproteins that cover the embryo before fertilization until hatching. The ZP plays a significant role in maintaining the integrity of the cell to prevent some harsh disturbances that may threaten the growth of the embryo (Ballesta *et al*., 2011). Before hatching, the blastocyst undergoes series of contractions and expansions that cause a decrease in the thickness of the ZP until it becomes almost invisible (Johansson *et al*., 2001). In mice, the ZP is digested by the synthesis of the enzyme trypsin in localized sites of the trophectoderm. A similar enzyme may be produced by human and animal blastocyst from the whole of its surface. Expansion and contraction of the blastocyst seem not to be the only predominant factor in the shedding of the ZP (Montag *et al*., 2000).

All mammalian eggs are covered by a thick layer called the ZP, and the cover play crucial roles during the process of oogenesis until pre-implantation. Glycoproteins are responsible for species-restricted binding of sperm to unfertilized eggs, inducing sperm to undergo acrosomal exocytosis, and preventing sperm from binding to fertilized eggs. The ZP has different roles including oocyte development, protection during growth and transport, fertilization, preventing polyspermy and preventing premature implantation (Wassarman, 2008).

The ZP of murine embryos is made of three different glycoproteins, the ZP1, ZP2 and ZP3 (Wassarman, 2008). In mice, the ZP2 and ZP3 constitute about 80% of the total mass of the ZP

(Debra *et al*., 2008). The ZP of the mouse embryo at an early stage of development is approximately 6.2 µm in thickness and it increases its thickness during development to the blastocyst stage. After reaching the blastocyst stage, the thickness of the ZP should decrease so as to allow the easy tearing of the ZP to allow hatching. The impaired ZP affects negatively on the hatching process of the embryo (Wassarman, 2008).

There are four different assisted hatching techniques that have been developed and these include mechanical, chemical, laser and enzymatic (Elhelw *et al*., 2004). A sufficient thinning of the inner layer of the entire ZP is very important for optimal and easy assisted hatching. Moreover, there is a possibility that an extrusion of the blastomeres or whole embryo from a large slit may be induced by contractions of the reproductive tract. In contrast, smaller holes of the ZP trap embryos during the hatching process and prevent their implantation. 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 ZP thickness, the area of thinning treatment, the thickness of the hole created, mechanical damage to the embryo by manipulation, chemical damage by acid solution and the technical skill of the operator (Das *et al*., 2009).

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 of monozygotic twinning after assisted hatching. There is still no clear-cut consensus over which technique of assisted hatching will be more efficient to increase the implantation rates. Drawbacks to mechanical zona pellucida opening are its technical complexity and the inability to produce reproducible uniform opening (Wassarman, 2008).

Culture media are used for embryo culturing and are very essential for both animal and clinical assisted reproduction. Several studies showed the significant role in improving assisted reproduction (Biggers, 2003). The use of Ham's F10 and TCM-199 in other studies showed the quality and improved embryo development. Media were also used to prevent contaminations (Quinn, 2004). These media are widespread used in both humans and animals to prompt the positive increase in improving the results of quality reliable results (Mauri *et al.*, 2001). There is no reviewed information concerning the superiority of one medium over the other, however, all are good enhancers of high fertilization and implantation (Nedambale *et al*., 2004).

There is a need for good hatching embryos to enhance implantation following blastulation; thus, it is necessary to improve hatching rate of embryos by artificial techniques. Moreover, it was described that both mechanical and chemical openings or thinning of the ZP, cause a significant change in both the window period of hatching and the rate of blastocyst hatching. Successful hatching of the embryos from the ZP was described as a prerequisite for better implantation in all animals and this was significant in animal production (Cohen *et al*., 1992).

The thickening and hardening of the ZP mostly arise due to the basal FSH level (Carroll *et al*., 1990). Blastocyst hatching and implantation may be impaired in some animals due to reproductive disorders like animals whose ZP was too thick or hard, especially in the horse (Cohen *et al*., 1992). The lowest percentage of implantation was attributed by the impaired hatching of the blastocyst embryo from the ZP in the both animals and humans, especially *in vitro* produced embryo (Cohen *et al*., 1991).

Assisted hatching (AH) can enhance implantation of the blastocyst-stage embryos, by both catalyzing the time and rate of hatching. However, it was also indicated that use of mechanical AH and laser AH have is no benefit in old age animals. Currently, there are high controversial outcomes of AH in animals and there was a debate against the other as to whether AH really has advantages in certain species of animals (Cohen and Feldberg., 1991).

On day four of embryonic development, the mouse embryo will be at the blastocyst stage, and expected to hatch from the ZP and continue with the process of implantation on day five. The ZP is made of glycoproteins synthesized by cells in the mural trophoblast (Wassarman, 2008), and hatching take place after uterine enzymes have digested the ZP. The escape or bursting of the embryo from the ZP may also be facilitated by rhythmic expansion and contraction of the blastocyst. Hatching of the embryo from the ZP is independent of the uterine environment; however, the disorders in the reproductive physiology, for example, hypertension of the mouse, will disturb the hatching process (Wassarman, 2008).

1.2 Problem statement

The environmental conditions such as high temperatures (above normal) and low temperatures (below normal) are a threat to livestock production. As climatic changes cause environmental stress to livestock, reproductive physiology of livestock become affected. The effect of reproductive physiology also affects the reproductive rate of livestock, especially in beef cattle production. This is because the ZP becomes so much harder than normal, thus it fails to hatch as the embryo reaches the blastocyst stage. Many livestock like cattle require assisted reproduction, especially during the hatching from the ZP. In ruminant livestock, it is natural for the ZP of the embryo, to tear about a week after fertilization, when the embryo emerges in order to attach to the endometrium. This tearing process may in some female animals, be inhibited by age and other factors. However, a small opening can be made in the outer layer of the embryos, to assist in tearing of the ZP to assist the hatching of the embryo. Assisted hatching is especially needed where hardening of the ZP does occur or hatching fails to occur due to age factor.

1.3 Justification

The research study is important because of the environmental stress has on livestock. This is because, as earlier mentioned, physiologically, abnormal temperatures have adverse effects on the reproductive efficiency of livestock. The high unexpected above and below normal ambient temperatures affect the physiology of the body, and metabolism, reproduction of the animals, thus leading to diminished hatching of the embryos before implantation. The project is therefore very important to the extent that it will assist the farmers, both communal and commercial in assisted hatching of the embryos to prevent poor implantation. The outcomes of the research study will benefit the animal breeders both communal and commercial. Results will be used to increase the production rate of livestock. The study was also aligned with the research niche of improving agriculture in South Africa, as the scientific information based on culturing embryos and assisted hatching will improve pregnancy until calving in cattle, where the as mouse was used as a model in the *in vitro* environment. Despite the assisted hatching techniques, challenges remain to be experienced and there is limited number of studies that have examined the effect of various assisted hatching techniques and the use of different media to assist hatching of murine embryos in Africa. Moreover, there is no report that have studied interactions of AH techniques and culture media.

1.4 Objective of the study

The main aim of the study was to find a suitable culture medium, number of embryos in culture droplets and to improve embryo hatching for improved implantation in mice blastocyst stage.

The specific objectives are to:

i. To compare two culture media on mice embryo development.
ii. To compare four assisted hatching techniques on mice embryo development and hatching rate from the zona pellucida (ZP).
iii. To compare the different embryo grouping on hatchability.

1.5 Hypothesis

The hypotheses set in pursuit of the above specific objectives were:

i. The two culture media have no an effect on mice embryo development.
ii. The four assisted hatching techniques cannot have an effect on mice embryo development and hatching rate from the ZP.
iii. The four different embryo groupings have no effect on hatchability.

CHAPTER 2

LITERATURE REVIEW

2.1. Embryo assisted hatching using mice as model

Prior to fertilization, the zona pellucida (ZP) surrounding the mammalian oocyte acts as a species-specific sperm barrier and is involved in sperm binding. Therefore, following fertilization, the ZP plays a significant role in blocking polyspermy (fertilization by more than one sperm) fertilization; consequently, it protects the integrity of the preimplantation embryo during early embryonic development until hatching in vivo. Zona pellucida experience hardening and it occurs naturally after fertilization. During embryo development, combination of lysine produced by the cleaved embryo or the uterus and physical expansion then reduces the zona thickness in preparation for hatching. Thus, ZP hardening is not quantifiable; however, might also be induced by *in vitro* culture environment and or by in vivo aging. Prolonged exposure of oocytes and embryos to artificial culture conditions seems to impair their ability to implant. Therefore, it is necessary to assist embryos to hatch in order to improve implantation (De Vos and Van steirteghem., 2000).

Failure of the blastocyst embryos to extrude from the zona pellucida (ZP) causes the failure of implantation in animals (Andras, 2003). The disruption of the outer cover known as the ZP is simply defined as assisted hatching and has been introduced as the techniques to play significant role in improving the implantation rate of both animals and humans embryos (Park *et al*., 2014). It is the order of reproduction that the ZP should first shed to allow the easy escaping of the blastocyst embryo to move out on day four after fertilization for implantation on day five in mouse reproduction. Assisted hatching (AH) is the reproductive technique that increases the hatching percentage and by so doing lead to high embryo implantation (Cohen *et al*., 2009). It was also described as the technique that enhances the early communication between the embryo and the endometrium (Cohen *et al.,* 2009).

Assisted hatching was described by Choi *et al*. (2013) who reported the pregnancy after assisted hatching. The AH has been introduced to enhance the capacity of the embryos to implant within a time frame (Schimmel *et al*., 2014). Assisted hatching can be done in a variety of techniques, these include the use of chemical, mechanical, enzymatic and laser assisted hatching techniques (Schimmel *et al*., 2014).

In mice reproduction, blastocyst stage was reached mostly on day four and the AH was expected to be performed on the same day to allow implantation to take place on day five as the blastocyst embryo will be ready for implantation on day five. Hatching of the embryo from the ZP can be affected from hatching by many factors, environmental factors, age, physical factor and diseases. Prior to hatching of the embryo from the ZP, the blastocyst will start to move in rhythmic expansion and construct (Kubota *et al*., 2014).

The advantage of mice is that, they are easier to handle and hence cheaper to raise than other species. They breed more rapidly with a short gestation period. Transgenic mice are thought to share around 80% of the genes of the humans and most of that of the livestock. Genetic engineers spend most of their time designing transgenic mice, so to be used to study certain disease and disorders affecting other animals. The gestation periods for laboratory mice vary amongst the strains from 18 to 21 days and reach maturity at 4-6 weeks of age (Dressman *et al*., 2007).

Hatching of the embryo from the ZP is independent of the uterine environment. The uterine wall reacts in a way that allows easy implantation, but mutant mice with non-functional Leukemia inhibiting factors cannot support the implantation. Normally, the trypsin enzyme synthesized by cells in the mural trophoblastic play a role in enhancing the hatching process, but due to defects of the above factors, hatching cause a problem to animal reproduction (Wasserman, 2008).

2.2 Structure and the role of the ZP

Murine embryos are fully coated by the ZP which is made of three glycoproteins layers and other substances such as carbohydrates. The ZP plays a significant role during fertilization and in embryo development. It is involved in the protection of the embryo from early fertilizations to the blastocyst stage towards the implantation stage (Kubota *et al*., 2014).

The ZP is essential for maintaining the integrity of embryo cells. Hardening of the ZP occurs by virtue of many natural and non-natural factors. The hardness of the ZP increases with age and is also influenced by other body abnormalities, poor hormonal response and diseases in animals (Schiewe *et al*., 1995).

2.3 Embryo hatching process

At the blastocyst stage in a normal process, the enzymes such as lysine ‘proteases’ produced by the cleaved embryos are involved in the dissolution of the ZP. Moreover, it happens to be the physical expansion of the embryonic mass that also involved in reducing the ZP

thickness for efficient hatching process, to allow implantation to occur. Hatching of the embryo can take place artificial or naturally. This is a process that takes place after the embryo has reached the expanded blastocyst stage, as the expanded blastocyst undergoes a series of changes until the ZP becomes almost invisible. Assisted hatching techniques enhance time and rate of occurrences of these processes, hence described as the prerequisite for implantation (Strohmer and Feichtinger., 1992).

Studies were carried out to observe the hatching sites of human embryo and mouse embryo. Results showed that blastocysts in humans have a natural hatching site which usually develops in close proximity to the inner cell mass (ICM), whereas in mice, the hatching site was observed to be opposite the ICM (Strohmer and Feichtinger., 1992). Therefore, Hirotoshi *et al*. (2010) hypothesized that the choice of the AH site could be important in the development of a blastocyst embryo for hatching rate.

2.4 Mechanism of assisted hatching

Although the mechanism by which assisted hatching technique improve the implantation rate of murine embryo remains unclear, there are possible explanations based on human reproduction that still try to give good and clear background use and benefits of assisted hatching. There is a window period of implantation that is referred to as the ‘implantation window which is the essential period when the uterus gets ready to accept the blastocyst on its wall for implantation. The blastocyst embryos are then expected to undergo the hatching process just before the attachment to the uterine wall.

The ZP with artificial gaps made on it, tends to initiate the hatching process earlier than the ZP without artificial disruptions. Therefore, it was postulated that assisted hatching facilitates the hatching process, thus, speeding up the implantation by allowing earlier stage embryos to attach to the uterine wall. Theoretically, AH allows crucial nutrients to be acquired by the blastocyst in the media, thus enhance embryo development and blastocyst hatching from the ZP (Sawada *et al*., 1990).

So far only two techniques of ZP manipulation were used more often. When compared to the use of others for the ability to enhance embryos to hatch, mechanical and chemical were used often. The two techniques have been used to create a small slit in the ZP of murine embryos as to facilitate the hatching process (Cohen *et al.*, 1990). Currently, laser technique has been commonly used in medical practices and it was defined as the best because of its mode of action (Welch *et al.*, 1991).

2.5 Techniques of assisted hatching

Though mechanical assisted hatching was the first technique to be used, nowadays there are other approaches that are used in the assisted hatching. Many of the assisted hatching experiments are done using murine as the experimental animal. Techniques include: mechanical, chemical, chemical, enzymatic with trypsin and laser assisted hatching. The study focus was on four assisted hatching techniques (mechanical, chemical, enzymatic with trypsin and laser assisted hatching techniques).

2.6 Mechanical hatching technique

The technique of assisted hatching using dissection of the ZP by creating a slit or an opening on the ZP of the blastocyst was previously described by Cohen *et al*. (1990). In this technique, the embryo is held stable by use of a holding pipette, and the ZP is pierced with a micro needle through the space between the ZP and blastomeres. The blastocyst embryo is released from the holding pipette and a small part of the ZP trapped against the micro needle in rubbed against the holding pipette, thus making an opening area between the two sides.

The mechanical technique using zona dissection is quick and easy to perform, but production of variable holes thickness may not always be optimal for the proper hatching of the blastocyst from the ZP. The mechanical technique, allows the creation of larger openings while permitting the protection of the embryo by the ZP. Nijs *et al*. (1993) described a ZP rubbing technique to assist hatching, by reducing the thickness of ZP to its invisibility by rubbing with a micro needle.

2.7 Chemical hatching techniques

Acidified tyrode's is the acid used in the chemical assisted hatching technique and it has been reported in detail by Cohen *et al.* (1990). The blastocyst embryo is secured with the holding pipette and a micro-needle. The micro-needle is preloaded with acidified tyrode's acid using a mouth-controlled suction. The acidified solution is expelled with high precision over a targeted area until the ZP is drilled or bleached. Suction is applied just immediately after bleaching of the ZP to prevent excess acid entering the perivitelline space (Lanzendorf *et al*., 2007). The chemical assisted hatching technique requires very quick handling in order to avoid unnecessary exposure of the embryo to the acidified tyrodes. The acid may be detrimental to the blastomeres which are

just adjacent to the drilled part of the ZP. Crucial thinning of the ZP with acidified tyrode's has been described in mice and humans (Lanzendorf *et al*., 2007).

2.8 Enzymatic hatching technique

The main mechanism in enzymatic hatching is by lysis of the ZP using trypsin enzyme. A trypsin-like protease enzyme plays a big role in digestion of murine blastocyst stage ZP and it has been known to play an important role in hatching. Perona and Wassarman. (1986) reported that trypsin is found in cells of mural trophectoderm and that the hatching of murine blastocyst *in vitro* was initiated by the ZP region overlying the mural trophectoderm. Germond *et al*. (1995) again reported that trypsin like protease, is detected in the culture medium and mentioned that it plays a significant role in murine blastocyst hatching. The enzyme like trypsin does not fully dissolve the ZP during digestion, but instead, it partially, digests the ZP to allow easy escaping of blastocyst embryo (Fabres *et al.,* 2010).

2.9 Laser hatching technique

Hellebaut *et al*., (1996) and Kort *et al*., (1988) were the first authors to report the drilling and laser assisted hatching techniques. The laser technique puts an ideal tool for microsurgical procedures, as the energy is easily emitted and focused, directly to the targeted area on the ZP to create a hole or just a drill to allow the easy escape of the blastocyst from the ZP. Laser assisted hatching technique, used for drilling the ZP can be done with high precision and repeatability, and is reported that there is no side effect on embryo development. The safety of the 1.48-µm diode laser beam has been evaluated in murine and human oocytes and zygotes.

2.10 *In vitro* culture media

The *in vitro* processes involve the use of culture media, which play an important role in the maturation and development of embryos. Biotechnology laboratories use culture media for many processes and this lead to the conclusion that the selection of good, reliable and clean media is a basic information. Many studies that involve culture media use TCM-199 and Ham's F10 as culture media. Murakami *et al*. (2002) reported that the use of TCM-199 increased the blastocyst formation of embryos during culture. The same authors reported again on the effect made by the constituents of the type of culture medium, that it makes a big effect on the

development of cultured embryos. Serum-free medium was reported to have an effect on the development of oocytes maturation in *in vitro* culture, but no effect of blastocyst formations. Hypoxanthine, which was present in the complex Ham's F10 medium reported to have the capability to block the development in murine embryos (Loutradis *et al*., 1987).

The successful development of murine embryo is more influenced by the type of the culture medium used (Ham's F10 and TCM-199). The volume of the medium again plays a significant role in the development and hatching of the murine embryos. The number of embryos per unit volume has the greatest effect on the embryonic development, because of autocrine factor that are secreted by the embryos before hatching for the assistance of hatchability. Studies by Loutradis *et al*. (1987) indicated that, smaller volumes of the medium lead to improved development of murine embryos and the visa versa. The autocrine factors have a great effect on the development, thus the smaller the volume, the less chances of dilution of autocrine factors that influence the development of the embryos.

According to De Wit *et al*. (2000) embryo maturation under *in vitro* depend on the culture media with the main effect by addition of hormones. There are many culture media for selection and this include TCM-199 (Kharche *et al*., 2006) and Ham's F10 (Tamilmani *et al*., 2005) and both can be used for mammalian embryo maturations. Culture media usually differ in their ion concentration and in the concentration of the energy sources. The constituents like physiological saline with pyruvate, lactate and glucose play role in culture media while other media contain vitamins and purines. Differences in embryo maturation are due to the culture media (Nandi *et al*., 2002) while prolonging of culture under suboptimal media adversely affects the thickness of ZP.

CHAPTER 3

MATERIAL AND METHODS

3.1 Description of the study

The study was conducted in the Biotechnology Laboratory of the Centre of Excellence in Animal Assisted Reproduction (CEAAR), Department of Animal Science, School of Agriculture, University of Venda, Thohoyandou, Vhembe District Municipality of Limpopo Province, South Africa, 22°58′32″S 30°26′40″E. The F1 generations of C57BL/6 and BALB/c mice were raised until 6 weeks of age, then used for blastocyst-stage embryo production.

3.2 Experimental design

Three factors were investigated. These were AH techniques, *in vitro* culture medium used and group culture. The first factor involved four AH techniques plus control; No AH (control), laser, mechanical, chemical, and enzymatic. The second factor involve *in vitro* culture media; TCM-199 and Ham's F10. The third factor involve grouping of embryos; one embryo, two embryos, three embryos and four embryos per drop. The experiment was set up as 5 x 2 x 4 factorial design arranged in a completely randomized design (CRD) to determine the significant differences. There were 40 combinations of treatments.

Technique	Media	Blastocyst-stage embryos	No. of drops	Embryos per drop				Culture-duration after AH	Staining after culturing
Control group	Ham's F10	40	4	1	2	3	4	24 h	0-4 h
	TCM-199	40	4	1	2	3	4	24 h	0-4 h
Chemical techniques	Ham's F10	40	4	1	2	3	4	24 h	0-4 h
	TCM-199	40	4	1	2	3	4	24 h	0-4 h
Mechanical techniques	Ham's F10	40	4	1	2	3	4	24 h	0-4 h
	TCM-199	40	4	1	2	3	4	24 h	0-4 h
Laser techniques	Ham's F10	40	4	1	2	3	4	24 h	0-4 h
	TCM-199	40	4	1	2	3	4	24 h	0-4 h
Enzymatic techniques	Ham's F10	40	4	1	2	3	4	24 h	0-4 h
	TCM-199	40	4	1	2	3	4	24 h	0-4 h

TCM-199= Tissue culture medium (Highveld Biological, South Africa), Ham's F10 (Highveld Biological, South Africa), h= hour and AH= Assisted hatching

3.3 Description of animal and experiment

3.3.1 Animal and breeding house condition

Mice were housed in cages with sawdust and grouped into two per cage. They were raised, and fed with mouse pellets and water *ad libitum*. The sawdust in the cages was changed every 5 days. The light in the breeder house was regulated day and night. The light was on from 5 am until 7 pm (14 h) and was switched off from 7 pm until 5 am (10 h). A constant temperature of 24 °C was maintained in the breeder house.

3.3.2 Production of F1 generation

Black female mice (C57BL/6) were mated with white male mice (BALB/c) and were kept together in the breeding cage for 7 days. After 7 days the males were removed and returned to their original cages. The F1 offspring were born 21 days after mating. The offsprings were raised until they were 6 weeks old.

3.3.3 Injections preparation

Folligon

Folligon is available in a bottle of 1000 international units (IU) each (Folligon, Intervet SA, MSD Animal Health). One bottle of 1000 IU was diluted with saline using a syringe to give a working 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.

Chorulon

Chorulon is available in a concentration of 1500 (IU) of hCG (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.

3.3.4 Superovulation and mating

Female mice were injected (peritoneal) with 0.1 ml (5IU) of eCG into the abdominal cavity with 1 ml syringe and 0.5 x 16 mm needle, and 46-48 h later, 0.1 ml (5 IU) of hCG was injected. The eCG was used to stimulate follicular growth and hCG was used to mimic luteinizing hormone (LH) to cause ovulation, maintains the corpus luteum and stimulates it to secrete progesterone

for maintenance of pregnancy. After the injections, the male and female mice were allowed to mate overnight and the next morning the vaginal plugs were observed to confirm mating. The mice with vaginal plugs were kept separately for the collection of blastocyst-stage embryos on day four following successful mating.

3.3.5 Collection of blastocyst stage embryos

On day four after mating, the mouse was taken from the cage and placed on the cage bars, so that it can grip the bars with its front paws. The cervical vertebra was separated by applying firm pressure at the base of the head (back of the neck). The mouse was laid on its back on the absorbent paper and its abdomen soaked with 70% ethanol to attain sterility. A fine cut was made in the middle of the abdomen by using a sterile surgical scissor, while holding the skin firmly above and below the incision. The skin was pulled apart using watchmaker forceps.

3.3.6 Removal of the uterus and blastocyst-stage embryo

The uterine horns were held with the forceps and the uterus was cut off at the junction with fine scissors. The uterus was pulled upwards in order to trim the membranes close to the wall of the uterine horns. The uterine horns were then cut below their junction through the body of the uterus and placed in the 35-mm Petri dish containing D-PBS. The same procedure was used for all of the mice during the experiment.

The blastocyst-stage embryos were collected from the uterine horn by flushing the embryos using the D-PBS loaded in the tip of a pipette inserted onto a 1 ml syringe. A 26-gauge needle was inserted into the tip of the uterine horn (from the ovarian side) to flush blastocyst embryo towards the uterine body. A 1 ml syringe with a pippete tip was used to pick up the blastocyst-stage embryos and washed three times in droplets of D-PBS and culture media as prepared in the tables below.

Table 3.1. Ham's F10 *in vitro* culture medium (Highveld Biological, South Africa)

Ham's F10	4.920 ml
Heparine	0.05 ml
Bovine serum albumin (BSA)	0.03 ml
Total	5 ml

Table 3.2. TCM-199 *in vitro* culture medium (Highveld Biological, South Africa)

TCM-199	4.920 ml
Heparine	0.05 ml
Bovine serum albumin	0.03 ml
Total	5 ml

The BSA powder (1.2 g) was dissolved in 10 ml of saline and stored in 0.5 ml eppendorf vials at a temperature of -20 °C. (Heide, 2005)

3.4 Assisted hatching procedures

All the assisted hatching techniques (Mechanical, chemical, enzymatic and laser) were done in TCM-199 and Ham's F10 and eighty embryos were randomly used per treatment with 40 embryos for each technique per medium. A control group of embryos without AH were applied in the two culture media to compare their results and the groups with AH. AH was done before *in vitro* culture.

Before the assisted hatching were performed, the ZP thickness were measured by use of NIS-Elements AR 4.10.00 software program. The program was part of the Micromanipulator computer connection. The ZP was measured through projection of the embryos on the screen monitor connected to micromanipulator computer series. The NIS-Elements AR 4.10.00 program was opened by clicking on it and go to 'measure' icon. The measure icon was set to give readings in micrometers by selecting the reading type. A mouse control curser was used to make measurements by putting the curser at the inner case of the inner layer of the ZP and dragged toward the end of the outer layer. The measurements were done in two opposite side and the means were recorded. All embryos were subjected to initial ZP measurements before and after AH.

3.4.1 Mechanical assisted hatching technique (partial zona dissection)

Four 50 µL drops of culture medium were prepared in a 35 mm petri dish. The drops were covered with sterile mineral oil to prevent contamination and drying of the medium. One washed embryo was transferred to the first drop, two embryos to the second, three embryos to the third drop and four embryos to the fourth drop. The dish containing the embryos was then placed onto the micromanipulation microscope.

The embryo was held by a holding pipette held at 30° from the left and the partial zona dissection (PZD) needle was inserted from a right of the embryo. The ZP of the embryo was pierced with the PZD needle with care in order to avoid damage to the inner cell mass. The needle was removed after making a hole in the ZP. The same procedure was repeated on the remaining embryos in the two different media with four replicates of each petri dish.

3.4.2 Chemical assisted hatching technique (acidified tyrode's solution)

A concentration of 0.125% tyrode, pH of 2.5- 3 and 99.875% of culture medium (TCM-199 and Ham's F10) was used. Chemical assisted hatching used acidified tyrode's solution to digest the ZP. Refrigerated acidified tyrode's solution was allowed to reach room temperature while setting up the microscope. A total of 50 µL of culture medium with 10 blastocyst-stage embryos was placed in the four petri dish, and covered with oil before it was placed on the stage of the microscope. The holding pipette was lowered into a drop containing blastocyst-stage embryos to be bleached in order to control the movement of embryos.

Embryos were made to rest stable in the culture medium by this holding pipette. The injection needle with a small break on its tip was filled with acidified tyrode's solution. The new injection needle was removed from the packet and the tip of the needle was broken to allow easy suction of acidified tyrode's solution inside the injection needle. The injection needle was connected with the homemade mouth-control delivery system. The mouth-controlled delivery system was placed on the left hand side of the micromanipulator. A bleaching of the outer layer of the ZP was done using a mouth-controlled homemade delivery system to blow the volume of 20 µL acidified tyrode's solution over the outer surface of the ZP. The embryos were then washed two times in the culture medium. Embryos were then grouped according to the design of the study and cultured for 24 hours.

3.4.3 Laser assisted hatching technique

The dish containing the mouse blastocyst stage was placed on the micromanipulator. 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 guided by safety circles which help the person to choose the area of interest and the target was positioned on the ZP making sure 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. One embryo was fired by one laser beam following the other until all embryos are fired by the laser beam. Entire thickness of the ZP was fired by the laser beam without breaking the inner layer. The same procedure was repeated for all the embryos in TCM-199 and Ham's F10.

3.4.4 Assisted hatching techniques using Trypsin

A medium with the concentrations of 0.5% trypsin, pH of 6.8-7.2 and 0.1% of EDTA was used in the digestion of the ZP. Assisted hatching technique using the above medium was prepared in a 35 mm petri dishes and 50 µL droplets of trypsin was placed in a petri dish and covered with a light mineral oil. Embryos were placed in the drops for one hour to allow partial zonal digestion. Digestion of the ZP was done under mineral oil in atmosphere at 37 °C on the IVF workstation.

3.5 Culture of the blastocyst-stage embryos

After the assisted hatching techniques were completed, the harvested blastocyst-stage embryos were cultured in TCM-199 or Ham's F10. The window period of 24 hours of culture was allowed for all embryos to undergo hatching after assisted hatching was performed. A different number of blastocyst-stage embryos from one blastocyst to four blastocyst per drop was located in different droplets, as described above, to determine the effect of the number of embryos per volume of culturing droplets on hatching. Ham's F10 (Table 3.1) and TCM-199 (Table 3.2) were used as culture media. After 24 hours of *in vitro* culture, the zonal thickness of all hatched embryos were measured and recorded.

3.6 Determination of number of blastomeres in blastocyst stage

After 24 hours of embryo culture, the hatched blastocysts were stained with Hoechst 33258. The hatched blastocysts were removed from the culture droplets and placed on a glass slide. The medium was aspirated using a micro-pipette in order to remove most of the volume of medium. The staining was done in the 4-well plate where well one and well two were filled with 0.5 ml of phosphate buffered saline and polyvinyl pyrrolidon medium, well three filled with 0.5 ml fixative solution and well four filled with Hoechst 33258 of concentration 0.5 µg/mL in saline.

The hatched blastocyst-stage embryos were washed in well one and fixed with fixative solution (paraformaldehyde in D-PBS, 40 mg/ml) in well three for 30-60 minutes. After fixation in well three, embryos were stained in well four for a period of 20-30 minutes and washed again in

well two before transferred to the slide. Stained embryos were placed on a new slide and labelled. A cover slip was placed to cover the embryo on the slide with vaseline placed at each corner of the slip. The cover slip was used to cover the embryos in order to prevent foreign particles and spread the blastomeres.

Stain solution was added under the cover slip in order to cover the embryo. The stain is kept in the dark place at 2-8°c (SIGMA ALDRICH Co, Intervet South Africa). Freshly made stain was used once per day. The Cutex nail polish (M.A.C Studio Nail Lacquer) was used to cover all the sides of the cover slip to prevent the hatched embryos to escape from underneath the slide. The labelled slide was turned upside down for counting the blastomeres under fluorescent UV light within 4 hours using an inverted microscope under X400 magnification (Nikon eclipse TI, Narishige co., Ltd.USA).

3.7 Statistical analysis

Data collected were subjected to analysis of variance using PROC General Linear Model of SAS version 9.4, where samples were randomly collected from the F1 generation mice. Where significant differences between groups was detected, and the Tukey's test was used to separate the means.

The following statistical model was used:

$Y_{ijkl} = \mu + T_i + M_j + N_k + (TM)_{ij} + (TN)_{ik} + (MN)_{jk} + (TMN)_{ijk} + Є_{ijkl}$

Where,

Y_{ijkl} =the observations

μ = overall mean

T_i= effect of the i^{th} techniques

M_j=effect of the j^{th} culture media

N_k = effect of the k^{th} number of embryos

TM_{ij}=interaction of i^{th} techniques and j^{th} culture media

TN_{ik}= interaction of i^{th} techniques and k^{th} embryos

MN_{jk}= interaction of j^{th} culture media and k^{th} number of embryos

TMN_{ijk}= interaction of i^{th} techniques, j^{th} media and k^{th} number of embryos

$Є_{ijkl}$ = random error

3.8 Ethical considerations

The ethical clearance certificate referenced (SARDF / 15 / ANS / 03 / 0110) was obtained from the University of Venda Research Ethics Committee.

CHAPTER 4

RESULTS

This study was done to compare the effect of two *in vitro* culture media and four assisted hatching (AH) techniques on blastocyst hatching rate using mice as the model. A total of 400 mice blastocyst-stage embryos were harvested and subjected to treatment groups in two *in vitro* culture media. Four techniques of AH in two culture media plus two controls (one for each culture medium) were used with 40 embryos in each group. Four AH techniques and four culture micro droplets with different grouping of embryos per drop were done to test the effect on hatchability and number of blastomeres were counted after staining. Control measures were done for all assisted AH techniques and culture media. The results are presented in Tables 4.3 to 4.10 and Figures 4.1 to 4.11.

Table 4.3. Effect of Assisted hatching techniques

AH techniques	ZP thickness before AH(µm) & ±SD	Hatchability (%) & ±SD	ZP thickness after culture (µm) & ±SD	Number of blastomeres & ±SD
Control	11.45^{b}±0.74	18.75^{b}±32.65	11.03^{b}±0.70	74^{a}±9.18
Laser	11.57^{b}±1.22	51.81^{a}±39.95	11.27^{b}±1.22	72^{a}±8.59
Mechanical	12.84^{a}±0.97	51.55^{a}±37.31	12.49^{a}±1.02	73^{a}±11.56
Enzymatic	11.50^{b}±0.92	34.10^{ab}±36.31	7.31^{c}±1.42	70^{a}±9.59
Chemical	11.52^{b}±0.82	33.59^{ab}±36.89	10.65^{b}±0.77	74^{a}±8.42

Different superscripts (a, b and c) within the same line indicate significant differences among means (P<0.05), %= percentage, AH= assisted hatching, ZP= zona pellucida, ±SD= standard deviation, TCM-199= Tissue culture media 199 and Ham's F10

Table 4.4. Effect of *in vitro* culturing media

Culture media(CM)	ZP thickness before AH(µm) & ±SD	Hatchability (%) & ±SD	ZP thickness after culture (µm) & ±SD	Number of blastomeres & ±SD
Ham's F10	11.76^{a}±1.30	36.87^{a}±37.56	10.86^{a}±1.83	71^{a}±8.27
TCM-199	11.80^{a}±0.81	40.00^{a}±39.32	10.25^{b}±2.18	75^{b}±10.46

Different superscripts (a, b and c) within the same line indicate significant differences among means (P<0.05), %= percentage, CM= Culture media, ZP= zona pellucida, ±SD= standard deviation, TCM-199= Tissue culture media 199 and Ham's F10

Table 4.5. Effect of number of embryos per drop

Number of embryos per drop (E)	ZP thickness before AH(µm) & ±SD	Hatchability (%) & ±SD	ZP thickness after culture (µm) & ±SD	Number of blastomeres & ±SD
1	11.70^{a}±1.06	20.00^{c}±40.50	10.68^{a}±1.85	71^{a}±9.11
2	11.75^{a}±1.15	28.75^{bc}±29.71	10.48^{a}±2.07	73^{a}±8.66
3	11.82^{a}±1.14	59.14^{a}±38.84	10.52^{a}±2.16	75^{a}±9.82
4	11.83^{a}±0.99	43.75^{ab}±32.39	10.53^{a}±2.09	73^{a}±10.42

Different superscripts (a, b and c) within the same line indicate significant differences among means (P<0.05), %= percentage, E= number of embryos per drop, ZP= zona pellucida, ±SD= standard deviation, TCM-199= Tissue culture media 199 and Ham's F10

Comparing the AH techniques, it was observed that the thickness of the ZP before AH to the thickness of ZP after culture for 24 hours was significantly different (P<0.05). The control group showed no variation on the ZP thickness before and after 24 hours of culture when compared to the ZP of the AH techniques (Table 4.3 to 4.5).

There was no significant difference (P>0.05) between the two *in vitro* culture media (TCM-199 and Ham's F10). Considering the function of grouping embryos in different numbers per drop, a high significant difference (P<0.05) was observed (Table 4.3 to 4.5).

There was a significant difference (P<0.05) observed in the number of blastomeres counted after staining (Table 4.3 to 4.5).

The results on the summary of the significant level of AH techniques, *in vitro* culture media and number of embryos per drop and their interactions are presented (Table 4.6).

Table 4.6. Summary for significant levels of control hatching, AH techniques, culture media, number of embryos per drop, AH x CM, AH x E, C M x E and AH x CM x E

Significance	ZP thickness before AH	Hatchability (%)	ZP thickness after Culture	Number of blastomeres after staining
AH	**	**	**	NS
CM	NS	NS	**	*
E	NS	**	NS	NS
AH x CM	**	NS	**	NS
AH x E	NS	NS	NS	NS
CM x E	NS	NS	NS	NS
AH x CM x E	NS	NS	NS	NS

AH= assisted hatching, CM= Culture media, E= number of embryos per drop, AH x E= interaction between AH techniques and number of embryo per drop, CM * E= interaction between culture media and number of embryos per drop, AH x CM x E= interaction among assisted hatching techniques, culture media and number of embryos per drop, NS= not significant (P>0.05), **= highly significant difference (P<0.01), *= significant difference (P<0.05) and ZP = zona pellucida

The results indicate that there was a highly significant difference (P<0.01) between the thickness of the ZP measured before AH and after culture. There was no significant difference (P>0.05) on the number of blastomeres counted after staining. *In vitro* culture media, indicated a highly significant difference (P<0.01) in the number of counted blastomeres and a significant difference (P<0.05) on ZP thickness after culture. The number of embryos grouping per drop, were highly significant different (P<0.01) on hatchability.

Interaction between AH techniques and *in vitro* culture media had highly significance difference (P<0.01) on the thickness of the ZP after culture except in all other parameters, whereas interaction amongst (AH techniques and number of embryos per drop, *in vitro* culture media) and (number of embryos per drop, AH and culture media) did not show any significance difference (P>0.05) on all parameters.

The results of the effect of interaction between AH techniques and culture media, evaluated on the ZP thickness before AH, hatchability (%), ZP thickness after 24 hours culture and number of blastomeres after staining are presented in Table 4.7.

Table 4.7. Effect of interaction between AH techniques and culture media

AH Techniques	Culture media	ZP thickness before AH (µm) & ±SD	Hatchability (%) & ±SD	ZP thickness after culture (µm) & ±SD	Number of blastomeres & ±SD
Control	Ham's F10	11.20^{c}±0.64	13.02^{b}±27.71	10.84^{bc}±0.65	75±8.09
	TCM-199	11.70^{bc}±0.78	24.48^{ab}±36.94	11.21^{bc}±0.71	74±10.40
Laser	Ham's F10	11.82^{bc}±1.54	46.86^{ab}±37.12	11.51^{b}±1.56	69±6.35
	TCM-199	11.35^{bc} ±0.78	56.25^{ab}±43.30	11.04^{bc}±0.75	75±9.65
Mechanical	Ham's F10	13.39^{a}±1.02	51.07^{ab}±40.19	13.10^{a}±0.98	70±13.10
	TCM-199	12.31^{ab}±0.51	52.55^{a}±35.50	11.87^{b}±0.62	75±11.87
Enzymatic	Ham's F10	11.12^{c}±0.79	39.05^{ab}±35.83	8.44^{d}±0.91	69±8.44
	TCM-199	11.88^{bc}±0.91	49.16^{ab}±37.26	7.89^{e}±0.80	71±6.19
Chemical	Ham's F10	11.27^{bc}±0.80	33.85^{ab}±37.50	10.39^{c}±0.85	72±10.39
	TCM-199	11.78^{bc}±0.78	33.32^{ab}±35.50	10.92^{bc}±0.60	76±10.92

Different superscripts (a, b and c) within the same line indicate significant differences among means (P<0.05), %= percentage, AH= assisted hatching, ZP= zona pellucida, ±SD= standard deviation, TCM-199= Tissue culture media 199 and Ham's F10

The ZP thickness after culture and before AH in the two culture media

There was a significant difference (P<0.05) observed between the thickness of ZP thickness before AH and after 24 hours of culture. The thickness of the ZP decreased with assisted hatching techniques.

Effects of culture media and AH method on the hatchability of the embryos

There was no significance difference (P>0.05) among hatchability. Also, the interaction between AH techniques and *in vitro* culture media was found to be significantly different (P<0.05) on hatchability, but with higher hatchability in laser AH techniques (56.25±43.30%) in TCM-199 culture medium followed by mechanical AH techniques at 52.55±35.50% in the same *in vitro* culture medium. The lowest hatchability (33.32±35.50%) was recorded in the chemical AH technique group

Effects of culture media and AH technique on the number of blastomeres

The results indicate that there is no significance difference (P>0.05) on the blastomeres counted after staining. The number of blastomeres counted under interaction of AH techniques and culture media was not significantly different (P>0.05), with the values ranging from 69±6.35 to 76±10.92 blastomeres for hatched embryos.

The results of the effect of interaction between *in vitro* culture media and number of embryos per drop evaluated on the ZP before AH, hatchability (%), ZP after culture and blastomeres after staining are represented in Table 4.8.

Table 4.8. Effect of interaction between culture media and number of embryos per drop

Culture Media	Number of embryos per drop	ZP thickness before AH (µm) & ±SD	Hatchability (%) & ±SD	ZP thickness after culture (µm) & ±SD	Number of blastomeres & ±SD
Ham's F10	1	11.78±1.20	20.00^{c}±41.04	11.03^{a}±1.61	70±6.70
	2	11.69±1.40	27.50^{bc}±30.24	10.69^{ab}±2.00	72±9.01
	3	11.70±1.45	54.97^{ab}±37.88	10.70^{ab}±2.10	74±7.73
	4	11.87±1.23	45.00^{a-c}±32.04	11.02^{a}±1.67	69±8.98
TCM-199	1	11.62±0.93	20.00^{c}±41.04	10.33^{ab}±2.04	73±10.89
	2	11.82±0.89	30.00^{a-c}±29.91	10.27^{ab}±2.17	73±8.50
	3	11.95±0.74	63.31^{a}±40.31	10.34^{ab}±2.27	76±11.70
	4	11.79±0.70	42.50^{a-c}±33.54	10.04^{b}±2.38	75±10.93

Different superscripts (a, b and c) within the same line indicate significant differences among means (P<0.05), %= percentage, AH= assisted hatching, ZP= zona pellucida, ±SD= standard deviation, TCM-199= Tissue culture media 199 and Ham's F10

The ZP thickness after culture and before AH in the two culture media

The results showed no significant difference (P>0.05) in the thickness of the ZP in TCM-199 and Ham's F10 before assisted hatching was performed, but, the thickness was significantly affected after culturing in both media.

Effects of culture media and number of embryos per drop on hatchability

There was a significant difference (P<0.05) on hatching percentage in relation to the grouping of the number of embryos per drop. Embryos grouped into three were the ones that yielded the highest percentage in Ham's F10 compared to TCM-199, with 54.97±37.88% and 63.31±40.31% respectively. The number of blastomeres ranged from 69±8.98 to 76±11.70. There was an exponential relationship between hatching and increase in the number of embryos per drop with slight decrease of hatchability in the four embryos grouped together.

Effects of culture media and number of embryos per drop on the number of blastomeres

Interactions had no significant effect on the *in vitro* culture media and the number of embryos per drop with significant difference (P<0.05) in the blastomeres counted after staining.

The results of the effect of interaction between AH techniques and number of embryos per drop, evaluated on the ZP thickness before AH, hatchability (%), ZP thickness after culture and blastomeres after staining are represented in Table 4.9.

Table 4.9. Effect of interaction between AH techniques and number of embryos per drop

AH Techniques	Number of embryos per drop	ZP thickness before AH(µm) & ±SD	Hatchability (%) & ±SD	ZP thickness after culture (µm) & ±SD	Number of blastomeres & ±SD
Control	1	11.33^{ab}±0.83	12.20^{bc}±35.35	10.93^{c-e}±0.72	73±9.41
	2	11.49^{ab}±0.99	6.25^{a-c}±17.68	11.04^{a-e}±0.90	74±7.72
	3	11.66^{ab}±0.76	37.49^{a-c}±45.20	11.28^{a-e}±0.78	77±11.56
	4	11.32^{ab}±0.30	18.75^{a-c}±22.16	10.86^{de}±0.32	74±9.11
Laser	1	11.42^{ab}±0.78	37.50^{a-c}±51.75	11.31^{a-e}±0.84	73±11.30
	2	11.41^{ab}±1.25	57.50^{a-c}±23.15	10.96^{a-e}±1.10	69±10.96
	3	11.64^{ab}±1.46	74.98^{ab}±34.51	11.32^{a-e}±1.53	75±11.31
	4	11.81^{ab}±1.49	56.25^{a-c}±39.52	11.52^{a-e}±1.49	71±11.52
Mechanical	1	12.94^{ab}±0.98	12.50^{bc}±35.35	12.61^{ab}±0.90	67±12.61
	2	12.87^{ab}±1.22	43.75^{a-c}±32.04	12.57^{a-c}±1.15	74±12.56
	3	12.99^{a}±0.94	73.12^{a}±17.29	12.65^{a}±0.91	79±12.65
	4	12.58^{ab}±0.84	71.88^{a-c}±20.86	12.11^{a-d}±1.19	71±12.11
Enzymatic	1	11.28^{ab}±0.99	25.00^{a-c}±46.29	7.77^{f}±1.42	70±7.96
	2	11.45^{ab}±0.88	18.75^{a-c}±25.87	7.13f±1.21	69±6.90
	3	11.64^{ab}±0.92	45.81^{a-c}±43.40	7.27^{f}±1.21	70±12.70
	4	11.64^{ab}±1.05	46.87^{a-c}±20.86	7.08^{f}±1.90	72±11.39
Chemical	1	11.55^{ab}±0.90	12.50^{bc}±35.35	10.78^{de}±0.73	72±10.67
	2	11.53^{ab}±0.93	37.50^{a-c}±35.35	10.72^{de}±0.75	76±8.27
	3	11.19^{b}±0.85	58.30^{a-c}±38.82	10.25^{e}±0.83	73±6.26
	4	11.83^{ab}±0.61	25.00^{a-c}±26.72	10.88^{c-e}±0.76	73±9.01

Different superscripts (a, b, c and d) within the same line indicate significant differences among means (P<0.05), %= percentage, AH= assisted hatching, ZP= zona pellucida and ±SD= standard deviation

The ZP thickness

The thickness of ZP before AH and the ZP thickness after 24 hours of culture showed a decrease.

Hatchability of the embryos

It was observed that the mean interaction between the assisted hatching techniques and the number of embryos per drop had highly significance difference (P<0.01). The interaction showed great effect on hatching percentage on the different grouping of embryos per drop. The effect is observed in all AH techniques with great effect on grouping of embryos by three per drop compared to other groupings

The *in vitro* culture of one embryo per drop showed poor hatchability, but increases with increase in number of embryos per drop. Grouping of embryos in four showed a slightly decline in hatching percentage. Hatchability ranged from 12.50±35.35 to 74.98±34.51% under AH techniques and a minimum of 6.25±17.68% in the control group. Laser AH technique had a

highest hatching percentage of 74.98±34.51%, compared to mechanical, enzymatic and chemical AH techniques. The results of hatchability on mechanical followed laser with maximum of 73.12±17.29%. Chemical AH technique recorded the second lowest percentage of the four techniques with maximum hatching percentage (58.30±38.82%) at grouping of three embryos per drop.

Number of blastomeres

It was also observed that there was no significance difference (P>0.05) on the interaction of AH and number of embryos per drop on the blastomeres counted

The results of the effect of interaction among main factors AH techniques, culture media and number of embryos per drop, evaluated on the ZP thickness before AH, hatchability (%), ZP thickness after culture and blastomeres after staining are represented in Table 4.10.

Table 4.10. Interaction among AH techniques, culture media and number of embryos per drop

AH techniques	Culture Media	Embryos per drop	ZP thickness before AH (µm) & ±SD	Hatchability (%) & ±SD	ZP thickness after culture (µm) & ±SD	Blastomeres & ±SD
Control	Ham'sF10	1	11.10^{ab}±0.41	25.00±50.00	10.79^{a-f}±0.50	73±11.19
		2	11.07^{ab}±0.96	23.89±32.41	10.70^{a-f}±0.94	78±8.80
		3	11.39^{ab}±0.84	8.33±16.65	11.02^{a-f}±0.87	79±5.50
		4	11.24^{ab}±0.40	18.75±23.93	10.87^{a-f}±0.43	70±4.92
Control	TCM-199	1	11.55^{ab}±1.15	23.00±15.36	11.08^{a-f}±0.96	73±8.99
		2	11.91^{ab}±0.97	12.50±25.00	11.39^{a-f}±0.84	70±4.65
		3	11.93^{ab}±0.66	66.65±47.14	11.54^{a-e}±0.68	74±16.27
		4	11.40^{ab}±0.18	18.75±23.93	10.85^{a-f}±0.26	78±11.38
Laser	Ham'sF10	1	11.64^{ab}±0.92	25.00±50.00	11.67^{a-d}±0.91	67±7.41
		2	11.65^{ab}±1.44	37.50±25.00	11.10^{a-d}±1.25	67±2.57
		3	11.83^{ab}±2.10	74.95±16.70	11.47^{a-f}±2.23	77±2.94
		4	12.17^{ab}±2.06	50.00±40.82	11.81^{a-f}±2.10	66±5.03
Laser	TCM-199	1	11.21^{ab}±0.66	50.00±57.74	10.94^{a-f}±0.68	80±11.17
		2	11.18^{ab}±1.20	37.50±25.00	10.82^{a-f}±1.10	71±2.98
		3	11.46^{ab}±0.70	85.00±50.00	11.16^{a-f}±0.68	74±8.28
		4	11.45^{ab}±0.76	62.50±43.30	11.23^{a-f}±0.73	76±14.43
Mechanical	Ham'sF10	1	13.53^{a}±0.75	25.00±50.00	13.23^{a}±0.61	69±5.80
		2	13.55^{a}±1.44	25.00±28.86	13.23^{a}±1.33	71±12.17
		3	13.55^{a}±1.04	83.30±19.28	13.20^{ab}±1.03	73±10.08
		4	12.92^{ab}±1.10	75.00±20.41	12.68^{a-c}±1.15	69±14.07
Mechanical	TCM-199	1	12.36^{ab}±0.88	67.00±35.50	11.99^{a-d}±0.70	64±6.40
		2	12.21^{ab}±0.45	62.50±25.00	11.86^{a-d}±0.18	77±15.41
		3	12.44^{ab}±0.37	74.95±16.70	12.10^{a-d}±0.26	85±9.12
		4	12.24^{ab}±0.37	68.75±23.94	11.52^{a-e}±1.03	74±13.49
Enzymatic	Ham'sF10	1	11.26^{ab}±1.14	25.00±50.00	8.86^{f-i}±0.85	69±6.58
		2	10.94^{ab}±0.59	25.00±28.87	7.94^{h-j}±1.06	67±3.79
		3	10.98^{ab}±0.50	49.98±43.02	8.03^{g-j}±0.86	71±11.97
		4	11.31^{ab}±1.04	56.25±12.50	8.92^{e-i}±0.64	70±14.52
Enzymatic	TCM-199	1	11.29^{ab}±0.99	25.00±50.00	7.25^{ij}±0.91	71±10.09
		2	11.97^{ab}±0.86	12.50±25.00	8.78^{ij}±0.75	71±9.36
		3	11.30^{ab}±0.74	41.65±49.98	7.68^{j}±0.51	70±15.25
		4	11.97^{ab}±1.08	37.50±25.00	8.10^{j}±0.89	74±9.20
Chemical	Ham'sF10	1	11.38^{ab}±1.00	46.25±21.25	10.59^{b-g}±0.84	69±1.82
		2	11.21^{ab}±0.92	50.00±40.82	10.44^{b-h}±0.87	77±10.78
		3	10.75^{b}±0.55	60.40±41.93	9.73^{d-h}±0.73	71±4.57
		4	11.74^{ab}±0.60	25.00±28.68	10.81^{a-f}±0.85	70±5.46
Chemical	TCM-199	1	11.72^{ab}±0.88	25.00±50.00	10.96^{a-f}±0.66	76±15.08
		2	11.85^{ab}±0.93	25.00±28.86	10.98^{a-f}±0.61	76±6.50
		3	11.63^{ab}±0.93	58.30±41.93	10.78^{a-f}±0.59	76±7.23
		4	11.91^{ab}±0.70	25.00±28.86	10.95^{a-f}±0.78	77±11.49

Different superscripts (a, b, c, d, e, f, g, h, I and j) within the same line indicate significant differences among means (P<0.05), %= percentage, AH=assisted hatching, ZP =zona pellucida, ±SD= standard deviation, TCM-199= Tissue culture media of 199 and Ham's F10

Effect of AH techniques and culture media and number of embryos on ZP thickness

The joint interaction between AH techniques, culture media and number of embryos had significant difference (P<0.05) on the thickness of the ZP after 24 hours of *in vitro* culture. The control group did show a slightly decrease in the thickness after culture. High effect of interaction was observed under use of enzymatic AH technique. The thickness of the ZP ranged from 10.75±0.55 to 13.55±1.44 µm before AH was performed. The results of ZP both before and after culture thickness of this study ranged from 7.25±0.91 to 13.23±1.33 µm including the control group.

Effects of AH techniques and culture media and number of embryos on hatchability

Interaction of main factors (culture media, grouping of embryos per drop and AH) had no significance difference (P>0.05) on the hatching percentage. The highest hatching percentage was observed with the use of laser AH techniques with TCM-199 culture medium as and on the grouping of three embryos per drop with 85.00±50.00%. The mechanical method of assisted hatching using Ham's F10 with grouping of three embryos per drop yielded the second best hatchability 83.30±19.28%. The third best hatching rate was attained from enzymatic method of AH using TCM-199 and three embryos per drop yielding 60.40±41.93%. The lowest hatchability was observed from the chemical method of AH with TCM-199 and grouping of two embryos per drop. The enzymatic AH, TCM-199 and grouping of three embryos to a culture drop had the lowest effect on hatchability with 41.65±49.98%. In all interactions of factors, hatchability increases with increase embryo grouping, but in this study the decline was observed when embryos are grouped into four per drop.

Effects of AH techniques and culture media and number of embryos on the number of blastomeres

Counted blastomeres of hatched blastocysts embryos after staining did not show any significance difference (P>0.05) amongst them as a results of the interaction of three factors. The blastomeres counted ranged from 64±6.40 and 85±9.12.

CHAPTER 5

DISCUSSION

The thickness of the ZP before assisted hatching was used as a control measure to view its thickness after *in vitro* culture. The thickness of the ZP at blastocyst stage of mice embryos ranged from 10.75±0.55 and 13.55±1.44 µm and the recorded results are similar to the report by Erdal *et al*. (2006) who record the thickness ranging from 11.7 to 12.1 µm. Moreover, prior to assisted hatching, the thickness was observed to be different amongst the experimental animals. The difference in the thickness of the embryos within a column could be attributed to the fact that the animals used in the present study were of different ages. This could be supported by the findings of Debra *et al*. (2008) who reported that age of mouse determines the thickness of the ZP. It was again observed that, the initial ZP thickness solely does not have effect on hatchability.

Wang *et al*. (2006) and Sung *et al*. (2014) found that the thickness of the ZP play a significant role in the hatching of murine embryos. Again, it was also recorded that blastocysts with the ZP thickness less than 16 µm had hatching rate of up to 69% whereas those with thickness greater than 17 µm had hatching rate up to 25%. A slight decrease in the thickness of the ZP was observed in non-assisted, mechanical, laser and chemical methods of AH with a huge decrease in the thickness observed in enzymatic technique.

High variation in the thickness of the ZP in enzymatic technique might be as a result of the fact that, the trypsin enzyme digest the ZP while other techniques only affected a portion of the ZP. There is a limited information based on the ZP thickness after culture. However, it was reported that the ZP thinner than 16µm hatch with ease (Sung *et al*., 2014). From the present study, hatchability of up to 85.00±50.00% was obtained on different interactions involving type of culture medium, number of embryos per drop and assisted hatching techniques where they played notable role especially with laser AH. More significant effects were observed in interactions between two or more factors than in individual factors.

Loret *et al.* (1997) found that the thickness of the ZP decreases in relation to embryo development until hatching takes place. The age of the animal has the positive relationship with the hardening of the ZP of murine embryos. Hence, there was a variation in the means of the thickness of the ZP before AH and after culture.

Laser assisted technique was simpler during data collection, hence considered as a good technique in this study. Furthermore, it was precise and not complex. During trials, the laser method of AH was seen to be an easier technique compared to the rest. Hence, the laser technique can be established to be a better option.

The hatching percentages obtained from the present study are similar to the findings of Hsieh *et al*. (2002) where they obtained a hatching percentage of 31.8% and 16.2% for laser and chemical techniques, respectively. This result makes laser AH technique more superior over mechanical, enzymatic and chemical assisted hatching techniques. Neev *et al*. (1995) findings are in support of the results of the current study. However, Neev *et al*. (1995) found that the use of laser AH technique enhances complete hatching up to 80%. Erdal *et al*. (2006) reported that there is a great accuracy and safety in the use of the ZP beaming compared to all other AH techniques (mechanical, enzymatic and chemical).

Earlier study conducted by Sung *et al*. (2014), obtained up to 85.1% hatchability with mechanical method of AH. The result is higher than the results obtained from the present study. The difference in these results may be due to the difference in the period of time the *in vitro* culture were carried out in both studies. The culture in the earlier study was carried out for 48 hours and 72 hours, whereas, the present study cultured the embryos for 24 hours.

The current study yielded 83.30±19.28% hatchability with the mechanical AH technique, which is lower compared to the laser AH technique, but greater than both chemical and enzymatic AH techniques. Makrakis *et al*. (2006) compared the mechanical and chemical AH techniques, where it was found that the mechanical AH technique yielded highest hatchability than the chemical AH technique. This is because the chemical AH technique has a likelihood to damage the inner cell mass due to the acidic solution used. From the study by Sung *et al*, (2014), 77.6% hatching percentage was reported using the mechanical AH technique.

In comparison of *in vitro* culture media between Ham's F10 and TCM-199, 36.87±37.56% and 40.00±39.32% hatching, respectively, were yielded without interactions of factors. Although, an increase in hatchability was observed when the media interacted with AH techniques and group culture of embryos. Nedambale *et al*. (2004), emphasized that the addition of constituents such as serum, to *in vitro* culture medium has a positive feedback for hatching, while serum free media lead to delayed hatching. Serum nutrients has got an effect on embryo development. According to Barnea *et al*, (2012), during embryo development, blastocyst expresses multiple factors and their receptors in response to sex steroids and growth factors, which in turn regulate

blastocyst growth and participate in the signal exchange with the receptive endometrium. Hormones and factors expressed in this process include the preimplantation factor, chorionic gonadotrophins, (Srisuparp *et al.,* 2001), leukemic inhibitory factor, (Movaghar. 2012), heparin-binding epidermal growth factor, (Yoshinaga. 2010), and Prostacyclin is the most abundant PGs produced by the mouse blastocysts.

Furthermore, it was reported that addition of serum in a culture medium has a potential to promote hatching of embryos by providing plasminogen that proteolytically degrades the ZP, hence facilitating hatching (Nedambale *et al*., 2004).

The differences between the results observed from TCM-199 and Ham's F10 *in vitro* culture media might be because TCM-199 contains glutamine and glucose. The presence of glucose is found to be essential to generate ATP via glycolytic metabolism while glutamine can feed into tricarboxylic acid cycle and serves as potential energy source (Downs and Verhoeven., 2003). The absence of glucose fails to support murine embryo development (Downs and Hudson., 2000). Teruel *et al*. (2005) obtained 49.66% hatching observed after 24 hours in Ham's F10 *in vitro* culture medium, while the current study obtained 36.87±37.56%.

The current study cultured blastocyst-stage embryos which were subjected to different assisted hatching techniques for a period of 24 hours before evaluation. TCM-199 and Ham's F10 were used as culture media and they yielded 36.87±37.56% and 40.00±39.32%, respectively. The results of this study indicated that the successful development of murine embryos is influenced by the culture media where Ham's F10 and TCM-199 played varied roles in hatchability. The results still align with what other researchers found (Hoelker *et al*., 2010).

Canseco *et al*. (1992) reported that the volume of *in vitro* culture medium and density of embryos in a drop has a significant role in embryonic development, such that an increase in the density of embryos in a drop also increases embryonic development at the blastocyst stage. This is probably by concentrating the trophic autocrine and paracrine factors (Richter. 2008). Fujita *et al*. (2006) supported the idea that the *in vitro* culture of an embryo alone in the drop does not fully enhance hatchability.

Culturing of murine embryos in groups under a 50 µL micro-droplet has a significant effect on the hatchability after 24 hours of culturing. The findings of this study do align with what other authors reported Vutyavanich *et al*. (2011), because higher hatching was seen when embryos are

cultured in groups than solely. Kato and Tsunoda. (1994) also agreed that the volume of *in vitro* medium also plays a significant role in the development of the murine embryos.

The interaction of assisted hatching technique and culture media, with laser using TCM-199 yielded the highest hatching percentages of 56.25±43.30% compared to all other interactions, whereas interaction method of AH with TCM-199 yielded the least hatching percent with 33.32±35.50%. The culture of embryos without AH yielded 24.48±36.94% with TCM-199 which was lower when compared to AH. The results show that the interaction between assisted hatching techniques and TCM-199 has a potential to initiate higher hatching percentages of murine blastocyst-stage embryos.

Interaction between assisted hatching techniques and number of embryos per drop, showed an overall hatching percentage ranging from 12.50±35.35 to 74.98±34.51%, with maximum recordings 74.98±34.51%, 73.12±17.29%, 46.87±20.86% and 58.30±38.82% on laser, mechanical, enzymatic and chemical, respectively. The highest percentages of hatching were observed where embryos were grouped into three embryos per drop. In contrast, higher hatching percent were seen when embryos were grouped into four embryos per drop with the enzymatic technique. Under non-assisted hatching technique groups, the lowest was 6.25±17.68% when two embryos were cultured per drop and highest was 37.49±45.20% hatchability when three embryos were cultured per drop.

Interaction of assisted hatching techniques, culture media and number of embryos per drop is represented on Table 4.8. Although it is a complex study having three interactions, the results of these interactions seem to be the best overall on hatching. Least hatching was observed as 12.50±25.00% and highest being 85.00±50.00%. Enzymatic x TCM-199 x grouping of two embryos per drop yielded the poorest results of all interactions (12.50±25.00%), while laser x TCM-199 x grouping of three embryos per drop yielded the best overall hatching (85.00±50.00%). Hatchability of 86.9% was obtained by Shan-Jun Dai *et al*. (2012) when embryos were grouped into nine in a 50 µL droplet. The results were slightly higher than that of the current study.

This might be because only three embryos were grouped which yielded 85.00±50.00% hatchability. Hatchability of 75.4% in AH was recorded by Shan-Jun Dai *et al*. (2012), with a slight decline to 70.8% when grouped in 12 embryos per drop. The same consistence of results was seen in this study with the grouping of four embryos per drop with a slight decline in hatchability to 75.00±20.41%. Grouping of embryos into three per drop using Ham's F10 showed good hatching under mechanical, enzymatic and chemical techniques with only TCM-199 using

grouping of three embryos per drop yielded the highest overall hatching in the laser technique from the current study. Canseco *et al*. (1992) and Salahuddin *et al*. (1992) also supported that the density of incubation of mouse embryos do have beneficiary effect on hatching rate.

On the blastomeres count after staining it was observed that the number of blastomeres do not influence hatchability. There was no significant difference among blastomeres counted after staining. The observations were supported by Sung *et al*. (2014) who also mentioned that there is no correlation between blastomeres and hatchability. Thouas *et al*. (2001) counted and recorded a total of 75 blastomeres in number for hatched blastocyst-stage embryo. The used specie was mouse and embryos were cultured for 24 hours with one embryo per drop.

It was my belief that the viability of embryos before implantation is measured by the number of blastomeres of the embryo. Thouas *et al*. (2014) recorded 75 blastomeres in an embryo stained before it reaches its blastocyst stage. According to Sung *et al*. (2014) the total blastomeres were high in the embryos which underwent laser assisted hatching with 88 and 83 number of blastomeres, when compared to the blastomeres in mechanical AH. Chemical AH have the ability to damage the embryo, thereby leading to the conclusion that the number of blastomeres are also affected. This indicates that if we compare blastomeres of hatched murine blastocysts produced from laser, mechanical and chemical AH techniques, the chemical technique will be expected to have lowest number of blastomeres.

Furthermore, Montag *et al*. (2000) found that the blastomeres counted do not have any relationship with the hatchability. In contrast to the current study, Lane and Gardner. (1997) found that the faster the embryo development, the higher the blastomeres count in the inner cell mass, of which automatically lead to higher total blastomeres counts. The Richter *et al*. (2011) study support that the number of blastomeres do not have any relationship with the hatching of the embryo, but rather the shape of the embryo play role in its hatching from the ZP.

The fact that other authors support that the number of blastomeres do not have a rigid exponential response to embryo grading, affirm the findings of this study that there is no significant difference in the blastomeres count among, the grouping of embryos, form of assisted hatching techniques and type of culture media. Lane and Gardner. (1997) reported that, there is no positive relationship between mouse blastocyst blastomeres count and hatching. The current study did not show any relationship between the hatchability and the number of blastomeres.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The comparison of the effect of assisted hatching techniques and *in vitro* culture media on blastocyst-stage embryo hatching rate using mice as the model were carried out in this study and thus, the following conclusions were made, even though AH remains complex procedure with limited studies done to date. This study lighten the conclusion that assisted hatching has significant effect on hatching percentage of blastocyst-stage embryos. Laser AH technique has higher hatchability compared to all other AH techniques. More embryos were recorded in TCM-199 medium compared to Ham's F10 medium. Furthermore, AH has the combined effects from group culture, *in vitro* culture media, ZP thickness and type of assisted hatching. Interaction of the following factors (AH techniques, group culture and *in vitro* culture media) showed more effect on hatching. The hatching percentage up to 85.00±50.00% observed in this study under AH techniques, especially laser and mechanical AH techniques is a benefit. It was also observed that, ZP does not solely affect hatchability.

6.2 RECOMMENDATIONS

Following the success of this study, it is therefore recommended that:

i. *In vitro* culture of the murine blastocyst-stage embryos is possible in both Ham's F10 and TCM-199.
ii. Assisted hatching can be done with interactions of grouping embryos per drop and *in vitro* culture media with three embryos per drop.
iii. The ZP of *in vitro* produced embryos should be laser rented and used for livestock purpose.
iv. Furthermore, it is recommended that more studies should be done to test assisted hatching techniques on 'different culture time' after assisted hatching.
v. More studies are needed to be conducted to test assisted hatching techniques and *in vitro* culture media in cattle embryos.

REFERENCES

Andras, N. **2003**. Manipulating the mouse embryo. *A Laboratory Manual*. John English New York, United States of America.

Barnea, E., Kirk, D and Paidas, M. **2012**. "Pre Implantation Factor (PIF) promoting role in embryo implantation: increases endometrial Integrin-$\alpha 2\beta 3$, amphiregulin and epiregulin while reducing betacellulin expression via MAPK in decidua," *Reproductive Biology and Endocrinology*. **10** (1), 50

Biggers, J.D. **2003**. Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues. *Human Reproduction*, **9**, 557-582.

Canseco, R.S., Sparks, A.E., Pearson, R.E. and Gwazdauskas, F.C. **1992**. Embryo density and medium volume effects on early murine embryo development. *Journal Assisted Reproductive Genetics*. **9**, 454-457.

Carroll, J., Depypene, H. and Mathews, C.D. **1990**. Freeze-thaw induced changes of the zona pellucida explain decreased rates of fertilization in frozen-thawed mouse oocytes. *Journal Reproduction Fertility,* **90**, 547-553.

Choi, D., An, S., Park, J., Kwon, H. and Lee, S. **2013**. Half assisted hatching significantly raise implantation rate in comparison with quarter assisted hatching. *Fertility and Sterility,* **100** (3), 296-297.

Cohen J., Alikani M., Trowbridge, J. and Rosenwacks Z. **1992**. Implantation enhancement by selective assisted hatching using zona drilling of human embryos with poor prognosis. *Human Reproduction,* **7**, 685-91.

Cohen, J. and Feldberg, D. **1991**. Effects of the size and number of zona pellucida openings on hatching and trophoblastic outgrowth in the mouse embryo. *Molecular Reproduction and Development*, **30**, 70-78.

Cohen, J., Elsner, C. and Kort, H. **1990**. Impairment of hatching process following IVF in the human and improvement of implantation by assisted hatching using micromanipulation. *Human Reproduction,* **5**, 7-13.

Das, S., Blake, D., Farquhar, C. and Seif, M.M.W. **2009**. Assisted hatching on assisted conception (IVF and ICSI). *Cochrane Database Systematic Review*. CD001894.

De Vos, A. and Van Steirteghem, A. **2000**. Zona hardening, zona drilling and assisted hatching: new achievements in assisted reproduction. *Human reproduction* **166** (2), 220-227.

De wit, A.A., Wurth, Y.A. and kruip, T.A. **2000**. Effect of ovarian phase and follicle quality on morphology and developmental capacity of the bovine cumulus-oocytes complex. *Journal Animal Science*, **78**, 1277-1283.

Debra, A., Gook, D.H., Edgar, J. and Borg, M. **2008**. Martic Detection of zona pellucida proteins during human folliculogenesis. *Human Reproduction,* **23** (2), 394-402.

Debrock, S., Spiessens, C., Peeraer, K., De Loecker, P., Willemen, D. and D'Hooghe, T.M. **2009**. Higher implantation rate using modified quarter laser-assisted zona thinning in repeate implantation failure. *Gynecologic and Obstetric Investigation,* **76**,127-133.

Downs, S.M. and Hudson, E.D. **2000**. Energy substrates and the completion of spontaneous meiotic maturation. *Zygote*, **8**, 339-351.

Downs, S.M. and Verhoeven. **2003**. Glutamine and the maintenace of meiotic arrest in mouse oocytes: influence of culture medium, glucose and cumulus cells. *Molecular Reproduction and Development*, **66**, 90-97.

Dressman, H.K., Muramoto, G.G., Chao. N.J, Meadows, S., Marshall, D., Ginsburg, G.S., Nevins, J.R and Chute, J.P. **2007**. Gene expression signatures that predict radiation exposure in mice and humans. PLOS Medicine, **4**

Elhelw, B.A., El Sadek, M.M. and El Nomrosy, K.M. **2004**. Assisted hatching: routine or selective application in IVF*. Middle East Fertility Society Journal*. **9** (3), 198-201.

Erdal Aktan, M.D., Aygul Demirol, M.D., Kaan Bozkurt, M.D., Dilek Ozer, M.D. and Timur Gurgan, M.D. **2006**. The effect of zona thinning size on implantation and pregnancy rates of ICSI-ET patients with advanced women age. Journal Middle East Fertility Society, **11** (3), 191-195.

Fabres, A., De Andrade, C., Guizzo, M., Sorgine, M., de O. Paiva-silva, G., Masuda, A., Da Silva Vaz, I and Logullo, C. **2010**. Effect of GSK-3 activity, enzymatic inhibition and gene silencing by RNAi on tick oviposition and egg hatching. *Parasitology,* **137** (10), 1537- 1546.

Feng, H., Hershlag, A., Scholl, G. and Cohen, M. **2009**. A retroprospective study comparing three different assisted hatching techniques. *Fertility and Sterility,* **91** (4), 1323-1325.

Fujita, T., Umeki, H., and Shimura, H. **2006**. Effect of group culture and embryo-culture conditioned medium on development of bovine embryos. *Journal Reproductive Development*, **52**, 137-142.

Gabrielsen, A., Agerholm, I.B. Toft, B., Hald, F., Petersen, K. and Aagaard, J. **2004**. Assisted hatching improves implantation rates on cryopreserved-thawed embryos. *A randomized prospective study. Human Reproduction*, **19** (10), 2258-2262.

Germond, M., Nocera, D., Senn, A., Rink, K., Delacnetaz, G. and Fakan, S. **1995**. Microdissection of mouse and human zona pellucida using a 1.48 Am diode laser beam: efficacy and safety of the procedure. *Fertility and Sterility,* **64**, 604-611.

Hammadeh, M.E., Fischer-Hammadeh, C. and Ali, K.R. **2011.** Assisted hatching in assisted reproduction: a state of the art. *Journal of Assisted Reproductive Genetics*. **28**,119-128.

Heiden, S. **2005.** *Germ cell protocol volume.* 1,170-175.

Hellebaut, S., De Sutter, P., Dozortsev, D., Onghena, A., Qian, C. and Dhont, M. **1996**. Does assisted hatching improve implantation rates after *in vitro* fertilization or intracytoplasmic sperm injection in all patients. A prospective randomized study. *Journal of Assisted Reproduction Genetics,* **13**, 19-22.

Hirotoshi, M., Hidehiko, M., Noriko, F., Junko, M., Azusa, K. and Tatsuhiro, T. **2010**. Relevance of the siteof assisted hatching in thawed human blastocysts: a preliminary report. *Fertility and Sterility*, In Press, Corrected Proof.

Hoelker, M., Rings, F., and Lund, Q. **2010**. Effect of embryo density on in vitro developmental characteristics of bovine pre implantative embryos with respect to micro and macro environments. *Reproductive Domestic Animals*, **45**, e138–145.

Hsieh, Y.Y., Huang, C.C., Cheng, T.C, Chang, C.C., Tsai, H.D. and Lee, M.S. **2002**. Laser assisted hatching of embryos is better than the chemical method for enhancing the pregnancy rate in women with advanced age. Fertility and Sterility, **78**, 179-182.

Izquierdo-Rico, M.J., Gimeno, L., Jiménez-Cervantes, C., Ballesta, C.J. and Avilés, M. **2011**. Biosynthesis of hamster zona pellucida is restricted to the oocyte. *Theriogenology,* **75** (3), 463-472.

Kato, Y. and Tsunoda, Y. **1994**. Effects of the culture density of mouse zygotes on the development *in vitro* and *in vivo*. *Theriogenology,* **41**, 1315-1322.

Kharche, S.D., Goel, A.K., Jindal, S.K. and Sinha, N.K. **2006**. *In vitro* maturation of caprine oocytes in different concentrations of estrous goat serum. *Small Ruminants Research*, **64**,186-189.

Kubota, T., Yoshimura, Y. and Sakumoto, T. **2014**. Validity for assisted hatching on pregnancy rate in assisted reproductive technology: Analysis based on results of Japan Assisted Reproductive Technology Registry System 2010. *Journal Obstetric Gynaecology Research,* **40** (6), 1653-1660.

Lane, M. and Gardner, D.K. **1997**. Effect of incubation volume and embryo density on the development and viability of mouse embryos *in vitro*. *Human Reproduction,* **7**, 558-562.

Lanzendorf, S.E., Ratts V.S., Moley, K.H., Goldstein, J.S, Dahan, M.D. and Odem, R.R. **2007**. A randomized, prospective study comparing laser-assisted hatching and assisted hatching using acidified medium. *Fertility and Sterility,* **87**, 1450-1457.

Loret, D.M., Ricardo, J., Garside, W.T., Bucchi, J., Tureck, R.W and Heynier, S. **1997**. Analysis of human zona pellucida during culture: correlation with diagnosis and the preovulatory hormonal environment. *Journal of Assisted Reproduction and Genetics,* **14** (6), 332-336.

Loutradis, D., John, D. and Kiessling, A.A. **1987**. *Hypoxanthine causes a 2-cell block in random bred mouse embryos*. *Biology of Reproduction,* **37**, 311-316.

Makrakis, E., Angeli, I., Agapitou, K., Pappas, K., Dafereras, A. and Pantsos, K. **2006**. Laser versus mechanical assisted hatching: A prospective study of clinical outcomes. *Fertility and Sterility*. **86**, 1596-1600.

Mauri, A.L., Petersen, C.G. and Baruffi, R.L. **2001**. A prospective, randomized comparison of two commercial media for ICSI and embryo culture. *Journal of Assisted Reproduction and Genetics,* **18**, 378-381.

Montag, M., Koll, B., Holmes, P. and Van Dee Ven, H. **2000.** Significance of the number of embryonic cells and the state of the zona pellucida for hatching of mouse blastocysts in vitro versus in vivo. *Biology of Reproduction*. **62**, 1738-1744.

Movaghar, B. **2012**. "Expression of e-cadherin, leukemia inhibitory factor and progesterone receptor in mouse blastocysts after ovarian stimulation," *Cell Journal*. **14** (3), 225–230.

Murakami, M., TOtoi, N.W., KOoka, A. and Tsuzuki. **2002**. *Effects of serum-free culture media on in vitro development of domestic Cat*: Embryos following *in vitro* maturation and fertilization. *Reproduction Domestic Animal,* **37**, 352-356.

Nandi, S., Raghu, H.M., Ravindranatha, B.M. and Chauhan, M.S. **2002**. Production of buffalo (bubalus bubalis) embryos *in vitro*: premises and promises. *Reproduction Domestic Animal*, **37**, 65-74.

Nedambale, T.L., Dinnyes, A. and Groen, W. **2004**. Comparison on *in vitro* fertilized bovine embryos cultured in KSOM or SOF and cryopreserved by slow freezing or vitrification. *Theriogenology,* **62**, 437-449.

Neev,J., Schiewe, M.C., sung, V.W., Kang, I.D., Berns, M.W. and Tadir,Y. **1995**. Animal investigations. Assisted hatching in mouse embryos using a noncontact Ho: YSGG laser system. *Journal of Assisted Reproduction and Genetics*, **12** (4).

Nijs, M., Vanderzwalmen, P. and Segal-Bettina, G. **1993**. A monozygotic twin pregnancy after application of zona rubbing on a frozen–thawed blastocyst. *Human Reproduction,* **8**,127-129.

Park, S., Kim, H., Choi, Y., Ahn, K., Lee, K., Yang, J., Yu, C. and Seo, B. **2014**. The effect of various assisted hatching techniques on the mouse early embryo development. *Clinical and Experiment Reproduction Medicine,* **41** (2), 68.

Perona, R. and Wassarman, P.M. **1986**. Mouse blastocysts hatch in vitro by using a trypsin-like proteinase associated with cells of mural trophectoderm. *Developmental Biology* .**114**, 42 52.

Quinn, P. **2004**. The development and impact of culture media for assisted reproductive technologies. *Fertility and Sterility,* **81**, 27-29.

Richter, K.S. **2008**. The importance of growth factors for preimplantation embryo development and in-vitro culture. *Obstetrics and Gynecology*, **20**, 292-304.

Salahuddin, S., Ookutsu, S. and Goto, K. **1995.** Effects of embryo density and co-culture of unfertilized oocytes on embryonic development of in-vitro fertilized mouse embryos. *Human Reproduction*, **10**, 2382-2385.

Sawada, H., Yamazaki, K. and Hoshi, M. **1990**. Trypsin-like hatching protease from mouse embryos: evidence for the presence in culture medium and its enzymatic properties. *Journal of Experimental Zoology,* **25**, 83-87.

Schiewe, M.C., Haze Léger, N.L., Sclimenti, C. and Balmacaan, J.P. **1995**. Physio-logical characterization of blastocyst hatching mechanisms by use of a mouse ant hatching model. *Fertility and Sterility,* **63**,288-294.

Schimmel, T., Cohen, J., Saunders, H., and Alikani, M. **2014**. Laser-assisted zona pellucida thinning facilitates hatching and may disrupt the *in vitro* hatching process: A morpho kinetic study in the mouse. *Human Reproduction,* **29** (12), 2670-2679.

Shan-Jun Dai, Chang-Long Xu, Jeffrey Wang, Ying-Pu Sun and Ri-Cheng Chian**. 2012**. Effect of culture medium volume and embryo density on early mouse embryonic development: Tracking the development of the individual embryo. Published online: Springer Science Business Media. *Journal of Assisted Reproduction and Genetics,* **29**, 617-623.s

Srisuparp, S., Strakova, Z and Fazleabas, A.T. **2001**. "The role of chorionic gonadotropin (CG) in blastocyst implantation," *Archives of Medical Research*. **32** (6), 627–634.

Strohmer, H. and Feichtinger, W. **1992**. Successful clinical application of laser for micromanipulation in an *in vitro* fertilization program. *Fertility and Sterility,* **58**,212-214.

Sung, B. P., Hye, J. K., Young, B. C., Kwang, H. A., Kee, H.L., Jung, B. Y., Chang, S. Y. and Byoung, B.S. **2014**. The effect of various assisted hatching techniques on the mouse early embryo development. *Clinical and Experimental Reproductive Medicine,* **41** (2), 68 74.

Tamilmani, G., Rao, B.S., Vegdevi R., Amarnath D., Naik, B.R., Mutharao, M. and Rao V.H. **2005**. Nuclear maturation of bovine oocytes in cultured preantral follicles. *Small Ruminants Research*, **60**,295-305.

Teruel. M.T., Catalano, R.C., Cabodevila, J.A. and Callejas, S.S. **2005**. Regulation of mouse embryo development by autocrine trophic factors. *BIOCELL*, **29** (2)183-186.

Thouas, G.A., Dominguez, F., Green, M.P., Vilella, F., Simon, C. and Gardner, D.K. **2014**. Soluble ligands and their receptors in human embryo development and implantation. *Journal of the Endocrine Society.* **36** (1), 92-130.

Thouas, G.A., Korfiatis, N.A., French, A.J., Jones, G.M. and Trounson, A.O. **2001**. Simplified techniques for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts. *Reproductive BioMedicine Online*, **3**, 25-29.

Vutyavanich, T., Saeng-Anan, U., Sirisukkasem, S. and Piromlertamorn, W. **2011**. Effect of embryo density and microdrop volume on the blastocyst development of mouse two-cell embryos, *Fertility and Sterility*, **95**, 1435-1439.

Wang, Y., Ock, S.A. and Chian, R.C. **2006**. Effect of gonadotrophin stimulation on mouse oocyte quality and subsequent embryonic development in vitro. *Reproductive Biomedicine Online*, **12**, 304-314.

Wassarman, P.M. **2008**. Zona pellucida glycoproteins. *Journal of Biological Chemistry*, **283** (36), 24285-24289.

Welch, A.J., Motamedi, M., Rastegar, S., Le Carpentier, G.L., and Jansen, D. **1991.** Laser thermal ablation. *Photochemistry and Photobiology*. **53**, 815-823.

Yoshinaga, Y. **2010**. “Research on blastocyst implantation essential factors (BIEFs),”*American Journal of Reproductive Immunology*. **63** (6), 413–424.

APPENDIX

Appendix 1. Mean squares of analysis of variance for ZP before AH, hatchability, ZP after 24 hours of *in vitro* culture and blastomeres after staining

Source of variations	DF	Zona pellucida before AH	Hatchability	Zona pellucida after culture	Blastomeres after staining
AH	4	11.4**	6257.54**	119.87**	91.02
CM	1	0.06	173.89	15.01**	406.41*
E	3	0.16	11859.90**	0.31	103.87
AH x CM	4	4.96**	591.59	10.73**	61.67
AH x E	12	0.35	1057.67	0.58	55.84
CM x E	3	0.36	215.56	0.85	62.42
AH x CM x E	12	0.21	1222.10	0.21	65.63
Error	120	0.93**	1188.66**	0.87**	95.78

DF= degree of freedom, AH= assisted hatching, CM= Culturing media, E= No. of embryos per drop, AH x E= interaction between AH techniques and no. of embryo per drop, CM * E= interaction between culturing media and no of embryos per drop, AH x CM x E= interaction among assisted hatching techniques, culturing media and no of embryos per drop, no significance difference (P>0.05), **= highly significant difference (P<0.01), *= significant difference (P<0.05) and ZP = zona pellucida

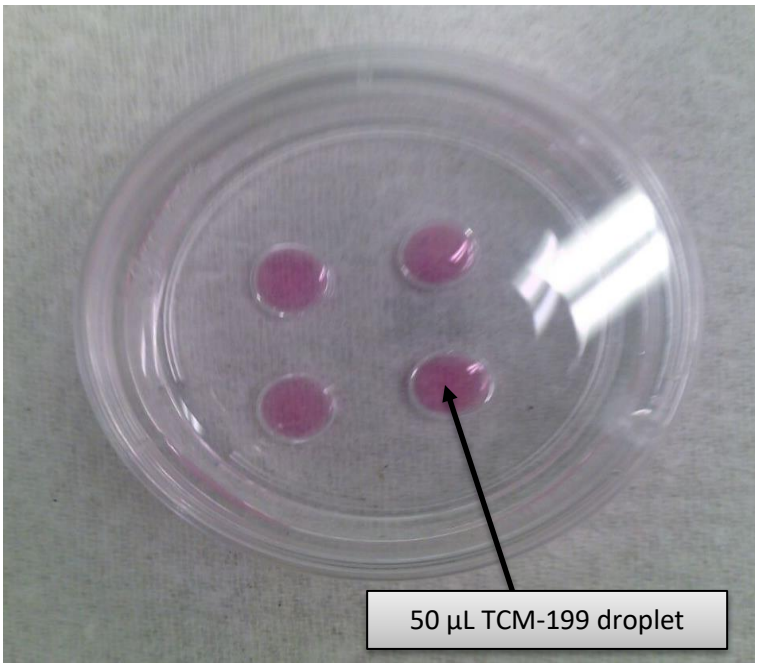

Figure 4.1 TCM-199 culture medium droplets used for culturing embryos

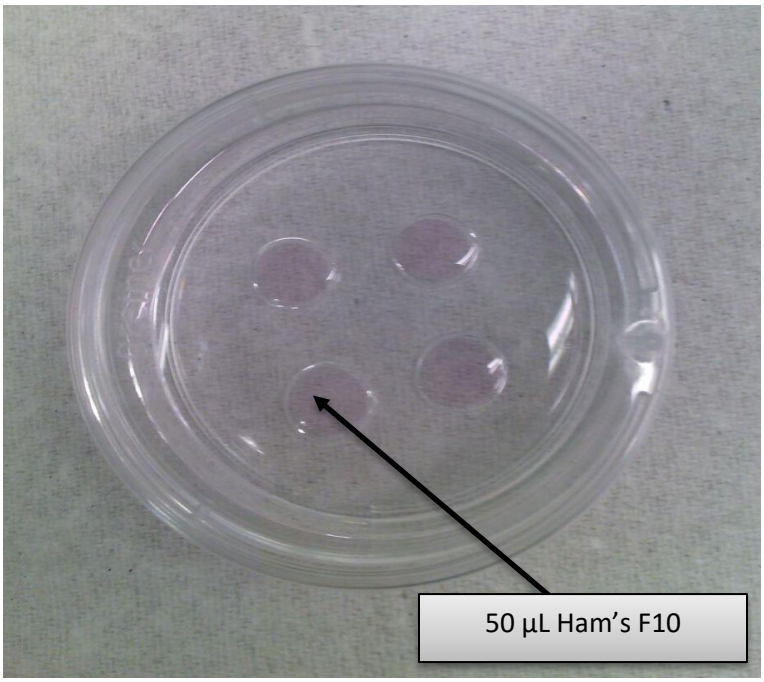

Figure 4.2 Ham's F10 culture medium droplets used for culturing of embryos

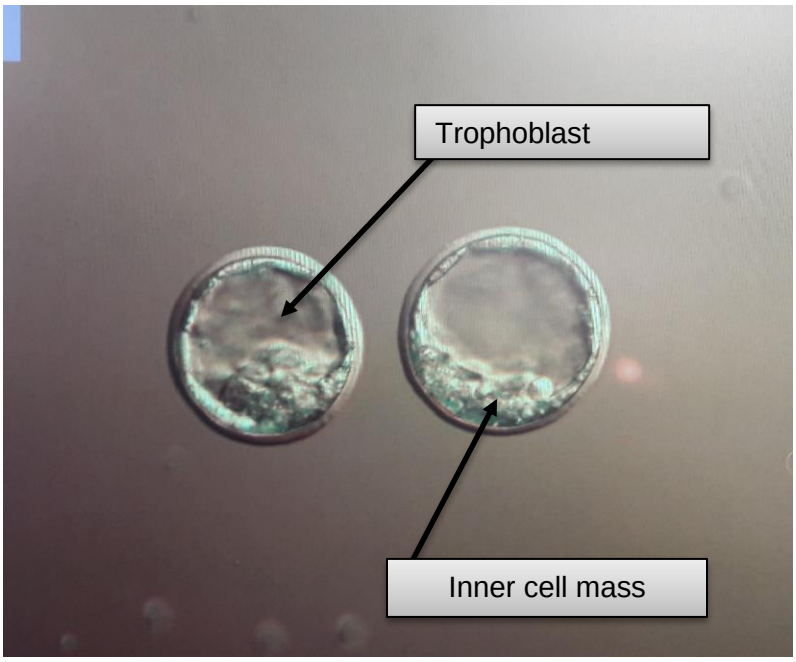

Figure 4.3 Blastocyst-staged embryos (X20 Magnification)

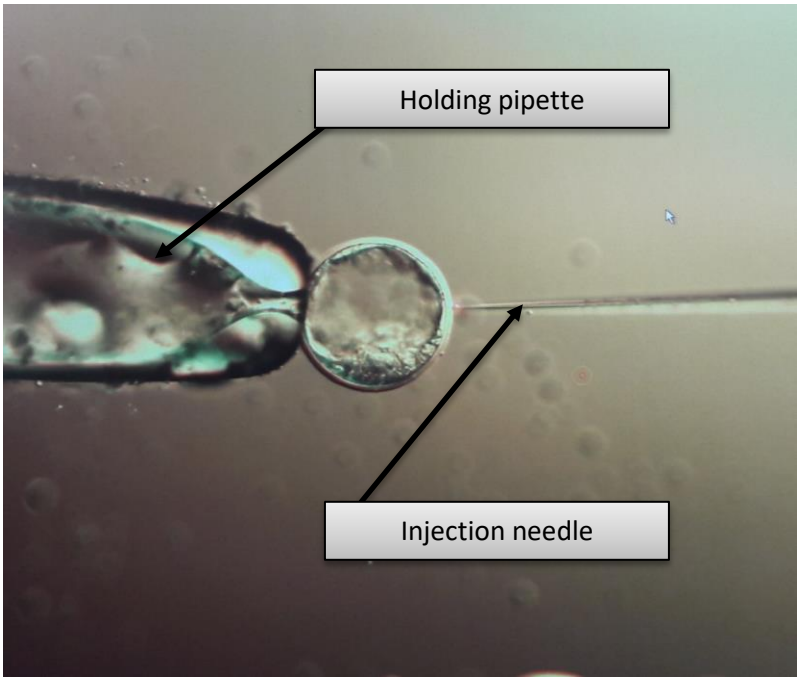

Figure 4.4 Mechanical assisted hatching technique (X20 Magnification)

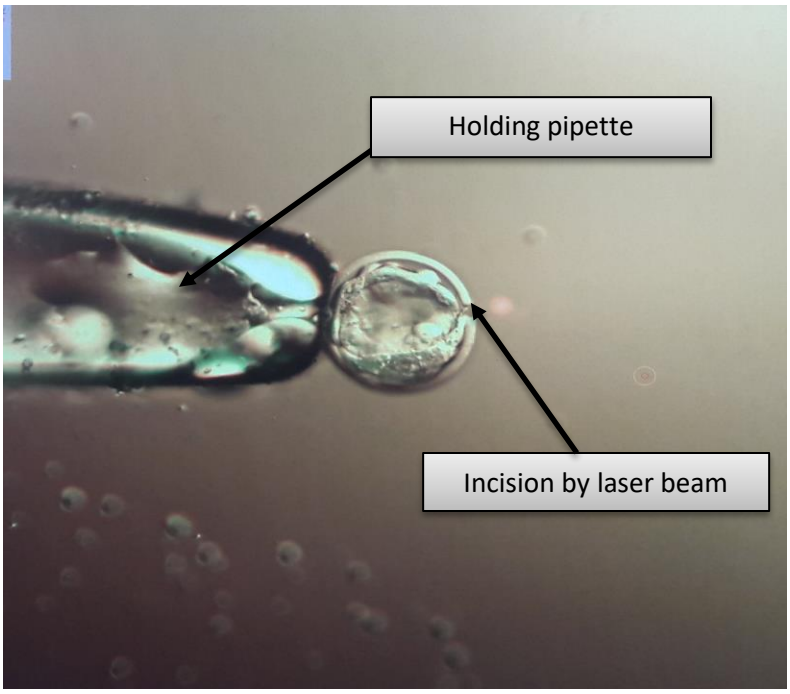

Figure 4.5 Laser assisted hatching technique (X40 Magnification)

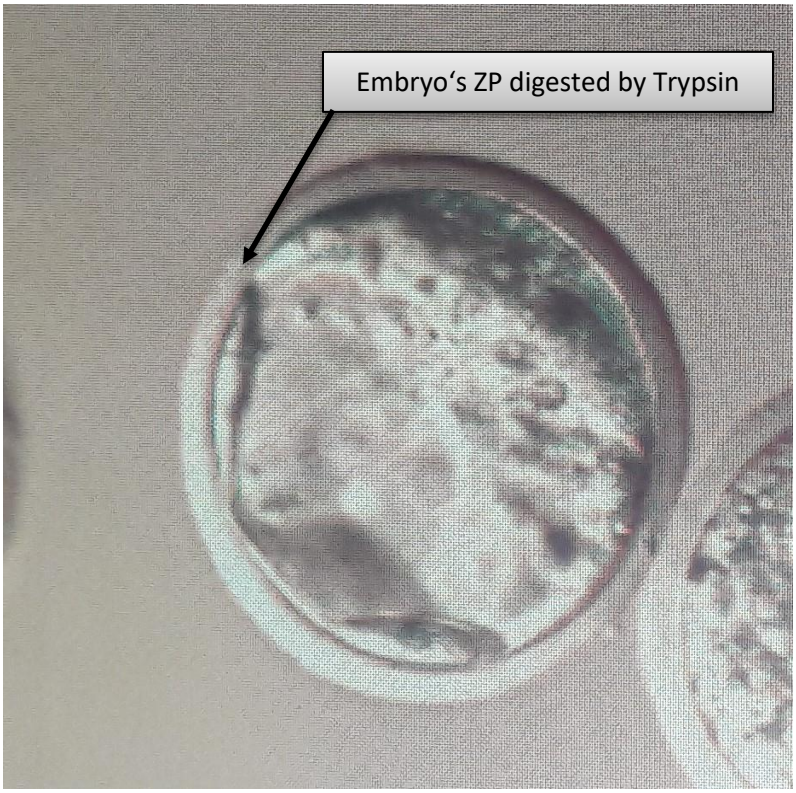

Figure 4.6 Enzymatic assisted hatching technique (X20 Magnification)

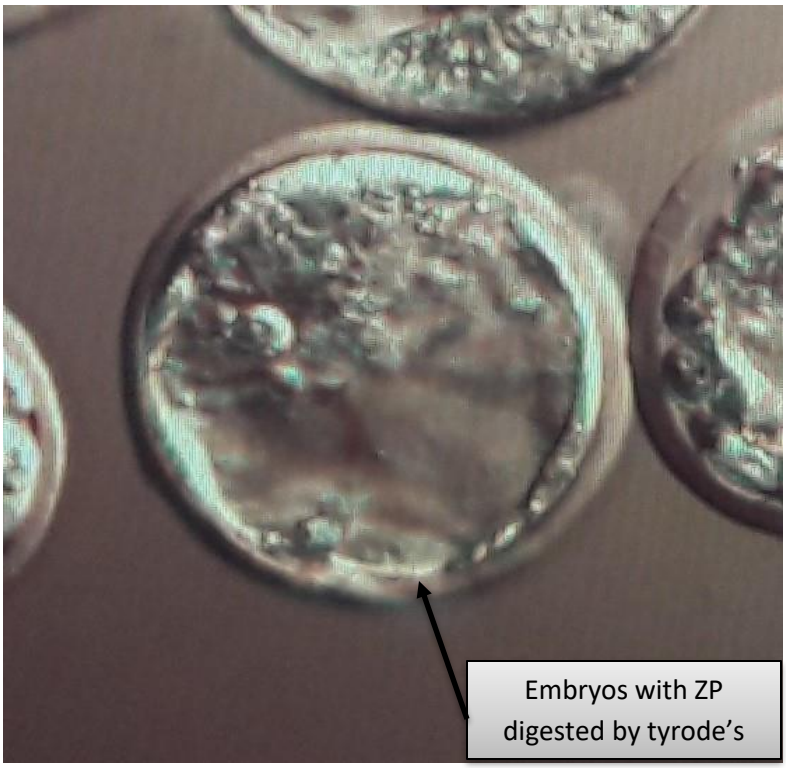

Figure 4.7 Chemical assisted hatching technique (X20 Magnification)

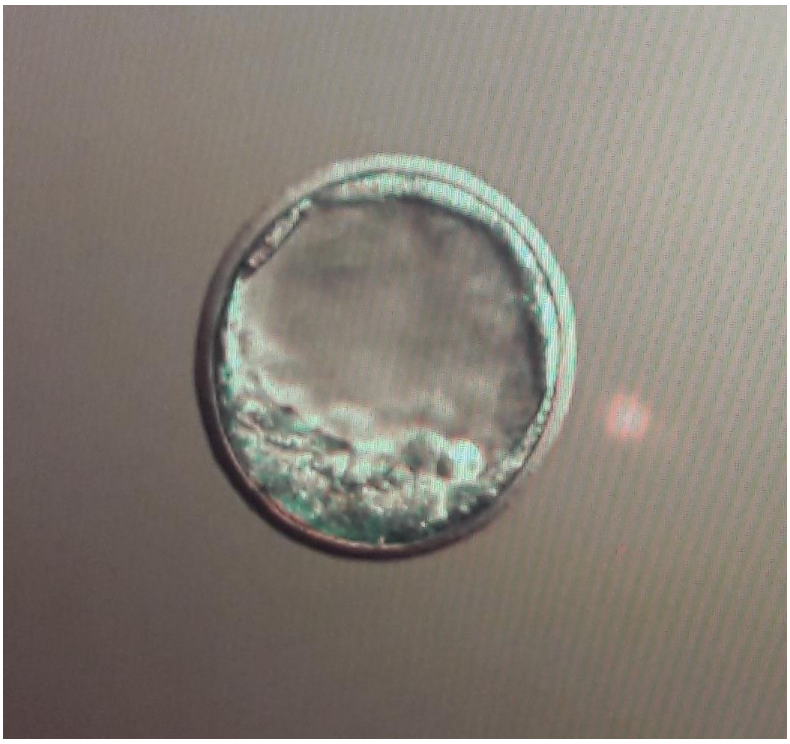

Figure 4.8 Non-assisted hatching used as the control (X20 Magnification)

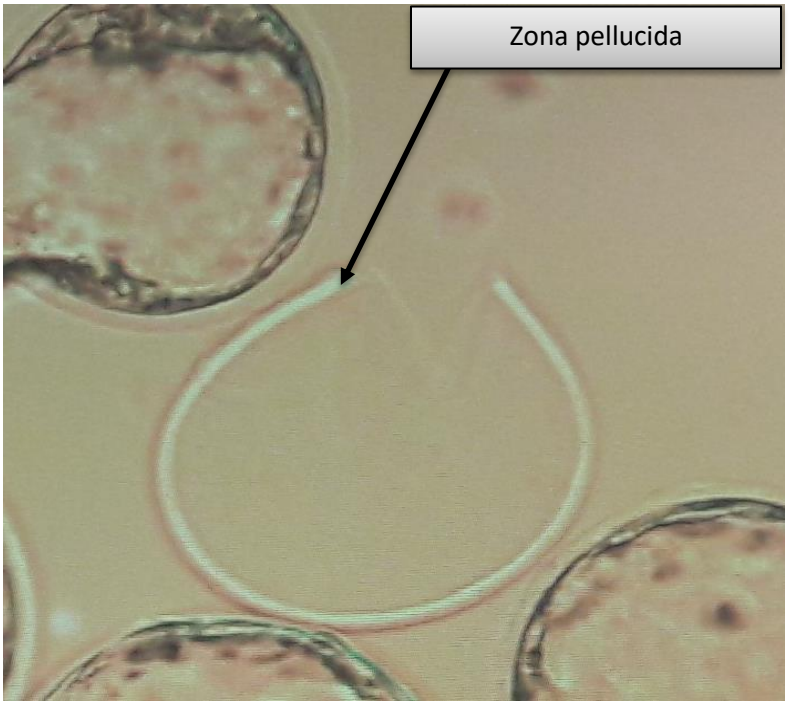

Figure 4.9 Empty zona pellucida after embryo hatching (X20 Magnification)

Figure 4.10 Hatched embryo (X20 Magnification)

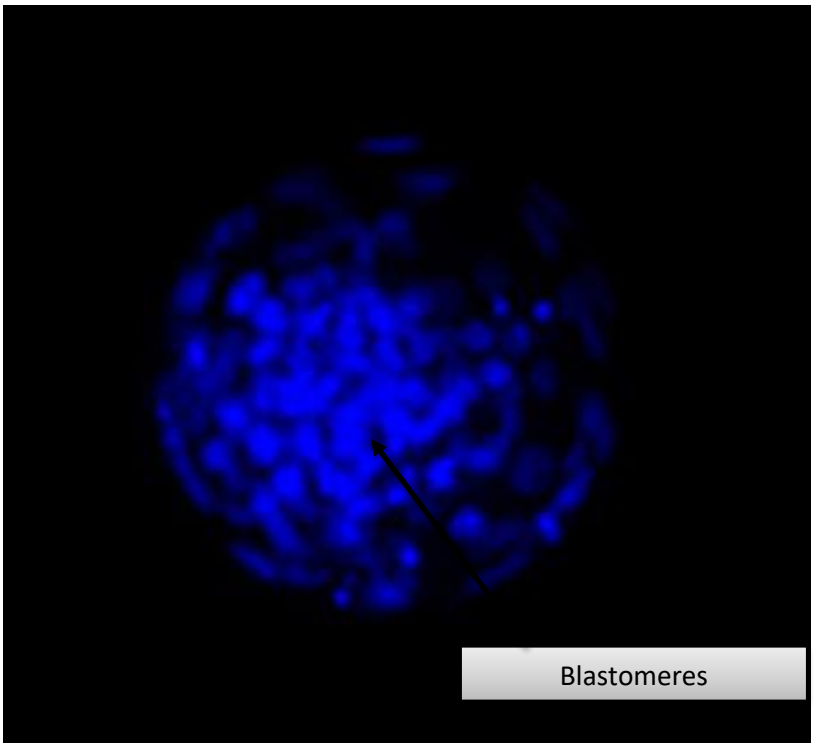

Figure 4.11 Hatched and stained embryo with 69 nuclei (X400 Magnification)