Vitrification of Day 5/6 Human Morulas/Blastocysts: A 10 year Retrospective Study in a Private Assisted ReproductiveTechniques [ART] Clinic

By Emma Wilson Poe

Thesis presented in partial fulfillment of the requirements for the Degree of Master of Science, at Stellenbosch University

Supervisor: Dr ML Windt De Beer

March 2015

Declaration

By submitting this thesis/dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

March 2015

Abstract

This study was designed to retrospectively evaluate the established embryo vitrification/warming programme currently implemented at Drs Aevitas Institute for Reproductive Medicine and to look at factors that might play a role in optimizing the pregnancy outcomes thereof.

Vitrification is the achievement of a "state of suspended animation" wherein molecular translational motions are arrested without structural reorganization of the liquid. In embryo vitrification it involves placement of the embryo in a very small volume of vitrification medium that must be cooled at extremely high cooling rates. The vitrification medium contains cryoprotectants to prevent any cryoinjury from occurring to the embryo.

This process was initially proposed to effectively manage supernumerary embryos, but it has also provided a viable method of reducing costs for additional embryo transfers as well as the reduction of the incidence of multiple births. Patients who are at risk of ovarian hyper stimulation syndrome (OHSS) can also have all of their embryos vitrified in advance to reduce the likelihood of adverse clinical symptoms if a pregnancy is established.

Throughout the period in which vitrification has been in practice, there have been advances in technology as well as continual research being conducted to establish whether newly suggested techniques do, in fact, optimize the outcomes of vitrification. Focus has subsequently been applied to the carrier device used for vitrification, the day on which the embryos are vitrified and stored, as well as the number of embryos transferred in each respective cycle, all to ensure the most favourable outcome.

This retrospective study confirmed the use of the Cryotop® as the most viable carrier device for successful survival and pregnancy outcomes. Transfer of day 5 vitrified embryos resulted in significantly higher pregnancy rates compared to day 6 vitrified embryos. Results also indicated that the number of embryos transferred does indeed have a significant effect on the pregnancy outcome and consequently we can possibly argue against the implementation of single embryo transfer in the vitrification programme. Investigation into the effect of female age, specifically oocyte age, on each of these categories indicated that reduced age can be associated with optimal outcomes; however this could not be proven statistically in this cohort of patients.

To further look at optimization of the vitrification/warming programme, a Literature Survey was conducted to ascertain the results after Assisted Hatching in frozen/warmed human embryos. Assisted Hatching has been proposed as a solution to Zona Pellucida hardening, which has been found to occur during vitrification. The need for further studies and a meta-analysis of the literature is confidently proposed, as well as a Prospective Study to evaluate the effect of Laser Assisted Hatching in the human blastocyst vitrification/warming programme at Drs Aevitas Institute for Reproductive Medicine.

Opsomming

Hierdie studie is ontwerp om die gevestigde embrio vitrifikasie/ontdooi program by Drs Aevitas Instituut vir Reproduktiewe Medisyne, retrospektief te evalueer en die faktore te optimaliseer wat swangerskap uitkomste kan beïnvloed.

Vitrifikasie is die proses waardeur die molekulere aktiwiteit binne die embrio in ‘n staat van arres gehou word sonder om die strukture binne die sitplasma te versteur. Dit behels die plasing van ’n embrio in 'n klein hoeveelheid vitrifikasie medium wat teen 'n hoë tempo afgekoel word. Die vitrifikasie medium bevat kriobeskermmiddels wat die embrio tydens die vitrifikasie proses teen moontlike skade beskerm.

Hierdie proses is aanvanklik voorgestel om oortollige embrio’s doeltreffend te bestuur. Dit bied ’n koste effektiewe metode vir embrio terugplasing, en verlaag die insidensie van veelvoudige swangerskap. Vitrifikasie bied pasiënte met ’n hoë risiko vir ovariale hiperstimulasiesindroom (OHSS) ‘n alternatief om nadelige kliniese simptome te vermy indien ’n swangerskap bereik word.

Tegnologiese vordering en voortdurende navorsing ondersoek voortdurend nuwe tegnieke vitrifikasie uitkomste te optimaliseer. Fokus word geplaas op die draertoestel wat gebruik word vir vitrifikasie, die dag waarop die embrio's gevitrifiseer en gestoor word, sowel as die aantal embrio’s wat met elke vitrifikasie siklus teruggeplaas word.

Hierdie retrospektiewe studie het bevestig dat die gebruik van die Cryotop® die mees suksesvolle toestel vir oorlewing en swangerskap uitkomste is. Die terugplasing van dag 5 gevitrifiseerde embrios het beduidende hoër swangerskapsyfers as dag 6 embrios tot gevolg gehad. Die resultate het ook aangedui dat die aantal embrio's wat teruggeplaas word 'n beduidende uitwerking op die swangerskapsyfer het. Daar kan dus moontlik teen die implementering van 'n enkel embrio-terugplasing neiging in die vitrifikasie program geargumenteer word. Resultate het ook getoon dat optimale uitkomste verwant is aan ’n laer oösiet ouderdom, alhoewel dit nie in die groep pasiente statisties bewys kon word nie.

'n Literatuurstudie oor AH (Assisted Hatching) op gevitrifiseerde/ontdooide menslike embrio’s is uitgevoer om die vitrifikasie/ontdooi program verder te optimaliseer. AH bied ‘n oplossing vir Zona pellucida verharding, wat tydens vitrifikasie plaasvind. Verdere studies, 'n meta-analise van die literatuur, sowel as 'n prospektiewe studie om die effek van laser AH in gevitrifiseerde/ontdooide menslike blastosiste by Drs Aevitas Instituut vir reproduktiewe medisyne te evalueer, word voorgestel.

Acknowledgements

This Master of Science Degree forms part of a two year theoretical and practical training programme to qualify as a Medical Biological Scientist in Reproductive Biology with the Health Professionals Council of South Africa [HPCSA]. Consequently, I wish to thank all the doctors, embryologists, nurses, administrative staff as well as the support staff at both *Drs Aevitas* Institute for Reproductive Medicine at *Life* Vincent Pallotti Hospital and Tygerberg Hospital's Fertility Unit, without whom this experience would not have been the remarkable journey that it was.

A very special thank you to Greg Tinney, Director of the Laboratory at *Drs Aevitas* Institute for Reproductive Medicine, who made each day of training an exciting and fun learning experience, and guided me with tremendous expertise and patience. There could be no better introduction to the world of Reproductive Science.

I wish to thank my supervisor, Doctor ML Windt De Beer, for her guidance and direction during this learning experience. She went above and beyond her duties as a supervisor on both a professional and personal level, for both of which I am extremely grateful. The submission of this thesis would not be possible without her.
I would also like to thank Prof TF Kruger for his advice and support throughout this period.

I want to thank my fellow trainee and MSc student, Estée van Zyl. Very few people are lucky enough to travel this road with the support of a true friend who also has such insight, as well as understanding, into of the field of study.

Thank you to my boyfriend, Antony Ashley, who challenged me to persevere and motivated me to achieve success.

And finally, to my parents, without whom I wouldn't be where I am today and to whom I owe everything. Thank You for your continuous support.

"The greater the obstacle, the more glory in overcoming it."

Molière

Table of Contents

List of Figures

Page

List of Tables

Page

List of Abbreviations

AH – Assisted Hatching

ART – Assisted Reproductive Technology

AS – artificial shrinkage

ASRM – American Society of Reproductive Medicine

BPD – broncho pulmonary dysplasia

CAH – chemically assisted hatching

CGH – Comparative Genome Hybridization

DMSO - dimethyl sulfoxide

DNA – Deoxyribonucleic acid

ET – embryo transfer

EDE – early dividing embryos

FET – frozen embryo transfer

FISH – Fluorescent *in Situ* Hybridization

GLY - glycerol

hCG – Human Chorionic Gonadotropin

HREC – Health Research Ethics Committee

ICM – inner cell mass

IMSI – intracytoplasmic morphologically selected sperm injection

IVF – in vitro fertilization

IVH – intraventricular haemmorage

ICSI – intracytoplasmic sperm injection

LAH – laser assisted hatching

LDE – late dividing embryos

MAH – mechanically assisted hatching

mCGH – metaphase Comparative Genomic Hybridization

OCC – oocyte-corona-cumulus complex

OHSS – Ovarian Hyper Stimulation Syndrome

PB – polar body

PB-I – polar body I

PB-II – polar body II

PB-III – polar body III

PDA - persistent ductus arteriosus

PGD – Pre-implantation Genetic Diagnosis

PGS – Pre-implantation Genetic Screening

PICSI – physiological intracytoplasmic sperm injection

PROH – propanediol

PSSC - premature separation of sister chromatids

PZD – partial zona dissection

RDS - respiratory distress syndrome

TE - trophectoderm

TLM – Time-lapse Monitoring

ZP – zona pellucida

ZP1 – zona pellucida protein 1

ZP2 - zona pellucida protein 2

ZP3 – zona pellucida protein 3

Chapter One

Literature Review

1.1. Short History of Assisted Reproductive Technology [ART]

The history of ART and embryo transfer (ET) dates back to as early as 1890. Walter Heape, a professor and physician at the University of Cambridge, England, who had been conducting research on reproduction in a number of animal species, reported the first known case of embryo transplantation in rabbits. This was long before the applications to human fertility were even suggested.

However, it was not until 1959 that indisputable evidence of in vitro fertilization (IVF) was obtained by Chang (Chang, 1959) who was the first to achieve births in a mammal (a rabbit) by IVF. The newly-ovulated oocytes were fertilized, in vitro by incubation with capacitated sperm cells in a small Carrel flask for 4 hours, thus opening the way to assisted procreation.

1978 marked the first ever human IVF birth (Louise Brown), which occurred in Oldham, England on July 25. This birth was the result of the collaborative work of Patrick Steptoe and Robert Edwards (Steptoe and Edwards, 1978). Robert Edwards received the Nobel Prize for Physiology and Medicine in 2010 for this development of IVF and ET to treat infertility in women with non-patent oviducts. (Biggers, 2005). IVF and ET involves four main aspects: i) the acquisition of viable, mature oocytes ii) the fertilization of these mature oocytes *in vitro* iii) the culture of the fertilized and thus preimplantation embryos and iv) the transfer of the embryos into the uterus of the mother (Biggers, 2012). Ultimately human IVF and ET were built upon extensive research produced by many investigators for over a century (Biggers, 2012). Robert Edwards was able to build on his earlier work and that of his colleagues, and in turn he was able to make major scientific contributions in the field of Reproductive Biology. Today there are accounts of over 4 million IVF/ET births worldwide (Biggers, 2012).

1.2. Multiple Gestations in ART

With the refinement of extended culture systems (Kader *et al*. 2009), the occurrence of full term IVF pregnancies, and thus live birth rates increased dramatically. A significant complication of ART is multiple gestations. Approximately half of all children born subsequent to ART result from a plural gestation (Nakhuda and Sauer, 2005). Furthermore, the majority of triplets and higher order births are the product of ART. The risks for multiple pregnancies vary with practice patterns and the techniques used to achieve pregnancy. A healthy child delivered at term is the ideal endpoint of treatment for an infertile patient. Despite this general acknowledgment the current standards of care favour the risk of multiple pregnancies over the risk of failing to conceive (Nakhuda and Sauer, 2005).

Multiple births are increasingly large contributors to the preterm and low birth weight population (Bryan, 2003) and always present a greater risk for mother and child (Scholz *et al*., 1999). The average duration of pregnancy is 37 weeks for twins, 33.5 for triplets and 31.5 for quads. About 40% of twins and nearly all triplets and quads are born preterm (<37 weeks). 50% of twins, 90% of triplets and nearly all quads have a birth weight of less than 2500g. Half of the quadruplets weigh less than 1500g compared to a quarter of triplets, 1 in 10 twins and 1 in a 100 singletons (Macfarlane *et al*. 1990). Several studies have found that IVF twin infants have a still greater tendency to prematurity and low birth weight than those that are naturally conceived (Lambalk *et al*., 2001).

Levene showed in 1991 that due to prematurity and low birth rates, mortality and morbidity rates are much higher in triplets than in singletons. Scholz *et* al. (1999) reported that postnatal problems result from multiple birth (29%) and prematurity (23%) and include: respiratory distress syndrome (RDS), persistent ductus arteriosus (PDA), bronchopulmonary dysplasia (BPD), septicaemia, intraventricular haemorrhage (IVH) and convulsions (Wilcox *et al*., 1996). Infants also later show developmental delay, cerebral palsy and visual and hearing impairment (Gonen *et al*., 1990).

There is also the social, psychological and financial burden of multiple births. Unexpected multiple births directly affect the available space and finances of the family and there are obligations to meet the needs of all the children, which results in lack of sleep and higher stress levels (Scholz *et al*., 1999). The risk of depression has been shown to increase with the number of multiple births (Thorpe *et al*., 1991) and the prognosis for both parents and children is often disappointing (Scholz *et al*., 1999).

Given the personal and societal costs associated with the complications of multiple births, practice patterns of reproductive endocrinologists have public health implications (Nakhuda and Sauer, 2005). Techniques to reduce or eliminate the incidence of multiple gestations needed to be considered. Although techniques such as embryo freezing have been suggested, efforts to implement them into practice met resistance (Nakhuda and Sauer, 2005).

1.3. Embryo Selection Methods – A solution to multiple gestations in ART

Identifying the embryos with optimized implantation competence for transfer, and those that have the highest probability of developing into a live baby, has been an issue of debate and continuous research. Embryo selection can take place throughout the development process.

1.3.1. Embryo Development

Insemination of the oocyte with the sperm cell is considered to be day 0. Fertilization is assessed on day 1, which is usually checked between 16-18 hours after the sperm cell is added to the oocyte. Normal and successful fertilization is evident if two pronuclei and two polar bodies can be seen. The pronuclei contain small nucleoli, which contain the genetic information from each respective parent. The pronuclei will eventually disappear with the union of two gametes, which is known as syngamy. The zygote is now on its way to becoming an embryo. Approximately 48 hours after the ova were first inseminated, the resulting embryos will usually have reached the 4 cell stage and after 72 hours the 8 cell stage. If the patient will be having pre-implantation diagnosis to test the genetic makeup of the embryo, the biopsy (removing a cell from the embryo to test) will usually be performed on this day.

The embryo continues to divide, and there is increased cellular adhesion until the distinction between the blastomeres becomes difficult to see. The embryo at this stage is called a morula or a compact embryo and can be seen on day 4.

The morula will continue to develop and start to cavitate. The embryo begins to turn into a hollow ball of cells known as a blastocyst. A blastocyst is achieved 5 or 6 days after fertilization has taken place. The blastocyst consists of two main parts; an area denser with cells called the inner cell mass (ICM) which will eventually form the foetus, and the trophectoderm which lines the cell membrane and will go on to become the placenta.

1.3.2. Embryo Selection Methods

Implantation failure can be as a result of poor embryo quality and transfer techniques, endouterine abnormalities as well as immunologic dysfunction. Identifying the embryos with optimized implantation competence for transfer, and those that have the highest probability of developing into a live baby, has been an issue of debate and continuous research. Both invasive and non-invasive techniques have been established to facilitate a more effective selection process in order to improve transfer results.

Non-invasive techniques include gamete/embryo grading and morphology, analysis of biochemical markers in culture media and culture to the blastocyst stage for selection. Earlier studies reported that morphological evaluations will furnish clues that enhance the ability to identify the best embryos for transfer (Shoukir *et al.*, 1997) and pronuclear morphology has now been indicated as a method of selection for transfer (Scott *et al.*, 2013). However, all these methods have significant limitations when it comes to predicting the likelihood of successful implantation and live births.

Research also shows that fast cleaving embryos resulted in a higher degree of developmental competence than slower ones (Windt *et al., 2004;* Kotze *et al*., 2013). Fast cleaving or early cleavage, is defined as the timing at which the first mitotic division, post fertilization takes place (Shoukir *et al., 1997*). This has been extensively studied to be used as additional criteria for embryo selection prior to transfer, as well as to predict embryo development potential and improved implantation/pregnancy rates (Kotze *et al*., 2013). Windt *et al*. (2004) performed a retrospective study to assess the effect that early dividing embryos (EDE) had on clinical pregnancy outcomes when compared to embryo transfers using late dividing embryos (LDE). It became evident that EDE used for embryo transfer showed increased ongoing and clinical pregnancy rates than those using LDE. This result was also replicated in the case of live births. Statistical evaluations also considered the number of embryos transferred and subsequently found EDE to be significantly favourable. Edwards and Beard (1999) also commented on the importance of the first cleavage and suggested that early selection criteria up to and including the first division may have advantages (Windt *et al*., 2004).

Daily embryo scoring is used as another morphological evaluation. Many scoring methods have been published and cleavage stage morphological assessment is based mainly on blastomere number relative to time and the degree of fragmentation (Veeck, 2003). Blastomeres often divide evenly, although asynchronously, so it is not uncommon for embryos to have an odd number of cells and uneven blastomeres. The embryo is capable of repairing this itself. We can also see various indicators of poorer quality in the embryo at this stage; such as fragments or multinucleation of blastomeres.

Although many selection methods are promising, grading systems based on morphology remain the preferred way of assessing embryonic competence (Kirkegaard *et al*., 2012). The result of embryo scoring can change markedly within just a few hours (Montag *et al*., 2011) thus limiting the evaluation of a dynamic process by a few snap shots at discrete time points (Kirkegaard *et al*., 2012). The embryo scoring also relies on analysis outside of the incubator, which results in undesirable changes in temperature, humidity and gas composition (Zhang *et al*., 2010). More recently, Time-lapse monitoring (TLM) has been developed as a way of overcoming these obstacles. It involves cameras that are incorporated into the incubators and provides a promising clinical method of extending and refining morphological assessments that capture dynamic parameters (Kirkegaard *et al*., 2012). TLM subsequently has the ability to maintain samples in an optimal culture environment during the entire time of data acquisition (Wong *et al*., 2013). This novel, non-invasive technique ultimately increases the precision and sensitivity (Kirkegaard *et al*., 2012) of the favourable morphological evaluation technique.

Invasive techniques, however, provide information about the chromosomal status of the embryo or specifically the ploidy (Kotze *et al*., 2012).

Pre-implantation Genetic Diagnosis (PGD) was introduced in 1990 and involved using Fluorescent *in Situ* Hybridization (FISH) to identify aneuploidy and known single gene disorders in order to prevent their transmission. A few years later Kallioniemi *et al*. (1992) developed comparative genome hybridization (CGH) to screen the whole genome's Deoxyribonucleic acid (DNA) in tumours. This technique was modified to study the DNA of single cells like blastomeres (Kotze *et al*., 2013).

Evidence shows that embryonic aneuploidy screening can improve clinical outcomes. Biopsy for PGD can be done at one or more of four possible developmental stages: (1) first polar body (PB) from oocyte (2) second PB from pronuclear embryo (3) blastomere biopsy obtained at cleavage stage or (4) trophectoderm biopsy at the blastocyst stage (Kotze *et al*., 2013). It also needs to be considered whether screening is being done at the correct time and thus whether the abnormalities are being accurately identified, or whether it is too early to target the required genes. Subsequently there are queries as to whether the abnormalities identified correctly reflect a corresponding abnormality in the embryo or whether the embryo still has the ability to self-correct; whether the biopsy can be carried out with enough time to select for the most viable embryo and finally; whether the biopsy itself can compromise the embryo quality.

The polar bodies are obtained early in the process and therefore allow more time for analysis. Evidence shows that 10% of PB abnormalities are as a result of nondysjunction and 90% are attributed to premature separation of sister chromatids (PSSC). These abnormalities will ultimately self-correct. PB screening indicates that 55% of embryos are anueploid. However, this means that 45% of this is due to PSSC and therefore provides suboptimal levels of diagnostic precision. PB biopsy also fails to identify approximately 40% of genetic errors (Scott *et al*., 2013).

The safety of PB biopsy requires more evaluation. It is considered to be less invasive as it does not require removal of any portion of the actual embryo, even though it may play a role in its early organization. However, a recent study by Levin *et al*. (2012) showed that embryos that had been biopsied had more fragmentation and were less cellular but the critical end

point of implantation was not addressed and previous studies showed no adverse effect on implantation rates.

Embryo biopsy is definitely more invasive than PB biopsy. At this stage, however, meiosis has been completed and both maternal and paternal errors can be detected, which overcomes some of the limitations of PB biopsy. In most circumstances a single blastomere biopsy is conducted and thus the detection of mosaicism is highly variable. It is assumed that the biopsy is an accurate representation of the entire embryo; however mosaicism has been shown to impact as much as 29% of all embryos. These embryos seldom implant, however it is one of the primary reasons why women who conceive with anueploid identified embryos should undergo antenatal aneuploidy screening (Levin *et al*., 2012).

Biopsy is either conducted at the cleavage stage (day 3) of *in vitro* culture or at the blastocyst stage (day 5 or day 6). Day 3 biopsy still allows for a fresh transfer while only clinics with "in house" PGD laboratories will be able to continue with a fresh cycle following blastocyst biopsy. Both fresh cycle and vitrification cycles have shown to yield similar implantation and pregnancy rates. There is no clinically significant research to show that mosaicism is more or less prevalent during cleavage or blastocyst stage and is therefore a nonfactor in determining the optimal time for biopsy (Scott *et al*., 2013). The potential for self-correction at the embryo level should not influence the decision about the optimal time to biopsy.

Embryos biopsied at the cleavage stage sustained implantation rates of 30% in comparison to 50% in non-biopsied embryos. This indicates sufficient harm rendering them incapable of implanting and progressing to term. A similar evaluation showed equivalent results for trophectoderm biopsy at blastocyst stage, however is it important to note that the only randomized controlled trials to ever demonstrate a beneficial effect both used trophectoderm biopsy. As of present, cleavage stage biopsy can be considered harmful while trophectoderm biopsy appears to be safe (Scott *et al*., 2013).

Trophectoderm biopsies also contain multiple cells with an increased number of DNA copies for evaluation. This may help in reducing the nonresult screening rate and it is thus recommended that PGD biopsies be conducted through trophectoderm biopsy at the blastocyst stage.

Previous invasive methods such as FISH showed that greater than 50% of embryos considered for transfer were in fact aneuploid. FISH however is limited by the amount of fluorochromes available and therefore only 12 of the 23 sets of chromosomes can be analysed at once. Problems such as mosaicism in day 3 blastomeres is also significant (Kotze *et al*., 2013).

Comparative Genomic Hybridization (CGH), however, allows for the entire karyotype to be analysed simultaneously. Polyploidy and balanced translocations cannot, however, be detected reliably and the hybridization process initially took up to 72 hours, which meant that embryos analysed for PB-I and PB-II ploidy could still undergo fresh transfers, whereas embryos biopsied on day 3 needed to be vitrified and transferred at a later stage (Kotze *et al*., 2013).

Potentially detrimental effects of cryopreserving Pre-implantation Genetic Screening (PGS) embryos have been suggested by Zheng *et al*. (2005). Researchers reported a 30%-40% reduction in their implantation potential. However, the recent the introduction of blastocyst vitrification (ultra-rapid freezing) has significantly improved the gamete/embryo cryopreservation process and consequently the survival of warmed embryos. Sher *et al*. (2007) and Kotze *et al*. (2012) reported more than 50% live birth rates after the transfer of PGS vitrified/warmed blastocysts.

The time taken to perform CGH required that biopsied blastocysts be cryopreserved and held until the results of CGH testing are available. Lately, a more rapid and detailed technology has been developed, namely CGH Array, allowing for the analysis of PB-I, PB-II, day 3 blastomeres and trophectoderm cells (Hu *et al*., 2004; Wells *et al*., 2004; Le Caignec *et al*., 2006; Treff *et al*., 2010) and also sebsequently avoiding the need for embryo cryopreservation.

Metaphase CGH, or rather mCGH, uses this array technique, however it requires a far less amount of DNA, and can therefore effectively evaluate PBs.

An article by Kotze *et al*. (2012) focuses on the significance of using oocyte/embryo ploidy status to achieve implantation and subsequent pregnancy rates. They therefore aimed to correlate first and second polar body ploidy, as well as day 3 blastomere ploidy and embryo morphology, with blastocyst development and *in vitro* pregnancy outcome. 57 oocytes from donors were analysed with the majority of which being aneuploid for PB-I, PB-II and day 3 blastomere biopsies. 54.5% of the aneuploid embryos originated from the best graded group of embryos. Using mCGH they found a significant association between the CGH results of PB-I and PB-II respectively therefore indicating that each phase provides nearly the same information regarding their ploidy status. This significant association was also evident

between the PB-I and blastomere biopsies as well as with the PB-II and blastomere biopsies, however, there was no association with subsequent blastocyst development with 62% of aneupoloid embryos reaching blastocyst stage. They therefore suggested that an embryo's morphology cannot be correlated with its ploidy prior to transfer and the best way to select euploid embryos for transfer would be a combination of both morphologic and genetic testing.

An interesting study conducted by Mertzanidou *et al*. (2013) showed that around 70% of good-quality embryos carry chromosomal abnormalities, including structural aberrations. In this study they analysed the majority of the blastomeres from top-quality embryos that originated from a cohort of embryos showing normal developmental rates and high implantation potential.

With the development of extended embryo culture to the blastocyst stage there has also been much debate surrounding transfer at the cleavage or blastocyst stage. Both clinical pregnancy and live birth rates appear to be significantly higher in the latter group (Papanikolaou, 2008).

We can conclude that a combination of both invasive and non-invasive techniques, such as those afore mentioned, as well as using biological markers as indicators will aid in selecting for the most optimal embryo for transfer.

1.4. Blastocyst Culture and Development of Sequential Culture Mediums

Optimized blastocyst culture and transfer has also been shown to be a solution for multiple gestations. This is a result of being able to selectively choose, with greater integrity, the most viable embryo for transfer.

Despite the fact that the first IVF pregnancy ever reported was from the transfer of a blastocyst (Edwards and Brody, 1995), the transfer of cleavage stage embryos dominated IVF for decades and resulted in implantation rates between 5% and 30% (Gardner *et* al., 1998). This was mainly due to difficulties in successfully culturing embryos to the blastocyst stage as the culture media used were not complex and did not completely support normal blastocyst development. In the early 1990s, knowledge of the metabolic requirements of the developing embryo increased and both co-culture techniques and sequential media were introduced (Hardarson *et al*., 2012; Gardner and Lane, 1998) and resulted in refined culture of embryos to the blastocyst stage. Implantation rates attained with the culture and transfer of human blastocysts are higher than those associated with the transfer of cleavage stage embryos to the uterus (Gardner *et al*., 2002; Papanikolaou, 2008). Such increases in implantation rates have facilitated the establishment of high pregnancy rates while reducing the number of embryos transferred (Gardner *et al*., 2002).

Culture media aims to create an *in vitro* environment for the embryo which mimics that of the *in vivo* environment. The metabolic requirements of the embryo changes during development and Biggers et al. (2005) stated that the idea that sequential media is required for optimal embryo development therefore has an intuitive appeal. It is, however, not supported by direct

experimental evidence. It has, however, been suggested that a sequential media system is in fact used for embryo development to the blastocyst stage (Gardner and Lane, 1997). The composition of the culture media used for embryo culture from day 1 to day 3 of development will differ from that of the culture media used for subsequent culture to the blastocyst stage (Gardner and Lane, 1997; Gardner and Lane, 1998) in a sequential media culture system.

Sepulvéda *et al*., (2009) however conducted a study to compare the *in vitro* development and pregnancy rates for embryos cultured in a single medium, with medium renewal on day 3, or in sequential media. All oocytes were obtained from young anonymous donors to minimize variability in the study. They found that embryos cultured in a single culture medium showed development that was morphologically just as good, if not better than those cultured in sequential media. After transfer of two morphologically comparable groups, implantation rates were recorded as being higher for those embryos cultured in the single culture medium as opposed to those in the sequential media system (Sepulvéda *et al*., 2009). The use of a sequential media culture system is therefore highly questionable (Sepulvéda *et al*., 2009).

A study by Reed *et al*. (2009) also showed that for embryo transfer on day 5, there was a significantly higher mean number of blastocysts on day 5 in the single medium compared with sequential media ($P<.05$), and a significantly higher number of blastocysts were selected for transfer from the single medium compared with the sequential media, at approximately a 2:1 ratio ($P<.01$). Single culture mediums ensure less handling errors as well as reduced temperature and pH fluctuations (Lane and Gardner, 2005) and there is no apparent advantage in using sequential media systems (Sepulvéda *et al*., 2009).

This improvement in embryo culture dramatically increased the proportion of embryos developing to the blastocyst stage and therefore the application of blastocyst transfer in clinical IVF. The main objective of blastocyst culture was to increase the success rate of IVF because of better embryo selection after genomic activation and/or better endometrial synchronicity (Hardarson *et al*., 2012). Blastocyst culture has also been used to facilitate the morphological selection of the most viable embryo. This reduces the number of embryos transferred (Liebermann, 2009) and was proposed to reduce the incidence of multiple gestations. The ultimate goal of ART is to achieve a singleton, ongoing pregnancy.

1.5. Blastocyst Development and Grading

As the popularity of blastocyst culture increased, so did the need for an effective morphological scoring system. One of the blastocyst grading systems introduced by Gardner and Schoolcraft in 1999 was quickly adopted by the majority of IVF laboratories (Veeck, 2013). Although the system does not cover all aspects of blastocyst morphology, especially aberrant morphology, it has been very useful in classifying the degree of blastocyst expansion as well as the morphological appearance of the inner cell mass (ICM) and the trophectoderm (TE) cells (Hardarson *et al*., 2005). The blastocyst is therefore graded according to the degree of expansion, the ICM morphology and the TE morphology [Addenda 2 and 3].

A defining moment in embryonic development is when fluid starts to accumulate between cells at the morulae stage of development. As the fluid's volume increases, a cavity appears and gradually forms the blastocoel. This normally happens between Days 4 and 5 in human embryos *in vitro* and marks the beginning of the blastocyst stage. As the fluid inside the newly formed blastocyst increases, so does the number of cells, and the combination of these two features causes a progressive enlargement of the blastocyst and it's cavity with a consequent progressive thinning of the zona pellucida (ZP). Finally, the blastocyst breaks free of the ZP through a process called hatching (Hardarson *et al*., 2005).

Blastocyst formation is initiated through an initial secretion between the morula cells and this small cavity is then maintained and increased by actions of the membrane channels. Residing Na/K-ATPase channels raise the salt concentration within the embryo, attracting water through osmosis (Watson et al., 2004). This increased water pressure gradually increases the size of the cavity which continues throughout the blastocyst stages (Hardarson *et al*., 2005).

Embryos are graded according to the *Gardner and Schoolcraft* grading system (Veeck, 2003) [Figure 1] and the degree of expansion is noted accordingly [Addendum 2 and 3].

Once the blastocyst has reached an expansion grade of 3 or more, a clear distinction can be made between the two newly formed cell populations (Hardarson *et al*., 2005), namely the ICM and the TE. The destiny of the ICM is to become the embryo proper and its associated extra-embryonic structures. Morphologically, the ICM can range from being very large with tightly packed cells to almost non-existent with loosely bound cells (Hardarson *et al*., 2005; Veeck, 2003) [Addenda 2 and 3].

The number of cells composing the ICM can vary as well as the morphology of the cells within the ICM (Hardarson *et al*., 2005). Several studies have shown a positive correlation between the morphological appearance of the ICM to clinical outcome; the hypothesis being that the larger the ICM, the better the chances of a successful implantation (Balaban et al., 2000; Richter et al., 2001). ICM cell numbers were found by Hardy *et al. (1989) to* double between days 5 and 6 (20.4 +/- 4.0 and 41.9 +/- 5.0, respectively) and remain virtually unchanged on day 7 (45.6 +/- 10.2). The shape of the ICM has been observed to be quite variable in appearance. It has been reported that the optimal shape of the ICM with respect to implantation potential is more oocyte-like in shape rather than the rounder or more elongated forms (Richter et al., 2001).

Veeck (2003) [Figure 1] describes the ICM grading [graded as A, B C or D – Addendum 2 and 3] as being either tightly packed or compacted cells, larger loose cells, where there is no discernible ICM and lastly whether the cells of the ICM appear degenerative.

The TE cells can be clearly distinguished from the ICM cells as the blastocyst begins to expand (Hardarson *et al*., 2005). The role of the TE cells in the early stages of blastocyst development is not entirely clear but their role in creating the fluid filled blastocoel may be a key parameter in ICM determination. The role of the TE cells, however, is better understood during and after implantation as they play a key role in apposition, adhesion and invasion of the endometrium, thus allowing the blastocyst to embed in the uterus. The TE cells also produce several molecular factors that aid in the implantation process (Aplin, 2000). Without properly functional TE cells, the embryo would remain within the ZP as these cells are actively involved in breaking free of the ZP (Sathananthan et al., 2003). The ultimate fate of the TE cells is to become the foetal extra-embryonic membranes as well as the placenta. Ahlstrom *et al*. (2011) have shown that for the first time the predictive strength of TE grade has a far greater predictive strength over ICM grading for selecting the best blastocyst for embryo transfer. It may be that, even though ICM is important, a strong TE layer is essential at this stage of embryo development, allowing successful hatching and implantation as opposed to a few cells that are compacted at one side or cells that appear degenerative. Hill *et al*. (2013) also stated that is has been increasingly shown that it's the trophectoderm morphology grading rather than the inner cell mass morphology grading that significantly correlated with implantation and live birth after single-blastocyst transfer. This could reflect a positive embryonic condition in the interaction with the endometrium at the implantation site.

Veeck (2003) [Figure 1] describes the trophectoderm [graded as A, B C or D – Addendum 2] as having either many healthy cells forming a cohesive epihelium; a few, but healthy cells, that are large in size; very large or unevenly distributed cells which may appear as a few cells that are compacted at one side or cells that appear degenerative.

1.6. Day 3 vs Day 5 Embryo Transfer

Assisted reproduction programmes still lack high implantation rates but extending the duration of embryo culture to the blastocyst stage gives several theoretical advantages over the transfer of cleavage stage blastocysts. These include higher pregnancy rates, the opportunity to select the most viable embryo for transfer, the potential decrease in the number of embryos transferred, as well as better synchronization between the embryo and the endometrium at the time of synchronization (ASRM Practice committee 2014; Papanikolaou *et* al., 2008). It is generally assumed that blastocysts that develop *in* vitro in a timely manner are of better quality than those that develop more slowly (Veeck, 2003). Also, embryos with reduced viability will arrest in development and will not be cryopreserved (Alikani *et al*., 2000).

With the development of extended embryo culture to the blastocyst stage there has also been much debate surrounding transfer at the cleavage or blastocyst stage. The activation of the embryonic genome occurs three days post oocyte fertilization, after which the 8 cell stage has been achieved (Liebermann, 2010). Embryos transferred during and after this period are therefore known to have undergone genome activation (Desai *et al*. 2010; Papanikolaou *et* al., 2008). Blastocysts develop following this 8 cell stage. Blastocysts increase the ability of a more accurate selection and therefore a lower number of embryos can be transferred, which will result in fewer high-order multiple pregnancies and increased implantation rates (Liebermann, 2009).Whether blastocyst-stage transfer offers any real benefit to infertile couples was debated in a Cochrane report by Blake *et al*. (2004). In an updated Cochrane review published in 2012 both clinical pregnancy and live birth rates appear to be significantly higher in the blastocyst group (Blake et al., 2012) and made blastocyst culture a

popular embryo selection choice. We can speculate that this was as a result of increased numbers as well as refined culture systems.

A prospective randomized controlled trial by Papanikolaou *et* al. (2008) demonstrated that, if a patient has at least four morphologically good embryos on the third day of embryo culture, extending culture to day 5 and transferring two blastocysts results in significantly higher ongoing pregnancy and live birth rates than when two embryos are transferred at the cleavage stage. However, this was at the cost of almost half of the patients in the blastocyst group initially having a multiple pregnancy (42.9 versus 29.6%, $P > 0.05$), which stresses the higher implantation potential of day 5 embryos in a selected patient population.

This strategy, is reinforced by the widely acknowledged limitations of the morphological criteria used for the selection of cleavage-stage embryos (Rijnders and Jansen, 1988), and also by the large proportion of morphologically normal day-3 embryos that are chromosomally abnormal (Magli *et al*., 2000; Staessen *et al*., 2004). Papanikolaou (2008) stated that patients, however, whose embryos do not reach the blastocyst stage, are deprived of the chance of an embryo transfer and, potentially, a pregnancy (Jones *et al*., 1998a; Tsirigotis, 1998). It has, however, been shown that there is a positive correlation between the number of blastocysts developed and the number of eight-cell embryos formed on day 3 (Jones *et al*., 1998b). This can therefore be used as an indication whether a blastocyst will develop. Furthermore, it has been suggested that patients with more than three 8-cell embryos on day 3 should be offered a blastocyst embryo transfer (Racowsky *et al*., 2000).

Optimized embryo culture provided advances in embryo selection protocols. However, with this, came the problem of supernumerary embryos/blastocysts and freezing protocols were required to manage the numbers responsibly (Veeck, 2003).

1.7. The Effect of the Number of Embryos Transferred

As mentioned previously, the goal of any fertility treatment is to achieve a single, ongoing pregnancy. Cryopreservation has allowed for single embryo transfer (SET) to occur by facilitating the freezing of supernumerary embryos for transfer at a later stage. This has also resulted in selective SET. Studies have been done to compare the outcome of single vs. multiple embryo transfer to determine the optimal number of embryos to transfer.

The American Society for Reproductive Medicine reported in 2012 that in Europe, during 2005, 20% of all ETs were SET. They summarized that there was no difference between the pregnancy rates and live birth rates of SET and double embryo transfers (DET). They also noted that SET was a more viable option for good prognosis patients (<35 years) than using selective methods for the most optimal embryo for transfer, and therefore confirming this exact guideline proposed by the ASRM in 2009.

Jones *et al*. (1998b) reported that, in their study, the number of embryos transferred had a significant effect on clinical pregnancy. A greater chance of a clinical pregnancy was associated with an increase in the number of embryos transferred. Le Lannou *et al*. (2006) showed that the pregnancy rate was 27.6% in SET and 36.9% in DET in fresh cycles. SET was significantly lower ($p<0.05$) and was consistent with pregnancy rates of 14.4% and 23.5% in frozen embryo transfers respectively. Gelbaya *et* al. (2010) confirmed this with a systematic review and meta-analysis where elective SET was reported to significantly reduce the probability of live births rates when compared to DET.

This is confirmed in a recent summary of a Cochrane review (Pandian *et al*., 2014), which reiterates that SET is associated with fewer live births.

1.8. Blastocyst Cryopreservation

1.8.1 Background

The purpose of any cryopreservation technique is to ensure high survival rates of living cells after warming (Liebermann, 2003), and therefore to establish a protocol that does as little damage as possible to the embryo. Human blastocyst cryopreservation was first reported in 1985 and was performed using glycerol in a series of ten increasing concentrations (Cohen *et* al., 1985). Blastocyst cryopreservation was initiated as a viable method of managing numbers of supernumerary embryos.

Generally, cryopreserved embryos have a lower rate of pregnancy than fresh embryos (Hiraoka *et al*., 2008), which is thought to be as a result of ZP hardening during cryopreservation (Das *et al.*, 2009). This would then create a negative effect on the natural hatching process of the embryo *in vitro.* More recent research, however, shows that the vitrified-warmed blastocyst transfer cycles resulted in statistically significantly higher clinical pregnancy rates as well as implantation rates, when compared to fresh cycles (Zhu *et al*. 2011; Wu *et al*., 2014).

Successful cryopreservation programs can also increase the treatment success of infertile patients since supernumerary embryos will be available for transfer at low additional cost. In fact, as a result of recent findings, frozen embryo cycle transfers are now suggested irrespective of supernumerary embryos, as they have been shown to lower multiple pregnancy rates while also improving clinical pregnancy outcome (Wu *et al*., 2014). Additionally, patients who are at risk of ovarian hyper stimulation syndrome (OHSS) can

vitrify all of the embryos in advance and reduce the likelihood of adverse clinical symptoms if a pregnancy is established (Veeck, 2003; AbdelHafez *et al*., 2010).

The transfer of cryopreserved-warmed/warmed embryos account for more than 20% of ETs worldwide (Lieberman *et al*., 2003).

1.8.2 Comparing Slow Rate Freezing and Vitrification

The freezing procedure ultimately suspends all chemical and biological reactions, and under extremely cold liquid nitrogen storage (-196˚C) the embryos are able to undergo very long term storage. The total cryopreservation procedure involves the initial exposure to a cryoprotectant, cooling to sub-zero temperature, storage, warming, dilution and removal of the cryoprotectant, as well as the return of the biological material to a physiological environment (Liebermann, 2010).

Initially slow rate freezing methods were used and were designed to create a delicate balance between damaging factors such as ice crystal formation, fracture, toxicity and osmotic damage (Vajta and Kuwayama, 2006). In the slow freezing method, done in a specifically designed machine, low concentrations of cryoprotectant are used and the cooling rate is relatively slow at 1.0°C/minute. The formation of ice crystals is a genuine threat and great care has to be taken to prevent them as they can be lethal (AbdelHafez *et al*., 2010).

Slow freezing utilizes the equilibrium process to drive cellular dehydration, while slowly decreasing the temperature. Cooling rates lie between -0.5 - -5^{o}C/min. This allows cryoprotectants to permeate the cell and ensure complete dehydration without

ice crystal formation. The embryos are cooled until their temperature has passed below -30ºC, before being stored in liquid nitrogen at -196 ºC. Low concentrations of cryoprotectants in addition to varying sucrose or glycerol concentrations are used in the process (Youssry *et al*., 2008). Cryoprotectants can be toxic and non-toxic and also vary in permeability. Permeable cryoprotectants include Dimethyl Sulfoxide (DMSO), Propanediol (PROH), Glycerol and Ethylene Glycol. Sucrose is a non-permeable cryoprotectant. It is however soluble in water and creates dehydration in the cell by increasing the extracellular fluid as a result of diffusion. Sucrose also plays an important role in stabilizing the lipid bilayer and osmolarity. An increasing concentration of a cryoprotectant will result in an increasing toxicity level. A lower toxicity level cryoprotectant, however, does not vitrify the liquid as well. A perfect balance between reduction of intracellular ice formation and low toxicity needs to be created. Glycerol is incapable of completely vitrifying water and as a result is used in conjuction with other cryoprotectants. PROH has been found to be the most effective cryoprotectant (Lucci *et al*., 2002) with the highest the survival rate, blastocyst formation rate and blastocyst hatching rate of embryos (Wei-Xin *et al*., 2011) when compared to glycerol and DMSO. A successful combination is when PROH is used and the more toxic glycerol is added at a later stage during the process.

Seeding is a crucial step that occurs during the freezing process. Supercooling is the ability of an aqueous solution, to cool down it's normal freezing point, without changing it's state; from liquid to solid (Özkavukcu and Erdemli, 2003). Seeding is where the solution is super cooled to below its freezing point, crystallization is introduced, and as a result the soloution immediately returns to its freezing point. Crystallization can be induced by particles within the medium, mechanical vibration or through rough surfaces (Özkavukcu and Erdemli, 2003). Seeding allows the temperature to remain relatively constant during the phase change from liquid to solid (ice).

Seeding prevents damaging effects from occurring to the embryo. Seeding is initatited to propagate a series of latent heat peals down the vertical straw towards the bottom meniscus where the embryo is found. The ice moves along the wall and to the meniscus and then surrounds the embryo.

Kuleshova and Lopata (2002) showed that the success of blastocyst cryopreservation with slow freezing protocols was not very successful, although a publication by the Alpha scientists group clearly shows that cryopreservation of human blastocysts are performed very successfully either by slow freezing or vitrification across the globe (Alpha Scientists in Reproductive Medicine, 2012). Following their consensus meeting it was published that the fundamental principles of cryobiology suggest that the mechanisms leading to high survival of vitrified cells are similar, if not identical to the mechanisms resulting in survival of slow-frozen cells.

Edgar and Gook (2012) also concluded that although slow cooling of blastocysts has been reported to be inferior in some studies, others comparing the two approaches in the same clinical setting have demonstrated comparable results (Liebermann and Tucker, 2006). Ultimately successful blastocyst cryopreservation may be more consistently achieved with vitrification but slow cooling can produce similar results (Edgar and Gook, 2012).

The development of a more rapid freezing method, otherwise known as vitrification, showed greatly improved outcomes in many studies (AbdelHafez *et al*., 2010; Mukaida and Oka, 2012). AbdelHafez *et* al (2010) confirmed the findings by Loutradi *et* al. (2008) stating that vitrification resulted in significantly higher post warming survival rates when compared to slow freezing. They subsequently verified higher implantation rates as well as ongoing

pregnancy rates. Stehlik *et* al. (2005) demonstrated that vitrification of blastocysts showed significant improvement when compared to slow freezing of human blastocysts. Vitrification has higher efficacy and utilization rates (Liebermann, 2003), it maximizes the efficiency of IVF cycles (Liebermann, 2009), it is more time efficient and it is more cost effective as it does not involve expensive instrumentation (AbdelHafez *et al*., 2010). A thorough systematic meta-analysis conducted by AbdelHafez *et al*. (2010) shows that slow programmed freezing is still predominant; however there is a trend towards acceptance of vitrification in ART laboratories worldwide.

Vitrification has been described as the achievement of a "state of suspended animation" wherein molecular translational motions are arrested without structural reorganization of the liquid. It involves placement of the embryo in a very small volume of vitrification medium that must be cooled at extremely high cooling rates (Liebermann, 2010). Vitrification has been endorsed by some to be the most promising method of preservation for tissues (Fahy *et al*., 2006). It was developed not only as a pure optimization of the slow freezing protocol; being quicker, less expensive, and reducing the limitations of the protocol to several cell types, but essentially to eliminate the incidence of cryoinjury. The most common of cryoinjuries are intracellular icicle formation and exposure to high salt concentrations, which needed to be eliminated. Cryoinjury may also occur as a result of osmotic swelling, osmotic shrinkage, fracture damage, chemical toxicity of the cryoprotectant, as well as extracellular ice formation (Liebermann, 2003). A typical embryo vitrification process cools the cells in the embryo at rates close to 20,000°C/minute (Loparatova *et al*., 2006) to a temperature of -.

The vitrification medium will contain an intracellular cryoprotective agent (typically Ethylene glycol, DMSO and Sucrose), which will readily withdraw water from inside the cell as a result of a higher extracellular concentration of the cryoprotectant (Mazur, 1984).

Cryoprotectants also have the ability to lower the freezing point of a solution (Veeck, 2003). Vitrification cryoprotectants are highly soluble in water and form stable hydrogen bonds with the water molecules. This prevents a marked increase in salt concentration that would occur when freezing, by increasing the unfrozen fraction of water molecules and therefore preventing osmotic stress. Vitrification requires much higher concentrations of cryoprotectants than that used for slow freezing. Typically, embryos that are vitrified are exposed to 5-10 times more cryoprotectant than slow frozen embryos. The actual concentration depends on how fast the embryo can be cooled, and the faster the rate of cooling, the less cryoprotectant that has to be used. Of course biologists everywhere are sensitive to keeping cryoprotectant exposure to an absolute minimum; so much effort has gone into developing faster and faster cooling methods.

The process of vitrification has 3 critical components. First, embryos/blastocysts are exposed to high concentrations of cryoprotectants to allow rapid dehydration of cells. Second, the embryos/blastocysts are loaded into tiny storage devices (specifically straws at *Drs Aevitas* Institute for Reproductive Medicine) that will facilitate ultra-rapid cooling, and third, the straws containing the embryos/blastocysts are cooled as fast as possible, typically at thousands of degrees per minute.

Ultimate survival of cryopreserved cells and tissues may be more dependent on the rate at which the specimen is warmed rather than cooled (Leibo and Pool, 2011). A recent study showed that very rapid warming of slow frozen oocytes resulted in better outcomes (Parmegiani *et al.*, 2014) with survival rates increasing significantly from 75% to 90.6%. The oocyte still remains the most fragile human cell with the highest risk of ice crystal formation and therefore cryoinjury during freezing/warming, due to an unfavourable nucleus/cytoplasm ratio and elevated intracellular water concentration (Parmegiani *et al*., 2014). Since the rapid

warming procedure proposed in their study is viable for oocytes, they suggest its potential for being applicable to other less fragile reproductive cells such as embryos. A recent study by Kojima *et al*. (2012) reiterated just that in a prospective study on slow frozen pronuclear embryos. They confirmed a positive effect on embryo development using the rapid warming protocol.

Vajta and Kuwayama (2006) stated that vitrification can only be induced in exceptional situations, specifically with dangerously-high concentrations of cryoprotectants and/or with extreme increase of the cooling and warming rates (Vajta and Kuwayama, 2006).

1.8.3. Factors Affecting the Success of Vitrification

The success of a vitrification protocol depends on: (i) an optimal process of dehydration when cells are exposed to hypertonic conditions; and (ii) an ideal penetration rate of a non-toxic cryoprotectant that is sufficient to generate an intracellular environment that will vitrify and remain vitrified for a defined cooling–warming rate (Alpha Scientists in Reproductive Medicine, 2012). The addition as well as the removal of the cryoprotectant causes successive phases of shrinkage and re-expansion due to movement of water as well as movement of the cryoprotectant across the cell membranes that are linked with several biophysical parameters (Alpha Scientists in Reproductive Medicine, 2012).

1.8.3.1. Cooling rate and storage device

Vitrification can be achieved either by direct (open system) or indirect (closed system) contact with liquid nitrogen (Papatheodorou *et* al. 2013) and by making use of varying loading devices and methods. As mentioned previously, a high cooling rate at vitrification, as well as a high warming rate at warming is required to facilitate the process successfully. Several devices have been designed to facilitate these high cooling rates and ideally there

should be no barrier between the oocyte/embryo and the liquid nitrogen (Vanderzwalmen *et* al., 2007; Alpha Scientists in Reproductive Medicine, 2012).This effort has yielded many storage devices that are only marginally bigger than the embryos being vitrified, to allow the fastest possible rate of cooling while keeping the embryos safely contained. The straws that are used to hold embryos during vitrification and storage are very small compared to the large ½ and ¼ cc straws that are used with slow freezing. The amount of fluid surrounding a vitrified embryo is 100-200 fold less than what would surround a slow frozen embryo. Loading devices available today, to name but a few, include the Cryoloop, Cryotop® , Cryotip, Cryolock, Cut Standard Straws, Cryoleaf™, Vitrisafe©, as well as High Security Straws ™ (Kader *et al*. 2009). Recently it has been reported that the open system is more successful than the closed system with respect to survival, fertilization, cleavage and clinical pregnancy rates, probably due to higher cooling rates (Paffoni *et* al. 2011). There are however safety concerns regarding the direct exposure of the embryos to liquid nitrogen (Papatheodorou *et al*. 2013) specifically due to the possibility of contamination and disease transmission (Vajta and Kuwayama, 2006). It has been proposed that contamination can be avoided by sterilized filtration of the liquid nitrogen (Lin *et al*. 2001), ultraviolet radiation (Parmegiani *et al*. 2010) and the storage of samples in vapors of nitrogen (Cobo *et al*. 2008; Eum *et al*. 2009). As a result, numerous amounts of research compare the open and closed systems. As mentioned previously research conducted by Paffoni *et* al. (2011) favoured the open system of vitrification. Nonetheless, Papatheodorou *et al*. (2013) showed that there is no significant difference between the two protocols and due to the safety concerns surrounding direct liquid nitrogen contact, suggest that the ultra-rapid vitrification may be replaced by aseptic vitrification without affecting clinical efficiency. The Vitrisafe© method is a closed system or direct method and makes use of a device consisting of two parts: a carrier called the Vitrisafe© and a 0.3ml high security straw (Papatheodorou *et* al. 2013). It consists of a

large gutter in which a small quantity of cryoprotectant (<1µl) containing the blastocysts can be deposited. Before placing the biological material into liquid nitrogen, the Vitrisafe© is inserted into the high-security 0.3 ml straw. For warming, the top of the outer straw is cut and the Vitrisafe© device is removed without contact with the liquid nitrogen

The successful vitrification protocols for blastocyst cryopreservation have contributed to ever increasing rates of successful vitrification outcomes. Masashige Kuwayama was a pioneer in oocyte vitrification (Kuwayama, 2005) and also showed that the Cryotop® method of vitrification was the most successful (Kuwayama, 2007). This method is where vitrification occurs in <0.1µl medium droplet on the surface of a specially constructed fine polypropylene strip attached to a plastic handle. This was also reported by Vanderzwalmen *et al*. (2007) and along with other confirmations (Vajta and Kuwayama, 2006), it has resulted in its subsequent implementation today for blastocyst vitrification.

1.8.3.2. Warming rate

As mentioned previously, rapid cooling and warming rates are required for the induction of vitrification (Vajta and Kuwayama, 2006). Warming is conducted at 37°C. Vajta and Kuwayama (2006) also reported that as the cooling rate increases the cryoprotectant concentration can be lowered. Another characteristic of vitrification is that for any given concentration of cryoprotectant, the warming rates are much faster than the critical cooling rates (Fahy *et al*., 1987). These high warming and cooling rates are what eliminate, either completely or partially, any chilling injury (Vajta and Kuwayama, 2006). This is possible due to the sample passing through the dangerous temperature zone too quickly for any damage to develop.

1.8.3.4. Blastocyst quality

The selection criteria for the optimal embryos for cryopreservation and ultimately transfer, must include not only the daily grading procedures but also the original quality of the early-stage embryo as well as the time taken to reach each stage of development (Liebermann, 2009).

Freezing protocols, as mentioned above, were designed to ensure optimal survival rates and to prevent any of the previously mentioned forms of cryoinjury, however, it is imperative that the blastocysts are of good quality before undergoing cryopreservation (Sunkara *et al*., 2010). Frozen–warmed blastocysts undergo multiple morphological changes including the collapse of the blastocoele cavity along with cellular lysis and degeneration (Alpha Scientists in Reproductive Medicine, 2012), which also needs to be considered.

The blastocyst quality is determined morphologically by grading methods described, for example, by *Gardner and Schoolcraft* (Veeck, 2003) [Addenda 2 and 3]. It is commonly accepted that blastocysts which exhibit a clear, well-defined ICM and have an adequate total cell count are of better quality and deemed more viable for cryopreservation (Alpha Scientists in Reproductive Medicine, 2012).

Some studies consider the time in which the embryo takes to reach blastocyst stage as the most important indicator of viability, while others argue that embryo viability is determined more accurately by the stage of development (Liebermann and Tucker, 2006).

1.8.3.5. Technical skill

As with all laboratory procedures the skill of the technician plays a vital role. The experience as well as talent of the technician will contribute towards the success. Vitrification requires excellent skill, especially, to load the blastocysts onto the storage device with as little surrounding cryoprotectant as possible (FertiPro VitriFreeze^{TM}-VitriWarming^{TM}). This is essential to the vitrification success.

1.8.3.6. Blastocoel collapsing

A factor limiting the survival rate of the blastocyst during vitrification is the blastocoel. As can be expected, the formation of intracellular ice crystals is directly proportional to the volume of the blastocoel (Youssry *et al*., 2008). It was shown that mechanically collapsing the blastocyst, described previously (Vanderzwalmen *et* al., 2002), facilitates the successful outcome of blastocyst vitrification (Vanderzwalmen *et al*., 2002; Son *et* al., 2003; Hiraoka *et* al., 2004; Mukaida *et al.*, 2006*).* Vanderzwalmen *et al*. (2002) analysed the effectiveness of reducing the blastocoel volume, or rather, inducing the collapse of the blastocoel. The fluid in the cavity dilutes the cryoprotectant making it harder to expose cells inside the cavity to the full cryoprotectant concentration. Artificial shrinkage (AS) or collapsing is used to evade this concern. A small hole is made with a needle between two outer cells in the embryo to allow the fluid to escape from the cavity. As the fluid escapes, the embryo collapses in on itself giving the procedure its name. An increase in the survival rate of up to 70.6% was noted (Vanderzwalmen *et al*. 2002) and pregnancy rates also improved after the AS procedure. Other methods of AS also proved to be beneficial. In September 2004 a laser pulse generated by a laser system ZILOS-tk^{TM} (Hamilton Thorne Bioscience Inc., Beverly, MA, USA) was introduced to perform AS as an alternative to micro-needle puncture and is described by

Mukaida *et al*. (2006): The ICM should be located away from the targeted point of the laser pulse. One single laser pulse (200 ms) targeted at the cellular junction of the TE creates a hole to induce collapsing of the blastocoelic cavity. ICM and TE gradings are described in Addenda 3 and 4. Ideally you require a tight, compact ICM to significantly reduce the chance of damage caused by the laser or needle. The blastocoele of the expanded blastocyst shrinks immediately. This laser system does not require location and holding of the expanded blastocyst with a holding pipette or being connected to a micromanipulator. There were no statistically significant findings to differentiate between the micro-needle or laser pulse techniques (Mukaida *et al*., 2006), and although this technique (AS by means of laser) is notably simpler and convenient it is definitely a more expensive procedure.

1.8.3.7. Day 5 vs day 6 Vitrification

There was an increasing production of mid to fully expanded blastocysts on the 5^{th}, 6^{th} and 7^{th} day of embryo culture (Alpha Scientists in Reproductive Medicine, 2012), in which the expanded blastocoel cavity was evident.

Some studies show that embryos frozen on day 5 have better implantation and clinical pregnancy rates in comparison to those frozen on day 6. Liebermann and Tucker (2006) conducted a retrospective study which expressed vitrified-warmed day 5 embryos having survival, implantation and pregnancy rates as 95.9%, 33.4% and 48.7%. In comparison, those embryos frozen on day 6 resulted in rates of 97.5%, 25.9% and 42.8% respectively.

However, cryopreservation on day 6 is still encouraged as it does result in live births (Liebermann, 2010). Richter *et al*. (2006) showed the embryos frozen at the expanded blastocyst stage had the similar viability, implantation potential and pregnancy outcome,

whether they were frozen on day 5 (32%) or day 6 (28%). However, the implantation and pregnancy rates for expanded day 7 blastocysts were lower (15%). McVearry *et al*. (2004) showed that these rates were still much higher than day 7 embryos transferred in a fresh cycle.

Sunkara *et al*. (2010) conducted a systematic review and meta-analysis of controlled studies to compare pregnancy outcomes following transfer of warmed blastocysts that were frozen either on Day 5 or Day 6 following fertilization *in vitro*. They showed that ongoing pregnancy and live birth rates were higher for those embryos frozen on day 5 and a significantly higher clinical pregnancy rate for embryos frozen on day 5 was also reported. They also showed that there was no significant difference between the post-warming survival rates or the miscarriage rates of the two groups. It is, however, important to note that embryos having the same morphological grading, frozen on day 5 or day 6, showed no difference in clinical or ongoing pregnancy rates as well as live birth rates (Sunkara *et al*., 2010). It is clearly evident that studies still yield inconsistent results (Liebermann and Tucker, 2006; Richter *et al*., 2006; Shapiro *et al*., 2008).

1.8.3.8. Survival rate

Implantation and Clinical Pregnancy rates have also been shown to rely largely on survival rates. The probability of implantation drops with a decrease in survival rates. Shipley *et al*. (2006) showed that implantation is relatively high when the majority of, if not all, cells survive, but begins to drop as cell survival declines below 95%. When fewer than 80% of cells survive, implantation potential appears minimal.

1.8.3.9. Assisted hatching

Over the past 35 years there have been many advances in optimizing the treatment course for fertility, and despite this being highly beneficial it is still imperfect (Kissin *et* al., 2014). Kissin *et* al. (2014) describes Assisted Hatching (AH) as the purposeful disruption of the zona pellucida (ZP) layer surrounding the embryo and has been proposed as a method of improving favourable outcomes. A Cochrane review emphasized that research conducted on fresh embryos showed no increase in favourable outcomes (Das, 2009), however it has been shown to benefit those women of increased age (Das, 2009; Liebermann, 2010) and in those women with repeated cycle failures (Chao *et al*., 1997; Das, 2009; Martins *et al*. 2011). It was noted in the Cochrane Review that there was a wide variation amongst the trials reported on in the review (Kissin *et* al., 2014). Limited research has been conducted with regards to AH in frozen warmed embryos, however, the available literature shows the procedure as being both beneficial (Tucker *et al*., 1991; Vanderzwalmen *et al*., 2003; Kung *et al*., 2003; Gabrielsen *et al*., 2004; Balaban *et al*., 2006; Petersen, 2006; Hiraoka *et al*., 2008; Valojerdi *et al*., 2008; Ge *et al*., 2008; Wan *et al*., 2014), with increased implantation rates (IR), pregnancy rates (PR) and LBR (live birth rates), as well as being non-beneficial (Edirisinghe *et al*., 1999; Ng *et al*. 2005; Sifer *et al*., 2006; Petersen, 2006; Aran *et al*., 2012).

Chapter Two

Assisted Hatching on Vitrified/Warmed Embryos:

A Survey of the Literature

2.1. Introduction

A blastocyst is comprised of an inner cell mass, a blastocoel fluid cavity, and a trophectoderm layer lining the inside of the outer ZP. The ZP is a glycoprotein layer; made from acidic glycoproteins namely zona pellucida proteins 1, 2 and 3 (ZP1, ZP2 and ZP3), and surrounds the embryo during the preimplantation period (Veeck, 2003). This outer layer plays a vital role in maintaining the three dimensional structure of the precompacted embryo (Cohen, 1991). The ZP is known to prevent polyspermy, to protect the embryo from mechanical and immunological damage, and to preserve the integrity of the embryo during cleavage. It also plays a role in sperm cell binding and penetration as well as the induction of the acrosome reaction (Bleil and Wassarman, 1983; Bielfield *et al*., 1994). Bleil and Wasserman (1983) showed that mouse sperm recognize and bind ZP3 and that the binding thereof leads to the induction of the acrosome reaction. Experiments conducted by Bielfield *et* al. (1994) confirms that human ZP-sperm binding induces activation of multiple second messenger pathways, involving kinases, during the acrosome reaction.

After the embryo compacts and the formation of cell junctions occur, blastocyst development follows and the ZP needs to be breached for the natural hatching process to occur. Hatching is described as the blastocyst escape through the ZP. It is a prerequisite for normal implantation as it facilitates trophoblastic invasion of the uterine endometrium (ASRM, 2008). As the blastocyst expands during development there is an accumulation of fluid in the blastocoel, which results in increased pressure on the trophectoderm and ZP. The trophectoderm is also proliferating rapidly to form a cohesive monolayer. Thinning of the zona occurs due to these physical pressures (Stein *et al*. 1995), however, it has been proposed that lytic enzymes released by the developing blastocyst may also play a combined role in the

hatching process (Schiewe *et al,* 1995). Gradual thinning of the zona, as well as changes in diameter before expansion occurs, are evidence of enzymes being involved. *In vivo* studies state that it is possible that the uterine enzymes are partly responsible for the complete shedding of the ZP (Lin *et al*. 2001). Hatching sites are variable in humans but usually develop relatively close to the inner cell mass, in proximity to the polar trophectoderm. Small membrane vesicles can protrude from the ZP during *in vitro* culture, however they are not necessarily indicative of the subsequent hatching site. Once rupture of the ZP occurs the opening consists of one quarter to one third of the ZP, thus facilitating rapid and easy escape. The blastocyst begins to protrude from the ZP, while following the path of least resistance, but other mechanisms are thought to be involved in completing the hatching process. Actin polymerization is believed to play a role as actin filaments are highly concentrated on the trophectoderm cells, especially at the hatching site. When blastocysts hatch *in vitro*, small trophectoderm projections are observed outside of the ZP, which have been suggested to serve as the first contact between the blastocyst and the uterus, since they are localized to the precise region that will ultimately attach to the uterine epithelium (Veeck, 2003). *In vitro* hatching of healthy human blastocysts typically occurs on day 6 or 7 of culture (Sathananthan *et* al., 2003) otherwise on day 5 if the zona has been manipulated (Das *et al*., 2009). Hardening of the ZP is speculated to occur spontaneously during *in vitro* culture as well as during cryopreservation (Vanderzwalmen *et al*., 2003) due to the cryoprotectant (Larman *et al*., 2006). Zona hardening is a result of the fusion of cortical granules to the plasma membrane and the release of their contents into the ZP layers (Matson *et* al., 1997). This hardening may impair successful embryonic hatching and implantation (Shi *et* al., 2013).

Larman *et al*. (2006) conducted an experiment in mice to compare the effects of two commonly used cryoprotectants used in vitrification procedures, namely DMSO and ethylene glycol, on ZP hardening. Both cryoprotectants were found to cause a large transient increase

in intracellular calcium concentration in mouse metaphase II (MII) oocytes, which was comparable to the initial increase triggered at fertilization. When they removed the extracellular calcium from the medium surrounding the oocytes, the increase in intracellular calcium was unaffected in the presence of DMSO. It was, however, significantly reduced in the presence of ethylene glycol. This suggests that the source of the DMSO-induced calcium increase is solely from the internal calcium pool, as opposed to ethylene glycol that causes an influx of calcium across the plasma membrane from the external medium. They also carried out vitrification in calcium-free media which resulted in significantly reduced ZP hardening and subsequent fertilization and development to the two-cell stage was significantly increased.

This supports the idea that ZP hardening is an early activation event that is triggered by the sperm-induced calcium increase observed at fertilization (Homa and Swann, 1994) and that the addition of cryoprotectants serves to exacerbate this process.

Since it is widely accepted that the implantation potential of a frozen warmed embryo is lower than that of a fresh embryo (Debrock *et* al., 2011) and that this may be a result of ZP hardening, AH of the ZP was introduced into fertility treatment programmes to breach the hardened ZP and promote the natural process of hatching (Gabrielsen *et al*., 2004). AH was first introduced in the 90's using Acid-Tyrode's solution (Cohen, 1991).

2.2. Assisted Hatching of Blastocysts

2.2.1. General

AH involves creating an artificial hole or thinning in the ZP to facilitate the natural hatching process while *in vitro*. It was originally suggested due to an observation that microsurgically fertilized embryos with artificial gaps in their ZP appeared to have higher rates of implantation (Cohen *et* al., 1989). Cohen *et al*. (1990) and Kissin *et al*. (2014) accurately described the benefits and disadvantages of AH and how the adoption of new technologies should involve a thorough and proven risk/benefit ratio for validity and efficacy prior to implementation, but the rate of scientific progress often supersedes the ability to do so. Retrospective studies have therefore been of great scale on this topic. A thorough analysis of retrospective data was reviewed on AH in fresh cycles in the United States over the years 2000-2010 (Kissin *et* al., 2014). Kissen *et* al. (2014) showed that cycles involving AH increased significantly from 25 724 to 35 518 over the 10 year period (p=0.002). Despite the increase in its implementation, they reported that AH was associated with decreased odds of implantation, clinical pregnancy, live births and an increase in miscarriage rates. In the case of poor prognosis patients there was no significant difference in pregnancy outcomes. As mentioned previously, in a Cochrane review no significant difference in live birth rates between assisted hatching groups and control groups in fresh ART cycles was found (Das, 2009). This was supported in a systematic review and meta-analysis conducted by Martins *et* al. (2011).

2.2.2. Controversial Findings

There is however evidence that shows that AH is beneficial after numerous failed fresh and frozen warming IVF cycles (Chao *et* al., 1997; Das, 2009; Martins *et al*., 2011) as well as in women of increased age (Das, 2009; Liebermann, 2010). The improved clinical pregnancy rates were 74% in frozen cycles and 25% in fresh cycles. Smaller numbers were used, however, and they insist that a randomized control trial is necessary to be able to conclude whether AH is beneficial in frozen warming embryo cycles. Martins *et al.* (2011), however, found no significant advantage associated with women of increased age.

2.2.3. Types of AH

Hatching of the zona has been performed mechanically (Cohen *et al*., 1990), chemically (Cohen *et al*., 1992), or by the use of laser (Palankar *et al*. 1991; Antinori *et al*., 1996). Otherwise denoted as mechanically assisted hatching (MAH), chemically assisted hatching (CAH) and laser assisted hatching (LAH). Acid tyrodes, proteinases, piezon vibrator manipulators and lasers are used (Hammadeh e*t al*., 2010). An increased implantation rate after mechanical thinning of the ZP was first reported in 1990 (Cohen *et al.*, 1990) and CAH using acidified Tyrode's solution to drill through the ZP suggested improved implantation rates when selectively performed on poor prognosis patients (Cohen *et al*., 1992). LAH is more widely used because it is easier to control and is more precise (Sagoskin et al., 2007), whereas the chemical and mechanical techniques require extensive technical skill (Hammedeh *et al*., 2010). Hammedeh *et al*. (2010) also emphasized the need to minimize the time that the embryo is kept outside the incubator and therefore to optimize the methodologies to reduce both pH and temperature variations that could be detrimental to embryo development. MAH involves the

use of a microneedle or, more recently, piezo technology. In the case of a microneedle, Partial Zona Dissection (PZD) is performed while the embryo is stabilized using a holding pipette and then pierced with the microneedle tangentially through the space between the ZP and the blastomeres until it pierces through the ZP again (Cohen *et al*., 1990). The small part of the ZP that is trapped against the microneedle is then rubbed against the holding pipette, and therefore creating an opening between the two sides that have been pierced (Hammedeh *et al*., 2010). Piezo technology was later introduced for ZP drilling (Nakayama *et al*., 1999). Nakayama *et al*. (1999) described how, while a holding pipette anchors the embryo, vibratory movements produced by a piezo-electric pulse regulated by a controller are used to carve a limited conical area in the ZP. Numerous applications in adjacent areas serve to produce a large hole, which facilitates complete hatching of the blastocyst (Hammedeh *et al*., 2010). CAH is done using acidified Tyrode's solution and has previously been described in detail by Cohen *et al*. (1990; 1992). They explain that, as with MAH, the embryo is secured using a holding pipette and the microneedle is applied to a blastomere-free area. The microneedle is preloaded with acid Tyrode's solution using mouth-controlled suction. The solution is expelled gently and once the ZP has been breached suction is applied to prevent any excess solution entering the perivitteline space. The ZP dissolves instantly and the embryo is then immediately removed to be washed and therefore remove any trace of the acid (Hammedeh *et al*., 2010). Finally, LAH, was first reported in 1991 (Palankar *et al*., 1991). After advances in the technology, there is a general preference of a non-contact laser mode using a 1.48 µm diode laser, which was reported by Blake *et al*. (2001). Hammedeh *et al*. (2010) stated in their summary of the literature that LAH would appear to be associated with the lowest potential risk and that it is relatively simple to perform with consistency between operators. Initially a complete hole was made to breach the ZP until improved pregnancy outcomes were reported by Blake *et al*. (2001) where they used the laser to thin the ZP rather than producing a full thickness hole. This was then confirmed in a

subsequent study by Mantoudis *et al*. (2001). There is no ultra-structural damage associated with LAH, which was confirmed using both light electron microscopy and scanning electron microscopy (Obtruca *et al*., 1997). AH has, however, been associated with an increased risk of monozygotic twinning (Hershlag *et al*. 1999; Schieve *et al*. 2000). Ultimately, results between these methodologies vary and are likely due, at least in part, to variations in the level of experience and hatching methods (ASRM, 2008).

All these methods aim to create a hole in the ZP (Primi *et al*., 2004; Valojerdi *et al*., 2008) or can also be used purely as a thinning technique (Debrock *et al*., 2011) to facilitate the escape from a possibly altered ZP. This will assist the embryo in hatching out of the ZP once the blastocyst has expanded and is ready to implant. This also ensures an earlier contact time with the endometrium (Hammedeh *et al*, 2010). Liu *et al*. (1990) showed that early implantation is associated with subsequent viable pregnancies, while delayed implantation is associated with a high incidence of miscarriage.

2.3. Assisted hatching in vitrified warmed cleavage stage embryos and blastocysts – results from the literature

To overcome the occurrence of zona pellucida hardening (Larman *et al*., 2006) and to therefore circumvent the resulting impairment of successful hatching and implantation, AH was introduced as a possible solution and became implemented in IVF programmes worldwide. Research was conducted on embryos, first, during fresh cycles (Chao *et al*., 1997; Das, 2009; Leibermann 2010) and, as previously mentioned, it was only shown to be beneficial in repeated cycle failures as well as in women of increased age.

The benefit of AH on frozen warmed embryos is more inconclusive. Some studies show it to be beneficial (Tucker *et* al.,1991; Vanderzwalmen *et al*., 2003; Kung *et al*., 2003; Gabrielsen *et al*., 2004; Balaban *et al*., 2006; Petersen, 2006; Hiraoka *et al*., 2008; Valojerdi *et al*., 2008; Ge *et al*., 2008; Wan *et al*., 2014), while others do not (Edirisinghe *et al*., 1999; Ng *et al*., 2005; Sifer *et al*., 2006, Petersen, 2006; Aran *et al*., 2012). The Cochrane Review on Assisted Hatching in IVF and ICSI in 2006 showed that there is a benefit in women with repeated cycle failures as well as in those women with poor prognosis embryo transfer cycles, however, this is inconclusive (Das *et* al., 2009).

We conducted a survey of the literature and report back on 16 studies that have been carried out on frozen warmed embryos. There is very limited research that has been conducted and as a result we have included data on cleavage stage embryos as well as blastocysts, which also allows a comparison thereof. The results have been summarized and are presented in Tables 2.3.1, 2.3.2 and 2.3.3.

Table 2.3.1: Summary of descriptive information from publications found in a literature search regarding assisted hatching [AH] on vitrified/warmed embryos

Article	Number of Cycles		Age at Freezing		Embryo Stage
	CTRL	AH	CTRL	AH	
Tucker et al., 1991	63	64	no data	no data	blastocyst
Edirisinghe *et al*., 1999	no data	no data	no data	>38	blastocyst
Vanderzwalmen *et al*., 2003	49	36	27-41	27-41	blastocyst
Kung *et al*., 2003	NA	89	NA	31.70	cleavage
Gabrielsen *et al*., 2004	117	136	32.8	33.10	cleavage
Ng *et al*., 2005	80	80	34.00	34.00	cleavage
Sifer *et al*., 2006	61	64	32.00	32.30	cleavage
Balaban *et al*., 2006	183	183	32.7	32.40	cleavage
Petersen, 2006	110	110	32.5	31.70	cleavage
Petersen, 2006	19	19	30.1	32.10	cleavage
Hiraoka *et al*., 2008	30	40	31.00	32.00	blastocyst
Hiraoka *et al*., 2008	30	31	31.00	33.00	blastocyst
Valojerdi *et al*., 2008	90	90	31.79	31.18	cleavage
Ge *et al*., 2008	100	100	30.66	31.84	cleavage
Wan *et al*., 2014	102	96	32.60	33.10	blastocyst
Zhou *et al*., 2014	335	480	29.90	29.73	cleavage

Table 2.3.2: Summary of descriptive information from publications found in a literature search regarding assisted hatching [AH] on vitrified/warmed embryos (continued)

Article	Number of blastocysts transferred		ICSI/IVF	Type of AH	AH before (B) or after (A) vitrification	Complete Hole (CH) or Partial Thinning (PT)
	CTRL	AH				
Tucker et al., 1991	no data	no data	no data	mechanical	A	PT
Edirisinghe *et al*., 1999	no data	no data	ICSI	mechanical	A	no data
Vanderzwalmen *et al*., 2003	2.00	2.00	ICSI/IVF	mechanical	A	PT
Kung *et al*., 2003	NA	2.50	ICSI/IVF	laser	B	PT
Gabrielsen *et al*., 2004	1.91	1.94	ICSI/IVF	chemical	A	PT
Ng *et al*., 2005	1.00	1.00	ICSI/IVF	laser	A	CH
Sifer *et al*., 2006	2.00	2.00	ICSI/IVF	chemical	A	PT
Balaban *et al*., 2006	3.10	2.90	ICSI	laser	A	PT
Petersen, 2006	2.75	2.69	ICSI	laser	A	PT
Petersen, 2006	2.60	2.9	ICSI	laser	A	PT
Hiraoka *et al*., 2008	2.70	2.6	ICSI	laser	A	PT (40µm)
Hiraoka *et al*., 2008	2.70	3.00	ICSI	laser	A	PT (50%)
Valojerdi *et al*., 2008	3.14	3.29	ICSI/IVF	laser	A	CH
Ge *et al*., 2008	2.46	2.33	ICSI/IVF	laser	A	PT
Wan *et al*., 2014	max 3 pp	max 3 pp	ICSI/IVF	laser	A	CH
Zhou *et al*., 2014	2.48	2.48	ICSI/ IFV	laser	A	PT

Table 2.3.3: Summary of descriptive information from publications found in a literature search regarding assisted hatching [AH] on vitrified/warmed embryos (continued)

Article	IR (%)		PR (%)		LBR (%)		Beneficial (Y/N)
	CNTRL	AH	CNTRL	AH	CNTRL	AH	
Tucker et al., 1991	9.00	16.00	no data	no data	no data	no data	Y
Edirisinghe *et al*., 1999	13.30	0.00	16.70	5.00	no data	no data	N
Vanderzwalmen *et al*., 2003	13.00	22.00	19.00	38.00	no data	no data	Y
Kung *et al*., 2003	NA	16.90	NA	31.40	no data	no data	Y
Gabrielsen *et al*., 2004	5.80	11.40	11.10	17.60	no data	no data	Y
Ng *et al*., 2005	6.80	9.00	15.00	12.50	no data	no data	N
Sifer *et al*., 2006	9.20	9.60	17.20	18.00	no data	no data	N
Balaban *et al*., 2006	9.90	20.10	27.30	40.90	no data	no data	Y
Petersen, 2006	8.60	8.10	18.20	18.20	no data	no data	N
Petersen, 2006	14.30	27.80	31.60	47.40	no data	no data	Y
Hiraoka *et al*., 2008	10.00	27.00	17.00	43.00	no data	no data	Y
Hiraoka *et al*., 2008	10.00	52.00	17.00	74.00	no data	no data	Y
Valojerdi *et al*., 2008	4.20	12.80	11.10	31.20	no data	no data	Y
Ge *et al*., 2008	7.30	16.70	14.00	25.00	no data	no data	Y
Wan *et al*., 2014	23.60	34.20	35.30	51.00	28.40	40.60	Y
Zhou *et al*., 2014	16.95	31.85	33.43	53.96	23.88	43.58	Y

Table 2.3.1, 2.3.2 and 2.3.3 show that the available data from the literature included 16 control and 16 matched AH cycles. One study had no control group and compared their AH results to previous data and literature. Statistics were calculated using the Independent t-Test. The average female age for the control group was 31.92 and 32.37 for the AH group. There was no significant difference between the ages of the women in the different studies or the number of embryos transferred in each cycle (P=0.9). The average number of embryos transferred in each cycle in the control group was 2.39 and 2.42 for the group undergoing AH. Again, there was no significant difference between the two groups.

62.5% [10/16] of the studies performed AH on cleavage stage embryos. 75% [12/16] of the studies showed that AH is beneficial in frozen warmed embryos (including the study where AH was performed prior to vitrification). This is statistically significant with a P value of 0.0356. Only one of the included studies did AH before cryopreservation and did not include a control group. Of the studies showing no benefit for AH in frozen warmed embryos, 75% were associated with AH being performed in the cleavage stage, however this is non-significant (P= 0.35). Within this group there was no association to the type of AH performed as well as to whether a complete hole was created during AH or if only a portion of the ZP was thinned during AH. Where LAH was performed, there was an 81.81% [9/11] benefit, MAH had a 66.67% [2/3] benefit and CAH had a 50% [1/2] benefit for frozen warmed embryos, and subsequently no significant association was reported.

In the case of Partial Thinning, 83.33% [10/12] of studies had beneficial results, and where a complete hole was created, a 66.67% [2/3] benefit was observed. The most viable methodology, however, could not be statistically identified (P=0.53).

Edirisinghe *et al*. (1999) as well as Ng *et al*. (2005), Sifer *et al*. (2006), Petersen (2006) and Aran *et al*. (2012) showed that AH was not beneficial for frozen warmed embryos, however it was also found in several other studies that AH on frozen warmed embryos improved implantation rates and pregnancy rates respectively (Tucker *et al*., 1991; Vanderzwalmen *et al*., 2003; Kung *et al*., 2003; Gabrielsen *et al*., 2004; Balaban *et al*., 2006; Petersen, 2006; Hiraoka *et al*., 2008; Valojerdi *et al*., 2008; Ge *et al*., 2008; Wan *et al*., 2014).

From the tables shown we calculated the average implantation rate for the control group to be 10.35% compared to an increase to 18.9% for the AH group. This increase was also apparent for pregnancy rates: 19.2% vs 32.3%. The Independent T Test calculated the P values to be 0.53 and 0.47 respectively and the differences are therefore non-significant. In two studies where live birth was recorded AH of frozen warmed embryos also showed increased rates 28.4% vs 40.6% for control and AH cycles respectively. This would indicate a benefit, however the numbers too small to be conclusive.

From this data we can identify trends; however, the numbers of studies, to date, are too small to viably and significantly be compared. As a result, it is still controversial as to whether assisted hatching does indeed play a vital role in the implantation and survival of the frozen-warmed embryo, and consequently there is a need for numerous more studies on a larger scale. A meta-analysis should also be conducted on the collected data. Most of the research to date has been conducted on cleavage stage embryos and is therefore more conclusive (Kung *et al*., 2003; Gabrielsen *et al*., 2004; Ng *et al*., 2005; Sifer *et al*., 2006; Balaban *et al*., 2006; Petersen, 2006; Petersen, 2006; Valojerdi *et al*., 2008; Ge *et al*., 2008; Zhou *et al*., 2014). To clarify whether AH on frozen warmed blastocysts, however, is indeed beneficial we plan to design a prospective study in which this can be evaluated.

Chapter Three

Aim and Objectives

The aim of this study is to optimize the outcomes of human blastocyst vitrification in an ART programme. To do so, we have designed a Retrospective Study to report back on the established Vitrification/Warming Programme in place at *Drs Aevitas* Institute for Reproductive Medicine over a period of 10 years. A thorough analysis of the data, and comparison with available literature, will allow us to identify strengths as well as weaknesses within the programme and suggest ways of improving and therefore optimizing the outcomes. Consequently we have also designed a Prospective Study to confirm our speculations and ultimately further refine the programme.

Chapter Four

Materials and Methods

4.1. Retrospective Study

Title:

Human blastocyst Vitrification: A retrospective review of outcomes at an Assisted Reproductive Technology (ART) clinic [2004-2014]

The study was conducted using the database of patient files at *Drs Aevitas* Institute for Reproductive Medicine at *Life* Vincent Pallotti Hospital. Patients are included over a period of ten years from 2004-2014. Ethical Approval from the Health Research Ethics Committee of the University of Stellenbosch (HREC) was obtained [S13/05/102] on 18 September 2013 and all data obtained from the patient files remained anonymous in the study. No funding was required for data collection.

The Materials and Methods described in this Chapter pertain to standard protocols and procedures that patients frequenting the clinic undergo during Assisted Reproductive cycles.

Detailed methodologies are presented as Addenda in Chapter Nine.

4.1.1. Methods

4.1.1.1. Patients

Standard IVF and intracytoplasmic sperm injection (ICSI), physiological intracytoplasmic sperm injection (PICSI) and intracytoplasmic morphologically selected sperm injection (IMSI) patients with stored, vitrified blastocysts were included. All female ages and all semen profiles were included.

4.1.1.2. Ovarian Stimulation and oocyte aspiration

Different but standard, routine procedures were followed for ovarian stimulation. A routine oocyte aspiration protocol (using a sonar guided method) was followed [Addendum 7].

4.1.1.3. Semen preparation and insemination

Standard procedures for semen production and preparation were followed. Both the swim up and gradient centrifugation preparation methods were done depending on the semen characteristics [Addenda 5 and 6].

Standard IVF and ICSI insemination procedures were followed [Addenda 8 and 9].

4.1.1.4. Embryo culture and evaluation

All embryos were cultured and graded using standard protocols used at *Drs Aevitas* Institute for Reproductive Medicine [Addendum 2]. Blastocysts were graded according to the *Gardner and Schoolcraft* [Addenda 3 and 4] grading method (Veeck, 2003).

4.1.1.5. Morula and Blastocyst Vitrification

Supernumerary embryos/blastocysts of good quality after fresh embryo transfer were vitrified on day 5 or day 6 (and in some instances on day 7), following a modified protocol designed for Cryotop® (Kitzimato®) vitrification (Cobo *et al*., 2008) with accordance to the *Ferti-Pro® Vitri Freeze kit* protocol [Addendum 10], and currently being used at *Drs Aevitas* Institute for Reproductive Medicine.

4.1.1.6. Blastocyst Vitrification [Addendum 12]

- Vitrification was performed at room temperature and it was therefore ensured that the heated stage was turned off.
- Two pulled glass pipettes were made and used for embryo manipulation.
- A coloured Cryotop® was chosen and labelled with the patient's initials and surname, ID number and date of vitrification on alternating sides at the top of the Cryotop® using an IVF-safe permanent marker.
- Blastocysts with an expansion grading of a 2 or higher were collapsed using a collapsing dish and micromanipulation needles. Collapsing was done at 37°C in drops of flushing medium covered with oil on a heated stage [Addendum 13].

  Note: it is not necessary to collapse a hatched or hatching blastocyst.
- Vitrification media (Fertipro® "VitriFreeze") was aliquotted into labelled Eppendorf tubes.

  1: VitriFreeze Pre-incubation medium (200µl per patient)

  2: VitriFreeze Freezing medium1 (100µl per patient)

  3: VitriFreeze Freezing medium2 (100µl per patient)

4.1.1.7. Blastocyst Warming [Addendum 14]

The blastocysts were warmed, again according to the routine, standard protocol in accordance with the *Ferti-Pro Vitri Warming* kit protocol (Addendum 4) used at *Drs Aevitas* Institute for Reproductive Medicine.

- Warming was performed on a heated stage measuring 37°C.
- Warming medium was warmed a minimum of 1 hour before (or the day before) to ensure a temperature of 37°C.
    - VitriWarming 1 (1000µl per patient)
    - VitriWarming2 (200µl per patient)
    - VitriWarming3 (200µl per patient)
    - VitriWarming4 (200µl per patient)
- A centre well dish was also incubated to 37°C in the same fashion.
- Enough pulled pipettes were available for blastocyst manipulation.

4.1.1.8. Blastocyst Vitrification and Warming (Vitriplug Method)

In earlier years, more specifically from 2004 up until 2008, the Vitriplug method of cryopreservation and warming was used in our laboratories. As a result, data collected from the initial cycles will have followed this alternate procedure. A detailed description can be found in Chapter 9 [Addendum 15].

4.1.1.9. Embryo Transfer [Addendum 16]

A minimum incubation time of three hours post warming was utilized prior to blastocyst transfer. This allowed adequate time to ensure survival of the frozen/warmed blastocyst.

A standardized blastocyst transfer method (using sonar guidance) was performed. Blastocysts were transferred with the patient having been instructed to present with a full bladder.

4.1.1.10. Human Chorionic Gonadotropin (HCG) Levels

A quantitative βHCG was performed to determine early pregnancy, which can be extrapolated to an implantation rate. Blood was drawn according to standard procedure [Addendum 17] using a Vacutainer © on days 10 and 14 following embryo transfer.

4.1.1.11. Recording of Patient Information

Patient files included a daily embryo scoring sheet and embryo freezing and transfer is duly noted in the respective files. Forms for embryo freezing [Addendum 11] were also completed and stored separately in folders, along with duplicated patient information and consent forms respectively. A thorough analysis of prospective patient files and Embryo Vitrification folders was conducted. The Excel spread sheet created (including data of the fresh, cryopreservation and warming cycles) allowed for analysis of:

1) *Descriptive Statistics*

Incidence of survival rates [2004-2014] after warming of vitrified blastocysts.

Biochemical Pregnancy Rates [2004-2014]

Clinical Pregnancy Rates [2013-2014]

Incidence of female (oocyte) age in vitrification/warming cycles [2004-2014]

Incidence of vitrification in warming cycles on different days of blastocyst development [2004-2014]

Incidence of Donor Oocyte vitrification cycles [2004-2014]

Number of embryos transferred/cycle [2004-2014]

Incidence of Good Quality Embryos before Vitrification [2004-2014]

Incidence of Good Quality Embryos post Warming [2004-2014]

Incidence of Fresh Pregnancy Cycles in patients with frozen embryo transfers [2004-2014]

The incidence of FET pregnancy outcome in cycles where pregnancy in the initial fresh cycle was also achieved [2004-2014]

2) *The Effect of storage device on Pregnancy outcomes (Survival, Biochemical Pregnancy and Clinical Pregnancy rates) [Vitriplug vs. Cryotop®]*
3) *The Effect of oocyte age on Pregnancy outcomes [Cryotop® data only]*
4) *The Effect of day of vitrification on Pregnancy outcomes [Cryotop® data only]*
5) *The effect of the number of embryos transferred on Pregnancy outcomes [Cryotop® data only]*
6) *The effect of using donor oocytes on Pregnancy outcomes*

4.1.2. Statistical Analysis

Professor Martin Kidd, Director of the Centre for Statistical Consultation (CSC) at the University of Stellenbosch analysed the retrospective data. *STATISTICA 12 (ANOVA)* was used to generate the results described in Chapter 5. The results are expressed as the mean ± standard deviation of the mean and significance is accepted at $p \leq 0.05$.

4.1.2.1. Definitions

Survival Rates: (no. of embryos survived/no. of embryos warmed*100)

Biochemical Pregnancy Rates: (no. of cycles with positive βhCG/total no. of embryo transfer cycles*100)

Biochemical Pregnancy: A cycle recording a positive quantitative βhCG recording [Addendum 17].

Clinical Pregnancy Rate: (no of cycles with clinical pregnancy/total no. of embryo transfer cycles*100)

Clinical Pregnancy: A clinical pregnancy is defined as a positive sonographic fetal heart beat at 7 weeks gestation.

Good quality Embryo Classification: (Veeck, 2003)

Day 5: minimum expansion embryo grading of 1; no C grading for ICM or TE

Day 6: embryo expansion grading of 2-3; no C grading for ICM or TE

Day 7: minimum embryo expansion grading of 3; no C grading for ICM or TE

Chapter Five

Results

Data recorded in patient files obtained from *Drs Aevitas* Institute for Reproductive Medicine over the period of 2004-2014 was collated and analysed retrospectively in this study. All patients enrolled at the Institute undergoing an FET cycle were considered for the study. Those patients that did not receive an embryo transfer were excluded from the outcomes analysis of the study. Cycles with missing data had to be excluded from the study for an accurate comparison to be conducted. As a result, 231 cycles were eligible for statistical analysis. Over the years, recording of data and outcomes has become more formalised, resulting in the later years having a greater representation in the results. Positive Biochemical Pregnancies are defined with an βhCG >10 and a proceeding βhCG of at least double the original value. Clinical Pregnancies were only calculated for the years 2013-2014, again due to the more recent implementation of a more structured recording system. Positive Clinical Pregnancies are defined as the presence of at least one fetal heart at 7 weeks. For the period 2004-2008 the Vitriplug® storage device was used which was then replaced by the Cryotop® device for the remainder of the study. No of obs indicates the Number of Observations.

STATISTICA 12 (ANOVA) was used to analyze the results of the study, which are presented in Figures 5.1-5.24 and included:

1)Descriptive Statistics

2)The Effect of storage device on Pregnancy outcomes (Survival, Biochemical Pregnancy and Clinical Pregnancy rates) [Vitriplug vs. Cryotop®]

3)The Effect of oocyte age on Pregnancy outcomes [Cryotop® data only]

4)The Effect of day of vitrification on Pregnancy outcomes [Cryotop® data only]

5)The effect of the number of embryos transferred on Pregnancy outcomes [Cryotop® data only]

6)The effect of using donor oocytes on Pregnancy outcomes

5.1. Descriptive data

The total number of vitrification cycles (all patient cycles that had embryos vitrified) for the study period was 818. 417 cycles, of which, only underwent vitrification whilst the remaining 401 cycles underwent both vitrification and warming. 1817 blastocysts were vitrified and 856 blastocysts were warmed for embryo transfer.

As mentioned previously, FET cycles with no embryo transfer were excluded from the study, as well as those cycles in which there was missing data. Consequently N=231 cycles were evaluated and the results reflect the vitrification/warming programme and thus the pregnancy outcomes of the aforementioned cycles.

The overall survival rate for those cycles that underwent embryo transfer was calculated at 86.8%.

The Descriptive Data is displayed in Figures 5.1-5.11.

Figure 5.1 shows the incidence of 100% survival (the survival of all embryos in each respective transfer cycle) as well as the incidence of <100% survival. 78% [181/231] of the warming cycles had a survival rate of 100% while 22% [50/231] had a <100% survival.

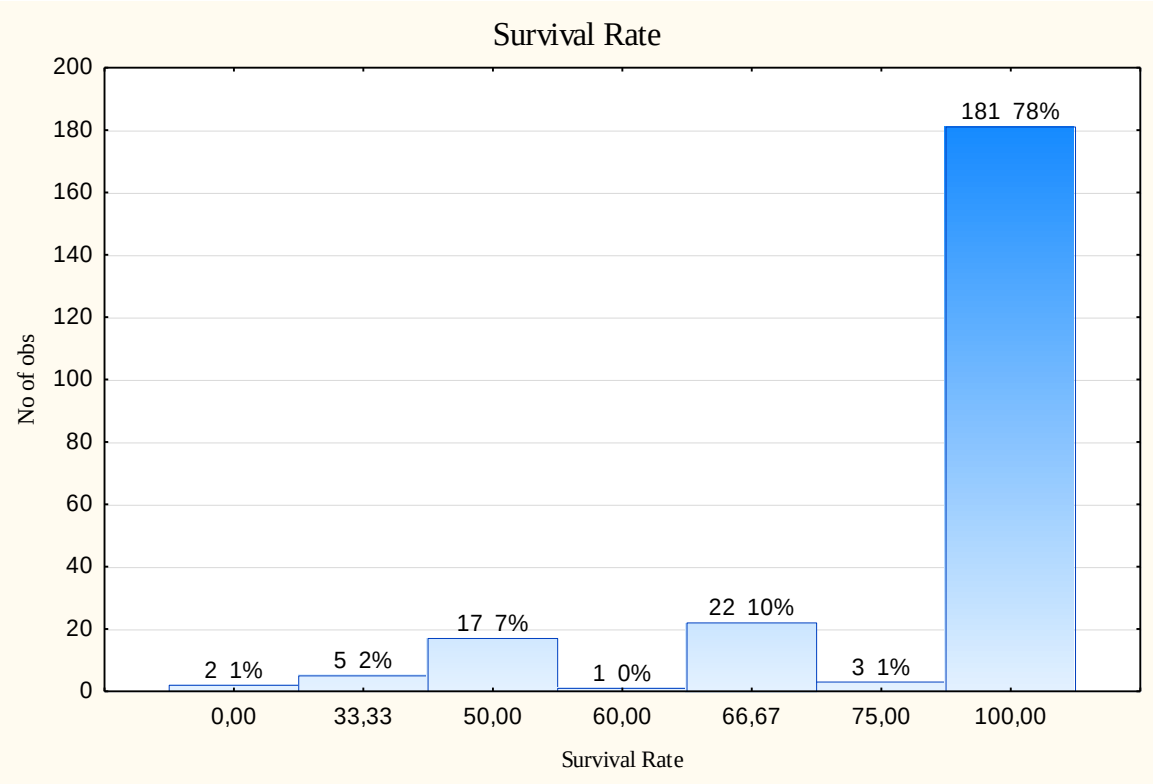

Figure 5.1: Incidence of survival rates [2004-2014] after warming of vitrified blastocysts

No of obs = Number of vitirifcation/warming cycles

Figure 5.2 shows that, overall, a biochemical pregnancy rate of 35% [80/231] was observed.

Complete data to calculate clinical pregnancy for 2004-2012 was unavailable.

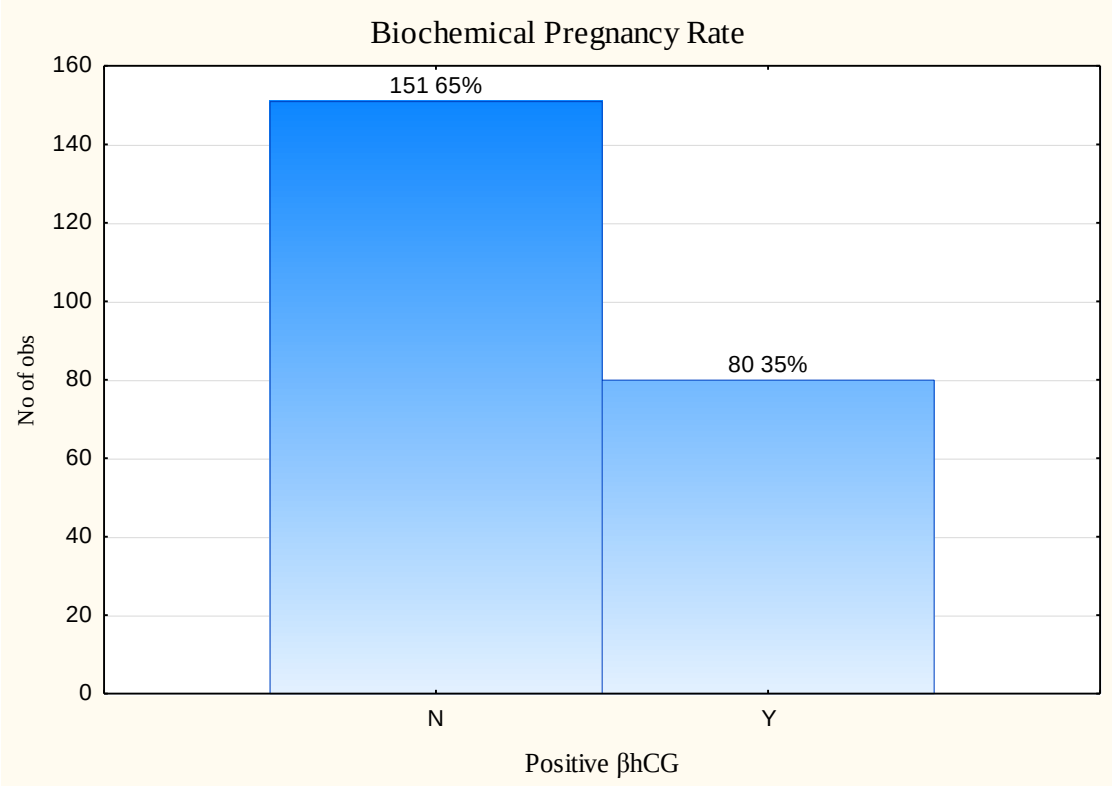

Figure 5.2: Biochemical Pregnancy Rates [2004-2014] (+ βHCG/ itirifcation/warming embryo transfer cycle)

No of obs = Number of vitirifcation/warming cycles

Figure 5.3 shows that the Clinical Pregnancy rate for the period 2013-2014 was 47% [18/42].

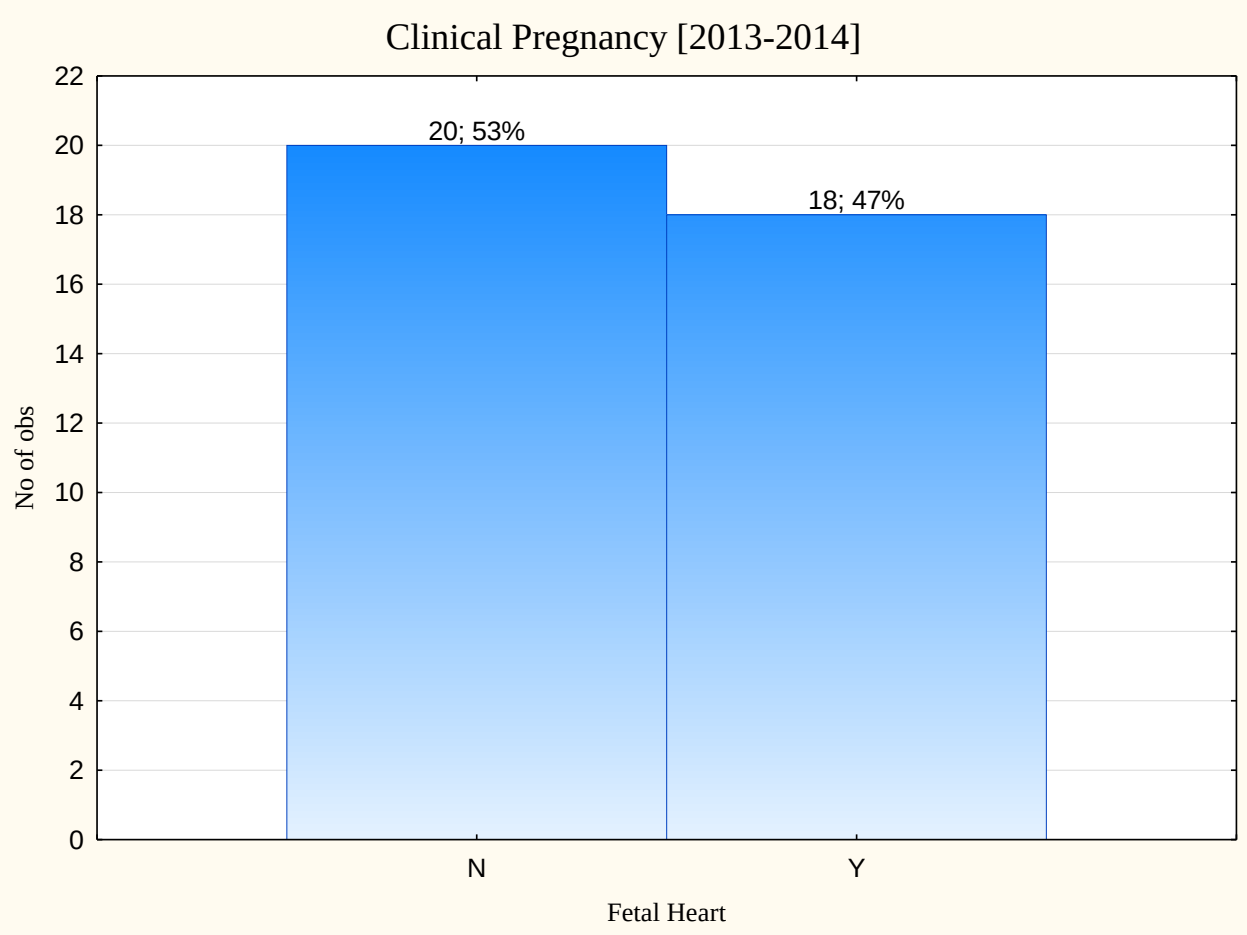

Figure 5.3: Clinical Pregnancy Rates [2013-2014] (fetal heart/ itirifcation/warming embryo transfer cycle)

. No of obs = Number of vitirifcation/warming cycles

Figure 5.4 shows that median and mean female (oocyte) age was calculated to be 31±5.3 years. The average age range was 20.0 – 46.0 years. [Number of cycles (N) = 231]

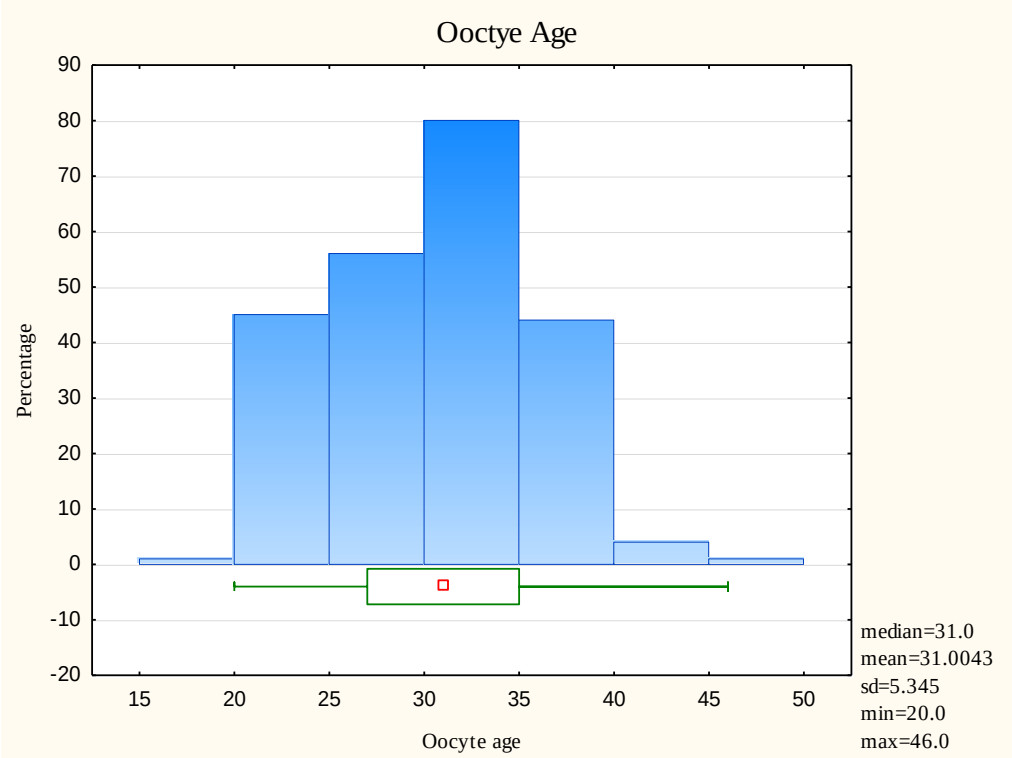

Figure 5.4: Incidence of female age (oocyte age in years) in vitrification/warming cycles [2004-2014]

Figure 5.5 shows that 15% [35/231] of the warming cycles had embryos frozen on Day 5, 77% [177/231] on Day 6 and 8% [19/231] on Day 7.

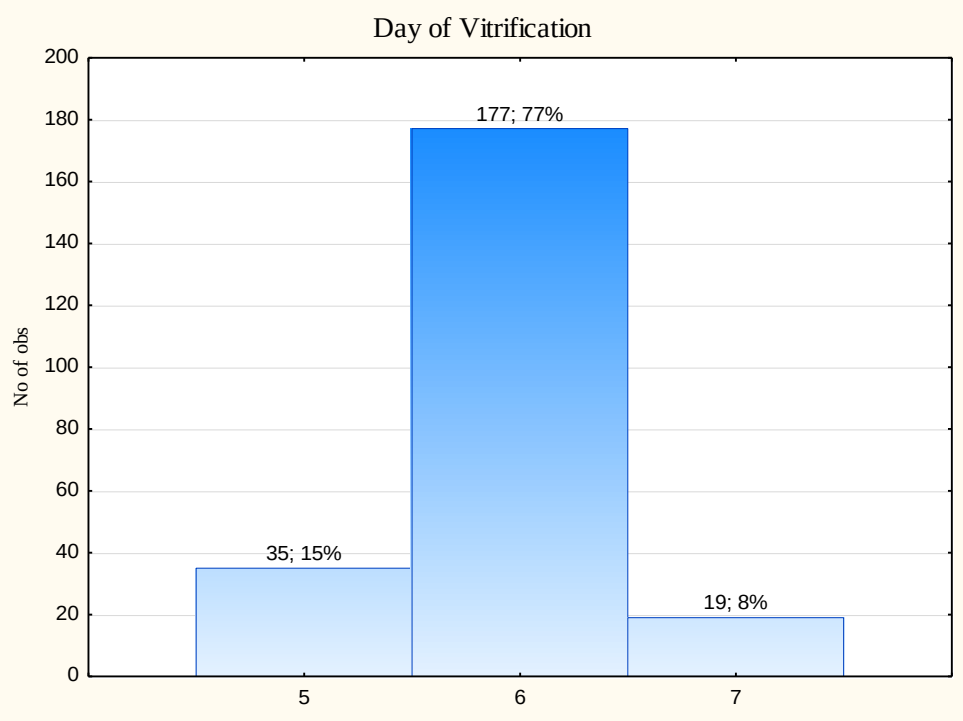

Figure 5.5: Incidence of vitrification in warming cycles on different days of blastocyst development [2004-2014]

No of obs = Number of vitirifcation/warming cycles

Figure 5.6 shows that 27% [63/231] of the warming cycles made use of donor eggs.

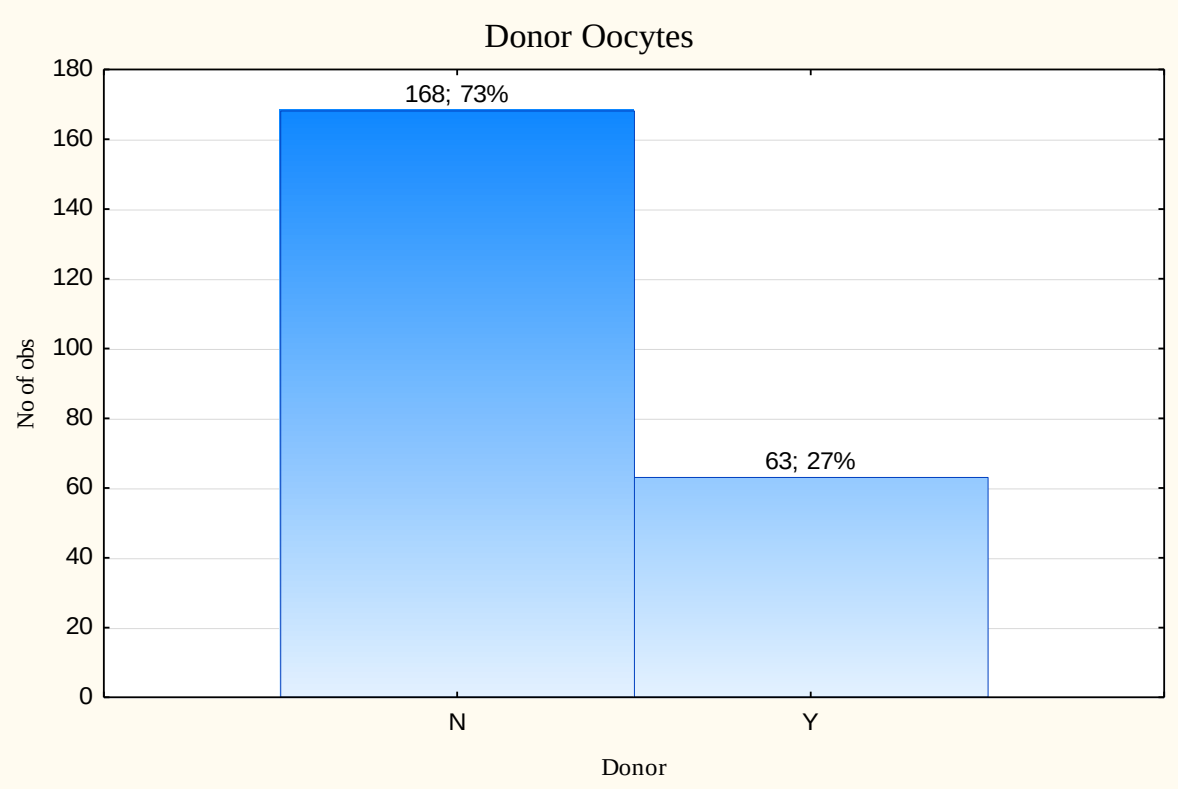

Figure 5.6: Incidence of Donor Oocyte vitrification cycles [2004-2014]

No of obs = Number of vitirifcation/warming cycles

Figure 5.7 shows that in 37% [86/231] of the warming cycles only 1 embryo transferred, while in 48% [111/231] cycles 2 embryos were transferred, in 14% [32/231] 3 embryos were transferred and in only 1% [2/231] 4 embryos were transferred.

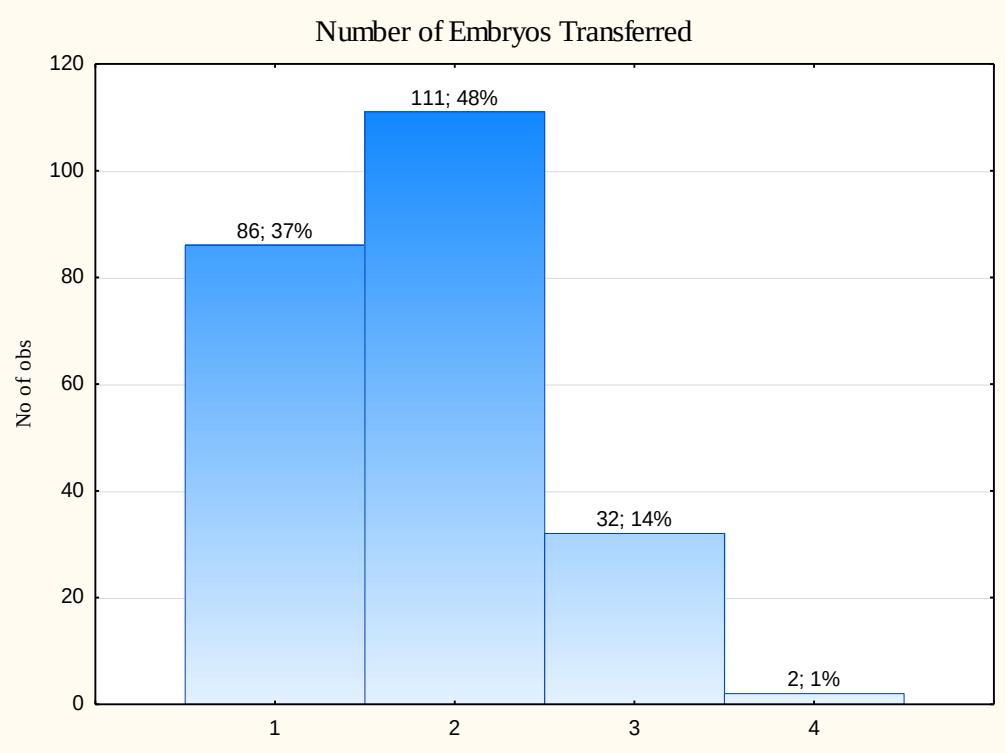

Figure 5.7: Number of embryos transferred/cycle [2004-2014]

No of obs = Number of vitirifcation/warming cycles

In each vitrification/warming cycle a total of either 1, 2, 3 or 4 embryos were vitrified respectively. Figure 5.8 shows that in 37% [85/231] of the cycles none of the embryos available to be vitrified were considered to be of good quality. 39% [89/231] of the cycles had only 1 embryo classified has good quality, 19% [43/231] had 2 good quality embryos, 5% [12/231] had 3 good quality embryos and only 1% [2/231] of the cycles had all four embryos transferred classified as good quality.

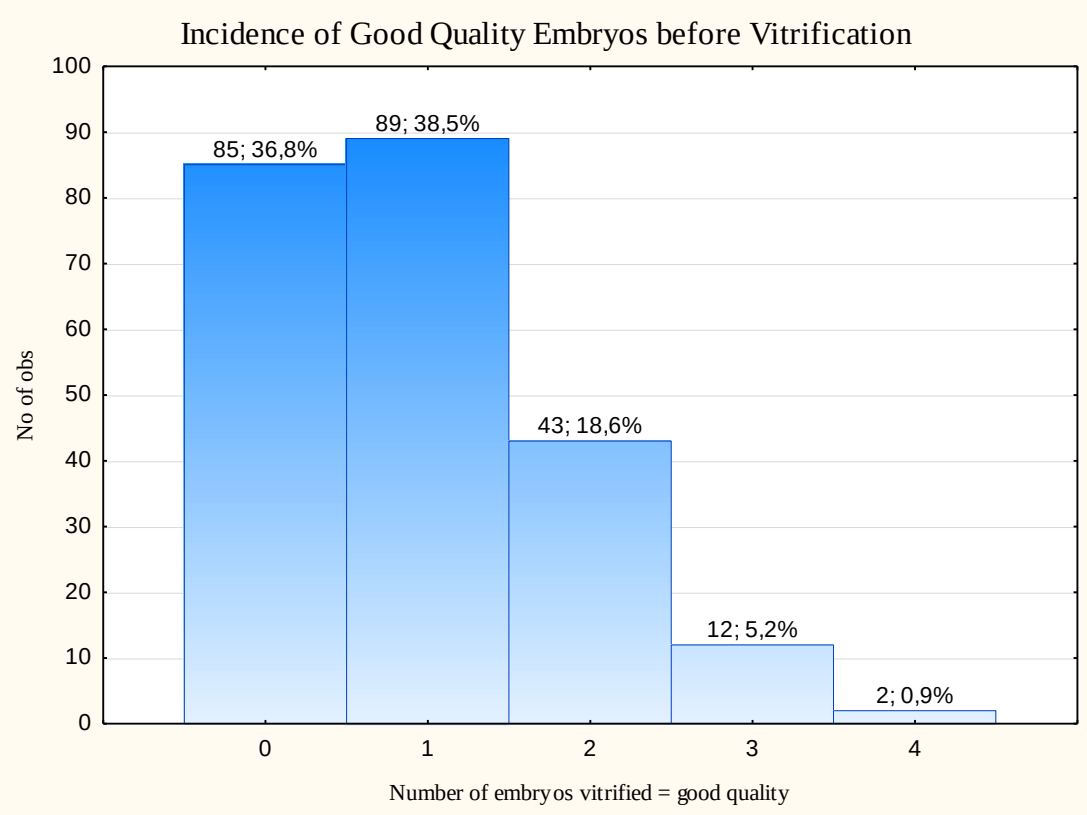

Figure 5.8: Incidence of Good Quality Embryos before Vitrification [2004-2014]

No of obs = Number of vitirifcation/warming cycles

Consistent with results from Figure 5.8, Figure 5.9 shows the incidence of good quality embryos in each cycle post warming. In 41% [95/231] of the warming cycles none of the embryos were considered to be of good quality. 42% [96/231] of the cycles had only 1 embryo classified has good quality, 16% [37/231] had 2 good quality embryos, 1% [2/231] had 3 good quality embryos and only 1 of the cycles had all four embryos transferred classified as good quality.

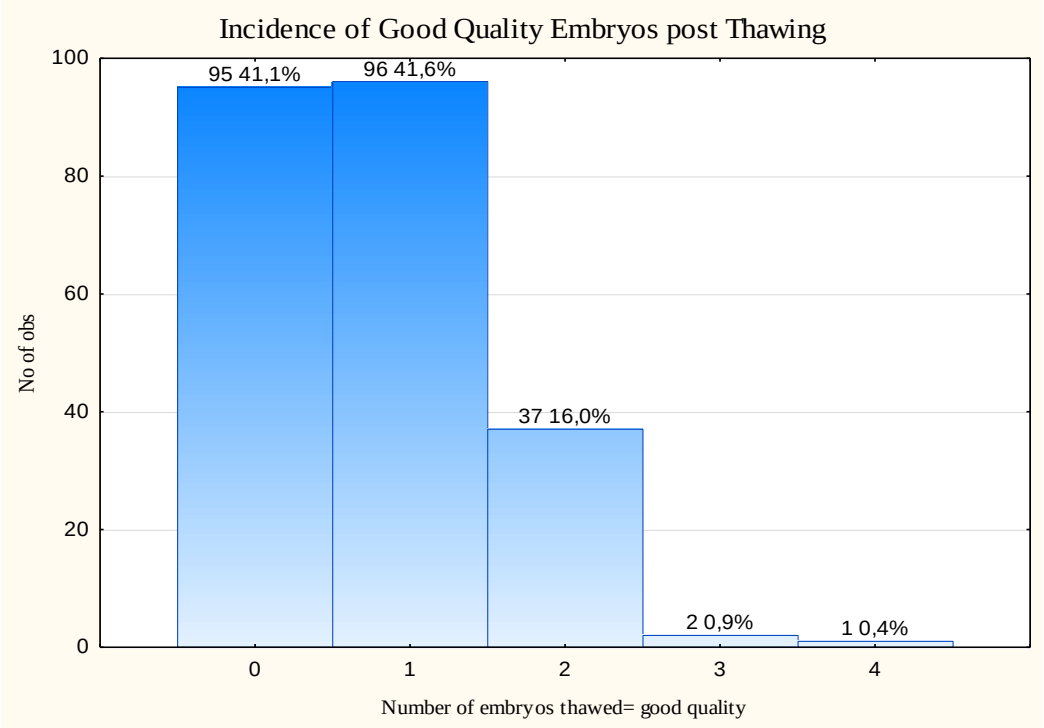

Figure 5.9: Incidence of Good Quality Embryos post Warming [2004-2014]

No of obs = Number of vitirifcation/warming cycles

An increased percentage of good quality embryos available for transfer was associated positively (but not significantly) with an increased biochemical pregnancy rate (Fishers Exact Test, one tailed, p=0.17). Good quality embryos prior to vitrification were also confirmed as having better survival rates post warming (p=0.17), although this was not statistically significant.

Figure 5.10 shows that 16% [38/231] of the warming cycles also had previous fresh cycle pregnancies.

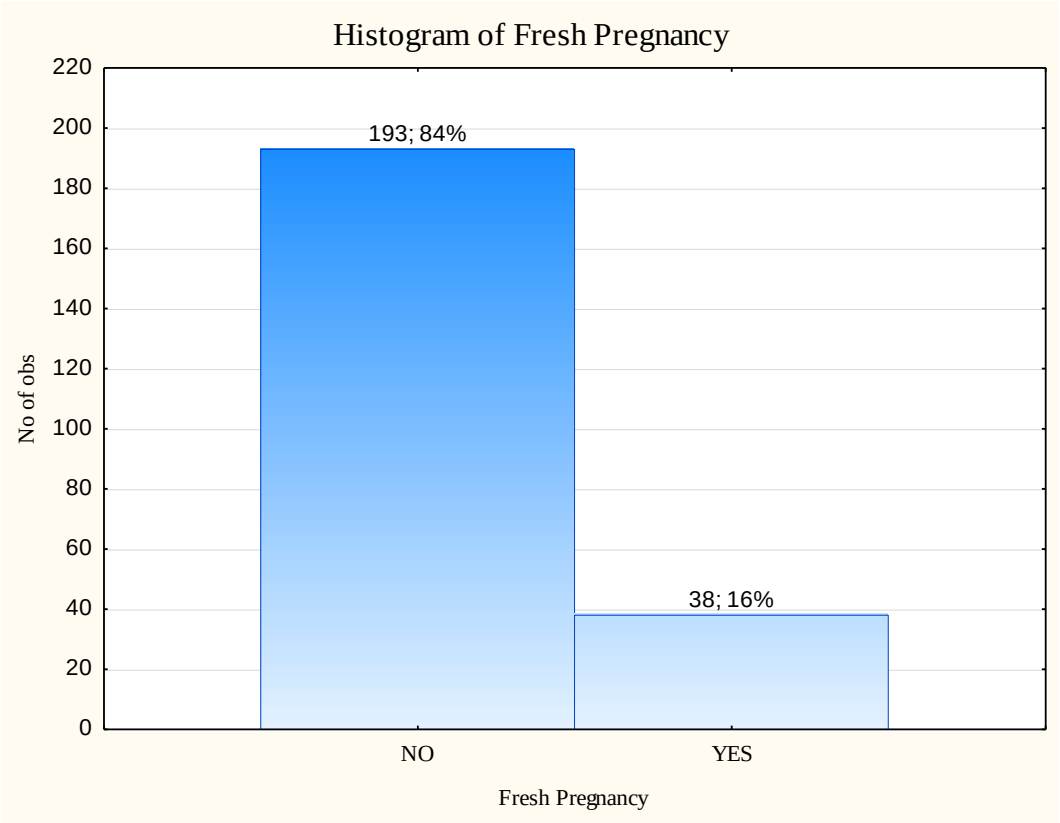

Figure 5.10: Incidence of Fresh Pregnancy Cycles in patients with frozen embryo transfers [2004-2014]

No of obs = Number of vitirifcation/warming cycles

Figure 5.11 shows that in the case of a fresh pregnancy where supernumerary embryos were vitrified, 41% [14/34] of the cycles went on to have positive pregnancy in subsequent FET cycles. In those cycles with no previous fresh cycle pregnancy, 37% [64/231] went on to have a positive pregnancy outcome in subsequent FET cycles. This, however, was non-significant (Fishers Exact Test, one tailed, p=0.39124).

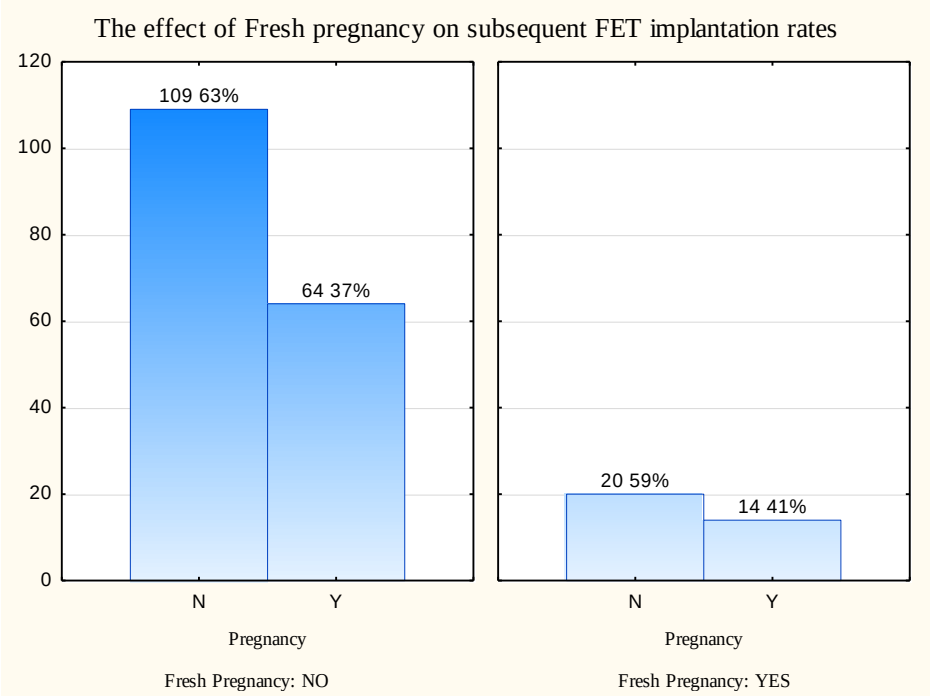

Figure 5.11: The incidence of FET pregnancy outcome in cycles where pregnancy in the initial fresh cycle was also obtained [2014-2014]

No of obs = Number of vitirifcation/warming cycles

5.2. The Effect of storage device on Pregnancy outcomes [Vitriplug® vs. Cryotop®]

The number of warming cycles analysed making use of the Vitriplug® device were significantly lower [n=24] than those using the Cryoptop® device [n =207]. This is due to the lower number of FET cycles being performed in the years before the Vitriplug® was replaced by the Cryoptop® as well as being due to the exclusion data.

The mean female ages (oocyte age) were 31.0±4.54 and 30.9±5.4 for the Cryotop® and Vitriplug® devices respectively.

It can be seen in Figure 5.12 that 80% [166/201] of the warming cycles vitrified with the Cryotop® device were shown to have 100% survival of embryos warmed per cycle. Only 63% [15/24] of those vitrified using the Vitriplug® device had a 100% survival rate. The fishers exact test found this to be significant at p=0.047, therefore the Cryotop® device had significantly better survival rates than the Vitriplug® device. 91.7% 166/181] of 100% survival in the total warming cycles was attributed to the Cryotop® device and 8.28% [15/181] was attributed to the Vitriplug® respectively.

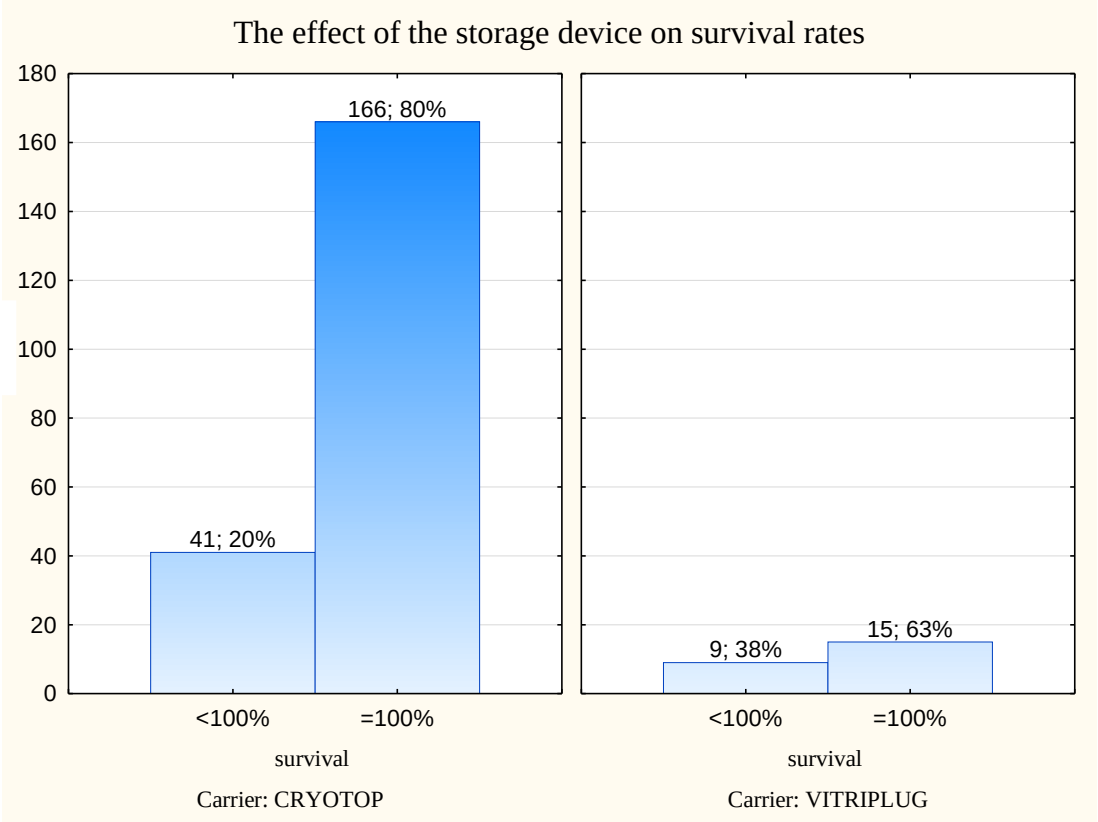

Figure 5.12: The effect of the storage device Cryotop® vs Vitriplug® on post warming survival rates [2014-2014]

No of Obs = Number of vitirifcation/warming cycles

This graph [Figure 5.13] shows that 38% [78/207] of the warming cycles where the Cryotop® device was used for vitrification were shown to have a positive βhCG. Only 8% [2/24] of cycles using the Vitriplug® device for vitrification had a positive βhCG. The Cryotop® device vitrification cycles resulted in a significantly higher biochemical pregnancy rate compared to that in the Vitriplug® device cycles (Fishers Exact Test, one tailed, p=0.0025). 97.5% [78/80] of the biochemical pregnancies in the total warming cycles was attributed to the Cryotop® device and 2.5% [2/80] was attributed to the Vitriplug® respectively.

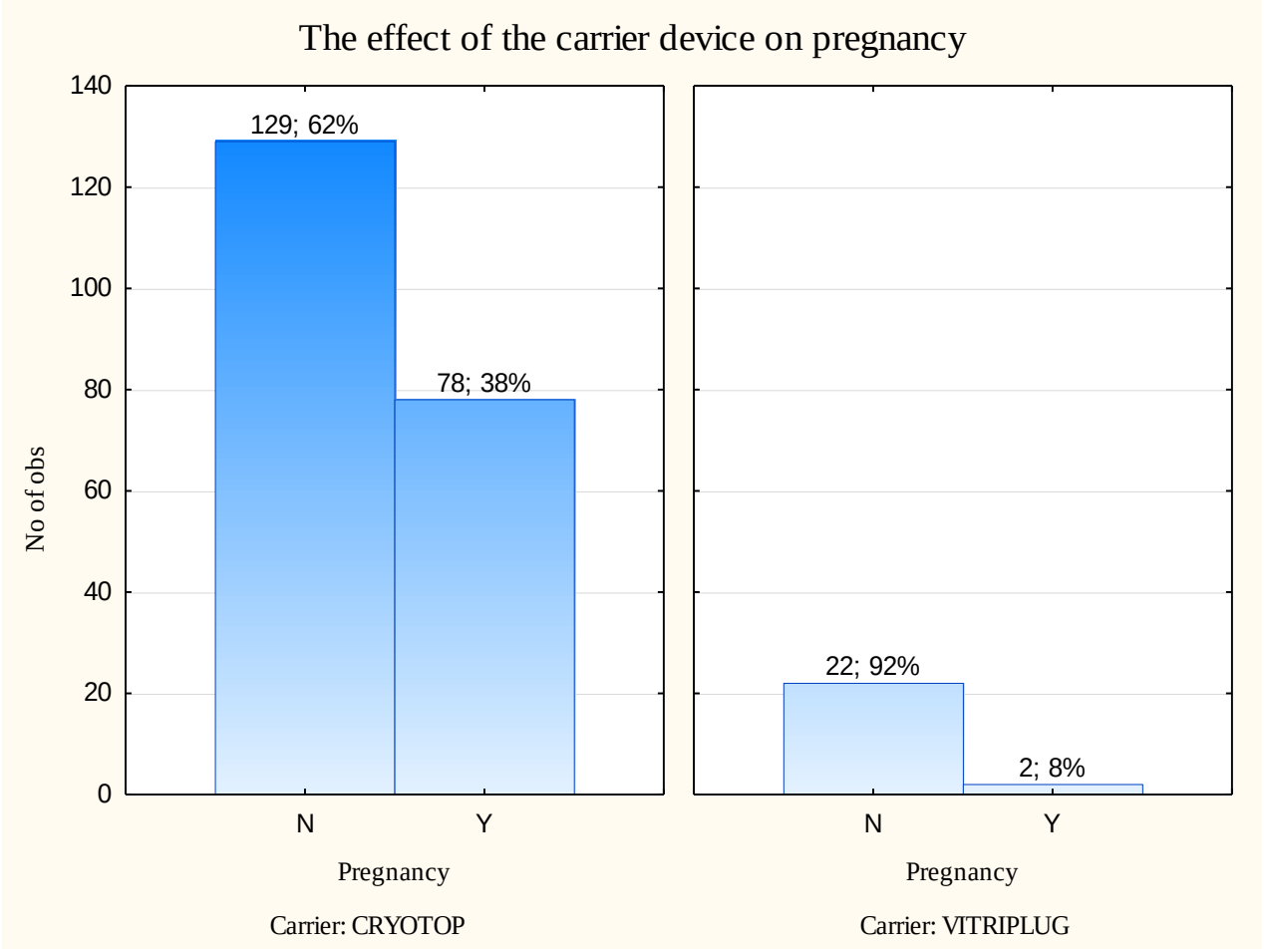

Figure 5.13: The effect of the storage device on biochemical pregnancy [2004-2014]

N= No (Negative Biochemical Pregnancy)

Y= Yes (Positive Clinical Pregnancy)

No of obs = Number of vitirifcation/warming cycles

5.3. *The Effect of Female age (oocyte age) on Pregnancy outcomes (post warming Survival, Biochemical Pregnancy and Clinical Pregnancy rates)*

In Figure 5.14 (Box-and-Whisker plot) it is shown that there is a strong trend, however it is non-significant (p=0.23), towards a decreased female age (oocyte age) being associated with the 100% embryo post warming survival group (31.5914).

[<100%: n=50; +100%: n= 181]

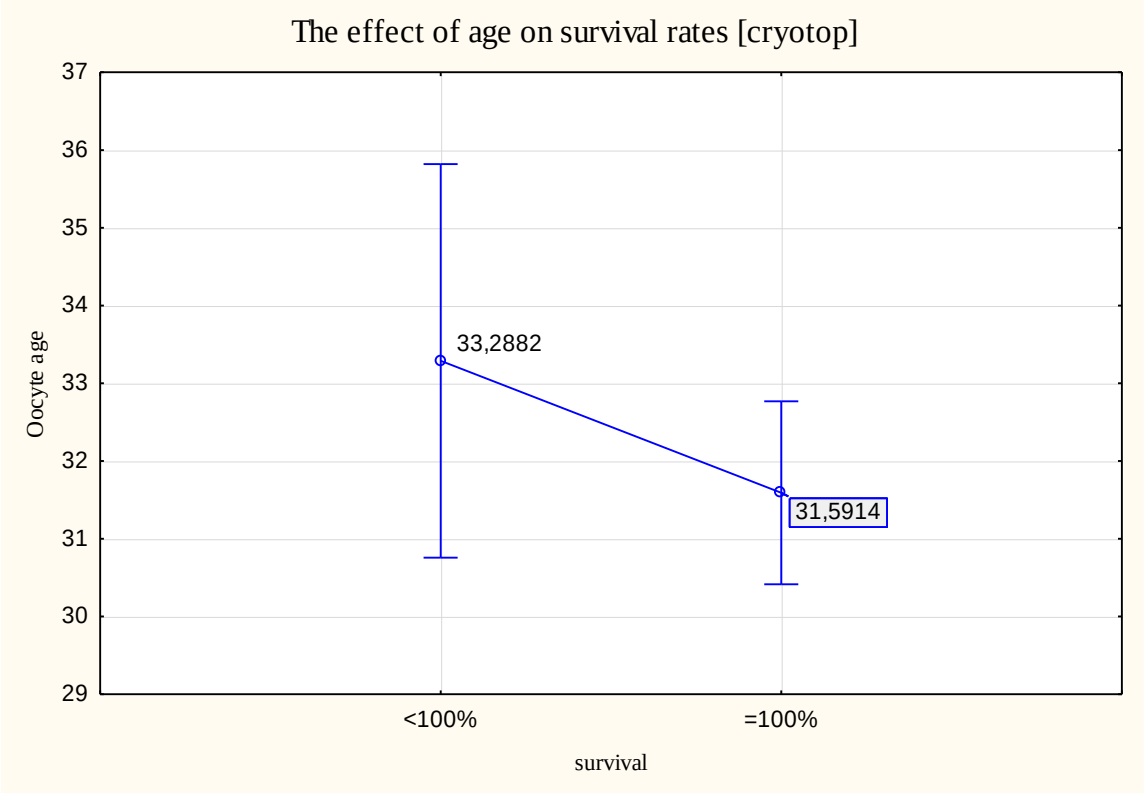

Figure 5.14: The effect of female age (oocyte age in years) on post warming embryo survival rates [2008-2014]

This Box-and-Whisker plot [Figure 5.15] shows that there is a strong trend (but not significant) towards a decreased female age (oocyte age = 30.4625) being associated with a positive biochemical pregnancy (Mann- Whitney u test, p=0.34).

[not pregnant: n=128; pregnant n=78]

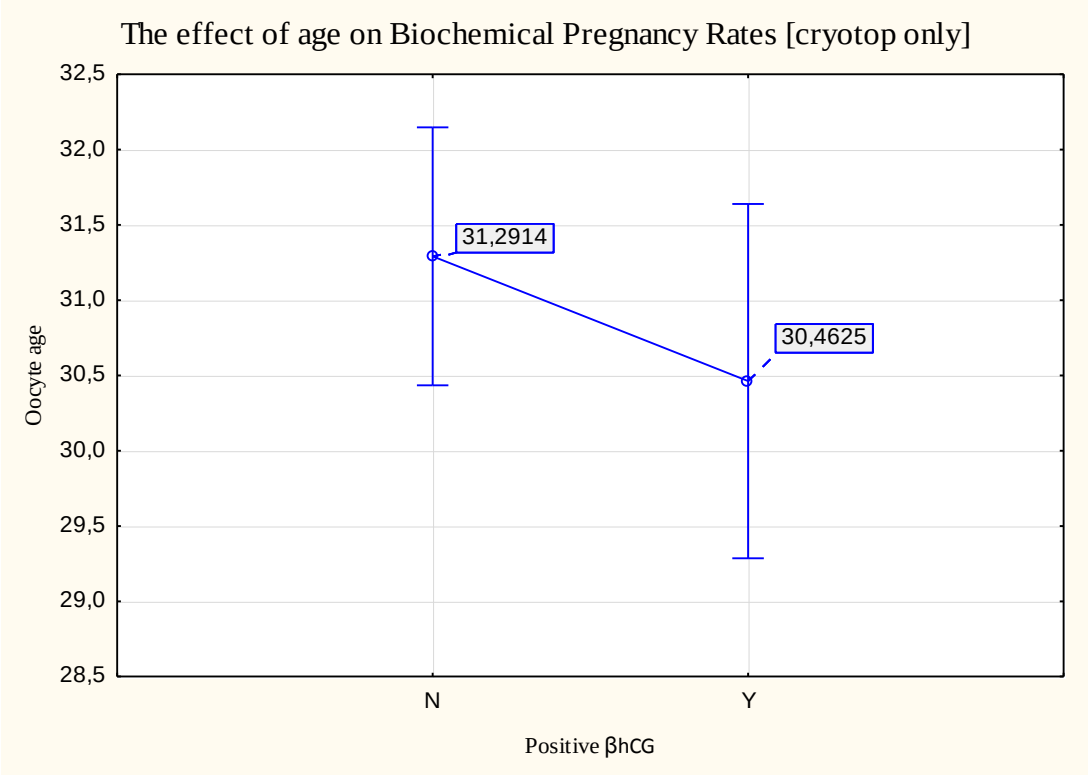

Figure 5.15: The effect of female age (oocyte age in years) age on Biochemical Pregnancy Rates [2008-2014]

N= No (Negative Biochemical Pregnancy)

Y= Yes (Positive Biochemical Pregnancy)

This Box-and-Whisker plot [Figure 5.16] shows that there was no effect of female age (oocyte age) on Clinical Pregnancy (p=0.90) between the 2 groups (30.75 vs 30.5556).

[fetal heart present: n=18; fetal heart not present: n=20]

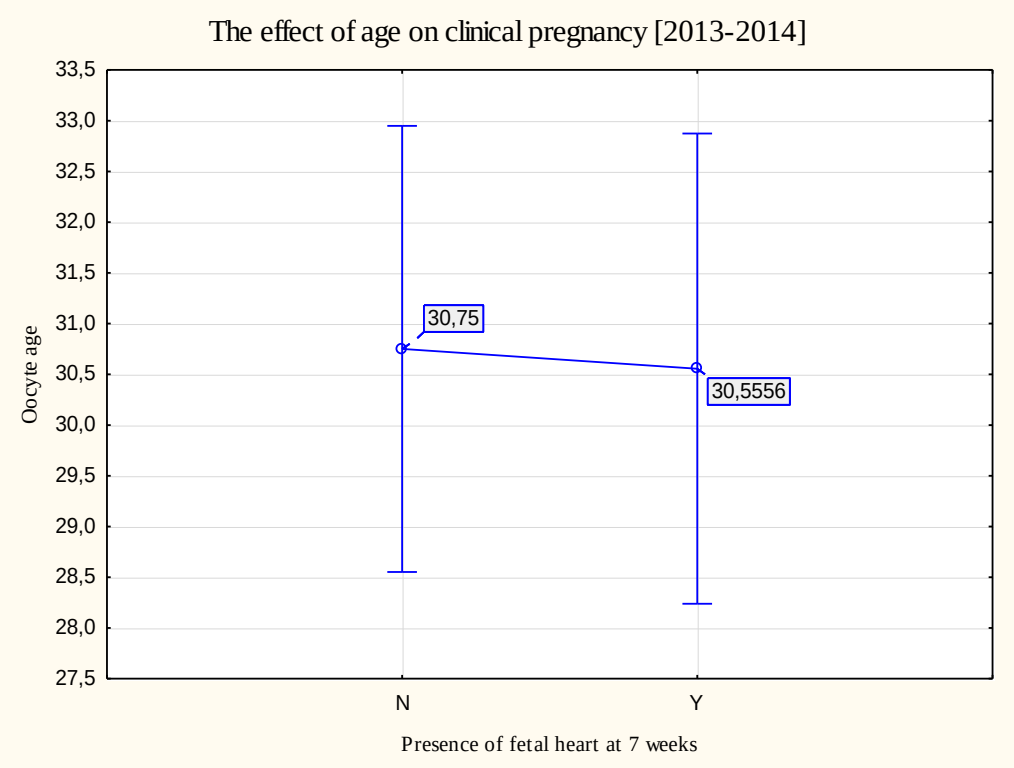

Figure 5.16: The effect of female age (oocyte age in years) on Clinical Pregnancy Rates [2013-2014]

5.4. *The Effect of day of vitrification on Pregnancy outcomes [Cryotop® data only]*

Figure 5.17 shows that 83% [24/29] of cycles having embryos vitrified on day 5 were associated with 100% survival while 79% [127/161] of the cycles with embryos vitrified on day 6 were associated with 100% survival. Fishers Exact Test (one tailed) reported this as being non-significant (p=0.42395), therefore the day of vitrification did not affect the survival outcome.

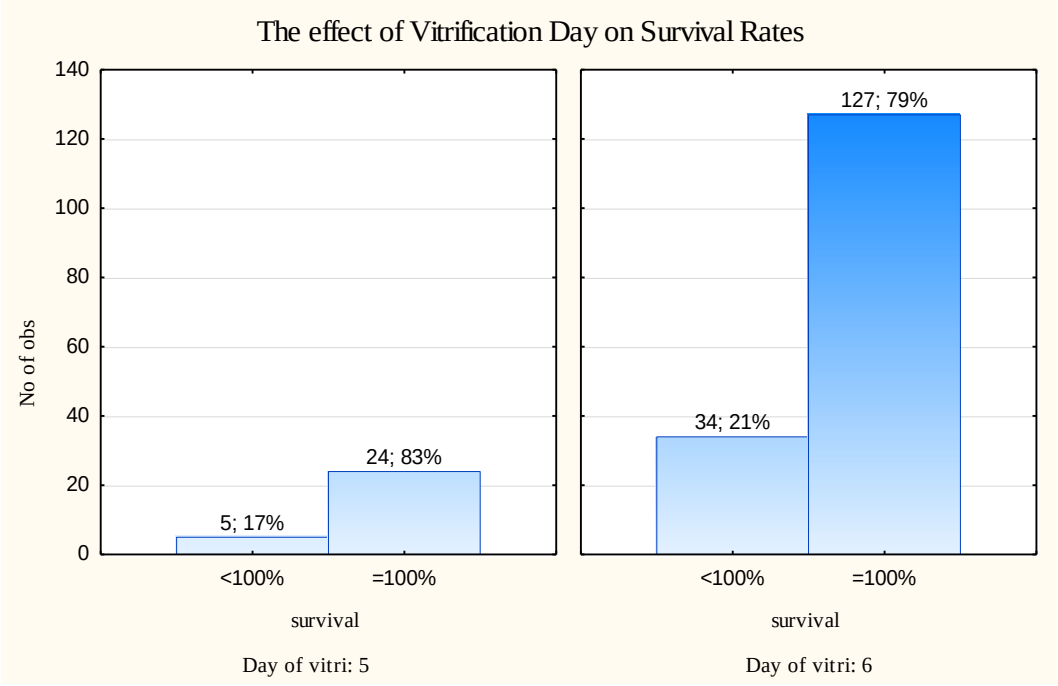

Figure 5.17: The effect of Vitrification Day (Day 5 or 6) on post warming Survival Rates [2008-2014 – Cryotop® data only]

No of obs = Number of vitirifcation/warming cycles

Figure 5.18 shows that 48% [14/29] of the cycles with embryos vitrified on day 5 had biochemical pregnancies, while only 36% [58/161] of those with vitrification on day 6 had biochemical pregnancies. The day of vitrification did not have a significant effect on the pregnancy outcome, with a Fishers Exact Test (one tailed) reporting a p value of 0.14840.

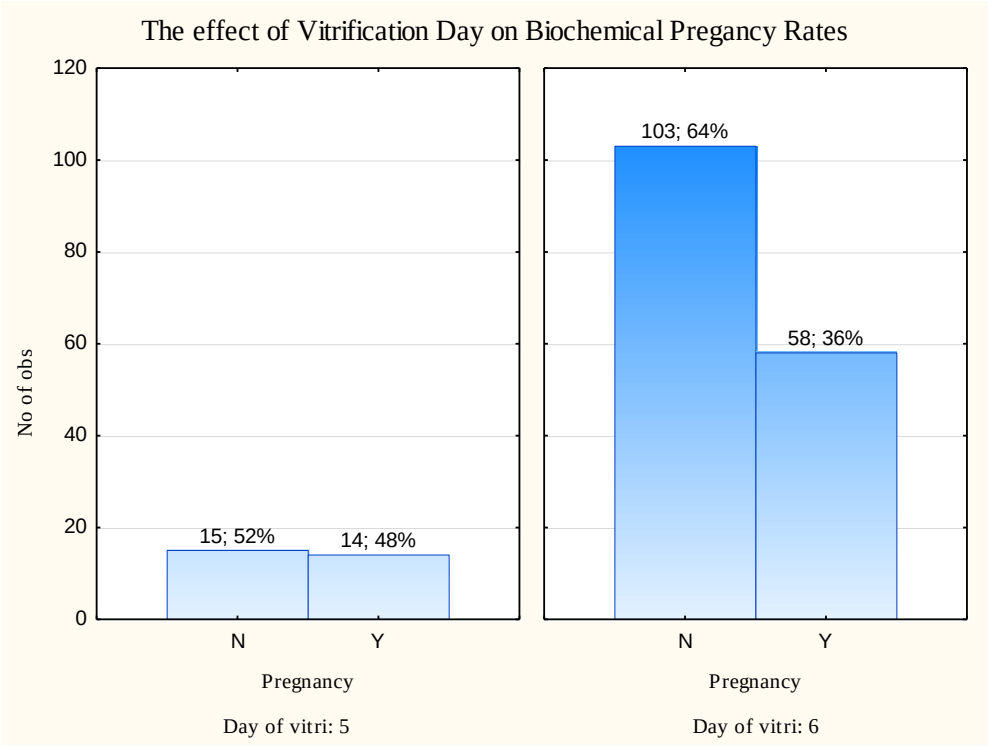

Figure 5.18: The effect of Vitrification Day (Day 5 or 6) on Biochemical Pregnancy rate Rates (2008-2014 – Cryotop® data only)

N= No (Negative Biochemical Pregnancy)

Y= Yes (Positive Biochemical Pregnancy)

No of obs = Number of vitirifcation/warming cycles

Figure 5.19 (2013-2014 data) shows that 57% [4/7] of the warming cycles with embryos vitrified on day 5 had clinical pregnancies, while only 45% [14/31] of those vitrified on day 6 had clinical pregnancies. Again this was non-significant (Fishers Exact Test, one tailed, p=0.43735) indicating that the day of vitrification did not affect the clinical pregnancy outcome.

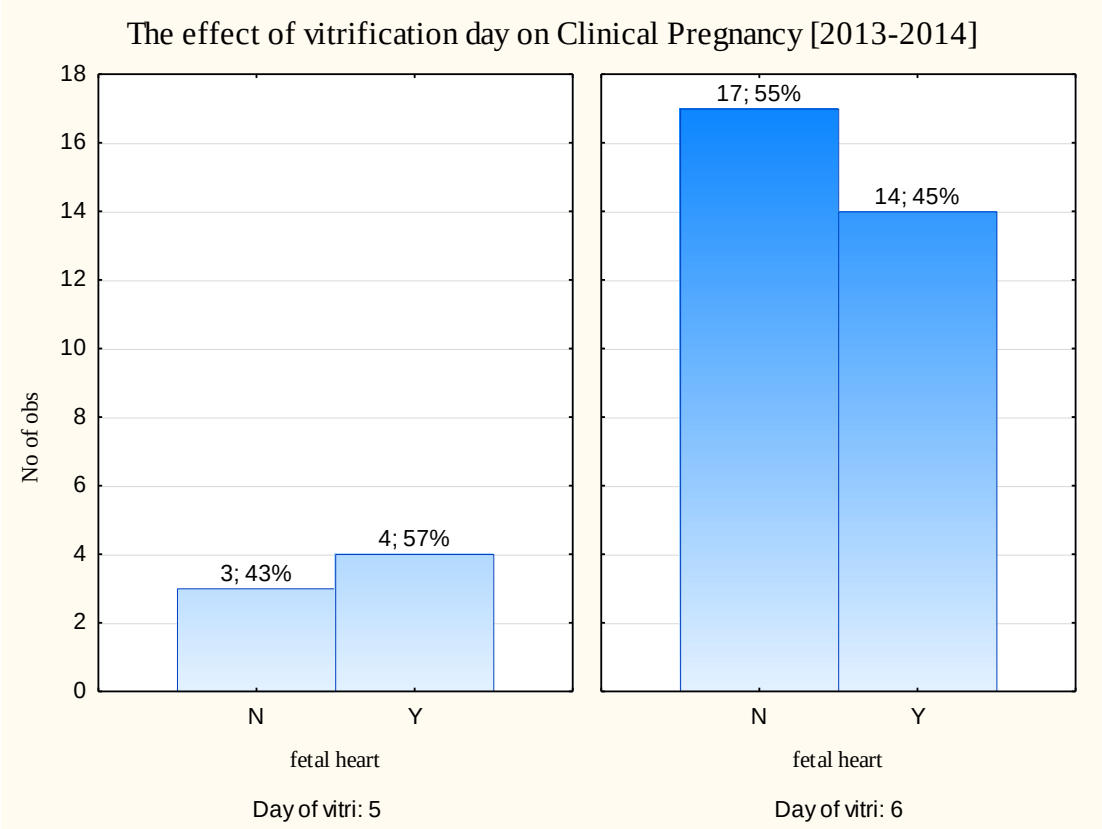

Figure 5.19: The effect of Vitrification Day (Day 5 or 6) on Clinical Pregnancy [2013-2014]

N= No (Negative Clinical Pregnancy)

Y= Yes (Positive Clinical Pregnancy)

No of obs = Number of vitirifcation/warming cycles

This Box-and-Whisker plot [Figure 5.20] shows that cycles having embryos vitrified on day 6 were significantly associated with patients of a lower age (31) than those (32.97) having vitrification on day 5 (p=0.03). The more favourable outcomes associated with vitrifying on day 5 are therefore not associated with a decreased female age (p=0.38).

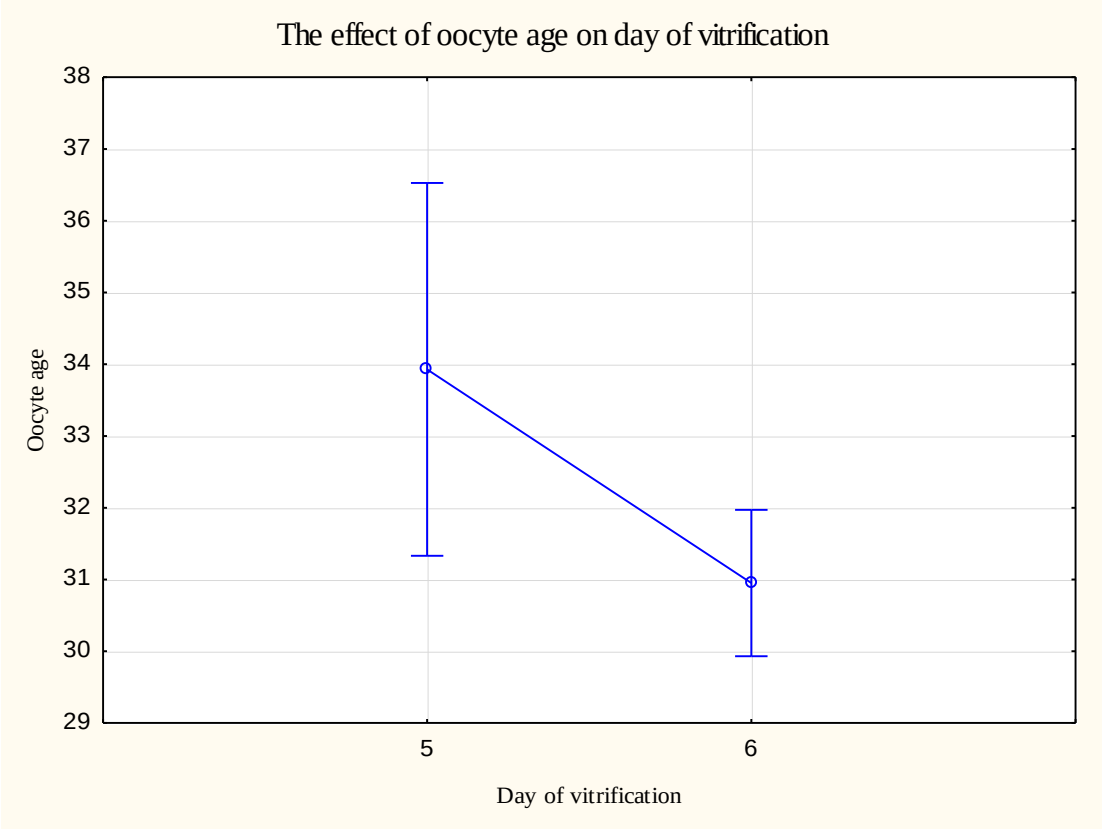

Figure 5.20: The association of female age (oocyte age in years) with the Day of Vitrification (day 5 or 6) (2008-2014 – Cryotop® data only)

Figure 5.21 indicates that within each group (Day 5 or Day 6 of vitrification) there was a trend towards a decreased female (oocyte) age being associated with a biochemical pregnancy. This Box-and Whisker plot shows that despite the trend, the oocyte age played no significant role in effecting the success on each day of vitrification (p=0.972).

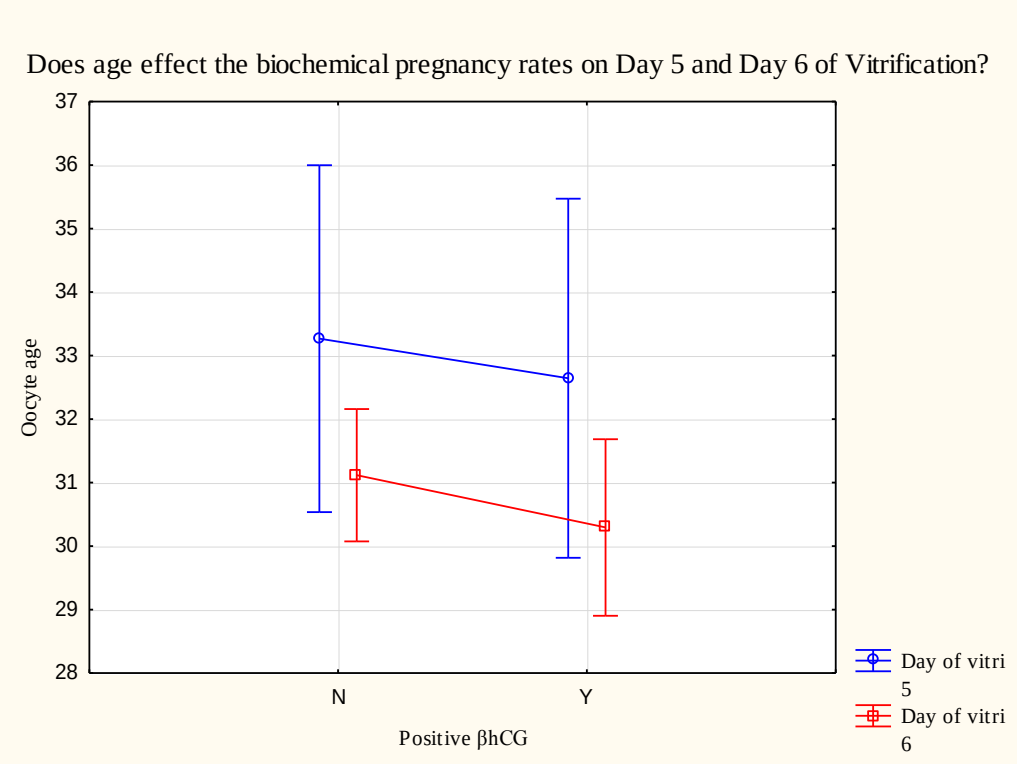

Figure 5.21: The effect of female age (oocyte age in years) on biochemical pregnancy rates specific to the Day of Vitrification (2008-2014 – Cryotop® data only)

N= No (Negative Biochemical Pregnancy)

Y= Yes (Positive Biochemical Pregnancy)

5.5. *The effect of the number of embryos transferred on pregnancy outcomes*

Figure 5.22 shows that as there was an increase in the number of embryos transferred in each cycle, so was there an increase in the incidence of a biochemical pregnancy. We can therefore say that the number of embryos transferred is significantly associated with an increase in biochemical pregnancy rates (Fishers Exact Test, one tailed, p=0.007). There was no correlation between the female age (oocyte age) and the number of embryos transferred (Pearsons Correlation Coefficient reported as R=-0.08).

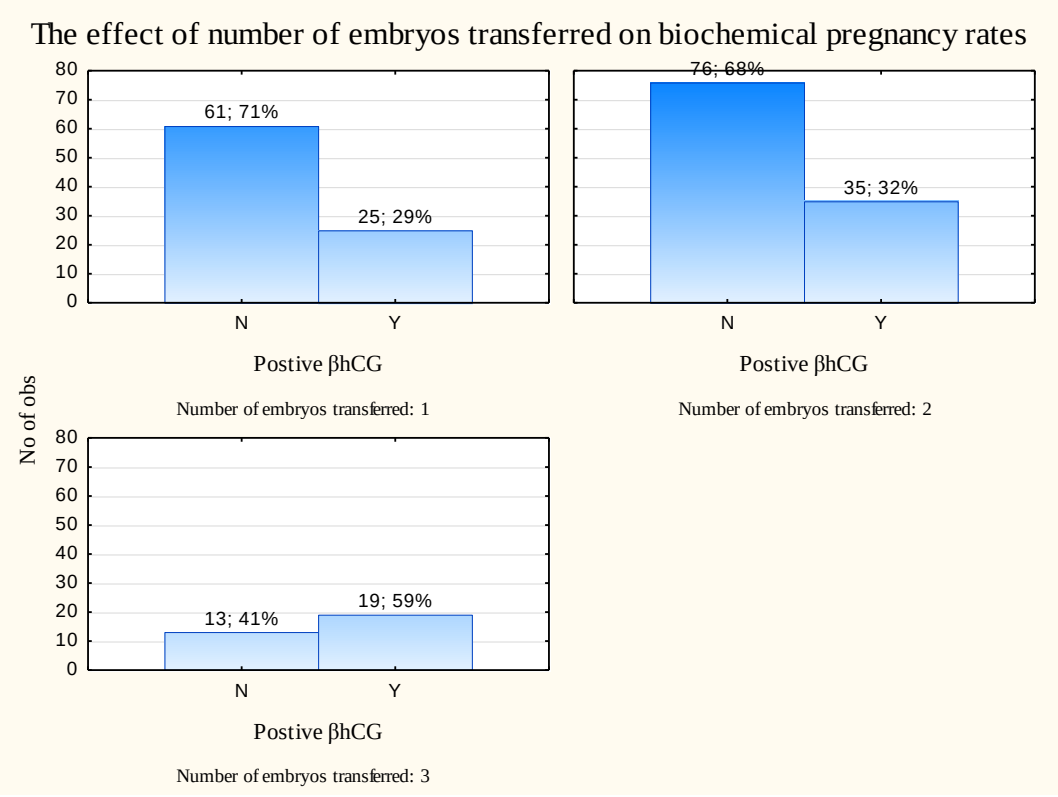

Figure 5.22: The effect of the number of embryos transferred on biochemical pregnancy rates [2004-2014]

N= No (Negative Biochemical Pregnancy)

Y= Yes (Positive Biochemical Pregnancy)

No of obs = Number of vitirifcation/warming cycles

5.6. The effect of using donor oocytes on Pregnancy Outcomes

Figure 5.23 shows that 82% [49/60] of the cycles using donated eggs had 100% survival when warmed. 80% [117/147] of the cycles not using donor oocytes reported 100% survival. Fishers Exact Test (one tailed) showed that the use of donor oocytes did not significantly affect the chance of 100% survival (p=0.44782).

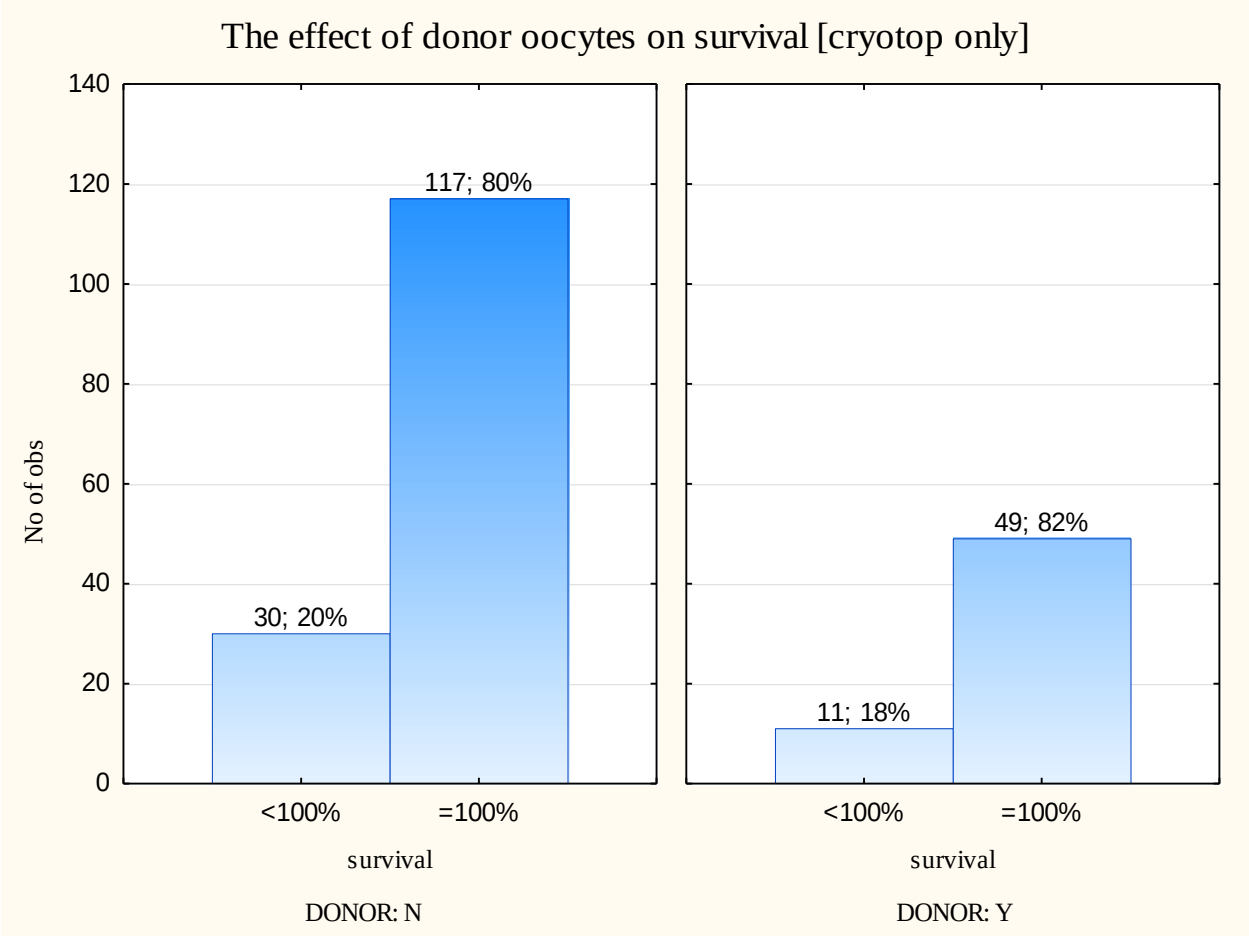

Figure 5.23: The effect of donor oocytes on post warming survival [2004-2014 - Cryotop® only]

No of obs = Number of vitirifcation/warming cycles

Figure 5.24 shows that Biochemical Pregnancies were observed in 40% [24/60] of the warming cycles using donor eggs. 37% [54/147] of the cycles not using donor eggs reported Biochemical Pregnancies. Fishers exact Test (one tailed) showed that the use of donor oocytes did not play a significant role on the pregnancy outcome (p=0.38720).

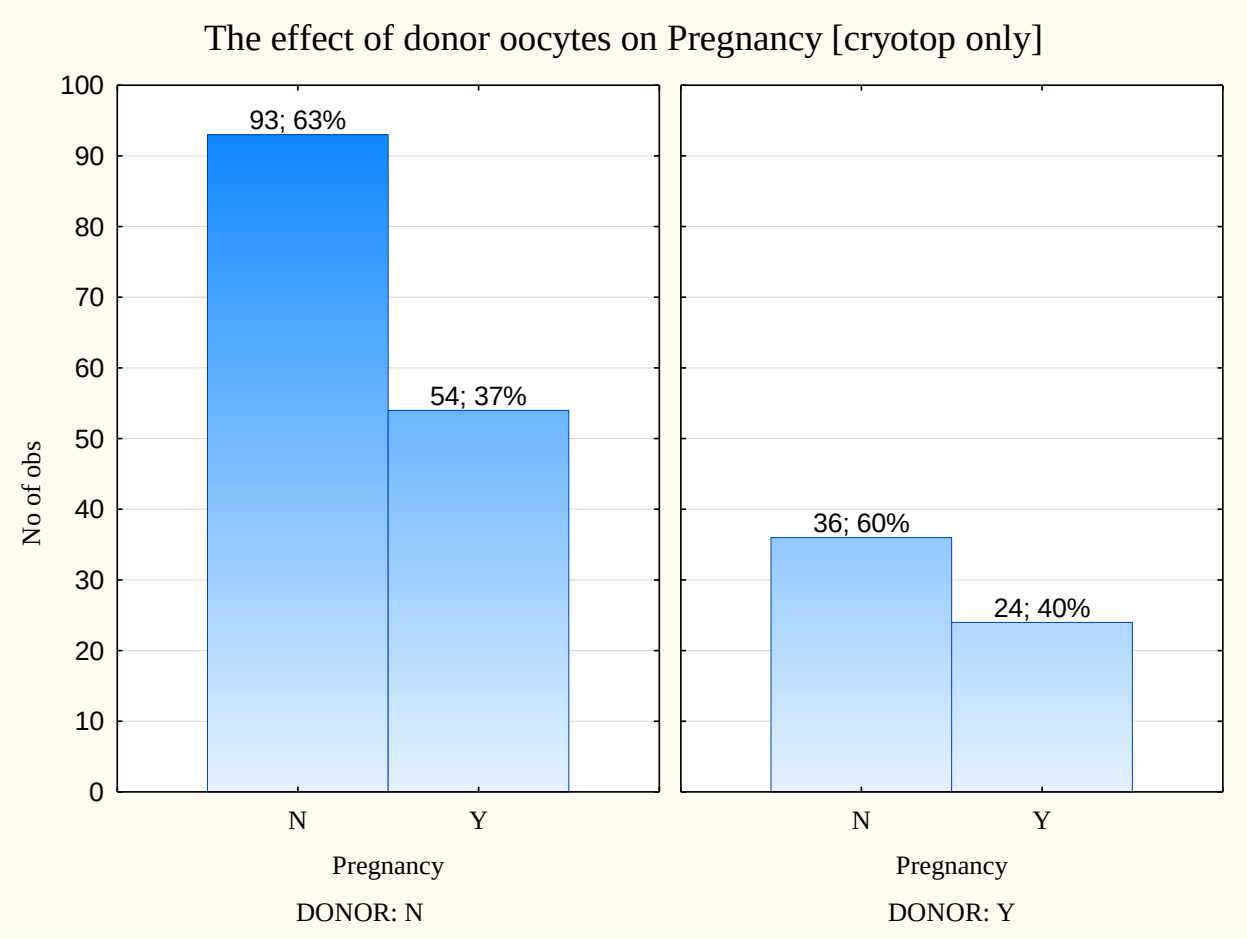

Figure 5.24: The effect of donor oocytes on pregnancy [2004-2014 – Cryotop® only]

N= No (Negative Biochemical Pregnancy)

Y= Yes (Positive Biochemical Pregnancy)

No of obs = Number of vitirifcation/warming cycles

Chapter Six

Discussion and Conclusion

6.1 Discussion

The objective of this dissertation was to look at the possible factors that can optimize vitrification/warming protocols using an established vitrification programme in place at *Drs Aevitas* Institute for Reproductive Medicine.

The main purpose of the vitrification program at *Drs Aevitas* Institute for Reproductive Medicine is for the management of supernumerary embryos. We can report that during 2013, for all age groups, the Clinical Pregnancy rate in fresh cycles was 42.4% [n=239/564] , which was slightly higher than the 40% [n=24/60] in frozen cycles. During 2014, again for all age groups, fresh cycles had a 35.8% (n=182/508) Clinical Pregnancy rate, which was marginally lower than the frozen cycles, which had a 36.5% [n=38/104) Clinical Pregnancy rate.

There is, therefore, no difference associated between the outcomes of the fresh and frozen cycles, which is consistent with recent literature (Roy *et al*., 2014). It is then plausible to state that there is a successful FET programme in practice at *Drs Aevitas* Institute for Reproductive Medicine.

As a result we wanted to analyse the outcomes, over a period of years, to determine which variables were involved.

Another objective was to determine ways in which the programme could be improved and consequently, we researched and proposed future studies.

To do this we designed a Retrospective Study to allow for data collection, and the analysis thereof, over the period of years dating 2004-2014. We have also designed a Prospective Study, which was carefully constructed after a thorough literature survey [Chapter Two] was conducted to address the continual need for improving outcomes in any Assisted Reproductive Technology programme.

Our study had limited numbers due to exclusion factors, and as a result we decided to concentrate on the vitrification/warming cycles as our primary objective was to analyse the outcomes. The exclusion factors included patient cycles where information was missing. This was advised by the statistician, to allow for a more precise and accurate comparison between all varying categories. As previously mentioned, over the years the recording of data and outcomes have become more formalised, resulting in the later years having a greater representation in the results. This representation is also due to the increase in the number of FET cycles performed in more recent years. Clinical Pregnancies were only calculated for the years 2013-2014, again due to the more recent implementation of a more structured recording system.

This study was also initially proposed including a Prospective Study that is divided into two phases. Due to a stipulation by The Health Research and Ethics Committee of the University of Stellenbosch, approval from the Minister of Health had to be obtained [2012 Amendment to the NATIONAL HEALTH ACT 61 OF 2003, Chapter 8, Section 57(4)] before continuing with the study. As a result of the novel process that it involves, and therefore the implications that it had on our time restraint, the prospective study could not be carried out yet and we have included the proposal as part of proposed Future Studies [Chapter Seven].

As the outcomes were the main focus of the study, emphasis was placed on the effect of female age (oocyte age); the vitrification carrier device; the day of vitrification; and the number of embryos transferred. We found that the carrier device as well as the number of embryos transferred had a significant effect on the outcomes.

6.1.2 The effect of female age (oocyte age) on vitrification/warming cycle outcomes

In 1994, Navot *et al*. conducted a study to evaluate the contribution of the uterus to age-related reproductive failure in women. To do this they harvested oocytes from a single donor during a specific cycle and distributed them evenly between young recipients and old recipients. The two recipient groups were classified according to female age, specifically 35.8 +/- 3.1 years and 44.0 +/- 3.1 years respectively. They found that 21.6% of the Clinical Pregnancies achieved were observed in the young recipients and 23.5% of Clinical Pregnancies were attributed to the older recipients. This indicates that it is the oocyte [donor] age and not the recipient female age that plays a role in the capacity to conceive and carry a conception to term. This was supported by Abdalla *et al*. (1997) who stated that the decline in fecundity with age cannot be explained by uterine factors alone. In 63 of the 231 [63/231] of the analyzed cycles donor oocytes were involved. In our study we therefore considered female age to be the age of the oocyte source instead of the recipient age of those embryos selected to by cryopreservation.

Although it is widely accepted that a reduced female age (oocyte age) would result in more favourable pregnancy outcomes (Piette *et al*., 1990; Van Noord-Zaadstra *et al*., 1991; Gilbert *et al*., 1999), our results only found strong trends towards this association rather than a significantly significant association. A reduced female age (ooctye age) was associated with the 100% post warming survival group with a P value of 0.23, and again the reduced female age (ooctye age) was associated with a positive biochemical pregnancy with a P value of 0.34.

The Clinical Pregnancies calculated for the period 2013-2014 showed no significant difference between positive and negative resultant Clinical Pregnancies (p=0.90). It is, however, important to note that the mean age of the entire sample was 30.5 ± 4.6 years. Those patients with a clinical pregnancy had a mean age of 30.5 ± 3.8 years and patients without a clinical pregnancy had a mean age of 30.75 ± 5.2 years. From the start, however, there is no significant difference in the age range between the pregnant and non-pregnant groups.

6.1.3 The effect of the day of vitrification on vitrification/warming cycle outcomes

As with female age (ooctye age) only strong trends were identified to indicate that the day of vitrification (day 5 or 6) played a role in the vitrification outcomes. This is evident with respect to the post warming survival as well as the biochemical and clinical pregnancies:

When clinical pregnancies from day 5 vitrified blastocysts were compared to those that resulted from Day 6 vitrified blastocysts , the outcome of the Day 5 group was superior but not significantly so (83% vs 79% respectively).

Biochemical pregnancies were shown to be unaffected by the day of vitrification (day 5 or 6) with observed rates of only 48% and 36% respectively.

When clinical pregnancies observed on day 5 were compared to those clinical pregnancies resulting from day 6 vitrification, day 5 resulted in a higher rates, however, this too was non-significant.

The data showed that there was a significant association between patients of reduced female age (oocyte age) and vitrification on day 6 ($p=0.03$). This indicates that the favourable outcomes seen in those patients having embryos vitrified on day 5, could not be attributed to female age.

From the retrospective data we calculated that 15% [35/231] of the vitrification/warming cycles had embryos frozen on day 5, while 77% [177/231] of vitrification/warming cycles had embryos vitrified on day 6.

Vitrification on Day 5 was therefore shown to be more favourable, however, non-significantly so. Liebermann and Tucker (2005) as well as Sunkara *et al*. (2010) showed that

Day 5 vitrification had higher success rates, although other studies present inconsistent results (Liebermann and Tucker, 2006; Richter *et al*., 2006; Shapiro *et al*., 2008).

It has to be emphasized however that, at *Drs Aevitas* Institute for Reproductive Medicine, day 6 vitrification mainly takes place when the embryo grading on day 5 is not regarded as optimal (Addendum 4). The embryos are left to develop another 24hours to facilitate a more efficient selection process. The majority of blastocysts vitrified on day 6 may therefore have reduced potential. This is different from a situation where day 6 vitrification takes place regardless of the quality of the embryo on day 5. This may be an explanation for our results. It also has to be noted that in practice, the laboratory personnel, in conjunction with the fertility specialist, make the final decision as to which day the embryos are vitrified. It is also common practice to delay vitrification to day 6 if it is thought that the blastocysts may develop further to better quality embryo a day later. This could account for the discrepancy in our results, where high percentages of embryos were considered to be poor quality according to the strict grading, however they underwent vitrification.

It is important to note that, for example, a compact (or morula) stage embryo on day 5 is classified as poor quality (Veeck, 2003), however they have been shown to produce pregnancies in our clinic and it often also vitrified. This suggests that embryos graded as poor quality do not necessarily effect the pregnancy outcomes.

6.1.4. The effect of the storage device on vitrification/warming cycle outcomes

The Vitriplug® vitrification cycle numbers are significantly lower than the Cryotop® numbers due to the aforementioned exclusion criteria as well as a reduced number of FET cycles being performed over the period of 2004-2008 when this storage device was used.

It is important to note that the culture conditions were consistent over the 10 year period. The same dishes as well as culture medium were used, with the same culture conditions subsequently being followed throughout.

The low sample number makes it difficult to accurately compare the two groups, however, significantly higher survival ($p=0.047$) and pregnancy rates ($p=0.0025$) were reported in the cycles using the Cryotop® device. The mean female ages (oocyte ages) were 30.9 (SD=5.4) and 31.0 (SD=4.54) for the Vitriplug® and Cryotop® devices respectively. This indicates that the difference in pregnancy outcomes could not be associated to female age (oocyte age) and therefore is due to the device itself.

Better results with the Cryotop® device were to be expected as it was designed with the purpose of, and later confirmed, to improve the success rates of vitrification (Vajta and Kuwayama, 2006; Kuwayama, 2007; Vanderzwalmen *et al*., 2007). The improved outcomes could also be attributed to improved technique and the technician skills. The construction of the Vitriplug® device meant that a larger volume of liquid surrounded the embryo. It was later shown that minimal surrounding liquid is essential to the successful vitrification process (Vanderzwalmen *et al*., 2007), which also requires excellent technical skill. The Cryotop® device, being much smaller in construction, ensures that a smaller volume of liquid surrounds the embryo (Vajta and Kuwayama, 2006; Vanderzwalmen *et al*., 2007). Consequently higher cooling and warming rates can be achieved, which is essential for success in the vitrification

process. With the evidently higher success rates depicted in our results, we too can confirm this phenomenon along in conjunction with the literature.

6.1.5. The effect of the number of embryos transferred on vitrification/warming cycle outcomes

The data shows that an increase in the number of embryos transferred had a positive association with an increased incidence of a biochemical pregnancy (p=0.007).

The American Society for Reproductive Medicine concluded that there was no difference between the pregnancy rates and live birth rates of single embryo transfers (SET) and double embryo transfers (DET). They also noted that SET was a more viable option for good prognosis patients (<35 years) than using selective methods for the most optimal embryo for transfer, and therefore confirming this exact guideline proposed by the ASRM in 2009.

Our results, however, are consistent with Jones *et al*. (1998b) who reported that, in their study, the number of embryos transferred had a significant effect on clinical pregnancy. A greater chance of a clinical pregnancy was associated with an increase in the number of embryos transferred. Le Lannou *et al*. (2006) also showed that the pregnancy rate was 27.6% in single embryo transfers and 36.9% in double embryo transfers in fresh cycles. Single embryo transfer pregnancy was significantly lower ($p<0.05$) and was consistent with pregnancy rates of 14.4% and 23.5% in frozen embryo transfers respectively (Le Lannou *et al*., 2006). It is important to note that 8 cell embryos were transferred in this study. In a Cochrane Review Summary conducted by Pandian *et al*. (2014) SET was associated with a lower live birth rate than DET in fresh cycles. Berin *et al*. (2011), however, compared SET and DET of frozen blastocysts transfers. Clinical pregnancy rates (50.4% vs. 34.7%), live birth rates (45.8% vs. 30.6%), and twin live birth rates (19.3% vs. 0) were significantly higher in the DET versus SET group respectively ($p < 0.05$). This too is consistent with our results.

6.2. Conclusion

We can therefore conclude that the Cryoptop® device (or similar, facilitating high cooling and warming rates) should be used in vitrification procedures, supporting the current implementation thereof at *Drs Aevitas* Institute for Reproductive Medicine.

We can suggest that embryos, consistent with the literature, be vitrified on Day 5 for more favourable pregnancy outcomes – but that day 6 vitrified embryos also contributes significantly to the positive outcomes in our clinic and should always be considered.

There are increasing amounts of Reproductive Institutions adopting SET approaches in both fresh and frozen cycles. From the data, and supporting literature previously mentioned (Jones *et al*., 1998b, Le Lannou *et al*., 2006, ASRM, 2012), we wouldn't propose the implementation of SET into routine cycles for optimal pregnancy outcomes.

When the total pregnancy outcomes at *Drs Aevitas* Institute for Reproductive Medicine for the period 2013-2014 were compared, it was evident that the pregnancy rate which resulted from FET cycles were higher than that of those that were achieved in fresh cycles. There is evidence to suggest that freezing all resulting embryos of sufficient quality in each respective cycle, for transfer at a later date, may be beneficial (Roque *et al*., 2013)

To further optimize the outcomes of the vitrification/warming programme we researched possible techniques that could facilitate this process. Assisted Hatching was found to be beneficial in numerous failed fresh cycles (Chao *et* al., 1997; Das, 2006; Martins *et al*., 2011) as well as in -women of increased age (Das, 2006; Liebermann, 2010). 11.68% [27/231] of patients in our established vitrification/warming programme had an oocyte age ≥38 years indicating that this could be a viable option.

Chapter Two identified trends to show the benefit of AH in frozen warmed embryos as well, but emphasised that the number of studies, to date, are too small to viably and significantly be compared. To clarify whether AH on frozen warmed blastocysts is indeed beneficial we have designed a prospective study, in which this can be tested, which is proposed in Chapter Seven (Future Studies).

Chapter Seven

Future Studies

7.1. Introduction

In order to confirm the benefit of AH in frozen warmed blastocysts, more evidence is required. We were able to identify trends in the Literature as well as in Chapter Two. This research encompasses varying types of Assisted Hatching performed at various embryo development stages, however, we have designed a Prospective Study to accurately test the hypothesis that Laser Assisted Hatching is beneficial in vitrified/warmed human blastocysts.

7.2. Prospective Study Phase I

Title:

Phase I: Prospective Study on Donated, Vitrified-Warmed Human Blastocysts

The aim of this study will be to compare the hatching and survival outcomes in the control and LAH groups to determine whether LAH is either detrimental, has no impact, or is beneficial in donated, vitrified-warmed human blastocysts.

The embryos are donated for research by patients who have already undergone treatment at the facility.

The Materials and Methods described in this Chapter pertain to standard protocols and procedures that patients frequenting *Drs Aevitas* Institute for Reproductive Medicine undergo during Assisted Reproductive cycles.

Detailed methodologies are presented as Addenda in Chapter Nine.

7.2.1. Methods

7.2.1.1. Patients

Standard IVF and ICSI, PICSI and IMSI patients with stored, vitrified blastocysts who have consented to donating their embryos for scientific research [Addendum 18] will be included in the study.

7.2.1.2. Ovarian Stimulation and oocyte aspiration

Different but standard, routine procedures were followed for ovarian stimulation. A routine oocyte aspiration protocol (using a sonar guided method) was followed [Addendum 7].

7.2.1.3. Semen preparation and insemination

Standard procedures for semen production and preparation were followed. Both the swim up and gradient centrifugation preparation methods were done depending on the semen characteristics [Addenda 5 and 6].

Standard IVF and ICSI insemination procedures were followed [Addenda 8 and 9].

7.2.1.4. Embryo culture and evaluation

All embryos were cultured and graded using standard protocols used at *Drs Aevitas* Institute for Reproductive Medicine [Addendum 2]. Blastocysts were graded according to the *Gardner and Schoolcraft* [Addenda 3 and 4] grading method (Veeck, 2003).

7.2.1.5. Blastocyst Vitrification

Supernumerary embryos/blastocysts of good quality after fresh embryo transfer were vitrified on day 5 or day 6 (and in some instances on day 7), following a modified protocol designed for Cryotop® (Kitzimato®) vitrification (Cobo *et al*., 2008) with accordance to the *Ferti-Pro® Vitri Freeze kit* protocol [Addendum 10], and currently being used at *Drs Aevitas* Institute for Reproductive Medicine.

7.2.1.6. Blastocyst Vitrification [Addendum 12]

- Vitrification was performed at room temperature and it was therefore ensured that the heated stage was turned off.
- Two pulled glass pipettes were made and used for embryo manipulation.
- A coloured Cryotop® was chosen and labelled with the patient's initials and surname, ID number and date of vitrification on alternating sides at the top of the Cryotop® using an IVF-safe permanent marker.
- Blastocysts with an expansion grading of 2 or higher were collapsed using a collapsing dish and micromanipulation needles. Collapsing was done at 37°C in drops of flushing medium covered with oil on a heated stage [Addendum 13].

  Note: it is not necessary to collapse a hatched or hatching blastocyst.
- Vitrification media (Fertipro® "VitriFreeze") was aliquotted into labelled Eppendorf tubes.

  1: VitriFreeze Pre-incubation medium (200µl per patient)

  2: VitriFreeze Freezing medium1 (100µl per patient)

  3: VitriFreeze Freezing medium2 (100µl per patient)

7.2.1.7. Blastocyst Warming [Addendum 14]

The blastocysts were warmed, again according to the routine, standard protocol in accordance with the *Ferti-Pro Vitri Warming* kit protocol (Addendum 4) used at *Drs Aevitas* Institute for Reproductive Medicine.

- Warming was performed on a heated stage measuring 37°C.
- Warming medium was warmed a minimum of 1 hour before (or the day before) to ensure a temperature of 37°C.
    - VitriWarming 1 (1000µl per patient)
    - VitriWarming2 (200µl per patient)
    - VitriWarming3 (200µl per patient)
    - VitriWarming4 (200µl per patient)
- A centre well dish was also incubated to 37°C in the same fashion.
- Enough pulled pipettes were available for blastocyst manipulation.

7.2.1.8. Laser Assisted Hatching on Donated Warmed Human Blastocysts [Addendum 19]

During a standard FET cycle, embryos are incubated for a minimum of three hours post warming before transfer. This allows for an accurate assessment of survival. In this experiment, the control embryos will be transferred to a NUNC dish for incubation in blastocyst medium, as described in the warming protocol [Addendum 14]. The embryos will be cultured for 24 hours at the standard conditions used in the ART laboratory. Both hatching and survival status, or more specifically whether or not the embryo has indeed survived or not

or hatched or not, will be noted after the 24 hour incubation period. Survival is defined as having fully expanded and is not yellow in colour or degenerate.

Experiment embryos to undergo LAH should be transferred to flushing drops (SAGE Quinn's Advantage ® Medium with HEPES + SAGE Quinn's Advantage ® Human Serum Albumin) covered with oil and then moved to the microscope fitted with laser and heated stage.

The laser method of assisted hatching will be performed on donated, vitrified blastocysts to ascertain hatching outcome and survival rates. The Vitrification files of the consenting patients will be collected and two groups will be identified. The two groups will be designed so that similar blastocyst qualities and development stages are represented equally in both groups.

Group 2 (LAH) we will follow the procedure below:

- During the last warming step of the warming protocol [Addendum 14] when blastocysts are in the 0.125M sucrose solution and still collapsed, blastocysts (in a HEPES buffered medium), will be transferred to the microscope equipped with the laser already set up for the laser procedure. The microscope makes use of a Zona Laser Treatment System (ZLTS) from Hamilton Thorne Research (Beverly, MA) (Tinney *et al*., 2005).
- All procedures will be done at 37ºC on heated stages.
- The zona pellucida will be lasered according to the protocol described in Addendum 19. In summary, 4-6 consecutive shots are fired to create an opening of roughly 1/8 of the circumference. This hole is created on the opposite to where the ICM is positioned to avoid any damage from occurring.

- Lasered blastocysts will then be transferred into the last medium of the warming kit and the rest of the procedure continued routinely [Addendum 14], cultured for 24 hours and hatching and survival rate will be noted.

7.2.1.9. Ethical Considerations

- The LAH method is a well published and implemented method worldwide with no known negative effects (some studies indicated positive outcomes)
- *Consideration of the implications of the National Health Act with regards to research involving embryos:*

  Phase I approval from the Minister of Health will be applied for once ethical approval from HREC of Stellenbosch University has been obtained.

  Participating patients will receive information and counselling before being included and will give informed consent [Addendum 18].

  Each patient will be allocated a number, to which only laboratory personnel can associate a name. This will ensure anonymity and confidentiality in the results, as well as in the retrospective portion of the study.

7.2.2. Statistical Analysis

Professor Martin Kidd, Director of the Centre for Statistical Consultation (CSC) at the University of Stellenbosch will be approached to analyze the data.

7.3. Prospective Study Phase II

Title:

Phase III: Prospective Randomized Control Trial

The aim of this study will be to compare the pregnancy outcomes in the control (non LAH) and LAH groups to determine whether LAH is beneficial in vitrified-warmed human blastocysts.

All patients undergoing an FET cycle will be counselled and made aware of the benefits and implications associated with LAH. All consenting patients will then be randomized according to a table [Addendum 21] to determine which patients undergo LAH and those who do not.

The Materials and Methods described in this Chapter pertain to standard protocols and procedures that patients frequenting *Drs Aevitas* Institute for Reproductive Medicine undergo during Assisted Reproductive cycles.

Detailed methodologies are presented as Addenda in Chapter Nine.

7.3.1. Methods

7.3.1.1. Patients

Standard IVF and ICSI, PICSI and IMSI patients with stored, vitrified blastocysts who have consented to LAH [Addendum 20] will be considered for the study. Patients will be counselled extensively in the benefits and implications of LAH before consenting.

7.3.1.2. Ovarian Stimulation and oocyte aspiration

Different but standard, routine procedures were followed for ovarian stimulation. A routine oocyte aspiration protocol (using a sonar guided method) was followed [Addendum 7].

7.3.1.3. Semen preparation and insemination

Standard procedures for semen production and preparation were followed. Both the swim up and gradient centrifugation preparation methods were done depending on the semen characteristics [Addenda 5 and 6].

Standard IVF and ICSI insemination procedures were followed [Addenda 8 and 9].

7.3.1.4. Embryo culture and evaluation

All embryos were cultured and graded using standard protocols used at *Drs Aevitas* Institute for Reproductive Medicine [Addendum 2]. Blastocysts were graded according to the *Gardner and Schoolcraft* [Addenda 3 and 4] grading method (Veeck, 2003).

7.3.1.5. Blastocyst Vitrification

Supernumerary embryos/blastocysts of good quality after fresh embryo transfer were vitrified on day 5 or day 6 (and in some instances on day 7), following a modified protocol designed for Cryotop® (Kitzimato®) vitrification (Cobo *et al*., 2008) with accordance to the *Ferti-Pro® Vitri Freeze kit* protocol [Addendum 10], and currently being used at *Drs Aevitas* Institute for Reproductive Medicine.

7.3.1.6. Blastocyst Vitrification [Addendum 12]

- Vitrification was performed at room temperature and it was therefore ensured that the heated stage was turned off.
- Two pulled glass pipettes were made and used for embryo manipulation.
- A coloured Cryotop® was chosen and labelled with the patient's initials and surname, ID number and date of vitrification on alternating sides at the top of the Cryotop® using an IVF-safe permanent marker.
- Blastocysts with an expansion grading of 2 or higher were collapsed using a collapsing dish and micromanipulation needles. Collapsing was done at 37°C in drops of flushing medium covered with oil on a heated stage [Addendum 13].

  Note: it is not necessary to collapse a hatched or hatching blastocyst.
- Vitrification media (Fertipro® "VitriFreeze") was aliquotted into labelled Eppendorf tubes.

  1: VitriFreeze Pre-incubation medium (200µl per patient)

  2: VitriFreeze Freezing medium1 (100µl per patient)

  3: VitriFreeze Freezing medium2 (100µl per patient)

7.3.1.7. Blastocyst Warming [Addendum 14]

The blastocysts were warmed, again according to the routine, standard protocol in accordance with the *Ferti-Pro Vitri Warming* kit protocol (Addendum 4) used at *Drs Aevitas* Institute for Reproductive Medicine.

- Warming was performed on a heated stage measuring 37°C.
- Warming medium was warmed a minimum of 1 hour before (or the day before) to ensure a temperature of 37°C.
    - VitriWarming 1 (1000µl per patient)
    - VitriWarming2 (200µl per patient)
    - VitriWarming3 (200µl per patient)
    - VitriWarming4 (200µl per patient)
- A centre well dish was also incubated to 37°C in the same fashion.
- Enough pulled pipettes were available for blastocyst manipulation.

7.3.1.8. Laser Assisted Hatching on Consenting, Randomized Patients [Addendum 19]

As with the experimental (LAH) embryos in Phase II, those patients whose embryos are randomly allocated to the LAH group, in this phase, will be transferred to a NUNC dish for incubation in blastocyst medium, as described in the warming protocol [Addendum 14].

Embryos to undergo LAH should be transferred to flushing drops (SAGE Quinn's Advantage ® Medium with HEPES + SAGE Quinn's Advantage ® Human Serum Albumin) covered with oil and then moved to the microscope fitted with laser and heated stage.

The laser method of AH will then be performed according to the protocol described in Addendum 19.

The LAH embryos will follow the procedure below:

- During the last warming step of the warming protocol [Addendum 14] when blastocysts are in the 0.125M sucrose solution and still collapsed, blastocysts (in a HEPES buffered medium), will be transferred to the microscope equipped with the laser already set up for the laser procedure. The microscope makes use of a Zona Laser Treatment System (ZLTS) from Hamilton Thorne Research (Beverly, MA) (Tinney *et al*., 2005).
- All procedures will be done at 37ºC on heated stages.
- The zona pellucida will be lasered according to the protocol described in Addendum 19. In summary, 4-6 consecutive shots are fired to create an opening of roughly 1/8 of the circumference. This hole is created on the opposite to where the ICM is positioned to avoid any damage from occurring.
- Lasered blastocysts will then be transferred into the last medium of the warming kit and the rest of the procedure continued routinely [Addendum 14], and left to culture for 3 hours to ensure survival before ET.

7.3.1.9. Embryo Transfer [Addendum 16]

A minimum incubation time of three hours post warming was utilized prior to blastocyst transfer. This allowed adequate time to ensure survival of the frozen/warmed blastocyst.

A standardized blastocyst transfer method (using sonar guidance) will be performed. Blastocysts will be transferred after the patient has been instructed to present with a full bladder.

7.3.1.10. Human Chorionic Gonadotropin (HCG) Levels

A quantitative βHCG will performed to determine early pregnancy, which can be extrapolated to an implantation rate. Blood was drawn according to standard procedure [Addendum 17] using a Vacutainer © on days 10 and 14 following embryo transfer.

7.3.1.11. Pregnancy

Patients will be continuously monitored throughout their pregnancy to determine the outcome.

7.3.1.12. Ethical Considerations

- The LAH method is a well published and implemented method worldwide with no known negative effects (some studies indicated positive outcomes)
- *Consideration of the implications of the National Health Act with regards to research involving embryos:*

  Phase II approval from the Minister of Health will be applied for once ethical approval from HREC of Stellenbosch University has been obtained.

  Participating patients will receive information and counselling before being included and will give informed consent [Addendum 20].

  Each patient will be allocated a number, to which only laboratory personnel can associate a name. This will ensure anonymity and confidentiality in the results, as well as in the retrospective portion of the study.

7.3.2. Statistical Analysis

Professor Martin Kidd, Director of the Centre for Statistical Consultation (CSC) at the University of Stellenbosch will be approached to analyze the data.

Chapter Eight

References

- Abdalla H, Wren M, Thomas A *et al*. Age of the uterus does not affect pregnancy or implantation rates; a study of egg donation in women of different ages sharing oocytes from the same donor. Human Reproduction 1997;12:827-829.

- AbdelHafez F, Desai N, Abou-Setta A *et al*. Slow freezing, vitrification and ultra-rapid freezing of human embryos: a systematic review and meta-analysis. Reproductive Biomedicine Online 2010;20:209-222.

- Ahlstrom A, Westin C, Reismer E *et al*. Trophectoderm morphology: an important parmeter for predicting live birth after single blastocyst transfer. Human Reproduction 2011;26:3289-3296.

- Alikani M, Calderon G, Tomkin G *et al*. Cleavage anomalies in early embryos and survival after prolonged culture in vitro. Human Reproduction 2000;15:2634-2643.

- Alpha Scientists in Reproductive Medicine. The Alpha consensus meeting on cryopreservation key performance indicators and benchmarks: proceedings of an expert meeting. Reproductive Biomedicine Online 2012;25:146–167.

- Antinori S, Selman H, Caffa B *et al*. Zona opening of human embryos using a non-contact UV laser for assisted hatching in patients with poor prognosis of pregnancy. Human Reproduction 1996;11:2488-2492.

- Aplin JD. The cell biological basis of implantation. Baillieres Best Practical Resource for Clinical and Obstetrical Gynaecology 2000;14:757–764.

- Aran B, Sole M, Rodrigues-Pizà *et al*. Vitrified blastocysts from Preimplantation Genetic Diagnosis (PGD) as a source for human Embryonic Stem Cell (hESC) derivation. Springer Science and Business Media LLC (2012).

- American Society for Reproductive Medicine (ASRM). Role of Assisted hatching in in vitro fertilization: A review of the literature. A committee opinion. Fertility and Sterility 2008;90:196-198.

- ASRM. Guideline of number of embryos transferred. Fertility and Sterility 2009;92:1518–1519.

- ASRM Practice Committee. Role of Assisted hatching in in vitro fertilization: A guideline. Fertility and Sterility 2014;102:348-351.

- Balaban B, Urman B, Sertac A *et al*. Blastocyst quality affects the success of blastocyst-stage embryo transfer. Fertility and Sterility 2000;74:282–287.

- Balaban B, Urman B, Yakin K *et al*. Laser assisted hatching increases pregnancy and implantation rates in cryopreserved embryos that were allowed to cleave in-vitro after warming: a prospective randomised study. Human Reproduction 2006;21:2136-2140.

- Biggers J, McGinnis L and Lawitts J. One-step versus two-step culture of mouse preimplantation embryos: is there a difference? Human Reproduction 2005;20:3376–84.

- Berin I, McLellan ST, Macklin EA *et al*. Frozen-thawed embryo transfer cycles: clinical outcomes of single and double blastocyst transfers. Journal of Assisted Reproduction and Genetics 2011;28:575–581.

- Biggers J. IVF and embryo transfer: historical origin and development. Reproductive BioMedicine Online 2012;25:118–127.

- Bielfield P, Faridi A, Zaneveld LJ *et al*. The zona pellucida-induced acrosome reaction of human spermatozoa is mediated by protein kinases. Fertility and Sterility 1994;61:536-541.

- Bjercke S, Tanbo T, Dale P *et al*. Human chorionic gonadotrophin concentrations in early pregnancy after in-vitro fertilization. Human Reproduction 1999;14:1642-1646.

- Blake D, Forsebrg A, Johansson B *et al*. Laser zona pellucida thinning-alternative approach to assisted hatching. Human Reproduction 2001;16:1959-1964.

- Blake D, Proctor M and Johnson N. The merits of blastocyst versus cleavage stage embryo transfer: a Cochrane review. Human Reproduction 2004;19:795–807.

- Blake D, Glujovsky D, Bardach A *et al*. Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology: A Cochrane review. Wiley Online Library (2012).

- Bleil J and Wassarman P. Sperm-egg interactions in the mouse: Sequence of events and induction of the acrosome reaction by zona pellucida glycoprotein. Developmental Biology 1983;95(2):317-324.

- Braunstein G. Evidence favouring human chorionic gonadotropin as the physiological "rescuer" of the corpus luteum during early pregnancy. Early Pregnancy 1996;2:183–190.

- Bryan E. The impact of multiple preterm births on the family. BJOG: an International Journal of Obstetrics and Gynaecology 2003;110:24-28.

- Chao K, Wu M, Chen S *et al*. Assisted hatching increases the implantation and pregnancy rate of in vitro fertilization (IVF)-embryo transfer (ET), but not that of IVF-tubal ET in patients with repeated IVF failures. Fertility and Sterility 1997;67:904-908.

- Chang MC. Fertilization of rabbit ova in vitro. Nature 1959;8:184 466-467.

- Cobo A, Bellver J, Domingo J *et al*. New options in assisted reproduction technology: the Cryotop® method of oocyte vitrification. Reproductive BioMedicine Online 2008;17:1:68-72.

- Cohen J, Simons R, Edwards R *et al*. Pregnancies following the frozen storage of expanding human blastocysts. *Journal of In Vitro Fertilization and Embryo Transfer* 1985;2:59-64.

- Cohen J, Inge K, Suzman M *et al*. Video cinematography of fresh and cryopreserved embryos: a retrospective analysis of embryonic morphology and implantation. Fertility and Sterility 1989;51:820-827.

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

- Cohen J. Review: Assisted Hatching of Human Embryos. Journal of In Vitro Fertilization and Embryo Transfer 1991;8:179-190.

- Cohen J, Trowbridge J and Rosenwaks Z. Implantation enhancement by selective assisted hatching using zona drilling of human embryos with poor prognosis. Human Reproduction 1992;7:5:685-691.

- Das S, Blake D, Farquhar C *et al.* Assisted hatching on assisted conception (IVF and ICSI) (Review). The Cochrane Collaboration. John Wiley & Sons Ltd. Issue 1 (2009).

- Edwards R and Brody S. History and ethics of assisted human conception. In: Brody SA (ed.). Principles and Practice of Assisted Human Reproduction. Philadelphia, USA: WB Saunders, 1995,17– 47.

- Debrock S, Peeraer K, Spiessens C *et al*. The effect of modified quarter laser-assisted zona thinning on the implantation rate per embryo in frozen/vitrified-warmed/warmed embryo transfer cycles: a prospective randomized control trial. Human Reproduction 2011;26:1997-2007.

- Desai N, Filipovits J and Goldfarb J. Secretion of soluble HLA-G by day 3 human embryos associated with higher pregnancy and implantation rates: assay of culture media using a new ELISA kit. Reproductive Biomedicine Online 2006;13:272-277.

- Edgar D and Gook D. A critical appraisal of cryopreservation (slow cooling versus vitrification) of human oocytes and embryos. Human Reproduction Update 2012;18: 536-554.

- Edirisinghe W, Ahnonkitpanit V, Promviengchai S *et al*. A study failing to determine significant benefits from Assisted Hatching: Patients Selected for Advanced Age, Zonal Thickness or Embryos, and Previous Failed Attempts. Journal of Assisted Reproduction and Genetics 1999;16:294-301.

- Edwards R and Beard H. Blastocyst stage transfer: pitfalls and benefits. Is the success of human IVF more a matter of genetics and evolution than growing blastocysts? Human Reproduction 1999;14:1-4.

- Eum J, Park J, Lee W *et al*. Long-term liquid nitrogen vapor storage of mouse embryos cryopreserved using vitrification or slow cooling. Fertility and Sterility 2009;91:1928-1932.

- Fahy G, Levy D, and Ali S. Some emerging principles underlying the physical properties, biological actions, and utility of vitrification solutions. Cryobiology 1987;24:196–213.

- Fahy G, Wowk B and Wu J. Cryopreservation of complex systems: the missing link in the regenerative medicine supply chain. Rejuvenation Research 2006;9:279

- France J, Keelan J, Song L *et al*. Serum concentrations of human chorionic gonadotropin and immunoreactive inhibin in early pregnancy and recurrent miscarriage: a longitudinal study. Australian and New Zealand Journal of Obstetrics and Gynaecology 1996;36:325.

- Gabrielsen A, Agerholm I, Toft B *et al*. Assisted Hatching improves implantation rates on cryopreserved-warmed embryos: A randomized prospective study. Human Reproduction 2004;19:2258-2262.

- Gardner D and Lane M. Culture and selection of viable blastocysts: a feasible proposition for human IVF? Human Reproduction Update 1997;3:367–382.

- Gardner D and Lane M. Culture of viable human blastocysts in defined sequential serum-free media. Human Reproduction 1998;3:148–59.

- Gardner D, Schoolcraft B, Wagley L *et al*. A prospective randomised trial of blastocyst culture and transfer in in-vitro fertilization. Human Reproduction 1998;13:3434-3440.

- Gardner DK and Schoolcraft WB. In vitro culture of human blastocysts. In: Jansen R, Mortimer D (eds). Toward Reproductive Certainty: Fertility and Genetics Beyond 1999. UK: Parthenon Publishing London, 1999, 378 – 388.

- Gardner D, Lane M and Schoolcraft W. Physiology and culture of the human blastocyst. Journal of Reproduction Immunology 2002;55:85-100.

- Ge H-S, Zhou W, Zhang W *et al*. Impact of assisted hatching on fresh and frozen–warmed embryo transfer cycles: a prospective, randomized study. Reproductive BioMedicine Online 2008;16:589-596.

- Gelbaya T, Tsoumpou J and Nardo L. The likelihood of live birth and multiple birth after single versus double embryo transfer at the cleavage stage: a systematic review and meta-analysis. *Fertility and Sterility* 2010;94:935-945.

- Gilbert W, Nesbitt T and Danielsen B. Childbearing Beyond Age 40: Pregnancy Outcome in 24,032 Cases. The American College of Obstetricians and Gynecologists 1999;93:9-14.

- Gonen R, Heyman E, Asztalos V et al. The outcome of triplet, quadruplet and wuintuplet pregnancies managed in a perinatal unit: Obstetric, neonatal, and follow-up data. American Journal of Obstetrics and Gynaecology 1990;162:454-49.

- Hammadeh M, Fischer-Hammadeh C and Ali K. Assisted hatching in Reproduction: A state of the Art. Journal of Assisted Reproduction and Genetics 2010;28:119-128.

- Hardarson T, Van Landuyt L and Jones G. The blastocyst. Human Reproduction 2012;27:72-91.

- Hardy K, Handyside AH and Winston RM. The human blastocyst: cell number, death and allocation during late preimplantation development in vitro. Development 1989;107:597-604.

- Hershlag A, Paine T, Cooper GW *et al*. Monozygotic twinning associated with mechanical assisted hatching. Fertility and Sterility 1999;71:144-146.

- Hill M, Richter K, Heitman R *et al*. Trophectoderm grade predicts outcomes of single-blastocyst transfers. Fertility and Sterility 2013;99:1283-1289.

- Hiraoka K, Hiraoka K, Kinutami M *et al*. Blastocoele collapse by micropipetting prior to vitrification gives excellent survival and pregnancy outcomes for human day 5 and 6 expanded blastocyst. Human Reproduction 2004;19:2884-2888.

- Hiraoka K, Fuchiwaki M, Hiraoka K *et al*. Effect of size of zona pellucida opening by laser hatching and clinical outcome of frozen embryos that were cultured to blastocysts after warming in women with multiple implantation failures of embryo transfer: a retrospective study. Journal of Assisted Reproduction and Genetics 2008;25:129-135.

- Homa S and Swann K. Fertilization and early embryology: A cystolic sperm factor triggers calcium oscillations and membrane hyperpolarizations in human oocytes. Human Reproduction 1994;9:2356-2361.

- Hu D, Webb G and Hussey N. Aneuploidy detection in single cells using DNA array-based comparative genomic hybridization. Molecular Human Reproduction 2004;10:283-289.

- Jones G, Trounson A, Gardner D *et al*. Evolution of a culture protocol for successful blastocyst development and pregnancy. Human Reproduction 1998a;13:169–177.

- Jones G, Trounson A, Lolatgis N *et al*. Factors affecting the success of human blastocyst development and pregnancy following in vitro fertilization and embryo transfer. Fertility and Sterility 1998b;70:1022–1029.

- Kader A, Choi A, Orief A *et al*. Factors affecting the outcome of human blastocyst vitrification. Reproductive Biology and Endocrinology 2009;7:99.

- Kallioniemi A, Kallioniemi O, Sudar D *et al*. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science Magazine 1992;258:818-821.

- Kirkegaard K, Agerholm I and Ingerslev H. Time-lapse monitoring as a tool for clinical embryo. Human Reproduction 2012;27:1277-1285.

- Kissin D, Kawwass J, Monsour M *et al*. Assisted hatching: trends and pregnancy outcomes, United States, 2000-2010. Fertility and Sterility 2014;102:795-801.

- Kojima E, Fukunaga N, Nagai R *et al*. The vitrification method is significantly better for warming of slow-freezing embryos. Fertility and Sterility 2012;98:124.

- Kotze D, Keskintepe L, Sher G *et al*. A linear karyotypic association between PB-I, PB-II and blastomere using sequentially performed comparative genome hybridization with no association established between karyotype, morphologic, biochemical (sHLA-G expression) characteristics, blastocyst formation and subsequent pregnancy outcome. Gynecologic and Obstetrics Investigation 2012;74:304-12.

- Kotze D, Kruger T, Sher G *et al*. A Review of Non Invasive and Invasive Embryo Characteristics to identify the most competent Embryo for Transfer. British Journal Medicine and Medical Research 2013;15;2910-2930.

- Kratzer P and Taylor R. Corpus luteum function in early pregnancies is primarily determined by the rate of change of human chorionic gonadotropin levels. American Journal of Obstetrics and Gynecology 1990;163:1497–1502.

- Kuleshova L and Lopata A. Vitrification can be more favourable than slow cooling. Fertility and Sterility 2002;78:449-454.

- Kung F, Lin Y-C, Tseng Y-J *et al*. Transfer of frozen–warmed blastocysts that underwent quarter laser-assisted hatching at the day 3 cleaving stage before freezing. Fertility and Sterility 2003;79:893-899.

- Kuwayama M. Highly efficient vitrification method for cryopreservation of human oocytes. Reproductive Biomedicine Online 2005;11:300-308.

- Kuwayama M. Highly efficient Vitrification method for cryopreservation of human oocytes and embryos: The Cryotop® method. Theriogenology 2007;67:73-80.

- Lambalk C and van Hooff M. Natural versus induced twinning and pregnancy outcome: a Dutch nationwide survey of primiparous dizygotic twin deliveries. Fertility and Sterility 2001;75:731– 736.

- Lane M and Gardner D. Understanding cellular disruptions during early development that perturb viability and fetal development. Reproductive Fertility Development 2005;17:371–378.

- Larman M, Sheehan C and Gardner D. Calcium-free vitrification reduces cryoprotectant-induced zona pellucida hardening and increases fertilization rates in mouse oocytes. Reproduction 2006;131:153-161.

- Le Caignec C, Spits C, Sermon K *et al*. Single-cell chromosomal imbalances detection by array CGH. Nucleic Acids Research 2006;34:68.

- Leibo S and Pool T. The principal variables of cryopreservation: solutions, temperatures and rate changes. Fertility and Sterility 2011;96:269-276.

- Lenton E, Neal L and Sulaiman R. Plasma concentrations of human chorionic gonadotropin from the time of implantation until the second week of pregnancy. Fertility and Sterility 1982;37:773.

- Le Lannou D, Griveau J-F, Laurent M-C *et al*. Contribution of embryo cryopreservation to elective single embryo transfer in IVF−ICSI. Reproductive Biomedicine Online 2006;13:368-375.

- Levene M. Assisted Reproduction and its implications for paediatrics. Archives of Disease in Childhood 1991:66;1-3.

- Levin I, Almog B, Schwartz T *et al*. Effects of laser polar-body biopsy on embryo quality. Fertility and Sterility 2012;97:1085-1088.

- Liebermann J. Recent developments in human oocyte, embryo and blastocyst vitrification: where are we now? Reproductive Biomedicine Online 2003;7:623-633.

- Liebermann J and Tucker M. Comparison of vitrification and conventional cryopreservation of day 5 and day 6 blastocysts during clinical application. Fertility and Sterility 2006;86:20–26.

- Liebermann J. Vitrification of Human Blastocytsts. Reproductive Biomedicine online 2009;19:4.

- Liebermann J. More than six years of blastocyst Vitrification – What is the Verdict? US Obstetrics and Gynecology 2011;5:14-17.

- Lin S, Lee R and Tsai Y. In vivo hatching phenomenon of mouse blastocysts during implantation. Journal of Assisted Reproduction and Genetics 2001;18:341-345.

- Liu H and Rosenwaks Z. Early pregnancy wastage in in-vitro fertilization patients. Journal of In Vitro Fertilization and Embryo transfer 1990;8:65-72.

- Liu H, Pyrgiotis E, Davis O *et al*. Active corpus luteum function at pre-, peri- and post-implantation is essential for a viable pregnancy. Early Pregnancy 1995;1:281–287.

- Loutradi K, Kolibianakis E, Venetis C *et al*. Cryopreservation of human embryos by vitrification or slow freezing: a systematic review and meta-analysis. Fertility and Sterility 2008; 90:186-193

- Lopatarova M, Cech S, Holy L and Dolezel R. The effect of vitrification in open pulled straws on pregnancy rates after transfer of in vivo produced bovine embryos. Veterinarni Medicina 2006:51;454-460.

- Lucci CM, Kacinski MA, Lopes LHR *et* al. Effect of different cryoprotectants on the structural preservation of follicles in frozen zebu bovine (Bos indicus) ovarian tissue. Theriogenology 2004:61;1101-1114.

- Macfarlane AJ, Price FV, Bryan EM and Botting BJ. Early days. In: Botting BJ, Macfarlane AJ, Price FV, editors. Three, Four and More. London: HMSO, 1990:80– 98 (Chapter 6).

- Magli M, Jones G, Gras L *et al*. Chromosome mosaicism in day 3 aneuploid embryos that develop to morphologically normal blastocysts in vitro. Human Reproduction 2000;15:1781–1786.

- Malter H and Cohen J. Blastocyst formation and hatching in vitro following zona drilling of mouse and human embryos. Gamete Research 1989;24:67-80.

- Mantoudis E, Podsiadly B, Gorgy A *et al*. A comparison between quarter, partial and total laser assisted hatching in selected infertility patients. Human Reproduction 2001;16:2182-2186.

- Martins W, Rocha I, Ferriani R *et al*. Assisted Hatching of human emnryos: a systematic review and meta-analysis of randomized control trails. Human Reproduction Update 2011;17:438-453.

- Matson P, Graefling J, Junk S *et al*. Cryopreservation of oocytes and embryos: use of a mouse model to investigate effects upon zona hardness and formulate treatment strategies in an in vitro fertilization programme. Human Reproduction 1997;12:1550-1553.

- Mazur P. Freezing of living cells: mechanisms and implications. American Journal of Physiology 1984;247:125-142.

- McVearry I, Shipley S, Richter K *et al*. Examination of success rates of frozen/warmed blastocyst transfer cycles according to the day of blastocyst cryopreservation reveals surprisingly high success rates among blastocysts frozen on day 7 after oocyte retrieval. Fertility and Sterility 2004;82:148–149.

- Mertzanidou A, Wilton L, Cheng J *et al*. Microarray analysis reveals abnormal chromosomal complements in over 70% of 14 normally developing human embryos. Human Reproduction 2013;28:256-264.

- Montag M, Liebenthron J and Koster M. Which morphological scoring system is relevant in human embryo development? Placenta 2011;32:252 –256.

- Mukaida T, Oka C, Goto T *et al*. Artificial shrinkage of blastocoeles using either a micro-needle or a laser pulse prior to the cooling steps of Vitrification improves survival rate and pregnancy outcome of vitrified human blastocysts. Human Reproduction 2006;21:3246-3252.

- Mukaida T and Oka C. Vitrification of oocytes, embryos and blastocysts. Best Practice and Research: Clinical Obstetrics 2012;26:789-803.

- Nakhuda G and Sauer M. Addressing the Growing Problem of Multiple Gestations Created by Assisted Reproductive Techniques. Seminars in Perinatology 2005:29;355-362.

- Nakayama T, Fujiwara H, Yamada S *et al*. Clinical application of new assisted hatching method using a piezo-micromanipulator for morphologically low quality embryos in poor prognosis infertile patients. Fertilty and Sterility 1999;71:1014-1018.

- Navot D, Drews M, Bergh P *et al*. Age-related decline in female fertility is not due to diminished capacity of the uterus to sustain embryo implantation. Fertility and Sterility 1994;61:97-101.

- Ng E, Naveed F, Lau E *et al*. A randomized double-blind controlled study of the efficacy of laser-assisted hatching on implantation and pregnancy rates of frozen–warmed embryo transfer at the cleavage stage. Human Reproduction 2005;20:979-985.

- Obruca A, Strohmer H, Blaschitz A *et al*. Ultrasctructural observations in human oocytes and preimplantation embryos after zona opening using an erbium:yttrium-luminium Garnet Er: (YAG) laser. Human Reproduction 1997;12:2242-2245.

- Özkavukcu S and Erdemli E. Cryopreservation: Basic Knowledge and Biophysical Effects. Journal of Ankara Medical School 2003:24:187-196.

- Paffoni A, Guarneri C, Ferrari S *et al*. Effects of two vitrification protocols on the developmental potential of human mature oocytes. Reproductive Biomedicine Online 2011;22:292-298

- Palankar D, Ohad S, Lewis A *et al*. Technique for cellular microsurgery using the 193nm excimer laser. Lasers in Surgery and Medicine 1991;11:580-586.

- Pandian Z, Marjoribanks J, Ozturk O *et al*. Number of embryos for transfer following in vitro fertilisation or intra-cytoplasmic sperm injection: summary of a Cochrane review. Fertility and Sterility 2014;102:345-347.

- Papanikolaou E, Kolibianakis E, Tournaye H *et al*. Live birth rates after transfer of equal number of blastocysts or cleavage-stage embryos in IVF. A systematic review and meta-analysis. Human Reproduction 2008;23:91-99.

- Papatheodorou A, Vanderzwalmen P, Panagiotidis Y *et al*. Open versus closed oocyte vitrification system: a prospective randomised sibling-oocyte study. Reproductive BioMedicine Online 2013;26:595-602.

- Parmegiani L, Tatone C, Cognigni GE *et al*. Rapid warming increases survival of slow-frozen sibling oocytes: a step towards a single warming procedure irrespective of the freezing protocol. Reproductive Biomedicine Online 2014; 28:614-623.

- Petersen C. Laser-assisted hatching of Cryopreserved warmed embryos by thinning one quarter of the zona. Reproductive Biomedicine Online 2006;13:668-675.

- Piette C, de Mouzon J, Bachelot A *et al*. In-vitro fertilization: influence of women's age on pregnancy rates. Human Reproduction 1990;5:56-59.

- Primi M, Senn A, Montag M *et al*. A Eurpoean multicentre prospective randomized study to assess the use of assisted hatching with a diode laser and the benfit of an immunosuppressive/antibiotic treatment in different patient populations. Human Reproduction 2004;10:2325-2333.

- Racowsky C, Jackson K, Cekleniak N *et al*. The number of eight-cell embryos is a key determinant for selecting day 3 or day 5 transfer. Fertility and Sterility 2000;73:558–564.

- Reed M, Hamic A and Thompson D. Continuous uninterrupted single medium culture without medium renewal versus sequential media culture: a sibling embryo study. Fertility and Sterility 2009;92:1783–1786.

- Richard C, Kubik C and DeLoia J. Physiological range of human chorionic gonadotropin for support of early human pregnancy. Fertility and Sterility 2001;76:988-993.

- Richter K, Harris D, Daneshmand S *et al*. Quantitative grading of a human blastocyst: optimal inner cell mass size and shape. Fertility and Sterility 2001;76:1157–1167.

- Richter K, Shipley S, McVearry I *et al*. Cryopreserved embryo transfers suggest that endometrial receptivity may contribute to reduced success rates of later developing embryos. Fertility and Sterility 2006;86:862–866.

- Rijnders P and Jansen C. The predictive value of day 3 embryo morphology regarding blastocyst formation, pregnancy and implantation rate after day 5 transfer following in-vitro fertilization or intracytoplasmic sperm injection. Human Reproduction 1998;13:2869–2873.

• Roy T, Bradley C, Bowman M *et al*. Single-embryo transfer of vitrified-warmed blastocysts yields equivalent live-birth rates and improved neonatal outcomes compared with fresh transfers. *Fertility and Sterility* 2014: 101:1294-301.

• Roque M, Lattes K, Serra S *et al*. Fresh embryo transfer versus frozen embryo transfer in *in vitro* fertilization cyclesa systematic review and meta-analysis. *Fertility and Sterility* 2013;99:156-162.

• Sagoskin A, Levy M, Tucker M *et al*. Laser Assisted Hatching in poor prognosis patients undergoing IVF-embryo transfer: randomized control trial. Fertility and Sterility 2007;87:283-287.

• Sathananthan H, Gunasheela S, Menezes J *et al*. The mechanics of human blastocyst hatching in vitro. Reproductive BioMedicine Online 2003;7:228–234.

• Schieve L, Meikle S, Petersen H *et al*. Does assisted hatching pose a risk for monozygotic twinning in pregnancies conceived through in vitro fertilization? Fertility and Sterility 2000;74:288-294.

• Schiewe M, Arcujo E Jr, Asch R *et al*. Enzymatic characterization of zona pellucida hardening in human eggs and embryos. Journal of Assisted Reproduction and Genetics 1995;12:2-7.

• Scholz T, Bartholomaus S, Grimmer I *et al*. Problems with multiple births after ART: medical, psychological, social and financial aspects. Human Reproduction 1999;14:2932-2937.

• Scott K, Hong K and Scott R. Selecting the optimal time to perform biopsy for implantation and genetic testing. Fertility and Sterility 2013;100:608-614.

• Sepulvéda S, Garcia J, Arriage E *et al*. In vitro development and pregnancy outcomes for human embryos cultured in either a single medium or in a sequential media system. Fertility and Sterility 2009;91:1765-1770.

• Seeber B. What serial hCG can tell you, and cannot tell you, about an early pregnancy. Fertility and Sterility 2012;98:1074-1077.

• Shapiro B, Daneshmund S, Garner F *et al*. Contratsing patterns in in vitro fertilization pregnancy rates among fresh autologous, fresh oocyte donor, and cryopreserved cycles with

the use of day 5 or day 6 blastocysts may reflect differences in embryo-endometrium synchrony. Fertility and Sterility 2008;89:20-26.

• Sher G, Keskintepe L, Keskintepe M *et al*. Oocyte karyotyping by comparative genomic hybridization [correction of hybrydization] provides a highly reliable method for selecting "competent" embryos, markedly improving in vitro fertilization outcome: a multiphase study. Fertility and Sterility 2007;87:1033-1040.

• Shi W, Zhang S, Zhao W *et al*. Factors related to clinical pregnancy after vitrified warmed embryo transfer: a retrospective and multivariate logistic regression analysis of 2313 transfer cycles. Human Reproduction 2013;28:1768-1775

• Shipley S, Richter K, Tucker M *et al*. Single embryo transfer of frozen-warmed blastocysts: proportion of cells surviving the cryopreservation process is indicative of implantation potential. Fertility and Sterility 2006;86:532.

• Shoukir Y, Campana A, Farley T *et al*. Early cleavage of in-vitro fertilized human embryos to the 2-cell stage: a novel indicator of embryo quality and viability. Human Reproduction 1997;12:1531-1536.

• Sifer C, Sellami A, Poncelet C *et al*. A prospective randomized study to assess the benefit of partial zona pellucida digestion before frozen-warmed embryo transfers. Human Reproduction 2006;21:2384-2389.

• Son W, Yoon S, Yoon H *et al*. Pregnancy outcome following transfer of human blastocyst vitrified on electron microscopy grids after induced collapse of the blastocoele. Human Reproduction 2003;18:137-139.

• Staessen C, Platteau P, Van Assche E *et al*. Comparison of blastocyst transfer with or without preimplantation genetic diagnosis for aneuploidy screening in couples with advanced maternal age: a prospective randomized controlled trial. Human Reproduction 2004;19:2849–2858.

• Stehlik E, Stehlik J, Katajama P *et al*. Vitrification demonstrates significant improvement versus slow freezing of human blastocysts. Reproductive Biomedicine Online 2005;11:53-57.

- Stein A, Rufas O, Shoshana A *et al*. Assited hathcingby partial zona dissection of human pre-embryos in patients with recurrent implantation failure after in vitro fertilization. Fertilty and Sterility 1995;63:838-841.

- Steptoe P and Edwards R. Birth after the reimplantation of a human embryo. Lancet 1978;12:2366.

- Sunkara S, Siozos A, Bolton V *et al*. The influence of delayed blastocyst formation on the outcome of frozen-warmed blastocyst transfer: a systematic review and meta-analysis. Human Reproduction 2010;25:1906–1915.

- Thorpe K, Golding J, Macgllivray I *et al*. Comparison of prevalence of depression in mothers of twins and mothers of singletons. British Medical Journal 1991;302:875-878.

- Tinney G, Windt M, Kruger T *et al*. Use of zona laser treatment system in assisted hatching: optimal laser utilization parameters. Fertility and Sterility 2005;84:1737-1741.

- Treff N, Su J, Tao X *et al*. Accurate single cell 24 chromosome aneuploidy screening using whole genome amplification and single nucleotide polymorphism microarrays. Fertility and Sterility 2010;94:2017-2021.

- Tsirigotis M. Blastocyst stage transfer: pitfalls and benefits. Too soon toabandon current practice? Human Reproduction 1998;13:3285–3289.

- Tucker M, Cohen J, Massey J *et al*. Partial dissection of the zona pellucida of frozen-warmed human embryos may enhance blastocyst hatching, implantation and pregnancy rates. American Journal of Obstetrics and Gynaecology 1991;165:341-345.

- Valojerdi M, Eftekhari-Yazdi P, Karimian L *et al*. Effect of laser zona pellucida opening on clinical outcome of assisted reproduction technology in patients with advanced female age, recurrent implantation failure, or frozen-warmed embryos. Fertility and Sterility 2008;90:84-91.

- Vajta G and Kuyawama M. Improving cryopreservation systems. Theriogenology 2006;65:236–244.

- Vanderzwalmen P, Bertin G, Debauche C *et al*. Birth after vitrification at morula and blastocyst stage: effect of artificial reduction of blastocoelic cavity before vitrification. Human Reproduction 2002;17:744-751.

• Vanderzwalmen P, Bertin G, Debauche C *et al*. Vitrification of human blastocysts with the hemistraw method: application of assisted hatching after warming. Human Reproduction 2003;18:1504-1511.

• Vanderzwalmen P, Ebner T, Zech N *et al*. One decade of experience with vitrification of human embryos in straws, hemi-straws and high security vitrification straws. In: Tucker MJ, Liebermann J. (Eds.) Vitrification in Assisted Reproduction: A User's Manual and Troubleshooting Guide. 2007; Informa Healthcare, London, pp. 195–217.

• Van Noord-Zaadstra B, Looman C, Alsbach H *et al*. Delaying childbearing: effect of age on fecundity and outcome of Pregnancy. British Medical Journal 1991;302:1361-1365.

• Veeck L. An Atlas of the Human Blastocysts: The Encyclopedia of Visual Medicine Series. Parthenon Publishing Group, 2003.

• Watson A, Natale D and Barcroft L. Molecular regulation of blastocyst formation. Animal Reproduction Science 2004;82–83:583–592.

• Wan C, Cheng S, Diao L *et al*. Laser-assisted hatching improves clinical outcomes of vitrified-warmed blastocysts developed from low-grade cleavage stage ebryos: a prospective randomized study. Reproductive Bomedicine Online 2014;28:582-589.

• Wei-Xin L, Hua L, Meng-Jun L and Liang-Zhi X. Effects of Different Cryoprotectants and Cryopreservation Protocols on the Development of 2-4 Cell Mouse Embryos. Cryoletters 2011:32;240-247.

• Wells D, Chu L, Weier U *et al*. Recent Advances in Prenatal Genetic Papp Z RC, editor. Bologna: Medimond; 2004.

• Wilcox L, Kiely J, Melivic L *et al*. Assisted Reproductive Technologies: estimates of their contribution to multiple births and newborn hospital days in the United States. Fertility and Sterility 1996:65;361-366.

• Windt M-L, Kruger T, Coetzee K *et al*. Comparative analysis of pregnancy rates after the transfer of early dividing embryos versus slower dividing embryos. Human Reproduction 2004;19:1155-1162.

• Wong C, Chen A, Behr B *et al*. Time-lapse microscopy and image analysis in basic and clinical embryo development research. Reproductive BioMedicine Online 2013;26:120–129.

• Wu K, Zhao H, Liu H *et al*. Day 3 ET, single blastocyst transfer (SBT) or frozen-warmed embryo transfer (FET): which is preferable for high responder patients in IVF/ICSI cycles? Journal of Assisted Reproduction and Genetics 2014;31:275-278.

• Youssry M, Ozmen B, Zohni K *et al*. Current aspects of blastocyst cryopreservation. Reproductive Biomedicine Online 2008;16:311-320.

• Zhang J, Li X, Peng Y *et al*. Reduction in exposure of human embryos outside the incubator enhances embryo quality and blastulation rate. Reproductive Biomedicine Online 2010;20:510-515.

• Zheng W, Zhuang G, Zhou C *et al*. Comparison of the survival of human biopsied embryos after cryopreservation with four different methods using non-transferable embryos. Human Reproduction 2005;20:1615-1618.

• Zhou H, Zao W, Zhang W *et al*. No adverse effects were identified on the perinatal outcomes after laser-assisted hatching treatment. Reproductive Biomedicine Online 2014;29:692-8.

• Zhu D. Vitrified-warmed blastocyst transfer yield higher pregnancy and implantation rates compared with fresh blastocyst transfer cycles-time for a new embryo transfer strategy. Fertility and Sterility 2011;95:1691-1695.

Websites Accessed

- Becton Dickinson. 2014. Available at http://www.bd.com/vacutainer/hcw_safety/pdfs/wall_chart.pdf

  [Accessed 25 August 2014]

- Becton Dickinson. 2014. Available at http://www.bd.com/vacutainer/hcw_safety/pdfs/PBBCS_NE_Study_Pstr_VS7787.pdf

  [Accessed 25 August 2014]

- Egg Technology United Kingdom

http://www.eggtech.co.uk/resources/TechnicalNotesManualSeeding.pdf

[Accessed 05 February 2015]

- Pacific fertility centre. 2014. Available at:

http://www.pacificfertilitycenter.com/treatment-care/vitrification

[Accessed 27 August 2014]

- Yale School of Medicine, Yale Fertility Centre. 2013. Available at: http://www.yaleobgyn.org/yfc/ourservices/invitro/development.aspx

[Accessed 04 November 2014]

Chapter Nine

Addenda

1) *Ethical Approval from the Health Research Ethics Committee [HREC] of the University of Stellenbosch*
2) *Embryo culture and pre-blastocyst grading used at Drs Aevitas Institute for Reproductive Medicine*
3) *Embryo Grading: Drs Aevitas Institute of Reproductive Medicine*
4) *Gardner & Schoolcraft Embryo Grading System (Veeck, 2003)*
5) *Swim Up Method for Semen Preparation*
6) *Sil-select Gradient Method for Semen Preparation*
7) *Oocyte Retrieval Protocol used at Drs Aevitas Institute for Reproductive Medicine*
8) *IVF Procedure used at Drs Aevitas Institute for Reproductive Medicine*
9) *ICSI Procedure used at Drs Aevitas Institute for Reproductive Medicine*
10) *FertiPro® VitriFreeze/VitriWarming Protocol*
11) *Vitrification Consent Forms used at Drs Aevitas Institute for Reproductive Medicine*
12) *Modified Vitrification Procedure used at Drs Aevitas Institute for Reproductive Medicine*
13) *Collapsing protocol used at Drs Aevitas Institute for Reproductive Medicine*
14) *Modified Blastocyst Warming Procedure used at Drs Aevitas Institute for Reproductive Medicine*
15) *Vitriplug Method of Blastocyst Cryopreservation and Warming*
16) *Embryo Transfer Protocol used at Drs Aevitas Institute for Reproductive Medicine*
17) *Protocol for obtaining Human Chorionic Gonadotropin (HCG) Levels*
18) *Proposed Phase II Informed Consent Form*
19) *Protocol for Laser Assisted Hatching used at Drs Aevitas Institute for Reproductive Medicine*

1) Ethical Approval from the HREC of the University of Stellenbosch

Approval Notice
New Application

15-Oct-2013
WILSON-POE, Emma Josephine

Ethics Reference #: S13/05/102

Human Blastocyst vitrification: A retrospective review of outcomes in an assisted reproductive technology (ART) clinic (2004 - 2013).

Dear Miss Emma WILSON-POE,

The **New Application** received on **16-May-2013**, was reviewed by Health Research Ethics Committee 2 via Committee Review procedures on **18-Sep-2013** and has been approved.

Please note the following information about your approved research protocol:

Protocol Approval Period: **18-Sep-2013 -18-Sep-2014**

Present Committee Members:
Davids, Mertrude MA

Fernandez, Pedro PW

Kruger, Mariana M

Bardien-Kruger, Soraya S

Barsdorf, Nicola

De Roubaix, Malcolm JAM

Moller, Marlo M

Engelbrecht, Susan S

Willett, Derrick DWE

Edwards, C E

Rohland, Elvira EL

Botha, Matthys MH

Please remember to use your **protocol number** (**S13/05/102**) on any documents or correspondence with the HREC concerning your research protocol.
Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:
Please note a template of the progress report is obtainable on www.sun.ac.za/rds and should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of
Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Helene Visser at City Health (Helene.Visser@capetown.gov.za Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.

For standard HREC forms and documents please visit: www.sun.ac.za/rds

If you have any questions or need further assistance, please contact the HREC office at 0219389207.

Sincerely,

Mertrude Davids

HREC Coordinator

Health Research Ethics Committee 2

2) *Embryo culture and pre-blastocyst grading used at Drs Aevitas Institute for Reproductive Medicine*

- Following oocyte aspiration and retrieval, and prior to insemination, the oocytes are denuded in those patients undergoing ICSI treatment [Addendum 9].
- The oocytes are then cultured Quinn's Advantage ® Protein Plus Fertilization Medium, which is covered by Sage ® Oil for Tissue Culture. Insemination then occur [Addenda 8 and 9].
- Incubate overnight at 37°C, 5% CO_2
- Day 1: Fertilization Check

  (In the case of IVF patients the oocytes are cleaned using denuding pipettes and rinsed well [Addendum 8] before checking for fertilization.)

  Check for PN and PB number on the inverted microscope fitted with heated stage. (Take note whether PN numbers are abnormal at > or < 2, or any other anomalies). Fertilization is indicated by the presence of 2PN. Transfer to preincubated Quinn's Advantage ® Protein Plus Cleavage Medium drops covered with Sage ® Oil for Tissue Culture in a greiner dish and incubate overnight at 37°C, 5% CO_2
- Embryos are graded according to the number of cells present and the quality thereof. A scale ranging from 1-5 is used to grade the quality. 1 being of worst quality and 5 being of the best. Cells are ideally equal in size with no fragments.

Figure 9.1:4 cell stage embryo with quality grade of 5

http://www.yaleobgyn.org/yfc/ourservices/invitro/development.aspx

Figure 9.2: 7 cell stage embryo with quality grade of 5

http://www.yaleobgyn.org/yfc/ourservices/invitro/development.aspx

- Day 2: 2-4 cell

  On day 2 the embryos should have cleaved to a 2-4 cell stage and are graded accordingly. Slow dividers are usually evident of genetic abnormalities.

- Day 3: 6-8 cell

  By Day 3 the embryos should have cleaved to a 6-8 cell stage and are graded accordingly.

- Day 4: The cells within the embryo continue to grow and begin to tightly align themselves against each other to form a compact ball of cells, known as a morula or compacting embryo. The cells are no longer distinguishable from each other.

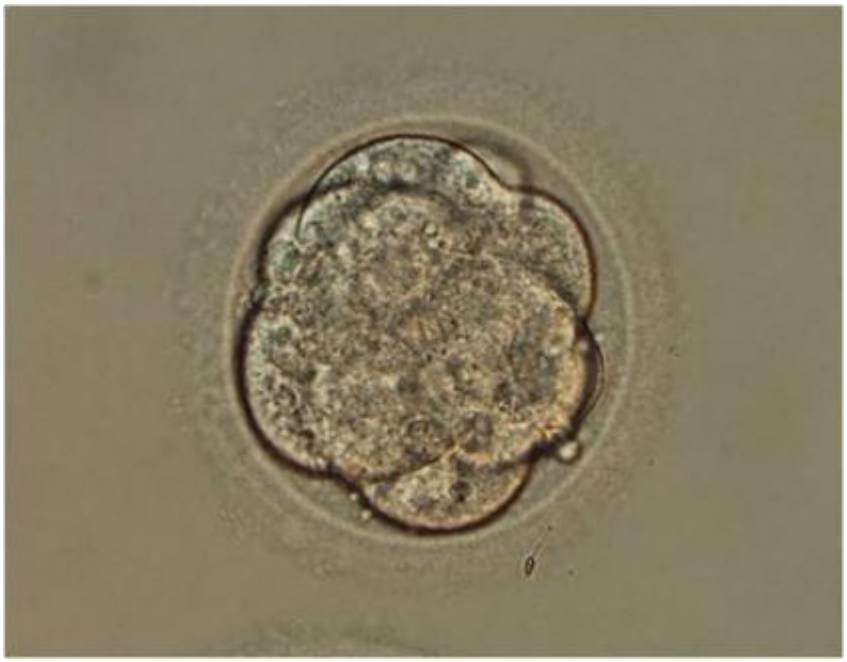

Figure 9.3: Compacting embryo/Morula

http://www.yaleobgyn.org/yfc/ourservices/invitro/development.aspx

- Day 5: Blastocyst Stage [Addenda 3 and 4]

3) Blastocyst Grading: Drs Aevitas Institute of Reproductive Medicine

Blastocyst Grading

Degree of Expansion and hatching status

1 Early blastocyst, the blastocoel filling more than half the volume of conceptus, but no expansion in overall size as compared to early cleavage stage embryos

2 Blastocyst, the blastocoel filling more than half of the volume of conceptus, with slight expansion in overall size and notable thinning of the zona pellucida.

3 Full blastocyst, a blastocoel more than 50% of the conceptus volume and overall size fully enlarged with a very thin zona pellucida

4 Hatching blastocyst, non-preimplantation genetic diagnosis. The trophectoderm has started to herniate

5 Fully hatched blastocyst, non-preimplantation genetic diagnosis. Free blastocyst fully removed from the zona pellucida.

6 Hatching or hatched blastocyst, preimplantation genetic diagnosis.

Inner cell mass (ICM) grading

A Tightly packed, compacted cells

B Larger loose cells

C No ICM distinguishable

D Cells of ICM appear degenerative

Trophectoderm grading

A Many healthy cells forming a cohesive epithelium

B Few, but healthy cells, large in size

C Poor, very large or unevenly distributed cells, may appear as few cells squeezed to the side

D Cells of the trophectoderm appear degenerative

4) *Gardner and Schoolcraft Blastocyst Grading System (Veeck, 2003)*

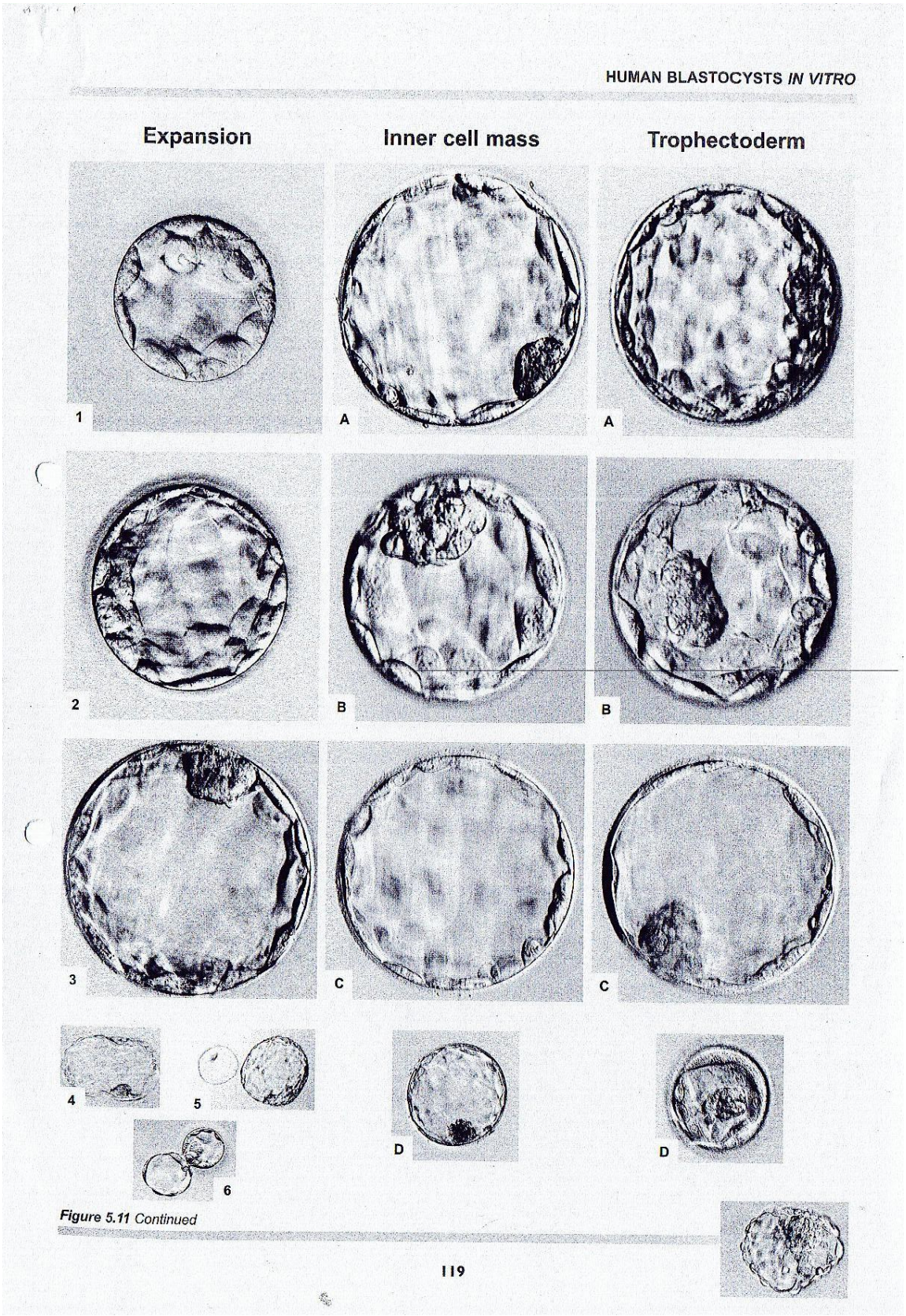

Figure 9.4: Gardner and Schoolcraft Blastocyst Grading System (Veeck, 2003)

5) Swim Up Method for Semen Preparation

MATERIALS:

- Sperm Preparation Medium

METHOD:

Semen Collection and Analysis

- Obtain semen sample by masturbation
- Allow sample to liquefy for 20 minutes
- Estimate the count, motility and forward progression of the sperm

Semen Preparation

1. Dilute 0.5-1ml semen with 2ml warm sperm preparation media
2. Vortex and centrifuge the sample at 450g for 10 minutes
3. Remove the supernatant and resuspend the pellet again in 2ml of warm sperm preparation medium.
4. Vortex and centrifuge at 450g for 10 minutes
5. Remove the supernatant and gently overlay 0.5ml of sperm preparation medium
6. Place the tube at an angle in the incubator set to 37°C for 60 minutes
7. After the 60 minutes, gently remove the 0.5 ml of sperm preparation medium and place in a clean tube
8. Make a wet-preparation slide of the sample and assess as outlined by the WHO criteria (WHO, 2010)

6) Sil-Select Gradient Method for Semen Preparation

MATERIALS

- SilSelect Media (Fertipro, Beernem, Belgium)
- Sperm Washing Medium (SAGE In-Vitro Fertilization Inc., Cooper Surgical Company, Trumball, CT)

METHOD

Semen collection and analysis

- Obtain semen sample by masturbation into sterile plastic containers
- Allow fresh sample to liquefy and prepare within one hour of ejaculation
- Make a wet preparation of the sample and evaluate the count, motility and forward progression

Gradient Preparation

- Discontinuous density gradients can be prepared with either two or three layers

  2 layers: 90% and 45% SilSelect

  3 layers: 90%, 70% and 45%
- To prepare the gradient, 1ml of the SilSelect dilution with the highest density (90%) is placed at the bottom of a sterile conical gradient tube
- Gently overlay with an equal volume of 45% SilSelect if a two-layer gradient is required, or 70% and then 45% SilSelect when a three layer gradient is required
- The gradient is left to equilibrate at 37°C
- Gently layer the semen onto the prepared, warmed gradient
- Centrifuge at 400g for 18 minutes

- Following centrifugation, carefully aspirate the supernatant from the meniscus down towards the 90% SilSelect layer using a small bore-holed pipette, such as a glass pipette. Gently aspirate the top third of the 90% SilSelect layer leaving behind the sperm pellet and those sperm cells that may still be suspended in the remaining 90% SilSelect
- Using a new pipette, remove the pellet from the gradient tube and place into a sterile conical-bottom tube.
- Resuspend the remaining 90% SilSelect media containing sperm with 0.3ml sperm preparation media and flush (by aspirating in and out) the area of the tube where the bottom layer of the gradient was to capture any sperm stuck to the sides of the tube
- Remove the suspension and transfer into the clean tube where the sperm pellet was placed
- Add 2ml of warm sperm preparation medium to the suspension to dilute out the 90% SilSelect
- Centrifuge at 450g for 10 minutes
- Remove the supernatant and resuspend the pellet again in 2ml of warm sperm preparation medium
- Centrifuge at 450g for 10 minutes
- Remove the supernatant and resuspend the pellet in 0.2ml of warm sperm preparation medium
- Make a wet preparation slide of the sample and assess count motility and forward progression as outlined by the WHO criteria

7) Oocyte Retrieval Protocol

Oocyte Pickup

-Follicular fluid is aspirated using a long 16 gauge aspiration needle into sterile tubes and sent through to the laboratory.

-The fluid is poured into large petri dishes under the stereomicroscope fitted with a heated stage.

-Follicular fluid must be examined immediately after follicular aspiration and not at 37°C for later examination because red and white blood cells tend to adhere strongly to the cumulus cells.

-If the cumulus is heavily stained with blood then those areas of cumulus can be removed with sterile needles because it is well established that the blood interferes with the fertilization rate and subsequent embryo quality.

-The oocyte-corona-complexes (OCC) are identified, graded (MI vs MII) and collected with a sterile, rounded, wide-bore glass pipette.

-The OCC are transferred into test tubes (MI and MII) into approximately 2.0ml fresh, warmed flushing medium to wash them of excess blood.

-The OCC are then transferred into Greiner dish with 2.0ml gassed fertilization medium at 37°C and incubated until denuding or insemination time.

8) IVF Procedure used at Drs Aevitas Institute for Reproductive Medicine

Semen Preparation

- See Addenda 4 and 5

Medium preparation

- Medium is prepared the previous day in a CO_2 incubator for equilibration

Aspiration

- Check suction pump (100-120mmHG)
- Prepare glass polished pipettes for pick up
- Place pick-up tubes in heated block
- Hand theatre medium tube to sister when needed
- Place aspirated follicular fluids in heated block and examine for oocyte-cumulus complexes using a large petri dish on a heated stage of a dissection microscope
- Determine maturity (GV, MI, MII)
- Put the complexes in the pick-up tubes (MI and MII separate)
- When done, rinse all complexes in small petri dish with gassed fertilization medium – check number obtained
- Transfer to 4 well NUNC dish with gassed fertilization medium – maturities separate and not more than 5 complexes per well
- Incubate in the CO_2 incubator until insemination

Insemination

- Inseminate complexes with the correct number/volume of prepared sperm cells according to the sperm count after semen preparation
- Do insemination ±40 hours post HCG administration if possible
- Incubate overnight at 37°C, 5% CO_2
- The next morning, clean oocytes with denuding pipette (Cook, Australia) and check for fertilization

9) ICSI Procedure used at Drs Aevitas Institute for Reproductive Medicine

Prior to Injection

Denuding of oocytes

Cumulus cells are removed using SAGE ® hyaluronidase solution and fire drawn and polished glass Pasteur pipettes.

Corona cells are then removed using denuding pipettes with a stripper pipette attached.

Intracytoplasmic Sperm Injection

The microinjection procedure is performed on an inverted microscope equipped with:

1. A heated microscope stage (37°C)
2. Two coarse and fine control manipulators
3. Two micro injectors, one for the holding pipette and the other for the injection pipette.

The holding pipette is inserted into the pipette holder and manually positioned so that the angled section is perpendicular to the microscope stage. The pipette is positioned using the coarse manipulators. The microinjection pipette is inserted into the pipette holder and manually positioned so that the angled section is at an angle approximately 20° to the microscope stage. The angel is to ensure that the tip of the microinjection needle will touch

the surface first. It must also be ensured that the two pipettes (holding and injection) move in line with each other.

The microinjection dish is made according to the configuration of your microinjection system and the experience of the technician. The dish must, however, contain certain basic elements; an oocyte droplet (flushing medium), spermatozoa (in sperm prep medium) and a PVP droplet.

The microinjection pipette is first carefully lowered into the clean PVP droplet. Before the manipulation of sperm is attempted PVP medium is repeatedly aspirated and expelled to coat the inside of the microinjection pipette. A large enough volume (for optimal control) of PVP is aspirated and the pipette is lifted and moved to the sperm storage droplet.

Sperm selection and Immobilization

The motile spermatozoa pipetted into the storage droplet (medium or PVP) swim to the outer perimeter of the droplet. When selecting a sperm cell preference is given to the sperm which appear morphologically normal and swim progressively forward. The motile sperm is aspirated into the injection pipette, the pipette is lifted and then moved to the clean PVP droplet. The pipette is lowered to just above the surface (in the PVP droplet), the sperm is slowly ejected moving up or down so that the sperms tail is transversely positioned to the pipette. The pipette tip is lowered onto the section of the tail just below the midpiece. The pressure and the movement of the microinjection needle on and over the sperm tail destabilizes the sperm membrane system and immobilizes the sperm. Destabilizing the sperm membrane before ICSI appears to be of crucial importance for decondensation of the sperm head and pronuclei formation. It must be ensured that the sperm is immotile before injection, as a motile sperm may cause structural damage within the oocyte ooplasma. The sperm is aspirated and ejected repeatedly to ensure that the sperm cell can be ejected during the

injection procedure. The selected immobilized sperm is aspirated tail first into the microinjection pipette. The microinjection pipette containing the sperm is lifted and moved to the oocyte droplet.

Oocyte Microinjection

An oocyte is added to each oocyte droplet in the dish.

Using the microinjection pipette the oocyte is rotated to locate the polar body at the 12 o'clock or 6 o'clock position. The holding pipette is lowered and the oocyte held by gentle suction. The microscope is focussed on the oocytes equatorial plane and the internal lumen of the holding pipette. The microinjection pipette is lowered into the same focus plane at the 3 o'clock position. The plane of the microinjection pipette can be corrected by gently pushing on the ZP. The sperm is carefully brought forward to the point of the microinjection pipette and the pipette is pushed carefully though the ZP and through the oolemma and ooplamsa. The successful penetration of the oolemma is indicated by the ability to aspirate ooplamsa into the pipette. The ooplasma and sperm cell are then carefully ejected into the oocyte and the microinjection pipette is then withdrawn and the oocyte released from the holding pipette. The process of penetrating the oolemma and the aspiration of a small volume of ooplasma also helps to activate the oocyte which is essential for the normal process of fertilization to occur. Care must be taken not to eject a large volume of PVP medium into the oocyte, because this inevitably leads to oocyte degeneration. The whole procedure is repeated for all the oocytes in the microinjection dish.

10) FertiPro® VitriFreeze/VitriWarming Protocol

VitriFreeze™

VitriWarming™

Intended Use

VitriFreeze™ and VitriWarming™ are a set of ready-to-use media for vitrification and warming of human embryos.

Background

Vitrification which is preservation of cells at extremely low temperatures without ice crystal formation and can be more favourable than slow cooling (Kuleshova and Lopata, 2002). Due to variable results after application of slow-freezing methods for blastocysts, vitrification was introduced as an alternative approach. The success rates of vitrification have been increased with the use of ultra-rapid vitrification procedures. For several years, vitrification of blastocysts and embryos using different embryo carriers have resulted in many pregnancies. In order to accomplish aseptic vitrification of blastocysts, the HSV High security Vitrification Kit (Cryo Bio System) was developed (Vanderzwalmen *et al.*, 2000). The tip of the HSV straw is designed to hold the blastocysts in a very small volume of vitrification solution allowing very fast cooling and warming rates as compared to those achieved by immersion of sealed 0.25ml plastic straws into liquid nitrogen. An alternative, aseptic method is now available in the shape of the VitriSafe straw (available from FertiPro N.V.).

Composition

VitriFreeze/Warming are DMSO/ethylene glycol based vitrification media that also contain PBS, sucrose, Ficoll and human serum albumin (10-20g/Liter). VitriFreeze/Warming do not contain antibiotics.

Material Included with the Kit

VitriFreeze™ kit (VF_KIT1)

- 1 vial of VitrFreeze Pre-incubation medium (5ml)
- 1 vial of VitriFreeze Freezing medium 1 (1ml)
- 1 vial of VitriFreeze Freezing medium 2 (2ml)

VitriWarming™ kit (VF_KIT1)

- 1 vial VitriWarming Warming Medium 1 (5ml)
- 1 vial VitriWarming Warming Medium 2 (1ml)
- 1 vial VitriWarming Warming Medium 3 (1ml)
- 1 vial VitriWarming Warming Medium 4 (1ml)

The media should be used in the order displayed above.

Material Not Included in the Kit

- well dishes (egNunc 144 444)
- freezing tank with liquid nitrogen
- water bath (able to hold 37◦C)
- attenuated pipettes
- forceps
- vitrification device (HSV device, Cryo Bio System)
- LAF-bench (ISO Class 5)
- Microscope
- Lab timer

VitriFreeze/Warming and embryo culture

VitriFreeze/Warming can be used in combination with FertiCult media (Flushing, IVF and G3) before freezing and after warming.

Product Specifications

- Chemical Composition
- pH between 7,20-7,40
- Osmolality

  VitriFreeze Pre-Incubation medium: 270-290 mOsm/kg

  VitriWarming Warming Medium 4: 270-290 mOsm/kg

  VitriWarming Warming Medium 1: 805-845 mOsm/kg

  VitriWarming Warming Medium 2: 535-565 mOsm/kg

  VitriWarming Warming Medium 3: 405-435 mOsm/kg
- Sterility: SAL 10^{-3}

- Endotoxins: <0,25 EU/ml
- Mouse Embryo Assay (blastocysts after 96h) ≥ 80%
- Use of PhEur or USP grade products if applicable
- The certificate of analysis and MSDS are available upon request

Pre-Use Checks

- Do not use the product if it becomes discoloured, cloudy, or shows any evidence of microbial contamination
- Do not use the product is seal of the container is opened or effect when the product is delivered.

Storage Instructions

- Store between 2-8◦C
- Do not freeze before use
- Keep away from sunlight
- The products can be used safely us to 7 days after opening, when sterile conditions are maintained and the products are stored at 2-8◦C.
- Do not use after expiry date

Warnings and Precautions

Standard measures to prevent infections resulting from the use of medicinal products prepared from human blood or plasma include selection of donors and screening of individual donations and plasma pools for specific markers. Despite this, when medicinal products prepared from human blood or plasma are administered, the possibility of transmitting infective agents cannot be totally excluded. This also applies to unknown or emerging viruses and other pathogens. There are no reports of proven virus transmissions with albumin manufactured to European Pharmacopoeia specifications by established processes. Therefore, handle all specimens as if capable of transmitting HIV or hepatitis. Always wear protective clothing when handling specimens. Always work under strict hygienic conditions (e.g. LAF-

bench ISO Class 5) to avoid possible contamination. Only for intended use. The long term safety of embryo vitrification on children born following this procedure is unknown.

Method

Ensure all media are well mixed before use. We strongly advise to read through all the steps of the vitrification/warming procedure before starting the procedure.

Preliminary Steps

In a 4-well dish fill the first well with 300µl of Pre-Incubation medium, the second with Vitri-Freeze 2 solution. Next open as many HSV devices as will be required for the vitrification step, taking into account that 1 HSV device can hold up to two embryos. Conveniently place the separate parts of the HSV device on the work bench for easy access later in the procedure.

Freezing Procedure

Transfer the embryos from the blastocyst cell culture medium into each of the VitriFreeze solutions using the following scheme:

Stage	Pre-Incubation	VitriFreeze 1	VitrFreeze 2	Temperature
Early Blastocyst Morulae	2’	2’	30”	Room temperature
Blastocyst – Expanded Blastocyst	2’	3’	30”	37°C
Blastocyst – expanded blastocyst and artificial shrinkage	2’	2’	30”	Room temperature

*Before starting the vitrification procedure, in order to reduce the negative effect of the blastocoel, expanded blastocysts should be collapsed by reducing artificially with a glass

pipette the volume of the blastocoel (Vanderzwalmen et al., 2002; Son et al., 2003; Hiraoka 2004).

Vitrification

1. Using an attenuated pipette or an equally suitable device, deposit maximum 2 blastocysts in a volume of approximately 0.3µl of VitriFreeze2, in the gutter of the tip of the vitrification straw.
2. Place the vitrification straw in the outer sheath and seal it as indicated in the instructions for use of the HSV device.
3. Plunge the sealed device into the liquid nitrogen.

Warming

1. Remove vitrification straw from the outer sheath as indicated in the instructions for use of the HSV device.
2. Immediately plunge the vitrification straw into pre-heated VitriWarming Warming medium 1 (37°C) and leave in warming 1 for 3 minutes.
3. Transfer into VitriWarming Warming medium 2 (37°C) and leave in this medium for 2 minutes.
4. Transfer into VitriWarming Warming medium 3 (37°C) and leave in this medium for 2 minutes.
5. Finally transfer into VitriWarming Warming medium 4 (37°C) and wash for at least 1 minute.
6. Transfer into blastocyst culture medium for continued cell culture.

11) Vitrification Consent Forms used at Drs Aevitas Institute for Reproductive Medicine

INSTITUTE FOR REPRODUCTIVE MEDICINE
TYGERBERG & VINCENT PALLOTTI HOSPITALS

VITRIFICATION OF HUMAN EMBRYOS
INFORMATION & CONSENT

- Vitrification is a process similar to freezing.
- Vitrification is a standard and routine method used all over the world to successfully preserve and store human embryos and especially blastocyst stage embryos.
- Only embryos/blastocysts of top quality can be vitrified and warmed (warmed) successfully.
- Human embryos/blastocysts can be stored in vitrified state for a number of years.
- Some embryos/ blastocysts can be affected by the vitrification-warming process and may therefore not survive and not be available for transfer.
- A warmed embryo/blastocyst cannot be vitrified a second time.
- The transfer of surviving, warmed embryos/blastocysts does NOT guarantee a successful pregnancy.

We have implemented a vitrification program at our clinic and need your consent to treat your supernumerary embryos, should vitrification be possible.
Please note that only if embryos are vitrified will a form with the results be posted to you.

Please complete the following form.

We, _____ (Husband/Partner) [I.D. No._____ _____]

and _____ (Wife/Partner) [I.D. No._____]

referred by _____ (Doctor), request Aevitas clinic to vitrify and store our embryos, according to the provisions stated below:

1. An **initial fee** for the vitrification procedure and an **annual storage fee** (*pro rata*) is payable to the AEVITAS clinic;

2. The AEVITAS clinic must be informed of any **changes in personal details** (eg. postal address; telephone numbers). If patients cannot be contacted using the latest personal details on record after a period of 1 year, the stored embryos will be warmed.

3. **Failure to pay** the annual fee within 1 year after the account has been issued, will result in the stored embryo/s being warmed.

4. Embryos will be stored for a maximum period of 3 years (**for non-pregnant cycles)**. Thereafter they will be warmed.

5. Embryos will be stored for a maximum period of 5 years (**for pregnant cycles)**. Thereafter they will be warmed.

6. Vitrified – stored embryos must be warmed and/or transferred before a new stimulation and oocyte aspiration cycle will be attempted.

- ❖ We understand that the vitrified-stored embryos may not survive the warming process in preparation for embryo transfer, and that surviving, transferred embryos might not result in a pregnancy.

- ❖ We understand the necessity of selecting embryos of sufficient quality for vitrification and give the doctor and laboratory staff the authority to make this decision.

- ❖ We acknowledge that if, at any time, we wish to have our vitrified embryo/s warmed, and we **both** must give written consent to the AEVITAS clinic by means of the form which is available from the clinic.

- ❖ In the event of the death of one or both husband and wife, the stored embryos must be:

(Please mark and initial the selected option)

In the event of wife's / partner's death:	
assigned to the care of my husband/partner	
husband's / partner's name:	I.D. No.
Warmed (warmed)	
used for scientific research	
offered for donation to a couple	

In the event of husband's / partner's death:	
Assigned to the care of my wife/partner	
wife's / partner's name:	I.D. No.
Warmed (warmed)	
used for scientific research	
offered for donation to a couple	

In the event of death of both husband & wife:	
Warmed and (warmed)	
used for scientific research	
offered for donation to a couple	

Signed at …… on the ……. day of ……..…….. 20 ……..

Husband (signature) _____ (print name) _____

Wife (signature) _____ (print name) _____

Witness (signature) _____ (print name) _____

INSTITUTE FOR REPRODUCTIVE MEDICINE
TYGERBERG & VINCENT PALLOTTI HOSPITALS

STORAGE OF / VITRIFIED EMBRYOS
RESULT OF VITRIFICATION PROCESS

Dear Mr & Mrs _____ Date _____

Result of vitrification process

Number of embryos vitrified	*Cell stage*

Signed at …… on the ……. day of ……..…….. 20 ……..

Laboratory technologist _____ _____
(signature) (print name)

Head clinical technologist _____ _____

(signature) (print name)

INSTITUTE FOR REPRODUCTIVE MEDICINE
TYGERBERG & VINCENT PALLOTTI HOSPITALS

WARMING OF VITRIFIED EMBRYOS

If you no longer wish to continue the storage of your vitrified embryo/s, please complete the following form and return it to the clinic.

*Please note that **BOTH** partners **MUST** sign this form before any embryos can and will be discarded.*

Date: _____

We hereby request that our vitrified embryos should be warmed and discarded.

HUSBAND / PARTNER

Surname: _____

First name(s): _____

Identity number: _____

Signature: _____

WIFE / PARTNER

Surname: _____

First name(s): _____

Identity number:

Signature:

Institute for Reproductive Medicine

- Tygerberg Hospital, 3rd Floor, Green Avenue, Room 22.
  Tel: (021) 938 5173 Fax: (021) 933 3084
- Vincent Pallotti Hospital, Alexander Road, Pinelands.
  Tel: (021) 531 6999 Fax: (021) 531 7919
- PO Box 19058, Tygerberg, Cape Town, South Africa, 7505.

12) Modified Vitrification Procedure used at Drs Aevitas Institute for Reproductive Medicine

Vitrification Procedure used for the outcomes in the retrospective study

- Done at room temperature
- In an ICSI dish make a large drop of VitrFreeze Pre-incubation medium and a small drop adjacent to it.
- Using a pulled pipette place the embryos into the smaller drop of medium and start the countdown timer at 7 minutes.
- Aspirate clean medium from the big drop and then transfer the embryos from the small drop to the surface of the big drop, allowing them to float down.
- Fetch enough liquid nitrogen in a deep polystyrene container to cover the clearly labelled Cryotop® straws.
- Plunge the Cryotop® cover straw into the liquid nitrogen.
- When there are 30 seconds left on the timer make 2 drops as before with VitriFreeze Freezing medium 1.
- Once the 7 minutes of equilibration are over transfer the blastocysts from VitrFreeze Pre-incubation medium to VitriFreeze Freezing medium 1 as described above.
- The blastocysts remain in VitriFreeze Freezing medium 1 for 2 minutes.
- Repeat the process of making drops with of VitriFreeze Freezing medium 2 and culture the embryos in this medium for 30 seconds.
- When there are about 15 seconds left on the timer aspirate the blastocysts with as little medium as possible and pick up ~~of~~ the Cryotop® using your free hand.
- Move the dish away and focus on the Cryotop® tip. Place the blastocysts [1, 2 or 3] onto the Cryotop® and aspirate as much surrounding medium as possible. Do not place more than three embryos on one straw.
- Plunge the tip of the Cryotop® quickly into the liquid nitrogen, stirring it for 5 seconds.
- While working under the liquid nitrogen, replace the cover straw over the Cryotop® ®.
- Store the Cryotop® combination in a liquid nitrogen tank at -196°C.
- Do all the necessary paperwork – note the storage place, the colour of the Cryotop® and the date of vitrification.
- Make sure all consent forms are in order.

13) Collapsing Protocol used at Drs Aevitas Institute for Reproductive Medicine

- Place a holding and collapsing pipette into the ICSI manipulators.
- Place a single blastocyst [grade 2 and 3) into each collapsing droplet [oil covered HEPES buffered flushing medium – SAGE] in a small petri dish.
- Place the dish on the heated stage and lower the collapsing needle into the flushing medium of the dish. Aspirate flushing medium into the needle and bring the meniscus into your field of view as to see where suction begins.
- Gently aspirate the blastocyst onto the holding pipette ensuring that the inner cell mass (ICM) is at 9 o'clock position
- Insert the collapsing needle into the embryo with a quick, piercing movement through the zona into the blastocoele and ensuring not to touch the opposite side of the trophectoderm.
- Gently aspirate with the collapsing needle, watching the meniscus slowly moving upwards. Aspirate as much fluid as possible, moving the needle into the area of fluid. Take care not to aspirate the trophectoderm or the ICM.
- Once complete, remove the collapsing needle and release the collapsed blastocyst.
- Once all the blastocysts are collapsed move the dish to the cooled, room temperature stage of the dissecting microscope.

14) Modified Blastocyst Warming Procedure used at Drs Aevitas Institute for Reproductive Medicine

Blastocyst warming procedure used for the retrospective study

- Collect the patient's Cryotop® straw from liquid nitrogen storage in a deep polystyrene container, ensuring that the straw is submerged at all times.
- Bring all warming media to 37°C
- Fill a centre well dish with VitriWarming Medium 1 and place on the heated stage under the microscope.
- Whilst working under the liquid nitrogen, remove the Cryotop® straw cover.
- Quickly transfer the tip of the Cryotop® loaded with embryos to the VitriWarming Medium 1 in the centre well dish.
- Look under the microscope to ensure that the blastocysts are dislodged from the Cryotop® into the medium.
- Start the countdown timer for 3 minutes.
- When there are about 30 seconds left make a large drop of VitriWarming Medium 2 in a small petri dish and a small drop adjacent to it.
- Using a pulled glass pipette remove the blastocysts from the centre well dish ~~into~~ and place them into the smaller of the two drops.
- Start the countdown timer for 2 minutes.
- Aspirate clean medium from the big drop and then transfer the blastocysts from the small drop to the big drop.
- Again, when there are about 30 seconds left, make drops for VitriWarming Medium 3 as previously described for Medium 2.
- Follow this same procedure for VitriWarming Medium 4
- The blastocysts must incubate in Medium 4 for at least 1 minute.
- Embryos should then be transferred into a NUNC® 4 well dish containing blastocyst medium to be incubated, and later checked for survival.
- A minimum incubation time of 3 hours is required before embryo transfer can occur.

15) Vitriplug Method of Blastocyst Freezing and Warming

MEDIUMS

Product	Company	Cat. No	Tel/fax/email
Dulbeccoss PBS	LSS (Invitrogen)	14040-083	
Ethylene glycol	Sigma	E 9129	
DMSO	Sigma	D 2650	
Ficoll 400	Sigma	F 8636	
Sucrose	Sigma	S 1888	
HSA	Cooper Surgical		
Filters 0.22µm	Sartorius	16534	
Hemistraws (VitriPlug)	Astro Medtec	VIT 100	astromed@utanet.at
Steel balls	Astro Medtec	VIT-B-100	astromed@utanet.at
Sperm straw 133 mm 0.5 ml	Astro Medtec	CBS-011455	astromed@utanet.at
Flexible conventional straws 133 mm e	CryoBioSystem	014103	bl@cryobiosystemimv.com
CBS 0.3 ml straw	CryoBioSystem	CBS-010288	bl@cryobiosystemimv.com
Liquid Nitrogen			
LN_2 storage container			
Marker pen			
LN_2 vitrificatio container			Peter Rowley
Culture media	Cooper Surgical		

Solutions

Make up stock solutions

Stock A

5 ml DMSO
6 ml Dulbeccos PBS
1.5 ml HSA (100 mg/ml)

- Mix DMSO and PBS thoroughly FIRST
- Then add HSA
- Filter sterilize

(can keep for 2 weeks at 4ºC)

Stock B

5 ml ethylene glycol [EG]
6 ml Tobaccos PBS
1.5 ml HSA (100 mg /ml)

- Mix thoroughly
- Filter sterilize

(can keep for 2 weeks at 4ºC)

Stock C (20% HSA/PBS)

2 ml HSA (100 mg/ ml)

8 ml PBS

- Mix thoroughly
- Filter sterilize

(can keep for 2 weeks at 4ºC)

Equilibration medium (E Medium)

2 ml of A

2 ml of B

4 ml of C

 Mix thoroughly

(can keep for 2 weeks at 4ºC)

10% EG, 10% DMSO

Vitrification medium (V Medium)

3 ml of A

3 ml of B

1.53 g Sucrose

60 mg Ficoll 400

 Mix thoroughly

(can keep for 2 weeks at 4ºC)

20% EG, 20% DMSO, 25 µmol/L Ficoll, 0,75 mol/L sucrose

Method

- Label VitriPlug
- Prepare LN_2
- Prepare cover straw (CBS-010288)
- Cut back part (± 2.5 cm)
- Put Cover straw in LN_2
- Find a storing space for embryos
- Fill out all appropriate forms

For all cell types:

- Remove embryo from culture medium
- Wash in 2 drops (50 µl) of PBS/HSA20 at 37ºC

Then do vitrification for different embryo stages as follow:

Embryo stage	E Medium	V Medium	Temperature
Morula and Early blastocyst	2 minutes	30 seconds	25ºC [room temperature]
Blastocyst	3 minutes	30 seconds	37ºC
Expanded blastocyst	4 minutes	30 seconds	37ºC
Collapsed expanded blastocyst	2 minutes	30 seconds	25ºC [room temperature]
Hatching/hatched blastocyst	2 minutes	30 seconds	25ºC [room temperature]

- Aspirate embryo with mouth pipette (maximum 2 embryos/ VitiPlug)
- Place in small volume (0.1-0.3µl) onto the VitriPlug tip
- Plunge tip into LN_2 and move gently around for a few seconds
- Keep under LN_2 and insert into precooled cover straw (held with a metal pincette)
- Plunge into LN_2
- Store securely in documented storage place (under LN_2)

WARMING METHOD

To be done 24 hours before embryo transfer (ET)

Solutions

- 0.5 M sucrose in PBS/HSA20

  1.71 g sucrose in10 ml PBS/HAS20

- 0.25 M sucrose in PBS/HSA20

  2 ml 0.5 M sucrose + 2 ml PBS/HSA20

- 0.125 M sucrose in PBS/HSA20

  1 ml 0.5 M sucrose + 1 ml PBS/HSA20

METHOD

- Place ± 3 ml of the 0.5 M sucrose solution in a small petri dish at 37ºC
- In a separate petri dish put 2 drops of 0.25 M, 0.125 M and PBS/HAS.
- Remove VitriPlug from storage tank
- Remove cover straw very carefully
- Insert VitriPlug tip with embryos directly into the 3 ml 0.5M sucrose at 37ºC
  Stir for a few seconds
- Make sure embryo/s are dislodged from the VitriPlug tip and free-floating
- Incubate the embryos for 2, 3 or 4 in minutes in the 0.5M sucrose solution the medium.
- Transfer to the big drop of 0.25M sucrose at room temperature for 2 minutes (I minute in each drop)
- Repeat for the 2 drops of 0.125 M sucrose (2 minutes/ room temperature)
- Transfer to the PBS/HSA20 drops for 2 minutes at room temperature
- Transfer now to culture medium drop under oil at 37ºC (for blastocyst – to blastocyst medium)
- Incubate for 24 hours before transfer

16) Embryo Transfer Protocol used at Drs Aevitas Institute for Reproductive Medicine

Embryo Transfer Protocol

- Place 0.7ml of blastocyst medium into wells 2 and 4 of a 4-well NUNC® dish and incubate in a CO_2 incubator for a minimum of 3 hours.
- 10 minutes before the transfer, place the selected embryos/blastocysts into well 4 of the NUNC® dish and return the dish back to the incubator.
- Supply the doctor with speculum rinsing medium at 37°C
- Once the doctor has positioned the speculum take the syringe and soft catheter from theatre and move through to the laboratory.
- Remove the NUNC® dish from the incubator an start the timer (transfer should take no longer than 2 minutes)
- Use the syringe to aspirate all the medium from well 2 of the NUNC®.
- Hold the syringe upright (tip up), tap it so any air bubbles move to the top and expel the bubbles.
- Attach the Efficiere Series catheter (Cooper surgical) to the syringe and expel all the medium in the syringe through the soft catheter back into well 2 in order to rinse and fill the soft catheter with medium.
- Lift the top of the soft catheter out of the medium and aspirate a small air bubble at the tip of the catheter.
- Place the tip of the catheter into well 4, away from the embryos and aspirate first, a small amount of medium, followed by the embryos and more medium (0.2ml in total). You must ensure to visualize the embryos entering the tip of the soft catheter.
- Double check well 4 to make sure that all the embryos for transfer are no longer there.
- Move through to theatre and carefully hand the soft catheter and syringe to the doctor.
- Once the doctor has indicated that the catheter is in place - as seen on the sonar image - slowly push in the syringe plunger in a controlled manner and thereby the medium containing the embryos into the uterus, trying to visualize the “bubble” of medium on the sonar image.
- Hold the plunger of the syringe in for about 5 seconds before releasing it.
- The timer can be stopped at this point.

- Take the catheter back to the laboratory once the doctor has removed it.
- While holding the tip of the catheter over the overturned lid of the NUNC® dish, remove the syringe from the catheter in order for the remaining liquid to be expelled. Aspirate air into the syringe and reattach to the catheter in order to expel any further medium that has not flowed out of the catheter.
- Under the microscope check the expelled medium to ensure that all embryos have been transferred successfully.

17) Protocol for obtaining Human Chorionic Gonadotropin (HCG) Levels

Human Chorionic Gonadotropin (hCG) is a glycoprotein hormone comprising 2 subunits, namely alpha and beta, which are joined non covalently. The corpus luteum is responsible for the production of progesterone during the luteal phase of the menstrual cycle. In the absence of fertilization, the corpus luteum undergoes the degradation process known as luteolysis, and, as a consequence, progesterone levels decline (Richard *et al*., 2001). In the event of fertilization, the hormone hCG is secreted by the implanting embryo and interacts with the Leutenizing Hormone Choriogonadropin Receptor to maintain the corpus luteum and consequently secrete progesterone. The progesterone enriches the uterus with a thick lining of blood vessels and capillaries so that it is able to sustain the developing foetus. Due to its highly negative charge, hCG may repel the immune cells of the mother, protecting the fetus during the first trimester.

Although the timing from fertilization to the initial appearance of hCG can differ between assisted and unassisted pregnancies, the amounts and rate of increase per day of hCG levels are similar (Lenton *et al., 1982*). Implantation in patients usually occurs within 10 days after embryo transfer (Liu *et al*., 1995) and is the day on which the first hCG measurement is obtained. The maternal plasma levels of hCG continue to double about every 2 days in a normally developing intrauterine pregnancy (Seeber, 2006), and peak at 6–8 weeks after conception, when production of progesterone is taken over by the placenta (Braunstein, 1996). Failed pregnancies have been correlated with a low amount of hCG (France *et al*., 1996) and with a slow doubling time during pregnancy (Kratzer and Taylor., *1990*). Comparisons show that hCG mean levels are several times higher in successful pregnancies than in unsuccessful pregnancies (Seeber, 2006; Bjerke *et al*., 1999).

The Vacutainer© needle has a sharp point at both ends, covered by a rubber sheath, with one end being shorter than the other. The long end of the needle (distal) is used for penetrating the vein, the shorter end (proximal) is used to pierce the rubber stopper of the vacuum tube. The sheath makes it possible to draw several tubes of blood by preventing leakage of blood as tubes are changed, which is called a multi-draw.

As the name implies, there is a vacuum in the collection tube. The vein is punctured with the distal hypodermic needle and when the sheathed proximal needle pierces the color-coded rubber stopper, blood is sucked into the tube. That assumes that the distal end of the needle has successfully pierced the vein.

A positive βhCG recording is considered to be when the first reading (day 10) is >10 and the second reading (day 14) is at least double the amount recorded in the first reading. A Biochemical Pregnancy is defined as a Positive βhCG reading.

18) Proposed Phase I Informed Consent

DRS AEVITAS
VINCENT PALLOTTI HOSPITAL

DONATION OF FROZEN EMBRYOS FOR RESEARCH

If you no longer wish to continue the storage of your vitrified embryo/s and wish to donate them to the clinic for research, please complete the following form and return it to the clinic.

*Please note that **BOTH** partners **MUST** sign this form before any embryos can be used.*

Date:

We hereby give permission that our embryos be used by Drs Aevitas clinic for research purposes. We understand that the embryos will not be used for cloning research nor will they be offered to other patients.

WIFE / PARTNER

Surname:

First name(s):

Identity number:

Signature:

Vincent Pallotti Hospital, Alexander Road, Pinelands.
Tel: (021) 531 6999 Fax: (021) 531 7919
PO Box 112, Howard Place, Cape Town, South Africa, 7450.

19) Protocol for Laser Assisted Hatching used at Drs Aevitas Institute for Reproductive Medicine

Laser Procedure

- Turn on heated stage, microscope light and computer
- Slide laser box into microscope and let it "click" back into alignment
- Open ZILOS programme on computer

Laser Alignment

- Put cover slip with black marker onto heated stage
- Click on "Setup" and "Begin target alignment"
- Click on "Fire"
- Use the cursor to click on the centre of the fired circle which will appear in the black marker. This will move the red arrow to the centre of that circle.
- Repeat roughly 3 times until the hole that is shot is always at the red arrow.
- Click "Alignment OK"

Performing Laser Assisted Hatching

- Transfer one embryo into a small drop of flushing medium under oil.
- Transfer dish to heated stage.
- Find the embryos location by looking through the microscope eye pieces.
- Centre embryo on screen
- Take measurements and picture 1
- Move embryo so that the area where you are going to laser is positioned under the red arrow. The ICM should be positioned on the opposite side of the embryo and a large perivitteline space is preferable. An eight of the circumference of the embryo is required to be lasered.
- Take photograph2
- Transfer the embryo back into the culture drop

20) Proposed Phase II Informed Consent

INFORMATION AND CONSENT

Human Blastocyst Vitrification: Optimizing outcomes in an Assisted Reproductive Technology (ART) programme

Emma Wilson Poe (BSc Hons); Dr De Beer (PhD); Prof Kruger (MD, PhD, DSc) & Mr Greg Tinney (MSc)

Dept of Obstetrics and Gynecology, Stellenbosch University/Dr Aevitas Fertility Clinic, Pinelands.

Please read the following and feel free to ask any questions. Participation is **entirely voluntary** and you are free to decline to participate. If you say no, it will not affect your treatment negatively in any way whatsoever. You are also free to withdraw from the project at any point.

To implant into the uterus the embryo must escape from its outer covering, the zona pellucida. Assisted hatching involves creating a hole in the zona pellucida to facilitate this natural hatching process while *in* vitro. Some studies have shown that laser assisted hatching (whereby the hole is created by use of a laser) on frozen-warmed embryos can POSSIBLY increase implantation, pregnancy and live birth rates after transfer of such embryos. There have been no reports of any adverse effects. We are investigating this practise in our clinic.

The frozen embryos in storage will be warmed before transfer. Patients will be randomized into two groups: in one group the zona pellucida of all the warmed embryos will be treated with laser to make an opening; and in the other group it will not be done. In both groups warmed embryos will be transferred using the routine standard embryo transfer method of the Dr Aevitas Clinic.

DECLARATION BY COUPLE:

We _____ *(wife/partner)*[ID No: _____]

_____*(husband/partner)*[ID No.:_____]

agree to take part in a project entitled *Human Blastocyst Vitrification: Optimizing outcomes in an Assisted Reproductive Technology (ART) programme.*

- We have read or have had read to us the information on the consent form and it is written in a language with which I am fluent and comfortable.
- We have had a chance to ask questions and all my questions have been adequately answered.
- We understand that taking part in this project is **voluntary** and I have not been pressurised to take part.
- We may choose to leave the project at any time and will not be penalised or prejudiced in any way.
- This procedure will be at no additional cost to the patient.

Signatures of participants

Signature of witness

Signed at (*place*) _____ on (*date*)____ / _____ / 2014.

21) *Randomized Table for Patient Distribution in Randomized Control Trial*

Laser Assisted hatching – vitrified –warmed blastocysts			
		NAAM	PR
1	A		
2	A		
3	B		
4	B		
5	B		
6	A		
7	B		
8	A		
9	A		
10	A		
11	B		
12	B		
13	A		
14	A		
15	A		
16	B		
17	B		
18	A		
19	B		
20	B		
21	A		
22	B		
23	A		
24	A		
25	B		

A LAH

B Control