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Factors affecting the survival and implantation of human blastocysts following vitrification

Hamish Barblett
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FACTORS AFFECTING THE SURVIVAL AND IMPLANTATION OF HUMAN BLASTOCYSTS FOLLOWING VITRIFICATION

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B App Sci (Biol), MRepMed

This thesis is presented in partial fulfilment of the
degree of

Master of Science (Human Biology)

Supervised by:

Associate Professor Peter Roberts and Professor Phillip Matson

School of Medical and Health Sciences,
Edith Cowan University

2019

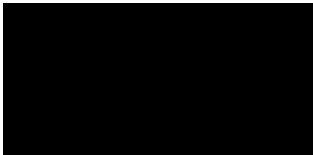
ABSTRACT

The increased cell numbers, presence of the blastocoel and rapid cell re-organisation have required the development of specific survival criteria post warm to effectively select the most viable blastocyst for transfer. Pre-freeze blastocyst expansion and post warm re-expansion have been shown to contribute significantly to the chances of an implantation and subsequent live birth. The aim of this study was to explore factors that influence the outcome of blastocyst transfers after vitrification and warming, and hopefully improve outcomes by further applying improvements in future cycles. Variables from 8 years of vitrified/warmed blastocysts were retrospectively compiled and analysed to determine the most significant contributors to outcome. There were 2466 transfers of either 1 or 2 vitrified/warmed blastocysts resulting in 796 (32.3%) clinical pregnancies and 751 (30.5%) live born babies. The patient/cycle specific variables of age: ≤ 38 years (OR: 2.01, 95% CI:1.48-2.73), transfer order: ≤ 2 (OR:1.32, 95% CI:1.10-1.59) and cycle type: non-HRT (OR: 1.38, 95% CI:1.15-1.66) significantly influenced the live birth outcome. Blastocysts vitrified on day 5 of development had significantly improved outcomes to day 6 blastocysts (OR: 1.80, 95% CI: 1.37-2.35). A greater degree of blastocyst expansion on Day 5 further improved these outcomes (OR: 1.47, 95% CI:1.17-1.86). A grade 1 morphology rating significantly improved the outcomes of day 5 expanded blastocysts (OR: 1.51, 95% CI:1.24-1.85). The composition of the warming media and possibly the concentrations of osmotic buffer contributed to the survival of warmed blastocysts. Post warming assessment of the blastocyst showed that if the level of cell degeneration in the surviving and transferred embryo was less than 5%, this significantly influenced the outcome (OR:1.57, 95% CI:1.22-2.03). There was no significant difference if a blastocyst with $\geq 95\%$ cell survival commenced re-expansion within 30 or 60 minutes after the warm (OR: 1.13, 95% CI:0.87-1.46). This study highlights the significance of even a small number of degenerative cells in the warmed blastocyst despite early commencement of re-expansion and warrants further prospective analysis.

DECLARATION

I certify that this thesis does not, to the best of my knowledge and belief:

- Incorporate without acknowledgment any material previously submitted for a degree or diploma in any institution of higher education;
- Contain any material previously published or written by another person except where due reference is made in the text of this thesis; or
- Contain any defamatory material



Hamish Barblett

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I would like to express immense gratitude to my supervisors Associate Professor Peter Roberts and Professor Phillip Matson. They have been more than forthcoming in providing me timely advice and counsel during a protracted period of part-time study. Their experience in experimental design, science communication and learning in addition to thesis structure and composition has kept me focussed on the challenging task of putting thoughts to paper. In addition to support from my supervisors I would also like to thank the critical appraisal of both reviewers of my proposal and this thesis.

As a part-time student I am immensely grateful for the contributions made by my colleagues at Hollywood Fertility Centre. Dr Guy Callender that shared his wisdom after completing a PhD part-time. I can now relate to those 4am starts to help build a solid block of writing! It is also pertinent to mention two laboratory managers I have worked with during this project. Both Natalie Moska and Itziar Rebollar-Lazaro were more than willing to provide an ear when I needed to bounce ideas off them. They were also both very supportive with flexitime. In addition to the managers I would like to thank also the embryologists I have worked with during the 4 years of this project. They are a constant reminder why it is important, regardless of experience, to remain competent in all procedures being performed in the IVF laboratory especially when methodologies are changing at a rapid rate. Of these embryologists I would like to especially thank Megan Weybury for assisting me with her input on the clinical and laboratory protocol summaries.

The biggest sacrifice when embarking on such a project over a considerable amount of time has been made by my immediate family. To my wife Robyn and our children Charles, Laura, Jessica and Sarah I am especially thankful. Robyn, a full time professional herself, kept the cogs turning on the home front in addition to her own study. Without her patience, support and motivation during the last 4 years, this idea of post-graduate research would have been short-lived. Our children have also been very forgiving when Dad has been either at work or locked away in the study most of the time. They endured quite a few very short conversations as I laboured through some very frustrating periods of research. Thanks fam.

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ABBREVIATIONS

ART	Assisted Reproductive Technology
ATP	Adenosine Triphosphate
CC	Clomiphene Citrate
CAC	Chemical Air Contaminants
COC	Cumulus Oophorus Complex
CPA	Cryoprotective Agent
DMSO	Dimethyl Sulphoxide
EG	Ethylene Glycol
EBSS	Earls Balanced Salt Solution
ET	Embryo Transfer
FSH	Follicle Stimulating Hormone
GIFT	Gamete Intra-Fallopian Transfer
hCG	Human Chorionic Gonadotrophin
HEPA	High Efficiency Particulate Air
HSA	Human Serum Albumin
ICM	Inner Cell Mass
ICSI	Intra-Cytoplasmic Sperm Injection
IVF	In vitro Fertilisation
FET	Frozen Embryo Transfer
GM-CSF	Granulocyte-macrophage colony stimulating factor
hCG	Human Chorionic Gonadotrophin

hMG	Human Menopausal Hormone
LH	Luteinising Hormone
LN ₂	Liquid Nitrogen
NGS	Next Generation Sequencing
NIR	Near infrared
NPESU	National Perinatal Epidemiology and Statistics Unit
OCC	Oocyte Cumulus Complex
OHSS	Ovarian Hyperstimulation Syndrome
OPS	Open Pulled Straws
OR	Odds Ratio
PGS	Pre-implantation Genetic Screening
PN	Pronucleus
PROH	Propylene Glycol (1, 2 propanediol)
PROST	Pronuclear Stage Embryo Transfer
PVA	Polyvinyl alcohol
PVS	Perivitelline Space
RE	Re-expansion
RNA	Ribonucleic Acid
RTAC	Reproductive Technology Accreditation Committee
SET	Single Embryo Transfer
SSV	Solid Surface Vitrification
TC	Thermoconductivity

TE	Trophectoderm
TET	Tubal Embryo Stage Transfer
TLS	Time-lapse Imaging Systems
VOC	Volatile Organic Compounds
ZIFT	Zygote Intra-Fallopian Transfer
ZP	Zona Pellucida

PUBLICATIONS AND PRESENTATIONS

The following publication and presentations are a direct result of the work submitted in this thesis.

Publication

Barblett, H., Roberts, P., Matson, P., & Turner, S. (2018). P-002 - Selection of the selected: a retrospective cohort comparison of blastocyst warming media and its influence on survival and live birth rates following vitrification, warming and single embryo transfer. *Reproductive biomedicine online*, 37, e4.

doi:<https://doi.org/10.1016/j.rbmo.2018.04.003>

Oral presentations

Barblett, H., Roberts, P., and Matson, P. (2015). Early indicators of blastocyst viability after vitrification and warming: A retrospective analysis. Presented at the Scientists in Reproductive Technology (SIRT) Western Australia Post Graduate Seminar Evening, The University Club of Western Australia, Crawley, Australia.

Barblett, H., Roberts, P., Matson, P. and Turner, S. (2017) Time-lapse incubation as a tool for diagnosing blastocyst survival after vitrification, warming and prior to single embryo transfer. Presented at the SIRT mid-year meeting, Auckland, New Zealand.

Barblett, H., Roberts, P., Matson, P. and Turner, S. (2019) Development Rate of Human Blastocysts Before Vitrification Is a Significant Predictor of Outcome After Warming and Transfer On Day 5 of a Programmed Cycle: A Retrospective Analysis of Embryo-Specific Criteria In 2311 Single Embryo Transfer Cycles. Presented at the Fertility Society of Australia Annual Conference, Hobart, Australia.

Poster presentations

Barblett, H., Roberts, P., Matson, P., Moska, N., Ussher, C., and Turner, S. (2017) Before/After: The use of Time-lapse incubation for diagnosing blastocyst survival after vitrification, warming and prior to single embryo transfer. Fertility Society of Australia annual conference. Adelaide, Australia.

Barblett, H., Roberts, P., Matson, P., and Turner, S. (2018). Selection of the selected: a retrospective cohort comparison of blastocyst warming media and its influence on survival and live birth rates following vitrification, warming and single embryo transfer. Alpha Scientists in Reproductive Medicine, 12th Biennial Conference, Reykjavik, Iceland.

1 INTRODUCTION

1.1 Background to the Study

A review of 25 population surveys in 2007 showed a global prevalence of infertility to be 9% of the population (Boivin, Bunting, Collins, & Nygren, 2007) with estimates of 1 in 6 couples requiring medical intervention to achieve conception during their reproductive lifetime. A report from the World Health Organisation published in 2012 concluded that prevalence estimates had changed little over the previous two decades (Mascarenhas, Flaxman, Boerma, Vanderpoel, & Stevens, 2012). Despite the stable infertility prevalence there is increasing use of Assisted Reproductive Technology (ART) to procreate. The proportion of Australian births in 2016 that required some form of ART treatment was 4.4% (Australian Institute of Health and Welfare, 2018). This compared to 1992 when *In vitro*-fertilisation (IVF) treatment accounted for 0.9% of births (Lancaster, Shafir, & Huang, 1995). The latest report from the National Perinatal Epidemiology and Statistics Unit (NPESU) describes ART as the most common form of treatment for infertility, with over 81,000 ART cycles initiated in Australia and New Zealand in 2016 (Fitzgerald, Paul, Harris, & Chambers, 2018). Despite a significant utilisation of IVF technology, the summary embryo data from this report reveals that less than 10% of embryos created implant into the uterus. This compares to a reproductive efficiency of a fertile couple in their mid-20's of 25% (Wang. et al., 2003). Decreased implantation rates of embryos grown *in vitro* and the use of single embryo transfer (SET) have led to the development of strategies to improve the culture environment and selection of embryos prior to transfer.

Development of nutrient gradients in culture media to mimic the passage of the embryo through the fallopian tube and into the uterus has allowed extended use of culture beyond the cleavage stage to the blastocyst stage of development (Gardner, 1998). This has assisted with embryo selection as only 40-50% of fertilised oocytes reach this stage of development *in-vitro* (Schoolcraft et al., 1999a). Differentiation of the blastocoel, trophectoderm (TE) and inner cell mass (ICM) as morphological markers on Day 5 and 6 of culture also enable a more informative assessment of embryo morphology. It is also relevant that the physiological processes of the cleavage stage embryo depend on

translation of stored maternal rather than embryonic RNA (Telford, Watson, & Schultz, 1990).

Cryopreservation of excess embryos after selection for fresh transfer is well established as a successful process, with the first pregnancy in the world for the cleavage stage achieved in Australia (Trounson & Mohr, 1983). Vitrification has been the cryopreservation procedure of choice for excess blastocysts (Kuwayama, Vajta, Kato, & Leibo, 2005) and the recovery of the embryo from this procedure introduces further assessment criteria of the warmed blastocyst. The proportion of cell degeneration and further division is easily calculated in warmed cleavage stage embryos due to the relatively low number of cells (2-8) but the increased cell numbers in blastocysts (100-150) makes it difficult and inaccurate to perform this analysis. During the equilibration and vitrification of blastocysts the blastocoel collapses as the TE breaks contact with the zona pellucida (ZP). After warming, the blastocoel re-expands in some embryos and not in others, usually within a 3-4 hour period (Desai & Goldfarb, 2005) with better implantation results when re-expansion has occurred prior to the frozen embryo transfer (FET).

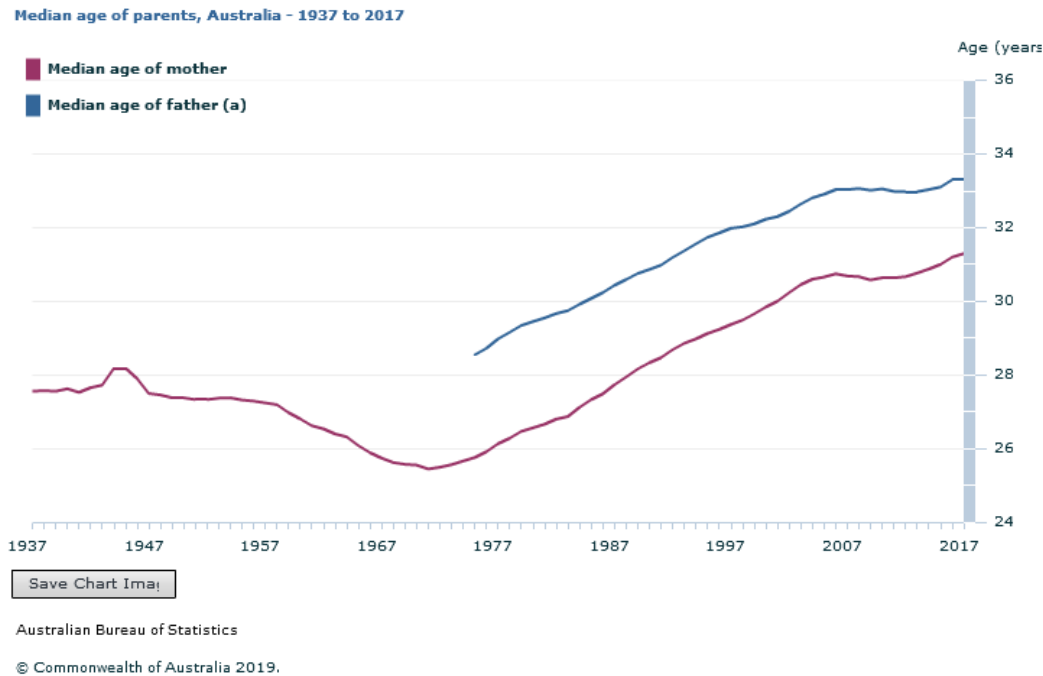
The maximum time required for a blastocyst to commence this re-expansion and successfully implant is unknown. Identifying this time point would help ensure embryologists provide the best possible embryo for transfer to the patient. The endometrium in frozen embryo transfer cycles is synchronised with the age of the embryo which for blastocysts is Day 5 or 6 post ovulation and therefore the *in vitro* time available for blastocyst assessment is limited. This is further exacerbated by embryos being frozen singularly and assessments made one embryo/warm at a time.

1.2 Prevalence of infertility and ART trends in Australia

Despite the prevalence of infertility remaining constant over 2 decades there are some important shifts in society that has seen an increasing use of ART (Mascarenhas et al., 2012). In 2017 (Figure 1-1) the median maternal age of child birth of 31.3 years compares to 29.8 in the year 2000 and 25.4 in 1971 (Australian Bureau of Statistics, 2018). Women are choosing to bear children later. The latest NPESU report for ART cycles in 2016 shows that the highest live delivery rate per embryo transfer for fresh

autologous cycles (36.9%) and autologous thaw cycles (33.3%) was for women aged less than 30 years.

Figure 1-1 Median age of parents, Australia-1937 to 2017



This success rate declined with advancing women's age with a rate of 1.3% for women aged over 44 (Fitzgerald et al., 2018). These figures demonstrate that maternal age has a significant impact on fertility and the delaying of conception results in an increased utilisation of ART. The same report revealed that more than 1 in 4 women using IVF technology to attempt a conception in 2016 were aged 40 or older. There were 15,198 babies born due to ART in Australia and New Zealand in 2016 according to the NPESU. Trends in the provision of ART are detailed in section 7 of the NPESU report. There has been a shift in the utilisation of extended blastocyst culture rather than shorter term cleavage culture prior to embryo selection for transfer and cryopreservation between 2009 (49.8%) and 2016 (78.4%). This trend is accompanied by an increase in the proportion of cycles not proceeding to embryo transfer from 23.4% to 49.0%. This is mainly due to an increasing trend in freeze-all cycles to avoid a transfer in a fresh cycle. The proportion of blastocyst warm cycles utilising the vitrification technique for cryopreservation increased from 33.2% in 2009 to 87.8% in 2016.

The number of SET procedures has increased from 69.7% in 2009 to 87.7% in 2016 resulting in a drop in multiple deliveries from 8.2% to 3.8%. These achievements have occurred while improving live birth rates per embryo transfer from 21.2% to 26.2%.

1.3 Ovarian stimulation

The first successful birth utilising IVF technology involved retrieving an oocyte from a natural menstrual cycle (Steptoe & Edwards, 1978). To increase the probability of successful fertilisation, embryo development and implantation it has been necessary to induce multiple dominant folliculogenesis in the ovaries to produce supernumerary oocytes for insemination (Claman, Domingo, Garner, Leader, & Spence, 1993). There are differing drug regimens that have been utilised to produce this follicular response.

1.3.1 Clomiphene Citrate

It was discovered during treatment of endometrial hyperplasia and breast cancer with the oral anti-oestrogen Clomiphene Citrate (CC) that it also resulted in ovulation induction (Kistner & Smith, 1961). The oral delivery, preservation of the steroid negative feedback and low cost made CC an attractive option for ovarian stimulation during early applications of IVF technology (Trounson, Leeton, Wood, Webb, & Wood, 1981). Anti-oestrogenic effects localised to the uterus, the recruitment of only 1-2 follicles and inhibited cervical mucus production are effects of CC that limit the pregnancy rates of this therapy (Gelety & Buyalos, 1993). Various modifications were developed to combat these effects including the use of lower doses (Quigley, Maklad, & Wolf, 1983) and supplementing with gonadotrophins (Li et al., 2015). Due to the reliance on a functional hypothalamic pituitary ovarian axis to exert its oestrogen receptor effects, CC is not suitable for patients with hypo or hypergonadotrophic hypogonadism (Practice Committee ASRM, 2013).

1.3.2 Gonadotrophins

Gonadotrophic preparations are the most commonly utilised agents for ovarian stimulation in IVF (Lunenfeld, 2004). Follicle Stimulating Hormone (FSH), Luteinising Hormone (LH) and human Chorionic Gonadotrophin (hCG) have evolved in purification level and source, from human pituitary gonadotrophin (Gemzell, Diczfalusy, & Tillinger, 1958) and placental cells (Seegar-Jones, Gey, & Ghisletta, 1943) to recombinant FSH

(Howles, 1996; Olijve, de Boer, Mulders, & van Wezenbeek, 1996) and hCG (International rhCG Study Group, 2001). The endogenous response to gonadotrophin therapy is monitored with the use of peripheral oestradiol measurements and ovarian ultrasonography (Speroff & Fritz, 2005). There are many factors influencing ovarian response including age of the patient and the proportion of antral follicles prior to the commencement of gonadotrophin (Rutherford et al., 1988).

1.3.3 Agonist

A problem occurring in cycles augmented with exogenous gonadotrophins is the spontaneous LH surge that can lead to ovulation prior to oocyte collection in 25-30% of cases (Hillier, Afnan, Margara, & Winston, 1985). The development and application of gonadotrophin releasing hormone (GnRH) agonists allowed physicians to flood the GnRH receptors in the pituitary with an initial activation followed by desensitization effect, thereby suppressing endogenous gonadotrophin release hence preventing premature ovulation (Conn & Crowley, 1994). An added benefit to this pituitary suppression was the ability to schedule oocyte retrievals rather than accessing facilities on an emergency basis (Rogers et al., 1986).

1.3.4 Antagonist

GnRH antagonist analogues act by binding to pituitary receptors excluding endogenous GnRH and suppressing pituitary gonadotrophic production with immediate effect and recovery. The rapid effect without a stimulation phase of gonadotrophins allows short term suppression of the endogenous LH surge and hence prevention of premature ovulation during ovarian stimulation for IVF (Olivennes et al., 1994). Other advantages of GnRH antagonist use in IVF treatment include: reduced costs; shorter duration of treatment; less patient discomfort and less gonadotrophins required to produce the oocytes (Al-Inany & Aboulghar, 2002; Fauser & Devroey, 2005).

1.3.5 Mild Stimulation

The use of gonadotrophins with GnRH analogues has enabled ovarian stimulation protocols to produce multiple oocytes for use in IVF but there are also significant side effects (Macklon, Stouffer, Giudice, & Fauser, 2006). High cost of medications, patient discomfort from injections, inconvenience due to monitoring strategies and elevated

risks associated with ovarian hyperstimulation syndrome (OHSS) are some of the issues associated with ovarian stimulation. To address these sequelae and make stimulation regimes more patient friendly, milder strategies have been developed to produce a reduced number of oocytes (Verberg et al., 2009).

1.4 Endometrial Preparation

Embryo(s) developed *in vitro* are transferred into a receptive uterus during the proliferative/ secretory phase of the endometrium. This can be in the same cycle the oocytes were retrieved from or in a subsequent monitored cycle specifically designed for the transfer of a frozen/warmed embryo.

1.4.1 Fresh embryo transfer cycles

A significant contributor to the failure of early IVF attempts was the method used to extend the shortened luteal phase in cycles stimulated with gonadotrophins (Edwards, 1973). Eventually problems associated with the luteal phase were bypassed in fresh embryo transfers by retrieving an oocyte in a natural menstrual cycle and utilising a natural luteal phase to nurture the embryo (Edwards, Steptoe, & Purdy, 1980). Cancellation and success rates per cycle start however were reduced when compared to cycles utilising ovarian stimulation with gonadotrophins (Fahy, Cahill, Wardle, & Hull, 1995). The use of ovarian stimulation regimes in assisted reproduction and in particular GnRH agonists disrupts the activity of the corpus luteum and endometrial preparation for implantation (Smitz et al., 1988). Luteal supplementation has been shown to improve endometrial quality and subsequent rates of embryo implantation (Soliman, Daya, Collins, & Hughes, 1994).

1.4.2 Frozen embryo transfer cycles

The use of ovarian hyperstimulation and the production of multiple oocytes and subsequent embryos resulted in supernumerary embryos after completion of the fresh embryo transfer (Van den Abbeel et al., 1988). Cryopreservation at various stages of embryo development has been successful in rescuing these extra embryos (Troup et al., 1991). Cycles utilising embryos previously cryopreserved require synchronisation of the endometrium with the developmental stage of the embryo (Devroey & Pados, 1998). Patients with regular cycles and an endometrium receptive to implantation may have a

natural cycle monitored to target ovulation (Sathanandan et al., 1991). Patients with irregular cycles can be administered hormone replacement therapy (Pattinson, Greene, Fleetham, & Anderson-Sykes, 1992) or mild stimulation by gonadotrophins to produce a receptive endometrium (Lornage et al., 1990). The successful use of the frozen embryo transfer cycle has led to some freeze all strategies where no fresh embryo transfer is performed so as to not reduce the chance of embryo implantation by transferring into an endometrium compromised by excessive doses of gonadotrophins (Shapiro et al., 2011).

1.5 Collection of oocytes and fertilisation

1.5.1 Oocyte Collection

After exogenous ovarian stimulation by gonadotrophins with some form of pituitary desensitisation (GnRH antagonist/agonist) the ovaries hopefully produce supernumerary follicles. Follicular development criteria are calculated in individual clinics according to their stimulation regimes but in general when 2-3 follicles $\geq 17\text{mm}$ are observed with ultrasound monitoring after 7-12 days of stimulation, ovulation is triggered by the administration of hCG (Tan et al., 1992). The timing of the egg collection procedure subsequent to the hCG trigger can vary between 35-40 hours (Templeton et al., 1986). Human oocytes were originally collected using a laparoscopic technique utilised at the time for various gynaecological surgeries (Lopata et al., 1974; Steptoe & Edwards, 1970). Due to the general anaesthesia required, difficult access due to obstructive pelvic adhesions and long recovery times, an alternative approach using an abdominal transducer was developed (Lenz, Lauritsen, & Kjellow, 1981). This procedure soon evolved after the development of vaginal transducers to a technique that was quicker and less painful requiring shorter recovery times and able to be performed as a day procedure (Wikland, Enk, & Hamberger, 1985). There are however still clinical cases that require approaching the ovary trans abdominally (Yovich, Matson, & Yovich, 1989). Transvaginal oocyte aspiration (TVOA) requires a 16-17-gauge needle, 30-35 cm in length threaded through a guide attached to a transducer that is introduced into the vaginal canal. Negative pressure (100-200 mm Hg) applied using a vacuum pump controlled by a foot pedal aspirates the contents of follicles 10mm or greater (Blackledge et al., 1986). The contents of the follicle are then examined using a stereo

dissecting microscope and oocyte cumulus complexes (OCC) identified, rinsed in culture media and placed in culture at 37 degrees Celsius utilising a physiological bicarbonate pH buffering system to maintain the pH at 7.2-7.3.

1.5.2 Sperm preparation

Seminal fluid contains 90% secretions from the accessory glands in addition to Sertoli cell secretions and spermatozoa from the testes (Kierszenbaum & Tres, 2012). Alkaline bases present in these substances provide the spermatozoa with protection from acidic denaturation in the vagina (Mann, 1964). The inhibitory effects of seminal fluid also prevent sperm from prematurely becoming acrosomally responsive (Cross, 1996). Due to these factors sperm must separate from seminal fluid to achieve capacitation and subsequent fertilisation (Austin, 1952). This separation is achieved prior to IVF often by either using a swim up procedure, density gradient centrifugation or a combination of both (Carrell et al., 1998).

1.5.3 Insemination

Insemination methods are usually confined to either conventional IVF where a number of sperm are placed around the OCC for a period of time, or intracytoplasmic sperm injection (ICSI) where individual sperm are isolated and injected into a metaphase II oocyte that has been denuded of its cumulus (Palermo, Joris, Devroey, & Van Steirteghem, 1992).

1.5.3.1 IVF

Currently in most IVF programmes the IVF fertilisation process remains blinded to the embryologist due to the presence of the corona radiata that surrounds the secondary oocyte. Oocytes retrieved 35-40 hrs after a trigger injection are briefly rinsed in culture media and placed in a dish containing media before washed sperm is added.

Insemination times are usually co-ordinated to enable an embryologist to be available 18-20 hours later to perform a fertilisation check prior to syngamy. An abbreviated co-incubation time is practiced in some centres and a systematic review and meta-analysis of studies on abbreviated insemination times has shown that as little as one hour's exposure of oocytes to sperm can improve clinical pregnancy rates without compromising fertilisation rates (Zhang et al., 2013). Co-incubation times even as short as 30 seconds have been shown to reduce polyspermy rates from 7.2% down to 2.8%

without compromising fertilisation, embryo development and pregnancy rates (Bungum, Bungum, & Humaidan, 2006). The removal of cumulus cells after the abbreviated co-incubation period (3-6 hr post insemination) has been performed successfully in some centres (Guo et al., 2016; Jin et al., 2014; Xiong et al., 2011; Xue et al., 2013).

1.5.3.2 ICSI

It is difficult to estimate the proportion of the various types of aetiologies presenting at ART units across Australia and New Zealand as it is based on clinical diagnosis that may vary between clinicians (Fitzgerald et al., 2018). According to reported data in 2016, 10.7% of initiated cycles had a male infertility diagnosis alone with 12.2% having both male and female causes of sub-fertility (Fitzgerald et al., 2018). Male infertility is defined in the WHO manual (World Health Organization., 2010) as simply one or more abnormalities in the semen. The most successful solution to achieving fertilisation with reduced sperm parameters in IVF was described in 1992 as ICSI (Palermo et al., 1992). ICSI requires the removal of the cumulus oophora soon after egg collection using a brief exposure to exogenous hyaluronidase along with mechanical pipetting using various gauge pipettes. Mature metaphase II oocytes are injected with viable sperm that have been immobilised by rupturing the plasma membrane around the distal portion of the flagellum. ICSI requires specialised micromanipulation equipment attached to an inverted microscope and performed by an experienced clinical embryologist. Progressive motility is not an essential pre-requisite for fertilisation after ICSI and therefore sperm can be sourced from both the epididymis and testicular tissue (Devroey et al., 1994; Silber, Ord, Balmaceda, Patrizio, & Asch, 1990). To aid in the manipulation of sperm prior to and during the ICSI process, a high molecular weight polymer, polyvinylpyrrolidone (PVP) or a more natural component of the extra-cellular matrix, Hyaluronic acid is used (Liu, Feenan, Chapple, Roberts, & Matson, 2017).

1.5.4 Fertilisation

Fertilisation after conventional IVF insemination does not require the sperm to travel through the various regions of the female reproductive tract. In-vivo numbers of sperm reaching the cumulus oophorus complex (COC) are thought to be an overestimate of a hundred or so with 10-20 reaching the ZP (De Jonge, 2005). The numbers used for gamete co-incubation *in vitro*, especially with poor starting sperm parameters have been

reported up to 1.2 million/ml (Trounson, 1994). As sperm enters the egg vestments, hyaluronic activity and hyperactive motility enables acrosome intact sperm to proceed through the extra cellular matrix and make surface contact with the ZP (Huszar et al., 2003). Exposure to zona proteins leads to an acrosome reaction assisting passage through the ZP into the perivitelline space (PVS) and the sperm plasmalemma subsequently fuses with the oolemma of the oocyte (Chen & Sathananthan, 1986). Oocyte activation soon follows with repetitive cytoplasmic oscillations, elevation of intracellular calcium levels and the migration of cortical granules towards the oolemma (Sathananthan et al., 1994). Cortical granules release enzymes that modify zona proteins and prevent further sperm entering the PVS (Ducibella, 1996). Meiosis II resumes with the extrusion of the second polar body and the sperm head chromatin decondenses. Nuclear envelopes form and the male and female pronuclei migrate toward each other with microtubules inherited from the sperm centrosome (Schatten, 1994).

1.6 Embryo culture, zygote to blastocyst

1.6.1 Culture Media

Early culture media formulations used for human IVF were based on simple salt solutions such as Earls Balanced Salt Solution (EBSS) or more complex recipes such as Hams F10 inherited from animal somatic cell culture systems. Tyrodes T6 and Whittens WM1 were also inherited from animal ART laboratories (Mahadevan, Fleetham, Church, & Taylor, 1986). By the mid 1980's a medium more imitative of the fallopian tube environment named human tubal fluid medium was formulated (Quinn, Kerin, & Warnes, 1985). Some 10 years later media formulations were altered according to different metabolic requirements of the human embryo from zygote to blastocyst (Leese, 1995). The energy substrate glucose was found to be at a lower concentration mid-cycle when compared to the follicular or luteal phases (Gardner, Lane, Calderon, & Leeton, 1996). This has also been augmented with improved embryo quality when glucose is removed from culture media between the pronuclear (Day 1) and cleavage stages (Day 2-3) (Coates, Rutherford, Hunter, & Leese, 1999). It was also demonstrated that only a simple set of non-essential amino acids were required during Day 1-3 of culture (Lane & Gardner, 1997) and that possibly they could act as modulators of

intracellular pH (Bavister, 1993). Later stages of development benefit by the inclusion of the essential amino acids (Gardner et al., 1998).

All embryo culture requires a buffering system to maintain a stable physiological pH. It has been shown that ionic exchange mechanisms within human embryos can maintain a pH between 7.0 and 7.3 over significant changes in external pH (Dale, Menezo, Cohen, DiMatteo, & Wilding, 1998). To avoid stress on these mechanisms the best strategy is to keep culture pH in a similar range. Commonly used buffers in ART include NaHCO_3 and N-2-hydroxyethylpiperazine-N'-2ethansulfonic acid (HEPES). The former requires gassing with CO_2 while the latter is used when handling gametes and embryos in ambient air. The use of NaHCO_3 as a non-toxic and physiological buffer also provides nutrient to the culture environment and allows manipulation of the pH through the adjustment of the concentration of the buffer or the CO_2 percentage (Carney & Bavister, 1987). The recommendation for human embryos based on the Henderson-Hasselbach equation in a culture with 25mM NaHCO_3 at sea level is to provide 6% CO_2 at 37°C. Reduced oxygen tension from atmospheric (20%) to 5% in the gas provided to human embryos *in vitro* has been shown to benefit growth (Catt & Henman, 2000; Dumoulin et al., 1999).

The majority of embryo culture is performed with some form of macromolecule supplementation in the media. Fertilisation through to cleavage and pregnancy is possible in protein-free media but consistent performance is improved with supplementation (Caro & Trounson, 1986). Whole serum has been used as a protein source in culture media but the presence of unknown elements and patient to patient variation can affect the quality of embryo culture (Gardner, 1994). Today the most commonly used protein ingredient is human serum albumin that was shown to improve results when compared to whole serum (Staessen et al., 1990). Due to the biological source of these supplements, the risk of transmitting disease and unidentified protein components there has been a focus on the development of recombinant and alternative macromolecule protein substitutes (Dyrlund et al., 2014). These have already been shown to maintain embryo quality and success rates when compared to HSA (Bungum, Humaidan, & Bungum, 2002) but the cost of regular use in IVF culture media has prevented significant uptake. Other macromolecule substitutes have also been explored

including polyvinyl alcohol (PVA), dextran and hyaluronan (Gardner, Rodriegez-Martinez, & Lane, 1999; Pool & Martin, 1994).

Cytokines and growth factors present in the female reproductive tract play a multifunctional role in supporting blastocyst development, implantation and ongoing foetal health (Kane, Morgan, & Coonan, 1997). One of these cytokines, Granulocyte-macrophage colony stimulating factor (GM-CSF) has been identified and shown through knock out mouse studies to be a significant contributor to ICM size, birth weight and subsequent mortality (Robertson, Roberts, Farr, Dunn, & Seamark, 1999). An increase in proportion of blastocyst development and subsequent cell number was shown when supernumerary human embryos were cultured in medium supplemented with recombinant GM-CSF (Sjoblom, Wikland, & Robertson, 1999). A randomised multi-centre trial found addition of GM-CSF to culture media increased the survival of transferred human embryos to live birth and may be beneficial for women with previous miscarriage (Ziebe et al., 2013).

1.6.2 Oil Overlay

It is common practice to use oil to overlay culture media to avoid rapid changes in osmolality, temperature and pH (Brinster, 1963). Media that can be expensive when obtained ready made through a commercial source can be used in smaller volumes with oil overlay due to the protection from evaporation. The oil also provides a physical barrier to airborne particulate matter. Mineral oil is a product of fractional distillation of crude petroleum. Further refinement to remove unsaturated hydrocarbons produces paraffin oil that is less likely to oxidise and be less reactive. Peroxidation of mineral oil leads to the formation of hydroperoxide that can combine with hydrogen to produce free radicals that can alter the function of the lipid component of the embryo plasma membrane (Otsuki, Nagai, & Chiba, 2007). The degree of peroxidation is dependent on exposure to heat, UV light, extended storage, lot number and manufacturer (Otsuki, Nagai, & Chiba, 2009). Manufacturers need to ensure these elements are controlled during production and distribution to IVF clinics.

1.6.3 Incubators

One of the most important elements in an ART laboratory is the incubation system. This is where the oocytes/embryos are cultured for an extended pre-implantation period from day 0 when oocytes are collected through to Day 3-6 when embryos are removed, transferred to the patient or cryopreserved. There have been novel approaches to design a system that nurtures the growing embryo to a standard close to the female reproductive tract. These include a submarine incubation system where a pre-gassed sealed bag containing the embryos are placed under water in a water bath (Vajta et al., 2004) and intravaginal culture where embryos are encapsulated and inserted into the vagina of the patient for incubation (Ranoux et al., 1988). These approaches have not been widely adopted due to inherent technical difficulties in achieving culture consistency. Traditionally large, often water jacketed incubators (150-200 litres) with CO₂, temperature and humidity control were used for human embryo culture. These incubators were gassed from a 100% CO₂ cylinder and an internal solenoid in the incubator-controlled gas injection to maintain a 5% CO₂ level with the balance in ambient air. These incubators struggled to maintain constant conditions due to the repetitive opening required in a busy IVF unit and the smaller volumes used when compared to somatic cell culture. Humidity levels especially would struggle to remain high during these periods leading to inaccurate CO₂ readings as older thermal conductivity sensors (TC) required stable humidity and temperature conditions to work correctly. Reducing the volume of these units (<50 litres) allowed for quicker recovery times and improved blastocyst development (Avery & Greve, 1992). Reducing the incubation space even further to less than 0.5 litres in bench top incubators improved temperature recovery times from 20 minutes to just 5 minutes (Cooke, Tyler, & Driscoll, 2002). The smaller volume incubation chambers can have a constant feed of pre-mixed gas that enables a cost-effective method of supplying 6% CO₂ with reduced oxygen (5%) and the balance in N₂. Bench top incubators also have customised grooved culture plates that allow direct transfer of heat to the culture ware rather than relying on convection in a larger box incubator (Fujiwara et al., 2007). Smaller bench top incubators are now produced with the additional tool of time-lapse imaging integrated into the instrument that has the additional benefit of reduced embryo handling (Cruz et al., 2011).

1.6.4 Laboratory Air

Gametes and embryos are vulnerable to the presence of volatile organic compounds (VOC) and chemical air contaminants (CAC) in the immediate environment. The air within an IVF laboratory may have elevated proportions of these compounds despite the use of high efficiency particulate air filters (HEPA) installed (Cohen, Gilligan, Esposito, Schimmel, & Dale, 1997). Full elimination of these contaminants is very difficult in IVF laboratories inheriting the workspace used previously for different purposes, but specific design-built IVF laboratories require an extensive air management system. This system should not only provide HEPA filtration but also reasonable protection from VOC by using rapid air exchange and carbon/potassium permanganate filtering. Contaminants can also come from within the workspace from new equipment, disposable tissue culture ware, compressed gas, sterilising agents and people traffic (Hall, Gilligan, Schimmel, Cecchi, & Cohen, 1998). These laboratory items require significant off gassing time and personal cosmetics and perfumes should be avoided in the IVF laboratory. The effect of VOC on embryo growth is not always obvious and can be realised through poor embryo quality and implantation rates (Boone, Johnson, Locke, Crane, & Price, 1999).

1.6.5 Culture platforms

There has been extensive research into the chemical requirements of human embryos in culture with various culture media formulations being proposed. It is important also to consider the physical environment surrounding the developing embryo. The *in vivo* embryo travels constantly through the fallopian tube and is therefore exposed to microvilli contact and movement. These factors contrast significantly to culture platforms used *in vitro* (Swain & Smith, 2011). Inert culture dishes made of a plastic polymer are in common use in IVF laboratories. The configuration of the culture is varied with isolated culture (Rijnders & Jansen, 1999), isolated but shared media (Vajta et al., 2008), or group culture (Moessner & Dodson, 1995). Various volumes and oocyte/embryo densities are used to take advantage of autocrine/paracrine effects (Katz-Jaffe, Schoolcraft, & Gardner, 2006). To create a more dynamic culture the application of external forces such as shaking (Isachenko et al., 2006), tilting (Matsuura et al., 2010), vibrating (Isachenko et al., 2011) and fluid flow (Heo et al., 2010) have been used to mimic the dynamic environment of the fallopian tube. These physical forces can

refresh the chemical constitution of the immediate environment of the embryo (Smith, Takayama, & Swain, 2012).

1.7 Embryo Selection

Although there were early attempts at IVF using gonadotrophic ovarian stimulation there was still no success at achieving ongoing pregnancies and live births (Talbot et al., 1976). After the success in the UK of a natural IVF cycle producing the world's first IVF baby, natural cycles hopefully producing one oocyte and one embryo transferred were used (Edwards et al., 1980). Success rates were low however and there were significant practical limitations due to the requirement to detect the LH surge and perform the egg collection procedure at any point in a 24 hr period (Lopata, 1980). Soon after the first baby was born there were normal pregnancies reported in controlled cycles using the anti-oestrogen Clomiphene (Trounson et al., 1981). The increasing successful use of Clomiphene alongside human menopausal gonadotrophin (hMG) and hCG to produce multiple oocytes and embryos ultimately resulted in the transfer of more than one embryo in a fresh IVF cycle (Edwards, Lobo, & Bouchard, 1996). The elevated proportion of multiple births and the associated perinatal complications ultimately resulted in the reduction of the number of embryos transferred (Templeton & Morris, 1998). The latest figures in Australia and New Zealand for cycles in 2016 show that 87.7% of embryo transfer procedures were of 1 embryo (Fitzgerald et al., 2018). This compares to less than 10% in 1993 (Lancaster et al., 1995). The widespread use of SET without a reduction in implantation rate has been the result of improved culture conditions and embryo selection strategies implemented in the laboratory.

1.7.1 Morphology

Appearance and rate of development has been the mainstay of embryo selection since ovarian stimulation increased the pool available for utilisation (Cummins et al., 1986). This strategy is easy to implement with standard microscopy and embryo handling equipment but still requires training, competency and precision of the clinical embryologist (Braude, 2013; De los Santos et al., 2016). Depending on the facilities available, clinic philosophy and local legislation there is an emphasis on selection and transfer at different developmental stages of the embryo. This selection uses various

scoring systems based on morphological features of the embryo relative to time (Hossain, Phelps, Agarwal, Sanz, & Mahadevan, 2016).

1.7.1.1 Pronucleate Embryo (Day 1)

The single cell embryo is dependent on oocyte reserves during the early developmental stages and therefore oocyte quality is a significant ingredient in any assessment of the pronucleate embryo (Braude, Bolton, & Moore, 1988). Fertilisation is dynamic and difficult to assess with static observations, but various methodologies have been proposed to select embryos with high implantation potential at this stage of development. These methodologies are based on the orientation of the pronuclei relative to each other and polar body position (Garello et al., 1999), the number and distribution of nucleoli within the pronuclei (Tesarik & Greco, 1999) and the timing of pronuclear fading and cytokinesis (Scott, Alvero, Leondires, & Miller, 2000). Grading, selection and transfer of pronucleate embryos is rarely used in contemporary IVF laboratories with the advent of blastocyst culture (Reh et al., 2010). In some circumstances when there is a history of embryo fragmentation early selection and transfer may be beneficial (Sermondade et al., 2012)

1.7.1.2 Early Embryo Cleavage (Day 2-3)

For much of the past 4 decades of IVF the morphology and rate of division of cleaving embryos has been used as a measure of implantation potential (Hossain et al., 2016; Stylianou, Critchlow, Brison, & Roberts, 2012). Common to many different grading schemes is the assessment of cell number relative to insemination time, comparative blastomere size and the proportion of fragmentation (McKiernan & Bavister, 1994; Van Royen et al., 1999). These elements are often combined into a cumulative embryo score to choose the embryo most likely to implant (Visser & Fourie, 1993). However the various scoring systems can be confusing when trying to compare embryo quality results and an international consensus may be able to address this (Alpha Scientists in Reproductive Medicine, 2011). The validity of these scoring systems have been questioned due to the difficulty of tracing embryo implantation when more than one embryo is transferred (Hoover, Baker, Check, Lurie, & O'Shaughnessy, 1995).

1.7.1.3 The Blastocyst (Day 5-6)

All stages of blastocyst development share 3 common morphological features in the TE, ICM and blastocoel and they form the basis of the most common grading system for the selection of viable blastocysts (Gardner & Schoolcraft, 1999a). The first morphological signs of cell differentiation occur when blastocoel formation begins approximately 16-20 hrs after compaction and 95-103 hrs after insemination (Campbell et al., 2013). It results in the first dimensional changes to the originating oocyte as the actions of Na^+/K^+ ion pumps located basolaterally in the forming TE cells create an osmotic gradient (Watson & Barcroft, 2001). This gradient leads to a passive movement of fluid into the extracellular space within the blastocyst which is then contained by tight junctions between TE originally formed during compaction. The blastocoel fluid containment, restricting fluid leakage by paracellular routes, ultimately results in expansion of the blastocyst (McLaren & Smith, 1977). During the expansion process the PVS disappears and the ZP thins from a 10-16 μm thickness (Balaban et al., 2002) to just an outline when fully expanded prior to hatching. The embryo increases in diameter from the 110-120 μm average of the secondary oocyte (Griffin, Emery, Huang, Peterson, & Carrell, 2006; Payne, Flaherty, Barry, & Matthews, 1997) to an average 265 μm of the expanded blastocyst (Richter, Harris, Daneshmand, & Shapiro, 2001). TE cells are the first differentiated cell line of the embryo and grow rapidly in number after the onset of cavitation with numbers in excess of 200 in the fully hatched blastocyst (Hardy, Handyside, & Winston, 1989). Soon after blastulation has begun it is evident that a clump of cells form within the blastocoel at one pole of the blastocyst. These ICM cells are pluripotent and range in morphology from a large, highly compacted group of cells correlating with an improved implantation potential (Richter et al., 2001) to a small to non-existent group of loosely compacted cells. The work of Richter et al (2001) emphasised the importance of quantifying blastocyst parameters to determine their individual influence on implantation outcome. They determined the ICM as the most important morphological element when selecting viable blastocysts. Recent studies have challenged this work suggesting more emphasis should be placed on the blastocoel and the TE rather than the ICM (Ahlstrom, Westin, Reimer, Wikland, & Hardarson, 2011; Ebner et al., 2016; Hill et al., 2013; Thompson, Onwubalili, Brown, Jindal, & McGovern, 2013).

1.7.1.4 *Morphokinetics*

Time-lapse imaging systems (TLS) have developed from bespoke setups (Payne et al., 1997) to ready built time-lapse incubators (Meseguer et al., 2011) enabling many embryos from multiple patients to be monitored simultaneously. It is now possible to replace static observations with images taken every few minutes in multiple focal planes without removing embryos from the incubator and without an embryologist present. New phenomena in embryo development can now be observed that previously were not captured. These include 2nd polar body extrusion (post ICSI), pronuclear formation and fading, cleavage patterns, compaction, cavitation, blastocyst expansion and hatching dynamics. A combination of observations at various developmental time points can be packaged into algorithms to aid in embryo selection (Liu, Chapple, Feenan, Roberts, & Matson, 2015). Software recognition of developmental milestones can also semi-quantify parameters enabling complex embryo selection algorithms to be applied (Conaghan et al., 2013). Despite these advances in technology, in 2014 a Cochrane review showed there was little peer reviewed evidence that outcomes are improved based on better embryo selection criteria in TLS (Armstrong, Arroll, Cree, Jordan, & Farquhar, 2015) and that any improvements could be attributed to incubator design. This opinion paper has since been followed with others supporting the hypothesis that improved outcomes can be realised by using a combination of TLS and traditional morphological assessments compared to traditional methods alone (Adamson et al., 2016). It is possible that these benefits may only be realised with cleavage stage embryo transfers rather than extended culture blastocyst transfers (Goodman, Goldberg, Falcone, Austin, & Desai, 2016). Another recent prospective, observational, two-centre pilot study with a matched control group showed that TLS selected embryos did not have significantly improved outcomes compared to static morphological assessments alone (Kieslinger et al., 2016). This study however did show that utilising TLS for embryo selection enabled the transfer of equally successful embryos with decreased morphological scores.

1.7.2 **Extended Culture**

The first IVF successes from ovarian stimulation were through the selection and transfer of cleavage stage embryos (Trounson et al., 1981). These early attempts however

utilised Clomiphene Citrate and hCG yielding low oocyte numbers. Super ovulation and LH suppression with agonists often result in a greater number of oocytes (Rutherford et al., 1988) and more confidence in extending the culture period to the blastocyst stage. Despite the greater number of embryos available the blastocyst formation and implantation rates were reduced during early attempts at extended culture in clinical programmes (Bolton, Wren, & Parsons, 1991). The development of complex stage specific culture media improved the outcomes of extended culture leading to greater clinical implementation (Gardner, 1998) and the most common form of embryo selection in use today (Wang, Kovacs, & Sullivan, 2010).

1.7.3 Genetic Screening

Advances in the techniques used for genetic analysis of embryos has enabled increasing use of pre-implantation genetic screening (PGS) to ensure the transfer of only euploid embryos to the patient (Munné, 2006). If supplemented with extended culture and blastocyst biopsy there is evidence that 52% of blastocysts may be euploid compared to 30% of cleavage stage embryos (Fragouli et al., 2011). The use of high resolution next generation sequencing (NGS) has also helped with the identification of levels of mosaicism believed to be up to 30% in blastocysts (Grifo et al., 2015).

1.7.4 Biochemical markers of viability

There has been much work on the identification of bio-markers of viability for human embryos that are non-invasive and better quantify evidence for embryo selection. Oocyte respiration analysis has shown a variation of ATP production in unfertilised oocytes in an IVF programme and may be a future tool for embryo selection (Obeidat et al., 2018). The measurement of proteins in the supernatant of culture media used for embryo culture has identified possible indicators of embryo viability (Katz-Jaffe & Gardner, 2008; Warner, Lampton, Newmark, & Cohen, 2008). Amino acid production and depletion of embryos as measured in culture media has much potential in the selection of viable embryos (Sturmey, Brison, & Leese, 2008). Raman (low frequency) and near infrared (NIR) spectrophotometric analysis of the culture medium surrounding embryos has enabled the quantification of the secretome (ATP, fatty acids, glucose, cholesterol, hormones and other signalling molecules) providing a snapshot of embryo

health that has shown some correlation to embryo implantation potential (Nagy, Sakkas, & Behr, 2008).

1.8 Embryo transfer

Embryo transfer techniques in common use today involve access to the uterus via the cervical canal and have changed little since the early pregnancies in IVF (Trounson et al., 1981). A retrospective study of embryo transfer procedures with difficulty achieving passage through the cervical canal has shown poorer outcomes compared to easier transfers (Tomás, Martikainen, Tapanainen, Tikkinen, & Tuomivaara, 2002). The addition of ultrasound guidance using an abdominal transducer has however improved the accuracy and outcome of the procedure especially when access is difficult (Kan et al., 1999; Sallam & Sadek, 2003). The use of ultrasound guidance has enabled the documentation of embryo positioning and a position closer to the fundus has also been shown to positively influence outcomes (Wong et al., 2016). Surgical transfer techniques have been explored in the past due to perceived problems with this approach, including the induction of contractions in the myometrium possibly moving embryos into the cervix or upward to the fallopian tubes (Righini et al., 1998). Contamination of the uterine environment from microbes present in the cervical mucus and adhering to the transfer catheter is also considered a risk (Tan, Bennett, & Parsons, 1990). Surgical approaches included gamete intra-fallopian transfer (GIFT), suitable for patients without tubal occlusion, where a preparation of sperm and oocytes are introduced abdominally via laparoscope to the fallopian tube (Asch, Balmaceda, Ellsworth, & Wong, 1985). If sperm parameters were suboptimal then oocytes were fertilised *in vitro* and transferred to the fallopian tube by zygote intra-fallopian transfer (ZIFT), pronuclear stage embryo transfer (PROST) or tubal embryo transfer (TET) (Balmaceda et al., 1988; Devroey et al., 1986; Yovich et al., 1987). The utilisation of tubal transfers has progressively been replaced with the earlier cheaper and less invasive cervical procedures due to other advances in ART. These include the development of complex culture media enabling blastocyst transfers, ICSI and assisted hatching (Ménézo & Janny, 1996). The use of media supplementation with adherence compounds such as hyaluronan has been shown to offer some benefit to the embryo transfer procedure (Bontekoe, Blake, Heineman, Williams, & Johnson, 2010).

1.9 Embryo cryopreservation

1.9.1 Early work with sperm and mouse embryos

The original challenge in the cryopreservation of living cells was to avoid intracellular ice formation and avoid the deleterious effects of increasing extracellular solute concentrations due to ice formation (Mazur, 1963; Muldrew, 2008). Non-toxic, low molecular weight, water soluble molecules that disrupt hydrogen bonds between water are common in animal and plant species that are required to survive sub-zero temperatures (Gosden, 2011). The use of 10% dilutions of cryoprotectant agents (CPA) glycerol, propylene glycol or ethylene glycol (EG) to achieve this during the slow freezing of semen to -79 °C showed good recovery rates (Polge, Smith, & Parkes, 1949). However, each cell type has different requirements in cooling rates depending on cell membrane permeability and CPA used. The work of Whittingham and colleagues on the successful cryopreservation of mouse embryos at various stages laid the foundation for many of the slow freezing protocols used today (Whittingham, Leibo, & Mazur, 1972). They showed superior results using Dimethyl Sulphoxide (DMSO) with slow rates of freezing for mouse embryos rather than the use of glycerol. DMSO (C_2H_6OS) with a molecular weight of 78.13 g/mol has a freezing point of 18.5°C but the freezing point in solution (eutectic point) is less than -60°C (Kleinhans & Mazur, 2007).

1.9.2 Slow freezing trials of Human Embryos

The use of gonadotrophins for ovarian stimulation often produced more embryos than could be transferred to the patient safely in the fresh cycle and this scenario introduced embryo cryopreservation as a technique that IVF laboratories were obliged to incorporate into their services (Trounson, Wood, & Leeton, 1982).

1.9.2.1 Pronucleate and Cleavage Stage

A trial of 1M glycerol vs 1.5M DMSO as a CPA on day 2-3, 4-8 cell embryos demonstrated the superiority of DMSO, most likely due to the reduced permeability of glycerol into the large blastomeres of the cleavage stage embryo (Trounson & Mohr, 1983). Serial dilutions at room temperature were used prior to sealing in a glass ampoule and reducing the temperature with liquid nitrogen and a programmable freezer. The transfers were scheduled 12 hrs following the warm with a blastomere survival rate of

≥50% considered suitable for transfer. The use of propylene glycol, also referred to as 1,2 propanediol (PROH), was also trialled as a CPA for cleavage stage embryos (Lassalle, Testart, & Renard, 1985). PROH was considered a better option than DMSO due to reduced toxicity with increasing exposure time (Renard & Babinet, 1984) and reduced ice crystal formation on removal from LN₂ (Boutron & Kaufmann, 1979). Increasing use of PROH showed better survival of embryos during day 1-2 of culture rather than latter stages and freezing at the pronucleate stage became a commonly used technique (Testart et al., 1986). Supplementation of a non-permeating CPA such as sucrose at 0.1M in addition to PROH during freezing was shown to improve embryo survival by assisting in cell dehydration, membrane stability and increasing CPA concentration within embryonic cells (Mandelbaum et al., 1987). The hypertonic concentration of sucrose prevents the rapid rehydration of the embryo which impedes the passage of CPA out during warming (Leibo & Mazur, 1978).

1.9.2.2 Blastocyst Stage

The use of glycerol as a CPA for blastocysts was successful at producing pregnancies and a live birth in a trial including DMSO (Cohen, Simons, Edwards, Fehilly, & Fishel, 1985). A subsequent comparison of cryopreservation at the cleavage and blastocyst stages concluded that a protocol of fresh transfer of cleavage stage embryos and cryopreservation of blastocysts was most efficient in their IVF programme (Fehilly, Cohen, Simons, Fishel, & Edwards, 1985). Equilibration of blastocysts in glycerol required increasing concentration steps for 10min each at room temperature prior to containment in an ampoule and loading into a programmable freezer. Blastocyst warming also required time consuming equilibration to remove the CPA. Reflective of culture conditions of the time, the blastocyst cryopreservation rate was reduced (183/784; 23%). Survival of blastocysts after thawing was based on the re-expansion of the blastocoel during a subsequent culture period (2-20 hrs). Cleavage stage embryos with ≥50% of blastomeres intact were transferred 2-43 hrs after thawing. The survival rate was 66% in blastocysts compared to 35% in cleavage stage embryos. Subsequent to this trial the use of PROH and non-permeating cryoprotectants further improved the success of slow cooling cleavage stage embryos (Testart et al., 1986).

1.9.3 Embryo Vitrification

Various improvements have been made to slow freezing protocols with the use of 0.25ml cryostraws that increase the warming rate to 300°C/min rather than large cumbersome ampoules (Renard & Babinet, 1984). Adding of the osmotic buffer sucrose in freezing and warming solutions and the acceleration of freezing between -35°C and -80°C also allowed the duration of the procedure to be shortened. However, the major improvements have come in the form of vitrification. The term vitrification, as used for contemporary embryo cryopreservation, refers to the process in which the entire specimen, including intra and extra cellular space forms an amorphous glassy solid (Gosden, 2011). Vitrified embryos were used in 83.7% of the 29,820 frozen warmed embryo transfer cycles in Australia in 2016. The live delivery rate per embryo transfer was 29.0% in autologous cycles with 82.8% of these transfers being SET (Fitzgerald et al., 2018).

1.9.3.1 Oocytes, Pronucleate and Cleavage Stage Embryos

As IVF clinics were implementing slow freezing techniques in their laboratories animal reproductive biologists were trialling rapid freezing techniques using much higher concentrations of solutes (Rall & Fahy, 1985). However, initial attempts at duplicating this methodology in human embryos was disappointing (Quinn & Kerin, 1986). In addition to the challenges of intracellular ice formation, fracture from extracellular ice precipitation and swelling during CPA removal there was the problem of toxicity of the elevated CPA concentrations used (Mukaida et al., 1998). The poor results from attempts at slow human oocyte cryopreservation when compared to embryo stages, despite the use of ICSI and PROH (Porcu et al., 1997; Tucker, Morton, Wright, Sweitzer, & Massey, 1998), motivated scientists to trial vitrification as a solution for human oocytes (Kuleshova, Gianaroli, Magli, Ferraretti, & Trounson, 1999). This group used 10% EG for 40s, 20% for 30s and 40% for 60s all at 37°C to equilibrate oocytes before loading into open pulled straws (OPS) (G. Vajta et al., 1998). Sucrose was also added to the 40% EG solution at 0.6 mol/l. Straws were then plunged directly into liquid nitrogen (-196°C) giving a cooling rate of 20 000°C/min. Warming was directly into decreasing sucrose concentrations from 0.4mol/l to 0.125mol/l at 37°C. Variations of this methodology also

produced pregnancies after vitrification of pronuclear and cleavage stage embryos (El-Danasouri & Selman, 2001; Selman & El-Danasouri, 2002).

1.9.3.2 Blastocysts

The use of extended culture and vitrification techniques has made frozen embryo transfer implantation rates as successful as those of fresh (Roy, Bradley, Bowman, & McArthur, 2014). The development of unique vessels to carry the blastocysts through the vitrification process with a reduced volume as low as 0.1µl has enabled cooling/warming rates in excess of 20 000°C/min and lower concentrations of CPA to be used (Arav, Shehu, & Mattioli, 1993; Choi et al., 2000; Kuwayama, 2007; Lane, Schoolcraft, & Gardner, 1999; Martino, Songsasen, & Leibo, 1996; Roy, Brandi, et al., 2014; G. Vajta et al., 1998). A vitrification method utilising a thin polypropylene strip 0.1mm in depth, 0.4mm wide and 20mm in length protected by a 30mm long sheath has proven to produce significantly improved results for the cryopreservation of blastocysts (Kuwayama, Vajta, Ieda, & Kato, 2005). Accelerated cooling and warming rates up to 40 000 °C/min, a reduced volume <0.1µl, versatility, safety and ease of use has made this device efficient for oocytes, zygotes, cleavage and blastocyst stages of human embryos (Kuwayama, 2007). EG and DMSO have been shown to be easily permeable beyond the plasma membrane (Fahy, Levy, & Ali, 1987) and they were used first successfully as an equal proportion combination in mice (Ishimori, Takahashi, & Kanagawa, 1992). This combination along with an osmotic buffer such as sucrose or trehalose has now become ubiquitous amongst IVF centres for the vitrification of human embryos including blastocysts (Argyle, Harper, & Davies, 2016).

1.9.4 Survival

Survival of embryos after cryopreservation can only be determined at the time of the warm. A blastomere survival rate $\geq 50\%$, e.g. 2/4 cells, is considered sufficient to diagnose a cleavage stage embryo as survived and select for transfer. It is important, however, to distinguish between those embryos with 100% blastomere survival and those with partial survival (Edgar, Bourne, Speirs, & McBain, 2000). Efficacy of cryopreservation methods for the blastocyst (survival) can be assessed by normal response to osmotic changes (re-expansion) during the dilution process at the time of the warm (Kuwayama, Vajta, Ieda, et al., 2005). A unique feature of the blastocyst is the

presence of the blastocoel which can present problems in the evacuation of water and the influx of cryoprotectant during vitrification. The possibility of ice crystal formation is directly proportional to the volume and inversely proportional to the viscosity and cooling rate (Liebermann et al., 2002). The artificial reduction of the blastocoel prior to vitrification showed some success at increasing the survival rate of expanded blastocysts from 30 to 60% (Vanderzwalmen et al., 2002). Some protocols now routinely use collapsing prior to vitrification and assisted hatching prior to transfer of warmed blastocysts (Desai et al., 2016). The advantage of oocyte, zygote and cleavage stage cryopreservation is that survival can be determined by assessing further development from 24 hrs through to the blastocyst stage on day 5-6 (Liebermann & Tucker, 2002). These embryo stages often have less than 9 cells to assess and survival criteria can be based on further cytokinesis of blastomeres. There have been attempts to replicate this methodology for blastocysts, but they have many cells, are rapidly re-organising their cell structure and are often already hatching and expected implantation is imminent. This requires observation over a shorter time-span to assess cell survival, expansion and hatching prior to selection for transfer (Yeoman et al., 2001). Blastocyst cryo-survival has been defined as cell survival immediately after warming and the absence of dark granular cells and areas of degeneration being the essential criteria without reference to expansion (Desai et al., 2016). Survival of cells is difficult to assess objectively and accurately however unless blastocysts are fixed and stained with nuclear and vitality stains (Lopes et al., 2015). There is a greater emphasis on re-expansion of blastocysts and cell-reorganisation in some protocols (Ahlström, Westin, Wikland, & Hardarson, 2013). Zech et al warmed blastocysts 18-22 hrs prior to transfer, assessed under 200x magnification immediately after warming and again prior to transfer. If at 24hrs there was evidence of a blastocoel and structural integrity of the ICM, the embryo was transferred (Zech, Lejeune, Zech, & Vanderzwalmen, 2005). This strategy has also been used for day 4 morula stage embryos warmed 48 hrs prior to transfer (Ishimori et al., 1992). These methods may however require the warming of more than one blastocyst to ensure the greatest possibility of the transfer of a viable blastocyst in a synchronised cycle. If a blastocyst is deemed not to have survived, there is not enough time to perform another warm and assessment prior to transfer (Ahlström et al., 2013). Wirleitner et al (2016) defined survival as re-expansion and further development 3 hours

after warming. Slow and incomplete re-expansion within 3 hours and poor morphology grading relative to pre-freeze morphology was regarded as delayed recovery. There was no transfer of a blastocyst when there was no re-expansion after 3 hours and cells showed signs of degeneration and lysis (Wirleitner, Schuff, Stecher, Murtinger, & Vanderzwalm, 2016). Shu et al retrospectively compared two groups of warmed blastocysts and their resulting implantation rate. They allocated the groups based on fast and slow re-expansion but defined these categories by the proportion of re-expansion at the end of the 2-4 hour period prior to transfer (Shu et al., 2009). They found significantly higher rates of implantation (26.7% vs. 11.3%) when the transferred blastocyst had a fast re-expanding blastocoel compared to a slow re-expanding blastocoel.

The Alpha (Scientists in Reproductive Medicine) consensus on survival of blastocysts after cryopreservation is that $\geq 75\%$ of cells are intact after warming. Although re-expansion after 2 hours was considered it was not included due to discrepancies in the time available post warming to make such an assessment (Alpha Scientists in Reproductive Medicine, 2012). Despite this there are groups using different criteria such as $\geq 50\%$ morphological survival (cells intact) and signs of re-expansion before transfer (Van Landuyt et al., 2015).

2 SUMMARY and AIMS

The transfer of blastocysts that have a low implantation potential due to damage following vitrification and warming will result in poor pregnancy rates (Ahlström et al., 2013). This is a waste of resources, gives patients false hope, and results in treatment taking longer without any improved outcome. This present study aims to retrospectively identify variables from a blastocyst vitrification programme that were significantly associated with survival and live birth outcomes. These associations may then be utilised to modify existing protocols and better manage patient expectations of a successful outcome. This would potentially decrease the number of embryo transfer procedures required to achieve a pregnancy.

3 MATERIALS and METHODS

This is a retrospective cohort study of blastocysts that have been vitrified and warmed during the period from 2008 to 2015. The primary outcome measure is a live birth from those embryos deemed to have survived and subsequently transferred to the patient. A comparison is made between different variables that may influence this primary outcome including vitrification and warming techniques used over time.

3.1 Ethics

All research involving human participants requires adherence to the National Statement on Ethical Conduct in Human Research that is a series of guidelines made in accordance with the National Health and Medical Research Council Act 1992. Research undertaken at Hollywood Fertility Centre required approval from the Hollywood Private Hospital Ethics Committee and this was obtained (Appendix 7.1.1). Ethics approval was also granted by the Edith Cowan University Research Ethics Committee (Appendix 7.1.2). The research method identified for this project is the analysis of retrospective data involving human beings. Important issues covered in the ethics application include:

3.1.1 Consent to the use of Data

An acknowledgement and agreement was sighted and signed by the gamete providers of the embryos involved in this study. This agreement outlined the potential use of data collected during treatment for research and quality improvement processes.

3.1.2 Risk

As this was a retrospective data analysis, the only identified risk was the disclosure of sensitive personal information. This risk was managed by de-identifying data prior to analysis and an employer/employee confidentiality agreement as the principal researcher is a full-time employee of the IVF unit.

3.1.3 Benefit

The negligible risk was outweighed by the benefits associated with the data analysis. These include identification of blastocyst survival criteria associated strongly with live birth outcomes. These benefits are realised by improved outcomes for patients, more efficient use of resources, advancement of knowledge and benefits to society in improving the outcomes of ART.

3.1.4 Data and Privacy

The data used in this study was categorised as re-identifiable coded information sourced from a database used during the normal course of an IVF cycle and stored on the secure servers at Hollywood Fertility Centre. The Hollywood database is a Microsoft SQL Server 2008 R2 database with security managed via Windows Active Directory accounts and groups. The only patient information that leaves the Hollywood Fertility Centre's premises is encrypted with 256-bit encryption solely for offsite disaster recovery purposes.

3.1.5 Translation into Clinical Practice

Any significant findings from this work will be published and presented at conferences to highlight the importance of looking more closely at the changing blastocyst after vitrification and warming. These protocols can then be assessed by prospective clinical trials and implemented into clinical practice if proved beneficial by improved outcomes.

3.2 Data retrieval

The data collection period commenced in February 2008 as the embryo cryopreservation systems moved to 100% vitrification at this time. The end date chosen was May 2015 due to moving to a different data base making consistent data entry and retrieval difficult. 17 fields of data were collected for each blastocyst warmed, 20 for each transferred and 25 for each birth outcome (Appendix 7.2). Blastocysts that had been biopsied, vitrified, warmed and transferred as known euploid were removed from the data set to avoid bias. Data was imported into a Microsoft Excel document that is securely stored using Windows Active Directory on the Hollywood Fertility Centre Servers. Data was retrieved for 2977 blastocysts that were vitrified and warmed.

3.3 Data analysis

Categorical variables were analysed using Pearson's chi square test or Fisher's exact test with Bonferroni correction for multiple comparisons. Independent t-test or Mann-Whitney U-test was used for continuous variables depending on the normality of the distribution. Normality was assessed using the Shapiro-Wilk test. Statistical significance was defined as $P < 0.05$ for single comparisons and adjusted lower for multiple comparisons (Bonferroni correction). Simple and multiple logistic regression was used to

calculate odds ratios. All analyses were performed with the use of SPSS 22 Statistical software (IBM, Armonk, New York), vassarstats.net.

3.4 Risks and limitations

This study included a retrospective analysis of data with an outcome measure that was known at the commencement of the study. Retrospective studies are vulnerable to sources of bias and confounding and results should be treated with caution. Most evident confounders were controlled for by multivariate analysis, but unknown sources could cause residual confounding. The period of the study may include other changes in protocols and procedures which may influence the outcome. Only good quality blastocysts are considered for cryopreservation, warming and transfer and therefore results cannot be generalised for all blastocysts.

3.5 Patient Population and Setting

This study was performed in a private specialist medical centre treating patients presenting with infertility. The outcomes of blastocysts vitrified and warmed from 2008 to 2015 were examined.

3.6 IVF Treatment

3.6.1 Ovarian Stimulation

All patients starting stimulation had a BMI <35 kg/m², P₄ < 5.0 nmol/l and FSH ≤ 15 IU/ml. Ovarian stimulation was initiated by the administration of recombinant follicle stimulating hormone (rFSH) injections 125 – 300IU/daily (follitropin- α , Gonal F[®], Merck Serono Australia or follitropin- β , Puregon[®], MSD), beginning day 3 of the menstrual cycle. Follicular response was assessed via vaginal ultrasound on days 2, 6 and 11. Plasma oestradiol (E₂), progesterone (P₄) and luteinising hormone (LH) were also measured on days 2, 6 and 11. Dosage was altered accordingly depending on ovarian response.

To prevent premature ovulation the GnRH antagonist Orgalutran[®] (Merck Sharp and Dohme, Australia) was administered at 0.25 mg/ daily when the leading follicle diameter reached 14 mm. Final follicular maturation (trigger) was achieved by the administration of 0.25 mg Ovidrel[®] (Merck Serono Australia) when at least three follicles had reached a

mean diameter of 17mm. Trans-vaginal oocyte aspiration (TVOA) was performed 35-38 hours after Ovidrel® was administered.

3.6.2 Blastocyst Culture

Insemination was performed, 41-44 hours post trigger via conventional IVF or where indicated, ICSI. Gamete co-incubation occurred in Sydney IVF Fertilisation Media (COOK® Medical, Australia) and fertilisation check occurred 16-18 hours post insemination. Zygotes were moved into 20µl droplets of Cleavage Media (COOK® Medical, Australia) with no more than 5 per droplet. Embryos were cultured in the MINC incubator (COOK® Medical, Australia) in 5% O₂, 6% CO₂, and 89% N₂. Embryonic development was assessed on days 3, 5 and 6 of culture, with embryos from day 3 forward being cultured in a separate 20µl droplet of Blastocyst Media (BM, COOK® Medical, Australia). Cleavage stage division of embryos was examined using a 2-factorial system 68 ± 1-hour post insemination. Cell number and degree of cytoplasmic fragmentation was examined for each embryo. One hundred and sixteen hours ± 2 hours post insemination (Day 5) embryos were graded using a two-component grading system; 1) degree of expansion as indicated by the presence of a blastocoel cavity and 2) ICM and TE quality. The assessed ICM and TE cells were assigned a numerical grade. Grades 1-2 indicate a discernible to distinct ICM and cohesive to loose layer of TE cells. Any embryo with no visible ICM or few or uneven layer of TE was not considered suitable for transfer or vitrification on day 5 and remained in culture for reassessment on day 6.

3.6.3 Blastocyst Vitrification and Warming

During the data collection period there were two protocols used for blastocyst vitrification utilising different devices, media, equilibration and vitrification techniques.

3.6.3.1 Hollywood Modified CVM™ Method.

The CryoLogic Vitrification Method (CVM™) is a commercially available vitrification kit incorporating the use of a Fibreplug™ and the solid surface vitrification (SSV) method (Appendix 7.3.1). A modified version of this protocol was used incorporating equilibration and vitrification solutions from supplier A and warming solutions from suppliers A, B and C. (Appendix 7.3.6).

3.6.3.1.1 Fibreplug™ Vitrification (Detail Appendix 7.3.2)

Blastocysts were equilibrated singularly for 2 minutes at 37°C in a solution of 8% EG/DMSO containing osmotic buffer while the blastocoel was collapsed using a 200 µm pipette. The blastocyst was then moved to a vitrification solution of 16% EG/DMSO for 30 seconds prior to dispensing onto the Fibreplug™ in a 3 µl drop. The Fibreplug™ was then placed against a stainless-steel vitrification block, pre-equilibrated in liquid nitrogen where the drop formed a glass like bead. Once vitrified the Fibreplug™ was placed inside a pre-cooled sheath and stored in LN₂.

3.6.3.1.2 Fibreplug™ Warming (Detail Appendix 7.3.3)

Fibreplugs were removed from LN₂ and plunged into a solution pre-incubated at 37°C containing an osmotic buffer (sucrose or trehalose). Once identified, the blastocyst was immediately washed free of the vitrification media and moved through a series of solutions with decreasing concentrations of osmotic buffer (Appendix 7.3.8) in 5-minute intervals. The blastocyst was then moved to culture in BM prior to assessment for survival.

3.6.3.2 *Hollywood Modified Cryotop® Vitrification Method (CT)*

The Cryotop® Vitrification Method (CT) is a commercially available kit with media and devices (Appendix 7.3.4). A modified version of the CT method utilising the Cryotop® Vitrification device, the CryoLogic Vitrification block (SSV) and the equilibration and vitrification solutions from supplier A and C were used. The solutions from suppliers A, B and C were used for warming (Appendix 7.3.7).

3.6.3.2.1 Cryotop® Vitrification (Detail Appendix 7.3.5)

Blastocysts were equilibrated singularly for 15 minutes at room temperature in a solution of 8% EG/DMSO containing osmotic buffer. Unlike the CVM protocol there was no artificial collapsing of the blastocoel. The blastocyst was then transferred to a vitrification solution containing 16% EG/DMSO with a higher concentration of osmotic buffer. After rinsing for 60 seconds the blastocyst was aspirated in a volume < 0.1 µl and placed on the tip of the Cryotop® sheet. Excess vitrification solution was removed leaving a planar droplet containing the blastocyst. The sheet was then placed against a stainless-

steel vitrification block, pre-equilibrated in liquid nitrogen. Once vitrified the sheet was inserted into a pre-cooled sheath and stored in LN₂.

3.6.3.2.2 Cryotop® Warming (Detail Appendix 7.3.6)

Single blastocyst warming commenced a minimum 3 hrs prior to the scheduled transfer to allow enough time for embryo assessment. Cryotop® sheets holding the blastocysts were removed from LN₂ and plunged into a solution pre-incubated at 37°C containing an osmotic buffer (sucrose or trehalose) for 1 minute. The blastocyst was then moved through a series of solutions with decreasing concentrations of osmotic buffer prior to moving to BM for culture and survival assessment.

3.6.4 Survival Assessment

Blastocysts were observed briefly at 30- and 60-minutes post warming on an inverted microscope for commencement of re-expansion defined as the appearance of a cavity and/or lineation of trophectoderm cells. Cell degeneration assessment occurred at the same time and was observed as highly granular cytoplasm without distinct cell membranes. During the first 2 years of the study period, if there was estimation that the proportion of viable cells was less than 70%, the blastocyst was deemed not to have survived and was not transferred. Due to poor outcomes from blastocysts assessed as survived using this criterion alone but not commenced re-expansion within 60 minutes, the latter assessment method became the sole survival criterion.

3.6.5 Endometrial Preparation and Embryo Transfer

Frozen embryo transfer cycles (FET) were managed either by hormone replacement therapy (HRT), low dose stimulation (LDS) or natural (NAT) cycle monitoring. HRT cycles involved administration of 4mg TDS Progynova® (Bayer, Australia) for a minimum of 10 days. Serum E2 was measured on day 10 of the cycle and endometrial quality was examined via vaginal ultrasound. When E2 was greater than 800 pmol/l and endometrial thickness greater than 8mm, progesterone pessaries 400mg TDS (Orion, Balcatta Western Australia) were administered by the patient 3 times daily. The FET was scheduled on the sixth day of pessaries. Pessaries were continued until indicated otherwise by negative pregnancy result or placental support was initiated. Serum E2 and P4 levels were observed 3 days post transfer.

Gonal F® was administered for LDS cycles and response monitored using serum E2 levels on days 2 and 9. Once E2 level exceeded 500 pmol/l, vaginal ultrasound was used to monitor follicular and endometrial growth. When serum E2 levels reached 800IU and endometrial thickness reached 8mm, 5000IU hCG (Pregnyl™, human chorionic gonadotrophin) was used to trigger ovulation with embryo transfer scheduled for 6 days later. Gonadotrophic support was administered, 1500IU/day Pregnyl™ corresponding with days 17, 20, 23 and 26 of patient cycle. Serum E2 and P4 were examined 8 days post trigger.

All embryo transfers were conducted using a Guardia™ Access Embryo Transfer Catheter from COOK® medical. A 1ml syringe filled with equilibrated culture media was attached to the inner sheath and stored in 6%CO₂, at 37°C until required. At time of transfer, a blastocyst was moved from 20µl droplet into 900µl of culture medium. The embryo was loaded into the catheter by filling the inner sheath with pre-equilibrated medium, introducing a small air bubble, drawing approximately 5µl of medium into the catheter, followed by the embryo with another 5µl of media and another small air bubble. The total volume for transfer was approximately 10-15µl. The outer sheath of the catheter was placed through the cervix and under ultrasound guidance; the inner sheath was passed through and 20-35µl of medium containing the embryo deposited 1 cm back from the fundus. The inner and outer sheaths were checked by the scientist post transfer to ensure the successful transfer of the embryo.

Human chorionic gonadotrophin hormone levels higher than 25IU/l indicated a positive biochemical pregnancy. Oestradiol and progesterone support was continued in patients to ensure adequate support before the placental hCG production shift. Pregnancy monitoring continued per practice protocol. Weekly blood tests of serum E2, P4 and hCG continued until week 7 of pregnancy when clinical pregnancy was confirmed by the presence of a gestational sac and fetal heart via ultrasound.

4 RESULTS

4.1 Patient and Cycle Demographics

4.1.1 Number of Embryos Transferred

There were 2466 transfers of either 1 or 2 vitrified/warmed blastocysts resulting in 796 (32.3%) clinical pregnancies and 751 (30.5%) live born babies. Less than 7% of embryo transfers were of 2 blastocysts due to either advanced maternal age or previous failed attempts. Table 4-1 shows the proportion of multiple pregnancies was significantly greater when 2 blastocysts were transferred compared to one, despite the overall clinical pregnancy rate not being significantly greater. The individual embryo outcomes (Table 4-2) demonstrate that the embryo implantation rate in the patients receiving two blastocysts was significantly lower than those receiving one, however they were also significantly older (Table 4-1). Genetically tested, imported, and embryos derived from donor oocytes were excluded from the data set to avoid any bias.

Table 4-1: Pregnancy and multiple rates according to the number of warmed blastocysts transferred. Pregnancies were identified with a positive pregnancy test at week 4 and confirmed as viable with one or more fetal hearts present at 7 weeks gestation.

Parameter	No. embryos transferred		
	1	2	p-value
Age at transfer (mean years)	34.5 ± 4.5	36.0 ± 4.4	<0.0001
No. embryo transfers	2311	155	
No. positive pregnancy tests*	961 (41.6%)	74 (47.7%)	>0.133
No. viable pregnancies*	735 (31.8%)	61 (39.3%)	>0.052
No. multiple pregnancies**	18 (2.4%)	15 (24.6%)	<0.0001

*per transfer; **per viable pregnancy

Table 4-2: Embryo implantation and singleton birth details according to the number of warmed blastocysts transferred.

Parameter	No. embryos transferred		
	1	2	p-value
No. embryos transferred	2311	312	
No. fetal hearts	754 (32.9%)	76 (24.4%)	<0.003
No. babies born*	684 (29.6%)	67 (21.5%)	<0.005
No. boys:girls	344:340	34:33	= 1.0
Weight at birth (g)	3346 ± 639	3186 ± 683	>0.052
Gestation (weeks)	38.9 ± 2.4	38.4 ± 2.2	>0.101

* number of babies born per embryo transferred

The average gestational age of a multiple delivery was 36 ± 2.1 weeks compared to 39 ± 2.3 weeks for singleton deliveries. Average birthweight for multiples ($2261\text{g} \pm 498\text{g}$) was also significantly reduced ($p < 0.0001$) when compared to singletons ($3376\text{g} \pm 607\text{g}$).

Double embryo transfers were excluded from further analysis due to the reduced proportion (6.3%), significantly different outcomes and the inability to trace individual blastocysts.

4.1.2 Type of Transfer Cycle

Almost half (47.7%) of single embryo transfer cycles were prepared using HRT, 40.0% LDS and the remainder being NAT cycles (12.3%). HRT cycles produced significantly less viable pregnancies per SET compared to LDS and NAT cycles combined, however the age of patients in this group was significantly older (Table 4-3).

Table 4-3: Single embryo transfer pregnancy rates and outcomes according to the type of transfer cycle. Cycles were following hormone replacement therapy (HRT), low dose stimulation (LDS) or unstimulated (natural).

Parameter	Cycle type			
	HRT	LDS	Natural	p-value
Age at transfer (mean years)	35.2 ± 4.6	33.9 ± 4.1	33.5 ± 4.5	<0.0001**
No. transfers	1103	925	283	
No. positive pregnancy tests	422 (38.3%)	406 (43.9%)	133 (47.0%)	<0.006*
No. viable pregnancies	302 (27.4%)	321 (34.7%)	112 (39.6%)	<0.0001*

*based on Bonferroni sig level $p=0.017$ for multiple comparisons, LDS ($p=0.0111$, 0.0004) and Natural ($p=0.0080$, <0.0001) cycles produced significantly greater positive pregnancy tests and viable pregnancies respectively than HRT cycles. There was no significant difference in positive pregnancy tests ($p<0.3961$) and viable pregnancies ($p<0.1542$) between LDS and Natural cycles.

** The average age of patients was significantly different between both LDS ($p<0.0001$) and Natural ($p<0.0001$) compared to HRT cycles however there was no significant difference ($p>0.3730$) between LDS and Natural cycles.

The average weight at birth and gestational period was not significantly different when comparing the type of transfer cycle but the number of fetal hearts and babies born in proportion to SET was significantly lower in the HRT group (Table 4-4).

Table 4-4: Embryo implantation and birth details according to the type of transfer cycle. Cycles were following hormone replacement therapy (HRT), low dose stimulation (LDS) or unstimulated (natural).

Parameter	Cycle type			
	HRT	LDS	Natural	p-value
No. embryos transferred	1103	925	283	
No. fetal hearts	311 (28.2%)	327 (35.4%)	116 (41.0%)	<0.0001*
No. babies born	280 (25.4%)	299 (32.3%)	105 (37.1%)	<0.0001*
Weight at birth (g)	3307 ± 673	3355 ± 595	3423 ± 666	0.2767
Gestation (weeks)	38.8 ± 2.8	38.9 ± 1.9	39.1 ± 2.5	0.5475

*based on Bonferroni sig level $p=0.017$ for multiple comparisons, LDS ($p<0.0007$, <0.0007) and Natural cycles ($p<0.0001$, <0.0001) produced a significantly greater proportion of fetal hearts and babies born respectively than HRT cycles. There was no significant difference in the proportion of fetal hearts ($p<0.0985$) and babies born ($p<0.1563$) between LDS and Natural cycles.

4.1.3 Aetiology of Infertility

The cause of infertility, as diagnosed prior to the commencement of IVF treatment, did not significantly influence the outcome of single frozen embryo transfers (Table 4-5). More than half the embryos were from couples with male infertility only or idiopathic infertility.

Table 4-5: The relationship between aetiology of infertility and pregnancy following a single transfer of a warmed blastocyst.

Aetiology	Transfers	Viable pregnancies*	Deliveries**
Male and female factors	467 (20%)	127 (27.2%)	122 (26.1%)
Female factors only	447 (19%)	138 (30.9%)	117 (26.2%)
Male factors only	638 (28%)	216 (33.9%)	192 (30.1%)
Tubal factors	118 (5%)	44 (37.3%)	41 (34.7%)
Unexplained	637 (28%)	210 (33.0%)	190 (29.8%)
Total	2311	735 (31.8%)	662 (28.6%)

* $p > 0.0912$, ** $p > 0.1977$

Based on Bonferroni sig level $p < 0.005$ for multiple comparisons (10) there was no significant difference in viable pregnancies ($p > 0.0216$) or deliveries ($p > 0.0797$) relative to patient aetiology.

4.1.4 Transfer Attempt

The number of previous embryo transfers a patient had received, whether successful and producing a viable pregnancy, or not implanting, is associated with the implantation rate. 1st transfer patients have not had a prior fresh transfer either due to patient choice or medical reasons. An increased progesterone level (prior to hCG administration) and risk of OHSS are the most common reasons for a freeze all cycle. Table 4-6 describes the reduced proportion of deliveries as the transfer attempts increase.

Table 4-6: Impact on outcome relative to the rank of transfer attempt

Parameter	Single embryo transfer rank				Total
	1	2	3-5	6-20	
Average age (mean years)	32.4 ± 4.2	33.1 ± 4.5	33.6 ± 4.5	35.0 ± 3.9	33.5 ± 4.5
No. transfers (SET)	287	802	958	264	2311
No. viable pregnancies*	120 (41.8%)	264 (32.9%)	287 (30.0%)	64 (24.2%)	735 (31.8%)
No. deliveries*	111 (38.7%)	242 (30.2%)	254 (26.5%)	55 (20.8%)	662 (28.6%)

Based on Bonferroni sig level $p < 0.0083$ for multiple comparisons there was no significant difference in age between 1st and 2nd ($p < 0.0951$) or 2nd and 3-5th ($p < 0.0821$) transfer attempts. All other group comparisons of age were significant ($p < 0.0003$). 1st transfer viable pregnancy and delivery rate was significantly ($p < 0.0002$, $p < 0.0001$) greater than 3-5 or 6-20 transfer attempts. All other transfer rank comparisons were not significant ($p > 0.0084$).

4.1.5 Impact of Age

The proportion of embryo transfers resulting in viable pregnancies and deliveries according to the woman's age is shown in Table 4-7. Whilst there was no difference between the women <30 yrs and 30-34 yrs, there were progressive and significant reductions in rates of pregnancy and delivery for those aged 35-39 yrs and ≥ 40 yrs.

Table 4-7: Impact of the age of the woman at vitrification upon blastocyst survival and implantation.

Parameter	Age of woman at vitrification (years)				Total
	<30	30-34	35-39	≥40	
No. transfers (SET)	465	844	794	208	2311
No. viable pregnancies*	173 (37.2%)	291 (34.5%)	229 (28.9%)	42 (20.2%)	735 (31.8%)
No. deliveries*	166 (35.7%)	265 (31.4%)	199 (25.1%)	32 (15.4%)	662 (28.6%)

* $p < 0.0001$

Based on Bonferroni sig level $p < 0.0083$ for multiple comparisons there was no significant difference in viable pregnancies ($p > 0.3537$) or the number of deliveries ($p > 0.1277$) between patients aged less than 30 and between 30 and 34. The number of deliveries between all other age groups was significantly different ($p < 0.0053$).

4.1.6 Logistic Regression of patient and cycle parameters.

Maternal age, cycle type, and transfer order independently influence embryo transfer outcomes as shown by the odds ratios (Table 4-8). Patients less than or equal to 38 years of age at the time of vitrification are more than twice as likely to achieve the delivery of a baby than those older than 38. This trend is maintained despite adjusting for cycle type and transfer attempt (Table 4-9). The shift in odds ratio for all three independent variables is less than 10% using multiple regression analysis. This indicates the effect of confounding between cycle type, age and transfer attempt is minimal.

Table 4-8: Simple regression results for age, cycle and transfer rank.

Predictor	Value	p-value	OR	CI	
Age	≤ 38 years	< 0.0000	2.1752	1.6020	2.9534
Cycle	No HRT	< 0.0000	1.4770	1.2327	1.7696
Transfer attempt	≤ 2	< 0.0002	1.4034	1.1732	1.6788

Table 4-9: Multiple logistic regression results controlling for age, cycle and transfer rank.

Predictor	Value	p-value	OR	CI	
Age	≤ 38 years	< 0.0000	2.0086 (7.7%)	1.4755	2.7344
Cycle	No HRT	< 0.0005	1.3832 (6.4%)	1.1517	1.6611
Transfer attempt	≤ 2	< 0.0026	1.3220 (5.8%)	1.1027	1.5850

Percentage in parentheses represents the deviation from simple regression (Table 4-8) and multiple regression controlling for the other 2 variables. If >10% there is evidence of confounding.

4.2 Blastocyst Specific Variables

4.2.1 Age of embryo at time of vitrification (Day 5 vs Day 6)

In a fresh cycle, embryo selection for transfer and vitrification occurs initially on Day 5 and if nothing is suitable, subsequently on Day 6. A day 5 embryo has a typical age of 118-125 hrs post insemination and a day 6 embryo 138-143 hrs post insemination. The survival, viable pregnancy and delivery rates are significantly greater for Day 5 embryos within the context of this protocol (Table 4-10).

Table 4-10: The survival after warming and subsequent implantation of blastocysts vitrified on Day 5 vs Day 6

	D5	D6	P value
# warmed	2397	580	
#survived	2130 (88.9%)	493 (85%)	<0.0102
#SET	1887	424	
#viable pregs	643 (34.1%)	92 (21.7%)	<0.0000
#deliveries	580 (30.7%)	82 (19.3%)	<0.0000

4.2.2 Blastocyst grade and expansion level relative to day of vitrification.

In addition to the age of the blastocyst, the morphological variables, cell quality (grade 1-2) and the level of expansion differed at the time of vitrification. There was no significant difference in survival ($p > 0.6458$) or live birth rate ($p > 0.3161$) between grade 1 and 2 blastocysts at the early stages of expansion on day 5 (Table 4-11). The difference in live birth rate between grade 1 and 2 blastocysts became significant for more expanded blastocysts on day 5 of development. There were too few blastocysts at the early expansion stages vitrified on day 6 to make a comparison with just one grade one blastocyst being transferred (Table 4-12). There were no significant differences in survival or live birth rate between grade 1 or 2 blastocysts at the more expanded stages on day 6. The proportion of grade 1 blastocysts on day 5 (31.5%) was significantly greater ($p < 0.0000$) than that of day 6 (10.3%).

Table 4-11: The survival after warming and subsequent implantation of blastocysts transferred on Day 5. Embryos stratified as either non-expanded or expanded/hatching and grade 1 or 2.

Expansion	Early blastocyst (EB) to Blastocyst(B)		Expanded Blastocyst (XB) to Fully Hatched Blastocyst (FHB)	
Grade	1	2	1	2
# warmed	92	566	663	1076
#survived	84 (91.3%)	508 (89.8%)	591 (89.1%)	947 (88.0%)
#SET	75	432	547	833
#viable pregs	23 (30.7%)	109 (25.2%)	236 (43.1%)	275 (29.0%)
#deliveries	21 (28.0%)	98 (22.7%)	216 (39.5%)*	245 (25.8%)*

* $P < 0.0007$

Table 4-12: The survival after warming and subsequent implantation of blastocysts transferred on Day 6. Embryos stratified as either non-expanded or expanded/hatching and grade 1 or 2.

Expansion	EB to B		XB to FHB	
Grade	1	2	1	2
# warmed	1	31	59	489
#survived	1 (100%)	29 (93.5%)	52 (88.1%)	411 (84.0%)
#SET	1	26	47	350
#viable pregs	0 (0%)	4 (15.4%)	14 (29.8%)	74 (21.1%)
#deliveries	0 (0%)	4 (15.4%)	11 (23.4%)	67 (19.1%)

4.2.3 Logistic Regression of Embryo Specific Variables.

The day of vitrification, blastocyst grade and degree of expansion are independently associated with live delivery as an embryo transfer outcome as shown by the odds ratios in Table 4-13. Using multiple regression analysis, Day 5, Grade 1 Blastocysts that have reached the expanded/hatching stage have an 80% greater chance of continuing to a baby than Grade 1 expanded blastocysts on Day 6 (Table 4-14). Grade 1, Day 5 Blastocysts that have reached the expanded/hatching stage have a 51% greater chance of continuing to a baby than Grade 2 Blastocysts that are also at the expanded/hatching stage on Day5. Blastocysts that have reached the expanded/hatching stage on Day 5 and are grade 1 have a 47% greater chance of continuing to a baby than non-expanded blastocysts. There was a significant reduction (13.5%) in the OR of the morphology grade when controlling for day of vitrification and expansion level suggesting some confounding (Table 4-14).

Table 4-13 Simple regression results for a comparison of day of vitrification, embryo grade and expansion level on delivery rates.

Predictor	Value	p-value	OR	95% CI	
Day of vitrification	5	< 0.0000	1.8508	1.4270	2.4006
Morphology Grade	1	< 0.0000	1.7473	1.4417	2.1175
Expansion level	≥XB	< 0.0009	1.4626	1.1675	1.8323

Table 4-14 Multiple logistic regression results controlling for day of vitrification, embryo grade and expansion level relative to delivery rates.

Predictor	Value	p-value	OR	95% CI	
Day of vitrification	5	<0.0000	1.7962 (-3.0%)	1.3728	2.3503
Morphology Grade	1	<0.0001	1.5120 (-13.5%)	1.2373	1.8475
Expansion level	≥XB	<0.0012	1.4747 (+0.7%)	1.1663	1.8647

Percentage in parentheses represents the deviation from simple regression (Table 4-13) and multiple regression controlling for the other 2 variables. If >10% there is evidence of confounding.

Comparisons using binary logistic regression to identify individual confounding showed a significant reduction in the OR of day of vitrification when controlling for morphology grade. Day 5 grade 1 blastocysts have a 64% greater chance of continuing to a baby than day 6 grade 1 blastocysts (Table 4-15). However, a greater proportion of day 6 blastocysts are morphology grade 2 (376/424, 89%) compared to day 5 blastocysts (1265/1887, 67%). Within blastocysts vitrified on Day 5 the morphology grade 1 blastocysts had a 64% greater chance to continue to a baby than morphology grade 2 blastocysts on the same day (Table 4-15).

Table 4-15 Binary logistic regression of day of vitrification and morphology grade relative to delivery rates

Predictor	Value	p-value	OR	95% CI	
Day of vitrification	5	<0.0002	1.6404 (-11.4%)	1.2604	2.1349
Morphology Grade	1	<0.0000	1.6299 (-6.7%)	1.3406	1.9816

More expanded grade 1 blastocysts had a 32% greater chance of continuing to a baby than non-expanded grade 1 blastocysts (Table 4-16). Morphology grade had more than double the effect of expansion as grade 1 expanded blastocysts had a 67% greater chance of continuing to a baby than grade 2 expanded blastocysts.

Table 4-16 Binary logistic regression of expansion level and morphology grade relative to delivery rates

Predictor	Value	p-value	OR	95% CI	
Expansion level	≥XB	<0.0173	1.3216 (-9.6%)	1.0504	1.6628
Morphology Grade	1	<0.0000	1.6731 (-4.2%)	1.3762	2.0339

Expanded blastocysts on day 5 were more than twice as likely to continue to a baby than expanded blastocysts on day 6 of development (Table 4-17). Expanded blastocysts on day 5 of development had a 63% greater chance of continuing to a baby than non-expanded blastocysts on day 5. However, a greater proportion of day 6 blastocysts were expanded (397/424, 93%) compared to day 5 blastocysts (1380/1887, 73%).

Table 4-17 Binary logistic regression day of vitrification and expansion level relative to delivery rates

Predictor	Value	p-value	OR	95% CI	
Day of vitrification	5	<0.0000	2.0100 (+7.9%)	1.5456	2.6140
Expansion level	≥XB	<0.0000	1.6313 (+10.3%)	1.2976	2.0509

4.2.4 Ranking of implantation potential

Combining the survival rates and subsequent SET live delivery rates it is possible to construct a ranking table that provides a guide as to the probability of success of an embryo prior to choosing it to warm (Table 4-18). This table shows the improved success rates according to the day of vitrification, the morphology grade and to a lesser degree, expansion level.

Table 4-18 Blastocyst rankings according to blastocyst specific variables

Category of embryo	SET	Viable pregnancies	Deliveries	Delivery rate of warmed blastocysts
D5HB1	195	92 (47.2%)	84 (43.1%)	39.9%
D5XB1	342	140 (40.9%)	128 (37.4%)	32.6%
D5B1	67	22 (32.8%)	20 (29.9%)	27.1%
D5XB2	641	214 (33.4%)	193 (30.1%)	26.4%
D5HB2	187	61 (32.6%)	52 (27.8%)	24.6%
D5EB2	87	26 (29.9%)	25 (28.7%)	23.4%
D6HB1	21	6 (28.6%)	5 (23.8%)	20.2%
D5B2	345	83 (24.1%)	73 (21.2%)	19.5%
D6XB1	24	7 (29.2%)	5 (20.8%)	18.7%
D6XB2	195	41 (21.0%)	38 (19.5%)	16.7%
D6HB2	128	28 (21.9%)	25 (19.5%)	15.8%
D6B2	24	4 (16.7%)	4 (16.7%)	15.5%
D6FHB2	27	5 (18.5%)	4 (14.8%)	13.5%

D5/D6 refers to day of vitrification

EB early blastocyst, B blastocyst, XB expanded blastocyst, HB hatching blastocyst, FHB fully hatched blastocyst

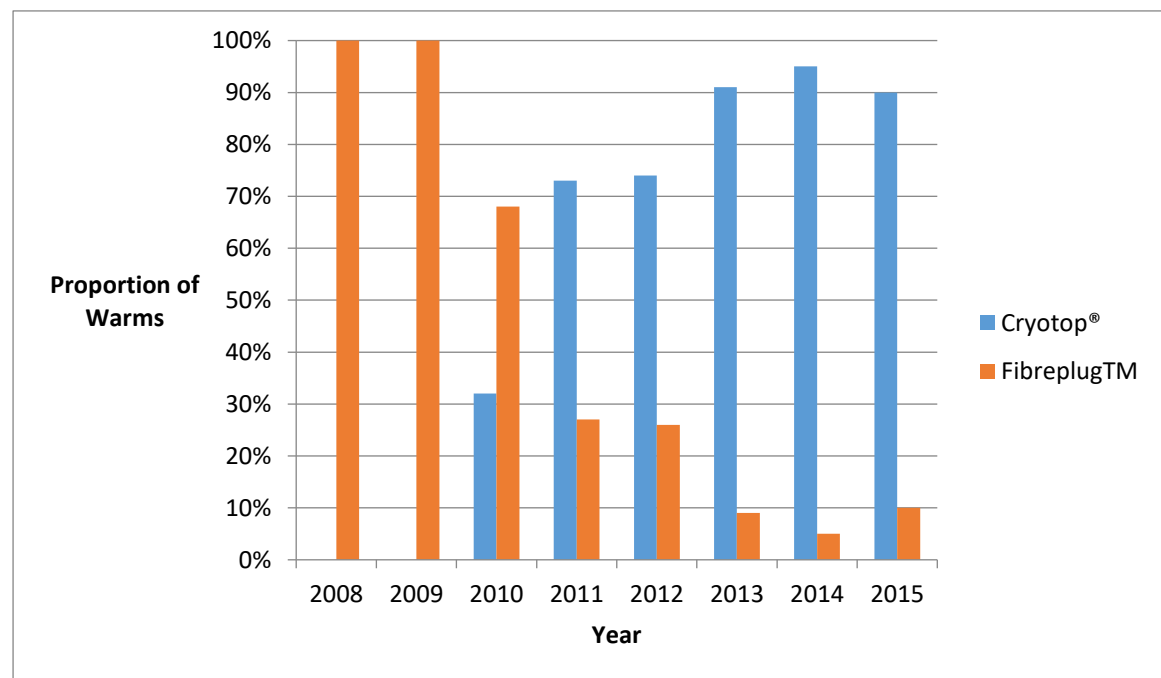
1/2 refers to embryo grade

4.3 Vitrification/Warming Technique Results

4.3.1 Vitrification Device

The change in vitrification device and technique during the data collection period resulted in an increasing proportion of CT warms occurring from 2010 through to 2015 (Figure 4-1). This was expected as there is a delay in pregnant patients returning to use any remaining embryos.

Figure 4-1 The proportion of warms with the Cryotop® and Fibreplug™ devices.



The overall survival rate of blastocysts vitrified on the Cryotop® device was 91.2% (1577/1730) which was significantly greater ($p < 0.0001$) than the 83.9% (1046/1247) for blastocysts vitrified on the Fibreplug™ device. The live delivery rate from SET's however was not significantly different ($p > 0.3196$) between the two devices (Cryotop® 422/1436, 29.4%; Fibreplug™ 240/875, 27.4%). The average age of the patients at the time of vitrification was also not significantly different ($p > 0.2273$) between groups (Cryotop®, 33.5 ± 4.5 ; Fibreplug™, 33.7 ± 4.4). The significant improvement in survival rate was not seen when controlling for media type ($p > 0.2016$). The survival rate of blastocysts vitrified and warmed using only media from supplier A on the Cryotop device was 86.2%. Using the same media combination with Fibreplug™ the survival rate was 83.8%.

4.3.2 Vitrification/Warming Media

Vitrification and warming media used for both devices were from three different suppliers during the data collection period (Appendix 7.3.7). All vitrification media contained the cryoprotectants ethylene glycol and DMSO with either sucrose or trehalose being used as the osmotic buffer. The warming media consisted of a base media with differing concentrations of osmotic buffer. Most blastocysts vitrified on the Fibreplug™ with media from supplier A were warmed using media from the same supplier and therefore it was not possible to make comparisons with media suppliers using this device (Table 4-19).

Table 4-19 The survival and implantation of blastocysts vitrified on the Fibreplug™ device using vitrification media from supplier A. Blastocysts were then warmed in media from suppliers A, B and C.

Parameter	Warming Media			Total
	A	B	C	
Age (av.yrs.)	33.8 ± 4.4	32 ± 3.2	32.4 ± 3.6	33.7 ± 4.4
# warmed	1185	12	50	1247
#survived	993	11	42	1046 (83.9%)
#SET	828	11	36	875
#viable pregs	241 (29.1%)	4 (36.4%)	16 (44.4%)	261 (29.8%)
#deliveries	222 (26.8%)	4 (36.4%)	14 (38.9%)	240 (27.4%)

Vitrification on the CT device used media from suppliers A (Table 4-20) and C (Table 4-21) with subsequent warming with media from suppliers A, B, C and B and C respectively. There was no significant difference in delivery rates between any of the media however the survival rate was significantly greater using warming media from supplier B. This difference persisted whether vitrification media from supplier A ($p < 0.0000$) or C ($p < 0.0195$) was used.

Table 4-20 The survival and implantation of blastocysts vitrified on the Cryotop® device using vitrification media from supplier A. Blastocysts were then warmed in media from suppliers A, B and C.

Parameter	Warming Media			Total
	A	B	C	
Age (av.yrs.)	33.6 ± 4.4	33.1 ± 4.2	33.2 ± 4.8	33.3 ± 4.5
# warmed	558	316	334	1208
#survived	481 (86.2%)*	301 (95.3%)*	304 (91.1%)	1086 (89.9%)
#SET	423	277	273	973
#viable pregs	127 (30.0%)	97 (35.0%)	97 (35.5%)	321 (33.0%)
#deliveries	109 (25.8%)	89 (32.1%)	85 (31.1%)	283 (29.1%)

*Based on Bonferroni sig level $p < 0.0167$ for multiple comparisons the survival rate using warming media from supplier B was significantly higher ($p < 0.0000$) than that using warming media from supplier A. There was no significant difference in delivery rate between any of the groups ($p > 0.0867$).

Table 4-21 The survival and implantation of blastocysts vitrified on the Cryotop® device using vitrification media from supplier C. Blastocysts were then warmed in media from suppliers B or C.

Parameter	Warming Media		Total
	B	C	
Age (av.yrs.)	34.0 ± 4.2	33.5 ± 4.8	33.7 ± 4.6
# warmed	190	332	522
#survived	185 (97.4%)*	306 (92.2%)*	491 (94.1%)
#SET	176	287	463
#viable pregs	66 (37.5%)	87 (30.3%)	153 (33.0%)
#deliveries	60 (34.1%)	79 (27.5%)	139 (30.0%)

* $p < 0.0195$

4.4 Embryo assessment prior to transfer

During the early part of the data collection period, 2008-2009, the sole survival criterion was the cell degeneration level. Any blastocyst with less than 70% cell survival was considered not to have survived and therefore was not transferred. Blastocysts with levels of cell degeneration greater than 5% had significantly poorer success rates than those with less than 5% (Table 4-22). This form of assessment was accompanied by observations of re-expansion (Figure 4-2). Re-expansion became the sole assessment tool for survival in 2010. It was easier to quantify, and early observations revealed extremely poor success with blastocysts that took longer than 60 minutes to begin the re-expansion process (Table 4-23, Figure 4-3). This effect was also seen on outcomes when comparing blastocysts that commenced re-expansion within 30 minutes to those commencing between 30-60 minutes. The commencement of re-expansion was demonstrated by the development of a blastocoel and/or lineation of trophectoderm cells (Figure 4-4).

Table 4-22 Proportion of cells surviving relative to the implantation and pregnancy outcome of warmed blastocysts.

Parameter	Cell survival (%)			p-value
	70-79	80-94	95-100	
Age (av.yrs.)	34.4 ± 4.7	33.9 ± 4.4*	33.3 ± 4.5*	0.0082
#warmed	71	634	1912	
#SET**	47	561	1692	
#viable pregs	5 (10.6%)	132 (23.5%)	593 (35.0%)	<0.0000
# deliveries	5 (10.6%) **	119 (21.2%) **	533 (31.5%)	<0.0000

* Based on Bonferroni sig level $p < 0.0167$ for multiple comparisons there was a significantly greater proportion of older patients in the 80-94% group than the 95-100% group ($p < 0.0165$).

**There was no significant difference in the delivery rate between blastocysts with 70-79% cell survival and those with 80-94% survival ($p > 0.0839$). Blastocysts with 95-100% survival had a significantly greater delivery rate than the 70-79 group ($p < 0.0023$) and the 80-94 group ($p < 0.0000$). ** x11 cell survival unknown as not recorded.

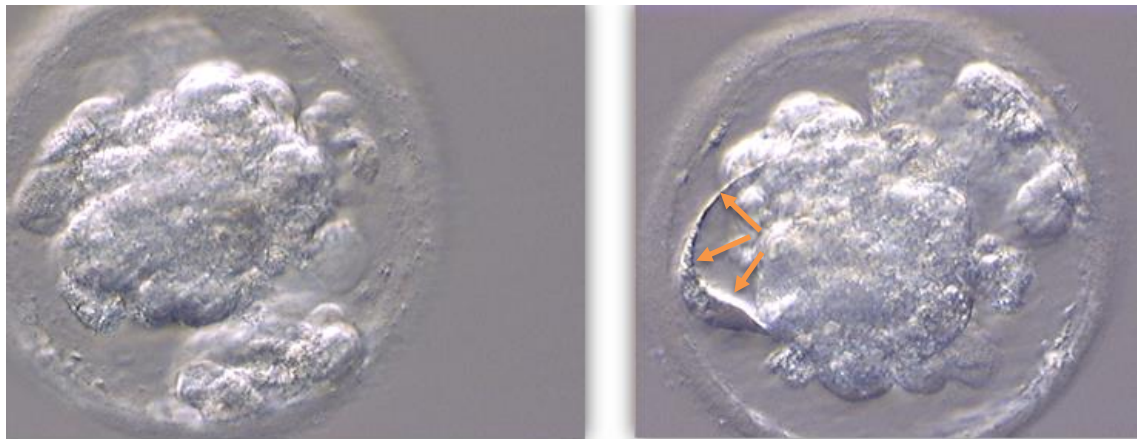
Table 4-23 Time taken to begin re-expansion after warming relative to the implantation and pregnancy outcome of warmed blastocysts (only recorded 2010 onwards).

Parameter	Time to start re-expansion (mins)			p-value
	<30	30-60	>60	
Age (av.yrs.)	33.4 ± 4.5	33.2 ± 4.3	33.3 ± 4.0	0.7268
#warmed	1475	456	391	2322
#SET	1329 (90.1%)	411 (90.1%)	25 (6.4%) ⁺	1765
#viable pregs	458 (34.5%)	120 (29.2%)	1 (4%)	<0.0012
# deliveries	410 (30.9%)*	105 (25.5%)*	1 (4%)	<0.0024

*Blastocysts commencing re-expansion within 30 minutes post warm had a significantly greater delivery rate than those commencing between 30- and 60-minutes post warm ($p < 0.0399$).

⁺ The practice of transferring blastocysts that commenced re-expansion more than 60 minutes after the warm ceased early in the study period due to the poorer results with these embryos.

Figure 4-2 Commencement of re-expansion



Arrows indicate evidence of re-expansion commencing.

Figure 4-3 A blastocyst failing to commence re-expansion after warming.

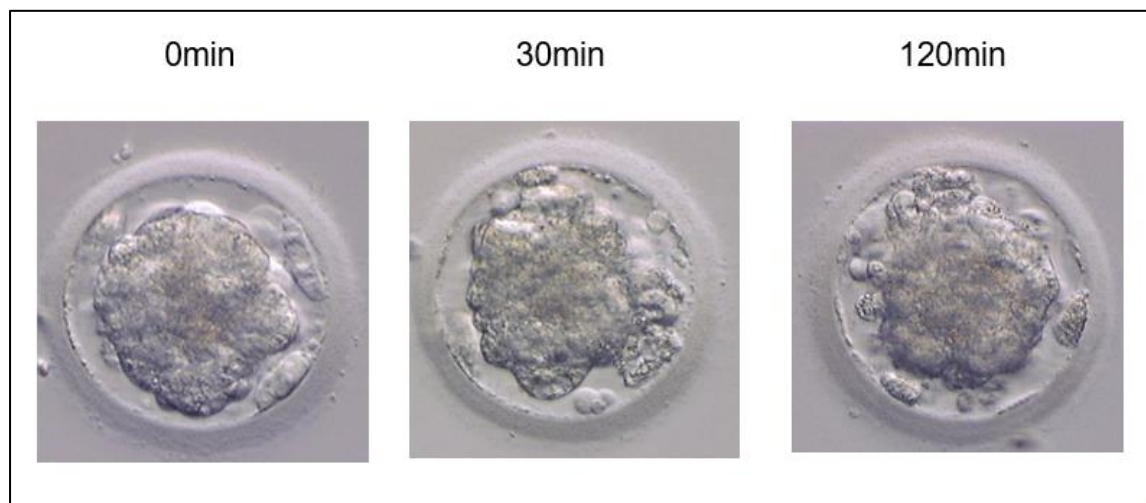
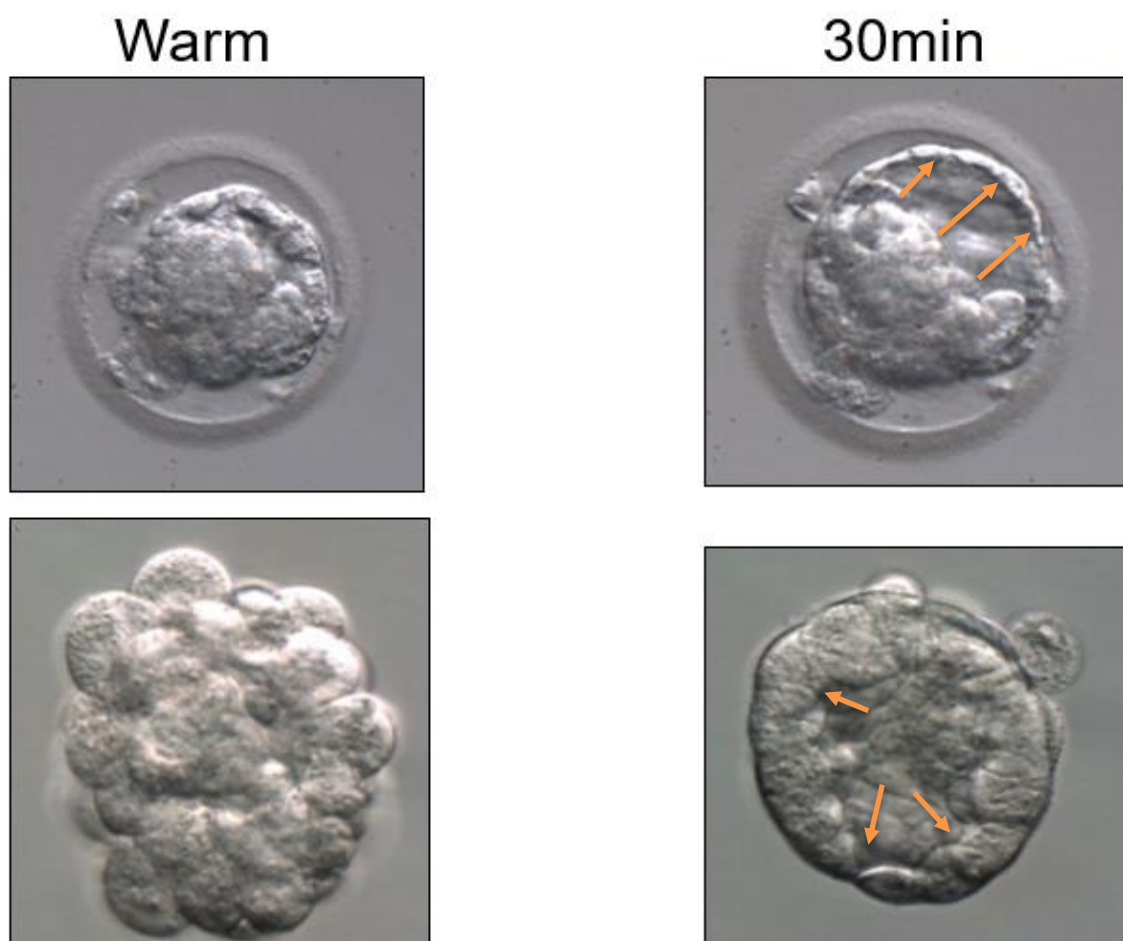


Figure 4-4 Re-expansion within 30 minutes for a full blastocyst (top row) and a fully hatched blastocyst (bottom row)



The rate of re-expansion for fully hatched blastocysts was relatively slower than the earlier stages of blastocyst development and had the greatest proportion (89%) that commenced re-expansion between 30 and 60 minutes. (Figure 4-5).

Figure 4-5 Time to commence re-expansion across different blastocyst stages

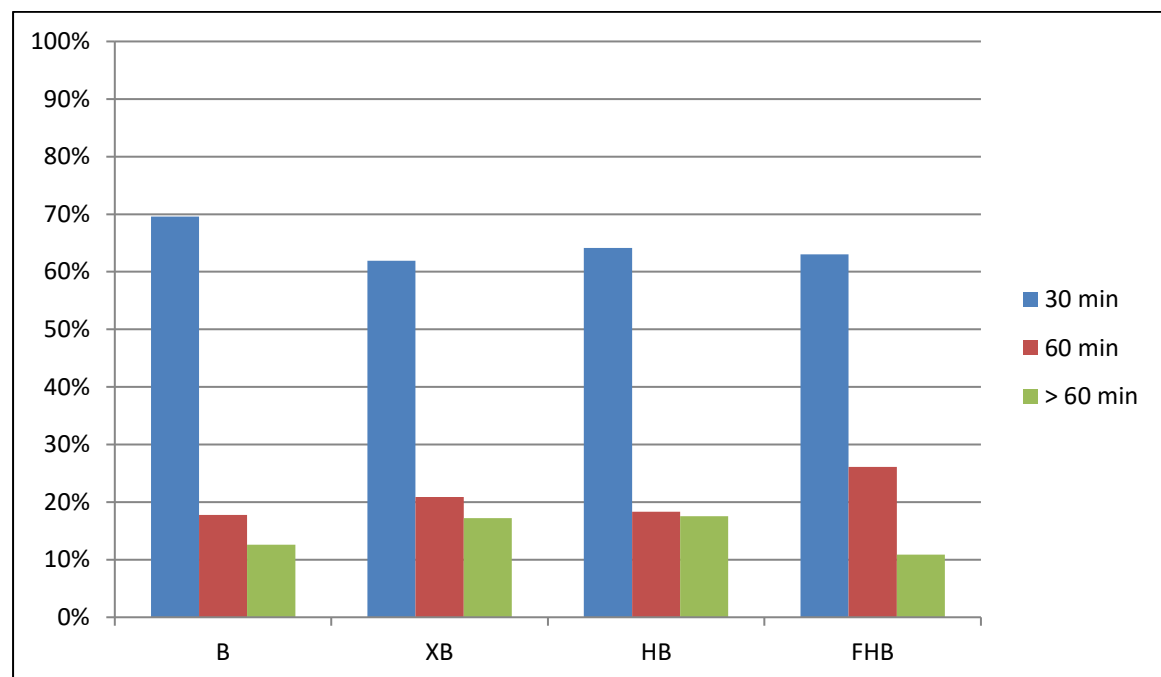
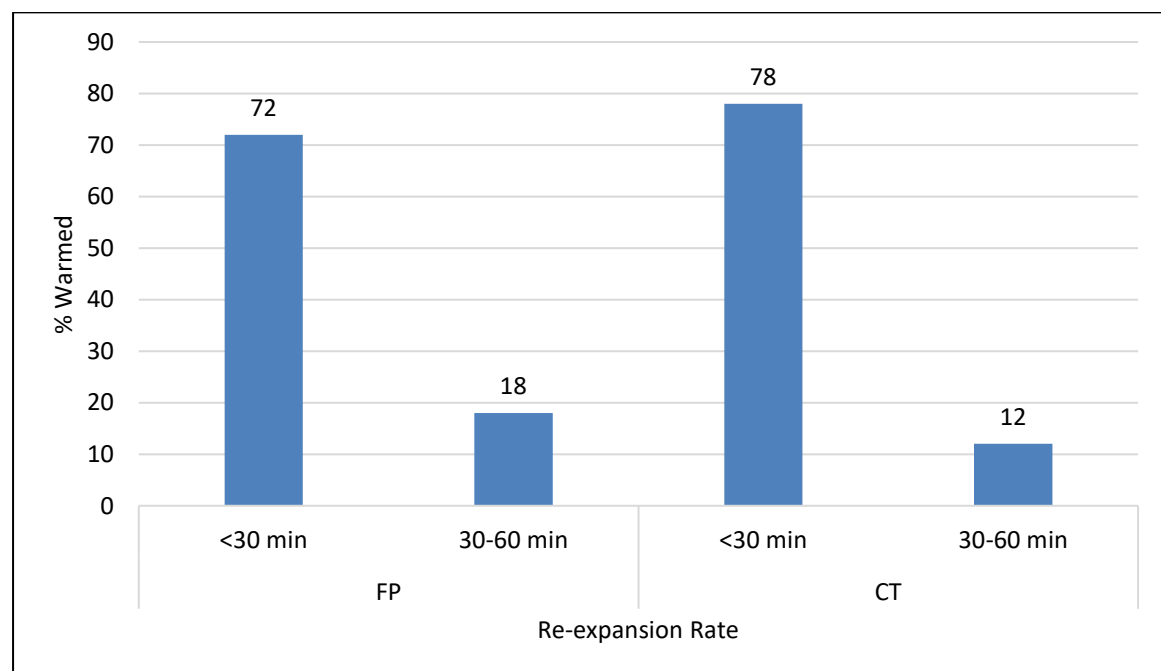


Figure 4-6 Rate of re-expansion relative to vitrification device.



A significantly ($p < 0.03$) greater proportion of blastocysts vitrified on the Cryotop® (CT) device re-expanded within the first 30 minutes post warming when compared to those vitrified on the Fibreplug™ (FP)(Figure 4-6).

4.4.1 Multiple Logistic Regression of embryo assessment criteria

Simple regression analysis of cell degeneration and re-expansion shows that both variables are significantly associated with delivery rates (Table 4-24). Binary logistic regression controlling for each variable, however, shows significant confounding between the two variables (Table 4-25). Within blastocysts that re-expand in ≤ 30 minutes, those with $\geq 95\%$ cell survival after warming have a 57% greater chance of progressing to a baby than those with between 70 and 94% cell survival. Within blastocysts that have $\geq 95\%$ cell survival, however, there is no significant difference in delivery rate between those that commence re-expansion in ≤ 30 minutes to those that commence re-expansion between 30 and 60 minutes.

Table 4-24 Simple regression results for a comparison of cell degeneration and re-expansion assessment criteria on delivery rates.

Predictor	Value	p-value	OR	95% CI	
Cell degeneration	$\geq 95\%$	<0.0000	1.7950	1.4372	2.2420
Re-expansion	$\leq 30\text{min}$	<0.0399	1.3002	1.0122	1.6701

Table 4-25 Binary logistic regression results controlling for cell degeneration and re-expansion.

Predictor	Value	p-value	OR	95% CI	
Cell degeneration	$\geq 95\%$	<0.0005	1.5719 (12.4%)*	1.2185	2.0280
Re-expansion	$\leq 30\text{min}$	>0.3499	1.1308 (13.0%)*	0.8738	1.4634

4.5 Time interval between warm and transfer

An advantage of assessing blastocysts after warming compared to cleavage stage embryos is the early evidence of the re-commencement of cellular activity. This gives an embryologist the ability to select or de-select a blastocyst within 60 minutes of warming it. Despite this shorter time frame, embryo transfer appointment times should allow \geq 3hrs for a suitable blastocyst to be assessed and selected for transfer. This provides for the possibility of needing to warm subsequent blastocysts. The warming of subsequent blastocysts and different scheduling times alters the time between the warm and the transfer (Table 4-26).

Table 4-26 The impact of time between starting the warm and transfer of vitrified blastocysts.

Parameter	Time between warm and transfer (hrs)						
	0-1	2	3	4	5	6	≥ 7
#SET	68	433	708	580	336	86	100
#viable pregs	20 (29.4%)	139 (32.1%)	218 (30.8%)	201 (34.7%)	104 (31.0%)	26 (30.2%)	27 (27.0%)
# deliveries	19 (27.9%)	124 (28.7%)	192 (27.1%)	182 (31.4%)	96 (28.6%)	23 (26.7%)	26 (26.0%)

Coefficient of determination, $R^2=0.1288$

$p=0.4291$

There was no linear relationship when comparing the time difference between warming a blastocyst and its eventual transfer. Only 13% of the variance in transfer outcome could be explained by the time interval between the warm of the blastocyst and its transfer.

5 DISCUSSION

5.1 Clinical value of embryo cryopreservation

The original clinical benefit of embryo cryopreservation was to utilise all embryos produced from an oocyte collection cycle using exogenous gonadotrophins without increasing the probability of a multiple pregnancy (Cohen et al., 1985). This strategy also improved the cumulative pregnancy rate from an oocyte retrieval cycle and reduced overall treatment costs (Kjellberg, Carlsson, & Bergh, 2006). In this present study the majority (> 80%) of embryos cryopreserved were from cycles that also had fresh embryo transfers and embryos were vitrified for future use. Embryo cryopreservation is also utilised for oocyte donor/recipient cycles to assist with cycle synchronisation but early attempts proved difficult to justify due to poor embryo survival rates of less than 50% (Devroey & Pados, 1998). More recently with improved cryopreservation techniques and better survival rates above 90%, embryo cryopreservation in donor/recipient cycles is more commonly used (Cobo et al., 2012). Of the 2821 oocyte recipient cycles performed during 2016 in Australia and New Zealand, 53.5% were thaw cycles and the live delivery rate was higher from thaw (26.2%) than fresh (14.5%) cycles (Fitzgerald et al., 2018). The use of thaw cycles for oocyte/embryo donation also allows for a quarantine period to reduce the risks of transmission of infectious diseases from donor to recipient (Hamer, Horne, Pease, Matson, & Lieberman, 1995). In this present study, donor recipient embryos were excluded to avoid bias. If facilities and logistics did not allow for blastocyst culture that can further discriminate viable embryos, cryopreservation at early embryo stages was useful (Mandelbaum et al., 1987). It has also been used to rescue cleavage stage embryos that were classified as unusable due to poor morphological grades. If they developed to blastocysts they could be cryopreserved at the blastocyst stage and utilised in a later cycle if needed (Hartshorne, Elder, Crow, Dyson, & Edwards, 1991). During this present study facilities were available for safe blastocyst culture and greater than 90% of embryo transfers were of blastocysts. Embryo cryopreservation has also been utilised to supposedly improve embryo/endometrium synchronicity. Asynchrony due to a pre-ovulatory progesterone rise during ovarian stimulation has been shown to reduce the success of fresh embryo transfers (Bosch et al., 2010) and be improved by embryo cryopreservation and

subsequent transfer in a cycle specifically programmed for embryo transfer (Shapiro et al., 2010). Data in this present study did not discriminate for freeze all indications, however it is estimated that less than 5% of embryos were cryopreserved due to a premature progesterone rise. Slower developing blastocysts have been shown to have improved outcomes by cryopreserving and replacement in a later more synchronised cycle (Kaye et al., 2017). In this present study slower developing blastocysts (day 6) had a significantly reduced delivery rate despite being transferred in a day 5 endometrium of a replacement cycle (19.3% vs 30.7%). Embryo cryopreservation can also be used to preserve fertility in patients with impending treatment for conditions requiring the use of gonadotoxic agents that increase the risk of developing premature ovarian failure and infertility (Bedoschi & Oktay, 2013). This may be neoplasia or non-oncological systemic autoimmune or haematological conditions (Gidoni, Holzer, Tulandi, & Tan, 2008). The increasing success of oocyte vitrification has led to more fertility preservation occurring at the oocyte level. This can be performed for younger patients as it avoids the need for a donor/partner at the time of preservation and also avoids the problem of having excess embryos in storage after completing a family (Robertson, 2014; Seshadri et al., 2018). The concordant advances in IVF of extended culture, trophectoderm biopsy, next generation sequencing (NGS) techniques for genetic screening of all 24 chromosome types and blastocyst vitrification have resulted in the increasing use of freeze-all cycles for pre-implantation genetic testing (Coates et al., 2017). Coates et al (2017) performed a randomised controlled trial of fresh vs frozen euploid blastocyst transfers and found no significant difference in live birth rates but more patients reached the stage of euploid embryo transfer as both test results for day 5 and 6 blastocysts were available. As the analysis took 17 hrs, only day 5 blastocysts could be used for biopsy and transferred fresh prior to 12 pm on day 6. The costs to the patient are kept to a minimum as biopsied cells can be sent to larger volume genetic testing laboratories where batching of samples is possible. In Australian and New Zealand IVF units there was an increase of 26.8% in cycles using pre-implantation genetic testing (PGT) during 2016 and 39.2% were the result of freeze-all cycles (Fitzgerald et al., 2018). In this present study, biopsied embryos were excluded to avoid the bias from improved outcomes of known euploid embryos.

The most prevalent use of the freeze-all embryos strategy is in managing the iatrogenic complication of ovarian stimulation, ovarian hyperstimulation syndrome (OHSS) (Horwath, Check, Choe, Wilson, & Amui, 2005; Shin, Jeong, Nho, & Jee, 2018). The incidence of mild to severe OHSS has been reported as being between 3.1% and 8% of IVF cycles (Delvigne & Rozenberg, 2002), however the validity of reporting is questionable for mild to moderate forms of the syndrome (Fernandez-Sanchez et al., 2019). In Australia and New Zealand 0.5% of cycles proceeding to egg collection in 2016 were reported as being admitted to hospital for treatment and the frequency of admissions was closely correlated with the number of oocytes collected (Fitzgerald et al., 2018). A freeze-all approach to managing OHSS reduces endogenous hCG exposure and subsequent elevation of VEGF (vascular endothelial growth factor) from the corpora lutea and is one of the most effective strategies to prevent severe forms of OHSS (Griesinger et al., 2007; Nelson, 2017). More than 12% (287/2311) of single embryo transfers in this present study were first time transfers and therefore from freeze all cycles. Subsequent transfers could also be from an original pool of embryos from a freeze all cycle.

5.2 Vitrification as a method of choice

Slow freezing of human embryos takes more than 2 hours and requires the use of a relatively expensive programmable freezer (Mukaida et al., 1998). Soon after the first pregnancy utilising slow freezing in human embryos, shorter protocols were being devised using an interrupted cooling step and ultrarapid freezing (Gordts, Roziers, Campo, & Noto, 1990; Trounson & Mohr, 1983; Trounson, Peura, Freemann, & Kirby, 1988). Poor survival and implantation rates along with regulatory constraints on human embryo cryopreservation trials resulted in most IVF units deferring to the more successful slow freezing techniques at this time (Friedler, Giudice, & Lamb, 1988; Trounson & Sjoblom, 1988).

Due to the advantage of possibly avoiding ice crystal formation during vitrification, further studies were being conducted on other mammalian species to refine the concentrations and types of CPA's being used (Kasai, 1996). At a similar time blastocyst culture became more prevalent with the introduction of stage specific media, the CPA combination of EG and DMSO with a step-wise exposure was proving successful for

blastocyst vitrification (Lane et al., 1999; Mukaida et al., 2001; Reed, Lane, Gardner, Jensen, & Thompson, 2002). Stage specific media was used for the culture of the embryos in this present study with EG and DMSO as the cryoprotectants. Despite consistent use of these products there was no consistency in the methodology being used for the clinical application of blastocyst vitrification and implementation was minimal (Vajta & Nagy, 2006). The development of suitable commercially available carrier devices that minimised the volume of the suspension containing the embryo and hence increased cooling and warming rates ultimately resulted in a widespread adoption of vitrification of human embryos (Kuwayama, 2007). Current vitrification techniques utilising these devices also provide for a reduced footprint, take up less storage space and reduce risk by reducing embryo handling (Liebermann, 2017). The Fibreplug vitrification system for blastocysts was established at Hollywood Fertility Centre in 2007.

5.3 Patient and Cycle Demographics

5.3.1 Single Embryo Transfer

There is an ongoing trend of safer embryo transfer practices in Australia. The proportion of thaw cycles with a double embryo transfer (DET) in Australia and New Zealand during 2016 was 8.2% (Fitzgerald et al., 2018). This is a direct result of supportive public funding for ART and to a lesser extent, clinical guidelines and educational campaigns (Chambers et al., 2013). All ART units in Australia are required by federal law (Research Involving Human Embryos Act 2004) to be issued a license to practice by the Reproductive Technology Accreditation Committee (RTAC). License holders comply with the RTAC Code of Practice that includes measures to reduce the incidence of DET. The latest version of the code requires units to recommend only one embryo be transferred to patients aged less than 35 years in their first cycle. During the data collection period of this present study (2008 to 2015) the proportion was 6.3%. Despite the additional embryo, the delivery rate for the Australia and New Zealand data was lower than SET (26.3% vs 28.6%). The main reason for this is that a higher proportion of patients 35 years of age or older received a DET (15.7%) compared to those under 35 (7%) (Fitzgerald et al., 2018). The multiple pregnancy rates for SET vs DET in this present study were 2.4% and 24.6% respectively and this closely aligns with national data (Fitzgerald et al., 2018). Further efforts are required in selecting the right patients for DET, if any. The

use of a prediction model applying age, embryo score, ovarian sensitivity and treatment history has been shown to reduce the multiple rates from 25.2% to 3.8% for early day 2 embryo transfers (Vaegter et al., 2019).

5.3.2 Perinatal outcomes

The average gestational period (38.9 weeks) and birth weight (3346g) for SET singleton pregnancies in this present study was similar to that of all singleton births in the Australian population (38.6 weeks and 3372g) (Australian Institute of Health and Welfare, 2018). Despite this similarity the average maternal age of the patients with a singleton delivery in this cohort was 33.6 years at the time of the transfer compared to 30.5 years in the Australian population. Both the gestational period (38.2 weeks) and average birth weight (3295g) of deliveries from blastocyst thaw cycles across Australia and New Zealand in 2016 were shorter and lighter respectively than the parameters from this present study. However, the average maternal age in Australia and New Zealand was almost 2 years older (35.4 years). The similar average gestation and birth weight to the Australian population contradicts some previous reports that birth weights from ART cycles are lower (Hansen et al., 2009; Z. Li, Wang, Ledger, & Sullivan, 2014). This could possibly be explained as mothers utilising ART are less likely to have smoked and more likely to be first time mothers (Fitzgerald et al., 2018). Interestingly, the sex ratio of babies from this present study was 100 males for every 100 females compared to 109 for ART in Australia and New Zealand and 106 for all Australian births in Australia in 2016 (Australian Institute of Health and Welfare, 2018; Fitzgerald et al., 2018). Blastocyst culture and cryopreservation have been implicated in reports of adverse neonatal outcomes, however the findings of preterm birth for blastocyst culture and large birthweight for cryopreserved blastocysts were not replicated in the study population of this present study (Maheshwari, Kalampokas, Davidson, & Bhattacharya, 2013; Maheshwari, Raja, & Bhattacharya, 2016). There is significant variance in the reporting of adverse outcomes between clinics and regions and large meta-analyses cannot capture the various protocol differences. It has been well described in a review that different culture conditions can adversely influence embryo metabolism and subsequent extended culture outcomes (Wale & Gardner, 2015). It is not clear as to the embryo culture conditions of the units contributing data to the study by Maheshwari et

al, (2013). A survey of similar units however, revealed the majority were using atmospheric oxygen (20%) as opposed to reduced oxygen tension (5-6%) during embryo culture (Christianson et al., 2014). The use of atmospheric oxygen for embryo culture ignores the negative effects identified in many mammalian species including humans (Gardner, 2016). Blastocyst culture in this present study was consistently with a low oxygen tension of 6.0% with occasional embryo handling at atmospheric oxygen levels. This could possibly explain the relatively normal singleton outcomes after blastocyst culture and cryopreservation compared to those reporting for the Maheshwari et al 2013 and 2016 meta-analyses.

5.3.3 Maternal Age

It is generally accepted that the human oocyte arrests at the prophase stage of the first meiotic division between the fourth and sixth months of life in utero (Johnson, 2018). Despite claims of potential postnatal oogenesis (Johnson, Canning, Kaneko, Pru, & Tilly, 2004), there remains little consensus on the existence of stem cells in the adult ovary (Sophie, Céline, Marina, Sylvie, & Célia, 2019). So, unlike most somatic cells, oogonia are potentially immortal until recruited by folliculogenesis that occurs every day from before birth to menopause (Gougeon, 1996). Unfortunately, this finite population of ovarian follicles and senescence over time appears irreversible and is the primary cause of ovarian aging and reduced fecundity with age (Kirkwood, 1998). Even ART cannot compensate for the 30-50% drop in fecundity caused by delaying attempts to conceive until a maternal age >35 years (Templeton, Morris, & Parslow, 1996; Wang et al., 2017). The most significant predictor of the outcome after a warmed blastocyst transfer in this present study was maternal age at the time of vitrification. Results showed those patients ≤ 38 years were more than twice as likely to achieve a live birth than those > 38 years after controlling for the cycle type and transfer attempt. The patients in this present study had already generated quality blastocysts in a fresh cycle for cryopreservation but maternal age still has an influence on the aneuploidy of blastocysts despite good morphology (Cimadomo et al., 2014).

5.3.4 Embryo Transfer Cycle

5.3.4.1 *Endometrial preparation and timing of embryo transfer*

Sufficient hormonal preparation of the endometrium is essential to provide an adequate environment for the developing embryo. Successful implantation requires both endometrial receptivity and synchronicity between both the embryo and the endometrium (Devroey & Pados, 1998).

5.3.4.1.1 Modified natural cycles

In patients who have regular cycles, it is possible to prepare the endometrium with the endogenous hormones produced by the naturally developing follicle. This requires thorough monitoring of LH levels and endometrium thickness to ensure the accurate timing of ovulation and subsequent transfer (Casper & Yanushpolsky, 2016). Although sometimes described as a simpler form of endometrial preparation, it can require frequent clinic visits with less cycle control and more cancellations (Sathanandan et al., 1991). An LH rise signifies ovulation in the following 36 to 40 hours (Andersen, Als-Nielsen, Hornnes, & Franch Andersen, 1995) although definitions of an LH rise vary between centres (Mackens et al., 2017). To avoid asynchrony that could result from a missed LH surge, modified natural cycles use an exogenous trigger of ovulation when a follicle reaches 16mm or greater in size. A reduced proportion of modified NAT cycles (12.3%) were used in this present study as it required frequent clinic visits and a significant number of patients were based in rural areas. Despite this difficulty, the outcomes from these cycles were significantly better than those managed by HRT. Of the 283 embryos transferred in modified NAT cycles, 105 (37.1%) babies were born. This is significantly greater than the 25.4% (280/1103) from HRT cycles (Table 4-4). Using multiple regression controlling for age and transfer rank, non HRT cycles are almost 38% more successful at producing a live born baby compared to the HRT cycles (Table 4-9). Despite individual studies demonstrating a similar conclusion (Alama et al., 2007; Chang et al., 2011; Xiao, Zhou, Xu, Yang, & Xie, 2012), repeated reviews in the literature, however have found no evidence that favours one cycle type over another (Groenewoud, Cantineau, Kollen, Macklon, & Cohlen, 2013; Groenewoud, Cohlen, & Macklon, 2018).

5.3.4.1.2 Low dose ovarian stimulation cycles

The use of gonadotrophins to control a cycle for the transfer of a frozen-thawed embryo has diminished after repeated studies showed no difference to natural, modified natural and HRT cycles (Ghobara & Vandekerckhove, 2008). Recent meta-analyses have not even included gonadotrophin stimulated cycles as part of the analysis (Groenewoud, Cantineau, Kollen, Macklon, & Cohlen, 2017; Mackens et al., 2017). In this present study however, 40% of cycles were prepared by a low dose gonadotrophin stimulation protocol. Clinical review within the unit concluded that the added cycle control alleviated problems with timing of ovulation and subsequent FET that are afforded to natural cycle management. Older patients also benefited from augmentation of spontaneous endometrial enrichment during the follicular phase. There was no significant difference between the 32.3% live birth rate of LDS cycles compared to the 37.1% success rate of modified natural cycles in this present study.

5.3.4.1.3 Hormone Replacement Cycles

Most of the cycles in this present study were prepared with the use of sequentially delivered exogenous oestrogen and progesterone supplementation (47.7%). One of the greatest benefits of the hormonally controlled cycle is that the proliferative phase can be adjusted without compromising the implantation window (Simon et al., 1999; Soares et al., 2005). This is convenient for programming cycles and suitable for those anovulatory patients and those with irregular cycles. Unlike the consensus in the literature (Groenewoud et al., 2018), the outcomes from HRT cycles in this present study were significantly ($p < .0001$) poorer than those cycles prepared by natural means or supplemented with gonadotrophins (25.4% vs 32.3% and 37.1%). There is little agreement regarding the timing of embryo transfer for HRT cycles (Nawroth & Ludwig, 2005) but a Cochrane review concluded that starting progesterone supplementation on the day equivalent to the day of egg collection or the day after resulted in greater success than commencing progesterone a day before the theoretical day of egg collection (Glujovsky et al., 2010). In this present study both D5 and D6 blastocysts were transferred on the 6th day of progesterone supplementation. This timing is considered too late by some authors and therefore a trial of transfer on the 5th day could be

considered (Escriba et al., 2006; Glujovsky et al., 2010; Mackens et al., 2017; van de Vijver et al., 2017).

5.3.4.2 Embryo transfer rank

In chapter 9 of the 2018 NPESU report, data is presented from an ongoing longitudinal study of patients commencing autologous treatment at the start of 2014, following their progress up to the end of 2016 (Fitzgerald et al., 2018). This provides an indicator of the pathway of treatment a patient takes if they are unsuccessful with their first embryo transfer. It excludes freeze-all cycles and patients drop out of the study when they have a successful delivery. Subsequent cycles maybe frozen or fresh transfers. The data shows a significantly reducing success rate as the transfer rank increases. In this present study there is also a significant reduction in success rates with subsequent embryo transfers. A direct comparison cannot be made as the NPESU data excludes subsequent cycles after a delivery and it is per cycle data rather than the per transfer data of this present study. However, the downward trend still exists in both data sets. In this present study more than 50% of embryo transfers were of the order of 3 or greater and the live delivery rate of 1st embryo transfers (38.7%) was significantly greater than the 26.5% and 20.8% of the 3-5 and 6-20 transfer rank groups (Table 4-6). Before the use of blastocyst culture became prevalent, studies showed that repeated cycles had similar success rates (Meldrum, Silverberg, Bustillo, & Stokes, 1998). This was however disputed for those that attained a pregnancy and were returning for another (Molloy, Doody, & Breen, 1995). Shapiro et al. (2001) reported contradicting significant declines in success rates for repeated cycles and suggested an explanation of improved culture techniques and blastocyst transfer. They showed an implantation rate of 30% for 1st transfers, 18% for 2nd transfers and 8% for 3rd (Shapiro, 2001). In this present study the equivalent implantation rate was 41.8%, 32.9% and 30.0% respectively. The reduced decline in comparison can be explained as this data set is for a period of 8 years and allows for patients returning after pregnancy to be included in the study. It is also only for vitrified blastocysts that come from cycles with improved outcomes (Wang et al., 2008). Vitrified blastocyst outcomes also benefit from the inclusion of freeze all patients that have been shown to have an increased proportion of patients that are good responders after ovarian stimulation (Taratzis, Grimbizis, Bosdou, Kolibianakis, & Venetis, 2019). In a

recent multicentre, non-blinded, randomised controlled trial in 21 academic fertility centres in China it was demonstrated that there was a 26% improvement in the freeze all singleton live birth rate compared to fresh cycles (Wei et al., 2019).

5.4 Blastocyst Morphology and Development Rate

5.4.1 Day 5/6

Development rate has been the most prevalent of embryo assessment criteria (Cummins et al., 1986; Hossain et al., 2016). This criterion holds for both the cleavage stage embryo and the blastocyst stage (Shapiro, Richter, Harris, & Daneshmand, 2001). In this present study, blastocysts were selected for vitrification either on day 5 or day 6 of development after insemination (Day 0). Controlling for morphology and the degree of expansion, day 5 blastocysts had an 80% greater chance of resulting in a live birth compared to day 6 blastocysts. Previous comparisons of fresh day 5 vs day 6 transfers have shown a similar or greater difference in success rates (Barrenetxea et al., 2005; Dessolle et al., 2011; Schoolcraft et al., 1999b; Shapiro et al., 2001). These studies relied on non-elective selection of the day 6 blastocyst transferred and therefore represented developmentally delayed blastocysts that did not meet the requirements of selection on Day 5. A subsequent study comparing elective Day 6 transfers showed the odds ratio of day 6 transfers to be 0.34 (Poulsen, Ingerslev, & Kirkegaard, 2017). They suggested the difference was likely to be the detrimental effects of prolonged culture or asynchrony with the endometrium in a fresh cycle. Some studies on replacement of frozen day 5/6 blastocysts in subsequent controlled cycles have shown little difference when blastocyst quality is similar (Richter, Shipley, McVearry, Tucker, & Widra, 2006; Sunkara et al., 2010). Sunkara et al., (2010) in a meta-analysis of studies comparing day 5 and 6 blastocysts concluded that if the morphology grade was controlled day 5/6 outcomes were comparable in subsequent programmed frozen cycles. They speculated that the endometrial-embryonic synchronisation could be more important than the blastocyst development rate in contributing to treatment outcome. This present study however contradicts these findings after controlling for blastocyst grade (OR 1.64) and expansion (OR 2.01). More recent studies comparing Day 5/6 outcomes from vitrified/warmed blastocysts also confirm that despite good quality blastocysts on Day 6 they do not

achieve the same outcomes as blastocysts vitrified on Day 5 (Ferreux et al., 2018b; Haas et al., 2016). However this does not fully remove the possibility of the differently primed endometrium playing a role in the implantation potential of the blastocyst (Weimar et al., 2012). Berrin et al (2018) also vitrified blastocysts developing on Day 7 and found similar implantation rates between Day 6 and 7 blastocysts (Berrin et al., 2018).

5.4.2 Pre-vitrification Expansion and Morphology

The assessment of blastocysts for vitrification used a combined ICM and trophectoderm score (Roy, Bradley, et al., 2014). Expansion level assessment is an indicator of embryo competence as it requires significant energy for sodium/potassium ATPases and the multi-protein tight junctions between trophectoderm cells to form a barrier (Alpha Scientists in Reproductive Medicine, 2011; Gardner & Schoolcraft, 1999a, 1999b). This criterion was first designed to be used as a rapid assessment tool under a dissecting microscope. The morphology grade, however, is easier to allocate at the more expanded stages as the visibility of the distinct cell types is clearer using an inverted microscope. The results in this present study confirm this by showing no significant influence of morphology grade on survival or live birth rate until there was significant expansion in the embryo on Day 5 (Table 4-11). Unexpanded blastocysts with a morphology grade 1 on day 5 had a greater delivery rate compared to grade 2 (28.0% vs 22.7%) however the difference was not significant as very few unexpanded blastocysts were given a morphology grade of 1. Similar findings from Ahlstrom et al (2011) showed that trophectoderm morphology was the strongest morphology predictor only when there was significant expansion in the blastocyst (Ahlstrom et al., 2011). In this present study, despite expansion on day 6, the significance of embryo grade is reduced as the development stage becomes the most significant indicator of viability. The ranking of embryos by combining survival and live birth outcomes in the present study confirms the importance of development rate (day 5/6) and the expansion level/morphology grade on day 5 and provides a useful tool for managing patient expectations and prioritising embryos for warming and transfer.

5.5 Vitrification Technique

The cooling/warming rates are critical criteria affecting blastocyst survival after vitrification in addition to the cryoprotectant concentrations, exposure time and

temperature (Kader, Choi, Orief, & Agarwal, 2009). Although there was no significant difference in live birth rates between techniques in this present study, the survival rate determines the embryos that make it to transfer for that calculation. This is an important factor often missed when looking at meta analyses suggesting the benefits of freeze all policies (Wei et al., 2019).

5.5.1 Vitrification Device

Although the cooling/warming rates using the Fibreplug™ are unpublished it is assumed to be slower than the Cryotop® device as the volume of the sample is 20 to 30 times greater and the cooling/warming rates are inversely proportional to volume and risk of ice crystallisation (Vanderzwalmen et al., 2002). The reduced cooling/warming rates of the Fibreplug™ in this present study could be an explanation of the lower blastocyst survival compared to the Cryotop (83.9% vs 91.2%). When vitrification of mouse embryos was first achieved in 1985, they were limited by cooling and warming rates <3000°C/min using a 45 µl drop drawn into a 0.25 ml plastic straw (Rall & Fahy, 1985). They manipulated the warming rate while keeping the cooling rate consistent and found significant differences in survival rates from 0% to 87.8%. Although there was success with the use of these straws for human blastocysts (Yokota, Sato, Yokota, Yokota, & Araki, 2001) the first birth from vitrified human blastocysts came from a group in Seoul, Korea using an electron microscope (EM) copper grid (Choi et al., 2000). The 26 µm bar diameter and 38 µm pore size of the 3mm diameter EM grid allowed direct exposure to liquid nitrogen with a more rapid cooling rate. The grid was subsequently inserted into a cryo vial pre-filled with liquid nitrogen and mounted on a cryo cane for storage. The survival rate of 51.6% was most likely more related to the use of EG as a single CPA rather than slower cooling/warming rates. Other devices also focussing on a reduced volume of the sample to be vitrified include the open pulled straw (OPS) and the cryoloop (Lane et al., 1999; Mukaida et al., 2003; Reed et al., 2002; G. Vajta, Holm, Greve, & Callesen, 1997). Despite the success of these devices, the rapid cooling rate (>20,000°C) and rapid warming rate (>40,000°C) of the Cryotop® due to the very small volumes (< 1µl) needed to contain the embryo have not been surpassed for clinical use on human embryos (Kuwayama, Vajta, Kato, et al., 2005). All these methods do however utilise direct exposure to liquid nitrogen to enhance their cooling and warming rates.

Due to concerns over contamination (Bielanski, Bergeron, Lau, & Devenish, 2003) from liquid nitrogen the development of closed systems have been explored (Papatheodorou et al., 2013). Both devices used during this present study were open systems with exposure to liquid nitrogen in the storage phase but utilised a sterilised vitrification block to vitrify the blastocysts prior to storage. The manufacturers of both devices have subsequently offered closed alternatives (Huang, 2016; Gábor Vajta, Rienzi, & Ubaldi, 2015).

In addition to the difference in shape between the two carrier devices there were also variations in the associated protocols that could explain an improved survival rate with the Cryotop device (Table 5-1). The equilibration time was significantly shorter with the Fibreplug (2 min vs 15 min) and temperature was 37 degrees Celsius compared to 19-23 degrees Celsius with the Cryotop. Due to multiple variances it is difficult to determine which of them would influence survival more than the other. A conclusion on blastocyst survival cannot therefore be attributed to the device only and it is important to further analyse differences in the pre-handling of the blastocyst (artificial collapsing) and the vitrification/warming media used.

Table 5-1 Cryotop® and Fibreplug™ Methods

	Fibreplug™	Cryotop®
Equilibration temp	37°C	19-23°C
Equilibration time	2 min	15 min
Artificial collapsing	YES	NO
Visualisation	NO	YES
SSV	YES	YES
Cooling rate	Unpublished	22,800°C/min (Kuwayama, Vajta, Kato, et al., 2005)
Warming rate	Unpublished	42,100°C/min (Kuwayama, Vajta, Kato, et al., 2005)
Vitrification volume	3 µl	<0.1 µl
Warming temp	37°C	37°C
Re-equilibration temp	37°C	19-23°C

5.5.2 Blastocyst Collapsing

In the Fibreplug™ group of this present study the reduction of the blastocoel was performed by manual pipetting of the blastocyst through a reduced diameter pipette prior to exposure to the final vitrification solution. Previous studies had shown that blastocyst survival rates were reduced due to the expanded blastocoel not completely being devoid of water and replaced with CPA, increasing the risk of ice crystal damage. Blastocoel reduction by micro-needle aspiration, laser, hyperosmotic pressure (sucrose) and the manual pipetting were performed (T. Ebner & Shebl, 2018; Hiraoka, Hiraoka, Kinutani, & Kinutani, 2004; Kovačič, Taborin, & Vlasiavljević, 2018; Son, Yoon, Yoon, Lee, & Lim, 2003; Vanderzwalm et al., 2002). In a study with mouse blastocysts, Frank et al (2019) concluded that the Na⁺/K⁺-ATPase driven water transport mechanisms were responsible for the majority of the re-expansion process after vitrification and warming

(Frank et al., 2019). They explained that a decreased cell survival due to not performing artificial collapsing prior to vitrification lead to these mechanisms being compromised and having to rely on passive mechanisms as measured through aquaporin transcripts Aqp3 and 8. They suggested this to be the mechanism resulting in artificially collapsed blastocysts re-expanding faster than those that were not artificially collapsed, however their study utilised laser collapsing as opposed to the manual pipetting method used in this present study. Their finding conflicts with the assessment in this present study that blastocysts vitrified on the Cryotop® device, that were not pre-treated with artificial collapsing, re-expanded quicker than those blastocysts vitrified on the Fibreplug™ (Figure 4-6). Artificial collapsing was not performed in the Cryotop® group as the blastocoel collapses and partially re-expands spontaneously during the first equilibration phase of the vitrification process. Equivalent success compared to fresh blastocyst transfer has been published using this method (Roy, Bradley, et al., 2014) but some groups still routinely perform this pre-treatment prior to vitrification with the Cryotop® (Du et al., 2016). As part of a study on aseptic vitrification, Vanderzwalmen et al (2009) concluded that the longer equilibration using lower concentrations of cryoprotectants at room temperature allowed sufficient cryoprotectant to enter the blastocoel to avoid ice crystal formation (Vanderzwalmen et al., 2009). A prospective RCT investigating the effect of artificial shrinkage (collapse) on the implantation potential of vitrified blastocysts found the artificial shrinkage by laser-induced collapse did not significantly increase the implantation rate per transferred collapsed blastocyst compared with non-collapsed blastocysts, however there was a significant improvement in survival rate (Van Landuyt et al., 2015). Although there is no evidence artificial blastocoel collapse can negatively affect survival and implantation outcomes, it was concluded for the protocols using Cryotop® in this present study that the procedure was an added handling risk and unnecessary. The extended equilibration time in the Cryotop protocol allows for gradual blastocoel collapse due to the removal of water and re-expansion as the blastocoel fluid is replaced with cryoprotectants.

5.5.3 Vitrification/Warming Media

The successful formula for vitrification media for human oocytes and embryos has remained consistent for the past decade with most formulations consisting of EG and

DMSO in equal proportions along with a carbohydrate acting as an osmotic buffer (Argyle et al., 2016). Each cryoprotectant has a role to ensure the formation of a solidified amorphous liquid state of high viscosity at a low temperature that spans both the intra and extra-cellular space (Fuller & Paynter, 2004). The concentrations of the permeable low molecular weight cryoprotectants used in this present study are the result of many years of trials with animal and human embryos (Leibo & Pool, 2011). They are similar for both sources of vitrification media used in this present study (8-16% v/v DMSO, 8-16% v/v EG) and are used with similar concentrations in many other centres worldwide (Table 5-2). Although the concentrations are similar, the temperature and duration of exposure differs between the two vitrification methods in this present study (Table 5-1). Permeability coefficients and associated activation energies of water and various CPA's differ with the changing surface area to volume ratio from the oocyte through to the blastocyst (Pedro et al., 2005). Jin et al (2013) showed in the pig blastocyst that the activation energy for permeability to water, glycerol, DMSO, and EG was markedly lower for blastocysts compared to oocytes (Jin et al., 2013). The exposure to CPA's at 37°C in the Fibreplug protocol of this present study, although for a short period, could explain the difference in survival rates between the two techniques considering the CPA concentrations were identical.

Although the blastocyst survival rate was significantly greater with the Cryotop device in the present study, this difference was not maintained when controlling for the media type used during warming. This suggests a possible influence on blastocyst survival due to the composition of the warming media. Carbohydrates such as sucrose and trehalose are also used to supplement the handling media during warming. The increased solute concentration provided by the carbohydrate reduces the osmotic gradient across the cell membrane as the cryoprotectant permeates to the extracellular space (Dinnyés, Dai, Jiang, & Yang, 2000). In this present study, warming solutions with different formulations from 3 different suppliers were used. Carbohydrate levels as high as 1 M were used to reduce the speed and magnitude of cellular swelling in the blastocyst (Liebermann, Dietl, Vanderzwalmen, & Tucker, 2003). It is thought that the higher osmotic buffer level compensates for the increased permeability of CPA's through aquaporin water channels in the blastocyst (Pedro et al., 2005). Initially single step

carbohydrate dilutions were used during warming but multiple step reductions were shown to improve outcomes (Cho, Son, Yoon, Lee, & Lim, 2002). Early studies on mouse zygotes show significant changes in survival to blastocyst after EG exposure based on the use of differing levels of sucrose (Oda, Gibbons, & Leibo, 1992). Results for both vitrification devices in this present study favour the use of lower concentrations of osmotic buffer, however it is difficult to determine whether the effect is coming from the carbohydrate or other additions to the media.

Table 5-2 Published Blastocyst Vitrification Methods

Reference	Collapse	CPA	Time	Temp	Device	Warming Media	Time	Temp	Culture	Survival
(Zhao, Yan, Huang, Sun, & Li, 2019)	Yes Laser	7.5% EG/DMSO 15% EG/DMSO 0.5M Sucrose	8-10 min 1 min	Not stated	Cryoloop	1 M Sucrose 0.5 M 0 M	2 min 3 min 10 min	RT RT 37°C	3 hrs	Not stated
(Berrin et al., 2018)	Yes Laser	7.5% EG/DMSO 15% EG/DMSO 0.5M Sucrose	6-10 min 80-110 s	RT	Cryoleaf	1 M Sucrose 0.5 M 0 M	1 min 4 min 8 min	37°C RT RT	4 hrs	Not stated
(Kovačič et al., 2018)	Yes vs No	7.5% EG/DMSO 15% EG/DMSO 0.5M Sucrose	10 min 1 min	RT	HSV Spatula	1 M Sucrose 0.5 M 0 M	1 min 4 min 8 min	37°C RT RT	>140 min	97.2-100%
(Cimadomo et al., 2018)	Yes	7.5% EG/DMSO 15% EG/DMSO 0.5% Sucrose	14 min 1 min	RT	Cryolock	1 M Sucrose 0.5 M 0 M	1 min 3 min 5 min	37°C RT RT	>120 min	99%
(Ferreux et al., 2018b)	Yes vs No	7.5% EG/DMSO 15% EG/DMSO 0.5% Sucrose	14 min Not stated	RT	CBS-Vit-HS	1 M Sucrose 0.5 M 0 M	3 min 4 min 3 min	37°C RT RT	120-240 min	95%
(Coello et al., 2017)	No	7.5% EG/DMSO 15% EG/DMSO 0.5% Sucrose	12 min 1 min	RT	Cryotop	1 M Sucrose 0.5 M 0 M	1 min 3 min 6 min	37°C RT RT	120 min	97.2%
(Ebner et al., 2017)	No	10% EG/DMSO 20% EG/DMSO 0.5% Sucrose	1-3 min 1 min	RT	Cryotop	0.5 M Sucrose 0.25 M 0.125 M 0 M	3 min 2 min 2 min 1 min	37°C RT RT RT RT	Average 4.5 hrs	87.8%

5.6 Embryo assessment post warming

The estimation of the proportion of cell degeneration in a collapsed blastocyst after warming is difficult and the methodology is not described well in the literature (Edgar & Gook, 2012). Despite this paucity in methodology, early reports used the survival criteria also used for cleavage stage embryos that at least 50% of the trophectoderm and ICM must have survived to proceed to transfer (Behr, Gebhardt, Lyon, & Milki, 2002). In this present study it was found that the commencement of re-expansion within 60 minutes of the warm was a more reliable indicator of survival than the estimation of cell degeneration and was used as the sole survival criterion for most of the study. This requirement persisted regardless of the size of the cavity prior to vitrification. Re-expansion has been identified as important in recent studies but this present study is the first describing the time to commencement of re-expansion as the sole determinant of blastocyst survival (Coello et al., 2017; Ebner et al., 2017; Ferreux et al., 2018a; Marren et al., 2016). Kovačič et al., (2018) included this criterion along with at least 50% cell survival (Kovačič et al., 2018) when assessing blastocysts after warming. The classification of a warmed blastocyst as survived may vary in different laboratories and in the literature (Edgar & Gook, 2012). These variations are due to assessments being done at different times according to different protocols (Ebner et al., 2009). It is possible also that if embryologists are put under a time pressure scenario there may not be enough time available to warm further blastocysts and this could bias the survival assessment (Alpha Scientists in Reproductive Medicine, 2012). For this reason, it is preferable for an IVF unit to be flexible as to when a warmed blastocyst can be transferred to ensure the best outcome for the patient. Some authors recommend warming 24 hrs prior for this reason, however a significant negative effect of day 6 blastocysts would make this difficult to justify (Sunkara et al., 2010; Vanderzwalmen et al., 2003). The Alpha Consensus on Cryopreservation does not allow for re-expansion assessment; however, it does include a transfer rate KPI in addition to a survival KPI (Alpha Scientists in Reproductive Medicine, 2012). This present study did not find any detrimental effects from the length of time between the warm and the transfer, when the transfer was performed on the same day as the warm.

Of the blastocysts deemed to have survived in this present study, records were kept of the proportion of degenerated cells and whether they commenced re-expansion within 30 or 60 minutes. It is difficult to obtain consistency amongst embryologists in estimating the proportion of degenerated cells so any estimations of 100% survival were combined with those of 95% and these compared with 70 to 94% assessments. A simple comparison of 30 min and 60 min commencement of re-expansion showed a significantly improved delivery rate of blastocysts commencing re-expansion within 30 minutes. This difference however became insignificant when controlling for the proportion of cell degeneration.

5.7 Summary

5.7.1 Findings

Double embryo transfers, despite strict criteria for inclusion, still produced unacceptable multiple pregnancy rates in this present study. Singleton deliveries from SET's of a vitrified/warmed blastocyst have a similar gestational age and weight at birth to the Australian population whether ART was used or not. The sex ratio (M: F) of these babies in the present study was 100:100 compared to 109:100 of ART births in Australia and New Zealand in 2016 and 106:100 in the Australian population of all births.

Maternal age is the strongest predictor of birth outcome and developmental age of the blastocyst post insemination remains the strongest embryo specific variable to influence outcome, even when all blastocysts were replaced on Day 5 in a controlled cycle.

Blastocyst morphology grade does not influence the live birth rate unless the blastocyst is at the expanded stage or greater.

Survival rates are improved when the vitrification volume is reduced, no artificial collapsing is performed and a longer equilibration period at room temperature is used. Osmotic buffer levels in warming media may contribute to blastocyst survival rate. The commencement of blastocyst re-expansion is a good indicator of blastocyst survival at 60 minutes and easier to measure than the proportion of cell degeneration. Evidence of cell degeneration greater than 5 % significantly affects the live birth rate.

5.7.2 Implications

Current double embryo transfer decisions need refining to reduce the multiple pregnancy rate for those procedures in this present study. The similar gestational period and birth weight compared to all singleton births contradicts reports of different outcomes for the ART population. Patients should be fully informed of the poorer outcomes from blastocysts utilised on Day 6 whether in a controlled fresh or frozen cycle. Blastocyst grading of ICM and trophectoderm cells should be reserved for blastocysts that are expanded or greater. Patients are best informed of the proportion of degenerative cells to be able to decide if they wish to improve their chances of a live birth by warming another embryo. If an embryologist has doubt about the proportion of degenerative cells in the warmed blastocyst then the early commencement of re-expansion is a quick confirmation within one hour of the warm.

5.7.3 Limitations

This present study was retrospective, so unknown residual confounding factors could be influencing the results and subsequent findings. The study period was extensive and there may be uncontrolled variables from changes in protocols, including blastocyst grading expertise over time. These could be clinical, or laboratory related. Morphology grading in this study was a combined ICM and trophectoderm score and therefore a grade 2 blastocyst may contain a grade 1 ICM or trophectoderm. The conclusions made in this study reflect the protocols used in one IVF unit and may not be able to be extrapolated to other units. This study targets vitrified/warmed blastocysts of a good grade only and therefore findings are restricted to this embryo population only. Due to the difficulty in cell degeneration assessment there may be some inter-operator variability in embryo assessment post warm. The data in this study is at the embryo level and therefore a single patient may be in the data set more than once and patient specific factors may influence the results. Day 6 development age blastocysts are used for transfer only when the supply of suitable day 5 blastocysts is exhausted. This may negatively influence conclusions about transfers with Day 6 embryos. All neonatal outcomes in this study were obtained from patient questionnaires rather than medically validated birth reports and may influence the birth outcome records.

5.7.4 Future Research

Better criteria for double embryo transfer should be explored to reduce multiple birth rates or have the practice eliminated. Adjustments and comparisons of the different replacement cycle control methods could further improve synchronisation of the endometrium to the blastocyst. Controlled comparisons of warming solutions with different concentrations/type of carbohydrate osmotic buffer but same base media should be performed as there are a wide range of concentrations currently in use in clinical practice. The feasibility of altering the logistics of blastocyst thaw and transfer timing should be explored to allow for sufficient time for embryo assessment prior to transfer. The use of more recent data using cell specific grading, time-lapse culture and automated vitrification could validate the conclusions in this study. D5/6 vitrification and transfer decisions could be validated by more accurate timings obtained from time lapse data.

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7 APPENDICES

7.1 Ethics approval Letters

7.1.1 Hollywood Private Hospital Research Ethics committee



7.1.2 Edith Cowan University

Mail - HBARBLET@our.ecu.edu.au

Page 1 of 2

Project 17409 BARBLETT Ethics Approval

Research Ethics

Wed 5/04/2017 12:22 PM

To: Hamish BARBLETT <HBARBLET@our.ecu.edu.au>;

Cc: Peter ROBERTS <p.roberts@ecu.edu.au>; Phillip MATSON <p.matson@ecu.edu.au>; Research Assessments <researchassessments@ecu.edu.au>;

1 attachments (47 KB)

Conditions of approval.pdf;

Dear Hamish

Project Number: 17409 BARBLETT

Project Name: EMBRYO SELECTION FOR VITRIFICATION, THAWING AND TRANSFER IN AN IVF PROGRAMME.

Student number: 10390155

The ECU Human Research Ethics Committee (HREC) has reviewed your application and has granted ethics approval for your research project. In granting approval, the HREC has determined that the research project meets the requirements of the *National Statement on Ethical Conduct in Human Research*.

The approval period is from 5 April 2017 to 1 September 2018.

The Research Assessments Team has been informed and they will issue formal confirmation of candidature (providing research proposal has been approved). Please note that the submission and approval of your research proposal is a separate process to obtaining ethics approval and that no recruitment of participants and/or data collection can commence until ethics approval has been granted, your research proposal has been approved and formal confirmation of candidature has been received.

All research projects are approved subject to general conditions of approval. Please see the attached document for details of these conditions, which include monitoring requirements, changes to the project and extension of ethics approval.

Please feel free to contact me if you require any further information.

Regards

Kim

Kim Giffkins, Senior Research Ethics Advisor, Office of Research & Innovation, Edith Cowan University, [270 Joondalup Drive, Joondalup, WA 6027](#)

Email: [\[research.ethics@ecu.edu.au\]research.ethics@ecu.edu.au](mailto:[research.ethics@ecu.edu.au]research.ethics@ecu.edu.au) Tel: +61 08 6304 2170 | Fax: +61 08 6304 5044 | CRICOS IPC 00279B

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16/06/2018

7.2 Data Field Table

Table 7-1 Data Fields Retrieved from Hollywood database

Field Name	Definition	Format	Content
Patient Identification code	Unique patient identification code used for de-identifying purposes	Numerical	5 figure number
Aetiology	Underlying cause of infertility	Nominal	Male factors only, Multiple causes, Other female only, Tubal only, Unexplained
Fertilisation code	Single number representing unique fertilisation event	Numerical	1-4
Transfer code	Single number representing unique transfer event	Numerical	1-7
Age at time of Cryopreservation	Maternal age at the time the embryo was vitrified	Numerical	Age in years

Date of Cryopreservation	Date of Vitrification	Date	dd/mm/yyyy
Straw Number	Unique embryo identifier	Numerical (2 part with year and number)	yy/0001
Blast Stage	Stage of blastocyst at time of vitrification	Nominal	EB,B,XB,HB,FHB
Day 5/6	Day of vitrification	Numerical	5/6
ICM	ICM Grade	Numerical	1-2
TE	TE Grade	Numerical	1-2
Blast	Blastocyst Grade	Numerical	1-2
Date of Warm	Date of Warm	Date	dd/mm/yyyy
Time of Warm	Time of Warm	Time	hh:mm
Exp	Time till expansion commences	Time (minutes)	mmm
Survival	Proportion of cells survived	Numerical (%)	%%

Fate	Whether Transferred or Discarded	Nominal	R or D
Date of Transfer	Date of Embryo Transfer	Date	dd/mm/yyyy
Time of Transfer	Time of embryo transfer	Time	hh:mm
#ET	Number of embryos transferred	Numerical	1-3
Cycle	Type of replacement cycle	Nominal	LDS,NAT,HRT
qhCG	Quantitative hCG level at pregnancy test	Numerical	Nnn (IU)
SACS	Number of sacs on US	Numerical	
FH	Number of heart beats on ultrasound	Numerical	
LMP	Date of last menstrual	Date	dd/mm/yyyy

	period (start of cycle)		
DOB	Date of Birth	Date	dd/mm/yyyy
1SEX	Sex of Baby1	Nominal	Male or Female
1BW	Weight of Baby1	Numerical	grams
1BIRTH	Mortality of Baby1	Nominal	Live or Still
1GEN	Any genetic malformations of Baby1?	Nominal	Yes or No
2SEX	Sex of Baby2	Nominal	Male or Female
2BW	Weight of Baby2	Numerical	grams
2BIRTH	Mortality of Baby2	Nominal	Live or Still
2GEN	Any genetic malformations of Baby2?	Nominal	Yes or No
3BW	Sex of Baby3	Nominal	Male or Female
3SEX	Weight of Baby3	Numerical	grams
3BIRTH	Mortality of Baby3	Nominal	Live or Still

3GEN	Any genetic malformations of Baby3?	Nominal	Yes or No
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7.3 Product Inserts and Detailed Protocols

Appendix 7.3 is not available in this version of the thesis.