DETERMINATION OF NON-INVASIVE VIABILITY MARKERS FOR HUMAN EMBRYOS IN IN VITRO FERTILIZATION

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Abstract

The major challenge currently facing human in vitro fertilization (IVF) programs is the prevention of multiple gestation pregnancies. The replacement of multiple embryos to fertility patients is common practice to increase pregnancy rates. However this introduces a significant risk of a resultant multiple pregnancy (25-50%), with associated increased complications such as perinatal morbidity, mortality and long-term disabilities, as well as significant maternal complications. The transfer of a single embryo during IVF treatment is the only acceptable way to overcome the issues related to multiple gestation pregnancies. To encourage the replacement of only one embryo, pregnancy rates must be able to be maintained. There is therefore a great need for clinical laboratories to use markers that can identify highly viable embryos.

Current laboratory embryo selection techniques involve selecting an embryo based on its appearance. However it is well established that this is only weakly linked to pregnancy success. Therefore other less subjective quantitative markers are required to select embryos that are the most viable within a patient's cohort. Any quantitative techniques which provide additional information to combine with current morphological assessment protocols must be rapid, simple, non-invasive and highly discriminating. This thesis describes the investigations of candidate non-invasive viability markers to assess the competence of the oocyte/embryo.

In order to track investigations of individual oocytes and embryos, single embryo culture is a prerequisite. Mouse and human embryo development and implantation were studied as a prelude to investigations of biomarkers of human embryo viability. No differences were observed in human embryo development or viability after single or group culture. Mouse embryos cultured singly were found to have a reduced cell number, however this did not affect implantation or fetal viability. Interestingly, mouse placental weights were reduced. This and a lack of power in the human studies suggest that single embryo culture in the human needs further long-term examination.

Following preliminary investigations of single embryo culture, morphological, biochemical and metabolic studies were undertaken as potential biomarkers of oocyte/embryo viability in the human. Firstly, a new morphological scoring system was described for day 4 human embryos, a stage of embryo development that has previously been overlooked. This morphological scoring system was easy to implement in the clinic and able to improve implantation rates over morphological scoring of cleavage stage embryos. It has subsequently been adopted in several clinics. Secondly, the reduction-oxidation (REDOX) state of cumulus cells surrounding oocytes retrieved for IVF was measured and retrospectively found to correlate with subsequent embryo viability. Finally, a direct measure of metabolism was also investigated in cumulus-oocyte complexes as well as cleavage stage embryos with determination of

metabolic turnover of glucose, lactate, pyruvate and alanine, with pyruvate uptake by the early embryo and pyruvate: alanine turnover of the cumulus-oocyte complex being related to viability.

Overall, these experiments showed that some metabolic parameters were correlated with subsequent viability. Biochemical markers in combination with current morphological measures show promise in selecting the most viable embryo for replacement. Further validations of these markers in randomized controlled trials are the next step in the introduction of these technologies to improve success rates of single embryo transfers in fertility treatment.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Publications arising from this thesis

1. **FEIL D,** HENSHAW RC & LANE M. (2008) Day 4 embryo selection is equal to Day 5 using a new embryo scoring system validated in single embryo transfers. *Human Reproduction 23 (7) p1505-10*.

2. **FEIL D**, HAMILTON H, RUSSELL D & LANE M. Cumulus cell REDOX levels correlate with subsequent embryo viability following single embryo transfer. *In preparation.*

3. **FEIL D,** MITCHELL M, THOMPSON JG & LANE M. Single embryo culture equals group embryo culture with implantation and development in an optimised culture system. *In preparation.*

Abstracts arising from this thesis

1. **FEIL D,** HAMILTON H, RUSSELL D & LANE M. (2008) Human cumulus cell REDOX state is predictive of subsequent embryo viability in single embryo transfers. Obex Research Day. Auckland, New Zealand.

2. **FEIL D**, HENSHAW RC & LANE M. (2007) Day 4 embryo transfer is a successful alternative to blastocyst transfer. 23rd Annual Meeting of the European Society of Human Reproduction and Embryology, Lyon, France. *Abs O-020.*

3. HAMILTON H, **FEIL D,** BARRY M, RUSSELL D & LANE M. (2006) Cumulus cell REDOX state is predictive of subsequent embryo quality. Annual Meeting of the Fertility Society of Australia. Sydney, Australia.

4. **FEIL D,** THOMPSON JG & LANE M. (2006) Placental size can be influenced by pre-implantation culture conditions. Healthy Development Adelaide Research Symposium. Adelaide, Australia.

5. **FEIL D** & LANE M. (2006) Retrospective analysis of the implementation of a single embryo transfer policy in an IVF program. Australian Society for Medical Research Scientific Meeting, Adelaide, Australia.

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Abbreviations

°C	degrees celsius
ADP	adenosine diphosphate
ANOVA	analysis of variance
AREG	amphiregulin
ART	assisted reproductive technologies
ATP	adenosine triphosphate
β-hCG	beta-human chorionic gonadotrophin
BCL2L11	BCL2-like 11
BDNF	brain-derived neurotrophic factor
BMP-15	bone morphogenic protein-15
Ca-125	cancer antigen 125
CEA	carcinoembryonic antigen
CO_2	carbon dioxide
COC	cumulus-oocyte complex
Complex I	NADH dehydrogenase
Complex II	succinate dehydrogenase
Complex III	cytochrome bc1 complex
Complex IV	cytochrome c oxidase
CX43	gap junctional alpha-1
Cyt C	cytochrome c
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
e-	electron
EGF	epidermal growth factor
FSH	follicle stimulating hormone
g	gram

g	gravity
G	gauge
G6PDH	glucose-6-phosphate dehydrogenase
GDF-9	growth differentiation factor-9
GIFT	gamete intrafallopian tube transfer
GPT	glutamate-pyruvate transaminase
GREM1	gremlin 1
h	hours
H^+	proton
HAS2	hyaluronan synthase 2
hCG	human chorionic gonadotrophin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSA	human serum albumin
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
IFFS	International Federation of Fertility Societies
IGF-1	insulin-like growth factor 1
IGF-2	insulin-like growth factor 2
IGFBP-1	insulin-like growth factor 1 binding proteins
IGFBP-2	insulin-like growth factor 2 binding proteins
IMM	inner mitochondrial membrane
IMS	intermembrane space
IU	international units
IVF	in vitro fertilization
L	litre
LDH	lactate dehydrogenase
LSD	least significant difference
LH	luteinizing hormone

n	nano
N_2	nitrogen
NADH/NAD+	nicotinamide adenine dinucleotide
NADPH/NADP+	nicotinamide adenine dinucleotide phosphate
NFIB	nuclear factor 1B gene
NIR	near infra-red
nM	nanomolar
µL	microlitre
µg	microgram
M	molar
m	mol
mg	milligram
mm	millimetre
mM	millimolar
mL	millilitre
mmHg	millimetres of mercury
MOPS	3-(N-morpholino)propanesulfonic acid
NPBs	nucleolar precursor bodies
NHMRC	National Health and Medical Research Council
O_2	oxygen
O_2^-	superoxide
OCP	oral contraceptive pill
OPU	oocyte pick up
p	probability
p	pico
PCK1	phosphoenolpyruvate carboxykinase 1
PCOS	polycystic ovary syndrome
PGD	pre-implantation genetic diagnosis

PI	propidium iodide
PN	pronucleus
PTGS2	prostaglandin-endoperoxide synthase 2
PTX3	pentraxin-3
PVP	polyvinylpyrrolidone
Q	ubiquinone
r	Pearson's correlation coefficient
R^2	coefficient of determination for linear regression
ρ	rho, Spearman's correlation coefficient
REDOX	reduction-oxidation
ROS	reactive oxygen species
SCD1	delta-9 desaturase 1
SCD5	delta-9 desaturase 5
SEM	standard error of the mean
SET	single embryo transfer
SOD	superoxide dismutase
SPSS	Statistical Package for Social Sciences
STAR	steroidogenic acute regulatory protein
TAC	total antioxidant capacity
TdT	terminal deoxynucleotidyl transferase
TE	trophectoderm
TGFβ1	transforming growth factor beta 1
TIFF	tagged image file format
TNF-α	tumour necrosis factor alpha
TNFαIP6	tumour necrosis factor alpha inducible protein 6
TUNEL	terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling
UV	ultra violet
VEGF	vascular endothelial growth factor

CHAPTER 1

SELECTION OF THE MOST VIABLE HUMAN EMBRYO: THE RATE LIMITING FACTOR IN THE ADOPTION OF SINGLE EMBRYO TRANSFER

1.1 INTRODUCTION

A major challenge in human assisted reproductive technologies (ART) is the selection of embryos with the highest implantation potential. This ability to identify the most viable embryo for replacement is essential to both maintain pregnancy rates but also to reduce the number of embryos transferred and the number of resultant multiple gestation pregnancies. Current protocols to select embryos for transfer are based on either single or a combination of morphological parameters (Edwards et al. 1984, Cummins et al. 1986, Hill et al. 1989, Steer et al. 1992). In general, the number and quality of cells in an embryo are assessed as well as the absence of fragmentation and multinucleation (Gerris et al. 1999, Van Royen et al. 2003). Additional observations have been more recently integrated into the clinical assessment of human embryos with the recognition that a single static observation is likely not indicative of the developmental potential of an embryo (Bavister 1995). These inclusions have been based on the initiation of key events during gamete and embryo development, in a time-dependent fashion, and incorporate; oocyte morphology (Ebner et al. 2000, Shen et al. 2005, Ebner et al. 2006, Montag et al. 2006, Madaschi et al. 2008), pronuclear morphology (Tesarik and Greco 1999, Scott et al. 2000) early cleavage assessment (Shoukir et al. 1997, Sakkas et al. 1998, Sakkas et al. 2001) and blastocyst development (Gardner et al. 2000a, Gardner et al. 2002). However, maximal implantation rates have likely been reached using an 'observation only' approach, and have been deemed unacceptable by both clinicians and patients, as demonstrated by the continuing practice of replacing multiple embryos in a treatment cycle to increase pregnancy rates. Given the known risks associated with multiple pregnancies (Dare et al. 2004), the ultimate goal of ART should be to achieve singleton pregnancies. The only way to reliably achieve this (other than selective fetal reduction) is by the transfer of a single embryo. Therefore other indicators of viability as defined by the ability to establish and maintain pregnancy need to be explored.

ART procedures, including in vitro fertilization (IVF) and gamete intrafallopian tube transfer (GIFT), were established prior to the ability to manipulate a menstrual cycle. As such, these procedures were performed during a woman's own natural menstrual cycle, aspirating the dominant follicle from the ovary following the luteinizing hormone (LH) ovulatory surge. As generally single oocytes were collected, single oocytes (GIFT)/embryos (IVF) were replaced, and therefore multiple gestations were rare. The ability to stimulate a woman's ovaries to produce multiple oocytes for collection was a major breakthrough in fertility treatments (Trounson et al. 1981), which also created the circumstance of having surplus oocytes/embryos. Cryopreservation was a viable option for surplus reproductive material (Trounson and Mohr 1983), however the preferred choice was to transfer a higher number of oocytes/embryos to maximize the chances of

achieving pregnancy. Due to the low pregnancy rates in the early stages of IVF, transferring more embryos was an acceptable practice as high order multiple gestations were uncommon.

Historically GIFT was introduced as it allows for in vivo fertilization, providing a physiological environment i.e. the fallopian tube, for gametes to be placed for normal fertilization to occur (McLaughlin et al. 1987). GIFT was introduced in Australia in 1985 and was initially preferred to IVF as it was simpler and more successful, and few clinics had the technology and personnel able to perform IVF (Seibel 2004). This technique is still preferred by some patients today for religious reasons. However, GIFT requires at least one normal fallopian tube to be present, and for patients with bi-lateral tubal occlusion, IVF was their only option to become pregnant (Seibel 2004). Today, IVF is more routine, less invasive, applies to a greater range of infertility diagnoses and is more successful than GIFT, and thus the demand for GIFT has sharply declined in recent years and now only accounts for a small proportion of ART cycles ((Wang et al. 2009), see Figure 1.1). In addition, GIFT usually involves transfer of multiple oocytes into the fallopian tube with processed spermatozoa and thus has a high risk of multiple pregnancy, with one in four pregnancies in 2006 being a multiple pregnancy in Australia and New Zealand (Wang et al. 2008).

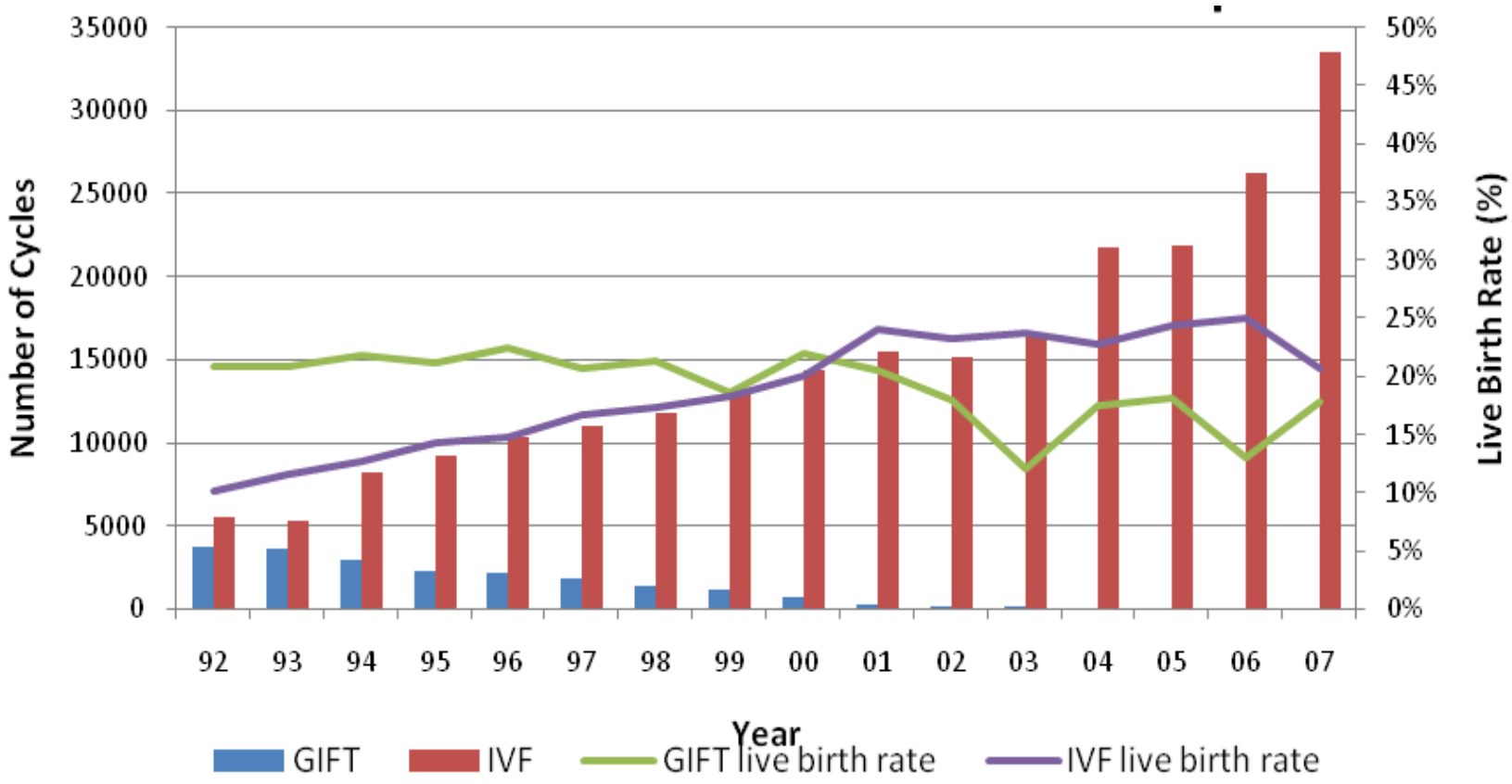


Figure 1.1 Number of GIFT and IVF cycles performed and live birth rates in Australia and New Zealand assisted conception 1992-2007.
Constructed from AIHW NPSU reports (Lancaster et al. 1995, Hurst et al. 1997, Lancaster et al. 1997, Hurst et al. 1999, Hurst and Lancaster 2001a, Hurst and Lancaster 2001b, Dean and Sullivan 2003, Bryant et al. 2004, Wang et al. 2006, Waters et al. 2006, Wang et al. 2007, Wang et al. 2008, Wang et al. 2009).

From its first successes in 1978, IVF embryos were replaced into the uterus soon after the first cleavage cycles were observed, as in vitro culture techniques were too primitive to maintain embryo viability (Gardner et al. 1996a). Improvements in embryo culture systems through investigations into oviduct and uterine fluids and the nutritional requirements of a developing embryo (Gardner et al. 1996a) led to the inception of sequential culture media to support the growth of embryos in vitro to the blastocyst stage (Gardner and Lane 1997, Gardner and Lane 1999, Schoolcraft and Gardner 2000, Pool 2002). The introduction of blastocyst stage embryo replacements into clinical IVF programs resulted, in many units, in an increase in pregnancy rates (Gardner et al. 2000a, Wilson et al. 2002). The ability to develop to the blastocyst stage in vitro provides a tool to select the most competent embryo from a cohort, as an extended period in vitro allows further differentiation between embryos, in particular embryos with little developmental potential. The replacement of blastocysts played a significant role in improving IVF pregnancy rates, for which the number of embryos being replaced was not compensated, contributing further to the increase in multiple pregnancy rates (Gardner et al. 2000a).

IVF pregnancy rates have improved with the advancement of technology and techniques, while the transfer of multiple embryos has remained common practice. This resulted in a large increase in the proportion of multiple pregnancies following IVF (Callahan et al. 1994, Wilcox et al. 1996). Many clinicians and patient couples underestimate the negative consequences associated with multiple pregnancies, including twins (Gleicher et al. 1995, Olivennes 2000). A higher rate of prematurity and low birth weights in babies born following a multiple pregnancy contribute to significant perinatal morbidity and mortality (Baird et al. 1998, Senat et al. 1998, Russell et al. 2003, Wang et al. 2009) including: admission to neonatal intensive care and extended length of care; respiratory distress; cerebral palsy and developmental delay (Williams and O'Brien 1998, Dani et al. 1999, Ross et al. 1999, Stromberg et al. 2002). Increased rates of maternal hypertension and pre-eclampsia are also associated with multiple as opposed to singleton pregnancies (Tallo et al. 1995), all of which significantly contribute to health costs (Ledger et al. 2006). In addition, there is now significant evidence that low birth weight singletons have increased risk of long-term adverse health outcomes, particularly cardiovascular disease, type 2 diabetes, hypertension and metabolic syndrome (Barker 1998). Although this phenomenon is yet to be investigated in multiple births, it suggests that the 96.1% of triplets and 61.4% of ART twins that are born prematurely in Australia and New Zealand will have considerable health and economic consequences.

A variety of methods have been proposed as potentially useful to evaluate embryo viability in human IVF programs (Ebner et al. 2003). Ultimately, for practical purposes, the techniques involved need to be non-invasive, timely and require simple operation as well as being highly discriminating. The employment of

such techniques to assist in selection of the most viable embryo from a cohort can only improve pregnancy rates, which would lead to the much anticipated reduction in the number of multiple embryo transfers and therefore the number of multiple pregnancies and births. The following review of the literature describes parameters that have been investigated to assess the viability of an embryo and the barriers of those parameters to date and for implementation in human IVF programs in the future.

1.2 CURRENT STATUS OF SINGLE EMBRYO TRANSFERS

The number of embryos replaced during an IVF treatment cycle has reduced from 3-4 to 2-3 since the publication of the International Federation of Fertility Societies (IFFS) Surveillance 01 (Jones and Cohen 2001, Cohen and Jones 2004). Legislation in countries such as Denmark, Hong Kong, Saudi Arabia, the United Kingdom, Switzerland and Brazil limits the number of embryos for replacement, with potentially severe penalties if violated. In countries without legislation limiting the number of embryos for replacement, national guidelines are generally in place, however there are a small number of countries which are regulated by neither legislation nor guidelines (Jones and Cohen 2001). In Australia and New Zealand, the number of embryos replaced during IVF has steadily decreased over the last decade (see Figure 1.2), with 63.7% of all embryo replacements in 2007 being of single embryos, 35.9% being of two embryos and <1% being of three or more embryos (Wang et al. 2009). The reduction in the number of embryos replaced is often a result of prolonged high pregnancy rates and a high proportion of multiple gestations within individual clinics (if not legislated). The change can clearly be seen in Australia and New Zealand (see Figure 1.2) where the proportion of patients having three or more embryos replaced between 1998 and 1999 was reduced, and similarly the proportion of patients having two embryos replaced between 2001 and 2002 reduced to a greater proportion of single embryo transfers (see Figure 1.2, (Wang et al. 2008)). Although explanations for the shift in the number of embryos transferred are not discussed in the Australia and New Zealand ART report (Wang et al. 2008), these two time periods were greatly influenced by the improvement in embryo culture systems (Gardner et al. 1996a, Gardner and Lane 1999, Pool 2002) and the introduction of blastocyst transfer as a selection tool (Gardner and Lane 1997, Schoolcraft and Gardner 2000), which resulted in an improvement in pregnancy rates and also an increase in the incidence of multiple gestations (see Figure 1.3). Despite the reduction in the number of embryos being transferred to patients in some countries, there is still a need to further reduce the number of embryos being replaced, in the pursuit for the majority of patients to have a single embryo replaced during fertility treatment. Investigations into improved embryo selection methods will allow this goal to be achieved, with patients, their pregnancy and child being the ultimate beneficiaries.

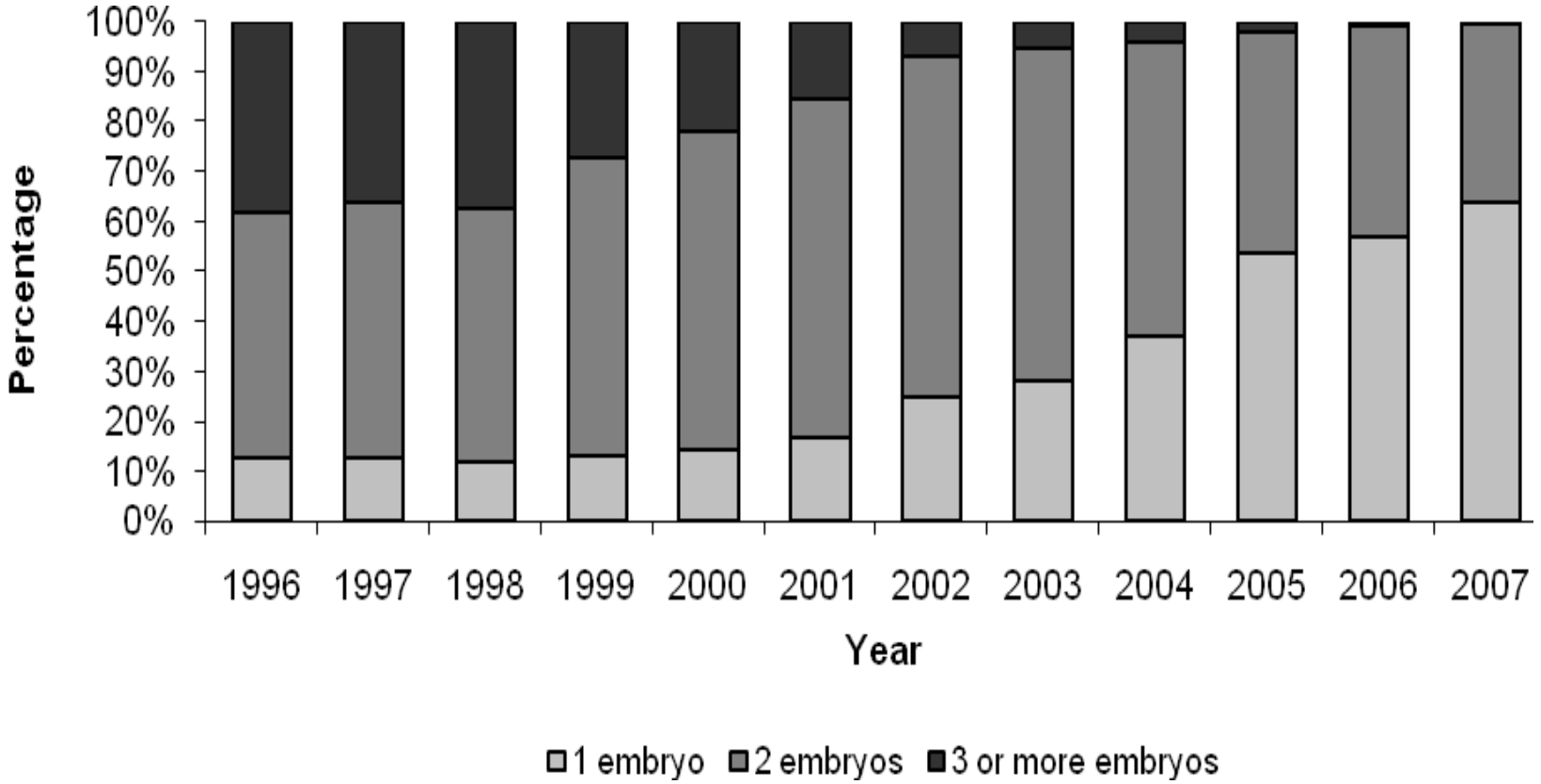


Figure 1.2 Proportion of embryo transfer cycles transferring one, two and three or more embryos in Australia and New Zealand. Adapted from (Wang et al. 2006, Waters et al. 2006, Wang et al. 2007, Wang et al. 2008, Wang et al. 2009).

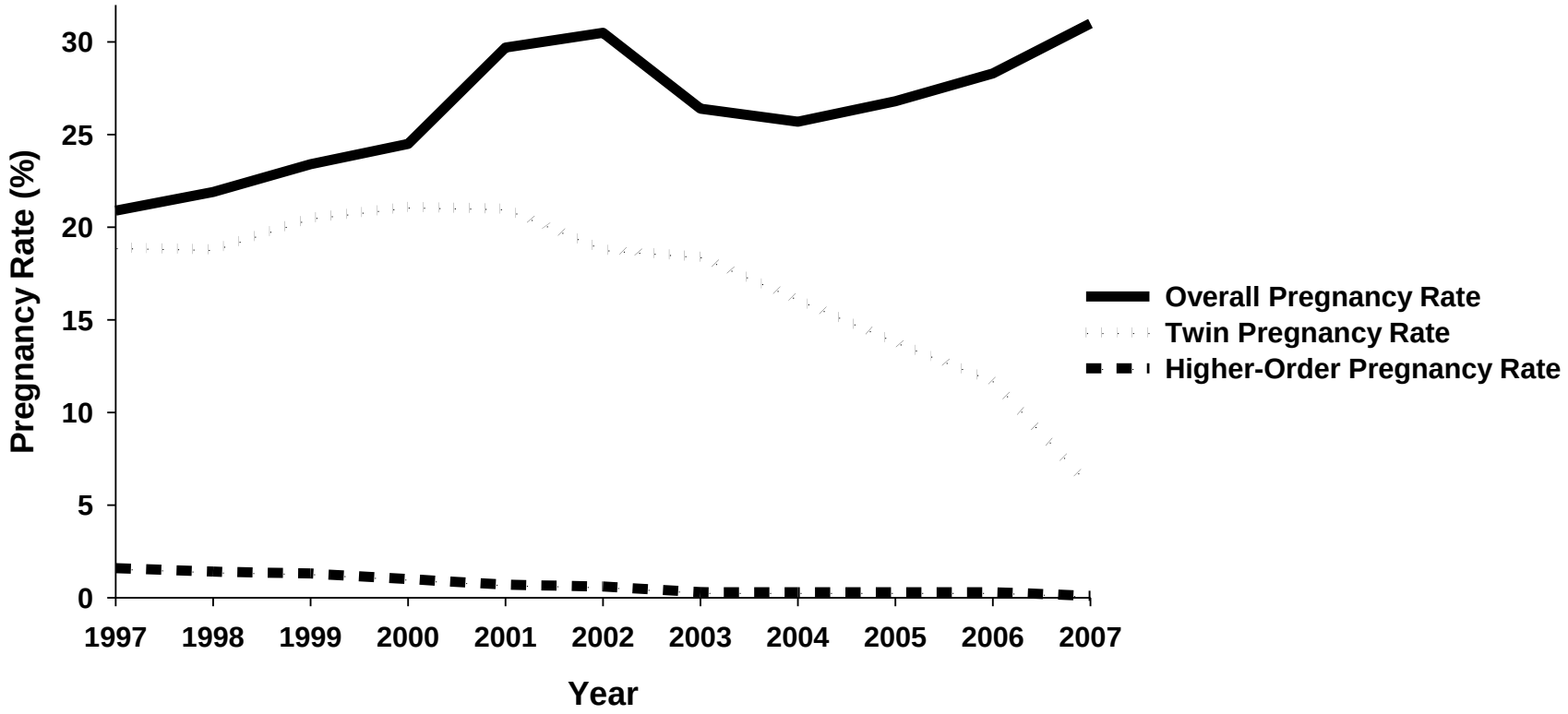


Figure 1.3 Pregnancy rates in Australia and New Zealand 1997-2006.
Data obtained from (Bryant et al. 2004, Wang et al. 2009).

1.3 EMBRYO ASSESSMENT

As mentioned earlier, the ability to generate multiple oocytes for IVF treatments resulted in multiple embryos available for utilisation. Hormonal stimulation of the ovary to produce many oocytes bypasses the natural atresia which a large number of oocytes undergo (Chun et al. 1996) prior to ovulation of the dominant follicle. Fertility treatments generate a heterogeneous cohort of meiotically mature sibling oocytes with differing levels of developmental competence (Pellicer et al. 1987, Stouffer and Zelinski-Wooten 2004, May-Panloup et al. 2005, Scott et al. 2008). Therefore resultant embryos in a cohort are highly variable in quality.

1.3.1 Morphological Parameters

Observational criteria are currently the only existing routine measures available to estimate embryo quality, excluding pre-implantation genetic diagnosis (PGD). Relatively low implantation rates worldwide result from the current use of morphological assessment of embryos, with the most likely explanation for the failure of this system being that the quality of an embryo at a single point in time is not representative of the developmental history of the gametes and resultant embryo and can therefore be misleading with regard to embryo viability. In addition, "on time" cleavage gives a measure of how successful an embryo is at overcoming any stressors it is exposed to and not the toll that those stressors are taking physiologically, which may not be observed until cleavage arrest or implantation failure result. Attempts around the world to improve human IVF pregnancy rates have led to the investigation of additional morphological markers including oocyte and pronuclear morphology, early cleavage status and blastocyst culture (Scott 2003a, Scott 2003b, Rienzi et al. 2005, Sakkas and Gardner 2005).

1.3.1.1 Cleavage Stage Embryos

Traditional criteria commonly used to assess a cohort of cleavage stage embryos (day 2 or 3 of development) incorporate cell number, integrity of cell membranes, quality of the cytoplasm, and the absence of both fragmentation and multinucleation (Gerris et al. 1999, Racowsky et al. 2000, Van Royen et al. 2003). Of these parameters it has long been known that ***cleavage speed*** (the appropriate cell divisions occurring at the correct rate) is of paramount importance to animal (Van Soom et al. 1992, Grisart et al. 1994, Gonzales et al. 1995) and human embryo competence (Cummins et al. 1986, Van Royen et al. 1999, Racowsky et al. 2000). When human embryos containing 4 blastomeres on day 2 (40 hours post-insemination) are transferred, a pregnancy rate can be obtained which is 2-fold higher than the combined pregnancy rate in cases where embryos containing 2, 3, 5 and 6 blastomeres are transferred (15.6% v

7.4%, (Giorgetti et al. 1995)). Similarly, on day 3 (64 hours post-insemination), when human embryos with greater than 7 blastomeres are replaced, implantation rates have been shown to significantly improve (49% v 12%, see Table 1.1, (Van Royen et al. 1999)). These windows of assessment for 'on-time' development are a critical component of the only tool that embryologists have available to assess the quality of embryos.

In addition to the number of blastomeres present during assessment, the degree of ***fragmentation*** in an embryo is an important morphological factor of embryo scoring systems to select the most suitable embryos for transfer. Cellular fragmentation is common in human embryos, with the presence of up to 20% small scattered fragments within the zona pellucida not impacting on further development or implantation rates (Ziebe et al. 1997, Alikani et al. 1999). However, a high proportion of fragmentation (>35%) is associated with chromosomal abnormalities such as mosaicism, polyploidy and haploidy in 70-90% of embryos (Munne et al. 1995, Magli et al. 2001). The detrimental effect of fragmentation on embryo quality originates from the loss of large volumes of cytoplasm from the remaining, viable blastomeres. In particular, the release of large fragments at an early stage of pre-implantation embryo development depletes the embryo of critical organelles such as mitochondria (Trounson and Sathananthan 1984), essential for energy generation for further development. Microsurgical removal of fragmentation has been performed and it has been hypothesised that removal of extracellular fragments reinstates the spatial communication between blastomeres, facilitating compaction, cavitation and blastocyst formation. In addition, a second hypothesis suggests that removal of degenerating cellular material from the embryo prevents the degeneration of adjacent blastomeres (Sathananthan et al. 1990). Fragment removal in embryos affected by up to 35% fragmentation, has been shown to alleviate the reduced implantation rates of these embryos, to be equivalent to the implantation rate of embryos without fragmentation. However embryos with >35% fragmentation do not show improved developmental competence from this technique, most likely due to the high incidence of chromosome abnormalities in these embryos (Alikani et al. 1999). Observations using time-lapse photography have shown creation, as well as evidence for the resorption of fragments (Hardarson et al. 2002), supporting the concept that low levels of fragmentation are a healthy indication, and do not affect the viability of the embryo. In addition, embryos that develop in vitro with a low to moderate degree of fragmentation have been shown to extrude those fragments when the blastocyst forms, with the fragments being retained in the zona pellucida when the blastocyst undergoes hatching (see Figure 1.4, (Veeck and Zaninovic 2003)). This is evidence that the embryo itself is able to manage low level fragmentation without compromising its developmental potential.

Table 1.1 Morphological measures used as assessment tools to select embryos.

Reference	Assessment Tool	Selected Patients (Y / N)	Implantation Rates (measure v comparison)*	Significant Improvement in Implantation Rates (Y / N)
(Scott and Smith 1998)	Pronuclear state	N (n=97)	28% v 2%	Y
(Ludwig et al. 2000)		N (n=74)	22% v 4%	Y
(Scott et al. 2000)		N (n=597)	31% v 19%	Y
(Tesarik et al. 2000)		Y (n=380)	30.2% v 11.2%	Y
(Wittemer et al. 2000)		N (n=262)	25.8% v 11.8%	Y
(De Placido et al. 2002)		Y (n=154)	15.7% v 7.1%	N
(Balaban et al. 2001)		Y (n=86)	42.2% v 8.3%	Y
(Montag and van der Ven 2001)		Y (n=512)	20.5% v 15.7%	Y
(Shoukir et al. 1997)	Early cleavage	Y (n = 143)	23.6% v 7.5%	Y
(Bos-Mikich et al. 2001)		N (n = 74)	18% v 8%	Y
(Lundin et al. 2001)		N (n = 727)	28% v 19.5%	Y
(Petersen et al. 2001)		Y (n = 200)	17.5% v 5.9%	Y
(Sakkas et al. 2001)		N (n = 230)	26.1% v 17.3%	Y
(Fenwick et al. 2002)		Y (n = 70)	21.4% v 6.0%	Y
(Salumets et al. 2003)†		**Y (n = 178)**	**50% v 26.4%**	**Y**
(Steer et al. 1992)	Day 3 morphology	Y (n = 390)	33% v 8%	Y
(Van Royen et al. 1999)		Y (n = 221)	49% v 12%	Y
(Desai et al. 2000)		N (n = 93)	35% v 12%	Y
(De Placido et al. 2002)		Y (n = 154)	15.4% v 4.0%	N
(Hardarson et al. 2001)	Even cell division	N (n = 378)	36.4% v 23.9%	Y
(Ziebe et al. 1997)	Fragmentation	Y (n = 1001)	21% v 5%	Y
(Alikani et al. 1999)		N (n = 1727)	31.8% v 6.4%	Y

Table 1.1 continued

Reference	Assessment Tool	Selected Patients (Y / N)	Implantation Rates (measure v comparison)*	Significant Improvement in Implantation Rates (Y / N)
(Racowsky et al. 2000)	Rate of development	Y (n = 362)	24.2% v 7.9%	Y
(Shapiro et al. 2000)		N (n = 93)	43% v 17%	Y
(Rijnders and Jansen 1998)	Blastocyst formation and morphology	Y (n=48)	30.1% v 0%	n/a
(Scholtes and Zeilmaker 1998)		N (n = 1532)	23% v 6%	Y
(Balaban et al. 2000)		Y (n = 350)	44.9% v 7.1%	Y
(Balaban et al. 2001)		N (n = 320)	21% v 11.6%	Y
(Milki et al. 2000)		Y (n = 100)	47% v 20%	Y
(Fisch et al. 2001)	Combined morphlogy scores	Y (n = 109)	39% v 24%	Y
(De Placido et al. 2002)		Y (n = 154)	15.5% v 5.3%	N

* test measures were only occasionally compared to standard practice, and often compared implantation rates between more than 2 groups, therefore the highest and lowest implantation rates are reported in this summary for each study. Implantation rates presented may be a comparison between high and low scores of different parameters e.g. pronuclear score or blastocyst score.
†Significantly, this study is the only study reported above that was performed using single embryo transfers.

More recently, ***blastomere size*** has been recognised as an important measure of embryo viability. Blastomeres of uneven size result when cells do not divide equally, and embryos with disproportionate cell cleavage have been shown to have a lower developmental capacity when compared to evenly cleaved embryos (Giorgetti et al. 1995, Hardarson et al. 2001). The reduced viability of embryos with unevenly cleaved blastomeres can in part be explained by unequal division of cellular organelles, which increases the likelihood of fragmentation as well as a higher degree of both ***multinucleation*** and aneuploidy (Hnida et al. 2004).

The criteria outlined above are measures that are common in most all IVF laboratories that transfer cleavage stage embryos. This system is valuable in the elimination of embryos with little developmental potential but is limited in its ability to predict viability (see Figure 1.5, (Langley et al. 2001). This is evidenced by the large number of high morphological quality cleavage stage embryos transferred that do not establish a pregnancy. As a result there have been attempts to increase the discriminatory nature of the morphological scoring system by including one or more additional observations such as pronuclear morphology, assessment of the timing of the first cleavage (early cleavage) and ability to develop to the blastocyst stage, in an attempt to gain additional information to assist in the selection of the most viable embryos, these are briefly described below.

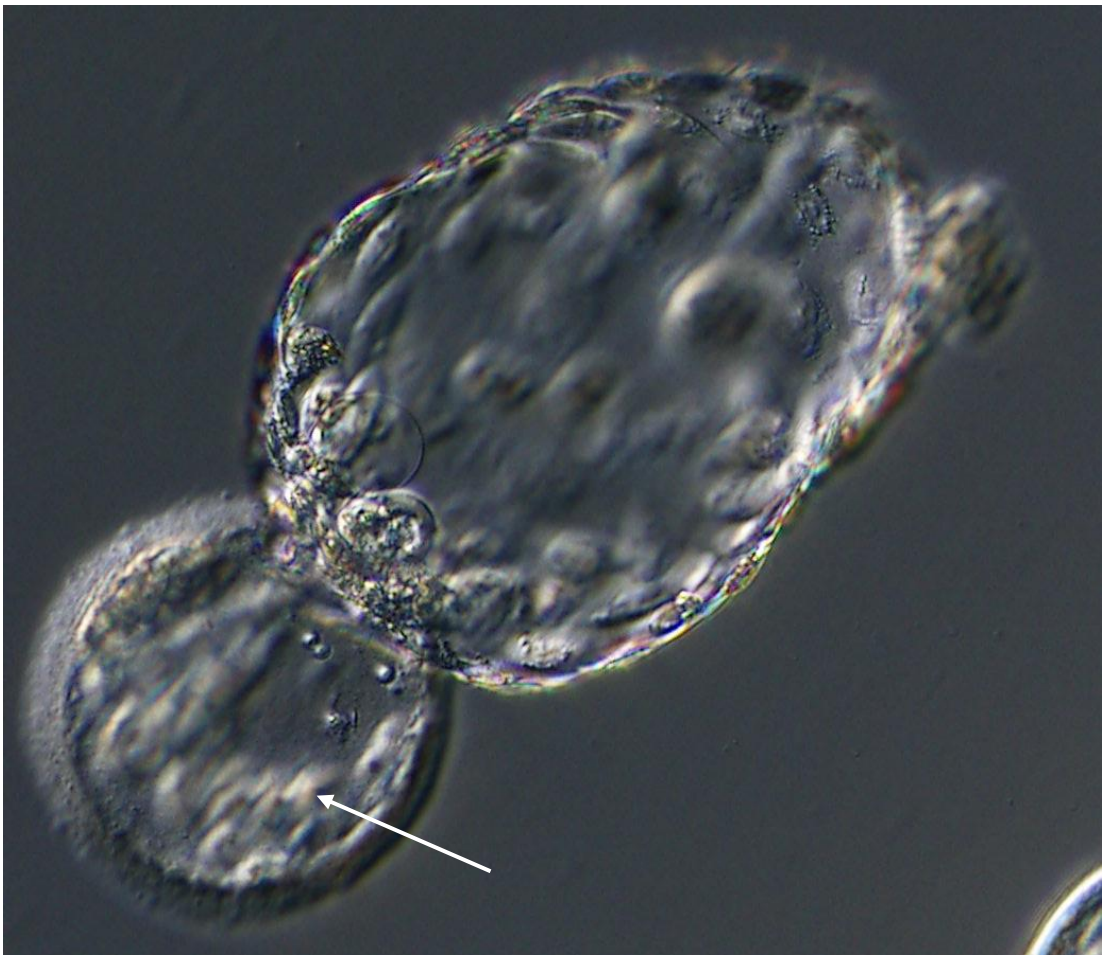


Figure 1.4 Hatching human blastocyst with fragmentation and cellular debris remaining in the zona pellucida as indicated.

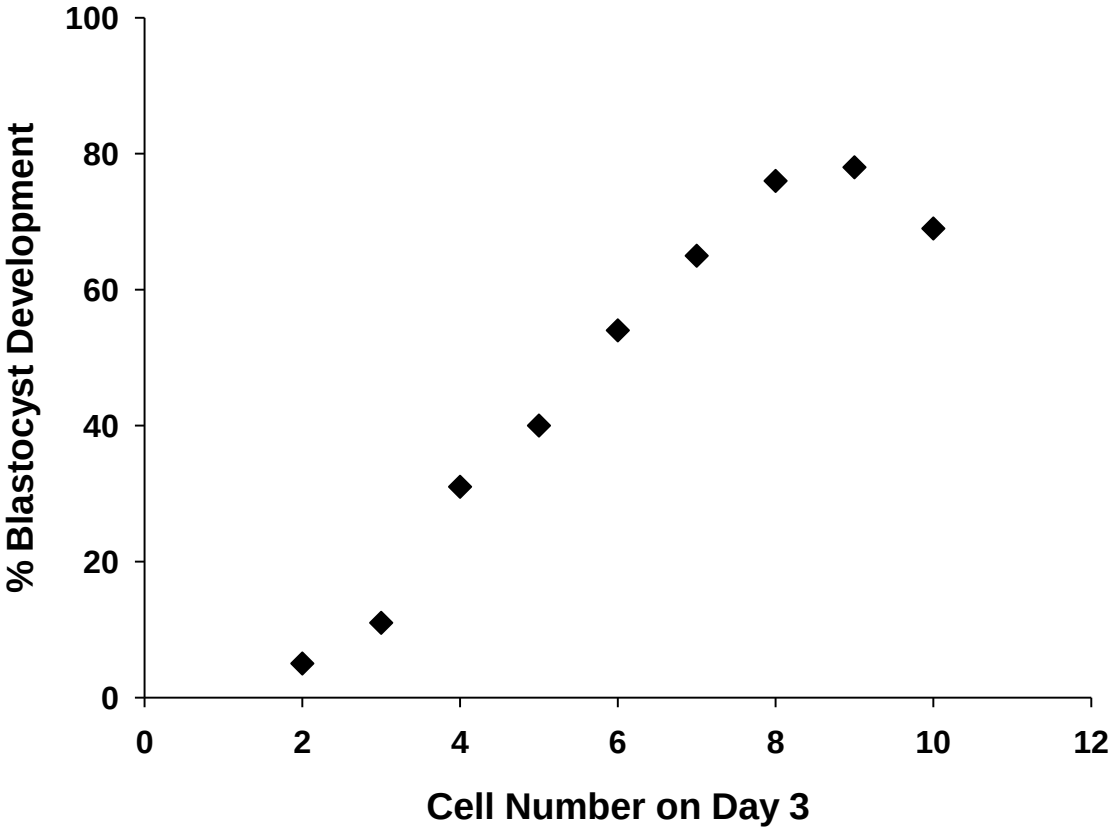


Figure 1.5 Relationship between day 3 cell number and blastocyst development. Adapted from (Langley et al. 2001).

1.3.1.2 Pronuclear Morphology

Pronuclear morphology is the earliest point at which the newly formed embryo can be assessed. Assessment of normal oocyte fertilization 16-19 hours following insemination involves the visualisation of two pronuclei (PN) and two polar bodies. The appearance, pattern and polarity of the pronuclei and nucleolar precursor bodies (NPBs, see Figure 1.6) has been suggested to be an important indicator of zygote quality and therefore predictive of embryo quality and viability (see Table 1.1, (Scott and Smith 1998, Tesarik and Greco 1999, Tesarik et al. 2000, Wittemer et al. 2000, Balaban et al. 2001, Montag and van der Ven 2001). The number of NPBs and the presence or absence of NPB polarisation has been retrospectively linked to the developmental fate of human embryos (Tesarik and Greco 1999). However, the sequence and timing of the morphological events of fertilization have been observed with time lapse imaging and vary between and within good and poor morphology embryos (Payne et al. 1997). Payne et al. (1997) demonstrated that in the majority of cases, asynchronous pronuclei development does not occur, which suggests that the assessment window for pronuclear morphology predicting implantation potential is extremely fluid. Other laboratories have been unable to demonstrate any value in determining nucleolar distribution or alignment after assessing more than 2000 fertilized oocytes (Clarke et al. 2001). In addition, Veeck and Zaninovic (2003) in another large study of 258 patients and more than 2000 zygotes observed few differences in the different classifications of pronuclear score. A zygote sub-type with scattered NPBs was more likely to undergo early cleavage, while a sub-type with aligned NPBs was more likely to result in a good quality embryo on day 3 of development. Furthermore, no zygote morphology classification was correlated with development to blastocyst stage, however as no embryo transfers were reported in this study, implantation ability is unknown (Veeck and Zaninovic 2003). As a result, some clinics have deemed pronuclear morphology to confer no benefit for the selection for transfer or prediction of subsequent in vitro development.

1.3.1.3 Early Cleavage

The rate at which the first cleavage division in human embryos occurs has also been used as an added measure to morphological scoring systems. Human embryos which have undergone the first cell division by 24-27 hours following insemination, show improved implantation rates (see Table 1.1, (Shoukir et al. 1997, Sakkas et al. 1998, Bos-Mikich et al. 2001, Lundin et al. 2001, Salumets et al. 2003)). Furthermore, this parameter is one of the few markers of viability that has been assessed in an elective single embryo transfer program which showed a near 2-fold increase in implantation rates when an embryo which had undergone the first cleavage by 25-27 hours post-insemination was transferred, compared to an embryo which had not cleaved at that time point (Salumets et al. 2003). A significant barrier to the application of

'early cleavage' as a predictive parameter is the susceptibility of the embryo to its culture system. For example: the gonadotrophin stimulation regime; the use of different commercial culture media; maintenance of correct temperature and pH during and following oocyte retrieval and handling between retrieval, insemination and fertilization assessment, can greatly influence the rate of embryo development, rendering a standard 'early cleavage' time point in one laboratory, ineffective in another. Differences in a laboratory's culture system, therefore require a new validation of the early cleavage 'window', which may differ between clinics. This creates a significant logistic exercise in a clinical setting.

1.3.1.4 Blastocyst Culture

The majority of embryos in the first two decades of IVF were transferred at the pronucleate or cleavage stage of development. This stems from the poor capacity of embryo culture systems to support the development of embryos beyond this point. However, with the advent of improved culture systems (Gardner et al. 1996a, Gardner and Lane 1997, Gardner and Schoolcraft 1999, Schoolcraft and Gardner 2000, Pool 2002), extended culture of embryos to the blastocyst stage (day 5/6) has been employed as an alternative clinical procedure in many IVF clinics, with the goal of transferring fewer embryos of higher viability, to reduce multiple births (Gardner et al. 2000a, Wilson et al. 2002). The ability of an embryo to develop to the blastocyst stage in vitro has been proposed as an indicator of embryo viability (Gardner et al. 2002) and a valuable selection technique, as after 5 days in culture embryos begin to differentiate themselves within a cohort as arrested or developmentally slow, with on average only 50% of embryos developing to the blastocyst stage. In addition, the degree of expansion of the blastocyst and differentiation into the inner cell mass and trophectoderm cell types of the blastocyst allow for further discrimination into the quality of individual embryos. The transfer of blastocysts has shown improved implantation rates in many clinics (see Table 1.1, (Dokras et al. 1993, Gardner and Lane 1997, Milki et al. 2000, Schoolcraft and Gardner 2000, Langley et al. 2001)), which is a prerequisite in the pursuit towards single embryo transfers. In contrast, other clinics have reported no change in implantation rates (Coskun et al. 2000, Huisman et al. 2000), and one clinic reported a decrease in implantation rates (Levron et al. 2002). The reasons for the mixed results following blastocyst culture have been discussed in more detail elsewhere (Blake et al. 2002, Gardner and Sakkas 2003).

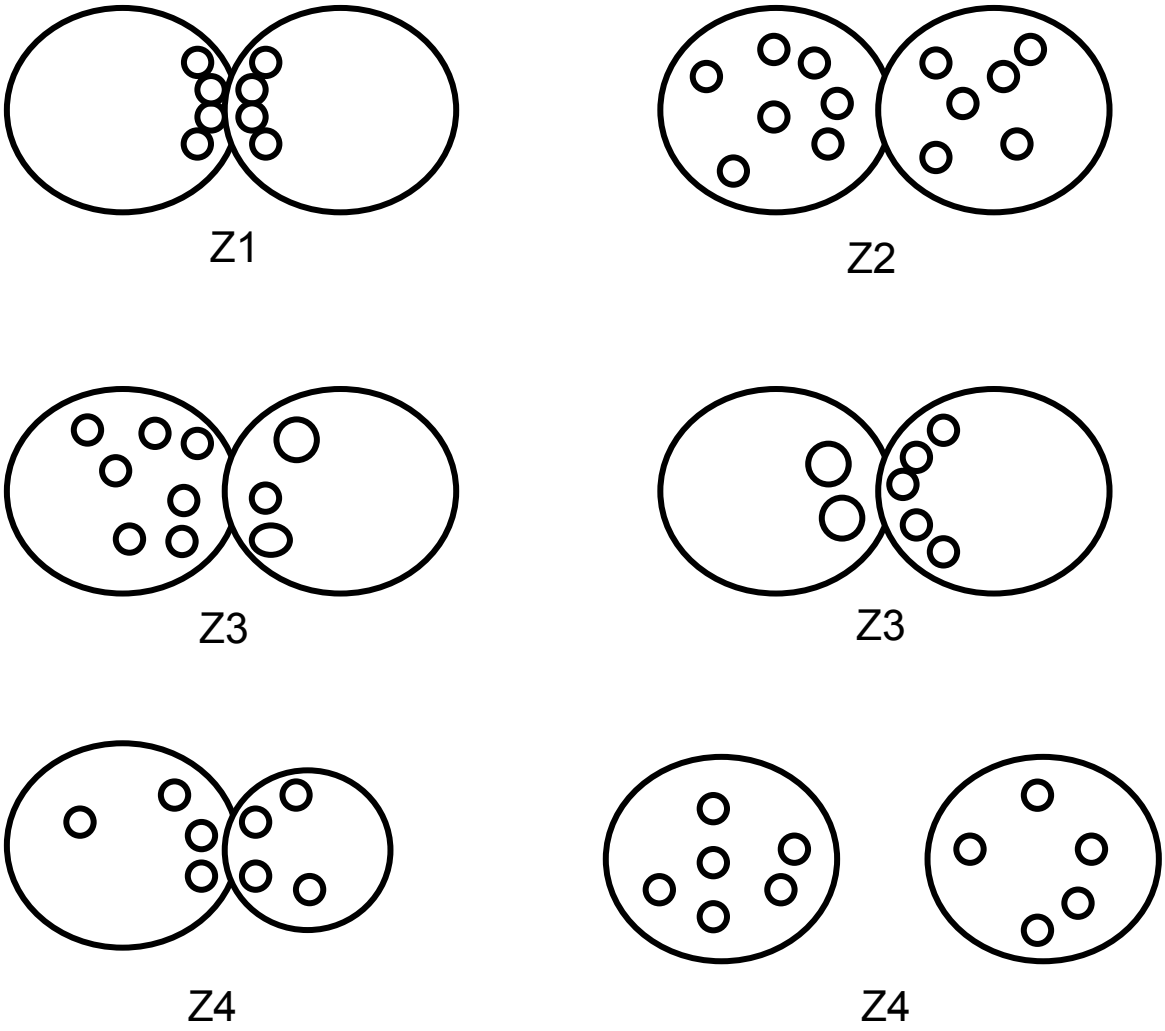


Figure 1.6 Z-scoring system for zygotes.

Z scores describe the number, size and position of the nucleoli and the equality between the nucleoli. Z1 zygotes have equal numbers of nucleoli which are aligned at the pronuclear junction. Z2 zygotes have an equal size and number of nucleoli that are not yet aligned. Z3 zygotes have nucleoli that are unequal in alignment, number or size. Z4 zygotes are grossly abnormal with unequal pronuclei and unequal nucleoli in size and number, which are unaligned (Scott et al. 2000).

Blastocyst culture is more demanding on personnel and resources, as it requires a multistep culture regimen for optimal development. This may explain some of the variations in results (Gardner et al. 1998a, Coskun et al. 2000, Karaki et al. 2002, Levron et al. 2002, Rienzi et al. 2002, Utsunomiya et al. 2002, Van der Auwera et al. 2002, Bungum et al. 2003, Emiliani et al. 2003, Frattarelli et al. 2003, Margreiter et al. 2003). Furthermore, the introduction of extended culture has been met with some reluctance relating to the unknown long term effects of culturing human embryos for up to 6 days in the laboratory (Leese et al. 1998, Martin 2004) as there has been reports that mouse embryos cultured to the blastocyst stage in poor culture conditions, exhibited perturbed gene expression of imprinted genes (Doherty et al. 2000), and that longer time periods of in vitro culture result in an increase in aberrant imprinting (Rivera et al. 2008). Currently, the data collected on imprinted disorders in the human does not discriminate between cleavage or blastocyst stage transfers, however, as these studies incorporate cohorts of children born prior to the year 2000 (reviewed by (Manipalviratn et al. 2009)), it could be presumed that the effect of blastocyst culture and imprinting disorders has not yet been adequately investigated in children born following blastocyst transfer. However, studies to date have concluded that due to the low incidence of imprinting disorders (<1:12000 births) large cohort studies are required to determine an association between imprinting disorders and ART.

Blastocyst culture and transfer are expensive and labour intensive for both the patient and laboratory staff, and for this reason many clinics around the world are unable to provide extended culture as an option to patients for either legal or practical reasons. Therefore viability determinants that can be easily incorporated into *any* IVF clinic worldwide are highly desired.

It is important to note that the majority of studies which have explored additional morphological measures to select the most viable embryo for transfer are confounded by the practice of multiple embryo transfer. Some studies manage this by analysing patients who have only embryos of one distinct description transferred. However, the intrauterine communication between multiple embryos and the endometrium compared with a single embryo is different and therefore single embryo transfers are the only true validation of independent embryo viability markers in human IVF (Matorras et al. 2005).

1.4 OOCYTE HEALTH

Gamete quality is a crucial component of embryo health and therefore a viable pregnancy. As such the oocyte and its influencing factors are fundamental in the determination of viability markers (Hardy et al. 1995). The cumulus-oocyte complex (COC) is contained within the ovarian follicle where growth and development of the somatic cells and oocyte occur in a highly coordinated and mutually dependent fashion (see Figure 1.7, (Gilchrist et al. 2004)). A dynamic bi-directional communication exists between cumulus cells and the enclosed oocyte which is essential for oocyte viability. The oocyte secretes growth factors such as growth differentiation factor-9 (GDF-9) and bone morphogenic protein-15 (BMP-15), which act on the surrounding cumulus cells to regulate gene expression and metabolism which, in turn, impact on oocyte function and viability (Eppig 2001, Gilchrist et al. 2004, Sugiura et al. 2005). As evidenced by mouse and sheep gene knockout models, oocyte derived factors are essential for fertility (Dong et al. 1996, Galloway et al. 2000, McNatty et al. 2005).

The close proximity of the cumulus cells to the oocyte makes them an ideal candidate to investigate the potential viability of the oocyte. In addition, cumulus cells are not utilised during infertility treatments and are routinely removed and discarded during handling of oocytes. Researchers are now exploiting cumulus cells to discover markers of oocyte viability that can be implemented into infertility clinics to improve clinical outcomes. A marker of oocyte viability would have further additional value in countries where the number of embryos able to be created is restricted by legislation, as well as overcoming the moral dilemma of creating embryos, in many parts of the world.

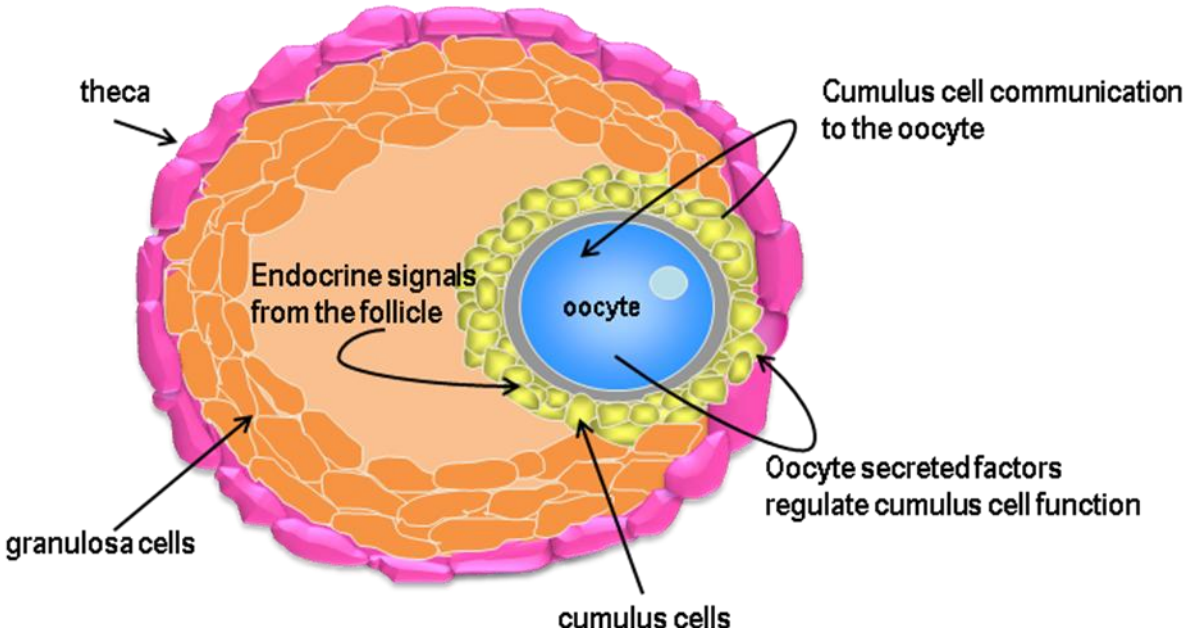


Figure 1.7 Follicle diagrammatic representing regulation of cumulus-oocyte complex health.
Schematic of the regulatory loop between the cumulus cells and oocyte. Oocytes secrete soluble growth factors (GDF-9 and BMP-15) which regulate cumulus cell gene expression and key cumulus cell functions. In addition, cumulus cells pass regulatory growth factors and small metabolites back to the oocyte via paracrine and gap-junctional signalling, with the influence of maternal signals such as Follicle Stimulating Hormone (FSH, (Eppig 2001, Gilchrist et al. 2004, Sugiura et al. 2005)).

1.5 FOLLICULAR FLUID

Follicular fluid provides an important environment for oocyte development (Downs and Eppig 1984, Naito et al. 1989), and is readily available as part of IVF treatments as it is aspirated to recover the oocyte. It is therefore a reasonable candidate to investigate its role in determining oocyte quality and subsequent developmental potential. It is known that altered follicular environments can influence oocyte quality (Nayudu et al. 1987, Nayudu et al. 1989, Artini et al. 1994, Gregory et al. 1994, Van Blerkom et al. 1997), however, to date no reliable biochemical factor able to determine oocyte viability has been identified. A recently published review has covered the parameters of follicular fluid that have been investigated as markers of oocyte quality in detail (reviewed by (Revelli et al. 2009)), including:

- Hormones – follicle stimulating hormone (FSH), LH, human chorionic gonadotrophin (hCG), growth hormone, prolactin, oestrogen and progesterone, cortisol and cortisone.
- Growth factors – Inhibin A and B, Activin A, Anti-Mullerian Hormone, BMP-15, Insulin-like Growth Factors 1 and 2 (IGF-1, IGF-2) and their binding proteins (IGFBP-1, IGFBP-2), amphiregulin (AREG) and vascular endothelial growth factor (VEGF).
- Interleukins – interleukin-1β, interleukin-2, interleukin-10 and interferon-γ, granulocyte colony-stimulating factor, interleukin-1α, tumor necrosis factor alpha (TNF-α) and leukotriene B4.
- Reactive Oxygen Species and antioxidants – reactive oxygen species (ROS), superoxide dismutase, selenium-dependent glutathione peroxidase, melatonin, 8-hydroxy-2-deoxy-guanosine (8-OHdG), total antioxidant capacity (TAC) and nitric oxide.
- Anti-apoptotic factors – tumor necrosis factor and its receptor and Fas-ligand and its receptor.
- Proteins – α-fetoprotein, carcinoembryonic antigen (CEA), cancer antigen 125 (Ca-125), antigen CD44, α-1-antitrypsin, leptin, endothelin-2, oocyte maturation inhibitor, homocysteine, β-endorphin, lactoferrin, angiotensin II and prorenin.
- Amino acids – glycine, alanine and aspartic acid.
- Sugars – hyaluronan and myo-inositol.
- Prostanoids – prostaglandin F2α and prostaglandin E2.

Despite the large number of biochemical factors that have been evaluated in the pursuit to identify a marker of oocyte quality, very few of these studies have correlated potential markers to the ultimate developmental competence of the oocyte and resultant embryo. Rather, many studies had endpoints of maturation, fertilization and occasionally embryo morphology (reviewed by (Revelli et al. 2009)). Follicular fluid factors that have correlated to pregnancy in human following IVF include increased LH (Mendoza et al. 2002),

decreased as well as increased levels of growth hormone (Tarlatzis et al. 1993, Mendoza et al. 2002) respectively), higher prolactin levels (Laufer et al. 1984, Mendoza et al. 2002), specific ranges of oestrogen and progesterone (Botero-Ruiz et al. 1984, Kreiner et al. 1987), increased cortisol/cortisone ratio (Keay et al. 2002, Lewicka et al. 2003), elevated inhibin A and B (Ocal et al. 2004), increased IGF-1/IGFBP-1 ratio (Fried et al. 2003), decreased interleukins (Mendoza et al. 2002, Ledee et al. 2008), increased ROS and TAC (Attaran et al. 2000, Pasqualotto et al. 2004), lowered VEGF (Ocal et al. 2004) and decreased leptin levels (Mantzoros et al. 2000). Many of these factors are controversial as predictors of IVF pregnancy establishment as observations were not able to be confirmed in other studies (Revelli et al. 2009). In recent years, the research in this area has progressed toward metabolomics i.e. analysis of all substances contained in a biological fluid. Thomas and colleagues (2000) demonstrated differences in oestrogen and progesterone levels between small and large antral follicles with this technique, however further analysis on oocyte developmental fate was not included in the study (Thomas et al. 2000). A subsequent investigation of follicular fluid demonstrated reliably produced unique 'fingerprints' that may be predictive of oocyte quality and successive pregnancy (Agarwal et al. 2006).

It is important to note that for follicular fluid to be related to resultant oocyte and embryo quality, follicles must be aspirated individually. This method requires flushing of the needle used for oocyte aspiration to ensure retrieval of the oocyte, if any, from each follicle, and requires multiple vaginal punctures with associated increased risk of vaginal bleeding and discomfort for the patient. In addition, it is not clear if follicular fluid parameters are a variable related to the quality of the follicle and potentially reflective of oocyte quality, or to the clinical characteristics of the patient, such as age, smoking habit or etiology. Therefore, the introduction of new techniques in this field require further analysis and validation to determine if a viable follicular fluid biomarker can be discovered.

1.6 CUMULUS CELLS

1.6.1 Cumulus Cell Gene Expression

Oocyte secreted factors are essential for appropriate cumulus cell gene expression in animal models (Gilchrist et al. 2004). In the final stages of oocyte maturation, the expression of several critical periovulatory cumulus cell genes are induced by oocyte secreted factors, which regulate the mucification of the cumulus-oocyte matrix, particularly in mouse and sheep (Dong et al. 1996, Juengel et al. 2002). These genes are crucial for cumulus expansion, ovulation and fertilization, which has been demonstrated by gene deletion

studies in mice and sheep, including tumour necrosis factor alpha inducible protein 6 (*Tnfαip6*), pentraxin-3 (*Ptx-3*) and prostaglandin-endoperoxide synthase 2 (*Ptgs2*) (Lim et al. 1997, Varani et al. 2002, Fulop et al. 2003, Ochsner et al. 2003, Salustri et al. 2004). The ability of cumulus cell gene expression to reflect oocyte competence in the human has been investigated by a small number of recent studies. Initially, McKenzie and colleagues (2004) retrieved cumulus cells from 8 patients undergoing intra-cytoplasmic sperm injection (ICSI) and determined that *PTGS2*, gremlin 1 (*GREM1*) and hyaluronan synthase 2 (*HAS2*) have increased expression in cleavage stage embryos with good morphological scores compared to embryos with a poor morphological score. This result was subsequently confirmed in 45 patients for HAS2 and GREM1 (Cillo et al. 2007). In contrast, it has been found that there was no predictive value of cumulus cell gene expression when investigating cleavage stage embryo quality, however those oocytes that went on to develop blastocysts were found to have reduced expression of steroidogenic acute regulatory protein (STAR), PTGS2, gap junctional α1 (CX43), AREG and delta-9 desaturase 1 and 5 (SCD1 and SCD5) in cumulus cells (Feuerstein et al. 2007). These observations, although of interest, are of limited value in a clinical setting. Embryo morphology can be determined in a timely manner by a simple observation without the requirement for the examination of gene expression levels. However, to further investigate the potential of such a technique, correlation of the findings to pregnancy establishment would be an extremely valuable study to undertake. Anderson and colleagues (2009) attempted to investigate this, though with the majority of patients in their study having two embryos transferred, clear analysis of pregnancy outcome was difficult. Despite this, PTGS2 expression was increased in cumulus cells that resulted in a good morphological quality embryo, a further validation of earlier results (McKenzie et al. 2004, Anderson et al. 2009). Furthermore, brain-derived neurotrophic factor (BDNF) expression of was also increased in cumulus cells that resulted in good quality cleavage stage embryos (Anderson et al. 2009). In another evaluation of cumulus cell gene expression, a microarray of pooled cumulus samples from 20 patients comparing resultant good morphological quality embryos to resultant poor quality embryos and unfertilized oocytes following IVF, 160 genes involved in cell cycle, cell proliferation, apoptosis, oncogenesis, energy metabolism and mitochondrial proteins were found to be differentially expressed (Zhang et al. 2005). A subsequent study determined by microarray that BCL2-like 11 (BCL2L11, an apoptosis promoting factor), phosphoenolpyruvate carboxykinase 1 (PCK1, an enzyme involved in gluconeogenesis) and nuclear factor IB (NFIB, a transcription factor) in cumulus cells were correlated with pregnancy, and importantly that the gene expression profiles of embryos with a good morphology on day 3 of development were different to the expression profiles of embryos which resulted in pregnancy (Assou et al. 2008). Multiple embryos were transferred in this study and thus to date the lack of single embryo transfer studies in this area of investigation mean the ability of cumulus cell gene expression markers to predict embryo competence, although promising still remains unanswered.

1.6.2 COC Metabolism

A vital function of the cumulus cells is to control the nutrient balance of the developing oocyte (reviewed by (Sutton et al. 2003a, Sutton et al. 2003b)). Glucose is the principal substrate of cumulus cells, which is metabolised to lactate and pyruvate, the predominant energy source of the oocyte (Gardner et al. 1996a). Oocytes utilise glucose poorly as an energy substrate and rely on cumulus cells to provide the products of glycolysis, such as pyruvate, by either secreting them or passing them through gap junctions that couple the two cell types (Biggers et al. 1967, Donahue and Stern 1968, Leese and Barton 1985, Sugiura et al. 2007). Paracrine factors secreted by the oocyte promote expression of glycolytic enzymes to regulate glycolysis in cumulus cells (Sugiura et al. 2005). A disruption in the balance of the consumption and production of carboxylic acids alters the metabolites available to the oocyte to support growth and maturation. Despite the cumulus cells playing such an important role in the utilisation of energy substrates by the oocyte, there is minimal information regarding the metabolic profile of the COC, with most studies focusing on the denuded oocyte.

Disruption of the carbohydrate balance in metabolism in mouse, pig and cow COCs perturbs resultant embryo developmental competence indicating that metabolic balance in the cumulus cells is essential for oocyte competence (Sutton-McDowall et al. 2004). It is however, likely that a disruption in the balance of glucose metabolism to pyruvate in human cumulus cells may similarly effect the viability of the human oocyte. While this has not been assessed directly, a study by Rice et al. (2005), demonstrated that cumulus cells from anovulatory women with polycystic ovary syndrome (PCOS) have perturbed levels of glucose conversion to lactate and the resultant oocytes produced embryos of lower morphological score (Rice et al. 2005). Therefore, it is possible that measurement of cumulus cell metabolism, especially the change in substrate levels found in medium used for short-term incubation of COCs, may be a useful marker of the conditions that the cumulus cells have developed under within the follicle.

1.6.3 COC Oxidative State

Oxidative stress results when an elevation in the level of reactive oxygen species (ROS) which exceeds a cell's level of antioxidant defence mechanisms (see Figure 1.8). The oxidative status of a cell can be a marker of both normal physiological processes and cellular stress. ROS result from external environmental factors as well as normal cellular metabolism (see Figure 1.8). Excessive ROS result in damage to lipids, proteins and DNA causing cellular damage and reducing viability. Bovine and human cumulus cells have antioxidant activity through enzymatic measures such as glutathione peroxidase, catalase and superoxide dismutases (SODs, (Cetica et al. 2001, Matos et al. 2009). Thus as the cumulus cells have the ability to regulate their oxidative state, this has potential as a marker of health of the follicular environment and

cumulus cells and subsequently the oocyte. The protective effect of the cumulus cells from ROS has been clearly demonstrated with denuded oocytes undergoing meiotic arrest and degenerating when exposed to ROS without the protective buffer of the cumulus cells (Tatemoto et al. 2000). Eight-hydroxy-2-deoxyguanosine (8-OHdG) is produced as a byproduct of DNA damage caused by reactive oxygen species (Floyd 1990), and is therefore a measure of oxidative stress in biological systems (Beckman and Ames 1997). In granulosa cells of patients undergoing IVF, 8-OHdG was found to adversely affect fertilization and subsequent embryo quality (Seino et al. 2002). Although granulosa and cumulus cells were separately investigated in this study, within patients the follicular aspirates were pooled so cumulus and granulosa cells from individual follicles and associated oocytes were not tracked. This is a technically and resource demanding task which is unlikely to be implemented in a clinical setting and therefore alternative methodology for such investigations would need to be explored.

Cumulus cells are a promising candidate as a marker of oocyte health as they are surplus to the IVF process. However, methods of collection and tracking need to be employed, that enable a single oocyte to be traced throughout the process and subsequently single embryo transfer assessment is required for the ultimate viability of such a test to be evaluated.

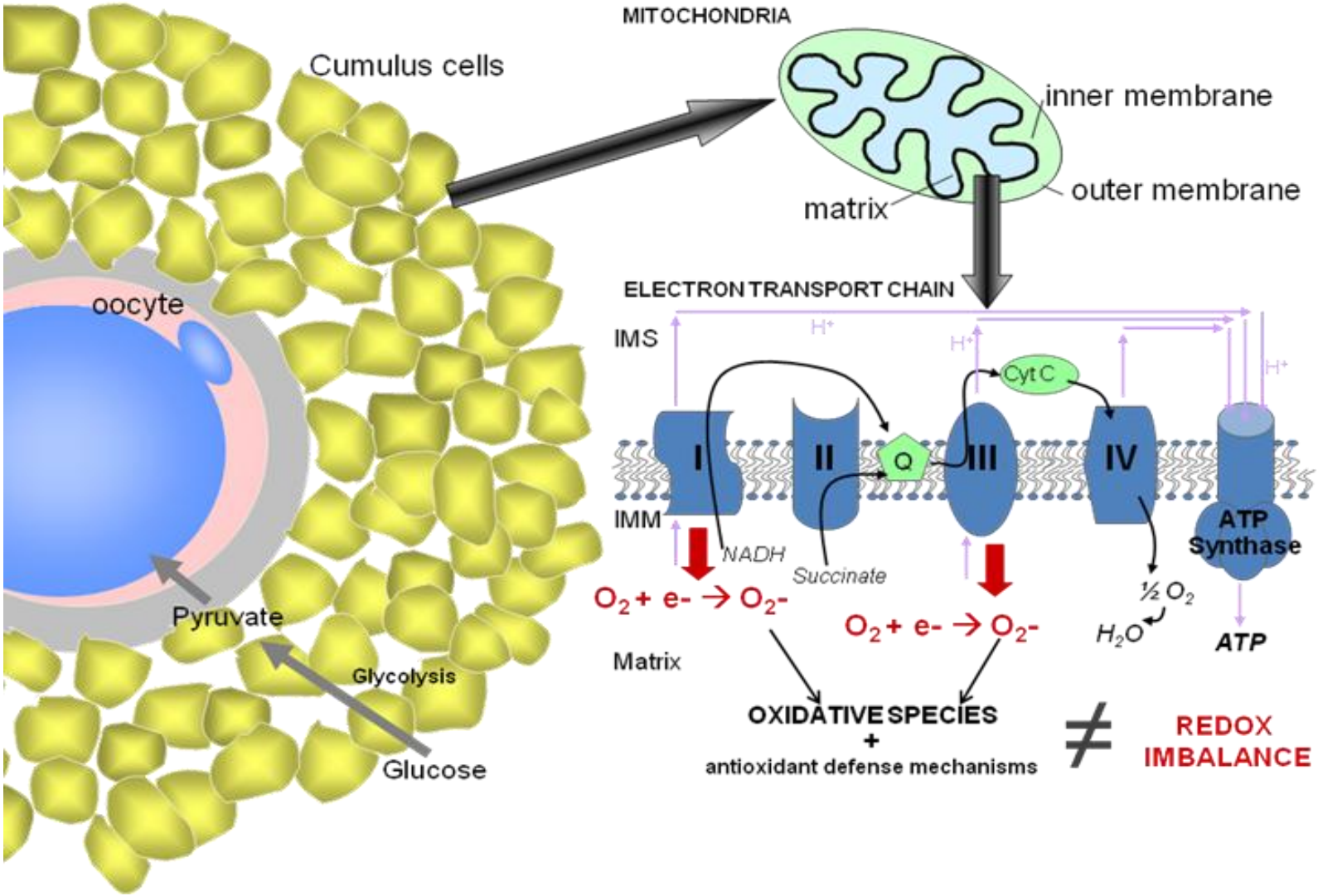


Figure 1.8 REDOX imbalance in mitochondria of cumulus cells.

Normal cellular metabolism and external factors produce reactive oxygen species (ROS), which are important second messengers in many processes. Antioxidant defence mechanisms neutralise high levels of ROS to prevent cellular damage and activation of the apoptotic cascade. Excess ROS production or reduced antioxidant capacity result in an oxidative state or REDOX imbalance in the cell which compromises viability. IMS – intermembrane space, IMM – inner mitochondrial membrane, complex I – NADH dehydrogenase, complex II – succinate dehydrogenase, complex III – cytochrome bc1 complex, complex IV – cytochrome c oxidase, Q – ubiquinone, Cyt C – cytochrome c, ATP – adenosine triphosphate, H^+ - proton, O_2 – oxygen, e- - electron and O_2^- - superoxide.

1.7 EMBRYO HEALTH

1.7.1 Embryo Metabolism

The pre-implantation embryo has a dynamic metabolism, which differs significantly from that of somatic cells. The mammalian oocyte and zygote have a requirement for pyruvate as the predominant energy substrate (Biggers et al. 1967). From the 2-cell stage lactate can be used as a substrate, while glucose is not the preferred substrate until the blastocyst stage (see Figure 1.9, (Leese and Barton 1984, Gardner and Leese 1986, Hardy et al. 1989, Gott et al. 1990, Rieger et al. 1992, Thompson et al. 1992, Gardner et al. 1993)). During this period of 4-6 days, the oocyte develops from a single cell that is quiescent and has a metabolic quotient similar to bone, to a multi-cellular blastocyst which has differentiated into 2 cell types, the inner cell mass (ICM) and the trophectoderm, and has a metabolic quotient similar to a tumour cell (Gardner and Lane 2003). Therefore, there are coordinated changes in the control of metabolism within the early embryo that must occur for normal metabolic capacity and developmental competence to be maintained. Metabolic control in animal embryos has been shown to be essential for the maintenance of viability (Gardner 1998, Gardner et al. 2000b, Lane and Gardner 2000a). Perturbations in metabolic parameters of embryos have been associated with a loss in viability (Lane 2001) and specific metabolic measures in animal models have been demonstrated to have a positive relationship with embryo viability (Renard et al. 1980, Gardner and Leese 1987, Lane and Gardner 1996). Therefore, the energy metabolism of human embryos in in vitro culture has the potential to be used to predict viability. Non-invasive measurements of carbohydrates and amino acids have been extensively studied in the human with varying success and have potential to be employed as viability markers in the clinic (Leese et al. 1993, Turner et al. 1994, Gardner et al. 2001, Houghton et al. 2002, Brison et al. 2004a, Houghton and Leese 2004).

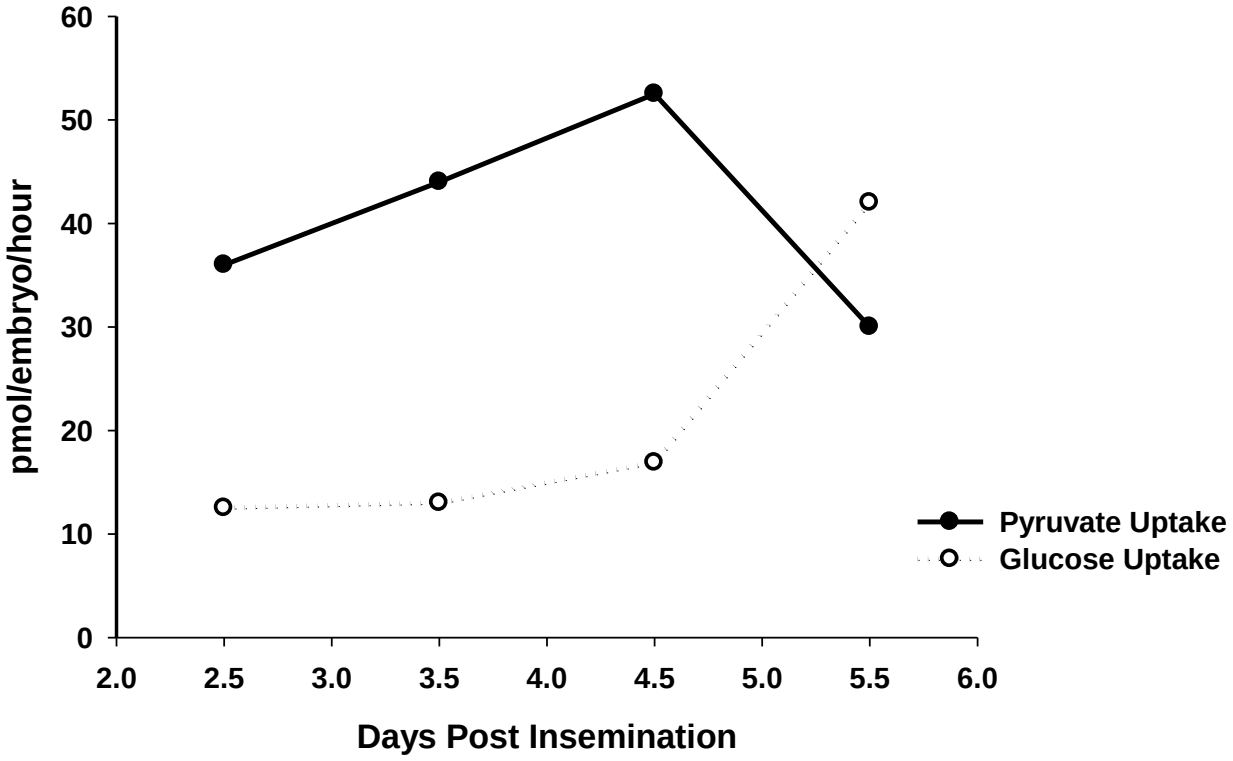


Figure 1.9 Consumption of pyruvate and glucose of the human pre-implantation embryo.
Pyruvate is the dominant substrate until the blastocyst stage when glucose uptake significantly increases. (data from (Gott et al. 1990)).

1.7.2 Carbohydrate Metabolism

Several studies have indicated that disrupted balance of carbohydrate metabolism is related to poor development in mouse and human embryos. A prospective study in the mouse with single embryo transfers was able to show that glycolytic activity (i.e. glucose uptake and lactate production) in blastocysts is a predictor of viability, where there was a 4-fold increase in viability in morphologically similar blastocysts selected using metabolic criterion over random selection. This demonstrates that metabolism can be used as a predictor of viability to differentiate morphologically similar blastocysts (Lane and Gardner 1996). In the human there have also been studies that have assessed embryo metabolism (Hardy et al. 1989, Gott et al. 1990, Conaghan et al. 1993b, Turner et al. 1994, Gardner et al. 2001, Seli et al. 2008). High levels of pyruvate uptake measured at intervals between day 2 of human embryo development and day 5 have shown improved development rates to the blastocyst stage (Hardy et al. 1989, Gott et al. 1990, Gardner et al. 2001). In further investigation of metabolic parameters with regard to pregnancy establishment, one study of 44 patients found that glycolytic activity did not predict pregnancy outcome (Jones et al. 2001). However, the implantation rates in this study were very low (14%), likely due to the incubation medium lacking key regulators such as pyruvate, lactate, amino acids and vitamins, which may have resulted in significant metabolic stress on the embryos and confounded the outcome. In contrast, a study of 42 patients, in complete medium, demonstrated that embryos with lower glycolytic activity had significantly higher pregnancy rates (Van den Bergh et al. 2001). This study did not alter the routine culture conditions for the analysis and the resultant implantation rates were in an acceptable clinical range (43%). In addition, glucose consumption on day 4 of human embryo development has been shown to be twice as high in embryos that subsequently form blastocysts, and within a patient's cohort, blastocysts with the same morphology score demonstrated a significant range of metabolic activity (Gardner et al. 2001), indicating as shown in the mouse, that metabolism can differentiate morphologically similar blastocysts with regard to viability. A limitation of this approach is that blastocyst culture is unlikely to be adopted as a routine treatment in the majority of clinics due to legal restrictions in some countries or technical and resource issues as highlighted previously. The majority of IVF transfers involve cleavage stage embryos so it is important to attempt to identify markers able to select the most viable embryo at the cleavage stage of development, or earlier in some cases, as legislation permits.

In the human cleavage stage embryo, pyruvate uptake was found to be approximately 20% less in embryos which arrest at the cleavage stage compared to embryos that subsequently developed to the blastocyst stage (Hardy et al. 1989). However, the small range of differences observed between embryos with differing potential to form blastocysts most likely would not be sufficiently critical to

implement as a robust marker of viability in the clinic (Hardy et al. 1989). A subsequent study of 116 patients with 243 embryos transferred, reported an inverse relationship between pyruvate uptake and pregnancy establishment (Conaghan et al. 1993b). Alternatively, Turner and colleagues (1994) found no difference in the mean pyruvate uptake of cleavage stage embryos, however the range in levels of pyruvate consumption was significantly larger in embryos which did not result in pregnancy. This study is particularly significant considering that single embryo transfers were performed on all 80 patients involved (Turner et al. 1994). Glucose uptake at the cleavage stage has been shown to be virtually identical between embryos that arrest and those that form blastocysts. If this study had been able to perform embryo transfers, subsequent blastocysts may have been able to be deemed viable or non-viable to further evaluate glucose uptake at the cleavage stage as a potential marker of embryo health (Hardy et al. 1989).

1.7.3 Amino Acid Metabolism

Amino acids maintain cellular functions, promote development and differentiation (Bavister 1995), and are present in high levels in the female reproductive tract (Fahning et al. 1967, Miller and Schultz 1987). Glycine, taurine, alanine, aspartate, glutamine and glutamate are present at the highest concentrations, which excluding glutamine and taurine, are part of Eagle's non-essential amino acids (Eagle 1959). Supplementation of amino acids in embryo culture medium improves blastocyst development in mouse (Dumoulin et al. 1992a, Dumoulin et al. 1992b, Gardner and Lane 1993, Gardner and Lane 1996), hamster (McKiernan et al. 1995), sheep (Gardner et al. 1994), cow (Takahashi and First 1992, Steeves and Gardner 1999b) and human (Devreker et al. 1998, Devreker et al. 1999, Devreker et al. 2001). More specifically, there is a shift in amino acid requirements in the pre-implantation embryo. Up to the 8-cell stage, non essential amino acids and glutamine enhance cleavage speed (Lane and Gardner 1997a, Steeves and Gardner 1999b), which is reflective of the amino acids found in high levels in oviduct fluid. After the 8-cell stage, these same amino acids promote blastocyst formation, while the essential amino acids stimulate cell division and inner cell mass development (Lane and Gardner 1994). Overall, and most importantly, amino acids improve the viability of in vitro embryos following transfer (Gardner and Sakkas 1993, Gardner et al. 1994, Lane and Gardner 1997a). Alternatively, addition of Eagle's essential amino acids reduces mouse blastocyst cell number (Lane and Gardner 1997b) and viability (Lane and Gardner 1994). Furthermore, morulae grown in medium lacking amino acids result in blastocysts which produce very high levels of lactate indicating impaired oxidative metabolism (Gardner and Leese 1990), whilst amino acid supplementation of the medium prevents excess lactate production (Gardner and Sakkas 1993). Pre-implantation embryo development is inhibited by a range of amino acids including proline, cysteine, arginine and phenylalanine, which has been demonstrated with hamster embryos (Bavister and Arlotto 1990). Subsequently, inclusion of non-essential amino acids and

glutamine at the cleavage stage and all 20 amino acids following the 8 cell stage have been shown to improve mouse and human embryo development and viability (Gardner et al. 1998a, Gardner et al. 1998b, Jones et al. 1998), and are now incorporated into complete sequential media formulations (Gardner and Lane 2004).

The laboratory of H. J. Leese has pioneered the profiling of amino acids as predictors of embryo quality and pregnancy outcome. Partridge and Leese (1996) showed that individual amino acids are depleted by bovine embryos throughout pre-implantation development and that amino acid requirements change during development, suggesting that determination of amino acid profiles from spent media may be a suitable marker of embryo physiology, health and viability. Following this, Houghton et al. (2002) was the first to demonstrate a link between non-invasive amino acid metabolism and embryo developmental competence by showing that amino acid profiles on day 2 of human embryo development were able to predict the ability of an embryo to form a blastocyst. Embryos which formed blastocysts consumed lower levels of arginine and glutamine, while producing lower levels of alanine and threonine compared to embryos which subsequently arrested development. Furthermore, the total amino acid turnover of embryos which developed to blastocysts was lower, and importantly, these metabolic measures were independent of morphological parameters of the embryos (Houghton et al. 2002). Consequently, Brison et al. (2004) performed a study investigating the relationship between day 2 human embryo amino acid profiles and the ability of the embryos to establish pregnancy and result in live birth. No amino acid metabolism was correlated with morphological embryo grade, however asparagine, glycine and leucine turnover were significantly associated with live birth (Brison et al. 2004a). Although some single embryo transfers were performed in this study, the mean number of embryos replaced in the study was 2.2, with up to three embryos being replaced at one time, which confounds accurate correlation of viability to individual embryos.

Much of the work on human embryos to date has been performed on embryos donated to research either fresh or following cryopreservation. This provides valuable information in the pursuit to understand human embryo development, however does not allow viability to be evaluated to the point of pregnancy and live birth.

1.7.4 Modern Metabolomics

In contrast to individual carbohydrate or amino acid measures, metabolomics is a technology able to identify and quantify low molecular weight metabolites. Metabolomics analyses entire profiles of metabolites giving an indication of the physiology of an embryo with large numbers of metabolites being analysed simultaneously and the relationship between those metabolites being characterised. Most

recently, the use of infrared spectroscopy and other methods of multiple substrate measurement have demonstrated that this approach can distinguish embryos of different competency by the analysis of the medium used to culture them (Singh and Sinclair 2007, Botros et al. 2008). Spent media from oocyte culture prior to ICSI for 11 patients has shown that a resultant relative viability score following near infra-red (NIR) spectroscopy analysis correlates with subsequent day 3 embryo morphology, day 5 blastocyst development and implantation ability with multiple embryos transferred (Nagy et al. 2009). Other investigations have determined that the embryo metabolome is independent of embryo morphology (Seli et al. 2009b), and that embryos with the same appearance have different viability scores following metabolomics analysis (Sakkas et al. 2009). In addition, metabolome profiles of embryos from single embryo transfers on day 2, 3 and 5 have been retrospectively correlated with implantation (Go et al. 2009, Henman et al. 2009, Makinen et al. 2009). Furthermore, two prospective multi-centre trials have demonstrated in day 5 single embryo transfers that viability scores resulting from metabolome profiles were correlated with implantation independent of embryo morphology as well as different commercially available culture media (Botros et al. 2009, Seli et al. 2009a). Despite promising technology, such approaches to date have yet to openly reveal the most significant changes to medium components which determine developmental competence.

With the exception of metabolomics, a major limitation to all studies that have been performed to determine viability markers of human embryos is that single embryo transfers have not been undertaken, rendering information gained scientifically interesting, but limited in value for application in a clinical setting. In addition, the development of culture media systems over time has superseded many studies of metabolism as the analysis of nutrient consumption and production was largely investigated prior to the knowledge of a more physiological in vitro system.

1.8 SUMMARY

Morphological measures of oocyte, zygote and embryo assessment are quick and relatively simple to perform and as such are extremely popular tools in the clinic. Morphological assessments are valuable and are therefore unlikely to ever be superseded. Additional potential measures to supplement morphology include **follicular fluid** analysis, which with modern metabolomics may be a more promising tool than has been previously demonstrated. However, single follicle aspiration is technically demanding and a higher risk procedure for the patient. It is currently unknown, but highly likely that there is significant contribution of maternal factors that may confound the ultimate viability determination of resultant embryos with follicular fluid analysis.

Cumulus cell parameters are promising viability candidates due to their close proximity and influential dynamic with the oocyte. In addition, single culture and tracking of COCs can commence once in the laboratory following the aspiration procedure. The discovery of such a technique will be particularly important in clinics which are restricted by legislation to inseminate minimal numbers of oocytes, and thus selection of the oocyte is required. However. any assessment tool which is performed prior to embryonic genome activation may have a limited viability prediction value. Similarly, **oocyte metabolism** as an evaluation tool has many of the benefits and disadvantages of cumulus cell analysis, with only the maternal contribution to the potential embryo being accounted for, but with tracking of individual oocytes able to begin in the laboratory. With any parameter that occurs prior to fertilization, some results obtained will be redundant, as those oocytes that do not fertilize have had a significant amount of investigation undertaken which will not provide benefit to the IVF cycle. Such parameters may only be useful in clinics which are restricted by legislation to inseminate minimal numbers of oocytes, in order to select the most viable oocytes to inseminate.

The earliest stage at which to assess the embryo is currently by **pronuclear morphology**. Morphological assessments are quick and relatively easy to perform, and again are valuable in countries with limitations on embryo in vitro culture. Following this point, many morphological measures have been introduced to aid selection including **early cleavage** status, **cleavage stage morphology** and **blastocyst morphology**. An extension of embryo morphology is an analysis of **embryo metabolism** at the cleavage and blastocyst stages. Metabolomics may result in easier incorporation of such techniques into the clinic and as such are quite promising. As previously mentioned, embryonic genome activation occurs in the cleavage stages of the human embryo and therefore metabolism of the cleavage stage embryo may have limited predictability. Ultimately, blastocyst culture allows for extended

morphological assessments and embryonic genome activation. Therefore, metabolic measures in addition to the current techniques have potential to improve an already high implantation rate. However implementation of blastocyst culture and transfer in many clinics is not a viable option.

1.9 CONCLUSION

Given the known risks associated with multiple pregnancies, the ultimate goal of ART must be to achieve singleton pregnancies. The only effective way to achieve this is by the transfer of a single embryo. The long-established reluctance to transfer a single embryo due to fear of reduced pregnancy rates highlights the need to identify factors that are prognostic of outcome. Transfer of only one embryo makes embryo selection of utmost importance. Current embryo selection techniques are valuable in selecting embryos with little developmental potential, but information is required which can supplement what is observed, to select the most viable embryo from a cohort. In recent years there has been a substantial body of evidence that has determined several factors that are associated with increased viability of animal embryos. However, due to the various limitations of animal models, few of these markers have also been used prospectively in the human.

1.10 HYPOTHESIS

We propose that the potential of an embryo to result in a viable pregnancy is multifactorial, and influenced by dynamic interactions between the cells within the follicle, the bi-directional communication of the cumulus-oocyte-complex and the ability of the resulting embryo to maintain normal development in an environment which may be less than optimal. Therefore markers of health of the oocyte and pre-implantation embryo, which take these factors into account, may result in the ability to select the most developmentally competent embryos from a cohort.

1.10.1 Specific Hypotheses and Aims

Hypothesis 1: Group embryo culture provides no additional benefit to embryo development and viability than single embryo culture.

- *Determine cleavage and blastocyst development rates in mouse embryos grown individually and in groups.*
- *Analyse viability of mouse embryos grown individually or in groups following transfer to recipients, through implantation rates, fetal development rates and fetal and placental parameters.*
- *Analyse implantation rates of human embryos grown individually or in groups prior to embryo transfer.*

Hypothesis 2: Extended culture of human embryos to day 4 of embryo development confers a selection advantage and improved pregnancy rates over cleavage stage embryo assessment and viability.

- *Describe a scoring system for day 4 of human embryo development.*
- *Implement scoring system in a clinical setting and analyse resultant implantation rates with regard to morphology grade.*
- *Analyse implantation rates of day 4 embryo transfers compared to cleavage and blastocyst stage embryo transfers.*

Hypothesis 3: Cumulus cell REDOX levels correlate with resultant embryo viability.

- *Establish the measurement of REDOX levels in human cumulus cells.*
- *Assess REDOX levels in human cumulus cells with relation to resultant fertilization ability, embryo morphology and viability.*

Hypothesis 4: Metabolism of the COC and early embryo correlate with embryo viability.

- *Assess spent media from COC and embryo culture for metabolites that may indicate embryo viability.*
- *Analyse metabolites with respect to embryo quality and pregnancy establishment following single embryo transfers.*

CHAPTER 2

MATERIALS AND METHODS

2.1 HUMAN PROCEDURES

Human procedures are summarised in Figure 2.1. All research was approved by the NHMRC sanctioned Human Ethics Committee, Women's and Children's Hospital, Adelaide, South Australia.

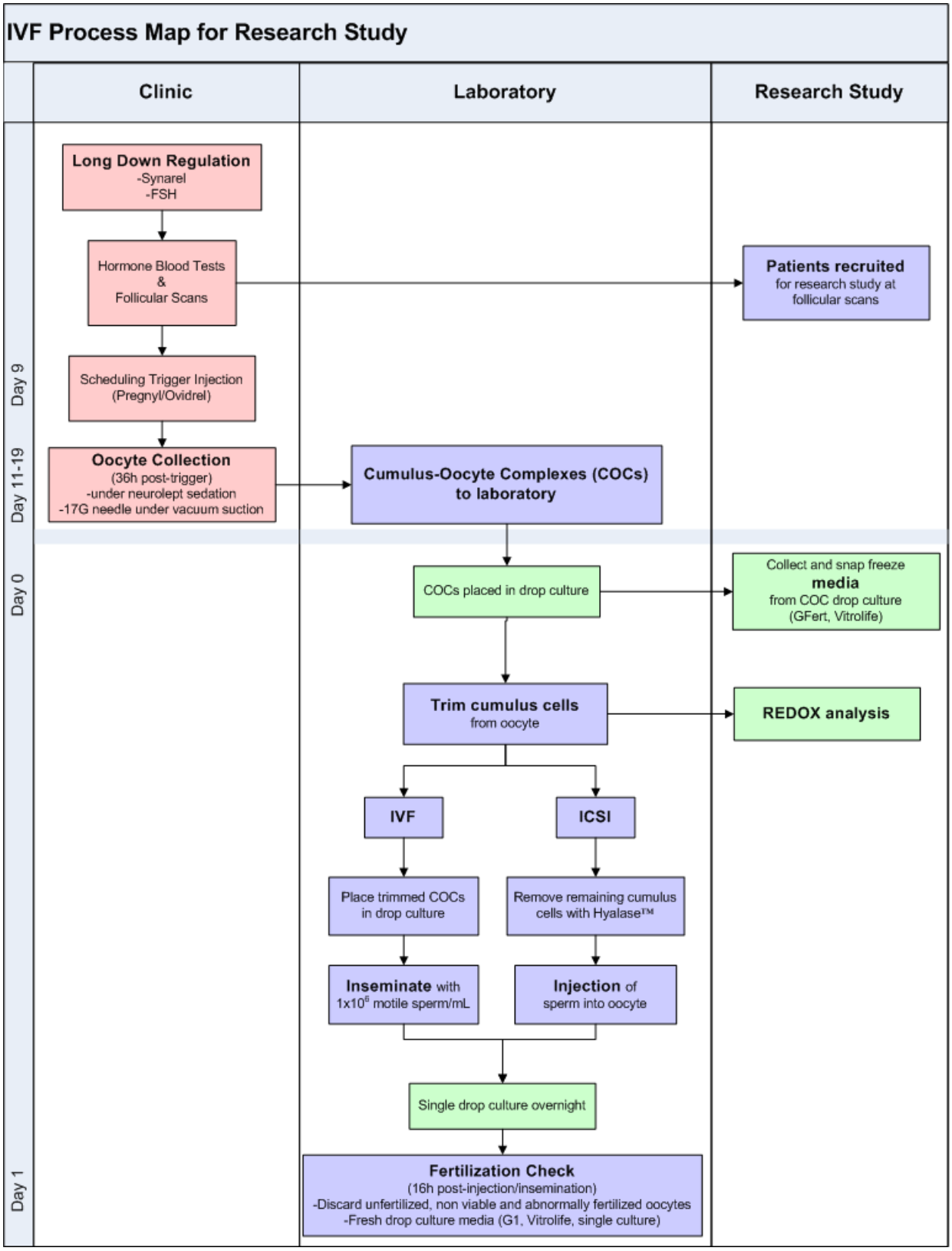
2.1.1 Patient Consent

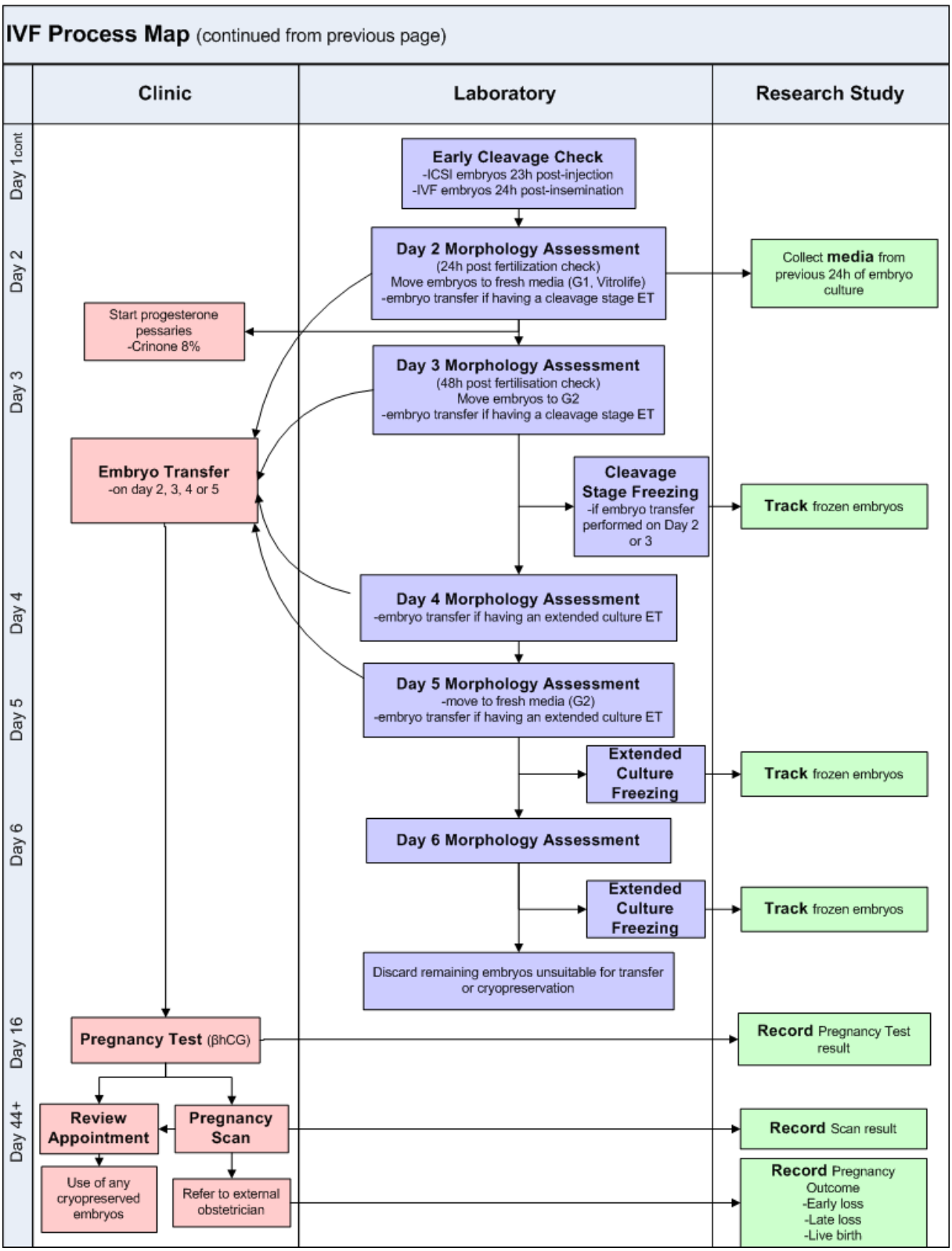
All patients seeking IVF treatment at Repromed, Adelaide were screened for eligibility to the study prior to attendance at the clinic for their first follicular scan. Patients were approached by the primary researcher on the project (D.Feil) to explain the details of the study, including the points at which samples would be collected and what type of information would be obtained from the samples collected (see Figure 2.1). The couples approached were given a Patient Information Sheet and Consent Form (see Appendix 1) and were then followed up at their next clinic appointment (blood test, scan or oocyte retrieval) where any questions were addressed and informed consent was obtained from both partners. In all cases consent was obtained prior to attendance in theatre for the oocyte retrieval. Separate cohorts of patients were recruited for each chapter detailed in this thesis, with some overlap occurring only between chapters 3 and 6 where single embryo culture and metabolites in spent media were assessed.

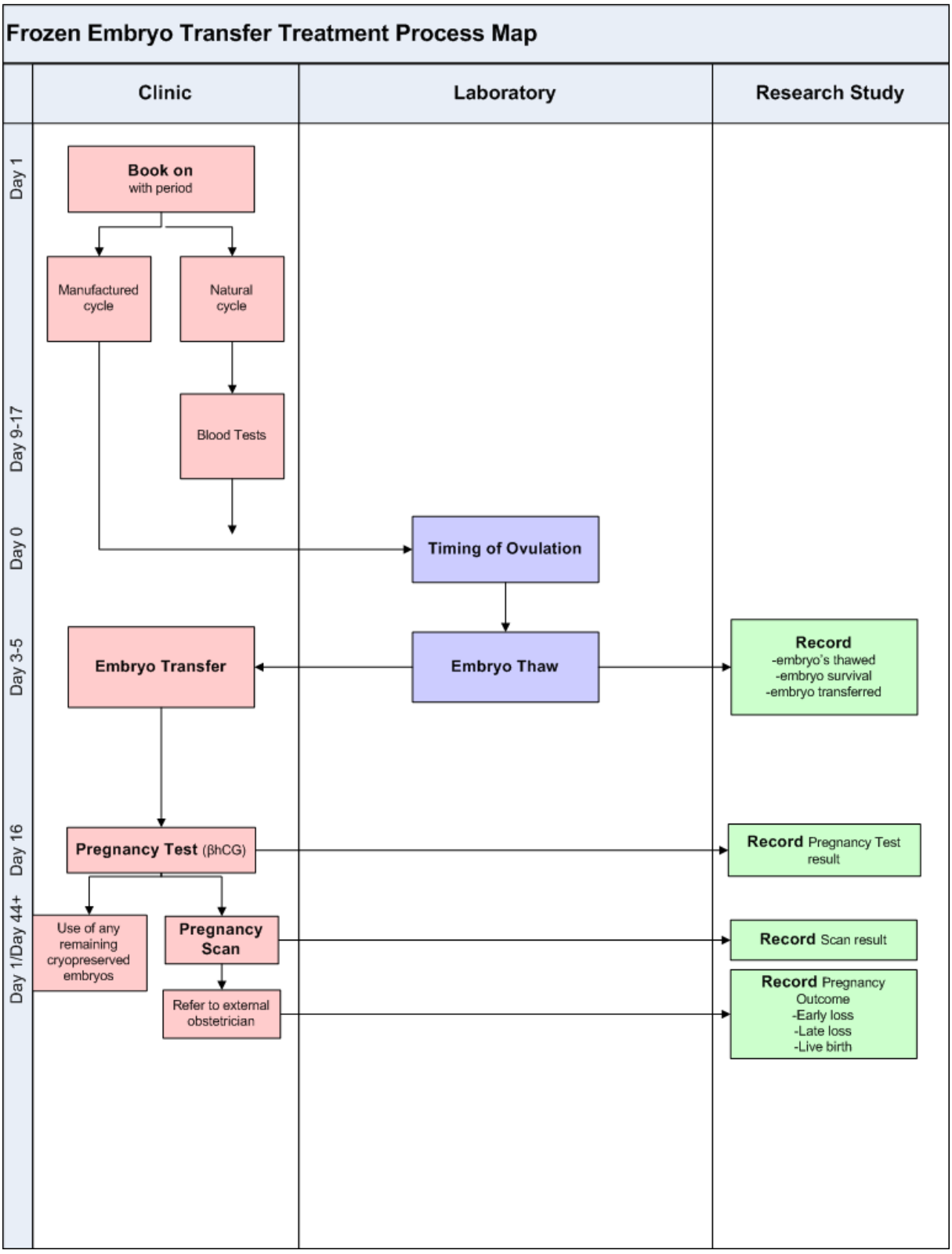
2.1.2 Patient Stimulation

Patients were stimulated using a long down-regulation protocol. For patients requiring menstrual cycle manipulation, the oral contraceptive pill (OCP, Marvelon, Organon, Sydney, Australia) was initiated on day 3 of the cycle and continued for 14-28 days. The administration of a Gonadotrophin Releasing Hormone analog (Nafarelin acetate (Synarel 2mg/ml), Pharmacia Australia, Leuprolide acetate (Lucrin), Abbott Australasia) was combined with the OCP for 6 days (termed crossover). Following at least 14 days of analog, down regulation was confirmed when blood oestrogen and progesterone levels were below 0.2nM/L and 4.0nM/L respectively. Patients were then administered recombinant Follicle Stimulating Hormone (FSH, Gonal-F, Serono, Sydney, Australia or Puregon, Organon, Sydney, Australia), doses ranging from 150IU to 300IU (see Appendix 2) for 9-12 days.

Figure 2.1 Research study process map.




IVF Process Map (continued from previous page)
Clinic
Laboratory
Research Study
Day 1cont
Day 2
Day 3
Day 4
Day 5
Day 6
Day 16
Day 44+
Early Cleavage Check
-ICSI embryos 23h post-injection
-IVF embryos 24h post-insemination
Day 2 Morphology Assessment
(24h post fertilization check)
Move embryos to fresh media (G1, Vitrolife)
-embryo transfer if having a cleavage stage ET
Collect media from previous 24h of embryo culture
Start progesterone pessaries
-Crinone 8%
Day 3 Morphology Assessment
(48h post fertilisation check)
Move embryos to G2
-embryo transfer if having a cleavage stage ET
Embryo Transfer
-on day 2, 3, 4 or 5
Cleavage Stage Freezing
-if embryo transfer performed on Day 2 or 3
Track frozen embryos
Day 4 Morphology Assessment
-embryo transfer if having an extended culture ET
Day 5 Morphology Assessment
-move to fresh media (G2)
-embryo transfer if having an extended culture ET
Extended Culture Freezing
Track frozen embryos
Day 6 Morphology Assessment
Extended Culture Freezing
Track frozen embryos
Discard remaining embryos unsuitable for transfer or cryopreservation
Pregnancy Test (βhCG)
Record Pregnancy Test result
Review Appointment
Pregnancy Scan
Record Scan result
Use of any cryopreserved embryos
Refer to external obstetrician
Record Pregnancy Outcome
-Early loss
-Late loss
-Live birth



Legend:

Pink boxes indicate routine clinic activities. Blue boxes indicate routine laboratory protocols. Green boxes indicate the incorporation of activities performed as part of this research study, which are not routine protocols. Processes are linked as indicated with arrows. The progression of time is displayed in the far left column. h=hours.

2.1.3 IVF Process

2.1.3.1 Oocyte Retrieval Scheduling

Follicle growth was monitored from day 9 by transvaginal ultrasound and serum oestrogen levels, until the lead ovarian follicle was a size of 18mm. Patients were then given human Chorionic Gonadotrophin (5,000IU hCG, Pregnyl, Organon, Sydney, Australia) and the oocyte collection scheduled for 36 hours later.

2.1.3.2 Oocyte Pick Up (OPU)

Patients were anaesthetized with neurolept sedation (fentanyl and midazolam) for the oocyte collection by an anaesthetist. Follicles were imaged by transvaginal ultrasound with an attached guide for the ovum collection needle (17-gauge, COOK, Australia) which punctured the vaginal wall to access the ovary. Follicle aspiration was performed using suction generated by a vacuum pump (-120mmHg, COOK, Australia). Fluid was aspirated from follicles greater than 14mm and collected in warmed 14mL tubes batch tested for embryo toxicity. An enclosed environment maintained at 37°C, 6% CO_2 was used for microscopic evaluation (magnification x6.7-40) of the follicular fluids to reveal any cumulus-oocyte complexes (COCs) present. COCs were removed from the follicular fluid using a polished glass Pasteur pipette and washed twice through glucose supplemented (2.5mM) GFert Plus v3 (Vitrolife, Kungsbacka, Sweden) to remove any follicular fluid and blood cells, and pooled in a final volume of 1mL of glucose supplemented GFert Plus v3 medium. The oocyte collection was preceded by flushing of the collection needle and line with HEPES buffered handling medium (Quinn's Advantage Medium, Gytech, Australia). Following aspiration of all follicles present in each ovary the collection needle and line were again flushed with HEPES handling media to ensure they did not contain any COCs, before terminating the procedure. All COCs collected were maintained in 1mL glucose supplemented GFert Plus v3 and incubated at 37°C, 6% CO_2, 5%O_2, 89%N_2 until further manipulation was required.

2.1.3.3 Semen Preparation

Sperm for all patients was obtained from fresh ejaculate produced by masturbation. No donor gamete treatments were assessed as part of these studies.

Semen samples with greater than 1 million sperm per mL and greater than 10% progressive motility were washed by gradient centrifugation. In a 15mL conical tube, 1 mL of 40% SpermGrad (Vitrolife) in HEPES (Gytech) was layered on top of 1mL of 80% SpermGrad to create 2 layers. Up to 1mL of neat semen was then layered on top of the gradient preparation. The sample was then centrifuged at 100g

for 20 minutes. The pellet was removed from the conical tube and transferred to a fresh 6mL tube and mixed with approximately 3mL of GSperm Plus v3 (Vitrolife) and centrifuged at 300g for 10 minutes. The supernatant was then removed and the pellet resuspended in approximately 0.5mL GSperm Plus v3. A final count and motility analysis were performed and the sample diluted to 1 million/mL in preparation for insemination.

2.1.3.4 Insemination

For both IVF and ICSI, COCs were trimmed of their outer layers of cumulus cells with an insulin syringe and 30G needle, post-OPU. For IVF insemination, trimmed COCs (see Figure 2.2) were placed either singly in 10µL drops of glucose supplemented GFert Plus v3 or in pairs in 50uL drops of the same medium under oil for 16 hours, and were co-incubated with 1,000 and 5,000 motile sperm respectively.

Following removal of the outer layers of cumulus cells, for ICSI insemination, remaining cumulus cells were denuded in 75IU Hyaluronidase (Hyalase®, Aventis Pharma Pty Ltd, Australia) in glucose supplemented GFert Plus v3. Between 4 and 6 hours post-OPU ICSI was performed. Individual oocytes were placed in 5µL drops of G1.3 Plus medium under oil, in the same dish as 5µL of polyvinylpyrrolidone (PVP, ICSI™, Vitrolife) with approximately 0.2µL of final sperm preparation (concentration $1.0x10^6$/mL). Sperm that appeared to have a normal morphology (as determined by head, neck, and tail appearance, as well as quality of motility, (World Health Organisation 1999)) were selected under magnification x200, and immobilized by a sharp downward action with the glass injection pipette (The Total Pipette Company, Australia) on the midpiece of the sperm. This action was repeated to ensure destabilisation of the sperm plasma membrane. The selected sperm was then aspirated into the injection pipette containing PVP. The oocyte was then positioned with the single polar body at 12 o'clock (see Figure 2.3) and held in place with light suction on the glass holding pipette (The Total Pipette Company, Australia). The injection pipette was then guided into the oocyte through the zona pellucida and oolemma, and the inner membrane was broken by aspirating a small volume of cytoplasm into the injection pipette. The aspirated cytoplasm was then returned to the oocyte with the sperm, and the injection pipette removed from the oocyte (see Figure 2.3). Injected oocytes were cultured either individually in 10µL drops of G1.3 Plus under oil, or in groups of 4 in 50µL drops of the same medium for the next 16 hours.

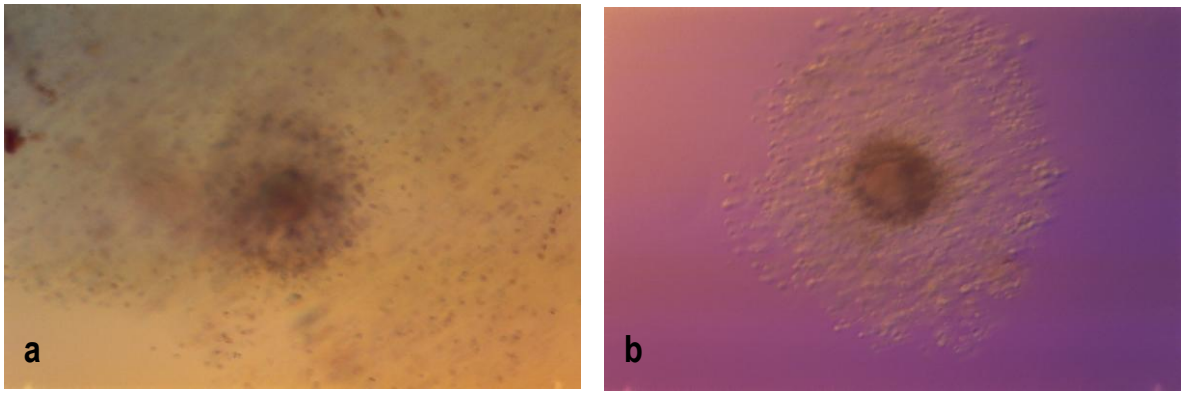


Figure 2.2 Cumulus-oocyte complex prior to and following trimming of cumulus cells in preparation for insemination.

a) Cumulus-oocyte complex following retrieval from the ovary and washing. b) Cumulus-oocyte complex following trimming of outer layers of cumulus cells, prior to insemination. Magnification of images x100.

Figure 2.3 Microinjection of an oocyte with a single spermatozoa (ICSI).

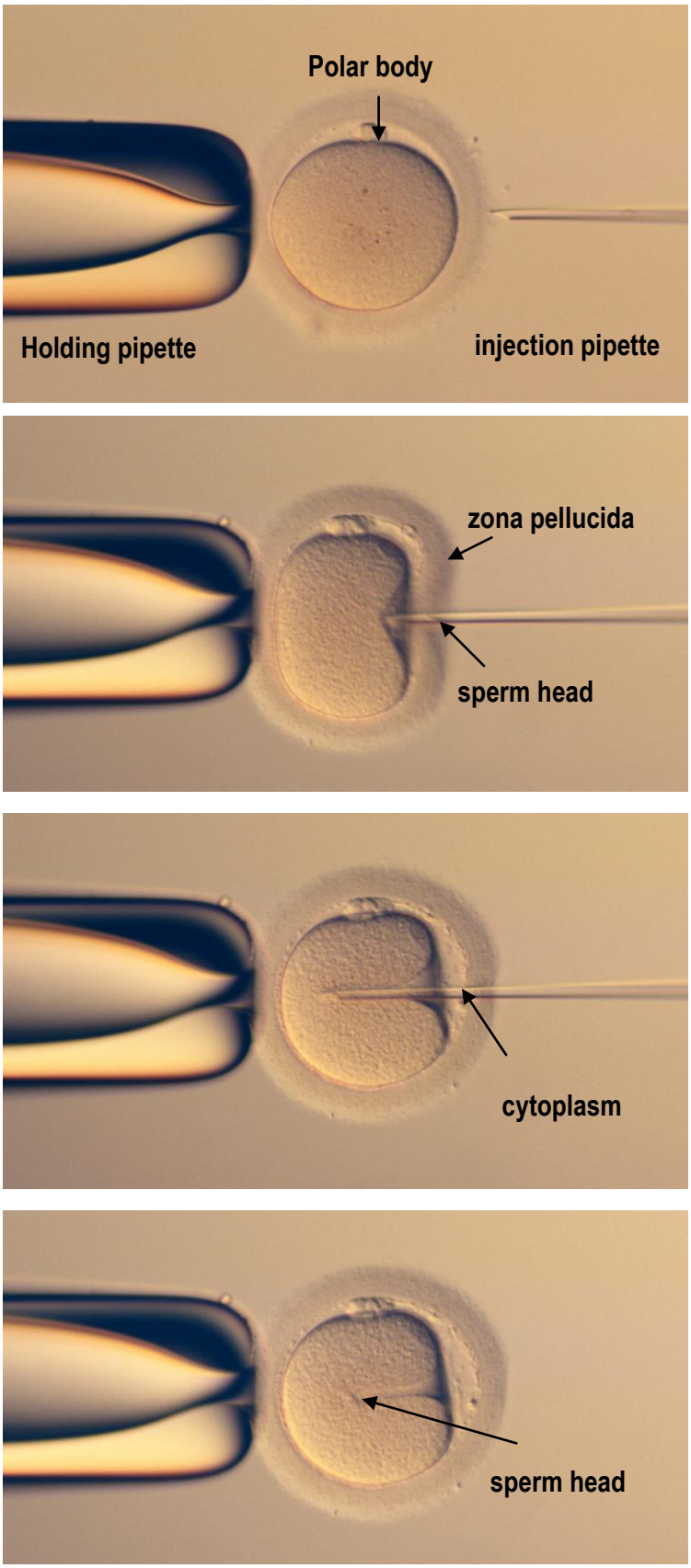
a) positioning of the oocyte with the polar body at 12 o'clock, sperm located in injection pipette

b) sperm at end of injection pipette and forced through the zona pellucida against the oolemma

c) oolemma broken, cytoplasm aspirated and then expelled back into the oocyte

d) completed injection of oocyte

Magnification x200 for all images.



2.1.3.5 Fertilization

The morning following insemination, IVF oocytes were mechanically denuded of their remaining cumulus cells and washed thoroughly through G1.3 Plus medium prior to fertilization assessment.

For both IVF and ICSI, all normally fertilized embryos with two pronuclei and two polar bodies present were cultured individually in 10µL drops, or in groups of 4 in 50µL drops, of G1.3 Plus medium under oil for the next 48 hours. Other oocytes/zygotes present at fertilization check were discarded including; unfertilized oocytes, abnormally fertilized zygotes, lysed and non-viable oocytes and immature oocytes (see Figure 2.4).

2.1.3.6 Embryo Culture

Human oocytes and embryos were cultured in the sequential GIII Series Plus Medium (Vitrolife, Kungsbacka, Sweden) and overlayed with Ovoil (Vitrolife). All oocyte and embryo culture was performed at 37°C, 6% CO_2, 5%O_2, 89% N_2 in a humidified atmosphere. All manipulations of oocytes and embryos occurred at 37°C, 6% CO_2 (excluding cryopreservation, thawing of embryos and embryo transfer procedures). All consumables in contact with gametes or embryos at any point during treatment were tested for embryo toxicity using a 1-cell mouse assay.

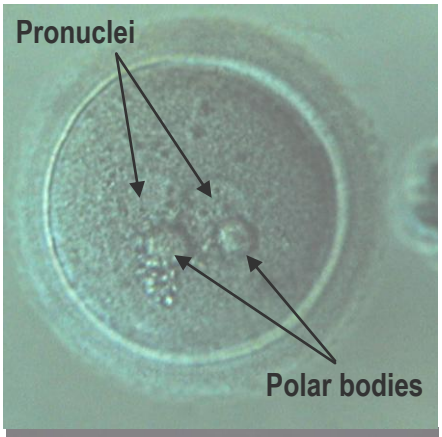
2.1.3.7 Embryo Morphology Assessments

During culture, embryo development was assessed at various critical time points (see Figure 2.5). Early Cleavage checks were performed 23 hours post-insemination for ICSI patients and 24 hours post-insemination for IVF patients. These checks assessed whether the zygotes had undergone the first cell division or syngamy by a set time point (see Figure 2.5).

For patients having a cleavage stage embryo transfer (day 2 or 3), embryos were assessed on the morning of transfer according to a morphological scoring system. This scoring system is based on the number and quality of blastomeres, the degree of fragmentation and the presence of multinucleated cells. Good quality embryos were determined to have 4-cells on day 2 of culture (41±1 hours post-insemination) or 7-8 cells on day 3 of culture (65±1 hours post-insemination), with <10% fragmentation and the absence of multinucleated blastomeres (see Figure 2.5 and Appendix 3). For patients having an extended culture embryo transfer (day 4 or 5), embryos were assessed on the morning of transfer according to the appropriate scoring system. A new scoring system was described for day 4 embryos (see Chapter 4) which assesses the degree of compaction and early cavitation of the embryo, as well as the number of blastomeres present. Day 5 embryos were assessed as per the grading system

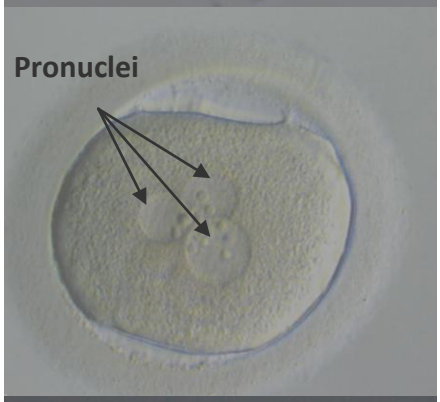
Figure 2.4 Fertilization assessments in human embryos.

a) normally fertilized oocyte
(2 pronuclei and 2 polar bodies present)

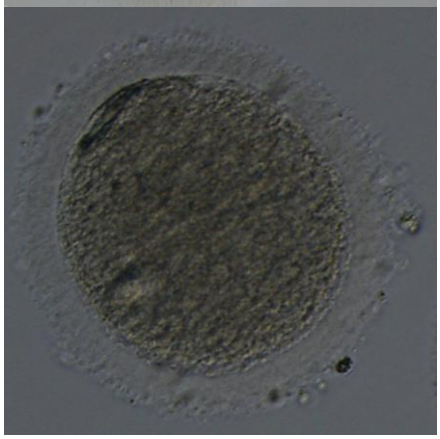


b) unfertilized oocyte
(single polar body, absence of pronuclei)

c) abnormally fertilized oocyte
(3 pronuclei visible)



d) Lysed/degenerate oocyte



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described by Gardner and Schoolcraft (Gardner and Schoolcraft 1999). Briefly, the degree of expansion and morphology of the inner cell mass and trophectoderm were visually assessed (see Appendix 3). Independent of day of transfer, embryo morphology was assessed using a scale of 1-4, where grade 1 indicated the best morphological quality and grade 4 indicated a poor morphological quality embryo.

2.1.3.8 Embryo Transfer

Embryo selection for transfer was based purely on assessment of morphology, and blinded to any quantitative measures. In all cases the single highest morphological quality embryo, as determined by the scoring system applicable to the stage of embryo development, dependent on the day of transfer (cleavage stage, day 4 or day 5 of embryo culture), was transferred. Embryos selected for transfer were incubated in EmbryoGlue® (Vitrolife) for 0.5-4 hours before transfer and transferred into the uterus in a volume of approximately 10µL, using an embryo transfer catheter (COOK, Sydney, Australia) under the guidance of abdominal ultrasound.

The day in which individual patients had their transfer was determined prior to the commencement of treatment in a clinic appointment with their clinician, where all decisions regarding the upcoming treatment were decided upon, and consent forms signed to reflect these decisions.

Patients were consented to have either a cleavage (day 2, 3) or extended culture (day 4, 5) transfer. After the commencement of treatment and scheduling of the patient for oocyte collection in theatre, embryo transfer appointments were allocated based on the patient's own clinician being available for transfer, and avoiding embryo transfers on Sundays. These reasons alone determined whether a cleavage stage transfer was performed on day 2 or day 3 and whether an extended culture stage transfer was performed on day 4 or day 5 of embryo culture.

Figure 2.5 Critical cleavage stage time points for checking human IVF embryos.

Early Cleavage Check

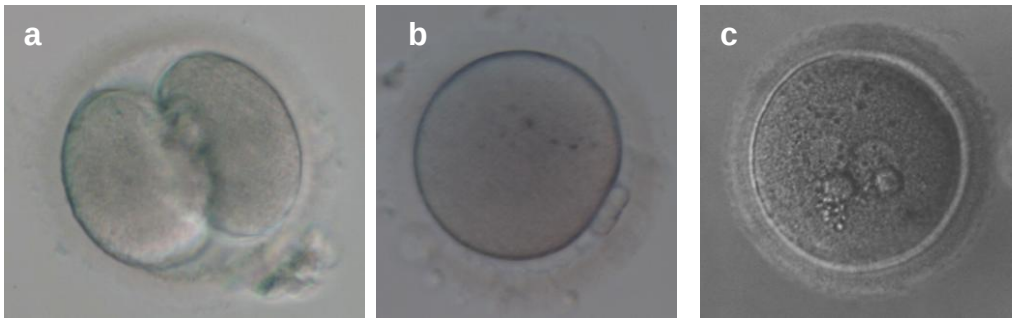
ICSI 23±0.5 hours post-injection

IVF 24±0.5 hours post-insemination

a) 2 cell embryo (early cleaver)

b) 1 cell embryo, no pronuclei present (syngamy)

c) 1 cell embryo, pronuclei present



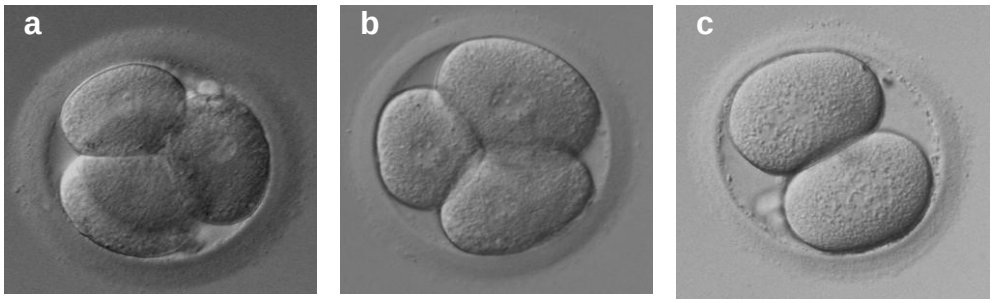
Day 2 Cleavage Check

40-42 hours post insemination

a) 4 cell embryo, <10% fragmentation

b) 3 cell embryo, <10% fragmentation, even cell division

c) 2 cell embryo, <10% fragmentation



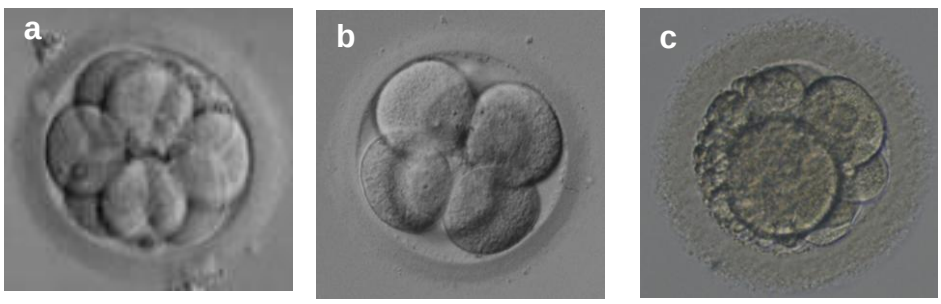
Day 3 Cleavage Check

64-66 hours post-insemination

a) 8 cell embryo, <10% fragmentation

b) 6 cell embryo, <10% fragmentation, uneven division of cells

c) 6 cell embryo ~20% fragmentation



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2.1.3.9 Embryo Cryopreservation

Assessment of surplus embryos for cryopreservation took place on days 3, 5 and 6 when a cleavage stage embryo transfer was performed, and on days 5 and 6 when either a day 4 or 5 embryo transfer was performed. Only embryos of good quality (Grade 1 or 2) were eligible for freezing.

Cleavage stage embryos underwent preparation for cryopreservation by transferring embryos to be frozen to warmed (37°C) HEPES buffered handling medium with 10% human serum albumin (HSA, HEPES/HSA) and then placing the dish containing the embryos at room temperature for 10 minutes. Embryos were then transferred to HEPES/HSA containing 1.5M propandiol for 15 minutes at room temperature before transferring into the final cryopreservation solution containing 1.5M propandiol and 0.1M sucrose and loading individually into cryostraws and placed in a programmable freezer (Cryologic Freeze Controller) at a start temperature of 20°C. Embryos were cooled at a rate of 2°C per minute to -7°C with seeding performed manually at this temperature. Embryos were then cooled at 0.3°C per minute to -35°C and then rapidly cooled at 4°C per minute to -196°C before storing in liquid nitrogen.

Blastocyst stage freezing was assessed on days 5 and 6. Cryopreservation was performed as described for cleavage stage embryos with embryos selected for cryopreservation being transferred to warmed HEPES/HSA, brought to room temperature then transferred to the first cryopreservation solution containing 0.69M glycerol and 0.1M sucrose for 10 minutes, then transferred to the final cryopreservation solution containing 1.37M glycerol and 0.2M sucrose for 7 minutes before being loaded individually into cryostraws and placed into a programmable freezer (Cryologic Freeze Controller) with a start temperature of -6°C, and seeded manually after 2 minutes at this temperature. Embryos were then cooled at rate of 0.5°C per minute to -35°C, and then rapidly cooled at 4°C per minute to -196°C before storing in liquid nitrogen.

2.1.3.10 Embryo Thawing

Individual cryostraws were selected and removed from liquid nitrogen storage and exposed to air temperature for 40 seconds and 30°C for a further 40 seconds. All embryos were thawed in HEPES buffered handling medium containing 10% HSA and varying concentrations of cryoprotectants as indicated below, at room temperature.

For cleavage stage embryos, the embryo was expelled from its cryopreservation straw into HEPES buffered handling medium at room temperature containing 10% HSA and 0.2M sucrose and 1.0M propandiol and incubated for 5 minutes. The embryo was then transferred to a variation of the same medium containing 0.2M sucrose and 0.5M propandiol for a further 5 minutes and then transferred to a

third thawing solution containing 0.2M sucrose in the absence of propandiol. Finally, the embryo was placed into a solution of HEPES handling medium and 10% HSA only, for 10 minutes at room temperature. Warming of the embryo to 37°C was then initiated for a period of 10 minutes in the same medium. An embryo with 50% survival or greater, at this point, was deemed to have survived the thawing procedure, and washed through the appropriate culture medium dependent on the age of the embryo (Day 2 embryo G1, Day 3 embryo G2), and placed in drop culture overnight. The following morning the embryo was assessed for development prior to embryo transfer.

For blastocyst stage embryos, the embryo was expelled from its cryopreservation straw into HEPES buffered handling medium at room temperature containing 10% HSA and 0.2M sucrose and 0.69M glycerol and incubated for 10 minutes. It was then transferred to a variation of the same medium containing 0.2M sucrose and no glycerol for a further 10 minutes, and then placed into a third thawing solution containing 0.1M sucrose. Finally, the embryo was placed into a solution of HEPES handling medium and 10% HSA only for 10 minutes at room temperature. Warming of the embryo to 37°C was then initiated for a period of 10 minutes. The embryo was then assessed for the proportion of blastomeres that had survived the cryopreservation and thawing procedure. An embryo with 50% survival or greater was deemed to have survived and washed through G2 culture medium and prepared for embryo transfer. Blastocysts were re-assessed 2-4 hours post-thawing to assess re-expansion of the blastocoel cavity.

2.1.3.11 Pregnancy Outcome

Pregnancy establishment was assessed by an initial β-hCG result >20 IU/L, 16 days after OPU. Subsequent blood tests were performed to determine ongoing pregnancy and diagnosis of ectopic pregnancy, as required.

A viable pregnancy was defined as the presence of an intrauterine fetal heart beat by ultrasound 5-6 weeks after embryo transfer. Ongoing pregnancies were tracked throughout gestation and outcomes analysed.

For the purposes of this thesis, pregnancy rates refer to the pregnant status of a patient irrespective of the number of embryos transferred or the presence or absence of a multiple gestation, while implantation rates refer to the number of intrauterine fetal heartbeats divided by the number of embryos transferred.

CHAPTER 3

SINGLE EMBRYO CULTURE AS A TOOL FOR EMBRYO SELECTION

3.1 INTRODUCTION

Despite the microlitre volumes of fluid that the embryo is exposed to in vivo (Leese 1988), embryos traditionally have been cultured in large volumes of media (≤1mL) (Lane and Gardner 1992). The beneficial effect of culturing mouse, sheep and cow embryos in groups or small volumes in simple style media for the entire culture period is well established (Wiley et al. 1986, Paria and Dey 1990, Canseco et al. 1992, Lane and Gardner 1992, Gardner et al. 1994, Kato and Tsunoda 1994, Keefer et al. 1994). In the human, group culture has been shown to enhance cleavage rates and embryo scores (Moessner and Dodson 1995). To date, there are conflicting results on the effect of grouping during culture with reports indicating an improvement in implantation rates following cleavage stage embryo transfer (Almagor et al. 1996), and others reporting no benefit in cleavage or blastocyst stage transfers (Rijnders and Jansen 1999, Spyropoulou et al. 1999). Similar to the animal studies, these human studies were performed in older style media systems (single tissue culture media with added serum at high oxygen concentrations) rather than the sequential culture systems which are now routinely used in human IVF protocols.

With research investigating the metabolic requirements for embryo development leading to advancements in embryo culture systems, the emphasis is now on how to select the most viable embryo from a cohort to maximize the chance of implantation and pregnancy. This is particularly so in human ART where multiple gestations have increased complications during and after pregnancy for both the mother and babies (Baird et al. 1998, Senat et al. 1998, Russell et al. 2003, Wang et al. 2009).

In clinical IVF, the assessment and selection of the most viable embryo from a cohort is paramount to maximizing the likelihood of successful implantation and pregnancy. Multiple morphological assessment criteria are now being employed at specific cleavage stages of pre-implantation embryo development, including oocyte and pronuclear morphology (Scott and Smith 1998, Wittemer et al. 2000), early cleavage (Shoukir et al. 1997, Shapiro et al. 2000, Lundin et al. 2001), on-time cleavage on days 2 and 3 of culture (Ziebe et al. 1997, Racowsky et al. 2000, Shapiro et al. 2000), assessment of fragmentation (Alikani et al. 1999) and cell quality parameters (Van Royen et al. 2003). Implementation of such assessment criteria, employed at numerous distinct time points, requires the tracking of individual embryos throughout development in culture thus the utilisation of single embryo culture is increasingly important.

Blastocyst stage embryo transfers were introduced as a selection tool, enabled by improvements in the quality of culture media and systems (Gardner and Schoolcraft 1999). The extended culture period required for development to the blastocyst stage presents further questions regarding the ideal environment for an embryo, in particular for the duration of the post-compaction period for which it has been suggested that embryo-derived growth or survival factor(s) exert an effect between the 8-cell and blastocyst stage in mouse (Paria and Dey 1990). In addition, it has also been demonstrated in mouse embryos that embryo-derived survival factors are required pre-compaction, although do not show an effect on development until the post-compaction stage (O'Neill 1998). Furthermore in human embryos, proteins for growth factors and their receptors are present in unfertilized oocytes and throughout pre-implantation development (Chia et al. 1995). Therefore a beneficial effect of group culture might be expected prior to, but particularly after the 8-cell stage in mouse and human embryos.

Despite the widespread implementation of both single and group culture in clinical IVF programs in physiological media, there is a current lack of understanding about the consequences of these protocols on embryo, fetal and placental development. Therefore the aim of this study was to explore the effects of in vitro single embryo culture with a consistent embryo: incubation volume by assessing cleavage and blastocyst stage pre-implantation development and subsequent viability in the mouse and human.

3.2 MATERIALS AND METHODS

3.2.1 Animal Procedures

3.2.1.1 Animals

All animals used in this study were bred at The University of Adelaide's Waite Campus, and housed at the Laboratory Animal Services Facility at The University of Adelaide Medical School. Animals were caged with access to food and water ad libitum, under a 14:10 light cycle, (700 hours to 2100 hours). C57BL6xCBA F1 hybrid male and females were used to generate embryo cohorts. Swiss Albino female mice were used as recipients for embryo transfer experiments. All procedures were approved by the Animal Ethics Committee, The University of Adelaide.

3.2.1.2 Superovulation/Mating

Virgin female 3-4 week old C57BL6xCBA F1 hybrid females underwent a superovulation regime involving an injection of 5IU equine chorionic gonadotrophin (Folligon, Intervet, Boxmeer, Holland), followed 48 hours later by 5IU of human chorionic gonadotrophin (hCG, Pregnyl, Organon, Sydney, Australia). Immediately following hCG administration, females were caged individually overnight with a male of the same strain. Mating was confirmed the following morning by the presence of a vaginal plug.

3.2.1.3 Collection of Zygotes

Twenty-two hours post-hCG females were humanely killed by cervical dislocation. The abdomen was sterilized with 70% ethanol and a small incision was made in the ventral midline. Either side of the incision the skin was held firmly and pulled apart towards the head and tail to expose the abdomen. The peritoneum was cut, and the visceral organs moved aside to expose the reproductive tract. The uterine horn and oviduct were located and a cut was made between the oviduct and ovary with a second cut being made through the utero-tubal junction. This process was repeated for collection of the second oviduct. Oviducts were placed into warmed HEPES (Quinn's Advantage Medium, Gytech, Australia) containing 5% HSA (Vitrolife). A dissecting microscope was used to visualize zygotes and surrounding cumulus cells in the distended ampulla of the oviduct which were released into HEPES/HSA medium by tearing open the oviduct wall close to the zygotes. Individual zygotes were denuded from surrounding cumulus by incubation in 50IU/mL hyaluronidase (Sigma-Aldrich Pty Ltd, Castle Hill, New South Wales, Australia) in HEPES/HSA.

Following dispersion of the cumulus cells, the remaining coronal cells were mechanically removed by pipetting. Zygotes were then washed 2-3 times in HEPES/HSA medium, and once in the final culture medium (G1v3, Vitrolife), before being placed into the final culture environment.

3.2.1.4 Collection of Blastocysts

Both uterine horns were removed by cuts below the utero-tubal junction and a second cut through the cervix. Uterine horns were collected 88 hours post-hCG and blastocysts were flushed by injecting HEPES/HSA handling medium through the cervical opening using a 32G needle.

3.2.1.5 Embryo Culture

Mouse embryos were cultured in the sequential GIII Series Plus Medium (Vitrolife) and overlayed with Ovoil (Vitrolife). All embryo culture was performed at 37°C, 6% CO_2, 5%O_2, 89% N_2 in a humidified atmosphere. All manipulations of embryos occurred at 37°C. Embryos were cultured under various grouping conditions, either (1) singly in 2µL drops, (2) grouped, 10 embryos per 20µL drop (3) singly to the 8 cell stage (in 2µL drops, up until 48 hours post-collection) and then grouped (10 embryos/20µL) for the final period of culture to the blastocyst stage. All consumables used for embryo collection and culture had been previously tested for embryo toxicity.

3.2.2 Assessment of Mouse Embryo Development In Vitro

3.2.2.1 Morphological Embryo Assessment

Embryo morphology was assessed at a magnification of 200x. All embryos were assessed for development to the 2-cell stage (41 hours post-hCG). Embryos failing to develop to a 2-cell were removed from culture. All embryos were assessed for developmental stage (2- to 8-cell stage, 65 hours post-hCG) and washed through G2v3 (Vitrolife), and placed in culture drops of the same medium, for the final 48 hours of culture to the blastocyst stage. The final morphological assessment took place 113 hours post-hCG. Embryos at the final assessment were considered blastocysts when they contained a blastocoel cavity greater than ¾ of the blastocyst in volume. Hatching blastocysts were identified by herniation of the trophectoderm through the zona pellucida.

3.2.2.2 Differential Cell Staining

Blastocyst cell allocation to the inner cell mass (ICM) or trophectoderm (TE) in mouse embryos was assessed using a previously described differential staining protocol (Mitchell et al. 2009). Briefly, 113 hours post-hCG embryos at the blastocyst stage were placed in 0.5% pronase (Sigma-Aldrich) until the zona pellucida was visually observed to dissolve, followed by a 10 minute incubation at 4◦C in 10mM 2,4,6-trinitrobenzenesulfonic acid (Sigma-Aldrich). Blastocysts were then transferred to 0.1 mg/mL anti-dinitrophenyl-bovine serum albumin (Sigma-Aldrich) for 10 minutes at 37◦C followed by a 5 minute incubation in 10µg/mL propidium iodide (PI, Sigma-Aldrich) in guinea pig serum (Sigma-Aldrich) at 37◦C. Blastocysts were then placed in 6µg/mL bisbenzimide (Sigma-Aldrich) in ethanol overnight at 4°C, followed by washing in 100% ethanol. Blastocysts were then mounted in glycerol on microscope slides and viewed under an ultra-violet (UV) filter on a fluorescent microscope (see Figure 3.1).

3.2.2.3 Assessment of Blastomere Apoptosis

Apoptotic DNA in blastomeres of blastocysts was detected using a TUNEL (terminal deoxynucleotidyl transferase (TdT) biotin-dUTP nick end labeling) technique, as previously described (Brison et al. 2004b), incorporating an In Situ Cell Death Detection Kit (Roche Molecular Biochemicals, Indianapolis, USA). Apoptotic cell nuclei were labeled with fluorescein and all cell nuclei were counterstained with propidium iodide (PI, see Figure 3.2). Positive controls were exposed to Dnase prior to TUNEL labeling and negative controls were incubated in the labeling mix minus TdT instead of TUNEL. The number of apoptotic cells was expressed as a percentage of the total cell number of each blastocyst to give the apoptotic cell index.

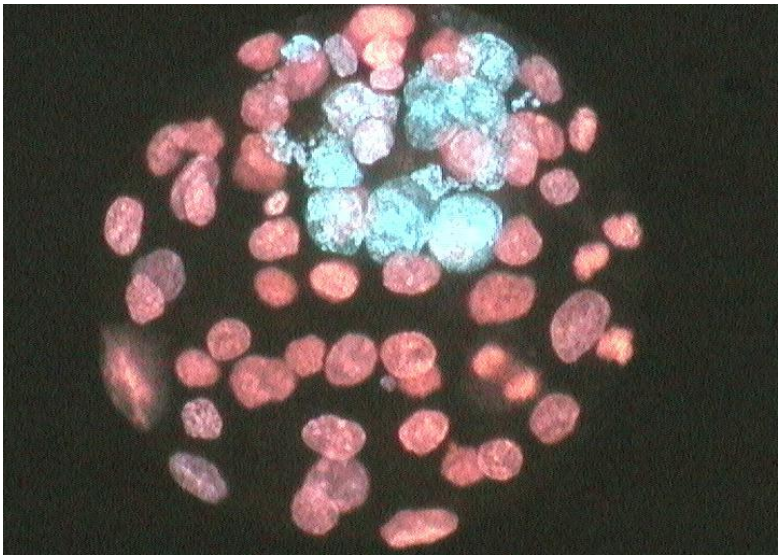


Figure 3.1 Differentially stained blastocyst.
Pink nuclei indicate trophectoderm cells and blue nuclei indicate inner cell mass cells.

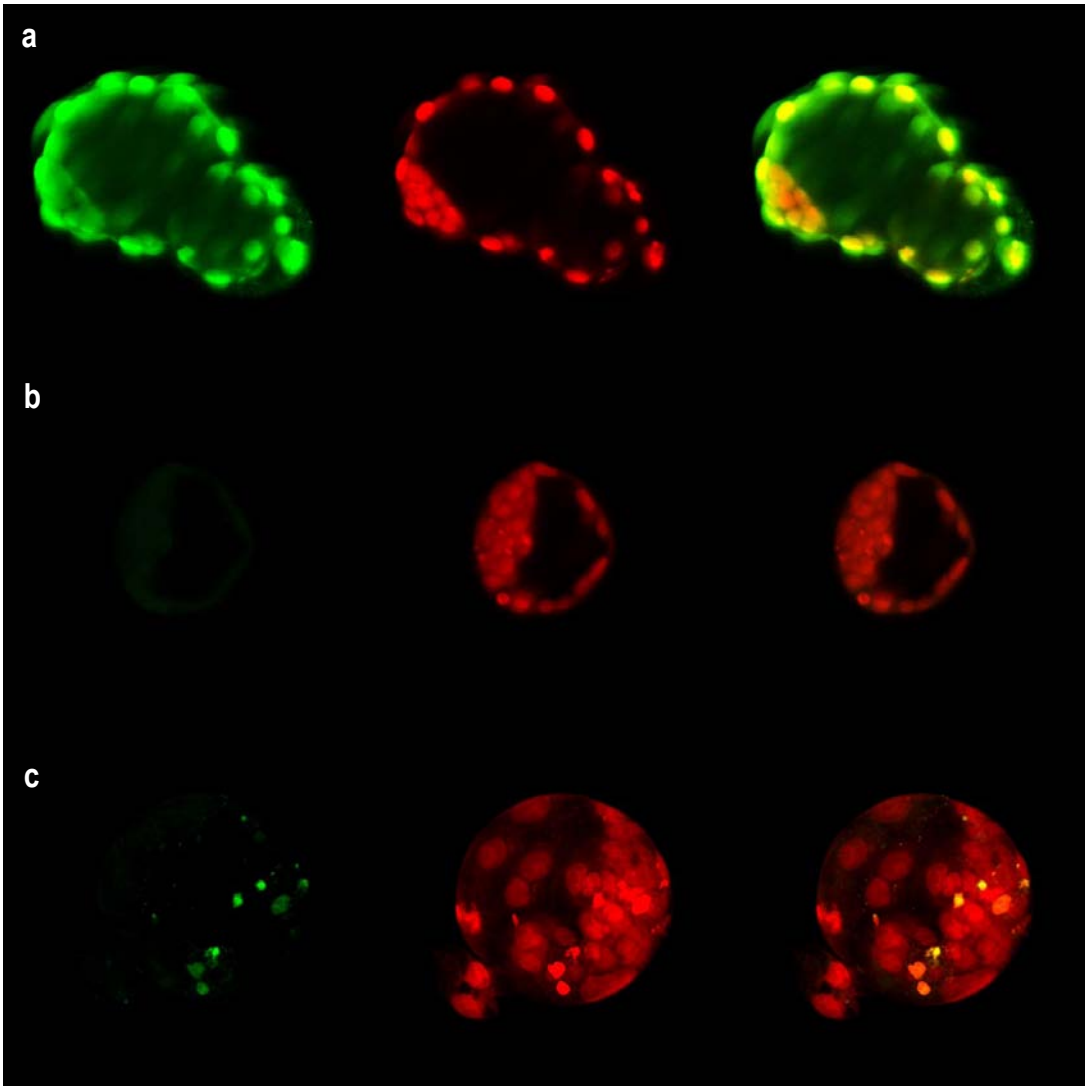


Figure 3.2 Imaging of apoptotic cells in mouse blastocysts with TUNEL and PI labeling.
a) positive control b) negative control c) treated blastocyst

3.2.3 Animal Surgical Procedures

3.2.3.1 Vasectomy

Male CBABL6xCBA F1 hybrid mice 6-8 weeks of age were anaesthetized by intraperitoneal injection of Avertin (0.02ml/g body weight, see Appendix 4). Each male had a horizontal incision made in the lower abdomen. Vasa deferentia were ligated at two places using silk suture and cut between the two ligations (Nagy et al. 2003). Incisions were closed with silk suture and mice were allowed to recover for 3 weeks. Test mating to superovulated females was performed to clear the vasa deferentia of any residual sperm. A second mating, 3-5 days later, was performed with superovulated females, and sterility confirmed by the presence of a vaginal plug the morning following mating and the absence of fertilized embryos collected 48 hours post-hCG.

3.2.3.2 Embryo Transfer

Female Swiss mice 10-12 weeks of age were used as recipients for embryo transfers. Natural matings were carried out with vasectomised males to induce pseudopregnancy. Mating was confirmed by the presence of a vaginal plug the morning following mating (day 0.5 of pseudopregnancy). Cleavage stage transfers were performed on day 3 of pseudopregnancy. Blastocyst transfers were performed on day 3.5 of pseudopregnancy.

Recipients were anaesthetized by intraperitoneal injection of Avertin (0.02ml/g body weight, see Appendix 4). A 10mm vertical incision was made in the dorsal midline below the ribs. The skin was moved to visualize the location of the ovary through the body wall. An incision was made in the peritoneum above the ovary and adjoining fat pad. The fat pad was secured with a clasp and removed from the body cavity with the ovary, oviduct and uterus. The embryos to be replaced (six per uterine horn) were picked up in a minimum volume of medium in a flame-pulled glass pipette, with a diameter slightly larger than the embryos. The upper end of the exposed uterus was pulled taut and a hole was made into the lumen of the uterus with a 26G needle. The pipette containing the embryos was then inserted into the hole in the uterine wall and the embryos expelled into the uterus. The pipette was then checked to ensure all the embryos had been transferred to the uterus. The uterus, oviduct and ovary were then eased back into the body cavity. This procedure was then repeated on the other uterine horn. The skin incision was sealed with clips and the female recipient placed on a heating stage at 37°C while recovering from the anaesthetic.

3.2.3.3 Post-Mortem

Embryo transfer recipients were humanely killed on day 18 of pregnancy by cervical dislocation. The numbers of viable or resorbing implantation sites was recorded. Fetuses and placentas were individually weighed and fetal crown-rump was measured.

3.2.4 Human Procedures

3.2.4.1 Patient Selection

Seventy-three patients undergoing routine IVF/ICSI with elective Single Embryo Transfer (SET) at Repromed's Adelaide clinic between May and December 2005 consented to be involved in research (Appendix 1). Ethical approval was obtained from the NHMRC sanctioned Human Ethics Committee of the Women's and Children's Hospital, Adelaide, South Australia. Consenting patients all underwent single culture of their embryos.

3.2.4.2 Embryo Culture

All protocols of the laboratory were followed as per standard practice, with embryos cultured individually in 10μL drops of the appropriate medium dependent on the stage of development. Single culture commenced following the collection of cumulus-oocyte complexes in theatre and was maintained throughout the culture period. All oocyte/embryo culture was performed at 37°C, 6% CO_2, 5%O_2, 89% N_2 in a humidified atmosphere. All manipulations of oocytes/embryos occurred at 37°C, 6% CO_2.

3.2.5 Statistics

All data are presented as mean ± standard error of the mean (SEM), with statistical analyses carried out using Statistical Package for the Social Sciences 13.0 for Windows (SPSS Inc., Chicago, IL). Replicate embryo development data was expressed as proportions and determined by Kolmogorov-Smirnov testing to be normally distributed therefore analysed using a univariate generalized linear model. Each replicate was treated as a covariate. Differential cell staining data was transformed for ICM and ICM: TE ratio for all data sets to be normally distributed and assessed using a univariate general linear model. Post-hoc tests as required were least significant difference (LSD) if data had equal variances as determined by Levene's test

of equality, or Dunnett's T3 for data sets with unequal variance. Cellular apoptosis was not normally distributed and was analysed by a Kruskal-Wallis non-parametric test.

Implantation rates were analysed by chi square testing. Fetal development rates were analysed using a univariate general linear model with embryo culture replicate and surrogate mother incorporated as covariates. A univariate generalized linear model was used to analyse fetal and placental outcomes with litter size incorporated as a covariate for fetal and placental outcome measures. Fetal and placental data that was not normally distributed was analysed by Kruskal-Wallis testing.

Human demographic data was not normally distributed and analysed by a Kruskal-Wallis test. Proportional data was analysed by a chi-square test. Significance was accepted at $p<0.05$.

3.3 EXPERIMENTAL DESIGN

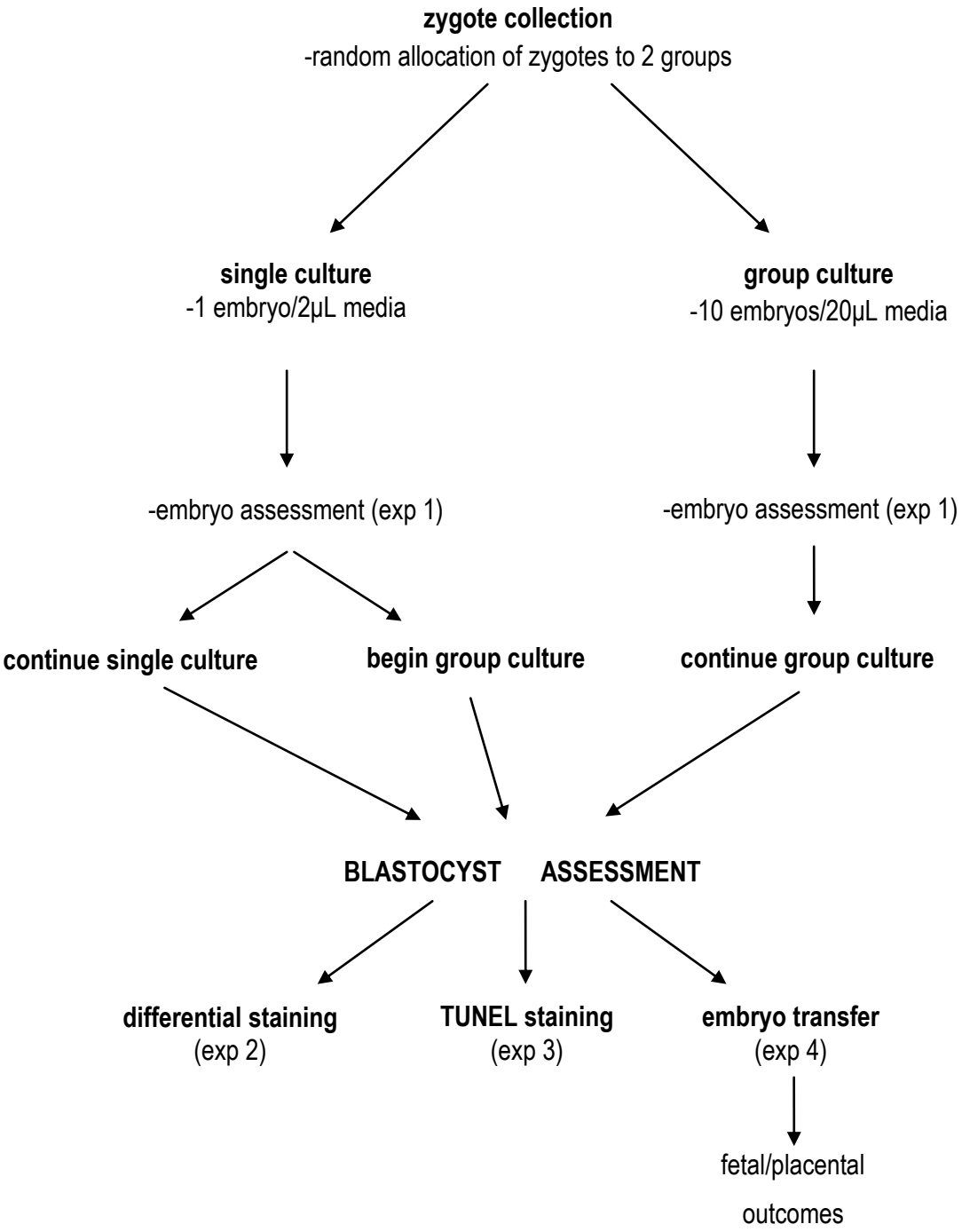


Figure 3.3 Animal experimental design detailing culture grouping under investigation while maintaining embryo density.
Experiment (exp) numbers indicated in parentheses.

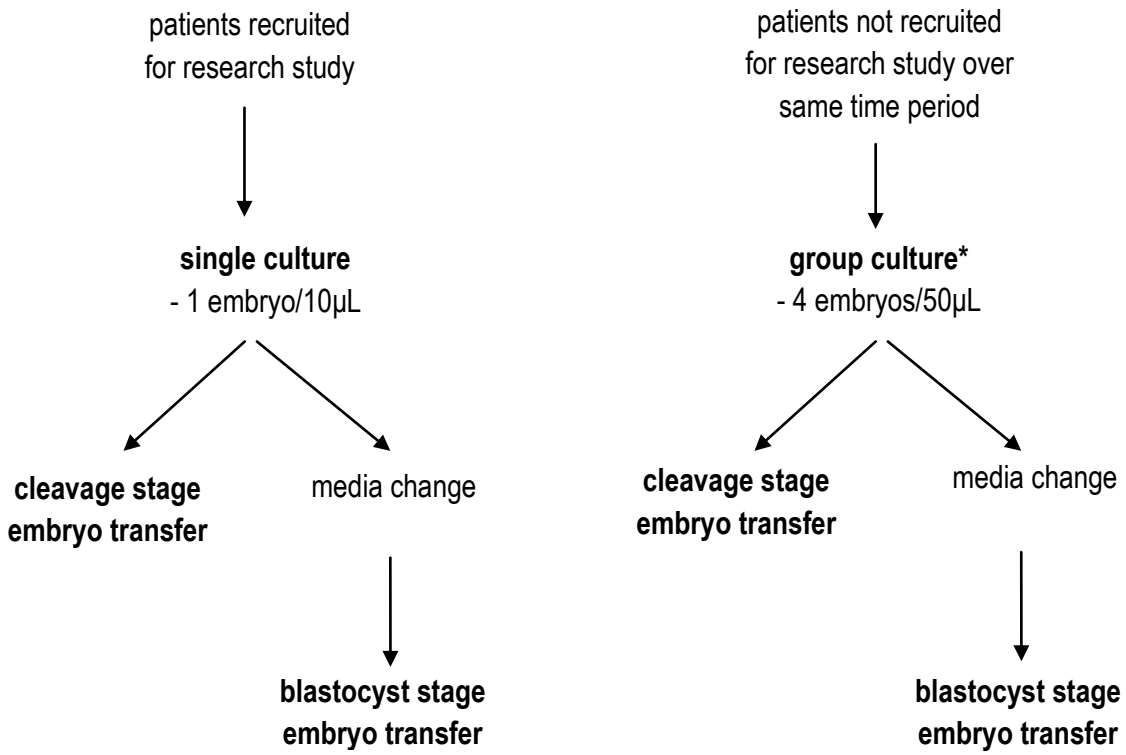


Figure 3.4 Human experimental design (experiment 5) detailing culture grouping under investigation.

* Patients not recruited for research underwent group culture as per the standard protocol of the laboratory at the time.

3.4 RESULTS

3.4.1 EXPERIMENT 1. Mouse embryo development rate.

Embryo development was assessed after 48 hours of culture (8-cell stage) and 90 hours of culture (blastocyst stage). There were no differences in embryo development rates between the two treatment groups (single and group culture) at 48 hours of culture (p=0.433) or between the three treatment groups (single culture, group culture or single → group culture) in the ability to form a blastocyst at 90 hours of culture (p=0.271, see Table 3.1). However, the proportion of blastocysts undergoing hatching at 90 hours was significantly lower when embryos were cultured singly, compared to grouped throughout culture (p=0.002), or cultured singly pre-compaction and grouped for the final period of culture (p=0.033, Table 3.1).

3.4.2 EXPERIMENT 2. Effect of embryo culture grouping on resultant blastocyst cell number and allocation.

Embryos cultured singly throughout the entire in vitro culture period had fewer inner cell mass (ICM) cells at the blastocyst stage (11.8 cells) when compared to embryos cultured either in groups for the whole period (18.0 cells, $p<0.001$) or singly pre-compaction followed by group culture post-compaction (16.0 cells, p=0.002, see Table 3.2). Furthermore, single culture had a reduced ICM: trophectoderm (TE) cell number compared to embryos cultured in groups throughout in vitro culture (p=0.047). The number of TE cells and total cell number were not altered by differing embryo culture groupings (p=0.32 and p=0.101 respectively).

3.4.3 EXPERIMENT 3. Effect of embryo culture grouping on apoptosis levels in blastocysts.

The proportion of apoptotic cells present in blastocysts resulting from embryos cultured in differing groups was not altered (p=0.372, Table 3.2).

Table 3.1 Effect of embryo grouping during in vitro culture on pre-implantation embryo development.

Culture Conditions	% development		
	8-cell stage (48 hours culture)	**Blastocyst** (90 hours culture)	**Hatching Blastocyst** (90 hours culture)
Group Culture	99.4 ± 0.01	95.5 ± 0.005	75.6 ± 3.2[a]
Single Culture	97.0 ± 0.01	95.7 ± 0.005	58.7 ± 4.4[b]
Single → Group Culture*	97.0 ± 0.01	98.9 ± 0.006	70.9 ± 3.6[a]

Data expressed as the number of embryos that were compacted or blastocysts respectively, as a proportion of the total number of embryos cultured ± SEM. Hatching blastocysts are expressed as a proportion of the embryos that were blastocysts ± SEM. Different superscripts within a column represent statistically significant differences. * Single → Group Culture represents embryos cultured singly for the pre-compaction period (48 hours) followed by grouping for the final period of culture. n=4 replicates.

Table 3.2 Effect of in vitro embryo culture grouping on blastocyst cell numbers.

Culture Conditions	n	ICM	TE	ICM:TE	TOTAL	% APOPTOSIS
Group Culture	31	18.0 ± 1.0[a]	47.1 ± 3.3	0.44 ± 0.04[a]	65.1 ± 3.4	1.8 ± 0.5 (37)
Single Culture	22	11.8 ± 1.3[b]	44.7 ± 3.7	0.30 ± 0.05[b]	56.5 ± 3.7	1.8 ± 0.3 (38)
Single → Group Culture	54	16.0 ± 0.9[a]	41.9 ± 1.6	0.39 ± 0.02[ab]	57.9 ± 2.2	2.3 ± 0.5 (35)

Different superscripts within a column represent statistically significant differences $p<0.05$. Data expressed as mean ± SEM. n values for % apoptotic cells indicated in parentheses. n=3 replicates.

3.4.4 EXPERIMENT 4. Effect of embryo culture grouping on embryo viability following transfer.

Embryo culture grouping was assessed following cleavage stage and blastocyst stage transfers. Cleavage stage embryo transfers assessed the viability of embryos grown either singly or in groups for the early cleavage stages of development. Blastocyst stage transfers assessed the viability of embryos grown;

- singly for the entire culture period,
- in groups for the entire culture period,
- singly for the pre-compaction stage (48 hours) and grouped for the final period of culture (single → group).

a) Cleavage stage embryo transfers (single v group culture)

Cleavage stage embryos were transferred to 19 recipients. In total, 28 uterine horns (14 from each treatment group) were studied. The remaining 4 non-pregnant females, and 2 non-pregnant uterine horns, which contained embryos from both treatment groups, were excluded from further analysis. It was assumed that lack of uterine receptivity played a role in females that were completely non-pregnant, and in the case of lack of pregnancy in individual uterine horns, technical error or uterine irritation during the transfer of embryos may have resulted in total absence of implantations. Implantation rates and the ability of a fetus to develop following implantation (viable fetuses/implantation) were not different between the two culture groups (p=0.890, Table 3.3). Placental weights were significantly reduced when embryos were cultured singly prior to transfer (p=0.021). Fetal weight, crown rump length and fetal: placental weight did not change as a result of culture treatment (p=0.394, p=0.455, p=0.142 respectively, Table 3.3).

b) Blastocyst stage embryo transfers (single v group v single → group)

Blastocysts were transferred to a total of 33 recipients, of which 27 were pregnant in at least one uterine horn on day 18 of pregnancy. Females who were not pregnant in both uterine horns contained embryos from all treatment groups (group = 5 horns, single = 2 horns, single → group = 5 horns), and were excluded from subsequent analysis. In addition, mothers who were completely non-pregnant in one uterine horn were also excluded from further analysis (single = 2 horns, single → group = 4 horns). In total, 48 uterine horns were studied (group n = 18, single n = 20, single → group n = 10).

Implantation rates were not altered as a consequence of embryo culture grouping when embryo transfers were performed at the blastocyst stage following group or single culture throughout development (p=0.865),

or if embryos were cultured singly initially followed by grouping (single → group, p=0.683 and p=0.843 respectively, Table 3.4). Fetal weight, crown rump length and placental weight were not altered between culture groups (p=0.475, p=0.701, p=0.792 respectively), and there was no effect of culture grouping on the fetal to placental weight ratio (p=0.843, Table 3.4).

Table 3.3 Effect of in vitro culture grouping on pregnancy outcomes with cleavage stage embryo transfers.

	Group Culture (14 uterine horns)	Single Culture (14 uterine horns)
Implantation rate [A] (%)	67.8 ± 5.0	73.3 ± 4.7
Viable fetuses/implantation [B] (%)	78.0 ± 5.4	80.3 ± 4.9
Fetal weight (mg)	792 ± 20	767 ± 20
Fetal crown-rump length (mm)	17.9 ± 0.2	17.6 ± 0.2
Placental weight (mg)	112 ± 3[a]	102± 3[b]
Fetal: placental weight ratio	7.3 ± 0.2	7.7 ± 0.2

Data expressed as mean ± SEM. Values represent 46 (group) and 49 (single) fetuses or placentas.

[A] Implantation rate - number of fetal/placental units or resorptions present as a percentage of the number of embryos transferred. [B] Viable fetuses/implantation - percentage of implanted embryos that developed into a viable fetus. Number of uterine horns per treatment indicated in parentheses beneath treatment group. Different superscripts within a row represent statistically significant differences $p<0.05$.

Table 3.4 Effect of in vitro culture grouping on pregnancy outcomes with blastocyst stage embryo transfers.

	Group (18 uterine horns)	Single (20 uterine horns)	Single → Group (10 uterine horns)
Implantation rate [A] (%)	81.3 ± 5.7	80.3 ± 5.3	77.7 ± 6.6
Viable fetuses/implantation [B] (%)	37.9 ± 6.5	46.5 ± 7.5	36.7 ± 9.5
Fetal weight (mg)	837 ± 26	864 ± 21	883 ± 30
Fetal crown-rump length (mm)	19.2 ± 0.3	18.7 ± 0.3	18.9 ± 0.3
Placental weight (mg)	117 ± 6	107 ± 5	117 ± 5
Fetal: placental weight ratio	7.6 ± 0.4	8.5 ± 0.3	7.8 ± 0.5

Data expressed as mean ± SEM. Values represent 29 (group), 39 (single) and 14 (single→group) fetuses or placentas
[A] Implantation rate - number of fetal/placental units or resorptions present as a percentage of the number of embryos transferred. [B] Viable fetuses/implantation - percentage of implanted embryos that developed into a viable fetus. Number of uterine horns per treatment indicated in parentheses beneath treatment group.

3.4.5 EXPERIMENT 5. Effect of embryo culture grouping on human embryo viability.

a) Cleavage stage embryo transfer

There was no significant difference in implantation rates when embryos were cultured in vitro in either groups or singly and transferred to patients at the cleavage stage (p=0.681, Figure 3.5).

b) Blastocyst stage embryo transfer

Transfer of embryos at the blastocyst stage following either single or group culture throughout the culture period, did not result in a significant difference in implantation rates (p=0.771, Figure 3.5).

Table 3.5 Patient demographic characteristics.

	Embryo Grouping		
	Single	**Group**	**Significance**
Number of patients	73	162	p=1.0
Maternal Age	34.2±0.4	34.1±0.4	p=0.511
% ICSI patients	72.6	77.2	p=1.0
Number of oocytes collected	9.7±0.6	8.5±0.4	p=1.0
Number of oocytes fertilized	5.4±0.4	4.9±0.3	p=0.746
% blastocyst transfer	23.0	25.9	p=1.0
Grade of transferred embryo	2.0±0.1	1.9±0.1	p=0.893
Infertility Diagnosis (%)*			p=0.585
- Male Factor	36 (51)	84 (53)	
- Endometriosis	3 (4)	14 (9)	
- Tubal	5 (7)	13 (8)	
- Ovulation/ovarian defect	13 (19)	25 (16)	
- Unexplained	12 (17)	17 (11)	
- Other	4 (6)	5 (3)	

There were no statistically significant differences between groups with any variables analysed. *Infertility diagnoses include both male and female factors, and are listed as the number of cases of each diagnosis. In some cases more than one category is applicable to a patient pair. Percentage infertility diagnoses are listed in parentheses.

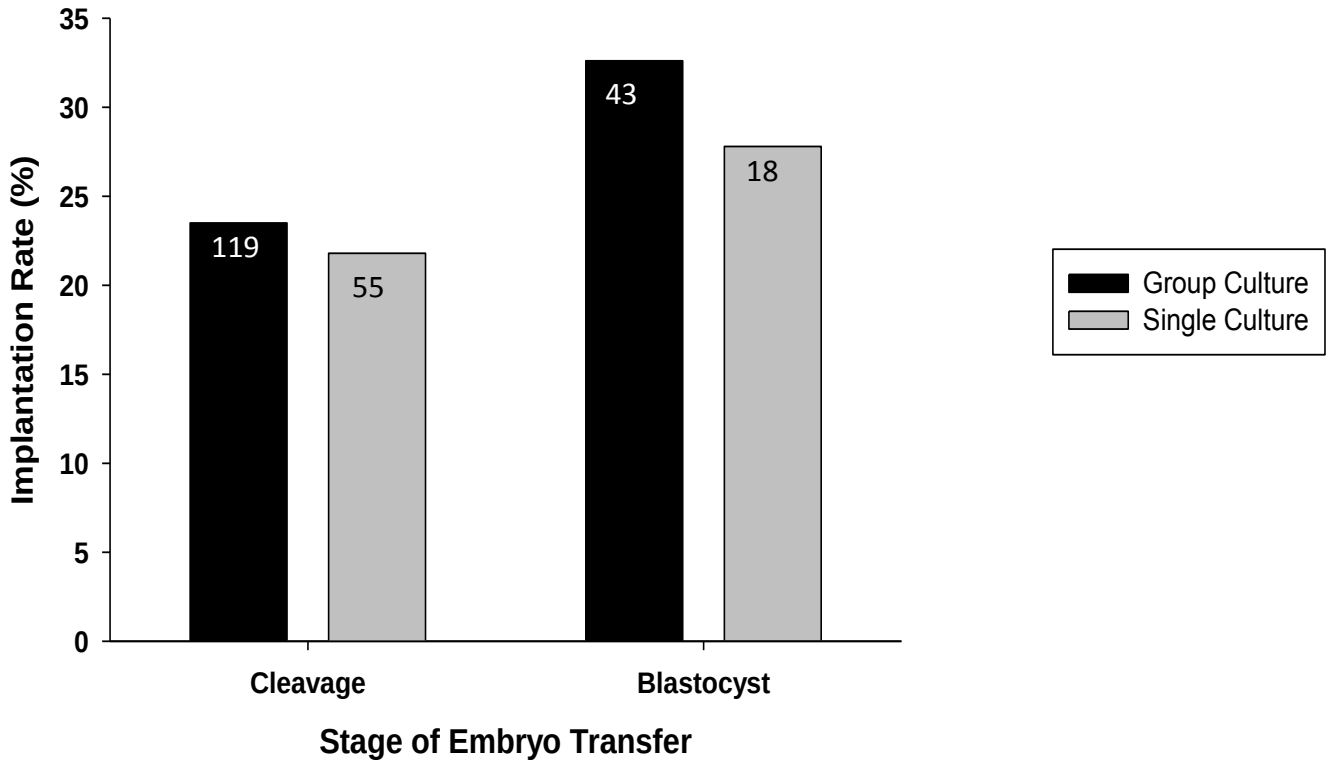


Figure 3.5 Human implantation rates following single and group culture with cleavage and blastocyst stage transfers. Number of patients in each group indicated in bars. All patients had elective single embryo transfers.

3.5 DISCUSSION

The widespread introduction of single embryo transfers in clinical IVF in order to reduce the incidence of multiple gestations is dependent on the ability to select the most viable embryo from a cohort for transfer. The ability to track single human embryos individually throughout development in the laboratory, significantly aids in the sequential assessment of development and therefore the choice of the highest quality embryo for transfer. This study aimed to assess the consequences of culturing embryos individually or in groups, and of cleavage and blastocyst stage embryo transfers under different grouping conditions, in mouse and human embryos, as well as investigating fetal and placental development following these in vitro culture conditions in the mouse. This data demonstrates that culture of mouse embryos singly in conditions that maintain the embryo: incubation volume to that of group culture did not affect the subsequent ability of the embryo to develop to the blastocyst stage or implant and produce a viable fetus compared to embryos cultured in groups. Additionally, human embryo implantation rates were not altered by in vitro culture groupings.

It has previously been demonstrated in animal models that in vitro culture in smaller volumes or in groups facilitates blastocyst formation, increases blastocyst development rate, blastocyst cell number and blastocyst viability (Schini and Bavister 1988, Paria and Dey 1990, Lane and Gardner 1992, Gardner et al. 1994, Keefer et al. 1994, Stoddart et al. 1996, O'Neill 1997). The current study contradicts many previous observations that single culture of mouse embryos during the pre-implantation period decreases blastocyst formation rates (Wiley et al. 1986, Paria and Dey 1990, Canseco et al. 1992, Lane and Gardner 1992, Keefer et al. 1994, Stoddart et al. 1996). No differences were observed in this study between cleavage rates and blastocyst development rates, which differs from the majority of the published literature performed in animals (Schini and Bavister 1988, Canseco et al. 1992, Lane and Gardner 1992, Gardner et al. 1994, Kato and Tsunoda 1994, Keefer et al. 1994, Fujita et al. 2006), although it should be noted that in many of these studies the methodologies differed in that embryos which were cultured in groups had smaller embryo: incubation volumes. Human studies (Almagor et al. 1996, Rijnders and Jansen 1999, Spyropoulou et al. 1999) and one bovine study (Carolan et al. 1995) that have been performed evaluating culture grouping are supportive of the observations in the present study with no difference being observed in cleavage rates and morphology. However these human studies observed embryos only until day 2 of development prior to embryo transfer, with the exception of Rijnders et al. (1999), who observed embryos to the blastocyst stage in a single tissue culture media system, and observed no difference in blastocyst development rates. This can potentially be explained by a number of factors incorporated in the culture system employed. Most significantly, embryos were cultured in medium containing pasteurized plasma as a protein source, which contains an undefined array of factors potentially capable of stimulating embryo growth. For

regulatory issues coupled with variations between batches, serum is not used in embryo culture systems today. This factor alone likely confounded the interpretation of the ability of embryos to develop to the blastocyst stage in group or single culture conditions as the milieu of factors in the medium is unknown.

The work presented in this chapter is the first density study to use a highly optimized sequential culture media, with recombinant albumin as a protein source, and to culture mouse embryos at low oxygen concentrations. This system could be expected to provide the embryo with greater developmental support than previously established simpler in vitro culture systems and may account for the success of the single culture embryos in this study. However, mouse blastocyst hatching rates were observed to be significantly reduced in singly cultured embryos in the present study. The apparent delay in blastocyst hatching of the singly culture embryos may be an indication of an overall delay in development, that has been previously reported for singly cultured embryos (Gardner et al. 1994, Keefer et al. 1994, Salahuddin et al. 1995). A reduction in blastocyst hatching rates has been observed in grouping studies in cases with and without maintenance of embryo density between singly and group cultured embryos. In single culture, when albumin or growth factors, epidermal growth factor (EGF) and transforming growth factor-β1 (TGFβ1) supplement the media, the proportion of blastocyst hatching is equivalent to group cultured embryos, while addition of these same factors to group culture does not further impact hatching rates (Keefer et al. 1994). This provides evidence for a role of autocrine factors in the stimulation of embryo development.

A prevalent argument in the literature suggests that the positive outcomes of culturing embryos in groups involves growth-promoting factors that one embryo can provide to another to assist in development (Schini and Bavister 1988, Paria and Dey 1990, Lane and Gardner 1992, Gardner et al. 1994, O'Neill 1998). Thus, if embryos are cultured singly, they would not benefit from any factors that may be secreted due to the lack of surrounding embryos in culture. Of the large volume of literature that has investigated culture groupings, very few of these studies deliberately maintained the embryo density of in vitro culture (Keefer et al. 1994, Fujita et al. 2006). As such, these are the only studies in which a direct comparison can be made regarding the production of any growth-promoting factor secreted by the embryo. In both studies, a decrease in blastocyst development rates was seen with single embryo culture compared to group culture. It can therefore be hypothesised that the benefit of stimulatory factors would likely only be evident in embryos which require additional support for development, in particular in meeting developmental checkpoints that are associated with development to the blastocyst stage. Thus the benefit of group culture may be that embryos with a higher intrinsic developmental capability are able to support the development of less capable sibling embryos through autocrine factors, to enhance their implantation potential. It is therefore expected that good quality singly cultured

embryos are producing these growth-promoting factors themselves, however in the case of poorer quality sibling embryos which might require additional stimulation, single culture is of no benefit and these embryos would have reduced developmental potential under such conditions.

Total cell numbers were not significantly altered by culture groupings in this study, however fewer ICM cells developed in embryos that had been cultured singly throughout the culture period, compared to embryos grouped either for the entire culture period, or only during the post-compaction period. Interestingly, the single → group culture blastocysts had an ICM cell number and total cell number lower than the group cultured embryos and higher than the single cultured embryos. This implies that either the post-compaction period is more critical for embryo density than the pre-compaction period or that crucial effects in the pre-compaction phase can partially be recovered post-compaction. This phenomenon of recovery has also been observed in bovine embryos where conditioned media was transferred from embryos grown in groups to embryos of the same developmental stage which were incubated singly. An improvement in blastocyst development rate was seen when the media of the singly cultured embryos was supplemented with media from group cultured embryos compared to embryos which were maintained in static single culture without media supplementation (Fujita et al. 2006). Multiple growth factor candidates have been shown to stimulate ICM development in embryos, which has been investigated through knockout mouse models (Feldman et al. 1995) and supplementation of growth factors to the embryo culture media (Harvey and Kaye 1990, Gardner and Kaye 1991, Rappolee et al. 1992, Feldman et al. 1995). Therefore with a reduction in ICM cell number observed, it is still possible that autocrine factors play a considerable role in optimal embryo development, however this role may not be a critical one. An alternative explanation for the reduction in ICM in singly cultured embryos may be that cells were undergoing apoptosis at an increased rate. This was determined not to be the case in the current study, with similar low levels of apoptosis observed throughout the blastocysts cultured individually and in groups. There is evidence that apoptosis in the pre-implantation embryo is regulated by growth/survival factors acting in an autocrine or paracrine manner such as transforming growth factor-α and members of the insulin-like growth factor family (Brison 2000). A reduction in ICM cell number has been associated with embryos cultured individually previously, compared to a larger number of embryos cultured in the same volume (Brison and Schultz 1997). However, in the same study a ~50% increase in cell death in the ICM and trophectoderm in embryos cultured singly was seen, which was not observed in the current study.

The most stringent test of embryo viability is to perform embryo transfers and investigate subsequent developmental outcomes. This study found no difference in the ability of embryos to implant when

cultured individually or in groups with the same embryo: incubation volume prior to transfer at the cleavage or blastocyst stage. It could have been expected from my results that embryos cultured singly would be less viable than their group cultured counterparts, as reduced ICM cell number and total cell counts are correlated with a reduction in embryo viability following transfer (Lane and Gardner 1997b). Therefore, it would not have been surprising given the reduced ICM, for singly cultured embryos to have reduced implantation or fetal development rates, however it has also been demonstrated that blastocyst viability is not always correlated with a greater ICM cell number (Banwell et al. 2007). It is feasible that there is a threshold proportion of ICM development and total cell counts required to adequately support development and although singly cultured embryos in the present study had significantly reduced ICM development compared to group cultured embryos, this was not adequate to impact on implantation and subsequent development. Further, it may be that the number of epiblast cells within the ICM were not altered between the treatments thus not affecting fetal development.

To establish the effect of single versus group culture on the pre-compaction stage of development, embryos were returned to the reproductive tract at the cleavage stage, to be exposed to a complex milieu of factors, unable to be replicated in vitro. Transfer of embryos that had been grouped or singly cultured at the cleavage stage of development, did not influence implantation or fetal development rates in human (with single embryo transfers) or in mouse. This supports a previous study of human embryos where despite the transfer of multiple embryos, no difference in implantation rate was observed following group or single culture (Spyropoulou et al. 1999). Alternatively, it has also been observed in human that group culture benefits embryos transferred at the cleavage stage with higher implantation rates (Moessner and Dodson 1995, Almagor et al. 1996). However, again multiple embryos were transferred in these studies, highlighting the requirement for a single embryo transfer study to be performed in this area. A further significant consideration in these studies is the volume of media that embryos were cultured in. Moessner et al. (1995) and Almagor et al. (1996) cultured embryos in 1000μL and 700μL of media respectively, either singly or grouped, which based on animal studies investigating the ideal embryo density for in vitro culture, is a large volume (Canseco et al. 1992, Lane and Gardner 1992, Gardner et al. 1994, Keefer et al. 1994). In addition, any autocrine stimulatory factors would most certainly have been significantly diluted in the single culture of an embryo in a volume as large as 700-1000μL, while in smaller volumes i.e. 20μL, stimulatory factors would have a higher likelihood of influencing quality. Furthermore, previous studies have incorporated up to 15% serum in their embryo culture systems (Moessner and Dodson 1995, Almagor et al. 1996, Rijnders and Jansen 1999), which contains a complex undefined array of growth factors, which may potentially make the embryo density less critical when investigating cellular development. This factor was considered by Spyropoulou et al.

(1999) who used a synthetic serum replacement as a protein source, and despite multiple embryo transfers, saw no difference in implantation rates between group and single culture.

Following transfer of blastocysts to both mouse and human after single and group culture, no differences in implantation rates were observed. Previously, one study in the human has explored the effect of embryo grouping and embryo culture volume on viability. This study grouped all embryos until day 3 of development and then investigated group and single culture in both large and smaller volumes of media for development to the blastocyst stage prior to embryo transfer (Rijnders and Jansen 1999). No difference in blastocyst formation or implantation rates for either grouping or volume of culture media was found which supports the current study, despite the employment of different protocols. Additionally, no effect was found on mouse fetal viability following embryo transfer of group or singly cultured embryos at the cleavage or blastocyst stage. However, placental weights were significantly reduced when embryos were cultured singly prior to cleavage stage embryo transfer. Interestingly, the blastocyst stage transfers did not have statistically significant differences in placental weights. Fetal weights were unaltered despite a difference in placental weight for embryos transferred at the cleavage stage, suggesting that compensatory changes occur in the placenta to maintain function (Constancia et al. 2002, Wyrwoll et al. 2009). However, the longer-term effect on the development of the offspring in this case is unknown and warrants further study.

This study requires further investigation into fetal outcomes to conclude the full impact of group and single culture of embryos. Live birth outcomes were not assessed in human or the mouse, which may provide valuable developmental data, specifically any effect of reduced placental weight on offspring. It would be valuable to assess human live birth data, as human and mouse placentation differ and the differences observed in mouse placental weights may not be applicable to humans. Alternatively, further investigations into the altered mouse placental weights could be undertaken including morphometry analysis to determine any impact on placental junctional zone size (exchange region) or anomalies. Furthermore, gene expression of placental growth related genes may be warranted.

Poor prognosis patients, particularly those with advanced maternal age and low quality oocytes, may potentially be further disadvantaged with the employment of single culture. Mitochondria play a vital role in the oocyte through provision of ATP for fertilization and preimplantation embryo development. Poor quality oocytes have been shown to have perturbed mitochondrial function which reduces developmental competence (Muller-Hocker 1996 Mol HR). Therefore the existence of any stimulatory

factors that one embryo may provide to another when cultured in groups may provide benefit to poorer quality embryos, while removal of these stimulants by performing single culture may impart a more stressful environment for low quality oocytes.

A small statistically insignificant difference was observed between single and group culture for the good prognosis patient population assessed in this study. Recent studies have illustrated that very large sample sizes are required to determine an effect of a subtle treatment such as embryo density, and often these are only found when assessing live birth rates (Meintjes et al. 2009). A power analysis based on the preliminary human data in this study, for patients with a maternal age less than 38 years, indicates for a prospective randomised controlled study 143 patients with cleavage stage transfers and 74 patients with blastocyst transfers would be required in each arm of the study to detect a significant difference between single and group culture.

Single culture provides the ability to track embryos throughout development in the clinical laboratory, significantly aiding in the sequential assessment of development from oocyte and pronuclear morphology through the early cleavage stages and therefore choice of the highest quality embryo for transfer. This study has demonstrated no difference between single and group embryo culture in human embryo implantation ability at the cleavage or blastocyst stage. In a mouse model, differences were seen in ICM cell number of singly cultured embryos, which did not appear to impact on subsequent ability to implant. Furthermore, a decrease in placental weights was observed with single culture of mouse embryos, which did not affect fetal weight however other consequences remain unknown. Neither of these parameters was able to be assessed in the human. Further studies would be required to determine the impact of these factors on offspring. Single culture of embryos with other than high intrinsic developmental potential may be more likely perturbed than if the same embryos were group cultured and able to take advantage of interactions between embryos. Therefore single culture of human embryos should only be employed with caution, particularly in women with advanced maternal age, for which it is known that embryo developmental potential is reduced.

CHAPTER 4

DAY 4 EMBRYO TRANSFER AS A MORPHOLOGICAL EMBRYO SELECTION TOOL

Portions of the data presented in this chapter have been published as follows;

Feil D, Henshaw R, Lane M (2008) Day 4 Embryo Selection is Equal to Day 5 using a new Embryo Scoring System Validated in Single Embryo Transfers. *Human Reproduction* 23 (7) p1505-1510. (see Appendix 5).

4.1 INTRODUCTION

In clinical IVF the majority of embryos are transferred on day 2 or 3 following insemination (Wang et al. 2009). Replacement of embryos at this stage involves transferring embryos that are governed by the maternal genome (Braude et al. 1988). Implantation is more likely to occur when an embryo that has developed beyond embryonic genome activation is transferred (Gardner and Lane 1997). Critical stages of development and indications that this has successfully occurred are compaction and blastulation of the embryo (Veeck and Zaninovic 2003). Furthermore, there is significant selection advantage, as embryos that are unable to activate the embryonic genome or complete compaction and/or cavitation can be easily identified and excluded for transfer (Balaban et al. 2000, Behr et al. 2000). It is reported that only 48% of embryos selected for transfer on day 3 are subsequently re-selected for transfer at the blastocyst stage (Graham et al. 2000), indicating the importance of the increased selection value of extended culture.

In animal models the majority of embryos arrest development following transfer of early developmental stages to the uterus (Noyes and Dickmann 1960, Moore and Shelton 1964, Newcomb and Rowson 1975). As a result, animal embryos, particularly from domestic species, are routinely transferred at the blastocyst stage (Hasler 2003). One of the clear advantages of blastocyst transfer is that the embryo is returned to the uterus at a stage of development where it would normally reside (Gardner et al. 2002). In vivo, the human blastocyst moves from the oviduct into the uterus on day 4 of development (Gardner et al. 2002), and at this stage the embryo has compacted and is developing a transporting epithelium (Biggers et al. 1988). This is a critical window in the development of the mammalian embryo, as acquisition of the ability to regulate its own physiology enables precise homeostatic control (Lane 2001). At this stage, as discussed earlier, the embryo has also activated its own genome. Therefore, it was postulated that the transfer of embryos on day 4 of development may result in the selection benefits of blastocyst transfer, while limiting the period of time in culture and burden on the IVF laboratory. Therefore, this study retrospectively assessed the effect of day 4 embryo transfer on implantation rates following single embryo transfer (SET) compared to cleavage stage SET (day 2 or 3) and day 5 SET.

4.2 MATERIALS AND METHODS

4.2.1 Patient Cohorts

Two cohorts of patients/embryos were assessed for this study. The first cohort of patients, treated between March-May 2006 (n=168 patients) had embryos observed only on day 4 of development, with no other change to clinic protocols. From the observations in cohort 1, a day 4 scoring procedure was described and the second cohort of patients included outcome assessment of day 4 embryo transfers. All patients in the second cohort underwent a SET between June 2006 and January 2007 at Repromed, Adelaide, Australia (n=705), excluding only treatment cycles that incorporated donor gametes or pre-implantation genetic diagnosis (PGD). The decision for a cleavage stage (day 2-3) or extended stage (day 4-5) embryo transfer was made prior to the commencement of treatment between the patient and their clinician. This decision was only altered if there was only a single embryo fertilized. Patients who had consented to an extended stage embryo transfer underwent a day 4 embryo transfer if their oocyte collection was on a Monday or Tuesday, while oocyte collections on Wednesday-Friday had a day 5 embryo transfer. All patients were stimulated using a long down-regulation protocol, as described in Chapter 2, Materials and Methods.

4.2.2 Culture Conditions

All laboratory procedures occurred as described in Chapter 2, Materials and Methods. Embryos were cultured in groups of 4 in 50µL drops of medium under oil. Cryopreservation for patients undergoing a day 4 embryo transfer was performed only on days 5 and 6 of development, as for day 5 embryo transfers.

4.2.3 Statistics

Data are presented as percentages or mean ± SEM, where appropriate. Proportion of patients undergoing ICSI, fertilization rates, infertility diagnoses and implantation rates underwent chi-square analysis. Remaining demographic data were analysed by One-Way ANOVA. Bonferroni post-hoc tests were applied, while Dunnett's T3 post hoc tests were applied for data that had unequal variances. Linear regression of implantation rates resulting from the transfer of embryos of different grades on day 4 of culture was assessed using Pearson's correlation coefficient test. P values <0.05 were considered to be statistically significant.

4.2.4 EXPERIMENT 1. Establishment of a day 4 morphological scoring system for human embryo development.

Morphology was assessed on day 4 of embryo development for all patients undergoing IVF treatment with extended culture (n=168 patients, 996 embryos) between March-May 2006. All embryos were observed and described by appearance to be classified into categories. These categories were subsequently used to develop a scoring system for day 4 human embryos.

4.2.5 EXPERIMENT 2. Assessment of the newly developed morphological grading system for day 4 human embryos.

The morphological grading system for embryos in Experiment 1 was implemented in a human IVF clinic to assess its effectiveness for embryo viability selection prior to embryo replacement. The developed grading system was distributed amongst embryologists in the clinic. Patients undergoing extended culture had an embryo replacement on either day 4 or day 5, dependent on the day of oocyte retrieval. On the morning of transfer for patients having a day 4 embryo transfer, embryos were graded according to the developed criteria and the highest morphological scoring embryo available was selected from the patient's cohort of embryos to be replaced. Similarly for day 5 embryo transfers, on the morning of transfer embryos were graded according to the scoring system developed by Schoolcraft and Gardner (see Appendix 3, (Gardner and Schoolcraft 1999)). Pregnancy was determined by a positive serum β-hCG result with viable implantation confirmed by ultrasound 5-6 weeks after embryo transfer.

4.2.6 EXPERIMENT 3. Comparison of implantation rates for day 4 embryo transfer to day 2-3 and day 5 embryo transfers.

Implantation rates for patients having a cleavage stage (day 2/3, n=388), day 4 (n=124) or day 5 (n=193) embryo replacement were compared for SETs in unselected patients. Pregnancy was determined by a positive serum β-hCG result with viable implantation being confirmed at ultrasound 5-6 weeks after embryo transfer.

4.3 RESULTS

4.3.1 EXPERIMENT 1. Establishment of day 4 morphological scoring system for human embryo development.

The range of embryo morphologies observed for embryos assessed on day 4 is detailed in Figure 4.1. There was a large range of developmental stages observed; a small number of embryos were undergoing cavitation and were able to be scored on a blastocyst scoring system (n=10, 1%), some embryos were yet to compact and still exhibited all the characteristics of a cleavage stage embryo (n=340, 34.1%) and other embryos appeared to have arrested (n=177, 17.8%). However, the majority of the embryos on day 4 were exhibiting some degree of compaction, either complete or partial (n=226, 22.7%, n=243, 24.4% respectively, Figure 4.1). A scoring system based on the range of morphologies observed on day 4 was developed to enable selection of high morphological quality embryos for replacement. The scoring system developed to grade embryos on day 4 of culture is shown in Figure 4.2.

4.3.2 EXPERIMENT 2. Assessment of the newly developed morphological grading system for day 4 human embryos.

The resultant implantation rates for the day 4 scoring system with regard to embryo stage are shown in Figure 4.3. A linear decrease in implantation rate with grade was observed on day 4 of culture (Pearson's coefficient r=-0.249, p=0.036). Interestingly, embryos with partial compaction on day 4 were shown to be as viable as completely compacted embryos when transferred, and as such have been included in the scoring system as eligible for assignment as a Grade 2 embryo, providing no additional morphological anomalies were present.

4.3.3 EXPERIMENT 2 and 3. Patient demographics.

Patient demographic characteristics are shown in Table 4.1. There was no difference in maternal age between treatment groups of day 2-3, day 4 or day 5 embryo transfers. The number of previous cycles undergone by patients and the rates of ICSI and IVF in the different embryo transfer day groups were also not significantly different. However, patients that had a transfer of a day 2/3 embryo had fewer oocytes collected and lower fertilization rates, compared to patients having a day 4 or 5 embryo transfer (p=0.0001, p=0.001 respectively). The grade of embryos transferred on day 5 (2.2±0.08) was significantly poorer than the grade of transferred embryos on days 2/3 or day 4 (1.6±0.05 and 1.5±0.05, respectively, p=0.0001). In addition, fewer embryos were frozen when embryos were transferred on day 4 or day 5 (3.0±0.2 and

2.7±0.2 respectively) compared to embryos being transferred at the cleavage stage (3.7±0.2, p=0.029 and p=0.0001 respectively).

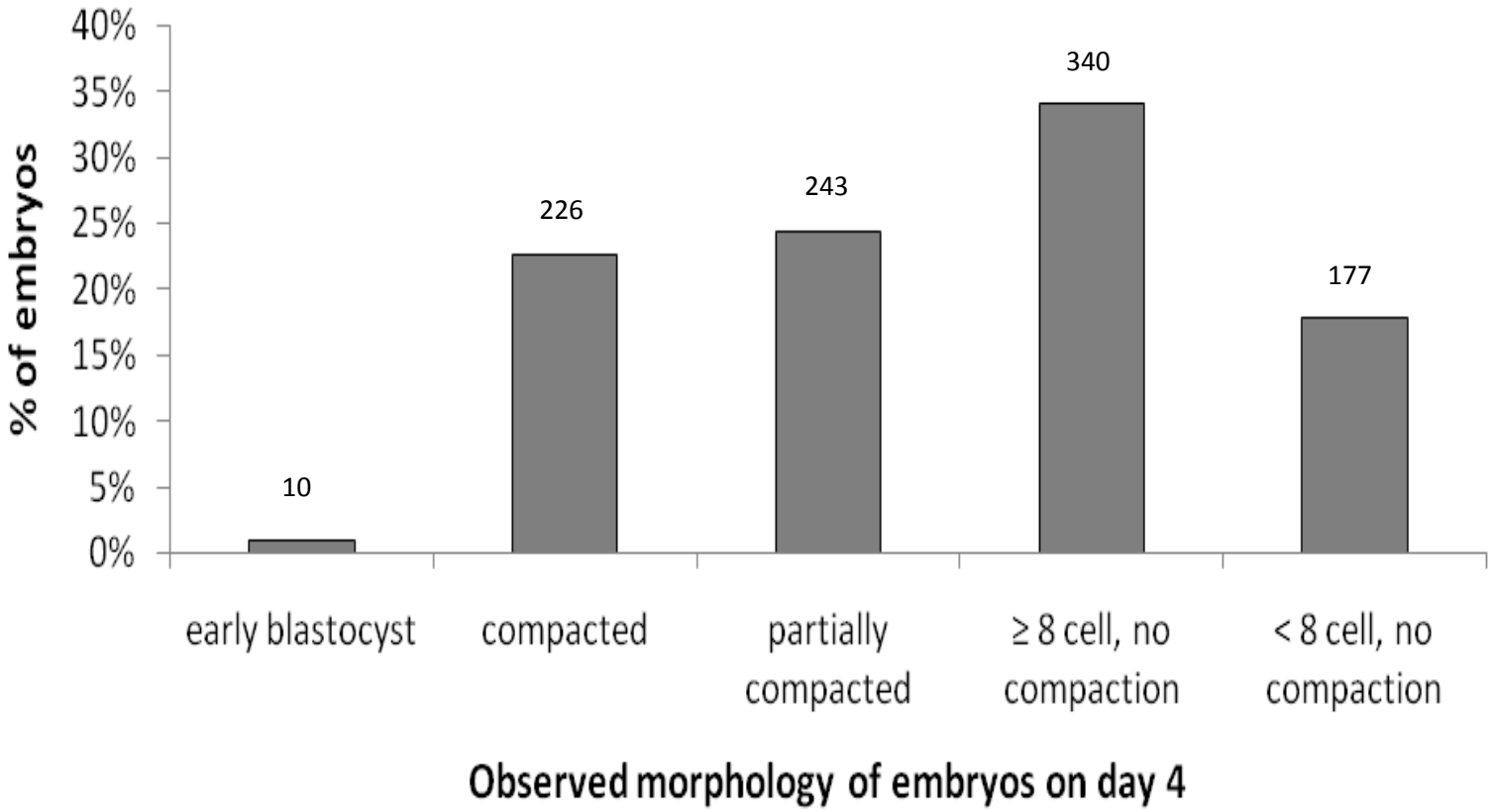


Figure 4.1 Range of morphologies observed on day 4 of human embryo development.
Numbers above columns indicate the number of embryos observed in each category.

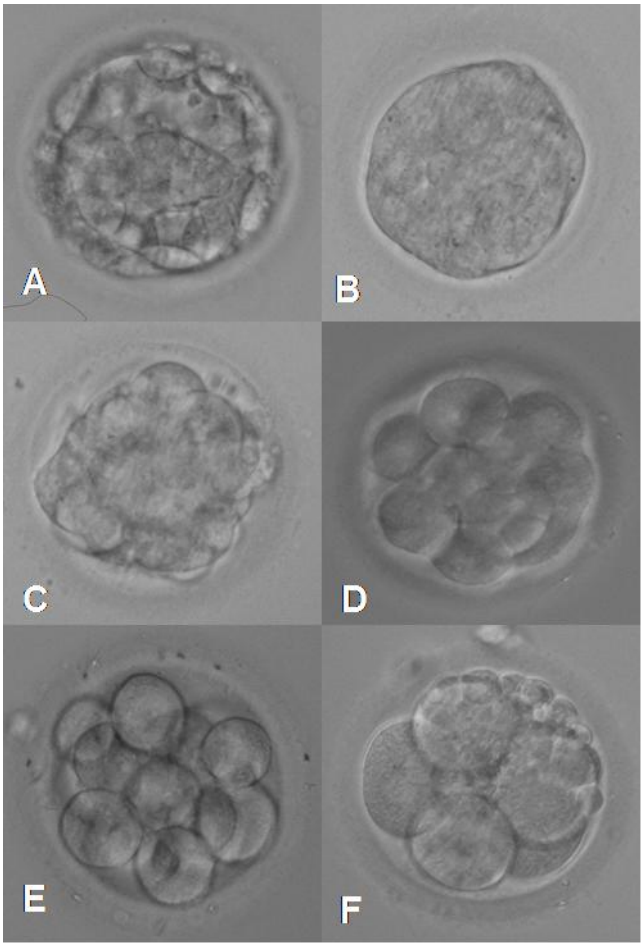


Figure 4.2 Scoring system developed for day 4 embryos.

Grade 1: Early blastocyst, visible signs of cavitation occurring **(A)** OR Completely compacted embryo, lacking morphological anomalies **(B)** e.g. Vacuolation, excessive fragmentation, large number of excluded cells, self cavitation of cells

Grade 2: Grade 1 compacted morula, with some morphological anomalies e.g. one of the following present, vacuolation, excessive fragmentation, excluded cells, self cavitation of cells OR At least an 8 cell embryo, with partial compaction present or the majority of cells at least showing signs of compaction **(C, D)**

Grade 3: Partially compacted embryo with vacuoles or excessive fragmentation present
OR 8 cell embryo or greater, with no signs of compaction evident **(E)**

Grade 4: Embryos with 8 cells or greater, with no sign of compaction and vacuoles or excess fragments OR Embryos with less than 8 cells, and no signs of compaction **(F)**

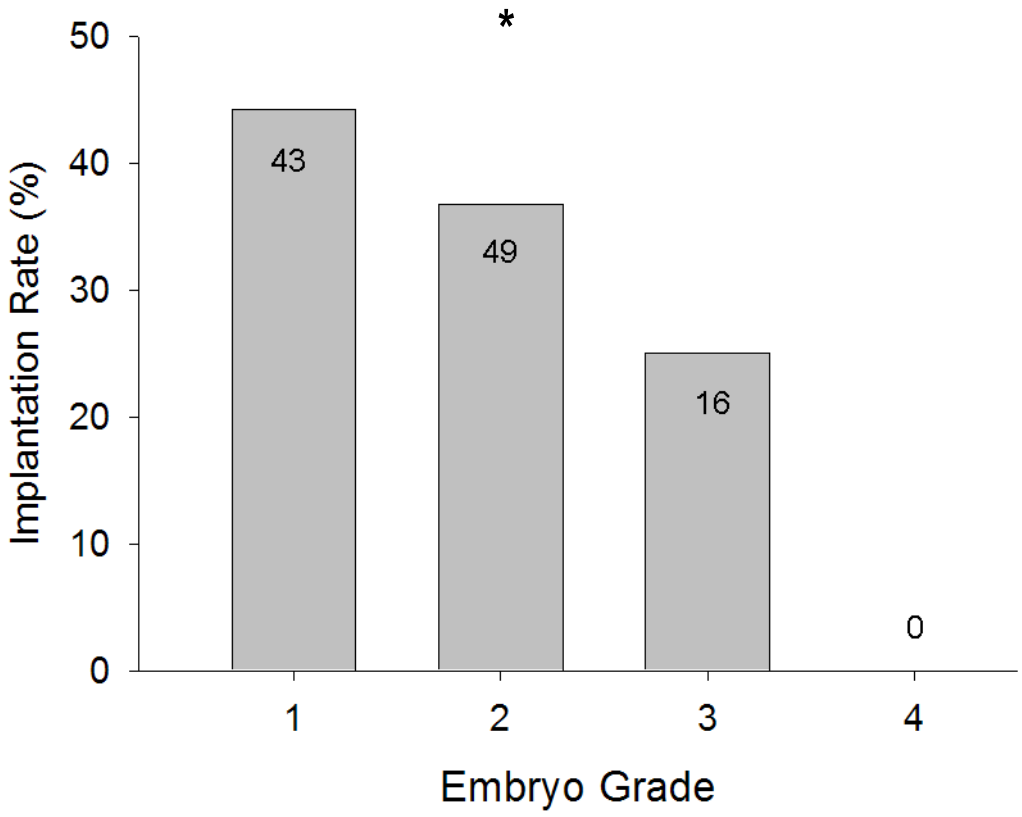


Figure 4.3 Implantation rates resulting from developed day 4 embryo scoring system with single embryo transfers.

* Pearson's coefficient r=-0.249, p=0.036. Numbers in columns indicate the number of embryos of each grade transferred to patients. No grade 4 embryos were selected for transfer during this study.

Table 4.1 Patient demographic characteristics for Experiment 2 and 3.

	Day 2/3 Transfer	Day 4 Transfer	Day 5 Transfer
Number of patients	388	124	193
Maternal Age	34.5±0.2	34.8±0.4	33.9±0.3
% ICSI	79.6%	82.3%	81.3%
Number previous IVF/ICSI cycles	0.88±0.08	1.25±0.14	0.94±0.10
Number of oocytes collected	8.8±0.3[a]	11.6±0.6[b]	11.8±0.4[b]
Percent fertilized	52.3%[a]	63.3%[b]	62.0%[b]
Grade of transferred embryo†	1.6±0.05[a]	1.5±0.05[a]	2.2±0.08[b]
Number of surplus embryos frozen	3.7±0.2[a]	3.0±0.2[b]	2.7±0.2[b]
Infertility Diagnosis % (n)*			
- Male Factor	58.5% (227)[ab]	63.6% (70)[a]	68.4% (132)[b]
- Endometriosis	8.8% (34)	9.1% (10)	9.3% (18)
- Tubal	10.6% (41)	13.6% (15)	9.8% (19)
- Ovarian	22.9% (89)	30.9% (34)	20.7% (40)
- Unexplained	16.5% (64)	16.4% (18)	12.4% (24)
- Other	2.8% (11)[a]	8.2% (9)[b]	2.6% (5)[ab]
Implantation rate‡	29.1% (113)	38.7% (48)	32.1% (62)

Variables with statistically significant differences are indicated by different superscripts within a row.
*Infertility diagnoses include both male and female diagnoses; in some cases more than one category is applicable to a patient pair. † It should be noted that different assessment systems were used to score embryos at each of the different days of transfer. ‡ Numbers in parentheses indicate the number of patients with an ongoing pregnancy.

4.3.4 EXPERIMENT 3. Comparison of implantation rates for day 4 embryo transfer to day 2-3 and day 5 embryo transfers.

Implantation rates for SETs at the cleavage stage (day 2/3), day 4 and day 5 are shown in Figure 4.4. Ongoing implantations were significantly increased following extended culture and embryo transfer on day 4 (38.7%) compared to cleavage stage transfer (29.1%, p=0.05). The higher implantation rate for extended culture with day 4 embryo transfer was further increased, compared to cleavage stage transfer, when implantation rates for only patients <38 years were examined (p=0.025, Figure 4.5). Implantation rates following extended culture of embryos and transfer on day 5 (32.1%) were not significantly increased compared to cleavage stage transfer (29.1%, p=1.0) in this study of SETs and unselected patients. Day 4 and day 5 embryo transfers combined also did not result in significant differences in resultant implantation rates to cleavage stage transfers (p=0.122).

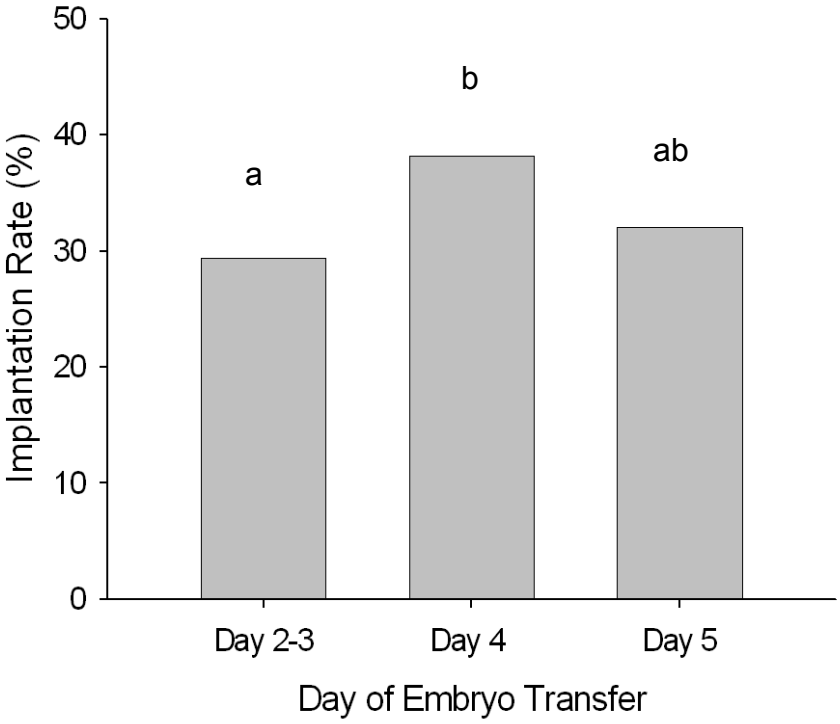


Figure 4.4 Implantation rates following single embryo transfer of cleavage and extended culture embryos.

Different superscripts indicate statistical significance, $p<0.05$.

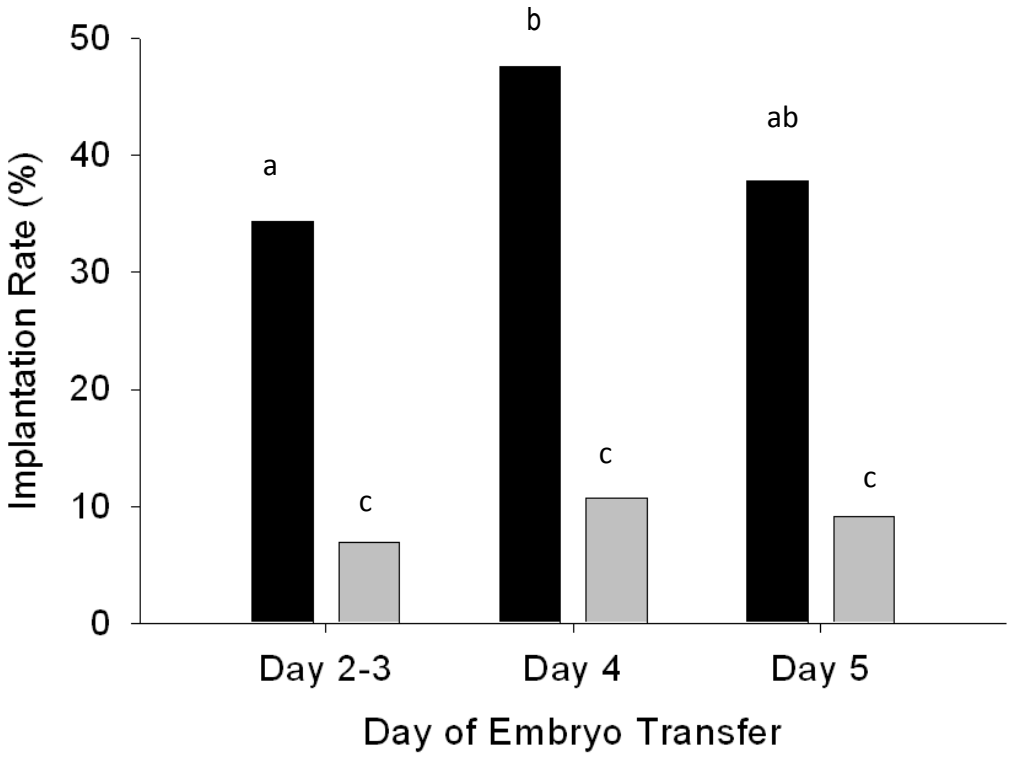


Figure 4.5 Effect of maternal age and day of transfer on implantation rates.
Dark bars indicate patients <38 years of age, light bars indicate patients ≥38 years of age. Different superscripts indicate statistical significance, $p<0.05$.

4.4 DISCUSSION

The ability to select the most viable embryo for transfer is an increasingly important aspect to an IVF cycle. Key considerations for selection of the most viable embryos include the assessment be non-invasive and that it occurs in a timely fashion that can easily occur within the operations of the clinical laboratory. As such, methods to increase selection based on embryo morphology are an attractive option. My data, though observational and retrospective, suggests that transfer of day 4 human embryos is a viable alternative to the transfer of embryos on day 5.

Human embryo development on day 4 has largely been neglected as a morphological marker of viability and was initially implemented following embryo biopsy for PGD, when embryo transfer required a delay in order for genetic testing to be performed and reported (Grifo et al. 1998, Gianaroli et al. 1999). Two previous morphological scoring systems have been described for human embryos on day 4. Huisman and colleagues (2000) described day 4 embryos as advanced (cavitating), average (compacted) or retarded (4-12 cells). Alternatively, a particularly detailed scoring system was described by Tao et al. (2002), which classified embryos initially as compacted or non-compacted embryos. This system further catalogued embryos with specific emphasis on the proportion of cells undergoing compaction, with consideration for the morphology of the compacted embryo, fragmentation and embryo quality on day 2/3 (Tao et al. 2002). Both previously described day 4 morphology scoring systems have assessed resultant pregnancy rates, however Tao et al. (2002) generally transferred 4 embryos, with occasions where 6 embryos were replaced, and Huisman et al. (2000) transferred 2 embryos to the majority of patients, and occasionally transferred a single embryo. The transfer of multiple embryos and mixed quality of embryos in previous studies make the validation of any scoring system difficult to analyse. Nevertheless, it was shown that the good quality graded embryos in both systems had a higher implantation rate than the poorly graded embryos (Huisman et al. 2000, Tao et al. 2002). In addition, a significantly higher implantation rate resulted from embryo transfer on day 4 compared to day 3, with fewer embryos being replaced for day 4 embryo transfers (Tao et al. 2002). The lack of implementation of a day 4 scoring system and embryo transfers in routine embryology thus far is likely based on the deficiencies of the morphological scoring systems that have been described to date. Any scoring system needs to be able to be easily incorporated into a laboratory while providing sufficient morphological description to differentiate embryos with higher viability from embryos with reduced potential. Previous attempts at describing morphological scoring systems have demonstrated that a compromise must be reached between ease of assessment and extremely detailed descriptions of embryo appearance. For example, blastocyst grading was first described by Dokras et al. (1993), which mainly incorporated the degree of blastocyst expansion (Dokras et al. 1993). Blastocyst morphology grading was further developed by Gardner and Schoolcraft (1999) before it became widely accepted, and included

assessment of expansion as well as visual estimation of ICM and TE cell number and quality (Gardner and Schoolcraft 1999). The day 4 scoring system described in the current study is simple to learn as it incorporates descriptive factors similar to a cleavage stage scoring system, however the diversity of morphological changes observed on day 4, in particular the degree of compaction, which is very easily observed, has a significant impact on the post-transfer outcome and is simple to teach. All embryologists in the clinic performed embryo assessments and selection of an embryo for transfer, which demonstrates the ease and consistency with which the grading system was introduced to the clinic. Furthermore, this grading system had a linear correlation between embryo grade and pregnancy rate, as well as improving embryo selection over cleavage stage embryo transfers.

The patients involved in this study were unselected and were comparable between groups. Patients having a cleavage stage transfer had fewer oocytes collected and a lower fertilization rate than patients having either a day 4 or day 5 embryo transfers, which was not caused by a difference in maternal age of patients or the proportion of patients undergoing ICSI or IVF. It could be postulated that the reduction observed in oocytes retrieved and fertilization rate in the cleavage stage embryo transfer group is likely a patient bias, where those who were expected to have a poor response to stimulation, as determined by ovarian reserve markers prior to treatment, were advised by their clinician to have a cleavage stage transfer. This would explain both the lower number of oocytes retrieved in this cohort of patients and the reduced fertilization rate (Lekamge et al. 2007), despite the similar maternal age to the other groups. Other differences that were observed between groups in the study were the grade of transferred embryo, which was significantly reduced in patients having an embryo transfer on day 5. This is not an unexpected result as different morphology grading systems were used for each stage of transfer, and a top quality blastocyst grading has extremely restrictive criteria. In addition, fewer embryos were frozen in both extended culture embryo transfer groups, which is result of both of these groups only having cryopreservation on days 5 and 6 of development, as day 4 cryopreservation was not implemented in this study. Furthermore, fewer embryos eligible for cryopreservation are expected as extended culture is a selection tool, with which fewer embryos are able to achieve good blastocyst formation (Blake et al. 2007).

This study is unique to the current literature as it is the first to analyse implantation rates following the transfer of single embryos on day 4 of development. Previous studies (Huisman et al. 2000, Tao et al. 2002, Margreiter et al. 2003, Skorupski et al. 2007) transferred up to four embryos to the uterus at one time, making the determination of which embryos resulted in viable implantations difficult. All SETs performed in the clinic were included in this study, excluding cycles that involved donor gametes or

PGD. Although patients included in the analysis for this study were non-selected, it must be noted that the clinic has a Single Embryo Transfer Policy, which recommends patients under the age of 38 have a single embryo transferred. However, unlike previous studies, the decision of whether a patient would have a transfer following extended culture, was not based on the patient's response to gonadotrophins, the number of pronuclear embryos available or embryo quality at the cleavage stage (Gardner et al. 1998a, Coskun et al. 2000, Karaki et al. 2002, Levron et al. 2002, Rienzi et al. 2002, Bungum et al. 2003, Emiliani et al. 2003, Frattarelli et al. 2003). Rather, this decision was made clinically, prior to the commencement of the patient's treatment. Patients were only converted from an extended culture transfer to a cleavage stage transfer in the event that there was no selection advantage to be gained by growing embryos for an extended period (i.e. there would be only one embryo in the patient's cohort).

The increased implantation rate previously described for blastocyst transfer on day 5 over cleavage stage embryo transfers was not observed for transfer of day 5 embryos in this study. The lack of difference between cleavage stage and day 5 embryo transfer pregnancy rates has previously been suggested to be due to patients having an embryo transfer regardless of whether there was a formed blastocyst on day 5 and therefore there were no transfer cancellations (provided the embryo had at least undergone compaction by day 5). However, if only the patients that had a good quality embryo replaced at day 5 or day 2/3 are compared (a blastocyst on day 5 or an 'on-time' unfragmented embryo at the cleavage stage), there was again, no difference in resultant implantation rates (37.0% compared to 35.1% respectively, data not shown). Therefore, in our clinic with unselected patients there is no added selection with blastocyst transfers compared to cleavage stage transfers, which further supports the proposal that blastocyst transfer is beneficial only in selected patient populations (Gardner et al. 1998a, Coskun et al. 2000, Karaki et al. 2002, Levron et al. 2002, Rienzi et al. 2002, Bungum et al. 2003, Emiliani et al. 2003, Frattarelli et al. 2003).

An increase in implantation rate was seen for embryos transferred on day 4 compared to cleavage stage transfers in the current study. However, day 4 embryo selection provided no additional selection advantage over day 5 embryo transfer. A previous study comparing cleavage stage (day 2/3) and extended stage (day 4/5) transfers in a prospective randomised multi-centre study showed an increase in implantation rate in extended stage compared to day 2/3 transfers (Margreiter et al. 2003). However, day 4 and day 5 pregnancy rates were reported as one result in this study, so a true comparison of day 4 and day 5 pregnancy rates cannot be made. Others have observed no improvement in pregnancy rates resulting from day 4 embryo transfers compared to cleavage stage transfers (Huisman et al. 2000, Tao et al. 2002). However, when Huisman and colleagues (2000) excluded transfers of embryos with

delayed developed from their analysis, a significant improvement in pregnancy rates for day 4 transfers was noted. Furthermore, with significantly fewer embryos being transferred, a similar pregnancy rate was achieved for extended culture compared to cleavage stage embryo transfers (Tao et al. 2002).

One of the clear physiological differences between cleavage and blastocyst stage transfers is the synchronisation of the embryo with the stage of the reproductive tract that the embryo would normally develop in vivo. The cleavage stage embryo would normally reside in the oviduct, and traverse to the uterus on day 4 of development (Gardner et al. 2002). The surroundings of the oviduct differ significantly from the uterus in nutrient levels, gas environment and pH (Fischer and Bavister 1993, Gardner et al. 1996a), and this change in nutrient levels is reflected in modern embryo culture systems with sequential media. In addition, the levels of growth factors such as cytokines that are produced in the oviduct and uterus differ (Kane et al. 1997), which provide a complex milieu of factors that are completely excluded from an in vitro system. The embryo undergoes physiological changes in its early development that reflect the differing environments of the oviduct and uterus. As such, it has been suggested that the premature transfer of the cleavage stage embryo to the uterus may induce stress on the embryo, in particular metabolic stress (Gardner and Schoolcraft 1998, Lane 2001). However, the human embryo has a high degree of plasticity (Gardner and Lane 2004) and as evidenced by the high implantation rates noted in the current study, any adaptation to the uterine environment made by cleavage stage embryos did not impact on viability. Synchronisation of the tract and embryo with a day 4 transfer may facilitate continued development and implantation. Although this is supported by the day 4 implantation rates compared to the cleavage stage, it would be expected that a similar advantage would be obtained with a day 5 embryo transfer, which was not the case in this investigation. Perhaps a potential advantage to replacement of a day 4 embryo to the uterus includes an additional ~24 hours of exposure to uterine components compared to a day 5 embryo transfer.

One of the key advantages of a day 4 embryo assessment appears to be in the wide range of observed embryo development on day 4, from cleavage stages through to early blastocysts. Therefore, it appears that this is a dynamic time-period of development and that assessment at this time may assist in selection of an “on-time” developing embryo. It is well documented in both animal models (McKiernan and Bavister 1994, Gonzales et al. 1995) and in the human (Cummins et al. 1986, Van Royen et al. 1999, Racowsky et al. 2000) that the speed of development is an indicator of viability, with embryos that reach defined periods of development at a rate more similar to in vivo development having greater viability. A further advantage of day 4 morphology is that the scoring system is founded based on a cleavage stage scoring system. Therefore, an embryo can be selected for transfer quickly with the added benefit that there is no requirement for extra training that is necessary with blastocyst embryo selection for transfer.

A demonstrated benefit of blastocyst culture and transfer is the ability to determine which embryos have not yet activated the embryonic genome, which occurs at the 4-8 cell stage in the human (Braude et al. 1988). Thus those embryos with limited developmental potential in a cohort can be identified and excluded. My data, along with others (Huisman et al. 2000, Tao et al. 2002, Skorupski et al. 2007) has demonstrated this increased selection is evident with a day 4 embryo transfer.

The implementation of embryo transfers on day 4 of development has enabled flexibility for embryo replacements to be scheduled to suit the patient, clinician and laboratory. The overall laboratory impact has been minimal, as only the transfer occurs on day 4 with cryopreservation still being performed on days 5 and 6. Thus the introduction of day 4 embryo transfers was purely a mechanism to improve the management of treatment by providing flexibility for the patient, their clinician and the laboratory. Cryopreservation of morulae has been investigated and shown to be a viable cryopreservation option (Tao et al. 2004). Mutliple IVF clinics in Australia have now implemented the culture and transfer of embryos on day 4 (Repromed Adelaide, Repromed Darwin, Flinders Reproductive Medicine and Fertility SA , personal experience Feil, D 2006-2009).

The transfer of day 4 embryos is beneficial in numerous ways; the embryo is returned to the uterus, to an environment where it would normally reside. This occurs post-genome activation to allow the embryo with the highest developmental potential to be selected from a cohort. An added advantage is being exposed to the uterine environment for the maximum time period, and an in vitro environment for a minimal time period before implantation. In addition, uterine contractility is reduced at this time (Fanchin et al. 1998, Lesny et al. 1998), all of which maximises the potential for implantation. The ability to transfer the embryo to the uterus on day 4 enables the benefits of embryo selection for transfer post-genome activation to be obtained with significantly less burden on the laboratory, and offers a viable alternative to day 5 transfers. It also has the advantage in a busy unit to enable flexibility as to the day of embryo transfer on day 4 or 5 without affecting ongoing pregnancy rates. Day 4 embryo selection appears to be an effective tool to maintain implantation rates in our laboratory, and as such the laboratory continues to perform day 4 embryo transfers to enable flexible treatment for patients. In order to further validate this observational data, a prospective randomised study should be undertaken to determine if day 4 embryo transfers result in improved pregnancy outcomes. A power analysis based on the preliminary data in this study, for patients with a maternal age less than 38 years, indicates for a prospective study 226 patients would be required in each arm of the study to detect a significant difference between day 4 and day 5 embryo transfers.

CHAPTER 5

HUMAN CUMULUS CELL REDOX LEVELS AS A MARKER OF EMBRYO VIABILITY

The work detailed in this chapter is covered by Australian provisional patent 2007904744, filed by Adelaide Fertility Centre and Adelaide Research and Innovation.

5.1 INTRODUCTION

Embryo quality and viability is ultimately determined by the health of the gametes from which the embryo is derived and therefore markers of oocyte quality are potential candidates to reflect the developmental potential of the resultant embryo (Hardy et al. 2002). Previous studies using oocyte donors or mathematical modeling have confirmed that the quality of the oocyte is a rate-limiting factor for the success of IVF procedures (Schoolcraft and Gardner 2000, Hardy et al. 2002). Therefore markers for embryo viability should include information on the quality of the oocyte. It is clear that although a large number of meiotically mature sibling oocytes (i.e. with an extruded first polar body) can be generated, these cohorts are heterogeneous with differing levels of developmental competence (Pellicer et al. 1987, Stouffer and Zelinski-Wooten 2004, May-Panloup et al. 2005, Scott et al. 2008). Therefore, it is likely that the molecular or metabolic profile of these oocytes differ.

A key component for the establishment of developmental competence during oocyte growth and maturation is the bi-directional communication between the oocyte and its surrounding cumulus cells (Sugiura et al. 2007, Gilchrist et al. 2008). The oocyte secretes factors (such as GDF-9 and BMP-15) that act on the surrounding follicular cumulus cells to stimulate growth and development and modulate their function. In turn, the cumulus cells signal to the oocyte to maintain development (Dong et al. 1996, Elvin et al. 1999a, Elvin et al. 1999b, Gilchrist et al. 2008). These oocyte derived growth factors regulate expression of several cumulus cell genes that control the metabolism of the cumulus cells (Elvin et al. 1999a, Elvin et al. 1999b). Therefore, the metabolic state of the cumulus cells surrounding the oocyte likely reflects the functioning of this communication loop, as well as the developmental environment of the follicle (Russell and Robker 2007). Evidence that the gene expression of the cumulus cells has the potential to indicate oocyte and embryo quality has recently been shown in patient cohorts (McKenzie et al. 2004, Cillo et al. 2007, Feuerstein et al. 2007, Assou et al. 2008, Hamel et al. 2008, Anderson et al. 2009). Further to this, cumulus cell gene expression has been correlated with pregnancy (Assou et al. 2008, Anderson et al. 2009), indicating cumulus cell parameters are reflective of oocyte quality and have potential as non-invasive biomarkers of implantation potential.

Normal cellular metabolism is dependent on enzymatically catalyzed reduction-oxidation (REDOX) reactions. The REDOX state refers to the ratio of oxidized and reduced forms of coupled reactions such as NADH/NAD+, NADPH/NADP+ and glutathione/oxidised glutathione and can be used to describe the cellular

environment (Dumollard et al. 2009). The cellular REDOX state is significantly influenced by factors that stimulate and neutralise reactive oxygen species (ROS). Oxidative regulation is important to protect cells from the harmful production of ROS, but also to ensure physiological levels of ROS are present as required, for involvement in some signaling pathways (Forman and Torres 2001). An imbalance in the production of ROS and the neutralizing antioxidant defence mechanisms of the cell results in oxidative stress (Agarwal et al. 2003, De Haan et al. 2003). The consequences of excessive ROS production and the resultant REDOX imbalance includes; mitochondrial damage, ATP depletion, embryo cell block and retardation, and apoptosis (reviewed by (Guerin et al. 2001)). Oxidative stress may be one of the factors that regulates ovarian follicle recruitment and growth, selection of the dominant follicle as well as follicular atresia (El Mouatassim et al. 1999, Goto et al. 2002, Lonergan et al. 2003). Furthermore, ROS have historically been of great interest in regards to pre-implantation development (reviewed by (Johnson and Nasr-Esfahani 1994, Guerin et al. 2001). In embryos, excessive ROS production is related to perturbed embryo development and viability (Nasr-Esfahani et al. 1990, Nasr-Esfahani and Johnson 1991). It is likely that disrupted REDOX levels in the cumulus cells would reflect a reductive or oxidative environment within the follicle that may subsequently reduce oocyte competence. It is proposed that a direct but non-invasive method to determine the functional vitality of oocytes is to measure cumulus cell physiology. Therefore the aim of this study was to retrospectively determine the REDOX state in cumulus cell masses from individual cumulus-oocyte complexes of patients undergoing IVF/ICSI. The REDOX state from each cumulus mass was correlated with embryo quality, by tracking the fate of each oocyte/embryo individually through single embryo transfer and birth outcome.

5.2 MATERIALS AND METHODS

5.2.1 Patient Selection

Twenty-six patients undergoing routine IVF/ICSI with Single Embryo Transfer (SET) at Repromed's Adelaide clinic between April and September 2006 consented for cumulus cells to be collected and analysed. All patients had treatment with their own gametes and were not candidates for pre-implantation genetic diagnosis. Ethical approval was obtained by the NHMRC sanctioned Human Ethics Committee of the Women's and Children's Hospital, Adelaide, South Australia.

5.2.2 Cumulus Cell REDOX Analysis

5.2.2.1 Cumulus cell collection and subsequent culture

COCs were isolated from follicular fluid as described in Chapter 2, Materials and Methods. For patients whose oocytes were destined to undergo either IVF or ICSI inseminations, COCs were trimmed of their outer layers of cumulus cells (n=250) with a 30G needle on a syringe, and cumulus masses for analysis were collected in GMOPS (Vitrolife) containing 5% HSA (Vitrolife) and maintained at 37°C (see Figure 5.1). Each cumulus mass and its respective oocyte were numbered to track which cumulus mass came from a particular oocyte. Oocytes undergoing ICSI (n=141) were then exposed to 75IU Hyaluronidase (Hyalase®, Aventis Pharma Pty Ltd, Lane Cove, Australia) in glucose supplemented GFert Plus medium and ICSI performed in G1.3 Plus prior to culture individually in 10µL drops of the same medium under oil for 16 hours, at 37°C 6%CO_2, 5%O_2, 89% N_2. Oocytes undergoing IVF (n=109) had the inner layers of cumulus left intact and were co-incubated with 1,000 motile sperm in 10µL drops of glucose supplemented GFert Plus under oil for 16 hours, at 37°C 6%CO_2, 5%O_2, 89% N_2. All oocytes underwent individual culture in order to track oocytes and resultant embryos with respect to fertilization, embryo quality, cryopreservation and embryos resulting in pregnancy following SET.

5.2.2.2 REDOX staining

Redox Sensor Red (2,3,4,5,6-pentafluorodihydrotetramethylrosamine, Redox Sensor Red CC-1, Molecular Probes, Invitrogen, Australia) was used as a fluorogenic indicator of cellular REDOX state (see Appendix 6). Redox Sensor Red enters cells passively and is oxidised to produce a fluorescent signal (Chintapalli et al. 2007). Human cumulus cell masses were incubated with 1µM Redox Sensor Red in GMOPS with 5% HSA (MOPS-HSA, Vitrolife), for 15 minutes at 37°C (see Figure 5.1). Cumulus masses were then washed twice

through MOPS-HSA and once through imaging medium (MOPS lacking protein) and placed onto glass coverslips in a minimal volume of imaging medium so the cumulus mass was able to be flattened for imaging with an Olympus BX51 microscope (Olympus, Melbourne, Australia) and MacProbe v 4.3 software.

5.2.2.3 Microscopy for REDOX analysis

Cumulus cell masses stained for REDOX analysis were imaged using an Olympus BX51 microscope at 100x magnification. Cumulus cell masses were located under bright field, and focus adjusted to have as many cells in focus as possible. Bright field light was then extinguished and a fluorescent filter (excitation 540nm, emission 600nm) applied. An image was then captured using an Olympus Optical BX51 camera (Olympus, Japan) and MacProbe v 4.3 software.

5.2.2.4 Analysis of REDOX state

Single images incorporated the majority of a cumulus cell mass for which average staining intensity was analysed using Adobe® Photoshop® v11.0 (see Figure 5.2). Images were saved in tagged image file format (TIFF) and the intensity of REDOX staining was quantified as the mean fluorescent pixel intensity of four random regions of the same size in each image. A fifth area of each image which did not contain cumulus cells was quantified for background staining and subtracted from the mean of the fluorescing areas. Thresholds were determined based on quartile analysis to segregate the cumulus cell complexes into three groups of low, intermediate or high REDOX state. Data was not normally distributed, with a positive skew. Intermediate REDOX range was assessed to be the central range between the 25th and 75th percentile. A Low REDOX state was classified as the lower quartile (up to the 25th percentile), and High REDOX the upper quartile (above the 75th percentile, see Figure 5.3).

The resultant REDOX value was retrospectively correlated with fertilization outcome, embryo quality on day 2 of development and embryo viability through single embryo transfer, as determined by fetal heart beat at an 8 week scan.

REDOX values were also retrospectively investigated on subsequent frozen embryo cycles (n=9) for patients who did not become pregnant on their initial cycle, or for those returning to attempt subsequent pregnancies using cryopreserved embryos. This enabled the calculation of cumulative implantation rates.

5.2.3 Statistics

Data are presented as percentages or mean ± SEM, as appropriate. Patient demographic data was analysed by one-way ANOVA if normally distributed, and Kruskal-Wallis test for data that was not normally distributed, followed by Mann-Whitney U analysis for between treatment comparisons. Proportion of patients undergoing ICSI, infertility diagnosis, implantation and live birth rates underwent chi-square analysis. Spearman's correlation coefficient was used to assess the relationship between REDOX level and fertilization rates or embryo grade. P values <0.05 were considered to be statistically significant.

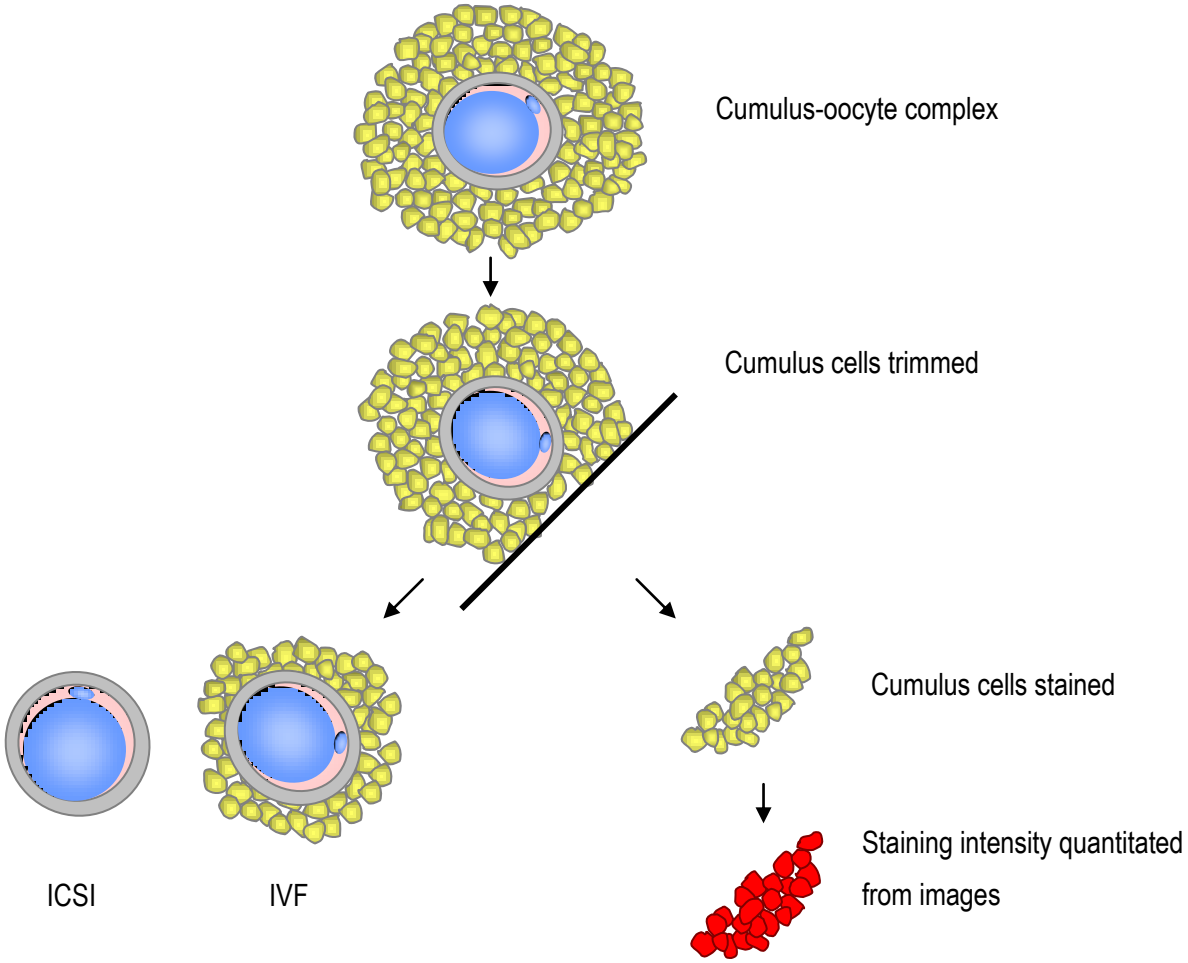


Figure 5.1 Procedure for determination of REDOX status of cumulus cells.

5.3 RESULTS

Patient demographic characteristics are detailed in Table 5.1. The mean maternal age of patients participating in this study was 33.6±0.7 years, who had undergone an average of 0.9±0.2 previous IVF cycles. The overall implantation rate following single embryo transfer for this group of patients was 30.8%. Patients had an average of 9.3±0.9 oocytes collected and 68% of patients underwent ICSI as the insemination method. All patients had a single embryo transferred (n=26). When patients were retrospectively segregated into the cumulus cell REDOX category that their transferred embryo had originated from, there was no difference in maternal age, number of previous cycles, number of oocytes collected or fertilized in each group (see Table 5.1).

5.3.1 EXPERIMENT 1. Establishment of REDOX levels in human cumulus cells.

A total of 250 cumulus masses were trimmed from oocytes and 208 (83%) of these were able to be assessed for REDOX level. Representative images for Low, Intermediate and High REDOX state cumulus masses are shown in Figure 5.2. The range of REDOX levels observed in the cumulus masses removed from oocytes for each patient is shown in Figure 5.3. This figure shows the REDOX level for every oocyte collected for each patient, regardless of ultimate fate. A range of cumulus cell REDOX levels was observed both between and within patients (see Figure 5.3). The majority of patients (n=25, 92.6%) had cumulus cell masses from their retrieved cohort that spanned more than one REDOX category.

5.3.2 EXPERIMENT 2. Effect of cumulus cell REDOX on fertilization potential of oocytes.

The REDOX state of a cumulus cell mass influenced the fertilization outcome of its corresponding oocyte. High REDOX levels in the cumulus cells resulted in a lower fertilization rate, compared to cumulus cell masses with Low REDOX levels (p=0.005, Figure 5.4a). The same pattern of fertilization rates with regard to REDOX level was maintained for both ICSI and IVF insemination techniques (see Figure 5.4b, 5.4c). As REDOX level in the cumulus cells increased, the overall resultant fertilization potential of the corresponding oocyte decreased (ρ correlation coefficient=-0.189, p=0.003, see Figure 5.4a). This result was also evident for oocytes inseminated by ICSI (ρ correlation coefficient=-0.201, p=0.033, see Figure 5.4c), while for IVF insemination the same pattern of fertilization rates with regard to

Table 5.1 Patient demographic characteristics.

	REDOX level of cumulus cells for the transferred embryo			Significance
	LOW	**INTERMEDIATE**	**HIGH**	
Number of patients	10	12	4	
Maternal age	33.9±1.2	33.4±1.2	35.1±1.7	p=0.759
% ICSI patients	70	58	75	p=0.778
Number previous IVF/ICSI cycles	0.7±0.3	1.1±0.3	1.3±0.8	p=0.687
Number of oocytes collected	7.9±1.7	9.3±1.2	10.3±1.8	p=0.642
Number of oocytes fertilized	3.9±0.7	5.9±1.2	6.0±1.4	p=0.361
Grade of transferred embryo	2.4±0.3	1.7±0.3	1.3±0.3	p=0.086
Number of embryos frozen	0.1±0.1	0.4±0.3	0.3±0.3	p=0.775
Infertility diagnosis*				p=0.413
- **Male factor**	5	4	1	
- **Endometriosis**	2	1	1	
- **Tubal**	1	2	-	
- **Ovulation/ovarian defect**	2	3	-	
- **Unexplained**	1	2	1	
- **Other**	-	-	1	

There were no statistically significant differences between groups with any variables analysed. *Infertility diagnoses include both male and female factors, and are listed as the number of cases of each diagnosis. In some cases more than one category is applicable to a patient pair.

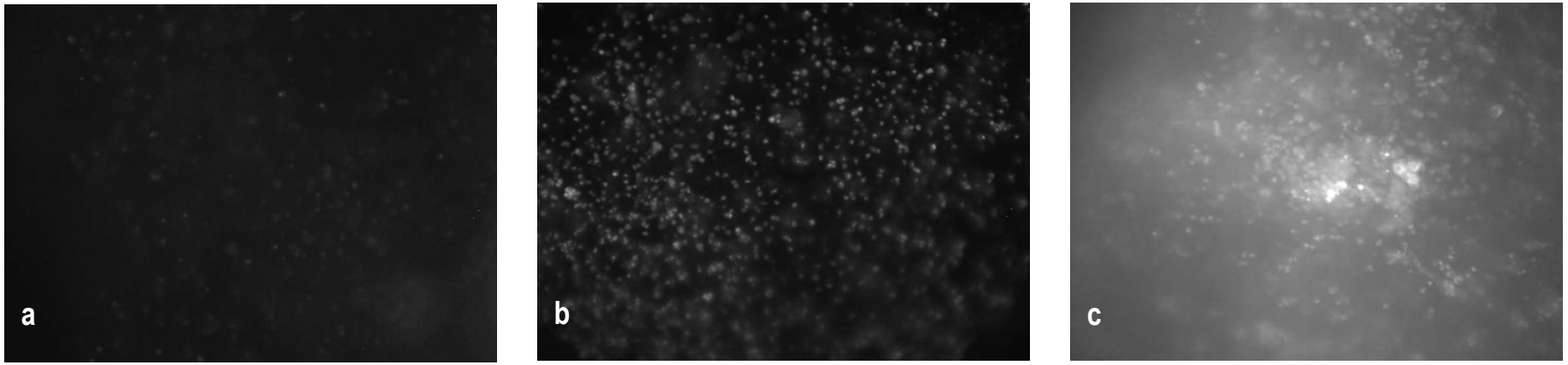


Figure 5.2 Representative examples of the REDOX levels observed in human cumulus cells.
a) Low b) Intermediate c) High. Magnification for all images x100.

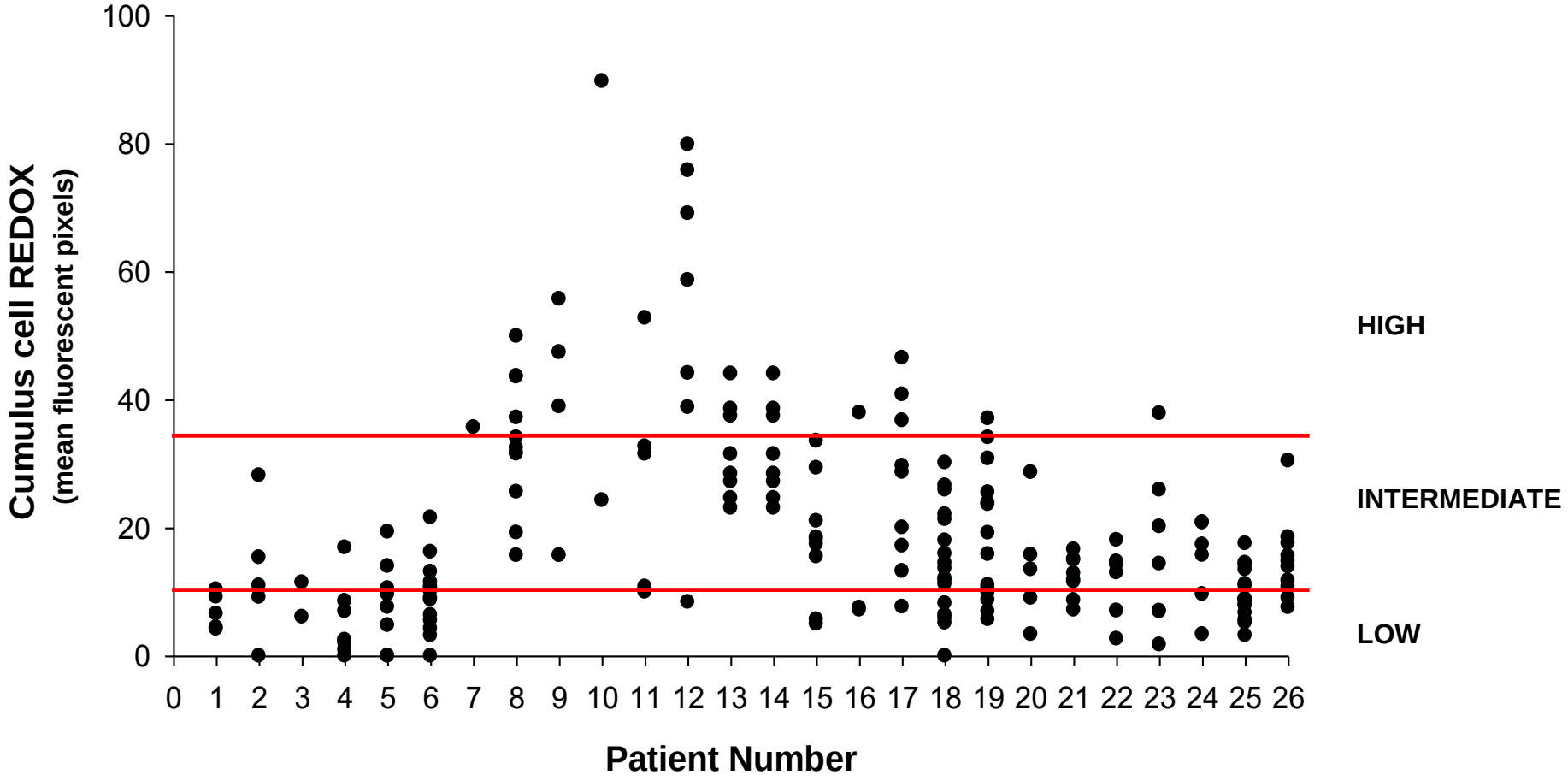
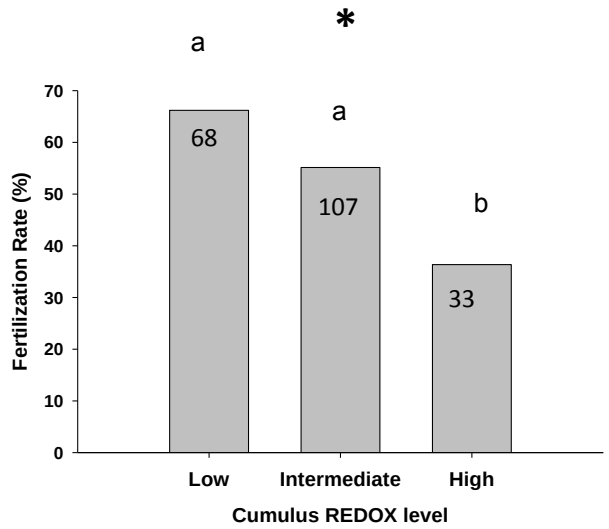
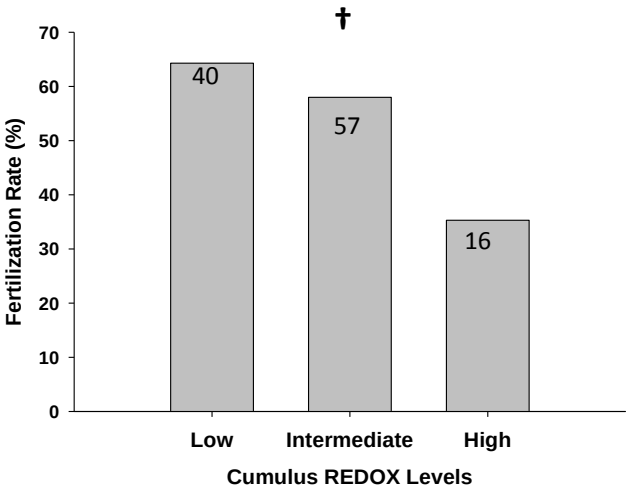


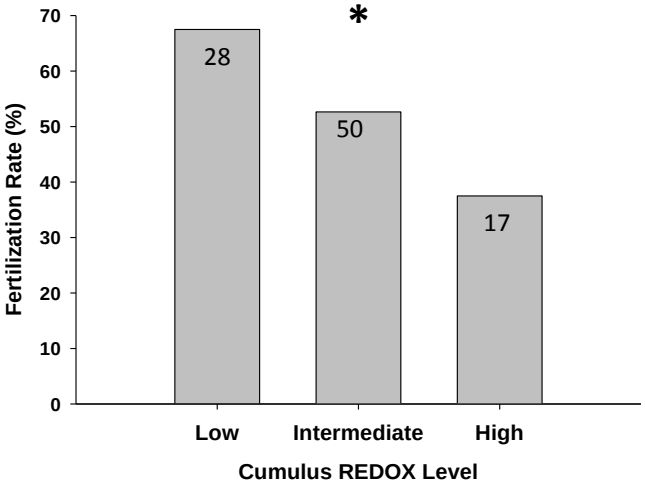
Figure 5.3 Range of REDOX levels observed in human cumulus cells.



a) overall fertilization rates for all patients



b) fertilization rates for patients undergoing IVF



c) fertilization rates for patients undergoing ICSI

Figure 5.4 Effect of REDOX state on fertilization rate with IVF/ICSI.

Data are expressed as the number of normally fertilized (2PN) zygotes per number of oocytes inseminated. Number of oocytes inseminated for each group is shown in bars. *significant correlation between REDOX level and fertilization potential of oocytes. † correlation between REDOX level and fertilization potential of oocytes undergoing IVF insemination p=0.084.

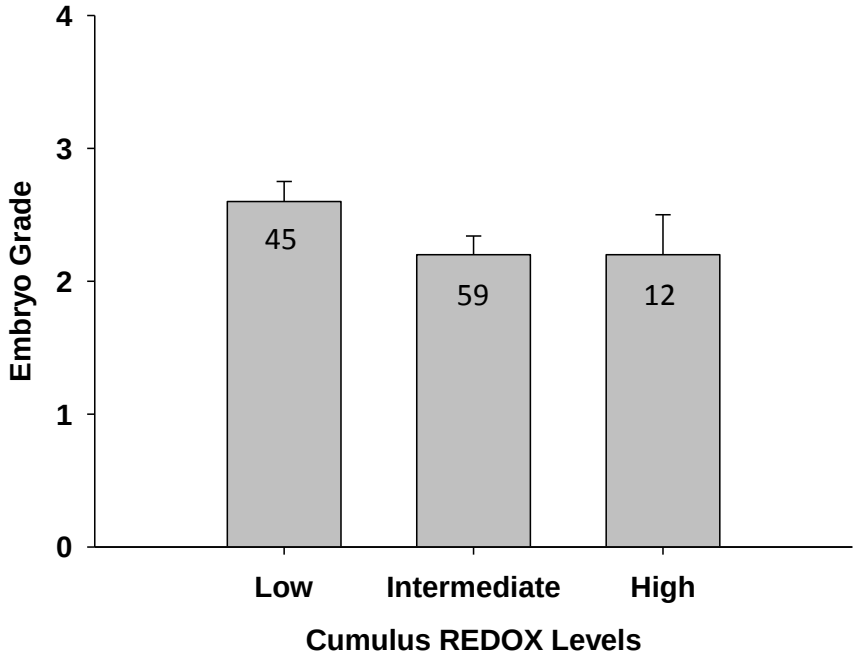
REDOX status was evident, but did not reach significance (ρ correlation coefficient=-0.178, p=0.084, see Figure 5.4b).

5.3.3 EXPERIMENT 3. Effect of cumulus cell REDOX on resultant embryo quality of oocytes.

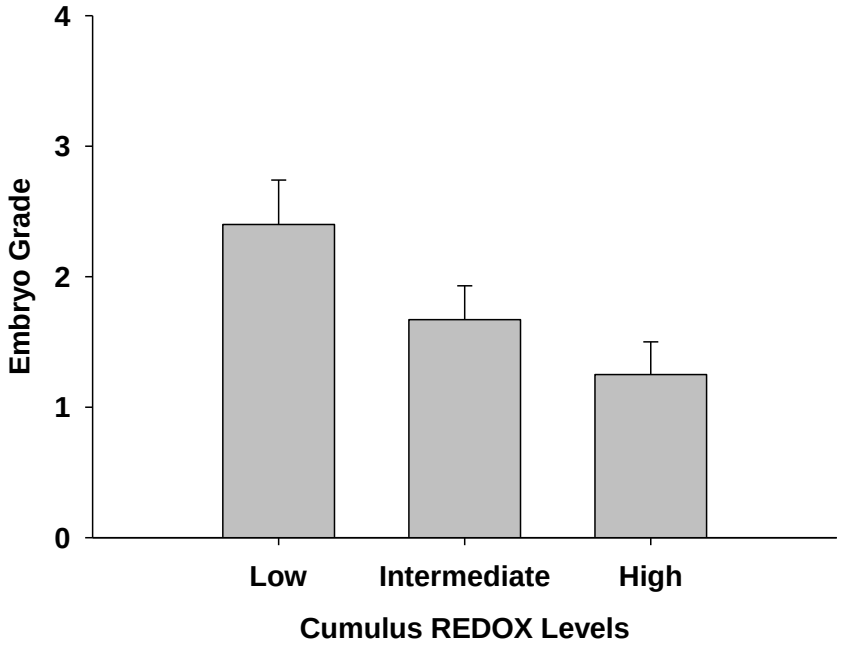
Cumulus REDOX state did not significantly affect resultant embryo quality, as indicated by cleavage stage embryo grade (p=0.082) when all cleavage stage embryos were included in the analysis (n=116, Figure 5.5a). Those embryos that were selected for transfer (n=26), also showed no significant difference between the REDOX groups with regard to embryo quality when assessed at the cleavage stage, however, though not statistically significant, it is interesting to note that there was higher levels of REDOX in cumulus cell masses with higher quality (grade 1) embryos at the cleavage stage (Figure 5.5b, p=0.091). Furthermore, the relationship between REDOX levels in cumulus cells and grade of the embryo which was selected for transfer was significantly negatively correlated with embryo morphological grade (ρ correlation coefficient=-0.442, p=0.016, see Figure 5.5b).

All patients had surplus embryos following embryo transfer, of which 15 patients had at least one good quality embryo available for cryopreservation. Patients having a cleavage stage embryo transfer (n=11) had high quality surplus embryos cryopreserved following transfer, as well as the remaining poorer quality embryos being re-assessed for cryopreservation at the blastocyst stage on days 5 and 6 of development. Patients having a blastocyst stage transfer (n=15) had embryos assessed for cryopreservation only on days 5 and 6 following their embryo transfer on day 5.

Overall, 38 of 116 surplus embryos were cryopreserved (33%). Of this, 35.6% (16/37) embryos in the Low REDOX group were suitable for cryopreservation, 28.8% (17/59) embryos in the Intermediate REDOX group, and 41.7% (5/12) embryos in the High REDOX group (see Figure 5.6).



a) Morphological grade of all embryos after classification into low, intermediate or high REDOX state of their cumulus cells.



b) Morphological grade of embryos that were transferred as part of a fresh IVF cycle after classification into REDOX state of their surrounding cumulus cells.

Figure 5.5 Morphological grade of embryos and associated REDOX state of cumulus cells.
***** significant correlation between REDOX level and embryo grade of transferred embryo. Numbers in bars indicate (a) the number of embryos assessed in each REDOX group and (b) the number of embryos transferred.

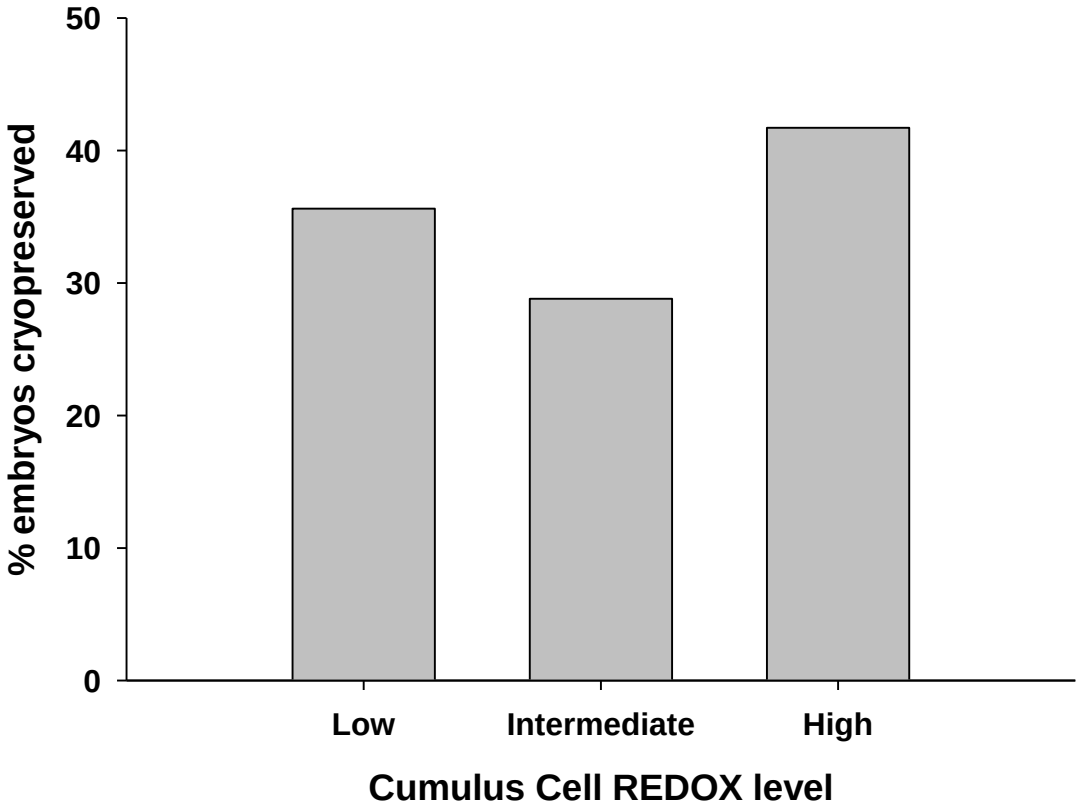


Figure 5.6 Distribution of embryos suitable for cryopreservation.*

* cryopreservation may have been performed at either the cleavage or blastocyst stage of development, depending on the stage of embryo transfer. Values in bars indicate the number of embryos cryopreserved in each group.

5.3.4 EXPERIMENT 4. Effect of cumulus cell REDOX on embryo viability following fresh and frozen embryo transfers.

Overall the fresh implantation rate as assessed by a viable fetal heartbeat for this group of patients, having a single embryo transfer was 30.8%. (8/26). There was no correlation with the morphological grade of the embryos on day 2 that were transferred and with subsequent pregnancy outcome (p=0.208, grade 1 41.7% implantation (n=12), grade 2 28.6% implantation (n=7), grade 3 20% implantation (n=5), grade 4 0% implantation (n=2)).

Implantation rates were clustered in an Intermediate range of cumulus cell REDOX levels (see Figure 5.7) when raw REDOX pixel fluorescence was analysed. Furthermore, implantation rates were statistically significantly higher, when an embryo resulting from an oocyte with an Intermediate level of REDOX staining in its corresponding cumulus cells was transferred, compared to cumulus masses that had Low REDOX staining (p=0.031, Figure 5.8). Due to the low number of patients with an embryo transferred originating from the High REDOX group, and the lack of pregnancies in this group no statistics have been performed comparing this group to the Low and Intermediate REDOX groups.

It should be noted that 11 pregnancies were actually obtained in this study of 26 patients, 3 of which were not viable, and have not been included in any analyses. One ectopic pregnancy in the High REDOX group, one pregnancy with no fetal heart in the Low REDOX group and a biochemical pregnancy in the Low REDOX group were obtained.

Following the initial treatment cycle of a fresh embryo transfer, 9 patients that either did not become pregnant from their fresh embryo transfer or those who had had a successful pregnancy, returned for frozen embryo transfer cycles, enabling the calculation of the cumulative implantation rate (see Figure 5.9). One frozen embryo pregnancy was a biochemical pregnancy, which originated from an embryo transferred from the Intermediate cumulus cell REDOX group, and was not included in any analysis for this study. Cumulus cells with Intermediate REDOX levels trended towards higher viability embryos than oocytes with cumulus cells that had Low REDOX levels (p=0.07). No viable implantations were achieved in the High REDOX group following frozen embryo transfer.

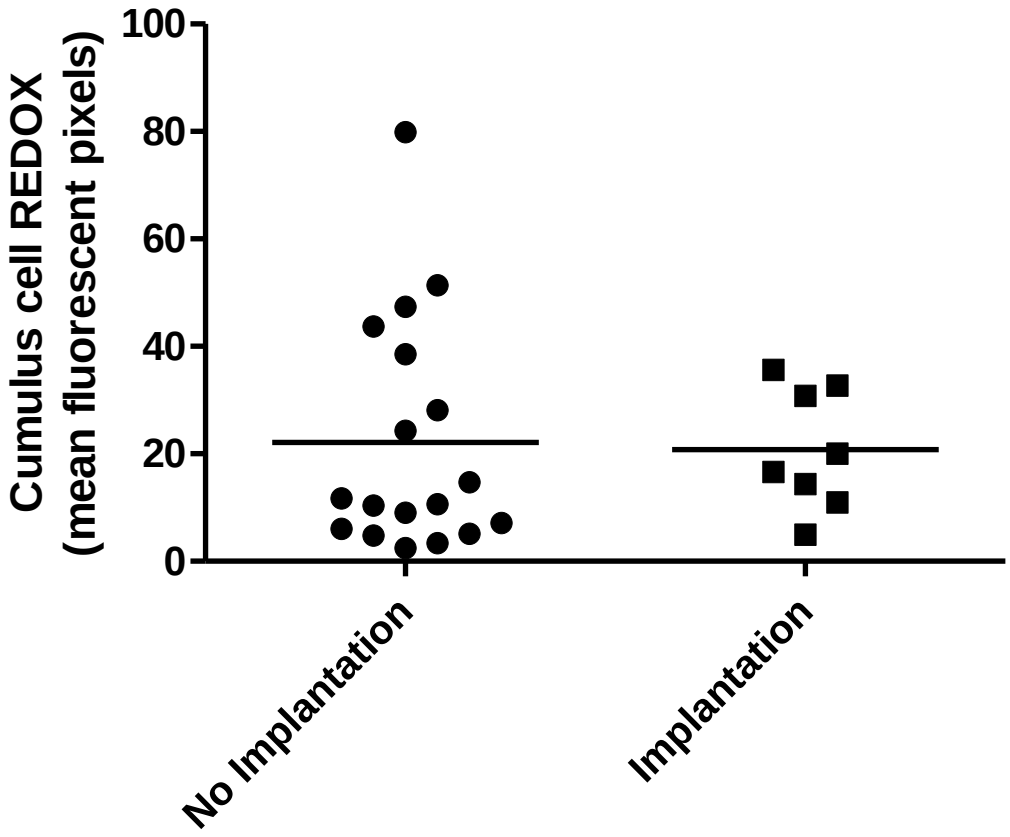


Figure 5.7 Range of REDOX levels observed in human cumulus cells and resultant implantation status of patients.

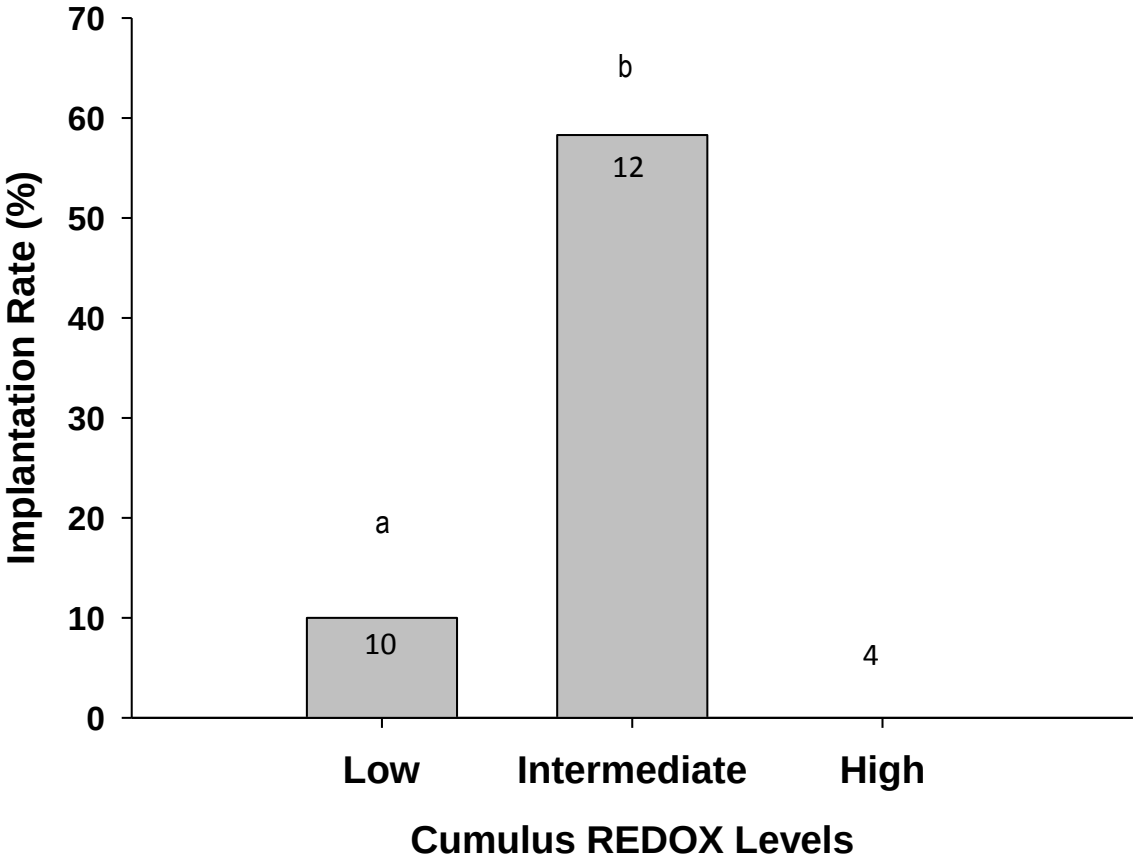


Figure 5.8 Cumulus cell REDOX levels and resultant implantation rates following fresh embryo transfer.

Number of patients in each group indicated in bars. Different superscripts indicate significant differences.

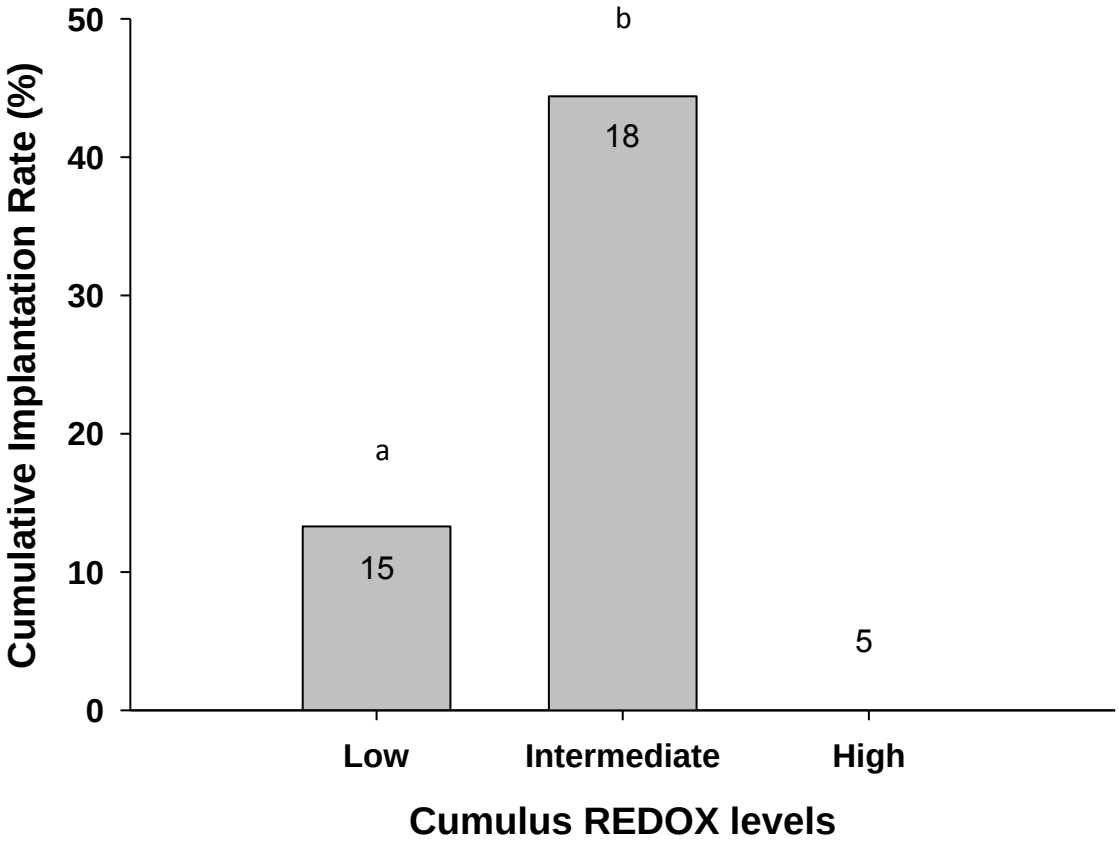


Figure 5.9 Cumulative implantation rates for embryos originating from oocytes with various cumulus cell REDOX levels.

Values in bars indicate the number of embryos transferred in each group. Different superscripts indicate a trend towards significance p=0.07.

5.3.5 EXPERIMENT 5. Effect of cumulus cell REDOX on cumulative birth outcomes.

Pregnancies were followed from positive scan result through to birth outcome. There was no significant difference in live birth rate between Low, Intermediate and High REDOX groups (p=0.09). One pregnancy following a fresh embryo transfer was lost in the Intermediate REDOX group by spontaneous abortion at 13 weeks gestation. The remaining pregnancies (n=7) that detected a fetal heart beat at their first scan resulted in successful live births. The sample size for number of births in this study is small, and the majority of births have resulted from the Intermediate REDOX group, therefore statistics have not been performed on birth outcomes (see Table 5.2).

Table 5.2 Cumulative implantation rate and live birth rate for embryos from cumulus-oocyte complexes with low, intermediate and high REDOX levels.

	REDOX level of cumulus cells transferred embryos		
	LOW	**INTERMEDIATE**	**HIGH**
Fresh implantation rate (%)	10[a] (10)	58.3[b] (12)	0 (4)
Cumulative implantation rate (%)	13.3[c] (15)	44.4[d] (18)	0 (5)
Cumulative live birth rate (%)	13.3 (15)	38.9 (18)	0 (5)
Average gestation (weeks)	38.3 ± 0.2 (2)	39.4 ± 0.3 (7)	-
Birth weight (g)	3660 ± 270 (2)	3389 ± 166 (7)	-

[a, b] superscripts within a row indicate significant difference. [c, d] superscripts within a row indicate a trend towards significance (p=0.07). Numbers in parentheses indicate the number of patients in each group.

5.4 DISCUSSION

The data collected during this study suggests that the level of REDOX measured in cumulus cells during IVF treatment may have predictive value with regard to embryo viability. Importantly, a range of REDOX levels were observed in cumulus cells within individual patients, suggesting that REDOX levels have the potential to be highly discriminating between cumulus-oocyte complexes. The majority of patients in this study had cumulus cell masses that spanned more than one REDOX category, which supports the concept that a heterogeneous population of sibling oocytes is obtained following gonadotrophin treatment which have varying developmental competence (Pellicer et al. 1987, Scott et al. 2008), and also have differing metabolic parameters.

Cumulus cells have been shown in animal models to provide protection to the oocyte and prevent oxidative-induced damage. ROS induce apoptosis in in vitro matured denuded porcine oocytes, whereas intact cumulus-oocyte complexes are able to more readily withstand exogenous ROS and successfully complete maturation (Tatemoto et al. 2000). In addition, bovine and porcine cumulus cells assist with the synthesis of glutathione and enhance the glutathione content of the oocyte, providing elevated antioxidant capacity, which is not observed in denuded oocytes (Yoshida et al. 1993, de Matos et al. 1996, Tatemoto et al. 2000).

The REDOX state of a cumulus cell mass influenced the fertilization outcome of its corresponding oocyte independent of insemination technique in this study. Complexes with a High REDOX state produced oocytes that had reduced fertilization potential compared to those with a Low or Intermediate REDOX state. Initially I predicted that IVF COCs would be more severely affected by a High REDOX state than ICSI oocytes. It was assumed that high intracellular REDOX levels would affect the extracellular environment, as oxidative species have been detected in follicular fluid and medium surrounding in vitro embryos (Bedaiwy et al. 2004, Das et al. 2006). Therefore spermatozoa, in the case of IVF, would need to penetrate a hostile cumulus matrix environment likely to cause significant damage to spermatozoa during their journey to the oocyte. Sperm are highly susceptible to oxidative-induced stress with in vitro exposure leading to impairment of fertilization through a decreased capacity to undergo the acrosome reaction (Griveau et al. 1995, Mammoto et al. 1996, Aitken et al. 1998, Twigg et al. 1998), although it is widely accepted that ROS formation is required for capacitation to occur ((de Lamirande et al. 1997b). However, in this study, ICSI and IVF fertilization outcomes appeared similarly affected by High REDOX levels in the cumulus cells. One of many potential explanations is that oocytes undergoing ICSI had compromised sperm decondensation following injection. In human and non-human primate oocytes that fail to fertilize there is often impairment in sperm decondensation (Simerly et al.

1995, Rawe et al. 2000). Glutathione is important for this process in many species (Mahi and Yanagimachi 1975, Perreault et al. 1988, Reyes et al. 1989, Yoshida et al. 1992) and a key antioxidant in the oocyte (de Matos and Furnus 2000). Glutathione content of the oocyte may be required as an antioxidant defence in the case of a High COC REDOX environment, rendering it unavailable, or only available in reduced concentrations, for sperm decondensation. In addition, cumulus cells play an important role in the synthesis of glutathione for accumulation in the oocyte prior to fertilization, as mentioned previously. Thus the altered REDOX state in cumulus cells may result in perturbed physiology and therefore an alteration of glutathione level in the oocyte. However, despite the insemination technique the fertilization rate and viability of resultant embryos was altered, which may reflect the exposure to oxidative stress in the follicle prior to cumulus cell removal in the IVF laboratory, or alternatively indicate the susceptibility of the COC to combat the three hour incubation in an in vitro environment in this study.

It is already clear that morphological assessment is able to distinguish embryos with no or very little developmental potential, as they can be easily differentiated by slow or arrested cleavage development. However, what is less clear is how to discriminate good morphological quality embryos into those which are viable and those which have limited developmental competence. In this study, using morphology as the assessment method, it is clear that although morphologically similar, only around one-third of embryos were capable of establishing a viable pregnancy whilst the remaining two-thirds resulted in failed pregnancy attempts. This study found that the morphological quality of the embryos observed was similar between the different REDOX groups. However, for those embryos that were selected for transfer, the higher morphological quality embryos had a High REDOX state in their cumulus cells. In contrast, when transferred these embryos proved to have reduced viability, although the numbers of embryos transferred from this group was low. The most viable embryos originated from the Intermediate REDOX group. Thus, as is desired for a viability marker, the REDOX measurement of the cumulus cells was better associated with implantation rate than morphology. The developmental competence of oocytes retrieved during IVF is contributed to by multiple factors, therefore in combination with established techniques, REDOX measurement of cumulus cells may improve embryo selection and resultant implantation success. The current study aimed to maximise the information available by performing single embryo culture and single embryo transfers to allow a true assessment of how the marker of interest (REDOX state of individual cumulus cell masses) influences the quality of the enclosed oocyte and the subsequent embryo, through to pregnancy establishment and live birth.

In the current study, an Intermediate level of REDOX in the cumulus cells was associated with the highest implantation potential. A recent study of human granulosa cells determined that a high percentage of cells producing ROS were associated with a diminished implantation rate (Jancar et al. 2007). The high levels of REDOX within the cumulus cells observed in this study suggest that there is either an increased level of ROS production in the cells or a reduction in the cell's antioxidant defenses, resulting in oxidative stress (De Haan et al. 2003). In animal models, experimental exposure of the pre-implantation embryo to culture environments that increase the levels of ROS in blastomeres are associated with reduced development rates and high rates of developmental arrest (Nasr-Esfahani et al. 1990, Nasr-Esfahani and Johnson 1991). Further it is known that oocytes with lower levels of glutathione, an oxidative defence mechanism, are also associated with lower rates of oocyte and embryo development (Gardiner and Reed 1994, Zuelke et al. 1997, Abeydeera et al. 1998, de Matos and Furnus 2000, de Matos et al. 2002).

A consequence of oxidative stress in many tissues is DNA damage. Detection of DNA damage by eight-hydroxy-2-deoxyguanosine (8-OHdG) in granulosa cells was shown to be associated with lower fertilization and reduced rates of growth in embryos (Seino et al. 2002). Further, this marker is associated with lower numbers of good morphological embryos after any insemination method (Seino et al. 2002). The current study has also concluded an increase in REDOX in the cumulus cells surrounding the oocyte is similarly associated with a reduction in developmental competence. These observations may suggest that the increase in REDOX and oxidative stress in the cumulus cells may also have resulted in an increase in DNA damage.

The oocyte and embryo are a source of ROS produced by the metabolism of oxygen to produce energy through mitochondrial oxidative phosphorylation (Biggers et al. 1967, Quinn and Wales 1973). The main sources of ROS in oocytes and embryos are produced by several pathways, namely, oxidative phosphorylation (Thompson et al. 2000), nicotinamide adenine dinucleotide phosphate oxidase (Manes and Lai 1995), and xanthine oxidase (Nasr-Esfahani and Johnson 1991) systems. ROS are produced as a normal by-product of metabolism and are important second messengers capable of modulating the expression of genes that govern physiological processes of gametes and embryos, essential for spermatozoa capacitation, acrosomal reaction, fertilization and cleavage development (de Lamirande et al. 1997a, Hensley et al. 2000, Droge 2002). A balancing act exists for aerobic metabolism with mitochondrial oxidative-phosphorylation reducing oxygen to water by ROS, while maximizing ATP synthesis and maintaining ROS production to only the amounts required for cell signaling. The vital roles of ROS production in a cell suggest that a critical threshold of ROS is important. The data from the

current study suggests that this may also be the case for cumulus cells and oocyte/embryo viability, as COCs with very low or high levels of ROS resulted in oocytes displaying lower developmental competence.

The concept that there is an optimal level of REDOX, with levels too low and too high resulting in perturbed outcomes has previously been reported for the oxidative state of follicular fluid. In a study that examined oxidative stress indices in follicular fluid, it was similarly shown that there was a reduction in associated embryo morphology for follicular fluid that had low or high oxidative levels, with optimal development occurring with moderate oxidative levels (Wiener-Megnazi et al. 2004). Wiener-Megnazi et al. (2004) examined oxidative state by thermochemiluminescence, which differs from the methodology of the current study, but suggests that various methods of detection may be feasible as biomarkers of oocyte/embryo health. A key advantage to examining cumulus cells rather than follicular fluid is that cumulus masses are removed from the follicle with the oocyte, making identification of individual COCs simple, and able to be performed following the completion of all oocytes being aspirated from the ovary.

By design, this study was retrospective in nature. It is clear that although this preliminary study is indicative of a correlation between optimal REDOX levels in cumulus cells and pregnancy, the potential of this as a diagnostic tool requires validation in a prospective study with much larger patient numbers. This will determine the true effectiveness of cumulus cell REDOX levels as a marker of viability and health.

The current study was focused in methodology and subsequent investigations should therefore explore alternative methods of determination of REDOX in cumulus cells. Redox Sensor Red CC-1 allows fluorogenic determination of the REDOX or ROS level by passively entering cells and being oxidised by superoxide anions and hydrogen peroxide (Chintapalli et al. 2007), which are two of the three major ROS produced by early mammalian embryos (reviewed by (Guerin et al. 2001). There are other commercially available candidate dyes which may be used for this purpose including others which detect hydrogen peroxide and superoxide similar to the dye in the current study, or dyes which detect the hydroxyl radical, hypochlorous acid, nitric oxide, peroxyl radical, peroxynitrite anion or singlet oxygen. Other reagents utilising conjugated dienes, lipid hydroperoxides, glutathione reductase markers, catalase markers and thiobarbituric acid-reactive substances may also be used to assess the metabolic state of cumulus cells. Indicators of DNA damage caused by oxidative stress such as 8-OHdG may also be used to determine the metabolic state of cumulus cells. Furthermore, there are

alternate detection methods which may be more accurate than the method employed in this study. The current method was limited as it is possible that cell density may be responsible for differences in intensity, as it is difficult to determine that the same amount of cells were being measured between samples, although all attempts to make this uniform were performed. Further analyses could be utilised in addition or preference to fluorescence microscopy including; flow cytometry, enzyme measurements, fluorescence assays, phosphorescence measurement and enzyme spin resonance spectrometric measurements.

Any potential viability marker needs to be assessed for its degree of contribution from maternal factors of infertility. The close relationship between the cumulus cells and the oocyte potentially provide a balance between maternal aetiology and oocyte developmental competence, compared to follicular fluid as a marker, which most likely has a mainly maternal contribution. The numbers in this study were too low to discern any correlations of REDOX state with any particular patient aetiology, however previously, enhanced oxidative stress has been shown in women with endometriosis (Portz et al. 1991, Shanti et al. 1999, Agarwal et al. 2003, Szczepanska et al. 2003). In addition, these women have a high incidence of apoptosis in their granulosa cells compared to patients with other infertility causes (Seino et al. 2002) and deregulation of the apoptotic cascade appears to be partly mediated by the effect of oxidative stress on cell membranes, DNA, and mitochondria (Yang et al. 1998).

Oxidative damage in cumulus cells appears to be a promising candidate biomarker that may affect the developmental potential of the enclosed oocyte. The availability of a diagnostic marker for oocytes has many benefits. As well as the potential to improve resultant embryo selection for transfer, such a tool could be employed at early points of development and determine which oocytes should be selected for insemination or cryopreservation, which is particularly important in countries which are limited in the number of oocytes they can legally inseminate. This study retrospectively determined a range of REDOX measurements for cumulus cells that was associated with an increased likelihood of pregnancy. Further investigation into this concept is required. The future goals of this work are to establish whether this concept can consistently differentiate oocyte competence in combination with the current capabilities of morphology to improve implantation rates beyond current capacities.

CHAPTER 6

METABOLITE ANALYSIS OF THE CUMULUS-OOCYTE COMPLEX AND CLEAVAGE STAGE EMBRYO AS PREDICTORS OF PREGNANCY

6.1 INTRODUCTION

Metabolic control in the embryo is essential to maintain viability (Gardner 1998, Gardner et al. 2000b, Lane and Gardner 2000a). Perturbations in metabolic parameters of embryos, in all species studied to date, have been associated with a loss in viability (Renard et al. 1980, Zhang and Armstrong 1990, Conaghan et al. 1993b, Gardner et al. 1994, Lane and Gardner 1996, Lane et al. 2001). The pre-implantation embryo has a dynamic metabolism which is significantly different to that of somatic cells, with the oocyte, zygote and cleavage stage embryo preferentially utilising pyruvate as a dominant substrate (Biggers et al. 1967, Hardy et al. 1989, Gott et al. 1990). From the 2-cell stage lactate can be used as a substrate, while glucose is not the preferred substrate until the blastocyst stage (Leese and Barton 1984, Gardner and Leese 1986, Hardy et al. 1989, Gott et al. 1990, Rieger et al. 1992, Thompson et al. 1992, Gardner et al. 1993). Further to a change in preference of carbohydrate substrates in pre-implantation development, there is also an alteration in preference of amino acids (Partridge and Leese 1996). Eagle's non-essential amino acids and glutamine are utilised at the cleavage stages while all 20 Eagle's amino acids are preferred following the 8 cell stage. Provision of amino acids in the culture media in this sequential fashion has been shown to improve mouse and human embryo development and viability (Gardner et al. 1998a, Gardner et al. 1998b, Jones et al. 1998).

Metabolites are the end product of cellular processes and indicate the response of the system to genetic and environmental influences, including nutrient exposure. Changes in gene expression do not necessarily correlate with a change in protein expression (Gygi et al. 1999), and in addition, are subject to post-translational modifications which can affect function, indicating that changes in gene and protein expression do not always correspond to cellular phenotype. However, metabolites can demonstrate functional alterations in gene/protein function. The early embryo modifies it's in vitro culture environment and as such, non-invasive measurements of metabolites, specifically carbohydrates and amino acids, have been extensively studied across species (Renard et al. 1980, Gardner and Leese 1987, Gardner et al. 1994, Turner et al. 1994, Lane and Gardner 1996, Houghton et al. 2002, Brison et al. 2004a). Several studies have indicated that disrupted balance of carbohydrate metabolism is related to poor development in embryos. Studies in a variety of species, including human, have shown improved blastocyst development and viability when embryos have a higher glucose or pyruvate utilisation (Renard et al. 1980, Rieger 1984, Gardner and Leese 1987, Hardy et al. 1989, Gott et al. 1990, Gardner et al. 2001). Lactate production has been used in conjunction with glucose consumption as a measure of glycolytic activity to prospectively select mouse blastocysts for transfer, resulting in a significantly improved implantation rates (Lane and Gardner 1996). However, rate of glycolytic activity in human embryos under varying culture conditions has

shown differing results. One study showed no relationship to pregnancy establishment, although the embryos were cultured in stressful culture conditions without amino acids and antioxidants (Jones et al. 2001). In contrast, a second study which assessed embryos in a complete media indicated that blastocysts with a lower glycolytic activity had improved viability after transfer (Van den Bergh et al. 2001). Most significantly, for glycolytic activity, bovine blastocysts after cryopreservation and thawing have shown distinct profiles of glucose consumption and lactate production between blastocysts able to survive thawing and undergo expansion and those blastocysts which were not viable (Gardner et al. 1996a). These results are desirable for any viability marker in order to conclusively separate oocytes/embryos from their cohort for selection.

Amino acids are important regulators of embryo development (Gardner 1998, Gardner et al. 2000b). Amino acid profiles differ with the stage of bovine embryo development, with alanine being the only amino acid that is consistently produced throughout development (Partridge and Leese 1996). Human embryos that produce high levels of alanine are less capable of forming blastocysts (Houghton et al. 2002). Furthermore, Leese and colleagues have related amino acid turnover by human cleavage stage embryos to blastocyst development (Houghton et al. 2002, Stokes et al. 2007), and following transfer, these profiles were able to predict embryos that were capable of establishing pregnancy (Brison et al. 2004a). While promising, this method currently requires a specific medium, specialised equipment and technical expertise that is not readily available in IVF clinics.

A great deal of research has been performed on the metabolism of the embryo and its potential to predict viability. In contrast, relatively little work has been performed on the metabolism of the oocyte or the cumulus-oocyte complex in terms of biomarkers of viability. Cumulus cell oxidative metabolism is a major contributor to energy production (Rieger and Loskutoff 1994), and influences the metabolic state of the oocyte (Downs and Utecht 1999). Oocytes from immature animals or those which are in vitro matured have altered metabolic activity and reduced developmental competence relative to oocytes from adult animals or those which are in vivo matured (O'Brien et al. 1996, Steeves and Gardner 1999a, Steeves et al. 1999, Khurana and Niemann 2000, Spindler et al. 2000), indicating the role of the cumulus cells in establishing an appropriate metabolic environment for the oocyte to maintain developmental potential.

Cumulus cells provide essential nutrients for oocyte growth (Donahue and Stern 1968, Leese and Barton 1985). Stimulated by paracrine factors from the oocyte, cumulus cell metabolic pathways are upregulated (Eppig et al. 2005, Sugiura et al. 2005). Further to this, glucose and lactate metabolism by the cumulus both play prominent roles in the physiology of the oocyte, including regulation of meiosis (Fagbohun and Downs 1992, Downs et al. 1998). Therefore the metabolic activity of the cumulus cells are likely reflective of the health and viability of the oocyte, making COC metabolism an interesting avenue to explore in the pursuit of a biomarker.

It is useful to attempt to identify markers able to select the most viable embryos at the cleavage stage of development or earlier, as early identification of viability would be most relevant for worldwide application. Furthermore, in order to identify the possibilities of metabolism as a marker of viability, a complete medium as used for in vitro oocyte/embryo culture in IVF clinics must be used for analysis, so as not to compromise the health of the oocytes/embryos being assessed (Conaghan et al. 1993a, Jones et al. 2001). Therefore, the present study was undertaken to determine if non-invasive analysis of cumulus-oocyte complex or cleavage stage embryo metabolism have potential to be indicators of resultant implantation potential, when analysing complete commercial embryo culture media.

6.2 MATERIALS AND METHODS

6.2.1 Patient Selection

Forty-two patients undergoing routine IVF/ICSI at Repromed's Adelaide clinic between May and November 2005 consented for spent media samples following cumulus-oocyte/embryo culture to be collected and analysed. Patients having PGD or using donor gametes/embryos were excluded from recruitment. Ethical approval was obtained by the NHMRC sanctioned Human Ethics Committee of the Women's and Children's Hospital, Adelaide, Australia.

6.2.2 Sample collection and processing

Spent media samples were collected at specific periods during the culture period:

1) Immediately following collection of COC's in theatre, COC's were transferred in 10µL of GFert Plus medium (Vitrolife), into individual 10µL drops of GFert Plus to have a final incubation volume of 20µL, under paraffin oil at 37°C, 6% CO_2, 5% O_2. COC's were incubated in these drops for 3 hours, prior to trimming for IVF or complete denudation for ICSI insemination. The spent media was then collected for analysis.
2) Normally fertilized zygotes were placed in individual 10µL drops of fresh media (G1v3, Vitrolife) 16 hours post insemination for continuing culture. After 24 hours incubation, media was replenished once again (G1) and the spent media for the previous 24 hour period, during which the embryos should undergo the first and second cell cycles, was collected for analysis.

Media samples were labeled to correspond with the appropriate oocyte/embryo from which they were sourced in order to relate metabolic analyses to individual oocytes/embryos. Media samples were frozen and stored at -80°C until required for analysis.

6.2.3 Microfluorometric Analysis

Quantitative microfluorimetry was employed to analyse the spent media for nutrient consumption and metabolite production by conventional biochemical assays on a smaller scale (Mroz and Lechene 1980, Leese and Barton 1984). The measurement of metabolites utilised and excreted by singly cultured embryos was able to be determined by performing a variety of metabolic assays using spent media and comparing to control media from the same patient which did not contain an embryo. Control media from each individual

patient's culture dish was collected to compare to the media containing an embryo allowing for spontaneous breakdown of metabolites as well as the metabolic activity of the embryo.

This technique was based on coupled reactions which generate or consume NADPH or NADH. Upon exposure to ultra-violet (UV) light (340nm), the reduced forms of these nucleotides fluoresce, the degree to which can be quantitated using a fluorescent microscope and photometer (Leica DMIRB and Leitz MPV-Combi, Leica, Wetzlar, Germany). Standard curves of fluorescence and substrate level were constructed for each set of assay analysis.

Assay conditions are optimized to favour the conversion of the substrate of interest to its product. The reaction volumes ranged from 5-60µL, depending on the assay being performed, on siliconised slides under mineral oil (Sigma Chemical Co.). The initial fluorescence of the 'cocktail' drop, containing all the co-factors and enzymes was determined under UV light. The sample 0.5-1µL was then added to the cocktail drop and after the reaction had reached completion the final fluorescence reading was taken. The change in fluorescence was then determined and the concentration of the substrate of interest was then quantified from a standard curve generated at the beginning of each assay.

6.2.4 Assay principles

6.2.4.1 Glucose

Assessment of glucose concentration, is based on the phosphorylation of glucose to glucose-6-phosphate by hexokinase and the oxidation of glucose-6-phosphate to 6-phosphogluconate by glucose-6-phosphate dehydrogenase (G6PDH) which is coupled to NADPH. NADPH is fluorescent, and can therefore be quantified by fluorometric analysis. An increase in glucose concentration results in an increase in fluorescence, through the increased production of NADPH, from the non-fluorescent NADP+.

$$\text{Glucose} + \text{ATP} \xrightarrow[\text{Hexokinase}]{} \text{Glucose-6-Phosphate} + \text{ADP}$$

$$\text{Glucose-6-Phosphate} + \text{NADP}^{+} \xrightarrow[\text{G6PDH}]{} \text{6-Phosphogluconate} + \text{NADPH} + \text{H}^{+}$$

A 0.5µL sample (GFert Plus diluted 1/10 (COC), G1.3 Plus undiluted (early embryo)) was added to a 5.0µL cocktail drop and 5 minutes allowed for the reaction to reach completion at room temperature.

Assay reagents and concentrations can be seen in Appendix 7. Mean R^2 for COC and early embryo glucose in spent media samples analysed was R^2=0.997 and R^2=0.995 respectively. An example standard curve generated from this technique for glucose can be seen in Figure 6.1a.

6.2.4.2 Pyruvate

Pyruvate concentration was determined by measuring the reduction of pyruvate to lactate by lactate dehydrogenase (LDH), via the disappearance of NADH as it is converted to NAD^+. Therefore this reaction produces a decrease in fluorescence as the concentration of pyruvate increases.

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow[\text{LDH}]{} \text{Lactate} + NAD^+$$

A 0.5µL sample was added to a 5.0µL cocktail drop and 10 minutes allowed for the reaction to reach completion at room temperature.

Assay reagents and concentrations can be seen in Appendix 7. Mean R^2 for COC and early embryo pyruvate in spent media samples analysed was R^2=0.999 and R^2=0.993 respectively. An example standard curve generated from this technique for Pyruvate can be seen in Figure 6.1b.

6.2.4.3 Lactate

Lactate concentration was assessed by the conversion of lactate to pyruvate, by lactate dehydrogenase (LDH). A higher lactate concentration in the sample results in an increase in fluorescence as NADH levels increase.

$$\text{Lactate} + NAD^+ \xrightarrow[\text{LDH}]{} \text{Pyruvate} + \text{NADH} + H^+$$

A 0.5µL sample (GFert Plus diluted 1/22.5, G1.3 Plus diluted 1/10) was added to a 5.0µL cocktail drop and 5 minutes allowed for the reaction to reach completion at room temperature.

Assay reagents and concentrations can be seen in Appendix 7. Mean R^2 for COC and early embryo lactate in spent media samples analysed was R^2=0.998 and R^2=0.990 respectively. An example standard curve generated from this technique for lactate can be seen in Figure 6.1c.

6.2.4.4 Alanine

The assessment of alanine concentration was via a two-step process. The initial step involves the conversion of alanine to pyruvate by glutamate-pyruvate transaminase (GPT). The final step of the reaction then follows the determination of pyruvate concentration (see above). The conversion of NADH in the final reaction allows a fluorescent measurement to be taken. An increase in alanine concentration produces a decrease in fluorescence.

$$\text{Alanine} + \alpha\text{-ketoglutarate} \xrightarrow[\text{GPT}]{} \text{Pyruvate} + \text{glutamate}$$

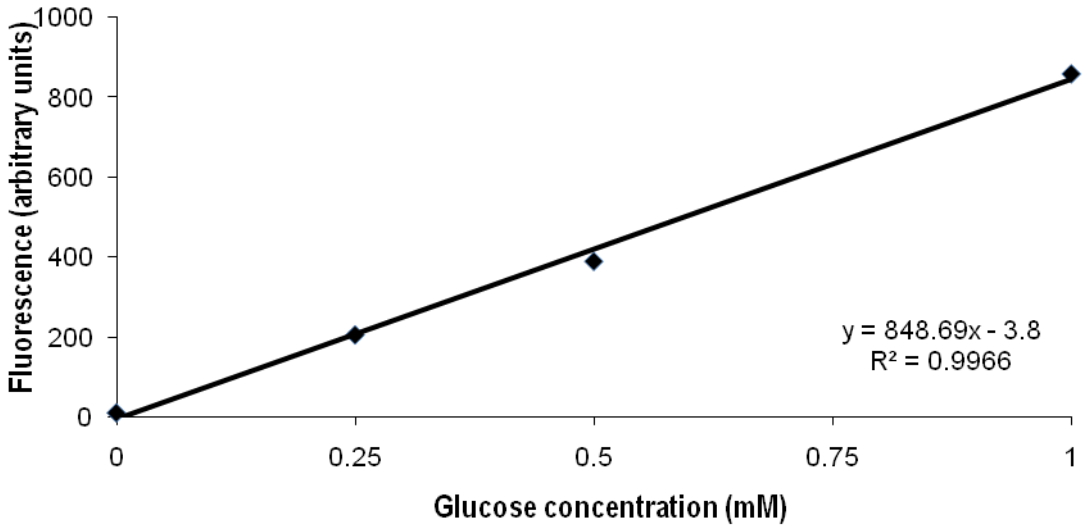
$$\text{Pyruvate} + NADH^{+} + H^{+} \xrightarrow[\text{LDH}]{} \text{Lactate} + NAD^{+}$$

A 1µL sample was added to a 60µL alanine cocktail drop and 60 minutes allowed for the reaction to reach completion at room temperature. A 1µL sample from the initial reaction drop was then added to a 10µL pyruvate cocktail drop for 10 minutes at room temperature for the final reaction to take place to produce NAD^{+}.

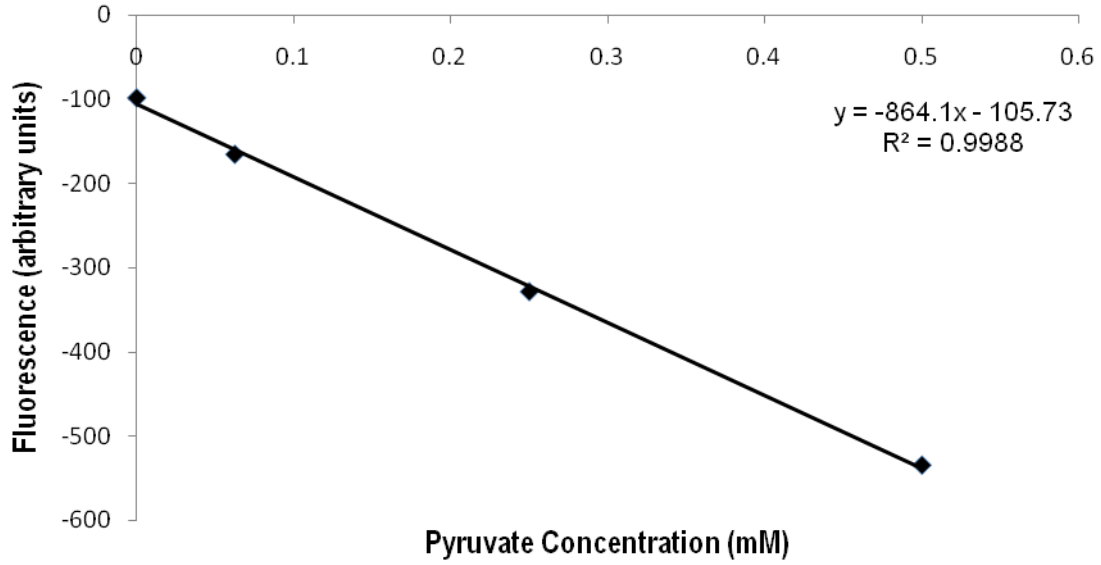
Assay reagents and concentrations can be seen in Appendix 7. Mean R^2 for COC and early embryo alanine in spent media samples analysed was R^2=0.997 and R^2=0.997 respectively. An example standard curve generated from this technique for alanine can be seen in Figure 6.1d.

Figure 6.1 Standard curves generated for microfluorometric assays.

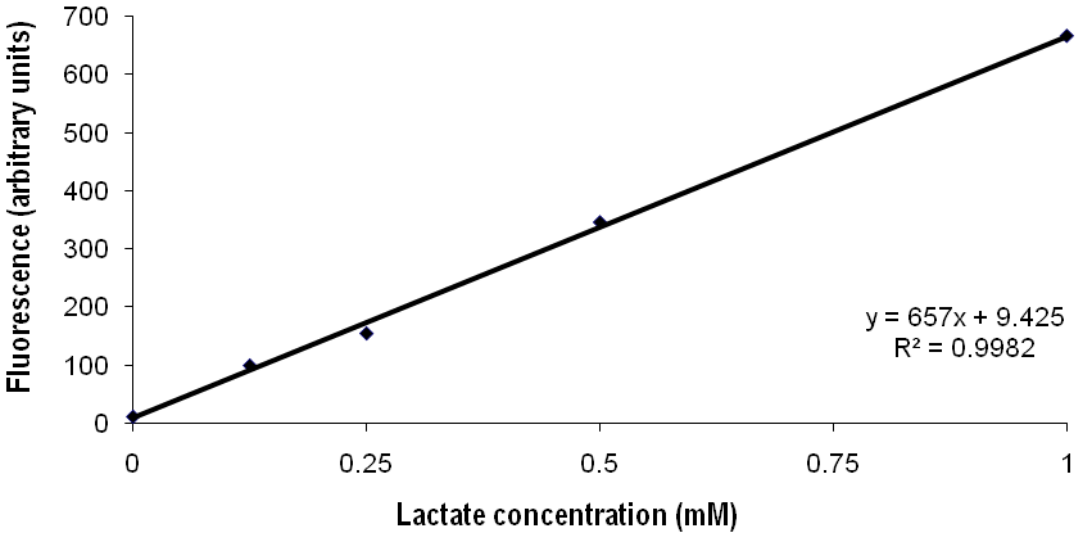
a) Glucose standard curve



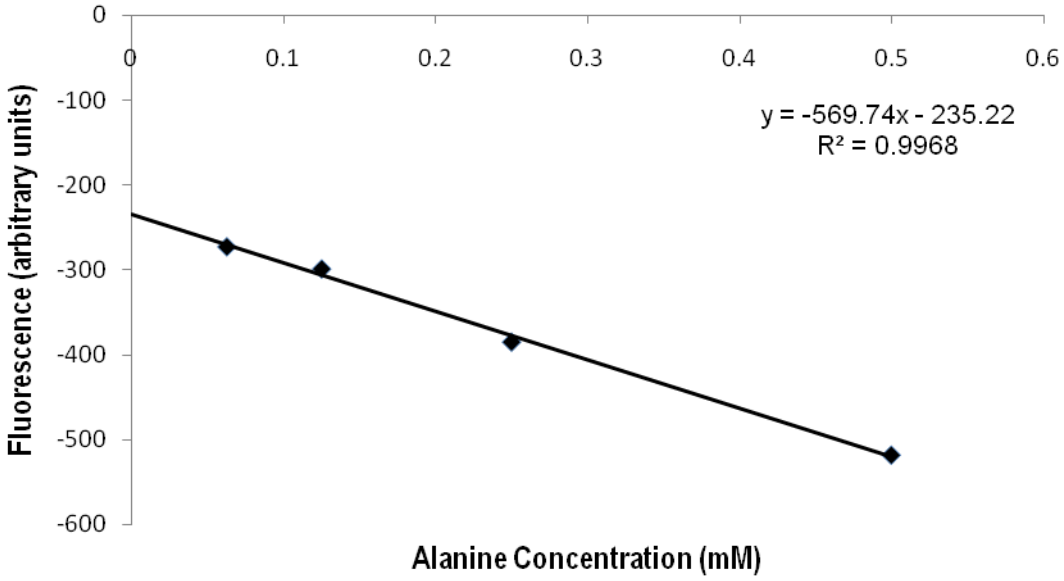
b) Pyruvate standard curve



c) Lactate standard curve



d) Alanine standard curve



6.2.5 Metabolic Analyses

Nutrient levels of interest were calculated in the spent media by analysis of the change in fluorescence during the assay, the standard curve generated for each assay and the dilution (if any) of the unknown media sample prior to assessment. This value indicated the concentration of the nutrient of interest in the spent media, which was then deducted from the concentration of the same metabolite in the control media to reflect the consumption or production of the nutrient of interest by a single embryo for the incubation period. Consumption or production of the metabolite was also able to be calculated per hour of incubation and compared retrospectively to the fate of oocytes following insemination, the cell number and morphological grade of an embryo 40 hours post insemination and the ability to establish a pregnancy. All analysis was conducted blinded to the quality of the resultant embryo.

Ratios of metabolites were also analysed to account for cell number in COC investigations. Individual cumulus masses were not quantitated therefore cell number was only incorporated by investigating the ratio of metabolites. In addition, for individual embryos, ratio of metabolites was investigated to assess whether the fate of a metabolite was correlated with viability.

6.2.6 Statistics

Metabolite data is presented in box-whisker plots. Boxes detail the median, 25th and 75th percentile, with whiskers indicating the 10th and 90th percentile. Outliers are also indicated. Metabolite data in tables is expressed as mean ± SEM. Patient demographic data is presented as either mean ± SEM or percentages as appropriate. Normally distributed data was analysed using a One-Way ANOVA and treatment comparisons performed using a bonferroni post-hoc test. Data that was not normally distributed was analysed using a Kruskal-Wallis test. Proportional data was analysed using chi-square analysis. Spearman's correlation coefficient was used to assess the relationship between alanine turnover and embryo grade. P values <0.05 were considered to be statistically significant.

6.3 RESULTS

6.3.1 Patient demographics

Patient demographic characteristics are detailed in Table 6.1. The mean maternal age of patients participating in this study was 33.4±0.6 years. The overall implantation rate following embryo transfer for this group of patients was 26.2%. Patients had an average of 10.6±0.7 oocytes collected and 71% of patients underwent ICSI as the insemination method. There was no correlation of embryo grade with implantation (grade 1 30.0% implantation (n=10), grade 2 25.0% implantation (n=24), grade 3 25.0% implantation (n=8)).There was no difference in any demographic parameters between patients who had embryos implant compared to those that did not become pregnant (see Table 6.1).

Table 6.1 Patient demographics.

	Pregnant	Non-pregnant	Significance
Number of patients	12	30	
Maternal Age (years)	32.8±1.2	33.7±0.6	p=0.471
% ICSI patients	75%	71%	p=0.794
Number of oocytes collected	9.5±0.6	11.0±0.9	p=0.321
Number of oocytes fertilized	5.4±0.5	5.8±0.6	p=0.741
Grade of transferred embryo	2.0±0.2	1.9±0.1	p=0.683
Number of embryos frozen	2.3±0.7	2.1±0.3	p=0.880
Infertility Diagnosis (%) *			p=0.829
- Male Factor	7 (58)	16 (48)	
- Endometriosis	1 (8)	3 (9)	
- Tubal	0 (0)	2 (6)	
- Ovulation/ovarian defect	1 (8)	3 (9)	
- Unexplained	3 (25)	8 (24)	
- Other	0 (0)	1 (3)	

There were no statistically significant differences between groups with any variables analysed.

*Infertility diagnoses include both male and female factors. In some cases more than one category is applicable to a patient pair. Numbers in parentheses indicate the proportion of infertility diagnoses.

6.3.2 EXPERIMENT 1. Relationship between metabolite concentrations in spent media and embryo grade.

a) following COC incubation

Forty-two patients had media collected following COC incubation. There was no significant relationship between COC metabolism of glucose or pyruvate and resultant embryo grade (p=0.500, p=0.528 respectively, see Figure 6.2 and Table 6.2). Lactate turnover of COCs trended towards significant differences between resultant morphological grade of embryos (p=0.053). While alanine production was significantly higher in COCs that produced an embryo of poorer grade (p=0.049), with grade 3 embryos producing more alanine than grade 1 embryos (p=0.06, see Figure 6.2b and Table 6.2). Furthermore, there was a significant correlation between COC alanine turnover and resultant embryo grade, with alanine turnover increasing with poorer embryo grades (correlation coefficient ρ=0.377, p=0.014, see Figure 6.2d).

b) following cleavage stage embryo incubation

Thirty-three patients had spent media collected following cleavage stage embryo incubation. Samples for nine patients were unable to be collected due to laboratory constraints.

There was no significant relationship between cleavage stage embryo metabolism and any of the metabolites analysed; glucose, lactate, pyruvate or alanine and resultant embryo grade (p=0.159, p=0.572, p=0.525 and p=0.945 respectively, see Figure 6.3 and Table 6.3). However, it was observed that the metabolism of pyruvate by cleavage stage embryos occurred over a large range for embryos of grade 3, while the majority of grade 2 embryos and all the grade 1 embryos had pyruvate metabolism that was more constant and clustered (see Figure 6.3c).

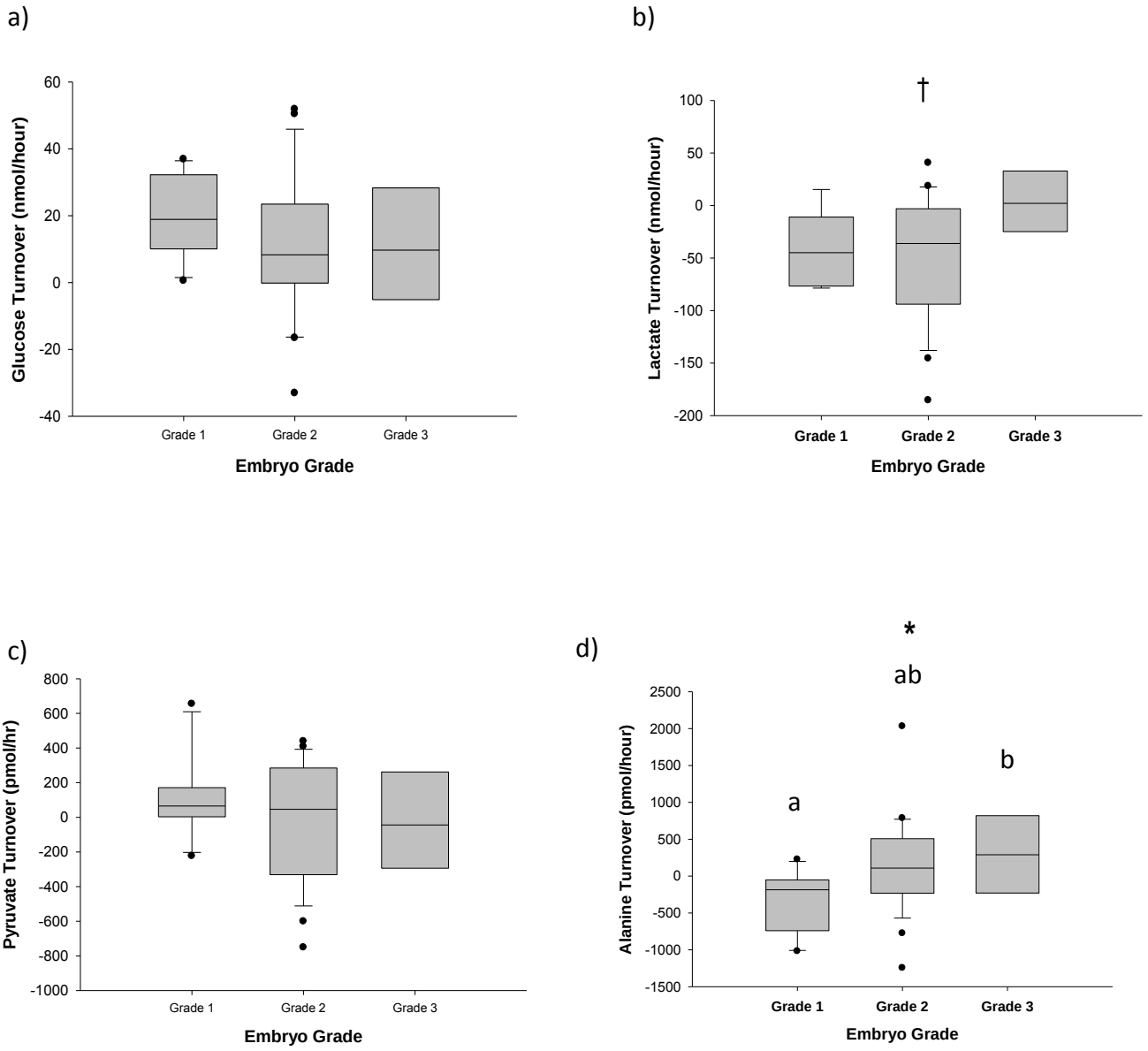


Figure 6.2 Metabolite turnover in individual cumulus-oocyte complexes prior to insemination relative to resultant embryo grade.

a) glucose turnover, b) lactate turnover, c) pyruvate turnover, d) alanine turnover. Different superscripts indicate statistical significance.*significant correlation between alanine turnover of COC and resultant embryo grade (correlation coefficient ρ=0.377, p=0.014). † indicates a trend towards significant difference p=0.053.

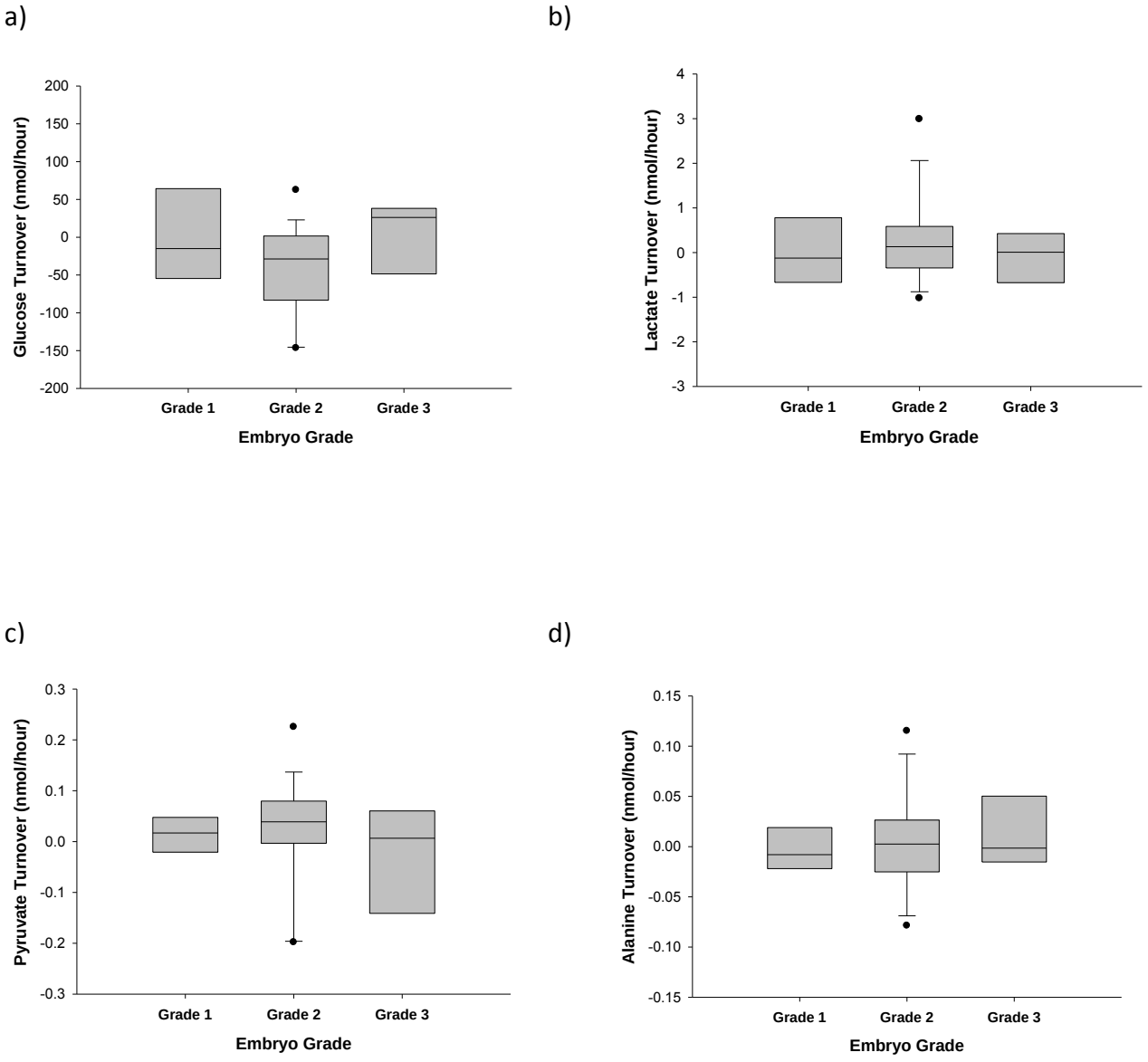


Figure 6.3 Metabolite turnover in individual cleavage stage embryos prior to embryo transfer relative to embryo grade.

a) glucose turnover, b) lactate turnover, c) pyruvate turnover, d) alanine turnover.

Table 6.2 Mean values of metabolite turnover by cumulus-oocyte complexes in relation to morphology of resultant embryos.

	COC Metabolite Turnover		
Metabolite	**Grade 1 embryos** (10)	**Grade 2 embryos** (24)	**Grade 3 embryos** (8)
Glucose (nmol/hour)	19.5 ± 3.8	11.9 ± 4.3	10.9 ± 5.8
Lactate† (nmol/hour)	-42.4 ± 12.0	-47.6 ± 11.9	4.3 ± 10.2
Pyruvate (pmol/hour)	101.9 ± 71.3	-25.22 ± 69.6	-20.33 ± 102.7
Alanine* (pmol/hour)	-361.0 ± 133.0[a]	114.3 ± 127.5[ab]	356.5 ± 285.6[b]

Numbers in parentheses indicate the number of embryos in each group. Different superscripts within a row indicate statistical significance. * positive correlation between alanine turnover and embryo grade, correlation coefficient ρ=0.377, p=0.014. † trend towards significance p=0.053.

Table 6.3 Mean values of metabolite turnover by cleavage stage embryos in relation to morphological grade.

	Embryo Metabolite Turnover		
Metabolite	**Grade 1 embryos** (8)	**Grade 2 embryos** (18)	**Grade 3 embryos** (7)
Glucose (nmol/hour)	5.2 ± 25.3	-43.7 ± 14.6	-6.1 ± 22.9
Lactate (nmol/hour)	-0.2 ± 0.4	0.3 ± 0.2	0.1 ± 0.6
Pyruvate (pmol/hour)	20.2 ± 15.5	28.3 ± 24.2	-21.9 ± 49.3
Alanine (pmol/hour)	-3.5 ± 9.9	3.5 ± 11.4	1.9 ± 21.9

Numbers in parentheses indicate the number of embryos in each group.

6.3.3 EXPERIMENT 2. Relationship between metabolite concentrations in spent media and implantation.

a) following COC incubation

There was no significant relationship between COC metabolism of glucose, lactate, pyruvate or alanine and resultant implantation potential (p=0.872, p=0.694, p=0.313, p=0.869 respectively, see Figure 6.4 and Table 6.4).

b) following cleavage stage embryo incubation

Pyruvate turnover of cleavage stage embryos trended towards a significant difference in resultant implantation (p=0.067), with higher turnover in embryos which resulted in implantations (see Figure 6.4c). There was no significant differences in cleavage stage embryo metabolism and implantation for glucose, lactate, or alanine (p=0.532, p=0.842, p=0.539 respectively, see Figures 6.4a,b,d and Table 6.5).

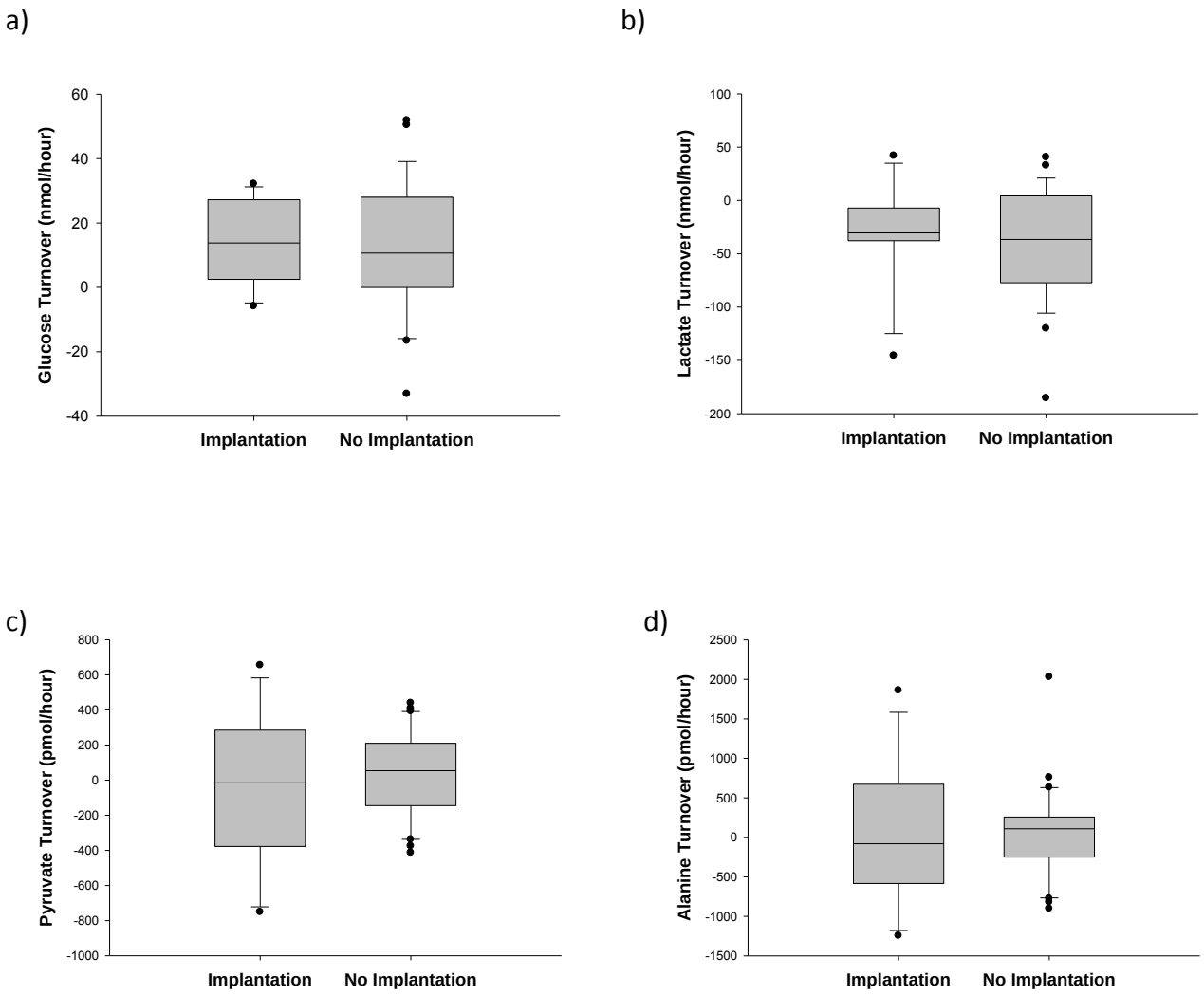


Figure 6.4 Metabolite turnover in individual cumulus-oocyte complexes prior to insemination relative to implantation.

a) glucose turnover, b) lactate turnover, c) pyruvate turnover, d) alanine turnover.

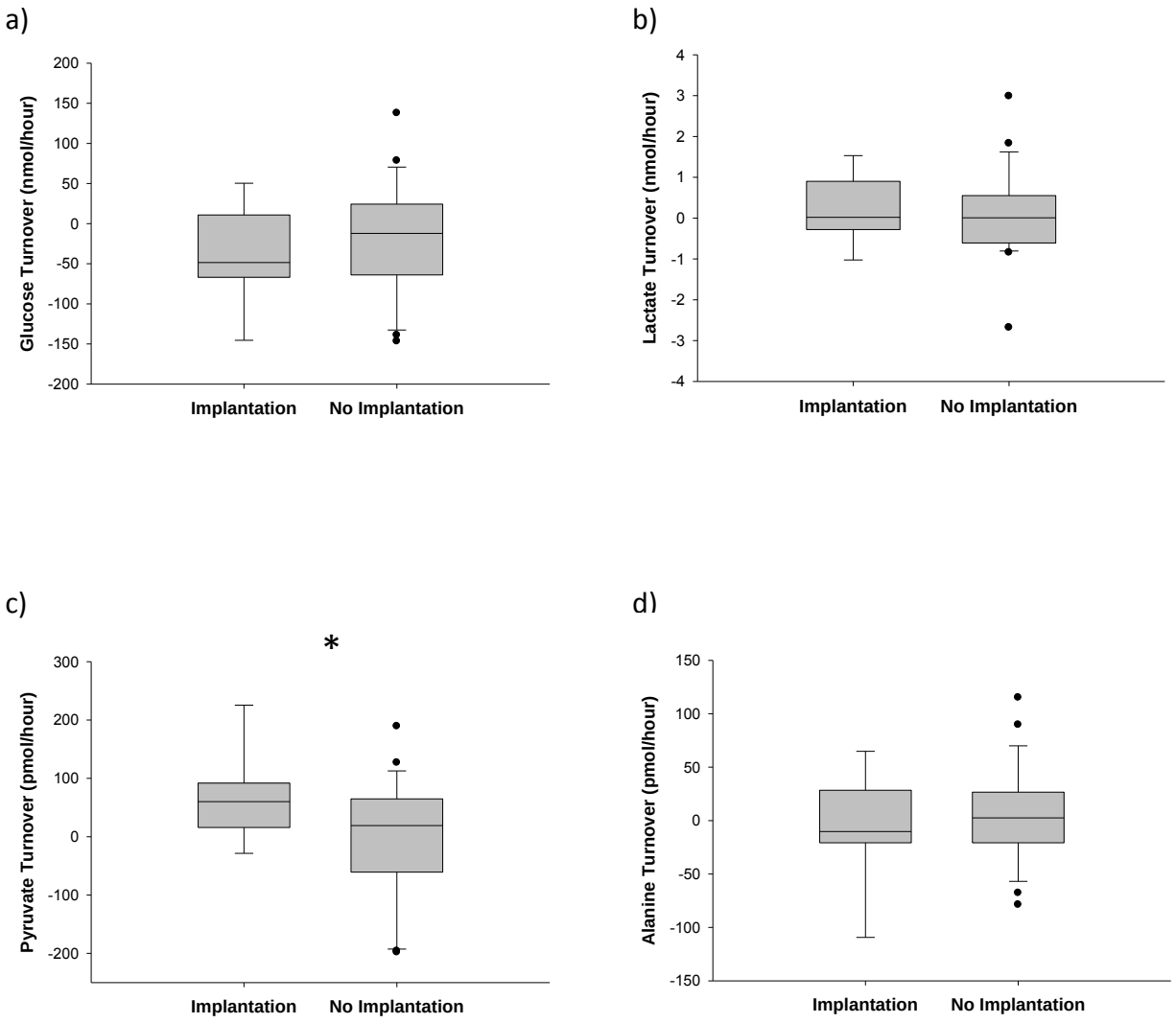


Figure 6.5 Metabolite turnover in individual cleavage stage embryos prior to embryo transfer relative to implantation.

a) glucose turnover, b) lactate turnover, c) pyruvate turnover, d) alanine turnover. * trend towards significant difference in pyruvate turnover of cleavage stage embryos and implantation potential (p=0.067).

Table 6.4 Mean values of metabolite turnover by cumulus-oocyte complexes in relation to implantation of resultant embryos.

	COC Metabolite Turnover	
Metabolite	**Implantation** (12)	**No Implantation** (30)
Glucose (nmol/hour)	14.3 ± 3.7	13.3 ± 3.7
Lactate (nmol/hour)	-32.0 ± 13.5	-39.0 ± 10.4
Pyruvate (pmol/hour)	-72.5 ± 126.0	35.8 ± 45.2
Alanine (pmol/hour)	20.3 ± 250.3	58.1 ± 103.9

Numbers in parentheses indicate the number of embryos/patients in each group.

Table 6.5 Mean values of metabolite turnover by cleavage stage embryos in relation to subsequent implantation potential.

	Embryo Metabolite Turnover	
Metabolite	**Implantation** (9)	**No Implantation** (24)
Glucose (nmol/hour)	-35.9 ± 19	-19.4 ± 14.2
Lactate (nmol/hour)	0.19 ± 0.27	0.11 ± 0.23
Pyruvate (pmol/hour)	66.6 ± 23.9[a]	-3.4 ± 20.6[b]
Alanine (pmol/hour)	-6.5 ± 16.1	4.8 ± 9.5

Different superscripts within a row indicate a trend towards significance p=0.067. Numbers in parentheses indicate the number of embryos/patients in each group.

6.3.4 EXPERIMENT 3. Ratio of metabolite concentrations in spent media and effect on embryo grade and implantation.

a) following COC incubation

Glucose and lactate metabolic profiles of COCs did not show any relationship with resultant embryo grade (p=0.218, see Figure 6.6a) or implantation success or failure (p=0.315, see Figure 6.7a). However, in contrast, the relationship between pyruvate and alanine metabolism of COCs showed a significant increase when subsequent embryos resulted in implantation compared to those which failed to implant (p=0.026, see Figure 6.7b). There was no association of morphological embryo grade with pyruvate: alanine turnover (p=0.722, see Figure 6.6b).

b) following cleavage stage embryo incubation

Cleavage stage embryos showed a significant negative correlation between embryo grade and glucose: lactate turnover (correlation coefficient ρ=-0.461, p=0.008, see Figure 6.8a). Higher morphological quality embryos (grades 1 and 2) had significantly higher glucose: lactate turnover compared to poorer quality embryos (grade 3, p=0.037 and p=0.0033 respectively, see Figure 6.8a). However, this correlation was not observed with regard to implantation ability. There was no difference in glucose: lactate profiles for embryos which implanted and those which did not implant (p=0.438, see Figure 6.9a).

Pyruvate and alanine metabolic profiles did not show any significant differences for morphological embryo grade (p=0.670, see Figure 6.8b), or for implantation (p=0.572, see Figure 6.9b).

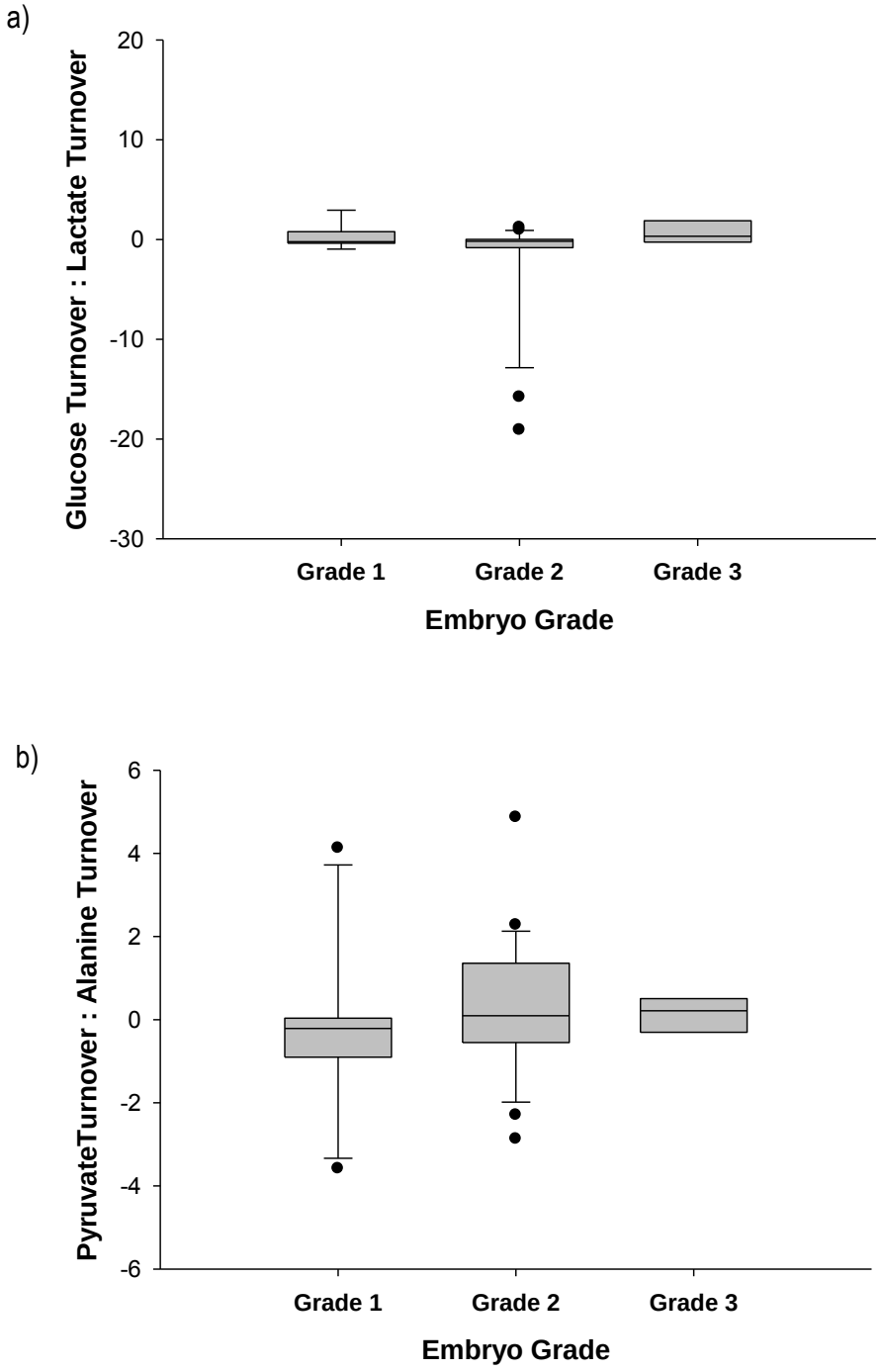


Figure 6.6 Cumulus-oocyte complex metabolite interactions and resultant embryo grade.
a) Glucose: Lactate turnover b) Pyruvate: Alanine turnover.

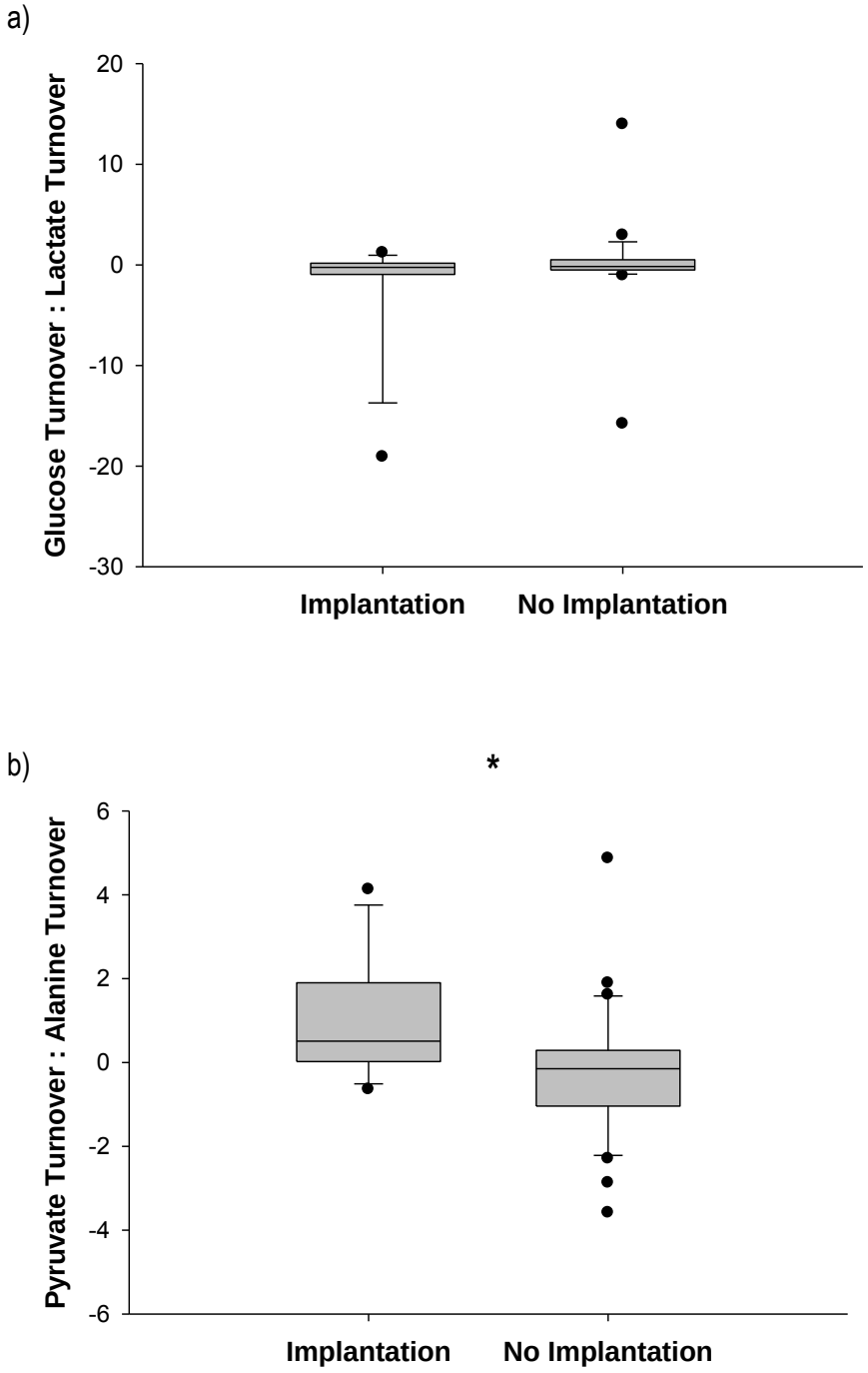


Figure 6.7 Cumulus-oocyte complex metabolite interactions and implantation ability.
a) Glucose: Lactate turnover b) Pyruvate: Alanine turnover.

* indicates statistically significant differences.

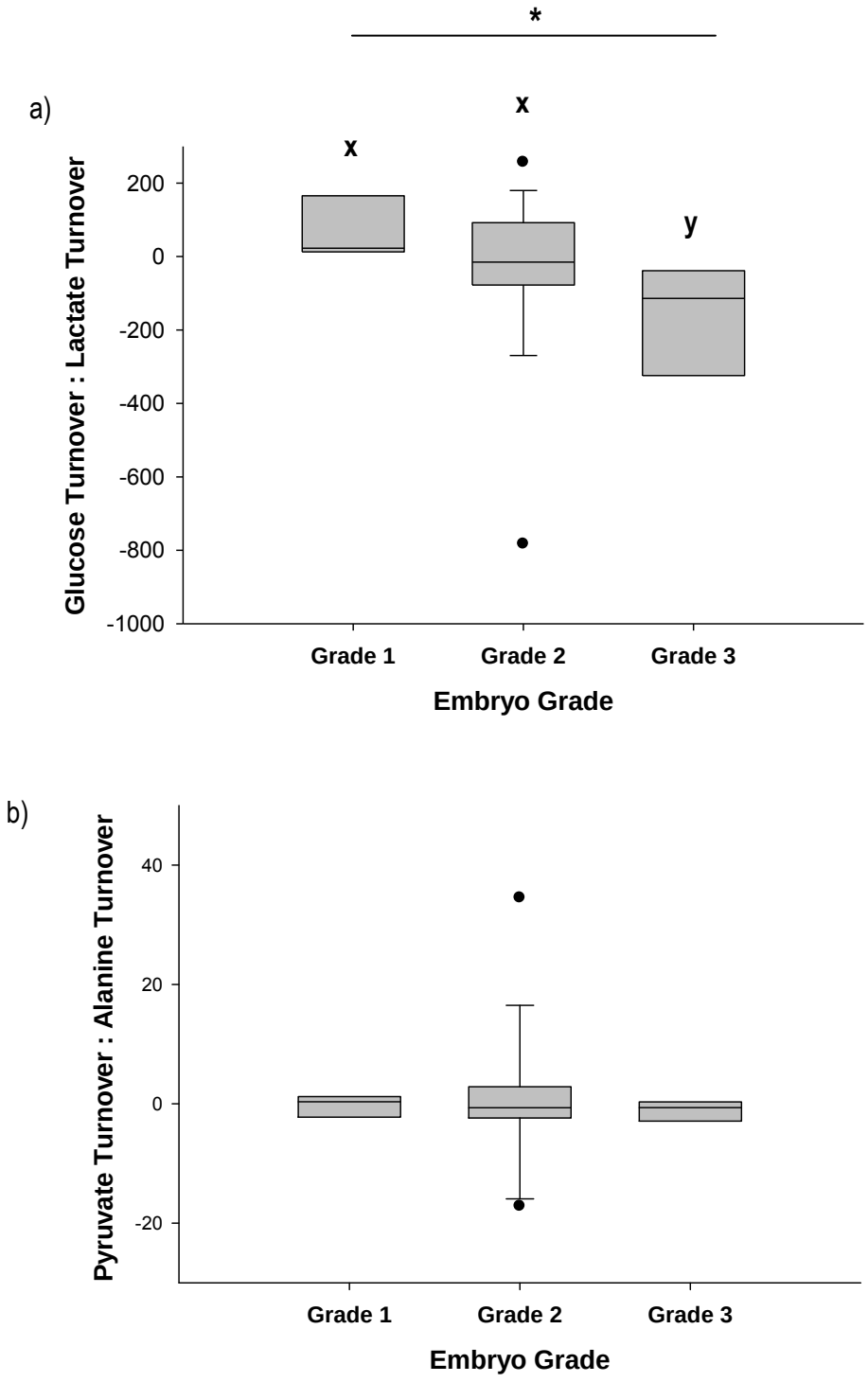
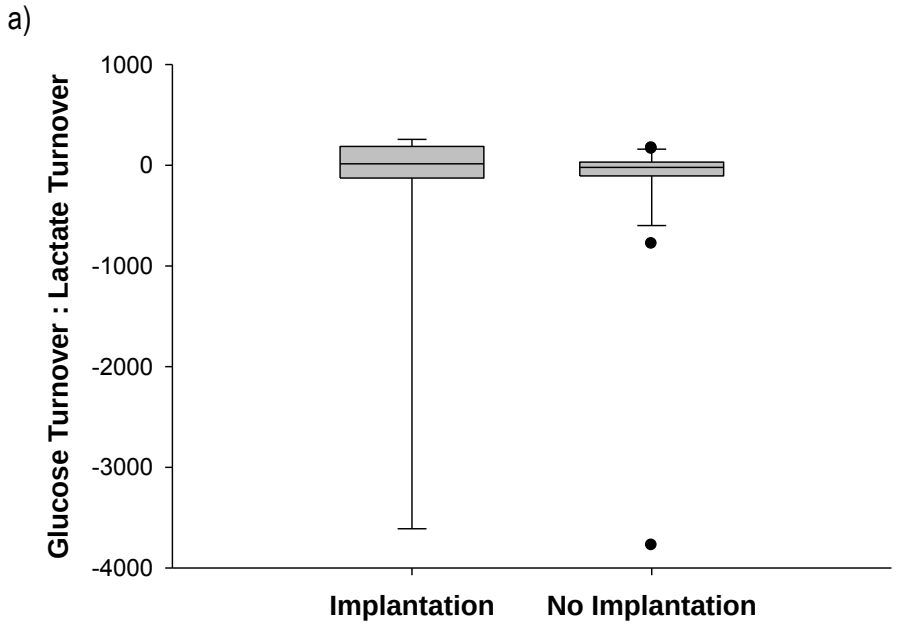


Figure 6.8 Cleavage stage embryo metabolite interactions and resultant embryo grade.
a) Glucose: Lactate turnover b) Pyruvate: Alanine turnover.

Different superscripts indicate statistical significance. *Significant correlation coefficient ρ=-0.416, p=0.008)



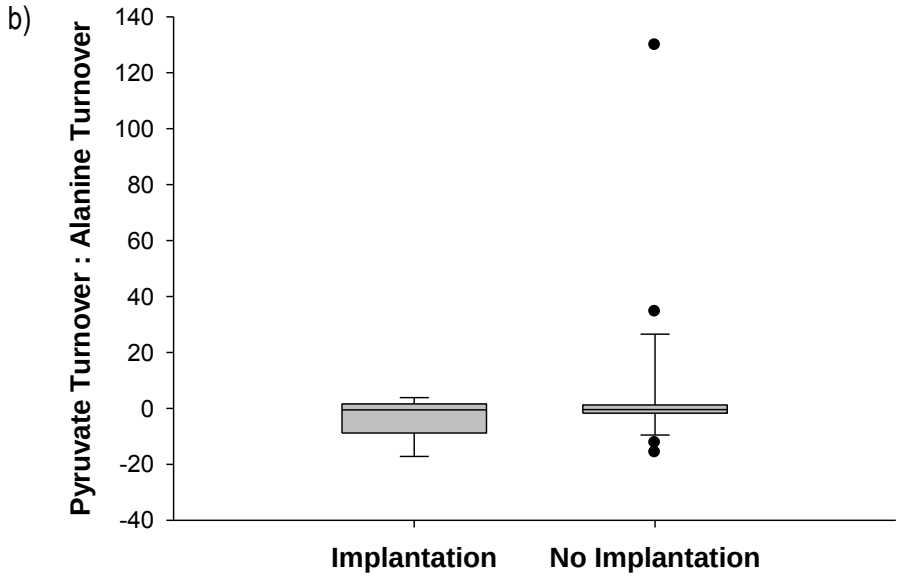


Figure 6.9 Cleavage stage embryo metabolite interactions and implantation ability.
a) Glucose: Lactate turnover b) Pyruvate: Alanine turnover.

6.4 DISCUSSION

Early determination of ideal embryo culture conditions involved investigation of the composition of reproductive tract fluids. These same methods have been extrapolated to examine spent media following embryo culture to attempt to differentiate embryos based on metabolic capacity and correlate this to implantation potential. A number of studies have considered the metabolism of embryos in in vitro culture as a potential measure to assess viability. The current study investigated metabolism of glucose, pyruvate, lactate and alanine of cumulus-oocyte complexes and cleavage stage embryos in a clinical setting to determine the potential of these metabolic measures in predicting viability of embryos. The main findings suggest that there is considerable variability in substrate uptake/production by both COCs and embryos under the conditions employed here. Nevertheless, there are some relationships between cleavage stage embryo metabolism and viability with pyruvate turnover trending to be different between embryos that ultimately produced a pregnancy compared to those that failed to implant. Similarly, the ratio of pyruvate to alanine turnover was significantly higher in COCs that produced a pregnancy compared to those that did not. However, the data collected during this study did not provide compelling evidence to support the use of metabolism of individual metabolites of COCs or cleavage stage embryos as absolute predictors with regard to embryo viability. Although there were some differences between the populations of embryos that implanted and produced a pregnancy compared to those that did not, such as pyruvate and alanine metabolism of the COC and pyruvate metabolism of the embryo, in all cases there was significant overlap within these populations. None of the metabolites investigated showed a pattern of turnover that was exclusive to embryos that had the ability to implant, which is required for an absolute viability marker. Therefore, such criteria should only be used in conjunction with other measurable parameters.

Lactate and alanine metabolism of the COC independently showed differences in the morphological grade of the resultant embryo. While this is interesting, it does not necessarily add further information to the viability assessment of IVF embryos. The morphological appearance of embryos is easily visually assessed therefore a marker which correlates with morphological appearance is redundant. A true viability marker is able to differentiate between embryos of similar morphological appearance and could therefore be used in conjunction with current morphological scoring systems.

Glucose metabolism was not indicative of embryo viability or morphology in the current study. Previously, Hardy et al. (1989) demonstrated that glucose uptake at the cleavage stage is almost identical for embryos that arrest and those which form blastocysts (Hardy et al. 1989). The data presented in this thesis chapter similarly showed no difference in glucose metabolism of COCs or

embryos, which, while not arrested in development, had similar metabolic profiles of glucose independent of implantation success or failure. It may not be completely surprising that cleavage stage embryo glucose metabolism is not a good candidate as a viability marker, as glucose utilisation of the embryo at this stage of development is relatively low. The higher rates of glucose uptake by the more advanced embryos (≥4-cells) that have entered the cleavage division to initiate the embryonic genome may reflect uptake to support advanced development while increased rates of uptake of the delayed embryos may reflect precocious uptake of glucose and disruption of metabolic balance.

Conversely, as pyruvate is the preferred substrate at these early stages it could be expected to give a better indication of embryo physiology and thus viability. Indeed, the current study showed a trend towards an increase in pyruvate turnover for embryos that implanted as opposed to those that did not. In the human cleavage stage embryo, pyruvate uptake has previously been shown to be approximately 20% less in embryos which arrest at the cleavage stage compared to embryos that subsequently develop to the blastocyst stage (Hardy et al. 1989), which is supported by the current study with embryos that were able to implant consuming a lower level of pyruvate compared to those which failed to implant. Hardy et al. (1989) demonstrated only a small range of pyruvate uptake between embryos with differing potential to form blastocysts/implant, which was supported by the current study which demonstrated small ranges of pyruvate metabolism in good morphology cleavage stage embryos. Alternatively, a previous study has demonstrated a large range of pyruvate consumption in embryos which failed to implant (Turner et al. 1994). In addition, Turner et al. (1994) also demonstrated that good morphology embryos with an intermediate uptake of pyruvate were more likely to implant compared to poor morphology embryos with intermediate pyruvate uptake. This is particularly significant considering that the potential of good morphology embryos was able to be differentiated and that morphology and metabolism were able to be used in combination to improve viability prediction. Furthermore, single embryo transfers were performed on all 80 patients involved (Turner et al. 1994), however these findings have not been able to be substantiated in other investigations, including the current study (Hardy et al. 1989, Devreker et al. 2000). In contrast, an inverse relationship between pyruvate uptake at the cleavage stage and pregnancy establishment has been reported (Conaghan et al. 1993a), although this was determined with up to three embryos being transferred. Alternatively, another study found no difference in the mean pyruvate uptake of early embryos in relation to implantation potential (Devreker et al. 2000). Despite interesting evaluations of metabolism of embryos under varied conditions, to date there does not appear to be a promising method of carbohydrate utilisation in predicting viability in human cleavage stage embryos. Further investigations into techniques, culture systems and stage of development are required in this field.

Considering the significant levels of alanine present in the reproductive tract (Fahning et al. 1967, Miller and Schultz 1987) and the consistent appearance of alanine in culture media of embryos throughout pre-implantation development (Partridge and Leese 1996, Donnay and Leese 1999, Houghton et al. 2002), an important role for this amino acid is suggested. The current study found an association between alanine turnover of cumulus-oocyte complexes and embryo grade, which has previously been reported in human cleavage stage embryos, where embryos which produced higher levels of alanine were more likely to arrest at the cleavage stage rather than form blastocysts (Houghton et al. 2002). However, the current observations did not extend to a difference in implantation potential of COCs or embryos exhibiting specific alanine turnover profiles. However, the ratio of pyruvate to alanine metabolism was investigated in the current study which revealed a significant association between COCs and implantation potential, suggesting that low pyruvate uptake to alanine production is a sign of cellular stress and reduced oocyte competence. Pyruvate is transaminated in embryos to produce alanine (Donnay et al. 1999), and this pathway is considered important in the clearance of ammonium generated through amino acid metabolism and protein degradation (Orsi and Leese 2004). Again, pyruvate: alanine metabolic profiles of COCs were not completely distinct for viable and non-viable resultant embryos. Further, COC metabolic profile determination has significant limitations for clinical implementation. It is a substantial undertaking to implement the retrieval of spent media following incubation of individual COCs as a reasonable number of the oocytes involved will not fertilize normally, or result in morphologically normal embryos likely to be selected for transfer. Ideally a metabolic marker would be useful after the determination of embryo morphology whether at the pronuclear, cleavage stage or blastocyst stage, when only embryos suitable for transfer need to be differentiated. Furthermore, cell counts of cumulus masses surrounding oocytes were not performed and the almost certain difference in cell numbers between cumulus masses would have influenced the determination of single metabolites in the spent media. In an attempt to control for this, the relationship between some metabolites was assessed. Although in ICSI oocytes cell counts could be performed, it is not feasible for IVF embryos. Therefore, metabolic assessment of the spent medium around the COC may be limited as a marker unless ratios of metabolites or pathway interactions are determined.

Several studies have indicated that disrupted *balance* of metabolism is related to poor embryo development. The relationship between glucose consumption and lactate production, or glycolysis, has been related to blastocyst viability markers previously (Gardner et al. 1996b, Jones et al. 2001, Van den Bergh et al. 2001). The glucose: lactate metabolic profiles of bovine blastocysts following cryopreservation have been shown to definitively identify blastocysts capable of re-expansion post-thawing as opposed to those blastocysts which subsequently died (Gardner et al.1996b). Significantly, the metabolic profiles of these viable blastocysts did not overlap with non-viable blastocysts, indicating that such parameters would be appropriate for prospective selection of embryos for transfer. Glucose

and lactate ratios were also assessed in the current study, with no association found between glucose and lactate metabolic profiles of COCs or embryos with regard to viability. A significant correlation was observed in the glucose: lactate metabolism of embryos and resultant morphological grade. However, as discussed previously, such an association does not confer benefit clinically in embryo selection. A prospective study with single embryo transfers in the mouse has shown that the balance of glucose uptake and lactate production in blastocysts is a predictor of viability (Lane and Gardner 1996). When mouse blastocysts from this study were prospectively selected for transfer based on glycolytic activity, fetal development increased four-fold (80%) over random blastocyst selection (20%). Furthermore, blastocysts with abnormal metabolic activity had a fetal development rate of only 6% (Lane and Gardner 1996). At the blastocyst stage the embryo exhibits a more somatic cell–like physiology and has the ability to utilise a wider array of nutrients. The embryo has also increased its metabolic quotient and there is a significant increase in biosynthetic activity, all of which increase energy demands required for essential activation of the embryonic genome, compaction and blastocyst formation. As a result it may be that metabolic prediction of viability may be more predictive at these later stages of development. Human morulae and blastocysts, with a more dynamic metabolic rate between days 4 and 5 of development have shown lower glycolytic activity in embryos which implant (Van den Bergh et al. 2001), and may be a more appropriate stage of embryo development to investigate this. Further, it may be logical to assume that the quantification of metabolic pathway flux, as opposed to nutrient utilisation or consumption alone, is more likely to reflect cellular function and thus viability however this remains to be determined.

The great variability and lack of consistency in results between different culture systems for metabolic measures to be used as biomarkers of embryo viability requires significant advancement before such technology could be considered as a clinical tool. In addition, it is widely known that the presence and concentration of one nutrient in the environment around the embryo can have a significant effect on the utilisation of other nutrients (Gardner and Leese 1988, Lane and Gardner 2000b). The embryo is readily able to adapt to its environment, and change its pattern of nutrient turnover, reflecting its plasticity. Any adaptation by the oocyte/embryo to less than optimal culture conditions would be expected to result in a level of physiological stress to the oocyte/embryo which would ultimately result in a reduction in viability (Menke and McLaren 1970, Gardner and Leese 1990) due to the allocation of resources to repair and maintenance processes. This may prove to be a negative influence in the pursuit to establish a biomarker such as metabolism unless such patterns are clearly described and accounted for. A significant barrier to metabolic viability measures may involve the varied compositions of commercial media altering measured parameters between media systems.

Since the beginning of my studies, a new area of metabolomics has emerged that may provide a promising future for the determination of a metabolic marker. Investigation into entire profiles of metabolites where data is relatively easily obtained is likely to determine a relationship, between one or more metabolites/metabolic pathways that are able to predict the implantation capacity of embryos. It has already been demonstrated that metabolomic data does not correlate with embryo morphology (Vergouw et al. 2008, Seli et al. 2009b), and profiles of embryos from single embryo transfers on day 2, 3 and 5 have been retrospectively correlated with implantation (Go et al. 2009, Henman et al. 2009, Makinen et al. 2009). Furthermore, two prospective multicentre trials have demonstrated in day 5 single embryo transfers that viability scores resulting from metabolome profiles were correlated with implantation, independent of embryo morphology, as well as in different commercially available culture media (Botros et al. 2009, Seli et al. 2009b).

For metabolic parameters to be a candidate in viability determination of embryos, technology requires significant advancement. Metabolomic techniques are promising but require further assessment and validation of algorithms generated between and within clinics. The ideal use of a metabolic marker would be in a quick, simple assessment of only the embryos that have been morphologically screened to have some viability. Metabolic data would then be additive in the selection of the most viable embryo from a cohort. If the metabolic screening of embryos could be developed for varying stages of oocyte/embryo development it would be extremely valuable.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

Due to the medical, social, ethical and economic consequences associated with multiple pregnancies, IVF clinics are under pressure to change clinical practice to the replacement of a single embryo rather than multiple embryos. This requires superior selection techniques in the laboratory to choose the embryo with the greatest viability from a patient's cohort. Currently no such validated diagnostic test exists, with embryo morphology being the primary selection tool available, which is only weakly correlated with pregnancy rates. Viability markers for human embryos must be non-invasive and reliable and ideally should be rapid, simple and relatively low in cost with robust instrumentation for widespread use in IVF clinics. The relatively high rate of single embryo transfer that is performed in Australia makes this one of the few countries where this research is possible.

Culture of embryos singly is a prerequisite of a number of evaluation techniques for individual embryos so that the embryo can be tracked. This raises one of the first challenges for a viability marker as there are several studies in the literature that indicate that embryo development and viability is enhanced when embryos are grown in groups. However, all of these studies were performed in conditions that we now understand cause stress to the embryos and do not reflect the sequential media conditions that are currently used in IVF clinics. Therefore, in chapter 3, the grouping environment of mouse and human embryos was assessed in sequential culture systems and using a methodology that maintained the embryo density and assessed the effects of single culture in the pre and post-compaction stages of the embryo. Morphologically, no differences in mouse or human embryos were observed at the cleavage stage or with blastocyst development rates. However, single culture of mouse embryos reduced the proportion of blastocysts undergoing hatching. In addition, singly cultured blastocysts had fewer inner cell mass cells, which have previously been reported to correlate with reduced fetal viability. In this study, resultant implantation rates or fetal viability were not affected in mouse embryos, when transferred at either the cleavage or blastocyst stage. Furthermore, human implantation rates were unaffected by group culture or single culture of embryos to the cleavage or blastocyst stage. Placental weights were reduced in conceptii derived from single culture of mouse embryos that were transferred at the cleavage stage, although in this case there did not seem to be an effect on resultant fetal weight. Although this finding cannot be directly extrapolated to human clinical embryology, this observation requires further investigation in both species. Placental function normally impacts fetal development and intrauterine growth restriction is a common fetal outcome that results from a small or insufficiently formed placenta. Early developmental insults have long-term health implications (Barker 1998), as recognised by the developmental origins of adult disease hypothesis, which states that not only circumstances during fetal life, but as early as the peri-conception period have been associated health issues in adult life (Fernandez-Gonzalez et al. 2007). Human birth outcomes were not assessed in the

study detailed in Chapter 3 and may warrant further investigation for a technique that is likely to become essential in IVF clinics.

It is possible that the group culture of embryos with high developmental competence stimulates the growth of embryos which have a lower developmental competence through autocrine factors, whereas embryos grown singly are dependent only on their own inherent viability. This potential cooperative agent(s) between human embryos is to date undescribed however may have implications in human IVF, where any stimulatory action should be harnessed and exploited, particularly for patients with reduced numbers of embryos or embryos with known reduced developmental competence, such as patients with advanced maternal age. This study only assessed what can be described as good prognosis patients and ideally a study on donated human embryos from a cohort of patients with varied maternal ages could be conducted to further assess the effect of maternal age on the developmental effects and outcomes of group and single embryo culture. This could also incorporate birth outcomes and follow up which were omitted from the current study.

One of the advantages of blastocyst morphology as a selection tool is that single culture of embryos is not essential. Embryo selection at the cleavage stage may benefit from multiple observations of embryos and thus single culture may be advantageous. However, a single static observation of human embryos on day 5 of in vitro culture is able to greatly differentiate blastocysts resulting in high implantation rates of up to 80% in donor oocyte cycles where multiple embryos are transferred (oocytes collected from young fertile women) (Schoolcraft and Gardner 2000). However, to date most studies comparing cleavage stage and blastocyst culture and transfer have been confounded by the replacement of multiple embryos. Therefore, to assess this knowledge gap, experiments in chapter 4 detail assessment of morphology at the cleavage stage compared to extended culture in single embryo transfers. However, blastocyst culture does require significantly more resources and space than cleavage stage culture, which is not an option for all clinics. Therefore, a scoring system for the morphology of day 4 embryos was developed, for which embryos have also undergone important developmental checkpoints such as embryonic genome activation, and are progressively compacting and beginning the formation of a blastocoel cavity. This was able to reduce the burden on the laboratory from the 5-6 days of culture required for day 5 blastocyst transfers. Day 4 embryo replacement improved implantation rates compared to cleavage stage transfers, indicating that extended culture and assessment of embryos on day 4 benefits embryo selection. Day 5 embryo transfers did not add any further advantage to embryo selection, and in fact were not significantly higher than implantation rates for embryos selected at the cleavage stage of development. Day 4 embryo transfer may have an added

advantage of exposure to the complex milieu of the uterine environment unable to be replicated in vitro, which is somewhat reduced when an embryo replacement is performed on day 5. Day 4 embryo selection and replacement may provide a balance between embryo selection post-genome activation at a dynamic period of development that morphological quality is easily determined and incorporating maximal exposure to the uterine environment prior to implantation.

To maximise implantation rates beyond what can be achieved with morphological observations, at any stage development, likely requires a combination of morphological observations with biochemical or molecular markers of developmental potential. This study is the first to describe and assess a day 4 morphological scoring system in single embryo transfers. Embryo morphological assessments at all stages of development are now available for implementation and thus future development of assessment markers will be able to be used in combination with a selection of developmental stages. This has wide reaching relevance to incorporate clinics employing blastocyst or extended stage culture and transfer of embryos, as well as those clinics who require embryo selection at the cleavage or pronuclear stages of embryo development.

Selection of an oocyte presents added challenges in IVF. Currently oocytes are scored based on the presence of negative morphological characteristics such as excessive granularity, presence of vacuoles, refractile bodies, perivitteline space and polar body morphology, as well as zona pellucida characteristics which have poorer developmental consequences (Alikani et al. 1995, Serhal et al. 1997, Kahraman et al. 2000, Rienzi et al. 2008). Chapter 5 aimed to investigate a biochemical marker of oocyte viability by using cumulus cells, a by-product of IVF treatment. In a retrospective study, REDOX status of cumulus cell masses were shown to be negatively correlated with fertilization potential of oocytes undergoing either IVF or ICSI. In addition, and most significantly, REDOX status of cumulus cells shows great promise as a candidate biomarker of implantation potential by being associated with a significantly improved implantation rate following single embryo transfer, when embryos resulting from oocytes with REDOX levels in cumulus masses measured at an intermediate level were selected for transfer. Importantly, a range of REDOX levels was observed within and between patients, and REDOX status did not correlate with embryo morphology, likely making these parameters additive in predictive ability of implantation. One of the benefits of this type of categorical analysis on oocytes is that embryos can be grouped into 3 categories, reducing the requirement for single culture and allowing for embryos from the same category to be cultured together in groups. The data in this chapter represents a promising development in the search for a viability marker and warrants further investigation in a prospective randomised controlled trial.

A field with potential in the pursuit to identify a biomarker of viability is the assessment of embryo metabolism. Being a product of the functional quality of the oocyte/embryo, and a combination of genetic and environmental factors, metabolism of individual oocytes and embryos has strength in the identification of embryos with high developmental potential. The data presented in Chapter 6 of this thesis, did determine that there were some relationships between metabolic activity and embryo morphology and importantly implantation. However, it also provided evidence of the potential limitations of using cleavage stage embryo metabolism and individual metabolites as a marker for subsequent implantation. It is likely that the best application of this type of metabolic assessment might be at the later stages of development where there is an increase in the metabolic activity of the embryo at the morula and blastocyst stages. Assessment of post-compaction embryos incorporates the contribution of the paternal genome (i.e. sperm) to the embryo, as the embryonic genome is activated just prior to this stage. One of the further studies that might be able to be undertaken is metabolic analyses in conjunction with the day 4 assessment described in chapter 4, as embryos would be at a dynamic stage of development, post-genome activation and more metabolically active than cleavage stage embryos, without the burden of blastocyst culture.

Advancements in technologies have resulted in the emerging field of metabolomics for embryos, which uses platforms such as mass spectrometry, nuclear magnetic resonance spectroscopy and near infrared spectroscopy to determine a broad profile of the metabolites from the medium, rather than the method used in this study, and others to assess known/single pathways. This method may be advantageous in that it measures many different metabolites simultaneously and currently shows potential for implementation in IVF clinics with a small device engineered to determine from extremely small volumes of media, an almost immediate output profile (Dunn and Ellis 2005).

Of the viability markers that have been investigated in these studies and many others to date, there has been no clear identification of a candidate marker which is consistently associated with implantation. Morphology and cleavage rate are likely to remain the mainstay of embryo assessment, being relatively quick and simple, and able to eliminate embryos with very little developmental potential from selection for transfer. The development of more quantitative methods of assessing the developmental potential of an embryo would further increase success rates following IVF. Many markers have shown promise, however there is constantly overlap in the measures of those embryos which implant or fail to establish pregnancy. No test is ever likely to be 100% reliable, and non-embryo-related factors in the process of implantation also require consideration. In addition, any biochemical or molecular marker will be influenced by multiple factors and the establishment of reference ranges may therefore be problematic

in a field such as IVF. Superovulation regimens, changes in embryo culture systems and differing aetiology of patients would all be expected to induce variation in any biomarkers that are discovered thus much work remains in this field to clarify these situations. The implications of this may be that we are able to evaluate the competency of the embryo, but need to consider that there are other significant interactions which are currently being disregarded i.e. patient factors such as uterine receptivity. A compromise that must be made in studies of this type are that viability markers are likely to provide a ranking of competency within a patient's cohort of embryos, rather than being ultimately predictive. Such a scheme would then enable selection of an individual patient's most viable embryo, which may still have a less than ideal ranking, but is that patient's best chance at pregnancy for that treatment cycle. The difficulties with such studies begin with the recruitment of patients undergoing an emotive medical treatment when large numbers of participants are required to elucidate and establish new technologies. Further, the collection of samples from all retrieved oocytes/embryos when only a few candidates will likely be eligible for measurement at the time of transfer is not an efficient use of resources.

The studies described in this thesis are the first to assess a range of candidate viability markers using single embryo transfers within a single IVF clinic. Our primary interest following these investigations is to validate and implement the techniques that appear to be promising and have industry applicability. Prediction of embryo implantation potential with the employment of multiple measures from the same patients and embryos in one study, using advanced statistical analysis, will hopefully reveal the possibilities available in pursuit of identifying the most viable embryo in a patient cohort. The preliminary operations for this require large scale, prospective randomised trials, with multi-centre investigations being advantageous to explore validity of any potential measures between IVF centres. Combinations of morphological and biological markers can only improve the success of fertility treatments by utilising more information about the health of the embryos we create than we are currently aware. Improvements in success rates will encourage clinicians, scientists and patients to transfer single embryos and reduce the incidence of multiple gestations and their associated negative outcomes which is the most significant current challenge to the industry.

Fertility has been a rapidly advancing scientific discipline since the inception of human IVF, however there is still much to explore. The discovery of technology of this kind generates with it volumes of knowledge which can only serve to educate clinicians, patients and scientists. The ultimate beneficiaries of such technology are the patients and their much desired single, healthy child.

CHAPTER 8

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APPENDICES

Appendix 1 – Ethics Patient Information and Consent Form

THE UNIVERSITY OF ADELAIDE AUSTRALIA

Women's & Children's Hospital ADELAIDE

Patient Information Sheet for the Research Study:

Predicting Pregnancy Potential of Human Embryos

Why do we want to do this study?

Thank you for reading this information sheet to consider the donation of fluids, cells and media that result from your IVF/ICSI cycle for research.

The goal of this study is to improve infertility treatment for couples by optimising the outcome of treatment with a single healthy baby. Currently, embryos are selected for transfer by an embryologist according to the cell characteristics of the embryo. By increasing our understanding of what factors contribute to embryo viability we will be able to select and transfer a single embryo based on more scientific information. This may result in an increase in the ability of that embryo to implant and produce a single healthy baby.

In the future we hope this knowledge will lead to an increase in pregnancy rates following IVF treatment. Therefore single embryos will be able to be transferred to all patients, reducing the risk of multiple pregnancies which pose serious health risks for both the mother and babies.

Who can take part?

Any woman having an IVF/ICSI cycle will be invited to join the study.

How does the study work?

During your IVF cycle you will have an egg retrieval in theatre. During this procedure, your eggs are removed from follicular fluids and placed in a culture medium during their time in the laboratory. These fluids are normally discarded immediately following your egg retrieval, however we would like to analyse the content of these fluids for factors that might contribute to producing a healthier egg.

The cells surrounding the eggs when they are collected in theatre, are removed during the IVF procedure. These are also normally discarded. However, recent studies in animal models have determined that these cells contain information about the health of the egg.

Finally, the culture media your eggs and embryos are bathed in needs to be changed regularly to maintain the correct nutrient levels. When your eggs and embryos are moved to new media, the used media is discarded. This media will be examined for nutrient uptake and production to investigate the metabolic pathways of healthy embryos.

Who will manage my treatment if I take part?

Your own doctor will continue to oversee your treatment.

How do I participate in this study?

If you choose to donate these excess materials involved in your treatment cycle for this study, a consent form must be signed by both partners having treatment and witnessed by a doctor/nurse or scientist at Repromed. The consent form must be signed before you have your operation.

What will I get out of the study?

The results of this study may not be of any direct benefit to you. However we hope the knowledge we gain from this research will increase our understanding of how to select the most viable embryo for transfer and result in positive outcomes from infertility treatment for future patients.
There will be no payment for your participation in this study.

Are there any risks to me or my embryos if I take part in the study?

There will be no additional risks or discomforts to you, other than those that would normally be experienced during an IVF cycle at Repromed. Your embryos will not be affected by the study as only discarded fluids, cells and media are being investigated. Embryos that are not transferred or frozen as part of your cycle are not available for research.

What happens if I don't want to take part?

Before deciding whether or not to take part in this trial, you may wish to discuss the matter with a relative, friend, your doctor, nurse or counselor. Arrangement can be made for such discussion. It is important that you understand that your participation in this trial is voluntary, and if you do not wish to take part you are under no obligation to do so.
If you decide to take part but later change your mind, you are free to withdraw at any stage. Your decision to take part, not to take part, or to withdraw, will not affect your routine medical treatment or your relationship with those treating you or your relationship with the Unit.

Assurances of confidentiality

Your confidentiality will be maintained regarding all records kept as a result of this study. Your identification number and initials only will be used in place of your full name in the case report forms and all other data storage forms for your privacy.
When the study is complete, all results will be pooled together to be analysed and published, subject to acceptance, in a scientific journal. This will be done in an anonymous way so no individual patient's information can be traced.

What if I have a question about the study?

If you have any questions regarding this study, you are welcome to contact the

Principal Scientific Investigator, Dr. Michelle Lane, on 8222 8409, or
the Repromed embryologist collaborating with Dr Lane, Deanne Feil, on 8333 8111.

The Women's & Children's Hospital's Ethics Committee has approved this study.

Should you wish to speak to a person not directly involved, in particular in relation to matters concerning policies, information about the conduct of the study or your rights as a participant, or should you wish to make a confidential complaint, you may contact the

Research Secretariat, Ms. Brenda Penny, on 8161 6521.

Thank you for reading this information sheet.

WOMEN'S & CHILDREN'S HOSPITAL RESEARCH

ETHICS COMMITTEE Consent Form

Women's & Children's Hospital ADELAIDE

I _____&_____

hereby consent to involvement in the research project entitled:

Predicting Pregnancy Potential of Human Embryos

1. The nature and purpose of the research project described on the attached Information Sheet has been explained to me. I understand it, and agree to take part.
2. I understand that I may not directly benefit by taking part in this study.
3. I acknowledge that the possible risks and/or side effects, discomforts and inconveniences, as outlined in the Information Sheet, have been explained to me.
4. I understand that while information gained in the study may be published, I will not be identified and information will be confidential.
5. I understand that I can withdraw from the study at any stage and that this will not affect medical care or any other aspects of my relationship with this hospital.
6. I understand that there will be no payment to me for taking part in this study.
7. I have had the opportunity to discuss taking part in this research project with a family member or friend and/or have had the opportunity to have a family member or friend present whilst the research project was being explained by the researcher.
8. I am aware that I should retain a copy of the Consent Form, when completed, and the Information Sheet.
9. I consent to samples of follicular fluid, cells and media being taken during my treatment cycle for use in the above project.

Signed: .....……Date: .....

Full name of patient: .....……

Signed: ……...Date: ……..…

Full name of patient: ……..

I certify that I have explained the study to the patients and consider that he/she understands what is involved.

Signed: .....…….. Date: ……

Name: .....…….....

Appendix 2 - IVF Cycle Stimulation

Stimulation Guidelines during an IVF cycle†

For patients on their first cycle of treatment*:

a) **Long Downregulation 150IU FSH**
used if **ALL** of the following present;

- Maternal age $\leq$ 35 years
- Both ovaries present and normal
- Ovarian volume > 4 mL
- Antral follicle count > 6 (2-5mm)
- Day 3 FSH < 8
- AMH > 8 pmol/L (if test has been performed)

b) **Long Downregulation 200-225**
may be used if **ANY** of the following present;

- Maternal age 36 – 38 years
- Day 3 FSH = 8-10
- One significantly compromised ovary

c) **Long Downregulation 300IU**
may be used if **ANY** of the following present;

- Maternal age $\geq$ 39 years
- Day 3 FSH $\geq$ 11
- One ovary or smallest ovarian volume < 3mL
- Severe endometriosis
- AMH < 8 pmols/L
- Antral follicle count < 6 (2-5mm)

*In **second and subsequent cycles** the stimulation dose was determined by the patient's response on their previous cycle and modified if required to aim for 8-12 oocytes.

† with thanks to Repromed Adelaide, for use of their patient stimulation criteria.

Appendix 3 – Morphology Scoring Systems

Overall Grades for all pre-implantation embryo stages

Grade 1 – excellent quality embryo, suitable for cryopreservation
Grade 2 – satisfactory quality embryo, suitable for cryopreservation
Grade 3 – poor quality embryo, not suitable for cryopreservation
Grade 4 – very poor quality embryo, likely arrested in development, not suitable for cryopreservation

Cleavage Stage Embryos* (Day 2,3)

	Fragmentation			
	<10%	*10-20%*	*20-50%*	*>50%*
On-time cleavage 40-42h 4-5 cell 64-66h 7-9 cell/compacted	1	2	3	4
Other delayed cleavage accelerated cleavage	3	3	4	4

Additional morphological anomalies known to affect embryo quality (multinucleation, uneven cell division, irregular or abnormal cytoplasm) result in a down grading per presence of anomaly.

* with thanks to Repromed Adelaide, for use of their morphology scoring criteria.

Blastocyst Stage Embryos* (Day 5, 6)

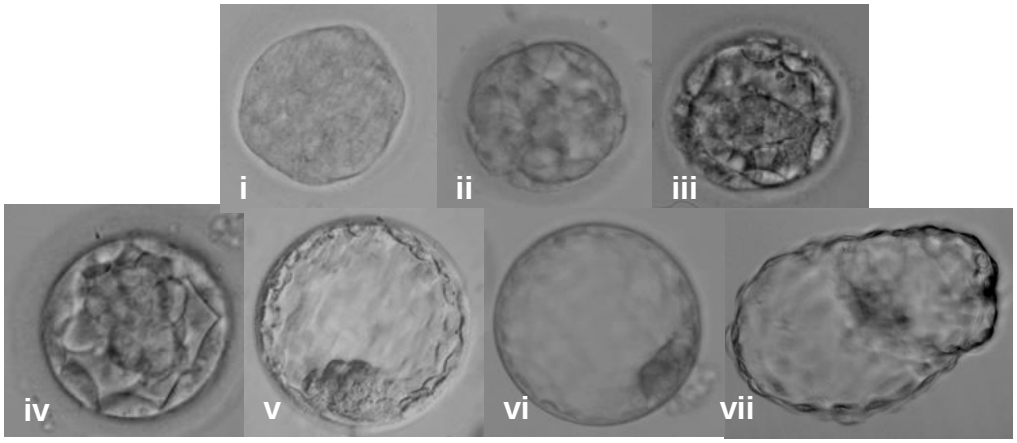

Figure		
i	**0** – morula or lesser stage	no blastocoel cavity seen
ii	**1** – early blastocyst	blastocoel less than half the volume of the embryo
iii	**2** – blastocyst	blastocoel ≥ half of the volume of the embryo
iv	**3** – full blastocyst	blastocoel completely fills the embryo
v	**4** – expanded blastocyst	zona thinning and overall increase in size
vi	**5** – hatching blastocyst	trophectoderm has started to herniate through the zona
vii	**6** – hatched blastocyst	blastocyst has completely escaped from the zona

Inner Cell Mass (ICM) Scoring
for embryos ≥ grade 3
A – Tightly packed, many cells
B – Loosely grouped, several cells
C – Very few cells

Trophectoderm (TE) Scoring
for embryos ≥ score 3
A – Many cells forming a cohesive epithelium
B – Few cells forming a loose epithelium
C – Very few cells

	Grade 1	Grade 2	Grade 3	Grade 4
Day 5 and Day 6	• Full blastocyst (≥3) with two 'A' grades for both ICM and TE	• Full blastocyst with at least one 'B' grade for ICM or TE	• Full blastocyst with a 'C' ICM or TE • Early blastocysts ('1' or '2')	• Compacted morula • Uncompacted embryos

* with thanks to Repromed Adelaide, for use of their morphology scoring criteria.

Appendix 4 – Avertin Formulation

(2,2,2-tribromoethanol, 2-methyl-2-butanol)

Stock Solution

0.12g 2,2,2-tribromoethanol

1mL butan-2-ol

The stock solution was prepared and stored in the dark at room temperature for up to 6 months.

Working Solution

0.12mL stock solution

10mL sterile saline

The working solution was prepared from the stock solution and allowed to dissolve at room temperature and stored at 4°C for up to 3 weeks.

Dosage

The dose of Avertin given to animals was based on the body weight of the mouse, where 0.02mL was given for each gram of body weight.

Appendix 5: Day 4 Embryo Selection is Equal to Day 5 using a New Scoring System Validated in SETs

Feil, D., Henshaw, R.C. and Lane, M. (2008) Day 4 embryo selection is equal to Day 5 using a new embryo scoring system validated in single embryo transfers.
Human Reproduction, v. 23 (7), pp. 1505-1510, July 2008

Appendix 6 – Redox Sensor Red

Redox Sensor Red (2,3,4,5,6-pentafluorodihydrotetramethylrosamine, Redox Sensor Red CC-1, Molecular Probes, Invitrogen, Australia) is a fluorogenic indicator of oxidative activity in proliferating and quiescent cells (Chen CS 2000 Free Radic biol med 1266). Redox Sensor Red enters cells passively and is oxidised by various oxidants including peroxynitrite, nitric oxide, nitrite, fatty acid hydroperoxide, hydrogen peroxide/superoxide radical and cytochrome c (Chen CS 2000 Free Radic biol med 1266). Once oxidised the indicator produces a fluorescent signal which has a linear relationship with the level of oxidant encountered.

Redox Sensor Red was supplied as 50µg vials which were prepared by addition of 115µL of dimethyl sulfoxide (DMSO) to produce a 1mM solution, which was stored at -20°C for up to 3 months. Working solutions were prepared by addition of 999µL of GMOPS medium with 5% HSA (Vitrolife) to 1µL of diluted Redox Sensor Red. The working solution of Redox Sensor Red was warmed to 37°C prior to use.

Appendix 7 - Ultramicrofluorometric Assays: Materials and Recipes

Glucose Concentration Assay

Cocktail

EPPS Buffer	0.75mL
5mM DTT	0.1mL
37mM $MgSO_4.7H_20$	0.1mL
10mM ATP	50µL
10mM NADP	150µL
Hexokinase/G6PDH	50µL

Freeze in 0.5mL aliquots.

EPPS Buffer

EPPS	2.52g
Penicillin	10mg
Streptomycin	10mg

Make up in 150mL H_20. pH to 8.0 with 1M NaOH. Dilute to 200mL.

Solutions

5mM DTT	7.72mg in 10mL H_20
37mM $MgSO_4$	91.2mg in 10mL H_20
10mM ATP	30.3mg in 5mL H_20
10mM NADP	39.4mg in 5mL H_20

Preparation of 1mM Standard

Add 0.0180g of glucose to 100mL of water and dissolve.

Pyruvate Concentration Assay

Cocktail

EPPS Buffer	0.7mL
5mM NADH	15µL
LDH	20µL

Freeze in 0.5mL aliquots.

EPPS Buffer

EPPS	2.52g
Penicillin	10mg
Streptomycin	10mg

Make up in 150ml H_20. pH to 8.0 with 1M NaOH. Dilute to 200ml.

Solutions

5mM NADH	17.73mg NADH in 5mL water

Preparation of 1mM Standard

Add 0.0110g of pyruvate to 100mL of water and dissolve.

Lactate Concentration Assay

Cocktail

Glycine/Hydrazine Buffer	0.45mL
Water	0.40mL
Lactate Dehydrogenase	25µL

Add to NAD+ in eppendorf just prior to use.

Glycine/Hydrazine Buffer

Glycine	7.5g
Hydrazine	5.2g
EDTA	0.2g
2M NaOH	51mL

Suspend glycine, hydrazine and EDTA in water. Add NaOH. Dilute to 100mL.

NAD+

Prepare 40mg/mL solution and freeze in 75µL aliquots in eppendorf tubes.

Preparation of 1mM Standard

Add 0.009g of lactate to 100mL of H_20 and dissolve

Alanine Concentration Assay

Cocktail

Buffer	570µL
Enzyme	20µL

Buffer

Tris-hydroxymethyl-amino methane	1.2g
α-ketoglutarate	0.231g

Dissolve in 100mL of water. pH with HCl to 7.6. Store at 4°C.

Enzyme Preparation

10mg/ml Glutamate-Pyruvate Transaminase (GPT)
Dissolve 10mg in 1mL of water. Store 4°C.

Preparation of 0.5mM Alanine Standard

Add 0.0045g of alanine to 100mL of water and dissolve.