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FACULITY OF NATURAL & ENVIRONMENTAL SCIENCES

Centre for Biological Science

Effect of *In Vitro* Fertilisation (IVF) and Embryo Culture Duration on Mouse Development and Postnatal Health

by

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Thesis for the degree of PhD

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ABSTRACT

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EFFECT OF *IN VITRO* FERTILISATION (IVF) AND EMBRYO CULTURE DURATION ON MOUSE DEVELOPMENT AND POSTNATAL HEALTH

Anan Aljahdali

Since the advent of IVF (*in vitro* fertilisation) and assisted reproductive technologies (ART), several million babies have been born worldwide. However, reports link *in vitro* techniques with adverse short and long-term health outcomes. Using a mouse model, we have investigated the effect of IVF and culture on blastocyst development and cell number and on postnatal health of offspring. To explore the effect of different durations of embryo culture after IVF (as used commonly in clinical practice) and to evaluate the effect of embryo transfer itself plus the need for different controls, five treatment groups were generated as follow, each comprising 8-13 litters. NM (natural mating control, no ART treatment, non-superovulated); IV-ET-2Cell (2-cell embryos derived *in vivo* from superovulated (SO) mothers and immediately transferred (ET) to pseudo-pregnant recipients); IV-ET-BL (blastocysts derived *in vivo* from SO mothers and immediate ET); IVF-ET-2cell (2-cell embryos generated by IVF from SO mothers, short culture and ET); IVF-ET-BL (blastocysts generated by IVF from SO mothers, long culture and ET). Offspring were

weighed weekly, systolic blood pressure (SBP) taken at weeks 9, 15, 21 and LIFE (average), and glucose tolerance test (GTT) carried out prior to culling for organ collection at week 27. Serum glucose, insulin concentration and the G:I ratio were calculated, with serum and lung angiotensin converting enzyme (ACE) levels determined after collection and storage of serum and lungs following culling; with random effects regression statistical analysis used to assess independence of litter size and maternal origin. IVF blastocysts after prolonged culture developed slower and comprised reduced trophectoderm and ICM cell numbers compared with *in vivo* generated blastocysts ($P<0.05$; n= 50-87 per treatment; differential nuclear labelling). Offspring from IV-ET-2Cell (n= 57), IV-ET-BL (n= 47), IVF-ET-2Cell (n= 75) and IVF-ET-BL (n= 42) groups compared with NM controls (n=80), showed increased body weight, increased SBP, impaired GGT and abnormal organ:body weight ratios in both sexes ($P<0.05$), independent of litter size. At weeks 15, 21 and LIFE, SBP for IVF-ET-BL males was increased compared with IV-ET-BL males ($P= 0.003$, 0.014 and 0.001, respectively). At weeks 15, 21 and LIFE, IVF-ET-BL males had increased SBP compared with IVF-ET-2Cell males ($P=0.032$, 0.034 and 0.017, respectively). In addition, offspring from the IVF-ET-BL group had a significant increase in serum and lung ACE activity compared with the NM group ($P= 0.034$), ($P= 0.019$) respectively. Offspring from IVF-ET-BL group also had a significant increase in lung ACE activity compared with IV-ET-BL group ($P=0.042$), although, serum ACE activity tended to be higher than IV-ET-BL, but this did not reach statistical significance ($\dagger =0.070$). Selected correlations show that SBP at 21 weeks in male offspring from IVF-ET-BL were positively correlated with body weight at 9 weeks ($\dagger =0.051$), at 15 weeks ($P=0.018$) and at 21 weeks ($P=0.016$) with R^2 values of 0.046, 0.09 and 0.09 respectively. SBP at 21 weeks and LIFE were also positively correlated with lung ACE activity 0.002 and 0.009 respectively. However, glucose

concentration 2 hours after glucose injection and the AUC (area under curve) in the male IVF-ET-BL group was reduced compared with IVF-ET-2Cell males (P= 0.03, 0.003, respectively). In males, IV-ET-2Cell, IVF-ET-2Cell and IV-ET-BL offspring all demonstrate low G:I ratios in comparison to NM mice (P=0.005, P=0.001 and P=0.038; respectively). Selected correlations demonstrate that there is a relationship between weight and AUC, in which weight is positively correlated with AUC measurements in NM (P=0.001), IV-ET-2Cell (P=0.000), IVF-ET-2Cell (P=0.046), IV-ET-BL (P=0.013) and IVF-ET-BL offspring (P=0.002), with R^2 values of 0.2, 0.29, 0.13, 0.26 and 0.2, respectively. Male IVF-ET-BL heart:body weight ratio was increased and liver:body weight ratio reduced compared with IVF-ET-2Cell males (P=0.019, 0.023, respectively). No differences were evident between the four treatments groups for females. Our results suggest that reproductive treatments affect the development and potential of preimplantation embryos, influencing postnatal development and physiology compared with undisturbed reproduction. In particular, prolonged embryo culture (from 2-Cell to blastocyst), with normalised SO, IVF and ET, may adversely affect male offspring cardiovascular health, but improve the metabolic profile, compared with short culture (ET at 2-cell stage). However, female health is less sensitive.

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DECLARATION OF AUTHORSHIP

I, Anan Aljahdali

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

EFFECT OF *IN VITRO* FERTILISATION (IVF) AND EMBRYO CULTURE DURATION ON MOUSE DEVELOPMENT AND POSTNATAL HEALTH

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

Signed: Anan Aljahdali

Date: 23/09/2016

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Definitions and Abbreviations

ACE	Angiotensin Converting Enzyme
ART	Assisted Reproductive Technologies
CHD	Coronary Heart Disease
DOHaD	Developmental of Origins of Health and Disease
EGA	Embryonic Gene Activation
EPI	Epiblast
ET	Embryo Transfer
FSH	Follicle Stimulating Hormone
GTT	Glucose Tolerance Test
hCG	Human Chorionic Gonadotropin
HTF	Human Tubal Fluid
ICM	Inner Cell Mass
ICSI	Intracytoplasmic Sperm Injection
IQ	Intelligence Quotient
IVF	*In vitro* Fertilisation
IV-ET-2Cell	2-cell embryos derived *in vivo* from superovulated mothers and immediately transferred
IV-ET-BL	Blastocyst embryos derived *in vivo* from superovulated mothers and immediately transferred

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IVF-BL-2Cell	Embryos generated by IVF from superovulated mothers and cultured to 2-cell stage before transfer
IVF-ET-BL	Embryos generated by IVF from superovulated mothers and cultured to blastocyst stage before transfer
JAM	Junctional Adhesion Molecule
LPD	Low Protein Diet
NM	Normal Mating
PC	Periconceptional Period
PE	Primitive Endoderm
PMS	Pregnant Mare's Serum
SBP	Systolic Blood Pressure
SO	Superovulated
TE	Trophectoderm
TJ	Tight Junction

Chapter 1: Literature Review

Over the last 30 years, assisted reproductive technologies (ART) have continued to expand as a clinical procedure to overcome infertility and currently account for approximately 3-4% of births in Europe. Indeed, the 2010 Nobel Prize in Medicine was duly awarded to Prof Robert Edward from The University of Cambridge in England, who with Mr Patrick Steptoe was the pioneer of the first successful human ART birth. However, the long-term consequences of ART have yet to be fully defined. Research shows that ART is linked with some adverse perinatal and postnatal outcomes, including an increase in some congenital abnormalities, low birth weight and an increased risk of genomic imprinting disorders and altered DNA methylation ((Bower and Hansen 2005); (Basatemur and Sctcliffe 2008); (Manipalviratn et al. 2009b). Since DNA methylation patterns are extensively remodelled during preimplantation development, the disturbance in the natural environment of embryos by ART provides a potential window for induction of epigenetic dysregulation. The increased risk of imprinting disorders in ART children is particularly associated with Beckwith-Wiedemann and Angelman syndromes (Bowdin et al. 2007); (Manipalviratn et al. 2009b); (Odom and Segars 2010). However, definitive conclusions are difficult to substantiate due to the rarity of imprinting disorders and the variability of ART protocols.

Animal studies have shown that ART procedures can alter normal imprinting, specifically DNA methylation patterns (Manipalviratn et al. 2009b); (Grace and Sinclair 2009); (Market-Velker et al. 2010). Epigenetic disorders in rodent and domestic animal models induced by embryo culture show both fetal and placental tissues can be affected and that the condition is preserved into adulthood and can be associated with disease (Doherty et al. 2000);

(Young et al. 2001); (Mann et al. 2004) ; (Fernandez-Gonzalez et al. 2004) ; (Morgan et al. 2008) ; (Rivera et al. 2008a). Moreover, the use of animal models where lifespan measurements can be made more easily do suggest risks. Indeed, our own study in mice revealed sustained hypertension throughout adulthood coupled with cardiovascular and metabolic dysfunction induced by preimplantation embryo culture (Watkins et al. 2007). The impact of prolonged embryo culture is in particular a concern in clinical ART as women move towards childbearing in later life.

1.1 Preimplantation embryo and blastocyst formation

Once fertilisation has been achieved, the fertilised egg undergoes several significant morphological and molecular events during the first few days of mammalian development, resulting in the formation of a blastocyst. These events begin when the zygote undergoes a series of early cleavage divisions and consequently the number of cells increases without changing the overall size of the embryo. These smaller cells, which are produced from the cleavage of the zygote, are referred to as blastomeres (Cockburn and Rossant 2010b).

As the blastomeres divide through cleavage divisions, the transition from maternal to zygotic genomes begins. During this event, the maternal gene products present in the oocyte (mRNA) actively undergo degradation and from the 2-cell stage, new transcripts and new proteins are formed by the embryonic genome of the zygote (Bacharova 1985); (Bacharova and Moy 1985); (Hamatani et al. 2004); (Gasperowicz and Natale 2011).

Crucial events that determine the fate of different cell lineages of the embryo including cleavage, compaction and polarisation take place during the preimplantation period. Generally, the zygote divides to form a spherical blastocyst with the outer cells of the

embryo contributing to the unilaminar epithelium and, the trophectoderm (TE), which goes on to form the chorio-allantoic placenta after implantation. In contrast, the undifferentiated inner cells contribute to the Inner Cell Mass (ICM), and this clump of cells subsequently segregates into two lineages: the epiblast (EPI) and the primitive endoderm (PE). The EPI gives rise to the entire foetus and the PE contributes to the extra-embryonic yolk sac placenta; Figure 1.1 and Figure 1.2 (Cockburn and Rossant 2010b).

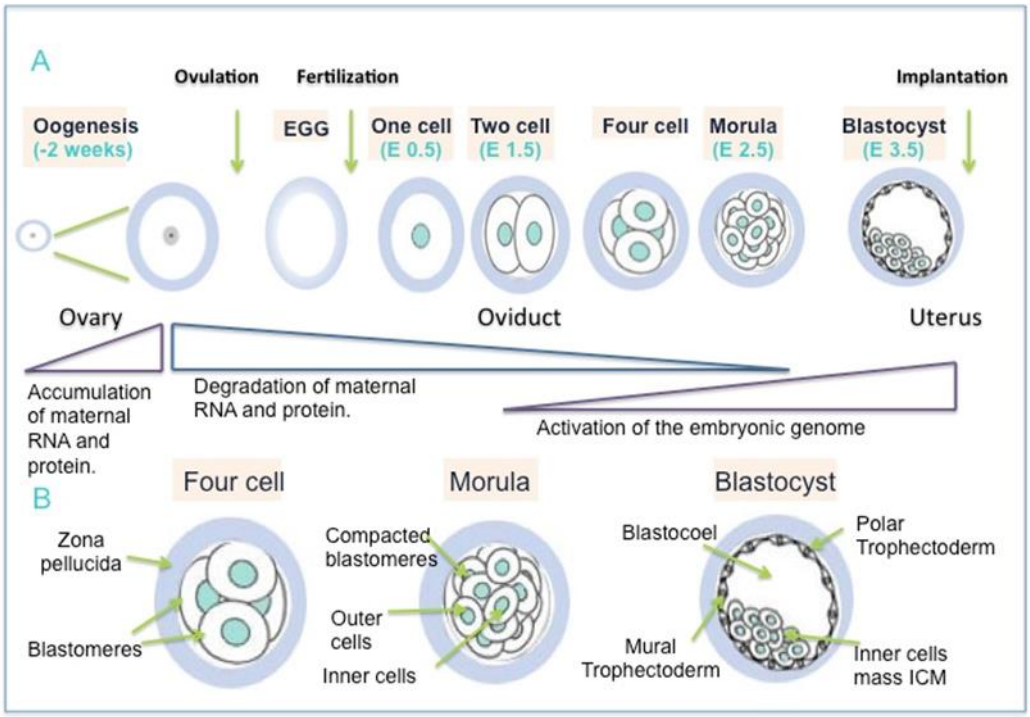


Figure 1.1 Stages of Preimplantation mouse embryo

A) Oogenesis and early development (from left to right) zygote, 2-cell, 4-cell, morula and blastocyst.
B) Late preimplantation development. At the 4-cell stage, blastomeres are encased within the protective confines of the zona pellucida. At the 8-cell stage, the embryo compacts to form the morula. Symmetric cell divisions lead to similar daughter cells, while Asymmetric divisions produces outer cells, which contribute to TE and inner cells, which establish the ICM of the fetus. Adapted from (Cockburn and Rossant 2010a).

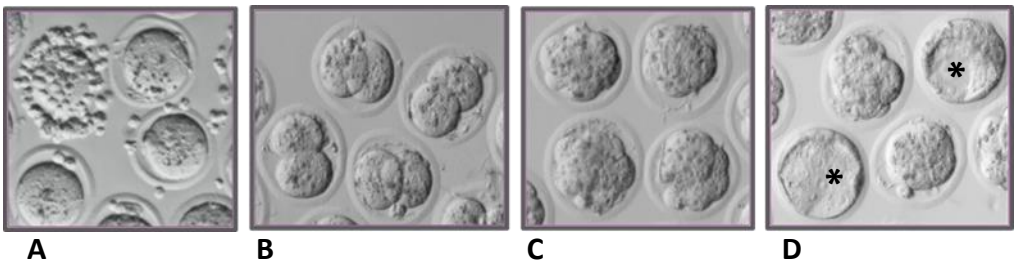


Figure 1.2 Preimplantation mouse embryo development stages

(From left to right) A- fertilisation (oocyte and sperm), B- 2-cell, C- morula and D- late blastocyst (*). In the late blastocyst, cells have committed to the TE and ICM. Micrographs taken during my IVF experiments. See Figure 3.3 in page 83 for scale bar, Fig 1.2 was edited from the Figure 3.3.

In mouse, the differentiation of the TE begins at compaction at the 8-cell stage. Compaction is the process of increasing intercellular adhesion, which results in a morphological change in the blastomeres and as a consequence they become smooth and flattened. Compaction is an important process, as it plays a role in the subsequent segregation of the embryonic and extra-embryonic lineages and later morphological events (Vestweber et al. 1987); (Van Roy and Berx 2008); (Cockburn and Rossant 2010b).

The increase in cell-to-cell adhesion at compaction is as a consequence of epithelial intercellular junction formation. E-cadherin is a transmembrane protein of the cadherin superfamily of adhesion molecules, which associates at its cytoplasmic region with a complex of several molecules including β-catenin, γ-catenin or plakoglobin and α-catenin to constitute the adherence junction (Eckert and Fleming 2008). In the mouse, at the 8-cell stage, E-cadherin becomes localised to regions of cell-to-cell contact. Once E-cadherin is functionally activated, the blastomeres adhere together to form a compact ball of cells (Vestweber et al. 1987); (Thomas et al. 2004); (Cockburn and Rossant 2010b); (Gasperowicz and Natale 2011). It has been shown that an epithelial tight junction (TJ) forms after compaction and composed of several interacting proteins localised as a belt around the

apicolateral contact site of each TE cell (Eckert and Fleming 2008). There are three main groups of transmembrane proteins identified within TJs: occluding (Furuse et al. 1993); claudin family members and junctional adhesion molecule (JAM) (Martin-Padura et al. 1998); (Bazzoni 2003) ; (Thomas et al. 2004). The TJ that forms between the outer cells of the TE stabilises the tightly packed arrangement, seals off the inside of the spherical embryo and is permissive for blastocyst cavity formation. All cells within the embryo also form gap junctions that enable small molecules and ions to pass between them (Wolpert et al. 2002); (Gilbert 2003).

In mice, 8-cell blastomeres become apicobasally polarised concomitant with cell adhesion at compaction. Polarisation occurs along the axis from cell contact to the free outer surface of the embryo, resulting in two domains: firstly, an outward-facing apical domain rich in microvilli and secondly, an inward-facing basolateral domain at contact sites with relatively few microvilli. Polarisation results in a radial polarity for the entire embryo. In addition, while the embryo at the 8-cell stage develops to the 16-cell stage, blastomeres undergo two types of cell division, mainly, associated with inheritance of the apical, microvilli-rich polar domain. The first division is parallel to the polarity axis (or inside-outside axis) and produces two outer (polar) daughter cells. Another division occurs perpendicular to the inside-outside axis, resulting in one outer (polar) cell and one inner (non polar) cell; Figure 1.3. As a consequence of these two types of division, there are two populations of cells: an outward daughter (polar) cells and an inward daughter (non-polar) cells at the 16-cell stage (Eckert and Fleming 2008) (Cockburn and Rossant 2010b); (Gasperowicz and Natale 2011); (Eckert et al. 2015).

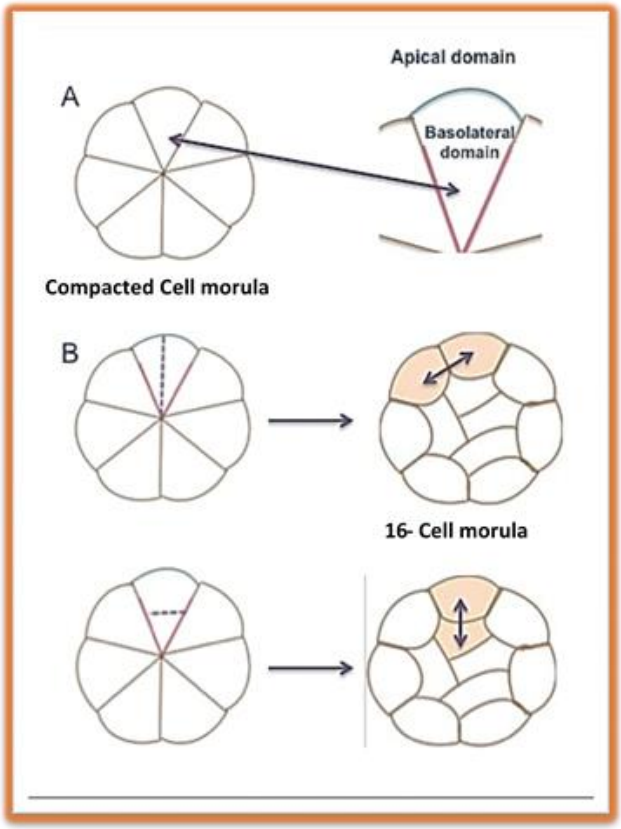


Figure 1.3 Polarity in the mouse preimplantation embryo

A) At the 8-cell stage: All blastomeres polarise along the axis of cell contact, forming outward, apical domains and inward-facing basolateral domains.
B) While the embryo at the 8-cell stage develops to the 16-cell stage; blastomeres that divide parallel to the inside-outside axis produce two outside, polar cells. Blastomeres that divide perpendicular to the inside-outside axis produce one outside, polar daughter cell and one non-polar, inside daughter cell. Because of these two types of cell divisions, there are two populations of cells: outside, polar cells and inside, non-polar cells; two types of cell division therefore occur as the embryo develops from 16 to 32 cells; adapted from (Cockburn and Rossant 2010a).

The mechanism of cell linage formation and emergence of different cellular fates in the embryo has been explained by several hypotheses; one of these is called the inside-outside microenvironment hypothesis. According to this hypothesis, differential fates result from different environments of the inner versus outer cells. Based on these different environments, the inner cells respond by becoming ICM, with the outer cells becoming TE. The origin of the two different fates is proposed by another hypothesis referred to as the polarisation model. This model puts the emphasis on cleavage planes of compacted morula cells, so the generation of outer (polar) cells and inner (non-polar) cells depend on the plane of division (parallel or perpendicular to the inside-outside axis) of previously asymmetric cells (Tarkowski and Wroblewska 1967);(Nagy et al. 2003); (Pfeffer and Pearton 2012); (Cockburn and Rossant 2010b).

It has been proposed that the morula does not have an internal cavity. When the TE secretes fluid into the morula, a cavity called the blastocoel is created through a process termed cavitation. This process occurs during blastocyst formation, which is regulated by the combined actions of ion transporters, water channels, and intercellular junctions. It has been hypothesized that blastocyst formation is regulated by the action of a polarised basolateral localised Na+/K+-ATPase that creates a trans-trophectodermal ion gradient. This Na+/K+-ATPase regulates water movement across the epithelium, in conjunction with aquaporin water channels resulting in blastoceolic fluid formation (Eckert et al. 2015). Consequently, the blastocyst expands during the continued movement of the fluid across the epithelium. As the blastocoel enlarges, the ICM is positioned on one side of the ring of TE cells (Wolpert et al. 2002); (Gilbert 2003); (Giannatselis et al. 2011).

At day E4.5 of mouse development, three cell lineages are apparent within the blastocyst; the epiblast, formed from the ICM and which will give rise to the entire fetus, and the TE

and primitive endoderm (PE) which both form extraembryonic tissues including the chorioallantoic placenta and visceral yolk sac respectively. In the same manner to the TE, the PE is an epithelial structure and lies over the free face of the ICM. At the same time, the TE becomes regionally specialised in term of its morphology and developmental potential. The TE cells which lie over the blastocoel cavity, known as mural TE cells, cease cell division, endoreplicate their DNA and become enlarged trophoblast giant cells. In contrast, TE cells that are in direct contact with the ICM, known as the polar TE, remain proliferative and go on to form extraembryonic ectoderm and ectoplacental cone see Figure 1.4; (Rossant 2015).

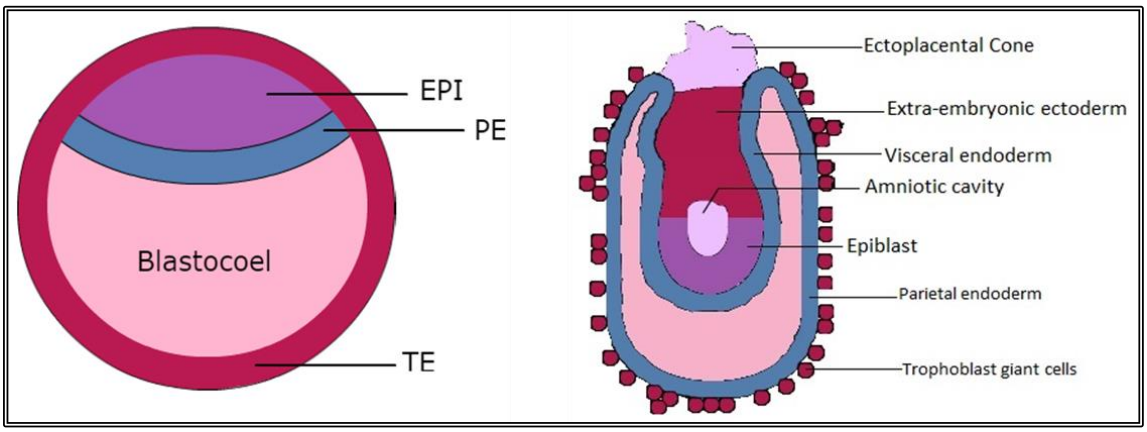


Figure 1.4 Blastocyst and early-post implantation mouse embryo development

The trophectoderm of the mouse blastocyst after implantation undergoes a proliferative phase stimulated by the epiblast, to form the extra-embryonic ectoderm (Red) and ectoplacental cone (light purple); adapted from (Rossant 2015).

It has been shown that the embryo initiates the developmental programme from a series of intrinsic mechanisms during zygotic cleavage that can occur independent of the maternal environment to include embryonic gene activation (EGA), cell cycling, cell lineage diversification and blastocyst morphogenesis including TE differentiation (Eckert and Fleming 2008). However, the embryo is also responsive to its surrounding or extrinsic

cues from the maternal environment to adapt or survive the course of development, with a range of cellular, physiological and epigenetic mechanisms (Watkins and Fleming 2009); (Fleming et al. 2015b).

The periconceptional period (PC) period, from before fertilisation and throughout preimplantation, is a potentially vulnerable period with respect to how external cues may affect the developmental programme. This question of PC vulnerability is relevant to The Developmental Origins of Health and Disease (DOHaD) hypothesis, discussed below, and particularly important in the context of ART treatments and the significant increase in the number of children born using these procedures, now amounting to some 5 million babies in developed countries.

1.2 The Developmental Origins of Health and Disease (DOHaD) hypothesis

The Developmental Origins of Health and Disease (DOHaD) approach evolved from epidemiological studies of infant and adult mortality. Professor David Barker produced a personal account of a program of epidemiological research of the geographic distributions of diseases across two hundred and twelve local authorities of England and Wales, which provided the countrywide data used by Barker and his colleagues to show that a large positive geographic correlation was found between ischaemic heart disease mortality rates in 1968-78 and infant mortality in 1921-25. An interpretation of the correlation between infant mortality and ischemic heart disease mortality rates was based on several factors, including the association of neonatal deaths in the 1920 with low birthweight (LBW). This led to the hypothesis that the geographic relationship of infant and adult death rates “reflects variations in nutrition in early life, which are

expressed pathologically on exposure to later dietary influences" (Barker and Osmond 1986); (Barker 2007).

According to (Barker 2007), to explore the developmental origins of cardiovascular disease required studies of a kind that had not previously been carried out. This study was initiated in Hertfordshire, UK, within a sample of adults (men born from 1911 to 1930). The study reported that men with the lowest birthweights had the highest death rates. On the contrary, those with the highest birthweights had the lowest death rates, and standardized death rates fell steeply with increasing weight at 1 year of age. This led to the hypothesis that "an environment which produces poor fetal and infant growth is followed by an adult environment that determines high risk for ischemic heart disease" (Barker and Osmond 1986); (Barker 2007).

The Dutch Hunger Winter is another relevant event that was studied to confirm Barker's hypothesis. In the winter of 1944 - 45, the German occupation of the Netherlands led to a blockade that cut off food from entering into the northern regions country around Amsterdam. The entire population was under severe caloric restriction, pregnant and lactating women were receiving about ~40% of the recommended calories during pregnancy (Parlee and MacDougald 2014). Based on the records kept by the Dutch, researchers have shown a link between gestational age at the time of the famine and later disease risk for cardiovascular disease and high body mass index (Ravelli et al. 1999); (Roseboom et al. 2001).

(Roseboom et al. 2001) showed that the long-term effects of intrauterine undernutrition, however, depend upon its timing during gestation and on the tissues and systems undergoing critical periods of development at that particular time. The study suggested

that risk factors for chronic diseases such as CHD, impaired glucose tolerance, hypercholesterolemia, raised blood pressure and obesity, which often co-exist, have their origins *in utero*, but are programmed at different times. The study reported that maternal malnutrition during gestation may permanently affect adult health without affecting the size of the baby at birth. This findings confirmed Barker's hypothesis that chronic diseases originate through adaptations made by the fetus in response to undernutrition (Barker 1997); (Fleming et al. 2015a).Barker's hypothesis stimulated a great deal of worldwide interest and activity in the area of developmental plasticity. This has led to the development of a theory based on the concept of predictive adaptive responses of the fetus to a variety of environmental cues and the consequences of a mismatch between prenatal and postnatal environments.

It has been shown that rats with Emb-LPD had significantly elevated blood pressure (Kwong et al. 2000). An early response by the embryo to an Emb-LPD maternal diet is to induce modulation of the extra-embryonic lineages to enhance compensatory maternal nutrient delivery during gestation, thereby contributing to developmental reprogramming (Fleming et al. 2011); (Fleming et al. 2015a). This may show that in a nutrient restricted environment, in order to survive, the preimplantation embryo adapts via various physiological mechanisms that when activated may also cause cardiovascular disease.

Similar findings were reported by (Watkins et al. 2008c) a study that investigated the sensitivity of preimplantation embryos to maternal protein undernutrition with respect to diverse aspects of postnatal phenotype and health using a mouse model. In their study, the pregnant mothers were supplied with a low protein diet (LPD) during conception, followed by a normal protein diet afterward (treatment group: Emb-LPD). They reported that the dietary-induced change in perinatal growth detected at the Emb-LPD group is a

strong predictor of those animals that will exhibit overgrowth and hypertension in later life. Moreover, female offspring were found to be more susceptible than males even when their mothers' diet were only altered during the periconceptional period (PC). In relation to the DOHaD hypothesis, this then questioned the safety of ART, which occurs during the preimplantation period, and whether ART would trigger environmental programming that would lead to chronic disease in later life. The relationship between the human PC environment and long-term health comes almost exclusively from epidemiological studies evaluating the health of children conceived via ART (Fleming et al. 2011); (Fleming et al. 2015a).

1.3 Assisted Reproductive Technologies (ART)

Since 1978 when the birth of the first "test tube baby" from an oocyte fertilised *in vitro* took place, it is estimated there have been approximately 5 million babies conceived from IVF procedures. In developed countries, 2-3% of births each year are through IVF, rising as high as 5% in Denmark and Belgium (Kamphuis et al. 2014). In the UK, 224,196 babies were born after IVF treatment between 1991 and 2014. According to a Human Fertilisation Embryology Authority (HFEA) report in 2016, the number of IVF performed each year in the UK has increased since 1991, and in 2014 approximately 52,288 women had a total of 67,708 cycles of IVF or ICSI treatment; See Figure 1.5.

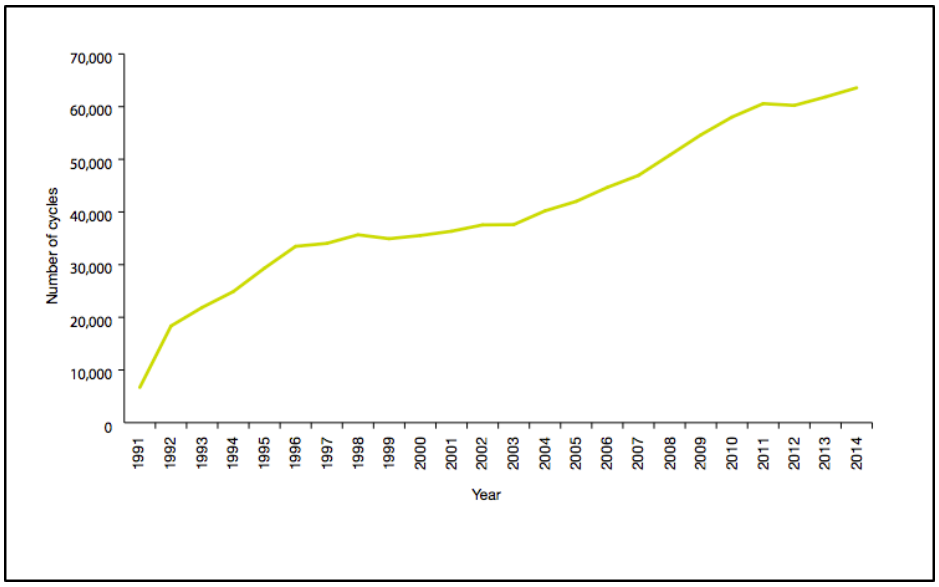


Figure 1.5 Number of IVF cycles performed each year in the UK from 1991 to 2014

Source: HFEA 2016 fertility trends report.

1.3.1 *What are assisted reproductive technologies (ART)?*

Over the past 30 years, ART has been a highly successful and widely used form of infertility treatment. It is a general term which refers to all treatment and procedures in which oocytes and sperm or embryos are handled or manipulated *in vitro* with the purpose of establishing a pregnancy (Chan and Dixon 2008). The most common procedures performed under the umbrella termed ART include *in-vitro* fertilisation – embryo transfer (IVF-ET), Cryopreservation and Intracytoplasmic sperm injection (ICSI).

1.3.1.1 In-vitro fertilisation IVF

IVF is considered a major treatment for infertility. This procedure includes the use of recombinant follicle stimulating hormone (FSH) to stimulate a female's ovaries to produce many eggs (oocytes). Following this, the oocytes are collected and added to a fertilisation culture medium, which already contains the sperm. The oocytes and sperm are co-cultured

in-vitro. Once fertilisation has occurred, one or more selected embryos (dependent on national regulations) are returned to the mother's or foster mother's uterus (embryo transfer ET) (Takeo and Nakagata 2011b); (Glujovsky et al. 2012).

Commonly, embryos are transferred into the uterus at E2 or E3 when the embryos are at the 2-cell to 8-cell stage (cleavage stage), as the uterus is able to provide the best environment for the embryo to develop (Laverge et al. 2001); (Glujovsky et al. 2012).

However, many recent studies indicate that embryos at E5 or E6 (64-cell or blastocyst stage) are at the best stage biologically to be transferred to the uterus, because the longevity of the culture may give scientists a greater chance to select higher quality embryos for transfer to the uterus (Edwards and Brody 1995); (Glujovsky et al. 2012).

1.3.1.2 Cryopreservation

Cryopreservation is a useful method that enables oocytes, sperm and embryos to be kept in store for many years, after which they can be thawed and fertilised if necessary using *in-vitro* fertilisation. Cryopreservation decreases costs and saves space (Takeo and Nakagata 2011b); (Ramon et al. 2013). Cryopreservation is a freezing procedure that removes free water to minimise water damage from ice crystal growth, without causing damage by uncontrolled removal of the bound water providing structural support to proteins, DNA and membranes. Dehydration and cryoprotectant uptake are enhanced by both slow and rapid cooling to reduce the likelihood of the free water forming large intracellular ice crystals, which are lethal to the cells (Shaw and Jones 2003). The intracellular ice crystals are prevented from forming by the cryoprotectant agent (CPA). The most common penetrating CPA used is made up of ethylene glycol (EG), dimethyl sulfoxide (DMSO) and

glycerol. Slow-freezing and vitrification procedures have different approaches to freeze cells or embryos using different CPAs.

1.3.1.3 Intracytoplasmic sperm injection (ICSI)

It is documented that hundreds of thousands of children have been born by ICSI since the first human baby was born using this technique in 1992 (Sancho et al. 2007); (Ramon et al. 2013). Intracytoplasmic sperm injection ICSI is one of the ART techniques, which consists of the microinjection of a single sperm across the membrane of a metaphase II oocyte, leading to fertilisation and bypassing the natural process of the sperm-oocyte interaction (Orgura et al. 2003); (Lopez-Saucedo et al. 2012).

1.4 Is there an increased risk after ART?

ART involves manipulating the many steps of conception and therefore may be associated with some adverse perinatal and postnatal outcomes. These manipulations include: the use of synthetic hormones to stimulate the ovaries into supernumerary oocyte production, *in-vitro* fertilisation (IVF), the direct injection of sperm using ICSI, cryopreservation of either gametes (sperm and oocytes) or embryos, the in-vitro culture of preimplantation embryos and the transfer of the embryo to the uterus (ET) (Manipalviratn et al. 2009b); (Talaulikar and Arulkumaran 2012). As a result, a great deal of research has been conducted to identify the potential risks associated with ART.

1.4.1 *Epigenetics, Imprinting and ART*

It is well known that developmental pathways in humans and animals are buffered against changes in genotype and environment. Therefore, it is not surprising that the vast

majority of children born after ART are healthy, although ART bypasses several biological filters and subjects the gametes and the early embryo to environmental stress (hormones, culture media and physical stress). If however, the buffer fails, the development of certain tissues or organs may follow an abnormal approach. Some studies have argued that the development of both normal and abnormal children conceived by ART can be explained by disturbance of epigenetic mechanisms, which control the initiation and maintenance of gene expression patterns in the placenta and foetus (Horsthemke and Ludwig 2005). A great body of research shows that genetic factors in infertile couples and environmental factors (superovulation, culture media and embryo transfer) can alter epigenetic processes that control gene expression associated with implantation, placentation, organ formation and fetal growth (Khosla et al. 2001); (Horsthemke and Ludwig 2005); (Kleijkers et al. 2015); (Sunde et al. 2016).

1.4.1.1 Genomic imprinting disorders and ART

The epigenome refers to the whole of epigenetic modification on the genome that regulate chromatin structure and accessibility of the DNA to the machinery regulating gene expression. Epigenetics is defined as heritable changes in gene expression that occur without any changes in gene sequence (Waddington 1942). Epigenetic modifications are heritable in the sense that the 'epigenetic status' of the chromatin is preserved during cell mitosis. It is shown that there are several types of epigenetic modifications that are known to affect gene expression, including changes in nucleosome positioning and conformation, and histone modifications such as acetylation, phosphorylation and methylation (Iliadou et al. 2011). DNA methylation is however the most thoroughly studied epigenetic modification in mammals (Iliadou et al. 2011).

Genomic imprinting is an epigenetic process by which the male and the female germ line of mammals confer a sex-specific mark (imprint) on certain chromosomal regions. As a consequence, the paternal and the maternal genome are functionally non-equivalent and both are required for normal embryonic development (McGrath and Solter 1984). The genomes of mature sperm and egg in mammals are highly methylated compared to genome methylation in somatic cells. Genomic imprints are deleted in primordial germ cells during later stages of germ cell development, and stably inherited through somatic cell divisions during post-zygotic development. In somatic cells, the imprint is read by the transcription machinery and used to regulate the parent-of-origin specific gene expression, hence only the paternal or the maternal allele of a particular gene is active and the inactive allele is epigenetically marked by histone modification, methylation or both (Reik et al. 2001). To date, there are approximately 150 imprinted genes that have been identified in mammals, and a majority of imprinted genes have roles in embryonic and placenta growth and development (Reik et al. 2001); (Iliadou et al. 2011); (Williamson CM 2013).

The parental differences in imprinted gene expression are epigenetic in nature. They are established throughout gamete differentiation by sex-dependent epigenetic chromatin modifications, particularly by the differential DNA methylation of promoter regions of imprinted genes or regulatory imprinted centres, which are further stably inherited in the somatic cells of the offspring. The epigenetic genome-wide reprogramming cycle involves two critical phases of DNA demethylation in mouse development; gametogenesis and preimplantation development (Iliadou et al. 2011); (Reik et al. 2001). As shown in Figure 1.6, a first wave of DNA demethylation takes place at embryonic day E10.5-13.5 in the male or female primordial germ cells (PGC) of the F1 individuals; this occurs

throughout the genome, including the imprinted and non-imprinted genes (Figure 1.6 -1). Accordingly, the genome of the gametes undergoes *de novo* methylation and during this period, sex-specific methylation of imprinted genes is established. In some imprinted genes, DNA methylation occurs exclusively in oogenesis, whereas in others it occurs during spermatogenesis (Figure 1.6 -2). A second wave of DNA demethylation, which involves somatic cells, occurs immediately after fertilisation in the F2 zygote at E0.5, demethylation in the paternal genome is rapid and active compared with the maternal genome. However, the paternal and maternal imprinted genes maintain their methylation pattern throughout this preimplantation reprogramming; this allows the inheritance of parent-specific monoallelic expression in somatic tissues of the F2 individual (Figure 1.6 -3). Lastly, genome-wide remethylation takes place in both parental genomes at about the time of implantation at E3.5 or later. It is important to note therefore that the very early embryonic development corresponds to an epigenomic reprogramming step, during which the new epigenetic modifications are more susceptible to being affected by the environment (Figure 1.6 -4). This may explain why the environment experienced during early development has an increased effect on the adult phenotype compared to that experienced later in life (Heijmans et al. 2009). Furthermore, it has been shown that the timing of the two global DNA demethylation and remethylation waves varies between male and female genomes, possibly explaining why they may be differently affected by a stress during these stages (Gabory et al. 2009).

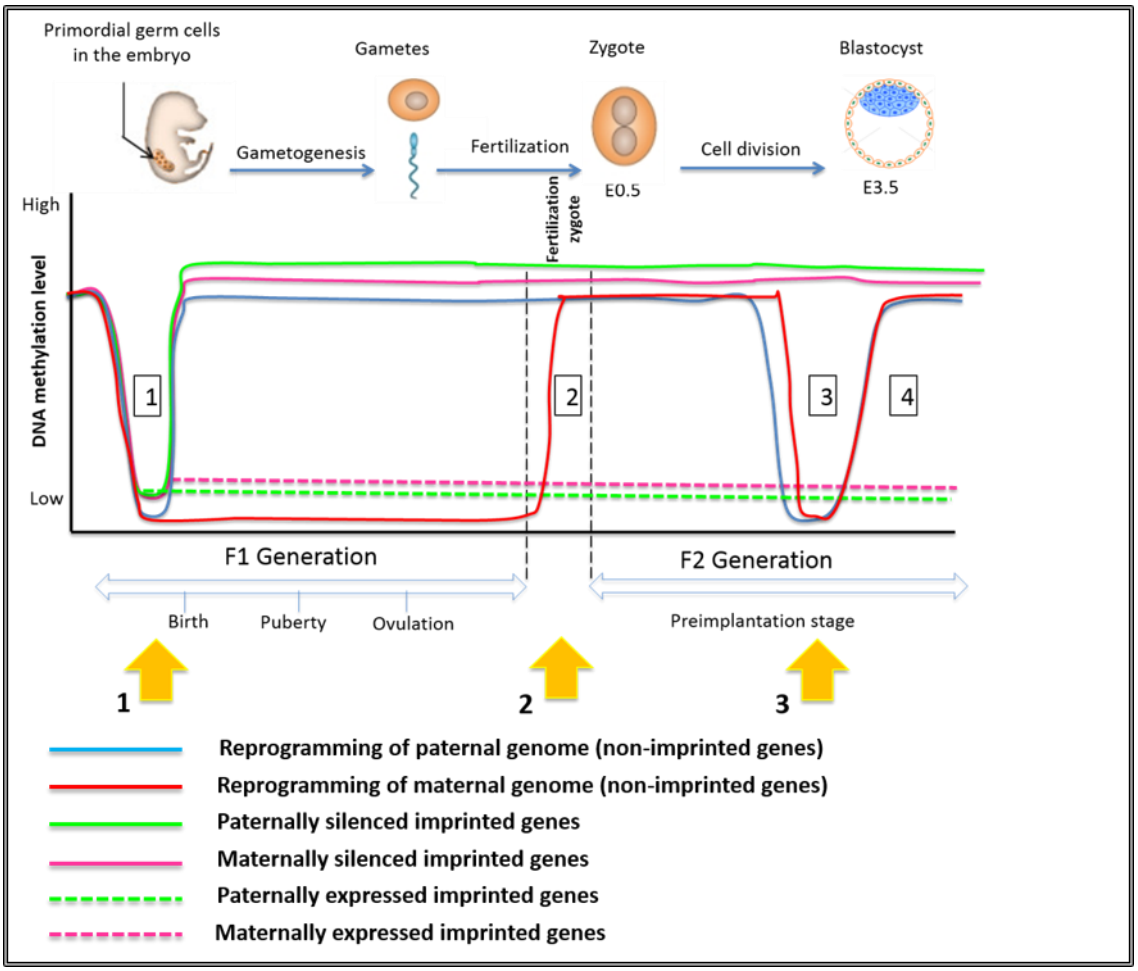


Figure 1.6 Methylation reprogramming in the germline and mouse embryo

Imprinted genes and non-imprinted sequences; male (blue curve) pattern or female (red curve) is shown during gametogenesis and early embryonic development. The horizontal axis indicates the time and the vertical axis indicates the level of DNA methylation. (E) is the embryonic day. Adapted from (Frésard et al. 2013) and (Iliadou et al. 2011);

1.4.1.2 Epigenetic risks related to ART

Recent research reported that there are two ways of considering imprinting problems associated with ART. Firstly, the imprinted disorders are associated with fertility problems of the parents, which involves at least four genes (*H19, LIT1, SNRP, UBE3A*) (Horsthemke and Ludwig 2005); (Whitelaw et al. 2014). Secondly, the differences in the methylation

indices are a consequence of the adaptation to the early environment, which differs in pregnancies conceived by ART (Tobi et al. 2014).

In mammals, the best-characterized epigenetic marks are the methylation of cytosines in cytosine-guanine (CpG) dinucleotides and the modification of histones that package the DNA. The silent state of certain regions of the genome is also marked by the methylation of cytosine residues located in CpG dinucleotides at the 5´ end of genes. When genes are switched on or off by a transcriptional activator or repressor at a specific time point during development, the activity states often persist for several rounds of cell divisions, even if the primary signal has faded away. The persistence of gene activity states is controlled by the epigenotype. These modifications are heritable during cell division, in particular mitosis, but potentially reversible. However, although these mechanisms are generally stable, environmental influences can cause changes in epigenetic marks (Iliadou et al. 2011); (Heijmans et al. 2009); (Horsthemke and Ludwig 2005).

Animal studies have reported that gametogenesis, fertilisation and early embryo development are stages susceptible to epigenetic dysregulation. The epigenome is known to be vulnerable to environmental changes and to have the potential to sustainably alter gene expression, most notably during embryonic development (Pinborg et al. 2016).

A growing number of studies suggest that the use of ART, such as ICSI and IVF, could cause aberrant DNA modification and result in *de novo* DNA methylation and histone methylation. In particular, reports suggest that ART might lead to aberrant genomic imprinting. Imprinted genes are a set of ~150 genes that exhibit monoallelic expression mediated through preferential silencing of one parental allele by epigenetic modifications; these genes have pivotal roles in regulating growth and placental function

during development (Kalish et al. 2014). Mis-expression of genomic imprints (eg, bi-allelic expression) occurring in response to ART include Angelman syndrome (AS) and Beckwith-Weidemann syndrome (BWS) (Bowdin et al. 2007); (Iliadou et al. 2011).

1.4.1.3 Gene imprinting from Angelman syndrome (AS) and Beckwith-Weidemann syndrome (BWA)

Angelman syndrome (AS) has emerged as a crucial neurogenetic syndrome because of its high prevalence and easier confirmation of the diagnosis by improved genetic testing. The estimated prevalence of AS is 1 in 10,000 among children and 1 in 2000 among adults (Williams 2005). AS is an example of genetic imprinting because the syndrome occurs due to the loss of maternal expression of *UBE3A* gene, which is located on chromosome 15q11-1339, known to be imprinted in the brain (Rougeulle and Lalande 1998). The four genetic mechanisms known to cause AS are deletion of maternal *UBE3A* (68% of cases), intragenic *UBE3A* mutation on the maternal allele (13% of cases), uniparental disomy (3% of cases), and an imprinting defect which switches off the maternal allele (6% of cases) (Williams 2005). It has been shown that two cases of AS post-ICSI to secondary male infertility, suggesting that ICSI enhanced the risk of imprinted defects (Cox et al. 2002).

Weidemann syndrome BWS is a clinically heterogeneous overgrowth syndrome associated with an increased risk for embryonal tumor development (Weksberg et al. 2005). BWS results from a variety of genetic or epigenetic alterations within two domains of imprinted growth regulatory genes on human chromosome 11p15. Domain 1 is the imprinting centre of differentially methylated region1 (DMR1), which regulates *IGF2* and *H19* (Weksberg et al. 2005). Domain 2 contains the imprinting centre of *DMR2 KCNQ1OT*, which has been shown that maternal methylation of *DMR2* normally silences *KCNQ1OT1*

on the maternal allele, consequently the paternal gene would be dominantly expressed. The reports from BWS cases show that there is loss of maternal methylation of *DMR.*

Gain of methylation at the normally unmethylated maternal allele of chromosome 11p15.5 IC1 DMR is linked with bi-allelic expression of the paternally expressed growth promoter *IGF2* and is linked with BWS. However, loss of methylation of the paternal allele at the same imprinting control centre is linked with loss of paternal allele *IGF2* expression and the phenotype of Silver-Russell syndrome (SRS), which is characterised by pre- and postnatal growth restriction (Weksberg et al. 2005); (Tee et al. 2013). Numerous studies suggested that the two imprinting disorders AS and BWS are associated with ART, hence this leading to the suggestion that ART may be associated with an increased susceptibility to imprinting errors.

1.4.1.4 Gene imprinting and its association with ovarian stimulation and embryo culture

ART involves manipulation of several steps associated with conception, such as hormone stimulation, *in vitro* fertilisation, embryo culture and embryo transfer, that might alter the normal imprinting processes. For example, these manipulations may include the use of hormones to down-regulate pituitary function and to stimulate multiple oocyte production, *in vitro* maturation of oocytes, use of donated sperm, *in vitro* fertilisation procedure and *in vitro* culture of preimplanted embryos. In (Figure 1.6), arrows 1, 2 and 3 indicate which stages in gametogenesis and early embryo development are specific phases in which ART might affect and disturb the processes of DNA methylation and/or demethylation (Iliadou et al. 2011).

Hormonal stimulation is frequently used to induce superovulation, and oocytes are retrieved directly from the ovaries prior to ovulation. ARTs also involve *in vitro* maturation of oocytes so oocytes are cultured *in vitro* for maturation then cryopreservation or *in vitro* fertilisation. These steps might influence and disturb the natural process of DNA re-methylation during late-stage oocyte maturation (Figure 1.6, arrow 1), subsequently affecting genomic imprinting of the maturing oocyte. One of the methods of assisted reproduction is ICSI. Concern has been raised regarding this procedure because it results in injection of culture medium into the cytoplasm along with the injected sperm (Figure 1.6, arrow 2). Moreover, current ART protocols tend to use a prolonged *in vitro* culture until the blastocyst stage before embryo transfer. Prolonged embryo culture results in higher pregnancy rates, allows for assessment of embryos and selection of a high quality embryo for transfer and facilitates the introduction of the trophectoderm (TE) lineage biopsy for the screening of genetic disease (See Chapter 4). However, extended exposure using different types of culture medium during early embryo development potentially influences the process of DNA re-methylation (Figure 1.6, arrow 3) (Lane and Gardner 1997); (Gardner et al. 2000); (Iliadou et al. 2011); (Pinborg et al. 2016).

Several animal studies have reported that the type of conditions encountered by the embryo during the pre-implantation stage may influence the phenotype of the adult, and epigenetic modification has been suggested as the possible mechanism (el Hajj and Haaf 2013). Animal studies show that the various steps of ART may affect the epigenome, as superovulation and *in vitro* culture of oocytes cause epigenetic changes in the embryos and offspring (Grace and Sinclair 2009).

Market-Velker et al showed that *in vitro* embryo culture conditions have an established epigenetic effect on cultured embryos in animal models (Market-Velker et al. 2010).They performed side-by-side comparisons of five commercial embryo culture systems (KSOMaa, Global, Human Tubal Fluid, Preimplantation 1/Multiblast, and G1v5PLUS/G2v5PLUS). Using a mouse model with embryos cultured from the 2-cell to the blastocyst stage, imprinted DNA methylation and expression were examined at three well-studied loci, including H19, Peg3, and Snrpn. The study showed that embryo culture in all commercial media systems resulted in imprinted gene methylation loss when compared to *in vivo*-derived embryos. Since the genes investigated in the study play an important role in early development, and since genetic and epigenetic perturbations lead to imprinting disorders, the data suggested that combined treatment of superovulation and embryo culture resulted in increased perturbation of genomic imprinting, above that from culture alone, indicating that multiple ART procedures further disrupt genomic imprinting (Market-Velker et al. 2010).

1.4.2 *Short term effect of ART*

Several studies have suggested that the embryo culture medium has an impact on the quality of embryos conceived through either IVF or ICSI (Batcheller et al. 2011a). The preimplantation mammalian embryo is sensitive to environmental conditions, which may influence immediate events of blastocyst morphogenesis and also the fetal or postnatal phenotype (Watkins et al. 2007). It has been reported that culture conditions contribute to a slower rate of blastocyst formation, reduce the proliferation rate and alter the embryo gene expression pattern and metabolic activity compared with *in-vivo* development (Manipalviratn et al. 2009b); (Batcheller et al. 2011a); (Kleijkers et al. 2016).

1.4.2.1 Preterm delivery and birth weight

A substantial amount of research shows that *in-vitro* culture during ART treatment may lead to adverse effects in humans, including an increase in the rates of preterm delivery, a low birth weight and perinatal mortality in singleton conceptions compared with natural pregnancy (Richter et al. 2001); (Hansen et al. 2002); (Olivennes et al. 2002b); (Schieve et al. 2002) ; (Kleijkers et al. 2016); (Sunde et al. 2016).

A growing body of research shows that ART is highly linked with preterm births. Preterm is defined as babies born alive before 37 weeks of pregnancy are completed. There are sub-categories of preterm birth, based on gestational age: extremely preterm (<28 weeks) very preterm (28 to <32 weeks) (World Health Organisation WHO, 2015). (Stojnic et al. 2013) reported that the preterm delivery rate of IVF pregnancies were significantly higher than the controls (9.3% vs. 5.85%, $p< 0.05$) especially with a gestation between 30-32 weeks even with the singleton (Schieve et al. 2002); (Wang et al. 2005); (Hansen et al. 2009); (Stojnic et al. 2013).

Apart from preterm birth, it has been reported that the use of ART such as, IVF and ICSI accounts for low birth weight and very-low birth weight infants ((Wang et al. 2005); (Källén et al. 2005); (Knoester et al. 2008); (Basatemur et al. 2010); (Kleijkers et al. 2016); (Sunde et al. 2016). An American study used population-based data to compare the rates of low birth weight (LBW) (<2500 g) and very low birth weight (<1500 g) babies among children born via ART with the rates in the general population in 1976 and 1977 and reported that the rate of low LBW was higher among singletons and twins born to nulliparous women and women who had previous ART procedures (Schieve et al. 2002). The rate of very low birth weight babies also varied with maternal and treatment-related factors, but were less

affected than a low birth weight. Singleton babies born using ART had a risk of term LBW that was more than twice that of singleton babies in the general population; they also had a significant increase in a risk of a preterm low birth weight. Among twins conceived via ART, the risk of term and preterm low birth weight were similar to infants in the general population (Schieve et al. 2002). Similar findings were reported by (Wang et al. 2005) that confirm that live-born ART singleton infants were more likely to be preterm and to have a LBW compared with the national Australian birth cohort. Furthermore, the study reported that female-factor infertility increased the likelihood of preterm birth and a LBW for babies conceived with ART. The female-factor infertility is more highly correlated with treatment by IVF procedure than ICSI, and it is the cause of infertility and not the type of ART procedures that resulted in LBW and preterm birth (Wang et al. 2005).

These findings were confirmed by (Shih et al. 2008), who showed that IVF and ICSI did not alter the embryos, but other possible factors, including surgical intervention on the woman may affect the endometrial receptivity, implantation, or early pregnancy and may be responsible for LBW with ART. However, the underlying biological mechanism of these complications in babies conceived with ART remains unresolved.

1.4.2.2 Psychological, medical and morbid congenital issues associated with ART

It has been shown that *in vitro* fertilisation is not associated with an increase in dysmorphic features. The positive association between time-to-pregnancy (TTP), which is considered as the main cause for severity of subfertility, and clinically relevant abnormalities suggests a role of the underlying subfertility and how determines the genesis of dysmorphic features (Seggers et al. 2012).

Even though the majority of children conceived by ICSI and IVF are healthy, a small but distinct percentage of these children suffer from a variety of significant morbid congenital issues. Children born through ICSI and IVF have twice the rate of major birth defects and congenital malformations when compared with babies born normally (Hansen et al. 2002). This data was confirmed by a cohort study from five European countries on 540 ICSI children at the age of 5 years. They reported that of the 540 ICSI children examined, (4.2%) had experienced a major congenital malformation compared with naturally conceived children. The ICSI children presented with more major congenital malformations and children born by ICSI and IVF were more likely to need health care resources than naturally conceived children (Bonduelle et al. 2005).

However, a review of studies on psychological well-being in children born following assisted reproductive technologies concluded that there were no differences in cognitive development between children conceived by IVF and naturally conceived children. The inconsistent outcomes from different cohorts might be due to different measurement techniques or improved ART over time (Zhan et al. 2013). Besides, the higher risk of morbid congenital issues reported in children conceived by ART, calls for more research and long-term follow-up.

1.4.3 *Long term effect of ART*

1.4.3.1 Cognitive and behavioral development

There are far fewer studies analysing the cognitive, behavioural and psychosocial development of children conceived by ART. Difficulties related to quantify and to control for the parental attitude and expectation of their children is one aspect of such outcome studies. The families of children conceived by IVF may differ in terms of family size,

parental expectation and paternal age. The cognitive and behavioral development evaluated at the age of 3 to 7 years has been shown to be normal among IVF children when compared with their spontaneously conceived peers (Cederblad et al. 1996). This fact was supported by a study by Wagenaar et al (2008) that reported there was no difference in the incidence of behavioral and socio-emotional problems in children at age of < 8 years, between IVF children and their naturally conceived peers. (Wagenaar et al. 2008). Subsequently, a study of cognitive, motor and emotional-behaviour psychology in children at age of 5 years showed that minimal differences were noted between ICSI conceived children and naturally conceived children (Hart and Norman 2013a). Ludwig et al (2006) concluded that regarding general health, growth, mental and psychomotor development IVF children do not differ from spontaneously conceived children. Children born after assisted reproductive techniques are healthy and develop similarly to children born after spontaneous conception (Ludwig et al. 2006).

However, it has been reported that there is a difference in intelligence quotient (IQ) scores in ICSI children at age of 8 years. The study reported that lower IQ scores, worse visual-motor ability or locomotor development and delayed receptive language competence were noted in the ART children group. Moreover, ICSI children have a higher risk of autism when compared to naturally conceived children; children conceived by IVF have a higher prevalence of behavioral problems compared to naturally conceived children (Zhan et al. 2013).

Apart from human studies, mouse models studying behaviour or morphology with histology techniques reported no significant differences. However, genome-wide DNA methylation changes were reported in the brain of IVF and ET mice where-small clusters of CpG islands and promoters were aberrantly methylated. Hypermethylation was more

common than hypomethylation in F1 and F2 generations. The study suggested that IVF may slightly modify the somatic methylation pattern and that some of this aberrant methylation might be inherited by the following generation. It is also suggested that possible marriages between ART-conceived individuals may be a cause of concern (Li et al. 2011).

1.4.3.2 Growth and development potential

One of the earliest studies following up IVF children from the age of 1 to 13 years reported that there was no significant pathological features concerning growth and physical development of IVF children (Olivennes et al. 1997). Another study was conducted to investigate the growth of children born in the UK following IVF and ICSI treatments up to the age of 12 years old. A total of 143 children born from IVF and 166 ICSI children were matched to 173 naturally conceived children were assessed (Basatemur et al. 2010). No significant difference was noted between the three groups regarding weight, length or head circumference at birth. However, it has been reported that as the children grew older, children conceived by IVF were significantly taller than naturally conceived children (by a mean of around 3 cm after adjustment for age and parental height (Basatemur et al. 2010) (Hart and Norman 2013b).

However, a recent study was performed to investigate the effect of culture medium during the first few days after fertilisation on prenatal growth and birth weight during the first 2 years of life. The main finding of the study was that *in vitro* culture of human embryos in medium from Cook resulted in singletons with lower birth weight during the first 2 years of life compared with singletons born after embryo culture in medium from

Vitrolife. This finding indicates an effect of culture medium on fetal development and birth weight (Kleijkers et al. 2014).

Using a mouse model, a study from our lab reported that the effect of *in vitro* culture and ET treatment on postnatal growth compared with *in vivo* development (independent of litter size) was minimal, and limited to reduced female growth in selected weeks (6, 7, 11, 12, and 13) (Watkins et al. 2007). These data are similar to those reported following mouse embryo culture in Whittens and KSOM media where no effect on postnatal growth was evident (Ecker et al. 2004).

1.4.3.3 Metabolic and cardiovascular effects

One of the earliest cohort studies of children born using IVF technique and followed into adulthood did not report a higher prevalence of being overweight or obese in IVF children when compared with normal children in the US population (Beydoun et al. 2010). However, after adjustment for antenatal, maternal and paternal factors it appears that children conceived by IVF, when assessed in late childhood and adolescence, have significantly more peripheral body fat deposits, regardless of minimal differences in BMI (Ceelen et al. 2007). A future study from this group suggested that despite early life catch-up growth, early childhood gain in height, weight and BMI were similar and appeared not to lead to an elevated in blood pressure in late childhood. However, those children having rapid weight gain in early childhood, but not late infancy, were at risk of developing high blood pressure in late childhood (Ceelen et al. 2009).

Children conceived by IVF at a mean age of 12.3 years had higher systolic and diastolic blood pressures than normal children (109 vs. 105 and 61 vs. 59 mmHg, respectively); in addition, IVF children had higher fasting serum glucose concentrations (Ceelen et al.

2008a). The blood pressure differences were also noted in a small case-controlled series of children conceived by IVF at a mean age of 8.8 years from Greece. However, there were no differences in any of the extensive metabolic parameters studies between the IVF and control groups (Sakka et al. 2010).

Using a mouse model, embryo culture from two-cell to blastocyst stages and ET led to an enhanced systolic blood pressure at 21 weeks compared with *in vivo* development independent of litter size, maternal origin, or body weight (Watkins et al. 2007).

They reported that female, but not male offspring from *in vitro* culture and ET, and *in vivo* developed and ET treatments exhibited elevated serum ACE activity compared with normal mating controls. ACE acts to convert angiotensin I to angiotensin II, a potent vasoconstrictor following binding to angiotensin II type 1 receptors in vascular smooth muscle and elsewhere, leading to elevation of blood pressure. (Watkins et al. 2007) suggested that one component underlying elevation in SBP in embryo-manipulated female offspring may derive from altered renin–angiotensin system. Angiotensin II has also been associated with endothelial dysfunction and heightened superoxide anion levels, which may further exacerbate hypertension (Watkins et al. 2007). See Figure 1.7 below, summary of potential pathways of disease programming by which ART factors may act on them mediated by epigenetics modifications.

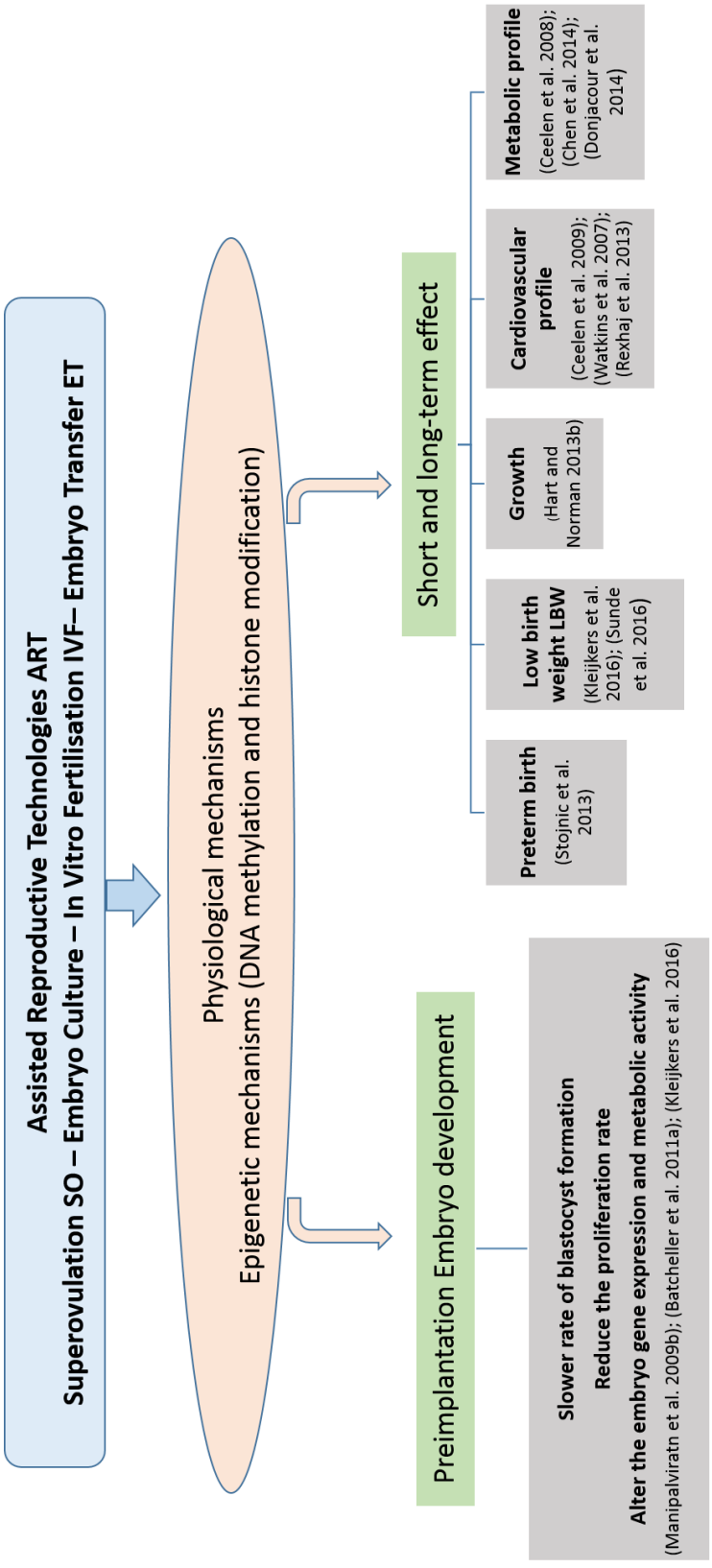


Figure 1.7 Overview of potential pathways of disease programming by which ART factors may act on them mediated by epigenetics modifications.

1.5 Mouse as a model for IVF

Animal models are vital in studying IVF since human studies are imitated due to ethical issue. Crucial studies have been conducted using either small or large numbers of animals to provide strong evidence that contribute to improving the mechanistic understanding of the developmental origins of disease, such as the causal relationship between early-life exposure and metabolism risk factors in later life (Young et al. 1998); (Watkins et al. 2007); (Watkins et al. 2008b).

1.5.1 *Advantages of using animal models in reprogramming*

Small animal models such as rodents have numerous advantages. The generation time, which is the time between a mouse being born and giving birth is short, usually around 8 to 10 weeks. This quick generation time and the relatively low cost makes them an ideal model for testing proof of principles, such as investigating whether an altered diet during pregnancy has an impact on the systolic blood pressure in either mice (Watkins et al. 2008b), or rats (Kwong et al. 2000). Furthermore, rodent studies involve relatively low costs even in maintaining large cohorts of animals. Mice models are also suitable for demonstrating specific parameters, such as body weight across their lifespan (Watkins et al. 2007); (Scott et al. 2010); (Donjacour et al. 2014).

Another advantage of small animal models is the ability to control any genetic and environmental variability. Researchers are able to control environmental factors such as, light, temperature, ambiance and diet even with a large cohort of animals. This enables the elimination of external factors that may contribute to any alterations in the data generated. Moreover, it is now possible with animal models to target specific genes and modulate their

expression to examine their role in a specific disease. The convenience of manipulating small animals allows particular treatments to be designed, such as IVF to mimic human procedures, which can later be translated to humans.

The murine IVF system and embryo has been widely used as a human IVF model for over 40 years (Edwards 1980); (Cockburn and Rossant 2010a). It has been shown that there is a close relationship in preimplantation development of mouse and human embryos *in vitro*, which supports the use of murine models for developmental studies. The rate of cleavage in mice (Harlow and Quinn 1982) and humans (Edwards 1981) for the first and second cell cycles has been shown to be around 24 hours from the time of conception (zygote and 2-cell stage), then approximately 12 hours for the later cycles, reaching a fully expanded blastocyst after 84-96 hours of culture, whereas human embryos take an additional 24-30 hours to reach the fully expanded blastocyst stage.

1.5.2 *Limitation in using animal models*

Animal models are a fundamental tool in the life sciences. They have enabled the scientific community to understand disease and test candidate therapies. However, no model is perfect; there are drawbacks in using small animal models in studies. One of the disadvantages is the large litter size (around 8 to 15 pups). It has been reported that mice that occupy the end of uterine horn are heavier at birth due to inconsistence distribution of nutrients to the litter (Ryan and Vandenbergh 2002).

Further limitation in small animal models is that they are altricial species. That means they are immature at birth when compared to large animal models and humans. At birth, mice and rat offspring are born with a poorly developed central nervous system, autocrine system and organs such as pancreas and kidneys. The implications in the programming of

disease continues into postnatal life. The period of susceptibility of the developing system therefore differs between species and interventions directed at the same developmental stage cannot be considered comparable (McMullen and Mostyn 2009).

1.6 Rationale

Mammalian preimplantation embryo development requires the coordinated activities of cellular proliferation and differentiation, changes in metabolism changes and a receptive synchronised uterine environment. At the preimplantation period, the embryo has two distinct cell lineages: the outer TE and ICM. In the blastocyst, the TE acts as a transporting epithelial barrier for the developing ICM by sealing it off from the surrounding environment and protecting it from disturbances in the environment. The two major morphological changes that occur in the embryo during the preimplantation period are compaction and blastocoel development. However, underlying these events is the sequential activation of the embryonic genome, with the subsequent inactivation of maternal mRNA and protein, culminating in an increase in biosynthetic activity.

Moreover, during this crucial period of prenatal development, the embryo has different metabolic and environmental needs at different stages of development. It is well known that the mother's reproductive tract is able to supply the embryo with these needs by providing an environment that is optimal in its pH, metabolite and nutrient levels as well as development cues. However, any alterations in these optimal factors can influence embryo development in a variety of ways, leading to impaired development, altered gene expression and potentially, increased risk of abnormal postnatal development mediated through altered epigenetic profiling.

Human studies show that the combined treatment of ART (including superovulation, *in vitro* fertilisation procedures, embryo culture and embryo transfer) may increase the risk of low birth weight, imprinting disorders and be associated with adverse perinatal outcome (Olivennes et al. 2002a); (Schieve et al. 2002); (Ludwig et al. 2006); (Manipalviratn et al. 2009a). IVF children have an increased risk of elevated blood pressure, high fasting glucose levels and an increase in total body fat composition (Ceelen et al. 2008a); (Hart and Norman 2013a, Kleijkers et al. 2014, Fauser et al. 2014).

Animal studies show that combined treatment of superovulation and embryo culture resulted in increased perturbation of genomic imprinting, above that from culture alone, indicating that multiple ART procedures further disrupts genomic imprinting (Market-Velker et al. 2010). Embryo culture can lead to abnormal postnatal behavior in mice (Ecker et al. 2004). Embryo culture or even simply embryo transfer in mice can lead to elevated blood pressure and dysfunctional cardiovascular cardiometabolic health in the offspring (Watkins et al. 2007).

1.7 Hypothesis

In this context, one critical use of a mouse model should be to evaluate the changes in clinical practice in current ART after IVF, concerning either a short duration embryo culture to early cleavage or a long culture to the blastocyst stage. Does the duration of embryo culture have an impact on long-term phenotype and health? Therefore, the hypothesis of this thesis is that the environment, in particular the length of embryo culture; short (2-cell stage) vs. long (blastocyst) in which the preimplantation embryo develops can not only affect blastocyst development, but may also affect aspects of

postimplantation and postnatal growth, physiology and metabolism. The main aims of this thesis are therefore:

- To investigate the effect of *in vitro* fertilisation IVF and prolonged embryo culture on the embryo developmental rate. Development of IVF embryos to morula and different stages of blastocyst (early, middle and late blastocyst) will be recorded from IVF and *in vivo* groups to determine the duration of time required for the embryo to form a blastocyst (Chapter - 3).
- To examine the effect of *in vitro* fertilisation IVF and prolonged embryo culture on lineage differentiation by analyzing cell number within ICM and TE and the ratio of ICM: TE in mouse blastocyst (Chapter - 3).
- To compare the pregnancy success rate after ET, ET efficiency (live birth), litter size, number of offspring and the male: female ratio between five treatment groups designed to evaluate the long-term effects of IVF and either short or long culture duration before ET; NM control (non-ART treatment, non-superovulated); (IV-ET-2Cell) 2-cell stage embryos derived *in vivo* from superovulated mothers and subsequently transferred immediately to pseudo-pregnant mothers; (IV-ET-BL) blastocyst embryos derived *in vivo* from superovulated mothers and immediately ET; (IVF-ET-2cell) 2-cell stage embryos generated *in vitro* by IVF from superovulated mothers and ET; and (IVF-ET-BL) blastocyst embryos generated *in vitro* by IVF from superovulated mothers and ET (Chapter - 4).

- To analyse the effect of IVF with either short or long embryo culture duration before ET on the growth and organ allometry of offspring using the five treatment groups (Chapter - 4).
- To analyse the effect of IVF with either short or long embryo culture duration before ET on the cardiovascular profile of offspring using the five treatment groups (Chapter - 5).
- To analyse the effect of IVF with either short or long embryo culture duration before ET on the metabolic profile of offspring using the five treatment groups (Chapter - 6).
- To investigate the relationship that exist between the parameters of growth, cardiovascular and metabolic profiles of offspring (Chapter – 5 and 6).

Collectively, postnatal factors such as growth rate, blood pressure SBP, a glucose tolerance test GTT and organ allometry will be monitored to assess the impact of prolonged embryo culture; short (2-cell stage) vs. long (blastocyst) (See Figure 1.8 for a time line of the proposed experiments).

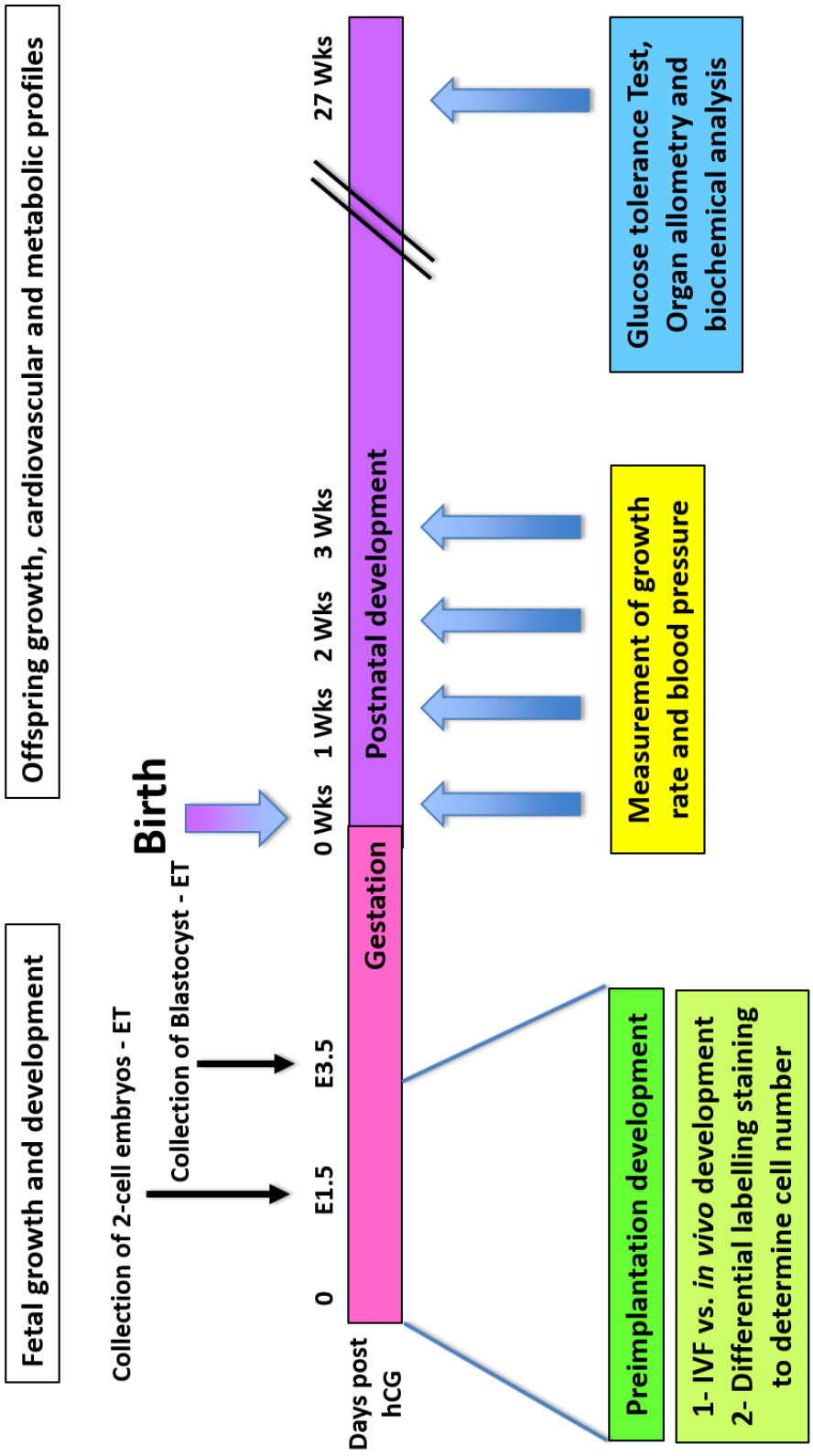


Figure 1.8 A Time line of the proposed experiments

1.8 Justification of experimental design

The experimental design was specified to best fit our main objectives, which are to investigate the effect of prolonged embryo culture on embryos and subsequently postnatal health. Five treatment groups were generated (see Table 4.1) and termed as (a) normally mated non-superovulated (undisturbed) (NM), (b) – embryos derived *in vivo* at 2-cell stage from superovulated mothers and immediately transferred to a recipient oviduct (IV-ET-2Cell), (c) embryos generated *in vitro* from superovulated mothers and cultured to 2-cell stage before transfer to the oviduct (IVF-ET-2Cell), (d) - embryos derived *in vivo* at blastocyst from superovulated mothers and immediately transferred to a recipient uterus (IV-ET-BL), (e) embryos generated *in vitro* from superovulated mothers and cultured to blastocyst before transfer to uterus (IVF-ET-BL). Various studies show that the main procedures used in the IVF technique, superovulation, embryo culture and embryo transfer, have adverse effects on the potential of embryos ((Rivera et al. 2008b); (Market-Velker et al. 2010); (Batcheller et al. 2011a). To examine the collective effects of the combined ART techniques used, the natural mating group in our study did not undergo the superovulation procedure. Similarly, within the four ART treatments groups, to ensure that we examine the effect of the length of culture specifically, both the *in vivo* groups (IV-ET-2Cell and IV-ET-BL) were superovulated and immediately underwent the embryo transfer procedure such that the only difference from the IVF groups (IVF-ET2Cell and IVF-ET-BL) was the duration of culture. Importantly, integration of the experimental treatments ensured offspring from the four treatment groups would be at the same age, analysed at the same time points from birth to week 27, and therefore experience the same environmental conditions (Figure 1.9).

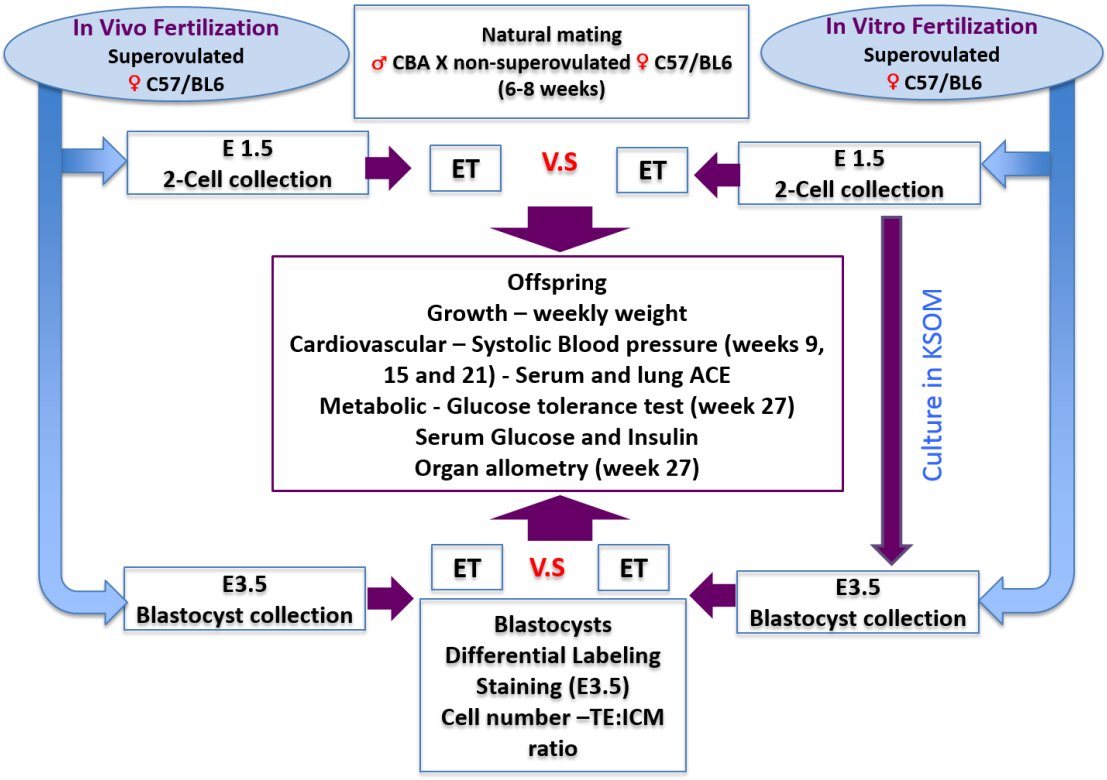


Figure 1.9 Experimental design

Chapter 2: Material and Methods

2.1 Animals

All mouse and experimental procedures were performed using protocols approved by, and in accordance with, the UK Home Office Animal (scientific procedures) Act 1986 and the local ethics committee at the University of Southampton under UK Home Office Licence PPL 30/3001. C57BL/6, CBA and MF1 mice were bred in-house (University of Southampton, Biomedical Research Facility) on a 07:00-19:00 light cycle, 24°C, fed *ad libitum* from weaning on a standard chow diet (CRM(X), 18% Protein, Special Diet Service, Cambridge, UK) and water.

2.1.1 ***Natural mating***

Virgin C57/BL6 inbred strain females from 6 to 8 weeks old (University of Southampton, Biomedical Research Facility) were naturally mated with CBA males aged between 6 to 10 months. A total of two females were allocated for one male each time. On the day of vaginal plug observation (E0.5), females were housed individually in a quiet room until they gave birth. Offspring were observed and had their own ID's dependent on pup number and gender. Offspring were weaned from mothers from an age of 3 weeks.

2.2 Embryo production and collection procedures

2.2.1 ***Superovulation***

Superovulation is a technique widely used to artificially induce ovulation of large numbers of oocytes from limited numbers of female mice, which reduces the total number of mice

required to provide eggs for manipulation. It is known that superovulation is achieved by the sequential administration of two hormones, pregnant mare's serum (PMS) and human chorionic gonadotropin (hCG) which mimic the activities of Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH), respectively.

C57BL/6 females at an age of 3 to 4 weeks, with a weight of 11g, (University of Southampton, Biomedical Research Facility) were superovulated to donate oocytes. Pregnant mare serum (PMS; Intervet, Cambridge, U. K.) was prepared in 1ml stock aliquots (1000 IU/ml PMS; appendix II), stored at -20°C and thawed for further dilution when required. Sterile saline 0.9 % (3 ml) was added to each aliquot to achieve a concentration of 250 IU/ml; 0.2 ml of the 250 IU/ml solution was aliquoted into 20 microfuge tubes (1.5-mL; Fisher Scientific) each containing 50 IU/ml and stored at -20°C until the day of mouse injection. In addition, 0.8 ml sterile saline was added to the 0.2 ml aliquot to achieve a concentration of 5 IU/ml. Each mouse was intraperitoneally (I.P.) injected with 0.1 ml of 5 IU/ml PMS using 1 ml syringe (BD Plastipak, Becton Dickinson S. A., Madrid, Spain). Females to be mated *in vivo* were superovulated at 1400 hours whereas IVF treatment females were superovulated at 1700 hours.

Human chorionic gonadotropin (hCG; Intervet, Cambridge, U. K.) was prepared in 0.1 ml stock aliquots (appendix II). The stock solution was stored at -20°C freezer until thawed for mice injection. We added 0.9 ml of 0.9% sterile saline to the previous aliquot. 1 ml hCG will be enough to superovulate 10 females. Each female was injected intraperitoneally 47 to 49 h after her last PMS injection. Female mice were either naturally mated with CBA males after the hCG injection (*in vivo* treatment), or were rested for 14 – 15 hours until oocyte collection the next morning (IVF treatment).

2.2.2 *In vivo embryo collection*

Embryos at 2-cell stage and blastocyst stage were obtained from virgin 3 to 4 weeks old C57/BL6 females individually mated with CBA males. The females were superovulated 2 days before mating (See section 2.2.1). On the day of vaginal plug observation (E0.5), females were housed individually in a quiet room. Then, on the afternoon of E1.5 and E3.5, females were killed by cervical dislocation and the embryos were flushed from the dissected oviduct or uterus depending on the stage of development required. Embryos were flushed using pre-warmed H6 medium supplemented with 4 mg/ml bovine serum albumin (BSA)(Sigma:9048-46-8, St. Louis, MO; embryo culture tested; H6 BSA; appendix I).

2.2.3 *In vitro embryo collection*

The IVF recovery procedure incorporating methyl-β-cyclodextrin and reduced glutathione is based on the work published by (Takeo and Nakagata 2011a, Ishizuka Y et al. 2013). Two types of media have been prepared in the *in vitro* fertilisation procedure, Human Tubal Fluid (HTF) medium and modified Krebs–Ringer bicarbonate medium (TYH+MBCD), Appendix IV.

2.2.3.1 Preparation of sperm dispersal dishes

A total of 90 µl TYH+MBCD was placed into the centre of a 35 mm Petri Dish (Falcon 351008) using a 200 µl pipette tip (Axygen Inc; T-400) and overlaid with mineral oil (Sigma) and equilibrated for 10-20 min at 37°C, in 5% CO_2 in air Figure 2.1.

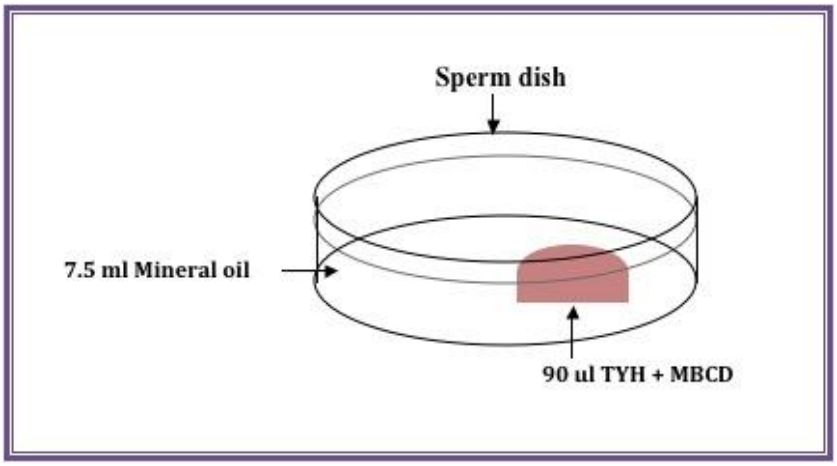


Figure 2.1 Preparation of the sperm dispersal dishes

2.2.3.2 Preparation of freshly harvested sperm samples

The selected CBA male was at least 8 weeks old, and was not used for mating for at least 3 days prior to sperm collection. Males were sacrificed and the cauda epididymides were dissected, and all adipose and vascular tissue cleaned from them under a dissecting microscope. Cauda epididymides were placed into the oil next to the sperm dispersal drop and the apex of the cauda epididymides was nicked using miniature scissors. A small "ball" of the sperm was teased out from the cauda epididymides and dragged into the sperm dispersal drop using watchmaker forceps Figure 2.2. Tissue was removed from the dish, and the sperm were allowed to disperse throughout the medium for approximately 60 minutes at 37°C, in 5% CO_2 in air.

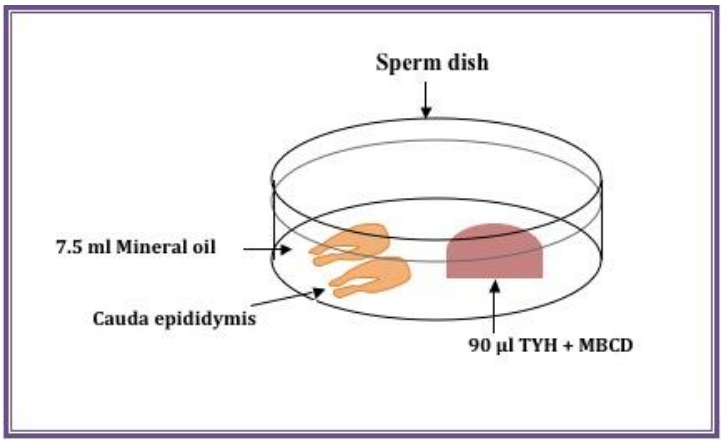


Figure 2.2 Preparation of freshly harvested sperm samples

2.2.3.3 Preparation of a fertilisation dish containing 1 mM reduced glutathione

Card medium was prepared by adding 0.5 ml HTF medium to a tube containing 15.35 mg reduced glutathione (GSH;Sigma: G4251); the medium and the powder were then mixed in the tube. Then, 25 µl of the GSH solution was added to 2.5 ml HTF medium and mixed together gently and filtered using a 0.22 µm syringe and filter. A drop of the card medium was placed in a 35 mm Petri Dish (Fertilisation dish; Falcon 351008) overlaid with mineral oil Figure 2.3, and incubated at (37°C, in 5% CO_2 in air) for 10-20 minutes.

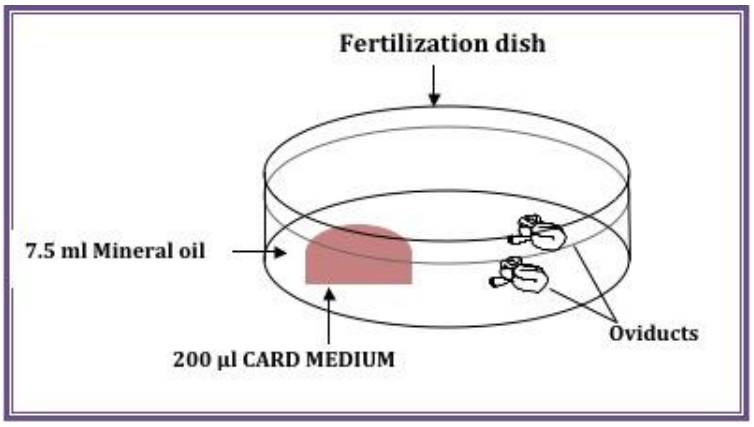


Figure 2.3 Fertilisation dish (Card medium)

2.2.3.4 Oocyte harvesting and in vitro fertilisation

For each IVF treatment, oviducts were dissected from three superovulated female mice (see 2.2.1 above) and transferred into the mineral oil overlaying the pre-incubated fertilisation drop that prepared in 2.2.3.3. Under a dissecting microscope, the oviduct was gently held down with forceps and the swollen ampulla was torn with a second pair of forceps to release the cumulus masses into the oil. The mass of cumulus oocyte complex (COCs) was dragged through the oil and into the fertilisation drop using the forceps and the oviduct was then removed from the dish (Figure 2.4 A+B).

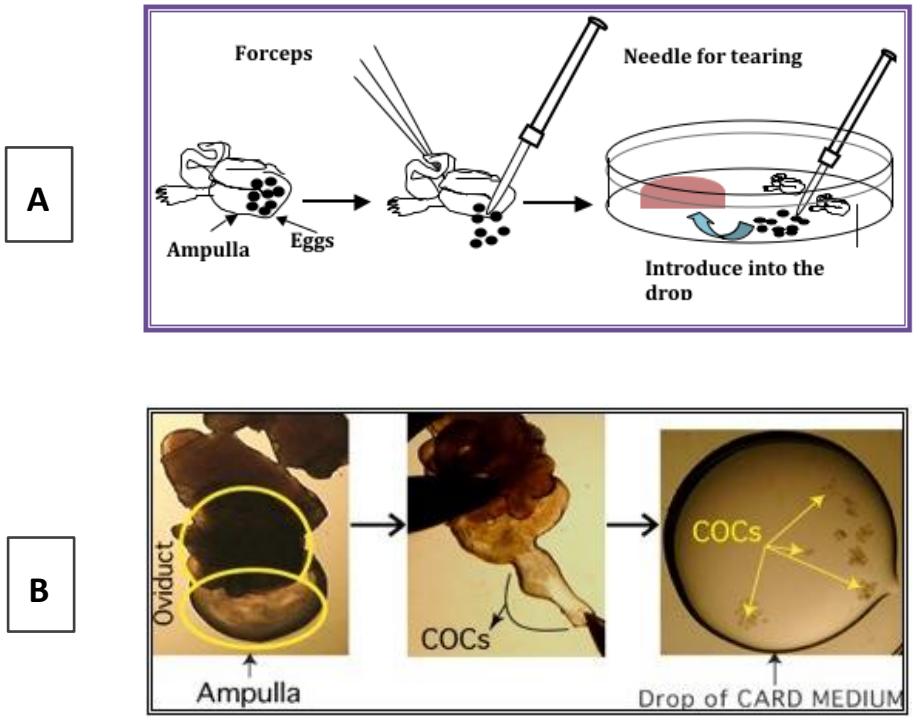


Figure 2.4 Oocyte collection.

A; collecting eggs by dragging cumulus oocytes complex into the fertilisation drop (Card medium) using forceps. B; pictures show how to drag COCs into card medium under a dissecting microscope.
Source: The manual for reproductive engineering techniques in mice (26 March 2016). Retrieved from
http://card.medic.kumamoto-u.ac.jp/card/english/sigen/manual/mouseivf.html

Next, 3-5 µl freshly harvested sperm from the TYH+MBCD medium in the sperm dish was added to the fertilisation drop using a Gilson pipette (Fig. 2.5). Steps 1-3 were repeated for each fertilisation dish in succession (i.e. complete all of the steps from collecting the oviduct to returning the fertilisation dishes to the incubator for one batch of females before starting the next batch). No more than 5 minutes was passed from collecting the oviducts to returning the fertilisation drop (including oocytes and sperm) to the incubator.

Dishes were incubated at 37°C, in 5% CO_2 in air for approximately 3-4 hrs to allow fertilisation to occur. After one hour of incubation, the dishes were checked to assess the motility and concentration of the sperm by assessing the cumulus cells around the eggs. If the motility and concentration of sperm was poor and few cumulus cells are being removed, 3 µl of the sperm suspension was added to the fertilisation drop, then the dish immediately returned to the incubator to allow the sperm to fertilise the eggs.

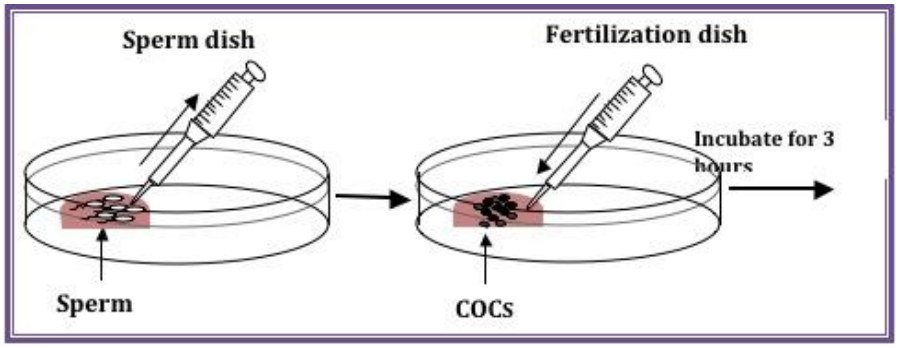


Figure 2.5 Addition of sperm to fertilisation drop using Gilson pipette

2.2.3.5 Washing and culturing the fertilised oocytes

Wash drops were prepared by placing four 150 µl drops of HTF (without GSH) in a 35 mm culture dish and covered with mineral oil (Figure 2.6). The dishes were incubated at (37°C) for at least 3 hours or overnight.

Fertilised oocytes, that had been incubated in the fertilisation dish for 3-4 hours, were washed with three wash drops to remove the cell debris, degenerating oocytes and dead sperm. Presumptive zygotes were removed into a fourth drop and cultured overnight. Next morning, the 2-cell embryos were separated from the unfertilised or degenerating oocytes and placed in a fresh drop.

The 2-cell embryos were separated into two groups: The first group was collected into a drop of pre-warmed M2 medium (Sigma; Cat No. M7167) (Appendix I) in a Falcon petri dish and transferred to the oviduct of 0.5 day pseudopregnant foster mothers (section 2.3). The second group was cultured in potassium simplex optimised medium (KSOM), which contains BSA (Sigma, See Appendex I), overlaid with mineral oil, and transferred as blastocysts to the uteri of 2.5 days pseudopregnant foster mothers (See section 2.3).

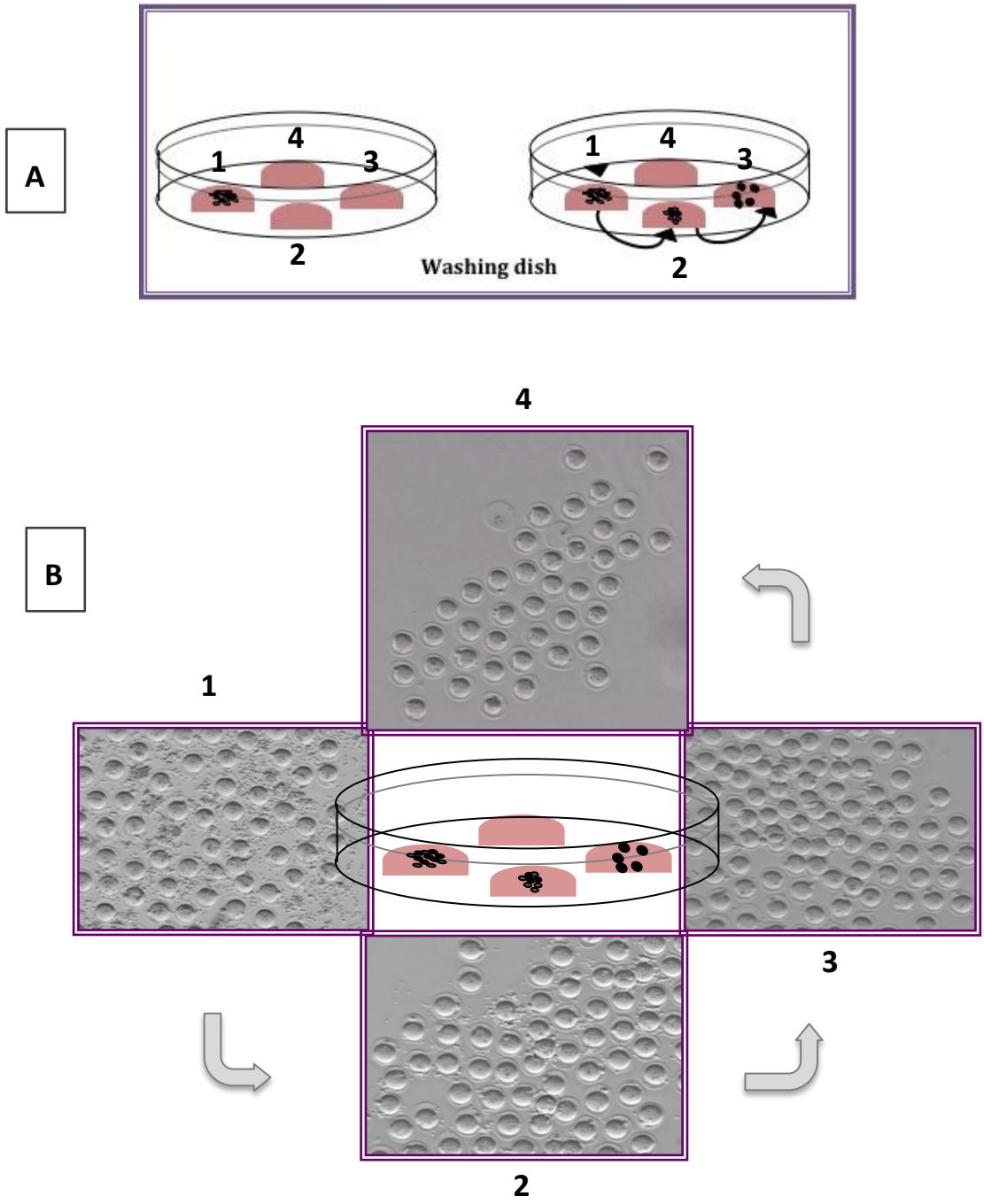


Figure 2.6 Washing drops

Zygotes were washed through the four washing drops of 150 µl of HTF medium to remove the cell debris, degenerating oocytes and dead sperm

2.3 Procedure for embryo transfer

2.3.1 *Generating pseudo pregnant females*

In this study, MF1 female mice were used as recipients for C57/BL6 X CBA embryos. MF1 have an average of 11.5 offspring per litter and have excellent productive and maternal characteristics (Harlan Laboratories report). It is the breed most commonly used in research for pseudo pregnant females and the most used outbred strain (Duah et al. 2013).

Virgin MF1 female mice at an age of 5 to 8 weeks and weighting 29 to 39 grams were naturally mated with MF1 vasectomised males (see below) and caged overnight. The allocation of females and duration of mating is explained in section 2.1.1. Positive plugged females which had copulated with the vasectomised males (determined by the presence of a vaginal plug the next morning) were individually housed for the embryo transfer procedure.

2.3.2 *Vasectoming males for generating pseudopregnant females*

Mrs. Caroline Mercer (Transgenic Facility Manger, Biomedical Research Facility) and Dr Bhav Sheth (Experimental Officer, CfBS, University of Southampton) conducted the procedure for the vasectomised males.

4-6 weeks old male MF1 mice (University of Southampton, Biomedical Research Facility) were moved to the operating theatre before surgery start. Mice were anaesthetised by an intraperitoneal injection of a ketamine (Fort Dodge) acepromazine (C-Vet) anaesthetic (appendix III). The mice were injected with approximately 7 µl /gm of body weight (i.e a

35 g mouse received ~245 µl of anaesthetic). The fur of the lower abdomen of the anaesthetised mouse was shaved, and the skin sprayed with a surgical scrub (7.5% Providone-Iodine, USP). Using fine dissection scissors (Fine Scientific Tools, F.S.T), a 1.5 cm transverse incision was made into the skin and body wall at a point level with the top of the legs. The right testicle, vas deferens and epididymis were exteriorized from the body cavity using blunt forceps. Using the forceps, the vas deferens was held and a portion (approximately 1 cm in length) removed using cautery. The two ends of the vas deferens were checked to ensure closure of the skin incision and carefully replaced back with the testicle and epididymis inside the body wall. The procedure was repeated on the left vas deferens. Using an absorbable suture (vicry1 absorbable suture, Johnson and Johnson), the hole in the body was sutured. The cut in the skin was sutured using a non-absorbable suture (ethilon non-absorbable suture, Johnson and Johnson), and a tissue adhesive (Vetbond, 3M Animal Care Procedure, USA) was used to cover the skin. After surgery, mice were intraperitoneally injected with 150 µl of analgesic (Temgesic 300 µg/ml; Reckitt & Colman Product, appendix III) and allowed to recover before being returned to their cages.

The sterility of the vasectomised males was assessed after two weeks of recovery. The vasectomised mice were mated overnight with 5-12 week virgin female CBAxC57/BL6 mice (University of Southampton, Biomedical Research Facility). The females were checked every morning for a vaginal plug (a sign of copulation). Each plug-positive female was housed and watched for the next 20 days until birth. Plug negative females were returned to the same male. To confirm sterility, each vasectomised male was mated with at least two females.

2.3.3 *Embryo transfer (ET)*

Dr Neil Smyth (Reader, Centre for Biological Sciences (CfBS), University of Southampton) and Dr Bhav Sheth (Experimental Officer, CfBS, University of Southampton) conducted the procedure of embryo transfer.

3-4 weeks old virgin female C57/BL6 mice were superovulated (See section 2.2.1). The superovulated females were either mated with CBA males for *in vivo* embryo collection or housed in a cage until the next morning to be used as donors for *in vitro* fertilisation. At the time of hCG injection, 6-8 week old (29-39 g) female MF1 mice were mated (without superovulation) with vasectomised MF1 males. Plug positive females which had copulated with vasectomised males (determined by the present of a vaginal plug the next morning) were either used on the same day of positive plug (day 0.5 of pseudopregnancy) or individually housed for 2 days (day 2.5 of pseudopregnancy).

To provide *in vivo* embryos (See section 2.2.2), the mice that had been superovulated and mated with a fertile CBA male were not flushed until an hour before the time of transfer (~23 hours post hCG for 2-cell stage ET and ~95 hours post hCG for blastocyst stage ET). Embryos were flushed from either the oviduct or uterus and kept in pre-warmed H6-BSA under mineral oil until transferred to the foster mother, approximately 1-2 hours after flushing.

To provide *in vitro* embryos (See section 2.2.3), the superovulated C57/BL6 females were killed (~15 hours post hCG) to harvest oocytes for IVF procedure. Two-cell stage and blastocyst stage embryos derived from an *in vitro* environment were kept in pre-warmed H6-BSA under mineral oil until being transferred to the foster mother, approximately 1-2 hours after collection. Before the surgery began, embryos were placed in drops of M2

medium, each drop contains 20 2-cell stage embryos for oviduct ET, 10 each side and 14 blastocysts for uterus ET, 7 each side.

Day 0.5 pseudopregnant females were anaesthetized by intraperitoneal injection of ketamine (Fort Dodge) acepromazine (C-Vet) anaesthetic (section 2). Dr Neil Smyth and Dr Bhav Sheth conducted the anaesthetics procedure.

The fur of the lower back of the anaesthetised mice was shaved, and skin cleaned using a surgical scrub (7.5% Providone-Iodine, USP). A 1 cm longitudinal incision was made in the skin at the level of the first rib on the right side. Connective tissues, which hold the skin to the body wall, were gently cut. A small cut was made in the body wall just over the right ovary using fine forceps and dissection scissors (Fine Scientific Tools, F.S.T). The left ovary was carefully exteriorized from the body via the fat pad and held outside the body using a serrated bulldog type serrefine (Fine Scientific Tools, F.S.T).

For the 2-cell stage ET, 10 embryos were taken from the M2 medium using a flame polished glass transfer pipette of smaller internal bore than the embryos, and attached to a mouth pipette. The flame-polished transfer pipette was made from glass capillaries (internal diameter 0.58 mm, external diameter 1.0 mm, Harvard Apparatus) pulled over an alcohol flame and broken in the centre to form two micropipettes. The micropipettes were then placed onto a microforge (De Fonbrune), re-broken after fusing allowed to cool onto a glass bead on the microforge filament, and finally flame polished to form the transfer pipette. Under a stereomicroscope (Zeiss) and using two pairs of fine forceps, the bursa surrounding the oviduct was gently torn open to allow access to the infundibulum, which is the opening to the oviduct. In the case of bleeding, a sterile cotton swab was gently used to blot away any excess blood. The tip of the loaded pipette was inserted in

the oviduct and embryos transferred; the pipette tip was checked underneath the microscope to make sure all the embryos had been transferred.

For blastocysts ET, 7 embryos were taken up from the M2 medium using a polished transfer pipette. Under a stereomicroscope (Zeiss), and using a 25 gauge needle, a hole was made in the top of the uterus a few millimetres down from the uteruo-tubal junction. While the uterus was held using forceps, approximately 5 mm of the transfer pipette was inserted into the hole and the blastocysts then transferred. The transfer pipette was checked underneath the microscope to ensure that all embryos had been transferred.

The ovary, oviduct and the uterus were replaced back inside the body wall taking great care not to handle or damage them. The hole in the body cavity was sutured using an absorbable suture (vicry1 absorbable suture, Johnson and Johnson). The procedure was repeated on the left side of the mouse. The cuts in the skin in both sides were sutured using a non-absorbable suture (ethilon non-absorbable suture, Johnson and Johnson), and a tissue adhesive (Vetbond, 3M Animal Care Procedure, USA) was used to cover the skin. After surgery, mice were intraperitoneally injected with 150 µl of analgesic (Temgesic 300 µg/ml; Reckitt & Colman Product, appendix III) and allowed to recover before being returned to their cages.

2.4 Anti-DNP protocol for differential cell staining of blastocysts

The protocol for differential labelling was based on study by (Handyside and Hunter 1984). Freshly collected blastocysts and IVF embryos at the blastocyst stage were analysed for cell numbers within the Trophectoderm (TE) and Inner cell mass (ICM) lineages by differential nuclear labelling. This protocol is based on the permeability

barrier created by TE cells forming a seal of zonular tight junctions. When embryos are incubated with a species-specific antiserum targeted to cell surface antigens (Anti-DNP) followed by complement treatment, the TE cells can be selectively lysed whereas the ICM cells are protected by the formed seal. The consequence of this is that only TE cells are stained with a membrane-impenetrable DNA stain, such as Propidium Iodide (PI), while both ICM cells and permeabilised TE cells are stained with a membrane-penetrable DNA stain, such as Hoechst (H). The two populations can be clearly distinguished by their nuclear colour using fluorescence microscopy, the blue ICM cell nuclei (stained only with H) and the pink TE cell nuclei (stained both with PI and H). The procedure to lyse the TE cells selectively is named immunosurgery.

The zona pellucida of blastocysts was removed with acid Tyrode's solution (pH 2.3, 37°C, appendix I; Sigma T1788-100ML) in a cavity block over ~30 secs. Embryos were immediately buffered after zona removal in a large volume of H6 medium with BSA (H6-BSA) in a cavity block, with 10-20 minutes at 37°C allowed for recovery. Blastocysts were incubated for 10-20 minutes at room temperature in a large drop (50 µl) of trinitrobenzenesulfonic acid (TNBS, Sigma; 1 ml TNBS plus 9 ml handling medium with PVP [H6-PVP] or PVA, pH adjusted to 7.4 [with NaOH, 1 µl at a time]), then washed in a small drop of the same medium. Blastocysts were then washed 3 times in 3 drops of 50 µl H6-PVP, until the medium was colourless. Blastocysts were incubated in a 40 µl drop comprising 20.8 µl Anti-Dinitrophenyl-BSA antibody solution (anti-DNP, ICN Biochemicals; 3 mg/ml in PBS with 0.1% PVP) in 29.2 µl H6-PVP for 10 minutes at room temperature after first washing them in a small drop of 10 µl of anti-DNP. Afterwards, embryos were washed in 3 drops of 50 µl of H6-PVP. Embryos were then incubated in 50 µl guinea pig complement protein (Cedarlane; 1:10 in H6 BSA) with 4 µl of Propidium Iodide (PI, Sigma;

1 mg/ml in distilled water) added at 37°C in incubator for 15 minutes. Embryos were washed 3 times in 50 µl drops of H6-BSA. Embryos were fixed in 10 µl Bisbenzimide H 33258 stain (Hoechst stain, Sigma, 2.5 mg/ml in distilled water) added to 990 µl absolute ethanol at 4°C for at least 30 minutes (maximum 2 days).

2.4.1 *Mounting*

Embryos were washed in absolute ethanol for 5-10 minutes, placed in groups of 3-5 in small drops of ultra pure glycerol (Amersham) on a slide and covered with a coverslip. Blastocysts were viewed and imaged using a Zeiss Axiovert 200 fluorescence microscope (Carl Zeiss Ltd, Welwyn Garden City, Hertfordshire, UK) and Metamorph® version 6.2r6 software (Universal Imaging Crop). Blue ICM and red TE cell nuclei were counted and recorded.

2.5 Analysis of postnatal growth and development

2.5.1 *Postnatal maintenance of offspring*

At the day of birth, litter size was adjusted to contain up to 8 pups per litter and if possible to contain similar number of males and females. At an age of week 1, offspring were weighed on an electronic balance taking great care not to transmit the smell of other mice onto the new born pups via wearing gloves and washing hands, because this may lead mothers to reject their pups. If the total number of pups was more than 8 per litter, the largest and smallest weight pups were culled to normalise the litter size. Offspring were weaned at an age of 3 weeks and housed in separate cages by gender per litter with access to standard chow and water *ad libitum*.

2.5.2 *Measurement of postnatal growth*

Pups were weighed from week 1 and then subsequently on the same day every week up to week 27, using gloves.

2.5.3 *Systolic Blood Pressure (SBP)*

Systolic blood pressure was determined at age of 9, 15 and 21 weeks by tail-cuff plethysmography using an Non-Invasive Blood Pressure Monitor (NIBP-8, Columbus Instruments, Columbus, OH, USA) SBP was measured at room temperature of 30-37°C, to allow the mice to acclimatise themselves for at least 90 minutes before readings were taken, and to the tail cuff apparatus for several minutes before use. This practice encourages vasodilatation, thus facilitating detection of SBP. Mice were restrained in a ventilated acrylic tube dependent on their weight (15-35 g or 35-50 g), see Figure 2.7. Mouse tails were threaded through the tail cuff Figure 2.8. Each mouse was allowed to acclimatize to the apparatus for 5 minutes prior to readings being taken. Five SBP recording with good waveforms and good overall quality were taken for each mouse, and the mean value of the three middle readings was calculated and recorded. If the mouse kept moving and did not allow a recording of five readings after 20 minutes, the mouse was released and allowed to recover before proceeding. Heart rate was monitored as an indicator of stress and if >500 beats per minute, readings were not taken until below this value.

Figure 2.7 Different sizes of ventilated acrylic tube.

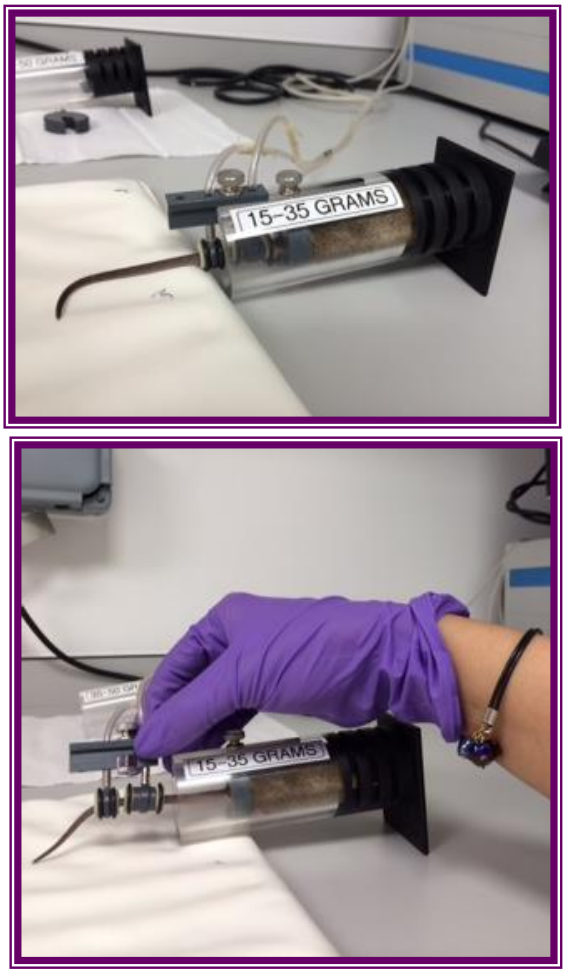


Figure 2.8 : Measuring SBP in the mouse using an acrylic tube and tail cuff.

2.5.4 *Glucose tolerance test (GTT)*

Offspring were fasted overnight for 15 hours. The next morning at 0900 hours, mice were weighed and their tails were marked with different colours for easier identification during the injection procedure. Glucose solution (D-glucose, embryo tested; G-6152 Sigma) was prepared in the morning before the procedure (20% glucose : 2g D-glucose + 10 ml distilled water). Syringes were filled with glucose solution and labelled with the same colour to each mouse (1 g D-glucose/kg body weight).

Anaesthetic (Xylocaine Gel 2%; lidocaine hydrochloride; Astra Pharmaceuticals) was applied to the tail of each mouse and mice were kept in their cages for 10 minutes before tail cutting. After 10 minutes, a small cut at the end of the tail was made using a razor blade and a drop of blood was gently squeezed out to conduct a glucose reading using the glucometer (AccuCheck Aviva, Roche). From each mouse, a duplicate reading was taken and the value of the glucose level (mmol/g) was recorded in an excel sheet. To perform the glucose tolerance test, after 15 minutes of the first reading (fasting reading), mice were intraperitoneally injected with glucose (1 g D-glucose/kg body weight). Blood collection was repeated at three further time points (at 30, 60, 120 minutes after glucose injection) and recorded as described above.

The glucometer was calibrated weekly before GTTs were carried out to ensure the reliability of the findings, using a calibrator provided by the manufacturer. Before opening the calibrator, the date and time were recorded, and the tip of the bottle was wiped. A drop of the calibrator was placed onto the test strip by squeezing the bottle gently. After a successful test, the glucometer turns off for 5 seconds once the test strip is removed.

2.5.5 *Culling and organ collection of 27 week old mice*

At 27 weeks, mice were sacrificed by cervical dislocation ensuring not to rupture any blood vessels. Mice were dissected ventrally and the rib cage was cut to expose the heart for blood collection. Blood samples were taken by puncturing the heart using a needle (30 gauge) attached to a syringe. Blood samples were placed in a small Eppendorf tubes, single tube per mouse, submerged in wet ice. After blood collection, the heart, liver, spleen, lung and both kidneys were dissected out, weighed and snap frozen in liquid nitrogen then stored at -80^{o}C. Blood samples were centrifuged at 10,000 g, 4^{o}C for 10 minutes. Serum was aliquoted into ten 3 µl samples and frozen at - 80ºC.

2.5.6 *Serum Glucose and Insulin*

Frozen serum samples collected after the animal culling procedure at the age of 27 weeks, were thawed by placing the samples (contained within Eppendorf tubes) in the fridge at -4^{o}C for 24 hours. The samples were then centrifuged at 10,000 rpm, at 4^{o}C for 1 minute. A total of 7 to 8 samples from each treatment, both male and female, were used for this experiment. Glucose concentration in offspring serum was measured using the glucometer and glucose strips as described in the GTT procedure (Section 2.5.4).

Insulin levels in the serum can be detected using an ELISA (enzyme-linked immunosorbent assay). The technique typically involves the use of two monoclonal antibodies in which one binds to and ‘captures’ the target antigen and the other covalently links and allows detection. A microtitre plate is commonly coated with the capture antibody and incubated with a sample containing test antigens that will bind to the antibody. A second antibody conjugated with an enzyme is then added, which also binds to the antigens. The mixture is incubated, washed and then a substrate is added. The substrate is then

hydrolysed in proportion to the concentration of antigen present due to the conjugate enzyme, and can be measured against a standard of known concentration. This sensitive technique is one of the most extensively used immunological assays which allows for the amplification, detection and quantitation of a range of antigens (Paulie and Perlmann 2001).

In our experiment, mouse serum insulin levels were determined using a specific ELISA kit (Mercodia, Sweden, Mouse: 10-1247-01) based on the manufacturer's instructions. Wash buffer solution was prepared by dilution of 35 ml of the wash buffer provided with the kit in 700 ml redistilled water, and stored at 4^{o}C for 8 weeks. Enzyme conjugate solution was prepared by mixing 100 µl enzyme conjugate with 1000 µl enzyme conjugate buffer for each strip (8 wells). When preparing enzyme conjugate for the whole coated-plate, all the required aliquots of enzyme conjugate buffer were aliquoted into the enzyme conjugate vial and mixed, 10 µl of each of the calibrators (Calibrator 0, 1, 2, 3, 4, and 5 ready to use) and serum samples were pipetted into appropriately coated microplate wells, followed by 100 µl of enzyme conjugate solution. After the plate was incubated on a plate shaker at room temperature at 750 rpm, for 2 hours, the wells were washed with wash buffer using a manual wash procedure. Reaction mixture was discarded by inverting the plate over the sink and 350 µl of wash buffer was added to each well and immediately discarded, the plate was then tapped on absorbent paper to ensure the removal of any excess liquid. The wash procedure was repeated five times. Next, 200 µl of substrate was added to each well followed by a second incubation for 15 minutes. Lastly, a 50 µl of stop solution was added and the absorbance was measured at 450 nm using a Varioskan Flash Multimode Reader (Thermo Scientific). The standard deviation and coefficient of variance was calculated for each sample. The sample was discarded if the coefficient of variation (CV%)

was higher than 20%. Using Prism software, the standard curve of the calibrators was created and insulin concentration was calculated. O.D values must be within the range of the standard curve and outlier values were not included in our calculations. Samples from the five treatment groups were run in three plates including optimising experiments. Each mouse serum sample was run in duplicate and mean insulin values calculated from these duplicate values.

2.5.6.1 Calculation of measurement of insulin resistance

Serum glucose and insulin values were measured as described above (Section 5.1) and the glucose/insulin ratio (G:I) ratio was calculated. The G:I ratio is a useful measurement for detecting insulin resistance (McAuley et al. 2001). The low G:I ratio indicates high insulin sensitivity (insulin resistance) in patients/animals where their body produces insulin but does not use it effectively (Chapter 4).

2.5.7 *Measurement of ACE activities in mouse serum*

The methods of (Hurst and Lovell-Smith 1981); (Forhead et al. 2000); (Watkins et al. 2007) were used to measure ACE activities with some modifications. The assay was based on the colorimetric determination of hippurate with cyanuric chioride/dioxan reagent. Hippurate is released from hippuryl-L-histidyl-L-leucine (HHL) by ACE in the presence of chloride ion. Samples were vortexed and centrifuged at 10,000 rpm, at 4^{o}C for 1 minute. Next, 25 µl of each sample was placed into an Eppendorf tube with 100 µl dH20 and 275 µl incubation buffer (0.2M H3BO3, 2M NaCl, pH 8.3, see appendix V), and incubated for 5 minutes at 37^{o}C. A total of 100 µl of 20 mmol/L Hippuryl-L-Histidine-L-Leucine (Sigma) in 20 mmol/L NaOH, see appendix V, warmed to 37^{o}C, was added to each sample. Samples were incubated at 37^{o}C for 15 minutes by placing in a water bath. Next, 500 µl of 1M HCl

was added to terminate the reaction, followed by 500 µl of 1M NaOH to neutralise. 2ml of KH2PO4 (0.2M) was added, followed by 1.5ml of 0.16 M cyanuric chloride (2-4-6 trichloro 1-3-5 triazine; Sigma, see appendix V) in 1,4-dioxane; (Sigma). Samples were then vortexed for 10 seconds, left to stand for 5 minutes before being vortexed again and then centrifuged for 10 minutes at 3000 rpm. Four 200µl aliquots for each sample were pipetted into a 96-well plate. Negative controls were created by duplicating samples and treating them exactly the same as the positives, except HCl and NaOH were added before the HHL. Hippurate standards of 20 µM, 40 µM, 60 µM, 80 µM and 100 µM (hippuric acid; Sigma, see appendix V), and a blank of just dH2O, were prepared and treated the same as the positives except for the addition of HHL. These were used to create a standard curve of known concentrations. Plates were analysed using a plate reader, and the absorbance at 380 nm was measured against the blank using a Varioskan Flash Multimode Reader (Thermo Scientific). Each sample was analysed in duplicate, and the average of these 8 readings was taken. ACE activity was expressed as the amount (in µM) of hippurate formed per ml of serum per minute (see Appendix VII). The method was based on the following studies (Hurst and Lovell-Smith 1981); (Forhead et al. 2000); (Watkins et al. 2007).

2.5.8 *Measuring ACE activities in the mouse lung*

Samples of lung were weighed to acquire 50mg (+/- 1mg) per sample. These were homogenised in 300 µl ice-cold boric buffer (H_3BO_3, 2M NaCl, pH 8.3, see appendix V) with a PowerGen homogeniser, then centrifuged at 16,400 rpm for 10 minutes at 4^{o}C. The supernatant was removed and stored at -80^{o}C. This process was then repeated with the remaining pellets after the supernatant had been aspirated off. Each pellet was

homogenised in 300 µl buffer. The homogenates were centrifuged as before and the supernatant removed and stored at -80^{o}C. Both supernatants were analysed.

A total 10 µl of each sample was placed into an Eppendorf tube with 20 µl dH_20 and 5 µl incubation buffer (0.2M H_3BO_3, 2M NaCl, pH 8.3, see appendix V), and incubated for 5 minutes at 37^{o}C. Next, 2 µl Hippuryl-L-Histidine-L-Leucine (20 mmol/L Hippuryl-L-Histidine-L-Leucine (HHL); Sigma in 20 mmol/L NaOH, see appendix VI), warmed to 37^{o}C, was added to each sample. Samples were incubated at 37^{o}C for 15 minutes by placing in a water bath. 100 µl of 1M HCl was added to terminate the reaction, followed by 100 µl of 1M NaOH to neutralise it. Then 400 µl of KH_2PO_4 (0.2M) was added, followed by 400 µl of (0.16 M cyanuric chloride(2-4-6 trichloro 1-3-5 triazine; Sigma) in 1,4-dioxane; Sigma, See appendix V). Samples were then vortexed for 10 seconds, left to stand for 5 minutes before being vortexed again and then centrifuged for 10 minutes at 3000rpm. Four 200 µl aliquots for each sample were pipetted into a 96-well plate. Negative controls were created by duplicating samples and treating exactly the same as the positives, except the HCl and NaOH were added before the HHL. Hippurate standards of 50 µM, 100 µM, 400 µM, 1M, 2M and 2.5M (hippuric acid; Sigma, see appendix V), and a blank of just dH2O, were prepared and treated the same as the positives except for the addition of HHL. These were used to create a standard curve of known concentrations. Plates were analysed using a plate reader, and the absorbance at 380 nm was measured against the blank using a Varioskan Flash Multimode Reader (Thermo Scientific). Each sample was analysed in duplicate, and the average of these 8 readings was taken. The total protein content of the samples was measured using a BioRad kit. ACE activity was expressed as amount (in nM) of hippurate formed per mg of protein, per minute (see Appendix VII).

2.6 Statistical analysis of data

Significance was taken as P ≤0.05 for all statistical analysis. If a p value of between 0.1 and 0.05 was observed, a trend was assumed to exist.

Blastocyst cell number and rates of blastocyst development were analysed using a one-way ANOVA (IBM SPSS Statistic 22). The litter size from the different treatment groups were analysed by one-way ANOVA followed by a pairwise t-test with Bonferroni correction analysis (IBM SPSS Statistic 22).

Data regarding litter size correlation were analysed for normality using the IBM SPSS Statistic 22. The correlation was assessed using a Pearson correlation if the distribution passed the normality test. However, if the normality test showed not a non-normally distributed sample, the correlation was analysed using a Spearman correlation.

All postnatal data comprising offspring weights, systolic blood pressure, glucose tolerance test, weights and ratios, post culling serum glucose and insulin and serum and lung ACE activities were analysed using a multilevel random effects regression model, which takes into account between-mother and within-mother variation. The model used was the following, with terms as defined in Table 2.1 shows explanations of the random effects model analysis:

$$y(1p) = a + b.treatment + u(1) + e(1p) + c$$

This programme estimates the variance of all the e(1p) terms. The random effects model analysis was performed in consultation with Professor Clive Osmond, Senior Medical Statistician at the Medical Research Council Epidemiology Unit at Southampton General Hospital.

Table 2.1 shows explanations of the random effects model analysis

Symbol	Definition
L	The litter
P	The pup
y(1p)	The response (outcome) variable as measured on pup p in litter 1
A	The intercept
treatment	The mouse group from different embryo origins e.g. control, *in vivo* and *in vitro*.
B	The regression which y is raised due to the treatment as opposed to the control treatment
u(1)	A random effect applying to all pups in litter 1, the litter tend to have large values of y, if u(1) is larger than all pups in this litter. The variance of all the u's that apply to the various litters estimated via the programme.
e(1p)	A random effect applying to all pups, it indicates measurement errors within pup variability and other uncaptured determinates of outcome.
C	The number of pups within the litter, either before or after correlation.

Chapter 3: Effect of *In Vitro* Fertilisation (IVF) and Embryo Culture Duration on Mouse Embryo Development

3.1 Introduction

Studies have shown that the preimplantation mammalian embryo is sensitive to environmental conditions that may impact blastocyst morphogenesis and also the fetal or postnatal phenotype (Fleming et al. 2004). *In vitro* cultured mammalian embryos develop slower than embryos developing *in vivo*. Embryos cultured from 2-cell stage to blastocyst in the optimum culture conditions are not more than 18-24 hours behind E4 *in vivo* blastocysts (Harlow and Quinn 1982). It is important to note that this thesis chapter is not on embryo culture media *per se* but on the environment of early development and how it may affect blastocyst formation and cell numbers.

Historically, embryo culture media has undergone many improvements in the attempt to optimise culture conditions, focusing on the development of a single medium to support all embryo development stages prior to implantation. Two types of media widely used for human IVF most recently are either 'simple' or 'complex'. The 'simple' media were those such as Earle's (Edwards 1981), modified Tyrode's medium T6 (used by (Dokras et al. 1993), and human tubal fluid (HTF) medium used

by (Quinn et al. 1985). These simple media are balanced salt solutions with added carbohydrate energy sources such as pyruvate, lactate and glucose, and are commonly supplemented with the patient's serum. Complex media, for instance Ham's F-10 and α-MEM, are specifically designed for the culture of somatic cells *in vitro* (Lane and Gardner 2007).

It has been reported that significantly more mouse zygotes have been developed in HTF medium than in T6 medium. Moreover, significantly more pregnancies (30% of 60 transfers) were obtained with HTF medium when compared with T6 medium (11% of 53 transfers). (Quinn et al. 1985) decreased the K^+ level in HTF medium to that level present in T6 medium and found that the number of mouse zygotes that developed in culture was significantly decreased, whereas modifying the Ca^{++} content of HTF had no effect. They hypothesised that the higher K^+ level in the HTF medium is initially responsible for the superiority of the HTF medium over the T6 medium (Quinn et al. 1985).

Recent studies show that adding reduced glutathione (GSH) to the fertilisation medium during IVF increases the fertilisation rate of fresh sperm (Ishizuka et al. 2013) and frozen/thawed sperm of various strains of mice (Takeo and Nakagata 2011b). GSH is a biological antioxidant that protects mammalian sperm against loss of DNA integrity and motility through oxidative stress. GSH-treated oocytes exhibit altered thiol status which leads to increased numbers of free thiols in the zona pellucida (ZP) and ZP expansion (Takeo and Nakagata 2011b). The mouse ZP consists primarily of three glycoproteins namely, ZP1, ZP2, and ZP3, which have 21, 20, and

12 cysteine residues, respectively, that form intra- or intermolecular disulfide bonds (Boja et al. 2003). The structure of the ZP is maintained by disulfide bonds, which cause its hardening. The impact of GSH on fertilisation may be caused by its ability to break the disulfide bonds and protect against ZP hardening. It has been demonstrated that ZP1-knockout mice produce a loosely organized ZP matrix and dramatic enlargement of the perivitelline space (Rankin et al. 1999).

In the mouse blastocyst, there are two distinct cell types, firstly the outer layer of the trophectoderm (TE), which is responsible for blastocoel fluid accumulation and is specialised for implantation. TE will only give rise to components of placenta and extraembryonic membranes after implantation. The second cell type is the inner cell mass (ICM), which is the mass of cells inside the embryo that will eventually give rise to the definitive structures of the fetus. The number of TE and ICM cells in mouse blastocysts developing *in vivo* have been demonstrated by differential labelling of nuclei with polynucleotide-specific fluorochromes (Handyside and Hunter 1986). (Handyside and Hunter 1986) demonstrated that in humans blastulation is initiated between the fourth and the fifth cleavage divisions and the minimum number of cells in normally fertilised, expanded blastocysts observed on E5 and E6 was 24 and 27; respectively. The overall cleavage rate of human embryos *in vitro* at the morula and blastocyst stages is similar to the previous cleavage divisions, with one division every 24 hours. In contrast, in the mouse, blastulation occurs at the completion of the fifth cleavage division. The cleavage rates over the morula stage are initially higher compared with human embryos, with about one division every 12 hours at

the morula stage. However, the rate decreases at the blastocyst (Hardy and Handyside 1993) stage due to a combination of giant cell formation in the mural TE , slower division and cell death in the ICM (Handyside and Hunter 1986).

Studies have been reported that the mouse preimplantation embryo is able to compensate for changes in its total volume and total cell number, whilst at the same time still being able to develop into a viable offspring (Hardy and Handyside 1993). In this study, they investigated the allocation in twin half mouse embryos produced by bisection at the 8-cell stage. Half embryos and intact zona-free control embryos were differentially labelled during cavitation, and the numbers of cells in the ICM and TE counted. They found that the numbers of ICM cells are not significantly different from those in intact controls, whereas the numbers of TE cells were significantly higher, producing a small increase in the total cell number of half embryos. However, there was no concomitant decrease in ICM cell number. The study proposed that as the surface area of a half embryo is greater than half an intact control, TE cells in half embryos divide faster in order to reach an optimal packing density similar to that observed in intact control embryos (Hardy and Handyside 1993).

A mouse study was performed to examine the ability of embryos to compensate for a loss of cells and to develop with body parts of normal size and normal proportions during post-implantation development. In this study, micro-manipulations were performed on 4-cell pre-implantation embryos to decrease the number of cells by either 25% (3/4 embryos) or 50% (2/4 embryos). It has been shown that blastocysts developed from these embryos showed a preferential loss of ICM population, and

fewer of them formed viable embryos after implantation. The 3/4 embryos size (post-implantation) was initially smaller than controls of the same gestational age, but compensatory growth, achieved by increasing cell numbers at above the normal rate and beyond the normal duration, resulting in a complete restoration of body size. It has been reported that embryos manipulated to contain decreased number of cells alter particular events of gastrulation and morphogenesis so as to keep the cell number and body size appropriate for each developmental stage (Biggers and Papaioannou 1991).

In humans, it has been shown that approximately 6 cells are allocated to the ICM, and 10 to the TE at the 16-cell stage, whilst at the 32-cell stage, the number was approximately 12 cells allocated to the ICM and 20 cells to the TE. The maximum number of TE cells in a human blastocyst was 170, whereas the maximum number of ICM was 113. In mouse, the numbers of cells allocated to ICM and TE are similar to the numbers reported in humans. The maximum number of TE cells in the mouse blastocyst was similar to the human blastocyst, 147, whereas the maximum number of ICM was smaller than the human blastocyst, 49 (Handyside and Hunter 1986).

There is growing evidence showing that *in vitro* cultured embryos develop at a slower rate and with a smaller total cell number compared with *in vivo*-derived embryos at the same time post-hCG (Harlow and Quinn 1982);(Gardner and Sakkas 1993); (Watkins and Fleming 2009). Despite the fact that several culture conditions such as amino acid composition, energy substrate, growth factors and cytokines have been shown to alter blastocyst cell number (Lane and Gardner 1997); (Kaye and

Gardner 1999); (Fleming et al. 2004), the long term effects of decreased blastocyst cell number have not been assessed extensively.

The hypothesis of this chapter is that the environment, in particular IVF and long embryo culture (blastocyst) in which the preimplantation embryo develops can slow down blastocyst development and redude trophectoderm TE, inner cell mass ICM and total cell number vs *in vivo* development.

The aim of the experiments in this chapter were to examine the effect of *in vitro* fertilisation and *in vitro* culture conditions on the development of the blastocyst and the cell numbers generated within ICM and TE and their ratio.

The first series of experiments scored the *in vitro* fertilisation (IVF) success by assessing the number of 2-cell stage embryos developed from zygotes after IVF (See section 2.2.3).The second series of experiments investigated the developmental rate of blastocysts between IVF and *in vivo* embryos. *In vivo* and IVF embryos were subsequently monitored to analyse the success rate of blastocyst formation. The final series of experiments examined the effect of IVF and subsequent culture on cell lineage numbers in blastocysts using differential labelling staining compared with *in vivo* controls (See section 2.4).

3.2 Methodology

3.2.1 *Superovulation and embryo collection*

Female C57/BL6 mice were superovulated and mated with CBA males (Section 2.2.1). Embryos were flushed at the blastocyst stage E3.5 (~96 hours post hCG; section 2.2.2).

3.2.2 *In vitro fertilisation embryos*

Eggs were harvested from superovulated C57/BL6 female mice and fertilised *in vitro* using CBA male sperm following the IVF procedure (Section 2.2.3). At 2-cell stage on E1.5, embryos were cultured in KSOM to the blastocyst stage E4.5.

3.2.3 *Successful rate of in vitro fertilisation*

In vitro fertilisation success was assessed at E1.5 by counting the number and proportion of 2-cell stage embryos formed (Section 2.2.3.5).

3.2.4 *Differential labelling staining*

Embryos were stained using a differential labelling staining technique at either the late blastocyst or expanded blastocyst stage (Section 2.4) and blue ICM and red TE cell nuclei were counted and recorded using Metamorph software (See section 2.4).

3.2.5 *Statistical analysis*

Data were analysed using a one-way analysis of variance (One-Way ANOVA test; IBM SPSS Statistic 22). Statistical significant was considered with a $P \leq 0.05$.

3.3 Results

3.3.1 *Effect of* in vitro *fertilisation and prolonged embryo culture on embryo development*

The developmental rates of IVF embryos (from zygote to blastocyst stage) is shown in Figure 3.1 and Table 3.1. Eggs were collected from a total of (n= 40) superovulated donors and used in 14 separate experiments. Collectively, from the eggs successfully fertilised and developed to 2-cell embryos (n= 1576), 78.13% of these developed to blastocysts, whereas 7.60, 8.07 and 6.20%, arrested at 2-cell, 4-cell and morula stages, respectively.

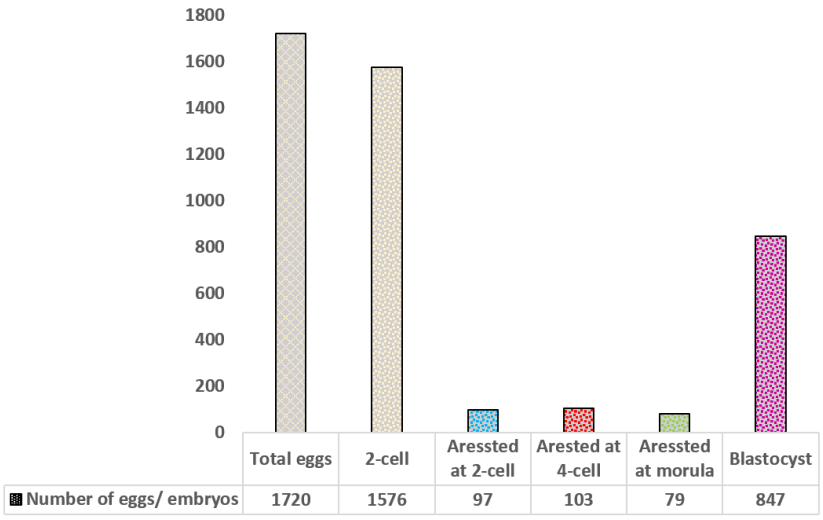


Figure 3.1 Effect of *in vitro* fertilisation and prolonged embryo culture on embryo development

Total number of eggs harvested (n= 1720) from (n= 40) donors; total number of 2-cell embryos (n= 1576), total number of arrested embryos at 2-cell stage (n= 97), total number of arrested embryos at 4-cell stage (n= 103), total number of arrested embryos at morula stage (n= 79), and total number of blastocyst embryos (n= 847); that developed *in vitro*.

Table 3.1 summarises results from the total number of IVF experiments performed in this study. From 14 separate experiments, and over 1,700 eggs used, the mean success rate of 2-cell embryo formation was 91.63%.

Table 3.1 Success rate of *in vitro* fertilisation experiments

Experiment No	Number of donors (mothers)	Number of 2-Cell embryos	Number of unfertilised eggs	Total	Success Rate %*
1	3	132	9	141	93.62
2	3	125	11	136	91.91
3	4	105	24	129	81.40
4	3	129	17	146	88.36
5	3	137	28	165	83.03
6	3	51	3	54	94.44
7	1	144	14	158	91.14
8	3	33	1	34	97.06
9	3	130	5	135	96.30
10	3	130	7	137	94.89
11	1	33	2	35	94.29
12	4	170	3	173	98.27
13	3	132	9	141	93.62
14	3	125	11	136	91.91
Total	40	1576	144	1720	91.63
Mean		112.57	10.29	122.86	91.63

*(total number of 2-cell embryos/total number of eggs * 100)

3.3.2 *Effect of* in vitro *fertilisation and prolonged embryo culture on embryo developmental rate compared with* in vivo *embryos*

Table 3.2, Figure 3.2 and Figure 3.3 represent the developmental rates of *in vivo* and IVF embryos from a total of 22 and 40 superovulated females, respectively used over 22 *in vivo* and 14 IVF separate experiments. From 469 *in vivo* generated embryos, at E3.5, different stages of development was achieved at morula (n=15; 3.14% of total), early-blastocyst (n=40; 8.74%), mid-blastocyst (n=114; 24.63%), late-blastocyst (n=295; 62.52%) stages and degenerated (n=5; 0.95%). For definition of these stages of blastocyst expansion, see Figure 3.3. In contrast, IVF embryos (n=662) at E3.5 showed delay in forming blastocysts and all were at the morula stage (100%). However, during later culture up to E4.5, IVF embryos underwent blastocyst formation, as shown in Figure 3.3.

Group	E3.5 (% ± SEM)						
	Total number of superovulated females	**Total number of embryos**	**Morula**	**Early blastocyst**	**Mid blastocyst**	**Late blastocyst**	**Degenerated**
in vivo	22	469	15 (3.14±0.71)	40 (8.74±0.94)	114 (24.63±1.1)	295 (62.52±1.29)	5 (0.95±0.38)
IVF	40	1076	1076 (100±11.14)	0	0	0	

Table 3.2 Percentage (% ± SEM) of developmental rate of *in vivo* and IVF embryos at E3.5 (96 hrs post hCG)

Embryos in culture develop slower than embryos *in vivo*.
Early blastocyst: has a blastocoel volume less than half of the total embryo volume.
Mid blastocyst: has a blastocoel volume equal to or larger than the total embryo volume.
Late blastocyst: blastocoel fully expanded within the embryo whilst the zona pellucida (ZP) is thinning.
Total number of embryos derived *in vivo* (n= 22) mothers.
Total number of embryos derived *in vitro* (n= 29) donors.

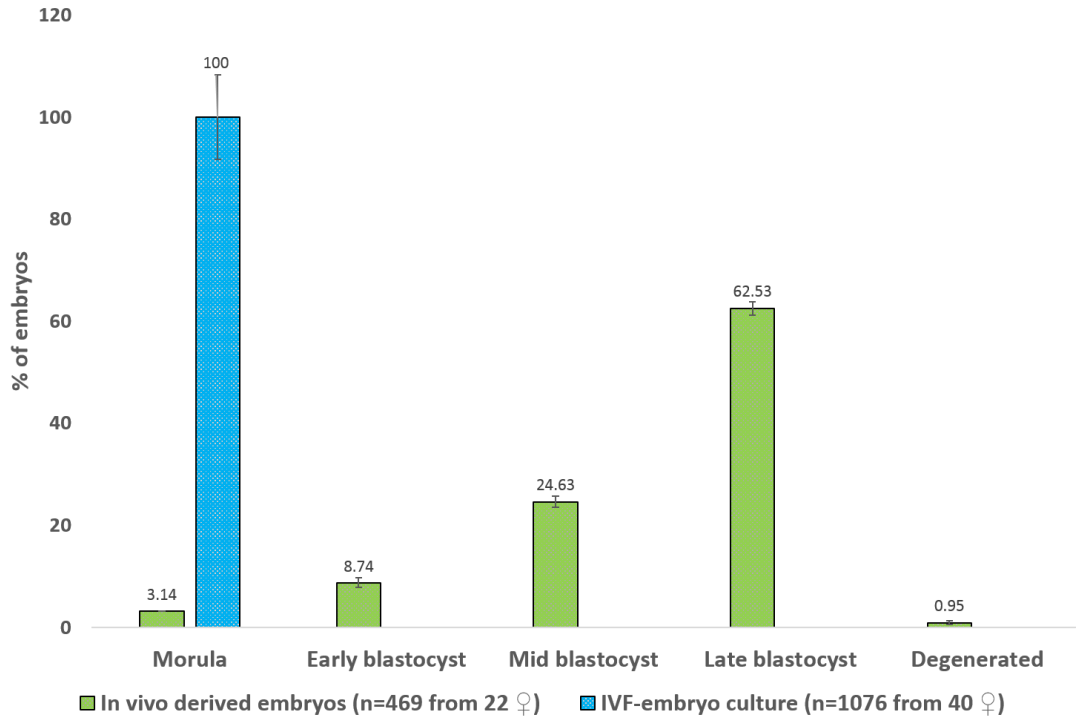


Figure 3.2 Effect of *in vitro* fertilisation and prolonged embryo culture on embryo developmental rate compared with *in vivo* embryos

Percentage (% ± SEM) developmental rate of IVF and *in vivo* embryos at E3.5 (96 hrs post hCG): IVF embryos develop slower than *in vivo* embryos.
Early blastocyst: has a blastocoel volume less than half of the total embryo volume, assessed by microscope observation.
Mid blastocyst: has a blastocoel volume equal to or larger than the total embryo volume.
Late blastocyst: a blastocoel fully expanded within the embryo whilst the zona pellucida (ZP) is thinning.

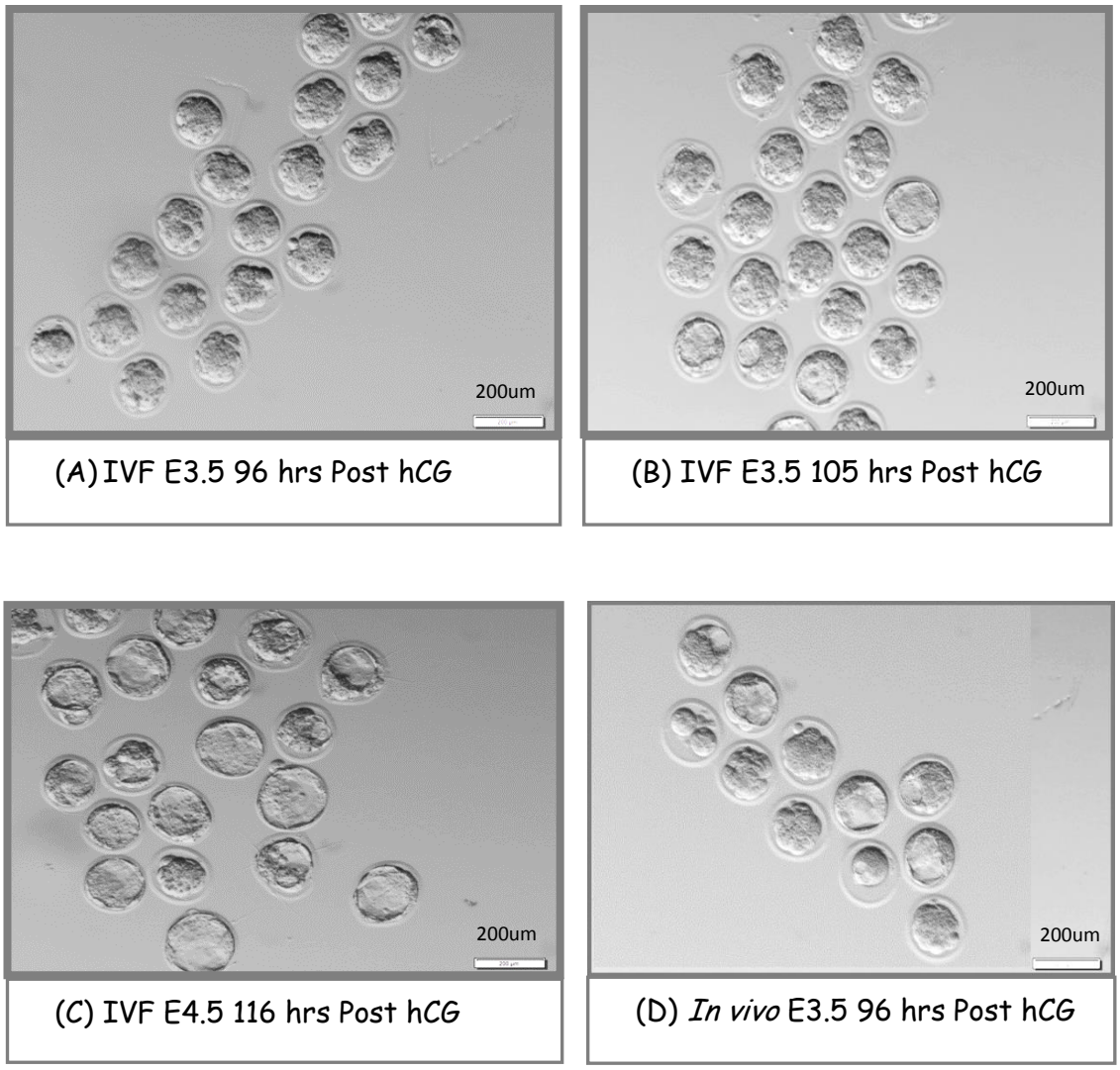


Figure 3.3 Images of IVF and *in vivo* embryos

A) IVF embryos at morula stage E3.5 (96 hrs post hCG)
B) IVF embryos at different stages E3.5 (105 hrs post hCG)
C) IVF blastocyst embryos at different stages E4.5 (116 hrs post hCG).
D) *in vivo* embryos at different stages (morula, early blastocyst, mid blastocyst and late blastocyst at E3.5 (96 hrs post hCG).

3.3.3 *Effect of in vitro fertilisation and prolonged embryo culture on blastocyst trophectodermTE, inner cell mass ICM and total cell number*

The effect of IVF and embryo culture on blastocyst cell lineage numbers is summarised in Figure 3.4 and Figure 3.5 including images of a representative *in vivo* blastocyst after differential nuclear labelling (See section 2.4). The TE, ICM and total cell numbers for *in vivo* (n=87) compared with *in vitro* embryos (n=50) at (E4.5) mid-expanded blastocyst stage was increased, although, the ICM:TE ratio did not differ between the two groups (Figure 3.4 B). The mean cell numbers in *in vivo* and IVF embryos, respectively were 13.82 ±0.62 vs. 9.16 ±0.33 within the ICM, 27.9 ±0.87 vs. 21.32 ±0.45 in the TE, whilst the total cell number was 41.77 ±0.99 vs. 30.54 ±0.62 which all showed significant differences ($P<0.05$).

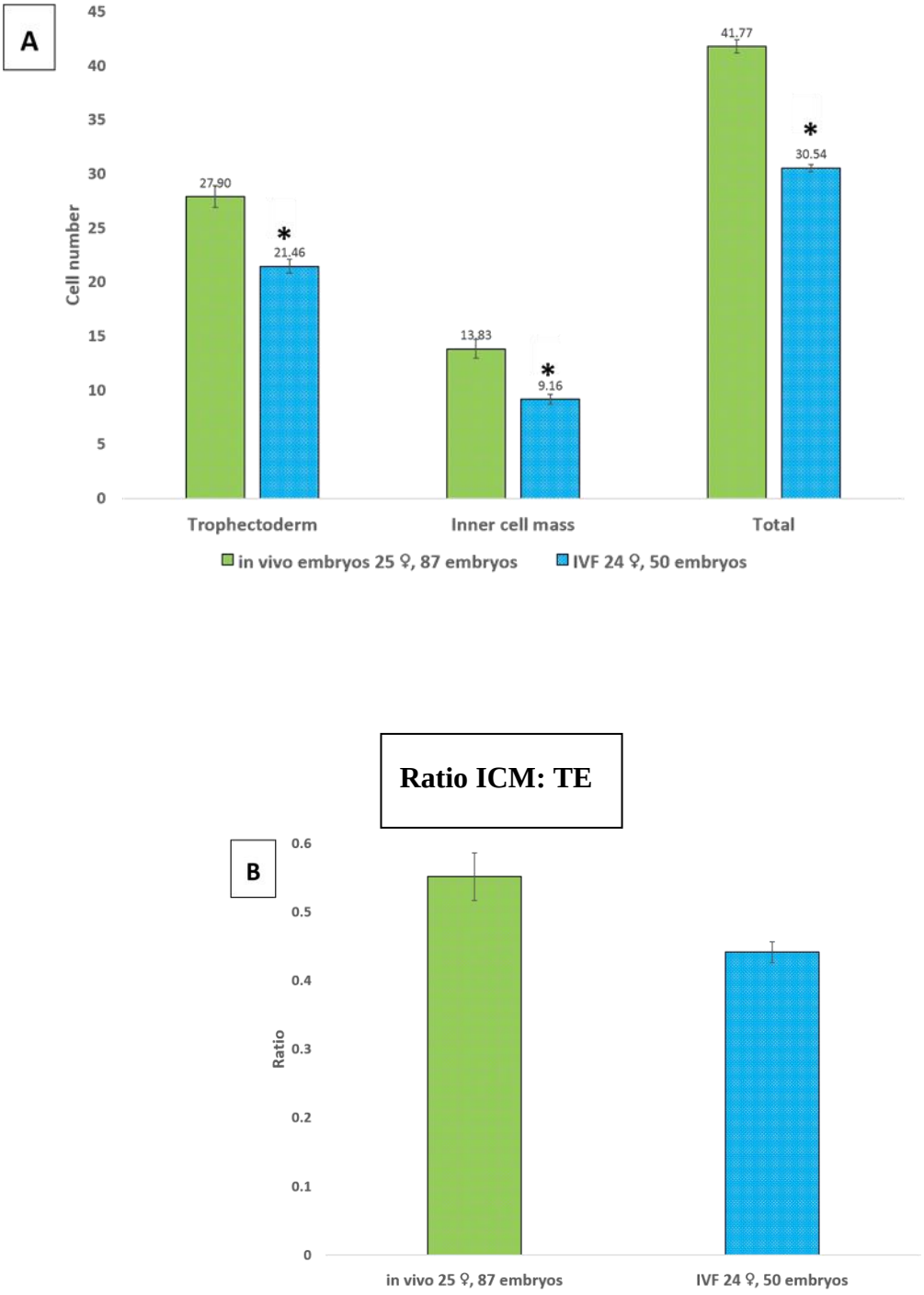
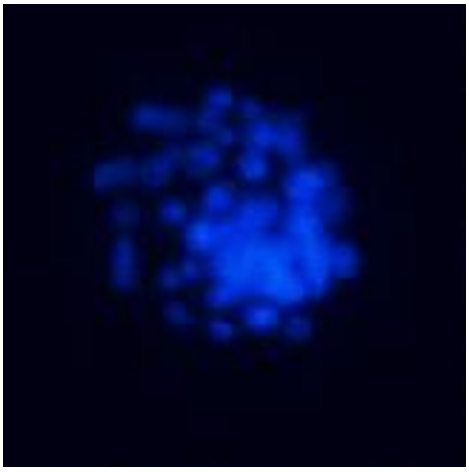
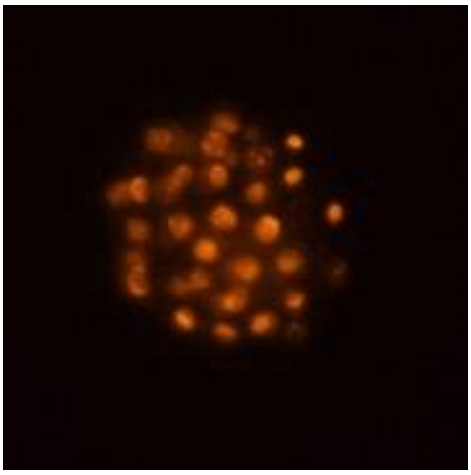


Figure 3.4 Effect of *in vitro* fertilisation and prolonged embryo culture on blastocyst cell number

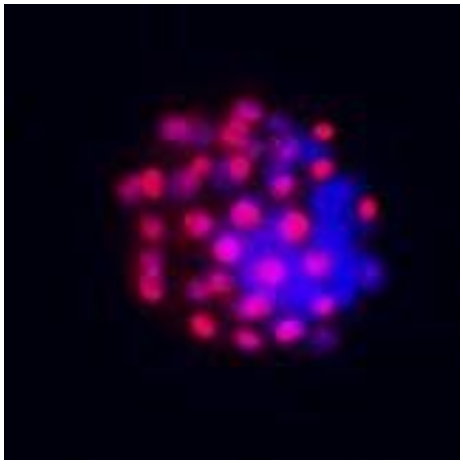
IVF embryos (n= 50) have fewer cells than *in vivo* embryos (n= 87) at blastocyst stage. (A) Mean (±SEM) blastocyst cell number for IVF compared *in vivo* embryos (P<0.05). (B) Mean (±SEM) ICM/TE ratio of blastocysts. IVF at (E4.5) and *in vivo* at (E3.5) embryos mid-expanded blastocyst stage.



(A) Blastocyst labelled with Hoechst



(B) Blastocyst labelled with Propidium Iodide



(C) Blastocyst labelled with Hoechst

Figure 3.5 Single mid-expanded blastocyst after differential nuclear labelling (section 2.4).

Images with filter settings for Hoechst (A), Propidium Iodide (B) with both channels combined in (C).

3.4 Discussion

In this study, the IVF procedure was performed with the addition of GSH to HTF medium, with the average fertilisation rate of all IVF experiment performed approximately 92%. This improvement in IVF reduced the number of donor mice used for egg collection, and the incidence of IVF procedures. It has been reported that adding 1.0 mM GSH to fertilisation medium (HTF) greatly enhanced the fertility of frozen/thawed C57/BL6 mouse sperm to approximately 90% when compared with HTF medium without GSH (approximately 62%) (Takeo and Nakagata 2011b). Fertilisation using *in vitro* matured (IVM) oocytes with fresh sperm was enhanced by the addition of GSH to the HTF medium, from 51 to 76% (Ishizuka et al. 2013).

There is evidence that addition of GSH to fertilisation media is not detrimental or may improve embryo development and potential. Thus 2-cell embryos that were generated *in vitro* in THF media with addition of 1.0 mM GSH developed normally in potassium simplex optimised medium (KSOM) into blastocysts (Takeo and Nakagata 2011b). The blastocyst developmental rates were similar to that of eggs fertilised without GSH; normal live pups were obtained after embryo transfer, and the birth rate was similar to that of the control group. In contrast, (Ishizuka et al. 2013) reported that the blastocyst rate significantly increased with the addition of 1.0 mM GSH compared with the control. However, the rate of offspring obtained after embryo transfer was similar to that of the control. The current study reports about 78% of 2-cell embryos generated by IVF with freshly harvested sperm in HTF medium with additional of 1.0 mM GSH normally developed to the blastocyst stage and, as reported in Chapter 4, live pups were obtained from these blastocysts after

embryo transfer (Chapter 4). These results are therefore in agreement with (Takeo and Nakagata 2011b) and (Ishizuka et al. 2013).

The effect of IVF and prolonged embryo culture on the embryo developmental rate compared with *in vivo* embryos are shown in Figure 3.2. Our findings show that at E3.5 (96 post hCG), 100% of IVF embryos were at the morula stage, whereas *in vivo* derived embryos showed different development stages morula, early blastocysts, middle blastocysts, late blastocysts and degenerated embryo (3.14%, 8.74%, 24.63%,62.52% and 0.95%; respectively). These results agree with a previous study which demonstrated that *in vitro* cultured embryos develop at slower rate when compared with *in vivo* derived embryos at the same time post hCG.

The differences in cell number between IVF and *in vivo* derived embryos are shown in Figure 3.4 A and B. Our results are in agreement with previous research, which reported that *in vitro* cultured embryos develop at slower rate and with a smaller total cell number compared to *in vivo* derived embryos at the same time post-hCG (Harlow and Quinn 1982); (Gardner and Sakkas 1993); (Watkins et al. 2007). *In vitro* cultured blastocysts have significantly less TE and ICM cells when compared to the *in vivo* derived embryos at 96 hours post-hCG. After a further 20 hours in culture (at 116 hours post post hCG), the *in vitro* embryos obtain comparable cell numbers to the *in vivo* derived embryos at 96 hours post-hCG. However, Figure 3.4 B, shows that the ICM: TE ratio did not differ between *in vitro* and *in vivo* treatment groups. Although the slower developmental rate of the *in vitro* cultured embryos, their ICM: TE ratio is unaltered. This would imply that whilst the rate of cell division might be slower, the allocation of TE and ICM cells and the processes employed to maintain the correct ICM: TE ratio is unaltered.

Embryos developing *in vitro* may have slower rates of cleavage when compared to *in vivo* derived embryos due to the culture medium providing less than optimal conditions for preimplantation embryos. In various species, it has been demonstrated that environmental factors, including growth factors, amino acids, glucose and many others, commonly affect embryo proliferation and blastocyst TE and ICM cell numbers (Fleming et al. 2004). Likewise, *in vivo* development rodent embryos in response to maternal age (Jurisicova et al. 1998); and periconceptional protein undernutrition (Kwong et al. 2000) are associated with the reduction of blastocyst cell numbers. Decreased TE and ICM cell numbers in rat blastocysts caused by a periconceptional low-protein diet is linked with postnatal abnormal growth and hypertension (Kwong et al. 2000). However, in the mouse model, Eckert et al (2012) investigated mechanisms of early induction of adverse long-term developmental programming mediated through an embryonic low protein diet Emb-LPD. They reported that (Emb-LPD) induced changes in maternal serum metabolites at the time of blastocyst formation E3.5, and notably reduced insulin and increased glucose levels, together with a reduction of free amino acids levels. These changes were accompanied by an increased proliferation of blastocyst trophectoderm and total cells, which subsequently increased the spreading of trophoblast cells in blastocyst outgrowths (Eckert et al. 2012).

It was demonstrated that prolonged culture of mouse embryos showed a reduction in cell number (Kiessling et al. 1991). In Kiessling study, embryos derived from superovulated female mice that developed *in vitro* from the 2-cell stage were compared with *in vivo* embryos with respect to yield of blastocysts, number and types of cells and DNA polymerase activities. They found that ICM cells comprised approximately 30% of total cells in late morula/early blastocyst stage embryos developed either *in vitro* or *in vivo*.

However, the *in vitro* embryos developed approximately half the number of total cells as *in vivo* embryos. DNA-dependent DNA polymerase activities in *in vitro* embryos reduced in correspondence with the reduction in cell number resulting in per cell levels comparable to *in vivo* embryos (Kiessling et al. 1991).

It was shown that bovine embryos with reduced cell number are able to give rise to fetuses (Iwasaki et al. 1990). However, it was reported that bovine embryos produced from 1/8 blastomeres often fail to develop an ICM (Loskutoff et al. 1993); these findings suggest that the ICM requires a certain cell mass to be able to develop further.

A study has shown that the manipulation of embryo cell number such as embryo splitting and biopsy leads to viable and healthy offspring (Illmensee et al. 2005). In Illmensee study, mouse embryos at the 2-cell, 4-cell, 6-cell and 8-cell stage were studied. Half the number of blastomeres were microsurgically biopsied and transferred into empty mouse zonae pellucidae and twin embryonic development was monitored during in-vitro culture. They found that the success rate for obtaining twin blastocysts from 2-cell and 4-cell split embryos was higher (about 75%) than twin blastocysts derived from 6-cell split embryos (about 65%). The success rate was less efficient for 8-cell embryo splitting (about 35%). However, non-operated control 2-cell embryos showed a blastocyst development rate of 91.4%. They reported that the morphological quality of twin blastocysts derived from 2-cell and 4-cell embryo splitting was clearly superior when compared to those obtained from 6-cell and, in particular, from 8-cell embryo splitting (Illmensee et al. 2005).

There is some evidence that blastocyst proliferative potential and relative lineage size affects future growth and viability. Richter et al (2001) reported the first evidence of a significant relationship between human blastocyst ICM size and shape before embryo

transfer and the implantation potential of human blastocysts. The study shows that quantitative measurements of the ICM are highly indicative of blastocyst implantation potential. Blastocysts with larger ICM size and/or slightly oval ICM shape are more likely to implant than other blastocysts (Richter et al. 2001).

Furthermore, studies have shown that *in vitro* culture medium are stimulated by amino acid availability. Optimal development *in vitro* and *in vivo* after embryo transfer obtains when embryos at cleavage stage were cultured with non-essential amino acids and glutamine for 48 h followed by development to the blastocyst stage with all 20 amino acids (Lane and Gardner 1997). Fetal development per blastocyst transferred was increased if the embryos were cultured with non-essential amino acids and glutamine for the first 48 hours of development (from E0 to E1.5) compared with either essential amino acids without glutamine or all 20 amino acids. However, culture with non-essential amino acids for the second 48 hours (from E1.5 to E3.5) did not affect fetal development. In contrast, culture with essential amino acids without glutamine or all 20 amino acids from the eight-cell stage to the blastocyst significantly increased both fetal development per blastocyst transferred and fetal mass (Lane and Gardner 1997).

In conclusion, since both IVF and *in vivo* groups were derived from superovulated mothers, the findings report within this chapter show that *in vitro* culture slows the developmental rate of preimplantation embryos when compared to *in vivo* derived embryos for the same developmental time post hCG. Despite this, slower rates lead to a significant reduction in TE and ICM and total cell numbers, although the ICM:TE ratio of the cells within these two linages is unaffected.

These findings have demonstrated that *in vitro* culture or manipulations to preimplantation mouse embryos can alter the TE and ICM and total cell numbers, whilst still allowing development to blastocyst stage. It would, therefore, be interesting to examine the effect of these manipulations upon subsequent fetal and postnatal development (Chapters 4, 5 and 6).

Chapter 4: Effect of *In Vitro* Fertilisation (IVF) and Embryo Culture Duration on Growth and Organ Allometry of the Offspring

4.1 Introduction

The IVF procedure used widely to treat human infertility, involves the culture of preimplantation embryos. It is common practice in human IVF to transfer embryos to the uterus on day 1.5 (around 2-cell stage), day 2 (around the 4-cell stage) or on day 3 (around the 8-cell stage) of development (Scholtes and Zeilmaker 1996); (Shoukir et al. 1997). However, it is crucial to note that *in vivo* embryos at such cleavage stages reside in the Fallopian tube and not in the uterus. The importance of this observation is that in other mammalian species, transferring embryos at cleavage stage to the uterus does not result in high pregnancy rates when compared with transferring embryos at post-compaction or at the blastocyst stage (Bavister 1995). It has been shown that the premature replacement of the human embryo to the uterus may lead to low implantation rates. Human implantation rates of 10 to 15% are routinely reported in the literature, with only ~10% of embryos transferred proceeding to term. The replacement of human embryos at the cavitating morula and/or blastocyst stage on E4 or E5 of development indicates that such embryos exhibit clear evidence of developmental progression and an increased implantation rate (Huisman et al. 1994); (Olivennes et al. 1994); (Menezo and Khalifa 1995); (Gardner and Lane 1997).

In the case of replacement of human blastocysts, (Scholtes and Zeilmaker 1996) reported that the implantation rate of blastocyst stage embryos was twice that of cleavage stage embryos. Such data are in agreement with (Buster et al. 1985) study who reported that human blastocyst developed *in vivo*, flushed from the uterus and transferred singly to recipient patients showed an implantation and pregnancy rate of 60% per blastocyst transferred. These findings therefore supported the hypothesis that the transfer of later stage embryos will increase the implantation and pregnancy rate per embryo transferred.

It has been reported that fertility clinics now tend to transfer blastocysts rather than earlier-cleavage stages due to potential advantages of blastocyst culture and transfer in human IVF. Firstly, synchronisation of the embryo with the mother's cycle, which leads to an increased implantation rate and therefore, reduces the need for multiple embryo transfer. Secondly, blastocyst transfer allows for assessment of embryos and selection of a high quality embryo for transfer (Menezo and Khalifa 1995); (Gardner et al. 2000); (Schultz and Williams 2002); (Lonergan et al. 2006). It is important to note that culture of human embryos after the 8-cell stage, which is the time of genome activation (Braude et al. 1988), will facilitate the quantification of true embryonic markers as opposed to those before the 8-cell stage, which are inherited from the oocyte. Indeed, embryos after the 8-cell stage are important for determining embryo physiology, whereas before this stage, the embryo reflects that of the oocyte (Gardner and Lane 1997). Lastly, blastocyst transfer facilitates the introduction of TE lineage biopsy for the screening of genetic diseases (Gardner and

Lane 1997). The TE biopsy indicates the earliest form of genetic diagnosis of non-embryonic material.

In contrast, other research reports that an earlier, cleavage-stage transfer of embryos may be preferable because of the inability to maintain the mammalian embryo in culture for more than a couple of days without compromising its viability. It is crucial to note that there is a difference between the ability of a certain culture medium to support blastocyst development *in vitro* and the ability of this culture medium to give rise to a viable embryo. It has been shown that although blastocyst formation rates of 47.4% were achieved using a more complex tissue culture medium, α-minimal essential medium (MEM), the resultant pregnancy rate from E5 transfer was 28.2%, lower than those obtained for E2 (33.3%) and E3 (37.5%) transfers in the same programme (Noda et al. 1994).

Moreover, it has been demonstrated that not all of pronuclear stage embryos conceived by IVF will be able to reach the blastocyst stage because a significant number of embryos will be abnormal and the majority of these abnormalities are chromosomal. Approximately 25% of oocytes are aneuploidy (Van Blerkom 1989), and this factor is exacerbated with maternal age (Janny and Menezo 1996).

Despite that, good rates of blastocyst development are achievable in culture. The viability of such blastocysts seems to be <60% implantation and the live birth rate reported by (Buster et al. 1985) for blastocysts developed *in vivo*. It has also been shown that different culture media, which supports equivalent rates of blastocyst

development can lead to very different pregnancy rates (Gardner and Sakkas 1993); (Gardner and Lane 1997).

On the other hand, studies have been reported that *in vitro* culture and embryo transfer associates with numerous effects on preimplantation, fetal and postnatal development. Thus, human studies have demonstrated that children obtained by IVF have an increased risk of adverse perinatal outcome, with the chance of low birth weight and perinatal mortality greater than in the general population (Olivennes et al. 2002a); (Schieve et al. 2002); (Ludwig et al. 2006) ; (Dumoulin et al. 2010). Recent research shows that there are increases in the occurrence of elevated blood pressure, high fasting glucose level, increase total body fat composition, elevation of bone age and potentially subclinical thyroid disorder in IVF children (Ludwig et al. 2006); (Ceelen et al. 2008b); (Hart and Norman 2013b); (Hart and Norman 2013a); (Kleijkers et al. 2014); (Fauser et al. 2014).

The hypothesis of this chapter is that the environment, in particular the length of embryo culture; short (2-cell stage) vs. long (blastocyst) in which the preimplantation embryo develops can not only slow down blastocyst development, but may increase postnatal growth and alterd organs allometry in the offspring.

The aim of the studies reported in chapters 4, 5 and 6 was to determine the effect of IVF and embryo culture upon several aspects of postnatal development, in particular for the first time to compare between the culture of IVF embryos for either a short (to 2-cell) or long (to blastocyst) period to reflect the differing strategies used in human IVF as discussed above. Five treatment groups were generated (see Table 4.1

below); and termed as (a) normally mated non-superovulated (undisturbed) (NM), (b) – embryos derived *in vivo* at 2-cell and immediately transferred to recipient oviduct (IV-ET-2Cell), (c) embryos generated by IVFand cultured to 2-cell stage before transfer to the oviduct (IVF-ET-2Cell), (d) - embryos derived *in vivo* at blastocyst and immediately transferred to a recipient uterus (IV-ET-BL), (e) embryos generated by IVF and cultured to blastocyst before transfer to the uterus (IVF-ET-BL). All four IV-ET and IVF-ET treatment groups were derived from superovulated mothers. In all, these five treatment groups allowed the comparison of postnatal effects depending on whether the embryo was derived from IVF or not, whether cultured for a short or long period before transfer, and additionally identifying effects that might be caused merely by embryo transfer itself. Thus, IV-ET-2Cell and IV-ET-BL act as direct controls for the IVF and culture treatments in IVF-ET-2Cell and IVF-ET-BL, respectively; similarly the NM group provides a control for all reproductive treatments (superovulation, IVF, culture and transfer).

Offspring from the five treatment groups underwent cardiovascular and metabolic profile assessments, including weekly measurements of offspring weight from week (1 - 27), systolic blood pressure at week 9, 15 and 21 and a glucose tolerance test on week 27. Offspring were sacrificed at age of 27 weeks for blood and organ collection (Chapter 2). Mean body weight, systolic blood pressure at the three time points, glucose tolerances test and organs weight between treatment groups for male and female offspring were analysed using a multilevel random effects regression model, which takes into account litter size and the maternal origin of the pregnancy.

Table 4.1 The five treatment groups of offspring examined in this project

Embryonic treatments, purpose of treatments, superovulation (SO), Embryo Transfer (ET) and litter size regulated at birth

Group name	**Embryo treatment**	**Purpose**	**Embryos/eggs derived from superovulated mothers (SO)**	**Embryo transfer (ET)**	**Litter size regulated at birth**
Normally mated (Undisturbed offspring) NM	None	Control for superovulation, culture and embryo transfer	No	No	No*
IV-ET-2Cell	*In vivo*-derived embryos collected and immediately transferred at 2-cell stage	Control for IVF and short culture	Yes	Yes	Yes
IVF-ET-2Cell	IVF derived embryos cultured to 2-cell stage before transfer	Effect of IVF and short culture	Yes	Yes	Yes
IV-ET-BL	*In vivo*-derived embryos collected and immediately transferred at blastocyst stage	Control for IVF and long culture	Yes	Yes	No*
IVF-ET-BL	IVF derived embryos cultured to blastocyst stage before transfer	Effect of IVF and long culture	Yes	Yes	No*

*** The reason for a no litter size control was because always less than 8 offspring.**

4.2 Methodology

4.2.1 *Production of naturally mating offspring*

10 female C57/BL6 mice at an age of 6-8 weeks were naturally mated with CBA males and allowed to develop to term with their pregnancies (See section 2.1).

4.2.2 *Production of in vivo derived embryos*

3-4 week old C57/BL6 female mice were superovulated and naturally mated with CBA male mice. Embryos were flushed from the oviduct at 2-cell stage and from the uteri at blastocyst stage (See section 2.2.2). Both were placed in pre-warmed H6-BSA under mineral oil for approximately 1-2 hours after flushing to buffer against pH changes until they were transferred to oviduct (2-cell) or uterus (blastocyst) of their respective foster mother.

4.2.3 *Production of IVF embryos*

Embryos were generated by IVF procedure as described in (See section 2.3.32.2.2) and cultured in KSOM for either ~24 h up to the 2-cell stage or for ~60 hours to the blastocyst stage. Both sets were then placed in pre-warmed H6-BSA under mineral oil for approximately 1-2 hours until transferred to the oviduct or uterus respectively of the foster mother.

4.2.4 *Embryo transfer*

The ovaries and the oviducts of anaesthetised 1.5 days pseudopregnant female MF1 mice were carefully exposed from the body cavity and ten (2-cell) embryos were transferred to each oviduct (See section 2.3.2-2.3.3). Litter size was regulated to eight offspring at birth. For the blastocyst embryo transfer, the ovaries and the uterus of anaesthetised 2.5 days pseudopregnant female MF1 mice were carefully exposed from the body cavity. Seven blastocysts were then transferred to each uterine horn (See section 2.3.3). The litter size in these two groups was less than eight offspring so litter size correction was not applied.

4.2.5 *Measurement of postnatal growth rates*

All offspring from the five treatment groups were weighed on day seven of birth (at age of week 1) and then subsequently on the same day for the next 27 weeks. Offspring were weaned at age of three weeks and then ear punched to mark each individual mouse (See section 2.5).

4.2.6 *Measurement of organ weight and serum collection*

Mice were weighed and sacrificed at 27 weeks. Blood was collected and centrifuged at 10,000g at 4°C for 10 minutes and then stored at -80°C. Organs (heart, lung, liver, kidneys and spleen) were dissected, weighed and snap frozen in liquid nitrogen and then stored at -80°C. Parts of the liver and lung were stored in (200 µl) RNA Later for further molecular analysis.

4.2.7 *Statistical analysis of data*

Pregnancy rate, embryo transfer efficiency and litter size from the different treatment groups were analysed using IBM SPSS Statistic 22.

The animal weight, organ weight and the organs weight ratios, were analysed using a multilevel random effect regression model, which took into account between-mother and within-mother variation in litter size and parameters measured from individual animals (See section 2.6). Thus, differences identified between treatment groups throughout the study are independent of the maternal origin of litter, gestational litter size. Statistical significance was $p \leq 0.05$. If a p value was between 0.1 and 0.051, it was considered as a trend.

Z-score analysis

The Z-score is a test of statistical significance that allows for the mean-normalisation of results within studies and for subsequent comparison of related data across studies. Z-scores are standardised scores (by the group mean and group standard deviation) and no assumption about normality is made. Z-score shows how far a particular score is away from the mean; it indicates how many standard deviations (σ) an observation (X) is above or below the mean of a control group (μ).

$$\mathbf{Z} = (X - \mu)/\sigma$$

X represents the individual data for the observed parameter, μ and σ represents the mean and the standard deviation for the control group, respectively.

Fractional weight change

Fractional weight change is a calculation that indicates changes in body weight expressed in percentage (%) as following:

$$\frac{\text{Weight at week (X) - initial weight (W1)}}{\text{Initial weight (W1)}} \text{ X } 100$$

When X = (time in weeks).

Results:

4.2.8 *Mean number of offspring*

A total of eight to thirteen litters were generated from each treatment. The pregnancy rate after embryo transfer (ET), litter size and number of offspring for the five treatment groups are shown in Table 4.2

The ET pregnancy rate was significantly higher in the IV-ET-BL group (81.82%) compared with IVF-ET-BL (48.27%). However, there was no significant difference in pregnancy rate between the IV-ET-2Cell and IVF-ET-2Cell groups.

ET efficiency was significantly lower in the IVF-ET-BL group compared with the IV-ET-BL group. Similarly, ET efficiency was significantly higher in the IVF-ET-2Cell group compared with the IVF-ET-BL group. Litter size in the ET groups IV-ET-2Cell, IV-ET-BL and IVF-ET-BL were significantly different from the normal mating (NM) group. The IVF-ET-BL mean litter size was significantly lower than the IVF-ET-2Cell group, comprising 8.22 and 3.23 respectively. However, the male: female ratio was not significantly different between any of the treatment groups.

Table 4.2 Comparison between the treatment groups for ET pregnancy rate, ET efficiency, litter size at birth and number of offspring

Group	ET pregnancy rate[1] % (dam numbers)	ET efficiency[2] % (pups/embryos transferred)	Litter size at birth[3] (Total litter number]	Offspring number	No of male pups	No of female pups	Ratio Male: Female
Normally mated (Undisturbed)	N/A	N/A	8 ± 0.42 [10] [a]	80	40	40	1
IV-ET-2Cell	88.88 (8/9)	31.66 (57/180) 20*9 = 189	7.12 ± 1.54 [8] [b]	57	32	25	1.28
IVF-ET-2Cell	73.33(11/15)	16.66 (75/450)[a] 30*15 = 450	8.22 ± 1.25 [9] [a1]	75	42	33	1.24
IV-ET-BL	81.82 (9/11)[a]	30.52(47/154)[a1] 14*11 = 154	5.87 ± 0.61 [8] [b]	47	22	25	0.88
IVF-ET-BL	48.27(14/29)[b]	9.05 (42/464)[b,b1] 16*29 = 464	3.23 ± 0.49 [13][b ,b1]	42	26	16	1.62

Data were analysed using a ANOVA (mean± SEM)

1 = dams that gave birth/total number of ETs performed.

2= total number of pups at birth (before litter size correction)/total embryos transferred (10 each side for IV-ET-2Cell, 7 each horn for IV-ET-BL whereas, 15 each side for IVF-ET-2Cell ET, 8-9 each horn for IVF-ET-BL)

3= calculated on dams with live pups at birth (before litter size correction); [a,b , a1,b1] Within a column, values with different letters are significantly different (P<0.05).

4.2.9 *Offspring body weight*

The mean weekly weights for all five groups from week 1 to 27 are shown in Figures 4.1, 4.2, 4.3 and 4.4 (mean ± standard error). In males, Figure 4.1 shows the IVF-ET and IV-ET groups have a significant effect on offspring body weight compared with the NM control at weeks 1 and 3. No significant difference was observed at week 2. The IV-ET-BL group was significantly heavier compared with the IV-ET-2Cell group at weeks 1 and 3. The IVF-ET groups, IVF-ET-2Cell and IVF-ET-BL, were significantly different at week 1 compared with their IV-ET groups, IV-ET-2Cell and IV-ET-BL. Moreover, the IVF-ET-BL group was significantly heavier (P=0.05) compared with the IVF-ET-2Cell group at week 2.

Figure 4.3 shows the IVF-ET and IV-ET groups for male offspring were significantly heavier compared with the NM control group from week 5 until week 27. The IV-ET-BL group was significantly heavier compared with IV-ET-2Cell group at weeks 3 to 10. The IVF-ET-BL and IV-ET-BL groups were only significantly different at week 7 and 9. However, the IVF-ET-BL was significantly heavier compared to IVF-ET-2Cell at week 4. From week 8 to 16 both IVF-ET groups were similar then at week 17, the IVF-BL shows a decrease in body weight compared to the IVF-ET-2Cell although, this did not reach statistical significance (P≤0.05).

In females, Figure 4.2 shows the IVF-ET and IV-ET groups have a significant effect on offspring body weight compared with the NM control from week 1 to 3. The IV-ET-BL group was significantly heavier compared with IV-ET-2-Cell group at weeks 1. The IVF-ET-BL and IV-ET-BL groups were only significantly different at week 1.

Meanwhile, the IVF-ET-2Cell and IV-ET-2-Cell groups were only significantly different at week 1. At week 3, IVF-BL offspring were significantly heavier than IVF-2Cell offspring.

Figure 4.4 shows the mean weekly weight for female offspring from 3 to 27 weeks of age, which exhibited a similar pattern to the males, the IVF-ET and IV-ET groups were significantly heavier compared with the NM control from week 4 until the end of week 27. However, the IV-ET-BL group was significantly heavier compared with IV-ET-2-Cell group at weeks 14 and 23 whereas the IVF-ET-BL was significantly heavier compared with IVF-ET-2Cell at weeks 3 and 4. This difference remains to the end of week 27 but did not reach statistical significance ($P \leq 0.05$).

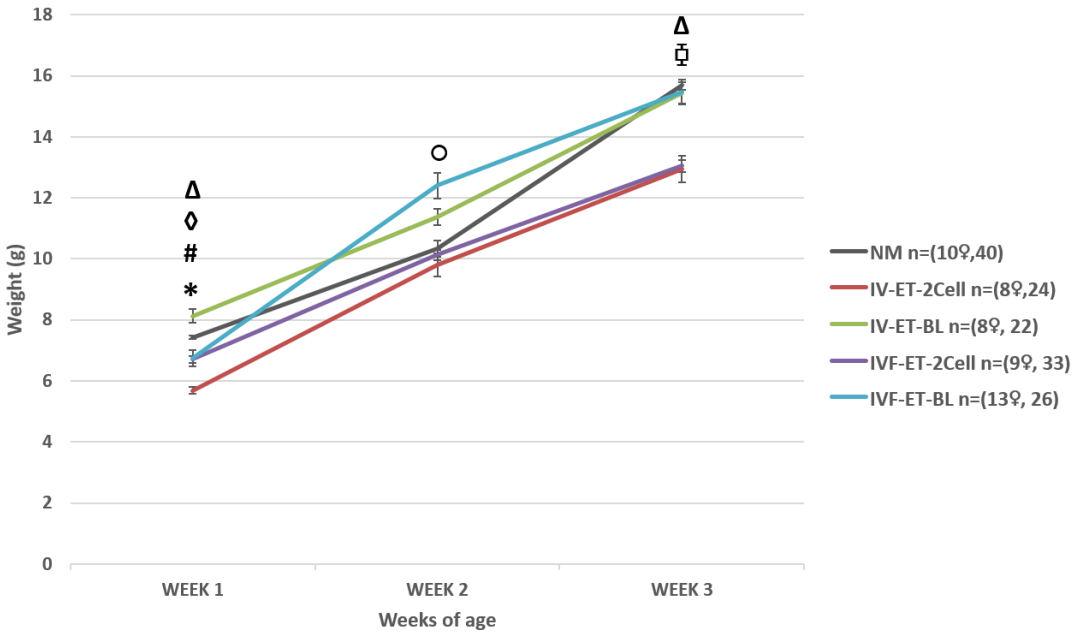


Figure 4.1 Effect of IV and IVF treatment groups and NM control group on male offspring weight from postnatal week 1-3.

* denote (P≤0.05) between NM and selected groups, ⍈ denote NM vs. (IV-ET-2Cell, IVF-ET-2Cell and IVF-ET-BL) at week 3, Δ IV-ET-2-Cell vs. IV-ET-BL, # IV-ET-2Cell vs. IVF-ET-2Cell, ◊ denote IV-ET-BL vs. IVF-ET-BL, and ○ denote IVF-ET-BL vs. IVF-ET-2Cell (P=0.05). Mean (±SEM) body weight from 1 to 3 weeks (from 8 – 10 litters); ((n) of mothers or foster mothers ♀, (n) of offspring).

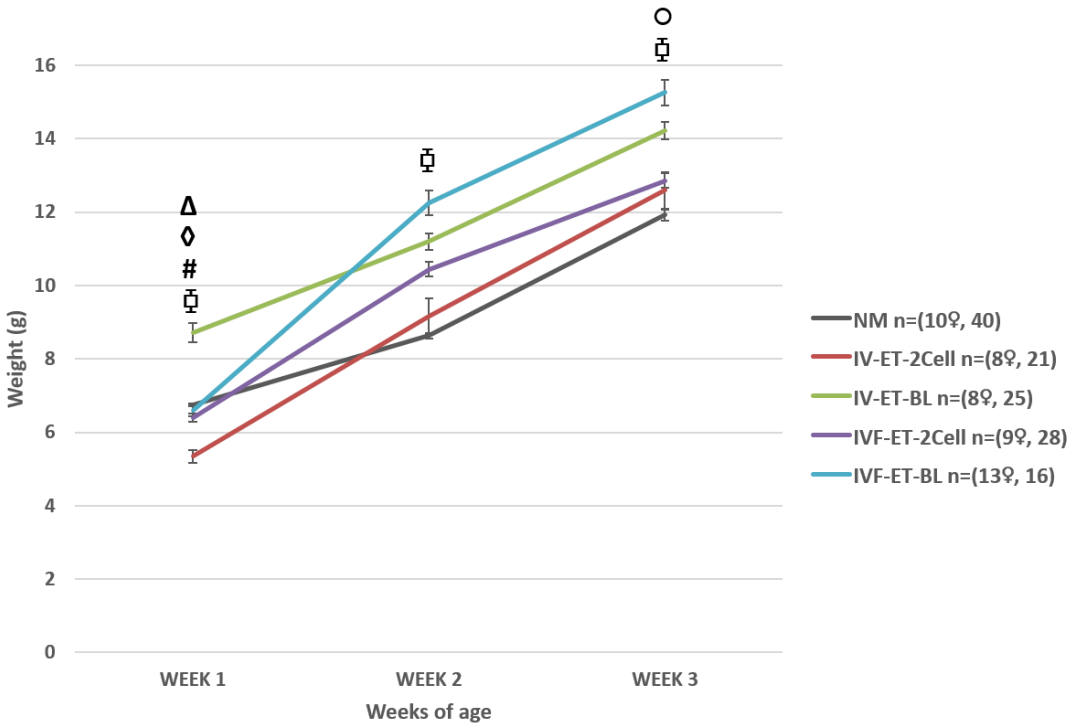


Figure 4.2 Effect of IV and IVF treatment groups and NM control group on female offspring weight from postnatal week 1-3.

* denote (P≤0.05) between NM and selected groups, ⌻ denote NM vs. IV-ET-BL at week 1, denote NM vs. (IV-ET-BL, IVF-ET-2Cell and IVF-ET-BL) at week 2, and denote NM vs. (IV-ET-BL and IVF-ET-BL) at week 3, Δ IV-ET-2-Cell vs. IV-ET-BL, # IV-ET-2Cell vs. IVF-ET-2Cell, ◊ denote IV-ET-BL vs. IVF-ET-BL and, ○ denote IVF-ET-BL vs. IVF-ET-2Cell (P=0.05). Mean (±SEM) body weight from 1 to 3 weeks (from 8 – 10 litters); ((n) of mothers or foster mothers ♀, (n) of offspring).

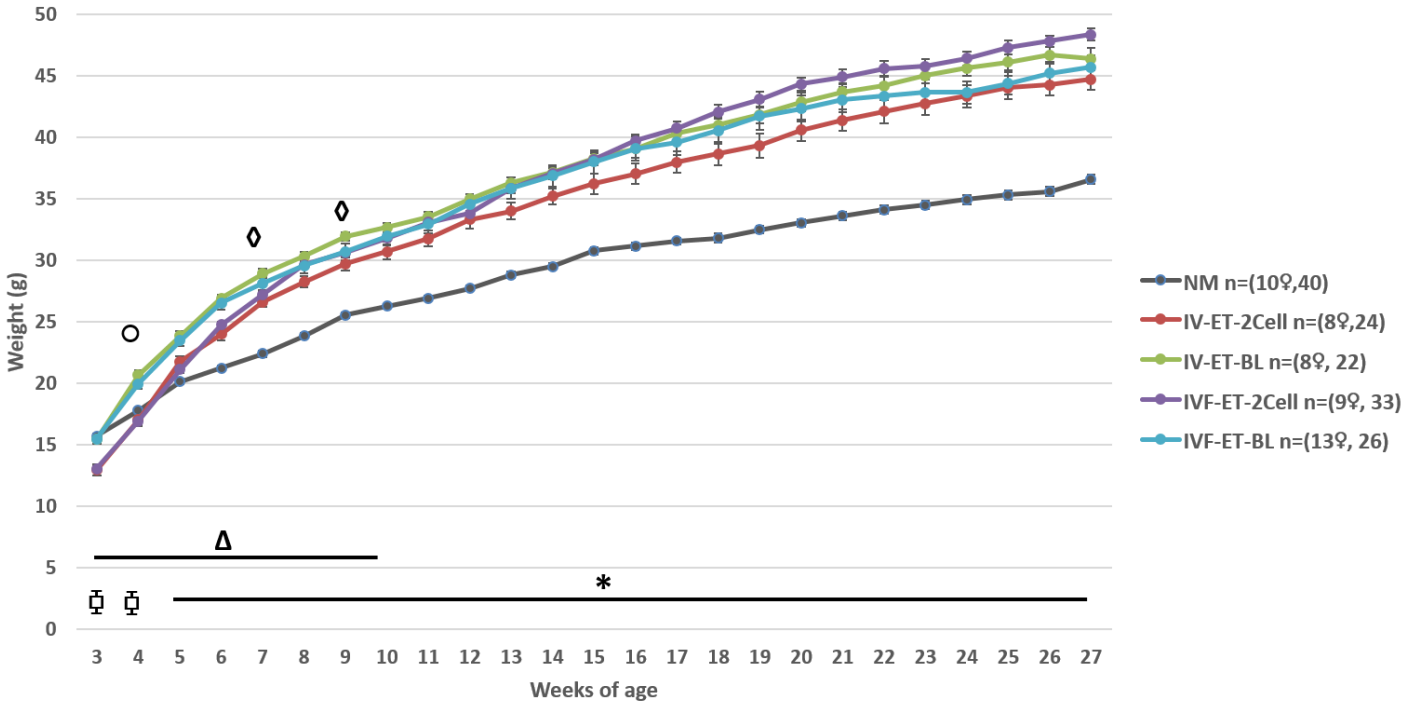


Figure 4.3 Effect of IV and IVF treatment groups and NM control group on male offspring weight from postnatal week 3-27.

* denote (P≤0.05) between NM and selected groups, ⌻ denote NM vs. (IV-ET-2Cell, IVF-ET-2Cell and IVF-ET-BL) at week 3 and denote NM vs. IV-ET-BL at week 4 (P≤0.05), Δ IV-ET-2-Cell vs. IV-ET-BL, # IV-ET-2Cell vs. IVF-ET-2Cell, ◊ denote IV-ET-BL vs. IVF-ET-BL, and ○ denote IVF-ET-BL vs. IVF-ET-2Cell (P=0.05). Mean (±SEM) body weight from 3 to 27 weeks (from 8 – 10 litters); ((n) of mothers or foster mothers ♀, (n) of offspring).

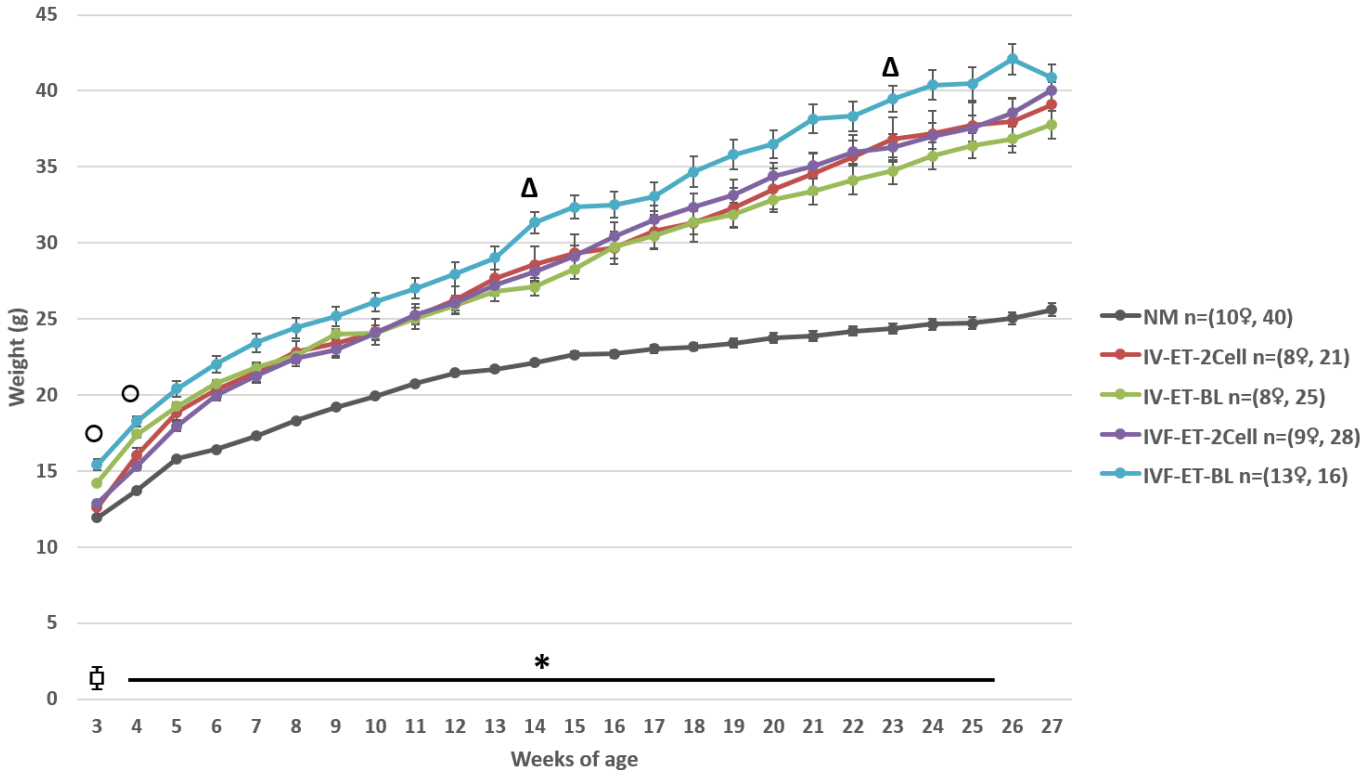


Figure 4.4 Effect of IV and IVF treatment groups and NM control group on female offspring weight from postnatal week 3-27.

* denote (P≤0.05) between NM and selected groups, ⏀ denote NM vs. (IV-ET-BL) and denote (NM vs. IVF-ET-BL) (P≤0.05), Δ IV-ET-2-Cell vs. IV-ET-BL, # IV-ET-2Cell vs. IVF-ET-2Cell, ◊ denote IV-ET-BL vs. IVF-ET-BL, and ○ denote IVF-ET-BL vs. IVF-ET-2Cell (P=0.05). Mean (±SEM) body weight from 3 to 27 weeks (from 8 – 10 litters); ((n) of mothers or foster mothers ♀, (n) of offspring).

The Z-score is a useful statistic that represents whether a particular score is equal to the mean, below the mean or above the mean for a set of scores. It can also indicate how far a particular score is away from the mean. The mean (±SEM) Z-score (Weight) from week 1 to 27 for males and females from all treatment groups are shown in Figures 4.5 and 4.6. In males, Figure 4.5 shows significant differences between NM and all treatment groups from week 1 to 27. There is a significant difference between IV-ET groups IV-ET-2Cell vs. IV-ET-BL from week 3 to 10. At week 1, there were significant differences between IVF-ET-2Cell and IV-ET-2Cell as well between IVF-ET-BL and IV-ET-BL.

Figure 4.6 shows significant differences between NM and all treatment groups from week 1 to 27 in females. There is a significant difference between IV-ET groups IV-ET-2Cell vs. IV-ET-BL at weeks 1, 14 and 23. At week 1, there were significant differences between IVF-ET-2Cell and IV-ET-2Cell and between IVF-ET-BL and IV-ET-BL. At weeks 3 and 4, there is a significant difference between IVF groups (IVF-ET-BL vs. IVF-ET-2Cell).

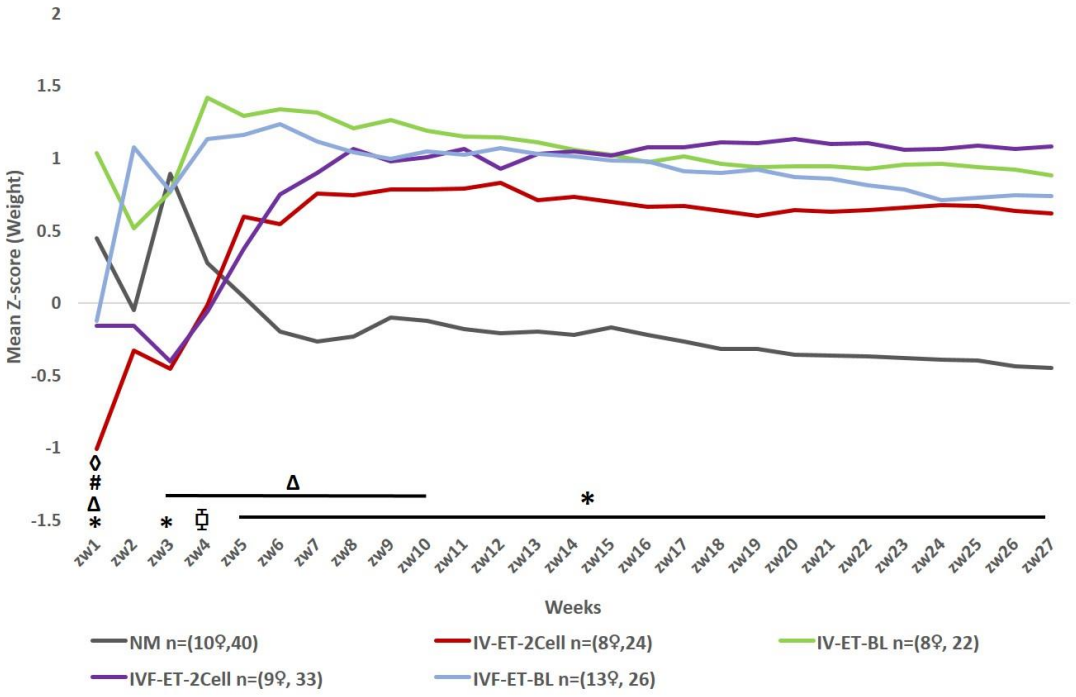


Figure 4.5 Graph of Z- score analysis for male offspring weight for the 5 groups during weeks 1 to 27

IVF and *in vivo* transfer groups in male offspring have a significant effect on postnatal growth compared to control groups. * denote (P≤0.05) between NM and selected groups, ♁ denote NM vs. IV-ET-BL, Δ IV-ET-2-Cell vs. IV-ET-BL, # IV-ET-2Cell vs. IVF-ET-2Cell, ◊ denote IV-ET-BL vs. IVF-ET-BL, and ○ denote IVF-ET-BL vs. IVF-ET-2Cell (P=0.05). Mean (±SEM) Z-score (Weight) from 1 to 27 weeks (from 8 – 10 litters); ((n) of mothers or foster mothers ♀, (n) of offspring).

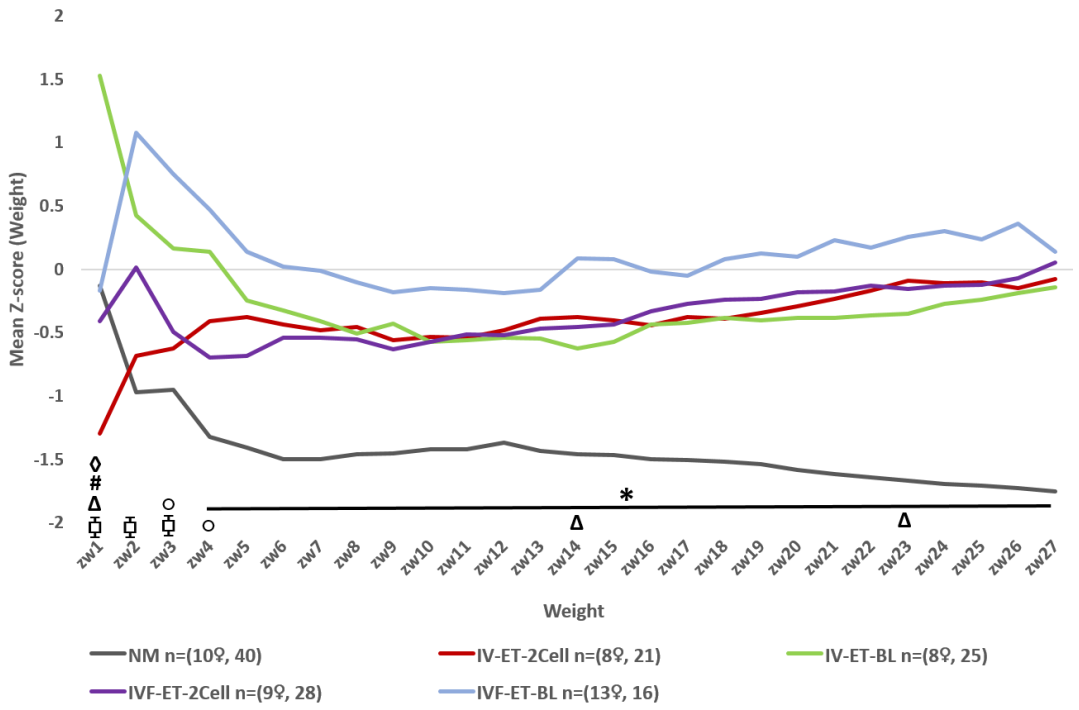


Figure 4.6 Graph of Z- score analysis for female offspring weight for the 5 groups during weeks 1 to 27.

IVF and *in vivo* transfer groups in female offspring have significant effect on postnatal growth compared to control groups.* denote (P≤0.05) between NM and selected groups, ⍈ denote NM vs. (IV-ET-2-Cell and IV-ET-BL) at week 1, NM vs (IV-ET-BL, IVF-ET-2Cell and IVF-ET-BL) at week 2 and NM vs (IV-ET-BL and IVF-ET-BL) at weeks 3, Δ IV-ET-2-Cell vs. IV-ET-BL, # IV-ET-2Cell vs. IVF-ET-2Cell, ◊ denote IV-ET-BL vs. IVF-ET-BL, and ○ denote IVF-ET-BL vs. IVF-ET-2Cell (P=0.05). Mean (±SEM) Z-score (Weight) from 1 to 27 weeks (from 8 – 10 litters); ((n) of mothers or foster mothers ♀, (n) of offspring).

The fractional weight change is a useful statistical calculation that shows changes in offspring body weight expressed in percentage (%). Offspring weight changes from week 1 to 3 and from week 1 to 27 for male and female offspring from all treatment groups are shown in Figures 4.7 and 4.8. In males, Figure 4.7 (A + B) shows significant differences between NM and all treatment groups from week 6 to 27. The mean (±SEM) body weight of NM, IV-ET-2Cell, IV-ET-BL, IVF-ET-2Cell and IVF-ET-BL male offspring at week 1 were (7.43 ± 0.05), (5.69 ± 0.12), (8.13 ± 0.22), (6.70 ± 0.11) and (6.75 ± 0.26) grams, respectively. The graphs show that offspring from all treatment groups gained weight from week 2 to 27. However, IV-ET-2Cell and IVF-ET-BL offspring gained weight at greater rate than NM, IV-ET-BL and IVF-ET-2Cell offspring from week 2 to week 5. Moreover, from week 1 to 27, there were significant differences between *in vivo* groups (IV-ET-2Cell vs. IV-ET-BL); and between IVF-ET-2Cell and IV-ET-2Cell and between IVF-ET-BL and IV-ET-BL. It is shown that IV-ET-2Cell offspring gained weight greater than all treatment groups, whereas, IV-ET-BL offspring group was the closest to the NM offspring. At weeks 2, 3, 5 and 6 there is a significant difference between IVF groups (IVF-ET-BL vs. IVF-ET-2Cell). However, no significant difference was noted between IVF long and short culture from week 7 to 27, offspring from IVF groups (IVF-ET-2Cell and IVF-ET-BL) gained weight greater than NM and IV-ET-BL but less than IV-ET-2Cell. Mean weight change (%) from 1 to 27 weeks (from 8 – 10 litters).

Similar to the male pattern, Figure 4.8 (A + B) shows significant differences between NM and all treatment groups for female offspring from week 24 to 27. However, there were differences between NM and all treatment groups except IV-ET-BL from

week 6 to 23. The mean (±SEM) body weight of NM, IV-ET-2Cell, IV-ET-BL, IVF-ET-2Cell and IVF-ET-BL female offspring at week 1 were (6.74 ± 0.04), (5.34 ± 0.17), (8.71 ± 0.26), (6.40 ± 0.11) and (6.59 ± 0.16) grams, respectively. The graphs show that offspring from all treatment groups gained weight from week 2 to 27 but IV-ET-2Cell and IVF-ET-BL offspring gained weight at a greater rate than NM, IV-ET-BL and IVF-ET-2Cell offspring from week 2 to week 5. From week 1 to 27, there were significant differences between *in vivo* groups (IV-ET-2Cell vs. IV-ET-BL); IVF-ET-2Cell and IV-ET-2Cell; and between IVF-ET-BL and IV-ET-BL. The graphs show that IV-ET-2Cell offspring gained weight at a greater rate than all treatment groups, whereas, no significant difference was detected between NM and IV-ET-BL from week 2 to 23. At weeks 2, 3 and 4 there is a significant difference between IVF groups (IVF-ET-BL vs. IVF-ET-2Cell. However, no difference was noted between IVF long and short culture from week 5 to 27. Mean weight change (%) from 1 to 27 weeks (from 8 – 10 litters).

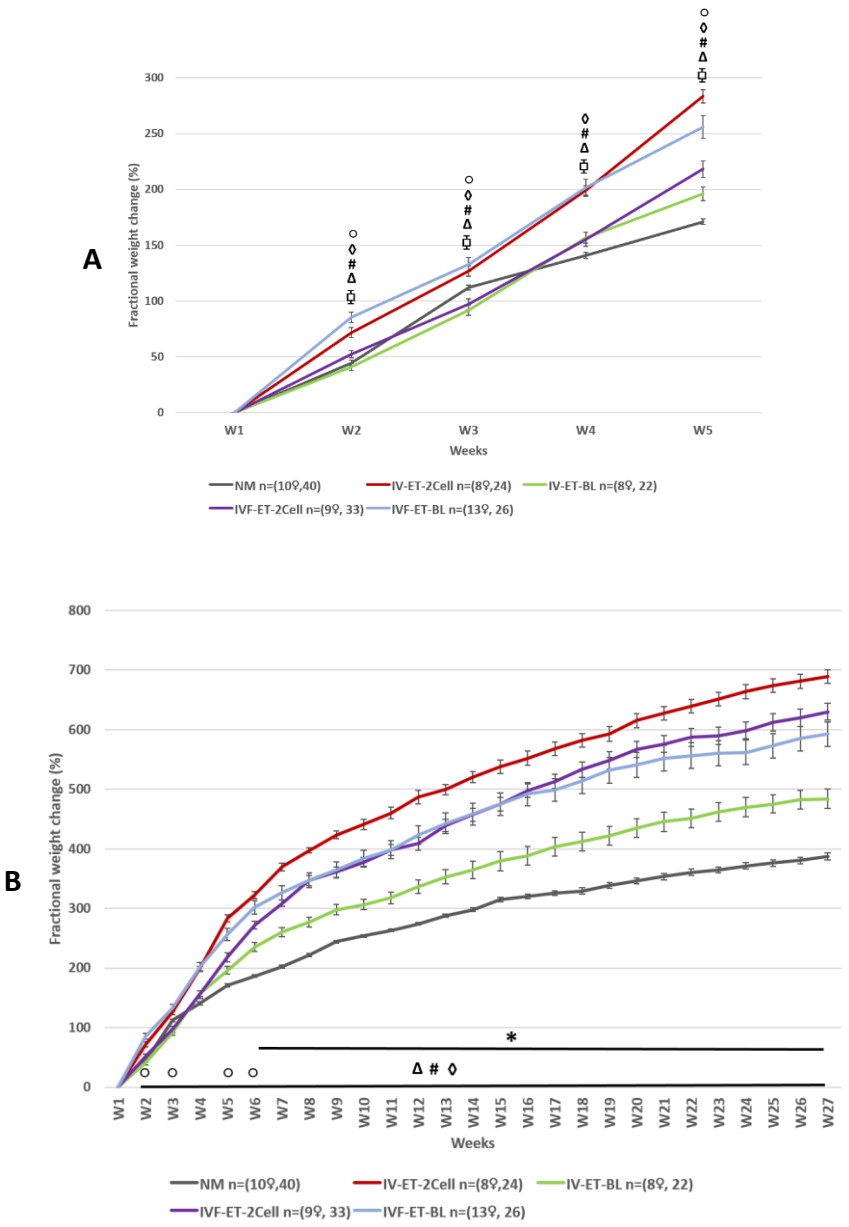


Figure 4.7 Fractional weight change for male offspring

Male offspring weight changes from week 1 to 3 (A) and from week 1 to 27 (B). Values are expressed as a percent of weight (%).* denote (P≤0.05) between NM and selected groups, ⍈ denote NM vs. IV-ET-2Cell and IVF-ET-BL from week 2 to 5, Δ IV-ET-2-Cell vs. IV-ET-BL, # IV-ET-2Cell vs. IVF-ET-2Cell, ◊ denote IV-ET-BL vs. IVF-ET-BL, and ○ denote IVF-ET-BL vs. IVF-ET-2Cell (P=0.05).Mean weight change (%) from 1 to 27 weeks (from 8 – 10 litters); ((n) of mothers or foster mothers ♀, (n) of offspring).

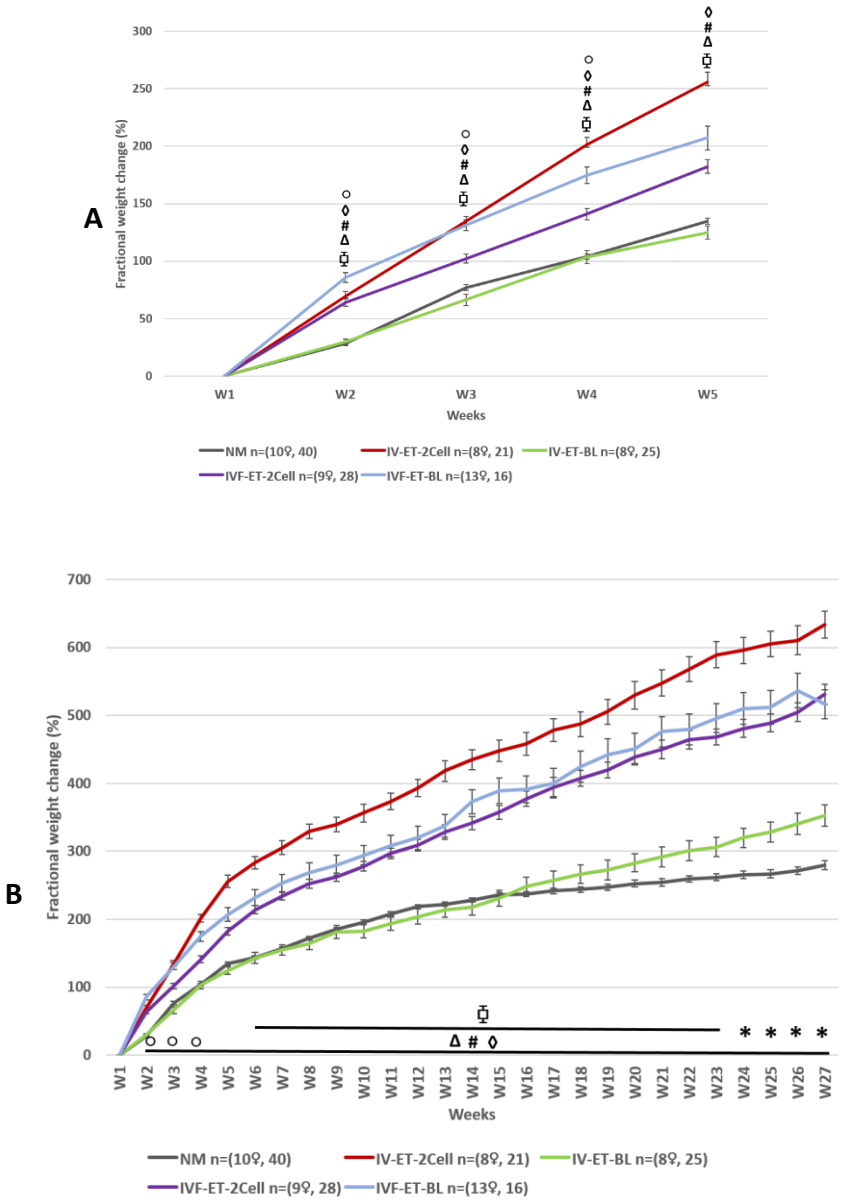


Figure 4.8 Fractional weight change for female offspring

Female offspring weight changes from week 1 to 3 (A) and from week 1 to 27 (B). Values are expressed as a percent of weight (%).* denote (P≤0.05) between NM and selected groups, ⊡ denote NM vs and all treatment groups except IV-ET-BL from weeks 2-27, Δ denote IV-ET-2-Cell vs. IV-ET-BL, # IV-ET-2Cell vs. IVF-ET-2Cell, ◊ denote IV-ET-BL vs. IVF-ET-BL, and ○ denote IVF-ET-BL vs. IVF-ET-2Cell (P=0.05). Mean weight change (%) from 1 to 27 weeks (from 8 – 10 litters); ((n) of mothers or foster mothers ♀, (n) of offspring).

4.2.10 *Offspring organ body weights and organ: body weight ratios*

Mean organ weights and mean organ: body weight ratios of offspring from the five treatment groups at week 27 are shown in Figures 4.9 to 4.12. In males, the mean weight of the lung, heart, right and left kidneys, spleen and liver in the NM control group were significantly smaller when compared to all four treatment groups (Figure 4.9). The right kidney and liver in offspring from IV-ET-2Cell were significantly smaller compared to offspring from IV-ET-BL and IVF-ET-2Cell groups (right kidney P= 0.03, 0.023; respectively), (liver P= 0.008, 0.000; respectively). However, the liver in IVF-ET-BL group was significantly smaller compared to IVF-ET-2Cell (P= 0.004).

In contrast, Figure 4.11 shows the lung: body weight ratio and heart: body weight ratio for the NM group was significantly higher than IVF-ET-2Cell. Meanwhile, the heart: body weight ratio for the NM group was significantly smaller but the right-kidney: body weight ratio was significantly higher when compared with the IV-ET-2-Cell group. The spleen: body weight ratio for the NM group was significantly smaller than IVF-ET-BL. Liver: body weight ratio for IV-ET-BL group was significantly higher than the IV-ET-2Cell group. Likewise, for the IV-ET-2Cell group, the lung: body weight ratio and the heart: body weight ratio were significantly higher but the liver: body weight ratio was significantly smaller when compared with IVF-ET-2Cell. The heart: body weight ratio for the IVF-ET-BL group was significantly higher but the liver: body weight ratio was significantly smaller than IVF-ET-2Cell group.

Figure 4.10 and 4.12 show mean organ weights and mean organ: body weight ratios for female offspring. In females, the mean weight of the lung, heart, right and left

kidneys, spleen and liver in the NM control was significantly smaller when compared with all four-treatment groups. However, the lung in the IV-ET-2Cell group was significantly higher when compared to IV-ET-BL group and IVF-ET-2Cell.

In contrast, Figure 4.12 shows the lung: body weight and heart: body weight ratios for control group were significantly higher than IV-ET and IVF-ET groups. Meanwhile, right and left: body weight ratios for the NM group were significantly higher than IV-ET-2Cell group (P= 0.028, 0.021; respectively). Spleen: body weight ratio for the NM group was also significantly higher than for either the IV-ET-2Cell or IVF-ET-2Cell group. Lung: body weight ratio for the IV-ET-2Cell group was significantly higher than IV-ET-BL and IVF-ET-2Cell groups (P= 0.015, 0.002; respectively). No significant differences were observed between groups for liver: body ratio in female offspring.

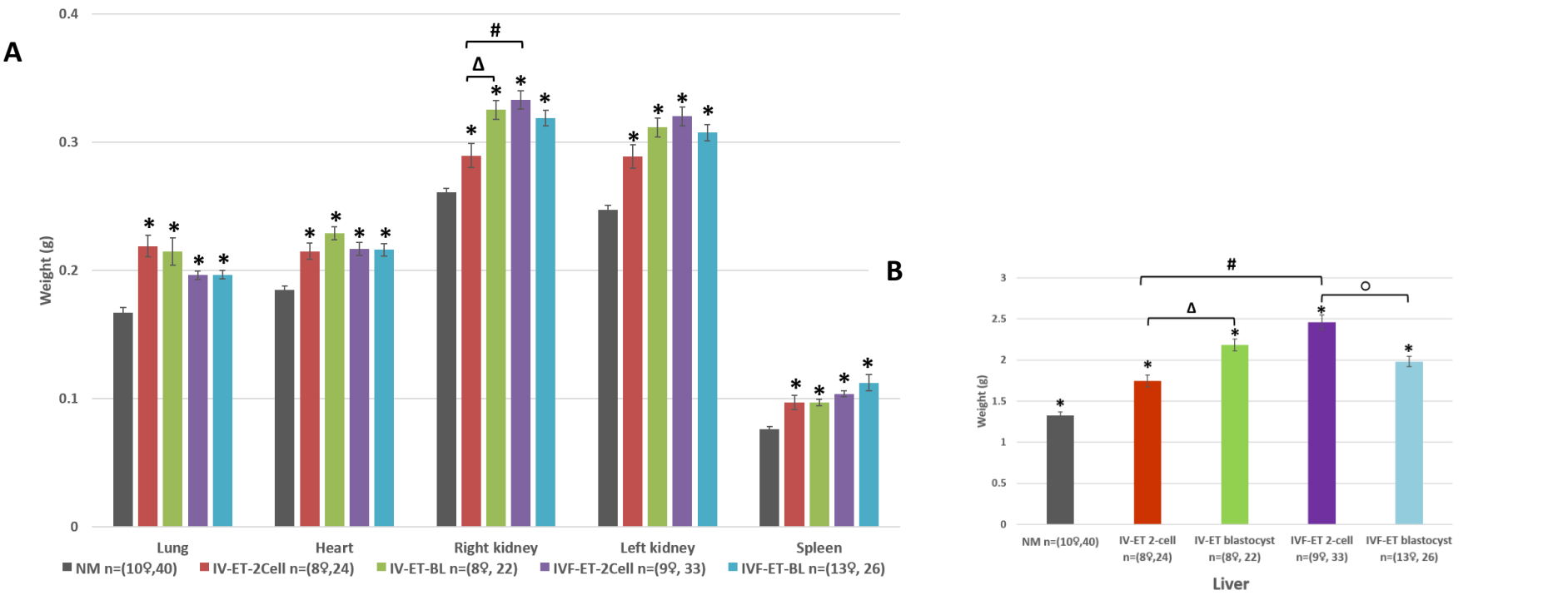


Figure 4.9 Effect of IV and IVF treatment groups and NM control group on offspring organ mean weight (A) lung, heart, kidneys, spleen and (B) liver in male offspring at 27 weeks

IVF and *in vivo* transfer groups in male offspring have significant effect on organ weight compared to control groups. * denote (P≤0.05) between NM and selected treatment group, Δ IV-ET-2-Cell vs. IV-ET-BL, # IV-ET-2Cell vs. IVF-ET-2Cell, ◊ denote IV-ET-BL vs. IVF-ET-BL, and ○ denote IVF-ET-BL vs. IVF-ET-2Cell (P=0.05). Mean (±SEM) for organ weight (from 8 – 10 litters); ((n) of mothers or foster mothers ♀, (n) of offspring).

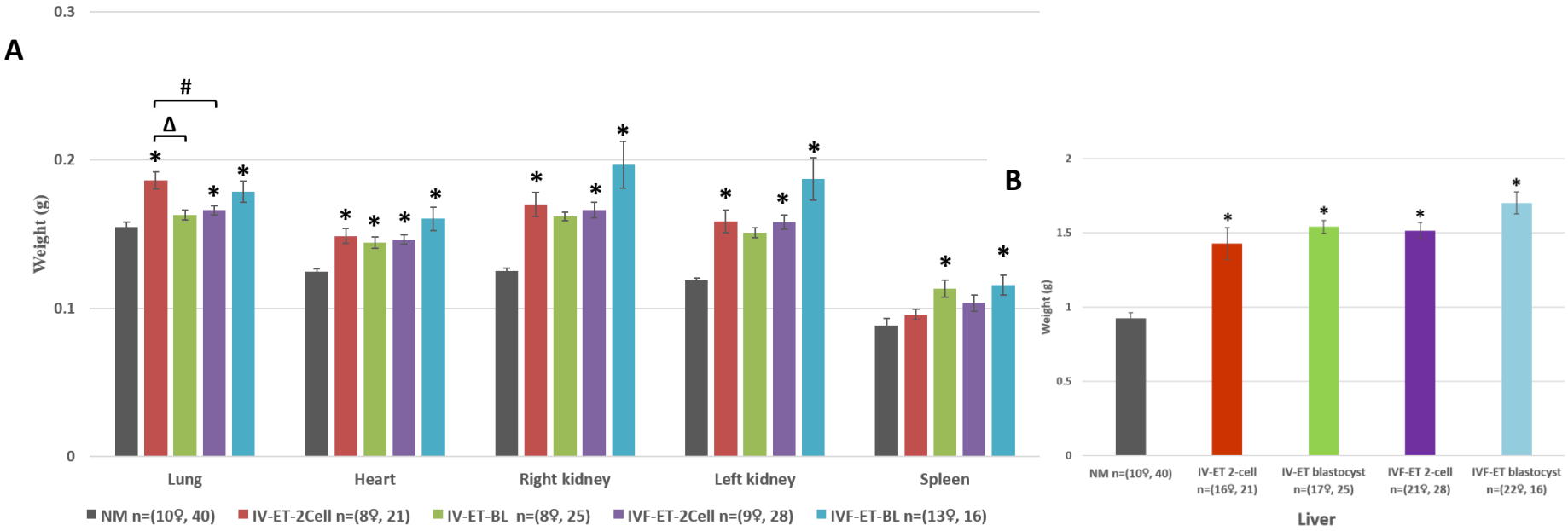


Figure 4.10 Effect of IV and IVF treatment groups and NM control group on offspring organ mean weight (A) lung, heart, kidneys, spleen and (B) liver in female offspring at 27 weeks.

IVF and *in vivo* transfer groups in female offspring have significant effect on organ weight compared to control groups. * denote (P≤0.05) between NM and selected treatment group, Δ IV-ET-2-Cell vs. IV-ET-BL, # IV-ET-2Cell vs. IVF-ET-2Cell, ◊ denote IV-ET-BL vs. IVF-ET-BL, and ○ denote IVF-ET-BL vs. IVF-ET-2Cell (P=0.05). Mean (±SEM) for organ weight (from 8 – 10 litters); ((n) of mothers or foster mothers ♀, (n) of offspring).

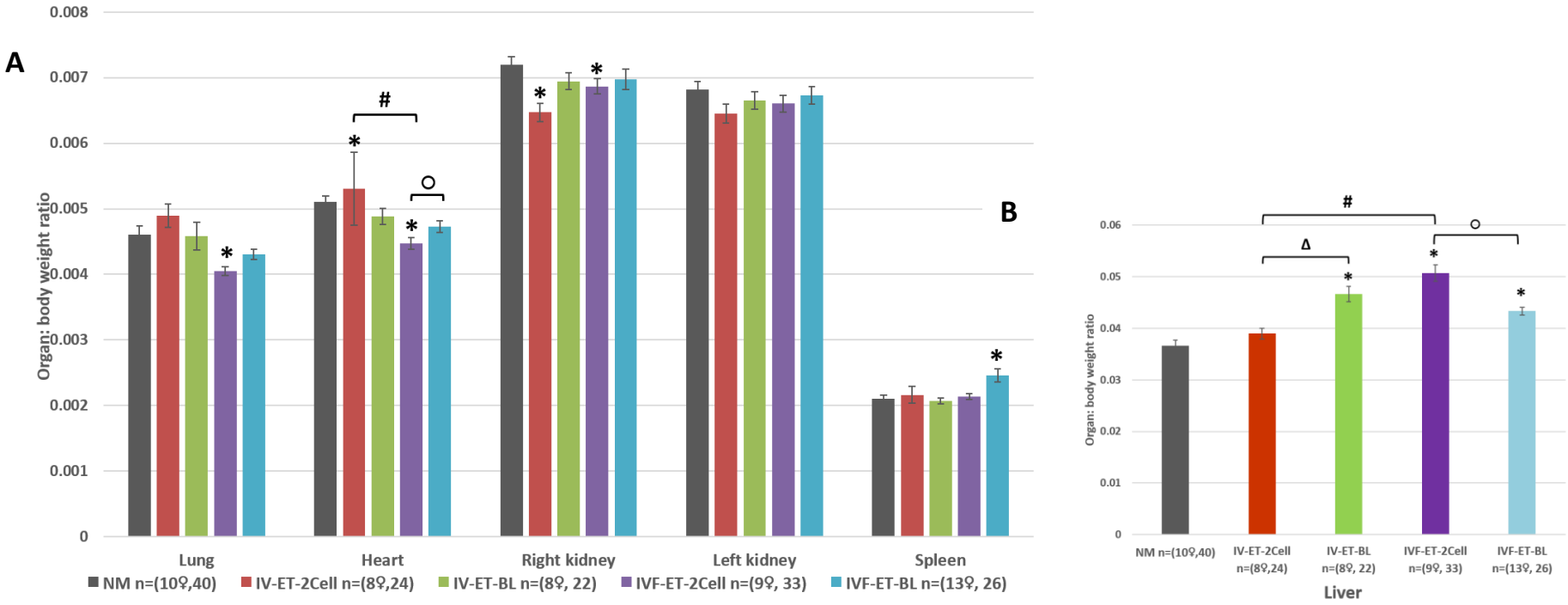


Figure 4.11 Effect of IV and IVF treatment groups and NM control group on offspring organ: body weight ratio (A); lung, heart, kidneys, spleen and (B) liver in male offspring at 27 weeks.

IVF and *in vivo* transfer groups in male offspring have significant effect on organ: body weight ratio compared to control groups. * denote (P≤0.05) between NM and selected treatment group, Δ IV-ET-2-Cell vs. IV-ET-BL, # IV-ET-2Cell vs. IVF-ET-2Cell, ◊ denote IV-ET-BL vs. IVF-ET-BL, and ○ denote IVF-ET-BL vs. IVF-ET-2Cell (P=0.05). Mean (±SEM) for organ weight (from 8 – 10 litters); ((n) of mothers or foster mothers ♀, (n) of offspring).

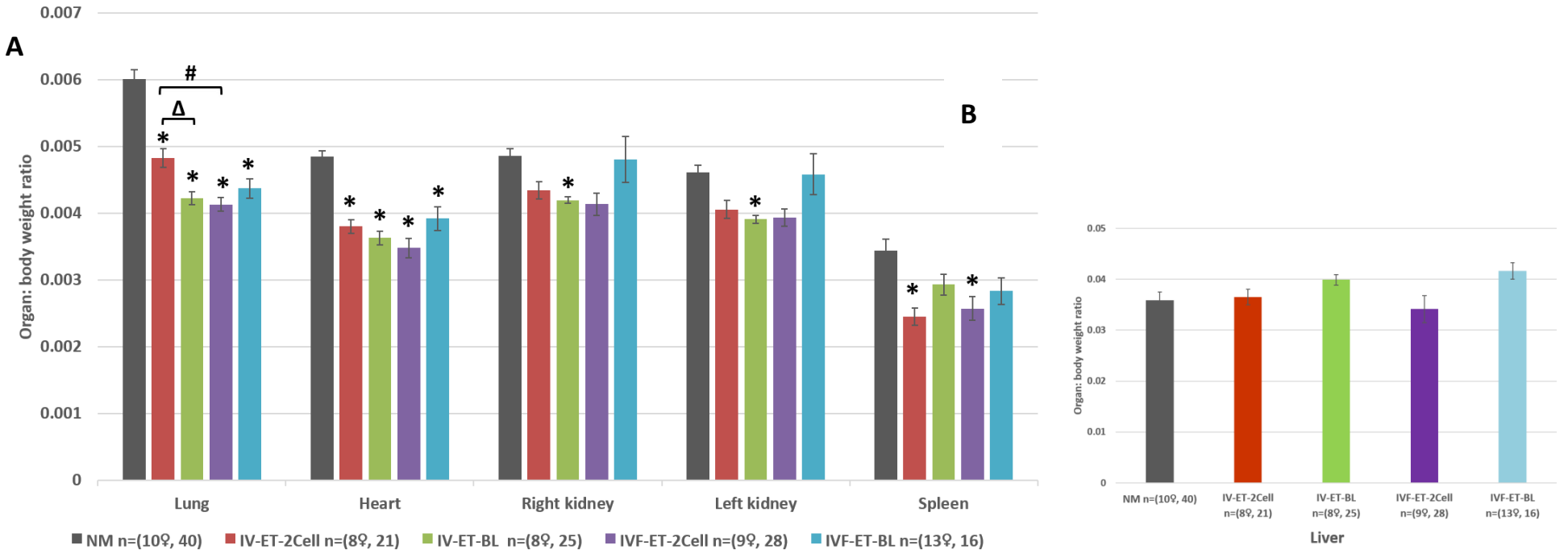


Figure 4.12 Effect of IV and IVF treatment groups and NM control group on offspring organ: body weight ratio (A); lung, heart, kidneys, spleen and (B) liver in female offspring at 27 weeks

IVF and *in vivo* transfer groups in female offspring have significant effect on organ: body weight ratio compared to control groups. * denote (P≤0.05) between NM and selected treatment group, Δ IV-ET-2-Cell vs. IV-ET-BL, # IV-ET-2Cell vs. IVF-ET-2Cell, ◊ denote IV-ET-BL vs. IVF-ET-BL, and ○ denote IVF-ET-BL vs. IVF-ET-2Cell (P=0.05). Mean (±SEM) for organ weight (from 8 – 10 litters); ((n) of mothers or foster mothers ♀, (n) of offspring).

4.3 Discussion

This study is the first report that specifically examines the long-term effect of the IVF procedure with either a short (2-cell) or prolonged (blastocyst) embryo culture before ET. In addition, the design also incorporates several important control treatment groups and evaluates offspring growth, cardiovascular and metabolic physiology using a mouse model.

Previous human (Denomme and Mann 2012); (Batcheller et al. 2011b) and mouse (Rivera et al. 2008b) studies have shown that assisted reproductive technologies affect preimplantation embryo development and postnatal development. Superovulation or embryo culture or just embryo transfer induces loss of genomic imprinting during development. Embryo culture can lead to abnormal postnatal behaviour in mice (Ecker et al. 2004). Embryo culture or even just embryo transfer in mice can lead to elevated blood pressure and dysfunctional cardiometabolic health in offspring (Watkins et al. 2007).

In the experiment reported within this chapter, it was observed that the four experimental treatments resulted in significantly reduced litter size when compared to naturally mated NM controls. It was also observed that the IVF-ET-BL group had significantly reduced litter size compared to the IVF-ET-2Cell group, which may be due to the total number of embryos transferred at 2-cell stage (15 embryos to each side) compared to total number of embryos transferred at blastocyst stage (8-9 embryos to each horn). The data in Table 4.2 also shows that the IVF-ET-BL group

had significantly lower ET pregnancy and ET efficiency when compared to IV-ET-BL. This may indicate that the IVF procedure and prolonged culture would reduce the implantation success rate for pregnancy in recipient mothers. Interestingly, the low ET efficiency was also observed in IVF-ET-BL group when compared to the IVF-ET-2Cell group which may indicate the negative effect of prolonged culture as the key factor in reducing litter size.

Body weight

In this study, male and female offspring in the IV-ET and IVF-ET groups showed differences compared to NM controls from week 4 through to week 27 of age. It can be seen that the mice from the largest litter (NM) have the lowest mean weight, whilst those mice from the smallest litter (IV-ET and IVF-ET) have the largest mean weight. However, the significant difference between the NM control and IV-ET and IVF-ET groups in terms of offspring weight after weaning was not due to differences in gestational litter size since our analysis was independent of litter size and maternal origin. This was also observed by (Watkins et al. 2007) and (Scott et al. 2010) in similar mouse studies, where a negative correlation was detected between litter size and body weight during specific periods of early postnatal growth and development. (Watkins et al. 2007) suggested that reduced body weight during early postnatal growth can be attributed to restricted growth in larger litters due to over-crowding within the uterus, which prevents the fetus reaching its maximum size. There would also be competition between them for maternal nutrients and blood supply. Reduced body weight in IV-ET and IVF-ET males during early postnatal period may indicate low birth weight, which is not measured in this experiment (Olivennes

et al. 2002a); (Schieve et al. 2002); (Dumoulin et al. 2010). In addition, the use of superovulation to stimulate oocyte maturation in all ET groups but not the NM control group may also act as a negative factor alongside embryo culture and transfer on embryo development, which all could result in a delayed implantation (Watkins et al. 2007); (Santos et al. 2010). Nevertheless, our results may in part offer an interpretation for delay in human embryonic development after IVF, and for low birth weight after ART as mentioned above (Olivennes et al. 2002a); (Schieve et al. 2002); (Watkins et al. 2007); (Santos et al. 2010).

Collectively, our finding has four possible causes: (1) the specific effect of superovulation; (2) the effect of combined ART treatments performed (superovulation; IVF procedure; embryo flushing, culture and transfer); (3) the exposure to an *in vitro* environment, regardless of the duration (60-120 minutes for the two IV-ET groups or for up to 24 and 69 hours post-hCG for IVF-ET-2Cell and IVF-ET-BL groups, respectively; (4) the different maternal environment experienced by post-implantation development as a potential contributory factor – MF1 for the 4 ET groups and C57BL6 for the NM group.

In male offspring, the mean weight from week 3 to 10 was significantly higher in the IV-ET-BL compared with IV-ET-2Cell group. The significant difference was also observed in female offspring only at week 14 and 23. IV-ET-2Cell offspring tended to have a larger litter size than IV-ET-BL offspring. As addressed above, restricted growth in larger litters caused by overcrowding within the uterus, preventing the fetus from growing to the maximal size and high competition for maternal nutrients and blood supply (Foxcroft et al. 2009), our finding suggests that the litter size effect

may remain up to late postnatal growth period. However, this does not affect our statistical findings because our statistical analysis was independent of gestational litter size.

Interestingly, male and female offspring were significantly heavier in the IVF-ET-BL group compared to the IVF-ET-2Cell group. These significant differences were observed in males at weeks 4, and female at weeks 3and 4; respectively. This increase in body weight remains up to week 27 in females but did not reach statistical difference ($P \leq 0.05$). As explained earlier, there is a significant difference in litter size between ET at 2-cell and blastocyst groups. Litter size in the IVF-ET-BL group was significantly smaller than in the IVF-ET-2Cell group, it might be anticipated that the former might be at a developmental disadvantage when compared to the latter. Studies have shown that *in vitro* culture can slow developmental rates, reduced total blastocyst cell numbers and can also even alter gene expression patterns (Watkins et al. 2007); (Giritharan et al. 2012), supporting this conclusion.

As preimplantation development is slower for IVF embryos cultured to blastocyst stage compared with *in vivo* derived blastocysts (Chapter 3), then the rate of postimplantation development may also be affected by the length of culture. In our study, birth size in the IVF-ET-BL group was almost half the birth size in the IVF-ET-2Cell group. In this case, reduced rates of development and low total cell numbers observed in IVF-ET-BL (Chapter 3) may compromise postimplantation development, so producing a small number of embryos that are able to implant and develop to viable offspring. Studies have demonstrated that foetuses derived from *in vitro*

derived embryos may have increased their rate of postimplantation development, compensation for initial slower preimplantation development (Rands 1986).

The assessment of overall growth rates was analysed by using Z-score analysis (Figure 4.5 and Figure 4.6), where the entire data set was transformed, so giving a mean value of (0) and standard deviation of (1). This revealed that the entire slopes were comparable for male and female IV-ET-2Cell, IV-ET-BL, IVF-ET-2Cell and IVF-ET-BL offspring but significantly different from male and female NM offspring, which remained smaller throughout the analysis period. This may reflect the difference in postnatal litter sizes between all treatment groups and identified as a significant dependent variable in the analysis.

In this study, male and female offspring in the IV-ET and IVF-ET groups gained weight greater when compared to NM controls overall, this indicated the effect of embryo manipulation (Superovulation, IVF, embryo culture (short and long) and ET) on the postnatal growth of offspring. However, there was considerable variability of these weight changes between IV-ET and IVF-ET groups. Interestingly, male and female IV-ET-2Cell offspring gained more weight than IV-ET-BL, which were not significantly different from NM female offspring and similar but not significant to NM male offspring, this may indicates the effect of ET at the early stage (2-Cell), which may alter the epigenetic processes that controls implantation, placentation, organ formation and fetal growth (See 1.4.1).

Organ Allometry

Previous studies have found a correlation between ART and organ weight and organ:body weight ratio in the mice offspring. In our study, the weight of the lung, heart, liver right and left kidneys and spleen were significantly smaller in the NM control group compared to the four ET groups in both male and female offspring. In mice, previous studies show that embryo culture have minimal effect on organ body weight including left and right kidneys and lung (Watkins et al. 2007). It is believed that is the phenomenon of large offspring syndrome (LOS) is the most dramatic display of how fetal and postnatal development can be affected in response to manipulating the preimplantation embryo. It has been shown that *in vitro* culture in the presence of serum leads to LOS in cattle and sheep (Sinclair et al. 1999). LOS is associated with significantly enhanced fetal and postnatal growth, altered organ sizing, changes in patterns of gene expression, and increased rates of perinatal death (Watkins et al. 2008a).

Our study demonstrated that the liver: body weight ratio in the NM control group was significantly smaller among all treatment group. The male offspring from IVF-ET-2Cell have the largest liver and highest liver: body weight ratio among all groups. The liver size and liver: body weight ratio values in the IVF-ET-2Cell group were even significantly higher than IVF-ET-BL. More analysis in our future work will be conducted to clarify the correlation between liver size, GTT and AUC.

Overall, from our results it would appear that male offspring exhibited significant differences detected between IVF-ET-BL and IVF-ET-2Cell groups, although this pattern was not observed in female offspring.

In conclusion, from the results reported within this chapter, the environment in which the embryo develops during the first 3.5 days can influence aspects of postnatal physiology and development of the offspring obtained. It would appear that exposure to an *in vitro* environment, whether long (E3.5) or short (E1.5), during preimplantation development can affect parameters of postnatal development in particular offspring growth patterns and organ ratios and weights. Exposure to an *in vitro* culture for a relatively short period (E1.5) can cause some of the same long-term effects as those observed from prolonged culture (E3.5). Interestingly, it would seem that exposure to an *in vitro* culture for long period (E3.5) can lead to significant long-term effects when compared to a short culture (E1.5). This would imply that the preimplantation mouse embryo is particularly sensitive to its surrounding environments, and that perturbations of normal patterns of development can have long-term consequences.

Chapter 5: Effect of *In Vitro* Fertilisation (IVF) and Embryo Culture Duration on Offspring Cardiovascular Health

5.1 Introduction

In the previous chapter, we discussed the effect of IVF and embryo culture duration on body weight and the organs weight of offspring. Our study demonstrated that male and female offspring conceived by IVF (IVF-ET-2Cell; IVF-ET-BL) or *in vivo* before immediate ET (IV-ET-2Cell; IV-ET-BL) were significantly heavier compared with normal mating (NM) offspring. A great deal of research shows that adults with diabetes are at a two- to-fourfold higher risk of CVD and an increased occurrence of elevated systolic and diastolic blood pressure (hypertension), with both factors appeared to be connected to excess weight and obesity, although this relationship is complex (Eckel et al. 2006). Moreover, poor fetal or adult cardiovascular and metabolic health, and particularly hypertension, constitute a common outcome phenotype from diverse preimplantation treatments (Watkins and Fleming 2009); (Eckert and Fleming 2011). It is therefore worth investigating the effect of IVF and embryo culture duration on cardiovascular profile. In this chapter, we studied the effect of IVF and embryo culture duration on Systolic Blood Pressure (SBP) and Serum and Lung Angiotensin Converting Enzyme (ACE) activities (See Section 2.5.3 and 5.2.7).

Human as well as animal studies surrounding IVF suggest that the technique may be one contributing factor to the development of cardiovascular disorders. For example,

human IVF offspring have demonstrated adverse metabolic profiles and increased blood pressure (Hart and Norman 2013a). In addition, children conceived through IVF at a mean age of 12.3 years had higher systolic and diastolic blood pressure than normal children (Ceelen et al. 2008a). Furthermore, a study in Greece has shown that such blood pressure differences were noted in a small case-controlled series of IVF children at a mean of age 8.8 years (Sakka et al. 2010).

Animal studies also demonstrated that mice conceived by IVF appear to have higher blood pressure compared with *in vivo* development. For example, (Watkins et al. 2007) reported that male and female offspring from embryo culture from 2-Cell stage to blastocyst and ET led to an enhanced systolic blood pressure at 15 weeks versus control (undisturbed) offspring. They also show that female, but not male, offspring from *in vitro* culture and ET have a significant elevation in blood pressure compared with *in vivo* development and ET, independent of litter size, maternal origin or body weight (Watkins et al. 2007).

(Watkins et al. 2007) also investigated serum ACE activities. They reported that female but not male mice derived from embryo culture from 2-cell stage to blastocyst and ET, and *in vivo* developed and ET treatments exhibited elevated serum ACE activity compared with normal mating controls. They suggested that one component underling elevation in SBP in embryo-manipulated female offspring may derive from an altered renin-angiotensin system.

As one of the parameters being investigated in our study is blood pressure, factors involved in directly regulating blood pressure will also be examined. It was decided to assay the activity of ACE within the serum and lungs collected at 27 weeks of age.

The renin–angiotensin system (RAS) is a major hormonal system involved in the pathophysiology of cardiovascular disease (CVD), and can act either as a systemic hormone (endocrine) or as a locally-generated factor (paracrine, autocrine). ACE is a zinc metallopeptidase and mammals produce two isozymes of ACE; somatic ACE, which plays an important role in the control of blood pressure, and testicular ACE, which is produced by male germ cells and plays important functional roles in male reproduction. ACE is an important regulator of the RAS through actions to convert angiotensin (I) into the vasoconstrictor angiotensin (II), which mediates its effects predominantly through angiotensin type 1 receptors. Angiotensin II is a potent vasoconstrictor which leads to an increased in blood pressure through vasoconstriction and salt and water retention, and contributes to cardiac remodelling, fibrosis, inflammation, thrombosis and plaque rupture (Patel et al. 2013).

Renin catalyses the conversion of a plasma protein called angiotensinogen (AGT) into angiotensin (ANGI). ACE then converts ANGI into angiotensin (II). Angiotensin II acts via receptors in the adrenal gland to stimulate the secretion of aldosterone, which stimulates salt and water reabsorption by the kidneys, and the constriction of small arteries (arterioles), which causes an increase in blood pressure. This process is summarised in Figure 5.1 (Unger 2002).

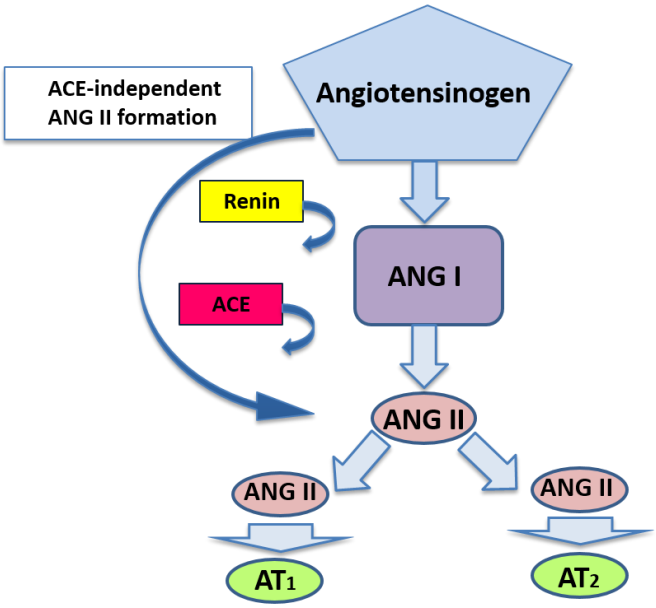


Figure 5.1 The role of the renin-angiotensin system in the development of cardiovascular disease

The renin-angiotensin (ANG) system: areas of potential blockade. ACE = angiotensin-converting enzyme; AT1 = angiotensin type 1 receptor; AT2 = angiotensin type 2 receptor; Adapted from (Unger 2002)

Somatic ACE consists of two homologous catalytic domains, a juxtamembrane stalk region, a hydrophobic transmembrane domain of 17 amino acids, and a 30-residue C-terminal cytosolic domain. Thus, ACE is primarily an integral membrane protein anchored to the plasma membrane by its C-terminal segment (Eyries et al. 2001). The N and C terminal domains can be cleaved to yield an active, soluble form of the enzyme that circulates within the blood stream known as serum ACE; See Figure 5.2. It is reported that there are a group of secretases that cleave the juxtamembrane stalk (Sadhukhan et al. 1998); (Woodman et al. 2000);(Eyries et al. 2001).

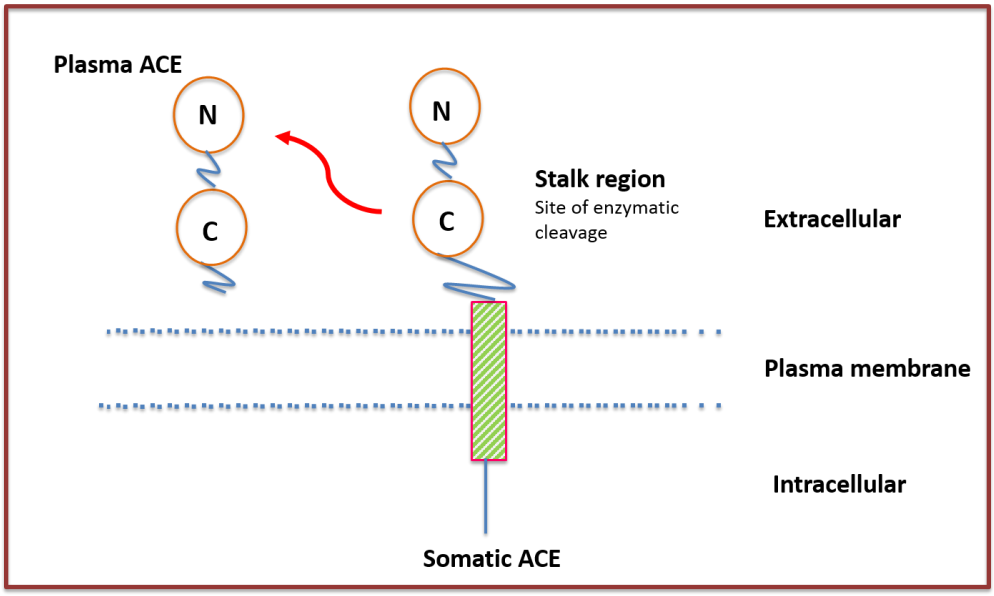


Figure 5.2 Diagram of somatic ACE showing the transmembrane region (green striped box) and the two extracellular domains (C and N).

The arrows indicate the region of enzymatic cleavage yielding a free-floating catalytic unit yielding serum ACE.

ACE is primarily localised (>90%) in various tissues and organs, most notably in many endothelial cells, indeed high specific activities of ACE have been found in the lung. Somatic ACE is now recognised as a key factor in cardiovascular and renal diseases. Endothelial dysfunction, in response to risk factors or injury such as hypertension and diabetes disrupts the balance of vasodilation and vasoconstriction, vascular smooth muscle cell growth, and is associated with activation of tissue ACE. It is shown that pathologic activation of local ACE can have adverse effects on the heart, vasculature and kidneys. The imbalance due to increased local formation of angiotensin II and increased bradykinin degradation favours cardiovascular disease (Dzau et al. 2001).

ACE activity or mRNA has also been seen in vessels or cultured endothelial cells from several animal or human fetal or adult sources (Metzger et al. 2011). In humans, ACE has been detected by immunohistochemistry in the lung and in small arteries and capillaries of a few studied organs (Sibony et al. 1993).

Animal studies have demonstrated the expression of ACE in several murine lung and kidney epithelial cell lines to identify a model system that could be useful for *in vitro* studies of ACE such as transcriptional regulation. The study confirmed the presence of ACE expression in adult mouse lung and kidney. They also found that ACE mRNA levels peak at embryonic day 18.5 in the mouse lung (Wiener et al. 2007).

Similarly, another study has investigated the role of the ACE gene in blood pressure control using mice generated to carry an insertional mutation that is designed to inactivate ACE. The study showed that heterozygous males but not females had blood pressures that were 15-20 mm Hg lower than normal, and both male and female heterozygotes had decreased serum ACE activity (Krege et al. 1995). Therefore, it would be of great interest to assay the activity of ACE within the serum and the lungs and examine their correlation to blood pressure.

The hypothesis of this chapter is that the environment, in particular the length of embryo culture; long (blastocyst) vs. short (2-cell stage) in which the preimplantation embryo develops cannot only slow down blastocyst development, but may also perturb cardiovascular phenotype by increasing SBP and increasing ACE activities in the offspring.

The aim of this chapter was to investigate the effect of IVF and embryo culture duration on cardiovascular profile by measuring systolic blood pressure (SBP) and examining the activity of ACE within the serum and lungs in offspring across the five treatment groups. In addition, we will examine the correlation between these factors with other postnatal factors such as growth weight (Chapter 4) and metabolic profile (Chapter 6).

SBP, ACE activity and correlations between treatment groups for male and female offspring were analysed using a multilevel random effects regression model, which takes into account litter size and the maternal origin of the pregnancy.

5.2 Methodology

5.2.1 *Production of naturally mated offspring*

10 female C57/BL6 mice at age of 6-8 weeks were naturally mated with CBA males and allowed to develop to term with their pregnancies (See section 2.1).

5.2.2 *Production of in vivo derived embryos*

3-4 week old C57/BL6 female mice were superovulated and naturally mated with CBA male mice. Embryos were flushed from the oviduct at 2-cell stage and from the uteri at blastocyst stage (See section 2.2.2). Both were placed in pre-warmed H6-BSA under mineral oil for approximately 1-2 hours after flushing to buffer against pH changes until they were transferred to the oviduct (2-cell) or the uterus (blastocyst) of their respective foster mother.

5.2.3 *Production of IVF embryos*

Embryos were generated by the IVF procedure as described in (See section 2.3.32.2.2) and cultured in KSOM either for ~24 h up to the 2-cell stage or for ~60 hours to the blastocyst stage. Both sets were then placed in pre-warmed H6-BSA under mineral oil for approximately 1-2 hours until they were transferred to the oviduct or the uterus, respectively of the foster mother.

5.2.4 *Embryo transfer*

The ovaries and the oviducts of anaesthetised 1.5 days pseudopregnant female MF1 mice were carefully exposed from the body cavity and ten (2-cell) embryos were

transferred to each oviduct (See section 2.3.22.3.3). Litter size was regulated to eight offspring at birth. For the blastocyst embryo transfer, ovaries and the uterus of anaesthetised 2.5 days pseudopregnant female MF1 mice were carefully exposed from the body cavity. Seven blastocysts were then transferred to each uterine horn (See section 2.3.3). The litter size in these two groups was less than eight offspring so litter size correction was not applied.

5.2.5 *Measurement of postnatal growth rates*

All offspring from the five treatment groups were weighed on day seven of birth (at age of week 1) and then subsequently on the same day for the next 27 weeks. Offspring were weaned at age of three weeks and then ear punched to mark each individual mouse (See section 2.5).

5.2.6 *Measurement of SBP*

Systolic blood pressure was determined at age of 9, 15 and 21 weeks by tail-cuff plethysmography (See section 2.5.3). The test was performed at the same time for the three time points, normally between 11am and 5pm, depending on the number of offspring on that specific day and mice behaviour (each mouse take around 10-15 minutes).

5.2.7 *Measurement of ACE activity*

Methods derived from (Hurst and Lovell-Smith 1981); (Forhead et al. 2000); (Watkins et al. 2007) were used with some modifications. The assay was based on the colourimetric determination of hippurate with cyanuric chloride/dioxan reagent.

Hippurate is released from hippuryl-L-histidyl-L-leucine by ACE in the presence of the chloride ion. Briefly, the method comprises serum incubation in Hippuryl-L-Histidine-L-Leucine acetate salt (HHLA; Sigma) solution in incubation buffer (0.2M H3BO3, 2M NaCl, pH 8.3, see appendix V) at 37^{o}C for 15 minutes followed by addition of cyanuric chloride (2-4-6 trichloro 1-3-5 triazine; Sigma, see appendix V) in 1,4-dioxane; Sigma) for a yellow coloration to develop. Four replicates per sample were analysed using a plate reader, and the absorbance at 380 nm was measured against the blank. Samples containing only serum and chloride buffer were used as negative controls. For each analysis, a standardised curve was prepared and treated exactly as the incubates. Serum ACE activity was expressed as amount (in µM) of hippurate formed per ml of serum, per minute (See section 2.5.7), whereas lung ACE activity was expressed as amount (in nM) of hippurate formed per mg of protein per minute (See section 2.5.8) and (see Appendix VII).

5.2.8 *Statistical analysis of data*

The animal SBP, ACE activity and correlations were analysed using a multilevel random effect regression model, which took into account between-mother and within-mother variation in litter size and parameters measured from individual animals (See section 2.6). Statistical significance was $P \leq 0.05$. If a P value was between 0.1 and 0.051, it was considered as a trend.

5.4 Results

5.4.1 *Offspring systolic blood pressure (SBP)*

The mean systolic blood pressure for the different groups at designated weeks (9, 15, 27 and LIFE; mean of 3 time points MEAN SBP) are shown in Figure 5.3 and Figure 5.4. In males, there was no significant difference for week 9 between groups. However, NM control males showed significant differences compared to the IVF groups (IVF-ET-2Cell and IVF-ET-BL) at weeks 15, 21 and life, and NM control males showed significant differences compared to the *in vivo* groups (IV-ET-2Cell and IV-ET-BL) at 21 and life. At weeks 15, 21 weeks and life, the systolic blood pressure for IVF-ET-BL group in males was significantly higher compared to the IV-ET-BL group (P= 0.003, 0.014 and 0.001; respectively). At weeks 15, 21 and life, the IVF-ET-BL group had elevated blood pressure when compared to the IVF-ET-2Cell (P=0.032, 0.034 and 0.017; respectively).

In the female offspring, NM control females showed significant differences between IV-ET and IVF-ET groups at weeks 15, 21 and life ($P<0.05$). However, there was no significant difference between the four treatment groups for females at designated weeks.

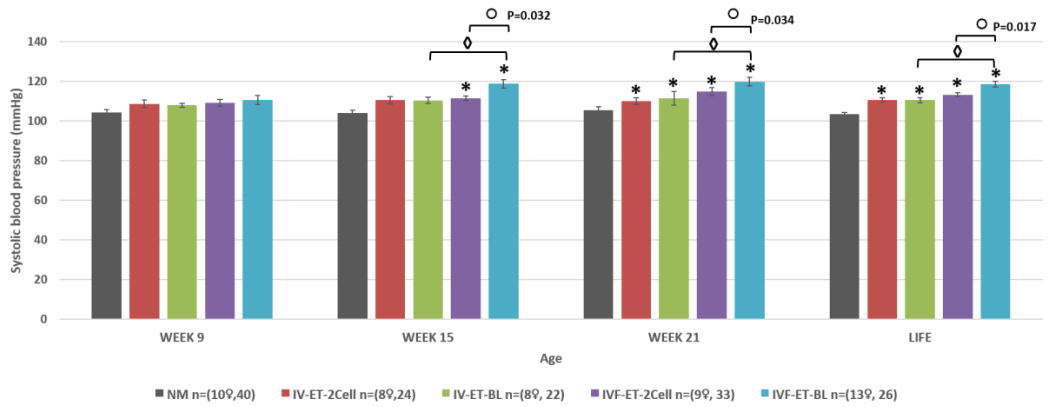


Figure 5.3 Effect of IV and IVF treatment groups and NM control group on SBP in male offspring for 3 designated weeks and life (±SEM of week 9, 15 and 27)

IVF-ET and IV-ET groups in male offspring have significantly higher blood pressure compared to the control NM group. IVF-ET-BL group has significantly higher SBP compared to IV-ET-BL and IVF-ET-2Cell groups. * denote (P≤0.05) between NM and selected groups, ◊ denote IV-ET-BL vs. IVF-ET-BL P<0.05 and, ○ denote IVF-ET-BL vs. IVF-ET-2Cell. Mean (±SEM) blood pressure for weeks 9, 15, 27 and life (from 8 – 10 litters); ((n) of mothers or foster mothers ♀, (n) of offspring).

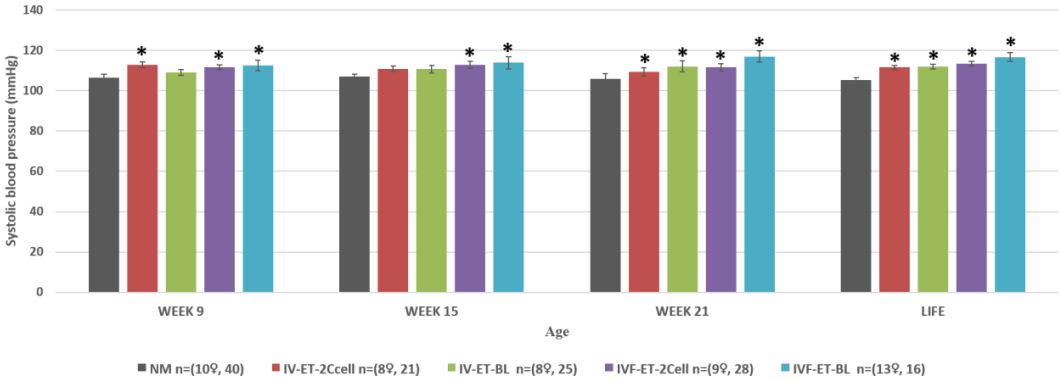


Figure 5.4 Effect of IV and IVF treatment groups and NM control group on SBP in female offspring for 3 designated weeks and life (±SEM of week 9, 15 and 27)

IVF-ET and IV-ET groups, female offspring have significantly SBP for weeks 9, 15, 21 and LIFE compared to NM control groups. * denote (P≤0.05) between NM and selected groups. Mean (±SEM) blood pressure for weeks 9, 15, 27 and life (from 8 – 10 litters); ((n) of mothers or foster mothers ♀, (n) of offspring).

5.4.2 *Serum ACE activity*

Serum ACE activity results for male and female offspring are shown in Figure 5.5 and Figure 5.6. In male offspring, the serum ACE activity in IVF-ET-BL group is significantly increased compared with the NM group (P= 0.034). The IVF-ET-BL group serum ACE activity also tended to be higher than IV-ET-BL, although this did not reach statistical significance (ţ =0.070).

In female offspring, the mean of IVF-ET-BL group serum ACE activity tended to be higher than NM (ţ =0.053).

5.4.3 *Lung ACE activity*

Lung ACE activity results for male and female offspring are shown in Figure 5.7 and Figure 5.8. In male offspring, lung ACE activity was significantly higher in IVF-ET-BL group compared with NM group (P= 0.019) and IV-ET-BL (P=0.042).

No significant differences were detected between the five treatment groups in female offspring.

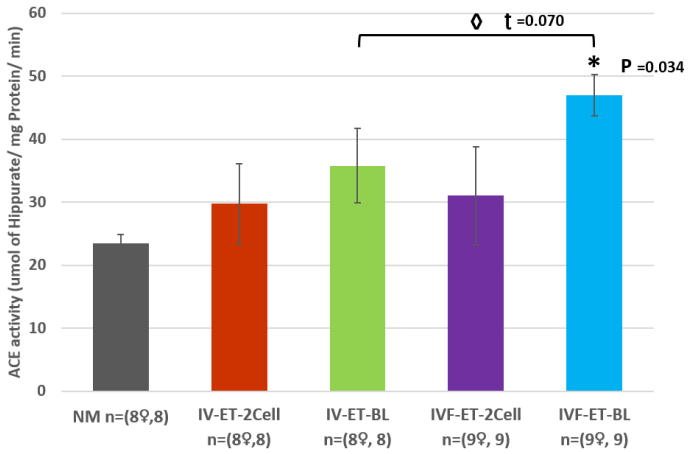


Figure 5.5 Effect of IV and IVF treatment groups and NM control group on serum ACE activity in male offspring

The IVF-ET-BL group has significantly increased serum ACE activity compared with NM (P= 0.034) and IV-ET-BL (P= 0.070, trend ţ <0.05). * denote (P≤0.05) between NM and selected groups, ◊ denote IV-ET-BL vs. IVF-ET-BL. Mean (±SEM) ACE activity (from 8 – 9 litters; one male per litter).

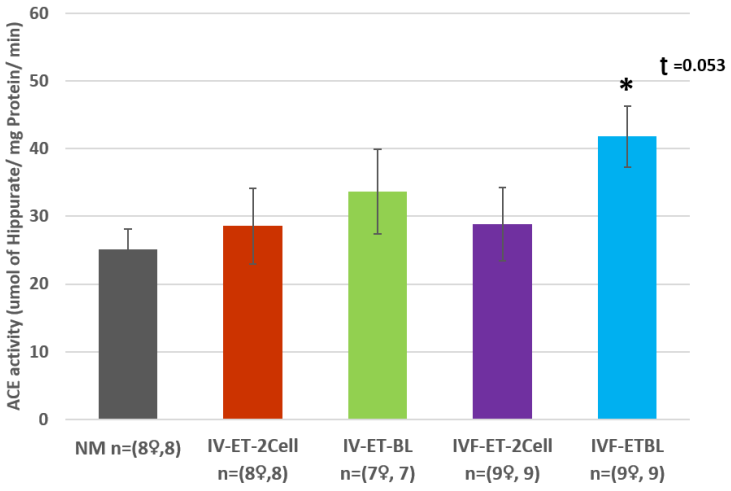


Figure 5.6 Effect of IV and IVF treatment groups and NM control group on serum ACE activity in female offspring

The IVF-ET-BL group has significant increased serum ACE activity compared with NM (ţ=0.053 trend ţ <0.05). * denote (P≤0.05) between NM and selected groups. Mean (±SEM) ACE activity (from 7 – 9 litters; one female per litter).

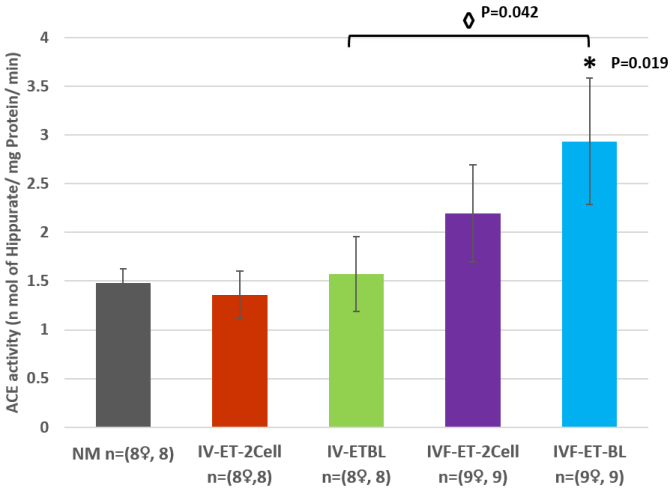


Figure 5.7 Effect of IV and IVF treatment groups and NM control group on lung ACE activity in male offspring

The IVF-ET-BL group has significantly increased lung ACE activity compared with NM (P=0.019) and IV-ET-BL (P= 0.042). * denote (P≤0.05) between NM and selected groups, ◊ denote IV-ET-BL vs. IVF-ET-BL. Mean (±SEM) ACE activity (from 8 – 9 litters; one female per litter).

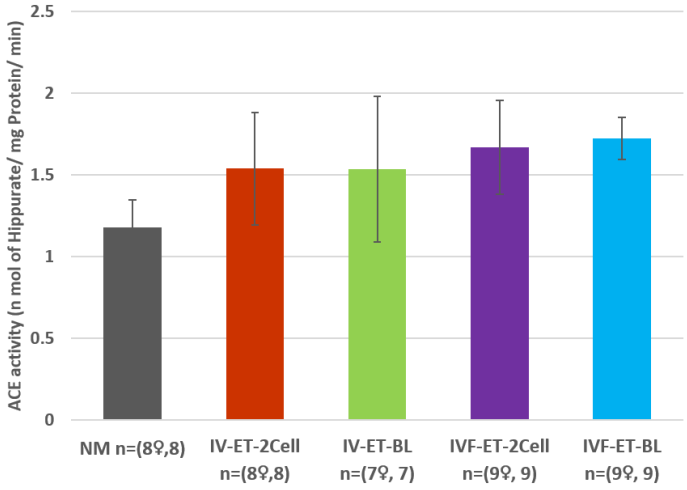


Figure 5.8 Effect of IV and IVF treatment groups and NM control group on lung ACE activity in female offspring

No significant differences were detected between the five treatment groups in female offspring. Mean (±SEM) ACE activity (from 7 – 9 litters; one female per litter).

5.4.4 *Selected correlation analyses of offspring cardiovascular phenotype with other postnatal data*

Weight at weeks 3, 9, 15, 21 and 27; systolic blood pressure at weeks 9, 15, 21 and the average over their life; fasting glucose levels and the glucose AUC were correlated for all offspring produced over the five treatments for evidence of associated effects. The only factors found to significantly correlate across all five treatments were SBP with weight and ACE activity, and weight at week 27 with glucose AUC (See Chapter 6). Interestingly, this relationship is only apparent in males.

5.4.4.1 Correlation between weight and SBP

Figure 5.9 shows the relationships between weight at weeks 9, 15 and 27 vs. SBP at week 21 in IVF-ET-BL male offspring; here, SBP at week 21 is positively correlated with weight measurements at 9 weeks (ţ =0.051), at 15 weeks (P=0.018) and at 21 weeks (P=0.016). With R^2 values of 0.046, 0.09 and 0.09 respectively. Figure 5.9 demonstrates that the gradient of the slopes at the three time points does not significantly differ, suggesting that for each week, SBP increased with weight by the same factor.

In Figure 5.10, the relationships between weight at 15 and 21 weeks vs. SBP LIFE (average of 9, 15 and 21 weeks) in IVF-ET-BL male offspring is presented, in which SBP LIFE is positively correlated with weight measurements at 15 weeks (ţ =0.066), at 21 weeks (P=0.026) with R^2 values of 0.041 and 0.026 respectively.

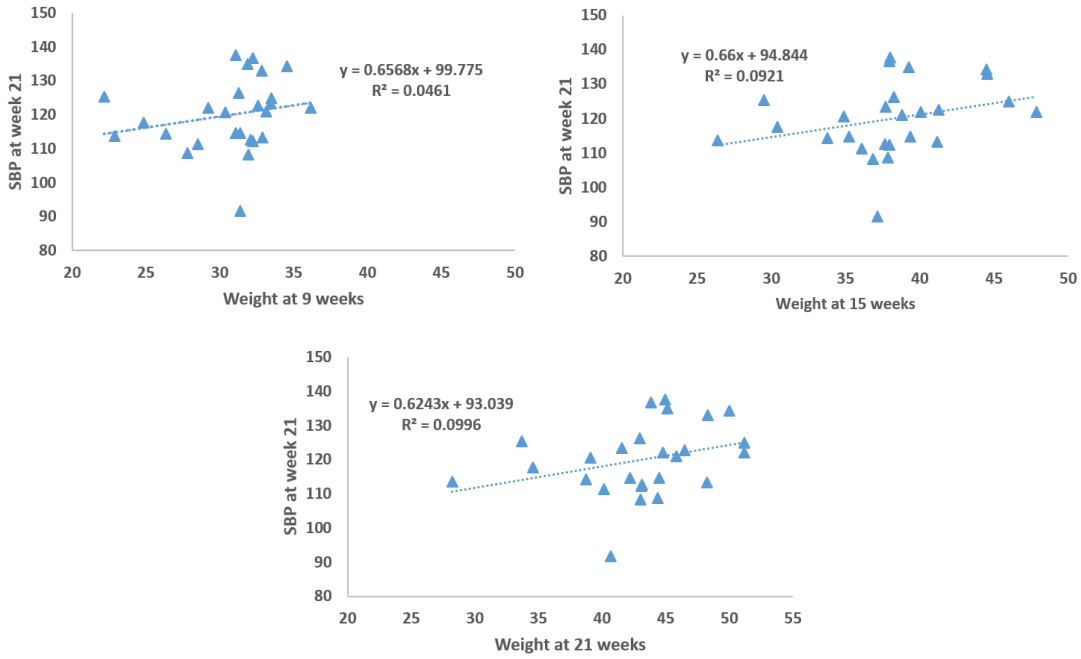


Figure 5.9 Correlation between SBP at week 21 and weight at 9, 15 and 27 weeks in IVF-ET-BL male offspring

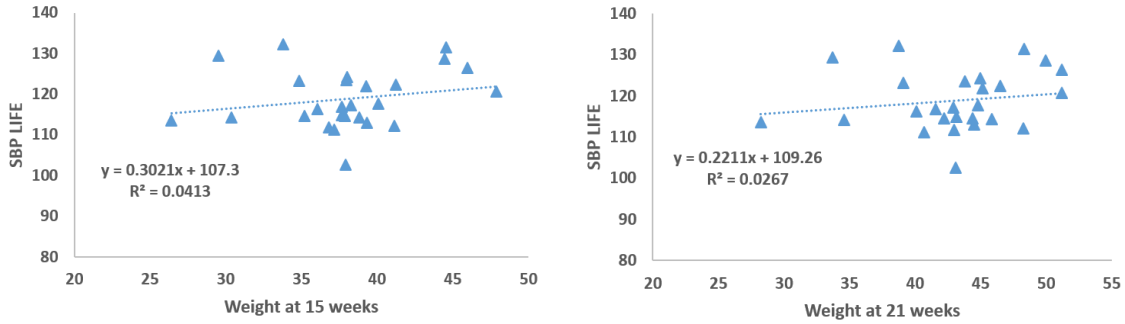


Figure 5.10 Correlation between SBP LIFE (Average of 9, 15 and 21 weeks) and weight 15 and 21 weeks in IVF-ET-BL male offspring

5.4.4.2 Correlation between SBP and ACE

The only relationships found were within the IVF-ET-BL male offspring. Male lung ACE activity positively correlates with SBP at 21 weeks of age and with LIFE SBP (average of 9, 15 and 21 weeks), with 50% and 81% respectively of the variation being explained by this model. In Figure 5.11, significant P values of 0.002 and 0.009 respectively validates that a relationship exists. As SBP levels increase, lung ACE activity also increases.

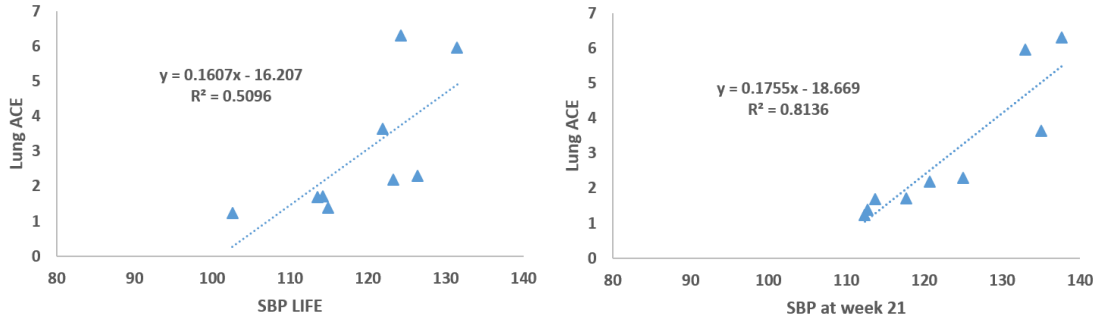


Figure 5.11 Correlation between Lung ACE and SBP at week 21 and LIFE (average of 9, 15 and 21 weeks) in IVF-ET-BL male offspring

5.5 Discussion

The aim of this chapter was to investigate the effects of IVF, length of embryo culture and embryo transfer on the postnatal health of offspring, particularly in terms of systolic blood pressure and ACE as an indicator of cardiovascular health.

Previous research has reported that the environment in which the preimplantation embryo develops can affect SBP in offspring. Studies investigating the blood pressure of adult mice have shown a similar mean SBP to those values reported for NM controls in our study (Table 5.1). In these studies, mean SBP ranges for similar strains of mice to those studied within this experiment, were found to be within 96-112 mmHg, depending on the method used to measure the SBP.

Table 5.1 Results from previous studies investigating SBP in mice

Strain of mice	Age of mice	Mean SBP (mmHg)	Measuring method	Reference
C57/BL6J	-	80	Tail cuff	1
C57/BL10J	-	93	Tail cuff	1
C57/BL6J	8-13 weeks	120	Catheterisation	2
C57/BL6	19-30 gm	98.4 (abdominal)	Radiotelemetry	3
C57/BL6	15,21 weeks	100 - 115	Tail cuff	4
C57/BL6	9,15,21 weeks	90 - 120	Tail cuff	5
C57/BL6	9,15,21 weeks	96 - 112	Tail cuff	6

References: (1) (Carlson and Wyss 2000); (2) (Schlager 1966); (3) (Mattson 2001); (4) (Watkins et al. 2007); (5) (Velazquez et al. 2016); (6) (Unpublished data, Khalif and Aljahdali).

Our results show that at most times analysed, offspring from the ET treatment groups have an elevated systolic blood pressure when compared to the NM control group. Therefore, even though for the two IV-ET groups (IV-ET-2Cell; IV-ET-BL), embryos experienced culture environment for only a short period of time (1 – 3 hours) before transfer, this appears to be sufficient to programme an elevation in blood pressure postnatally. It may also be the case that the duration of culture may exacerbate blood pressure with the mean SBP for males and females increasing in the order NM < IV-ET groups < IVF-ET-2Cell < IVF-ET-BL. As reported in Chapter 4, the mean body weight in the four ET groups for both male and female offspring was significantly higher compared with the NM control group. This may affect the SBP value.

Figure 5.3 and Figure 5.4 show that the male and female offspring of the four ET groups have significantly higher SBP compared with NM control offspring at weeks (9, 15 and 27) and LIFE. The high blood pressure observed in IV-ET and IVF-ET offspring was accompanied with increased body weight in male and female. However, previous experiments examining the effect of undernutrition (Watkins et al. 2008c) and *in vitro* culture (Watkins et al. 2007) during the preimplantation period in mice showed increased SBP in male and female offspring, but the increase in SBP was accompanied with noticeable change in body weight throughout postnatal development only in females. This may indicate that a combined effect of IVF procedure, culture and ET most probably are behind the increase in body weight and SBP in offspring regardless of sex.

The major finding of our study is that male offspring from IVF-ET-BL firstly had a significantly higher SBP at weeks 15, 21 and LIFE (P= 0.003, 0.014 and 0.001; respectively) compared with IV-ET-BL offspring and secondly, was significantly higher than that of IVF-ET-2Cell at weeks 15, 21 and LIFE (P=0.032, 0.034 and 0.017; respectively). This indicates that ART may act as a negative factor for adult hypertension with duration of embryo culture exacerbating the effect but with ET alone still contributing to this effect.

In support of our results, experimental evidence in rodent and ruminants has reported that altered phenotypes during postnatal life can be programmed during periconceptional period when crucial reproductive events such as preimplantation embryo development are exposed to effective environments such as undernutrition (Watkins et al. 2008c) and ART-related procedures (Watkins et al. 2007); (Rexhaj et al. 2013); (López-Cardona et al. 2015). (Watkins et al. 2007) has shown that preimplantation embryo culture and transfer led to elevation of systolic blood pressure in both males and females at week 15 compared to controls (undisturbed) offspring. (Watkins et al. 2007) cultured 2-cell embryos derived from CBAxC57/BL6 mice to the blastocyst stage and found that female offspring, but not male from *in vitro* culture and transfer have a significantly higher SBP at week 21 when compared to offspring derived *in vivo* and directly transferred to recipient mothers without prolonged culture. (Rexhaj et al. 2013) performed IVF using FVB mice and showed that male offspring had increased mean blood pressure at week 14 of age compared with normal mating controls.

It is well known that IVF media have undergone numerous changes aiming to improve success rates of IVF/ICSI. Commercial IVF media vary widely in their nutrient composition, so to evaluate possible consequences on embryo growth it is vital to know the composition of the media the embryo has been exposed (Sunde et al. 2016). However, it is still unclear how the composition of IVF media affects embryo development and what its adverse effects may be on postnatal health.

Culture media contain a number of components such as carbohydrates, amino acids, vitamins and growth factors, which may alter epigenetic or other reprogramming events, resulting in detrimental effects on the embryo and potentially to long-term health issues in the adult. The main component in the culture media is nutrients. In addition, amino acids and vitamins that are taken up by the embryo have been reported to cause direct epigenetic changes in the embryo. It is shown that early embryos undergo 'nutrient sensing' which means that embryos have the ability to monitor their environment in which they are developing. Glucose and amino acids are particularly crucial; both regulate embryo growth and development via epigenetic changes. Embryonic sensing of a low nutrient environment in culture media can therefore program the embryo to be thrifty in its metabolism, which may lead to programming a long-term thrifty phenotype (Sunde et al. 2016).

It is well known that the genome and epigenome of the embryo are completely remodelled during preimplantation period, making the embryo susceptible to its environment, particularly, at the stage of manipulation *in vitro* and culture during IVF. In our study, both IVF-ET-2Cell and IVF-ET-BL offspring were derived by IVF procedure and experienced embryo culture, however the only difference between

the two treatment groups was the length of culture. IVF-ET-BL offspring, which experienced long culture during their preimplantation development, showed higher SBP in their postnatal life compared with those that experienced short culture (IVF-ET-2Cell). This indicates that long culture can cause long term reprogramming of development and health, and this is likely mediated by epigenetic changes. Research shows that embryo culture specifically in suboptimal conditions disturbs the maintenance of the embryonic epigenome in the mouse and potentially in human preimplantation embryos (Market-Velker et al. 2010); (Velker et al. 2012); (White et al. 2015).

The possibility that exposure of embryos to an *in vitro* environment for either short (2-cell) or a prolonged period of time (blastocyst) could induce increased systolic blood pressure in the offspring is of potential concern. Despite of the fact that there are limited data regarding the postnatal development of children born by IVF, especially with regard to their systolic blood pressure, there is research showing that children conceived via ART are more likely to be born premature, to have some adverse effect such as low birth weight (<2500g), congenital abnormalities, an increase in the occurrence of elevated blood pressure, high fasting glucose level and an increase in total body fat composition (Ceelen et al. 2008b); (Hart and Norman 2013a); (Hart and Norman 2013b); (Kleijkers et al. 2014); (Fauser et al. 2014); (Watkins et al. 2007); (Kleijkers et al. 2016).

One important factor in the regulation of blood pressure is ACE. As mentioned above, a substrate for renin, secreted by kidneys, Angiotensinogen (AGT) is converted into the decapeptide angiotensin ANG I, and subsequently by ACE to the

octapeptide Ang II resulting in elevation in blood pressure (Unger 2002). By the action of ACE-2, Ang II activity is terminated by its conversion to Ang 1–7. Of note, both ACE-1 and ACE-2 are secreted by the lung ACE converts (See Section 5.1), Although the lung is a major source of ACE and most angiotensin I is converted to angiotensin II by a single pass through this organ, some studies show a functional role of locally-generated angiotensin II, predominantly in the brain, the heart and the kidney. A recent study reported that renal ACE and locally generated angiotensin II affect the activity of several key sodium transporters and the induction of sodium and water retention resulting in the elevation of BP (Bernstein et al. 2014). Therefore, we examined serum and lung ACE activity and the correlation between ACE and SBP in our study aiming to investigate the potential factor that led to increased SBP that observed in the offspring.

Our findings show that in male offspring, there are significant differences between the treatment groups in the activity of ACE within the serum and lung (Figure 5.5 and Figure 5.7). Our data demonstrated that male offspring from IVF-ET-BL group (which had the highest blood pressure at 15 and 21 weeks of age and LIFE) did have the highest serum and lung ACE activity at 27 weeks of age. However, in female offspring, the only different detected was between IVF-ET-BL group and NM within serum ACE activity and this did not reach significance (P= 0.053; Figure 5.6 and Figure 5.8). Thus, our data report that IVF and prolonged embryo culture can programme the development of high lung and serum ACE activity in adulthood in a sex-specific manner.

Interestingly, our findings showed several similarities with (Watkins et al. 2007) who found that female, but not male, offspring derived *in vitro* and ET, which had the highest blood pressure at 21 weeks of age had the highest serum ACE activity at 27 weeks of age. Similarly, (Krege et al. 1995) showed that all males but not females had blood pressures that were 15-20 mm Hg less than normal, and both male and female heterozygotes had decreased serum ACE activity.

These findings indeed are confirmed by our data shown in Figure 5.11. Our data reported that a positive correlation was detected between SBP at weeks 21 of age and LIFE, and lung ACE levels within IVF-ET-BL male offspring. Individuals of higher SBP demonstrate higher lung ACE values. It may therefore be hypothesised that increased level of ACE enzyme, or an increased activity of existing ACE, may result in an elevated blood pressure.

Interestingly, male offspring from IVF-ET-BL group shows a relationship between SBP at week 21 and LIFE, and body weight at weeks 9, 15 and 27 of age. Individuals of higher weight demonstrate higher SBP values (Figure 5.9 and Figure 5.10). These findings are similar to some human studies that have reported that serum ACE levels are significantly higher in hypertensive individuals compared to normotensive. The study also reported a positive correlation to body mass index (BMI) in males and females (Forrester et al. 1997). However, another recent study in North India investigated the correlation between BP, ACE and BMI. The study compared between 253 hypertensive individuals who were sub-categorised according to normal or high BMI and 125 normotensive. They found that ACE was significantly

associated with hypertension, but not associated with BMI (Rizvi et al. 2015). Further studies are needed to investigate the correlation between ACE and BMI.

As mentioned above, ACE is primarily an integral membrane protein anchored to the plasma membrane by its C-terminal segment (Eyries et al. 2001). The N and C terminal domains can be cleaved to yield a soluble active form of the enzyme that circulates within the blood stream and comprises the serum ACE. It may therefore be hypothesised that the more ACE that is expressed on the surface of the epithelia of the lung in male offspring, then the more ACE will be available for cleavage, so raising serum ACE protein and activity levels. To determine whether or not this is the case in our study we examine the correlation between lung and serum ACE. Our findings show that although male offspring from IVF-ET-BL have significantly increased serum and lung ACE activity at 27 weeks of age, there is no significant correlation observed between serum and lung ACE in these offspring.

ACE activity elevation has also been shown to correlate with hypertension in other models of early embryo programming, particularly in response to rat maternal protein undernutrition. Increased ACE activity of serum and lung has been observed in mice from mothers fed LPD either exclusively during preimplantation development or throughout gestation (Watkins et al. 2008a). In addition, increased ACE activity also occurs in mice *in vivo* in response to expression of the 'Ped' genes that contribute to embryo survival (Watkins et al. 2006). Furthermore, the use of the ACE inhibitor Captopril, significantly decreases the SBP in offspring of rats from LPD-fed mothers (Langley-Evans and Jackson 1995). RAS stimulation by ferusamide leads to significant elevation of BP in the sheep offspring of mothers with early gestational

nutrient restriction (Cleal et al. 2007). However, the extent to which maternal LPD can lead to development programing of the RAS in association with hypertension is not known. Also, the specific components of the RAS pathway that may be crucial in this programing are not well understood.

Along with this, a recent study investigated the hypothesis that maternal LPD in mice leads to sexually dimorphic developmental programming of the components of the pulmonary renin-angiotensin system, which may be vital in the maternal LPD-associated development of hypertension using real-time PCR and western immunoblot assays. The study reported that female mice offspring from LPD had significantly higher mean arterial blood pressure, compared to the offspring from dams on the normal diet. The study also showed that in response to maternal LPD, ACE-2 is not regulated at the transcriptional level, but rather programming occurs at the translational level. The ACE-2 mRNA levels were not significantly affected as a result of maternal protein deprivation, however, the ACE-2 protein levels were significantly lower in the maternal LPD females (Ravi Goyal 2015). They also reported that miRNA 429, which is complementary to ACE-2 was significantly upregulated, which is similar to other studies that have shown that miRNA are important in post-translational gene regulation, and play a crucial role in the developmental programming of hypertension. miRNA can bind to the 3’ UTR of a near-complementary sequence of mRNA and result in reduced translation (Goyal et al. 2011a); (Goyal et al. 2011b). (Ravi Goyal 2015) study suggested that maternal protein restriction leads to increased miR-429, which causes reduced production of ACE-2 protein. Decreased levels of ACE-2 results in reduced degradation of the

potent vasoconstrictor angiotensin II and therefore increased blood pressure. It is therefore worth investigating the mRNA and protein expression of the key components of the pulmonary, cardiac and renal renin-angiotensin system and to examine microRNA complementary to angiotensin converting enzymes ACE-2 in our stored tissue of offspring to illustrate the important of IVF and embryo culture duration in the genesis of hypertension in the adult offspring.

To conclude, our data presented in this chapter support the concept that adult CV function can be determined by developmental responses to embryo environment, and that if these predictions are not met, the adult may be maladapted and at greater risk of CV disease. Our findings show that the package of IVF treatments including, superovulation, IVF procedure, *in vitro* culture and ET or even just ET can alter cardiovascular health of offspring compared with NM control. Our study is the first to demonstrate that male offspring derived from IVF treatment and embryo culture to blastocyst (IVF-ET-BL) have a significant elevation in systolic blood pressure versus offspring derived from IVF treatment and embryo culture to 2-Cell stage (IVF-ET-2Cell). It is also shown that male IVF-ET-BL offspring have elevated serum and pulmonary ACE activity compared with IV-ET-BL. Our novel finding is in agreement with previous human and animal studies, which report preimplantation mammalian embryos from different species appear to be sensitive to their environment in which it develops, either *in vitro* or *in vivo*, for example, in response to culture conditions. This sensitivity may lead to long-term alterations in the characteristics of fetal and/or postnatal growth and phenotype, which have implications for clinical health and biotechnological applications. To our knowledge,

cardiovascular effects in response to ART treatment, especially in respect to the comparison between long and short culture approaches, have not been explored previously in human, partly reflecting the relatively young age of IVF offspring. However, our findings using an animal model cannot be extrapolated directly to human ART.

Chapter 6: Effect of *In Vitro* Fertilisation (IVF) and Embryo Culture Duration on Offspring Metabolic Health

6.1 Introduction

In the previous chapter, we discussed the effect of IVF and embryo culture duration on systolic blood pressure and ACE activity of offspring. Our study shows that male offspring conceived by IVF and cultured to blastocyst stage have significantly elevated blood pressure and ACE activity versus offspring from IVF embryos cultured to 2-cell stage, and with minimal effects observed on female offspring. A great deal of research shows that adults with diabetes are at a two- to four-fold higher risk of CVD and an increased occurrence of elevated systolic and diastolic blood pressure (hypertension); both factors also appear to be connected to excess weight and obesity, although this relationship is complex (Eckel et al. 2006). It is therefore worth investigating the effect of IVF and embryo culture duration on metabolic profile. In this chapter, we study the effect of IVF and embryo culture duration on glucose tolerance test (GTT) and insulin resistance by measuring serum glucose and insulin (See Section 2.5.6).

6.1.1 *Insulin resistance*

Glycogenolysis and gluconeogenesis processes are partly controlled by glucagon. Previous studies show that glucagon is an endocrine hormone released by the α-

cells found in the islets of Langerhans in the pancreas when blood glucose falls, activating the liver to increase glucose release into the blood. When blood glucose levels rise, insulin is secreted by the β-cells. This second hormone encourages uptake and storage of glucose (Makroglou et al. 2006). Insulin is defined as a vigilant regulator of plasma glucose levels and is not secreted if the blood glucose concentration is ≤ 3.3 mmol/l; it is however secreted in increasing amounts as glucose concentrations increase beyond this level. It is comprised of two polypeptide chains, each 51 amino acids long and acts on a variety of tissues through insulin receptors. When blood glucose is high, insulin will first bind to skeletal muscle, promoting its uptake of glucose. It then stimulates glycogenesis in the liver and inhibits glucagon release from the pancreas, causing a reduction in glycogenolysis and gluconeogenesis; see Figure 6.1 (Aronoff et al. 2004).

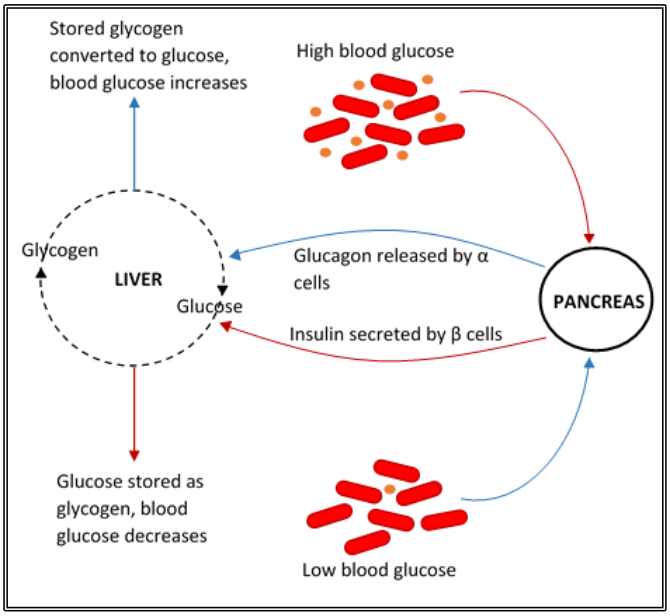


Figure 6.1 Interaction between glucose and insulin in regulating blood sugar levels. Adapted from Makroglou et al. (2006).

It was reported that dysregulation of glucose levels is often associated with metabolic disease, most predominantly diabetes mellitus. This is characterised by hyperglycemia (high blood glucose) due to either faults in insulin secretion, leading to type 1 diabetes, or the body's ability to react to this insulin (American Diabetes 2011). The latter is associated with type 2 diabetes and recognised as insulin resistance. This leads to hyperinsulinemia, in which insulin levels are raised in comparison to glucose present (Shanik et al. 2008). During a pre-diabetic state, individuals may present impaired fasting glucose (IFG) and impaired glucose tolerance (IGT). IFG shows continually elevated fasting glucose levels and IGT reveals intermediately elevated glucose levels 2 hours after glucose tolerance tests, such that the body is not removing glucose from circulation quickly enough (American Diabetes 2011). This pre-diabetic state has increased in prevalence in the US by 34.1% from 2007 to 2010, with prevalence at its highest amongst overweight and obese individuals (Abraham and Fox 2013). This has again been seen in the UK, with an increased prevalence from 11.6% to 35.3% from 2003 to 2011, with 50.6% of individuals over 40 who are classified as overweight being pre-diabetic (Mainous et al. 2014).

6.1.2 *Association between overweight, hypertension and insulin resistance*

As previously mentioned (See Section 1.4), numerous studies surrounding IVF suggest that the technique may be one contributing factor in the development of these disorders. For example, both animal and human IVF offspring have demonstrated adverse metabolic profiles, increased blood pressure (Hart and

Norman 2013a) and insulin resistance, leading to a higher risk of disease such as type 2 diabetes (Chen et al. 2014).

Studies showing overweight and/or obesity may correlate with diabetes, insulin resistance and hypertension in humans has raised deep concern (Chiarelli and Marcovecchio 2008); (Pires et al. 2015). A recent human study shows that obesity-related comorbidities are present in young children, thus providing a platform for early adult cardiovascular disorders (Pires et al. 2015). They reported that insulin resistance was present in 38.1% of obese children. Despite the small number in this study (n= 121), it provides a sufficient number of obese children with reliably high correlation with insulin resistance.

In another human study performed on obese UK children and adolescents, it was reported that the prevalence of the insulin resistance syndrome (IRS) in obesity starts as early as 2 years old. The study summarised the criteria for IRS that include abnormal BMI, abnormalities of glucose homoeostasis, hypertension and dyslipidaemia. They reported that 33% of obese children and adolescents of different ethnicities to have evidence of IRS (Viner et al. 2005).

Similarly, mouse studies have shown that significant correlation exists between perinatal growth and hypertension (Watkins et al. 2008b). In their study, they investigated the effect of maternal diet challenge during the peri-conception period (Emb-LPD) and revealed that offspring weight at week 3 (w3) is positively correlated with weight at week 27 (w27), and systolic blood pressure (the average of at weeks 9, 15 and 21) in male and female offspring. They concluded that perinatal growth

detected at w3 in the Emb-LPD group is a strong predictor of those animals that will exhibit overgrowth and hypertension in later life. (Watkins et al. 2008b). They suggested that this is due to the activation of the blastocyst response to Emb-LPD to stimulate growth which in turn predisposes to adult disease. Disease phenotype such as hypertension can therefore associate with being overweight as appears in Emb-LPD treatment in female offspring.

In another mouse study, female offspring from IVF exhibit weight increase at birth and at w3 compared with *in vivo* controls and these IVF offspring also displayed perturbed glucose homeostasis including higher glucose level after 45 minutes of glucose injection, a higher AUC GTT value, and higher fasting plasma insulin (Scott et al. 2010). The study suggested that the weight increase at w3 might be associated with IVF female offspring being insulin resistant. Collectively, the use of mouse models of embryo manipulation such as IVF (Scott et al. 2010) and alteration in diet such as Emb-LPD (Watkins et al. 2008b) support other studies indicating that manipulation during the peri-conception period may lead to perturbed postnatal development and with perinatal growth at week 3 a strong indicator for adulthood disease risk.

The hypothesis of this chapter is that the environment, in particular the length of embryo culture; long (blastocyst) vs. short (2-cell stage) in which the preimplantation embryo develops can slow down blastocyst development and reduce TE, ICM and total cell number, but may improve metabolism phenotype by reducing glucose AUC and serum Glucose: Insulin ratio in the offspring.

The aim of this chapter was to investigate the effect of IVF and embryo culture duration on metabolic profile by conducting glucose tolerance test (GTT) and measuring serum glucose and insulin in offspring across the five treatment groups. In addition, I have examined whether a correlation exists between these factors and other postnatal factors such as growth weight (Chapter 4) and cardiovascular profile (Chapter 5).

Glucose tolerances test, area under the curve (AUC) and serum glucose, insulin and correlations between treatment groups for male and female offspring were analysed using a multilevel random effects regression model, which takes into account litter size and the maternal origin of the pregnancy.

6.2 Methodology

6.2.1 *Production of naturally mated offspring*

10 C57/BL6 female mice at age of 6-8 weeks were naturally mated with CBA males and allowed to develop to term with their pregnancies (See section 2.1).

6.2.2 *Production of in vivo derived embryos*

3-4 week old C57/BL6 female mice were superovulated and naturally mated with CBA male mice. Embryos were flushed from the oviduct at 2-cell stage and from the uteri at blastocyst stage (See section 2.2.2). Both were placed in pre-warmed H6-BSA under mineral oil for approximately 1-2 hours after flushing to buffer against pH changes until transferred to oviduct (2-cell) or uterus (blastocyst) of their respective foster mother.

6.2.3 *Production of IVF embryos*

Embryos were generated by IVF procedure as described in (See section 2.3.32.2.2) and cultured in KSOM either for ~24 h up to the 2-cell stage or for ~60 hours to the blastocyst stage. Both sets were then placed in pre-warmed H6-BSA under mineral oil for approximately 1-2 hours until transferred to oviduct or uterus respectively of the foster mother.

6.2.4 *Embryo transfer*

The ovaries and the oviducts of anaesthetised 1.5 days pseudopregnant female MF1 mice were carefully exposed from the body cavity and ten (2-cell) embryos were transferred to each oviduct (See section 2.3.3). Litter size was regulated to eight offspring at birth. For the blastocyst embryo transfer, ovaries and the uterus of anaesthetised 2.5 days pseudopregnant female MF1 mice were carefully exposed from the body cavity. Seven blastocysts were then transferred to each uterine horn (See section 2.3.3). Litter size in these two groups was less than eight offspring so litter size correction was not applied.

6.2.5 *Measurement of GTT*

At 27 weeks, mice were fasted overnight (15 hours). Glucose tolerance value (0 minute) was measured by the means of two readings of collected blood samples from the tail vein using a glucometer. The same process was repeated for each time point (15, 30, 60 and 120 minutes) after intraperitoneal glucose injection (See Section 2.5.4).

6.2.6 *Measurement of serum insulin and glucose*

A total of 7 to 8 samples from each treatment, both male and female, were used to measure glucose and insulin levels by glucometer and ELISA kit respectively (See section 2.5.6).

6.2.7 *Statistical analysis of data*

Animal GTT, serum insulin and serum glucose, and correlations were analysed using a multilevel random effect regression model which took into account between-mother and within-mother variation in litter size and parameters measured from individual animals (See section 2.6). Statistical significance was $p \leq 0.05$. If a p value was between 0.1 and 0.051, it considered as a trend.

6.3 Results

6.3.1 *Offspring glucose tolerance test (GTT)*

Glucose tolerance test and AUC results for male and female offspring are shown in Figure 6.2 and Figure 6.3. In male offspring, fasting glucose level and glucose level after (15min, 30min, 1hr and 2hrs) of glucose injection showed significant differences between NM control and all treatment groups. Offspring from the NM control group have significantly the smallest AUC followed by offspring from IVF-ET-BL, IV-ET-2Cell, IV-ET-BL and IVF-ET-2Cell, respectively. The glucose level after 60 and 120 minutes of glucose injection and AUC in IVF-ET-2Cell group were significantly higher compared with IV-ET-2-Cell (P= 0.05, 0.004 ; respectively). Meanwhile, IVF-ET-BL had significantly reduced glucose level after 15, 30 and 120 minutes of glucose injection and significant smaller AUC compared with IVF-ET-2Cell (P= 0.01, 0.026, 0.03, 0.003; respectively). However, the mean IVF-ET-BL group glucose level tended to be higher than IVF-ET-2Cell at (GTT60), this did not reach statistical significance (P=0.06).

In female offspring, fasting glucose level and AUC showed significant differences between NM control and all treatment groups. Offspring from all treatment groups had a significantly higher glucose value compared to the NM control at different time points after glucose injection. At (GTT15), offspring from IV-ET-2Cell group had a significant higher glucose value compared with IV-ET-BL and IVF-ET-2Cell (P= 0.028, 0.02; respectively). However, the IVF-ET-2Cell group glucose level tended to be higher than IVF-ET-BL at (GTT60), this did not reach statistical significance (P=0.06).

AUC for female offspring is shown in Figure 6.3 (B). No significant differences were detected between the four treatment groups; offspring from IV-ET-2Cell had the largest AUC value followed by IVF-ET-2Cell groups then IVF-ET-BL, then IV-ET-BL and NM groups.

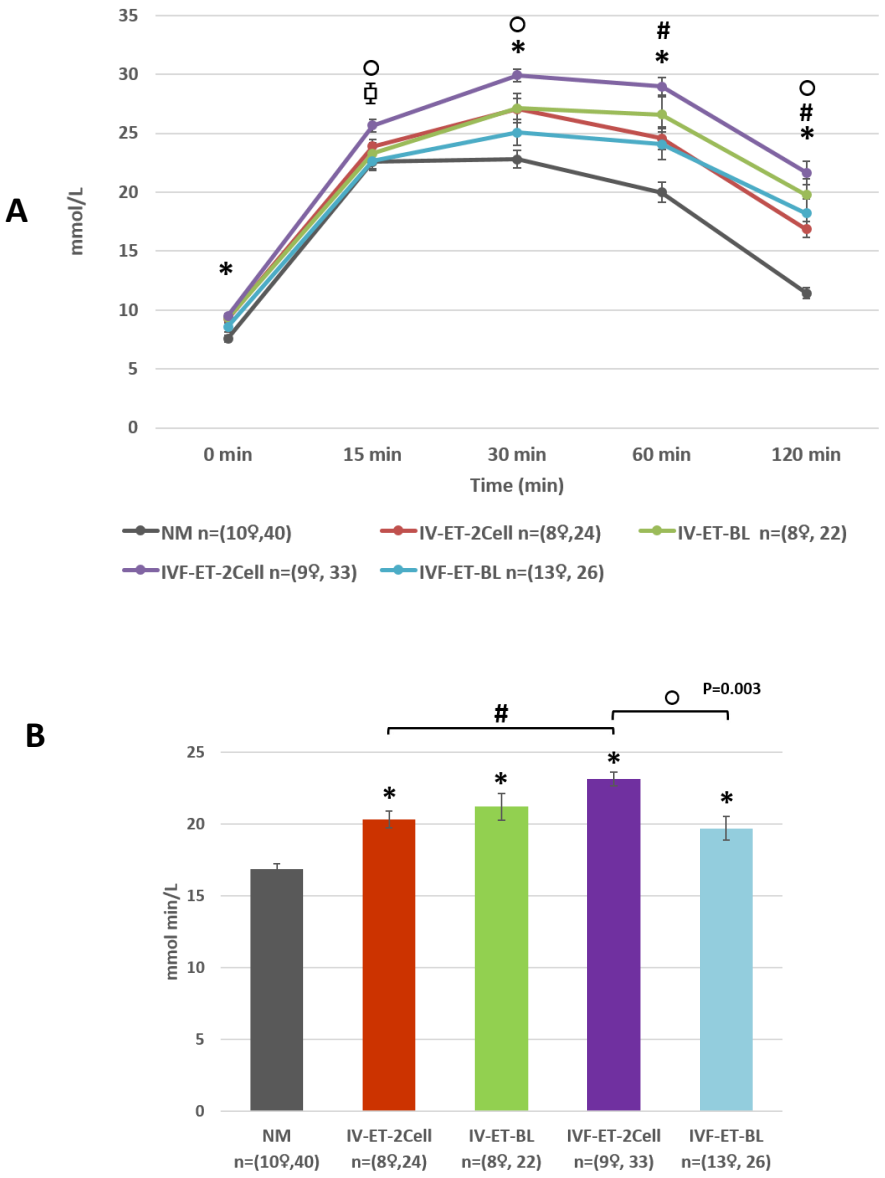


Figure 6.2 Effect of IV and IVF treatment groups and NM control group on male offspring glucose tolerance. (A) Glucose Tolerance Test (GTT) and (B) Area Under the Curve (AUC) at 27 weeks.

IVF-ET and IV-ET groups in male offspring have significant effect on glucose tolerance compared to NM control group. IVF-ET-2Cell group has significant effect compared with IV-ET-2-Cell and IVF-ET-BL groups. ⍈ denote NM vs. (IVF-ET-2Cell), * denote (P≤0.05) between NM and selected groups, # IV-ET-2Cell vs. IVF-ET-2Cell and ○ denote IVF-ET-BL vs. IVF-ET-2Cell. Mean (±SEM) for GTT0, GTT15, GTT30, GTT60, GTT120 and AUC (from 8 – 10 litters); ((n) of mothers or foster mothers ♀, (n) of offspring).

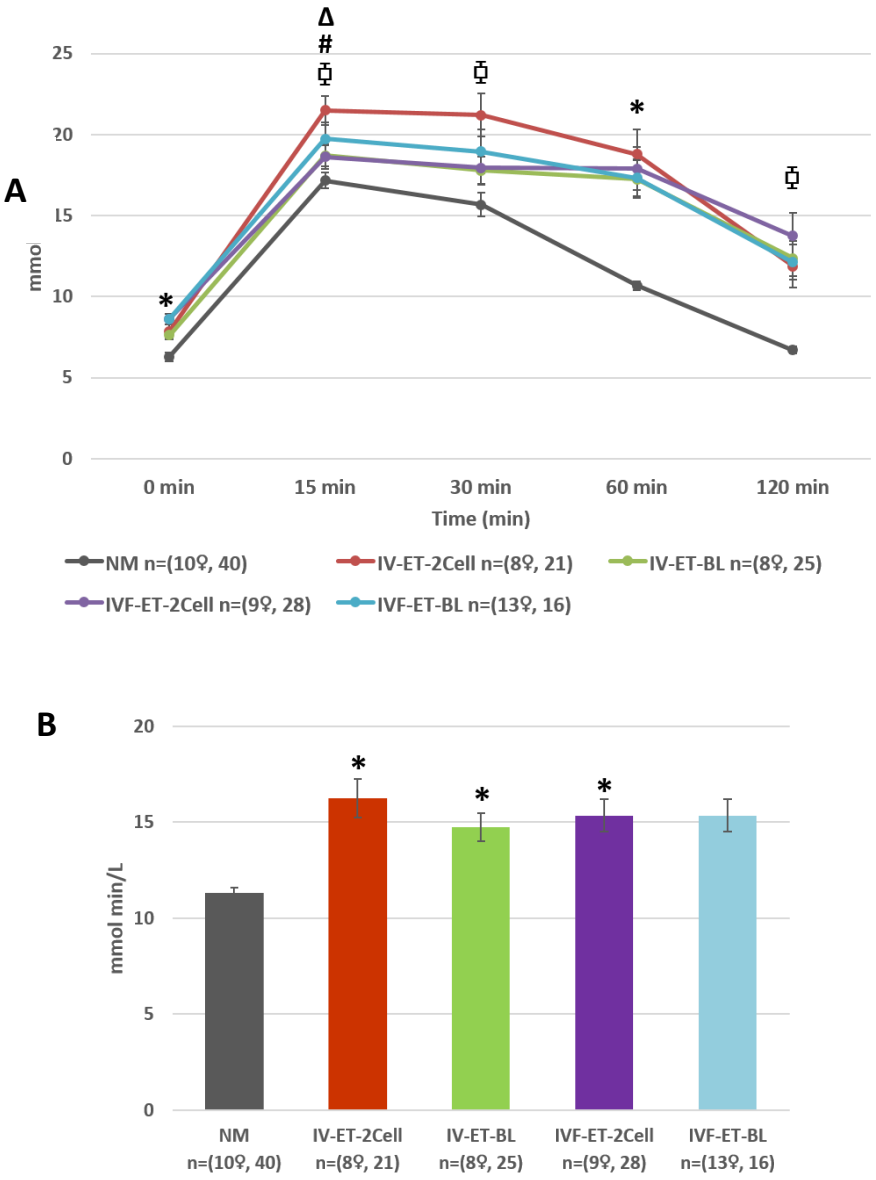


Figure 6.3 Effect of IV and IVF treatment groups and NM control group on female offspring glucose tolerance. (A) Glucose Tolerance Test (GTT) and (B) Area Under the Curve (AUC) at 27 weeks.

IVF-ET and IV-ET groups in female offspring have significant effect on glucose tolerance and AUC compared to NM control groups. * denote (P≤0.05) between NM and selected treatment group. ♁ at (GTT15 and GTT30) denote NM vs.IV-ET-2Cell, and at (GTT120) denote NM vs. all treatment groups except IVF-ET-B, Δ IV-ET-2-Cell vs. IV-ET-BL and # IV-ET-2Cell vs. IVF-ET-2Cell. Mean (±SEM) for GTT0, GTT15, GTT30, GTT60, GTT120 and AUC (from 8 – 10 litters); ((n) of mothers or foster mothers ♀, (n) of offspring).

6.3.2 *Offspring serum glucose, serum insulin and Glucose: Insulin ratio G:I*

Serum glucose, serum insulin and glucose: insulin ratio (G:I) for male and female offspring are shown in Figure 6.4 and Figure 6.5. The mean serum glucose concentration for the different groups of male offspring are shown in Figure 6.4 A, the graph shows that glucose measurements across males in all five treatments appear to be in a range from around 12-17 mmol/L. The least variation was detected within the NM group. IV-ET-BL offspring have a higher serum glucose level compared with IV-ET-2Cell and NM offspring (P=0.026, 0.019; respectively). Otherwise, glucose levels did not differ between groups.

Figure 6.4 B shows the mean serum insulin for the different groups in male offspring, IVF-ET-2Cell offspring have significantly higher insulin levels than NM (P= 0.000), IV-ET-2Cell (P=0.005), and IVF-ET-BL (P= 0.007) offspring. IV-ET-BL offspring also appear to have high insulin levels in comparison to NM offspring (P=0.026). Interestingly, all groups show increased standard error in relation to the NM group, reflecting a large variation between samples.

Calculating the G:I ratio provides information on how effective insulin is in regulating glucose levels with a low ratio indicating insulin resistance. In male offspring (Figure 6.4C), IV-ET-2Cell, IVF-ET-2Cell and IV-ET-BL offspring all demonstrate low G:I ratios in comparison to NM control mice (P=0.005, P=0.001 and P=0.038; respectively).

Female offspring serum glucose, serum insulin and G:I are shown in Figure 6.5 A,B and C respectively. The overall range of glucose levels (around 10-14 mmol/L) is

lower than in the males (Figure 6.4). No treatments were significantly different but the IV-ET-BL treatment showed the lowest serum glucose levels, whereas in males this treatment had the highest serum glucose level. There is a clear difference in glucose levels between genders.

In Figure 6.5B, female IVF-ET-2Cell offspring demonstrate high insulin levels, but this is only significant in comparison to NM offspring (P= 0.029). All other groups appear to have higher insulin concentrations in comparison to the NM group but did not reach statistical significance. All other treatments, as with the males, show quite large standard errors and therefore variation within the samples. In contrast, the range of insulin values within females (around 0.5ng/L to 2ng/L) is lower than in males (around 0.8ng/L to 14ng/L).

G:I ratio for female offspring is shown in Figure 6.5C, similar to male offspring, all female treatment groups had reduced G:I compared with NM controls and this was significant for IVF-ET-2Cell (P= 0.001) and IV-ET-BL (P= 0.048) offspring. Some treatments however, such as IV-ET-BL and IVF-ET-BL groups in males and IVF-ET-BL and IV-ET-2Cell in females, have large variation in G:I ratios and hence large standard errors.

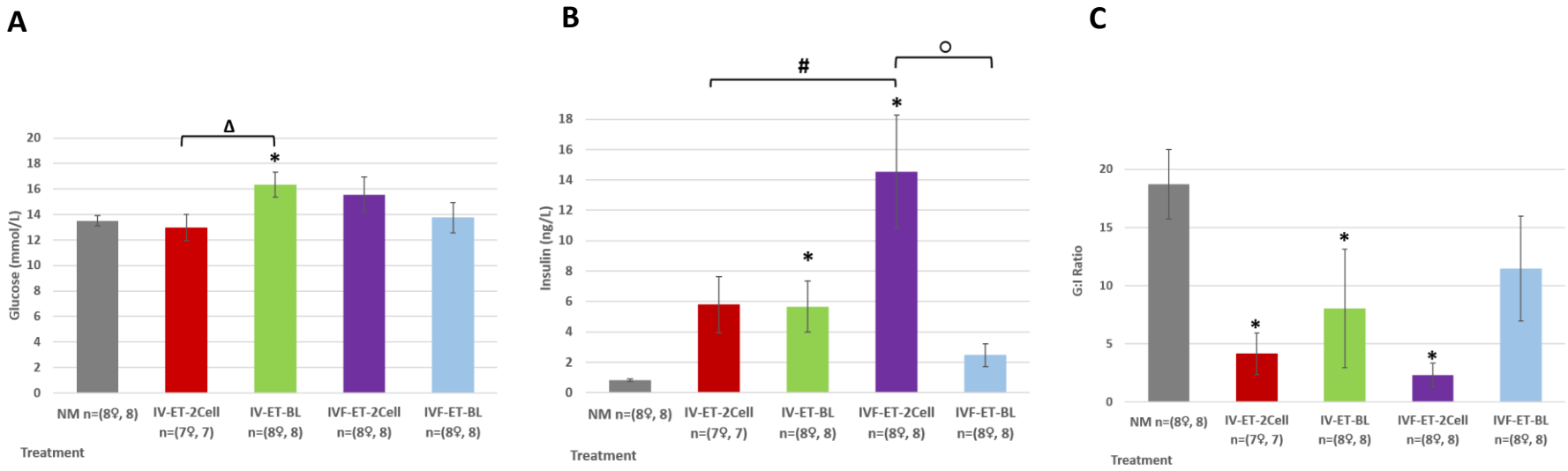


Figure 6.4 Effect of IV and IVF treatment groups and NM control group on serum glucose, insulin and G:I ratio in male offspring.

A) IV-ET- BL group has significant effect on serum glucose compared to NM and IV-ET-2Cell.

B) IV-ET-BL and IVF-ET-2Cell groups in male offspring induce elevation in serum insulin level compared to NM. IVF-ET-2Cell group has significant effect compared to IV-ET-2Cell and IVF-ET-BL.

C) IV-ET-2Cell, IV-ET-BL and IVF-ET-2Cell have significant differences in G:I ratio compared to NM.

* denote (P≤0.05) between NM and selected groups, Δ IV-ET-2-Cell vs. IV-ET-BL, # IV-ET-2Cell vs. IVF-ET-2Cell, and IV-ET-BL, and ○ denote IVF-BL vs. IVF-2Cell (P=0.05); Mean (±SEM) Serum glucose and insulin (from 7 - 8 litters).

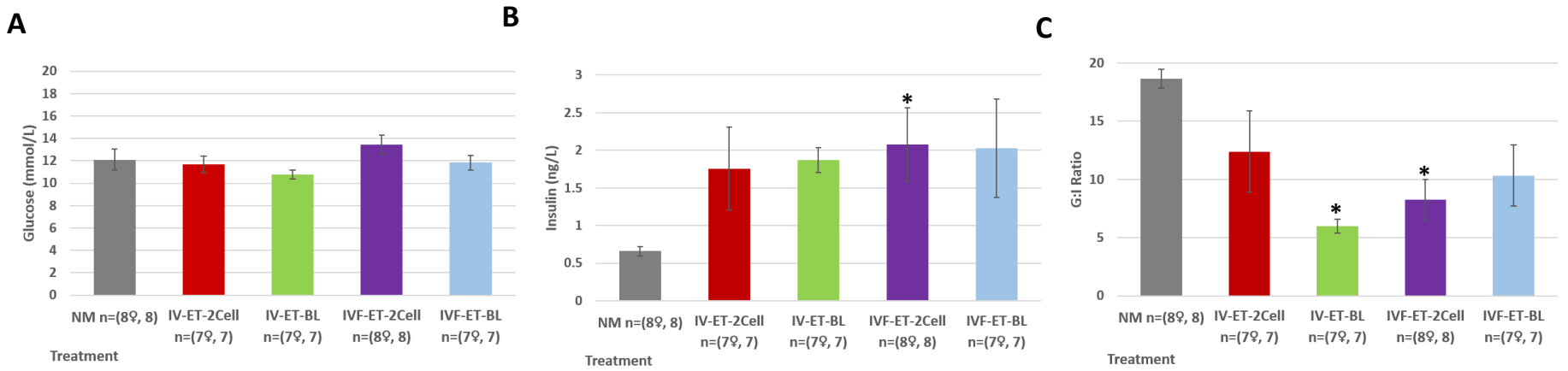


Figure 6.5 Effect of IV and IVF treatment groups and NM control group on serum glucose, insulin and G:I ratio in female offspring.

A) No significant differences in serum glucose level between all treatment groups.

B) IVF-ET-2Cell group in female offspring has elevated in serum insulin level compared to NM.

C) IV-ET-BL and IVF-ET-2Cell have significant differences in G:I ratio compared to NM.

* denote (P≤0.05) between NM and selected groups; Mean (±SEM) Serum glucose and insulin (from 7 - 8 litters).

6.3.3 *Selected correlation analyses on postnatal dataset following undisturbed, in vivo transfer and in vitro transfer*

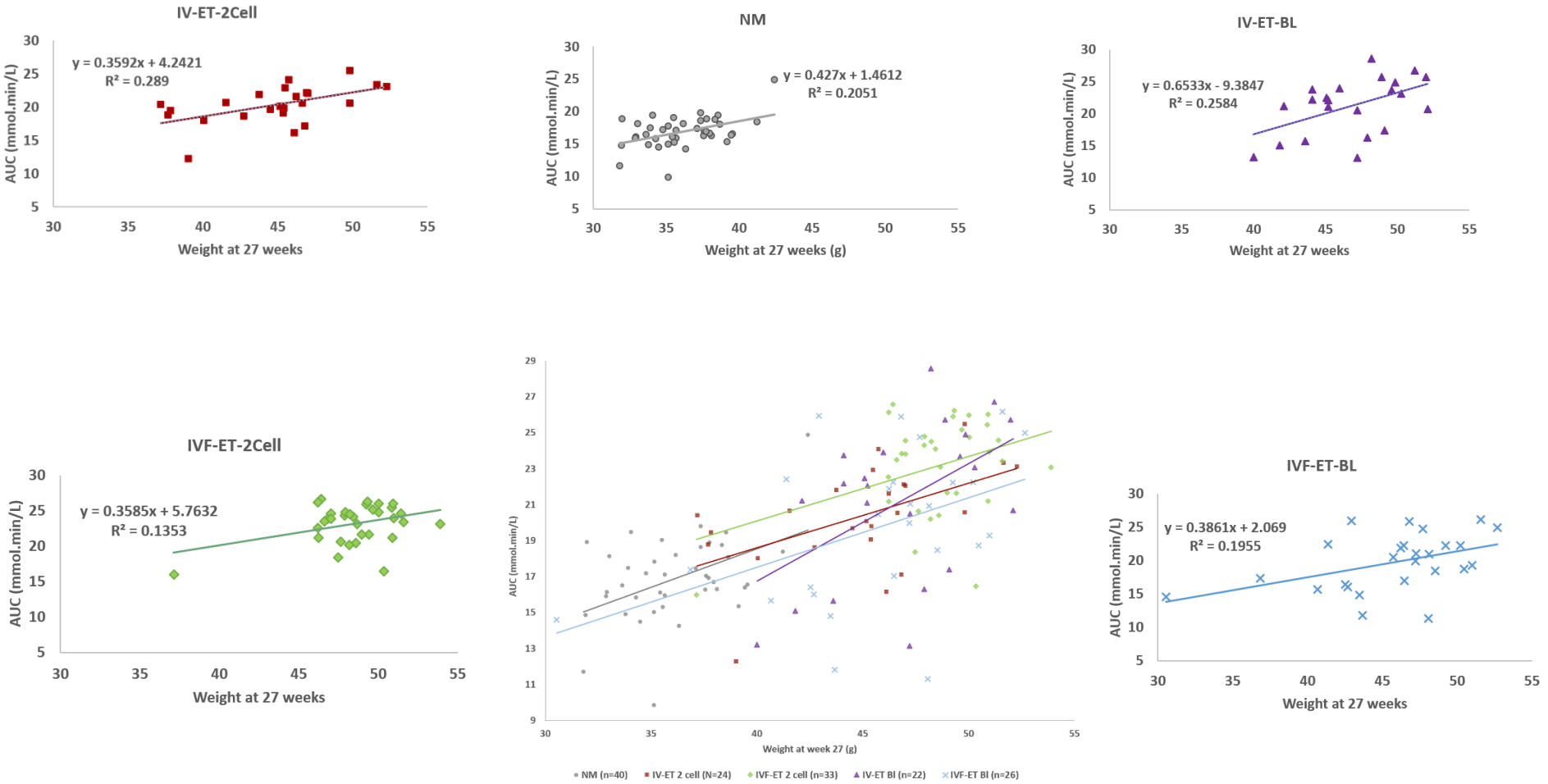
6.3.3.1 Correlation between weight and GTT AUC

Weight at weeks 3, 9, 15, 21 and 27; organs weight, systolic blood pressure at weeks 9, 15, 21 and the average over their life and ACE activities were correlated with GTT as well as serum glucose and insulin levels for all offspring produced over the five treatments. The only factors found to correlate across all five treatments were weight at week 27 with AUC. Interestingly, this relationship is only apparent in males. It is important to note that there was no correlation observed between SBP and GTT, or between SBP and serum glucose and insulin levels.

Figure 6.6 demonstrates the relationships between weight and AUC, in which weight is positively correlated with AUC measurements in normal mating (P=0.001), IV-ET-2Cell (P=0.000), IVF-ET-2Cell (P=0.046), IV-ET-BL (P=0.013) and IVF-ET-BL offspring (P=0.002) with R^2 values of 0.2, 0.29, 0.13, 0.26 and 0.2 respectively.

Figure 6.6 shows that the gradient of the slopes between each treatment does not significantly differ. This means that for each treatment, AUC increased with weight by the same factor. However, the elevation of the slopes for IVF-ET-2Cell and IVF-ET-BL offspring do differ (P=0.0094), with IVF-ET-2Cell offspring showing a higher elevation. This may indicate that, although the two groups increase by the same AUC unit per gram, IVF-ET-2Cell offspring show higher AUC measurements compared to IVF-ET-BL offspring of the same weight.

Figure 6.6 Correlation between weight at 27 weeks and GTT AUC in male offspring from five treatment groups



6.3.3.2 Correlation between serum glucose and insulin

Glucose and insulin values were correlated with health factors for the same pups, collected during their lifetime. These health factors were: weight at weeks 3, 9, 15, 21 and 27; systolic blood pressure at weeks 9, 15, 21 and the average over their life; fasting glucose levels and the area under the curve (AUC) obtained during a glucose tolerance test prior to culling.

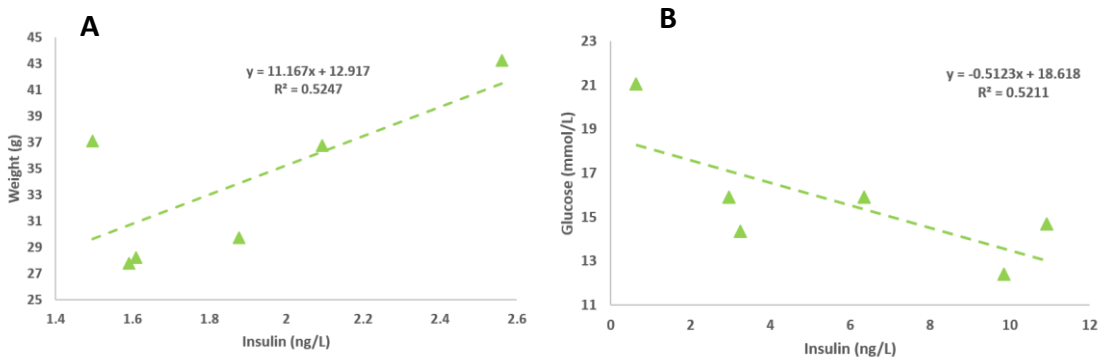


Figure 6.7 Correlation between serum insulin and body weight in IV-ET-BL female offspring (A), and between serum insulin and glucose in IV-ET-BL male offspring (B)

The only relationships found were within the IV-ET-BL male and female offspring. Female serum insulin levels appear to correlate with weight at 15 weeks of age, with 52% of the variation being explained by this model (Figure 6.7 A), a significant P value of 0.017 validates that a relationship exists. As insulin levels increase, weight appears to also increase.

In contrast, male serum concentrations appear to correlate with glucose concentrations, again with 52% of the variation being explained by the model,

(Figure 6.7 B). This relationship has a significant P value of 0.022, suggesting that as insulin levels increase, glucose levels appear to decrease.

6.4 Discussion

The aim of this chapter was to investigate the effects of IVF, length of embryo culture and embryo transfer on the postnatal health of offspring, primarily in terms of glucose tolerance (GTT) as well as glucose, insulin concentrations within serum and insulin:glucose ratio (G:I) as an indicator for insulin resistance.

Our study shows that in all groups analysed (NM, IV-ET-2Cell, IV-ET-BL, IVF-ET-2Cell and IVF-ET-BL) and all GTT conditions (GTT0, GTT15, GTT30, GTT60, GTT120), mean glucose levels in male were significantly higher than female. In male and female, glucose fasting level (GTT0) did not differ among all groups. This finding is similar to that reported by (Donjacour et al. 2014).

The novel finding of our study is that male offspring from IVF-ET-2Cell firstly were clearly glucose intolerant, as shown by the GTT (Figure 6.2). The glucose level after 60 and 120 minutes of glucose injection and AUC in IVF-ET-2Cell group were significantly higher compared with IV-ET-2-Cell (P= 0.05, 0.004 ; respectively) and secondly, was significantly higher than that of IVF-ET-BL after 15, 30 and 120 minutes of glucose injection and significant higher AUC (P= 0.01, 0.026, 0.03, 0.003; respectively). This indicates that in ART, in which the IVF procedure is coupled with a short duration of embryo culture prior ET may act as a negative factor for adult metabolism in a sex-specific manner compared with longer duration of culture.

Our study indeed showed some similarities to Donjacour et al (2014) who studied the effect of IVF on glucose metabolism using a mouse model. In their study, they

compared offspring conceived by IVF and cultured to blastocyst stage in either Whitten medium ((IVF_{WM}) or K simplex optimized medium (KSOM) with amino acids ($IVF_{KAA)}$ group against controls, which are normal mating NM and *in vivo* derived embryos and immediately ET at blastocyst stage (FB). The study reported that male IVF_{WM} mice (but not females) were larger during the first 19 weeks of life and showed glucose intolerance. They also examined the insulinogenic index, the amount of insulin secreted for a given glucose stimulus, which is calculated by dividing the area under the curve for insulin by the area under the curve for glucose and they found that the insulinogenic index was significantly greater in FB males than in either IVF_{WM} or IVF_{KAA} male. The study suggested that the IVF mice seemed to be slightly more sensitive to insulin. Moreover, the decrease in the insulinogenic index suggests a decreased ability to secrete insulin in response to glucose, likely because of beta cell insensitivity to glucose (Donjacour et al. 2014). The cause for glucose intolerance could be a pancreatic defect with altered insulin production (Tura et al. 2006).

Interestingly, our study and (Donjacour et al. 2014) study have reported that the type of conditions of environment encountered during the preimplantation period can affect adult metabolism. In addition, the postnatal glucose homeostasis following IVF may be sexual dimorphic. However, our study showed that male IVF-ET-2Cell were glucose intolerant compared with NM and IVF-ET-BL, whereas (Donjacour et al. 2014) have observed the glucose intolerance in IVF offspring, which is equivalent to our IVF-ET-BL, compared with NM and FB, which is equivalent to our IV-ET-BL. This indicate that ET at early stage of embryo development (2-cell stage)

and disturbance at this crucial stage, which denotes genome activation in mice (Niakan et al. 2012), may account as a crucial contributor to initiate the start of future metabolic dysregulation.

Moreover, in our study, both IVF-ET-2Cell and IVF-ET-BL offspring were derived by IVF procedure and experienced two types of embryo culture medium, HTF, which lacks amino acids (Kleijkers et al. 2016) and KSOM with amino acids; however the differences between the two treatment groups was the length of culture in KSOM and the stage of ET. It is important to note that IVF embryos were cultured in HTF medium from zygotes to 2-cell stage, then embryos were cultured in KSOM for either 4-5 hours prior to ET (IVF-ET-2Cell) or for approximately 68 hours (to blastocyst stage) prior to ET (IVF-ET-BL). The reason behind changing the medium in the IVF procedure that we conducted in our experiment is to improve fetal development. Studies have shown that *in vitro* culture medium which includes amino acids leads to improved fetal development. Optimal development *in vitro* and *in vivo* occur after embryo transfer when embryos at cleavage stage were cultured with non-essential amino acids and glutamine for 48 h followed by development to the blastocyst stage with all 20 amino acids (Lane and Gardner 1997). Fetal development per blastocyst transferred was increased if the embryos were cultured with non-essential amino acids and glutamine for the first 48 hours of development (from E0 to E1.5) compared with either essential amino acids without glutamine or all 20 amino acids. Furthermore, culture with essential amino acids without glutamine or all 20 amino acids from the eight-cell stage to the blastocyst significantly increased both fetal development per blastocyst transferred and fetal mass (Lane and Gardner 1997). It is

shown that amino acids are well characterised to affect cell function and differentiation in early embryogenesis with long lasting positive consequences on fetal growth (Eckert et al. 2015).

Along with this, in our study, IVF-ET-2Cell offspring, which experienced short culture during their preimplantation development and ET at early stage, showed high glucose intolerance in their postnatal life compared with those that experienced long culture and ET at blastocyst stage (IVF-ET-BL). Indeed, IVF-ET-2Cell offspring are derived from embryos generated by IVF and then cultured in medium lacking amino acids, then their development is disturbed twice, when they were cultured for short period of time 4-5 hours in KSOM and then ET at this crucial stage of development. Thus, the combined effects of all these factors close to the time of embryonic genome activation may therefore alter their metabolism more profoundly that the offspring of the IVF-ET-BL group.

As discussed in (Chapter 5), culture media can cause long term reprogramming of development and health, and this is likely mediated by epigenetic changes. Research show that embryo culture specifically in suboptimal conditions disturbs the maintenance of the embryonic epigenome in the mouse and potentially in human preimplantation embryos (Market-Velker et al. 2010); (Velker et al. 2012); (White et al. 2015). Therefore, both IVF-ET-2Cell and IVF-ET-BL embryos may be affected by culture conditions during their preimplantation development. However, IVF-ET-2Cell embryos underwent ET procedure after a short period of culture and at a crucial stage of development which may affect the metabolism profile of these embryos and

then show altered glucose tolerance later in life, whereas IVF-ET-BL have more time to adapt to culture conditions and then underwent ET at blastocyst stage. It is reported that embryos at the blastocyst stage are equipped with signalling pathways that can 'read' the metabolic cues and then change their developmental trajectory as a result. These changes in developmental plan are described as compensatory, optimizing the nutrient supply from mother to embryo to protect growth (Fleming et al. 2015a); (Sunde et al. 2016). Although this dialogue has reported to be the core component of embryo programming that may lead to adult onset disease phenotype such as cardiovascular disease that is observed in our IVF-ET-BL offspring, it could be beneficial to protect embryo's metabolic phenotype. This may interpret the glucose intolerance observed in our IVF-ET-2Cell vs IVF-ET-BL offspring; however, more studies are needed to define the long-term effects of IVF, duration of culture and ET at different stages of development on glucose metabolism.

Insulin resistance is generally regarded as a pathological condition in which cells fail to respond to the normal actions of the hormone insulin. Higher ACE, glucose and insulin values are suggestive of insulin resistance, but can only be confirmed by low G:I ratio. Individuals with higher AUC and glucose levels are indeed producing more insulin, but their bodies did not respond to the insulin secreted so they are resistant to insulin. Looking back to the G:I ratio in male offspring for IVF-ET-2Cell, IV-ET-2Cell and IV-ET-BL respectively, Figure 6.4, we can see a very low value indicative of a huge excess of insulin in relation to glucose present. This may indicate insulin resistance in IVF-ET-2Cell, IV-ET-2Cell and IV-ET-BL when compared to NM. This

further highlights the detrimental effect of IVF combined with ET at 2-Cell stage or ET *per se*.

In our study, male offspring from IVF-ET-2Cell initially appear to have normal glucose levels (Figure 6.4 A), but their insulin levels are dramatically increased (Figure 6.4 B). This again is reflected in their G:I ratio, which is markedly reduced (Figure 6.4 C). This indicates that IVF, short duration culture and ET at 2-cell stage can induce insulin resistance in a sex-specific manner. Our finding is supportive of (Chen et al. 2014) findings, which demonstrate the occurrence of insulin resistance in both human and mouse offspring derived from IVF and embryo culture. In their study, they examine insulin sensitivity and metabolic risk factors in young adults conceived by IVF or natural conception (NC) after consuming an energy-balanced diet (30% fat) and after 3 days of high-fat–overfeeding challenge (45% fat). In parallel, they compared key metabolic factors in adult male offspring that were generated by IVF and ET at blastocyst stage versus those that were naturally conceived using a mouse model (C57BL/6J mice). The study shows that adult humans conceived by IVF, the majority of whom were of normal weight, were more insulin resistant than NC individuals matched for BMI, sex, and age. Furthermore, mice conceived by IVF displayed increased fasting glucose levels, impaired glucose tolerance, and hepatic insulin resistance at both normal and high body weight after 8 weeks of consuming either a chow or high-fat diet (Chen et al. 2014).

In Figure 6.4 (A), glucose serum concentrations were at their highest level in IV-ET-BL males. This effect was not only observed in comparison to the NM control group, but

also to IV-ET-2Cell counterparts. However, there was no change in glucose levels of the IVF treatment male offspring. This may suggests that embryo transfer at a later developmental stage may lead to an increase in glucose concentrations in offspring during their later life.

Insulin measurements from the same serum allow us to understand the level of hormonal control underlying the glucose levels detected Figure 6.4 (B). Insulin levels of the same IV-ET-BL males were significantly higher than those from the NM males. This can then be followed into the low G:I ratio for the offspring (Figure 6.4 C). This may suggest that offspring from IV-ET-BL appear to be demonstrating some form on insulin resistance.

This relationship is even more apparent with the correlation of the two factors (Insulin and Glucose) for each individual, in which male offspring with very high insulin levels had lower glucose levels Figure 6.7 (B). Although it is difficult to assume causation, awareness of insulin's regulatory control over glucose (Aronoff et al. 2004) suggests that these individuals may be over producing insulin to reduce glucose levels. It is important to note that, even with excess insulin, the glucose levels found are still notably high. The increased insulin levels may still fail to maintain glucose levels within a normal range.

Our study shows that generally, females appear to have a more subtle response to ART procedures, with glucose levels all remaining within the same range of the NM group (Figure 6.5A), and all insulin levels remain reasonably low (Figure 6.5B). Similar to male offspring, IVF-ET-2Cell female offspring have higher insulin levels than the

NM group (Figure 6.5B), and then a significantly lowered G:I ratio (Figure 6.5C). A low G:I ratio was similarly observed in IV-ET-BL offspring, As these results, to varying extents, are also observed in females, it reinforces the ideas discussed above as to the main ART factors affecting glucose regulation.

Interestingly, male offspring from all treatment groups show a relationship between weight at week 27 and AUC. Figure 6.6 shows body weight at week 27 is positively correlated with AUC measurements in offspring from all treatment groups, Individuals of higher weights demonstrates higher AUC values. The AUC represents the average glucose concentration found in each mouse over the GTT. Higher AUC values are suggestive of insulin resistance. It is shown that body fat in IVF males, which were glucose intolerant, was greater than FB males (Donjacour et al. 2014). In addition, numerous epidemiologic studies have shown that the risk for diabetes, and presumably insulin resistance, rises as body fat content increases, measured by body mass index BMI from the very lean to the very obese, suggesting that the dose of body fat has an effect on insulin sensitivity (Kahn and Flier 2000). It is shown that by 3 days of HFD, body weight and adipose tissue mass, as well as insulin resistance, were clearly increased in wild-type mice (Lee et al. 2011). However, the correlated relationships within all the treatments do however have low R^2 values, this may suggest that there may be other elements interplaying with these factors. This emphasises the complex nature of metabolism and its regulation.

Female offspring from the IV-ET-BL group also demonstrate a potential relationship between insulin and weight. Within the group, individuals with higher insulin levels

also appear to weigh more at week 15 (Figure 6.7A). These individuals already appear to demonstrating insulin resistance, a symptom of type II diabetes. It is shown that type II diabetes is also commonly associated with obesity due to the increased uptake and storage of adipose as a result of insulin (Kahn and Flier 2000). In our study, this correlation observed in IV-ET-BL individuals therefore suggests that the raised insulin concentrations detected may be consistent throughout life, influencing weight gain and suggesting symptoms of diabetes or a pre-diabetic state within IV-ET-BL offspring. Although this is important to note as a possible concern, it is currently only based on correlation so cause cannot be deduced. Further study into whether these offspring are developing diabetes as a result of embryo transfer appears necessary.

Collectively, our data show that long-term effects of embryo culture (BL) associated with IVF can alter the offspring metabolic profile in mice, evident by the significant differences detected in IV and IVF treatment offspring when compared to NM control offspring. Our study is the first to demonstrate that mouse offspring derived from IVF treatment and embryo culture to 2-Cell stage (IVF-ET-2Cell) have a significantly poorer glucose homeostasis and insulin resistance phenotype versus offspring derived from IVF treatment and embryo culture to blastocyst (IVF-ET-BL). This may relate back to the environmental sensitivity known to exist in the pre-implantation embryo (Lonergan et al. 2006). The embryo, already developing in an unusual environment and therefore subject to maladaptation, is then transferred to another environment at the 2-cell stage, which denotes genome activation in mice (Niakan et al. 2012). Disturbance at this crucial stage whilst already vulnerable may

therefore be enough to initiate the start of future metabolic dysregulation. Mechanisms for this may include interference of epigenetic programming. Imprinting genes in particular are especially susceptible to methylation changes during this period (Khosla et al. 2001). Alteration in this modification has already been hypothesised to be a factor in the large offspring syndrome (LOS) observed in animals produced from *in vitro* culture and embryo manipulation, highlighting the possibility of these alterations being carried on into postnatal life (De Rycke et al. 2002). The removal of the embryo from an environment, specifically at crucial stage when genome activation occurs in order to implant into a new environment, in which they must re-establish, may therefore disturb one of the many following developmental events. Further study would be required to firstly examine this hypothesis, and secondly to see if this disturbance has the capacity to affect glucose control in later life. Importantly, one must use caution when extrapolating findings in mice to the human condition.

Chapter 7: General Discussion and Future Work

7.1 General Discussion

In vitro fertilisation (IVF) is a vital and crucial tool in reproductive technology to overcome infertility problems. However, research show that the IVF procedure is not risk-free and might have an immediate effect on embryo and/or long-term effect on offspring or babies born through IVF techniques. This study reports the effect of IVF and prolonged embryo culture using a mouse model on offspring cardiovascular health and it is the first report that evaluates the effect of IVF and prolonged embryo culture on offspring metabolic health. My study reports the effect of IVF and prolonged embryo culture on ET efficiency (implantation rate/ no of embryo transferred); TE, ICM and total cell number; offspring growth, systolic blood pressure (SBP), ACE activity, glucose tolerance test (including AUC), serum glucose and insulin levels and organ: body weight ratios after culling at 27 weeks in all groups. The study also correlates distinct parameters such as body weight, SBP, ACE, AUC, serum glucose and insulin levels across the treatment groups.

The first key point highlighted in this study is that the routine procedures used in ART, including ovarian stimulation, IVF technique, embryo culture and embryo transfer to generate mouse embryos can have lasting effects on postnatal physiology. Although culture has long been recognized to delay early development relative to *in vivo* environment (Harlow and Quinn 1982); (Fleming et al. 2004); (Watkins et al. 2007), the extent of postnatal consequences of the *in vitro*

environment, specifically the duration of embryo culture, has not been studied extensively (Fleming et al. 2004); (Watkins and Fleming 2009). We report that cardiovascular and metabolic changes in the adult may derive from *in vitro* embryo manipulation. Broadly, our findings also show that the postnatal consequences of ART are similar to those resulting from early gestational maternal undernutrition (Fleming et al. 2004); (Watkins and Fleming 2009); (Fleming et al. 2015a). Hence, abnormal early environment, whether *in vivo* or *in vitro*, may have long-term health outcomes.

7.1.1 *Effect of embryo manipulation on developmental rate and cell number*

The second finding in our study is that IVF and prolonged embryo culture affects embryo developmental rate compared with *in vivo* embryos. our result agrees with a previous study which demonstrated that *in vitro* cultured embryos develop at slower rate when compared with *in vivo* derived embryos at the same time post hCG (Watkins et al. 2007).

Another important finding is that IVF and long culture can induce a decrease in the number of cells at the blastocyst stage; ICM, TE and total cell number were significantly reduced in IVF embryos compared with *in vivo* derived embryos. Similarly, (Watkins et al. 2007) reported that changes in the number of cells of the ICM and TE lineages occur as a result of embryo culture. Decreased cell number in blastocysts has been associated with decreased placental and fetal weight in a mouse model of mitochondrial dysfunction (Wakefield et al. 2011). Fetal development was not examined in our study, but it has been reported that ART,

including certain culture media conditions, is associated with fetal growth restriction in early to mid-pregnancy, resulting in a substantial increase in placental size and accelerated fetal growth toward the end of gestation in all species (Bloise et al. 2014). In mice, it has been reported that *in vitro* embryo culture affects placental and fetal weights, as well as the placenta/fetus ratios (Delle Piane et al. 2010). We also did not analyse birthweight in our study, but it is important to note that male offspring from IVF-ET-BL and IVF-ET-2Cell treatments show significantly decreased body weight at week 1 compared with NM, IV-ET-BL and IV-ET-2Cell respectively ($P \leq 0.05$), this may indicate that offspring from IVF-ET-BL and IVF-ET-2Cell may have experienced low birthweight. Recent human research reported that there is a significant effect of embryo culture medium used in IVF on birthweight of offspring (Kleijkers et al. 2016). It has been reported that rodent offspring developing high blood pressure in adulthood demonstrated alteration in cell number at the blastocyst stage after experience of undernutrition or *in vitro* culture throughout their preimplantation period (Kwong et al. 2000); (Watkins et al. 2007). Interestingly, male offspring from IVF-ET-BL showed 'catch up' growth at week 2 compared with IVF-ET-2Cell ($P \leq 0.05$), this may also account for alteration in cardiovascular phenotype that is observed in their offspring later in postnatal life. This indeed is in agreement with Barker's hypothesis that showed that boys who were thin at birth but whose weight caught up in early years had increased coronary heart disease (Eriksson et al. 1999). Another recent finding supports rapid postnatal catch-up growth of LBW neonates as a more important factor than LBW alone in cardiovascular disease risk in later life (Kelishadi et al. 2015).

It is shown that embryos taken out of their normal *in vivo* environment within the mother and placed into *in vitro* culture often delay their developmental progress and change their gene expression and metabolic profile in accordance with the *in vitro* conditions (Bell et al. 2008). However, depending on the species, most embryos still retain their capacity to implant and continue developing into viable offspring after ET into the mother (Eckert and Fleming 2011). The question that arises from these findings is whether the embryos would be able to survive after ET as development might be compromised by IVF and length of culture.

7.1.2 *Effect of embryo manipulation on pregnancy rate, ET efficiency and litter size*

Our results in chapter 4, were able to answer the question, where we have shown the mean number of offspring obtained from *in vivo* and IVF groups. Despite the fact that the TE and ICM number were reduced in IVF embryos, they were still able to give rise to fetuses. This is similar with (Iwasaki et al. 1990) who reported bovine embryos with reduced cell number are able to give rise to fetuses. However, the mean number of offspring from IVF and *in vivo* groups, except the IVF-ET-2Cell group, were significantly reduced from the naturally mating (NM, undisturbed) group, so this might indicate that alteration in cell number affected the implantation or pregnancy rate in mouse. This was in agreement with (Iwasaki et al. 1990) who suggested that reduced ICM in bovine embryos following IVF may be the cause for low pregnancy rates. Our study showed that IVF and prolonged embryo culture affects ET efficiency (implantation rate/ no of embryos transferred) and litter size

Since most IVF children have not yet reached adulthood and follow-up studies are still limited by this, it is worth investigating the long-term consequences using a mouse model. There are a number of animal studies on altered metabolic (Donjacour et al. 2014) and cardiovascular (Rexhaj et al. 2013) physiology associated with *in vitro* fertilisation and altered normal genomic imprinting, specifically DNA methylation patterns (Manipalviratn et al. 2009b); (Grace and Sinclair 2009); (Market-Velker et al. 2010).

7.1.3 *Effect of embryo manipulation on postnatal growth and organ allometry*

Another important point is that the combination of IVF treatments (superovulation, IVF procedure, embryo culture and ET) affect offspring growth regardless of sex. This is in agreement with previous studies that reported that control mice tend to have bigger litters than those in ART groups (Watkins et al. 2007); (Scott et al. 2010); (Donjacour et al. 2014). This may be interpreted that restricted growth in larger litters can be attributed to overcrowding within the uterus, preventing the fetus from growing to maximum size, and high competition for maternal blood supply (Foxcroft et al. 2009). This however does not affect our statistical findings because although the controls were smaller in weight compared with all treatment groups, our statistical analysis was independent of litter size.

In this study, we showed that IVF and prolonged embryo culture altered organ weight of offspring in a non-specific manner. Our study showed that in males, the lung: body weight ratio and heart: body weight ratio for the NM group was significantly higher than IVF-ET-2Cell. Heart: body weight ratio for the IVF-ET-BL

group was significantly higher but the liver: body weight ratio was significantly smaller than IVF-ET-2Cell group. In females, lung: body weight ratio and heart: body weight ratio were significantly increased compared with the NM group across all treatment groups. In mice, previous studies show that embryo culture have minimal effect on organ body weight including left and right kidneys and lung (Watkins et al. 2007). It is believed that is the phenomenon of large offspring syndrome (LOS) is the most dramatic display of how fetal and postnatal development can be affected in response to manipulating the preimplantation embryo. It has been shown that *in vitro* culture in the presence of serum leads to LOS in cattle and sheep (Sinclair et al. 1999). LOS is associated with significantly enhanced fetal and postnatal growth, altered organ sizing, changes in patterns of gene expression, and increased rates of perinatal death (Watkins et al. 2008a).

7.1.4 *Effect of embryo manipulation on offspring systolic blood pressure SBP*

The vital finding in current study is that male and female offspring of the four ET groups have significantly higher SBP compared with NM control offspring at weeks (9, 15 and 27) and LIFE. Our data also showed that male and female from the four ET groups were heavier compared with NM controls. This may indicate that a combined effect of IVF procedure, culture and ET most probably are behind the increase in body weight and SBP in offspring regardless of sex. However, previous experiments examining the effect of undernutrition (Watkins et al. 2008c) and *in vitro* culture (Watkins et al. 2007) during the preimplantation period in mice showed increased

SBP in offspring form both genders, but only in females, the increase in SBP was accompanied with increased body weight during postnatal development.

Another novel finding is that male offspring from IVF-ET-BL firstly had a significantly higher SBP at weeks 15, 21 and LIFE compared with IV-ET-BL offspring and secondly, were significantly higher than that of IVF-ET-2Cell at weeks 15, 21 and LIFE. This indicates that ART may act as a negative factor for adult hypertension with duration of embryo culture exacerbating the effect but with ET alone still contributing to this effect. The effect of embryo culture on embryonic, perinatal and postnatal parameters has been documented in the literature, it is therefore not surprising that long embryo culture involved in IVF, with normalised SO, IVF and ET led to increased SBP in IVF-ET-BL offspring compared with IVF-ET-2Cell.

In support of our results, experimental evidence has reported that altered cardiovascular phenotype during postnatal life can be programmed during the periconceptional period when crucial reproductive events such as preimplantation embryo development are exposed to effective environments such as ART-related procedures in human (Hart and Norman 2013a); (Hart and Norman 2013b); (Sunde et al. 2016) and mice (Watkins et al. 2007); (Rexhaj et al. 2013). In mice, (Watkins et al. 2007) has shown that preimplantation embryo culture and transfer led to elevation of systolic blood pressure at week 15 compared to controls (undisturbed), regardless of gender. (Rexhaj et al. 2013) performed IVF using FVB mice and reported that male offspring had increased mean blood pressure at week 14 of age compared with normal mating controls. It is demonstrated that embryo culture

specifically in suboptimal conditions disturbs the maintenance of the embryonic epigenome in the mouse and potentially in human preimplantation embryos (Market-Velker et al. 2010); (Velker et al. 2012); (White et al. 2015). This indicates that long culture can cause long-term reprogramming of development and health, and this is likely mediated by epigenetic changes.

Our findings support the concept that embryo sensing of its environment can alter developmental programming (Fleming et al. 2015b); (Sunde et al. 2016). It has been shown that embryos at the blastocyst stage are equipped with signalling pathways that can read the metabolic cues and then change their developmental trajectory as a result. These changes in developmental plan have been described as compensatory, optimizing the nutrient supply from mother to embryo to protect growth (Fleming et al. 2015a); (Sunde et al. 2016). Embryonic sensing of a low nutrient environment in culture media can therefore program the embryo to be thrifty in its metabolism, which may lead to programming a long-term thrifty phenotype (Sunde et al. 2016). This dialogue may be the core component of embryo programming that leads to adult onset cardiovascular disease that we observe in our IVF-ET-BL offspring.

7.1.5 *Effect of embryo manipulation on offspring serum and lung Angiotensin Converting Enzyme ACE*

Angiotensin Converting Enzyme (ACE) is an important factor in the regulation of blood pressure. Therefore, we decided to examine the effect of IVF and prolonged culture on serum and lung ACE activity. Our study reported that male offspring from

IVF-ET-BL group (which had the highest blood pressure at 15 and 21 weeks of age and LIFE) did have the highest serum and lung ACE activity at 27 weeks of age. However, the only difference detected in female offspring was between IVF-ET-BL group and NM within serum ACE activity and this did not reach significance. We therefore report that IVF and prolonged embryo culture can programme the development of high lung and serum ACE activity in adulthood in a sex-specific manner. Hence, one factor behind increasing SBP in IVF-ET-BL male offspring may result from altered renin-angiotensin system. Here is another similarity with (Watkins et al. 2007) who reported that female, but not male, offspring derived *in vitro* and ET, which had the highest blood pressure at 21 weeks of age had the highest serum ACE activity at 27 weeks of age. Similarly, (Krege et al. 1995) examined the role of the ACE gene in blood pressure control and reproduction using mice generated to carry an insertional mutation, which is designed to inactivate both forms of ACE. They showed that heterozygous males but not females had blood pressures that were 15-20 mm Hg less than normal, and both male and female heterozygotes had decreased serum ACE activity.

Another novel finding is lung ACE level within IVF-ET-BL male offspring was positively correlated with SBP at weeks 21 of age and LIFE. Individuals of higher SBP demonstrate higher lung ACE values. It may therefore be hypothesised that increased level of ACE enzyme, or an increased activity of existing ACE, may result in an elevated blood pressure. However, ACE activity elevation has also been demonstrated to correlate with hypertension in other models of early embryo programming, particularly in response to maternal protein undernutrition. Increased

ACE activity of serum and lung has been observed in mice from mothers fed LPD either exclusively during preimplantation development or throughout gestation (Watkins et al. 2008a). Lessons from the maternal LPD model reported that maternal protein restriction leads to increased miR-429, which causes reduced production of ACE-2 protein, this decrease in ACE-2 levels results in reduced degradation of the potent vasoconstrictor angiotensin II and therefore increased blood pressure (Goyal et al. 2011a); (Goyal et al. 2011b). (Ravi Goyal 2015). It is therefore worth investigating the mRNA and protein expression of the key components of the pulmonary, cardiac and renal renin-angiotensin system and to examine microRNA complementary to angiotensin converting enzymes ACE-2 in our stored tissue of offspring to illustrate the important of IVF and embryo culture duration in the genesis of hypertension in the adult offspring.

Importantly, SBP at week 21 and LIFE in male offspring from IVF-ET-BL was positively correlated with their body weight at weeks 9, 15 and 27 of age. Individuals of higher weight demonstrate higher SBP.

This indeed is in agreement with human studies that have reported that serum ACE levels are significantly higher in hypertensive individuals and it is positively correlated with body mass index (BMI) regardless of sex (Forrester et al. 1997). However, this is in contrast with another recent study in North India that found that ACE was significantly associated with hypertension, but not associated with BMI (Rizvi et al. 2015). Further studies are needed to investigate the association between ACE and BMI.

7.1.6 *Effect of embryo manipulation on offspring glucose tolerance test GTT*

The next novel finding in current study is that male offspring from IVF-ET-2Cell firstly were clearly glucose intolerant. The glucose level after 60 and 120 minutes of glucose injection and AUC in IVF-ET-2Cell group was significantly higher compared with IV-ET-2-Cell and secondly, was significantly higher than that of IVF-ET-BL after 15, 30 and 120 minutes of glucose injection and significant higher AUC. This indicates that in ART, in which the IVF procedure is coupled with a short duration of embryo culture prior to ET may act as a negative factor for adult metabolism in a sex-specific manner compared with longer duration of culture. It is shown that the cause for glucose intolerance could be a pancreatic defect with altered insulin production (Tura et al. 2006). Our results are in agreement with (Donjacour et al. 2014) who reported that IVF male mice (but not females) showed glucose intolerance. Although our study and (Donjacour et al. 2014) study showed some similarities such as the effect of embryo culture media during the preimplantation period on adult metabolism and postnatal glucose homeostasis following IVF may be sexually dimorphic, there is one interesting difference between the two studies. Our study showed that male IVF-ET-2Cell were glucose intolerant compared with NM and IVF-ET-BL, whereas (Donjacour et al. 2014) have observed the glucose intolerance in IVF offspring, which is equivalent to our IVF-ET-BL, compared with NM and FB, which is equivalent to our IV-ET-BL. This indicate that ET at an early stage of embryo development (2-cell stage) and disturbance at this crucial stage, which denotes genome activation in mice (Niakan et al. 2012), may account as a crucial contributor to initiate the start of future metabolic dysregulation.

As discussed above, culture media can cause long term reprogramming of development and health, and this is likely mediated by epigenetic changes. Studies have reported that embryo culture specifically in suboptimal conditions disturbs the maintenance of the embryonic epigenome in the mouse and potentially in human preimplantation embryos (Market-Velker et al. 2010); (Velker et al. 2012); (White et al. 2015). Thus, embryos from both IVF-ET-2Cell and IVF-ET-BL may be affected by culture conditions during their preimplantation development. However, IVF-ET-2Cell embryos underwent ET procedure after a short period of culture (E0 – E1.5) and at a crucial stage of development which may affect the metabolism profile of these embryos and then show altered glucose tolerance later in life, whereas IVF-ET-BL exhibited longer period in culture (E0 – E4.5) and then underwent ET at blastocyst stage. This longer period in culture, including the embryonic sensing at the blastocyst stage (Fleming et al. 2015b), (Sunde et al. 2016) discussed above, could be beneficial to protect the embryo's metabolic phenotype. However, more studies are needed to define the long-term effects of IVF, duration of culture and ET at different stages of development on glucose metabolism.

7.1.7 *Effect of embryo manipulation on offspring serum glucose, serum insulin and Glucose: Insulin ratio G:I*

The following novel finding is offspring from IVF-ET-2Cell, IV-ET-2Cell and IV-ET-BL showed insulin resistance compared with NM. This further highlights the detrimental effect of IVF combined with ET at 2-Cell stage or ET *per se*. Male offspring from IVF-ET-2Cell initially appear to have normal glucose levels, however their insulin levels

are dramatically increased. This is reflected in their G:I ratio, which is markedly reduced, which indicates that IVF, short duration culture and ET at 2-cell stage can induce insulin resistance in a sex-specific manner. Our finding is supportive of (Chen et al. 2014) findings, which demonstrate the occurrence of insulin resistance in both human and mouse offspring derived from IVF and embryo culture.

Interestingly, male body weight at week 27 from all treatment groups was positively correlated with AUC, Individuals of heavier weight demonstrate higher AUC values. The AUC represents the average glucose concentration found in each mouse over the GTT. Higher AUC values are suggestive of insulin resistance. This indeed showed another similarity with (Donjacour et al. 2014) who demonstrated that body fat in IVF males, who were glucose intolerant, was greater than that of offspring derived *in vivo* and ET at blastocyst stage. Numerous epidemiologic studies have shown that the risk for diabetes, and presumably insulin resistance, rises as body fat content increases, measured by BMI from the very lean to the very obese, suggesting that the dose of body fat has an effect on insulin sensitivity (Kahn and Flier 2000). In addition, it is reported that by 3 days of HFD, body weight and adipose tissue mass, as well as insulin resistance, were clearly increased in wild-type mice (Lee et al. 2011).

An important point to be emphasized is that although IV-ET treatments (superovulation (SO) and ET) generally were less severe than the IVF, they still associate with many postnatal effects. This suggests that much of the phenotype generated is independent of the IVF and can be related to SO and/or ET. The use of SO and ET in our study most probably accounts for increased body weight, systolic blood

pressure and altered organs weight, which is in accordance with previous reports of enhanced postnatal body weight and systolic blood pressure in offspring of normally mated mice, coupled with SO and ET (Watkins et al. 2007). It is believed that zygotes are less sensitive than blastocysts to ET and that both synchronous (ET of blastocysts to the uterus or zygotes to the oviduct) and asynchronous (ET of blastocysts to the oviduct) blastocyst ET may have long-term effects on health, with possible impacts on body weight, arterial pressure, relative organ weight, and behaviour (López-Cardona et al. 2015). However, numerous studies have linked ovarian stimulation with epigenetic disorders on eggs and embryos, which are associated with developmental alterations in different mammal species. The analysis of the genomic imprinting of eggs obtained from SO demonstrated disorders in four imprinted genes: Peg1, Kcnq1ot1, Zac and H19 compared with eggs from natural ovulation (Ventura-Juncá et al. 2015). One recent human study showed that epigenetic alterations associated with gonadotropins also correlate with less fetal and placental development, as well as a smaller embryo during life (Grazul-Bilska et al. 2013). Animal studies also suggest that ovary hyper-stimulation in mice may affect embryo implantation by causing adverse changes in uterine receptivity (Fossum et al. 1989).

The question arises in the current study why environmental changes experienced by cells only during the preimplantation period may be 'memorised' and lead to long-term consequences later life? Despite the fact that direct mechanisms by which the postnatal consequences discovered result following ART are unknown at present, embryos may respond to diverse environmental stresses by epigenetic, metabolic,

cellular and physiological changes, all of which have potential to affect the developmental programme (Fleming et al. 2004); (Watkins et al. 2008a).

It is reported that imprinted genes tend to retain their mono-allelic DNA methylation pattern during the early preimplantation development; whereas the non-imprinted genes that become de-methylated and then later re-methylate, which make them more vulnerable. However, the worry would be loss of imprinting pattern by the environment of the embryo during the early preimplantation period (Fleming et al. 2004). It has been shown that culture environment during early embryogenesis may lead to epigenetic modification of imprinted and non-imprinted genes, and that these modifications may affect gene expression during later fetal development and result in conditions such as large offspring syndrome (LOS) (De Rycke et al. 2002). During preimplantation, extensive changes in genome-wide methylation occur, and any perturbation caused in this process may lead to deregulation of development at later stages. Imprinting genes are particularly vulnerable to methylation changes during early development (Fernández-Gonzalez et al. 2004). It is worth therefore to examine epigenetic changes in IVF-ET, IV-ET and NM offspring using stored organs, and the results might interpret the cardiovascular and metabolic consequences seen in our study.

In the current study, the number of male offspring within the IVF-ET-BL group was (n=26) from 13 foster mothers, which is almost double the number of female (n= 16) and the male/female ratio (1.62). This may be because of the ET procedure, in our experiments we selected IVF embryos at mid to late blastocyst stage for ET. Since it

is reported that males grow faster than females before implantation (Eriksson et al. 2010), it might be the blastocysts that we selected are more male than female. This is in agreement with (Eriksson et al. 2010), who reported that male foetuses have a more dangerous growth strategy than females in the womb. Males grow faster and invest less in placental growth, which makes them more susceptible to food shortages and puts them at crucial risk of becoming undernourished. (Eriksson et al. 2010) proposes that the ultimate manifestation of the male's more dangerous strategy may lead to adults with higher blood pressure and shorter lives than females.

7.1.8 *IVF, embryo culture duration and gender-specific*

Interestingly, in current study, we found that IVF and prolonged embryo culture affect was gender specific, See Table 7.1 and Table 7.2. Our study showed that male offspring are more susceptible than female. It is reported that male and female blastocysts show remarkable differences in gene expression (Bermejo-Alvarez et al. 2011). Sex chromosome complement, regardless of hormone exposure, may cause a large transcriptional dimorphism influencing both sex chromosome and autosome-encoded genes. Transcriptional sexual dimorphism influences different molecular pathways, which lead to functional consequences and varying vulnerability to environmental stressors. This can result in altered sex ratio by sex-selective embryo loss or to a sex-specific epigenetic response, which terminates in sex-dependent long term effects in offspring. Regarding the evidence discussed above, it is therefore not surprising that perturbation of physiologic signals caused by *in vitro* culture during the preimplantation period will lead to different health effects in the two sexes.

Table 7.1 Summary of the effect of IVF and embryo culture duration on sex-specific offspring: Shows the significant differences (P<0.05) between IVF and in vivo treatment groups vs normally mated offspring NM

Treatment	Challenge	Gender	
		Male	Female
IV-ET-2Cell	SO – Flushing – Culture 1-2hrs - ET	✓ Increased body weight ✓ Increased systolic blood pressure ✓ Impaired glucose tolerance test ✓ Insulin resistance ✓ Altered heart and right kidney: body weight ratios	✓ Increased body weight ✓ Increased systolic blood pressure ✓ Impaired glucose tolerance test ✓ Altered lung, heart and spleen: body weight ratios
IV-ET-BL	SO – Flushing – Culture 1-2hrs - ET	✓ Increased body weight ✓ Increased systolic blood pressure ✓ Impaired glucose tolerance test ✓ Insulin resistance ✓ Altered liver: body weight ratio	✓ Increased body weight ✓ Increased systolic blood pressure ✓ Impaired glucose tolerance test ✓ Altered lung, heart and both kidneys: body weight ratios
IVF-ET-2Cell	SO – IVF – Short culture (2-Cell) - ET	✓ Increased body weight ✓ Increased systolic blood pressure ✓ Impaired glucose tolerance test ✓ Insulin resistance ✓ Altered lung, heart, right kidney and liver: body weight ratios	✓ Increased body weight ✓ Increased systolic blood pressure ✓ Impaired glucose tolerance test ✓ Altered lung, heart and spleen: body weight ratios
IVF-ET-BL	SO – IVF – Long culture (BL) - ET	✓ Increased body weight ✓ Increased systolic blood pressure ✓ Elevated serum ACE ✓ Elevated lung ACE ✓ Impaired glucose tolerance test ✓ Altered spleen and liver: body weight ratios	✓ Increased body weight ✓ Increased systolic blood pressure ✓ Altered lung and heart: body weight ratios

Table 7.2 Summary of the effect of IVF and embryo culture duration on sex-specific offspring: Shows the significant differences (P<0.05) between IVF-ET-BL vs IVF-ET-2Cell

Treatment	Challenge	Gender	
		Male	Female
IVF-ET-2Cell	SO – IVF – Short culture (2-Cell) - ET	✓ Impaired glucose tolerance test ✓ Insulin resistance ✓ increased liver: body weight ratio	N/A
IVF-ET-BL	SO – IVF – Long culture (BL) - ET	✓ Increased systolic blood pressure ✓ increased heart: body weight ratio	N/A

Research shows that female IVF-ET mice, in contrast to males, did not show evidence of any specific alteration of hypertension or metabolic parameters, indicating a sexually dimorphic phenotype. Human and animals studies have demonstrated sexually dimorphic phenotype effects of in utero stress (Osmond et al. 1993) showing that women with low birth weight and higher weight at age of one year showed the highest incidence of cardiovascular mortality rates, a pattern not present in men. Only in women with a birth weight greater than normal (between 2.5 and 4 kg) did systolic blood pressure increase in parallel with birth weight (Gamborg et al. 2007). Animal models also show sexual dimorphic programming consequences. Thus, only female offspring of pregnant dams exposed to a high-fat diet exhibited hypertension in later life (Bubb et al. 2012) whereas only adult male rats exhibit alterations in triglycerides and expression of hepatic fatty acid enzymes after uterine artery ligation (Lane et al. 2001). Female offspring susceptibility also has been reported in the Emb-LPD mouse model with increased body weight affecting female offspring preferentially (Watkins et al. 2008b). Another possible and crucial reason behind gender specificity is sex

steroids. Sex steroids are known to alter adipose deposition and glucose clearance, with oestrogen increasing insulin sensitivity and testosterone having the opposite effect. This may take into account the higher insulin levels observed in males (Geer and Shen 2009). Our study may then imply that there may be multiple sexually dimorphic consequences of ART on energy homeostasis that need more comprehensive work.

In conclusion, the hypothesis of this thesis is that the environment, in particular the length of embryo culture; long (blastocyst) vs. short (2-cell stage) in which the preimplantation embryo develops cannot only slow down blastocyst development and reduce TE, ICM and total cell number, but may also affect aspects of postimplantation and postnatal growth, physiology and metabolism. The data described in chapters 3, 4, 5 and 6 are in support of our hypothesis, see Figure 7.1.

In this thesis, our results showed that the environment, in particular IVF and long embryo culture (blastocyst) slowed down embryo development and reduced TE, ICM and total cell number of embryos vs. *in vivo* embryos. The effect of embryo culture in particular the length of embryo culture; long (blastocyst) vs. short (2-cell stage) in which the preimplantation embryo develops cannot only slow down blastocyst development and reduce TE, ICM and total cell number, but also perturbed growth phenotype by increasing body weight and altering organs allometry, perturbed cardiovascular phenotype by increasing SBP and ACE activities but improving the metabolism phenotype by reducing glucose AUC and serum Glucose: Insulin ratio in the offspring.

It is now common practice for IVF clinics to transfer embryos at the blastocyst stage due to numerous studies demonstrating increased implantation rates, pregnancy and live births, along with the reduction of multiple births and ability to select high quality embryos for ET. However, our findings show that ET at this stage may have implications on long-term cardiovascular health leading to increased hypertension and poorer CV regulation in male offspring compared with ET at early cleavage (IVF-ET-2Cell). Interestingly, our data also show that IVF and ET at early stage (2-Cell) may have more sever long-term consequences on metabolic profile. Since we found that offspring from IVF-ET-2Cell and IV-ET-2Cell demonstrate abnormal G:I ratio when compared to NM control offspring, and do also demonstrate insulin resistance, this may indicate that embryo transfer at early stage (2-Cell) itself and not the short culture might be conductive to altered metabolic health during later life.

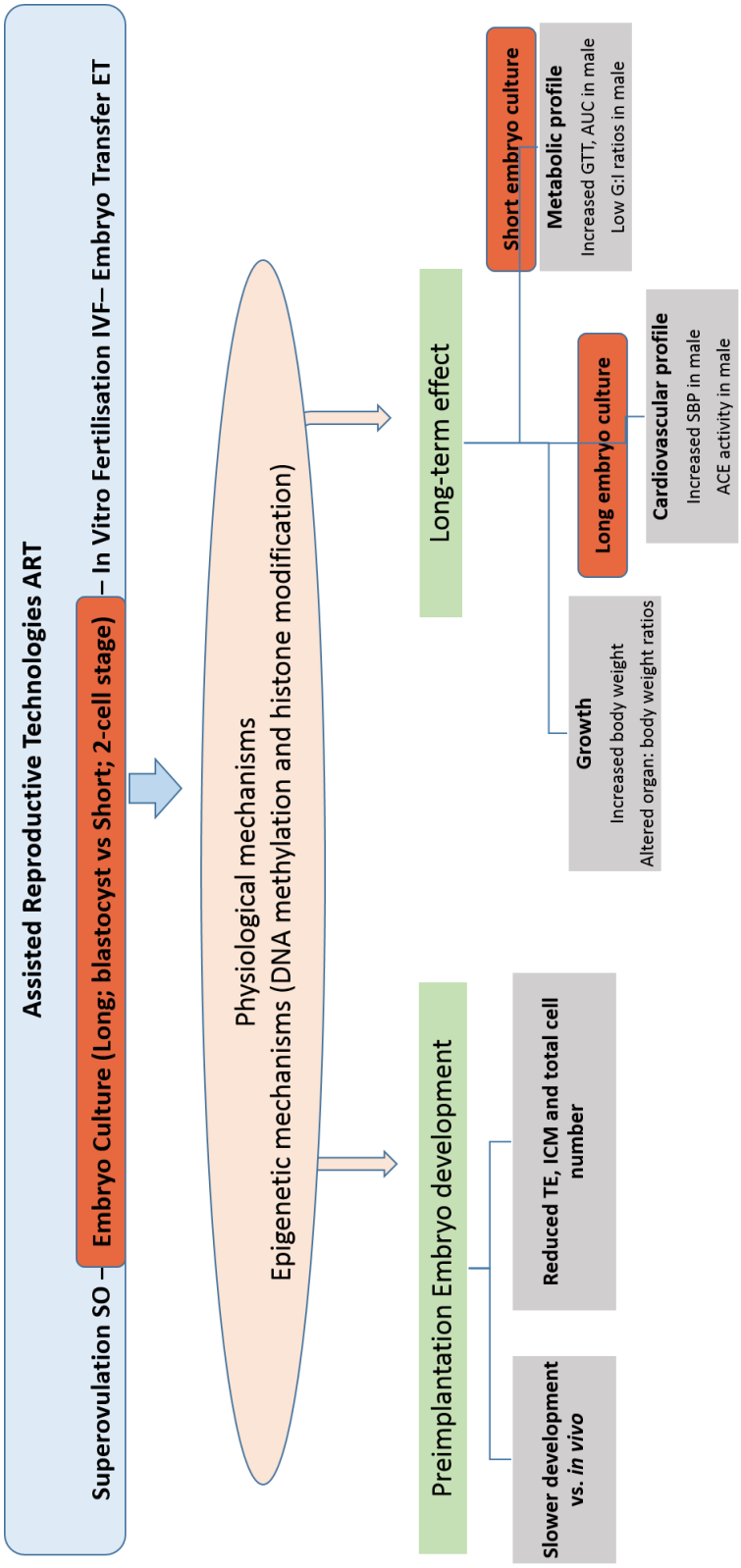


Figure 7.1 Summary of potential pathways of disease programming by which ART factors, in particular the duration of embryo culture may act on them mediated by epigenetics modifications.

Collectively, our data indicate that IVF techniques, including superovulation, IVF procedure, embryo culture and embryo transfer affect the postnatal growth, cardiovascular and metabolic profile because there were significant differences between the four IVF (IVF-ET-2Cell, IVF-ET-BL) and *in vivo* groups (IV-ET-2Cell, IV-ET-BL) when compared to NM group in any parameters analysed. Our data also show that ET procedure itself (where embryo culture has been reduced to a minimal period) may play a crucial role in postnatal phenotype programming for some of our outcomes. Furthermore, the ovarian stimulation condition of embryos in IV-ET and IVF-ET groups may lead to adverse outcomes.

Moreover, the major finding of this study is that prolonged embryo culture (from IVF to blastocyst) before transfer may affect cardiovascular health in males more so short culture (IVF to 2-cell). However, prolonged embryo culture appears to improve metabolic profile in male offspring. However, we did not observe correlations between metabolic (AUC, serum glucose and insulin levels) and cardiovascular (SBP and ACE) profiles in this study. No differences were evident between the four treatments groups for females.

Human studies have shown that ART is linked with some adverse perinatal and postnatal outcomes, including low birth weight (Hart and Norman 2013a); (Hart and Norman 2013b), an increase risk of genomic imprinting disorders and altered DNA methylation and hypertension and cardiovascular disease (Ceelen et al. 2008a). However, since ART offspring are relatively young, it is difficult to project health outcomes in later life hence the need for animal studies such as the current study.

Whilst, our data using animal model cannot be extrapolated directly to human ART, the long-term consequences observed here may be both important to rodents and may inform on potential outcomes relevant to humans. These studies therefore identify an area, which will require epidemiological and laboratory evaluation in the human.

7.2 Strengths and limitations

7.2.1 *Strength of present study*

One strength of current study is the novelty of the research. To our knowledge, this is the first study that reports the effect of IVF and duration of embryo culture on cell lineages at the blastocyst stage, systolic blood pressure and ACE activity, metabolic profile using a mouse model. The study was designed to fulfil the requirement of comparing the effect of the IVF procedure *per se* (IVF-ET-2Cell vs IV-ET-2Cell) and (IVF-ET-BL vs IV-ET-BL) and the effect of prolonged culture (IVF-ET-2Cell vs IVF-ET-BL) and the effect of other ART procedures such as superovulation, collection of embryos and ET (IV-ET vs NM controls).

Data obtained in this study consist of good numbers embryos for Chapter 3 and number of samples for both genders. The analysis of postnatal health was thorough and comprehensive. Chapters 4, 5 and 6 covered parameters on growth and organ: body weight ratio, systolic blood pressure SBP and ACE activity, glucose tolerance test GTT and serum glucose and insulin, respectively. This data analysis is vital in determining metabolism and cardiovascular disease. Repeated measurements on

the same animals for weekly body weight, systolic blood pressure taken on 3 different weeks, glucose tolerance test and organ allometry at week 27, and serum and lung analysis demonstrated different health parameters not only CVD. Importantly, our data analysis took confounders such as litter size and (foster) mother influences into account using a multilevel random effect regression model. Such confounders however have been reported to affect results (Scott et al. 2010); (Donjacour et al. 2014). In addition, to confirm the data we obtained from the GTT, a subset of serum samples collected after culling was analysed for glucose and insulin levels. Moreover, to confirm the findings we collected from the SBP, serum and lung ACE activity were analysed using serum and lung stored prior to culling at 27 week of age. Furthermore, our study correlated different physiological parameters (body weight, SBP, ACE, GTT and glucose/insulin homeostasis) to each other because we investigate them in the same animals or offspring.

Overall, we believe that the current study design is necessary to discriminate between ART factors (such as SO, ET and IVF procedure) and looks into the effect of the duration of culture *per se* on postnatal health. The study might contribute to new interest in discovering the mechanism that cause these significant postnatal differences.

7.2.2 *Limitation of present study*

There are some limitations and drawbacks in this study. Firstly, our data concluded that IVF and long embryo culture slows the developmental rate of embryos, and also significantly reduced the TE, ICM and total cell number compared with *in vivo*

derived embryos; however, mechanisms are still unknown. A more detailed analysis such as the developmental rate of IVF embryos using the time-lapse technique, the number within cell lineages (TE and ICM) at different time points, the proliferation and apoptosis rate by nuclear staining, and immunostaining for DNA visualisation (for spindle morphology) on IVF-2Cell and IVF-BL embryos are vital to understand better the effect of IVF and prolonged embryo culture in the future.

In addition, in our earlier experiments (Chapter 4), the birthweight of the pups was taken but we noticed that the mothers tended to eat the pups afterwards, even if we changed the gloves. So we decided to discontinue weighing at birth but continued monitoring the pups in their cages until week 3, when they were ready for weaning. However, it would be worthwhile if birthweight was considered in this study as it is one of the important main parameters and indicator for CVD. Numerous human and animal studies use birthweight as proxy to predict onset of adult disease risk (Watkins et al. 2007); (Ceelen et al. 2008a). Thus, having another set of data on birthweight would be an insightful perspective whether the birthweight would also be crucial indicator for future disease in IVF-ET groups, therefore strengthening our hypothesis. It would be worth also to investigate the behavioural and neurological parameters of the offspring since such studies are very limited in the literature.

We also encountered other limitations in the current study, such as relative lack of studies in the literature on the effect of SO and/ or ET, and embryo culture on embryos at early stage (2-cell), and offspring generated by IVF and ET at 2-cell stage. Also, there is a lack of biochemical and molecular studies on stored tissues.

7.3 Future Work

In this thesis, we present a thorough postnatal study on the effect of IVF and duration of culture using a mouse model by analysing the growth, cardiovascular and metabolic profile of offspring. Although answering some questions, this thesis has also raised many others. However, future work to support our results is necessary to further understand the mechanistic basis of ART treatments on embryos and their effect on gender specific adult phenotype.

From the results reported in Chapter 5, it would be of interest to investigate the differences in blood pressure further. It would be of interest to examine the expression profiles of proteins involved in blood pressure regulation such as type 1 angiotensin II receptor (AT1R) in our frozen tissues (lung, kidney and heart). As discussed above, it is well known that the effect of Ang II on blood pressure is mediated by angiotensin II type 1 receptors (AT1Rs). It is reported that AT1R is the "last decider" in the process of regulating BP and inhibiting or blocking AT1R reduces BP (Herichova I 2013).

In addition, it has been suggested that maternal protein restriction leads to increased miR-429, which causes reduced production of ACE-2 protein. Decreased levels of ACE-2 results in reduced degradation of the potent vasoconstrictor angiotensin II and therefore increased blood pressure (Ravi Goyal 2015). It is therefore worth investigating the mRNA and protein expression of the key components of the pulmonary, cardiac and renal renin-angiotensin system in our

stored tissue of offspring to illustrate the important of IVF and embryo culture duration in the genesis of hypertension in the adult offspring.

A report by (Chen et al. 2014) showed that the expression of the lipogenesis gene *Srebf1* was increased in IVF mice that were fed chow or high fat diet (HFD) compared with NM control mice. However, hepatic expression of the gluconeogenesis gene *G6pc* and mitochondrial biogenesis markers *Cpt1α*, *Pgc1α*, and *Tfam* were not different between groups or diets. HFD increased the expression of the glucokinase protein and decreased the expression of the gluconeogenic gene *Pck1* in all groups. It would be interesting therefore to investigate the expression of the gluconeogenesis gene *G6pc* and mitochondrial biogenesis markers *Cpt1α*, *Pgc1α*, and *Tfam* in our frozen liver samples and the results might explain the metabolic effect that we observed in the IVF-ET-2Cell offspring.

Collectively, the study of epigenetic alterations of the genome is becoming increasingly crucial in order to understand how environment and genetic background interact to build and regulate the functional genome. Due to global DNA methylation states used as a general measure of the methylome are cost-effective and rapid, such specific analytical tools would therefore be worth investigating the global DNA methylation of stored tissues using Luminometric Methylation Assay (LUMA) to illustrate the effect of IVF and embryo culture duration on the genome of adult offspring. The LUMA, a method which analyses global DNA 5-methylcytosine (5mC) via the use of restriction enzymes and detection with Pyrosequencing and it is able to simultaneously analyse up to 96 samples (Luttropp

et al. 2015). Although there are many methods for global DNA methylation and the majority of these methods focused on 5mC analysis, with appropriate enzymes, LUMA does not detect 5-hydroxymethylcytosine (5hmC), thus it is more specific than most 5mC techniques (Luttropp et al. 2015).

Hepatic phosphoenolpyruvate carboxykinase (PEPCK) activity

Hepatic phosphoenolpyruvate carboxykinase (PEPCK) is recognized as a crucial regulator of gluconeogenesis and the enzyme proved to be overexpressed in insulin resistance and diabetes (Magnusson et al. 1992); (Kwong et al. 2007). Furthermore, mice overexpressing PEPCK develop insulin resistance and diabetes (Valera et al. 1994). PEPCK would be a useful marker to correlate with glucose homeostasis and glucose resistance data that we presented in this thesis. (Watkins et al. 2007) reported that activity of enzymatic regulators of metabolic physiology such as hepatic PEPCK was significantly elevated at week 27 from female offspring from IV-ET (Embryos derived at 2-Cell then ET) and IVC-ET (Embryos derived at 2-Cell then cultured to blastocyst before ET).

Our study shows that glucose homeostasis was significantly different between NM control and IV and IVF groups in male and female offspring, and also was different between IVF-ET-2Cell and IVF-ET-BL. We believe that PEPCK is a useful marker for evaluating metabolic health in *in vivo* models for embryo environmental programming and could contribute to the effect of IVF and prolonged embryo culture on insulin resistance and diabetes.

Appendices

Appendix I

Component of H6 BSA and H6 PVP

Solution F (per 100 ml, osmolarity, 2555+/_ 20 mOsm)

Sodium chloride	4.720g
Potassium chloride	0.11g
Sodium dihydrogen othophosphate	0.06g
Magnesium chloride	0.1g
D- glucose	1.0g
DL- lactic acid (60%)	3.4g

Solution G (per 10 ml, osmolarity, 60+/_ 10 mOsm)

Pyruvic acid	0.03g
Penicillin	0.06g
Streptomycin	0.05g

Solution B (per 10 ml, osmolarity, 444+/_ 20 mOsm)

Sodium hydrogen carbonate	0.210g

Solution H (per 10 ml, osmolarity, 415+/_ 20 mOsm)

Calcium chloride 2-hydrate	0.26g

Solution E (per 50 ml, osmolarity, 354+/_ 20 mOsm)

Hepes	2.978g

Need pH to 7.4 with 5 M NaOH before checking mOsm

Phenol red	6 mg/ml
Sodium chloride	20%

Sodium chloride 15mg/ml

Embryo culture media

Stock	H6 BSA	H6 PVP
F	1.00 ml	1.00 ml
B	0.16 ml	0.16 ml
G	0.1 ml	0.1 ml
H	0.1 ml	0.1 ml
E	0.84 ml	0.84 ml
H_2O	7.8 ml	7.8 ml
BSA	40 mg	N/A
PVP	N/A	60 mg
20% NaCl	60 ul	60 ul
NaCl 15mg/ml	N/A	N/A
Phenol red	N/A	N/A

KSOM culture medium

2x stock KSOM

Sodium chloride NaCl	1.110 g
Potassium chloride KCl	0.0095 g
Magnesium sulphate 7-hydrate $MgSO_4.7H_2O$	0.0099 g
Lactic acid	0.362 ml
Sodium Pyruvate	0.0044 g
Glucose	0.0072 g
Sodium bicarbonate	0.42 g
Penicillin	0.01256 g
Streptomycin	1 ml
EDTA	4 ml
Tissue culture grade water	Made up to 100 ml

5 ml aliquots stored at -80°C for up to 2 months

1x stock KSOM (10ml)

The following was added to 5 ml of thawed 2x KSOM stock

Calcium chloride (100x stock 2 mg/ml)	100 µl
Non-essential amino acid (100x stock; 10mM)	50 µl
Essential amino acid (50x stock)	100 µl
L-glutamate (200 mM)	50 µl
Deionised water, sterile	4.75 ml
BSA	0.04 g

Sterile filtered (0.22 µm filter; osmolarity, 225+/_ 5 mOsm) with 20% NaCl; aliquots stored at 4°C for up to 1 week.

Acid Thyrode's solution (per 100 ml, pH 2.3)

NaCl	0.8 g
KCL	0.02 g
$CaCl_2 .2H_2O$	0.0265 g
$MgCl_2 .2H_2O$	0.01 g
D-glucose	0.1 g
PVP	0.4 g
Or	
$CaCl_2$	0.02 g

Appendix II

Component of PMS and hCG

Pregnant mare's serum (PMS)

1 vial (1000 i.u.) diluted in 20 ml 0.9% saline solution. Sterile filtered and aliquoted in 4 ml samples. Stored at -20°C.

Human chorionic gonadotrophin (hCG)

1 vial (1500 i.u.) diluted in 30 ml 0.9% saline solution. Sterile filtered and aliquoted in 4 ml samples. Stored at -20°C.

Appendix III

Anaesthetic for embryo transfer surgery

Saline (0.9%)	375 µl
Ketaset (Fort Dodge)	100 µl
Acepromazine	25 µl

Analgesic used after embryo transfer surgary

Temgesic (Reckitt & Colman Product Ltd). One capsule (300 µg/ml) diluted 1:10 in 0.9% saline solution to give a working concentration of 30 µg/ml.

Appendix IV

Preparation of IVF media (stored at 4°C up to 3 months)

Composition of high calcium Human Tubal Fluid (HTF) medium

1. All reagents were solubilised in Tissue Culture Water (TCW; Sigma W5500, LOT: RNBC4950).
2. Prepared 10 ml stock of each of the KCl (35 mg in 10 ml TCW), MgSO4.7H2O (49 mg in 100 ml TCW), KH2PO4 (54 mg in 100 ml TCW) and CaCl2.H2O (7.55 mg in 10 ml TCW) using a universal container.
3. 59.38 mg of NaCl was added to the container.
4. 1 ml from the 10 ml stock of each of the (KCl, MgSO4.7H2O, KH2PO4, CaCl2.H2O) were added in order.
5. 21 mg NaHCO3 and 5 mg Glucose were added immediately in order.
6. 34 µl of Na-lactate (liquid) was added.
7. Prepared 5 ml stock of Na-Pyruvate: (0.002 mg in 50 ml TCW), 1 ml from this stock was added to the medium.
8. Prepared 5 ml stock of Penicillin (0.004 mg in 50 ml TCW), 1 ml from the stock was added to the medium.
9. 10 ml stock of Streptomycin was prepared (50 mg in 100 ml of TCW) and 1 ml added to the medium immediately.
10. 0.040 g of BSA was added to the medium.
11. The medium was stirred on magnetic stirrer for 60 minutes.
12. Added 4 µl Phenol Red (0.5% soln) to the medium.

13. TCW was added to reach 10 ml.
14. HTF medium was stored at 4°C for up to 3 months, appendix IV.

Composition of sperm pre-incubation medium (TYH+MBCD) (stored at 4°C up to 3 months)

1. Reagents were solubilised in TCW.
2. Prepared 10 ml stock of each of KCl (35.6 mg in 10 ml TCW), MgSO4.7H2O (29.3 mg in 10 ml TCW), KH2PO4 (16.2 mg in 10 ml TCW) and CaCl2.H2O (25.1 mg in 10 ml TCW) using a universal container.
3. 69.76 mg NaCl was added to the container.
4. 1 ml from the 10 ml stock of each of the (KCl, MgSO4.7H2O, KH2PO4) was added in order.
5. 21.06 mg of NaHCO3 was added to the medium.
6. Prepared 5 ml stock of Na-Pyruvate: (0.002 mg in 50 ml TCW), 1 ml from this stock was added to the medium.
7. 10 mg Glucose was added to solutions.
8. 1 ml CaCl2.2H2O was added to solutions (from the previously prepared stocks).
9. 9.83 mg Methyl-β-cyclodextrin was added immediately to the medium.
10. 1 ml Penicillin was added to solution (from the previously prepared stocks).
11. 0.005 mg Streptomycin was added immediately to the medium.
12. 0.01 mg Polyvinylalcohol was added to the medium.
13. The medium was stirred on magnetic stirrer for 60 minutes
14. TCW was added to final 10 ml volume.
15. The (TYH+MBCD) medium was stored at 4°C up to 3 months.

Composition of high calcium HTF medium

Reagent Name	**Mg/10ml**	**10X stock**	**20x stock**
NaCl	59.38	-	
KCl	3.5	35mg/10ml	-
MgSO4.7H2O	0.49	49mg/100ml	9.8 mg
KH2PO4	0.54	54mg/100ml	10.8 mg
CaCl2.H2O	7.55	7.55mg/10ml	-
NaHCO3	21.00	-	-
Glucose	5.00	-	-
Na-lactate(ml)*	0.034	(34µl)	-
Na-Pyruvate	0.37	37mg/100ml	7.4 mg
Penicillin G	0.75	75mg/100ml	15.0 mg
Streptomycin	0.5	50mg/100ml	10 mg
BSA (Fraction V, Fatty Acid-Free)	40.00	-	-
Phenol Red (0.5% soln)	0.004	(4µl)	-
Note: Solubilise the CaCl2.2H2O separately from the other reagents before combining the solutions.			

Composition of high calcium TYH+MBCD medium

Reagent Name	**Mg/10ml**	**10X stock**
NaCl	69.76	-
KCl	3.56	35.6mg/10ml
MgSO4.7H2O	2.93	29.3mg/10ml
KH2PO4	1.62	16.2mg/10ml
NaHCO3	21.06	-
Na-Pyruvate	0.55	55mg/100ml
Glucose	10	-
CaCl2.2H2O	2.51	25.1mg/10ml
Methyl-β-cyclodextrin	9.83	-
Penicillin	0.75	75mg/100ml
Streptomycin	0.5	50mg/100ml
Polyvinylalcohol	10	-
Note: Solubilise the CaCl2.2H2O separately from the other reagents before combining the solutions.		

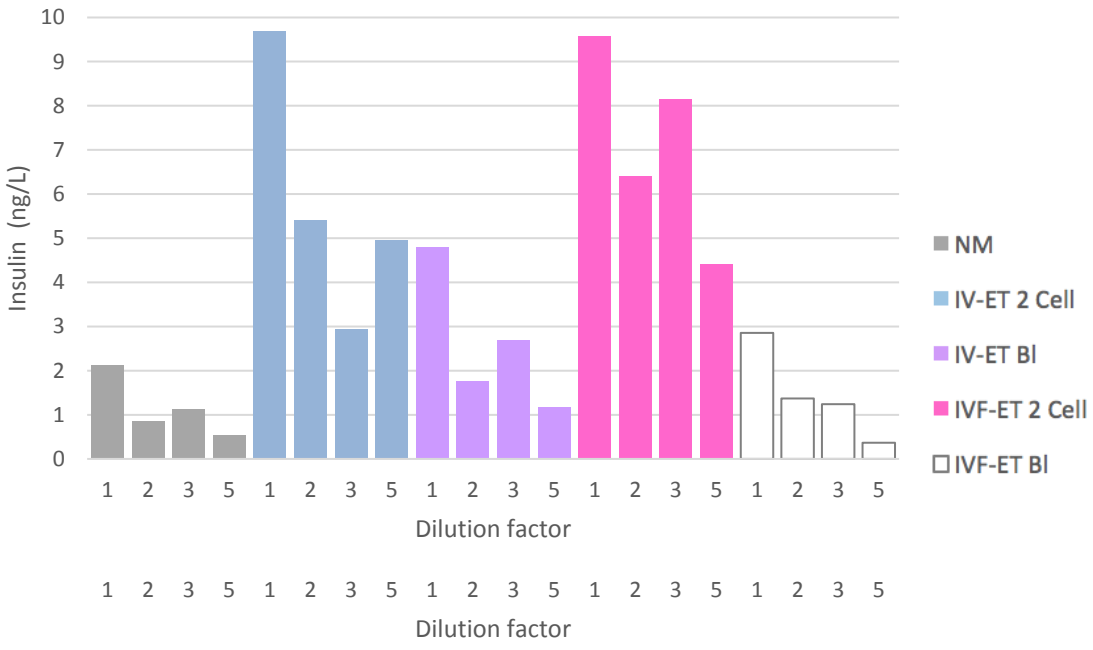
Appendix V

1) pH adjustments were at room temperature (23 ± 2ºC), with use of pH 7.41 and pH 10 calibration buffers.
2) Incubation buffer: Dissolve 3.09 g of H_3B0_3 (Sigma) and 29.22 g of NaC1 (Sigma) in 200 mL of distilled water. Adjust to pH 8.3 with 5 mol/L NaOH and dilute to 250 mL with distilled water. Store at room temperature.
3) Substrate solution: 20 mmolfL HHL (Sigma Chemical Co., St. Louis, MO 63178) in 20 mmolfL NaOH. Store frozen (-15 ºC).
4) Terminating solution: 1 mol/L HC1 (Sigma). Store at room temperature.
5) Neutralizing solution: 1 mol/L NaOH. Store at room temperature.
6) Diluent buffer: Dissolve 27.22 g of KH_2PO_4 (Sigma) in 900 mL of distilled water. Adjust to pH 8.3 with 5 mol/L NaOH and dilute to 1 L with distilled water. Store at room temperature.
7) Colour reagent: Dissolve 15 g of cyanuric chloride, practical grade (Sigma) in 500 mL of 1,4-dioxan (Sigma). Store at room temperature in a dark-brown glass bottle.
8) Hippurate standard: Dissolve 112 mg of hippuric acid (Sigma) in 250 mL of 20 mmol/L NaOH. Store at 4 ºC. Dilute as required with distilled water.

Appendix VI

Insulin Optimisation

Before the ultimate ELISA experiment was conducted, an optimisation test was carried out in which one male serum sample from each treatment was used without dilution, 1/2, 1/3 and 1/5. Some samples from the undiluted and 1/2 dilution resulted in absorbance values outside of the calibrator standard curve, reducing their reliability and eliminating the dilutions. Many of the values at a 1/5 dilution were close to zero, so a dilution of 1/3 was selected for the final ELISA experiment in order to best suit the range of insulin values within the samples.



Insulin concentrations of samples at different dilutions – a male serum sample was taken from each treatment, and an ELISA was performed on the undiluted serum and at dilutions of 1/2, 1/3 and 1/4. Insulin concentrations calculated by multiplying up to remove dilution factor.

Insulin

The ultimate ELISA experiment resulted in 9 samples with absorbance values outside of the standard calibrator curve due to high insulin concentration. These samples spanned across the treatments, so did not appear to have a relationship to a particular treatment. 11 samples were also found to have repeats more than 20% different to each other, suggesting a procedural error during the experiment, as both should theoretically provide the same value. These 11 samples were repeated at the same concentration, all of which produced two replicates with a difference of less that 20%.

The 9 samples out of the curve were repeated at dilutions of 1/10, 1/20 and 1/50 to account for the high insulin concentrations in the serum. An insulin concentration for each sample at each dilution was not obtained and those samples with values at more than one dilution did not have exactly the same value for each, presumable due to dilution error. To decide how to compile this data, five data sets were created. These data sets were: all insulin values with the 9 samples out of the curve excluded, an average of the dilution values for each sample, the average of the dilution values with any values more than 30% different from each other excluded and finally only the 1/20 or 1/50 dilutions used. The 30% limit was an arbitrary number selected to create a rule for all the data.

Figure 5 demonstrates the effects of handling the repeated data in these 5 different ways. Excluding all 9 samples appears to create a data set with the least standard error, however it was not selected due to the implications on sample numbers and therefore the validity of statistical testing carried out. All other methods appear to have little effect on the general trend of the data. The method of excluding dilution repeats that are more than 30% different from each other and then averaging was chosen. This is because it combines dilutions in order to negate some possible dilution effects and systematically removes values which can be considered outliers.

The huge amount of variation (large error bars) within all treatments besides the control raised the question of whether there are two populations within the treatment groups, one of high insulin values and one of low, causing this. Individual values were plotted in order to look for this trend. However, it appears that most samples lie within

a range of insulin values, with one or two samples having extremely high insulin levels, meaning two populations do not exist. As these samples were more than 2 standard deviations above the mean, they were discounted for the sake of statistical analysis. It is worth noting that there were no samples that were more than 2 standard deviations below the mean. Although theses samples appear as outliers and have been removed, they should still be considered. This is because they are not a product of human error, due to two repeats being carried out and falling within 20% of each other.

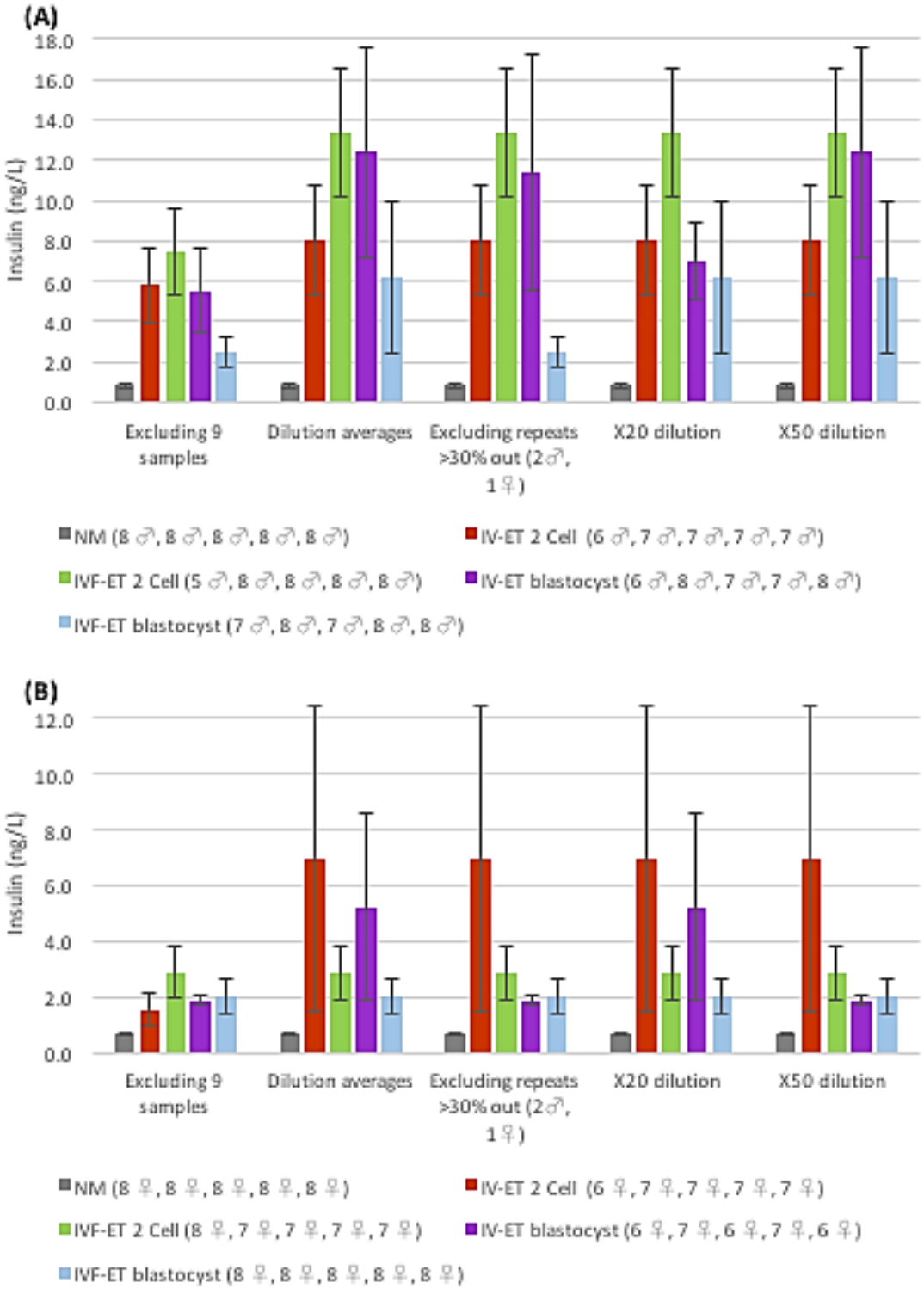
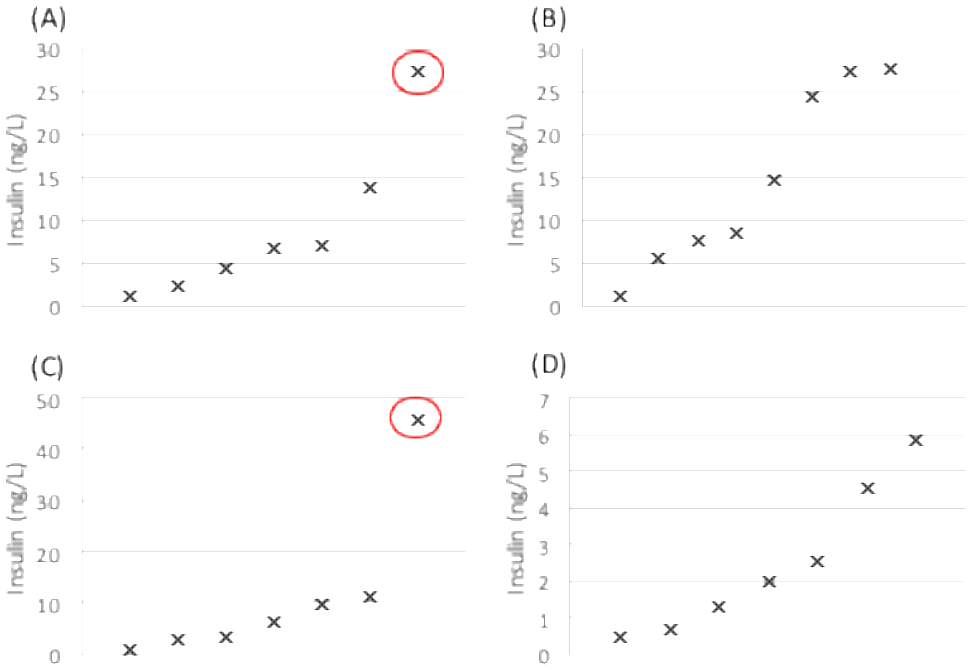
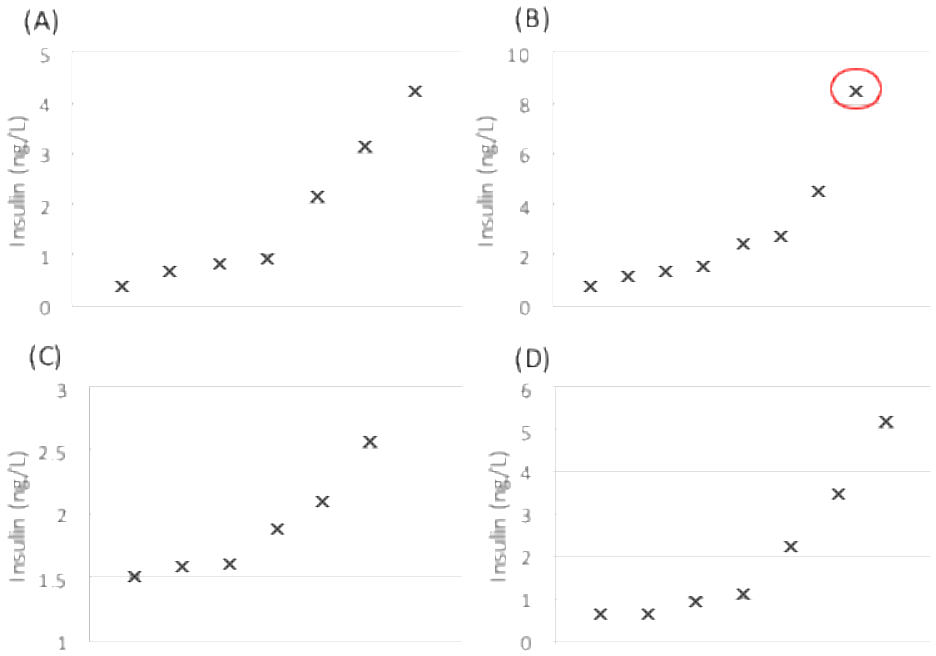


Figure 5: Serum insulin levels of (A) males and (B) females across five ART treatments. 9 samples repeated at three dilutions are either excluded, or incorporated through averaging, exclusion of repeats >30% or inclusion of 1/20 or 1/50 dilution data. NM=normal mating, IV-ET 2 Cell= In vivo embryo transfer 2 Cell, IVF-ET 2 Cell = In vitro embryo transfer 2 Cell, IV-ET Bl= In vivo embryo transfer blastocyst, IVF-ET Bl = In vitro embryo transfer BL.

The huge amount of variation (large error bars) within all treatments besides the control raised the question of whether there are two populations within the treatment groups, one of high insulin values and one of low, causing this. Individual values were plotted in order to look for this trend.



Individual insulin data points for males – (A) IV-ET 2 cell (B) IVF-ET 2 cell (C) IV-ET BI (D) IVF-ET BI Outliers are circled.



Individual insulin data points for females – (A) IV-ET 2 cell (B) IVF-ET 2 cell (C) IV-ET Bl (D) IVF-ET Bl Outliers are circled.

However, it appears that most samples lie within a range of insulin values, with one or two samples having extremely high insulin levels, meaning two populations do not exist. As these samples were more than 2 standard deviations above the mean, they were discounted for the sake of statistical analysis. It is worth noting that there were no samples that were more than 2 standard deviations below the mean. Although theses samples appear as outliers and have been removed, they should still be considered. This is because they are not a product of human error, due to two repeats being carried out and falling within 20% of each other.

Within males (Figure 6A), the IVF, 2 cell embryo transfer offspring have significantly higher insulin levels than control (P= 0.000), IVC-ET 2 Cell (P=0.005), IVC-ET Bl (P=0.015) and IVF-ET Bl (P= 0.007) offspring. Offspring conceived naturally and transferred at the blastocyst stage also appear to have high insulin levels in comparison to control offspring (P=0.026). Interestingly, all groups show an increased standard error to varying degrees in relation to the control, showing a larger amount of variation within these samples.

Similarly, the female IVF, 2 cell embryo transfer offspring also demonstrate high insulin levels (Figure 6B), but this is only significant in comparison to control offspring (P= 0.029). All other groups appear to have higher insulin concentrations in comparison to the control group, but not to a notable extent. All other treatments, as with the males, show quite large standard errors and therefore variation within the samples. In contrast, the range of insulin values within females (around 0.5ng/L to 2ng/L) is much lower than in males (around 0.8ng/L to 14ng/L).

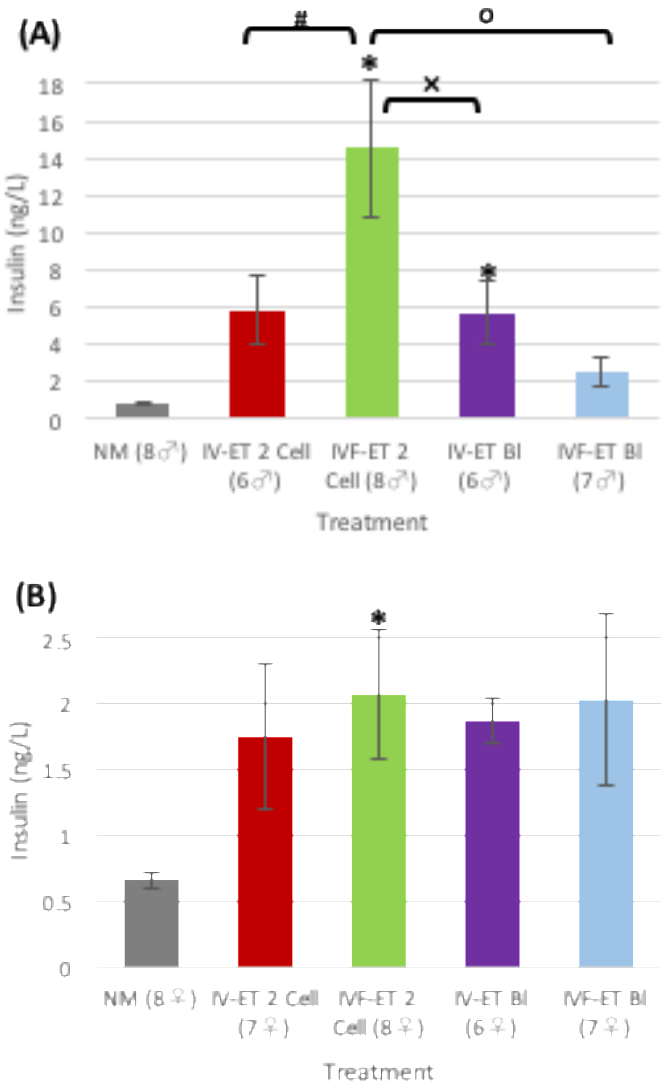


Figure 6: Average serum insulin measurement across five treatments.

(A) IVF combined with early embryo transfer may result in high insulin levels compared to all other treatments. Late embryo transfer also results in higher insulin levels than control mice in males. * = significant difference between normal mating and the treatment indicated. **o** = significant difference between IVF-ET 2 Cell and IVF-ET Bl. × = significant difference between IV-ET Bl and IVF-ET 2 Cell. # = significant difference between IV-ET 2 cell and IVF-ET 2 cell.

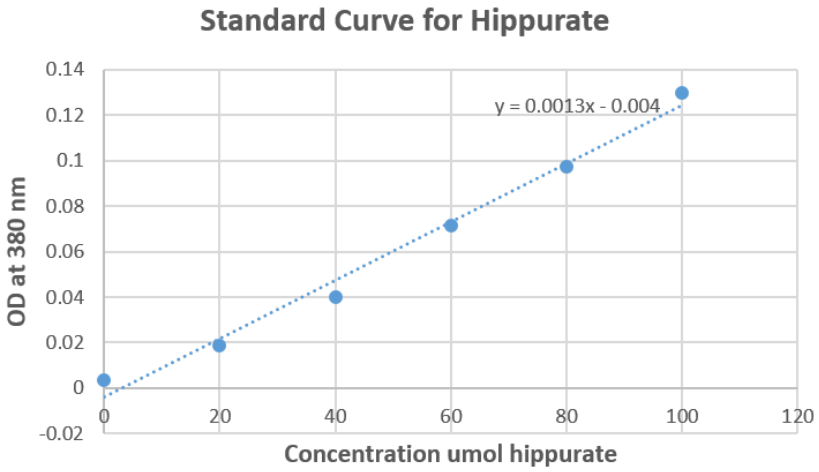
(B) IVF and short embryo culture may result in high insulin levels compared to control mice in females.
NM=normal mating, IV-ET 2 Cell= In vivo embryo transfer 2 Cell, IVF-ET 2 Cell = In vitro embryo transfer 2 Cell, IV-ET Bl= In vivo embryo transfer blastocyst, IVF-ET Bl = In vitro embryo transfer BL.

Appendix VII

ACE Assay

Standards

Standards were known concentrations of hippurate between 20um and 2.5mM. These were treated exactly the same as the samples and quantified spectrophotometrically. The absorbance of the standards was used to plot a graph of absorbance against concentration. The equation from this graph was used to determine hippurate levels in the samples.



Dilution

Standard curve created from known concentrations of hippurate between 2.5mM and 20uM

Undiluted serum and lung homogenate gave readings that were outside the standard curve ie higher values than the standards.

A 3x dilution was tried, but the readings were still too high.

Serum was given a 5x dilution and this led to samples falling within the standard curve

Standard curve was used to calculate total amount (in uM for serum, nM for lung) of hippurate in each sample

Protein Quantification

ACE in the lung homogenate was expressed in hippurate released per mg per minute

Needed to know total protein present in the samples

BCA assay performed

Coefficient of Variation

CV is defined as the ratio of the standard deviation to the mean

Expresses precision and repeatability of an assay

CV in this assay needed to be below 20% for accurate results

Tissue freeze/thaw

Serum and lung samples were only freeze-thawed once

List of References

ABRAHAM TM AND FOX CS. 2013. Implications of Rising Prediabetes Prevalence. Diabetes care 36: 2139-2141.

AMERICAN DIABETES A. 2011. Diagnosis and Classification of Diabetes Mellitus. Diabetes care 34: S62-S69.

ARONOFF SL, BERKOWITZ K, SHREINER B AND WANT L. 2004. Glucose Metabolism and Regulation: Beyond Insulin and Glucagon. Diabetes Spectrum 17: 183-190.

BACHAROVA R. 1985. Gene expression during oogenesis and oocyte development in mammals. Dev Biol (NY).

BACHAROVA R AND MOY K. 1985. Autoradiographic studies on the distribution of labeled maternal RNA in early mouse embryos. J Exp Zool.

BARKER DJ. 2007. The origins of the developmental origins theory. Journal of internal medicine 261: 412-417.

BARKER DJP. 1997. Maternal nutrition, fetal nutrition, and disease in later life. Nutrition 13: 807-813.

BARKER DJP AND OSMOND C. 1986. INFANT MORTALITY, CHILDHOOD NUTRITION, AND ISCHAEMIC HEART DISEASE IN ENGLAND AND WALES. The Lancet 327: 1077-1081.

BASATEMUR E AND SCTCLIFFE A. 2008. Follow-up of children born after ART. Placenta.

BASATEMUR E, SHEVLIN M AND SUTCLIFFE A. 2010. Growth of children conceived by IVF and ICSI up to 12 years of age. Reproductive BioMedicine Online 20: 144-149.

BATCHELLER A, CARDOZO E, MAGUIRE M, DECHERNEY AH AND SEGARS JH. 2011a. Are there subtle genome-wide epigenetic alterations in normal offspring conceived by assisted reproductive technologies? Fertil Steril 96: 1306-1311.

BATCHELLER A, CARDOZO E, MAGUIRE M, DECHERNEY AH AND SEGARS JH. 2011b. Are there subtle genome-wide epigenetic alterations in normal offspring conceived by assisted reproductive technologies? Fertility and sterility 96: 1306-1311.

BAVISTER BD. 1995. Culture of preimplantation embryos: facts and artifacts. Human reproduction update 1: 91-148.

BAZZONI G. 2003. The JAM family of junctional adhesion molecules. Current Opinion in Cell Biology 15: 525-530.

BELL CE, CALDER MD AND WATSON AJ. 2008. Genomic RNA profiling and the programme controlling preimplantation mammalian development. Mol Hum Reprod 14: 691-701.

BERMEJO-ALVAREZ P, RIZOS D, LONERGAN P AND GUTIERREZ-ADAN A. 2011. Transcriptional sexual dimorphism during preimplantation embryo development and its

consequences for developmental competence and adult health and disease. Reproduction 141: 563-570.

BERNSTEIN KE, GIANI JF, SHEN XZ AND GONZALEZ-VILLALOBOS RA. 2014. Renal angiotensin-converting enzyme and blood pressure control. Current opinion in nephrology and hypertension 23: 106-112.

BEYDOUN HA, SICIGNANO N, BEYDOUN MA, MATSON DO, BOCCA S, STADTMAUER L AND OEHNINGER S. 2010. A cross-sectional evaluation of the first cohort of young adults conceived by in vitro fertilization in the United States. Fertility and Sterility 94: 2043-2049.

BIGGERS JD AND PAPAIOANNOU VE. 1991. Postnatal compensatory growth of manipulated mouse embryos. Human reproduction (Oxford, England) 6: 36-44.

BLOISE E, FEUER SK AND RINAUDO PF. 2014. Comparative intrauterine development and placental function of ART concepti: implications for human reproductive medicine and animal breeding. Human reproduction update 20: 822-839.

BOJA ES, HOODBHOY T, FALES HM AND DEAN J. 2003. Structural characterization of native mouse zona pellucida proteins using mass spectrometry. The Journal of biological chemistry 278: 34189-34202.

BONDUELLE M ET AL. 2005. A multi-centre cohort study of the physical health of 5-year-old children conceived after intracytoplasmic sperm injection, in vitro fertilization and natural conception. Human Reproduction 20: 413-419.

BOWDIN S, ALLEN C, KIRBY G, BRUETON L, AFNAN M, BARRATT C, KIRKMAN-BROWN J, HARRISON R, MAHER ER AND REARDON W. 2007. A survey of assisted reproductive technology births and imprinting disorders. Human reproduction (Oxford, England) 22: 3237-3240.

BOWER C AND HANSEN M. 2005. Assisted reproductive technologies and birth outcomes: overview of recent systematic reviews

Reprod Fertil Dev.

BRAUDE P, BOLTON V AND MOORE S. 1988. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. Nature 332: 459-461.

BUBB KJ, KHAMBATA RS AND AHLUWALIA A. 2012. Sexual dimorphism in rodent models of hypertension and atherosclerosis. British Journal of Pharmacology 167: 298-312.

BUSTER JE, BUSTILLO M, RODI IA, COHEN SW, HAMILTON M, SIMON JA, THORNEYCROFT IH AND MARSHALL JR. 1985. Biologic and morphologic development of donated human ova recovered by nonsurgical uterine lavage. American journal of obstetrics and gynecology 153: 211-217.

CARLSON SH AND WYSS JM. 2000. Long-Term Telemetric Recording of Arterial Pressure and Heart Rate in Mice Fed Basal and High NaCl Diets. Hypertension 35: e1-e5.

CEDERBLAD M, FRIBERG B, PLOMAN F, SJÖBERG NO, STJERNQVIST K AND ZACKRISSON E. 1996. Children: Inteffigence and behaviour in children born after in-vitro fertilization treatment. Human Reproduction 11: 2052-2057.

CEELEN M, VAN WEISSENBRUCH MM, PREIN J, SMIT JJ, VERMEIDEN JPW, SPREEUWENBERG M, VAN LEEUWEN FE AND DELEMARRE-VAN DE WAAL HA. 2009. Growth during infancy and early childhood in relation to blood pressure and body fat measures at age 8–18 years of IVF children and spontaneously conceived controls born to subfertile parents. Human Reproduction.

CEELEN M, VAN WEISSENBRUCH MM, ROOS JC, VERMEIDEN JP, VAN LEEUWEN FE AND DELEMARRE-VAN DE WAAL HA. 2007. Body composition in children and adolescents born after in vitro fertilization or spontaneous conception. The Journal of clinical endocrinology and metabolism 92: 3417-3423.

CEELEN M, VAN WEISSENBRUCH MM, VERMEIDEN JP, VAN LEEUWEN FE AND DELEMARRE-VAN DE WAAL HA. 2008a. Cardiometabolic differences in children born after in vitro fertilization: follow-up study. The Journal of clinical endocrinology and metabolism 93: 1682-1688.

CEELEN M, VAN WEISSENBRUCH MM, VERMEIDEN JP, VAN LEEUWEN FE AND DELEMARRE-VAN DE WAAL HA. 2008b. Pubertal development in children and adolescents born after IVF and spontaneous conception. Human reproduction (Oxford, England) 23: 2791-2798.

CHAN WS AND DIXON ME. 2008. The "ART" of thromboembolism: a review of assisted reproductive technology and thromboembolic complications. Thrombosis research 121: 713-726.

CHEN M, WU L, ZHAO J, WU F, DAVIES MJ, WITTERT GA, NORMAN RJ, ROBKER RL AND HEILBRONN LK. 2014. Altered glucose metabolism in mouse and humans conceived by IVF. Diabetes 63: 3189-3198.

CHIARELLI F AND MARCOVECCHIO ML. 2008. Insulin resistance and obesity in childhood. European journal of endocrinology / European Federation of Endocrine Societies 159 Suppl 1: S67-74.

CLEAL JK ET AL. 2007. Mismatched pre- and postnatal nutrition leads to cardiovascular dysfunction and altered renal function in adulthood. Proceedings of the National Academy of Sciences of the United States of America 104: 9529-9533.

COCKBURN K AND ROSSANT J. 2010a. Making the blastocyst: lessons from the mouse. J Clin Invest 120: 995-1003.

COCKBURN K AND ROSSANT J. 2010b. Making the blastocyst: lessons from the mouse. J Clin Invest.

COX GF, BURGER J, LIP V, MAU UA, SPERLING K, WU BL AND HORSTHEMKE B. 2002. Intracytoplasmic sperm injection may increase the risk of imprinting defects. American journal of human genetics 71: 162-164.

DE RYCKE M, LIEBAERS I AND VAN STEIRTEGHEM A. 2002. Epigenetic risks related to assisted reproductive technologies: Risk analysis and epigenetic inheritance. Human Reproduction 17: 2487-2494.

DELLE PIANE L, LIN W, LIU X, DONJACOUR A, MINASI P, REVELLI A, MALTEPE E AND RINAUDO P. 2010. Effect of the method of conception and embryo transfer procedure on mid-gestation placenta and fetal development in an IVF mouse model. Human reproduction 25: 2039-2046.

DENOMME MM AND MANN MRW. 2012. Genomic imprints as a model for the analysis of epigenetic stability during assisted reproductive technologies. Reproduction 144: 393-409.

DOHERTY A, MANN M, TREMBLAY K, BARTOLOMEI M AND SCHULTZ R. 2000. Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. Biol Reprod.

DOKRAS A, SARGENT IL AND BARLOW DH. 1993. Fertilization and early embryology: Human blastocyst grading: an indicator of developmental potential? Human Reproduction 8: 2119-2127.

DONJACOUR A, LIU X, LIN W, SIMBULAN R AND RINAUDO PF. 2014. In vitro fertilization affects growth and glucose metabolism in a sex-specific manner in an outbred mouse model. Biol Reprod 90: 80.

DUAH OA, MONNEY KA, HAMBLY C, KRÓL E AND SPEAKMAN JR. 2013. Limits to sustained energy intake. XVII. Lactation performance in MF1 mice is not programmed by fetal number during pregnancy. The Journal of experimental biology 216: 2339-2348.

DUMOULIN JC ET AL. 2010. Effect of in vitro culture of human embryos on birthweight of newborns. Human reproduction (Oxford, England) 25: 605-612.

DZAU VJ ET AL. 2001. The relevance of tissue angiotensin-converting enzyme: manifestations in mechanistic and endpoint data. Am J Cardiol 88: 1l-20l.

ECKEL RH, KAHN R, ROBERTSON RM AND RIZZA RA. 2006. Preventing Cardiovascular Disease and Diabetes. A Call to Action From the American Diabetes Association and the American Heart Association 113: 2943-2946.

ECKER DJ, STEIN P, XU Z, WILLIAMS CJ, KOPF GS, BILKER WB, ABEL T AND SCHULTZ RM. 2004. Long-term effects of culture of preimplantation mouse embryos on behavior. Proceedings of the National Academy of Sciences of the United States of America 101: 1595-1600.

ECKERT JJ AND FLEMING TP. 2008. Tight junction biogenesis during early development. Biochimica et biophysica acta 1778: 717-728.

ECKERT JJ AND FLEMING TP. 2011. The effect of nutrition and environment on the preimplantation embryo. The Obstetrician & Gynaecologist 13: 43-48.

ECKERT JJ, PORTER R, WATKINS AJ, BURT E, BROOKS S, LEESE HJ, HUMPHERSON PG, CAMERON IT AND FLEMING TP. 2012. Metabolic Induction and Early Responses of Mouse Blastocyst Developmental Programming following Maternal Low Protein Diet Affecting Life-Long Health. PLoS ONE 7: e52791.

ECKERT JJ, VELAZQUEZ AM AND FLEMING PT 2015. Cell Signalling During Blastocyst Morphogenesis. In*:* LEESE, JH AND BRISON, RD (Eds.) Cell Signaling During Mammalian Early Embryo Development, New York, NY: Springer New York, p. 1-21.

EDWARDS R AND BRODY S. 1995. History and ethics of assisted human conception. Humam Reprodution.

EDWARDS RG 1980. Conception in the Human Female.

EDWARDS RG. 1981. Test-tube babies, 1981. Nature 293: 253-256.

EL HAJJ N AND HAAF T. 2013. Epigenetic disturbances in in vitro cultured gametes and embryos: implications for human assisted reproduction. Fertility and Sterility 99: 632-641.

ERIKSSON JG, FORSEN T, TUOMILEHTO J, WINTER PD, OSMOND C AND BARKER DJ. 1999. Catch-up growth in childhood and death from coronary heart disease: longitudinal study. Bmj 318: 427-431.

ERIKSSON JG, KAJANTIE E, OSMOND C, THORNBURG K AND BARKER DJP. 2010. Boys live dangerously in the womb. American Journal of Human Biology 22: 330-335.

EYRIES M, MICHAUD A, DEINUM J, AGRAPART M, CHOMILIER J, KRAMERS C AND SOUBRIER F. 2001. Increased Shedding of Angiotensin-converting Enzyme by a Mutation Identified in the Stalk Region. Journal of Biological Chemistry 276: 5525-5532.

FAUSER BC ET AL. 2014. Health outcomes of children born after IVF/ICSI: a review of current expert opinion and literature. Reprod Biomed Online 28: 162-182.

FERNANDEZ-GONZALEZ R, MOREIRA P, BILBAO A, JIMENEZ A, PEREZ-CRESPO M, RAMIREZ M, RODRIGUEZ D, PINTADO B AND GUTIERREZ-ADAN A. 2004. Long-term effect of in vitro culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior. Proc Natl Acad Sci USA.

FERNÁNDEZ-GONZALEZ R, MOREIRA P, BILBAO A, JIMÉNEZ A, PÉREZ-CRESPO M, RAMÍREZ MA, DE FONSECA FR, PINTADO B AND GUTIÉRREZ-ADÁN A. 2004. Long-term effect of in vitro culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior. Proceedings of the National Academy of Sciences of the United States of America 101: 5880-5885.

FLEMING TP, KWONG WY, PORTER R, URSELL E, FESENKO I, WILKINS A, MILLER DJ, WATKINS AJ AND ECKERT JJ. 2004. The Embryo and Its Future. Biology of Reproduction 71: 1046-1054.

FLEMING TP, LUCAS ES, WATKINS AJ AND ECKERT JJ. 2011. Adaptive responses of the embryo to maternal diet and consequences for post-implantation development. Reproduction, Fertility and Development 24: 35-44.

FLEMING TP, VELAZQUEZ MA AND ECKERT JJ. 2015a. Embryos, DOHaD and David Barker. Journal of Developmental Origins of Health and Disease 6: 377-383.

FLEMING TP, WATKINS AJ, SUN C, VELAZQUEZ MA, SMYTH NR AND ECKERT JJ. 2015b. Do little embryos make big decisions? How maternal dietary protein restriction can

permanently change an embryo's potential, affecting adult health. Reproduction, Fertility and Development 27: 684-692.

FORHEAD AJ, GILLESPIE CE AND FOWDEN AL. 2000. Role of cortisol in the ontogenic control of pulmonary and renal angiotensin-converting enzyme in fetal sheep near term. The Journal of Physiology 526: 409-416.

FORRESTER T, MCFARLANE-ANDERSON N, BENNETT FI, WILKS R, COOPER R, ROTIMI C, MORRISON L AND WARD R. 1997. The Angiotensin Converting Enzyme and Blood Pressure in Jamaicans*. American Journal of Hypertension 10: 519-524.

FOSSUM GT, DAVIDSON A AND PAULSON RJ. 1989. Ovarian hyperstimulation inhibits embryo implantation in the mouse. Journal of in vitro fertilization and embryo transfer : IVF 6: 7-10.

FOXCROFT GR, DIXON WT, DYCK MK, NOVAK S, HARDING JC AND ALMEIDA FC. 2009. Prenatal programming of postnatal development in the pig. Society of Reproduction and Fertility supplement 66: 213-231.

FRÉSARD L, MORISSON M, BRUN J-M, COLLIN A, PAIN B, MINVIELLE F AND PITEL F. 2013. Epigenetics and phenotypic variability: some interesting insights from birds. Genet Sel Evol 45: 16.

FURUSE M, HIRASE T, ITOH M, NAGAFUCHI A, YONEMURA S, TSUKITA S AND TSUKITA S. 1993. Occludin: a novel integral membrane protein localizing at tight junctions. J Cell Biol.

GABORY A, ATTIG L AND JUNIEN C. 2009. Sexual dimorphism in environmental epigenetic programming. Molecular and Cellular Endocrinology 304: 8-18.

GAMBORG M ET AL. 2007. Birth Weight and Systolic Blood Pressure in Adolescence and Adulthood: Meta-Regression Analysis of Sex- and Age-specific Results from 20 Nordic Studies. American Journal of Epidemiology 166: 634-645.

GARDNER D AND LANE M. 1997. Culture and selection of viable blastocysts: a feasible proposition for human IVF? Human reproduction update 3: 367-382.

GARDNER DK, LANE M, STEVENS J, SCHLENKER T AND SCHOOLCRAFT WB. 2000. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. Fertility and Sterility 73: 1155-1158.

GARDNER DK AND SAKKAS D. 1993. Mouse embryo cleavage, metabolism and viability: role of medium composition. Human Reproduction 8: 288-295.

GASPEROWICZ M AND NATALE DRC. 2011. Establishing three blastocyst lineages - then what? The Society for the Study of Reproduction, Inc.

GEER EB AND SHEN W. 2009. Gender Differences in Insulin Resistance, Body Composition, and Energy Balance. Gender medicine 6: 60-75.

GIANNATSELIS H, CALDER M AND WATSON A. 2011. Ouabain Stimulates a Na+/K+-ATPase-Mediated SFK-Activated Signalling Pathway That Regulates Tight Junction Function in the Mouse BlastocysT. PLoS One.

GILBERT S 2003. Developmental Biology. SINATUER ASSOCIATES, INC, Seventh ed, Sunderland USA: SINATUER ASSOCIATES, INC.

GIRITHARAN G, DELLE PIANE L, DONJACOUR A, ESTEBAN FJ, HORCAJADAS JA, MALTEPE E AND RINAUDO P. 2012. In vitro culture of mouse embryos reduces differential gene expression between inner cell mass and trophectoderm. Reproductive sciences 19: 243-252.

GLUJOVSKY D, BLAKE D, FARQUHAR C AND BARDACH A. 2012. Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology (Review). WILEY.

GOYAL R, LEITZKE A, GOYAL D, GHEORGHE CP AND LONGO LD. 2011a. Antenatal Maternal Hypoxic Stress: Adaptations in Fetal Lung Renin-Angiotensin System. Reproductive sciences 18: 180-189.

GOYAL R, LISTER R, LEITZKE A, GOYAL D, GHEORGHE CP AND LONGO LD. 2011b. Antenatal maternal hypoxic stress: adaptations of the placental renin-angiotensin system in the mouse. Placenta 32: 134-139.

GRACE KS AND SINCLAIR KD. 2009. Assisted reproductive technology, epigenetics, and long-term health: a developmental time bomb still ticking. Seminars in reproductive medicine 27: 409-416.

GRAZUL-BILSKA AT, JOHNSON ML, BOROWICZ PP, BARANKO L, REDMER DA AND REYNOLDS LP. 2013. Placental development during early pregnancy in sheep: effects of embryo origin on fetal and placental growth and global methylation. Theriogenology 79: 94-102.

HAMATANI T, CARTER M, SHAROV A AND KO M. 2004. Dynamics of global gene expression change during mouse preimplantation development. Dev Cell.

HANDYSIDE A AND HUNTER S. 1986. Cell division and death in the mouse blastocyst before implantation. Roux's Arch Dev Biol 195: 519-526.

HANDYSIDE AH AND HUNTER S. 1984. A rapid procedure for visualising the inner cell mass and trophectoderm nuclei of mouse blastocysts in situ using polynucleotide-specific fluorochromes. J Exp Zool 231: 429-434.

HANSEN M, COLVIN L, PETTERSON B, KURINCZUK JJ, DE KLERK N AND BOWER C. 2009. Twins born following assisted reproductive technology: perinatal outcome and admission to hospital. Human Reproduction 24: 2321-2331.

HANSEN M, KURINCZUK J, BOWER C AND WEBB S. 2002. The risk of major birth defects after intracytoplasmic sperm injection and in vitro fertilization. N Engl J Med.

HARDY K AND HANDYSIDE AH. 1993. Cell allocation in twin half mouse embryos bisected at the 8-cell stage: implications for preimplantation diagnosis. Molecular reproduction and development 36: 16-22.

HARLOW GM AND QUINN P. 1982. Development of preimplantation mouse embryos in vivo and in vitro. Australian journal of biological sciences 35: 187-193.

HART R AND NORMAN RJ. 2013a. The longer-term health outcomes for children born as a result of IVF treatment. Part II--Mental health and development outcomes. Human reproduction update 19: 244-250.

HART R AND NORMAN RJ. 2013b. The longer-term health outcomes for children born as a result of IVF treatment: Part I--General health outcomes. Human reproduction update 19: 232-243.

HEIJMANS BT, TOBI EW, LUMEY LH AND SLAGBOOM PE. 2009. The epigenome: Archive of the prenatal environment. Epigenetics 4: 526-531.

HERICHOVA I SK. 2013. angiotensin, angiotensin-converting enzyme, angiotensin-converting enzyme 2, angiotensin AT1 receptors, experimental model. Endocrine Regulations Vol47, No1, p39–52, 2013.

HORSTHEMKE B AND LUDWIG M. 2005. Assisted reproduction: the epigenetic perspective. Human reproduction update 11: 473-482.

HUISMAN G, ALBERDA AT, LEERENTVELD R, VERHOEFF A AND ZEILMAKER G. 1994. A comparison of in vitro fertilization results after embryo transfer after 2, 3, and 4 days of embryo culture. Fertility and sterility 61: 970-971.

HURST PL AND LOVELL-SMITH CJ. 1981. Optimized assay for serum angiotensin-converting enzyme activity. Clinical Chemistry 27: 2048-2052.

ILIADOU AN, JANSON PCJ AND CNATTINGIUS S. 2011. Epigenetics and assisted reproductive technology. Journal of internal medicine 270: 414-420.

ILLMENSEE K, KASKAR K AND ZAVOS PM. 2005. Efficient blastomere biopsy for mouse embryo splitting for future applications in human assisted reproduction. Reproductive BioMedicine Online 11: 716-725.

ISHIZUKA Y, NISHIMURA M, MATSUMOTO K, MIYASHITA M, TAKEO T, NAKAGATA, N HY AND M. A. 2013. The influence of reduced glutathione in fertilization medium on the fertility of in vitro-matured C57BL/6 mouse oocytes. Theriogenology.

ISHIZUKA Y, NISHIMURA M, MATSUMOTO K, MIYASHITA M, TAKEO T, NAKAGATA N, HOSOI Y AND ANZAI M. 2013. The influence of reduced glutathione in fertilization medium on the fertility of in vitro–matured C57BL/6 mouse oocytes. Theriogenology 80: 421-426.

IWASAKI S, YOSHIBA N, USHIJIMA H, WATANABE S AND NAKAHARA T. 1990. Morphology and proportion of inner cell mass of bovine blastocysts fertilized in vitro and in vivo. J Reprod Fertil 90: 279-284.

JANNY L AND MENEZO YJ. 1996. Maternal age effect on early human embryonic development and blastocyst formation. Molecular reproduction and development 45: 31-37.

JURISICOVA A, ROGERS I, FASCIANI A, CASPER RF AND VARMUZA S. 1998. Effect of maternal age and conditions of fertilization on programmed cell death during murine preimplantation embryo development. Molecular human reproduction 4: 139-145.

KAHN BB AND FLIER JS. 2000. Obesity and insulin resistance. J Clin Invest 106: 473-481.

KALISH JM, JIANG C AND BARTOLOMEI MS. 2014. Epigenetics and imprinting in human disease. International Journal of Developmental Biology 58: 291-298.

KÄLLÉN B, FINNSTRÖM O, NYGREN K-G AND OLAUSSON PO. 2005. In vitro fertilization (IVF) in Sweden: infant outcome after different IVF fertilization methods. Fertility and Sterility 84: 611-617.

KAMPHUIS EI, BHATTACHARYA S, VAN DER VEEN F, MOL BWJ AND TEMPLETON A. 2014. Are we overusing IVF? BMJ 348.

KAYE PL AND GARDNER HG. 1999. Preimplantation access to maternal insulin and albumin increases fetal growth rate in mice. Human Reproduction 14: 3052-3059.

KELISHADI R, HAGHDOOST AA, JAMSHIDI F, ALIRAMEZANY M AND MOOSAZADEH M. 2015. Low birthweight or rapid catch-up growth: which is more associated with cardiovascular disease and its risk factors in later life? A systematic review and cryptanalysis. Paediatrics and international child health 35: 110-123.

KHOSLA S, DEAN W, BROWN D, REIK W AND FEIL R. 2001. Culture of preimplantation mouse embryos affects fetal development and the expression of imprinted genes. Biol Reprod 64: 918-926.

KIESSLING AA, DAVIS HW, WILLIAMS CS, SAUTER RW AND HARRISON LW. 1991. Development and DNA polymerase activities in cultured preimplantation mouse embryos: comparison with embryos developed in vivo. J Exp Zool 258: 34-47.

KLEIJKERS SH ET AL. 2015. Differences in gene expression profiles between human preimplantation embryos cultured in two different IVF culture media. Human reproduction (Oxford, England) 30: 2303-2311.

KLEIJKERS SH ET AL. 2014. IVF culture medium affects post-natal weight in humans during the first 2 years of life. Human reproduction (Oxford, England) 29: 661-669.

KLEIJKERS SHM ET AL. 2016. Influence of embryo culture medium (G5 and HTF) on pregnancy and perinatal outcome after IVF: a multicenter RCT. Human Reproduction.

KNOESTER M, HELMERHORST FM, VANDENBROUCKE JP, VAN DER WESTERLAKEN LAJ, WALTHER FJ AND VEEN S. 2008. Perinatal outcome, health, growth, and medical care utilization of 5- to 8-year-old intracytoplasmic sperm injection singletons. Fertility and Sterility 89: 1133-1146.

KREGE JH, JOHN SW, LANGENBACH LL, HODGIN JB, HAGAMAN JR, BACHMAN ES, JENNETTE JC, O'BRIEN DA AND SMITHIES O. 1995. Male-female differences in fertility and blood pressure in ACE-deficient mice. Nature 375: 146-148.

KWONG WY, MILLER DJ, WILKINS AP, DEAR MS, WRIGHT JN, OSMOND C, ZHANG J AND FLEMING TP. 2007. Maternal low protein diet restricted to the preimplantation period induces a gender-specific change on hepatic gene expression in rat fetuses. Molecular reproduction and development 74: 48-56.

KWONG WY, WILD AE, ROBERTS P, WILLIS AC AND FLEMING TP. 2000. Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. Development 127: 4195-4202.

LANE M AND GARDNER DK. 1997. Differential regulation of mouse embryo development and viability by amino acids. Journal of Reproduction and Fertility 109: 153-164.

LANE M AND GARDNER DK. 2007. Embryo culture medium: which is the best? Best Practice & Research Clinical Obstetrics & Gynaecology 21: 83-100.

LANE RH, KELLEY DE, GRUETZMACHER EM AND DEVASKAR SU. 2001. Uteroplacental insufficiency alters hepatic fatty acid-metabolizing enzymes in juvenile and adult rats. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 280: R183-R190.

LANGLEY-EVANS SC AND JACKSON AA. 1995. Captopril normalises systolic blood pressure in rats with hypertension induced by fetal exposure to maternal low protein diets. Comparative biochemistry and physiology Part A, Physiology 110: 223-228.

LAVERGE HD, DE SUTTER P, VAN DER ELST J AND DHONT M. 2001. A prospective, randomized study comparing day 2 and day 3 embryo transfer in human IVF. Humam Reprodution.

LEE YS ET AL. 2011. Inflammation is necessary for long-term but not short-term high-fat diet-induced insulin resistance. Diabetes 60: 2474-2483.

LI L, WANG L, XU X, LOU H, LE F, LI L, SHENG J, HUANG H AND JIN F. 2011. Genome-wide DNA methylation patterns in IVF-conceived mice and their progeny: A putative model for ART-conceived humans. Reproductive Toxicology 32: 98-105.

LONERGAN P, FAIR T, CORCORAN D AND EVANS AC. 2006. Effect of culture environment on gene expression and developmental characteristics in IVF-derived embryos. Theriogenology 65: 137-152.

LÓPEZ-CARDONA AP, FERNÁNDEZ-GONZÁLEZ R, PÉREZ-CRESPO M, ALÉN F, RODRIGUEZ DE FONSECA F, ORIO L AND GUTIÉRREZ-ADÁN A. 2015. Effects of Synchronous and Asynchronous Embryo Transfer on Postnatal Development, Adult Health, and Behavior in Mice. Biology of Reproduction.

LOPEZ-SAUCEDO J, PARAMIO-NIETO MT, FIERRO R AND PINA-AGUILAR RE. 2012. Intracytoplasmic sperm injection (ICSI) in small ruminants. Animal reproduction science 133: 129-138.

LOSKUTOFF NM, JOHNSON WH AND BETTERIDGE KJ. 1993. The developmental competence of bovine embryos with reduced cell numbers. Theriogenology 39: 95-107.

LUDWIG AK, SUTCLIFFE AG, DIEDRICH K AND LUDWIG M. 2006. Post-neonatal health and development of children born after assisted reproduction: a systematic review of controlled studies. European journal of obstetrics, gynecology, and reproductive biology 127: 3-25.

LUTTROPP K, SJOHOLM LK AND EKSTROM TJ. 2015. Global Analysis of DNA 5-Methylcytosine Using the Luminometric Methylation Assay, LUMA. Methods in molecular biology (Clifton, NJ) 1315: 209-219.

MAGNUSSON I, ROTHMAN DL, KATZ LD, SHULMAN RG AND SHULMAN GI. 1992. Increased rate of gluconeogenesis in type II diabetes mellitus. A 13C nuclear magnetic resonance study. Journal of Clinical Investigation 90: 1323-1327.

MAINOUS AG, TANNER RJ, BAKER R, ZAYAS CE AND HARLE CA. 2014. Prevalence of prediabetes in England from 2003 to 2011: population-based, cross-sectional study. BMJ Open 4.

MAKROGLOU A, LI J AND KUANG Y. 2006. Mathematical models and software tools for the glucose-insulin regulatory system and diabetes: an overview. Appl Numer Math 56: 559-573.

MANIPALVIRATN S, DECHERNEY A AND SEGARS J. 2009a. Imprinting disorders and assisted reproductive technology. Fertility and sterility 91: 305-315.

MANIPALVIRATN S, DECHERNEY A AND SEGARS J. 2009b. Imprinting Disorders and Assisted Reproductive Technology

Fertility and Sterility.

MANN M, LEE S, DOHERTY A, VERONA R, NOLEN L, SCHULTZ R AND BARTOLOMEI M. 2004. Selective loss of imprinting in the placenta following preimplantation development in culture. Development.

MARKET-VELKER BA, FERNANDES AD AND MANN MR. 2010. Side-by-side comparison of five commercial media systems in a mouse model: suboptimal in vitro culture interferes with imprint maintenance. Biol Reprod 83: 938-950.

MARTIN-PADURA I, LOSTAGLIO S, SCHNEEMANN M, WILLIAMS L, ROMANO M, FRUSCELLA P, PANZERI C, STOPPACCIARO A, RUCO L AND VILLA A. 1998. Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. J Cell Biol.

MATTSON DL. 2001. Comparison of arterial blood pressure in different strains of mice*. American Journal of Hypertension 14: 405-408.

MCAULEY KA, WILLIAMS SM, MANN JI, WALKER RJ, LEWIS-BARNED NJ, TEMPLE LA AND DUNCAN AW. 2001. Diagnosing Insulin Resistance in the General Population. Diabetes care 24: 460-464.

MCGRATH J AND SOLTER D. 1984. Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell 37: 179-183.

MCMULLEN S AND MOSTYN A. 2009. Animal models for the study of the developmental origins of health and disease. The Proceedings of the Nutrition Society 68: 306-320.

MENEZO YJ AND KHALIFA MB. 1995. Cytogenetic and cryobiology of human cocultured embryos: a 3-year experience. Journal of assisted reproduction and genetics 12: 35-40.

METZGER R, FRANKE FE, BOHLE RM, ALHENC-GELAS F AND DANILOV SM. 2011. Heterogeneous distribution of angiotensin I-converting enzyme (CD143) in the human and rat vascular systems: Vessel, organ and species specificity. Microvascular Research 81: 206-215.

MORGAN H, JIN X, LI A, WHITELAW E AND O'NEIL C. 2008. The culture of zygotes to the blastocyst stage changes the postnatal expression of an epigentically labile allele, agouti viable yellow, in mice. Biol Reprod.

NAGY A, GERTSENSTEIN M, VINTERSTEN K AND BEHRINGER R 2003. Manipulating the Mouse Embryo: A Laboratory Manual. third ed: Gold Spring Harbor Laboratory Press.

NIAKAN KK, HAN J, PEDERSEN RA, SIMON C AND PERA RAR. 2012. Human pre-implantation embryo development. Development (Cambridge, England) 139: 829-841.

NODA Y, GOTO Y, UMAOKA Y, SHIOTANI M, NAKAYAMA T AND MORI T. 1994. Culture of human embryos in alpha modification of Eagle's medium under low oxygen tension and low illumination. Fertility and sterility 62: 1022-1027.

ODOM L AND SEGARS J. 2010. Imprinting disorders and assisted reproductive technology. Curr Opin Endocrinol Diabetes Obes.

OLIVENNES F, FANCHIN R, LEDEE N, RIGHINI C, KADOCH I AND FRYDMAN R. 2002a. Perinatal outcome and developmental studies on children born after IVF. Human reproduction update 8: 117-128.

OLIVENNES F, FANCHIN R, LEDEE N, RIGHINI C, KADOCH I AND FRYDMAN R. 2002b. Perinatal outcome and developmental studies on children born after IVF. Hum Rerod Update.

OLIVENNES F, HAZOUT A, LELAIDIER C, FREITAS S, FANCHIN R, DE ZIEGLER D AND FRYDMAN R. 1994. Fertiliza1tion and early embryology: Four indications for embryo transfer at the blastocyst stage. Human Reproduction 9: 2367-2373.

OLIVENNES F, KERBRAT V, RUFAT P, BLANCHET V, FANCHIN R AND FRYDMAN R. 1997. Follow-up of a cohort of 422 children aged 6 to 13 years conceived by in vitro fertilization. Fertility and sterility 67: 284-289.

ORGURA A, OGONUKI N, INOUE K AND MOCHIDA K. 2003. New microinsemination techniques for laboratory animals. Theiogenology.

OSMOND C, BARKER DJ, WINTER PD, FALL CH AND SIMMONDS SJ. 1993. Early growth and death from cardiovascular disease in women. BMJ : British Medical Journal 307: 1519-1524.

PARLEE SD AND MACDOUGALD OA. 2014. Maternal Nutrition and Risk of Obesity in Offspring: The Trojan Horse of Developmental Plasticity. Biochimica et biophysica acta 1842: 495-506.

PATEL SK, VELKOSKA E AND BURRELL LM. 2013. Emerging markers in cardiovascular disease: where does angiotensin-converting enzyme 2 fit in? Clinical and experimental pharmacology & physiology 40: 551-559.

PAULIE S AND PERLMANN H 2001. Enzyme-Linked Immunosorbent Assay. eLS: John Wiley & Sons, Ltd.

PFEFFER P AND PEARTON D. 2012. Trophblast development. Society for Reproduction and Fertility.

PINBORG A, LOFT A, ROMUNDSTAD LB, WENNERHOLM U-B, SÖDERSTRÖM-ANTTILA V, BERGH C AND AITTOMÄKI K. 2016. Epigenetics and assisted reproductive technologies. Acta Obstetricia et Gynecologica Scandinavica 95: 10-15.

PIRES A, MARTINS P, PEREIRA AM, SILVA PV, MARINHO J, MARQUES M, CASTELA E, SENA C AND SEIÇA R. 2015. Insulin Resistance, Dyslipidemia and Cardiovascular Changes in a Group of Obese Children. Arquivos Brasileiros de Cardiologia 104: 266-273.

QUINN P, KERIN JF AND WARNES GM. 1985. Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. Fertility and sterility 44: 493-498.

RAMON M, PEREZ-GUZMAN MD, JIMENEZ-RABADAN P, ESTESO MC, GARCIA-ALVAREZ O, MAROTO-MORALES A, ANEL-LOPEZ L, SOLER AJ, FERNANDEZ-SANTOS MR AND GARDE JJ. 2013. Sperm Cell Population Dynamics in Ram Semen during the Cryopreservation Process. PLoS One 8: e59189.

RANDS GF. 1986. Size regulation in the mouse embryo. II The development of half embryos 98: 209-217.

RANKIN T, TALBOT P, LEE E AND DEAN J. 1999. Abnormal zonae pellucidae in mice lacking ZP1 result in early embryonic loss. Development 126: 3847-3855.

RAVELLI AC, VAN DER MEULEN JH, OSMOND C, BARKER DJ AND BLEKER OP. 1999. Obesity at the age of 50 y in men and women exposed to famine prenatally. The American journal of clinical nutrition 70: 811-816.

RAVI GOYAL JV-W, DIPALI GOYAL AND LAWRENCE D. LONGO. 2015. Antenatal maternal low protein diet: ACE-2 in the mouse lung and sexually dimorphic programming of hypertension. BMC Physiology.

REIK W, DEAN W AND WALTER J. 2001. Epigenetic reprogramming in mammalian development. Science 293: 1089-1093.

REXHAJ E, PAOLONI-GIACOBINO A, RIMOLDI SF, FUSTER DG, ANDEREGG M, SOMM E, BOUILLET E, ALLEMANN Y, SARTORI C AND SCHERRER U. 2013. Mice generated by in vitro fertilization exhibit vascular dysfunction and shortened life span. The Journal of Clinical Investigation 123: 5052-5060.

RICHTER KS, HARRIS DC, DANESHMAND ST AND SHAPIRO BS. 2001. Quantitative grading of a human blastocyst: optimal inner cell mass size and shape. Fertility and Sterility 76: 1157-1167.

RIVERA R, STEIN P, WEAVER J, MAGER J, SCHULTZ R AND BARTOLOMEI M. 2008a. Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development. Human Mol Genet.

RIVERA RM, STEIN P, WEAVER JR, MAGER J, SCHULTZ RM AND BARTOLOMEI MS. 2008b. Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development. Human Molecular Genetics 17: 1-14.

RIZVI S, RAZA ST, SIDDIQI Z, ABBAS S AND MAHDI F. 2015. Association of Angiotensin-Converting Enzyme and Glutathione S-Transferase Gene Polymorphisms with Body Mass Index among Hypertensive North Indians. Sultan Qaboos University Medical Journal 15: e477-e485.

ROSEBOOM TJ, VAN DER MEULEN JH, RAVELLI AC, OSMOND C, BARKER DJ AND BLEKER OP. 2001. Effects of prenatal exposure to the Dutch famine on adult disease in later life:

an overview. Twin research : the official journal of the International Society for Twin Studies 4: 293-298.

ROSSANT J. 2015. Mouse and human blastocyst-derived stem cells: vive les differences. Development 142: 9-12.

ROUGEULLE C AND LALANDE M. 1998. Angelman syndrome: how many genes to remain silent? Neurogenetics 1: 229-237.

RYAN BC AND VANDENBERGH JG. 2002. Intrauterine position effects. Neuroscience & Biobehavioral Reviews 26: 665-678.

SADHUKHAN R, SEN GC, RAMCHANDRAN R AND SEN I. 1998. The distal ectodomain of angiotensin-converting enzyme regulates its cleavage-secretion from the cell surface. Proceedings of the National Academy of Sciences of the United States of America 95: 138-143.

SAKKA SD, LOUTRADIS D, KANAKA-GANTENBEIN C, MARGELI A, PAPASTAMATAKI M, PAPASSOTIRIOU I AND CHROUSOS GP. 2010. Absence of insulin resistance and low-grade inflammation despite early metabolic syndrome manifestations in children born after in vitro fertilization. Fertility and Sterility 94: 1693-1699.

SANCHO S ET AL. 2007. Effects of cryopreservation on semen quality and the expression of sperm membrane hexose transporters in the spermatozoa of Iberian pigs. Reproduction 134: 111-121.

SANTOS MA, KUIJK EW AND MACKLON NS. 2010. The impact of ovarian stimulation for IVF on the developing embryo. Reproduction 139: 23-34.

SCHIEVE LA, MEIKLE SF, FERRE C, PETERSON HB, JENG G AND WILCOX LS. 2002. Low and very low birth weight in infants conceived with use of assisted reproductive technology. New England Journal of Medicine 346: 731-737.

SCHLAGER G. 1966. Systolic Blood Pressure in Eight Inbred Strains of Mice. Nature 212: 519-520.

SCHOLTES M AND ZEILMAKER G. 1996. A prospective, randomized study of embryo transfer results after 3 or 5 days of embryo culture in in vitro fertilization. Fertility and sterility 65: 1245-1248.

SCHULTZ RM AND WILLIAMS CJ. 2002. The Science of ART. Science 296: 2188-2190.

SCOTT KA, YAMAZAKI Y, YAMAMOTO M, LIN Y, MELHORN SJ, KRAUSE EG, WOODS SC, YANAGIMACHI R, SAKAI RR AND TAMASHIRO KL. 2010. Glucose parameters are altered in mouse offspring produced by assisted reproductive technologies and somatic cell nuclear transfer. Biol Reprod 83: 220-227.

SEGGERS J ET AL. 2012. Dysmorphic features in 2-year-old IVF/ICSI offspring. Early human development 88: 823-829.

SHANIK MH, XU Y, SKRHA J, DANKNER R, ZICK Y AND ROTH J. 2008. Insulin resistance and hyperinsulinemia: is hyperinsulinemia the cart or the horse? Diabetes care 31 Suppl 2: S262-268.

SHAW JM AND JONES GM. 2003. Terminology associated with vitrification and other cryopreservation procedures for oocytes and embryos. Human reproduction update 9: 583-605.

SHIH W, RUSHFORD DD, BOURNE H, GARRETT C, MCBAIN JC, HEALY DL AND BAKER HWG. 2008. Factors affecting low birthweight after assisted reproduction technology: difference between transfer of fresh and cryopreserved embryos suggests an adverse effect of oocyte collection. Human Reproduction 23: 1644-1653.

SHOUKIR Y, CAMPANA A, FARLEY T AND SAKKAS D. 1997. Early cleavage of in-vitro fertilized human embryos to the 2-cell stage: a novel indicator of embryo quality and viability. Human Reproduction 12: 1531-1536.

SIBONY M, GASC JM, SOUBRIER F, ALHENC-GELAS F AND CORVOL P. 1993. Gene expression and tissue localization of the two isoforms of angiotensin I converting enzyme. Hypertension 21: 827-835.

SINCLAIR KD, MCEVOY TG, MAXFIELD EK, MALTIN CA, YOUNG LE, WILMUT I, BROADBENT PJ AND ROBINSON JJ. 1999. Aberrant fetal growth and development after in vitro culture of sheep zygotes. J Reprod Fertil 116: 177-186.

STOJNIC J, RADUNOVIC N, JEREMIC K, KOTLICA BK, MITROVIC M AND TULIC I. 2013. Perinatal outcome of singleton pregnancies following in vitro fertilization. Clinical and experimental obstetrics & gynecology 40: 277-283.

SUNDE A, BRISON D, DUMOULIN J, HARPER J, LUNDIN K, MAGLI MC, VAN DEN ABBEEL E AND VEIGA A. 2016. Time to take human embryo culture seriously. Human Reproduction.

TAKEO T AND NAKAGATA N. 2011a. Reduced glutathione enhances fertility of frozen/thawed C57BL/6 mouse sperm after exposure to methyl-beta-cyclodextrin. Biol Reprod 85: 1066-1072.

TAKEO T AND NAKAGATA N. 2011b. Reduced Glutathione Enhances Fertility of Frozen/Thawed C57BL/6 Mouse Sperm after Exposure to Methyl-Beta-Cyclodextrin. Biology of Reproduction 85: 1066-1072.

TALAULIKAR V AND ARULKUMARAN S. 2012. Reproductive outcomes after assisted conception.

TARKOWSKI AK AND WROBLEWSKA J. 1967. Development of blastomeres of mouse eggs isolated at the 4- and 8-cell stage. J Embryol Exp Morphol 18: 155-180.

TEE L ET AL. 2013. Epimutation profiling in Beckwith-Wiedemann syndrome: relationship with assisted reproductive technology. Clinical Epigenetics 5: 23-23.

THOMAS FC, SHETH B, ECKERT JJ, BAZZONI G, DEJANA E AND FLEMING TP. 2004. Contribution of JAM-1 to epithelial differentiation and tight-junction biogenesis in the mouse preimplantation embryo. Journal of cell science 117: 5599-5608.

TOBI EW ET AL. 2014. DNA methylation signatures link prenatal famine exposure to growth and metabolism. Nat Commun 5.

TURA A, KAUTZKY-WILLER A AND PACINI G. 2006. Insulinogenic indices from insulin and C-peptide: comparison of beta-cell function from OGTT and IVGTT. Diabetes research and clinical practice 72: 298-301.

UNGER T. 2002. The role of the renin-angiotensin system in the development of cardiovascular disease. The American Journal of Cardiology 89: 3-9.

VALERA A, PUJOL A, PELEGRIN M AND BOSCH F. 1994. Transgenic mice overexpressing phosphoenolpyruvate carboxykinase develop non-insulin-dependent diabetes mellitus. Proc Natl Acad Sci U S A 91: 9151-9154.

VAN BLERKOM J 1989. Developmental failure in human reproduction associated with preovulatory oogenesis and preimplantation embryogenesis. In*:* VAN BLERKOM, J AND MOTTA, P (Eds.) Ultrastructure of Human Gametogenesis and Early Embryogenesis: Springer US, p. 125-180.

VAN ROY F AND BERX G. 2008. The cell-cell adhesion molecule E-cadherin. Cellular and Molecular Life sciences.

VELAZQUEZ MA, SMITH CGC, SMYTH NR, OSMOND C AND FLEMING TP. 2016. Advanced maternal age causes adverse programming of mouse blastocysts leading to altered growth and impaired cardiometabolic health in post-natal life. Human Reproduction 31: 1970-1980.

VELKER BA, DENOMME MM AND MANN MR. 2012. Embryo culture and epigenetics. Methods in molecular biology (Clifton, NJ) 912: 399-421.

VENTURA-JUNCÁ P, IRARRÁZAVAL I, ROLLE AJ, GUTIÉRREZ JI, MORENO RD AND SANTOS MJ. 2015. In vitro fertilization (IVF) in mammals: epigenetic and developmental alterations. Scientific and bioethical implications for IVF in humans. Biological Research 48: 68.

VESTWEBER D, GOSSLER A, BOLLER K AND KEMLER R. 1987. Expression and distribution of cell adhesion molecule uvomorulin in mouse preimplantation embryos. dev Biol.

VINER RM, SEGAL TY, LICHTAROWICZ-KRYNSKA E AND HINDMARSH P. 2005. Prevalence of the insulin resistance syndrome in obesity. Archives of Disease in Childhood 90: 10-14.

WADDINGTON CH. 1942. The epigenotype. 1942. International journal of epidemiology 41: 10-13.

WAGENAAR K, HUISMAN J, COHEN-KETTENIS PT AND DELEMARRE-VAN DE WAAL HA. 2008. An overview of studies on early development, cognition, and psychosocial well-being in children born after in vitro fertilization. Journal of developmental and behavioral pediatrics : JDBP 29: 219-230.

WAKEFIELD SL, LANE M AND MITCHELL M. 2011. Impaired mitochondrial function in the preimplantation embryo perturbs fetal and placental development in the mouse. Biology of reproduction 84: 572-580.

WANG YA, SULLIVAN EA, BLACK D, DEAN J, BRYANT J AND CHAPMAN M. 2005. Preterm birth and low birth weight after assisted reproductive technology-related pregnancy in Australia between 1996 and 2000. Fertility and Sterility 83: 1650-1658.

WATKINS A, PLATT D, PAPENBROCK T, WILKINS A, ECKERT J, KWONG W, OSMOND C, HANSON M AND FLEMING T. 2007. Mouse embryo culture induces changes in postnatal phenotype including raised systolic blood pressure. PNAS.

WATKINS A, WILKINS A, OSMOND C, WARNER CM, COMISKEY M, HANSON M AND FLEMING TP. 2006. The influence of mouse Ped gene expression on postnatal development. The Journal of Physiology 571: 211-220.

WATKINS AJ AND FLEMING TP. 2009. Blastocyst environment and its influence on offspring cardiovascular health: the heart of the matter. Journal of Anatomy 215: 52-59.

WATKINS AJ, PAPENBROCK T AND FLEMING TP. 2008a. The preimplantation embryo: handle with care. Seminars in reproductive medicine 26: 175-185.

WATKINS AJ ET AL. 2008b. Adaptive Responses by Mouse Early Embryos to Maternal Diet Protect Fetal Growth but Predispose to Adult Onset Disease. Biology of Reproduction 78: 299-306.

WATKINS AJ ET AL. 2008c. Low protein diet fed exclusively during mouse oocyte maturation leads to behavioural and cardiovascular abnormalities in offspring. The Journal of Physiology 586: 2231-2244.

WEKSBERG R, SHUMAN C AND SMITH AC. 2005. Beckwith-Wiedemann syndrome. American journal of medical genetics Part C, Seminars in medical genetics 137c: 12-23.

WHITE CR, DENOMME MM, TEKPETEY FR, FEYLES V, POWER SG AND MANN MR. 2015. High Frequency of Imprinted Methylation Errors in Human Preimplantation Embryos. Scientific reports 5: 17311.

WHITELAW N, BHATTACHARYA S, HOAD G, HORGAN GW, HAMILTON M AND HAGGARTY P. 2014. Epigenetic status in the offspring of spontaneous and assisted conception. Human reproduction (Oxford, England) 29: 1452-1458.

WIENER RS, CAO YX, HINDS A, RAMIREZ MI AND WILLIAMS MC. 2007. Angiotensin converting enzyme 2 is primarily epithelial and is developmentally regulated in the mouse lung. Journal of cellular biochemistry 101: 1278-1291.

WILLIAMS CA. 2005. Neurological aspects of the Angelman syndrome. Brain and Development 27: 88-94.

WILLIAMSON CM BA, THOMAS S, BEECHEY CV, HANCOCK J, CATTANACH BM ET AL. 2013. Mouse Imprinting Data and References. MRC Harwell: Oxfordshire, UK http://wwwharmrcacuk/research/genomic_imprinting/.

WOLPERT L, BEDDINGTON R, JESSELL T, LAWRENCE P, MEYEROWITZ E AND SMITH J 2002. Principles of development. Second ed, New York USA: Oxford University Press.

WOODMAN ZL, OPPONG SY, COOK S, HOOPER NM, SCHWAGER SLU, BRANDT WF, EHLERS MRW AND STURROCK ED. 2000. Shedding of somatic angiotensin-converting enzyme (ACE) is inefficient compared with testis ACE despite cleavage at identical stalk sites. Biochemical Journal 347: 711-718.

YOUNG L, FERNANDES K, MCEVOY T, BUTTERWITH S, GUTIERREZ C, CARLON C, BROADBENT P, ROBINSON J, WILMUT I AND SINCLAIR K. 2001. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. Nat Genet.

YOUNG L, SINCLAIR K AND WILMUT I. 1998. Large offspring syndrome in cattle and sheep. Rev Reprod 3: 155-163.

ZHAN QT, PAN PP, XU XR, LOU HY, LOU YY AND JIN F. 2013. An overview of studies on psychological well-being in children born following assisted reproductive technologies. Journal of Zhejiang University Science B 14: 947-960.

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26 March 2016. The manual for reproductive engneering techniques in mice. http://cardmedickumamoto-uacjp/card/english/sigen/manual/mouseivfhtml.

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