UNIVERSITY OF SOUTHAMPTON

FACULTY OF NATURAL & ENVIRONMENTAL SCIENCES

School of Biological Sciences

The Effect of Vitrification on Embryo Development

And

Subsequently Postnatal Health using Mouse Model

by

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ABSTRACT
FACULTY OF NATURAL & ENVIRONMENTAL SCIENCES
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THE EFFECT OF VITRIFICATION ON EMBRYO DEVELOPMENT AND SUBSEQUENTLY POSTNATAL HEALTH USING A MOUSE MODEL
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Animal models have shown that vitrification impairs ultrastructure and developmental potential of the oocyte, embryo survival rate, pregnancy rate and results in low birth weight of offspring but any long term effects on offspring are still unknown. In this study, embryos were vitrified at the 8-cell stage and kept in LN_2. The first experiment investigated the effect of vitrification on numbers of surviving cells (comparing vitrified and non-vitrified embryos). The blastocysts developed from each group were analysed according to cell number and allocation to trophectoderm or inner cell mass (ICM). In the second experiment, embryos were warmed and cultured until they formed blastocysts before being transferred to foster mothers. Offspring were weighed weekly, systolic blood pressure (SBP) was taken at weeks 9, 15, 21, glucose tolerance tests (GTT), serum glucose and insulin concentration analyses (subset experiment) were carried out prior to culling for organ collection at week 27. Three different treatment groups were studied (10 litters in each group); 1) **control** (non-ART treatment) 2) embryos cultured from 8-cell to blastocyst stage followed by embryo transfer (ET) to pseudo-pregnant mothers; **non-vitrified transfer** (NVT) and 3) vitrified 8-cell embryos cultured to blastocyst followed by ET; **vitrified transfer** (VT).

In the first experiment, differential nuclear labelling of blastocysts showed vitrification significantly ($p<0.05$) reduced both trophectoderm and ICM cell numbers as compared to control (n=78 to 95 blastocysts per treatment). In the second experiment, in comparison to control (n=80), offspring body weight for both sexes showed a significant increase ($P<0.05$) in NVT (n=57) and VT (n=50) groups. Both male and female offspring demonstrated a significant increase of SBP in weeks 15, 21 and LIFE (average across all 3 time points) besides having a significantly higher glucose level as compared to control. Analysis of organ to body weight ratio found that liver, lung and heart were significantly heavier in both treatment groups for both male and female. A gender specific effect was only observed in a subset experiment of serum insulin concentration and G:I ratio in which the VT group (n=7) had significantly higher levels than the control (n=9), particularly in female offspring. However, there was no significant difference between the NVT and VT groups in terms of all parameters mentioned above. In addition, perinatal weight at week 3 (W3) is correlated significantly positive with SBP LIFE in female from the VT group ($r^2 = 0.1164$). There is also a positive correlation between W3 and GTT AUC in male from the VT group. The subset sample showed that there is a significant positive correlation between W3 and serum insulin concentration and a significant negative correlation between W3 and G:I ratio in the VT female offspring. These results indicate that VT offspring, especially female are more prone to elevate blood pressure or hypertension if they are overweight. In addition, overweight females from VT also result in a higher serum insulin concentration that suggests insulin resistance. Interestingly, male offspring from VT group showed that being overweight causes high glucose levels, which may lead to diabetes.

In conclusion, the results suggest that even though vitrification alters blastocyst development, it does not *per se* change postnatal body weight, blood pressure and glucose tolerance in mice, while *in vitro* culture or embryonic transfer procedures may alter these

parameters. Vitrification however, results in a correlation between being overweight and hypertension as well as insulin resistance, particularly in VT females. Further studies on key regulators such as ACE that regulate blood pressure and PEPCK, regulator of gluconeogenesis would further explain the link between vitrification and low surviving cell number and postnatal health effects including the observed significant correlations. The European Research Council (FP7-EPIHEALTH) and MARA, Malaysia, supported this work.

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DECLARATION OF AUTHORSHIP

I, Raja Ili Airina binti Raja Khalif declare that the thesis entitled

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I confirm that:

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Signed: ……..

Date:…….

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List of Abbreviations

ART	Assisted Reproductive Technology
WHO	World Health Organization
IVF	*In Vitro* Fertilization
ICSI	Intra-Cytoplasmic Sperm Injection
ET	Embryo Transfer
HFEA	Human Fertilisation Embryology Authority
GIFT	Gamete Intra-Fallopian Transfer
LBW	Low birth weight
DMSO	Dimethyl Sulfoxide
SET	Single Embryo Transfer
BWS	Beckwith–Weidemann Syndrome
AS	Angelman Syndrome
COH	Controlled Ovarian Hyperstimulation
PCR	Polymerase Chain Reaction
SNRPN	Small Nuclear Ribonucleoprtein Polypeptide N
DMR1	Differentially Methylated Region1
LOS	Large Offspring Syndrome
CPA	Cryoprotectant agent
EG	Ethylene glycol
T_g	Glass transition temperature
PI	Propidium iodide
HE	Haematoxylin and eosin
RER	Rough endoplasmic reticulum
MII	Metaphase II
BC	Blastocoel cavity
SL	Secondary lysosomes
LS	Litter size
BA	Born alive
EPI	Epiblast
TE	Trophectoderm
PE	Primitive endoderm
ZGA	Zygotic gene activation
EGA	Embryonic gene activation
DOHaD	Developmental Origins Of Health and Disease
LN_2	Liquid nitrogen
NVT	Non-vitrified transfer
VT	Vitrified transfer
SBP	Systolic blood pressure
GTT	Glucose tolerance test
AUC	Area Under The Curve
CVD	Cardiovascular disease
ACE	Angiotensin- converting enzyme
PEPCK	Hepatic phosphoenolpyruvate carboxykinase
HOMA-IR	Homeostasis Model Assessment-Insulin Resistance

Chapter 1 Literature review

1.1 DOHaD and its relation to identifying the critical period for reprogramming

The Developmental Origins of Health and Disease (DOHaD) hypothesis evolved from epidemiological studies on infant and adult mortality. From a study between 1911 and 1930 in Hertfordshire, it was reported that the highest death rates in men is due to their low weight at birth. On the contrary, those born with higher birth weights had lower death rates (Barker et al., 1989). Another study on the Dutch famine from October 1944 to May 1945 had confirmed the hypothesis. The Dutch famine described the link between maternal nutrition during gestation and the consequence of obesity during the adulthood. Due to extreme circumstances, pregnant and lactating women who were supposed to consume at least 800 calories a day as supplementary food were just given 40% of the required calories (Parlee & Macdougald, 2013). This phenomenon allowed the study of long-term effects on pregnant mothers with nutritional deprivation. The Dutch cohort analysis identified two groups of males whose obesity rates at the age of 19 were significantly higher than normal (Lumey et al., 1993). The hypothesis was further expanded on how foetal undernutrition at different gestation stages was affecting birth phenotype and metabolic abnormalities in adult which resulted in severe diseases such as heart disease (Roseboom et al., 2001).

In 2001, Roseboom et al., reported on the effects of timing of foetal undernutrition based on the Dutch cohort. The study showed that individuals who conceived prior to the famine were exposed to an energy-poor foetal environment during a later stage of pregnancy were susceptible to the increased risk of insulin resistance and impaired glucose tolerance. In contrast, for those conceived during the famine, had increased risk of high serum cholesterol and coronary heart disease during adulthood. This was supported by Barker et al. in 1997. They proposed that maternal undernutrition at the time of gestation reprogrammed the relationship between glucose and insulin as well as between growth hormone and insulin-like

growth factor (IGF). Nutrition restriction on specific times alters or changes the function, structure and metabolism of an individual thus increases the risk of coronary heart disease in later life.

These findings confirm the DOHaD hypothesis that nutritional deprivation or restricted environment during gestation leads to disturbance in foetal and infant growth and then affects the adult health where the risk to cardiovascular and heart disease is higher (Barker & Osmond, 1986; Barker et al., 1989; Roseboom et al., 2001). Also, DOHaD states that intrauterine or postnatal adaptations to the environment cause morphological, physiological and metabolic changes through structural, functional, and epigenetic modifications that influence health during adulthood (Wadhwa et al., 2009).

Barker's hypothesis stimulated a worldwide interest in the developmental plasticity field, which leads to the concept of foetal predictive adaptive responses towards a variety of environmental cues and the consequences of mismatch between prenatal and postnatal environments. In the last 10 years, the DOHaD concept was deduced to link with epigenetics in embryonic programming (McMullen & Mostyn, 2009; Wadhwa et al., 2009; Waterland & Jirtle, 2004).

Research conducted in a mouse model by Waterland and Jirtle, showed that a high-soy diet in $A^{vy/a}$ yellow agouti mice resulted in epigenetic changes, in which the methylation of CpG islands was increased during foetal development, affecting coat colour and reducing obesity (Waterland and Jirtle, 2004). The yellow agouti (A^{vy}) mice had mutation, which caused yellow hair pigmentation. They also investigated supplementary diet during pregnancy on pregnant mothers, and suggested that when the mothers received a standard diet supplemented with methyl donors, methylation of the A^{vy} gene increased and the coat-colour distribution phenotype shifted towards a brown phenotype.

Early nutrition is essential and plays an important role in DNA methylation because biological methylation reaction is highly dependent on dietary methyl donors and cofactor (Van den Veyver 2002). As an example, the dietary methionine and choline which are major sources of one-carbon units for folic acid, vitamin B12, and pyridoxal phosphate are the critical cofactors in methyl metabolism (Reik, 2007). The genome of the preimplantation mammalian embryo undergoes process such as demethylation, and after implantation takes place, cytosine methylation reestablishment occurs. These DNA methylation patterns must be maintained over many cycles of rapid cellular proliferation during foetal and early postnatal development. Over nutrition or undernutrition causes early methyl donor malnutrition which leads to "epigenetic aging," thereby enhancing the susceptibility to cardiovascular and metabolic diseases during adulthood (Van den Veyver 2002).

Watkins et al. (2008) reported that maternal diet changes can result in hypertension, abnormal hyperactive behaviour and alteration in postnatal growth in mice. They emphasised that female offspring are more susceptible than males, even when the mice diet was altered during the periconceptional period (PC) only. The pregnant mice in the treatment group were called Emb-LPD, these mice were supplied with a low protein diet (LPD) during conception, followed by normal protein diet afterwards. Kwong et al. obtained similar results in 2000, which observed a significantly elevated blood pressure in rats from the Emb-LPD group.

An early response to Emb-LPD includes the extraembryonic lineages modulation to enhance maternal nutrient delivery in late gestation, which becomes the potential cause of developmental reprogramming (Fleming et al., 2011). This demonstrates that in a nutrient restricted environment, in order to survive, the preimplantation embryo adapts with physiological mechanism activation, which may lead to cardiovascular disease.

In relation to the DOHaD hypothesis, the issue regarding the safety of assisted reproductive technology (ART) which takes place during the periconceptional period arises, and whether ART would trigger environmental programming which later leads to chronic disease. A comprehensive review from Fleming et al. (2011) stated that the relationship between human PC environment and long-term health comes almost exclusively from the epidemiological studies that evaluate the health of children conceived from ART.

1.2 The periconceptional period (PC) and blastocyst formation

The PC is very sensitive to its environment. The period of preimplantation from fertilisation up to implantation holds crucial events such as cleavage, compaction and polarisation, which determine the fate of different cell lineages. In general, three specific lineages will later develop to distinctive features. Epiblast (EPI) gives rise to foetus, whereas trophectoderm (TE) forms the placenta and primitive endoderm (PE) contributes to the yolk sac (Cockburn & Rossant, 2010).

Initially, the maternal mRNA provides protein synthesis in the zygote. Each cell or the blastomere and mature oocyte contain a storage of protein~~s~~ pool and degraded maternal RNA transcripts during ovulation. This maternal mRNA storage controls early embryonic development and is important in coding the enzymes required for metabolismto support the early stages of embryonic development prior to the activation of zygote genome (Cockburn & Rossant, 2010). The cell cycle of zygotic gene activation (ZGA), in which the zygotic genome is activated is the longest cycle in preimplantation development. Any delay occuring at this time will result in ~~a~~ decreased mRNA concentration below critical threshold.

ZGA occurs at a different time for mouse and human (Braude et al., 1988). In mouse, the initial burst of zygotic transcription occurs at the end of the one-cell stage followed by the second burst at the two-cell stage (Aoki et al., 1997; Hamatani et al., 2004). When it comes to

the two-cell stage, maternal transcripts degrade (Bachvarova, 1985; Paynton et al., 1988). In humans, ZGA occurs between four-cell and eight-cell stages (Braude et al., 1988). This proves the preimplantation timing difference between mouse and human, therefore the relatives role of maternal and zygotic transcripts in mouse and human are different too (Cockburn & Rossant, 2010).

The early cleavage process up to the eight-cell stage is followed by compaction, a process that involves increased intercellular adhesion. In mouse, compaction is associated with the formation of adherens and tight junctions between cells (Fleming et al., 1989). Tight junctions are formed between cells on the outside, protecting the cell by sealing off the inside part. Meanwhile, gap junctions are formed between the cells, enabling small molecules and ions to pass between the cells. A major component of the adherens junction is a Ca^{2+} dependent cell adhesion protein called E-cadherin. E-cadherin from the maternal supply knocks out embryos that normally compact at the eight-cell stage, hence the embryos fail to develop properly as blastocyst (Larue et al., 1994; Riethmacher et al., 1995). Conversely, embryos inherit E-cadherin deficiency are unable to compact at the eight-cell stage, but the compaction at 16-cell stage is rescued by zygotic expression of the paternal allele (De Vries et al., 2004).

1.2.1 Blastocyst formation in response to its surroundings

The embryo initiates the developmental programme comprised of embryonic gene activation (EGA), cell cycling, lineage diversification and blastocyst morphogenesis including TE differentiation during zygotic cleavage (Eckert & Fleming, 2008). However, the embryo seems to be responsive to its surroundings 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 & Fleming, 2009). The first differentiated cell type formed in embryogenesis is an epithelium, a single-layered tissue called trophectoderm (TE) which

is formed on the outside of embryo during cleavage and later gives rise to the chorioallantoic placenta in pregnancy (Eckert & Fleming, 2008).

Compaction transforms the cell phenotype from radially symmetrical to highly polarised or epithelioid as it maximises intercellular contacts. This polar phenotype is important as one of the daughter cells may inherit the basal domain and another inherit the apical domain (Zernicka-Goetz, 2004). The different domains lead to the development of different cell types in the blastocyst. The blastocyst comprises two distinctive cell types; an outer cell layer called the trophectoderm and an inner cell group called the inner cell mass (ICM). The ICM is adjacent to the blastocoelic cavity and in contact with the basal surface of one region of TE, the polar TE. The expansion of blastocoel, mediated by TE apicobasal transport of fluid, is stimulated by *EGF* and *TGF-α* growth factors (Zernicka-Goetz, 2004).

Figure 1.1 below briefly describes the embryonic development of zygote until late blastocyst. In the mouse embryos, *CDX2*-positive cell marker is detected early in the morula stage. However, in the human embryos, *OCT4* and *CDX2* markers are clearly distinguished in the late blastocyst.

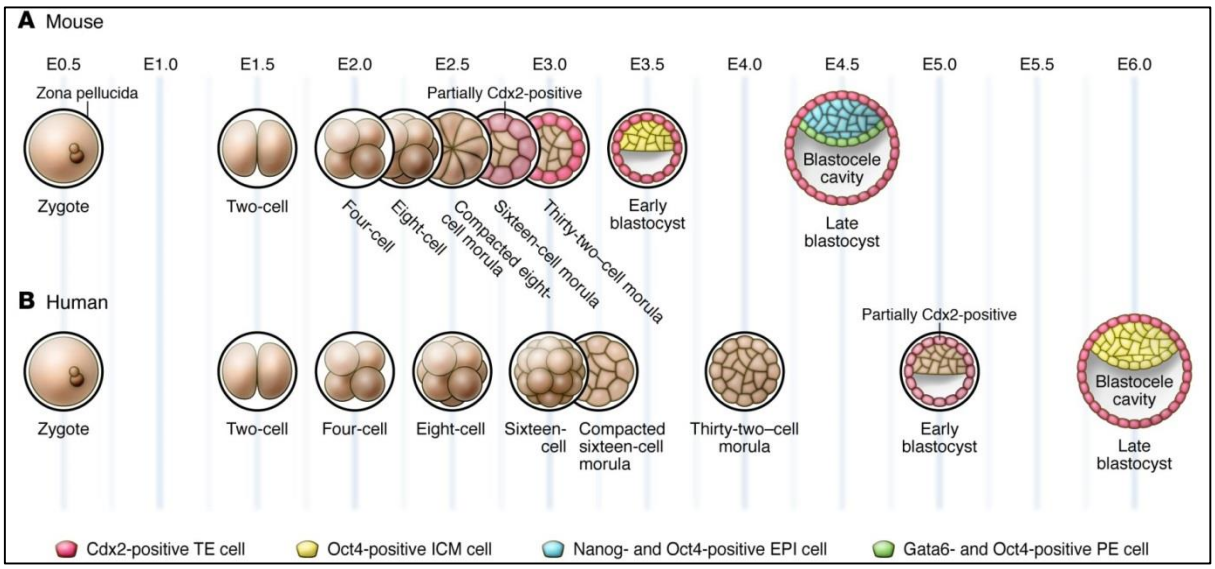


Figure 1.1: Development of embryos after fertilisation until preimplantation.
(Source from Cockburn & Rossant, 2010).

The trophoblast cells do not contribute to foetal development; instead, they give rise to foetal membrane, which is the chorion. The trophoblast lineage is characterised by the expression of *CDX2* and *EOMES* genes while the epiblast (embryonic portion of the ICM) is characterised by the expression of *NANOG* and *OCT* genes. Meanwhile, hypoblast (extra-embryonic portion of ICM) is characterised by the expression of *GATA6* gene and gives rise to the visceral yolk sac (Rossant et al., 2003).

The periconceptional (PC) period consists of orchestrated activation of development which later leads to blastocyst formation and eventually implantation. The DOHaD concept proposes that an external environment or disturbance during embryonic development would lead to alteration in offspring phenotype. However, the potential of ART procedure that have successfully produced 5 million babies to be the risk factor in generating children with cardiovascular and metabolic diseases requires further analysis.

1.3 Assisted Reproductive Technology (ART) and its contributions

Infertility is a growing problem in the world. In the analysis of 277 national surveys, the World Health Organization (WHO) reported the levels and trends, which cover 10-year time, span of infertility in 190 countries. The report claimed that in 2010, 1.9% of women in their 20s who wanted to have children were unable to have their first live birth (primary infertility) and 10.5% of women who had previously given birth were unable to have another baby (secondary infertility). This involved 48.5 million couples. Moreover, it was reported that almost 50 million couples worldwide were unable to have a child after trying for five years (Mascarenhas et al., 2012). The Human Fertilisation and Embryology Authority (HFEA) in 2012 reported 0.69% increase in the number of treatment cycles for *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI). Due to the increase in the number of couples who

find it difficult to conceive, along with the increase of childless women in the world, the need of ART such as IVF, cryopreservation, embryo transfer (ET) and ICSI is increasingly in demand.

1.3.1 Advances and implication of IVF in the human

IVF, which emerged 35 years ago, has proved successful in providing excellent pregnancy rates. The use of ART and IVF worldwide has increased exponentially since the first report in 1978. IVF was an alternative method of conception for about 10% couples worldwide (Boivin et al., 2007; Shufaro & Laufer, 2013). The HFEA in 2010 reported that there were 45,264 women who had IVF treatment in UK. In 2011, more than 2% of the babies born in UK were conceived through IVF treatment (Owen & Segars J., 2009). The number of babies born in the past 20 years was also reported to increase annually. It is estimated that 5 million babies have been born via ART method worldwide (Zegers-Hochschild et al., 2013).

ART, such as IVF and ICSI, is, however, also associated with higher incidence of induced labour and Caesarean section (Chian et al., 2014). Multiple maternal complications such as pre-eclampsia and gestational diabetes after ART raised concern regarding the safety of this procedure (Jackson et al., 2004). Other than that, possible risks on short term effect and the potential long term consequences on the use of ART towards IVF children have also been expressed (Bowdin et al., 2007).

1.4 Short term effect of ART

1.4.1 The effect on preterm delivery and birth weight

Reports from the analysis on the repercussions of ART procedures such as IVF (Sazonova et al. 2012; Miles et al. 2007; Seggers et al. 2012), ICSI (Leunens et al. 2006), *in vitro* culture medium (Nelissen et al. 2012), cryopreservation (Balaban et al. 2008; Pinborg et

al. 2004) and superovulation (Grace & Sinclair 2009) elucidated inconsistent and conflicting conclusions.

The first concern is that ART is highly associated with preterm birth (Hansen et al., 2009; Schieve et al., 2002; Stojnic et al., 2013; Wang et al., 2005). Preterm birth is considered as a live born or stillborn infant of gestation between 20 weeks and 37 weeks (Wang et al., 2005). Preterm delivery rate of IVF pregnancy is significantly higher for those with gestation of 30-32 weeks, even with singleton (Stojnic et al., 2013). Interestingly, preterm birth has resulted from the use of fresh embryos as compared with frozen embryos In contrast, a Norwegian study, which compared naturally born and ART-conceived siblings, showed no significant difference in terms of gestational age and preterm birth. They suggested that parents' infertility is the main factor of gestational age and preterm birth rather than because of the ART procedures (Romundstad et al., 2008).

IVF and ICSI procedures were also documented to affect birth weight in children (Basatemur et al., 2010; Källén et al., 2005; Knoester et al., 2008;Wang et al., 2005). It was reported that low birth weight (LBW) is attributed to higher frequency of multiple birth in IVF and ICSI children (Schieve et al., 2002). In the early 2000s, single embryo transfer (SET) was introduced to reduce the risks of pregnancy and perinatal complications. A cohort study in Australia compared ART singletons and normally conceived singletons, it was found that ART infants have a higher risk of LBW (Wang et al., 2005). LBW is 1.5 times more likely to occur among singletons conceived by transfer of fresh embryos, as compared with those of frozen embryos. The report showed that ART singletons have a 50% higher risk of having low birth weight (<2500 g). Interestingly, the reviews and meta-analyses of perinatal outcomes in ART singletons showed stronger difference between ART and non-ART singletons as compared to ART and non-ART twins (Ceelen et al., 2008a; McDonald et al., 2005).

In contrast, babies born from frozen embryos showed no difference in birth weight as compared to babies from fresh embryos (Shih et al., 2008). In fact, babies from fresh embryo transfer (ET) had a lower mean birth weight and z-score and tended to be born preterm than those from frozen ET (FET). Birth weight between fresh ART and FET is significant with 111 g difference. Even in twins, higher rate of LBW was reported from fresh ET as compared to frozen ET (55.2% vs 47.3% respectively). The report suggested that the freezing procedure *per se* did not affect the birth weight of babies, but other ART treatment such as gamete intra-fallopian transfer (GIFT), hyper stimulation and endometrial receptivity had contributed to LBW. Not only that, fresh embryos were reported to have a shorter gestation which lead to higher preterm rate and subsequently low birth weight (Shih et al., 2008). It has been proposed that the quality of eggs derived from patients and the idea that cryopreservation maybe filtering the low quality or weak embryos out, leaving all the good quality embryos before implantation was proposed (Källén et al., 2005; Schieve et al., 2002; Wang et al., 2005). Other than IVF and freezing procedures, i*n vitro* culture medium was also reported to affect the birth weight of new born babies (Nelissen et al., 2012).

1.4.2 Possible etiologies of preterm birth and LBW

Wang et al., (2005) indicated that female-factor infertility increased the likelihood of preterm birth and LBW for children conceived from ART. This is closely related to the finding that the IVF procedure, as compared with ICSI, resulted in a higher rate of preterm birth and LBW. The report concluded that female-factor infertility is highly correlated with treatment by IVF than ICSI and it is the cause of infertility, does not result in LBW and preterm birth. Similar findings were reported by (Shih et al., 2008) in which IVF and ICSI procedures did not affect the embryos, but other possible factors such as surgical intervention on woman affected the endometrial receptivity, implantation and early pregnancy and was responsible for LBW in

ART. However, the underlying biological mechanism of these complications in ART children remained unresolved.

1.4.3 Psychological, medical and morbid congenital problems associated with ART

A study comparing ICSI and normal children with matching sociodemographics according to age, number of siblings, profession of parents and location was conducted and in which no difference was found in the psychological or medical problems (Ludwig et al., 2009). Similarly, Seggers at al. in 2012 indicated that the *in vitro* procedure is not associated with an increase in dysmorphic features. They discovered that the positive association between time-to-pregnancy (TTP) as the main cause of the severity of subfertility, and clinically relevant abnormalities suggest a role of the underlying subfertility and its determinants in the genesis of dysmorphic features (Seggers et al., 2012).

Although the majority of ART babies are healthy, a small percentage of them suffer from a variety of significant morbid congenital problems. Children conceived by IVF or ICSI have twice the rate of major birth defects and congenital malformation as compared with babies conceived naturally (Hansen et al., 2002). These facts were supported by a cohort study on 540 ICSI 5-year-old children from five European countries. The report claimed that the odds of major malformation were 2.77 for ICSI children and 1.80 for IVF children as compared with naturally conceived children (Bonduelle et al., 2005). Furthermore, the higher rate observed in the ICSI group was due to an excess of malformation in male urogenital system (Källén et al., 2005).

This is similar to a report in Australia in which between 1993 and 1997, babies conceived from ICSI or IVF have twice the risk of having major birth defects as compared to naturally conceived babies. One year old babies were reported with defective organ systems such as cardiovascular, urogenital, and metabolic where 8.6% were from ICSI babies, and 9%

were the IVF babies, as compared to 4.2% in naturally conceived babies. They also found that there was a higher occurrence of major cardiovascular, urogenital, chromosomal, and musculoskeletal defects associated with babies conceived from ART. However, these findings should be interpreted with caution, since they are based on small numbers of infants in each group. The evaluation measurement in this study is biased because the number of babies conceived naturally was far higher, leading to a small percentage of babies with major birth defect. In contrast, Zhan et al. in 2013 concluded there were no difference in cognitive development between ART and normal children. The inconsistent outcomes from different cohorts is due to different evaluation measurement techniques. All studies suggested that follow-up study on IVF children should be carried out to give further understanding.

1.5 Long term effect of ART

1.5.1 Cognitive and behavioural development

Concerns about potential long term effects on ART children were raised and documented (Cederblad et al. 1996; Leunens et al. 2006). The cognitive and behavioural development following ART was assessed on aged 3 to 7 years. The results showed normal development in IVF children as compared to the naturally conceived children (Cederblad et al., 1996). However, Leunens et al. in 2006 found a difference in IQ of 8-year-old ICSI children. Interestingly, a follow-up study revealed that the previously reported difference of cognitive development between children born after ICSI and naturally conceived disappeared when they reached the age of 10 years. The result indicated that the effect of maternal educational level or home environment decreased in the ICSI group over time (Leunens et al., 2008). However, in a study conducted by Zhan et al. in 2013, it was found that children born from ART procedures had lower intelligence quotient (IQ) scores, lower visual-motor ability

or locomotor development, and delayed receptive language competence. Furthermore, ICSI children were found to have a higher risk of autism than the general population, and a higher prevalence of behavioural problems than the normal children (Zhan et al., 2013).

In a behavioural study including morphological and histological techniques, changes in genome-wide DNA methylation in the brain after IVF and ET was reported in mice (Li et al., 2012). The researchers suggested that small clusters of CpG islands and promoters were aberrantly methylated in IVF-ET mice, showing that hypermethylation was more common than hypomethylation over two generations (F1 and F2). This indicated that the somatic methylation pattern slightly changed due to ART and it is heritable. Research into the effect of ART on the transmission of epigenetic susceptibility to their offspring through non-genomic mechanisms is less established. However, there were no significant difference in behaviour or morphology observed between IVF-ET and naturally conceived mice (Li et al., 2011).

1.5.2 Growth and developmental potential

Some early studies on IVF children from the age of 1 to 13 years pointed out that there was no significant pathological feature concerning growth and physical development of IVF children (Olivennes et al., 1996; Olivennes et al., 1997; Wennerholm, 2000). Furthermore, the scholastic performance of IVF children was reported to be encouraging (Olivennes et al., 1997).

A prospective match-controlled but small study was performed to investigate the growth of children born in UK, aged 12 and below following standard IVF and ICSI compared to naturally conceived but no significant differences were found regarding their weight, length and head circumference at birth (Basatemur et al., 2010). However, it was found that as the IVF children grew older, they were significantly taller than those who were naturally

conceived after adjustment with age and parental height. There were no differences in the level of psychological or medical problems reported between the ICSI and naturally conceived children between the age of 4 to 6 years, however, it is too early to rule out the genetic interference and environmental factors which may also affect their reproductive health later on.

1.5.3 Cardiovascular and metabolite profile

Whether ART children are prone to cardiovascular diseases or related symptoms such as high blood pressure is also a major concern. In a study conducted by Ceelen et al. in 2008a, it was shown that the cardiometabolic health of IVF children was different as compared to naturally conceived children, where the former exhibited higher blood pressure and fasting glucose concentration. Similarly, Miles et al. in 2007 pointed out that ART children have significantly higher serum concentrations of insulin-like growth factor II and their serum fasting lipid profile comprised higher high-density lipoprotein and lower triglyceride concentrations.

1.5.4 Imprinting disorders and their association with ART

Reports on the increased incidence of imprinting disorder in children conceived by ART had also raised concern (Bowdin et al., 2007; Iliadou et al., 2011;Owen & Segars J., 2009). Particularly, reports suggested that IVF increases the risk of diseases caused by aberrant genomic imprinting (the preferential silencing of one parental allele by epigenetic modification), which includes Angelman syndrome (AS) and Beckwith–Weidemann syndrome (BWS) (Bowdin et al., 2007).

1.5.4.1 Epigenetics and ART

The fact is the phenotype of an individual is not exclusively determined by the genotype. In 1942, epigenetics was defined as heritable changes in gene expression which occur without any changes in gene sequence (Waddington, 1942). Epigenetic modifications are heritable in the sense that the 'epigenetic status' of a chromatin is preserved during cell mitosis. Epigenetic modifications are known to affect expression, including histone modifications such as methylation, phosphorylation, acetylation, ubiquitination and changes in nucleosome positioning and conformation (Iliadou et al., 2011). DNA methylation however is the most important and studied in mammals (Iliadou et al., 2011).

DNA methylation in mammals is exclusively restricted to CpG nucleotides. The methylation of CpG nucleotides represses transcription either by blocking the binding of transcription factors to the promoter or adding histone-modifying protein through a more condensed chromatin structure formation (Iliadou et al., 2011). The maintenance of methyltransferase 1 is upregulated and recruited to the replication fork during cell division. This is when it methylates CpG dinucleotides on the newly synthesised daughter strand. Methylation patterns and cellular identity are preserved throughout mitosis by the symmetric methylation of the newly synthesised strands (Berger, 2007).

Genomic imprinting refers to the phenomenon where certain genes are expressed in a parent-of-origin-specific manner (Iliadou et al., 2011). By definition, only one (maternal or paternal) allele is active, and the inactive allele is epigenetically marked by histone modification, DNA methylation or both (Reik et al., 2001). Currently, there are more than 80 imprinted genes detected in mammals, and the majority of them play their role in embryonic and placental growth and development. Abnormalities in genomic imprinting in humans cause conditions such as BWS and AS (Iliadou et al., 2011).

There are two critical periods where epigenetic modifications occur in mammalian reproduction, namely gametogenesis and preimplantation development. During gametogenesis, genome-wide demethylation occurs, followed by remethylation prior to the fertilisation process (Figure 1.2). Early embryogenesis is characterised by the second genome-wide demethylation event, a process which is sensitive to environmental factors (Santos et al., 2002).

Stages in germ-cell and early embryonic development are the specific periods in which ART will influence and disturb the methylation and/or demethylation processes (indicated by arrow in Figure 1.2). ART is involved throughout embryonic development until conception. Thus, one or more ART procedures may alter the normal imprinting process. For example, hormones are used to downregulate pituitary function and to stimulate multiple oocyte production, *in vitro* maturation of oocytes, *in vitro* culture of preimplantation embryos, and cryopreservation of either gametes or embryos (Iliadou et al., 2011).

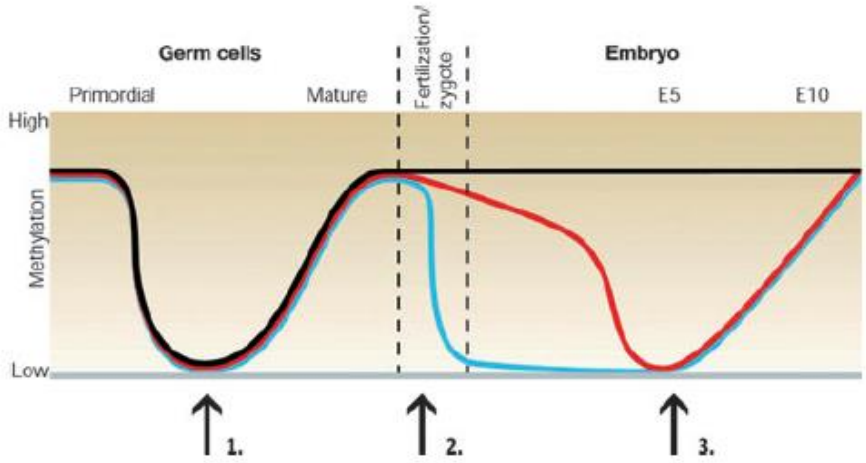


Figure 1.2: Methylation reprogramming in the germline and embryo. The methylation level of methylated (black) and non-methylated (grey) imprinted genes as well as non-imprinted sequences (red, maternal; blue, paternal) is shown during germ-cell and early embryonic development. The horizontal axis (time) and the vertical axis (methylation level) indicate the relative methylation on levels are not to scale by time. E=embryonic day. Arrows indicate stages in germ-cell and early embryo development in which assisted reproductive techniques

(ART) influence and disturb the processes of methylation and /or demethylation processes. Figure is modified from (Reik et al., 2001). Adapted from (Iliadou et al., 2011).

During ART, oocytes are retrieved via stimulated superovulation and hormonal stimulation from the ovaries prior to ovulation. Subsequently, oocytes are cultured *in vitro* for maturation before undergoing cryopreservation or fertilisation. These steps influence and disturb the natural remethylation process during the later stage of oocyte maturation (Figure1.2, arrow 1), thereby affecting genomic imprinting of the maturing oocyte (Iliadou et al., 2011). The current ART protocols favour ~~the~~ blastocyst stage for ET as it may result in a higher pregnancy rate. However, extended exposure to different types of culture medium during early embryonic development potentially influences the remethylation process (Figure1.2, arrow 3).

Using a mouse model, a recent study reported differential expressions of paternally expressed genes in testes of ART mice, mediated by methylation modification (Zhao et al., 2013). Semen quality, serum testosterone, histological analysis of testes and 6 paternally expressed imprinted genes in testes of adult F1 and F2 mice were investigated. During methylation analysis, there were significant differences in the methylation level which coincided with altered gene expression between natural mating (control group). *KCNQ1OT1, MEST, PEG3, PLAGL1* and *SNRPN* showed lower expression in ICSI mice as compared to control. Besides, the expression of *KCNQ1OT1, PEG3* and *SNRPN* in IVF conceived mice was lower than those in controlled ovarian hyperstimulation (COH) conceived mice but higher than ICSI mice. The F2 generation also had a significant difference between control and ICSI groups and between the IVF and ICSI groups. The study claimed that *in vitro* culture and mechanical stimulation were the main factors that induced the downregulation of paternally expressed imprinted genes in testes. Even though no phenotypic abnormality was detected,

both ICSI procedure and mechanical stimulation were shown to induce intergenerational transmission of the epigenetic changes.

1.5.4.2 Gene imprinting from Angelman syndrome (AS) and Beckwith–Weidemann syndrome (BWS)

Angelman syndrome is a rare neurogenetic syndrome with an estimated prevalence of 1 in 10,000 among children and 1 in 20,000 among young adults (Williams, 2005). Since 2002, there are reports of an increased incidence of children with AS and BWS following IVF or ICSI conception (Allen & Reardon, 2005). AS is a an example of genetic imprinting because the syndrome results from the loss of maternal expression of *UBE3A* gene which is located at the chromosome 15q11–1339, known to be imprinted in the brain (Matsuura et al., 1997; Rougeulle & Lalande, 1998). The four genetic mechanisms known to cause AS are deletion of maternal *UBE3A* (68% of cases), intragenic *UBE3A* mutation on maternal allele (13% of cases), uniparental disomy (3% of cases) and an imprinting defect which turns off the maternal allele (6% of cases) (Williams, 2005). A study on children post-ICSI was carried out to analyse the methylation patterns by PCR multiplex but, no abnormality at the 15q11-13 region was found (Bowdin et al., 2007). Cox et al. (2002) reported two cases of AS post-ICSI to secondary male infertility, suggesting that ICSI increases the risk of imprinting defects. Molecular analysis by methylation-specific PCR and Southern blotting with the small nuclear ribonucleoprotein polypeptide N (SNRPN) probe demonstrated that both cases showed hypomethylation of the SNRPN locus (Cox et al., 2002), a sporadic imprinting defect on the maternal chromosome. Given the low proportion of AS cases worldwide attributed to imprinting defects (estimated range of 4% to 6%), the incidence of AS with an imprinting defect due to the molecular etiology was approximated to be 1 in 300,000 (Cox et al., 2002). Thus, there were concerns raised on the association of AS and ICSI technique caused by imprinting defects.

BWS is an imprinting disorder which results from altered expression or mutation in imprinted genes that are critical for normal growth and development (Bowdin et al., 2007). In the majority of cases, BWS was due to imprinted genes from chromosome 11p15.5 and there was evidence to support the regulatory interactions between two imprinted domains (Weksberg et al., 2003). Domain 1 is the imprinting centre of differentially methylated region1 (DMR1) which regulates *IGF2* and *H19*. There were also reports of *H19*-dependent *IGF2* biallelic expression where methylation at the maternal *H19* and *DMR1* leads to loss of *H19* expression and biallelic *IGF2* expression (Weksberg et al., 2005). Domain 2 contains the imprinting center of *DMR2* and *KCNQ1OT*. Maternal methylation of *DMR2* normally silences *KCNQ1OT1* on the maternal allele so that the paternal gene would dominantly express. Most reports from BWS cases exhibit loss of maternal methylation of *DMR2*. Loss of methylation of *DMR2* results in reduced expression of *CDKN1C*, a potential negative regulator of foetal growth and is associated with *KCNQ1OT1* (Weksberg et al., 2005).

A British survey concluded that 2.9% BWS patients (6 out of 209) were conceived by IVF or ICSI. The value was compared to the estimated incidence of IVF/ICSI in the general UK population which was 0.8%, thus showing a higher frequency of IVF/ICSI than in the general population (Sutcliffe et al., 2006). Other than AS and BWS, several studies have suggested a possible link between ART and various imprinting disorders including Silver-Russell syndrome (SRS), maternal hypomethylation syndrome, and retinoblastoma (Iliadou et al., 2011; Owen & Segars J., 2009).

1.5.4.3 Gene imprinting associated with ovarian stimulation and embryo culture

Effects of ovarian stimulation and embryo culture medium on genomic imprinting in animal models were reported (Cox et al., 2002; Reik et al., 2001). An established report on large offspring syndrome (LOS) claimed that the loss of methylation at an imprinting region in the *IGF2R* gene is associated with LOS, a well-known phenotype of cattle born after *in*

vitro culture (Young 1998). It is characterised by foetal overgrowth, breathing difficulty and sudden perinatal death. Moreover, culture medium is the cause of epigenetic alteration that affects genomic imprinting, which plays a crucial role in foetal growth control (Neri et al., 2008).

Concern arises because ART procedures such as ovarian stimulation and preimplantation embryo manipulation occur during critical developmental period when genomic imprinting is shown to pose risk when studied on animals (Iliadou et al., 2011). This indicates that imprinting disorders are due to underlying subfertility or ovarian stimulation without subsequent *in vitro* procedure (Owen & Segars J., 2009). Up to now, the association between imprinting disorders and ART protocols remain unproven.

1.5.4.4 Infertility etiologies

Recently, the use of human ART has not been proven to be associated with the increased occurrence of epigenetic disorders. The relative contribution of pre-existing biology to epigenetic disturbances versus that added by ART is impossible to resolve. The rarity of imprinting disorders such as the Angelman syndrome, obesity and type II diabetes which are expressed in later developmental stage, cause no apparent link between ART and epigenetic changes. Besides, etiology is likely to be infertility inherited from the parents rather than the procedure, especially for children born with BWS after ART (Doornbos et al., 2007; Sutcliffe et al., 2006).

Reports established the link between infertility etiology with the increased occurrence of epigenetic anomalies in animal models (Grace & Sinclair, 2009); Houshdaran et al., 2007). Extensive animal studies were carried out to understand the mechanism underlying epigenetic reprogramming in ART offspring (Hasegawa et al., 2012; Li et al., 2005). It was considered whether the methylation pattern changes of various genes between ART and normal offspring

resulted from ART and/or the etiology of infertility (Horsthemke & Ludwig, 2005). Impaired male subfertility is the main factor for epimutations in sperm (Houshdaran et al., 2007). However, superovulation and *in vitro* culture were also shown to cause low birth weight in offspring, affecting epigenome and establishing epigenetic effects to the embryos or offspring (Grace & Sinclair, 2009; Market-Velker et al., 2010). These studies suggested that ART is not the sole reason for adverse health in ART offspring for animals.

1.6 Cryopreservation

Embryo freezing is a well-established technique as it forms an integral part of IVF routine worldwide. This procedure maximises the rate of conception per stimulation cycle or oocyte collection, reduces wastage of valuable embryos, minimises health risks and is more cost effective. Cryopreservation of supernumerary embryos obtained from a single IVF cycle allows single, double, and multiple ET, thus preventing multiple pregnancies and enhancing cumulative pregnancy rates. Concomitantly, embryo cryopreservation can be used to postpone embryo transfer in patients who are at high risk of ovarian hyperstimulation syndrome and preserve fertility option for patients who are preparing for chemotherapy or radiation therapy (Shin et al., 2011). Embryo cryopreservation has a been widely used to maintain genetic resources of domestic and wild animals, a cost effective approach to maintain scientifically important stocks, strains and lines (Boonkusol et al., 2006).

The first report of a successful vitrification was done using frog sperm (Basille & Eugune, 1938). The report emphasised the crucial role of 2 mol/L sucrose in water as a vitrification medium, and the usage of small drops had resulted in an increased medium viscosity. After the success of freezing the first cell using frog sperm, there were successful attempts to freeze mouse embryos in 1972 and calf embryos in 1973 (Whittingham et al., 1972; Wilmut & Rowson, 1973). Since then, exuberant successes on several species were reported using the slow freezing technique (Saragusty & Arav, 2011).

Cryopreservation for domestic and experimental animals requires DMSO, glycerol, or ethylene glycol but for human embryos, propanediol supplemented with sucrose was used as cryoprotectant at the early stage of embryonic development. With some modification on the cryoprotectant agent (CPA), the first child was born with embryo freezing in 1984 and in 1986, there was a child born from oocyte freezing (Chen, 1986; Zeilmaker et al., 1984). This then lead to the attempt to freeze human embryos or oocytes with some modifications (Mukaida & Oka, 2012).

1.6.1 Cryopreservation techniques

Cryopreservation is a freezing technique that removes free water to minimise water damage from ice crystal growth by uncontrolled removal of bound water, thus providing structural support to proteins, DNA and membranes. Dehydration and cryoprotectant uptake were enhanced by slow and rapid cooling to reduce the likelihood of free water to form large intracellular ice crystals, which are lethal to the cells (Shaw & Jone,s 2003). The cryoprotectant agent (CPA) is responsible in preventing formation of intracellular ice. The slow-freezing and vitrification techniques were different approaches of freezing cells or embryos by using different CPA.

1.6.2 Cryoprotectant agents (CPA)

CPA consists of different solutions that protect cells and embryos from freezing injury caused by intracellular ice formation. The combination of CPA solutions used is different between stages of embryos, oocytes and cells, but the common solution is made up of carrier solution (M2 or H6BSA) combined with a penetrating and a non-penetrating cryoprotectant (Chapman, 2007). Carrier solution which contains salts, osmotic agents, and pH buffers play a role in providing basic support for cells at near-freeze-temperature. Carrier solution is also

called base perfusate and usually presents at near isotonic concentrations (300 milliosmoles), preventing cells from shrinking or swelling.

The most common penetrating CPA is made up of ethylene glycol (EG), dimethyl sulfoxide (DMSO) and glycerol. In 1958, Sherman and Lin showed that equilibration of 5% glycerol was an important step in supercooling mouse oocytes at -20^{o}C, with 0.6^{o}C/min rate. However, cryopreserved oocytes faced another problem which is intracellular crystallization due to sudden low temperature (Sherman & Lin, 1958). EG is among the most effective cryoprotectant glycol tested for mouse embryos and commonly used as an antifreezing agent because of its low freezing point (Miyamoto & Ishibashi, 1979).

A combination of penetrating cryoprotectants enables a suitable CPA for cryopreservation. Dimethyl sulfoxide (DMSO), a small molecule, is used to cross cell membranes. Critser et al. in 1997 claim that when embryos are placed in a low concentration of DMSO (1 mol/L) at 20^{o}C, they would shrink by 25% in few seconds. The embryos would return to their original size within 20 to 25 minutes after an influx of DMSO into the cell, replacing the lost water. A higher concentration of DMSO (6 mol/L) also results in embryo shrinkage by 25% within a few seconds, but takes longer to return to their original size. They also reported that the degree of dehydration a cell could withstand is unknown, as DMSO is also associated with irreversible damage to the cell, resulting in collapsed and flattened embryos due to loss of free and bound intracellular water (Critser et al. 1997). Furthermore, another study from Anchordoguy et al. in 1991) reported that DMSO is attributed to electrostatic interaction between its polar sulphoxide moiety and phospholipid membrane in liposomes.

Another common CPA is non-permeating cryoprotectant such as sucrose, in which its large molecule inhibits ice growth. The use of non-permeating CPA is essential because it

increases the tonicity (osmotically active concentration) of CPA and prevents chilling injury. Another important factor is to avoid cell dehydration while maintaining a barrier or stabilising effect outside the cell membrane and reducing the CPA toxicity (Arav, 2014). The combination of DMSO in CPA then acts as a facilitator, acting as a carrier which enables other cryoprotectants to penetrate the cell without causing damage. One of the concerns with cryopreservation is the CPA, which leads to cell damage (Arav, 2014). Higher concentration solute results in high CPA toxicity, and this requires minimal exposure of the cryoprotectant in the cell. The permeable cryoprotectants are all toxic and can cause osmotic shock, particularly at high concentration. The CPA concentration and temperature play an important role in dictating the time length of embryos to be exposed to vitrification medium prior to the embryo freezing in LN_2. Temperature affects embryo permeability and CPA toxicity (Ménézo et al., 1992) . Toxicity is overcome by making up physiological solution consisting of one or two cryoprotectants capable of permeating the cell membranes (DMSO and EG) and non-penetrating solute such as sucrose (Anchordoguy et al., 1991; Crowe et al., 1988). The combination of solutes which complements the freezing mechanism is required as permeating cryoprotectants lower the freezing point and replace some bound water molecules in and around proteins, DNA, and other intracellular components. On the other hand, sucrose or other non-penetrating additives stay outside and aid in dehydration before and during preservation as they help to stabilize the phospholipid head groups. Figure 1.3 shows industrial CPA with a combination of different solutions to freeze cell.

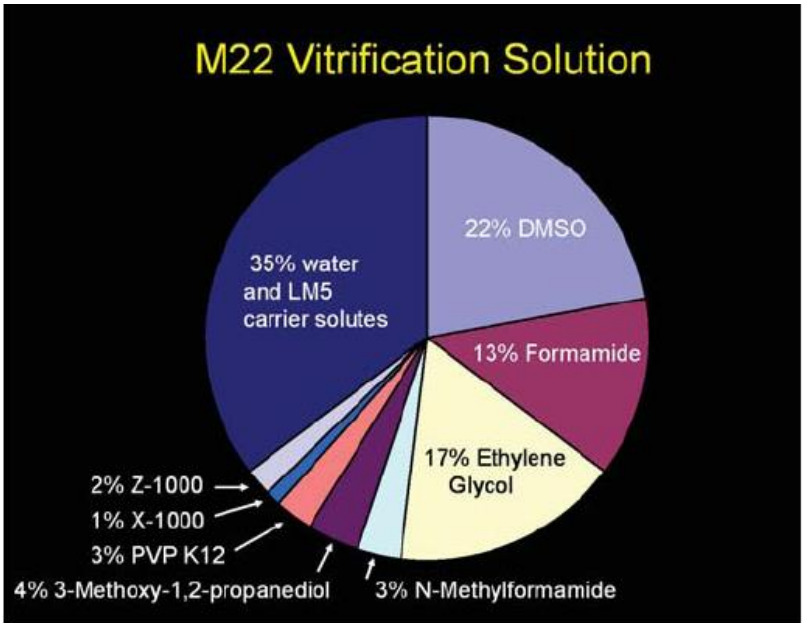


Figure 1.3: M22 vitrification solution is comprised of penetrating cryoprotectants, except for LM5 carrier solutes, Z-1000 and X-1000 ice blockers, as well as PVP K12 polymer (Chapman, 2007).

1.6.3 Slow freezing and ultra-rapid freezing (vitrification)

Embryo cryopreservation technique consists of two main categories; slow-freezing and vitrification. Slow-freezing method requires a gradual decrease of temperature with manual seeding at the temperature of -7^{o}C in a long duration, involving the use of expensive programmed instruments (Mukaida & Oka, 2012). Slow-freezing is still used today, and gives promise of good clinical outcome (Abdelhafez et al., 2010). However, a meta-analysis study claimed that embryo vitrification is superior to slow freezing based on embryo survival rate, clinical pregnancy rate, ongoing pregnancy and implantation rate (Desai et al., 2007). A more effective technique is by gradually increasing the cryoprotectant concentration compared to the single step of freezing method.

Vitrification was first used in mouse embryos back in 1985 (Rall & Fahy, 1985). In 2012, vitrification was reported as a better alternative than slow-freezing because it eliminates

injuries from ice formation (Mukaida & Oka, 2012). Vitrification reduces chilling sensitivity and crystallisation which are damaging to cells due to its rapid procedure (Arav, 2014).

The key factor to achieve a high survival rate for vitrification is to eliminate crystallisation and there are a few factors that need to be considered such as the CPA volume, the CPA viscosity and the cooling velocity rate (Figure 1.4). A smaller volume of CPA results in a higher heat transfer to the embryos, because smaller volume allows better heat transfer and cooling rate (Saragusty & Arav, 2011). Secondly, it is important to acquire a high CPA viscosity without increasing its concentration and avoiding toxicity in CPA. As mentioned earlier, the combination of different solutions is vital in lowering the toxicity and concurrently increasing the viscosity. A higher viscosity of cryoprotectant results in a high glass transition temperature (T_g) which reduces the formation of ice nucleation and crystallisation (Arav, 2014). Cooling and warming rate of the cell also plays an important factor. Cooling velocity depends on the thermal mass sample and surface area. Lower heat mass results in a higher surface area to volume ratio which is crucial for successful vitrification. If the vitrification procedure is carried out carefully, then the issues such as fracture, crystallisation and devitrification can be avoided (Saragusty & Arav, 2011).

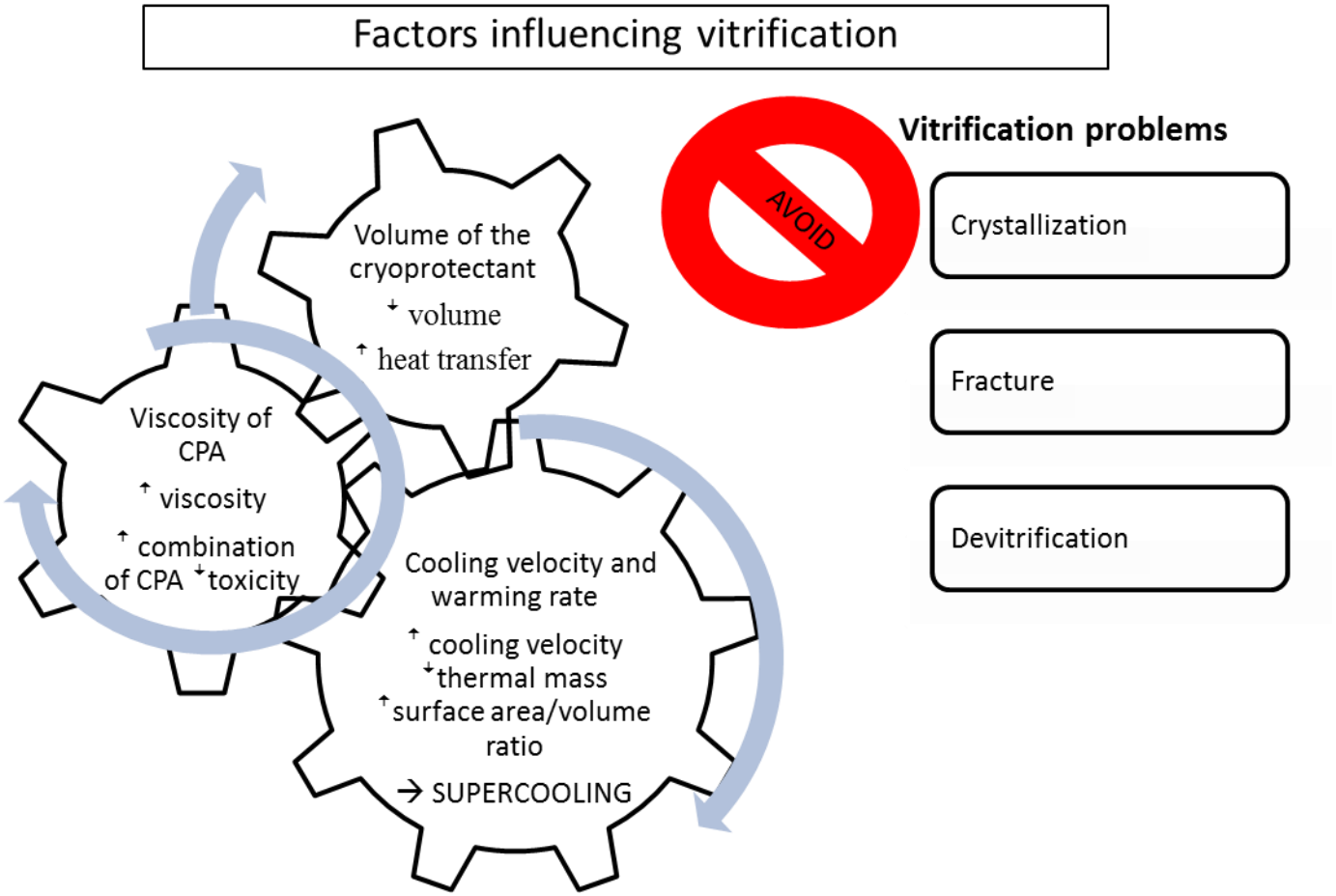


Figure 1.4: The important factors for vitrification are the CPA volume, CPA viscosity and the cooling velocity rate.

Vitrification uses very high concentration of cryoprotectants and rapid cooling rate which results in solidification of the solution, therefore preventing the formation of ice crystals and associated cellular damage. Vitrification involves extreme dehydration where cells are pre-equilibrated with low cryoprotectant concentration, then followed by high concentration of additive solutes such as sucrose for a better recovery rate from the treatment (Shaw & Jones, 2003). In the previous study, the equilibration medium was introduced to the embryos before a higher concentration of toxic CPA to avoid osmotic shock to the cell (Shaw & Jones, 2003).

Cryoprotectant at high concentration is toxic to the cells, especially to one-cell embryos and oocytes (Mukaida et al., 1998). Vitrification procedure provides better results with

warming as compared to the slow freezing. The cell shrinking and recovery rate depend on the osmolarity of the new and old solutions, cryoprotectant type, temperature and cell type. Nevertheless, cells which are exposed to physiochemical stress undergo physical change because of ice formation due to the extreme decrease of temperature during freezing. The sudden lowering of temperature leads to cold-shock injury in which cell structure and function are damaged. This also affects membrane permeability and causes cytoskeletal structural change (Shaw & Jones, 2003).

1.6.4 Vitrification in human embryos and oocytes

Although the vitrification procedure is able to minimise damage caused by ice crystal formation and growth, cryopreservation leads to various injuries, such as formation of intracellular ice in cells or embryos (Kasai, 1997). In recent years, vitrification is part of IVF because the technique is simple and provides high clinical efficiency along with a better clinical outcome. With CPA effectiveness, the method was refined and proved to be suitable for certain stages of human embryonic development. Mukaida et al. in 1998 reported that vitrification which used an ethylene glycol-based vitrification solution (EFS40) with conventional cryostraws was suitable for human embryos at the four-to-eight cell stage. There was another report on the effectiveness of ethylene glycol-based solution for vitrification at the eight-to-16-cell stage and morula stage (Saito et al., 2000; Yokota et al., 2001).

The use of CPA for vitrification is different in laboratory and domestic animals, as well as humans (Lassalle et al., 1985). CPA such as dimethylsulphoxide (DMSO), glycerol, or ethylene glycol are used commonly in animals, but for human embryos at the early cleavage stages, propanediol solution supplemented with sucrose is used while embryos at the blastocyst stage are frequently frozen with glycerol and sucrose (Hartshorne et al., 1991; Lassalle et al., 1985; Ménézo et al., 1992).

Protocols and clinical procedures have similar basic concepts and their outcomes are presented in Table 1.1. Vitrification is carried out by using the routine two-step vitrification procedure. The first step is to expose embryos with pretreatment equilibration medium, consisted of low CPA concentration and macromolecule such as Ficoll to promote vitrification (Kasai et al., 1990). The second step is to use cryostraw, cryoloop and cryotop as a container and the embryos are vitrified in ethylene glycol-based solution before being plunged in liquid nitrogen.

Table 1.1: Protocols: concentration, time and properties of the vitrification solution for day 2-3 human embryo cryopreservation

Author/ Reference	Mukaida et al., 2012	Desai et al., 2007	Rama Raju et al., 2005	Kuwayama et al., 2005
Type of container	Cryostraw	Cryoloop[a]	Cryoloop[b]	Cryotop
Temperature	Room (25-27ºC)	Warm stage (37ºC)	Warm stage (37ºC)	Room (25–27ºC)
Equilibration step	Ethylene glycol, Ficoll, sucrose 20 (2 min): 20% EG	7.5% ethylene glycol + 7.5% DMSO (2 min)	10.0% ethylene glycol	7.5% ethylene glycol+7.5% DMSO (5-10 min[c])
Vitrification step	Ethylene glycol, Ficoll, sucrose 40 (1 min): 40% EG	15% EG + 15% DMSO + F.S. (35 s)	40%EG + S. (30 sec)	15% ethylene glycol + 15% DMSO + sucrose (1 min)
Cooling system	Vapor phase LN2 (3 min), then plunged into LN_2	Plunged into LN2 (ultra-rapid cooling)	Plunged into LN2 (ultra-rapid cooling)	Plunged into LN2 (ultra-rapid cooling)
Warming step	One step 0.5 M sucrose (5 min)	Two steps 0.25 M S. (2 min) 0.125 M S. (3 min)	Four steps 1 M S. (2.5 min) 0.5 M S. (2.5 min) 0.25 M S. (2.5 min) 0.125 M S. (2.5 min)	Two Steps 1 M sucrose (1 min) 0.5 M sucrose (3 min)

DMSO, dimethylsulphoxide; LN_2, nitrogen oxide.
[a] Reported by (Desai et al., 2007)
[b] Reported by (Rama Raju et al., 2005)
Table is provided by (Mukaida & Oka, 2012)

Recent advances in culture system enable *in vitro* development of human embryos to blastocyst, hence promise a bright outcome for successful implantation with blastocyst

transfer. Accordingly, the need to cryopreserve the blastocyst is in high demand. Human blastocysts that are developed in the co-culture system and which are cryopreserved with slow freezing give 27% pregnancy rate and 17% implantation rate (Ménézo et al., 1992). However, the results by other studies have been inconsistent and this is due to different culture condition (Ludwig et al., 1999; Ménézo et al. 1992; Nakayama et al., 1995).

Vitrification of human blastocysts shows lower success rate as compared to the earlier stage vitrification, and this is because the blastocysts are less permeable to CPA and water, along with the formation of the blastocoel which induces intracellular ice crystal formation. Therefore, artificial shrinkage by puncturing the blastocoel with a micro-needle is applied prior to vitrification where this proves to be effective in improving the clinical outcome (Mukaida et al., 2006).

The establishment of oocyte vitrification provides several advantages in clinical practices (Mukaida & Oka, 2012). Oocyte vitrification gives rise to an egg bank which benefits the women who decide to have children. It allows ample time for genetic screening in donor oocyte programme. Plus, oocyte vitrification is another alternative for cancer patients to preserve fertility. Another important advantage that oocyte vitrification has over embryo vitrification is that this method is ethically sound, in comparison with the latter.

Unfortunately, there are drawbacks in term of clinical outcome. Oocyte vitrification results in low pregnancy rate and poor embryonic development (Mukaida & Oka, 2012). This is due to the fact that oocyte is a single cell whereas the embryo is multicellular. Embryos have the ability to compensate for any external change or internal injury such as embryo biopsy to develop further. It was also reported that oocyte vitrification affects membrane properties. CPA permeability and the capacity of oocyte membrane to withstand dehydration and rehydration are different to embryo vitrification. The oocyte has a higher cytoplasmic lipid

content which increases chilling sensitivity (Ghetler et al., 2005). Besides, it has lower submembranous actin and microtubules which makes it less robust. In contrast, embryonic membranes are less sensitive to chilling injury due to the release of cortical granules after fertilisation. hence, oocyte viability is more prone to be affected at high temperature, inducing irreversible damage of cytoplasmic lipid droplets, lipid-rich plasma membranes and microtubules (Ghetler et al., 2005).

Pre-treatment medium or equilibrium medium which acts as a buffer prior to the inclusion of higher toxicity CPA for embryos, is shown to act differently on oocytes. Oocyte undergoes osmotic shock at equilibration, resulting in oocyte shrinking and deformities, damaging the cytoskeleton and microfilament. Besides, osmotic swelling shock which can occur during the dilution (rehydration) step leads to extensive swelling and membrane rupture. This is related to microtubule depolymerisation, chromosome misalignment and the increased risk of aneuploidy, eventually resulting in lysis and the immediate death of the oocyte (Mukaida & Oka, 2012).

The vitrification technology is rapidly improving, ensuring the potential of oocyte vitrification as an alternative to pregnancy with a higher rate in the future. For the past 10 years, many studies have been carried out on oocyte vitrification with cryotop method (Kuwayamaet al., 2005a; Kuwayama et al., 2005b; Lucena et al., 2006). Lucena et al. in 2006 reported a success of 89.2% survival rate and 56.5% pregnancy rate (13 out of 23 women) with cryotop in oocyte vitrification.

Due to the concerns of ART procedures to human health, studies worldwide were carried out on animal models (Scott et al., 2010; Stout, 2012; Watkins et al., 2007). Reports on animal models such as murine, bovine, and porcine are important tool in investigating the effect of ART in IVF of animal models. The mouse model is a cheap resource and this is

necessary for the first approach in embryology such as embryo manipulation like intra-cytoplasmic sperm injection (ICSI).

1.6.5 The effect of cryopreservation on oocyte and embryos

Cryopreservation is proven to be a vital part in improving the success rate of embryo transfer, keeping genetic cell lines, and minimising the number of repetitive invasive procedures for patients. Unfortunately, this technology had been reported to affect the development, ultrastructure (Fabian et al. 2005) and gene imprinting (Zhao et al. 2012) of the frozen embryos.

In human studies, the largest study was conducted in Denmark and looked into 957 children born from cryopreserved embryos (Pinborg et al., 2010). This study also compared children born from non-ART (n= 4800), children born from fresh IVF (n=6904) and ICSI children (n=3425). The study shows that children born from the cryopreserved group weigh more than the non-ART and fresh IVF groups. No significant difference in birth defect, imprinting disorder or congenital malformation was found in the children of cryopreserved group as compared to others. However, the cryopreserved children had double perinatal mortality rate as compared to the non-ART children.

1.6.5.1 ***The effect of oocyte cryopreservation on animal models***

Using animal models, Diez et al., in 2005) reported that vitrification significantly affects the ultrastructure and developmental ability of bovine oocytes. The chilling sensitivity of the mammalian oocyte is well documented and the success of cryopreservation survival is limited (Mukaida & Oka, 2012; Woods et al., 2004). Aneuploidy and polyploidy occurred in

oocyte or cumulus cells, resulting from the cryopreserved medium, which led to oocyte cleaving, one of the leading causes of compromised developmental competence (Wu et al., 1999). This abnormality is associated with the temperature-sensitive nature of microtubules. Metaphase II (MII) spindles which are formed from microtubules undergo disassembly and reassembly during cooling and re-warming processes. Unfortunately, in some species such as bovine and human, MII spindles cannot completely reform after warming due to lack of pericentriolar material in some oocytes (Martino et al., 1996). The partially formed spindles will bring about chromosome disarrangement/displacement in some–oocytes, resulting in aneuploidy. The abnormality in spindle reformation also results in retention of the second polar body in the oocytes at the fertilisation time, which in turn gives rise to polyploid zygotes and embryos (Elst V. et al., 1993).

Men et al. in 2003 detected DNA fragmentation without alteration in the bovine oocyte morphology due to cryopreservation. The underlying mechanism for this damage is unknown. The osmotic stress during dehydration and rehydration also accounts for cryodamage (Arav et al., 1993). Fabian et al. in 2005 pointed out that there was an increase of fragmented DNA accumulation rate in the nuclei of porcine blastocysts through LM and TEM. The vitrified embryos which were left to culture for 24 hours showed 2/3 recovery from the forced collapse of blastocoel cavity (BC) as compared to embryos that were fixed immediately after vitrification, without leaving them to culture. General distension or shrinkage of mitochondria and massive increase in the amount of vesicles, vacuoles, and secondary lysosome (SL) was observed in the embryos without culture. There was a sign of direct osmotic injury. In contrast, embryos which were exposed to 24-hour culture showed normal morphology except there was perivitelline space widening, debris accumulation and partial distension of mitochondria. The act of warming porcine embryos immediately after vitrification process causes forced collapse of BC, severe subcellular damages and increased presence of nuclei with degraded

DNA without typical apoptotic morphology. This reflects the direct damaging effect of vitrification. In fact, 24 hours of culture allows a major portion of vitrified embryos to regenerate. Hence, the regenerated vitrified embryos manage to reach a higher number of nuclei. The need to replace the high amount of extruded and dead cells triggers the vitrified blastocysts to increase their mitotic activity and alter the expression of important genes in bovine (Fabian et al., 2005).

MII oocytes display a more flexible cytoskeleton indicating its less susceptibility to cryodamage (Hiraoka et al., 2004). Studies on chilling sensitivity conducted on MII stage oocytes found that the main damage occurred due to meiotic spindle disorganisation and then followed by microtubule depolymerisation (Men et al., 2003). Exposure to cryoprotectants in a vitrification solution affects mitochondria, vesicles, microvilli reducing the embryonic development of MII oocytes as a consequence of osmotic shock (Asada et al., 2002).

Van der Elst et al., in 1993 suggested that polyploidy in zygotes and embryos resulted from the retention of the second polar body of oocytes at the time of fertilisation. Aman & Parks in 1994 observed chromosomal dispersion during freezing and cooling of bovine oocytes, thus reducing meiotic spindles. In another cryopreservation study, DNA fragmentation of the oocyte was detected but no alteration on morphology was observed (Men et al., 2003).

1.6.5.2 The effect of embryo cryopreservation on animal models

Cryopreservation was reported to induce DNA damage in mouse embryos at the blastocyst stage (Kader et al., 2009). The susceptibility to DNA damage was greater on more expanded blastocysts during cryopreservation. Propanediol showed a greater impact as compared to glycerol when it came to slow freezing. The report suggested that the introduction of biological change related to metabolic changes such as the uptake of glucose

pyruvate and oxygen predominantly lead to DNA damage in slow freezing, whereas minimal or no effect was seen in vitrification (Kader et al., 2009).

The effect of cryopreservation and embryo transfer are well documented (Vajta et al. 1996; Mukaida & Oka 2012; Naik et al. 2005) but the long-term effect to the vitrified embryos remains unknown. Recently a study on rabbit embryos reported that cryopreservation had long-term and transgenerational effects on F1 and F2 female reproductive traits. Vitrification and transfer procedures were reported to cause a consistent increase in litter size and offspring mortality between F1 and F2 with their respective counterparts (Lavara et al., 2014). However, the underlying mechanism of the transgenerational effect is still unknown.

In 1995, Dulioust et al. investigated the long term effect of cryopreservation on mouse embryos. They suggested that frozen embryos were exposed to the changes of morphological and behavioural features in adult mice, and in which the effect may be delayed. Apart from its immediate toxicity, no detrimental effect was reported, as embryo cryopreservation did not induce major anomalies, even in senescence. The possible mechanisms that lead to the delayed or long term effect are the effect of vitrification on mitochondrial DNA and nuclear DNA, the nucleocytoplasmic interactions and initial alteration due to cryopreservation (covers nuclear mutation, mitochondrial mutation and epigenetic change), however these mechanisms are still unclear (Dulioust et al., 1995).

Similar outcomes were also observed in human oocytes and embryos. Vitrification of human blastocysts is increasingly used to cryopreserve supernumerary embryos following IVF. However, this technique lowers the normal rate of spindle formation in human. A study on the vitrification of 5-day human blastocysts showed no effect to the cytoskeleton and development, because the survival rate after thawing and the proportion of normal spindle/chromosome configuration is noticeably high (Chatzimeletiou et al., 2012). There was

a significant difference of abnormal spindle formation in the frozen embryos. Spindles with either focused or unfocused poles, chromosome bridging and disorganised middle spindle fibres at telophase were observed. It seems that the vitrified embryos in the mitotic stages are more vulnerable to abnormal spindles and adverse abnormalities on the chromosomal constitution of the developing blastocyst (Chatzimeletiou et al., 2012).

Moreover, the offspring sex ratio was significantly altered after vitrification-thawed blastocyst transfer (Lin et al., 2009). The live birth sex ratio is influenced by the blastocyst-stage embryo transfer. This correlates with top-quality embryo morphology and a higher rate of major postpartum haemorrhage in humans who have received vitrified blastocyst transfers (Wikland et al. 2010). Other than that, there are also reports suggesting that cryopreservation induces stress which results in an increase in apoptotic gene expression, DNA fragmentation and reduced developmental capacity (Shaw et al., 2003).

Epigenetics during cryopreservation and transfer procedures has long-term effect (Lavara et al., 2014). Events that occur during the preimplantation stage such as embryonic genome activation, compaction, lineage differentiation and blastocoel formation alter the later developmental processes (Zernicka-Goetz et al., 2009). Nevertheless, the vitrification effect on methylation, expression level of pluripotency and gene differentiation is poorly characterised. Further research in this area may help in understanding the epigenetic changes which occur in the vitrified blastocysts. The major effects of cryopreservation on blastocysts and postnatal growth are increasingly investigated and need to be evaluated in details.

Imprinting disorders occur during the critical preimplantation period of embryo development. A report showed that any alteration or changes caused by ART procedures during this critical window can alter the health and development of embryos and later effects through to adulthood (Grace & Sinclair, 2009). A similar concept, Developmental Origin of

Health and Disease (DOHaD) which was proposed 20 years ago, reported that environment perturbation during the preimplantation period can alter the genetic status and give a long term effect on health and cardiovascular metabolism (Watkins et al., 2007).

1.6.5.3 Oocyte and embryo morphological changes after cryopreservation in mice

Using mice, an in-depth study of cryopreserved oocytes and embryos was carried out. Light, fluorescence, and transmission electron microscopy were used to observe morphological changes after slow freezing and vitrification in mouse embryos (Coutinho et al., 2007). *Hoechst33342* and propidium iodide (H/PI) staining was used to evaluate the viability of the embryos. H/PI staining resulted in higher membrane permeability after vitrification (69.8%) as compared to only 48.4% in slow freezing. Morphological analyses using haematoxylin and eosin (HE) staining were performed in different techniques (*in situ*, paraffin and historesin in the presence of light and fluorescence microscopy). Cellular structures were better identified with historesin-embedded samples. Slow freezing and vitrification groups showed induction of pycnosis, revealed by nuclear evaluation in historesin. Cytoplasm evaluation indicated weak eosinophilic structures and degenerating cells indicated oncosis in the vitrification group, while the increase of eosinophilic intensity in slow freezing group showed sign of apoptosis. Transmission electron microscope was used to detect ultra-structural alterations. It was concluded that severe cellular alteration which occurred in vitrification reduced embryonic viability as compared to slow freezing. Cryopreservation techniques indeed resulted in oncosis and apoptosis injuries (Coutinho et al., 2007).

Coutinho et al. in 2007 studied the embryonic ultrastructure in the slow freezing and vitrification methods. The cytoplasm is filled with rough endoplasmic reticulum (RER), intact mitochondria and abundant nuclei with regular membrane surfaces (Fig 1.4). As expected, both methods showed sign of cell injury. Slow freezing method showed viable cells, mitotic pattern, vacuolised mitochondria, nuclei with irregular membrane surface, pycnosis, and

cytoplasm condensation (Figure 1.5). The reason for this finding was due to the use of low CPA concentration and gradual temperature reduction (Coutinho et al., 2007). The vitrification method however showed signs of oncosis in which there were disruption of plasma membrane, swelled and fragmented cytoplasm including RER reduction and vacuolized mitochondria. This was because the embryos were exposed to the high CPA concentration and high temperature increase, as indicated in Figure 1.6.

Mitotic patterns were only observed in the slow freezing method, suggesting that vitrification changes the nuclear status of the embryos. As expected, the vitrified embryos had a slower development as compared to slow freezing embryos. In 1997, this was supported by Critser et al. who reported that a higher CPA concentration leads to longer recovery time for embryos (Critser et al., 1997).

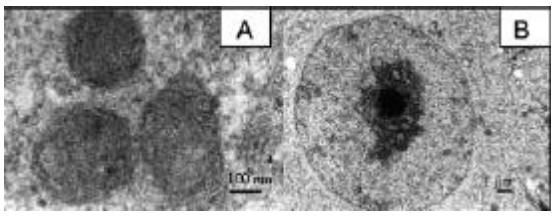


Figure 1.5 TEM micrographs of mouse embryos from slow freezing group. (A) Apoptotic bodies, 2000 x – Araldite embedding. (B) Mitotic pattern, 5000 x – Spurr embedding

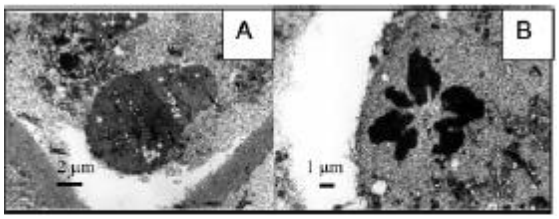


Figure 1.6: TEM of mouse embryos from control group: (A) intact mitochondria and (B) nuclei with regular membrane surface-Spurr embedding.

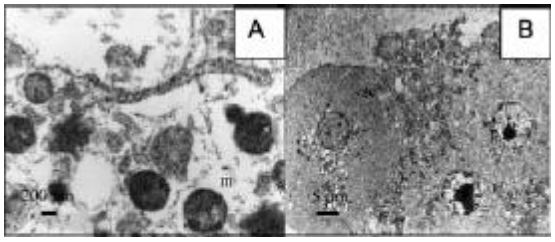


Figure 1.7 TEM micrographs of mouse embryos from vitrification group. (A) rupture of plasma membrane, mitochondrial vacuolization with high electrodensity (m), 25,000 x – Araldite embedding. (B) Swelling cytoplasm (sc) and pycnosis (p), 1,500 x – Spurr embedding. Figures 1.5 to 1.7 are sourced from Coutinho et al. in 2007.

1.6.6 Possible mechanism of cryoprotectant in preventing cell death following vitrification

Water solidification by freezing is expected to be lethal to living cells, however, freezing is the ultimate procedure that offers indefinite storage for future use (Mazur, 1984). In order to overcome the challenge faced in freezing living cells, the understanding of how freezing leads to cell damage is vital. Membrane lipids that are normally in a liquid crystalline state would solidify upon freezing, leading to alteration in cell membrane function. Freezing results in a decrease of temperature that causes intra-cellular imbalance such as the disintegration of metaphase spindle in oocytes. Overall, chilling injuries due to ice-crystal formation, solution effect and osmotic shock are the major consequences (Pereira & Marques, 2008).

In general, cell membranes consist of glycerophospholipids, molecules composed of glycerol, a phosphate group and two fatty acid chains. Glycerophospholipids are insoluble in water but they consist of hydrophilic phosphate head and hydrophobic hydrocarbon tails of fatty acids. Their main role is to act as barriers and gatekeepers for molecules to diffuse through the transmembrane proteins such as transporter and receptor protein. To prevent crystallisation and ice formation, cryoprotectant is required to permeate into the cell and replaces the water molecules inside the cells, without damaging or being toxic to the cell.

Simulation studies which are made by modulating the structures and properties of the cell membrane enable the understanding of the molecular mechanism in DMSO. DMSO, a small amphiphilic molecule is well-known as an antifreeze or cryoprotectant, as shown in Figure 1.8.

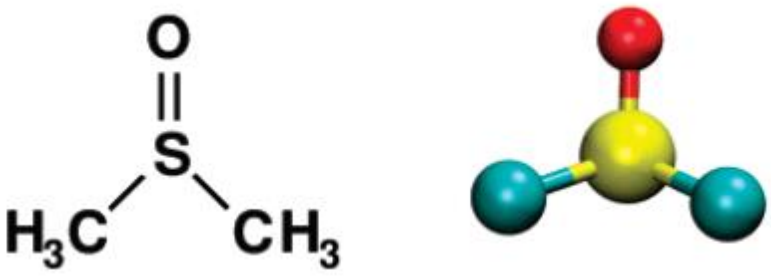


Figure 1.8: Chemical structure of a dimethyl sulfoxide (DMSO) molecule (*left*) and its united-atom representation (*right*). Source from Gurtovenko & Anwar, 2007).

DMSO's ability to promote the permeation of solutes was investigated and different modes of action relative to the DMSO concentration were shown (Gurtovenko & Anwar, 2007). Low DMSO concentration (less than 10%) induces membrane thinning and increases fluidity of the membrane's hydrophobic core. The report suggested that DMSO readily penetrates into the lipid/water interface and occupies a position beneath the lipid head-groups. DMSO also acts as a spacer between the lipids, destroying the coordination between phosphorus and nitrogen atoms on lipid head groups, thus increasing the average lateral distance between them. Then, DMSO induces expansion of the membrane and the membrane thickness concurrently decreases.

On the other hand, high DMSO concentration induces transient water pores into the membranes while the lipid molecules are released from the membrane, resulting in the membrane structure disintegration (Gurtovenko & Anwar, 2007). A further lateral expansion of the bilayer takes place, accompanied with loss of interaction between the head groups of different lipid molecules. DMSO molecules then force their way into the membrane interior and into the hydrophobic core. These factors allow the formation of transient water column in a few picoseconds.

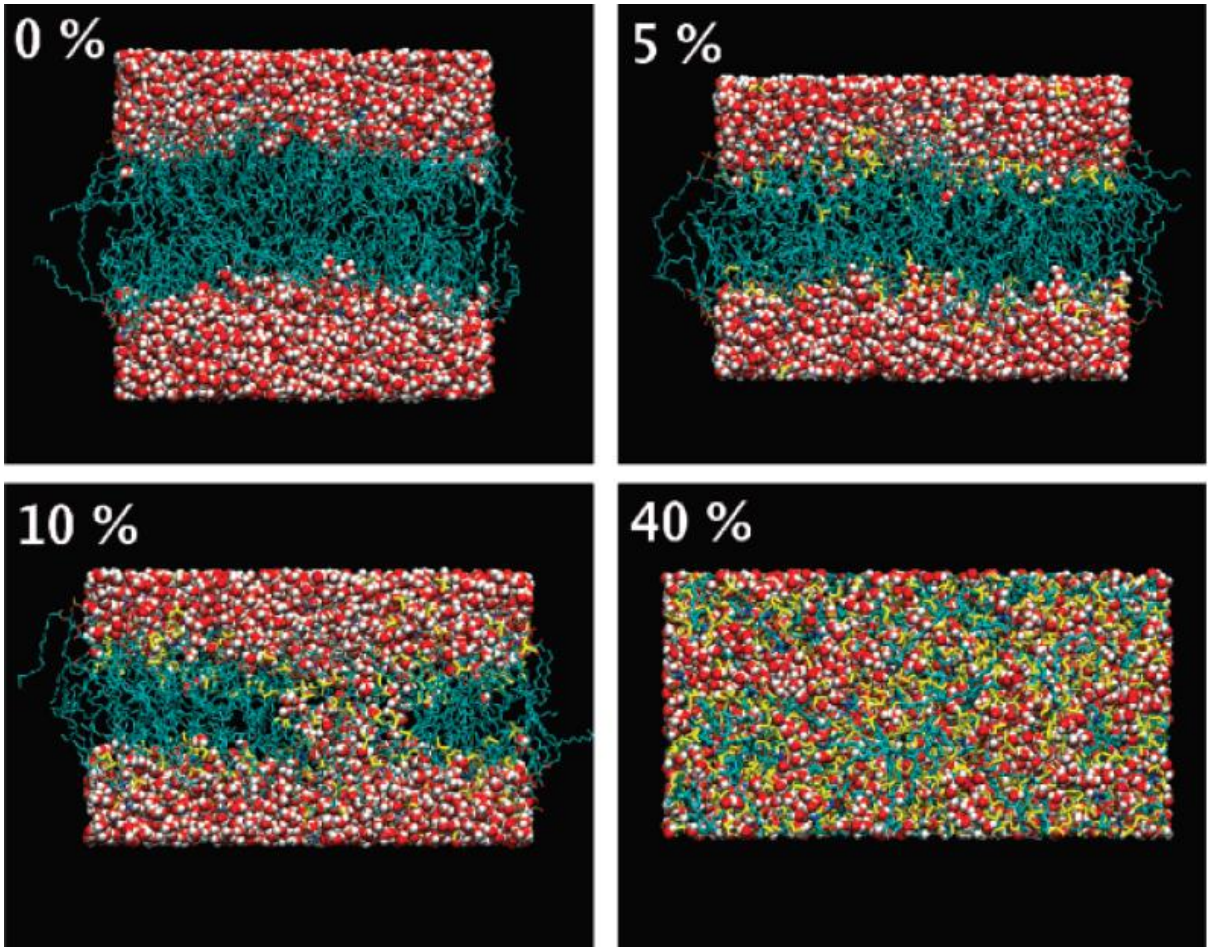


Figure 1.9: Distinct modes of action of DMSO on phospholipid membranes. Side views shown above are the final structures of the bilayer systems containing 0, 5, 10, and 40 mol % of DMSO (lipid-free basis). Lipids are shown in cyan, water in red and DMSO in yellow (Source from Gurtovenko & Anwar, 2007).

These findings showed the pore formation at different DMSO concentrations, in which10% DMSO gave the slowest formation (~20ns) whereas 20% DMSO showed the quickest formation (~3ns). In addition, multipores were observed in 12.5%, 15% and 20% of DMSO. In conclusion, DMSO particles partition into the membrane interior, reach the membrane equilibrium concentration and promote the formation of water column.

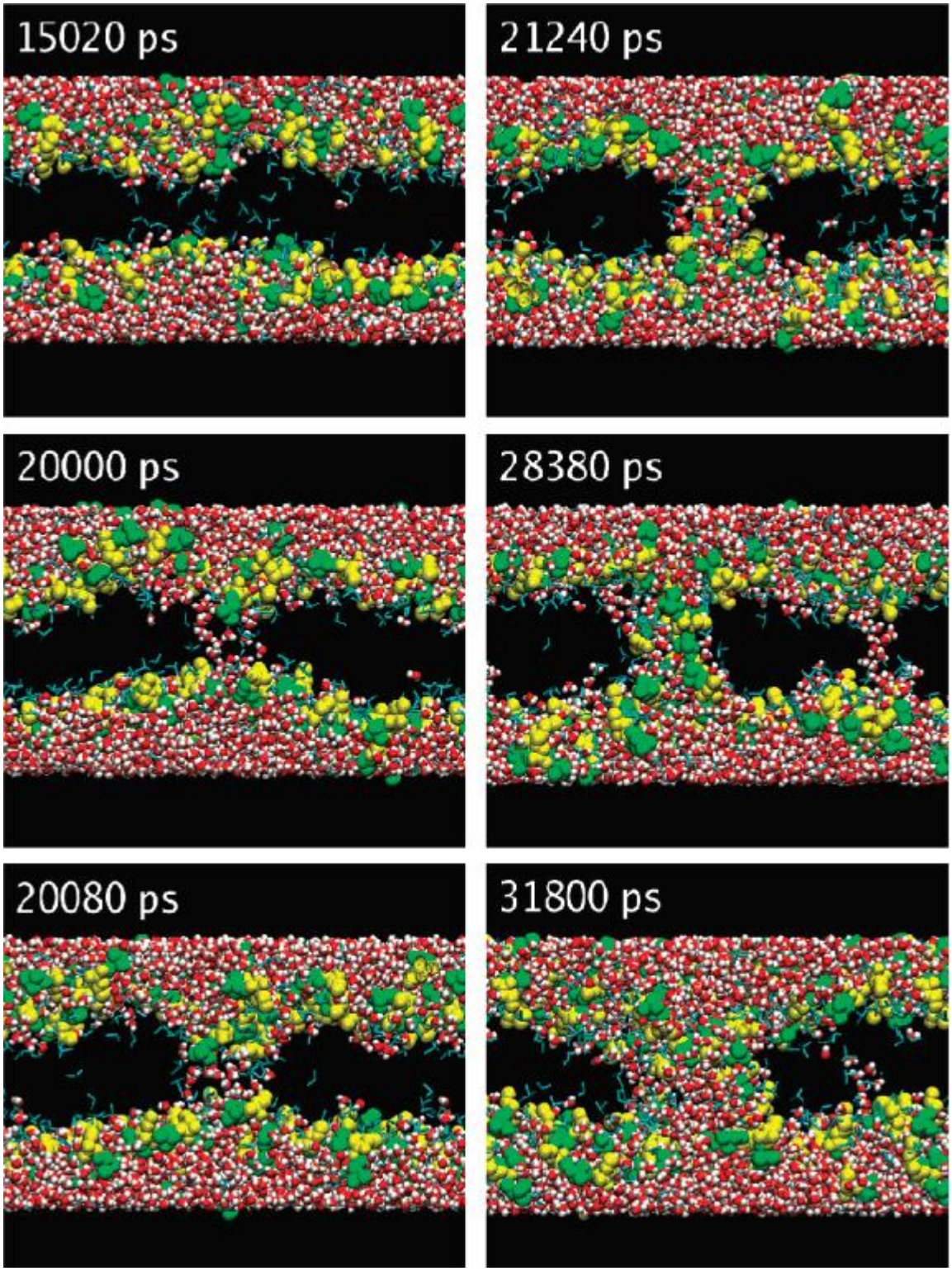


Figure 1.10: Pore formation in the bilayer system with 10 mol % of DMSO. Water is shown in red, DMSO in cyan, choline and phosphate groups of lipid headgroups in green and yellow, respectively. (Source from Gurtovenko & Anwar, 2007).

1.7 Animal models and their contribution improve understanding in the mechanisms of disease origin development.

Animal models are crucial since studies on humans exhibit limitation and bias that lead to discrepancies. Studies on animal model give the ability to test specific hypotheses while overcoming major limitations and variations between samples. There are important studies which used small and large animals that have contributed to the causal relationship between early-life exposures and metabolic risk factors in the later life (Rosenthal & Brown, 2007; Watkins et al., 2007; Watkins et al., 2008; Young, 1998).

1.7.1 Advantages of using animal models in developmental reprogramming

Small animal models such as rodents offer numerous advantages. The quick reproduction time and inexpensive cost make them an ideal model for biological testing as demonstrated in the effect of altered diet during pregnancy on the systolic blood pressure in mice (Watkins et al. 2008), and rats (Kwong et al., 2007). In addition, it is cheap to maintain large cohorts of animals if the studies are conducted on rodents. These animals are also suitable to investigate specific parameters such as the weight across their lifespan (Donjacour et al., 2014; Scott et al., 2010; Watkins et al., 2007). The data obtained can be correlated with different parameters such as weight vs. blood pressure to gain insights into possible predictors for the onset of disease in adults. Other than that, their short gestation period and lifespan make them preferable for transgenerational disease studies (Lavara et al., 2014; Mahsoudi et al., 2007; Zambrano et al., 2005).

Another advantage of using small animal models is that their genetic and environmental variability can be controlled. In a small animal house, for different treatments, it is easy to control environmental factors such as light, temperature, and ambiance, even with a large cohort of animals. This enables eradication of external factors that contribute to any changes

in data. Small animals also allow the manipulation of treatments (e.g. IVF) and experimental design in which the findings can later be translated to human.

1.7.2 Limitation in animal models

Animal models enable the scientific community to gain insight into how to tackle various diseases in human. However, there are drawbacks in using small animal models. One of them is large litter size, from 8 to 15 offspring. Mice that occupy the end of uterine horn have heavier birth weight due to inconsistent distribution of nutrients to litter (Ryan & Vandenbergh, 2002). Studies which investigate the effect on growth rate or distribution of nutrients during the gestation period are expected to avoid both ends of the uterine horn to reduce variability between groups. Foetal development is also affected by different levels of exposure to hormones (Vom S. et al., 1990).

Another limitation in small animals is that they are an altricial species. They are immature at birth as compared to large animals and the human. At birth, the offspring have a poorly developed central nervous system, autocrine system and organs such as the pancreas and kidneys. Thus, the ideal parameter for rodents is birthweight and subsequent weight up to weaning to show percentage of weight gain by mice from different groups. Studies of animal models have their advantages and disadvantages and the model which is the most appropriate for any particular study is dependent on the hypothesis (McMullen & Mostyn, 2009).

1.7.3 Long term effect studies of using animal models to be translated in human studies

Nutritional reprogramming in small animal models showed that alteration in maternal diet often occurs without affecting the foetal size at birth, indicating that the foetal growth restriction is not dependant to the prenatal dietary exposure and is common for CVD in postnatal outcome (McMullen & Mostyn, 2009). Long term effects in animal models are

observed throughout different species within a range of physiologically relevant factors. This is important in translating the findings to humans .

A follow-up study on the cardiometabolite health of children conceived from IVF reported that systolic and diastolic blood pressure levels were higher in IVF children than the normally born children (Ceelen et al., 2008a). It is important to note that the significant difference in blood pressure cannot be explained by current weight, birth weight or parental subfertility cause. This is similar to a study on mouse model which suggested that the prolonged culture of mice from 2-cell stage to blastocyst had significantly elevated blood pressure as compared to the control group (Watkins et al., 2007).

Reports from the past literature suggested the importance of further understanding on the effect of vitrification on embryo development and on cell number after they developed to blastocyst stage. Even though vitrification was reported to show morphological differences in mouse embryos compared to slow freezing technique and controls (Coutinho et al. 2007), reports on how vitrification affect the cell number of embryos are still limited. By investigating the effect of vitrification on cell number we might gain insight into the mechanisms how vitrification could reduce developmental capacity (Shaw & Jones 2003).

Other than that, the need to investigate the long term effect of vitrified embryos had been suggested from previous reports. There are follow up studies looking at different aspects of ART such as (Ceelen et al. 2008) that suggested IVF children had elevated blood pressure compared to naturally conceived children, but there are no report from children born from frozen thawed embryos.

Using animal models, we would be able to analyse the effect of vitrification on the embryo transfer (ET) efficiency and litter size of offspring from frozen thawed embryos

compared to fresh thawed embryos and in vivo (undisturbed). We would also analyse the postnatal health on growth, cardiovascular, metabolic and organ allometry. We would then be able to relate the results analysed from previous experiments on cell number with data from the postnatal health of offspring.

The objectives of my studies are

- To investigate the effect of vitrification on embryo developmental rate after vitrification. Development of vitrified thawed embryos to morula and different stages of blastocyst (early, middle and late blastocyst) will be recorded from each group to examine the duration of time needed for the embryos to form blastocyst.
- To examine the vitrification effect on lineage differentiation by analysing cell number within ICM and TE and the ratio of ICM:TE in mouse blastocyst
- To compare the pregnancy success rate after ET, ET efficiency (live birth), litter size, number of offspring and male: female ratio between controls (undisturbed), non-vitrified transfer (NVT) and vitrified transfer group (VT).
- To analyse the effect of embryo vitrification on growth, cardiovascular, metabolic and organ allometry of offspring.
- To investigate any gender-specific effect on postnatal health in offspring from undisturbed, NVT and VT groups.

Chapter 2 Materials and methods

2.1 Embryo collection and culture

2.1.1 Allocation of females for mating

Virgin female C57/Bl6 mice aged between 7 to 9 weeks old (University of Southampton, Biomedical Research Facility) were caged for 3 to 4 days continuously with male CBA/CA mice aged between 6 to 10 months. A total of two females were allocated for one male each time. The presence of a vaginal plug the following morning was taken as a sign of mating. Naturally mated females were used in all treatments throughout this study.

2.1.2 Collection and culture of embryos

Mice were killed on day 2 post mating by cervical dislocation and embryos were flushed from the dissected oviduct to obtain the embryos from 8-cell compacted or morula stages. Time of flushing was fixed approximately to 15.00 – 18.00 hours for all treatments. Embryos were flushed using pre-warmed M2 medium (with HEPES, penicillin and streptomycin, sterile-filtered; Sigma Cat No. M7167-100ml). Embryos were collected using a fine pulled glass pipette (disposable glass Pasteur pipette 230 mm; Volac series no. D812/HW) that attached to a mouth pipette. The pipettes were made by pulling glass Pasteur pipette over an alcohol flame; the resulting fine pipette was then broken in the centre to form two micropipettes. The pipette was retracted so that a clean break was formed at the tip.

After flushing, the embryos were pooled from different mothers before being divided into two groups; non-vitrified and vitrified. Embryos in the non-vitrified group were cultured immediately in potassium simplex optimised medium (KSOM: Appendix I). Embryos in the vitrified group underwent vitrification, and then were subsequently cultured in KSOM in the same way as the non-vitrified group. Approximately 30 µl droplets of KSOM were pipetted

into culture dishes (Falcon) under mineral oil (Sigma, Cat No. M8410-500ml; embryo culture tested). Culture dishes with KSOM were allowed to acclimatize within the incubator for at least 30 minutes before embryo culture. Embryos were cultured at 37ºC, 5% CO_2 in air until the blastocyst stage (E3.5).

2.2 Vitrification procedure

Initially, solid surface vitrification (SSV) was chosen as described previously by Mamo et al. (2006), because the 8-cell mouse embryos used by the researchers showed 100% survival rate after vitrification. However, the embryos (approximately 100 embryos, 6 replicates) used in this study did not survive the procedure. The blastomeres lysed immediately after being transferred into thawing medium. Thus, a toxicity test was run in the absence of vitrification procedure. The result remained the same (approximately 110 embryos, 8 replicates). So, troubleshooting was conducted as indicated in Table 2.1.

Table 2.1: Troubleshooting of vitrification procedure by Mamo et al. (2006)

Vitrification procedure	Troubleshooting
Equilibration medium (EM) - 4% EG for 5-10 mins	Prolonged the exposure in EM from 10 to 15 mins
Vitrification medium (VM) - 35% EG + 5% PVP + 0.4M trehalose for less than a minute	Reduce the cryoprotectant concentration from 35% EG to • 20% EG for 2 mins • 20% EG + 0.5M trehalose for 2 mins • 10% EG for 2 mins • 10% EG + 1.0M trehalose for 2 mins
Thawing medium (TM) – 0.3M trehalose for 1 min 0.15M trehalose for 2 min 0.075M trehalose for 2 min	Increase the trehalose concentration from 0.3M to • 0.4M for 1 min • 0.4M for 3 mins • 0.5M for 3mins • 1.0M for 3 mins

The exposure in EM is essential because it provides a preliminary step prior to vitrification procedure with a higher concentration of EG (Bagis & Mercan 2005). The penetration of cryoprotectant into the embryo is essential to achieve a vitrifiable concentration during the rapid cooling step. However, the EG concentration has to enable a short exposure to high cryoprotectant concentration without being too toxic to the cell (Arav, 2014). The change made for VM is to balance the EG concentration with trehalose as the combination of these cryoprotectants may lower the cell toxicity, resulting in a higher survival rate. In addition, the different concentration of TM is essential in reaching a suitable TM tonicity for the embryo to expand to the normal size. Unfortunately, embryo viability after this preliminary test was still unobtainable.

A Fertility Centre was then contacted to request for a medium sample (FCM). Surprisingly, the toxicity test on FCM worked well and 90% of the embryos (n= 25 embryos) survived and developed to blastocyst stage. Since FCM does not affect the embryonic development, vitrification in LN_2 was carried out. A total of 85% of the embryos (n= 23 embryos) survived and developed to blastocyst. A detailed list of protocol with FCM cryoprotectant was obtained and medium for vitrification was prepared (Table 2.2).

Table 2.2: Vitrification procedure and medium

Medium	Procedures
Equilibration medium (EM) (7.5M EG + 7.5M DMSO)	• Embryo was transferred and exposed in EM for 8 mins
Vitrification medium (VM) (15M EG + 15M DMSO +1.0M sucrose in M2)	• Embryo was washed 3 times and exposed in VM for less than 1 min • 8-10 embryos were transferred on the tip and plunged in LN_2
Thawing medium 1 (TM1) (1.0M sucrose in M2) Thawing medium 2 (TM2) (0.5M sucrose in M2)	• Embryo was incubated in cavity block with TM1 for 10 mins

	• Embryos were washed and exposed to TM2 for 3 mins before being transferred into KSOM for culture.

Vitrified group embryos were washed in M2 prior to vitrification. Vitrification procedures and references for medium were obtained from the Complete Fertility Clinic, Southampton. In this study, the medium was adapted to suit mouse embryos. The entire vitrification procedure consists of equilibrium, vitrification and thawing techniques. Before beginning the vitrification process, liquid nitrogen was decanted into a polystyrene box (20 x 15 x 8cm, w x h x d) to about half its depth. Embryos were equilibrated in 30 µl equilibration medium consisting of 7.5% ethylene glycol (EG; Sigma Cat No. 058-00986-500ml) and 7.5% dimethyl sulfoxide (DMSO; Sigma Cat No. D8418; Appendix I) in M2 for two minutes. Embryos were then transferred and washed through 3 drops of vitrification medium comprising 30 µl each (15% EG + 15% DMSO + 0.5 M sucrose in M2) for less than a minute before being transferred to a pipette plastic tip and plunged immediately into liquid nitrogen.

After the embryos were vitrified and left in the LN_2, the thawing procedure follows. Thawing solutions were placed in an incubator (37ºC, 5% CO_2,) at least 2 hours before the thawing procedure. The pipette plastic tip with the vitrified embryos on the tip was retrieved from the LN_2 using forceps after 10 to 15 minutes in the liquid nitrogen. The end of the tip with embryos was immediately submerged in 1ml thawing medium A (0.5 M sucrose; Sigma Cat No. S1888-500g in M2) for 10 minutes in a pre-warmed sterilised glass cavity block. The morphology of embryos in sucrose was observed every two to three minutes under the light microscope. Embryos were transferred to 30 µl thawing medium B (0.25 M sucrose in M2) for 4 minutes then washed in a few drops of 30 µl M2. The morphology of embryos was observed

and the development was recorded. Embryos were washed in KSOM three times and cultured until the blastocyst stage (section 2.1.2).

Initially, a spatula was used for the vitrification procedure. The spatula was placed on top of a cylindrical metal block that was submerged in liquid nitrogen. After the embryos were transferred, and washed through 3 drops of vitrification medium, the embryos were dropped directly on top of the spatula. Then, the spatula was picked up and the drop of vitrified embryos were transferred in a cryotube, and then left in the liquid nitrogen for approximately 10-15 minutes before thawing procedure. This method was discontinued because the percentage of embryo retrieval after vitrification was very low (less than 50% of embryo retrieval; approximately 64 embryos).

2.3 Differential nuclear staining

Differential labelling was referred from (Handyside & Hunter 1984) with slight modifications. Acid Tyrode's solution (pH 2.2, Appendix I) was incubated at 37^{o}C overnight in sterile cavity blocks prior to the experiment. The zona pellucida of mid-blastocyst embryos was removed by brief incubation (around 10-15 seconds) in acid Tyrode's solution. Embryos were allowed to recover in pre-warmed M2 in a sterile cavity block for a minimum of 20 minutes at 37^{o}C. Embryos were later treated at room temperature with 50 µl trinitrobenzenesulfonic acid (TNBS, Sigma Cat No. 92823; 1:10 dilution in phosphate buffered saline, PBS, with 0.1% PVP, adjusted to pH 7.4 with NaOH; stored in the dark) for 10 minutes then washed in 30 µl drops of pre-warmed H6 PVP (Sigma; embryo culture tested; H6 PVP, appendix I) until colourless. Next, the embryos were incubated in a 25 µl drop comprising 10.4 µl goat anti-Dinitrophenyl-BSA antibody solution (anti-DNP, Sigma Cat No. D9781; 3 mg/ml in PBS) in 14.6 µl H6 PVP for 10 minutes at room temperature. Embryos were rewashed in drops of H6 PVP, and then incubated in 8 µl of propidium iodide (Sigma Cat No. P4170; 1 mg/ml in distilled water) added to 25 µl guinea pig complement protein (Cedarlane Cat No.

CL4051; Diluted 1:10 in H6 BSA) for 10 minutes at 37°C, 5% CO_2 in air. Embryos were rewashed in several drops of H6 PVP before fixation in 990 µl absolute ethanol to which 10 µl BisBenzimide H33258 stain (Hoechst stain, Sigma Cat No. 861405; 2.5 mg/ml in distilled water) was added. Embryos were left at 4°C in four-well dishes for a minimum of 90 minutes, and a maximum of 1 week.

Approximately 5 µl of ultra-pure glycerol (Amersham) was placed onto a glass slide and three to four embryos were transferred from four-well dishes into the middle of the drop. A cover slip was placed, and firmly pressed, over the drop. The embryos were viewed under a fluorescence microscope (Leica), and once the embryos were detected, the image of embryos in Z-stack were recorded. Two standard filter sets were used (1) PI-labelled nuclei appear red at 450 nm wavelength and DAPI or bisbenzimide-labelled nuclei appear unlabelled, and (2) a U.V. citation PI-labelled nuclei and bisbenzimide-labelled nuclei appear blue at 410 nm. The number of nuclei was counted manually with Metamorph software.

2.4 Procedures of embryo transfer (ET)

2.4.1 Production of pseudo pregnant females

This study used MF1 females as recipient for C57/Bl6 embryos. MF1 have the average of 11.5 litter and have excellent reproductive and maternal characteristics (Harlan Laboratories report). It is the breed used in most reports for pseudo pregnant females, and is the most used for outbred strain (Duah et al. 2013).

Virgin female MF1 mice aged 5 to 8 weeks old that weigh 33 to 38 grams were mated with MF1 vasectomized males (see 2.4.2) and caged overnight. The allocation of females and duration of mating is explained in section 2.1.1. Females, which had copulated with the vasectomised males (determined by the presence of a vaginal plug the next morning), were individually housed for the next 3 days.

2.4.2 Production of vasectomized males

Mrs. Caroline Mercer, Transgenic Facility Manager from the Biomedical Research Facility, and Dr Bhavwanti Sheth, Transgenic Unit Manager, Centre for Biological Sciences, (both University of Southampton) carried out this procedure. Male MF1 mice aged 6 to 8 weeks old (Biomedical Research Facility, University of Southampton) were anaesthetized by an intraperitoneal (I.P.) injection of a ketamine (Fort Dodge) acepromazine (C-Vet) anaesthetic. The males received approximately 1 µl/gm of body weight (i.e. a 40 g mouse received approximately 40 µl of anaesthetic). The abdomen of the mouse was shaved, and the area cleaned using a surgical scrub (7.5% Providone-Iodine, USP). A 1.5 cm transverse incision was made into the skin and body wall using fine dissection scissors (Fine Scientific Tools, F.S.T), at a point level with the top of the legs. Using blunt forceps, the left testicle, vas deferens and epididymis were excised from the body cavity. A loop of approximately 1 cm of the vas deferens was held in the forceps then cauterized using red-hot forceps. The testicle, vas deferens and epididymis were carefully placed back inside the body wall, and the procedure was repeated on the right vas deferens. The incision in the body wall was sutured using an absorbable suture (vicryl absorbable suture, Johnson and Johnson). The cut in the skin was sutured (ethilon non-absorbable suture, Johnson and Johnson), and covered with tissue adhesive (Vetbond, 3M Animal Care Products, USA) to seal the wound.

The males were caged individually and allowed to recover for 2 weeks before their sterility was assessed. Virgin female MF1 mice aged 5 to 12 weeks (University of Southampton, Biomedical Research Facility) were housed overnight with the vasectomized males. Each male was mated with at least two females to confirm sterility. Each morning, the females were checked for a vaginal plug (taken as a sign of copulation). Plug positive females

were individually housed and monitored regularly for the next 20 days. This is to ensure the sterility of the males.

2.4.3 Embryo transfer in recipient females

This procedure was carried out by Dr. Neil Smyth from Centre for Biological Sciences, University of Southampton. Immediately prior to their transfer, mid-blastocyst embryos were removed from their *in vitro* culture conditions and placed into pre-warmed M2 in a culture dish under mineral oil for up to 3 hours, and kept at 37°C. This was to buffer against any pH changes until the embryos were transferred. Day 2.5 pseudo-pregnant female MFl mice (section 2.4.1) were anaesthetized by an intraperitoneal injection of a ketamine (Fort Dodge) acepromazine (C-Vet) anaesthetic. The lower back of the anaesthetized mice was then shaved, and the area cleaned using 100% ethanol. A small longitudinal incision (1 cm) was made in the skin near the rear leg. Connective tissue holding the skin to the body wall was carefully cut.

Using fine forceps and dissection scissors (Fine Scientific Tools, F.S.T), a small cut was made in the body wall just over the left ovary. The ovary was excised from the body via the fat pad taking great care not to handle or damage the ovary, oviduct or uterus. The ovary was held outside the body via the use of a serrated bulldog type serrefine (Fine Scientific Tools). Using a flame-polished transfer pipette (section 2.1.2; bore of pipette is made large enough to fit an expanded blastocyst) attached to a mouth pipette, six blastocysts were taken up. The transfer pipette was kept in place until required whilst the culture dish was replaced in the 37°C incubator. Under a stereomicroscope (Zeiss), a hole was made in the top of the uterus using a 25 gauge needle, a few millimetres down from the utero-tubal junction. Approximately 5 mm of the transfer pipette was inserted into the hole, and the blastocysts were transferred. The mouse was removed from the microscope and the hole in the body cavity

was sutured (vicryl absorbable suture, Johnson and Johnson). This procedure was repeated on the right hand side of the mouse. The cut in the skin was stapled with auto clip to close the opening.

Another limitation in obtaining the similar number of offspring from NVT and VT groups as compared to the undisturbed group was the lack of embryos obtained in one ET session. Ideally, two or more ET procedures should be performed for a session. As mentioned earlier, each ET requires 12 embryos (6 in each horn). Therefore, in total at least 24 or more viable blastocysts are required for ET. Considering that embryos from the VT group generally have 80% survival rate, at least 30 embryos are needed after flushing prior to the vitrification procedure.

In general, embryos obtained from C57Bl6 females comprised four to eight embryos from each female. If number 6 was considered as an average value, thus at least 5 females are required to carry out 2 ET per session. Initially, this was not feasible because there was insufficient number of mating female mated each day. Nevertheless, after troubleshooting and discussion, the number of mating female was increased to obtain at least 5 females each day, for 3 times of ET session a week (Figure 2.1).

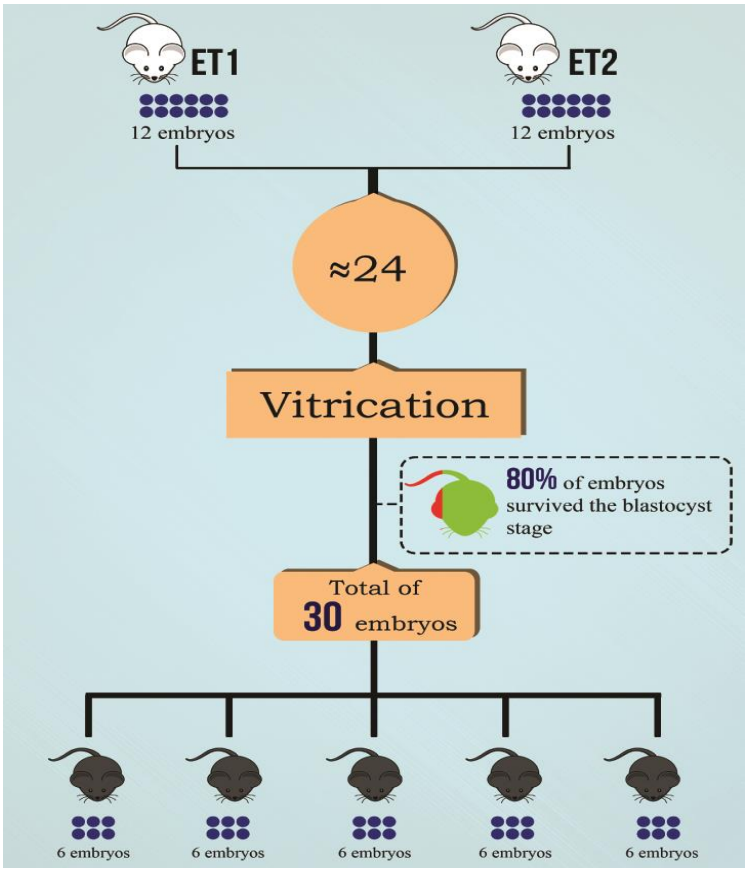


Figure 2.1 Flowchart on the number of embryos needed for one ET session. The black mice at the bottom are C57/Bl6 donors. Approximately 6 embryos were collected from donors prior to vitrification procedure. We estimated 80% of total embryos survived after vitrification, thus 24 embryos are ready for 2 ET in foster mother (white mice at the top).

2.5 Analysis of postnatal growth and development

2.5.1 Measurement of postnatal growth

Offspring were weighed starting on week 1 onwards. On day of weaning (week 3), offspring were weighed on an electronic balance, sexed and males and females subsequently kept in separate cages. Mice were ear punched to mark each individual animal. Each litter, and their cages, had individual ID numbers, and a maximum of five mice were left in each cage. Mice had access to standard chow and water *ad libitum*. Mice were weighed on day of weaning, and then subsequently on the same day every week for the next 27 weeks.

2.5.2 Measurement of systolic blood pressure (SBP)

Systolic blood pressure was determined at 9, 15 and 21 weeks of age by tail-cuff plethysmography using an ITC model 229 blood pressure monitor (Linton Instruments, Norfolk, UK). Mice were allowed to acclimatise for 10 to 15 minutes to a room temperature of 27 to 30°C. The mice were placed in a plastic container with a heat pad underneath for at least 10 minutes before readings were taken. Next, the mice were then taken out from the container and placed on the table without the heat pad and were restrained within a Perspex tube whilst the tail was placed into the tail cuff (Figure 2.2). The systolic blood pressure readings (5 readings per mouse) were taken on weeks 9, 15 and 21, at the same time as the weekly weighing of the mice. These readings were always taken after 1400 hours. The maximum and minimum values were discarded and the three remaining readings were averaged. If, after 30 minutes, all five recordings had not been taken, the mouse was released and allowed to recover for 30 minutes before obtaining the remaining blood pressure values. The mouse's heart rate was monitored as an indicator of stress. If the animal's heart rate rose above 600 beats per minute then readings were stopped until the heart rate had reduced.

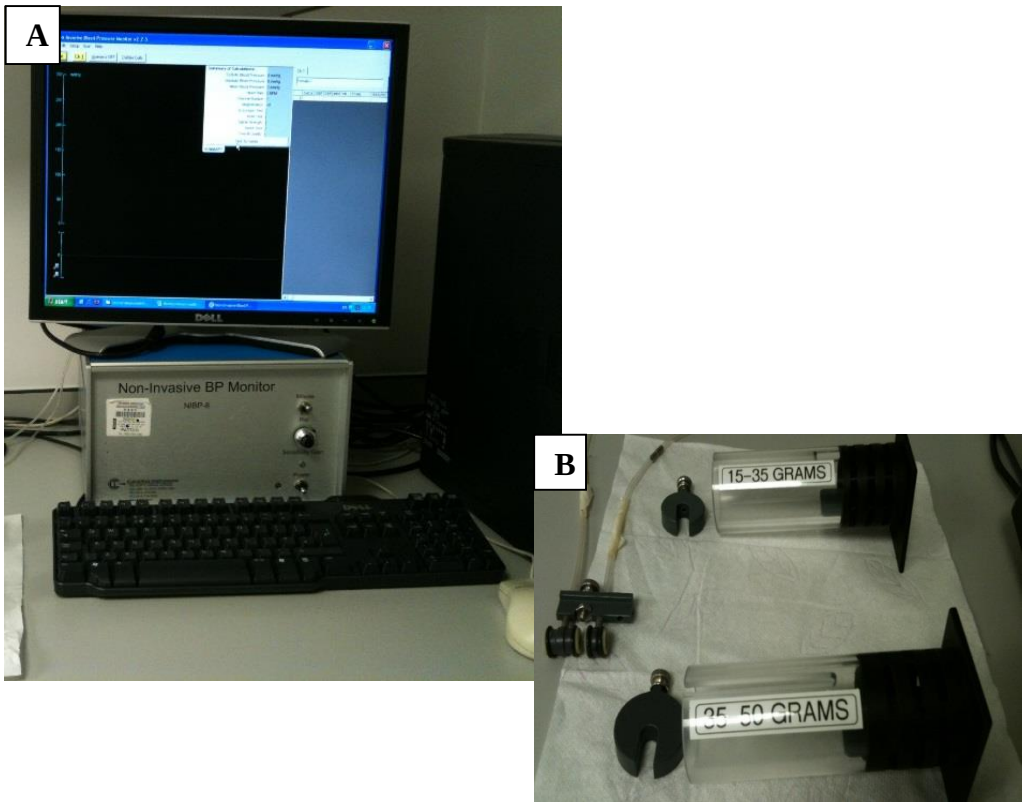


Figure 2.2: Non-invasive blood pressure monitor with Perspex tube for systolic blood pressure readings. (A) Non-invasive BP monitor. (B) Perspex tubes for 15 to 35 grams and 35 to 50 grams mice.

2.5.3 Glucose tolerance test (GTT)

Mice were fasted overnight for 15 hours. The next day at 0900 hours, mice were weighed and the tail was colour-coded for easier identification throughout GTT. Glucose (D-glucose, embryo tested; G-6152 Sigma) injection (2 g in 10 ml distilled H_2O solution) was prepared for 1 unit to 1 g of weight (36 grams equals to 36 ml of glucose solution). Mice were restrained and anaesthetic (Xylocaine Gel 2%; lidocaine hydrochloride; Astra Pharmaceuticals) was applied to its tail. After 10 minutes, a razor blade was used to make a small cut at the tail and a drop of blood was gently squeezed out to perform a glucose reading using the glucometer (AccuCheck Aviva, Roche). A duplicate reading was taken for each mouse. After 15 minutes, glucose tolerance tests were performed by intraperitoneal injection of glucose (1 g D-

glucose/kg body weight). Blood collection was performed three further times, at 30, 60 and 120 minutes after glucose injection. A timesheet was prepared to record the value of glucose level (mmol/g) for each time point.

The glucometer was calibrated to ensure the reliability of the results. Glucometer calibration was carried out each week, using a control solution provided by the manufacturer, before GTTs were carried out. The date and time were recorded when the control solution was opened. The tip of the bottle was wiped before the bottle was squeezed and the solution dropped onto the test strip. The button of the glucometer was then pressed twice to set the control level in the meter. After a successful test, the meter turns off 5 seconds after the test strip is removed.

A forecast calculation were carried out to determine the value of glucose concentration at 180 minutes after glucose injection between non-vitrified transfer and vitrified transfer groups. Another parameter that was investigated is the duration of time estimated for glucose level to return to its fasting glucose level (GTT0). The calculation use using FORECAST in Microsoft Excel and as the following:

$$a = \text{mean } y - b\,(\text{mean } x)$$

$$b = \frac{\sum (x - \text{mean } x)(y - \text{mean } y)}{\sum (x - \text{mean } x)^2}$$

2.5.4 Culling and organ collection of 27 week old mice

Mice aged 27 weeks were weighed before being 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. Blood samples were taken by puncturing the heart with a needle (30 gauge) attached to a syringe and kept in a small Eppendorf tube submerged in ice. Liver, left and right kidneys, spleen, heart and lungs were dissected out, weighed and snap frozen in liquid nitrogen, and stored at -80°C. Blood samples were centrifuged at 10,000 *g*, 4°C for 10 minutes, after which the serum was aliquotted into 10 µl samples and frozen at -80°C.

2.5.5 Serum glucose and insulin

A subset of serum from the whole sample that were frozen after animals being culled at 27 weeks were thawed and taken out to measure the glucose and insulin concentration across different groups. A total of 7 to 11 samples from each treatment in male and female were used for this experiment. Glucose concentration from serum were determine using glucose strip as described above (Section 2.5.3). Mouse serum insulin levels were measured using specific ELISA kits (Mercodia, Sweden, Mouse: 10-1247-01) according to the manufacturer's instructions. Briefly, enzyme conjugate and wash buffer solutions were prepared and 10 µl of calibrators or samples were pipetted into appropriate microplate wells, followed by 100 µl of enzyme conjugate. Following 1-2 hour incubation at room temperature with 750 rpm on plate shaker, wells were washed 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 and the plate was tapped on absorbent paper to remove any excess liquid. The wash procedure was repeated five times. A total of 200 µl of substrate was added followed by a second incubation of 15 minutes. A total of 50 µl of stop solution was added and absorbance was measured at 450 nm using Varioskan Flash Multimode Reader (Thermo

Scientific). The different concentration of substrate showed the level of insulin in the serum (Figure 2.3). Standard deviation and coefficient of variance was calculated for each sample. When the CV% was higher than 20%, the samples were discarded. OD values must be in the range of the standard curve and outliers were taken out from our data. All mouse samples were run on the three plates. Each sample was run in duplicate and mean insulin values calculated from these duplicate measurements.

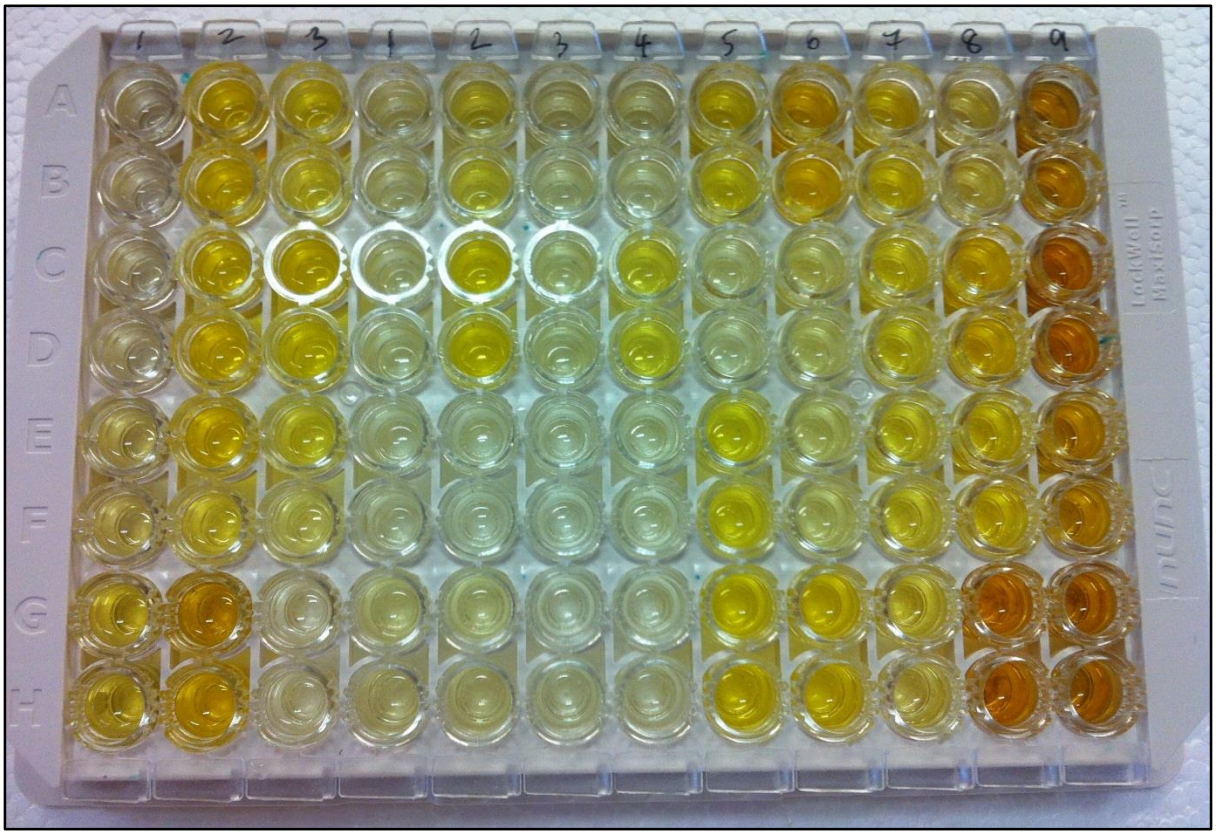


Figure 2.3 Elisa kit for insulin measurement

2.5.6 Calculation of measurement of insulin resistance

Serum glucose and insulin values can be used to calculate measures of insulin resistance with the glucose:insulin ratio. This ratio gives an indication of glucose homeostasis. From these value we could conclude that the lower the glucose:insulin ratio the more insulin insensitive

the patient/animal becomes as a higher insulin concentration is required to maintain the glucose concentration.

2.6 Justification of experimental design

The experimental design was structured to best fit our main objectives, which are investigating the effect of vitrification procedure on embryos and subsequently postnatal health. Several previous studies indicated that additional techniques used in ART alongside cryopreservation such as superovulation may have adverse effects on embryo potential (Santos et al. 2010; Huffman et al. 2015). However, to ensure that we focus on effects of the vitrification specifically, in our study the embryos were derived from naturally mated females without any superovulation procedures.

Second, we have used only a short duration of time (approximately 15 minutes) where the embryos are exposed to LN_2 for the vitrified group (Figure 2.4). This ensured suitable comparison could be made between treatment groups for blastocyst development and phenotype (Chapters 3 and 4). Also, blastocyst transfer to recipient mothers (section 2.4.3) from both treatment groups (non-vitrified and vitrified) could be achieved during the same ET session to avoid any variation in conditions. Similarly, offspring from both treatment groups would be approximately the same age and both groups thereby analysed at the same time points from birth to week 27, and are therefore experiencing the same environmental factors.

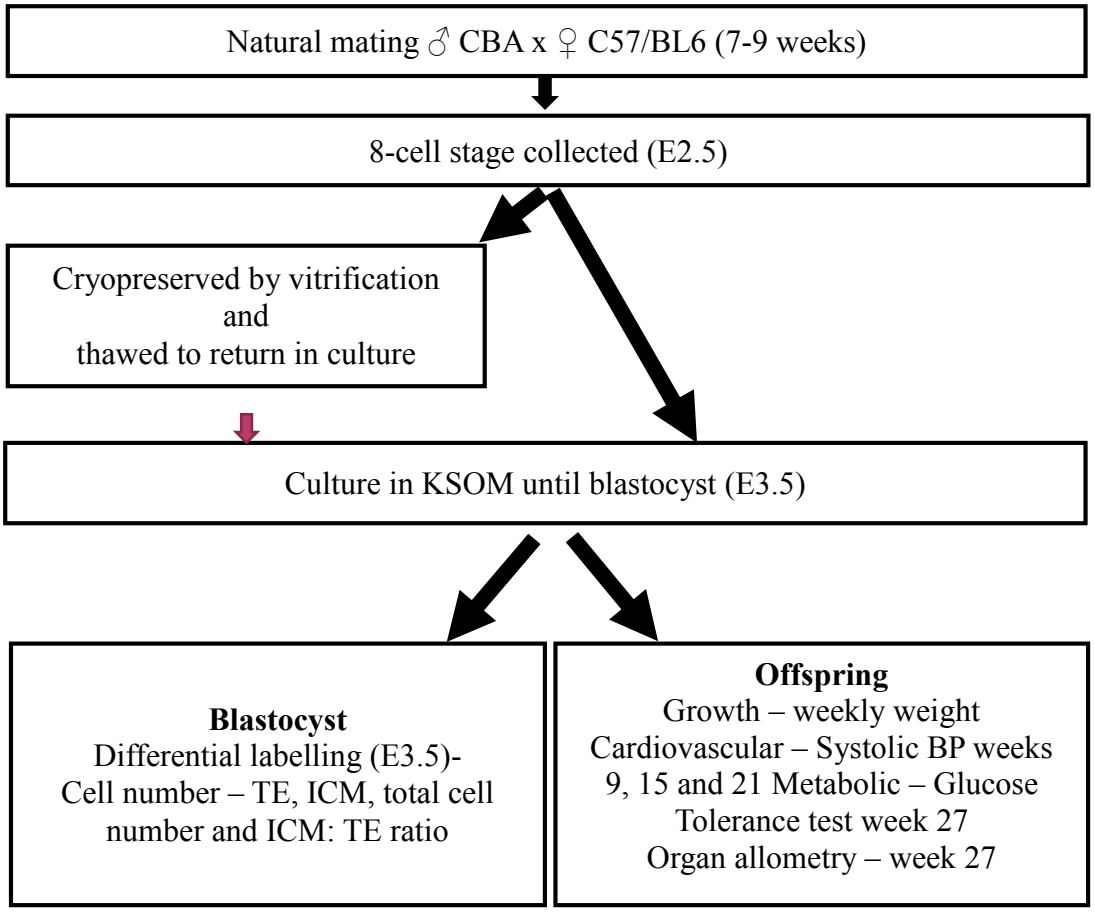


Figure 2.4 Experimental design

2.7 Statistical analysis

The random effects model analysis (SPSS; discussed below) was conducted in consultation with Prof Clive Osmond (MA, PhD, CStat); Medical Research Council Senior Scientist at the Epidemiology Unit, Southampton General Hospital and Professor of Biostatistics, University of Southampton. For all statistical analyses, significance was taken as $P \leq 0.05$. A trend was assumed to exist if a P value of between 0.1 and 0.051 was observed. All data regarding blastocyst cell numbers and rates of blastocyst development were analysed using a one way ANOVA (IBM SPSS Statistics 22). A one way ANOVA followed by a pairwise t-test with Bonferroni correction analysis was used to analyse the litter size from the different treatment groups (IBM SPSS Statistics 22).

All postnatal data regarding growth weights, blood pressures, glucose tolerance test (area under curve and individual time points), and organ weights and ratios were analysed using a multilevel random effects regression model.

The correlation analyses were also carried out using multilevel random regression within group. This model takes into account between-mother and within-mother variations in litter size and parameters measured from individual animals, thereby preventing differences that are due to abnormal litters. The model used was the following:

$$\mathbf{y(lp) = a + b.treatment + u(1) + e(lp) + c}$$

Where **1** is the litter, **p** is the pup, y(**lp**) is the response (=outcome) variable as measured on pup (p) in litter (1), a is the intercept, treatment is either the mouse group from the different embryo origins, **b** is the regression coefficient which measures the amount by which **y** is increased due to the treatment as opposed to the control treatment, **u(1)** is a random effect applying to all pups in litter **1**; if **u(1)** is large then all pups in that litter tend to have large values of **y**. The programme estimates the variance of all the 'u's that apply to the various litters e(lp) is a random effect applying to all pups, it represents measurement errors within-pup variability and other uncaptured determinants of outcome**. c** is the number of animals within the litter, either before and/or after correction The program estimates the variance of all the e(lp) terms.

Chapter 3 Effect of vitrification on blastocyst development and cell number

3.1 Introduction

Cryopreservation is widely utilized to conserve oocytes, embryos and sperm for animal husbandry and ART in human. However, the neutrality of cryopreservation technique is still debatable (Auroux et al. 2004). There are several reports on cryopreservation studies in human that focused on embryo survival and developmental potential after thawing (Loutradi et al. 2008; Kolibianakis et al. 2009).

Vitrification is preferable over traditional cryopreservation by slow freezing as it produces better rates of survival because the increased speed of temperature conduction eliminates ice crystal formation and reduces the chill-associated injuries. Comparison of the efficacy of human cleavage-stage embryo survival after vitrification to survival rates after slow freezing have shown significantly higher rate of survival in the vitrified group (Lin et al. 2010; Wong et al. 2014). Randomized controlled trials from four eligible studies also showed that vitrified human embryos resulted in a higher post-thawing survival rate compared to slow freezing (Loutradi et al. 2008). However, the drawbacks in these studies were the lack of an appropriate control group, the use of a retrospective study design and inadequate sample size (Loutradi et al. 2008).

Other than comparisons between the adequacy of vitrification and slow freezing techniques, vitrification on different stages of embryos were also studied (Fan et al. 2015). Pairwise comparison showed no difference ($P>0.05$) in blastocyst rate for vitrified mouse cleavage-stage when compared to fresh embryos. A significantly lower developmental rate was obtained with vitrified blastocysts compared to fresh controls.

There are also similarities with oocytes as Sripunya et al. (2010) reported on lower developmental rate of vitrified mouse oocytes compared to fresh controls. The damage in vitrified oocytes may be due to disruption of the meiotic spindles and other cytoskeletal elements (Chen et al. 2003), or the degradation of cytoplasmic mRNA levels (Succu et al. 2008) that is common in cryopreserved mammalian oocytes.

Regardless of the developmental potential of vitrified oocytes or embryos, we are still uncertain whether vitrification would affect the integrity of the embryos. Chatzimeletiou et al. (2012) analysed the effect of vitrification on cytoskeletal organisation and development of human blastocysts by assessing survival rate and chromosome configurations using fluorescence and confocal laser scanning microscopy. The high survival rate post-warming suggested that vitrified blastocysts do not diminished developmental potential. However, vitrified embryos seem to have a higher incidence of abnormal shaped spindles compared to fresh embryos. This often associates with chromosome lagging or congression failure. Abnormal spindles are spindles with a focused and unfocused pole, chromosome bridging and disorganized middle spindle fibres at telophase (Figure 3.1).

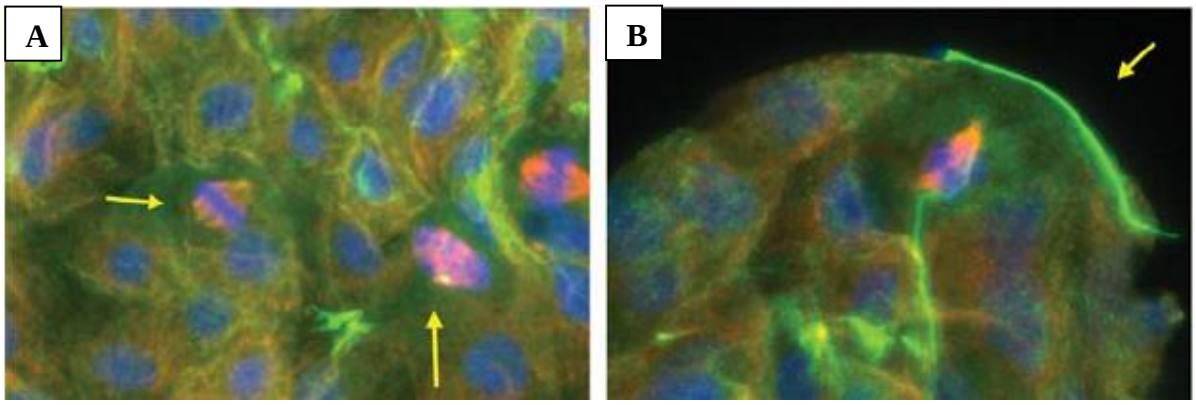


Figure 3.1**:** Confocal sections of human vitrified blastocysts showing (A) abnormally shaped spindle with a well-focused and an unfocused pole (short arrow) and monopolar spindle (long arrow). Note the strong staining of midbodies with acetylated tubulin (arrow heads), (B) abnormally shaped elongated spindle. Note the strong staining of sperm tails (arrow) and midbodies with acetylated tubulin (arrow in yellow). DAPI (blue), α-tubulin (red) acetylated tubulin (green). (Source from Chatzimeletiou et al. 2012)

Exposure to cryoprotectants, that cause the cells to shrink, may be the reason for abnormal shaped spindles observed. Abnormal spindles may associate with functional abnormality, thus creating the possibility of failed spindles. However, if the abnormal spindles progress through mitosis, the derivative cells may have abnormal chromosomes (Chatzimeletiou et al. 2012). Spindles that are associated with chromosome lagging resulted in chromosome loss that will affect derivative cells and later become monosomic (Chatzimeletiou et al. 2012). Although cell cycle checkpoints are thought not to operate during cleavage before global activation of the embryonic genome, during blastocyst stage, a functional spindle assembly checkpoint should minimize any deleterious effects. Mitosis was arrested until the defect was corrected, or by eliminating mitotically-arrested cells by apoptosis (Chatzimeletiou et al. 2012). These abnormalities in spindles reflect mechanisms that can lead to chromosome mosaicism in early human development.

Similarly, vitrification was suggested to lead to chromosomal abnormalities in mice (Shaw et al. 1991). The report elaborated that when comparing cryoprotectant concentrations, severe chromosome damage was found only in the vitrified group when compared to the fresh controls. The observation that embryo freezing can cause chromosomal damage but does not lead to immediate cell death has serious implications. It was reported that viability, both in vitro and in vivo, after embryo cryopreservation was reduced in mouse foetuses (Day 15) with major morphological abnormalities observed (Shaw et al. 1991).

As reports on chromosome and spindle integrity in the embryos were compromised due to vitrification, the question on whether vitrification may be lethal to embryos to such an extent that vitrified embryos would not develop into a normal foetus is still arguable. Fan et al. (2015) reported that there were no significant differences in live birth between vitrified hamster embryos (14/48) and fresh control embryos (19/48). In another report, vitrification was reported to have a higher implantation and clinical pregnancy rate compared to slow freezing

(Lin et al. 2010). This would then raise the question on the ability of vitrified embryos to implant and develop to a normal foetus.

Even though developmental potential between vitrified and fresh control in human embryos were the same, vitrification was believed to cause blastomere loss and degeneration. Guerif et al. (2002) reported that blastomere loss from vitrification is strongly associated with a reduction in implantation potential. The report is in agreement with (Lin et al. 2010) that reported that complete blastomere survival and total number of embryos appeared as the most important factor for pregnancy outcome. Edgar et al. (2004) also agreed and reported that intact thawed vitrified embryos showed the same developmental potential as fresh ones. The complete blastomere survival might also reflect a higher intrinsic embryonic quality before vitrification, allowing them to select only good quality embryo that was able to tolerate cryopreservation to survive and therefore implant (Guerif et al. 2002).

Cryopreservation of mouse oocytes or embryos was reported to decrease the cell numbers in resulting blastocysts (Uechi et al. 1999; Van der Elst et al. 1998). In contrast, using a different animal model, bovine embryos claimed that the inner cell mass (ICM), trophectoderm (TE) and total cell number in vitirifed embryos did not differ from fresh controls (Sripunya et al. 2010).

Another concern of vitrification is offspring abnormalities. Chatzimeletiou et al. (2012) reported that human spindle abnormalities resulting from vitrification probably would have a long term effect, but it depends on where the abnormalities occur, abnormal cell proportions and potential for further division. It was also reported that the majority of spindles analysed were located in TE, therefore it would be possible that there will be no detrimental effect as TE gave rise only to placenta. Abnormal spindles progressing into mitosis in the ICM, may also not be lethal if abnormal cells give rise to extra foetal membranes. However, adverse effects

may occur if the abnormal cells give rise to the foetus. However, low level mosaicism localized in specific tissues of the foetus may remain phenotypically invisible in adulthood.

3.2 Objectives

My aim in this chapter was to determine the effect of vitrification on blastocyst development, cell number (ICM and TE) and ICM: TE cell ratio. The first set of experiments focused on the developmental rate of blastocysts between vitrified and non-vitrified embryos. On E2.5, 8-cell embryos (n= 347 from 55 mothers) were flushed then either cultured in KSOM (+BSA,+L-glutamine,+Penicillin; Appendix I) and acting as a control (non- vitrified) group, or vitrified for the vitrified group (Section 2.1 and 2.2). Vitrified embryos were thawed shortly after freezing (10 to 15 minutes) to ensure both groups remained at approximately the same development stage. Embryos were monitored on E3.5 and E4.5 to analyse the success rate for blastocyst development for both groups.

The second set of experiments investigated the effect of vitrification on cell lineages of blastocyst, ICM and TE using differential labelling. The methodology was similar to that of the first experiment, explained above, but a total of only 173 embryos from 40 mothers were stained at the blastocyst stage from the vitrified and non-vitrified groups.

3.3 Methodology

3.3.1 Embryo collection

Female C57/BL6 mice were mated with CBA males (section 2). Embryos were flushed at the compacted 8-cell stage (E2.5) and non-vitrified embryos were directly cultured in KSOM to blastocyst stage (E3.5 or E4.5).

3.3.2 Embryo vitrification

Embryos were vitrified with equilibrium and freezing solutions, then plunged into liquid nitrogen before thawing after 10 to 15 minutes and subsequently cultured in KSOM (section 2). Non-vitrified embryos developed to middle and late blastocyst at E3.5, however, some vitrified embryos only developed to middle blastocyst at E4.5. Early and middle stage blastocysts comprise less than half, and more than half of blastocoel of a total embryo respectively. Late blastocyst is when the blastocoel fully occupy the embryo, and the zona pellucida is thinning,

3.3.3 Developmental rate for non-frozen and frozen embryos

Embryo development was assessed on E3.5 or E4.5. Stages and rate of embryo development were taken into consideration before differential labelling.

3.3.4 Differential nuclear staining

For the second set of experiments embryos were stained using differential staining technique only on E3.5 (section 2). Blastocysts that had delayed development (E4.5) were analysed only for development rate (Section 3.3.3). The numbers of ICM and TE nuclei were counted using Metamorph software with z-stack pictures. Initially, the staining of some embryos was indistinct only showing total nuclei stained with Hoescht without any TE nuclei stained by propidium iodide. These initial results may be the result of learning the new techniques required to remove the zona pellucida and handle embryos using a mouth pipette. Out of 255 embryos stained, only 173 (67.8%) embryos showed a whole embryo stained image and was selected for cell number counting. Another criteria essential for cell number counting is the embryo having a higher number of TE nuclei compared to ICM nuclei.

3.3.5 Statistical analysis

Means and SEMs were analysed by using a one way analysis of variance (one way ANOVA) test. For all statistical analyses, significance was taken as $p \leq 0.05$ (Section 2.5.5).

3.4 Results

Table 3.1 shows the developmental rates of non-vitrified and vitrified 8-cell embryos to blastocyst stage, from a total of 55 females. The embryo survival percentage from non-vitrified (98.7%; 150/152) was not significantly higher compared to vitrified embryos (87.2%; 170/195). However, vitrified embryos showed delay in forming middle or late blastocysts on E3.5 compared to non-vitrified. Non-vitrified group only had 5 and 10 embryos, that developed to late blastocyst and hatching blastocyst on E4.5; respectively. In contrast, the vitrified group have a total of 20 and 37 embryos developed to middle and late blastocyst on E4.5; respectively.

Figure 3.2a shows pictures of embryos reacting to equilibration and vitrification medium prior to vitrification procedure. The blastomere shrunk but maintain its cell membrane structure. After vitrification and thawing procedure, the blastomere returns to its original form (Figure 3.2b).

Figure 3.3 shows a blastocyst differentially labelled from the non-vitrified group. The TE, ICM, and total cell numbers for non-vitrified and vitrified embryos at the blastocyst stage, and the ICM to TE ratio is shown in Figure 3.3. The mean cell number in non-vitrified embryos (n=78 embryos) and vitrified embryos, (n=95 embryos) within the ICM (17.9±3.3 vs. 13.77±2.3), TE (26.5±4.05 vs. 19.07±2.89), and total cell number (44.4±4.94 vs. 32.63±3.32) showed significant differences ($p < 0.05$). Vitrified embryos possessed significantly reduced cell numbers within ICM, TE, and in total when compared with non-vitrified embryos. However, the ICM to TE cell number ratio showed no significant difference between the two groups.

Table 3.1**:** Developmental rates of non-vitrified and vitrified 8-cell mouse embryos to blastocyst stage.

			No. (% ± SEM) of embryos developing to					**No. (% ± SEM) of embryos developing to**				
Group	**E2.5**	**E2.5**	**E3.5**					**E4.5‡**				
	+ No. of embryos	**No. (% ± SEM) of embryos retrieve after thawing**	**Morula**	**Early blastocyst**	**Mid blastocyst**	**Late blastocyst**	**Degenerated**	**Morula**	**Early blastocyst**	**Mid blastocyst ‡**	**Late blastocyst ‡**	**Hatching blastocyst ‡**
Non-vitrified	152 (from 25 mothers)	Not applicable	0	15 (9.86 ± 5.3)	38 (25.0 ± 5.3)	97 (63.82 ± 5.3)	2 (1.32 ± 5.3)	0	0	0	5 (3.29 ± 0.32) ‡	10 (6.58 ± 0.32)
Vitrified	195 (from 30 mothers)	184 (94.4)	20 (10.87 ± 1.15)	37 (20.11 ± 1.15)	55 (29.9 ± 1.15)	58 (31.52 ± 1.15)	14 (7.69 ± 1.15)	0	0	20 (10.87 ± 1.87)	37 (20.10 ± 1.87)	0

Early blastocyst – have less than half of blastocoel of a total embryo
Middle blastocyst – have more than half of blastocoel of a total embryo
Late blastocyst - blastocoel fully occupy the embryo and zona pellucida (ZP) is thinning
Hatching blastocyst - Herniation of TE from ZP
+ Total number of embryos in each group derived from 55 females (n = 19 replicates)
‡ Developing embryos to blastocyst stage on E4.5 (at 1000 hours) from morula and early blastocyst E3.5 (at 1800 hours)

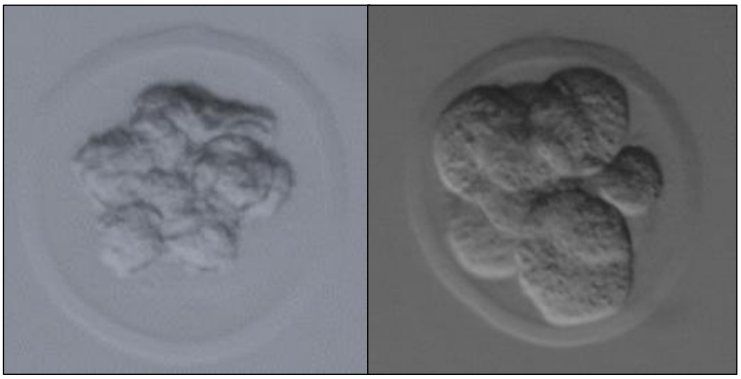


Figure 3.2: Embryos morphology in vitrification medium (a) and thawing medium (b).

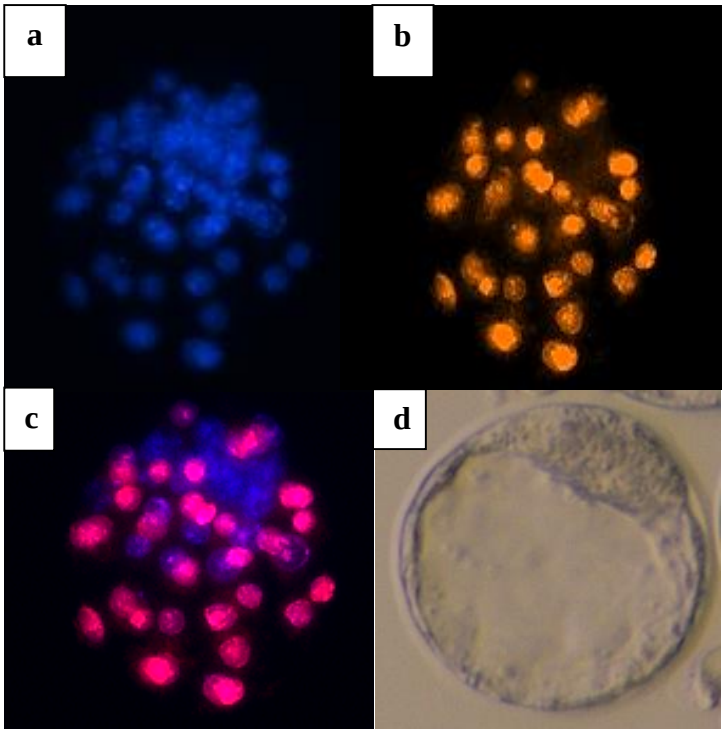


Figure 3.3: Blastocyst differentially labelled with Hoechst (a) and Propidium Iodide (b). Overlay shown in (c). Image of mid-stage blastocyst (d).

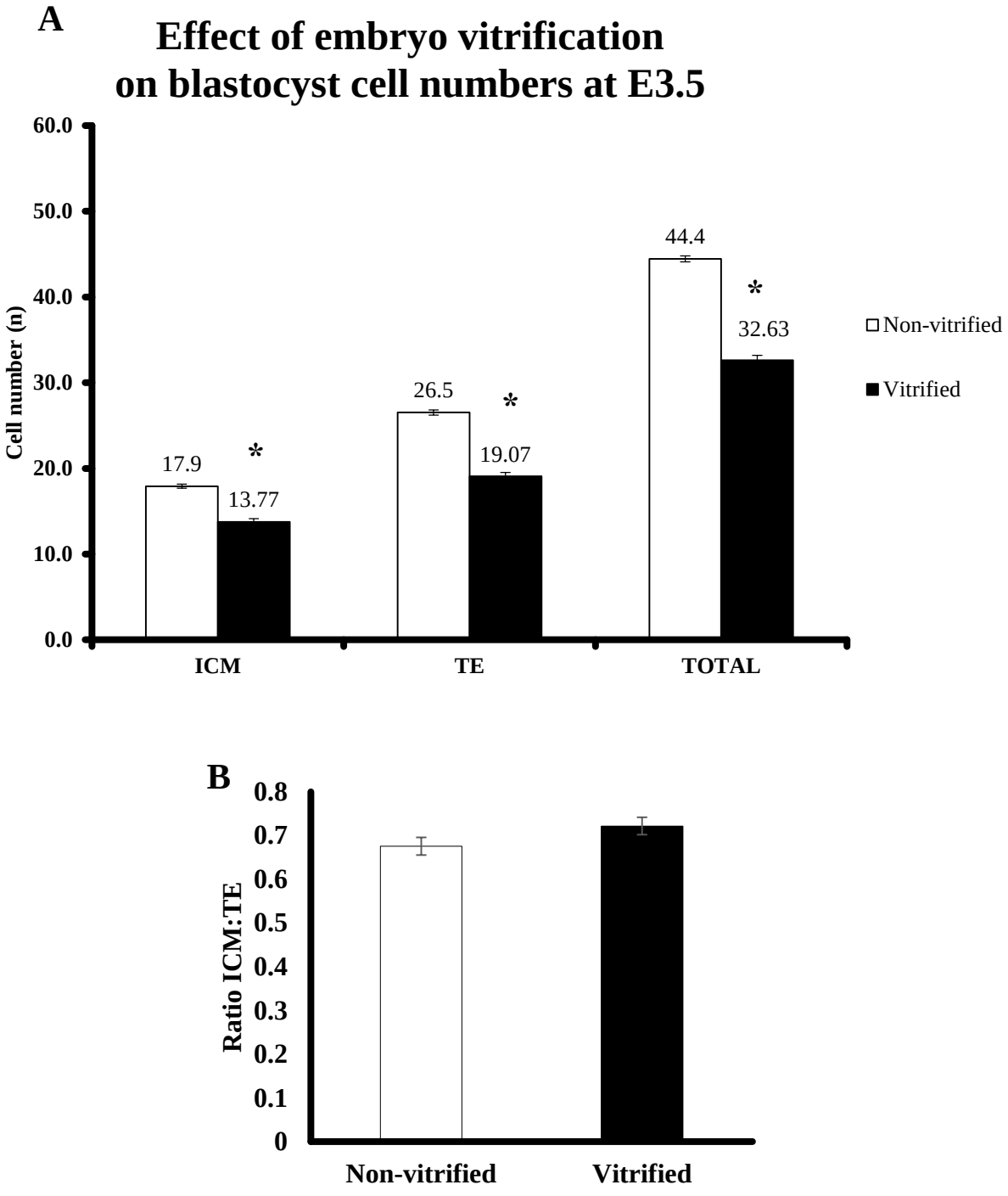


Figure 3.4**:** Vitrified embryos (n=95 embryos) have fewer cells than non-vitrified (n=78 embryos) at blastocyst stage. (A) Mean (±SEM) blastocyst cell numbers for non-vitrified compared with vitrified blastocyst. (B) Mean (±SEM) ICM/TE ratio of blastocysts in A. (∗, $P < 0.05$ compared with in non-vitrified group).

3.5 Discussion

This study indicated that developmental rates for vitrified and non-vitrified embryos were not different. This illustrates similarity with Fan et al. (2015) who reported a similar developmental rate to blastocyst stage for vitrified hamster 8-cell (80%) and morula (72%) embryos compared to fresh (non-vitrified) 8-cell (90%) and morula (92%) embryos, respectively. Shin et al. (2011) also reported that no differences were observed between control and frozen thawed mouse 8 cell embryos to blastocyst (92.9% vs. 97.4%; respectively). Permeability of embryo cell membranes to cryoprotectant might contribute to the high success rate for late cleavage stage (Jin et al. 2013). A study on cell membrane permeability concluded that the permeability of cryoprotectant and water increases further as the developmental stage proceeds. The high permeability of cryoprotectant (P_{EG} and P_{DMSO}) by simple diffusion through the lipid bilayer, or facilitated diffusion via channel processes, plays an important role ensuring the viability of the embryos after vitrification procedure (Jin et al. 2013).

On the other hand, the developmental rate of vitrified embryos to blastocyst was delayed and required an extra day (E4.5 compared to E3.5 in non-vitrified group - see Table 3.1). However, the delay was not significant from the controls. The same observation was recorded by Illmensee et al. (2005) who showed a 4 hour delay in 8- cell stages mouse embryos for the next cleavage stage after embryo splitting procedure. Thus, we may suggest that the development delay in our experiment most likely is due to the vitrification procedure.

It is important to note that the blastocysts derived from both groups were normal with respect to total cell number. Although the outcome on development rate from 8-cell stage to blastocyst stage between vitrified compared to non-vitrified groups were not significantly low, the subsequent experiments on blastocyst cell number showed that vitrification had significantly reduced cell numbers. This was similar with reports on mouse oocytes (Van der Elst et al. 1998) and embryos (Shin et al. 2011).

Reduction in cell number may be due to delayed cleavage and allocation of cells to ICM and TE (Van der Elst et al. 1998). Despite having an altered cell number, equivalent cleavage delay was found in TE and ICM cells. Since the ratio of ICM to TE is fairly constant throughout blastocyst development, this showed that vitrification process delayed cell division even though the control embryos compared to were predominantly at the same stage (mid-blastocyst stage).

The maintenance of a normal ICM:TE ratio after vitrification in my study is, however, in contrast with Van der Elst et al. (1993) where a significant lower value for ICM: TE ratio and reduced ICM cell numbers were observed between cryopreserved mouse embryos and controls. They suggested that disturbed allocation of cells to the ICM occurred in vitrified embryos. Inner and outer cell allocation in a compacted embryo must occur in a spatially and temporally synchronized way (Kelly et al. 1978; Lehtonen et al. 1988). The origin of outer TE and inner ICM is when compaction occurs at the late 8-cell stage (Ducibella & Anderson 1975) and when each cell forms a stable microvillus pole on its free outer surface (Handyside 1980). The polarized cells typically produce either one polar and one apolar daughter or two polar daughters, depending on the orientation of subsequent divisions (Johnson & Ziomek 1981). Polar cells remain external in the morula, whilst apolar cells come to reside internally and will give rise to ICM after cavitation occurs (Johnson & Maro 1984). We believe that the vitrification procedure may only delay development but not disrupt the allocation process in our experiments because we found no significant different in ICM: TE ratio. Van der Elst et al., (1993; 1998) use a higher concentration of DMSO (3.5M DMSO) with 2.5 minutes exposure to embryos sealed in a straw (close system) before being plunged into LN_2. On the other hand, our experiment only used 1.5M DMSO with addition 1.5M EG (see Section 2). The combination of these two cryoprotectants is essential to lower the toxicity of the cryoprotectant (Section 1.8.2). It is also worth noting that our experiment only exposed the

embryos to cryoprotectant for less than one minute before transferring them to a tip (open system) and plunging them directly to LN_2. These variables may then contribute to the different results in our cell number analysis.

Other than vitrification, prolonged culture of mouse embryos also showed a reduction in cell number (Watkins et al. 2007). It has also been previously shown that blastocysts developed *in vitro* consistently showed fewer blastomeres compared to blastocysts developed *in vivo* (Kiessling et al. 1991). This kinetic retardation was reported not reversible by alteration of protein source (Watkins et al. 2007) and growth factors (Kiessling et al. 1991) to the medium. The retardation of embryo development observed after vitrification was not shown in blastocysts exposed to only cryoprotectant and no vitrification (Kiessling et al. 1991). Overall, we can suggest that the vitrified-thawed and cultured embryos are more sensitive and are expected to have reduction in cell number compared to fresh controls.

It was reported that bovine embryos with reduced cell numbers are able to give rise to foetuses (Iwasaki et al. 1990; Tao & Del Valle 2008). However, it was documented that embryos produced from 1/8 blastomeres often fail to develop an ICM (Loskutoff et al. 1993) and this suggests that the ICM requires a certain cell mass to be able to develop further. Studies have also shown that manipulation of embryo in cell number such as embryo splitting and embryo biopsy still resulted in viable and healthy offspring (Illmensee et al. 2005).

Embryo biopsy is a common practice using one or two blastomeres for preimplantation genetic screening (PGD) for couples that may be genetic carriers for certain diseases, such as cystic fibrosis. The guidelines on embryo biopsies were standardized in the ESHRE guidelines for optimal results in Europe (Harton et al. 2011). PGD has been shown to be safe and efficient (Cockburn & Rossant 2010). The reduction of blastomeres by biopsy will likely reduce cell number at the blastocyst stage but embryo implantation is not compromised. A study showed

that embryo splitting at 8-cell stage resulted in blastocyst developmental rate of 32.1%. This study showed that the loss of half of the blastomeres at the 8-cell stage compromised their development into blastocysts, but the some of the embryos managed to survive and developed to viable offspring.

The study by Tsunoda & Mclaren (1983) emphasises the reduction in implantation rate and live foetuses in 8-cell compared to earlier cleavage stage. The number of viable live young produced from embryo splitting from 8-cell embryos is 15% compared to 2-cell split that gave 65%. They interpreted that because of the proportion of life foetuses from half embryos transferred to oviduct compared to uteri is significantly higher, the embryos need more time to increase their cell number before implantation (Tsunoda & Mclaren 1983). To relate these results with our study, even though embryo splitting and vitrification are different, vitrification might cause the loss of half or more blastomeres in some embryos. Thus, the low percentage of ET pregnancy rate, and ET efficiency, compared to the non-vitrified transfer group in our study (data in Chapter 4) can be explained by the impact that the loss of at least half of the embryo blastomeres has.

In conclusion, vitrification did not have any significant effect on the embryos' survival rate to blastocyst stage, but it altered the ICM, TE, and total cell number in the vitrified group. This raises the question of whether the vitrified embryos reach the critical mass for successful post-implantation embryo development subsequently perturbed the postnatal heath of offspring. This shall be investigated in Chapter 4.

Chapter 4 Effect of embryo vitrification on postnatal development and physiology

4.1 Introduction

4.1.1 Postnatal development and physiology

In the previous chapter, we discussed the effect of cryopreservation on the developmental potential of an embryo and cell number allocation within early cell lineages. Studies have shown that even though cryopreservation may alter DNA integrity (Kader et al. 2010), cytoskeleton formation (Chatzimeletiou et al. 2012), and cell number in the blastocyst stage (Chapter 3), embryo still developed to a viable embryo and had the potential and probability to form a whole foetus and term offspring. In this chapter, we investigate the effect of cryopreservation on postnatal health using the same mouse model.

Large cohorts and samples collected from patients worldwide have been analysed to evaluate and ensure that ART provides the best and safest option to have a healthy baby. However, there are reports that claimed ART procedure is not risk free and may have detrimental effects later in life (Rimm & Katayama 2012).

Using animal models, (Dulioust et al. 1995) reported on the long term effects of the slow freezing technique on mouse embryos. They concluded that embryo cryopreservation, without being severely detrimental, may have adverse effects later in adulthood. Weight of male offspring aged 67 weeks that were derived from cryopreserved embryos was significantly higher compared to the controls. However, there is no certainty that the intensity of a given effect would be the same in every species (Dulioust et al. 1995). In addition, the slow freezing technique has improved in the past 20 years, and better outcomes from the procedure have been reported (Abdelhafez et al. 2010). Thus, further study is needed to evaluate the effect vitrification on adulthood with animal models using the current cryopreservation approach.

Lavara et al. (2014) is the only report that studies the impact of embryo vitrification and transfer in the rabbit on long term reproductive physiology of offspring (F1) and whether the effect is transgenerational. The paper reported that F1 derived from vitrified embryos had a higher litter size and number born alive compared to F1 control females. There were also significant different in the same parameters between F2 vitrified group and F2 control females. They concluded that cryopreservation and transfer procedures have long-term effects on female reproductive traits (F1 females) and trans- generational effects on female F1 offspring (F2 females). It was suggested that these long-term effects could be provoked in part by altered epigenetic marks induced by the cryopreservation and transfer procedure (Lavara et al. 2014).

Unfortunately, this paper did not entirely show the effect of the cryopreservation technique on offspring because the control group was *in vivo* offspring (undisturbed) and not subjected to embryo transfer. Non-superovulated does were used in this study therefore, this would eliminate the superovulation effect on embryos and postnatal health. By adding another group to the study, a group of offspring derived from flushing and embryo transfer (ET) without vitrification, may then specifically address the effect of cryopreservation and eliminate the ET factor. This is because embryo transfer and culture medium was shown to have an impact on human embryos (Nelissen et al. 2012).

Cryopreservation is normally accompanied with superovulation because abundant numbers of embryos are required for animal studies. However, superovulation has been reported to have an immense effect on the development of embryos using mouse models (Huffman 2015). Other than superovulation, culturing embryos in culture medium was reported to have adverse effects on embryos (Nelissen et al. 2012). They reported that culture medium influences perinatal outcome of IVF children regardless of whether fresh or cryopreserved (slow freezing) embryos were transferred. They suggested that this might be due to methylation effect and altered expression of imprinted genes in several animal models (Young 1998;

Fauque et al. 2010). The paper suggested that *in vitro* culture might also lead to epigenetic disturbance that affect developmental programming in foetal (Nelissen et al. 2012).

Wennerholm et al. (2009) published a systematic review on postnatal health of ART children born from IVF/ICSI after cryopreservation (slow freezing and vitrification) of early cleavage stage embryos, blastocysts and oocytes. They reported that data from controlled studies showed that early cleavage embryos indicated a better, or at least as good, obstetric outcome, measured as preterm birth and low birth weight for children born after cryopreservation, as compared with children born after fresh cycles. Most studies found comparable malformation rates between frozen and fresh IVF/ICSI. However, reports on slow freezing of blastocysts, and for vitrification of early cleavage stage embryos, blastocysts and oocytes, and neonatal data are limited. No long-term child follow-up data were reported (Wennerholm et al. 2009).

In contrast, a study from Belgium (Belva et al. 2008) showed that cryopreserved-ICSI twins had significantly higher preterm birth, and very low birth weight rates, when compared to twins from fresh ICSI. Also, a higher rate of malformations was noticed for cryopreserved - ICSI as compared with fresh ICSI (Belva et al. 2008). The mechanism that may have induced the malformations is still unknown. This study then suggested that cryopreservation is responsible for high malformation in cryopreserved ICSI children.

Another report on population based registry studies from (Källén et al. 2005) and (Shih, Rushford, Bourne, Garrett, McBain, Healy & H. W. G. Baker 2008) claimed that there were no significant differences in malformation rates between cryopreserved-ET children and children conceived from non- cryopreserved -ET embryos. This was supported by one large registry studies, (Pinborg et al. 2010) that also showed no difference in malformation rates between cryopreserved children and children conceived from non-cryopreserved embryos.

Until now, data on growth, childhood morbidity, and mental development between children conceived from cryopreserved embryos and children conceived from non- cryopreserved embryos were limited. Follow up studies on children conceived from cryopreserved embryos is necessary for further investigation.

Although reports of increased prevalence of human perinatal problems and evidence obtained from animal experiments raise concerns, the risks identified in human studies do not seem to be associated with the techniques *per se*, but rather as being attributable to parental characteristics, and from clinical policies of transferring more than one embryo at the time, causing highly increased proportions of multiple pregnancies and deliveries (Amor & Halliday 2008). The occurrence of epigenetic anomalies identified in ART children might be increased as a result of infertility aetiologies, ovarian hyperstimulation, and extracorporeal handling and culture of gametes and embryos (Shufaro & Laufer 2013).

Until now, there are no studies on the effect of cryopreservation technique on phenotype through to adulthood using animal models. Thus, this chapter will investigate at the effect of cryopreservation on offspring cardiovascular and metabolic health.

4.1.2 Correlation between overweight, hypertension and insulin resistant

Studies between overweight or obesity being associated with diabetes, insulin resistance, and hypertension in humans raised concern (Chiarelli & Marcovecchio 2008; Pires et al. 2014). It was reported that obesity-related comorbidities are present in young obese children, thus, providing a platform for early adult cardiovascular disorders (Pires et al. 2014). There are significant correlation between the BMI in obese children (n=121) for HOMA-IR compared to normal BMI children (n=40). The homeostasis model assessment-insulin resistance (HOMA-IR) is used to diagnose insulin resistance in clinical studies. Even though

this study has a small control number, they provide a sufficient number for obese children with reliably high significant correlation.

Another report claimed that the prevalence of the insulin resistance syndrome (IRS) in obesity starts as early as 2 years old (Viner et al. 2005). The report summarised the criteria for IRS that include abnormal BMI, abnormalities of glucose homoeostasis, hypertension, and dyslipidaemia. It was also reported that the higher age increased the risk of IRS; however the syndrome was seen in 30% of children under 12 years.

Similarly, there are significant correlations detected between perinatal growth at week 3 with hypertension in mouse model (Watkins et al. 2008). The study that looked at the effect of diet alteration during the peri-conception period (Emb-LPD) suggested that weight at week 3 (W3) is significantly positive correlated with weight at week 27 (W27) and blood pressure life (SBP LIFE) in male and female offspring. They concluded that perinatal growth detected at 3 weeks of age in the Emb-LPD group is a strong predictor of those animals that will exhibit overgrowth and hypertension in later life (Fig. 3). They suggested that this is due to the activation of the blastocyst response to stimulate conceptus growth rather than its appropriateness with respect to later nutrient availability that predisposes to adult disease. This explains why disease phenotypes such as hypertension is accompanied by being overweight that appears in Emb-LPD treatment in females (Watkins et al. 2008).

In addition, there is another study that proved weight in the earlier stage is associated with perturb glucose homeostasis from IVF derived offspring in mice (Scott et al. 2010). The study indicated that there are significant differences at W3 between IVF and the control (*in vivo*) in female offspring. IVF offspring female were also reported to have a significantly higher glucose level after 45 minutes of glucose injection, a higher AUC GTT value, and a significantly higher fasting plasma insulin compared to the controls. The study shows that a

significant increase at week 3 might be associated with the female offspring from IVF group being insulin resistant. Studies using mouse model on embryo manipulation such as IVF (Scott et al. 2010) and alteration in diet such as Emb-LPD (Watkins et al. 2008) proved that manipulation during the peri-conception period may lead to perturbed postnatal development. These studies showed that perinatal growth at week 3 proved to be a strong indicator for adulthood disease due to altered diet and IVF procedure.

In this study, a specific correlation analyses between subset samples were carried out to determine the correlation between parameters for growth, cardiovascular and metabolic profile.

4.2 Objective

The aim of this study was to examine the effect of embryo vitrification upon aspects of postnatal development and physiology. Three treatment groups were classified as a) control (undisturbed offspring) b) non-vitrified transfer c) vitrified transfer (see Table 4.1 for treatment details). These three treatment groups will be used to compare the postnatal effects between embryos developing in vivo during the pre-implantation period and embryos developing in vitro, both vitrified and non-vitrified. This design (Table 4.1) should therefore discriminate between vitrification and other parameters of ART on offspring outcomes.

Table 4.1: Classification of treatment groups

Group Name	**Purpose**	**Embryo Treatment**	**Embryo transfer (ET)**	**Cryopreservation**
Control (undisturbed offspring)	Control for non-vitrified transfer group	None	No	No
Non-vitrified transfer	Control for vitrified transfer group	Derived from 8-cell, culture to blastocyst and ET to recipient	Yes	No
Vitrified transfer	Examine the effect of cryopreservation on offspring	Derived from 8-cell, cryopreserved and thawed, culture to blastocyst and ET to recipient	Yes	Yes

Parameters for growth, cardiovascular and metabolic profile comprise of weekly offspring weight, systolic blood pressure for weeks 9, 15 and 21, and a glucose tolerance test on week 27 will be obtain. Offspring will be sacrifice at week 27 for organ and blood collection. The mean organ weight and organ: body weight ratios, between treatments for male and female offspring, will be analysed (section 2.5).

Subsequently, using a subset from each group, serum, glucose, and insulin levels will be determine with a glucometer and ELISA kit (section 2.5.5). The correlation between parameters for growth, cardiovascular and metabolic profiles will be assess in all groups. To the author's knowledge, currently there are no reports on the cardiovascular system and metabolic health in animal models that investigate the long-term effect of embryo cryopreservation.

4.3 Methodology

4.3.1 Production of embryos for freezing

Female C57/Bl6 mice aged 7-9 weeks were mated with male CBA mice (section 2.1). For the non-vitrified transfer group 8-cell embryos were flushed, and cultured in KSOM for 24 hours until developed to the blastocyst stage (section 2.1.2), and did not experience vitrification or incubation in the vitrification solutions. For the vitrified embryos group, embryos were flushed from the oviduct at the 8-cell stage and subsequently, vitrified using the vitrification technique (section 2.2). After vitrification for 10 to 15 minutes, embryos were thawed and cultured in KSOM until the blastocyst stage. For the production of vitrified and non-vitrified ET offspring, 10 female recipients were allowed to develop to term with their pregnancies. Similarly, offspring from 10 control (undisturbed) pregnant females were generated.

4.3.2 Embryo transfer

This procedure was carried out by Dr. Neil Smyth from Centre for Biological Sciences, University of Southampton. The ovaries and top 1 cm of uterus of anaesthetised 2.5 day pseudo pregnant female MF1 mice (section 2.4.1) were carefully exposed from the body cavity. Six blastocysts were transferred to each uterine horn (section 2). Ten litters of each embryo treatment group were generated.

4.3.3 Measurement of postnatal growth rates

All of the offspring from recipient mothers were kept throughout the experiment. Mice were weighed starting from day of weaning, and then subsequently on the same day for the next 27 weeks (Section 2.5.1). Mice were ear punched to mark each individual animal.

4.3.4 Measurement of systolic blood pressure

Systolic blood pressure was determined at 9, 15, and 21 weeks of age by tail-cuff plethysmography (Section 2.5.2). Systolic blood pressure was normally measured between 11am and 5pm, depending on the numbers of offspring on that specific day.

4.3.5 Measurement of glucose tolerance test

Glucose tolerance test by tail cutting and glucose level measurement was taken twice for each time point using a glucometer at 27 weeks (Section 2.5.3). Glucose tolerance test starts after 15 hours fasting (around 1000 hours).

4.3.6 Measurement of organ weights and serum collection

At 27 weeks, mice were culled and organs were dissected out. This procedure normally started approximately at 1030 hours. Organs were weighed, and blood was collected, before being snap frozen in liquid nitrogen, and stored at -80^{o}C (section 2.5.4). Serum was collected after blood was centrifuged at 15000rpm for 10 minutes.

4.3.7 Measurement of serum glucose and insulin

Serum from 7 to11 samples from undisturbed, non-vitrified, and vitrified groups were used to measure glucose and insulin levels by glucometer and ELISA kit respectively (section 2.5.3).

4.3.8 Statistical analysis of data

Pregnancy rates between groups were analysed using t-test. Litter size variance from the different treatment groups was also analysed using t-test. Significant differences between NVT, VT and control groups on weight, blood pressure, glucose tolerance tests, serum analysis, organ weight and organ:weight ratio were analysed using ANOVA and multilevel random effects regression model; which takes into account between-mother, and within-mother,

variations in litter size and parameters measured from individual animals, thereby preventing differences that are due to abnormal litters (section 2.5.5). Correlations were using ANOVA that also looked into account between mother and individual litter. Statistical significance was $p \leq 0.05$.

4.4 Results

4.4.1 Mean number of offspring

A total of ten litters were generated from each treatment. The pregnancy rate, ET efficiency, litter size, and the number of offspring for the three groups are shown in Table 4.2. The non-vitrified transfer (NVT) and vitrified transfer (VT) groups had a reduced number of offspring per litter compared to the control however, it is not significant. There was no significant different between NVT and VT for ET pregnancy rate using t-test on ET efficiency, and litter size at birth. The male: female ratio and number of offspring for each treatment also showed no significant differences in all treatments.

Table 4.2: Comparison between control, non-vitrified transfer and vitrified transfer groups on ET pregnancy, ET efficiency and number of offspring.

Group[1]	ET pregnancy rate[2] (%)	ET efficiency[3] (%)	Litter size at birth[4] (No.) [Litter No.]	Offspring number	No of female (n)	No of male (n)	Male: Female ratio
Control (undisturbed)	0	0	8.0 ± 0.63 [10]	80	40	40	1.0
Non-vitrified transfer	54.5 (12/20)	24.58	5.7 ± 0.37 [10]	57	30	27	0.9
Vitrified transfer	42.3 (11/26)	11.71	5.0 ± 0.68 [10]	50	20	30	1.5

[1] = Embryos were vitrified, thawed and cultured to blastocyst (vitrified transfer), or only cultured to blastocyst (non-vitrified transfer)

[2] = Dams that gave birth/total number ET sessions performed. Analysis with t-test

[3] = Pups at birth/total embryos transferred. Analysis with t-test

[4] = Calculated on dams with live pups at weaning, Analysis with ANOVA.

Data were analysed with ANOVA (mean ± SEM) except where otherwise indicated.

4.4.2 Offspring body weight

The mean weekly weights for all three groups from week 3 to 27 weeks of age are shown in Figures 4.1 and 4.2. In males, the non-vitrified transfer (NVT) group was significantly heavier than controls from week 3 to weeks and 4, 7 to 27. The vitrified transfer (VT) group was significantly heavier at weeks 3, 5, 7, and 11 to 27 compared to the controls. NVT and VT group did not show any significant differences throughout life.

In females, the NVT group was significantly heavier compared with control at week 3, 6 to the end of week 27. Similarly, the control compared to VT groups were significantly smaller from weeks 6 to 27. NVT and VT group did not show any significant differences throughout the life. This was analysed using a multilevel random effects regression model which takes into account between-mother and within-mother variations in litter size and parameters measured from individual animals. All groups have been analysed against the control (undisturbed group). Statistical analysis ($p \leq 0.05$) was marked.

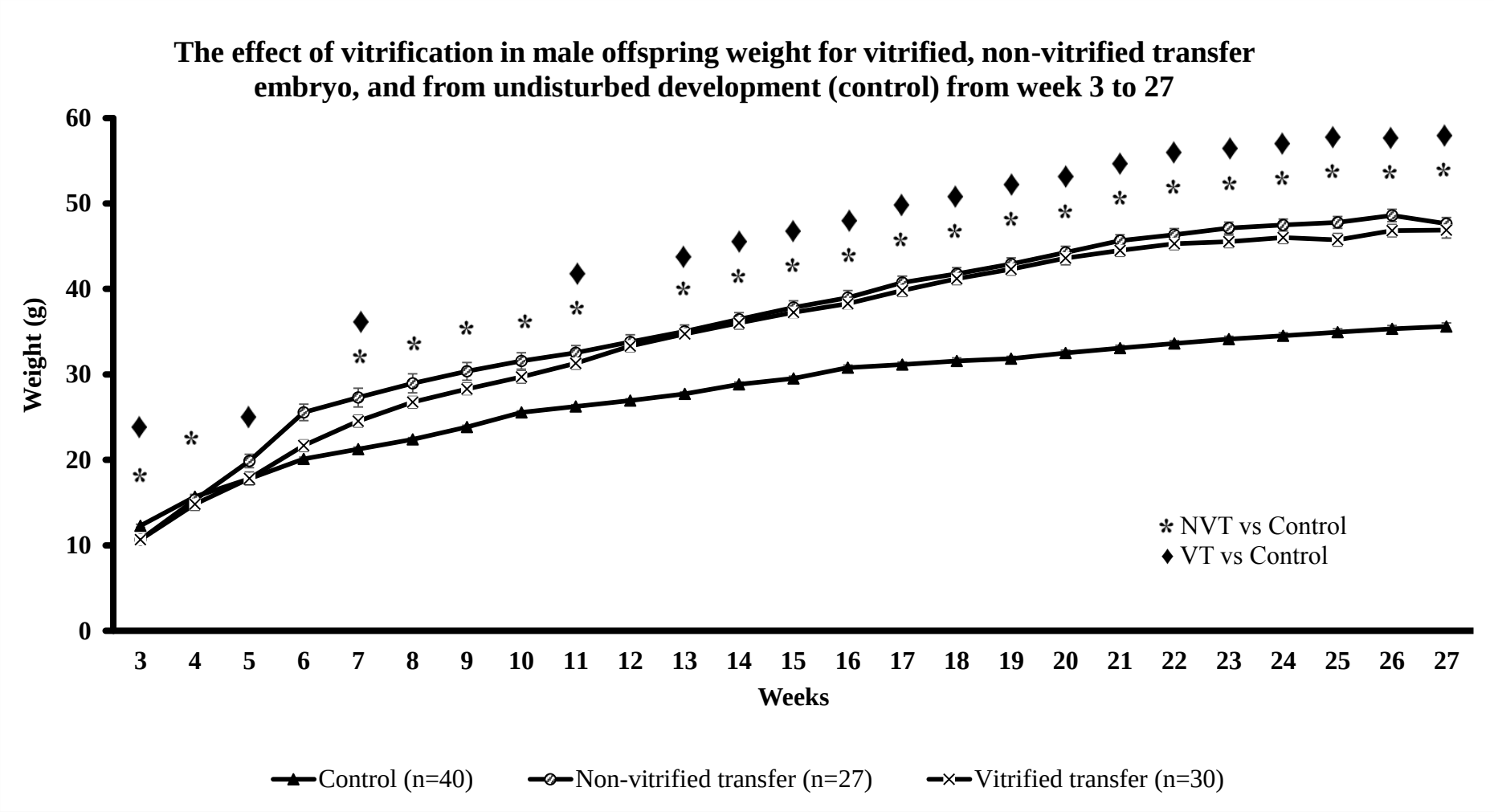


Figure 4.1**:** The graph showed the effect of vitrification in male offspring weight for 3 groups starting from week 3 to week 27. Vitrified transfer and non-vitrified transfer groups in male offspring have significant effect on postnatal growth compared to control groups. ***** denote ($P \leq 0.05$) between control and non-vitrified group and ♦ denote differences ($P \leq 0.05$) between control and vitrified group. Mean (±SEM) body weight from 1 to 27 weeks (from 10 litters).

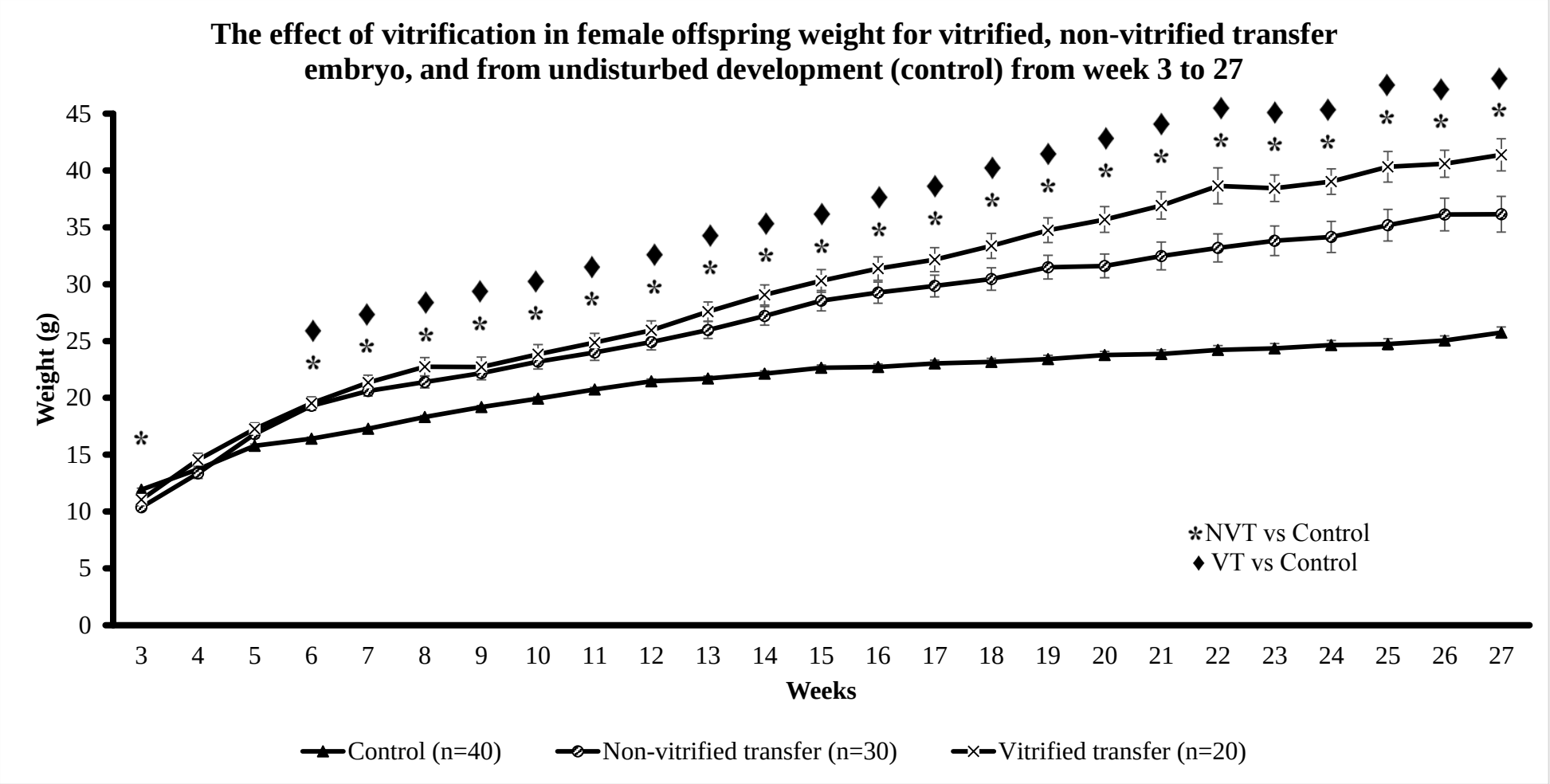


Figure 4.2: The graph showed the effect of vitrification in female offspring weight for 3 groups starting from week 3 to week 27. Vitrified transfer and non-vitrified transfer groups in male offspring have significant effect on postnatal growth compared to control groups. ***** denote ($P \leq 0.05$) between control and non-vitrified group and ♦ denote differences ($P \leq 0.05$) between control and vitrified group. Mean (±SEM) body weight from 1 to 27 weeks (from 10 litters).

4.4.3 Offspring systolic blood pressure (SBP)

The mean systolic blood pressures for the different groups at designated weeks (9, 15, and 21 weeks) are shown in Figures 4.3 and 4.4. In both male and female offspring, no significant differences were detected at week 9 between groups. However, at week 15, 21 and LIFE the NVT and VT groups had elevated blood pressure when compared to the control group ($p<0.05$). There was no significant difference between NVT and VT groups for male and female at designated weeks.

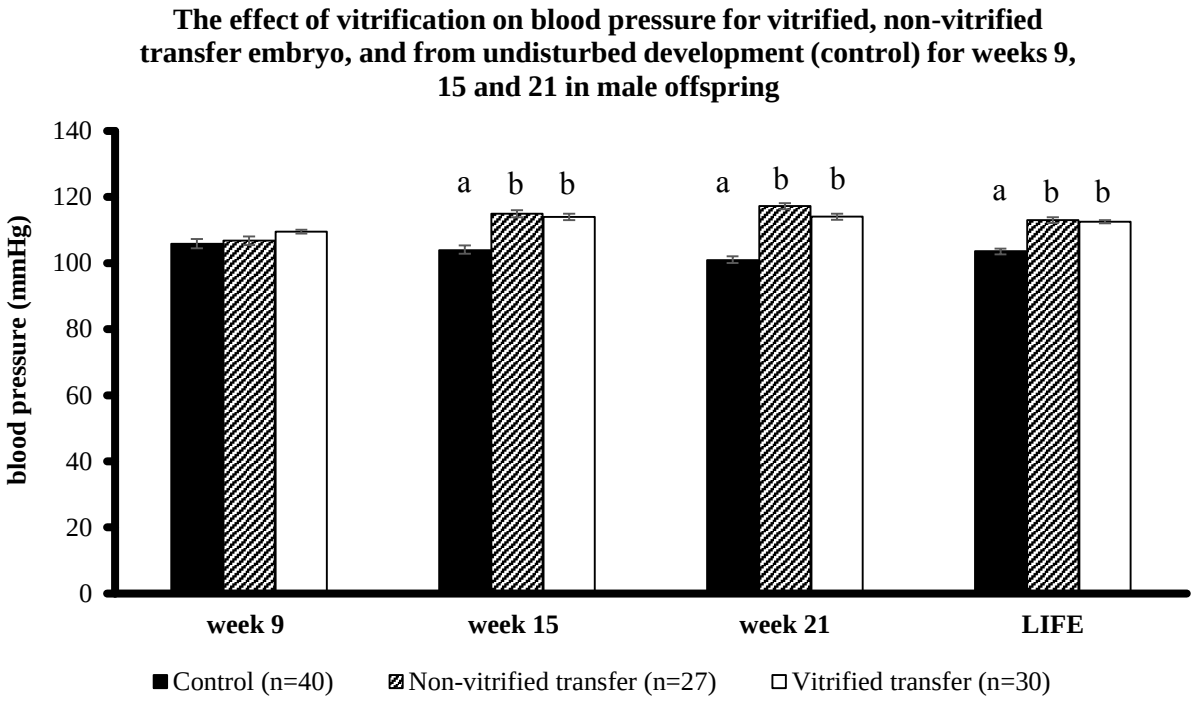


Figure 4.3: The graph showed the effect of vitrification in male offspring SBP for 3 designated weeks. Vitrified and non-vitrified groups in female offspring induce elevation in postnatal SBP for week 21 compared to control groups. Mean (±SEM) blood pressure for weeks 9, 15 and 21 (from 10 litters). Different letter denotes $P < 0.05$ independent of maternal origin, litter size, and weight.

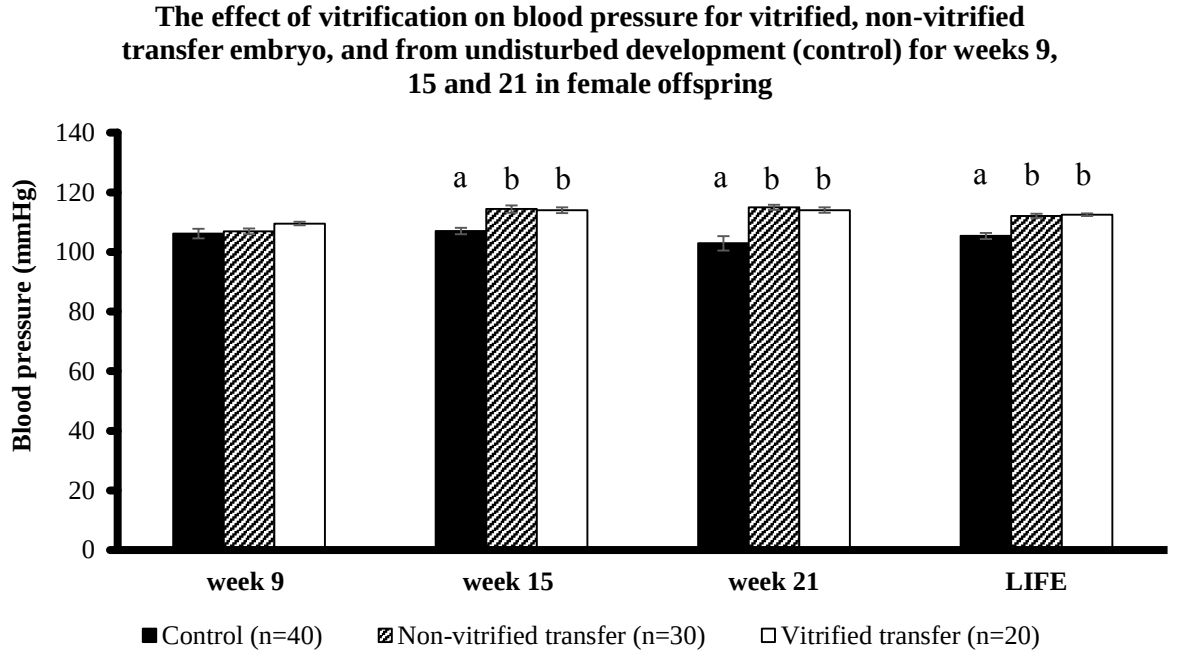


Figure 4.4: The graph showed the effect of vitrification in female offspring SBP for 3 designated weeks. Vitrified and non-vitrified groups in male offspring induce elevation in postnatal SBP for weeks 15 and 21 compared to control groups. Mean (±SEM) blood pressure for weeks 9, 15 and 21 (from 10 litters). Different letter denotes $P < 0.05$ independent of maternal origin, litter size, and weight.

4.4.4 Offspring glucose tolerance test (GTT)

Glucose tolerance test results, including area under the curve (AUC), for male and female offspring are shown in Figures 4.5 and 4.6. The results shows that males and females had the same pattern. Baseline glucose levels (GTT0) showed that NVT and VT had significantly higher glucose values compared to control. After 30 minutes of glucose injection (GTT30), offspring from NVT and VT groups had a significantly higher glucose value compared to the control and this continues after GTT60 and GTT120. GTT AUC for male and female offspring are shown in Figure 4.5B and Figure 4.6B, respectively. GTT AUC values for NVT and VT groups is significantly higher than control. There is no significant difference between NVT and VT.

The values for glucose level were also predicted by calculation (Section 4.3.5) at GTT180 between NVT and VT groups. With the estimated values, AUC for GTT0 to GTT180 were determined, but no significant differences between NVT and VT were observed in both males and females.

The readings to determine the amount of time needed for the glucose level to return to resting levels for NVT and VT groups were observed. Glucose levels were recorded throughout the experiment, and the AUC was calculated. However, there is no significant differences between the NVT and VT groups.

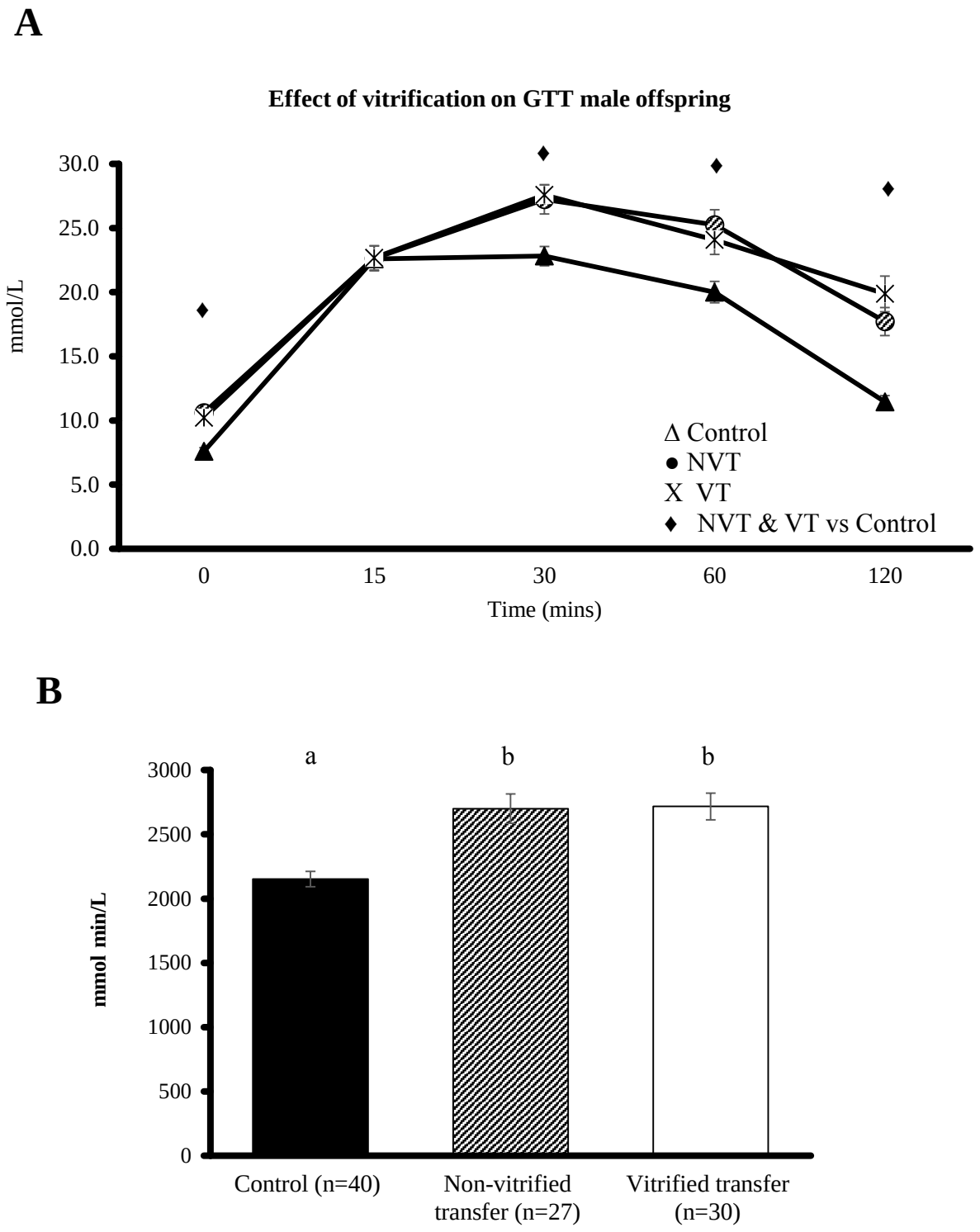
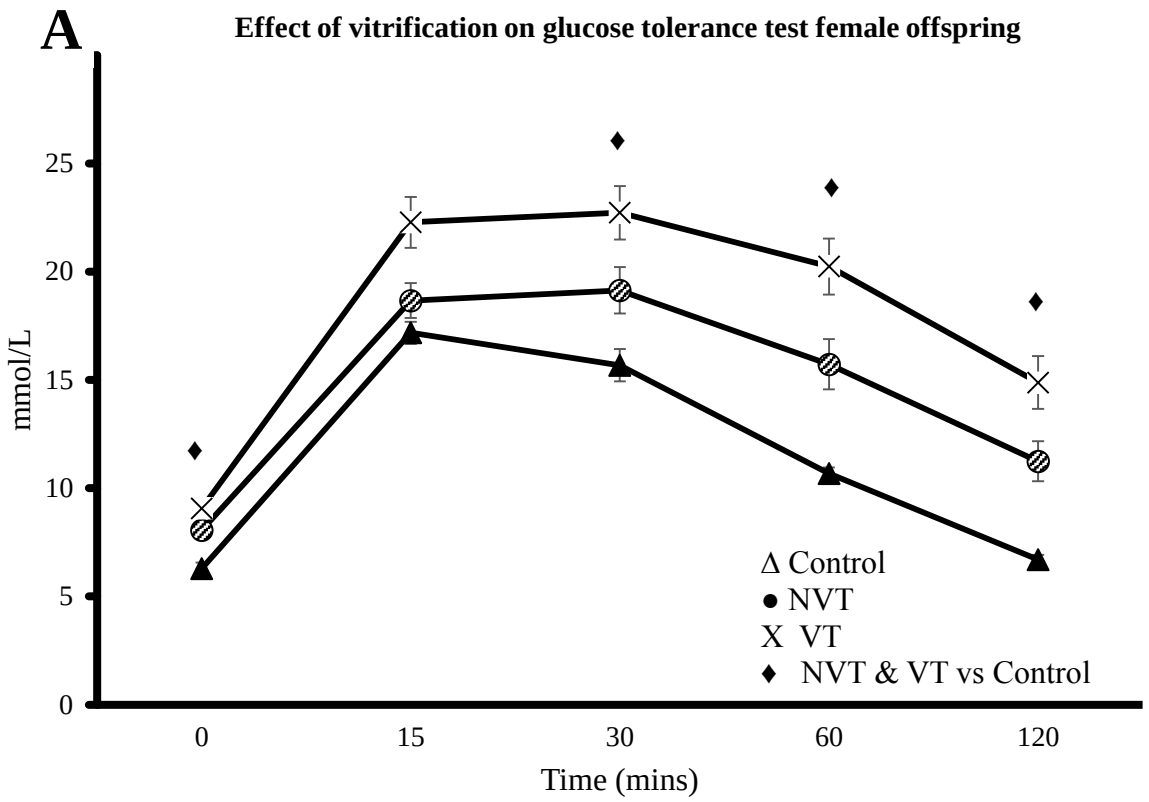


Figure 4.5: Effect of control, non-vitrified transfer and vitrified transfer treatments on male offspring glucose tolerance. (A) glucose tolerance test (GTT) and (B) area under curve (AUC) in male offspring at 27 weeks. Mean (±SEM) for GTT0, GTT15, GTT30, GTT60, GTT120 and AUC (from 10 litters). Different letter denotes $P < 0.05$ independent of maternal origin, litter size, and weight.



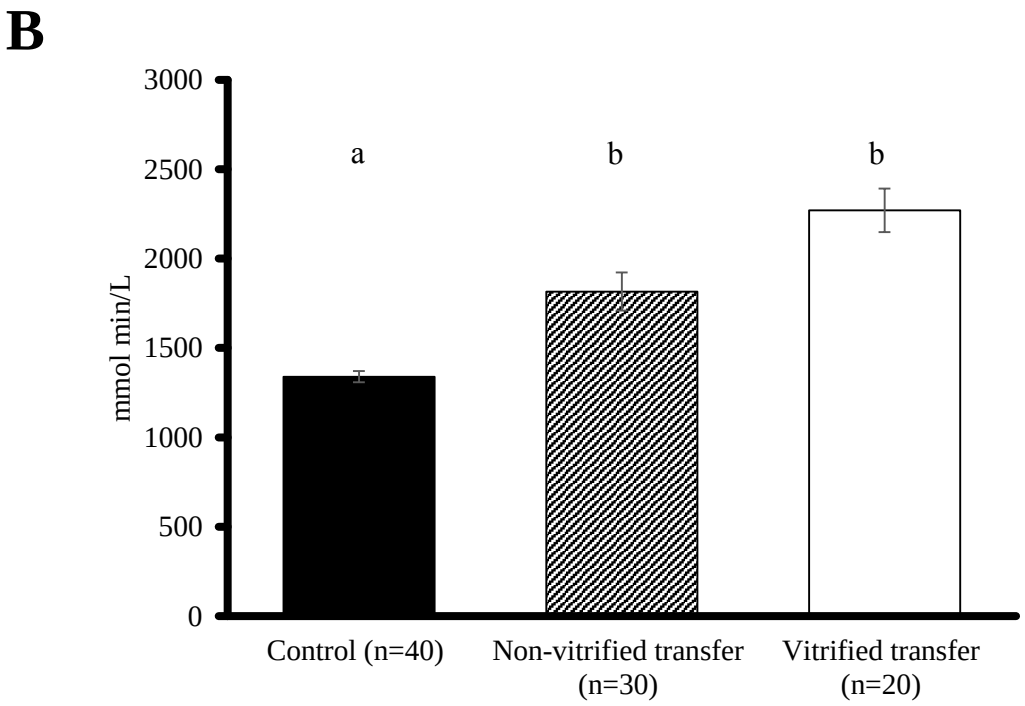


Figure 4.6: Effect of control, non-vitrified transfer and vitrified transfer treatments on female offspring glucose tolerance. (A) glucose tolerance test (GTT) and (B) area under curve (AUC) in male offspring at 27 weeks. Mean (±SEM) for GTT0, GTT15, GTT30, GTT60, GTT120 and AUC (from 10 litters). Different letter denotes $P < 0.05$ independent of maternal origin, litter size, and weight.

4.4.5 Offspring organ weights and organ:body weight ratio

Mean organ weights and mean organ: body weight ratios of offspring from the different treatment groups at week 27 are shown in Figures 4.7 to 4.10. Figure 4.7 shows significantly lower mean organ weight for males between control with NVT and VT groups. Right and left kidney weights from the control group was significantly lower compared to other groups. The same pattern was observed in spleen and heart where the VT and NVT groups were significantly heavier compared to the control ($p<0.05$). The liver mean weight in Fig 7B also showed that the controls are significantly smaller compared to the NVT and VT groups. Overall, the control offspring have the smallest organ weights compared to other groups. There is no significant differences between NVT and VT groups.

In females, the mean weight of the liver, and the right and left kidneys in the control group is significantly smaller compared to the VT and NVT groups (Figure 4.8). The weight of the heart was also significantly smaller compared to the VT group. There is no significant differences between the NVT and VT groups.

In Figure 4.9 the control groups in male lung: body weight ratio was significantly higher than the NVT and VT groups. The NVT group have a significantly smaller heart: body weight ratio compared to controls. Liver: body weight from the NVT and VT groups was also significantly smaller when compared to the control group (Fig. 4.9B). No significant differences between the NVT and VT groups.

In contrast, Figure 4.10 shows that the organ:body weight ratio for the control group was significantly higher than NVT and VT groups for lung, heart, spleen, right and left kidneys in female offspring. Meanwhile, the liver:body weight ratio from the NVT was significantly smaller compared to control group (Fig. 4.10B). There were no significant differences between NVT and VT groups.

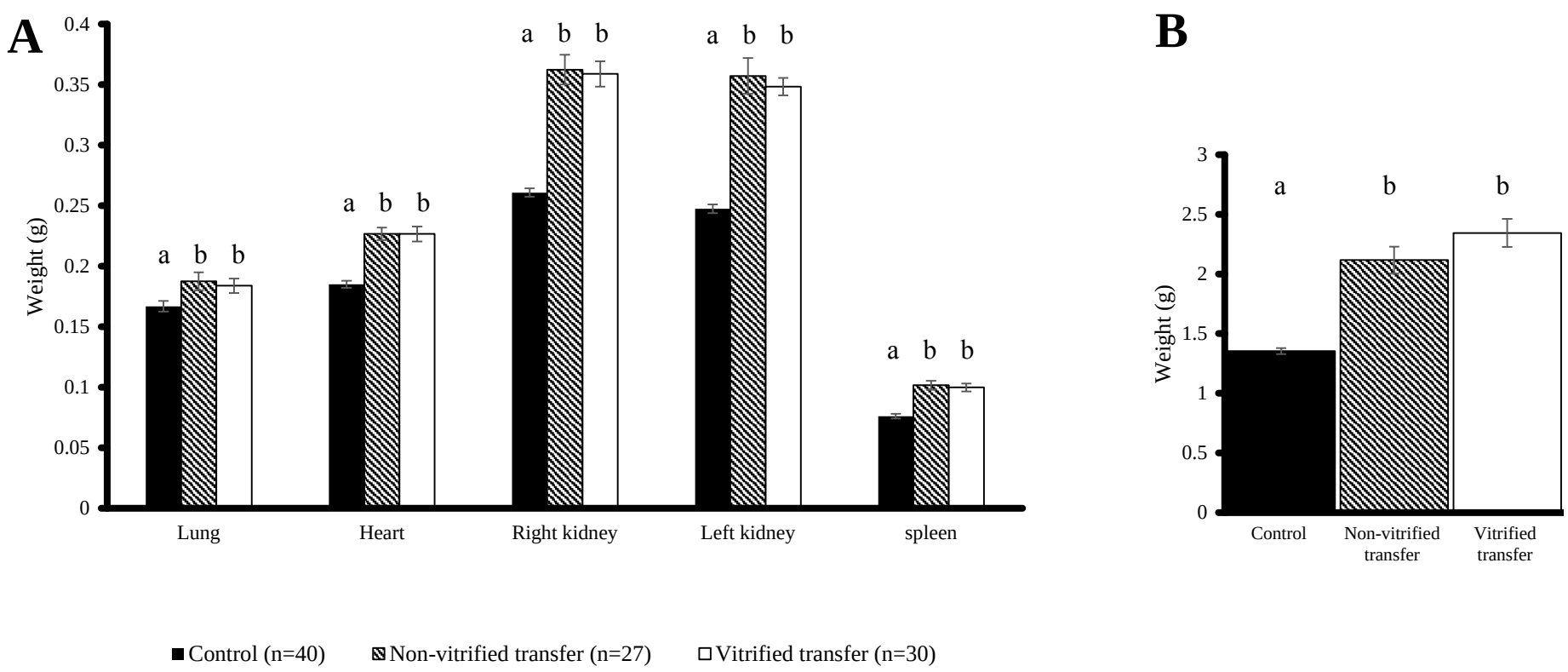


Figure 4.7: Effect of control, non-vitrified transfer and vitrified transfer treatments on male offspring organ mean weight. (A) lung, heart, kidneys and spleen (B) liver in male offspring at 27 weeks. Mean (±SEM) for organ weights (from 10 litters). Vitrified transfer and non-vitrified transfer groups in male offspring have a significantly higher mean weight for (A) right and left kidneys (B) liver at weeks 27 after culling compared to control groups. Different letter denotes $P < 0.05$ independent of maternal origin, litter size, and weight.

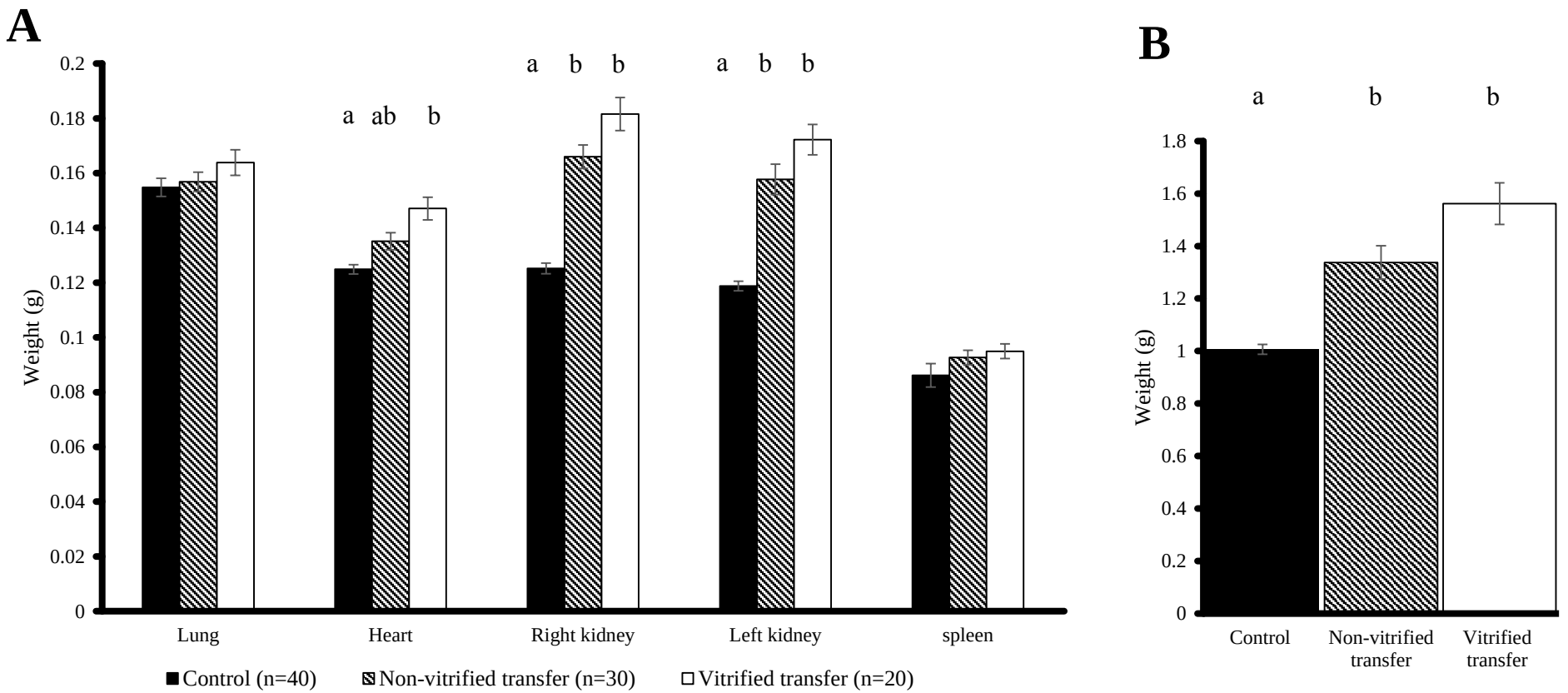


Figure 4.8: Effect of control, non-vitrified transfer and vitrified transfer treatments on female offspring organ mean weight. (A) lung, heart, kidneys and spleen (B) liver in male offspring at 27 weeks. Mean (±SEM) for organ weights (from 10 litters). Vitrified transfer and non-vitrified transfer groups in male offspring have a significantly higher mean weight for (A) right and left kidneys (B) liver at weeks 27 after culling compared to control groups. Different letter denotes $P < 0.05$ independent of maternal origin, litter size, and weight.

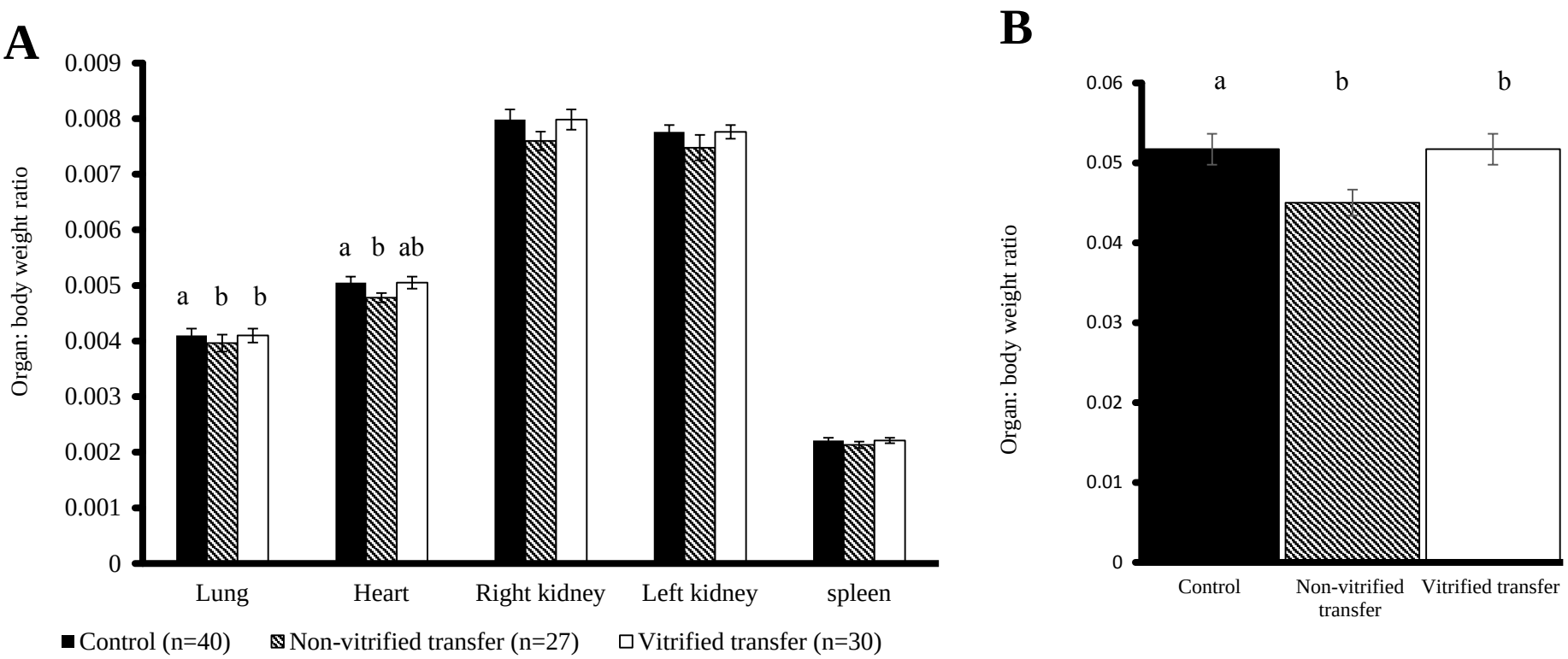


Figure 4.9: Effect of control, non-vitrified transfer and vitrified transfer treatments on male offspring organ:body weight ratio. (A) lung, heart, kidneys and spleen (B) liver in male offspring at 27 weeks. Mean (±SEM) for organ:body weight ratio (from 10 litters). Vitrified transfer and non-vitrified transfer groups in male offspring have a significantly lower mean body: weight ratio for (B) liver at weeks 27 after culling compared to control groups. Different letter denotes $P < 0.05$ independent of maternal origin, litter size, and weight.

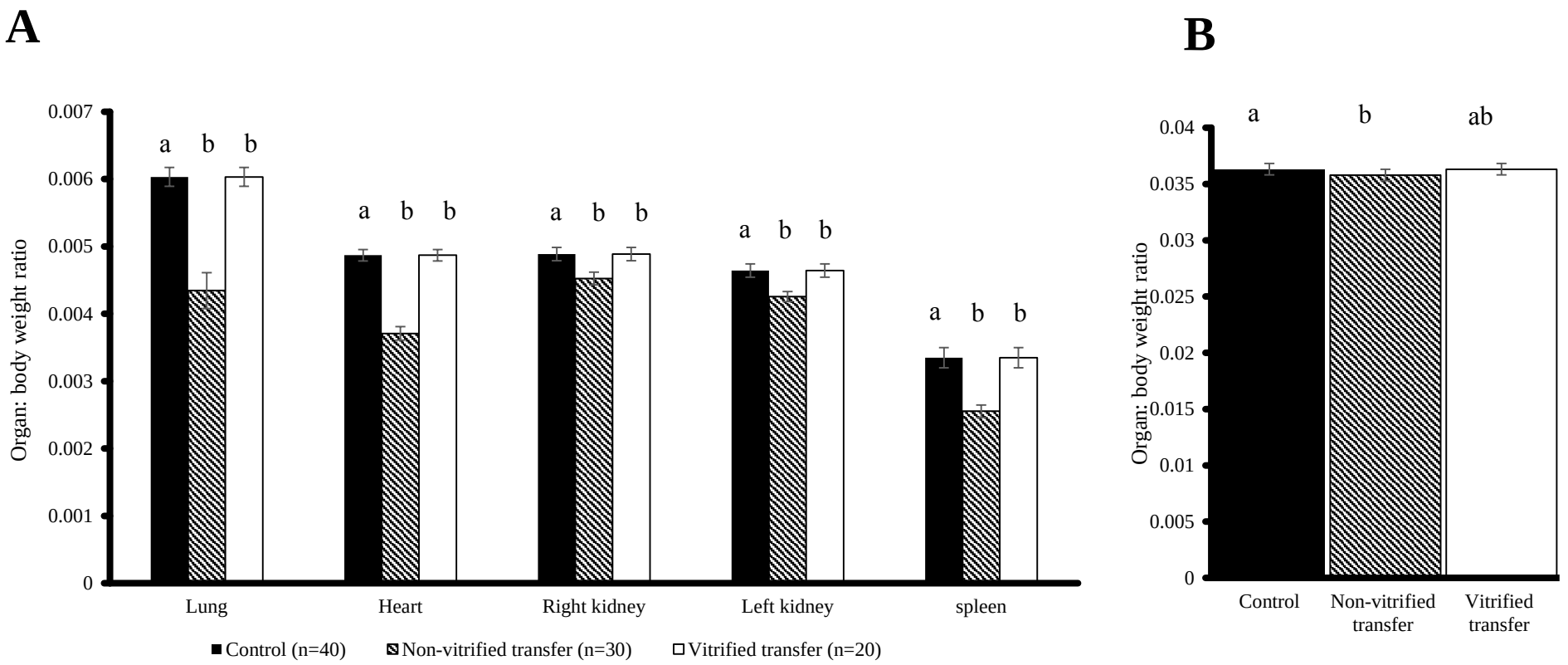


Figure 4.10: Effect of control, non-vitrified transfer and vitrified transfer treatments on female offspring organ:body weight ratio. (A) lung, heart, kidneys and spleen (B) liver in female offspring at 27 weeks. Mean (±SEM) for organ:body weight ratio (from 10 litters). Vitrified transfer and non-vitrified transfer groups in female offspring have a significantly lower mean body: weight ratio for (B) liver at weeks 27 after culling compared to control groups. Different letter denotes $P < 0.05$ independent of maternal origin, litter size, and weight.

4.4.6 Offspring serum glucose and insulin concentration

There were no significant differences in male offspring between any groups for serum glucose, insulin, and G/I ratio (Fig. 4.11). However, a trend (0.05< f <0.1; f in the graph) is observed in insulin values between the control and both the NVT and VT groups (Fig. 4.11A). In female offspring, VT group shows a higher serum insulin compared to control (Fig. 4.12A). In addition, G/I ratio value was significantly lower in the NVT and VT groups compared to the control (Fig. 4.12B). However, there were no significant differences between the NVT and VT groups in both male and female offspring.

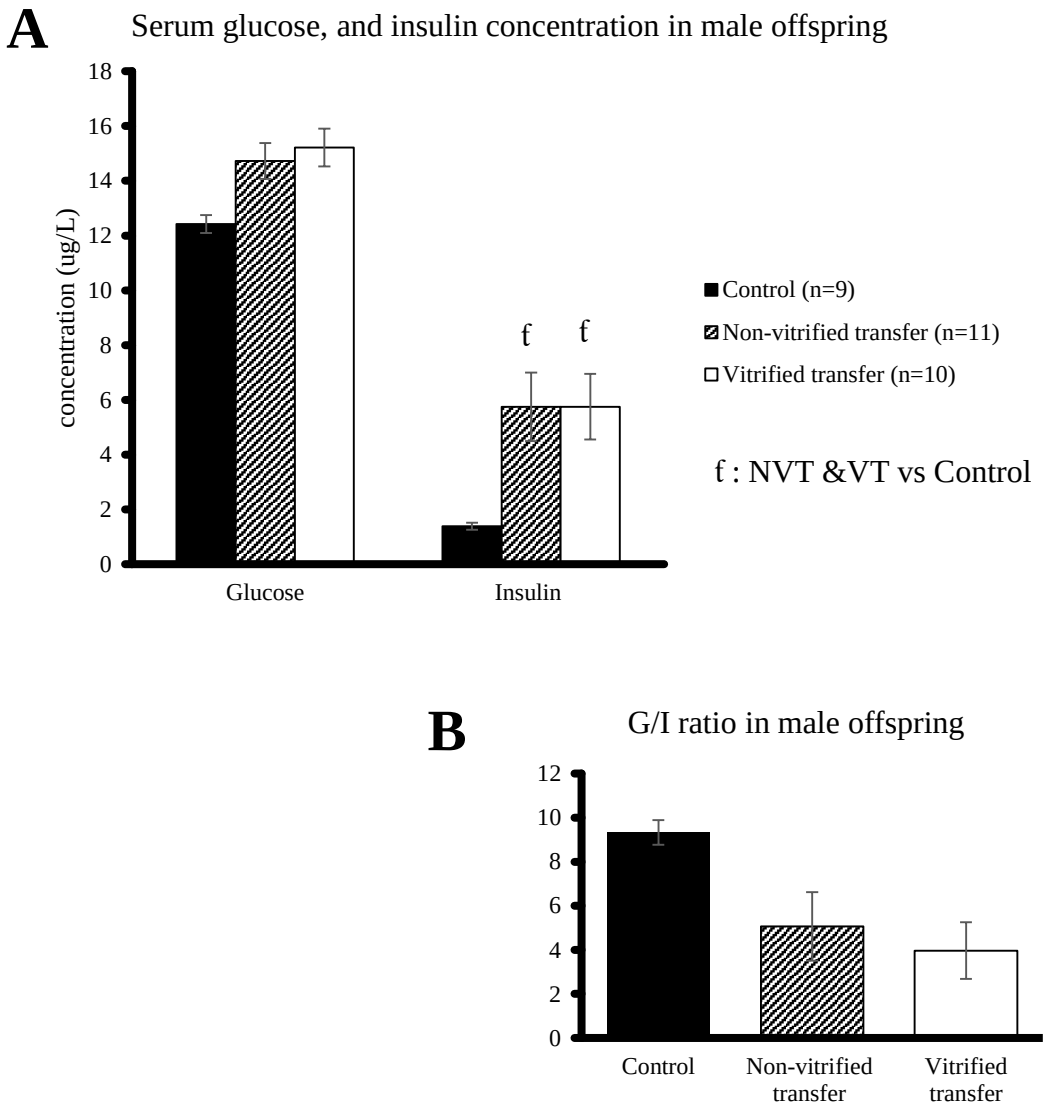


Figure 4.11: Effect on serum glucose, insulin and G:I ratio of control, non-vitrified transfer and vitrified transfer groups on male offspring. (A) Insulin level in non-vitrified and vitrified male showed a trend compared to control group. (B) No significance differences in G:I ratio between groups. f ($0.1 \leq f \geq 0.05$).

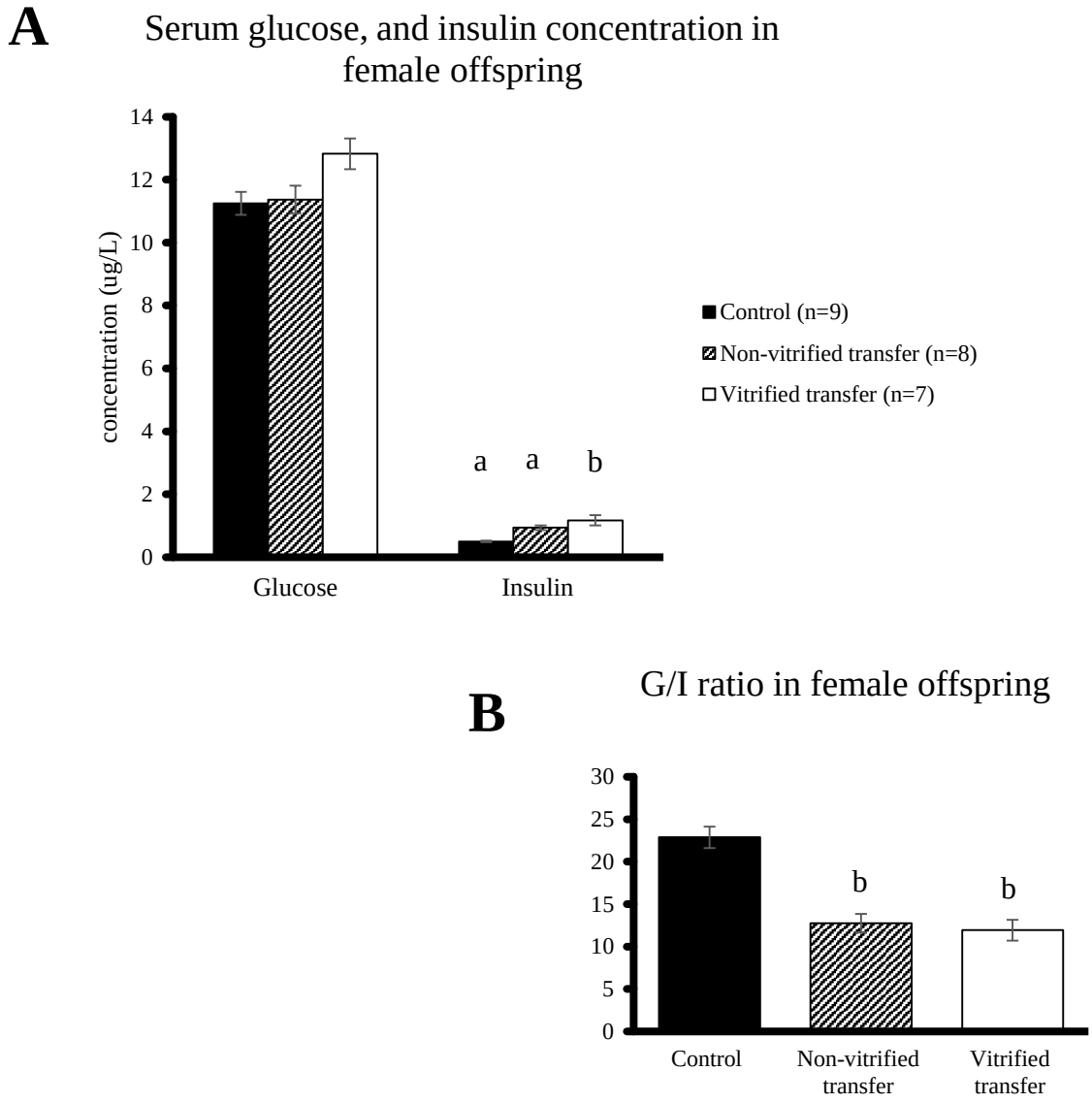


Figure 4.12: Effect on serum glucose, insulin and G:I ratio of control, non-vitrified transfer and vitrified transfer groups on female offspring. (A) Insulin level in VT female is highly significant (marked as b) compared to control group. (B) G:I ratio for NVT and VT group is significantly lower (marked as b) than control group.

4.4.7 Selected correlation analyses on postnatal dataset following undisturbed, non-vitrified transfer, and vitrified transfer.

The correlation between the weight at Week 3 (W3) with systolic blood pressure (SBP) from week 9, 15, 21 and LIFE were investigated (Table 4.3). There was a significant positive correlation observed between W3 and SBP LIFE in VT group only with female offspring. There were no significant correlations found in males. There is also no correlation detected for weight at week 9 (W9) with SBP9, weight at week 15 (W15) with SBP 15, and weight at week 21 (W21) with SBP 21.

Table 4.3: Correlation between offspring weight with systolic blood pressure (SBP) on specific week

Weeks	SBP (weeks)	r^2 value	Treatment
W3	9	NS*	All groups**
	15	NS	All groups
	21	NS	All groups
	LIFE	0.1164 (+ve)	VT – female ***
W9	9	NS	All groups
W15	15	NS	All groups
W21	21	NS	All groups
W27	LIFE	NS	All groups

* NS = no significant correlation
** all groups = undisturbed, non-vitrified transfer (NVT) and vitrified transfer (NVT) group
*** significant correlation within group ($p<0.05$)

In addition, correlations between W3 and W27 with homeostasis value from GTT were carried out (Table 4.4). The NVT male offspring showed a significant negative correlation between W3 and GTT30. The correlation between W3 and GTT AUC in males from VT male offspring showed a significant positive correlation. No significant correlations were found between W3 with glucose levels at any time point in GTT for the control group. There was also no correlations found between offspring weight at week 27 (W27) with glucose levels throughout GTT for all group (Table 4.4).

Table 4.4: Correlation between offspring weight with glucose homeostasis on specific week

Weeks	GTT (mins after injection)	r^2 value	Treatment
W3	0	NS*	All group**
	15	NS	All group
	30	0.2312 (-ve)	NVT – male ***
	60	NS	All group
	120	NS	All group
	AUC	0.0123 (+ve)	VT – male ***
W27	0, 15, 30, 60, 120, AUC	NS	All group

* NS = no significant correlation
** all groups = undisturbed, non-vitrified transfer (NVT) and vitrified transfer (NVT) group
*** significant correlation within group ($p<0.05$)

Plus, Table 4.5 shows significant positive correlations between W3 and W27, in male and female, from the VT group ($p\leq0.001$ and $p\leq0.01$ respectively). The same pattern was observed on correlation between SBP 9 and SBP LIFE in female from VT group ($p\leq0.01$).

Table 4.5: Correlation between different weeks on offspring weight and systolic blood pressure (SBP)

Weeks / SBP	Weeks / SBP	r^2 value	Treatment
W3	W27	0.1123 (+ve)	VT – male, female*
SBP9	SBP LIFE	0.1063 (+ve)	VT – female*

* significant correlation within group ($p<0.05$)

Table 4.6 shows the correlation between W3 and serum glucose, insulin, and glucose: insulin (G: I) ratio were observed from a subset of 7 to 11 samples from male and female offspring in each group (see Section 2.5.5). A significant positive correlation found between W3 and serum insulin concentration in VT female offspring (Fig 4.13). No correlation was observed between W3 and glucose value, but W3 and G: I ratio showed a significant negative correlation (Fig 4.14). No significant correlations were found in NVT and control groups.

Table 4.6: Correlation between offspring weight with serum glucose, insulin and G:I ratio (subset) on specific week

Weeks	Serum	r^2 value	Treatment
W3	Insulin	0.335 (+ve)	VT – female***
	Glucose	NS*	All group**
	G:I ratio	0.4191 (-ve)	VT – female

* NS = no significant correlation
** all groups = undisturbed, non-vitrified transfer (NVT) and vitrified transfer (NVT) group
*** significant correlation within group ($p<0.05$)

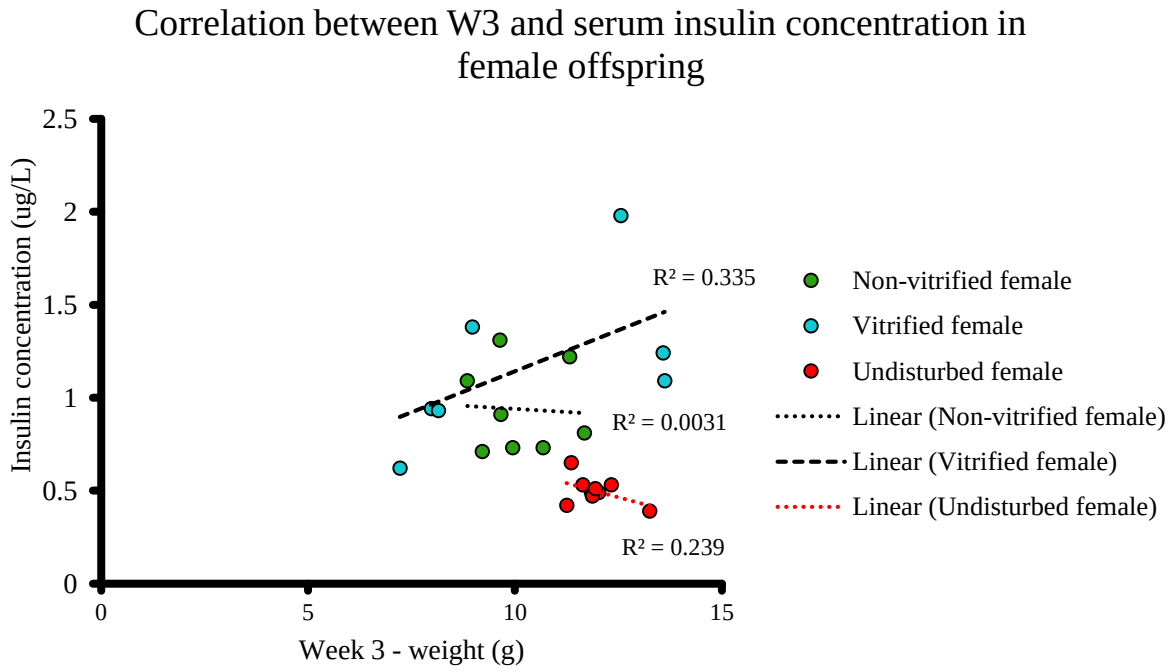


Figure 4.13 The correlation between W3 and serum insulin value in female offspring. Vitrified female shows significant positive correlation between weight at week 3 with insulin concentration (-----) with $r^2 = 0.335$.

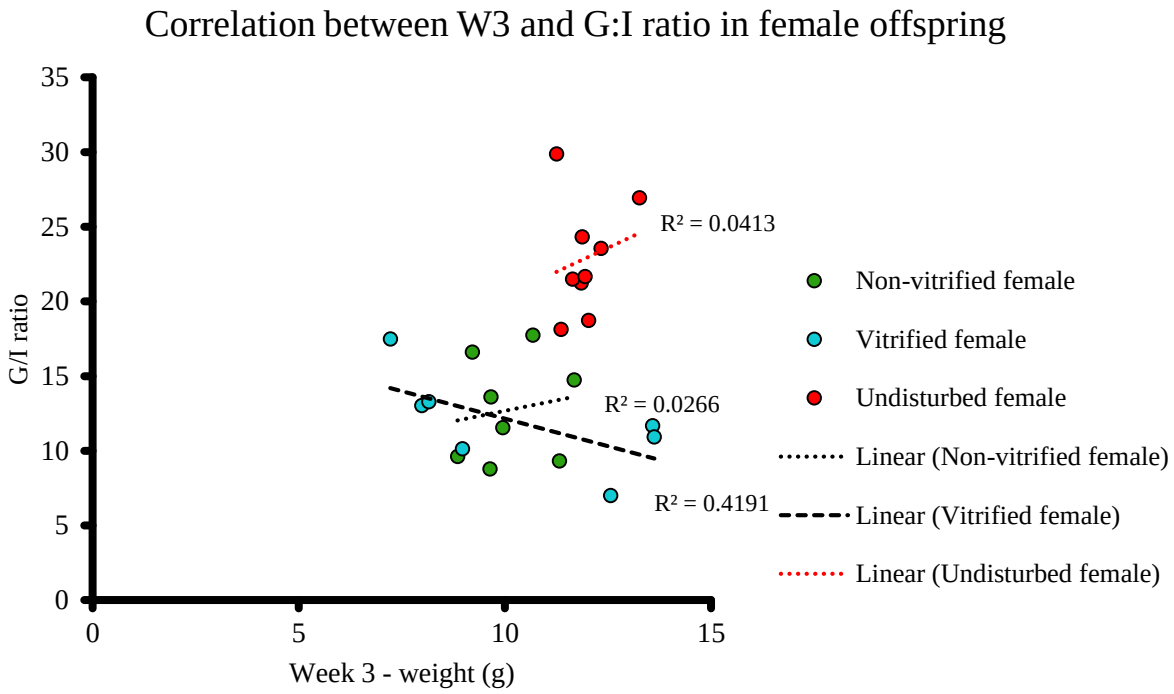


Figure 4.14: The correlation between W3 and G:I ratio in female offspring. Vitrified female shows significant negative correlation between weight at week 3 with insulin concentration (- - - - -) with $r^2 = 0.335$.

Correlation between serum insulin-glucose concentrations (from the subset experiment) in vitrified group was significantly positive in male offspring (Table 4.7). However, there were no significant correlations detected between serum glucose, serum insulin, and G: I ratio values with the fasting glucose value (GTT0) obtained from GTT at week 27.

Table 4.7: Correlation between offspring serum insulin and glucose homeostasis with serum glucose and G:I ratio (subset) on specific week

Weeks	Serum	r^2 value	Treatment
Insulin	Glucose	0.1221 (+ve)	VT – male***
	G:I ratio	NS*	All group**
GTT0	Insulin	NS	All group
(Fasting glucose)	Glucose	NS	All group
	G:I ratio	NS	All group

* NS = no significant correlation
** all groups = undisturbed, non-vitrified transfer (NVT) and vitrified transfer (NVT) group
*** significant correlation within group ($p<0.05$)

The correlation between W3 with GTT values were carried again within the subset. There are significant negative correlations observed between W3, GTT120, and GTT AUC in male offspring from VT group (Table 4.8).

Table 4.8: Correlation between offspring weight at week 3 (W3) with glucose homeostasis

Weeks	GTT (mins after injection)	r^2 value	Treatment
W3	0	NS*	All group**
	15	NS	All group
	30	NS	All group
	60	NS	All group
	120	0.4867 (-ve)	VT – male***
	AUC	0.7276 (+ve)	VT – male

* NS = no significant correlation
** all groups = undisturbed, non-vitrified transfer (NVT) and vitrified transfer (NVT) group
*** significant correlation within group ($p<0.05$)

4.5 Discussion

This study is the first report that evaluates the effect of vitrification on offspring cardiovascular and metabolite physiology using mouse models. We investigated the effect of vitrification from ET efficiency (implantation rate/no of embryos transferred), weekly weight, systolic blood pressure, glucose and insulin levels and organ: body weight ratio after culling at 27 weeks old in all groups. The study also correlates distinct parameters such as weight at week 3 (W3) with systolic blood pressure, glucose level and insulin level within group. Overall, there are significant differences between non-vitrified transfer (NVT) and vitrified transfer (VT) groups when compared to the controls in all parameters (explained below), however, no significant differences between the NVT and VT groups are found.

The first key point highlighted in the study is that the vitrification procedure does not affect the ET pregnancy rate, ET efficiency, litter size and male: female ratio. Even though, the combination of different embryo treatments (embryo flushing, culture, embryo transfer, freezing and vitrification) from VT would reduce the implantation and success rate for pregnancy in recipient mothers, the reduction is not statistically significant. Previously, Tamashiro et al. (2002) demonstrated that litter size is not solely responsible for effects on birth weight.

This was however, in contrast to other reports (Donjacour et al. 2014; Scott et al. 2010; Watkins et al. 2007). Control mice tend to have bigger litters than those in ART groups. This may be explained that restricted growth in larger litters can be attributed to overcrowding within the uterus, preventing the foetus from growing to maximal size, and high competition for maternal nutrients and blood supply (Foxcroft et al. 2009). This however does not affect our statistical finding because even though the controls were smaller in weight compared to the ART groups, our statistical analysis was independent of litter size. The male: female ratio presented in Table 4.2 shows that the VT group indicated a higher male: female ratio of 1.5.

The second finding in our study is vitrification does not affect the weight from weeks 3 to 27 in female and male offspring. This is similar with Scott et al. (2010) who reported no differences in ART derived offspring compared to controls in male offspring. Nevertheless, offspring from NVT and VT groups is significantly heavier than control offspring. The overall patterns of body weight in all groups are relatively similar. After weaning at week 3, offspring weights dramatically increase until week 6. The enhanced growth spurt was similar to that described by Scott et al. (2010) and Watkins et al. (2007).

In our study, the control groups for male and female were significantly smaller compared to NVT and VT (Week 3). This is similar to studies on IVF derived offspring (Scott et al. 2010). The study investigated the effect of in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI) and somatic cell nuclear transfer (SCNT) compared to *in vivo* offspring (undisturbed) on glucose clearance, body weight, and body composition of young adult mice until week 8. The report showed that in females, the IVF group were statistically significant at week 3 compared to all group. They suggest that studies have indicated that the practice of transferring multiple embryos to women who are attempting to conceive may be partially responsible for the low birth weights observed, because single embryo transfer is not associated with lower birth weights of IVF-conceived children (Ceelen et al. 2008).

Another important point to be emphasized is that male offspring from NVT was significantly heavier than control offspring starting from week 3 to 4, then week 7 until the end of the study. However, the VT group only showed significant differences at weeks 3, 5, 7 then persisted from 11 to 27. The slight differences that occurred in NVT group compared to VT group might be elucidated by a study from Shih et al. (2008). It has been suggested that the freezing procedure may be less harmful to embryo and offspring development than embryo transfer alone because cryopreservation may filter weak embryos, leaving all the competent

and healthy embryos to develop to blastocyst and thus, resulting in a normal development (Shih et al., 2008).

We found similarities with Scott et al. (2010) who reported heavier offspring derived from IVF and ICSI treatment from week 6 and the difference persisted until week 8. No data was obtained after week 8, because Scott et al. (2010) were looking at the effect of ART on mice only until week 8. The significance observed between the control, NVT, and VT groups was not due to different litter sizes, because our analysis was independent of litter size and maternal origin. So, the effect of the ART treatments used (embryo flushing, culture, and transfer) might affect the weight of NVT and VT offspring.

In human studies, there is limited yet conflicting evidence regarding postnatal growth, specifically the weight and height of children conceived from ART compared to naturally conceived children were recorded (Hyrapetian et al. 2014). Several studies looking at parental height, prematurity, and birth weight have shown that girls born after ART tend to be taller than naturally conceived children (Green et al. 2013; Miles et al. 2007). However, the small number of samples in these reports is a limitation for any firm conclusion.

Donjacour et al. (2014) proposed that alteration of the epigenome might be one possible mechanism that would affect adult growth and physiology. Imprinted genes are epigenetically regulated so their studies examined the expression of three candidate-imprinted genes (H19, Igf2, and Slc38a4) in multiple tissues of adult animals. However, no difference in expression between the offspring that was conceived from IVF and fresh embryos (similar as NVT group in our study) were found, indicating no widespread alterations in imprinted genes in these animals.

The third key point is vitrification does not affect systolic blood pressure. However, in males and females, NVT and VT groups were significantly higher at weeks 15, 21, and LIFE

compared to the controls. This shows that the effect of embryo transfer and/or vitrification might come at a later stage and can cause hypertension in mice. This finding is similar to that demonstrated by Watkins et al. (2007) on offspring from embryo culture and transfer (short culture) that showed significantly elevated systolic blood pressure in both females and males at week 15 compared with control offspring.

Watkins et al. (2007) also reported that at week 21 there were significant differences in male and female between *in vitro* culture and transfer offspring (prolonged culture) systolic blood pressure levels when compared to offspring that were derived from embryos that were directly transferred into recipient mothers (short culture). This study showed that culturing the embryos from two cells to blastocyst also resulted in high angiotensin converting enzyme (ACE) readings compared to the controls. ACE is found to convert ACE I to ACE II, which would lead to hypertension.

In addition, a study by Pires et al. (2004) claimed that obesity-related comorbidities present in young obese children potentially provide a platform for early adult cardiovascular disorders (Pires et al. 2014). Plus, studies on correlations between obesity and hypertension by Huang et al. (1998) showed that infant overweight would be a predictor to onset adult disease such as overweight.

Another novel finding in this study suggest that weaning in VT female offspring have significant positive correlation with systolic blood pressure (SBP LIFE). This suggest that overweight infant would be an ideal predictor for hypertension in adulthood. Similarly, Watkins et al. (2008) reported a positive significant correlation between weight after weaning at week 3 with SBP LIFE in female and male offspring from low protein diet embryos. The study suggested that early perinatal weight determined the phenotype at later stage, in this case, hypertension. In our study, VT group showed significant correlation, so we might suggest that

vitrification might have reprogrammed the embryos to adapt to the procedure but resulted in disease on later life.

Watkins suggested that the activation of the blastocyst response to stimulate conceptus growth rather than its appropriateness with respect to later nutrient availability that predisposes to adult disease. However, the disease phenotype appears exacerbated following Emb-LPD treatment in females because hypertension is accompanied by overweight.

A relevant study from Kumar et al. (2014), reported that cohort studies suggested that most prevalent types of cardiovascular disease (CVD) are associated with increased of absolute heart weight, even though the absolute weight of the human heart is influenced by various disease processes, in addition to body weight, gender, and age. Our finding might show that NVT and VT had an effect on absolute heart weight and thus make the mice prone to CVD, as in humans.

In addition, the absolute weight of both kidneys from the VT and NVT groups offspring were significantly higher from those in the control group. There were no reports suggesting that larger kidney contribute to cardiovascular disease but, we can hypothesize that the effect of embryo transfer and/or vitrification in offspring resulting in alteration in kidney sizing might lead to offspring more prone to cardiovascular health specifically hypertension.

The fifth key point to be highlighted is vitrification does not affect glucose levels in GTT, GTT AUCs and insulin levels. No differences was detected between NVT and VT groups.

In contrast, Donjacour et al. (2014) found significantly higher glucose levels at minutes 30, 60, and 120 after glucose injection from IVF derived male mouse from with Whitten's medium compared to fresh blastocysts, (similar to our NVT group). In addition, we also found that the NVT and VT groups have significantly higher GTT AUC in both male and female, while Donjacour et al. (2014) found none. Even though, Donjacour et al. (2014) is comparing IVF

derived offspring to fresh blastocyst, his results might also show the effect of perturbation during PC period (IVF), that might be similar to our work. They pointed out that ß-cell problem rather than a defect in peripheral tissues. This hypothesis was not disproven by the hyperinsulinemic-euglycemic clamp experiments in their study. Another cause for glucose intolerance in IVF derived offspring is pancreatic defect with altered insulin production. To support their point, insulinogenic index was reduced in both types of IVF mice at 19 wk of age compared to control. A decrease in the insulinogenic index actually proposed a reduced ability to secrete insulin in response to glucose, likely because of beta cell insensitivity to glucose (Donjacour et al. 2014).

Nevertheless, male and female offspring showed significantly higher values from fasting glucose, GTT30, GTT60, and GTT120 in the NVT and VT groups compared to the control groups. Similarly, Scott et al. (2010) suggested that females from a somatic cell nuclear transfer (SCNT) group has significantly higher glucose level after 60 and 120 minutes of glucose injection compared to other ART groups and the control group. The ART technique involved in SCNT might have some programming effect on the embryos, but the results also showed that changes can be seen as early as week 8 in females (Scott et al. 2010).

We decided to investigate whether there are any differences between NVT and VT groups if we were to extend the period after glucose injection to GTT180. Using FORECAST calculation (section 2.5.3), we were able to obtain GTT180 and GTT AUC readings however, there are no significant differences between NVT and VT groups. We also examined the duration of time needed for glucose to return to the fasting baseline levels after glucose injection. There were no differences between NVT and VT groups in male and female offspring.

The reason behind glucose intolerance in ART-derived offspring is still unknown. However, we would like to hypothesise that ART affects glucose tolerance by also altering the

liver weight in offspring. It was shown that liver weight in male and female offspring from the NVT and VT groups were significantly higher than those in the control group. Hwang et al. (2014) studied the effect of high-fat diet in mice and reported that glucose intolerant mice have a significantly heavier livers compared to normal mice. It was believed that the liver affected insulin response and efficiency.

The **sixth novel finding** that was observed was vitrification procedure plays a role in the correlation of infant weight and insulin resistant. It was statistically significant that overweight infant is a predictor for adult disease, specifically insulin resistance in VT female offspring. This however, is in contrast with another results that shows overweight infant have significantly negative correlation with glucose: insulin ratio.

In addition, we investigated the correlation between insulin and glucose concentration (from the subset experiment) in the VT group, and found significant positive correlation in male offspring. This suggests that male offspring from the VT group might be insulin resistant. This is due to the increased concentration of glucose observed even though insulin concentration is high. The values that showed no correlation with fasting glucose also elucidate that the level of fasting glucose is not correlated with insulin and glucose level in vitrified group.

This is similar in human studies that found 38% of the obese group individuals were considered insulin resistant (Pires et al. 2014). In the obese group, body mass index was directly correlated to the homeostasis model assessment-insulin resistance. Pires et al. (2014) had a lower prevalence of insulin resistance than published (50%-70%) in Chiarelli & Marcovecchio (2008). This discrepancy may be due to the different HOMA-IR cut off values used in the various reports to diagnose insulin resistance.

Interestingly, we found out that weight after weaning at week 3 is significantly correlated with glucose level from NVT and VT group in male offspring. Weight from VT group was

positively correlated with GTT AUC, but weight for NVT group was negatively correlated with glucose level after 30 minutes glucose injection. We are uncertain why overweight in infancy would resulted in a decrease glucose level. But, it is certain that the **seventh novel finding** is infant weight was also a predictor for impaired glucose tolerance in male offspring.

The same finding was confirm when we correlate weight after weaning with glucose level within the subset. It shows that overweight infant show a strong predictor for impaired glucose tolerance as significant correlations was observed at fasting glucose value, GTT120, and AUC in male offspring from vitrified group.

The next key point in this study is that vitrification *per se* did not alter organ weight and the organ: body weight ratio in offspring but ART did. The absolute weight of hearts in females was observed to be significantly higher in the ART group than the control group. Interestingly, the heart: body weight ratios in females for the ART groups were significantly smaller in comparison to the controls and the same pattern was found in male offspring. It seems that the ART derived offspring are bigger in weight but their organ are proportion to its body weight. The reason behind this is unknown.

The final novel finding shows that overweight infant tend to shows trajectory of overgrowth throughout life span ($p<0.01$) only for male offspring. However, female offspring derived from VT group tend to show trajectory of elevated high pressure throughout life span. Our results is similar to Watkins 2008 in male and female from control and low protein diet offspring.

Another **important finding** to be highlighted was the vitrification effect was gender specific. The infant weight proved to be an indicator for hypertension and insulin resistance only in female derived from vitrification procedure. However, in male offspring, weight is a significant predictor for impaired glucose tolerance. Similarly to Watkins et al. (2008), they

found that obesity affected female offspring preferentially. Although the basis for this gender distinction is not yet proven, possible causes may include gonadal hormone influences on appetite regulation (Watkins 2008). Interestingly, male and female blastocysts also show differences in gene expression (Bermejo-Alvarez et al. 2011). Therefore not surprising that any disturbance of physiologic signals because ART (in vitro culture, embryo transfer or vitrification) prior and during the preimplantation period will impacted in different health effects in the two sexes (Donjacour et al. 2014).

Other than that, behavioural differences seen in mice conceived using techniques associated with ART may be sexually dimorphic (Scott et al. 2010). A report from 22 claimed that mice generated through ICSI engaged in less exploration and locomotion and exhibited impaired performance on memory tasks, whereas no differences were seen in male mice generated by ICSI.

Another possible and important reason are sex steroids are known to affect adipose deposition and glucose clearance, with oestrogen enhancing insulin sensitivity and testosterone having the opposite effect (Geer & Shen 2009). This may account for the higher insulin levels observed in males and correlation significance. The present findings may then imply that there may be multiple sexually dimorphic effects of ART on energy homeostasis that require more future comprehensive work (Fernández-Gonzalez et al. 2004).

This data suggests a long-term effect of procedures associated with ARTs from NVT and VT groups such as the flushing, culturing, transferring, and vitrification of embryos on offspring growth, blood pressure, glucose homeostasis, and organ weight. We have found that the cardiovascular system is vulnerable to adverse programming during the critical peri-conception window. Phenotype alteration in animal models that resulted from treatments may be related to epidemiological studies concerning the developmental origin of adult chronic

disease in human. However, one must use caution when extrapolating findings in mice to the human condition. Prospective follow-up studies on gene expression are needed before any definitive conclusions are made. In conclusion, vitrification did not affect the postnatal health of offspring but we observed subtle significant for correlations VT group. However, ART seems to induce weight gain, elevated blood pressure, increased glucose and insulin levels, decreased G:I ratio and altered organ weight in male and female offspring.

Studies on correlations between obesity and hypertension (Huang et al. 1998), insulin resistant (Chiarelli & Marcovecchio 2008) and CVD (Pires et al. 2014) were reported. it is important to note that weight during at an early age such as after weaning would be a predictor to onset adult disease such as overweight, hypertension, impaired glucose tolerant, and insulin resistance.

Chapter 5 General discussion

5.1 General discussion

Vitrification is proved a vital and important tool in reproductive biology. However, the procedure may not be risk free and might have immediate effects on the embryo or long-term effects on offspring or babies derived from frozen embryos. The rationale behind the design of our study is to determine the differences between VT, NVT and control group from cell linage differentiation in blastocysts to adulthood.

In our study, we discussed that vitrification reduced the cell number of ICM and TE in blastocysts compared to the control. We suggested this may due to the delayed development or delayed in metabolic rate for vitrified embryos, thus, resulted in lower cell number. The question that arises from this result is whether the embryos would still be able to survive after embryo transfer as embryo development to full term might be compromised by vitrification.

Results obtained from chapter 4 than were able to answer that question, when we presented the mean number of offspring collected from each group. Although, the ICM and TE number were significantly reduced vitrified embryos can still give rise to foetuses. This is similar to (Iwasaki et al. 1990; Tao & Del Valle 2008) who reported bovine embryos with reduced cell numbers are able to give rise to foetuses.

The postnatal growth, systolic blood pressure at specific ages and glucose homeostasis as well as organ weight and organ: body weight ratio profiles for offspring from non-vitrified transfer (NVT) and vitrified-transfer (VT) group were all shown to be significantly different compared to the controls (undisturbed) group. This suggest that ART procedures such as embryo flushing, *in vitro* culture or ET play a role in determining the phenotype observed after ART (Nelissen et al. 2012; Watkins et al. 2007). However, it is worth noting that NVT and VT (ART) embryos were transferred into a different strain of foster mothers (MF1) compared to

control (C57Bl6) which may contribute to the significant differences found between control with ART groups.

Another important point is that specific phenotypes are gender specific. Females derived from vitrification confirmed that overweight infant proved to be a significant indicator for hypertension and insulin resistance in adulthood. However, in male offspring, weight is a significant predictor for impaired glucose tolerance. This is similar to Watkins et al. (2008).

In conclusion, the vitrification procedure *per se* does not affect the postnatal health of the offspring. Nonetheless, there was subtle differences observed within VT group that signified infant weight can indicate and determine adult diseases such as obesity, hypertension, impaired glucose tolerant and insulin resistance. This differences might also explain the significant reduction in cell number from vitrified embryos. We are however, still not sure of the mechanism involved. Future work to understand the mechanism behind vitrification procedure is vital.

5.2 Strength, limitations and future works

5.2.1 Strength of the study

One strength of this study is the novelty of the research. To our knowledge, this is the first study that reports on the effect of vitrification on cell lineages at the blastocyst stage and adulthood health using a mouse model. This study was designed to compare the effect of vitrification *per se* (NVT vs VT) and the effect of other ART procedures such as ET, embryo culture and collection of embryos (control vs NVT & VT). Furthermore, we did not superovulate our females to ensure that any effect on the postnatal health came solely from vitrification or other ET procedures excluding superovulation.

Data obtained in this study consist of good numbers of embryos for Chapter 3 and number of samples for both genders. The analysis of postnatal health was thorough and comprehensive.

Repeated measurements on the same animal for weekly weight, systolic blood pressure taken on 3 different weeks, glucose homeostasis test and organ allometry and serum analysis showed different health parameters not only CVD. The hierarchical data analysis took confounders such as litter size and (foster) mother influences into account, factors which have been shown to influence results (Donjacour et al. 2014; Scott et al. 2010). To confirm the data we obtained from the GTT, a subset of serum samples collected after culling was analysed for insulin and glucose levels. Collectively, the main effects were seen as a result of embryo transfer compared to controls and we found not different between NVT and VT groups. This suggests that vitrification had no effect on insulin and glucose levels.

The second point was the study was able to correlate different physiological parameters (SBP, weight, glucose/insulin homeostasis) to each other because we examined the same animals or offspring. With these analyses, we concluded that offspring from VT group show subtle differences in determining or predicting adult disease from infant overweight. Only the VT group had significant correlation between infant overweight and SBP, GTT and insulin value. None were observed from control group.

Overall, we strongly believe that our study design is the first study that discriminates between ART factors (such as ET) and looks into the effect of vitrification per se on postnatal health. This study might lead to new interest in discovering the mechanism that lies behind these significant postnatal differences.

5.2.2 Limitations and future works

However, there are limitations and drawbacks in this study. The results showed that vitrification significantly reduced ICM, TE and total cell number. It is concluded that the vitrification procedure affects the development of the vitrified embryos; however, mechanisms are still unknown. A more detailed analysis of the development rate, the number of cell lineage

(ICM and TE) at different time points, the proliferation and apoptosis rate by nuclear staining, and immunostaining for DNA visualisation (for spindle morphology) on vitrified and non-vitrified embryos are essential to understand better the effects of vitrification in the future.

For example, immunostaining to visualise DNA within vitrified blastocysts might clarify the morphology of DNA, spindle and tubulin in depth. Based on Chatzimeletiou et al. (2012), blastocyst immunostaining can be performed by using a primary rat monoclonal antibody specific for a-tubulin to visualise microtubules, combined with mouse monoclonal antibody specific for gamma-tubulin to identify spindle poles, acetylated tubulin to visualise spindle poles and mid bodies along with DAPI to visualise DNA. Blastocysts are then examined using the fluorescence and confocal microscope. An important criterion is classification of spindle abnormalities. According to Chatzimeletiou et al. (2012), normal spindle is classified as a spindle with astral shaped or fusiform poles with chromosomes aligned at the equator. Meanwhile, a spindle with one or two poorly defined or apparently absent poles, generally with misaligned chromosomes is classified as an abnormal spindle. Spindles with more than two clearly defined astral poles and having the 'Y' or 'X' shaped arrangement of DAPI- labelled chromosomes are classified as multipolar. Lagging chromosome or chromosomal loss is defined by misalignment of chromosomes with the other chromosomes on the spindle (Chatzimeletiou et al. 2005a). Such analysis would confirm whether vitrification leads to cell number reduction through spindle disintegration or congression failure.

Another additional experiment which would strengthen the results in this study is maintaining the procedure from our study but store the embryos in LN_2 for a longer period prior to differential labelling. If the results shows similarity with our study, it would confirm that vitrification for any length of time would affect the number of cells similarly. A study from (Sanchez-Osorio et al. 2010), concluded that no significant difference was detected between vitrified porcine embryos for different duration of time in LN_2.

We would suggest to also consider another crucial point which is to determine the different duration of time for LN_2 storage for the future study. (Sanchez-Osorio et al. 2010) considered the *in vitro* viability post-warming for 1 to 9 days, 10 to 30 days, 31 to 90 days and 1 to 3 years of LN_2 storage. Thus, it might be best to have groups on storage of vitrified embryos in LN_2 for 1 week (7 days), 1 month (30 days), 6 month (180 days) and 1 year (365 days) prior to differential labelling in the future.

In addition, it would be worth that birth weight is considered as one of the main parameters to study in the future. In our earlier experiments (Chapter 4), the birth weights of the pups were taken but we noticed that the mothers ate the pups afterwards. The weighing at birth was discontinued, continuing monitoring the pups in their cage until they were ready for weaning. Thus, there is no data to indicate whether vitrification would affect birth weight in offspring. Most studies use birth weight as proxy to predict the onset of adult disease (Ceelen, Mirjam M van Weissenbruch, et al. 2008; Wadhwa et al. 2009). So, having another set of data on birthweight would be an insightful perspective whether the birthweight would also be a significant indicator for future disease in VT group, thus strengthening our hypothesis.

In addition, our study only comprises half of the number of samples for females in NVT and VT compared to control groups. The ratio between male and female in VT group is 3:2, thus this resulted in a low yet fair number of samples in female offspring for comparison. The limitations in obtaining offspring from the VT group were described in detail in Section 2.4.3. This limitation however did not affect our statistical analysis.

Another important modification for future work is the serum sample analysis from offspring. Even though the analyses consisted of 4 to 5 litters each per group, it would be best in future to have the serum analysis from the entire offspring in all groups. From the correlation analyses, we saw a positive significant correlation between weights at week 3 with the level of

insulin in serum for the VT group but not in the control and NVT groups. This may illustrate that vitrification emphasises that overweight mice are prone to high insulin levels, thus being insulin resistant. .

It could also be worth to observe the same postnatal parameters in our study for a longer period (more than 27 weeks of age). In a study by Donjacour et al. (2014), the HOMA-IR value was observed between mice derived from IVF and control groups at two different times; weeks 19 and 40. Even though no significant differences were detected between groups, the values between weeks 19 were significantly higher compared to week 40 from both groups. This proves that a postnatal study on offspring up to 40 weeks might show that aged offspring derived from vitrified embryos gave a different result.

Aside from the comprehensive postnatal parameters that were observed in this experiment, investigating the transgenerational effect, to see whether these phenotypes (normal or altered) could be transferred to the progeny may also be crucial. A study in 2014, confirmed that vitrification and transfer group (similar to our VT group) had a significant increase in litter size (LS), number born alive (BA), in F1 and F2 female offspring compared to the control (similar to our control group) (Lavara et al. 2014). In the future, data on birthweight, LS, BA and ratio of male and female ratio may give additional input on the long-term and transgenerational effect of vitrification.

5.2.3 Future work : key regulators of blood pressure and glucose homeostasis.

We presented a thorough postnatal study on the effect of vitrification and transfer using a mouse model by comparing the cardiovascular and metabolite profile such as growth, blood pressure reading, glucose homeostasis and organ weights and ratio between groups. Future work to support our result is necessary to further understand the mechanistic basis of the subtle effects of vitrification on embryos into adulthood. For example, ACE and PEPCK are useful

markers for cardiovascular and metabolic health in in vivo models of embryo environmental programming (Kwong et al. 2007).

Watkins et al. (2007) reported that activity of enzymatic regulators of cardiovascular and metabolic physiology such as ACE and PEPCK were significantly elevated at week 27 from female offspring derived from 2-cell embryos and cultured to blastocysts before ET. Such perturbation could contribute to the significant elevation of systolic blood pressure reading (SBP) in female offspring. 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 (Skeggs et al. 1956).

Hepatic phosphoenolpyruvate carboxykinase (PEPCK) is recognized as a critical regulator of gluconeogenesis and the enzyme can be overexpressed in insulin resistance and diabetes (Magnusson et al. 1992; Kwong et al. 2007). Moreover, mice overexpressing PEPCK develop insulin resistance and diabetes (Valera et al. 1994). PEPCK would be a useful marker to correlate with our glucose homeostasis and insulin resistance data. In our study, glucose homeostasis was perturved in NVT and VT at GTT30 in male offspring. So, we believe that the enzymatic regulator PEPCK could contribute to the effect of vitrification on insulin resistance and diabetes.

Chapter 6 References

Abdelhafez, F.F., Desai, N., Abou-Setta, A.M., Falcone, T. & Goldfarb, J., 2010. Slow freezing, vitrification and ultra-rapid freezing of human embryos: A systematic review and meta-analysis. *Reproductive BioMedicine Online*, 20, pp.209–222.

Allen, C. & Reardon, W., 2005. Assisted reproduction technology and defects of genomic imprinting. *BJOG*, 112(12), pp.1589–1594. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16305560.

Aman, R.R. & Parks, J.E., 1994. Effects of cooling and rewarming on the meiotic spindle and chromosomes of in vitro-matured bovine oocytes. *Biology of reproduction*, 50(1), pp.103–110.

Amor, D.J. & Halliday, J., 2008. A review of known imprinting syndromes and their association with assisted reproduction technologies. *Human Reproduction*, 23(12), pp.2826–2834.

Anchordoguy, T.J., Cecchini, C.A., Crowe, J.H. & Crowe, L.M., 1991. Insights into the cryoprotective mechanism of dimethyl sulfoxide for phospholipid bilayers. *Cryobiology*, 28, pp.467–473.

Aoki, F., Worrad, D.M. & Schultz, R.M., 1997. Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo. *Developmental biology*, 181, pp.296–307.

Arav, A., 2014. Cryopreservation of oocytes and embryos. *Theriogenology*, 81(1), pp.96–102. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24274414.

Arav, A., Shehu, D. & Mattioli, M., 1993. Osmotic and cytotoxic study of vitrification of immature bovine oocytes. *Journal of reproduction and fertility*, 99, pp.353–358.

Asada, M., Ishibashi, S., Ikumi, S. & Fukui, Y., 2002. Effect of polyvinyl alcohol (PVA) concentration during vitrification of in vitro matured bovine oocytes. *Theriogenology*, 58, pp.1199–1208.

Auroux, M., Cerutti, I., Ducot, B. & Loeuillet, A., 2004. Is embryo-cryopreservation really neutral? A new long-term effect of embryo freezing in mice: protection of adults from induced cancer according to strain and sex. *Reproductive toxicology (Elmsford, N.Y.)*, 18(6), pp.813–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15279879 [Accessed September 25, 2014].

Bachvarova, R., 1985. Gene expression during oogenesis and oocyte development in mammals. *Developmental biology (New York, N.Y. : 1985)*, 1, pp.453–524.

Bagis, H. & Mercan, H.., 2005. Effect of Three Different Cryoprotectant Solutions in Solid Surface Vitrification (SSV) Techniques on the Development Rate of Vitrified Pronuclear-Stage Mouse Embryos. *Turk J Vet Anim Sci*, 29, p.7.

Balaban, B., Urman, B., Ata, B., Isiklar, A., Larman, M.G., Hamilton, R. & Gardner, D.K., 2008. A randomized controlled study of human Day 3 embryo cryopreservation by slow freezing or vitrification: Vitrification is associated with higher survival, metabolism and blastocyst formation. In *Human Reproduction*. pp. 1976–1982.

Barker, D.J.. & Osmond, C., 1986. Nutrition, and ischaemic heart disease in England and Wales infant mortality and childhood. *The Lancet*, p.5.

Barker, D.J.., Winter, P.. & Osmond, C., 1989. Weight in infancy and death from ischaemic

heart disease. *Lancet*, p.1335.

Barker, D.J.P., 1997. Maternal nutrition, fetal nutrition, and disease in later life. *Nutrition*, 13(9), pp.807–813.

Basatemur, E., Shevlin, M. & Sutcliffe, A., 2010. Growth of children conceived by IVF and ICSI up to 12years of age. *Reprod Biomed Online*, 20(1), pp.144–149. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20159000.

Basille, .J, L. & Eugune, R., H., 1938. Revival of spermatozoa vitrified in liquid air. *Proc Soc Exp Biol*, 39, p.2.

Belva, F., Henriet, S., Van den Abbeel, E., Camus, M., Devroey, P., Van der Elst, J., Liebaers, I., Haentjens, P. & Bonduelle, M., 2008. Neonatal outcome of 937 children born after transfer of cryopreserved embryos obtained by ICSI and IVF and comparison with outcome data of fresh ICSI and IVF cycles. *Hum Reprod*, 23(10), pp.2227–2238. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18628260.

Berger, S.L., 2007. The complex language of chromatin regulation during transcription. *Nature*, 447(7143), pp.407–412.

Bermejo-Alvarez, P., Rizos, D., Lonergan, P. & Gutierrez-Adan, A., 2011. Transcriptional sexual dimorphism during preimplantation embryo development and its consequences for developmental competence and adult health and disease. *Reproduction*, 141(5), pp.563–570.

Boivin, J., Bunting, L., Collins, J.A. & Nygren, K.G., 2007. International estimates of infertility prevalence and treatment-seeking: Potential need and demand for infertility medical care. *Human Reproduction*, 22, pp.1506–1512.

Bonduelle, M., Wennerholm, U.B., Loft, A., Tarlatzis, B.C., Peters, C., Henriet, S., Mau, C., Victorin-Cederquist, A., Van Steirteghem, A., Balaska, A., Emberson, J.R. & Sutcliffe, A.G., 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, pp.413–419.

Boonkusol, D., Gal, A.B., Bodo, S., Gorhony, B., Kitiyanant, Y. & Dinnyes, A., 2006. Gene expression profiles and in vitro development following vitrification of pronuclear and 8-cell stage mouse embryos. *Molecular Reproduction and Development*, 73(6), pp.700–708. Available at: <Go to ISI>://WOS:000237128200005.

Bowdin, S., Allen, C., Kirby, G., Brueton, L., Afnan, M., Barratt, C., Kirkman-Brown, J., Harrison, R., Maher, E.R. & Reardon, W., 2007. A survey of assisted reproductive technology births and imprinting disorders. *Hum Reprod*, 22(12), pp.3237–3240. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17921133.

Braude, P., Bolton, V. & Moore, S., 1988. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature*, 332, pp.459–461.

Cederblad, M., Friberg, B., Ploman, F., Sjöberg, N.O., Stjernqvist, K. & Zackrisson, E., 1996. Intelligence and behaviour in children born after in-vitro fertilization treatment. *Human reproduction (Oxford, England)*, 11(9), pp.2052–2057.

Ceelen, M., van Weissenbruch, M.M., Vermeiden, J.P., van Leeuwen, F.E. & Delemarre-van de Waal, H.A., 2008. Growth and development of children born after in vitro fertilization. *Fertil Steril*, 90, pp.1662–1673.

Ceelen, M., van Weissenbruch, M.M., Vermeiden, J.P.W., van Leeuwen, F.E. & Delemarre-van de Waal, H. a, 2008. Cardiometabolic differences in children born after in vitro

fertilization: follow-up study. *The Journal of clinical endocrinology and metabolism*, 93(5), pp.1682–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18285409 [Accessed October 6, 2014].

Chapman, J., 2007. Science of cryonics. *Cryonics*, 3, p.28.

Chatzimeletiou, K., Morrison, E.E., Panagiotidis, Y., Vanderzwalmen, P., Prapas, N., Prapas, Y., Tarlatzis, B.C. & Handyside, A.H., 2012. Cytoskeletal analysis of human blastocysts by confocal laser scanning microscopy following vitrification. *Hum Reprod*, 27(1), pp.106–113. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22028018.

Chen, C., 1986. Pregnancy after human oocyte cryopreservation. *Lancet*, 1, pp.884–886.

Chen, S.U., Lien, Y.R., Chao, K.H., Ho, H.N., Yang, Y.S. & Lee, T.Y., 2003. Effects of cryopreservation on meiotic spindles of oocytes and its dynamics after thawing: Clinical implications in oocyte freezing - A review article. In *Molecular and Cellular Endocrinology*. pp. 101–107.

Chian, R.-C., Wang, Y. & Li, Y.-R., 2014. Oocyte vitrification: advances, progress and future goals. *J Assist Reprod Genet*, 31(4), pp.411–420. Available at: <Go to ISI>://WOS:000333703100004.

Chiarelli, F. & Marcovecchio, M.L., 2008. Insulin resistance and obesity in childhood. *European Journal of Endocrinology*, 159(SUPPL. 1), pp.67–74.

Cockburn, K. & Rossant, J., 2010. Review series Making the blastocyst : lessons from the mouse. *The Journal of clinical investigation*, 120(4), pp.995–1003.

Coutinho, A.R.S., Mendes, C.M., Caetano, H.V.A., Nascimento, A.B. & Oliveira, V.P., 2007. Morphological Changes in Mouse Embryos Cryopreserved by Different Techniques. *Microscopy Research and Technique*, 301(December 2006), pp.296–301.

Cox, G.F., Bürger, J., Lip, V., Mau, U.A., Sperling, K., Wu, B.-L. & Horsthemke, B., 2002. Intracytoplasmic sperm injection may increase the risk of imprinting defects. *American journal of human genetics*, 71, pp.162–164.

Critser, J.K., Y., A. & Gunasena K.T., 1997. The cryobiology of mammalian oocytes. In *Reprod Tissue Banking*. pp. 329–357.

Crowe, J.H., Crowe, L.M., Carpenter, J.F., Rudolph, A.S., Wistrom, C.A., Spargo, B.J. & Anchordoguy, T.J., 1988. Interactions of sugars with membranes. *Biochimica et biophysica acta*, 947, pp.367–384.

Desai, N., Blackmon, H., Szeptycki, J. & Goldfarb, J., 2007. Cryoloop vitrification of human day 3 cleavage-stage embryos: post-vitrification development, pregnancy outcomes and live births. *Reproductive biomedicine online*, 14(2), pp.208–213.

Diez, C., Duque, P., Gómez, E., Hidalgo, C.O., Tamargo, C., Rodríguez, A., Fernández, L., De La Varga, S., Fernández, A., Facal, N. & Carbajo, M., 2005. Bovine oocyte vitrification before or after meiotic arrest: Effects on ultrastructure and developmental ability. *Theriogenology*, 64, pp.317–333.

Donjacour, A., Liu, X., Lin, W., Simbulan, R. & Rinaudo, P.F., 2014. In vitro fertilization affects growth and glucose metabolism in a sex-specific manner in an outbred mouse model. *Biology of reproduction*, 90(4), p.80. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24621920 [Accessed January 23, 2015].

Doornbos, M.E., Maas, S.M., McDonnell, J., Vermeiden, J.P.W. & Hennekam, R.C.M., 2007. Infertility, assisted reproduction technologies and imprinting disturbances: A Dutch study. *Human Reproduction*, 22(9), pp.2476–2480.

Duah, O. a, Monney, K. a, Hambly, C., Król, E. & Speakman, J.R., 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(Pt 12), pp.2339–48. Available at: http://jeb.biologists.org/content/216/12/2339.short.

Ducibella, T. & Anderson, E., 1975. Cell shape and membrane changes in the eight-cell mouse embryo: prerequisites for morphogenesis of the blastocyst. *Developmental biology*, 47(1), pp.45–58.

Dulioust, E., Toyama, K., Busnel, M.C., Moutier, R., Carlier, M., Marchaland, C., Ducot, B., Roubertoux, P. & Auroux, M., 1995. Long-term effects of embryo freezing in mice. *Proceedings of the National Academy of Sciences of the United States of America*, 92(2), pp.589–93. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=42787&tool=pmcentrez&rendertype=abstract.

Eckert, J.J. & Fleming, T.P., 2008. Tight junction biogenesis during early development. *Biochimica et biophysica acta*, 1778(3), pp.717–28. Available at: http://www.sciencedirect.com/science/article/pii/S0005273607003562 [Accessed September 8, 2014].

Edgar, D.H., Archer, J., Gook, D.A., Jericho, H., Wilton, L. & Bourne, H., 2004. Survival and developmental potential of stored human early cleavage stage embryos. In *European Journal of Obstetrics Gynecology and Reproductive Biology*.

Van der Elst, J., Amerijckx, Y. & Van Steirteghem, A., 1998. Ultra-rapid freezing of mouse oocytes lowers the cell number in the inner cell mass of 5 day old in-vitro cultured blastocysts. *Human Reproduction*, 13(6), pp.1595–1599.

Van der Elst, J., Nerinckx, S. & Van Steirteghem, A.C., 1993. Association of ultrarapid freezing of mouse oocytes with increased polyploidy at the pronucleate stage, reduced cell numbers in blastocysts and impaired fetal development. *Journal of reproduction and fertility*, 99, pp.25–32.

Fabian, D., Gjørret, J.O., Berthelot, F., Martinat-Botté, F. & Maddox-Hyttel, P., 2005. Ultrastructure and cell death of in vivo derived and vitrified porcine blastocysts. *Molecular Reproduction and Development*, 70, pp.155–165.

Fan, Z., Meng, Q., Bunch, T., White, K. & Wang, Z., 2015. Effective cryopreservation of golden Syrian hamster embryos by open pulled straw vitrification. *Laboratory animals*.

Fauque, P., Mondon, F., Letourneur, F., Ripoche, M.A., Journot, L., Barbaux, S., Dandolo, L., Patrat, C., Wolf, J.P., Jouannet, P., Jammes, H. & Vaiman, D., 2010. In vitro fertilization and embryo culture strongly impact the placental transcriptome in the mouse model. *PLoS ONE*, 5.

Fernández-Gonzalez, R., Moreira, P., Bilbao, A., Jiménez, A., Pérez-Crespo, M., Ramírez, M.A., Rodríguez De Fonseca, F., Pintado, B. & 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(16), pp.5880–5885.

Fleming, T.P., Lucas, E.S., Watkins, A.J. & Eckert, J.J., 2011. Adaptive responses of the embryo to maternal diet and consequences for post-implantation development. *Reprod Fertil Dev*, 24(1), pp.35–44. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22394716.

Fleming, T.P., McConnel, J., Johnson, M.H. & Stevenson, B.R., 1989. Development of tight

junctions de novo in the mouse early embryo: Control of assembly of the tight junction-specific protein, ZO-1. *Journal of Cell Biology*, 108, pp.1407–1418.

Foxcroft, G.R., Dixon, W.T., Dyck, M.K., Novak, S., Harding, J.C.S. & Almeida, F.C.R.L., 2009. Prenatal programming of postnatal development in the pig. *Society of Reproduction and Fertility supplement*, 66(1983), pp.213–231.

Geer, E.B. & Shen, W., 2009. Gender differences in insulin resistance, body composition, and energy balance. *Gender Medicine*, 6(SUPPL. 1), pp.60–75.

Ghetler, Y., Yavin, S., Shalgi, R. & Arav, A., 2005. The effect of chilling on membrane lipid phase transition in human oocytes and zygotes. *Hum Reprod*, 20(12), pp.3385–3389.

Grace, K.S. & Sinclair, K.D., 2009. Assisted reproductive technology, epigenetics, and long-term health: A developmental time bomb still ticking. *Seminars in Reproductive Medicine*, 27(5), pp.409–416.

Green, M.P., Mouat, F., Miles, H.L., Hopkins, S.A., Derraik, J.G.B., Hofman, P.L., Peek, J.C. & Cutfield, W.S., 2013. Phenotypic differences in children conceived from fresh and thawed embryos in in vitro fertilization compared with naturally conceived children. *Fertility and Sterility*, 99(7), pp.1898–1904.

Guerif, F., Bidault, R., Cadoret, V., Couet, M., Lansac, J. & Royere, D., 2002. Parameters guiding selection of best embryos for transfer after cryopreservation : a reappraisal. , 17(5), pp.1321–1326.

Gurtovenko, A. a & Anwar, J., 2007. Modulating the structure and properties of cell membranes: the molecular mechanism of action of dimethyl sulfoxide. *The journal of physical chemistry. B*, 111(35), pp.10453–10460.

Hamatani, T., Carter, M.G., Sharov, A.A. & Ko, M.S.H., 2004. Dynamics of global gene expression changes during mouse preimplantation development. *Developmental Cell*, 6, pp.117–131.

Handyside, A.H., 1980. Distribution of antibody- and lectin-binding sites on dissociated blastomeres from mouse morulae: evidence for polarization at compaction. *Journal of embryology and experimental morphology*, 60, pp.99–116.

Handyside, A.H. & Hunter, S., 1984. A rapid procedure for visualizing the inner cell mass and trophectoderm nuclei of mouse blastocysts insitu using polynucleotide-specific fluorochromes FLUOROCHROMES. *Journal of Experimental Zoology*, 231(3), pp.429–434. Available at: <Go to ISI>://WOS:A1984TJ37000016.

Hansen, M., Colvin, L., Petterson, B., Kurinczuk, J.J., De Klerk, N. & Bower, C., 2009. Twins born following assisted reproductive technology: Perinatal outcome and admission to hospital. *Human Reproduction*, 24, pp.2321–2331.

Hansen, M., Kurinczuk, J.J., Bower, C. & Webb, S., 2002. The risk of major birth defects after intracytoplasmic sperm injection and in vitro fertilization. *The New England journal of medicine*, 346, pp.725–730.

Harton, G.L., Magli, M.C., Lundin, K., Montag, M., Lemmen, J. & Harper, J.C., 2011. ESHRE PGD Consortium/Embryology Special Interest Groupbest practice guidelines for polar body and embryo biopsy for preimplantation genetic diagnosis/screening (PGD/PGS). *Human Reproduction*, 26(1), pp.41–46.

Hartshorne, G.M., Elder, K., Crow, J., Dyson, H. & Edwards, R.G., 1991. The influence of in-vitro development upon post-thaw survival and implantation of cryopreserved human blastocysts. *Human reproduction (Oxford, England)*, 6(1), pp.136–141.

Hasegawa, A., Mochida, K., Matoba, S., Yonezawa, K., Ohta, A., Watanabe, G., Taya, K. & Ogura, A., 2012. Efficient production of offspring from Japanese wild-derived strains of mice (Mus musculus molossinus) by improved assisted reproductive technologies. *Biol Reprod*, 86(5), pp.1–7,167. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22337332.

Hiraoka, K., Hiraoka, K., Kinutani, M. & Kinutani, K., 2004. Blastocoele collapse by micropipetting prior to vitrification gives excellent survival and pregnancy outcomes for human day 5 and 6 expanded blastocysts. *Human Reproduction*, 19(12), pp.2884–2888.

Horsthemke, B. & Ludwig, M., 2005. Assisted reproduction: The epigenetic perspective. *Human Reproduction Update*, 11(5), pp.473–482.

Houshdaran, S., Cortessis, V.K., Siegmund, K., Yang, A., Laird, P.W. & Sokol, R.Z., 2007. Widespread epigenetic abnormalities suggest a broad DNA methylation erasure defect in abnormal human sperm. *PLoS ONE*, 2(12).

Huang, Z., Willet, W.C., Manson, J.E., Rosner, B., Stampfer, M.J., Speizer, F.E. & Colditz, G.A., 1998. Body Weight,Weight Chnage, And Risk for Hypertension in Women. *JAMA: The Journal of the American Medical Association*, 128(11), pp.81–88.

Huffman, S.R., Pak, Y. & Rivera, R.M., 2015. Superovulation induces alterations in the epigenome of zygotes, and results in differences in gene expression at the blastocyst stage in mice. *Molecular Reproduction and Development*, 82(3), pp.207–217. Available at: http://doi.wiley.com/10.1002/mrd.22463.

Hwang, J., Park, J., Nam, M., Cho, H. & Han, I., 2014. Glucosamine Enhances Body Weight Gain and Reduces Insulin Response in Mice Fed Chow Diet but Mitigates Obesity, Insulin Resistance and Impaired Glucose Tolerance in Mice High-Fat Diet. *Metabolism*, pp.1–12. Available at: http://dx.doi.org/10.1016/j.metabol.2014.11.005.

Hyrapetian, M., Loucaides, E.M. & Sutcliffe, A.G., 2014. Health and disease in children born after assistive reproductive therapies (ART). *Journal of Reproductive Immunology*. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0165037814000667 [Accessed October 6, 2014].

Iliadou, A.N., Janson, P.C.J. & Cnattingius, S., 2011. Epigenetics and assisted reproductive technology. *Journal of Internal Medicine*, 270, pp.414–420.

Illmensee, K., Kaskar, K. & Zavos, P.M., 2005. Efficient blastomere biopsy for mouse embryo splitting for future applications in human assisted reproduction. *Reproductive biomedicine online*, 11(6), pp.716–725. Available at: http://dx.doi.org/10.1016/S1472-6483(10)61690-2.

Iwasaki, S., Yoshiba, N., Ushijima, H., Watanabe, S. & Nakahara, T., 1990. Morphology and proportion of inner cell mass of bovine blastocysts fertilized in vitro and in vivo. *Journal of reproduction and fertility*, 90, pp.279–284.

Jackson, R.A., Gibson, K.A., Wu, Y.W. & Croughan, M.S., 2004. Perinatal outcomes in singletons following in vitro fertilization: a meta-analysis. *Obstetrics and gynecology*, 103(3), pp.551–563.

Jin, B., Higashiyama, R. -i., Nakata, Y. -i., Yonezawa, J. -i., Xu, S., Miyake, M., Takahashi, S. -i., Kikuchi, K., Yazawa, K. -i., Mizobuchi, S., Niimi, S., Kitayama, M., Koshimoto, C., Matsukawa, K., Kasai, M. & Edashige, K., 2013. Rapid Movement of Water and Cryoprotectants in Pig Expanded Blastocysts via Channel Processes: Its Relevance to Their Higher Tolerance to Cryopreservation. *Biology of Reproduction*, 89(4), pp.87–87. Available at: http://www.biolreprod.org/cgi/doi/10.1095/biolreprod.112.107250.

Johnson, M.H. & Maro, B., 1984. The distribution of cytoplasmic actin in mouse 8-cell blastomeres. *Journal of embryology and experimental morphology*, 82, pp.97–117.

Johnson, M.H. & Ziomek, C.A., 1981. The foundation of two distinct cell lineages within the mouse morula. *Cell*, 24(1), pp.71–80.

Kader, A., Agarwal, A., Abdelrazik, H., Sharma, R.K., Ahmady, A. & Falcone, T., 2009. Evaluation of post-thaw DNA integrity of mouse blastocysts after ultrarapid and slow freezing. *Fertil Steril*, 91(5 Suppl), pp.2087–2094. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18774568.

Kader, A., Falcone, T., Sharma, R.K., Mangrola, D. & Agarwal, A., 2010. Slow and ultrarapid cryopreservation of biopsied mouse blastocysts and its effect on DNA integrity index. *J Assist Reprod Genet*, 27(8), pp.509–515. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20512616.

Källén, B., Finnström, O., Nygren, K.G. & Olausson, P.O., 2005. In vitro fertilization (IVF) in Sweden: Infant outcome after different IVF fertilization methods. *Fertility and Sterility*, 84, pp.611–617.

Kasai, M., 1997. Cryopreservation of mammalian embryos. *Molecular biotechnology*, 7(2), pp.173–179.

Kasai, M., Komi, J.H., Takakamo, A., Tsudera, H., Sakurai, T. & Machida, T., 1990. A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. *Journal of reproduction and fertility*, 89(1), pp.91–97.

Kelly, S.J., Mulnard, J.G. & Graham, C.F., 1978. Cell division and cell allocation in early mouse development. *Journal of embryology and experimental morphology*, 48, pp.37–51.

Kiessling, A.A., Davis, H.W., Williams, C.S., Sauter, R.W. & Harrison, L.W., 1991. Development and DNA polymerase activities in cultured preimplantation mouse embryos: comparison with embryos developed in vivo. *The Journal of experimental zoology*, 258(1), pp.34–47.

Knoester, M., Helmerhorst, F.M., Vandenbroucke, J.P., van der Westerlaken, L.A.J., Walther, F.J. & 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, pp.1133–1146.

Kolibianakis, E.M., Venetis, C.A. & Tarlatzis, B.C., 2009. Cryopreservation of human embryos by vitrification or slow freezing: which one is better? *Current opinion in obstetrics & gynecology*, 21, pp.270–274.

Kumar, N.T., Liestøl, K., Løberg, E.M., Reims, H.M. & Mæhlen, J., 2014. Postmortem heart weight: relation to body size and effects of cardiovascular disease and cancer. *Cardiovascular pathology : the official journal of the Society for Cardiovascular Pathology*, 23(1), pp.5–11. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24121021 [Accessed January 5, 2015].

Kuwayama, M., Vajta, G., Ieda, S. & Kato, O., 2005. Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. *Reproductive biomedicine online*, 11(5), pp.608–614.

Kuwayama, M., Vajta, G., Kato, O. & Leibo, S.P., 2005. Highly efficient vitrification method for cryopreservation of human oocytes. *Reproductive biomedicine online*, 11(3),

pp.300–308.

Kwong, W.Y., Miller, D.J., Wilkins, A.P., Dear, M.S., Wright, J.N., Osmond, C., Zhang, J. & Fleming, T.P., 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(1), pp.48–56.

Larue, L., Ohsugi, M., Hirchenhain, J. & Kemler, R., 1994. E-cadherin null mutant embryos fail to form a trophectoderm epithelium. *Proceedings of the National Academy of Sciences of the United States of America*, 91, pp.8263–8267.

Lassalle, B., Testart, J. & Renard, J.P., 1985. Human embryo features that influence the success of cryopreservation with the use of 1,2 propanediol. *Fertility and sterility*, 44(5), pp.645–651.

Lavara, R., Baselga, M., Marco-Jiménez, F. & Vicente, J.S., 2014. Long-term and transgenerational effects of cryopreservation on rabbit embryos. *Theriogenology*, 81(7), pp.988–92. Available at: http://www.sciencedirect.com/science/article/pii/S0093691X14000636 [Accessed September 24, 2014].

Lehtonen, E., Ordonez, G. & Reima, I., 1988. Cytoskeleton in preimplantation mouse development. *Cell Differ*, 24(3), pp.165–177. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=3061662.

Leunens, L., Celestin-Westreich, S., Bonduelle, M., Liebaers, I. & Ponjaert-Kristoffersen, I., 2006. Cognitive and motor development of 8-year-old children born after ICSI compared to spontaneously conceived children. *Human Reproduction*, 21, pp.2922–2929.

Leunens, L., Celestin-Westreich, S., Bonduelle, M., Liebaers, I. & Ponjaert-Kristoffersen, I., 2008. Follow-up of cognitive and motor development of 10-year-old singleton children born after ICSI compared with spontaneously conceived children. *Human Reproduction*, 23, pp.105–111.

Li, L., Wang, L., Xu, X., Lou, H., Le, F., Sheng, J., Huang, H. & Jin, F., 2011. Genome-wide DNA methylation patterns in IVF-conceived mice and their progeny: a putative model for ART-conceived humans. *Reprod Toxicol*, 32(1), pp.98–105. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21672625.

Li, L., Zhang, X., Zhao, L., Xia, X. & Wang, W., 2012. Comparison of DNA apoptosis in mouse and human blastocysts after vitrification and slow freezing. *Mol Reprod Dev*, 79(3), pp.229–236. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22213487.

Li, T., Vu, T.H., Ulaner, G.A., Littman, E., Ling, J.Q., Chen, H.L., Hu, J.F., Behr, B., Giudice, L. & Hoffman, A.R., 2005. IVF results in de novo DNA methylation and histone methylation at an Igf2-H19 imprinting epigenetic switch. *Mol Hum Reprod*, 11(9), pp.631–640. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16219628.

Lin, T.K., Su, J.T., Lee, F.K., Lin, Y.R. & Lo, H.C., 2010. Cryotop Vitrification as Compared to Conventional Slow Freezing for Human Embryos at the Cleavage Stage: Survival and Outcomes. *Taiwanese Journal of Obstetrics and Gynecology*, 49(3), pp.272–278. Available at: http://dx.doi.org/10.1016/S1028-4559(10)60060-5.

Loskutoff, N.M., Johnson, W.H. & Betteridge, K.J., 1993. THE DEVELOPMENTAL COMPETENCE OF BOVINE EMBRYOS WlTH REDUCED CELL NUMBERS. *Theriogenology*, 39, pp.95–107.

Loutradi, K.E., Kolibianakis, E.M., Venetis, C.A., Papanikolaou, E.G., Pados, G., Bontis, I. & Tarlatzis, B.C., 2008. Cryopreservation of human embryos by vitrification or slow freezing: a systematic review and meta-analysis. *Fertility and Sterility*, 90, pp.186–193.

Lucena, E., Bernal, D.P., Lucena, C., Rojas, A., Moran, A. & Lucena, A., 2006. Successful ongoing pregnancies after vitrification of oocytes. *Fertility and Sterility*, 85(1), pp.108–111.

Ludwig, A.K., Katalinic, A., Entenmann, A., Thyen, U., Sutcliffe, A.G., Diedrich, K. & Ludwig, M., 2009. Can we sense ART? The blinded examiner is not blind-a problem with follow-up studies on children born after assisted reproduction. *Fertility and sterility*, 92(3), pp.950–2. Available at: http://www.sciencedirect.com/science/article/pii/S0015028209001319 [Accessed October 6, 2014].

Lumey, L.H., Ravelli, A.C., Wiessing, L.G., Koppe, J.G., Treffers, P.E. & Stein, Z.A., 1993. The Dutch famine birth cohort study: design, validation of exposure, and selected characteristics of subjects after 43 years follow-up. *Paediatric and perinatal epidemiology*, 7(4), pp.354–367.

Magnusson, I., Rothman, D.L., Katz, L.D., Shulman, R.G. & Shulman, G.I., 1992. Increased rate of gluconeogenesis in type II diabetes mellitus. A 13C nuclear magnetic resonance study. *The Journal of clinical investigation*, 90(4), pp.1323–1327.

Mahsoudi, B., Li, a & O'Neill, C., 2007. Assessment of the long-term and transgenerational consequences of perturbing preimplantation embryo development in mice. *Biology of reproduction*, 77(5), pp.889–96. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17699738 [Accessed December 23, 2014].

Martino, A., Songsasen, N. & Leibo, S.P., 1996. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. *Biology of reproduction*, 54(5), pp.1059–1069.

Mascarenhas, M.N., Flaxman, S.R., Boerma, T., Vanderpoel, S. & Stevens, G.A., 2012. National, Regional, and Global Trends in Infertility Prevalence Since 1990: A Systematic Analysis of 277 Health Surveys. *Plos Medicine*, 9(12). Available at: <Go to ISI>://WOS:000312934200006.

Mazur, P., 1984. Freezing of living cells: mechanisms and implications. *The American journal of physiology*, 247(3 Pt 1), pp.C125–C142.

McDonald, S.D., Murphy, K., Beyene, J. & Ohlsson, A., 2005. Perinatel outcomes of singleton pregnancies achieved by in vitro fertilization: a systematic review and meta-analysis. *Journal of obstetrics and gynaecology Canada : JOGC = Journal d'obstetrique et gynecologie du Canada : JOGC*, 27, pp.449–459.

McMullen, S. & Mostyn, A., 2009. Animal models for the study of the developmental origins of health and disease. *The Proceedings of the Nutrition Society*, 68(3), pp.306–320.

Men, H., Monson, R.L., Parrish, J.J. & Rutledge, J.J., 2003. Detection of DNA damage in bovine metaphase II oocytes resulting from cryopreservation. *Molecular Reproduction and Development*, 64, pp.245–250.

Ménézo, Y., Nicollet, B., Herbaut, N. & André, D., 1992. Freezing cocultured human blastocysts. *Fertility and sterility*, 58(5), pp.977–980.

Miles, H.L., Hofman, P.L., Peek, J., Harris, M., Wilson, D., Robinson, E.M., Gluckman, P.D. & Cutfield, W.S., 2007. In vitro fertilization improves childhood growth and

metabolism. *The Journal of clinical endocrinology and metabolism*, 92(9), pp.3441–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17566097 [Accessed October 6, 2014].

Miyamoto, H. & Ishibashi, T., 1979. Effects of low temperatures on survival of frozen-thawed mouse embryos. *Experientia*, 35(11), pp.1505–1506.

Mukaida, T. & Oka, C., 2012. Vitrification of oocytes, embryos and blastocysts. *Best Pract Res Clin Obstet Gynaecol*, 26(6), pp.789–803. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22940094.

Mukaida, T., Oka, C., Goto, T. & Takahashi, K., 2006. Artificial shrinkage of blastocoeles using either a micro-needle or a laser pulse prior to the cooling steps of vitrification improves survival rate and pregnancy outcome of vitrified human blastocysts. *Human Reproduction*, 21(12), pp.3246–3252.

Mukaida, T., Wada, S., Takahashi, K., Pedro, P.B., An, T.Z. & Kasai, M., 1998. Vitrification of human embryos based on the assessment of suitable conditions for 8-cell mouse embryos. *Human reproduction (Oxford, England)*, 13(1O), pp.2874–2879.

Naik, B.R., Rao, B.S., Vagdevi, R., Gnanprakash, M., Amarnath, D. & Rao, V.H., 2005. Conventional slow freezing, vitrification and open pulled straw (OPS) vitrification of rabbit embryos. *Animal Reproduction Science*, 86, pp.329–338.

Nelissen, E.C., Van Montfoort, A.P., Coonen, E., Derhaag, J.G., Geraedts, J.P., Smits, L.J., Land, J. a, Evers, J.L. & Dumoulin, J.C., 2012. Further evidence that culture media affect perinatal outcome: findings after transfer of fresh and cryopreserved embryos. *Human reproduction (Oxford, England)*, 27(7), pp.1966–76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22552689 [Accessed September 25, 2014].

Neri, Q. V., Takeuchi, T. & Palermo, G.D., 2008. An update of assisted reproductive technologies results in the United States. In *Annals of the New York Academy of Sciences*. pp. 41–48.

Olivennes, F., Kerbrat, V., Blanchet, V., Fanchin, R., Frydman, R. & Rufat, P., 1997. Follow up of a cohort of 422 children aged 6 to 13 years conceived by IVF. *Fertility and Sterility*, 67(2), p.6.

Olivennes, F., Schneider, Z., Remy, V., Blanchet, V., Kerbrat, V., Fanchin, R., Hazout, A., Glissant, M., Fernandez, H., Dehan, M. & Frydman, R., 1996. Perinatal outcome and follow-up of 82 children aged 1-9 years old conceived from cryopreserved embryos. *Hum Reprod*, 11(7), pp.1565–1568. Available at: <Go to ISI>://WOS:A1996VD88000044.

Owen, C.M. & Segars Jr., J.H., 2009. Imprinting disorders and assisted reproductive technology. *Semin Reprod Med*, 27(5), pp.417–428. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19711252.

Parlee, S.D. & Macdougald, O.A., 2013. Maternal nutrition and risk of obesity in offspring: The Trojan horse of developmental plasticity. *Biochim Biophys Acta*, p.12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23871838.

Paynton, B. V, Rempel, R. & Bachvarova, R., 1988. Changes in state of adenylation and time course of degradation of maternal mRNAs during oocyte maturation and early embryonic development in the mouse. *Developmental biology*, 129, pp.304–314.

Pereira, R.M. & Marques, C.C., 2008. Animal oocyte and embryo cryopreservation. *Cell and Tissue Banking*, 9(4), pp.267–277.

Pinborg, A., Loft, A., Aaris Henningsen, A.K., Rasmussen, S. & Andersen, A.N., 2010. Infant outcome of 957 singletons born after frozen embryo replacement: The Danish National Cohort Study 1995-2006. *Fertility and Sterility*, 94(4), pp.1320–1327. Available at: http://dx.doi.org/10.1016/j.fertnstert.2009.05.091.

Pinborg, A., Loft, A., Rasmussen, S., Schmidt, L., Langhoff-Roos, J., Greisen, G. & Andersen, A.N., 2004. Neonatal outcome in a Danish national cohort of 3438 IVF/ICSI and 10 362 non-IVF/ICSI twins born between 1995 and 2000. *Human Reproduction*, 19, pp.435–441.

Pires, A., Martins, P., Pereira, A.M., Silva, P.V., Marinho, J., Marques, M., Castela, E., Sena, C. & Seiça, R., 2014. Insulin Resistance, Dyslipidemia and Cardiovascular Changes in a Group of Obese Children. *Arquivos Brasileiros de Cardiologia*, pp.266–273. Available at: http://www.gnresearch.org/doi/10.5935/abc.20140206.

Rall, W.F. & Fahy, G.M., 1985. Ice-free cryopreservation of mouse embryos at -196C by vitrification. *Nature*, 313(14), p.1985. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3969158.

Rama Raju, G.A., Haranath, G.B., Krishna, K.M., Prakash, G.J. & Madan, K., 2005. Vitrification of human 8-cell embryos, a modified protocol for better pregnancy rates. *Reproductive biomedicine online*, 11(4), pp.434–437.

Reik, W., 2007. Stability and flexibility of epigenetic gene regulation in mammalian development. *Nature*, 447(7143), pp.425–432. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17522676.

Reik, W., Dean, W. & Walter, J., 2001. Epigenetic reprogramming in mammalian development. *Science (New York, N.Y.)*, 293(5532), pp.1089–93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11498579 [Accessed July 9, 2014].

Riethmacher, D., Brinkmann, V. & Birchmeier, C., 1995. A targeted mutation in the mouse E-cadherin gene results in defective preimplantation development. *Proceedings of the National Academy of Sciences of the United States of America*, 92, pp.855–859.

Rimm, A.A. & Katayama, A.C., 2012. The impact of ART on major malformations is not known at this time. *Journal of assisted reproduction and genetics*, 29(7), pp.699–700. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3401255&tool=pmcentrez&rendertype=abstract [Accessed October 10, 2014].

Romundstad, L.B., Romundstad, P.R., Sunde, A., von Düring, V., Skjærven, R., Gunnell, D. & Vatten, L.J., 2008. Effects of technology or maternal factors on perinatal outcome after assisted fertilisation: a population-based cohort study. *The Lancet*, 372, pp.737–743.

Roseboom, T.J., Meulen, M.H. Van Der, Ravelli, a. C., Osmond, C., Barker, D.J.. & Bleker, O.., 2001. Effects of prenatal expossure to the Ductch famine on adult disease in later life: an overview. *Mol Cell Endocrinol*, 185, pp.93–98.

Rosenthal, N. & Brown, S., 2007. The mouse ascending: perspectives for human-disease models. *Nature cell biology*, 9(9), pp.993–999.

Rossant, J., Chazaud, C. & Yamanaka, Y., 2003. Lineage allocation and asymmetries in the early mouse embryo. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 358(1436), pp.1341–1348; discussion 1349.

Rougeulle, C. & Lalande, M., 1998. Angelman syndrome: how many genes to remain silent?

Neurogenetics, 1, pp.229–237.

Ryan, B.C. & Vandenbergh, J.G., 2002. Intrauterine position effects. *Neuroscience and Biobehavioral Reviews*, 26(6), pp.665–678.

Vom Saal, F.S., Quadagno, D.M., Even, M.D., Keisler, L.W., Keisler, D.H. & Khan, S., 1990. Paradoxical effects of maternal stress on fetal steroids and postnatal reproductive traits in female mice from different intrauterine positions. *Biology of reproduction*, 43(5), pp.751–761.

Sanchez-Osorio, J., Cuello, C., Gil, M.A., Parrilla, I., Alminana, C., Caballero, I., Roca, J., Vazquez, J.M., Rodriguez-Martinez, H. & Martinez, E.A., 2010. In vitro postwarming viability of vitrified porcine embryos: effect of cryostorage length. *Theriogenology*, 74(3), pp.486–490. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20452005.

Santos, F., Hendrich, B., Reik, W. & Dean, W., 2002. Dynamic reprogramming of DNA methylation in the early mouse embryo. *Developmental biology*, 241(1), pp.172–182.

Santos, M.A., Kuijk, E.W. & Macklon, N.S., 2010. The impact of ovarian stimulation for IVF on the developing embryo. *Reproduction*, 139(1), pp.23–34.

Saragusty, J. & Arav, A., 2011. Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. *Reproduction*, 141(1), pp.1–19. Available at: <Go to ISI>://WOS:000285641300001.

Sazonova, A., Kllen, K., Thurin-Kjellberg, A., Wennerholm, U.B. & Bergh, C., 2012. Obstetric outcome in singletons after in vitro fertilization with cryopreserved/thawed embryos. *Human Reproduction*, 27(5), pp.1343–1350.

Schieve, L.A., F., M.S., C., F., B., P.H., G., J. & S., W.L., 2002. Low and very low birth weight in infants conceived with use of assisted reproductive technology. *The new England Journal of Medicine*, 346(10), pp.731–737. Available at: <Go to ISI>://CCC:000324122800001.

Scott, K. a, Yamazaki, Y., Yamamoto, M., Lin, Y., Melhorn, S.J., Krause, E.G., Woods, S.C., Yanagimachi, R., Sakai, R.R. & Tamashiro, K.L.K., 2010. Glucose parameters are altered in mouse offspring produced by assisted reproductive technologies and somatic cell nuclear transfer. *Biology of reproduction*, 83(2), pp.220–7. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2907285&tool=pmcentrez&rendertype=abstract [Accessed January 23, 2015].

Seggers, J., Haadsma, M.L., Bos, A.F., Heineman, M.J., Keating, P., Middelburg, K.J., van Hoften, J.C., Veenstra-Knol, H.E., Kok, J.H., Cobben, J.M. & Hadders-Algra, M., 2012. Dysmorphic features in 2-year-old IVF/ICSI offspring. *Early Hum Dev*, 88(10), pp.823–829. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22795820.

Shaw, J.M. & Jones, G.., 2003. Terminology associated with vitrification and other cryopreservation procedures for oocytes and embryos. *Hum Reprod Update*, 9(6), p.23.

Shaw, J.M., Kola, I., Macfarlane, D.R. & Trounson, A., 1991. An association between chromosomal abnormalities in rapidly frozen 2-cell mouse embryos and the ice-forming properties of the cryoprotective solution. *Reprod Fertil*, 91, pp.9–18.

Sherman, J.K. & Lin, T.P., 1958. Survival of Unfertilized Mouse Eggs During Freezing and Thawing. *Exp Biol Med*, 98, pp.902–905.

Shih, W., Rushford, D.D., Bourne, H., Garrett, C., McBain, J.C., Healy, D.L. & Baker, H.W., 2008. Factors affecting low birthweight after assisted reproduction technology: difference between transfer of fresh and cryopreserved embryos suggests an adverse

effect of oocyte collection. *Hum Reprod*, 23(7), pp.1644–1653. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18442997.

Shih, W., Rushford, D.D., Bourne, H., Garrett, C., McBain, J.C., Healy, D.L. & Baker, H.W.G., 2008. Factors affecting low birthweight after assisted reproduction technology: difference between transfer of fresh and cryopreserved embryos suggests an adverse effect of oocyte collection. *Hum Reprod*, 23(7), pp.1644–1653. Available at: <Go to ISI>://WOS:000256519900026.

Shin, M.R., Choi, H.W., Kim, M.K., Lee, S.H., Lee, H.S. & Lim, C.K., 2011. In vitro development and gene expression of frozen-thawed 8-cell stage mouse embryos following slow freezing or vitrification. *Clin Exp Reprod Med*, 38(4), pp.203–209. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22384443.

Shufaro, Y. & Laufer, N., 2013. Epigenetic concerns in assisted reproduction: Update and critical review of the current literature. *Fertility and Sterility*, 99, pp.605–606.

Skeggs, L.T., Kahn, J.R. & Shumway, N.P., 1956. The preparation and function of the hypertensin-converting enzyme. *The Journal of experimental medicine*, 103(3), pp.295–299.

Sripunya, N., T., S., Y., I., T., N., IMAI, K. & R., P., 2010. A Comparison of Cryotop and Solid Surface Vitrification Methods for the Cryopreservation of In Vitro Matured Bovine Oocytes. *Journal of Reproduction and Development*, 56(1), p.6.

Stojnic, J., Radunovic, N., Jeremic, K., Kastratovic Kotlica, B., Mitrovic, M. & Tulic, I., 2013. Perinatal outcome of singleton pregnancies following in vitro fertilization. *Clinical and Experimental Obstetrics and Gynecology*, 40, pp.277–283.

Stout, T.A., 2012. Cryopreservation of equine embryos: current state-of-the-art. *Reprod Domest Anim*, 47 Suppl 3, pp.84–89. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22681302.

Succu, S., Bebbere, D., Bogliolo, L., Ariu, F., Fois, S., Leoni, G.G., Berlinguer, F., Naitana, S. & Ledda, S., 2008. Vitrification of in vitro matured ovine oocytes affects in vitro pre-implantation development and mRNA abundance. *Molecular Reproduction and Development*, 75, pp.538–546.

Sutcliffe, A.G., Peters, C.J., Bowdin, S., Temple, K., Reardon, W., Wilson, L., Clayton-Smith, J., Brueton, L.A., Bannister, W. & Maher, E.R., 2006. Assisted reproductive therapies and imprinting disorders - A preliminary British survey. *Human Reproduction*, 21(4), pp.1009–1011.

Tamashiro, K.L.K., Wakayama, T., Akutsu, H., Yamazaki, Y., Lachey, J.L., Wortman, M.D., Seeley, R.J., D'Alessio, D. a, Woods, S.C., Yanagimachi, R. & Sakai, R.R., 2002. Cloned mice have an obese phenotype not transmitted to their offspring. *Nature medicine*, 8(3), pp.262–267.

Tao, T. & Del Valle, A., 2008. Human oocyte and ovarian tissue cryopreservation and its application. *J Assist Reprod Genet*, 25(7), pp.287–296. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18670872.

Tsunoda, Y. & Mclaren, A., 1983. Effect of various procedures on the viability of mouse embryos containing half the normal number of blastomeres. *J Reprod Fertil*, 69, pp.315–322.

Uechi, H., Tsutsumi, O., Morita, Y., Takai, Y. & Taketani, Y., 1999. Comparison of the effects of controlled-rate cryopreservation and vitrification on 2-cell mouse embryos and

their subsequent development. *Human Reproduction*, 14, pp.2827–2832.

Vajta, G., Holm, P., Greve, T. & Callesen, H., 1996. Factors affecting survival rates of in vitro produced bovine embryos after vitrification and direct in-straw rehydration. *Animal Reproduction Science*, 45(3), pp.191–200. Available at: <Go to ISI>://WOS:A1996VZ04500003.

Valera, A., Valera, A., Pujol, A., Pujol, A., Pelegrin, M., Pelegrin, M., Bosch, F. & Bosch, F., 1994. Transgenic mice overexpressing phosphoenolpyruvate carboxykinase develop non-insulin-dependent diabetes mellitus. *Proc Natl Acad Sci U S A*, 91(19), pp.9151–9154.

Van den Veyver, I.B., 2002. Genetic effects of methylation diets. *Annual review of nutrition*, 22, pp.255–282.

Viner, R.M., Segal, T.Y., Lichtarowicz-Krynska, E. & Hindmarsh, P., 2005. Prevalence of the insulin resistance syndrome in obesity. *Archives of disease in childhood*, 90(1), pp.10–14.

De Vries, W.N., Evsikov, A. V, Haac, B.E., Fancher, K.S., Holbrook, A.E., Kemler, R., Solter, D. & Knowles, B.B., 2004. Maternal beta-catenin and E-cadherin in mouse development. *Development (Cambridge, England)*, 131, pp.4435–4445.

Waddington, C.H., 1942. The epigenotype. *Endeavour*, pp.18–20. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22186258.

Wadhwa, P.D., Buss, C., Entringer, S. & Swanson, J.M., 2009. Developmental origins of health and disease: brief history of the approach and current focus on epigenetic mechanisms. *Semin Reprod Med*, 27(5), pp.358–368. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19711246.

Wang, Y.A., Sullivan, E. a, Black, D., Dean, J., Bryant, J. & 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(6), pp.1650–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15950632 [Accessed January 6, 2015].

Waterland, R. & Jirtle, R.L., 2004. Early Nutrition, Epigenetic Changes at Transposons and Imprinted Genes, and Enhanced Susceptibility to Adult Chronic Diseases. *Nutrition*, 20(1), pp.63–68.

Watkins, A.J. & Fleming, T.P., 2009. Blastocyst environment and its influence on offspring cardiovascular health: the heart of the matter. *Journal of anatomy*, 215(1), pp.52–9. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2714638&tool=pmcentrez&rendertype=abstract [Accessed September 22, 2014].

Watkins, A.J., Platt, D., Papenbrock, T., Wilkins, A., Eckert, J.J., Kwong, W.Y., Osmond, C., Hanson, M. & Fleming, T.P., 2007. Mouse embryo culture induces changes in postnatal phenotype including raised systolic blood pressure. *Proceedings of the National Academy of Sciences of the United States of America*, 104(13), pp.5449–5454.

Watkins, A.J., Ursell, E., Panton, R., Papenbrock, T., Hollis, L., Cunningham, C., Wilkins, A., Perry, V.H., Sheth, B., Kwong, W.Y., Eckert, J.J., Wild, A.E., Hanson, M.A., Osmond, C. & Fleming, T.P., 2008. Adaptive responses by mouse early embryos to maternal diet protect fetal growth but predispose to adult onset disease. *Biology of reproduction*, 78(2), pp.299–306.

Weksberg, R., Shuman, C. & Smith, A.C., 2005. Beckwith-Wiedemann syndrome. *American*

journal of medical genetics. Part C, Seminars in medical genetics, 137C, pp.12–23.

Weksberg, R., Smith, A.C., Squire, J. & Sadowski, P., 2003. Beckwith-Wiedemann syndrome demonstrates a role for epigenetic control of normal development. *Human molecular genetics*, 12 Spec No, pp.R61–R68.

Wennerholm, U.-B., 2000. Cryopreservation of embryos and oocytes: obstetric outcome and health in children. *Human Reproduction*, 15(suppl 5), pp.18–25. Available at: http://humrep.oxfordjournals.org/lookup/doi/10.1093/humrep/15.suppl_5.18.

Wennerholm, U.B., Soderstrom-Anttila, V., Bergh, C., Aittomaki, K., Hazekamp, J., Nygren, K.G., Selbing, A. & Loft, A., 2009. Children born after cryopreservation of embryos or oocytes: a systematic review of outcome data. *Hum Reprod*, 24(9), pp.2158–2172. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19458318.

Wikland, M., Hardarson, T., Hillensjö, T., Westin, C., Westlander, G., Wood, M. & Wennerholm, U.B., 2010. Obstetric outcomes after transfer of vitrified blastocysts. *Human Reproduction*, 25(7), pp.1699–1707.

Williams, C.A., 2005. Neurological aspects of the Angelman syndrome. In *Brain and Development*. pp. 88–94.

Wilmut, I. & Rowson, L.E.A., 1973. Experiments on low-temperature preservation of cow embryos. *Veterinary Record*, 92(26), pp.686–690. Available at: <Go to ISI>://WOS:A1973P972000002.

Wong, K.M., Mastenbroek, S. & Repping, S., 2014. Cryopreservation of human embryos and its contribution to in vitro fertilization success rates. *Fertility and Sterility*, 102(1), pp.19–26.

Wu, B., Tong, J. & Leibo, S.P., 1999. Effects of cooling germinal vesicle-stage bovine oocytes on meiotic spindle formation following in vitro maturation. *Molecular reproduction and development*, 54, pp.388–395.

Yokota, Y., Yokota, H., Yokota, M., Sato, S. & Araki, Y., 2001. Birth of healthy twins from in vitro development of human refrozen embryos. In *Fertility and Sterility*. pp. 1063–1065.

Young, L., 1998. Large offspring syndrome in cattle and sheep. *Reviews of Reproduction*, 3(3), pp.155–163. Available at: http://www.reproduction-online.org/content/revreprod/3/3/155 [Accessed September 25, 2014].

Zambrano, E., Martínez-Samayoa, P.M., Bautista, C.J., Deás, M., Guillén, L., Rodríguez-González, G.L., Guzmán, C., Larrea, F. & Nathanielsz, P.W., 2005. Sex differences in transgenerational alterations of growth and metabolism in progeny (F2) of female offspring (F1) of rats fed a low protein diet during pregnancy and lactation. *The Journal of physiology*, 566(Pt 1), pp.225–36. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1464716&tool=pmcentrez&rendertype=abstract [Accessed September 22, 2014].

Zegers-Hochschild, F., Mansour, R., Ishihara, O., Adamson, G.D., de Mouzon, J., Nygren, K.G. & Sullivan, E.A., 2013. International Committee for Monitoring Assisted Reproductive Technology: world report on assisted reproductive technology, 2005. *Fertility and Sterility*.

Zernicka-Goetz, M., 2004. First cell fate decisions and spatial patterning in the early mouse embryo. *Semin Cell Dev Biol*, 15(5), pp.563–572. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15271302.

Zernicka-Goetz, M., Morris, S.A. & Bruce, A.W., 2009. Making a firm decision: multifaceted regulation of cell fate in the early mouse embryo. *Nature reviews. Genetics*, 10(7), pp.467–77. Available at: http://dx.doi.org/10.1038/nrg2564 [Accessed July 10, 2014].

Zhan, Q., Pan, P., Xu, X., Lou, H., Lou, Y. & 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(11), pp.947–60. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3829644&tool=pmcentrez&rendertype=abstract [Accessed October 6, 2014].

Zhao, X.-M., Du, W.-H., Hao, H.-S., Wang, D., Qin, T., Liu, Y. & Zhu, H.-B., 2012. Effect of vitrification on promoter methylation and the expression of pluripotency and differentiation genes in mouse blastocysts. *Molecular Reproduction and Development*, 79(7), pp.445–450. Available at: http://dx.doi.org/10.1002/mrd.22052.

Zhao, X.-M., Ren, J.-J., Du, W.-H., Hao, H.-S., Wang, D., Qin, T., Liu, Y. & Zhu, H.-B., 2013. Effect of vitrification on promoter CpG island methylation patterns and expression levels of DNA methyltransferase 1o, histone acetyltransferase 1, and deacetylase 1 in metaphase II mouse oocytes. *Fertility and Sterility*, 100(1), pp.256–261. Available at: <Go to ISI>://BIOSIS:PREV201300539156.

Appendix I

KSOM culture medium
2x stock KSOM
Sodium chloride (NaCl) 1.110 g
Potassium chloride (KCl) 0.0095 g
Magnesium sulphate7-hydrate (MgSO4.7H2O) 0.0099 g
Lactic acid 0.362 ml
Sodium pyruvate 0.0044 g
Glucose 0.0072 g
Sodium bicarbonate (NaHCO3) 0.420 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 -80ºC for up to 2 months.

1 x KSOM (10ml)
The following was added to 5 ml of thawed 2x KSOM stock:
Calcium chloride (CaCl2; 100x stock 25 mg/ml) 100 µl
Non-essential amino acids (100x stock; 10 mM) 50 µl
Essential amino acids (50 x stock) 100 µl
L-glutamine (200 mM) 50 µl
Deionised water, sterile 4.75 ml
BSA 0.040 g

Sterile filtered (0.22 µm filter); osmolarity adjusted to 255 ± 5 mOsm with 20% NaCl; aliquots stored at 4ºC for up to 1 week.

H6PVP medium

Stock F 1.0 ml
Stock B 0.16 ml
Stock G 0.1 ml
Stock H 0.1 ml
Stock E 0.84 ml
H_2O 7.8 ml
20% NaCl 60 µl
PVP 60 mg

Solution F (per 100 ml, osmolarity, 255 ± 20 mOsm
Sodium chloride 4.720 g
Potassium chloride 0.11g
Sodium dihydrogen orthophosphate 0.06g
Magnesium chloride 0.1 g
D-glucose 1.0 g
DL-lactic acid (60%) 3.4 ml

Solution G (per 10 ml, osmolarity, 60 ± 10 mOsm
Pyruvic acid 0.03 g
Penicillin 0.06 g
Streptomycin 0.05 g

Solution B (per 10 ml, osmolarity, 444 ± 20 mOsm
Sodium hydrogen carbonate 0.2106 g

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 2.9785 g

Acid Tyrode's solution (per 100ml, pH2.3)
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
$CaCl_2$ 0.02 g

DMSO
DMSO is a very hygroscopic liquid. The purity of the material was essentially unchanged per gas chromatographic analysis. DMSO is thermally stable. It can be heated to 150°C for 24 hours with less than 0.1% loss in purity