The impact of *in vitro* stress on pre-implantation embryo development, viability and mitochondrial homeostasis.

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Contents

Figures and tables

Abstract

It is recognised that the environment to which the fetus is exposed *in utero*, after implantation, can program longer term health outcomes and alter the possibility of disease onset later in life. It is becoming evident that the environment, to which the pre-implantation embryo is exposed, can also affect the ability of the embryo to form a viable pregnancy as well as altering fetal growth.

Despite this understanding, little is known about the mechanism by which the environment can 'program' the pre-implantation embryo. Using model stress systems, either ammonium or DMO in the culture medium, this thesis addressed the hypothesis that suboptimal environmental conditions may alter mitochondrial homeostasis and function and/or epigenetic parameters and these are the possible mechanisms responsible for the altered fetal outcomes seen.

While common measures of embryo quality such as on time blastocyst development were not affected by either stress, more in-depth investigations found several striking differences. Exposure to DMO significantly decreased blastocyst cell number and allocation to the inner cell mass and trophectoderm, as well as increased blastocyst apoptosis. After exposure to DMO, blastocysts were transferred to pseudopregnant recipients, and both the ability of the embryos to implant and develop into a fetus was impaired as well as fetal weights and crown rump length were significantly reduced indicative of altered growth. Similar results have also been demonstrated after pre-implantation embryos are exposed to ammonium *in vitro*.

Exposure to ammonium during pre-implantation embryo development also altered placental gene expression and function, indicating a possible mechanism of the observed reduced fetal growth parameters.

Interestingly, the pre-implantation embryo appears to be the most vulnerable to an environmental stress during the pre-compaction stage, in particular the zygote to 2-cell transition, as exposure to either stress during this stage alone shows similar perturbations to if the stress was present for the entire pre-implantation developmental period.

At this early stage of embryo development, mitochondria are the sole energy generators and are therefore critical for embryo function. This study determined that either ammonium or DMO stress exposure, during the first cleavage division, significantly perturbed mitochondrial distribution, membrane potential and ATP/ADP levels. Removal of the stress did not allow these effects to be completely reversed, implicating mitochondrial perturbations as a possible mechanism behind altered embryo programming.

During pre-implantation embryo development there are also significant epigenetic changes which are vital for re-programming the embryonic genome. Both *in vitro* stresses significantly altered DNA de-methylation at the 2-cell stage and reduced blastocyst gene expression levels of DNA methyltransferases (*Dnmt3a* and *Dnmt3b*), which are responsible for *de novo* methylation. Together these data highlight the importance of pre-implantation embryo development as a critical period of

growth in which the presence of environmental stress can have an impact on metabolic homeostasis and critical epigenetic events that may be responsible for the downstream effects seen on fetal growth. These results are not only important for assisted reproductive therapy, where the presence of an *in vitro* laboratory stress can potentially alter embryo programming, but are also important for *in vivo* embryo development where the health and wellbeing of the mother can also potentially influence the *in utero* environment and thus the long-term health outcomes of her child.

Declaration

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Publications arising from thesis to date

Referred journal articles

Zander DL, Thompson JG, Lane M. Perturbations in Mouse Embryo Development and Viability Caused by Ammonium are More Severe after Exposure at the Cleavage Stages. *Biol Reprod* 2006 Feb;74(2):288-94. Epub 2005 Oct 12.

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Zander-Fox DL, Mitchell M, Thompson JG, Lane M. Repercussions of a transient decrease in pH on embryo viability and subsequent fetal development. *Reprod Fertil Dev* 2007; Special Issue pg 112, Abstract 404

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'Life is not easy for any of us. But what of that? We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something and that this thing must be attained'. ***Marie Curie***

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Common abbreviations

ATP	Adenosine Triphosphate
ADP	Adenosine Diphosphate
BSA	Bovine Serum Albumin
DMO	5,5-Dimethyl-2,4-Oxazolidinedione
hCG	Human Chorionic Gonadotrophin
HSA	Human Serum Albumin
ICM	Inner cell mass
IVC	*In vitro* culture
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
i.p	Intraperitoneal
IU	International units
MMP/ $\Delta\Psi m$	Mitochondrial membrane potential
PBS	Phosphate Buffered Solution
pH_i	Intracellular pH
PI	Propidium Iodide
PMSG	Pregnant Mares' Serum Gonadotrophin
PUN	Plasma urea nitrogen concentration
PVP	Polyvinal-pyrrolidone
RDP	Ruman degradable protein
ROS	Reactive oxygen species
RUP	Ruman undegradable protein
TE	Trophectoderm

1 Literature review

1.1 Introduction

For centuries now, infertility has been a growing problem that mankind has been attempting to remedy by whatever means possible. As early as the 3rd century Jewish thinkers were discussing whether it was possible for a woman to become pregnant by accidental artificial insemination. During the 14th century, Arab cultures were using artificial insemination in their horse breeding programmes, and by 1777 an Italian Priest began experiments in reptiles and dogs using artificial insemination techniques. The first attempt at human insemination was made in 1785 by John Hunter, which resulted in a child being born that very same year (Morice *et al.* 1995). Thus treating infertility is by no means a new science.

The 20th century has seen a significant increase in the prevalence of human infertility (Forti and Krausz 1998), with the causes being divided into four categories: a female factor (35%); a male factor (30%); combined factors (20%); and unexplained infertility (15%). It has been suggested that the increase in infertility is a result of an increase in conceptual age, alterations in sperm quality due to environmental factors such as cigarette smoking and alcohol, as well as changes in sexual behaviour, although the exact changes in prevalence of infertility are increasingly difficult to establish (Brugo-Olmedo *et al.* 2001).

Because infertility is increasing—one in six couples now requires some form of assisted reproductive technology (ART)—considerable advances have been made in the area of embryo culture leading to the global application of *in vitro* fertilisation (IVF).

Despite being a wonder of modern medicine and technology, IVF is by no means a perfect science, with the development of the *in vitro* pre-implantation embryo being associated with reduced cleavage rates, perturbed mitochondrial metabolism, altered gene expression, decreased blastocyst development and viability (Biggers and Stern 1973; Gardner and Lane 1993b; Gardner and Leese 1990; Lane and Gardner 1994; Menke and McLaren 1970). The impact of the stress of *in vitro* culture has also been associated with the increased occurrence of gene imprinting defects leading to an increase in Angelman and Beckwith-Weiderman syndrome in children born from IVF (DeBaun *et al.* 2003; Ludwig *et al.* 2005).

Owing to the occurrence of these abnormalities as well as perturbed embryo development and decreased implantation rates after IVF, continual research is being conducted into both the identification of potential stress, the alleviation of stress and also into how this stress is inducing its impact on the embryo. It has been proposed that the development of culture conditions, which alleviate stress, will facilitate normal cell function and, in turn, will result in increased embryo viability and normality leading to an increase in normal resultant offspring (Lane and Gardner 2005b).

1.2 Oocyte maturation, fertilisation and pre-implantation embryo development

In mammals, fertilisation is dependent upon the coordination of a number of complicated processes. On the maternal side, it involves the growth, recruitment and maturation of oocytes under the influence of peripheral and follicular endocrine factors either directly or via interaction with the follicular granulosa cells, of which any deviation can lead to the production of inferior oocytes (Greve and Callesen 2001). On the paternal side, spermatozoa must progress to the ampulla of the oviduct, of which any alteration to the microenvironment can affect sperm release, transport and binding (Greve and Callesen 2001).

1.2.1 Oocyte maturation and ovulation

In all mammalian species, follicle and oocyte development follows a distinctive sequence of events that begins with the establishment of the ovary shortly after conception and ends with the repetitive ovulation of a mature, fertile, metaphase Π oocyte (Picton *et al.* 2008). Early in fetal development, primitive germ cells in the ovaries differentiate into oogonia, which rapidly divide into thousands of cells containing the full complement of chromosomes (23 pairs). Initially, all oocytes begin as primordial follicles, where they remain in growth arrest until they proceed to the primary follicle phase. This is mediated by a balance of stimulatory and inhibitory hormones and growth factors (Fortune *et al.* 2000). The follicles remain in the primary stage until the signal is received to begin the maturation process via a surge in follicle-stimulating hormone (FSH). At this stage communication between the oocyte and follicle are formed via rudimentary paracrine signalling (van den Hurk and Zhao 2005).

Following this the follicle continues to mature and becomes a secondary follicle, and more granulosa layers begin to form as well as theca layers between which capillary vessels form and circulate blood to and from the follicle. The follicle then becomes pre-antral, in which the oocyte is fully grown and surrounded by the zona pellucida, a glycoprotein polymer shell through which the sperm must penetrate (Peters *et al.* 1975; van den Hurk and Zhao 2005). Throughout the pre-antral phase of folliculogenesis, the development of the oocyte is dependent on, and concomitant with, follicular granulosa cell layers. Communications between the oocyte and these supporting cells is dependent on heterologous gap junctions. The communication between these cells is vital in sustaining oocyte growth and development, as the support cells provide all the nutrients for the developing oocyte (Picton *et al.* 2008).

Following pre-antral follicle development is tertiary/antral follicle development, which is marked by the formation of a fluid-filled cavity adjacent to the oocyte. At this stage the granulosa cells begin

to differentiate due to oocyte-secreted factors in particular *coronal radiata* cells, which directly surround the zona pellucida, and *cumulus oophorous* cells surrounding the *coronal radiata* cells.

During the course of follicle development, many follicles have stopped growing along the way and undergo atresia. Meanwhile approximately 5–7 follicles will compete to be the dominant follicle, by competing for FSH. At this stage FSH levels are decreasing and so follicles with less FSH receptors will display retarded development and will become atretic, with only one viable follicle remaining. The one remaining follicle will grow rapidly and become the pre-ovulatory follicle (Peters *et al.* 1975). Following the luteinizing hormone surge, the pre-ovulatory follicle will develop an opening called a stigma, and the oocyte, surrounded by its cumulus cells, is excreted in a process called ovulation. Up until the pre-ovulatory phase the follicle contains a primary oocyte that is arrested in prophase of meiosis I. During the late pre-ovulatory phase the oocyte resumes meiosis and becomes a secondary oocyte, arrested in metaphase Π. The secondary oocyte is now fully mature and is able to be fertilised. The ruptured follicle will then transform into the corpus luteum, which then secretes large amounts of progesterone that maintains the endometrial lining (Peters *et al.* 1975).

1.2.2 Fertilisation

After ovulation has occurred, fertilisation can now begin and occurs at the ampullary-isthmic junction. The successful interaction of the oocyte and sperm is mediated primarily by gamete surface proteins (Primakoff and Myles 2002). Initially when the sperm encounter the oocyte it is surrounded by cumulus cells and the sperm must first permeate this barrier. To do this sperm use hyperactivated activity and a gylcosylphosphatidylinositol-anchored surface hyaluronidase, the combination of which allows the sperm to digest a path through the extracellular matrix of the cumulus cells (Primakoff and Myles 2002). Sperm that reach the zona pellucida, bind to it via ZP3, one of the glycoproteins in the zona pellucida. They receive a signal to acrosome react, which means that there is a release of cortical granules (hyaluronidase) from the acrosome that assists in digesting the matrix of hyaluronic acid in the vestments surrounding the oocyte (Hunter and Greve 1998; Hyttel *et al.* 1988; Talbot 1985). The sperm then breaks through the zona pellucida by cutting a penetration slit as wide as the sperm head. This is done by a combination of motility, proteases and glycosidases (Primakoff and Myles 2002). The sperm, having penetrated the zona, binds to the egg plasma membrane and the now activated oocyte thickens its cell membrane to prevent the entrance of additional spermatozoa into the ooplasm (Talbot 1985). The binding of the sperm to the egg plasma membrane also induces an increase in intracellular calcium (Ca^{2+}). This rise in intracellular Ca^{2+} is necessary for the completion of all the events involved in egg activation, including the exocytosis of the cortical granules required to block polyspermy and resumption of the meiotic cycle, as well as pronuclear formation, and recruitment of maternal mRNAs (Cran *et al.* 1988; Malcuit *et al.* 2006; Schultz and Kopf 1995; Swann and Yu 2008). These Ca^{2+} oscillations also transfer immediately to the mitochondria where they directly stimulate

mitochondrial activity and adenosine triphosphate (ATP) production (Dumollard *et al.* 2004). Following decondensation of the sperm chromatin and pronuclei formation, the male and female pronuclei containing the sets of haploid chromosomes migrate to the centre of the zygote via the action of microtubules (Austin 1961). This is where they fuse, undergo DNA replication of the haploid chromosomes and prepare for the first mitotic division. The two nuclear envelopes subsequently break down, and the condensed chromosomes of both paternal and maternal origin align on a common spindle and move to opposing poles of the cell, completing meiosis. Cleavage then occurs, resulting in a 2-cell embryo with each cell containing a new diploid genome (Maro 1986) (**Figure 1-1**).

1.2.3 Embryo development

The conceptus continues its cellular division, the rate being specific for each particular species. Each cell undergoes a series of divisions called 'cleavage', during which time the total size of the embryo does not change. Thus with each division the size of the cells, or blastomeres, is reduced, which assists in restoring the exaggerated cytoplasmic:nuclear ratio back to normal (Johnson 1988).

Although the cytoplasm of the conceptus contains invaluable materials that assist in cleavage division, it has been shown that from as early as the 2-cell stage in the mouse (although some studies in the mouse have demonstrated that transcription can begin from as early as the zygotic stage, but the rates are extremely low) and between the 4 and 8 cell stage in the human, the actual genes of the conceptus itself play a larger role in development, as the embryo undergoes the switch from maternal genome control to embryo genome control with maternal transcripts being degraded and being replaced by those of embryonic origin (Flach *et al.* 1982; Schultz 1993; Schultz 2002; Schultz *et al.* 1999; Telford *et al.* 1990)(**Figure 1-1**). At these early stages of cleavage development significant epigenetic modifications also occur. At fertilisation the paternal genome exchanges protamines for histones and undergoes active DNA demethylation; whereas at this stage the maternal genome remains virtually unchanged (Morgan *et al.* 2005). As early cleavage division occurs there is also passive demethylation of the maternal genome and further modifications to histones, although imprinting is maintained (Morgan *et al.* 2005) (**Figure 1-2**).

During the cleavage stage development the cells of the embryo are undifferentiated and are largely insensitive to growth factors and hormones. The embryo is also completely reliant on its mitochondria to produce energy via oxidative phosphorylation of pyruvate and cannot metabolise glucose because of a possible block to the regulatory glycolytic enzyme phosphofruktokinase (PFK) (Barbehenn *et al.* 1974). Interestingly evidence has also shown that there may also be a block at the plasma membrane and/or hexokinase whose activity increases during development (Barbehenn *et al.* 1978). The block to glucose utilisation before blastocyst formation may actually be due to insufficient capacity of glucose transporters and hexokinase to produce enough glucose-6-phosphate to activate PFK allosterically and

that glucose transporter's may be important in determining metabolic priorities of the embryo (Pantaleon *et al.* 1997; Pantaleon and Kaye 1998).

The early embryo is also considered to be metabolically quiescent, in that it has a low metabolic rate and has low biosynthetic activity which results in a high ATP:ADP ratio. In fact its respiratory quotient is similar to that of bone (Leese 1991; Leese and Barton 1984). The cleavage stage embryo also appears to be more susceptible to stress exposure, as it has a limited ability to regulate against alterations in pH, osmotic stress and reactive oxygen species (Baltz *et al.* 1991b; Harvey *et al.* 1995; Lane 2001).

Following the cleavage stages, the pre-implantation embryo undergoes a change in morphology by compacting and forming a morula, at which stage the embryo has reached the cornua of the uterus. Compaction typically occurs at the 8-cell stage in the mouse and between the 8 and 12 cell stage in the human. It involves the blastomeres flattening into one another to maximise cell-cell contact, resulting in the polarisation of the cells (Gallicano 2001; Ziomek and Johnson 1980). This interaction is mediated by E-cadherin, which assists in the epithelial polarisation of the blastomeres (Khang *et al.* 2005). Here the embryo also shows a marked increase in its biosynthesis capacity, with net synthesis of RNA and protein increasing, transport of amino acids and nucleotides escalating, as well as changes in the synthetic patterns of phospholipids and cholesterol (Johnson 1988). The process of compaction results in the formation of a transporting epithelium, which creates a barrier between the reproductive tract and the internalised cells; however, despite there now being structural differences between the polar and apolar cells, all cells are still totipotent (Johnson and Ziomek 1982).

Once compaction has occurred the embryo now begins the process of cavitation, which results in the formation of the blastocyst. Initially the outer cells of the embryo begin to elaborate their junctional complexes, in particular ion transport systems and tight junctions in preparation for cavity formation (Watson *et al.* 2004). At this stage the Na/K-ATPase establishes and maintains an ionic gradient across the trophectoderm, which promotes water accumulation across the epithelium due to osmosis (Watson *et al.* 2004). This, combined with the tight junctions, maintains this water in the centre of the embryo, resulting in a fluid-filled cavity.

It is at this stage that the embryo undergoes cellular differentiation into two distinct cell types: inner cell mass (ICM) and trophectoderm (TE). The TE forms the outer rim of the embryo, surrounding the blastocoelic cavity, which contains blastocoelic fluid, and the ICM is located eccentrically within the blastocoelic cavity against the TE (**Figure 1-1**). As cell differentiation occurs, epigenetic events also transpire, as the genome is *de novo* methylated, with the trophectoderm being hypomethylated and the inner cell mass being hypermethylated. These early cell lineages have now set up the methylation status of their somatic and placental derivatives (Morgan *et al.* 2005).

The major difference between these two cell types is that the TE are now committed to certain differentiative pathways where they will eventually form extra-embryonic tissue (placenta and yolk sac) (Gardner 1975), whereas the ICM is still totipotent and will eventually form the fetus as well as contributing to the yolk sac and allantois (Gardner 1975; Gardner and Rossant 1979).

Throughout development the embryo remains encased in the zona pellucida, which has two roles. First, it prevents the blastomeres from separating during early cleavage, prior to compaction, which in turn assists in preventing the formation of two distinct cell groups. These could then result in the formation of monozygotic twins. Second, it prevents the adhesion of two genetically distinct embryos from sticking together and forming a single chimaeric embryo composed of two sets of cells, each with its unique genotype (Johnson 1988).

As the blastocyst enters the uterus it begins to interact with the uterine endothelium, and it is this local interaction that effects implantation. In the mouse the process of implantation begins with the loss of the zona pellucida, due to digestion by proteolytic enzymes secreted by the uterine endothelium or the trophectoderm (McLaren 1970; Sawada *et al.* 1990). It is also believed that hydrostatic pressure exerted by pulsations from the expanding and contracting blastocyst may also assist in zona pellucida shedding (Cole 1967). In the uterus the embryo begins to engage in an elaborate interaction with the maternal environment where messages are transmitted in a bi-directional loop. Initially the embryo establishes physical and nutritional contact with the maternal endometrium. The uterine environment contains oxygen and nutrients and the blastocyst actively acquires ions and nutrients via specific transport mechanisms, while oxygen and carbon dioxide exchange is diffusional. As mentioned above, at the site of implantation of the blastocyst the zona pellucida is digested, and trophectoderm cell processes can establish direct contact with the luminal epithelium and become firmly adherent to each other via interlocking microvilli (Johnson 1988). This interaction induces the initial stages of vascularisation and differentiation in the underlying stromal tissue leading to the establishment of the maternal component of the placenta (Johnson 1988).

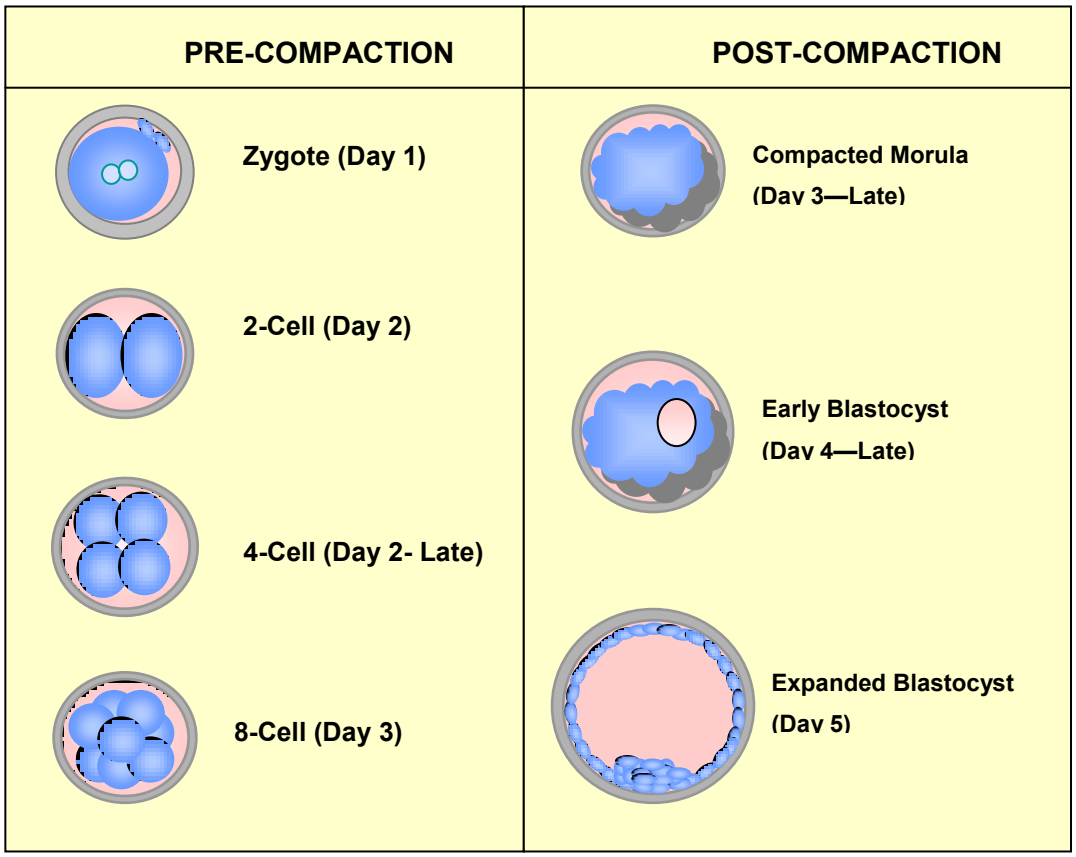

Figure 1-1: Diagram depicting cellular divisions of a mouse embryo from fertilisation on Day 1 to the Blastocyst stage on Day 5. Zygote (Day 1): fertilised egg, the second meiotic division is complete leading to the formation of the second polar body and the male and female pro-nuclei. 2-cell (Day 2): initial cellular division has occurred and genome activation occurs in the mouse. 4-cell (Day 2-late): cleavage to 4-cells and genome activation begins to occur in the human. 8-cell (Day 3): cleavage to 8-cells. Compacted moruls (Day 3-late): after the 8-cell stage the embryo undergoes compaction, cells polarise and flatten maximising cell contacts. Early Blastocyst (Day 4-late): Fluid is secreted internally to form a blastocoelic cavity and cell differentiation occurs. Expanded Blastocyst (Day 5): the blastocoelic cavity is enlarged and cell differentiation has occurred giving rise to two cell types; trophectoderm (TE) cells surrounding the exterior and an eccentrically located inner cell mass (ICM) cells. The TE gives rise to placenta tissue and the ICM gives rise to fetal tissue.

1.3 The *in vivo* embryo environment

The female reproductive tract is a dynamic and constantly changing environment. The composition of fluid changes between the oviduct and the uterus providing the embryo with nutrients that it needs to assist in development and differentiation in a stage-specific manner. Oviduct fluid is the environment in which the cumulus-oocyte complex is transported following ovulation, sperm transport, fertilisation and where embryo early development takes place, whereas the uterine environment is the site of compaction (species dependent), blastocyst development, implantation and invasion (Leese 1988).

As mentioned earlier, the early and late stage embryo differ in many aspects, including preferred metabolic substrate (Gardner and Leese 1988; Hardy *et al.* 1989; Leese *et al.* 1993), active genome (maternal or embryonic) (Braude *et al.* 1979), mitochondrial structure (Hillman and Tasca 1969) and level of oxidative capacity (Leese 1991). The pre-compaction stage embryo also lacks efficient regulatory mechanisms for pH (Baltz *et al.* 1991a; Baltz *et al.* 1991b; Lane *et al.* 1999b; Phillips and Baltz 1999a) and reactive oxygen species (ROS) (Harvey *et al.* 1995). Because of these differences in physiology it's logical to expect that the environment in which they develop are also different.

Two of the main differences between oviduct and uterine fluids are carbohydrate and amino acid content, which reflect the changes in the metabolic requirements of the developing embryo. The early embryo (pre-compaction) requires pyruvate and lactate as its primary source of energy, and, hence, the concentration of pyruvate and lactate are at their highest concentrations in the oviduct fluid and are around 0.3mM and 5mM, respectively (Gardner and Leese 1990; Leese and Barton 1984) (Figure 1-2). Previous research has shown that the cumulus mass surrounding the oocyte and early embryo metabolise glucose to further increase the concentration of pyruvate and lactate, with lactate being the primary metabolite produced (Leese and Barton 1985). This in turn maintains the early embryo in an environment rich in lactate and pyruvate until the cumulus disperses (Gardner and Leese 1990). The concentrations of pyruvate and lactate in human uterine fluid were found to be 0.1mM and 5mM, respectively, and did not vary with any stage of the cycle (Gardner *et al.* 1996). Therefore the pre-compaction stage embryo is exposed to decreasing concentrations of pyruvate and lactate as it moves from the oviduct to the uterus.

Glucose concentrations have similarly been measured in the oviduct and uterus. The concentration of glucose in the oviduct fluid of the mouse (Gardner and Leese 1990) human (Gardner *et al.* 1996), and pig (Nichol *et al.* 1992) has been measured to be between 1-3mM; however, this has been shown to be reduced on the day of ovulation to 0.5mM (Dickens *et al.* 1995). This reduction in glucose is consistent with glucose utilisation by epithelial cells of the tract due to increasing energy demands of secretory activity and muscular and cilia movement (Leese 1983; Leese 1988). The presence of the cumulus in the oviduct immediately after ovulation also further depletes glucose concentration (Gardner and Leese 1990; Leese and Barton 1985), and therefore the concentration of glucose in the oviduct fluid is at its lowest when the oocyte and early embryo are residing there.

Studies of uterine fluid have been determined in both the bovine and the human (Gardner *et al.* 1996; Hugentobler *et al.* 2008). Unlike the oviduct, the concentration of glucose does not appear to fluctuate and has been found to range between 3mM and 5mM (Gardner *et al.* 1996). Therefore, the pre-implantation embryo appears to be exposed to increasing concentration of glucose as it progresses along the reproductive tract, which reflects the change in energy requirements by the developing embryo as it gradually acquires the capacity to metabolise glucose as development proceeds (Figure 1-2).

Amino acids also play an important role in pre-implantation embryo development and are also found at varying concentrations within the reproductive tract. Multiple studies have assessed amino acid levels within the tract of several species, as well as within the embryo itself, and have found that levels are remarkably similar across all species, with the amino acid composition of the embryo also having a high degree of homology with the levels in the reproductive tract fluid.

Similar to carbohydrate levels, these studies showed that the amino acid content of the reproductive tract fluid changes significantly from the oviduct to the uterus. Interestingly, those amino acids that are at high levels in the oviduct have homology to Eagle's non-essential amino acids (defined for their inability to support growth of a cell line in culture), while those amino acids that are classified as essential were increased in the uterus (Eagle 1955; Gardner and Lane 1993b). Both essential and non-essential amino acids benefit pre-implantation development of embryos, albeit at different stages and by different mechanisms (Lane and Gardner 1997a)

Glycine was shown to be the most abundant amino acid, contributing to 30-60% of the total amino acid pool within the tract (dependent on day) (Jaszczak S 1972; Miller and Schultz 1987; Schultz *et al.* 1981). The oviduct and uterus amino acid pool also contains high levels of threonine, alanine, glutamate, glycine, glutamine, serine and taurine.

The amino acid concentrations of both the reproductive tract and the embryo change considerably as embryo development occurs. Glycine is present in high amounts within the reproductive tract at all stages of development and is also at high levels within the egg; however, in the mouse, the glycine concentration decreases dramatically as development to the blastocyst stage occurs. This is due to an increased capacity for glycine transport as well as utilisation of glycine in metabolism and molecular synthesis (Hammer *et al.* 2000; Hobbs and Kaye 1985; Schultz *et al.* 1981). Taurine levels are also relatively high within the tract and the oocyte, although they decrease as embryo development progresses and are believed to play an important role in capacitation and fertilisation as well as sperm motility (Mrsny *et al.* 1979).

It is also believed that the blastocoel cavity pool of amino acids also plays an important role in maintaining embryo development. The development of an epithelium on the trophectoderm allows the transport of amino acids to occur and maintains a dynamic amino acid pool within the blastocyst cavity, which is constantly changing as development progresses (Miller and Schultz 1983).

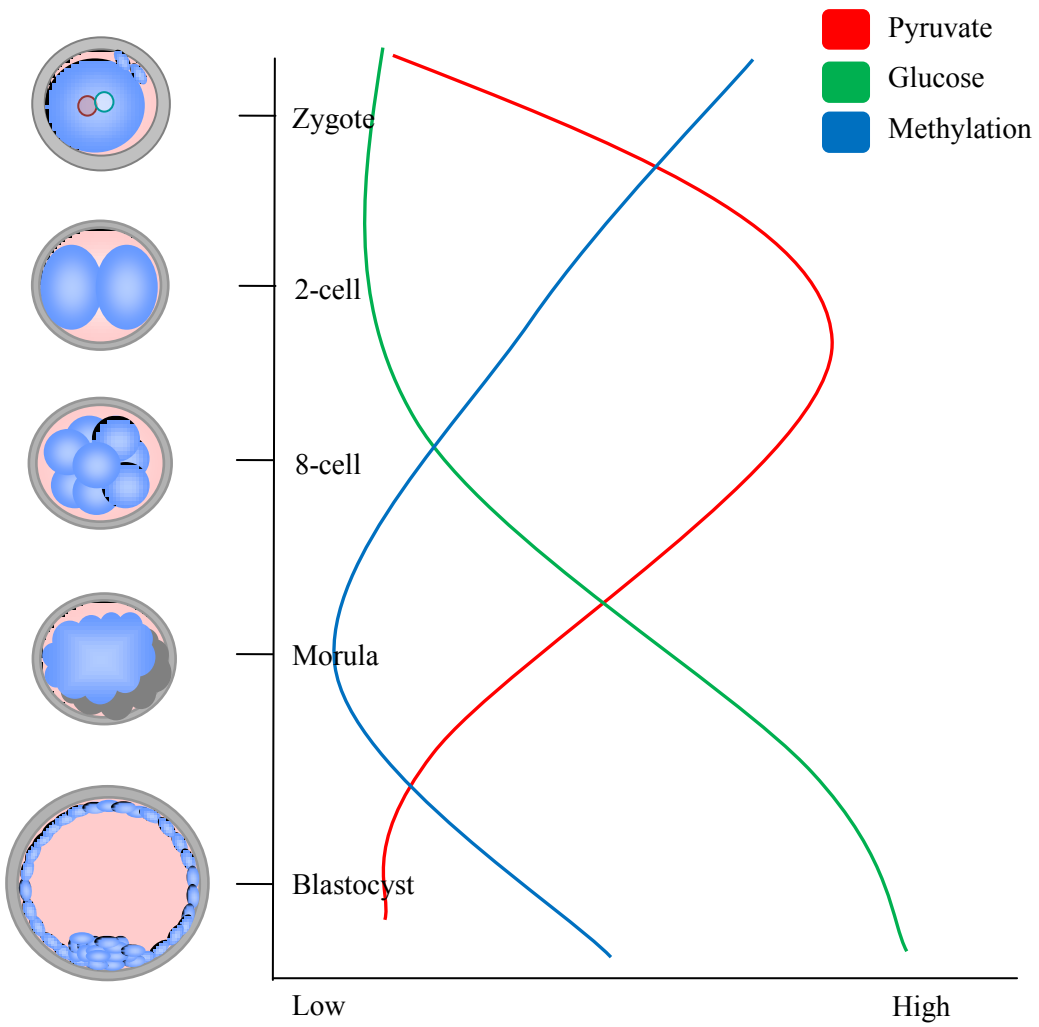

Figure 1-2: Schematic diagram depicting the changes in metabolic substrate requirements and methylation during pre-implantation embryo development.

1.4 Metabolism of the pre-implantation embryo

The *in vivo* developing pre-implantation embryo is contained in an undefined complex environment containing nutrients, including pyruvate, lactate and glucose, amino acids, ions and macromolecules, all of which are required to enable embryo development to occur (Leese 1988; Leese 1995). As mentioned earlier, pre-implantation embryo development can be divided into two different stages; pre-compaction and post-compaction. These stages roughly correlate with the time at which the embryo is in the oviduct and when it makes the transition into the uterus, respectively (Leese 1995). The early embryo is undifferentiated and divides repeatedly but displays no net growth, and genetic control relies largely on maternal mRNA before the embryonic genome is expressed (Brinster 1967; Leese 1995). At these early stages, metabolic activity in terms of oxygen consumption, DNA/RNA synthesis and protein synthesis is low (Leese 1991). In comparison, the later stages of embryo development display net growth and cell differentiation. Development is now regulated by the embryonic genome, and metabolism has increased dramatically (Leese 1995).

Cells generate energy by the hydrolysis of ATP, a product of the Embden-Meyerhof pathway of the TCA (tricarboxylic acid) cycle. Since ATP cannot be stored in the cell for any length of time, the changes seen in energy demand require changes in activity of energy-generating pathways. As the pre-implantation embryo develops, the energy requirements of the embryo also develop and increase. The oocyte and early embryo have the metabolic quotient similar to that of brain tissue (QO_2 of 4µg/mg dry weight/hour). In comparison, the blastocyst has a metabolic quotient similar to tumour cells and has increased six-fold compared to the early stages of development (Leese 1991). This increase in energy consumption is due to the increase in metabolically costly processes such as compaction and cavity generation.

1.5 Energy sources

1.5.1 Pyruvate and lactate

The pre-implantation embryo relies on different energy sources at different stages of development to generate ATP. As mentioned previously, the early stages of development require pyruvate as an energy source and it is the only know substrate, alongside of oxaloacetate, which can support the first cleavage division in the mouse (Biggers and Stern 1973). Pyruvate is oxidised directly by mitochondria via oxidative phosphosylation to yield ATP, and the early embryo can only utilise this mechanism to derive energy. As mentioned earlier, at these early stages, the embryo is unable to utilise glycolysis because of a possible lack of the regulatory glycolytic enzyme phosphofruktokinase and differential expression of glucose transporters (Barbehenn *et al.* 1974; Pantaleon and Kaye 1998).

Lactate can also be an important energy source; however, lactate cannot support the first cleavage division in the mouse embryo and causes abnormal changes in the pronuclei (Whittingham 1969).

Despite this, lactate can support development from the 2-cell stage and is a regulator of pyruvate uptake. Previous studies have shown that a greater proportion of 2-cell embryos develop when both pyruvate and lactate are in the media compared to when either energy source is present by itself (Brinster 1965c). This is because pyruvate facilitates the conversion of lactate to pyruvate, and, conversely, lactate regulates the uptake and metabolism of pyruvate and that regulation is based on the stage of embryo development (Biggers and Stern 1973; Lane and Gardner 2000a). Studies have demonstrated that pyruvate uptake at the pre-compaction stage is high and glucose uptake is very low; however, after compaction, a switch occurs and glucose uptake is high and pyruvate uptake decreases, with both these studies having high levels of lactate in the medium (Gardner and Leese 1986; Leese and Barton 1984). However, when pyruvate is present as the sole energy source and lactate is not present, the decrease in pyruvate uptake at the post-compaction stage is no longer evident. This demonstrates that metabolic switches in the mouse embryo can be dependent on the nutrients present in the culture media (Gardner and Leese 1988). Interestingly, these studies have been done using high concentrations of lactate, which do not represent the physiological condition *in vivo* (Gardner and Leese 1990). When physiological lactate concentrations are used, pyruvate uptake remains high at all stages of development (Gardner and Leese 1988) Similar outcomes have also been demonstrated in sheep and human models (Gardner *et al.* 1993; Gott *et al.* 1990; Hardy *et al.* 1989).

As mentioned earlier, the concentration of lactate can also influence the metabolic fate of pyruvate. Studies have demonstrated that increasing the concentration of lactate in the culture media results in a decrease in pyruvate oxidation at all stages of development. This is coupled with increased lactate oxidation, indicating substantial interaction between these two substrates in embryo metabolism (Wales and Whittingham 1973). One study has demonstrated that the early embryo and blastocyst have different abilities to metabolise pyruvate in the presence of lactate. For the zygote, increasing concentrations of lactate resulted in decreasing levels of pyruvate oxidation, conversely resulting in an increase in conversion of pyruvate to lactate. However, in the blastocyst, despite high concentrations of lactate, oxidative phosphorylation of pyruvate remained high. Therefore it was concluded that the zygote and blastocyst have different capabilities for regulating metabolism (Lane and Gardner 2000a). This variation in ability to utilise pyruvate at the earlier stages, in the presence of high levels of lactate, is believed to be due to competition for the carboxylic acid transporter—the higher the concentration of lactate, the more competition for the transporter, the less pyruvate can bind. Once pyruvate is inside the embryo, however, its fate is determined by cytosolic factors, which vary in the early stage embryo compared to the blastocyst, such as *in situ* regulation of the enzyme LDH (lactate dehydrogenase) which converts pyruvate to lactate and the ability to regenerate metabolic intermediates such as NAD+ and NADH (Lane and Gardner 2000a).

1.5.2 Glucose

Glucose metabolism is also used to generate energy; however, this occurs in the late stage embryo only. Glucose is used by almost all mammalian cells as the primary energy source and so the early embryo is unique in that it lacks the ability to metabolise glucose. Experiments have demonstrated that the zygote and 2-cell mouse embryo cannot develop in the presence of glucose alone (Biggers *et al.* 1967; Brinster 1965b; Whitten 1957). In contrast the 8-cell mouse embryo will develop in the presence of glucose alone, and in other species, such as the rabbit, only the blastocyst stage will develop in the presence of glucose alone (Brinster and Thomson 1966; Daniel and Krishnan 1967; Whitten 1956). Glucose is taken up by the cells and converted to pyruvate by a series of reactions of the Embden-Meyerhof pathway in the cytosol. This results in the production of 2 ATP molecules. Pyruvate is then converted to acetyl-CoA and enters the TCA cycle and, in the presence of oxygen, is oxidised to produce CO_2 and H_20 within the mitochondria. Complete oxidation of glucose produces 38 ATP molecules, two in glycolysis and 36 in the TCA cycle.

1.5.3 Oxygen consumption

The best indication of an embryo's ability to produce ATP is oxygen production, and the overall pattern of oxygen consumption across all species measured is very similar (Houghton and Leese 2004). Oxygen consumption remains relatively constant from the 1-cell stage up until compaction and then increases dramatically at the blastocyst stage (Houghton *et al.* 1996). Following implantation, oxygen consumption drops back down to the levels observed in pre-compaction. Interestingly, it has been shown that at the blastocyst stage, although glucose consumption increases, it accounts for only 17% of all ATP produced and oxidation of pyruvate accounts for approximately 40% of ATP produced (Houghton *et al.* 1996). Therefore the difference in ATP production is most likely due to the oxidation of other, as yet unidentified, sources such as serum albumin or endogenous fat (Houghton and Leese 2004). It is also believed that much of the residual oxygen utilised at the blastocyst stage may be accounted for by reactive oxygen species (ROS) formation. One study has shown that approximately 40% of oxygen consumed by the blastocyst was utilised via non-oxidative mechanisms such as ROS generation (Trimarchi *et al.* 2000).

1.5.4 Amino acids

Amino acid transport and metabolism plays a vital role in embryo development, as mentioned above; the embryo requires different amino acids at different stages of development. Multiple studies have assessed the activity of varying amino acid transporters within the developing embryo to determine their role in regulating amino acid uptake. Correlations between developmental changes in Na^+-dependent transport activities for taurine, glycine and aspartate and changes in internal content of

these amino acids within the embryo have been previously shown (Van Winkle 2001). Similarly, the amino acid content of the blastocyst increases when cultured in the presence of glycine, alananine, glutamine, taurine and glutamate from the 2-cell stage, and systems for their transport are present during pre-implantation development.(Van Winkle and Dickinson 1995; Van Winkle *et al.* 1991).

Different stages of development also coincide with differences in Na^+-dependent transporter expression. For example, Na^+-dependent glycine transporter is expressed throughout cleavage development but is not expressed at the blastocyst stage. Although there is no net protein increase during early embryo development, amino acids will be taken up and metabolised—a process which requires ATP (Houghton and Leese 2004). Interestingly, it has been demonstrated that amino acid metabolism is markedly different between those embryos that develop to the blastocyst stage and those that undergo developmental arrest, even though at the early stages they appear morphologically normal (Houghton and Leese 2004). It has been shown that embryos that are metabolically quiescent (have a lower amino acid turnover) are the ones that have the propensity to develop to the blastocyst stage, and those with high amino acid turnover tend to undergo development arrest (Houghton and Leese 2004).

1.6 pH

Intracellular pH (pH_i) is known to regulate numerous biochemical and enzymatic reactions and plays an important role in maintaining overall cellular homeostasis. In all cells alterations in pH can induce severe perturbations in enzyme activity, cell division, protein synthesis, and membrane transport (Boron 1986), and therefore inhibit normal cell function.

Robust regulation of pH_i within most cells occurs at the cell membrane via specific transporters, which is essential in maintaining optimal physiological activity (Roos and Boron 1981). Most mammalian cells utilise the Na^+/H^+ antiporter to regulate acid ranges, while HCO_3^-/Cl^- transporters regulate against alkaline loads (Boron 1986). Both of these regulatory mechanisms are very efficient and are able to return pH to homeostatic levels within two to three minutes (Boron 1986). Despite this, most cellular processes are exquisitely pH-sensitive, and cellular growth and proliferation can be significantly impaired if pH_i regulation is perturbed and pH_i is disrupted (Grinstein *et al.* 1989; Kapus *et al.* 1994). Also dysregulation in somatic cell pH_i is also associated with decreased cell survival (Pouyssegur *et al.* 1984).

The oocyte and pre-implantation embryo are significantly different from somatic cells in their ability to regulate pH_i. As mentioned earlier, mammalian oocytes grow within the ovarian follicle and are surrounded by granulosa cells. In the mouse, oocyte growth takes approximately 15 days during which time the oocyte grows from approximately 15μm in diameter to 70-80μm. This growth is associated with increased proliferation of the surrounding granulosa cells (Fitzharris and Baltz 2006). Studies have demonstrated that the smaller mouse oocyte has limited ability to regulate against

changes in pH_i and that it begins to acquire the mechanisms to regulate against alteration in pH_i as it grows and matures. It is the granulosa cells themselves that regulate against changes in pH and protect the oocyte for acid and base loads (Erdogan *et al.* 2005). By the time the oocyte is a fully grown germinal vesicle stage oocyte, it has developed robust mechanisms for regulating pH_i (Erdogan *et al.* 2005; Fitzharris and Baltz 2006). Interestingly, at the next stage of development, the MΠ stage, the oocyte has virtually no ability to regulate against changes in pH. Mouse MΠ oocytes completely lack HCO_3^-/Cl^- exchanger activity and cannot recover from alkalosis (Phillips and Baltz 1999a). This has also been demonstrated in the hamster oocyte; however, the human oocyte appears to be able to recover after alkaline exposure (Dale *et al.* 1998; Lane *et al.* 1999a). The MΠ mouse, hamster and human oocyte also lack the Na^+/H^+ exchanger and have no ability to regulate against acid loads (Fitzharris and Baltz 2009; Lane *et al.* 1999b; Phillips *et al.* 2000). So somewhat perplexingly the fully grown germinal vesicle stage oocyte is equipped with the ability to regulate pH_i and yet this ability is lost during meiotic maturation and is not regained until after fertilisation. It is believed that the MAPK cascade, that is necessary for maintaining meiotic arrest, may have a novel role in controlling the regulatory mechanisms for maintaining pH_i during meiosis; however, this requires significantly more investigation (Fitzharris and Baltz 2009).

The pre-implantation embryo also appears to have less efficient mechanisms for dealing with alterations in pH. Immediately after fertilisation, the early embryo does not appear to have any regulatory mechanisms for controlling pH_i, making it extremely susceptible to acid changes (Dale *et al.* 1998; Lane *et al.* 1999b). Following pronuclei formation these transporters are activated; however, their activity has been shown to be inefficient at removing acid load within the murine embryo (Baltz *et al.* 1990; Baltz *et al.* 1991b). Recent studies however have now suggested that the presence of H^+-monocarboxylate transporters may also be responsible for regulating against acid load in 2-cell mouse embryos (Gibb *et al.* 1997). It is believed that these H^+ lactate co-transporters efflux a proton with lactate or pyruvate to maintain intracellular pH in the early embryo. These studies also contradict previous research by demonstrating that Na^+/H^+ exchanger isoforms are present in the 2-cell embryo (Barr *et al.* 1998; Gibb *et al.* 1997).

In contrast to the apparent limited ability of the early embryo to regulate efficiently against acid loads, activity of the HCO_3^-/Cl^- transporters appears to be more robust and is able to mediate a rapid specific recovery from alkalosis (Zhao and Baltz 1996). This ability to regulate alkaline loads has been linked to the increase in pH that the embryo is exposed to as it moves from the oviduct and into the reproductive tract (Bishop 1957).

It has been suggested that the post-compaction stage embryo may be better able to deal with regulation of intracellular pH compared to the uncompacted embryo (Edwards *et al.* 1998b). Using a radiometric fluorophore and exposure to the weak acid lactate, Edwards *et al*. assessed the ability of embryos pre- and post-compaction to regulate pH_i. This study showed that at concentrations greater than 5mM, lactate caused the pH_i of the zygote to be significantly more acidic as well as showing that through their acidifying effects, weak acids also induce a reduction in glycolytic activity in the pre-

compacted embryo. In contrast, the pH_i of compacted embryo remained unchanged in the presence of 5mM lactate and incubation with a weak acid did not affect the rate of glycolysis within the morula. Furthermore, addition of cytochalasin B to decompact the embryo resulted in the morula losing the ability to regulate its intracellular pH, which demonstrates that the process of compaction enables the pre-implantation embryo to be better able to deal with regulation of pH_i (Edwards *et al.* 1998b). To further support this theory, results from another study have shown that 2-cell mouse embryos that were exposed to a weak acid (ammonium) were unable to recover their pH_i to normal levels even after 60 minutes (Lane and Gardner 2003).

Because of the inability of the pre-compaction stage embryo to regulate pH_i, studies have assessed the effects of an alteration of pH_i on cellular function. Perturbations in pH_i have also been linked to disruptions in cellular organisation where hamster 2-cell embryos were exposed to a weak acid or weak base that induced disruptions in mitochondrial distribution and microfilament organisation. Exposure to either the weak acid or weak base changed the nuclear:cortical ratio of mitochondria within the embryo, indicating that the mitochondria were dispersing away from the nucleus and into the intermediate cytoplasm. It has been hypothesised that this dispersion disrupts efficient delivery of ATP to the nucleus, which leads to a loss of energy needed to support transcriptional activity and developmental delay (Squirrell *et al.* 2001). These studies have also linked alterations in pH_i with reductions in the ability for embryos to develop normally in culture (Edwards *et al.* 1998b; Lane *et al.* 1998a; Squirrell *et al.* 2001).

It is however not always important to maintain constant pHi. As in somatic cells, pH_i also plays an important role in cell signalling within gametes and embryos. In later development, pH_i is a trigger for the later developmental events of neural induction and posterior axial development (Gutknecht *et al.* 1995; Sater *et al.* 1994). An acute rise in pH_i is also associated with fertilisation in the egg of the sea urchin among other species and is important for subsequent development (Dube and Eckberg 1997; Johnson and Epel 1976; Webb and Nuccitelli 1981).

These results combined suggest that control of pH_i is essential for normal gamete and embryo development and due to the fact that small changes pH_i of only around one tenth of a pH unit are correlated with very large changes in cell function and metabolism such as protein synthesis respiration, glycolysis and nucleic acid synthesis, it is very important that the embryo be maintained in an environment free from pH stress (Boron 1986; Busa and Nuccitelli 1984; Roos and Boron 1981).

1.7 Embryo culture

The ability to culture tissue samples was pioneered in the early 1900s, and during this time the importance of using a biologically defined media was acknowledged. Biologically defined media was beneficial in that it could be reproduced at different times and in different laboratories, as it followed a set formula. It can also be varied in a controlled manor and is free of enzyme activities that may

interfere with the responses being studied (Biggers 1971). The design of chemically defined media accelerated in the 1940s, as media was designed to support the growth of plant and animal cells. Following this the culture of mammalian pre-implantation embryos began in the late 1940s. At this time ill-defined culture media were used for embryo culture that primarily occurred in the rabbit (Biggers 1987).

The possibility of experimentally studying the mammalian pre-implantation embryo *in vitro* was first realised by Whitten, who reported that 8-cell mouse embryos could develop to the blastocyst stage when cultured in a simple chemically defined media based on Krebs-Ringer bicarbonate, supplemented with glucose, bovine plasma albumin, antibiotics and utilising a CO_2-bicarbonate buffering system (Whitten 1956). At this point embryo stages prior to the 8-cell would not grow; however, the crucial observation was made the following year that lactate was needed to support early embryo development, and, for the first time, 2-cell embryos were able to be grown to the blastocyst stage in culture (Brinster 1963; Whitten 1957). Subsequently, it was also shown that pyruvate, oxaloacetate and phosphoenolpyruvate could substitute lactate and that when used in combination, pyruvate and lactate in the media results in increased blastocyst yield from 2-cells when they are used alone (Brinster 1965c). These studies were of fundamental importance, as they demonstrated that the early embryo was unlike a somatic cell in that it cannot utilise glucose for development and, instead, requires a more simple sugar in the form of lactate and pyruvate. The following year the transfer of these blastocysts into surrogate mothers produced offspring that appeared outwardly normal, demonstrating that the culture environment produced viable blastocysts (McLaren and Biggers 1958) .

These pioneering studies lay the foundation for the discovery of the exact substrate requirements of the mammalian embryo and assisted in overcoming the developmental blocks seen in varying stages of embryo development. It was discovered that mouse embryos from the 1-cell to 2-cell stage require pyruvate to develop, and this requirement seems to be universal among all mammalian species: it is now an essential component of all media for pre-implantation embryo development (Whittingham and Biggers 1967).

During the 1960s and 1970s there were many detailed studies trying to understand more about basic embryo physiology. The involvement of energy substrates on epigenetic regulation was of particular interest. When pyruvate and lactate were supplied, mouse zygotes would cleave to the 2-cell stage; however, further development was blocked unless the 2-cells were then transferred to explanted oviduct cultures (Whittingham and Biggers 1967) and only pyruvate would support the first cleavage division and nuclear maturation of mouse oocytes. From this it was deduced that the metabolism of the oocyte and the 1-cell embryo were virtually the same. At this point it was also discovered that the ability of the embryo to survive the culture period was highly reliant of the mouse strain. Using F1 hybrids of inbred strains it was possible to grow embryos from the 1-cell to the blastocyst in simple media of a modified salt and protein composition (Whitten and Biggers 1968). However 1-cell embryos from random-bred strains would not develop and it took another 20 years of research before this problem was overcome. This research led to the discovery of the ‘substrate triad’ of pyruvate,

lactate and glucose, as together they could support the development of some inbred mouse strain embryos from the 1-cell to the blastocyst (Bavister 1995).

Subsequently, the focus began to shift to human research, as it was discovered that human embryos could be fertilised *in vitro* (Edwards *et al.* 1969). During this period a variety of media were used involving both simple and complex media formulations containing BSA or serum. As early as 1970 it was reported that the human embryo could be cultured up to the 16-cell stage (Edwards *et al.* 1970). Following this progress many studies were undertaken to try and support the *in vitro* development of species, other than the mouse and rabbit, using semi-defined serum-free media; however, this was met with limited success due to the poor knowledge of substrate requirements for each species. Most studies from this period appeared to assume that substrate requirements for all species were similar to that of the mouse and rabbit. But it has now become apparent that although some aspects are the same, each species needs to be assessed independently and the media modified depending on substrate requirements (Bavister 1995).

Although some success was found in growing embryos from a variety of species, such as monkey and cattle, these were mainly grown in complex media containing blood serum. They resulted in low blastocyst yield and did not increase the knowledge on the varying requirements of embryos from different species (Bavister 1995). It was also noted, then, that the majority of useful laboratory animals displayed 'blocks in development' that precluded complete pre-implantation development in culture. A variety of methods were trialled to try and overcome these blocks, such as transferring cytoplasm from non-blocking embryos into those displaying developmental blocking; however, the reason for these blocks was unknown (Muggleton-Harris *et al.* 1982).

The need to overcome these developmental blocks was the focus of research during the late 1980s onwards. This was mainly due to the need to obtain complete pre-implantation development *in vitro* for the livestock industry, in particular using oocytes from slaughterhouse cattle ovaries. Unlike in the human and primates, early cleavage stage embryos of most species will not survive if transferred into the uterus. Thus it is necessary to support *in vitro* development up to stages (morula and blastocyst) that are compatible with non-surgical transfer (uterine). Complete embryo development was also required to obtain comparative data between species, other than inbred mice and rabbits, for research purposes and also for other livestock industries.

The developmental blocks were attributed to artefacts of the culture environment, and the stage of the block appeared to be species-specific (Bavister 1995). It was also noted that the blocks are perhaps genetically derived. As mentioned previously, inbred mouse strains were able to develop from the 1-cell stage to the blastocyst; however, random-bred strains displayed the 2-cell block. Because these blocks were believed to be maternally derived, this has led to numerous advances in our understanding of the epigenetic regulation of embryo development. Interestingly, it was discovered that these blocks in development coincided with the activation of the embryonic genome, which occurs at different

stages depending on the species (Telford *et al.* 1990). It was also noted that some of these blocks also occur when the embryo would be transitioning from the oviduct to the uterus.

Many studies have shown that the environmental requirements of the pre-implantation embryo change as the embryo progresses along the female reproductive tract. Changes in morphology and ultrastructures accompany alterations in energy transport mechanisms and responsiveness to growth factors as the embryo develops and moves from the oviduct to the uterine environment (Bavister 1995). It has also been shown that changes in mitochondrial morphology accompany embryo development and that these changes may be particularly significant in relationship to the perturbed metabolism that is seen in cultured embryos (Hillman and Tasca 1969). From this it was hypothesised that perhaps the developmental blocks seen were due to inadequate energy production, as many studies have demonstrated that the addition of certain nutrients or energy substrates can overcome developmental blocks in embryo development (Brinster 1963; Whitten 1957).

Continual research into the requirements of the developing embryo then led to the generation of more defined culture media, which can support the growth of a mammalian embryo from the zygote to the blastocyst stage; however, this growth was associated with decreased development and viability along with altered gene expression and perturbed mitochondrial homeostasis (Gardner and Lane 1993b; Lane and Gardner 1994).

To try and alleviate these problems much research has been conducted into the formulation of culture media designed around the requirements of the embryos. One approach has been to develop media that more closely reflect the composition of the reproductive tract. As mentioned earlier, the initial culture media designed to support mouse embryo development was very simple in its design; however, over time culture media systems have been tailored to the metabolic and nutritional requirements of the embryo. Investigations into the components found in uterine fluid and the oviduct demonstrated that there are significant changes in the concentrations of metabolites and nutrients such as lactate, pyruvate and glucose within the tract, and that changes in these concentrations not only occur because of location but also during the stage of the menstrual cycle (Gardner and Leese 1990; Leese 1988).

Furthermore, it was also discovered that amino acids play a crucial role in embryo development, and their concentrations also vary at different regions in the tract, such that a specific group of amino acids are required for early embryo development; however, late stage embryo development requires a different subset of amino acids (Bavister and Arlotto 1990; Gardner and Lane 1996; Gardner *et al.* 1996; Gwatkin 1969).

These studies eventually lead to the design and implementation of sequential culture media, which was tailored to meet the metabolic and nutritional needs of specific stages of embryo development (Gardner 1998a). This culture media design was an improvement on the current human culture media designs, which were single phase, and, although they were able to support blastocyst development, they were based on somatic cell requirements and did not take into account the different nutritional needs of different stages of embryo development (Menezo *et al.* 1984; Quinn 1995; Quinn *et al.*

1985). Sequential media were then used to produce viable pregnancies and live deliveries after transfer of single human blastocysts, and the media were then marketed (Barnes *et al.* 1995; Pool 2004). Since then sequential culture media have been continually improved. Other single phase culture media have since been modified and altered into a sequential phase to try and mimic the *in vivo* environment, resulting in improved embryo homeostasis and viability followed by increased pregnancy and live birth rates after transfer (Gardner and Lane 1998; Lane *et al.* 2003).

1.7.1 Amino acids

As mentioned previously, the reproductive tract is a complex and dynamic environment and contains significant concentrations of amino acids (Fahning *et al.* 1967; Lane and Gardner 1997b; Miller and Schultz 1987). Furthermore, the oocyte and embryo itself maintain an endogenous pool of amino acids as well as possessing specific amino acid transporters (Van Winkle 1988). Therefore, many studies investigated the utilisation and importance of amino acids to the embryo and, in turn, their importance in embryo culture media (Bavister and Arlotto 1990; Brinster 1971; Carney and Bavister 1987; Gardner and Lane 1996; Gardner *et al.* 1994; Gwatkin 1969; Kane and Bavister 1988; Kane *et al.* 1986; Kaye *et al.* 1982; Lane and Gardner 1997a; Lane and Gardner 1997b; McKiernan *et al.* 1995; Pinyopummintr and Bavister 1996; Schultz *et al.* 1981). Multiple studies have shown that amino acid transport is critical for early embryo development, even a brief exposure to media without added amino acids can be detrimental to the developing embryo by reducing development to the blastocyst stage and decreasing blastocyst cell numbers (Gardner and Lane 1996).

Subsequently, the importance of amino acid inclusion in embryo culture media has been demonstrated in a variety of species, including the mouse (Biggers *et al.* 2000; Gardner and Lane 1993b; Ho *et al.* 1995; Lane and Gardner 1997a; Lee and Fukui 1996; Nakazawa *et al.* 1997; Summers *et al.* 2000), human (Devreker *et al.* 2001), sheep, cattle (Lee and Fukui 1996) and hamster (Kane and Bavister 1988; Kane *et al.* 1986). In particular, it has been shown that the addition of Eagle's non-essential amino acids and glutamine significantly increases the development of the mouse and hamster embryo from the zygote to blastocyst stage, alleviates the 2-cell block seen in CBAF1 mouse embryo development and increases the rate of compaction (Gardner and Lane 1993b; Gardner and Lane 1996; Lane and Gardner 1997b; McKiernan *et al.* 1995).

In contrast, zygotes cultured to the blastocyst stage in the presence of essential amino acids without glutamine show a significant reduction in blastocyst cell number (Gardner and Lane 1993b). It has also been shown that the presence of non-essential amino acids and glutamine from the zygote to 8-cell stage significantly increased fetal development after transfer. These studies have shown that while non-essential amino acids and glutamine support the highest levels of growth from the zygote to the 8-cell stage, the presence of essential amino acids for the first 48 hours of culture had no benefit to the embryo and reduced the stimulatory effect of the non-essential amino acids on development.

Furthermore after development to the 8-cell stage, non-essential amino acids and glutamine no longer stimulated cleavage or development of the ICM; however, they still promoted blastocyst development and hatching. In contrast to their lack of effect in earlier stages, essential amino acids from the 8-cell to blastocyst stage increased cleavage, development of the ICM and increased fetal development after transfer (Lane and Gardner 1997a). Similar results of improved embryo and blastocyst development and quality due to the addition of specific amino acids during different stages of embryo development have also been shown in the sheep and cow (Gardner *et al.* 1994; Pinyopummintr and Bavister 1996; Steeves and Gardner 1999; Thompson *et al.* 1995).

The beneficial effect of amino acids is not believed to be solely due to their use as a substrate but also because of their ability to act as osmolytes and regulators of intracellular pH (Edwards *et al.* 1998a; Van Winkle *et al.* 1990). Unicellular organisms routinely use amino acids as buffers of intracellular pH, and it has been hypothesised that the pre-compaction embryo also uses a similar mechanism owing to the individual nature of each cell within the embryo and the lack of a transporting epithelium (Gardner 2008). Studies have also demonstrated that amino acids also play an essential role as chelators and anti-oxidants and can regulate metabolism and cell differentiation (Gardner 1998a; Lane and Gardner 1997a; Lane and Gardner 2005a; Lindenbaum 1973; Liu and Foote 1995; Martin and Sutherland 2001).

Traditionally embryos are cultured in media at approximately 37 °C (with some variation in this temperature depending on species), which is required for optimal embryo development as this is the temperature that they would naturally grow at *in vivo*. However, at 37 °C components of the media such as amino acids and vitamins begin to break down spontaneously, and amino acids break down to form ammonium.

Initial studies that assessed the effect of amino acids on embryo development discovered that if embryos were cultured in the absence of amino acids, culture media renewal at 48 hours did not alter blastocyst development. In comparison, if amino acids were present in the culture media, blastocyst development was significantly increased if the culture media was renewed after both 48 and 72 hours when compared to 96 hours in the same media.

Embryo quality was also improved after media renewal, with an increase seen in blastocyst cell number (Gardner and Lane 1993b). A subsequent study demonstrated that media renewal also resulted in increased implantation and fetal weights after transfer and reduced fetal defects when comparing to embryo cultured in the same media continuously (Lane and Gardner 1994). These studies determined that spontaneous breakdown of amino acids and de-amination of amino acids by the embryos themselves resulted in a toxic build-up of ammonium. In particular, glutamine is considered to be the most volatile amino acid and is responsible for the majority of ammonium produced (Gardner and Lane 1993b; Lane and Gardner 1995).

1.7.2 Ammonia and ammonium

Ammonium is formed in culture media by the spontaneous breakdown of amino acids and by the de-amination of amino acids by the cells of the embryo (although this is a relatively small contribution to the overall ammonia concentration), where the amino group is removed from the amino acid and converted to ammonia. Ammonia (NH_3) is normally encountered as a gas; it is also a proton acceptor. In water (pH 7), a very small percentage of NH_3 is converted into the ammonium cation (NH_4^+). The degree to which ammonia forms, the ammonium ion increases upon increasing the pH of the solution, at 'physiological' pH (~7.4), about 99% of the ammonia molecules are protonated (converted to NH_4^+). Therefore in embryo culture media (pH 7.2–7.4 at 5–7% CO_2) the ammonium concentration can increase quite substantially, as NH_3 is converted to NH_4^+ (Gardner and Lane 1993b). Temperature and salinity also affect the proportion of NH_4^+. The ammonium ion can act as a very weak Brønsted-Lowry acid in the sense that it can protonate a stronger base using any one of its hydrogen (H^+) atoms and convert back to ammonia. This means that the ammonium ion is a conjugate acid of the base ammonia.

In culture media the majority of ammonia and ammonium production is believed to be due to the most volatile amino acid, glutamine. The toxicity of glutamine in tissue culture media is well known and is attributed to the accumulation of ammonium, which arises because of its metabolism or breakdown (Heeneman *et al.* 1993; McLimans *et al.* 1981; Visek *et al.* 1972). In the absence of living tissue, ammonia forms by the spontaneous breakdown of glycine into ammonia and 2-pyrrolidone-5-carboxylic acid (non-metabolic ammonia) (Vickery *et al.* 1935 CCCXXIII). When tissue is present, ammonium also forms owing to the metabolism of glycine and other metabolites (metabolic ammonia) (Newsholme and Newsholme 1989).

Ammonia and ammonium are also by-products of embryo culture systems caused by embryo metabolism and amino acid degradation. Thus they can build up in culture media as well as increase in concentration in the tract after high protein diets (Gardner 2004; Gardner and Lane 1993b). In culture media the concentration of ammonium can increase significantly over time; and more so in certain media types such as KSOM^{AA} that can reach over 170µm after only 24 hours and 545.2µm after 120 hours, the concentration increasing linearly over time (Lane and Gardner 2003). The majority of this ammonium production is because of the presence of glutamine, a highly unstable amino acid. However when substituted with alanyl-L-glutamine or glycyl-L-glutamine, di-peptides of glutamine which have increased stability in culture, will result in significantly lower levels of ammonium production such as 20.2µm and 10.5µm ammonium after 120 hours in either Quinn Advantage or G1.2/G2.2 culture medium (Gardner and Lane 1993b; Lane and Gardner 2003). Interestingly, embryo culture media, which do not contain the stable form of glutamine, are still routinely used in both the research setting and human embryo culture. These media may result in reduced embryo viability and pregnancy outcomes in the human, as well as possibly confound experimental results in the laboratory (Virant-Klun *et al.* 2006).

The increase in the waste products, ammonia and ammonium, has been the focus of much attention because of their effect on embryo development and fecundity. Many studies have highlighted the detrimental effects of these by-products on embryo viability (outlined in the following sections); however, there is constant confusion around whether ammonia or ammonium concentration was measured. In many papers these two terms are used interchangeably and it is unclear what was actually measured. Because at physiological pH and in the presence of living tissue, ammonia is almost completely converted to ammonium, it makes sense that ammonium would be measured; however, in the majority of *in vivo* studies, it appears that ammonia is measured. If an increase in ammonia is observed, this would most likely indicate a large increase in ammonium as well, but this is not usually addressed in the studies. As a result of the confusion around the terminology it is often difficult to determine what has actually been measured.

1.7.3 Ammonium in culture

As mentioned earlier, amino acids have been shown to be essential components of culture media, as they play a key role in maintaining embryo pH and metabolism (Edwards *et al.* 1998b; Gardner and Lane 1993a; Lane and Gardner 1998) as well as acting as osmolytes (Baltz 2001; Van Winkle *et al.* 1990), energy substrates (Rieger *et al.* 1992) and chelators (Lindenbaum 1973). One of the observed side-effects of amino acid supplementation into the culture medium was a significant increase in ammonium levels during the culture period (Gardner and Lane 1993b; Lane and Gardner 1994; Lane and Gardner 2003).

Previous research in the mouse has shown that the presence of ammonium in the culture media during pre-implantation development, from the zygote to the blastocyst stage, can have a detrimental effect on the embryo, as it can decrease embryo cleavage and blastocyst development, decrease blastocyst cell number, alter gene imprinting and metabolism and increase apoptosis in a concentration-dependent manner (Gardner and Lane 1993b; Lane and Gardner 2003). Concentrations as low as 18.8μM can decrease the number of inner cell mass cells (ICM) within the resultant blastocysts and increase apoptosis, and 37.5μM and above decreases total blastocyst cell number (Lane and Gardner 2003).

Additionally, exposure to ammonium during the entire pre-implantation stage also decreases implantation rates and fetal development rates as well as increasing fetal abnormalities and decreasing fetal maturity after transfer (Lane and Gardner 1994; Lane and Gardner 2003). There is also evidence that the presence of ammonium in culture media can also increase the occurrence of birth defects such as exencephaly (Lane and Gardner 2003; Sinawat *et al.* 2003).

The effects of ammonium has also been assessed in ruminant species, as high levels have been linked to decreased fecundity in cattle due to increased protein consumption (examined in the following section). The level to which bovine embryos are susceptible to ammonia/ammonium *in vitro* is dependent on concentration, duration and stage of exposure (Hammon *et al.* 2000b). Studies that

have assessed ammonia concentration in bovine follicles have found that levels are highest in follicular fluid from the smallest follicles and decrease as follicle diameter increases with the average concentration in the smallest follicles (≤1mm) being 366±51 μM down to 33±10μM in the largest follicles (10–15mm). This data indicates that immature oocytes and follicular cells develop in a microenvironment that contains levels of ammonia greater than that of normal somatic cell environments. The reason for the varying levels is unknown; however, the hypothesis is that during the early follicular development, the higher ammonia levels may be a by-product of the high metabolic activity during follicular development, which is lost after the rapid accumulation of fluid in the antrum at the later stages (Hammon *et al.* 2000a).

The effect of varying concentrations (29μM to 356μM) of ammonium chloride in *in vitro* maturation (IVM) media on bovine oocyte maturation and subsequent blastocyst development has also been assessed. Bovine oocytes exposed to varying concentrations of ammonium during IVM did not display any alteration in subsequent embryo development when compared to controls, indicating that the cumulus-oocyte-complex can tolerate high levels of ammonium during IVM. This may be due to the presence of the cumulus cells, as they have a protective effect on the oocyte by limiting the impact of toxins on the oocyte itself. However, these conclusions as to the effect of ammonium on oocyte development during IVM used blastocyst morphology as the sole end-point. It is now well established that blastocyst morphology is a poor marker of embryo viability as often embryos exposed to sub-optimal conditions can form morphologically normal blastocysts however they are compromised at the metabolic and cellular level (Lane 2001). Therefore, whether the presence of ammonium *in vitro* during oocyte maturation affects viability remains to be determined, as it is possible that these embryos had reduced viability that was not detectable using morphology as a marker but may have surfaced after cell allocation studies or transfer.

The effect of ammonium on bovine *in vitro* fertilisation and culture has also been assessed (Hammon *et al.* 2000b). Here it was demonstrated that exposure to moderate concentrations of ammonium chloride (29–88μM) during fertilisation increased blastocyst development and hatching rates, while continuous exposure of embryos to moderate to high concentrations of ammonium chloride (29–356μM) increased the number of degenerate ova and decreased blastocyst development and hatching rates. Interestingly, continuous exposure during IVM, IVF and *in vitro* culture (IVC) to moderate levels (88μM) increased development to the morula stage and did not affect blastocyst development at any concentration used, which is perhaps indicative of an adaptation process (Hammon *et al.* 2000b). However, as morphology is now believed to be a poor marker of blastocyst homeostasis and viability, the true effect of incubation with ammonium during bovine IVM, IVF and IVC on bovine embryo quality can only truly be determined by assessing implantation and fetal development after transfer.

As mentioned earlier, to alleviate the build-up of ammonium in culture media, culture systems have been developed that contain a di-peptide of glutamine, the most volatile amino acid, which keeps

ammonium build-up to a minimum (Gardner and Lane 2003). The sequential media system G1/G2 (Vitrolife Pty Ltd) has maximal ammonium build up of 10.5±2.3μM after 48 hours incubation (Lane and Gardner 2003). This is compared to a medium without the stable form of glutamine that had levels of ammonium of approximately 250μM after the same incubation time (Lane and Gardner 2003).

Therefore, both the storage of media containing glutamine and the length of time that a medium is placed into the incubator before embryo culture can have a significant effect on the levels of ammonium produced and therefore on the embryo development and viability outcomes (Lane and Gardner 2003). Two examples of such likely ammonium toxicity come from the work of Gardner *et al.*, (1993) and Ho *et al.* (1995). They clearly demonstrate a reduction in cleavage rate and development over time of embryos cultured in a medium containing glutamine, indicating the build-up of a component in the medium that is inhibitory to the embryo (Gardner and Sakkas 1993; Ho *et al.* 1995). It is likely that this is a result of a build-up of ammonium. Further evidence of this is a recent report by Biggers *et al*., who demonstrated in the same medium, K-simplex optimised media (KSOM), that the replacement of glutamine with a stable derivative significantly improved embryo development (Biggers *et al.* 2004).

Even though media systems exist with stable forms of glutamine and therefore low ammonium production, many animal research facilities and human IVF clinics still use single phase systems in which ammonium levels can build up as high as 178μM after only 24 hours of culture and can reach 545μM after 120 hours culture (Lane and Gardner 2003).

Although much work has been done on the effect of ammonium on mouse and ruminant embryos, little attention has been paid to the effect of ammonium in the human IVF setting, despite the fact that ammonium build-up in human IVF media is highly probable. One recent study assessed the increase in ammonium production in a sequential media system (Medi-cult) in a human IVF clinic. In this study ammonium concentration was measured in media (M2: Post-compaction stage media) from Day 4 embryos after 24 hours of culture where normal ammonium concentration was set to ≤119μmol/L as per a ROC curve analysis. (In the mouse ≥75μM is detrimental to blastocyst cell number, ICM cell number, apoptosis and fetal development (Lane and Gardner 2003). Increased ammonium was defined as ≥119μmol/L and was found in 62% of all samples measured. In this study a statistically significant negative correlation was found between ammonium concentration in the media on Day 4 and blastocyst development with a reduction by 26%, regardless of whether the cycle was stimulated or natural (Virant-Klun *et al.* 2006). Increased ammonium also significantly increased the number of arrested embryos by 16%. Although this study cultured varying numbers of embryos per drop (1–13 embryos per 500μl drop), no correlation was observed between the number of embryos per drop and the ammonium concentration, indicating that the majority of ammonium build-up was due to spontaneous de-amination of amino acids. However, the mean ammonium concentration in media incubated without embryos was 56μmol/L, indicating that embryo amino acid metabolism does contribute to the overall ammonium concentration. Nevertheless, in this study, ammonium concentrations were not correlated to pregnancy rates.

Despite its obvious effect on the embryo, the mechanism by which ammonium causes these perturbations are currently unknown. It has been suggested that one mechanism may be by reducing intracellular pH, but this remains to be elucidated.

1.7.4 Ammonia/ammonium and urea in livestock

In ruminants, ammonia (the conjugate base of ammonium) and urea concentrations, as well as decreased uterine pH, are also believed to be involved in the decreased fecundity often observed in cows fed a high protein diet to increase milk production.

In order to sustain optimum milk production during early lactation, industry practice is to feed dairy cows increased nutrient density (often involving increased protein levels); yet, this has been linked to a decrease in fertility (Elrod *et al.* 1993). Through normal ruminal fermentation, RDP (ruman degradable protein) provides a source of ammonia for microbial protein synthesis, but some of the ammonia escapes incorporation by microorganisms and diffuses into the blood and is then converted to urea by the liver. The second form of urea that is formed is due to the de-amination of amino acids found in RUP (ruman undegradable protein). This leads to production of ammonia and ammonium ions that are converted into urea and excreted. Although the production of these toxins can be minimised by balancing the levels of RDP with the level of RUP, most high producing cows consume protein in excess of the amount required. Consequently, ammonia, ammonium and urea concentrations increase in accordance with intake and are often measured by assessing plasma urea nitrogen concentration (PUN) (Butler 1998).

High protein diets in dairy cows have also been linked to a decrease in uterine pH, with these cows not displaying the usual increase in uterine pH that occurs in the luteal phase between estrus and Day 7. Whether this is due to the increase in urea and ammonia/ammonium is currently unknown; however, it has been postulated that it may be owing to excess ammonium that is derived from glutamine de-amination (Elrod and Butler 1993; Elrod *et al.* 1993). These cows also display a decrease in fecundity, linking high PUN levels, from cows on a high protein diet, to a lower conception rate (42%) after AI (artificial insemination). Cows displaying PUN levels ≥16mg/dL have conception rates 30% lower than those with PUN <16mg/dL (9.9–16mg/dL: 72.5% <9.9mg/dL: 87.5%) (Elrod and Butler 1993).

This decrease in uterine pH and increase in plasma urea levels has also been associated with a concomitant decrease in oocyte and embryo quality. Since the greatest changes in the uterine environment occur during the mid-luteal phase, which is a critical period for embryo development in the cow, this change in the environment of the reproductive tract may also have an impact on embryo survival and viability (Blanchard *et al.* 1990; Rhoads *et al.* 2006; Sinclair *et al.* 2000). Furthermore, ammonia concentrations in follicular fluid were significantly increased in the high protein diet cows when compared to control (Sinclair *et al.* 2000).

Interestingly, cows on a high protein diet do not appear to have impaired ovulation, fertilisation or alterations in actual embryo number collected when compared to control diet (Bishonga *et al.* 1996; Rhoads *et al.* 2006; Sinclair *et al.* 2000). However, what has been demonstrated clearly, in the *in vitro* studies on the effects of ammonium on the embryos, is that the greatest effects of ammonium are on a cellular level and are frequently not evident by an assessment of morphology alone.

There is conflicting evidence that embryo quality and viability is altered following a high protein diet, with some studies in sheep showing an increase in embryo developmental delay (Bishonga *et al.* 1996; McEvoy *et al.* 1997) and with others in cows showing no difference (Rhoads *et al.* 2006). Nevertheless, a decreased pregnancy rate in these high-protein-fed-cows was consistently observed, and was also seen in groups where embryos from high-protein-fed cows were transferred to recipients (Canfield *et al.* 1990; Elrod and Butler 1993; McEvoy *et al.* 1997; Rhoads *et al.* 2006). Interestingly, PUN concentration of the recipient did not have an effect on the ability of control embryos to form a pregnancy, indicating that the high PUN and ammonia concentrations appear to be having an effect on the oocytes and embryo before Day 7 of pregnancy (Rhoads *et al.* 2006)

1.8 Mitochondria

1.8.1 Origin

Mitochondria are a vital component of any living cell, as they produce the energy required for many diverse cellular functions. They also play an intrinsic role in Ca^{2+} signalling, apoptosis, reactive oxygen species (ROS) production, regulation of the cells' redox state and other functions such as steroid and heme synthesis (Dumollard *et al.* 2007a).

Phylogenetically, mitochondria are considered to be ancient. They are believed to be descendent from specialised bacteria that were endocytosed by another species of prokaryote incorporating them into its own cytoplasm, resulting in an organism that could now metabolise complex carbohydrate and produce hydrogen and CO_2, which the host needed to live. It is postulated that along with some genomic rearrangement and a possible third merger with another bacteria to provide cytoskeleton, this trimeric symbiosis was now able to survive in a more aerobic environment (Doolittle 1998; Jansen 2000). As evolution progressed and animals, plants and fungi evolved from this line of protoists the mitochondrion present in these organisms would utilise pyruvate as an energy source, and the by-product of oxidative phosphorylation, hydrogen ions, would be pumped into the intra-membrane space in such large amounts that they reverse an ATP requiring Na^+/H^+ pump, thus producing ATP for the host cell (Jansen 2000).

Interestingly mitochondria still contain a separate genome, despite the hypothesis that most of the protomitochondrion's genes were ceded to the nuclear genome during establishment of the successful symbiosis. The purpose for this is believed to be due to the need to increase efficiency and to prevent the symbiont from being able to replicate independently of the host (Jansen 2000). The remaining

genome (mtDNA) carries 37 genes and is only able to produce 13 of the 1500 mitochondrial proteins required, all of which are involved in oxidative phosphorylation. The hypothesis behind the persistence of the mtDNA is that the 13 proteins for which it encodes are very hydrophobic. Transport of these from the cytoplasm and into the mitochondrial matrix would be very difficult, and so production inside the mitochondria appears to be more efficient (May-Panloup *et al.* 2007).

1.8.2 Structure and purpose

Although mitochondria are most well known as the 'powerhouse' of the cell because of their vital role in the generation of ATP, they also have other important roles within the cell which are vital for cellular survival and homeostasis.

In addition to producing energy for the cell, they also have a vital role in Ca^{2+} signalling and Ca^{2+} handling, apoptosis and reactive oxygen species production, and there is also now some evidence of their role in cellular and organismal aging (Dumollard *et al.* 2007a).

In a typical eukaryotic cell the mitochondria are traditionally ellipsoid in shape. They have a smooth outer membrane and a highly folded inner membrane with numerous invaginations, known as cristae. The outer membrane contains many complexes of membrane proteins that form channels for the traffic of ions and molecules in and out of the mitochondria. The inner membrane contains the complexes involved in mitochondrial metabolism, Complex I (NADH dehydrogenase), Complex II (succinate dehydrogenase), Complex III (cytochrome c reductase), Complex IV (cytochrome c oxidase) and Complex V (ATP synthase). Inside the inner membrane is the mitochondrial matrix that contains a complex mixture of soluble enzymes involved in the respiration of pyruvic acid (**Figure 1-3**).

The primary role of the mitochondria within any cell is to generate energy, in the form of ATP, through oxidative phosphorylation. Oxidative phosphorylation involves five multisubunit enzyme completes that are composed of >80 different polypeptide units. The majority of the coding for these subunits is contained within the nuclear genome; however, 13 are encoded for by the mitochondria's own genome (mtDNA). This means that the successful oxidative phosphorylation requires the coordination of both the mitochondrial and nuclear genome for generation of all the subunits required to perform this task (Trounce 2000).

In somatic cells, the conversion of glucose to pyruvate, a process known as glycolysis, only produces two molecules of ATP per glucose molecule. The citric acid cycle and oxidative phosphorylation, which occurs also in somatic cells and at every stage of pre-implantation embryo development, converts pyruvate to carbon dioxide and water and yields 38 ATP molecules per glucose molecule and is very energy efficient.

Oxidative phosphorylation requires all five multimeric enzyme complexes to be present within the mitochondrial membrane. These enzymes, collectively called the electron transport chain, pass

electrons from NADH and $FADH_2$ to molecular oxygen so that the electronated oxygen products produced can enter the mitochondrial matrix and eventually be reduced to water. Complex I is the largest of the complexes and perhaps the least understood: it carries electrons from NADH to ubiquinone. Complex II contains only four subunits, all of which are encoded by the mtDNA, and it carries electrons from succinate to ubiquinone. This reduced ubiquinone is oxidised by Complex III.

The energy release from this is partly conserved by the pumping of hydrogen ions into the intermembrane space of the mitochondria creating a proton gradient. The presence of such a high concentration of protons causes the reversal of the ATP requiring Na^+/H^+ pump to produce ATP (commonly referred to as Complex V). The protons discharged into the mitochondrial matrix reduce the electron transport chain's electronated oxygen products (which potentially constitute reactive oxygen species) to form water (Trounce 2000) (**Figure 1-4**).

Figure 1-3: A three-dimensional diagram of a mitochondrion cut longitudinally. The F_0F_1 complexes (small red spheres), which synthesise ATP, are intramembrane particles that protrude from the inner membrane into the matrix. The matrix contains the mitochondrial DNA (blue strand), ribosomes (small blue spheres), and granules (large yellow spheres). (Figure from (Lodish 2000)

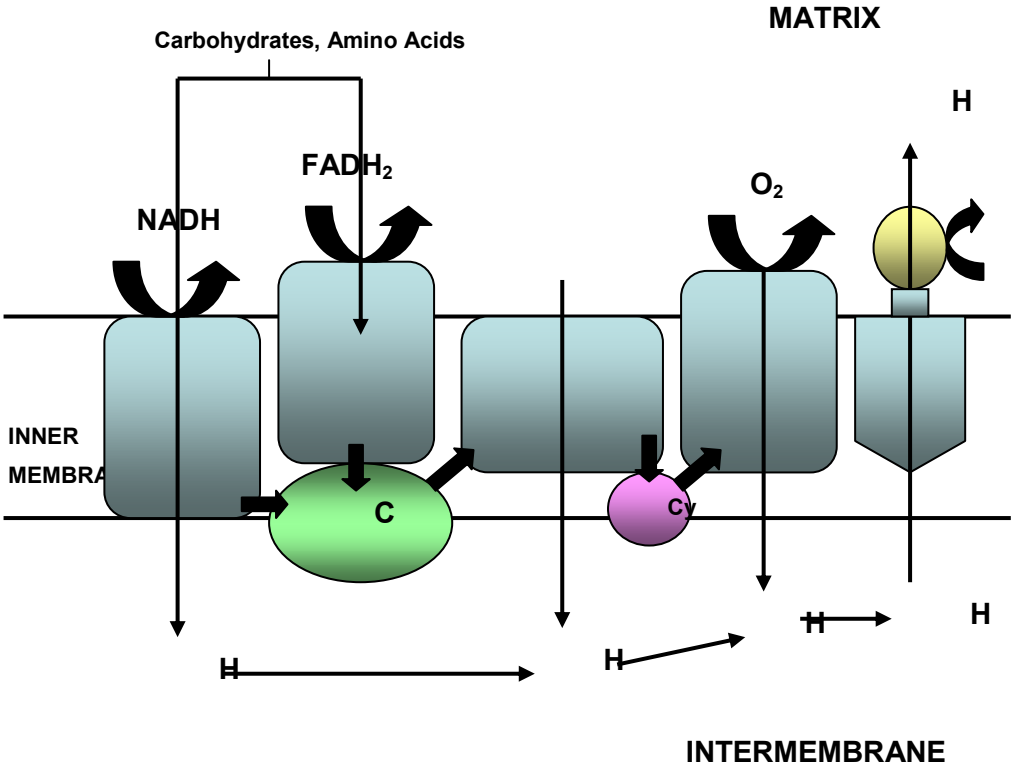

Figure 1-4: Diagram of the electron transport chain used to synthesise ATP. Figure adapted from (Naviaux and McGowan 2000).

1.8.3 Mitochondrial involvement in disease

It is known in other cell types that mitochondria are a primary intracellular target in the initiation of cell dysfunction, particularly in the process of cell damage associated with metabolic failure. Mitochondria are also recognised as not only indispensable for cell metabolic control but are also a critical component in cell regulation and cellular fate, such as their involvement in the regulation of apoptosis (Cho and Choi 2002).

As mentioned earlier, the primary function of the mitochondria is to provide the energy required for the cell to function and maintain homeostasis. Disruption to this energy supply can have devastating consequences to the cell, the organ and the individual. Over the last two decades, mutations in both mitochondrial DNA (mtDNA) and nuclear DNA have been implicated in many diseases and clinical syndromes (McFarland *et al.* 2007). There is significant evidence emerging that mitochondria play a key role in the aetiology of a number of neurological disorders such as Parkinson's and Alzheimer's disease. It is believed that mitochondrial dysfunction and oxidative stress play an important role in the pathogenesis of these diseases (McFarland *et al.* 2007). Studies have shown that cells obtained from Parkinson's disease patients display mitochondrial respiratory chain compromise and they have a higher level of mtDNA deletions (Bender *et al.* 2006).

Mitochondria have also been implicated in cancer pathology, with one study demonstrating that human colorectal carcinomas frequently display mutations in mtDNA (Polyak *et al.* 1998). Since then

mutations in mtDNA have also been discovered in a variety of tumours and leukaemias (Fliss *et al.* 2000; Jeronimo *et al.* 2001). Other diseases such as diabetes and hypertension also have mitochondrial point mutations, which have been associated with disease development. It has also been proposed that a common mtDNA variant (m.16189T>C) is associated with the onset of diabetes (Type 2) (Poulton *et al.* 2002).

Another process in which mitochondrial pathology has been implicated is the aging process. Theoretically, as cells age there is a progressive accumulation of somatic mutations in mtDNA leading to a decline in mitochondrial function (Harman 1992). The mitochondrial theory of aging is that ROS are generated at low levels during normal mitochondrial respiratory chain activity, and these ROS can directly attack DNA (including mtDNA) and cause DNA strand breaks and mutations. As this occurs continuously throughout life, more and more mutations develop, which will have an impact on mitochondrial metabolism and result in more ROS production and more DNA mutations and damage. This continual positive feedback mechanism will result in an exponential increase in oxidative damage during aging, resulting in eventual loss of cellular and tissue function because of decreased energy production, defects in signalling (as mentioned earlier Ca2+ signalling and ATP are intrinsically linked) and increased apoptosis (Harman 1992). At present this theory is being tested and knockin mouse models have supported the hypothesis that mtDNA mutations are associated with aging. Further studies are required in the human, however, to assess if mitochondrial mtDNA mutations occur in aged human tissue to the same degree as is seen in the mouse (Kujoth *et al.* 2005; Trifunovic *et al.* 2004).

1.9 Mitochondria in the oocyte and embryo

1.9.1 Mitochondrial numbers

Mitochondria are inherited maternally and independently of the nuclear genome. During oogenesis there is a mitochondrial replicative burst that ceases at the end of maturation, resulting in a large population of mitochondria in the mature egg. The number of mitochondria per egg has been analysed using electron microscopy or mtDNA copy number (with an estimate of 1–2 mtDNA copies per mitochondrion) and has shown species-specific differences, with mammals containing 150,000 to 800,000 mtDNA copies. Other species can contain smaller amounts, such as 18,000 mtDNA copies in nematodes (*C. elegans*) and 70–200 million copies in fish (Dumollard *et al.* 2006). Thus the large mitochondrial population in the mature egg all originate from a restricted founder population (from as low as 10–100 mitochondria in the human primordial germ cell (Poulton and Marchington 2002) providing a genetic 'bottleneck', ensuring a homoplasmic population of mitochondria within the resultant embryo (**Figure 1-5**). This is vital as the survival of each blastomere depends on the inheritance of a functional complement of mitochondria from the egg (Dumollard *et al.* 2007a).

During oocyte maturation, the growing population of mitochondria also segregate into varying regions of the cell, with the early phase of segregation appearing to be conserved between species and the final stages giving rise to the species-specific mitochondrial patterns that are observed in mature oocytes (Dumollard *et al.* 2007a; Dumollard *et al.* 2006).

Following fertilisation, in most species studied, the number of mitochondria remains reasonably static during pre-implantation embryo development; although in the bovine there does appear to be some variation in mtDNA content during pre-implantation development, and one study in the mouse has shown a small burst of mtDNA replication following fertilisation (May-Panloup *et al.* 2005; McConnell and Petrie 2004), with mitochondrial replication beginning after implantation (Dumollard *et al.* 2006).

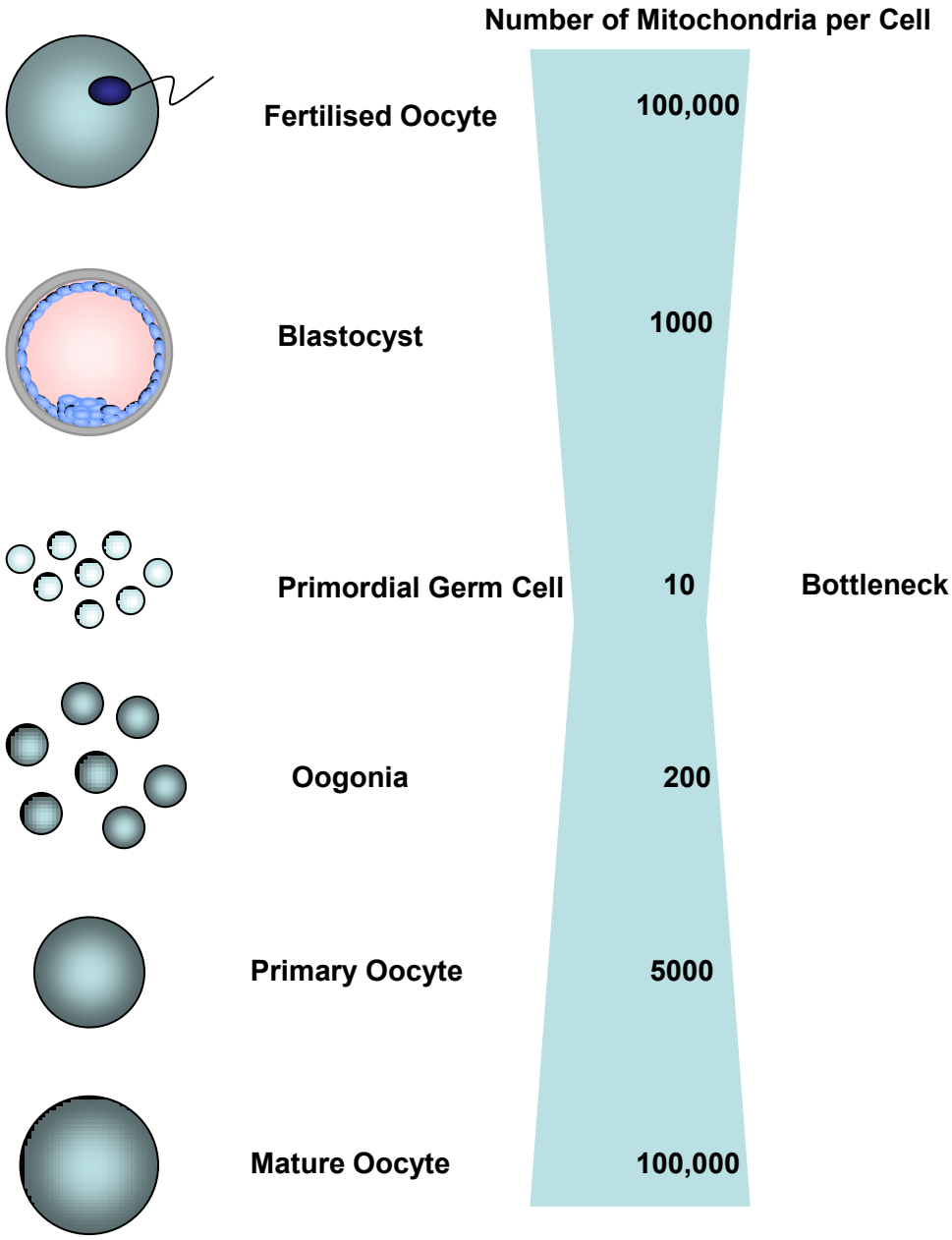

Figure 1-5: Diagrammatic representation of the changes in the number of mitochondria during development of the female germ line and the bottleneck concept. The estimate of the number of mitochondria per cell is indicated in the flowchart on the right. (Figure adapted from (Shoubridge and Wai 2007).

1.9.2 Mitochondrial structure and function

As well as number, mitochondrial structure also varies during germ cell development and pre-implantation embryo development, with these changes perhaps being reflective of the changing energy requirements of the embryo as it develops.

Mitochondria within ovulated oocytes and the pre-compaction stage embryo are condensed, have a vacuolated appearance and few concentric cristae (Hillman and Tasca 1969; Stern *et al.* 1971). This mitochondrial structure is more characteristic of that of primitive organisms rather than somatic tissue. It is not until the embryo compacts and develops to the blastocyst stage that the mitochondria begin to enlarge and take on a more orthodox configuration with more numerous and predominantly transversely arranged cristae. These changes in mitochondrial structure are paralleled by increases in the oxidative capacity of the embryo (Hillman and Tasca 1969; Plante and King 1994; Stern *et al.* 1971).

The metabolism of the mitochondria within the oocyte and embryo is also quite different from somatic cell metabolism. The majority of studies assessing metabolism in embryos has been done in mammalian embryos, which utilise nutrients found in their surrounding environment of the oviduct and uterine tract. In contrast, embryos from species such as fish, reptiles and birds consume internal stores that are present in the lipid and protein-rich yolk (Dumollard *et al.* 2007a).

As mentioned previously, many studies have determined the substrate requirements for mammalian embryo development and have demonstrated very specific nutrient requirements, accompanied by tightly controlled metabolism (Biggers *et al.* 1967). Initially, the early stage embryo derives its energy from oxidative phosphorylation of pyruvate, lactate and amino acids, and because of this the mitochondria are essential for energy production. At this stage of embryo development amino acids are oxidised to pyruvate, oxaloacetate or alpha-ketogluterate and are fed directly into the Krebs cycle (**Figure 1-6**). In comparison, the post-compaction stage embryo is able to utilise glycolysis and oxidative phosphorylation, and, at this stage, glycolysis increases sharply, coupled by an increase in oxygen consumption (Dumollard *et al.* 2007a). This increase in glycolytic rate is facilitated by the stage specific expression of high affinity, high capacity transport system for glucose which has been demonstrated to be crucial for this metabolic differentiation to occur (Pantaleon *et al.* 1997).

The long-established role of pyruvate metabolism by the mitochondria was recently confirmed using imaging techniques in living mouse oocytes and embryos (Dumollard *et al.* 2007b). By imaging functioning mitochondria in the oocyte and early embryo, using autofluroesence, it was discovered that pyruvate is rapidly metabolised by mitochondria whereas glucose is not. Interestingly, it was also discovered that pyruvate derived from lactate is poorly metabolised by the mitochondria (Dumollard *et al.* 2007b). After compaction there is a switch, and, as glucose is metabolised to pyruvate in the cytosol, by the process of glycolysis, glycolytic pyruvate is then fed directly into the Krebs cycle and metabolised by the mitochondria (**Figure 1-6**). Since the mitochondria are considered immature at the early stages of embryo development because of their sparse cristae and small surface area, oxygen

consumption and metabolism is considered to be low; this has been termed ‘quiet metabolism’ (Leese 2002). This ‘quiet metabolism’ may serve a purpose of lowering production of oxidants (ROS) and protecting the early embryo from oxidative damage (Houghton and Leese 2004).

Figure 1-6: Redox and energetic metabolism in the mammalian embryo. Schematic representation of the metabolic pathways producing NADH, NADPH and GSH functioning in the cytosol and mitochondria. Figure obtained from (Dumollard *et al.* 2007b).

1.9.3 Mitochondria and Ca^{2+} homeostasis

Mitochondria play a vital role within the somatic cell in modulating cellular Ca^{2+} homeostasis. They are responsible for the uptake and release of cellular Ca^{2+} and, in doing so, regulate cellular signalling (Jouaville *et al.* 1995). Notably, mitochondria are often located near Ca^{2+} release channels on the endoplasmic reticulum (ER) or Ca^{2+} influx channels located on the plasma membrane, which allows the mitochondria to capture the free-flowing Ca^{2+} from either the influx or release channels (Malli *et al.* 2003; Rizzuto *et al.* 1993).

Similar to somatic cells, the oocyte and embryo also require rigid Ca^{2+} homeostasis to modulate cellular signalling. During fertilisation, egg activation is accompanied by the triggering of Ca^{2+} waves

as the sperm enters (Dumollard *et al.* 2002). The sperm-triggered Ca^{2+} signals are controlled by IP3-dependent Ca^{2+} release from the ER. Like somatic cells, mitochondria are clustered around the ER and this allows for transmission of cytosolic Ca^{2+} into the mitochondria and the mitochondria supply the energy in the form of ATP to fuel the Ca^{2+} pump on the ER membrane (Dumollard *et al.* 2002). Sperm-triggered Ca^{2+} oscillations then directly stimulate mitochondrial metabolism and energy production. This results in an increase in O_2 consumption, as the signal is transmitted directly into the mitochondrial matrix, upregulating oxidative phosphorylation, which, in turn, maintains the Ca^{2+} oscillation (Dumollard *et al.* 2003). Therefore Ca^{2+} is directly linked to ATP supply and demand. When Ca^{2+} levels are low, oxidative phosphorylation is also low and ATP production reduced, and it is only after sperm penetration occurs that Ca^{2+} levels are increased, mitochondrial metabolism is triggered and ATP levels are increased to support post-fertilisation events that are initially stimulated by Ca^{2+} waves (Dumollard *et al.* 2006). Thus, the production of reactive oxygen species (ROS) by the electron transport chain is also minimised, as metabolism only occurs when necessary, minimising cellular and mitochondrial ROS-induced damage (Ramalho-Santos *et al.* 2009).

1.9.4 Mitochondrial distribution, ΔΨm and stress response

During oogenesis, the growing population of mitochondria are relocated into different regions of the cell. Throughout the early phases of oocyte growth, mitochondrial distribution appears to be similar between species, but in the later stages of growth the pattern of distribution becomes varied between species (Dumollard *et al.* 2006). In the mature egg there are specific differences between mitochondrial density at different poles of the egg, and, providing these asymmetric distributions are retained after fertilisation, it results in blastomeres containing varying mitochondrial load (Dumollard *et al.* 2007a). Studies have also demonstrated that as the oocyte and early embryo develops, the mitochondria undergo stage-specific distribution changes. In the mouse oocyte, mitochondria translocate to the perinuclear region of the cell, via microtubules, to form a sphere of organelles surrounding the chromosomes (Van Blerkom and Runner 1984). After fertilisation in the mouse, hamster and human, the mitochondria migrate to the perinuclear region to surround the pronuclei, and this transient perinuclear accumulation occurs after each blastomere division during the early cleavage stages (Bavister and Squirrell 2000; Van Blerkom *et al.* 2000; Van Blerkom and Runner 1984). It is believed that this spatial remodelling is used to direct mitochondria and ATP production to regions of the cell that have higher energy demands (Barnett *et al.* 1996).

Furthermore, studies show that mitochondrial trafficking and inheritance in daughter cells may be a crucial determinant of human embryo viability. Disproportionate mitochondrial segregation and inheritance during cell division can lead to the generation of cell lineages with lowered metabolism and ATP levels due to lower mitochondrial numbers, which can then result in arrested cell division and blastomere swelling and lysis (Van Blerkom *et al.* 2002).

Because mitochondrial distribution plays an important role in determining cellular viability, it is important to note that many culture-induced stresses, which result in perturbed embryo viability, can induce alterations to the spatial remodelling of mitochondria within the blastomeres. For example, the exposure of hamster 2-cell embryos to high levels of glucose and phosphate that cause the '2-cell block' resulted in mitochondria moving away from the normal perinuclear localization to a more homogeneous distribution in the cells (Barnett *et al.* 1997; Ludwig *et al.* 2001). It has also been demonstrated in other models of *in vitro* environmental stress such as altered pH as well as *in vivo* models such as high and low protein diets or omega-3 supplementation, mitochondrial distribution is altered in the resultant embryo and may potentially be a mechanism behind the decreased embryo viability seen (Mitchell *et al.* 2009; Squirrell *et al.* 2001; Wakefield *et al.* 2008).

Another important parameter in mitochondrial homeostasis is mitochondrial membrane potential ($\Delta\Psi m$). Mitochondrial membrane potential is generated by respiration, which involves the outward pumping of protons across the inner mitochondrial membrane. This, in turn, creates a proton gradient which has two parts: $\Delta\Psi m$ and a pH gradient. The energy that is stored in either component drives the conversion of ADP to ATP by the respiratory chain enzymes (Van Blerkom 2004). By utilising a fluorescent dye (JC-1), it is possible to measure the magnitude of the $\Delta\Psi m$ within the cell, and this technique has been used to establish the link between $\Delta\Psi m$ and embryo developmental competence (Acton *et al.* 2004; Smiley *et al.* 1991; Van Blerkom *et al.* 2002). Some studies have shown that the oocyte and embryo contain regions of highly polarised mitochondria that cluster in the pericortical region within the cell (Jones *et al.* 2004; Van Blerkom *et al.* 2003). It is hypothesised that these regions of highly polarised mitochondria may produce domains within the cell where differential metabolism could be established; however, this theory remains to be confirmed (Van Blerkom 2004). Interestingly, it has been discovered that a reduction in ATP production due to inhibition of the electron transport chain did not result in altered $\Delta\Psi m$, suggesting that highly polarised mitochondria do not necessarily indicate high energy production (Dumollard *et al.* 2004).

Despite this conflict in opinion, it has been shown that $\Delta\Psi m$ is related to developmental competence (Ahn *et al.* 2002). It has been reported that thawed embryos that failed to divide or that develop abnormally showed a loss of mitochondrial hyperpolarisation, and cryopreservation of oocytes is accompanied by a loss of hyperpolarised mitochondria in the pericortical region when compared to fresh oocytes (Ahn *et al.* 2002; Jones *et al.* 2004). Studies in the human have suggested that differences in the ratio of high to low polarised mitochondria may reflect perturbed mitochondrial distribution, which could result in metabolic defects leading to abnormal chromosomal segregation and chaotic mosaicism (Wilding *et al.* 2003; Wilding *et al.* 2002). Alterations in $\Delta\Psi m$ have also been associated with fertilisation and degree of embryo fragmentation (Acton *et al.* 2004). In this study it was shown that abnormally fertilised human embryos displayed $\Delta\Psi m$ similar to that of arrested 1-3cell embryos and that increased fragmentation was associated with high $\Delta\Psi m$.

It has also been demonstrated that the *in vivo* environment can also have an impact on alterations to ΔΨm, with decreased ΔΨm being observed in 2-cell embryos following administration of a high and low protein diet during peri-conception (Mitchell *et al.* 2009).

It is well known in other cell types that mitochondria are a primary target for the initiation of cellular dysfunction; in particular they are linked to cellular damage due to metabolic failure (Lane and Gardner 2005b). In the embryo, metabolic disruption is often a result of exposure to sub-optimal environmental conditions; in particular a disruption is seen between oxidative and glycolytic activity (Lane and Gardner 2003; Lane and Gardner 2005b). Exposure to *in vitro* stress such as lack of amino acids, ammonium, high osmolarity, and elevated oxygen all result in perturbations to metabolic control. In particular, the balance between mitochondrial and cytoplasmic metabolism is lost (Brinster 1965a; Gardner and Lane 1993a; Lane and Gardner 1998; Lane and Gardner 2000a; Lane and Gardner 2003; Lane and Gardner 2005b; Seshagiri and Bavister 1991). *In vivo* studies have also shown similar metabolic perturbations after exposure to altered nutritional levels; for example, a low protein diet during pre-conception resulted in significantly increased levels of ADP and ROS in the resultant 2-cell embryos, which is indicative of metabolic stress (Mitchell *et al.* 2009).

A side effect of metabolic stress is often ROS production, and it has been demonstrated that one of the perturbing effects of culture is the production of harmful reactive oxygen species (ROS) (Nasr-Esfahani *et al.* 1990b; Nasr-Esfahani and Johnson 1991). It is likely that a target of reactive oxygen species within the embryo is the mitochondria. As the early embryo contains mitochondria that have low surface area, they may be more sensitive to the effects of ROS. Evidence in support of this shows that the addition of components to the culture media, to reduce ROS production, had their greatest effect at the 2-cell stage by preventing developmental arrest (Nasr-Esfahani *et al.* 1990a; Nasr-Esfahani and Johnson 1992).

All of this evidence indicates that mitochondrial homeostasis is a target of environmental stress. The overall effect of these altered parameters is decreased metabolism and ATP production leading to disrupted cellular function. This would directly affect most cellular functions, including cell division, DNA replication and possibly DNA modification such as methylation: all processes which are energy requiring. This would then result in altered cellular viability and possibly have an impact on downstream events such as fetal and placental growth.

1.10 Epigenetics and DNA methylation

During cellular development, different cells and tissues acquire different programmes of gene expression, depending on specific requirements. It is believed that these differences in gene expression are primarily regulated by epigenetic modifications such as DNA methylation, histone tail modifications and non-histone proteins that bind to chromatin (Bird 2002; Li 2002). Thus each cell type in our body has its own epigenetic signature that reflects genotype, the environment which it is

exposed to, and developmental history, all of which is then reflected in the phenotype of the cell (Morgan *et al.* 2005).

1.10.1 Imprinting

For most cell types, these epigenetic marks become fixed after differentiation; however, in some circumstances such as cellular disease or fertilisation, epigenetic marks can be erased and new epigenetic marks established, a concept known as 'reprogramming' (Reik *et al.* 2001; Rideout *et al.* 2001).

Initially, in the germ line, genomic imprinting is established, which involves differential DNA methylation. Thus particular DNA regions become methylated in one sex germ line but not in the other so that after fertilisation and during development gene expression comes from either the maternal or paternal allele only (Young and Fairburn 2000). The DNA is also highly methylated in non imprinted gene regions as a mechanism of gene expression control.

1.10.2 Methylation in fertilisation and embryo development

After fertilisation occurs, both the paternal and maternal genomes undergo reprogramming to erase gamete epigenetic marks and reset the genome of the zygote for totipotency and later establishment of the embryo's own genetic marks (Hou *et al.* 2008). Initially the paternal genome is actively demethylated and then, around the time of the first cleavage division, the embryo undergoes passive DNA demethylation; however, imprints in certain 'imprinted genes' are maintained (Morgan *et al.* 2005). This rapid decline in methylation is due to the absence of the maintenance methylase, *Dnmt1* (**Figure 1-7**) (Carlson *et al.* 1992). Although this gradual loss of global DNA methylation is complete before DNA replication of the paternal pronucleus (between the 2-cell and 4-cell stage in the mouse), imprinted genes retain their characteristic methylation differences throughout pre-implantation development (Gardner and Lane 2005; Santos *et al.* 2002). *De novo* methylation is then initially established at the morula stage, as the cells begin differentiation into the two distinct cell lineages of the blastocyst and continue through implantation up until gastrulation. One important factor in the re-establishment of methylation, after cell differentiation, is DNA methyltransferases (*Dnmt* family), in particular *Dnmt3a* and *Dnmt3b* (**Figure 1-7**). These proteins are responsible for *de novo* methylation and are vital for normal fetal development, as *Dnmt3a*-null mice are runted and died at approximately four weeks of age and *Dnmt3b*-null mice have multiple mutations and none proceeded to birth (Okano *et al.* 1999).

It is believed that the purpose of this post-zygotic demethylation and remethylation wave is to erase male- and female-specific methylation patterns (excepting imprinted genes) and to allow resetting of the embryo's own genome (Shi and Haaf 2002). The epigenetic reprogramming during the

pre-implantation period controls expression of early embryonic genes, cell cleavage and cell determination (De Rycke *et al.* 2002).

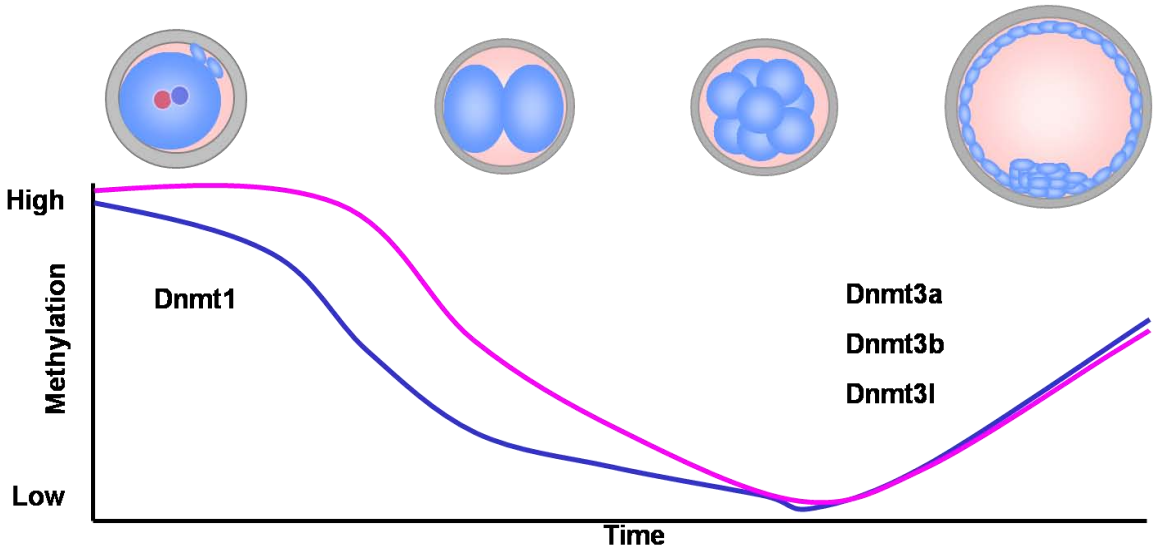

Figure 1-7: DNA methylation during embryo development. The pink line depicts maternal DNA and the blue line depicts paternal DNA.

1.10.3 Epigenetics, the Environment and Embryo Culture

For over three decades it has been known that the embryo is susceptible to its surrounding environment, which can influence not only physiology, metabolism and viability but also gene expression and imprinting (Biggers and Stern 1973; Doherty *et al.* 2000; Ho *et al.* 1994; Ho *et al.* 1995; Menke and McLaren 1970). Although other aspects of cellular trauma are of serious concern, such as increased apoptosis or decreased cell number, alterations in gene expression and imprinting have been of particular interest, as they may play a role in long term programming of the embryo that could have life-long effects and are also potentially inheritable (Gardner and Lane 2005).

As mentioned in the previous section, imprinting is an epigenetic process by which male and female gametes confer sex-specific marks (imprints) on certain regions of their DNA via alterations in methylation. This results in paternal and maternal alleles that are functionally nonequivalent and this is required for normal embryonic development (McGrath and Solter 1984; Surani *et al.* 1984). The genomes of mature sperm and oocytes are highly methylated and as DNA is replicated during early embryo development, this methylation is gradually lost, however imprinted regions maintain their methylation status. *De-novo* methylation begins as cellular differentiation occurs at the compaction

stage, so that by the peri-implantation period, methylation resembles that of a somatic cell (Reik *et al.* 2001).This reprogramming is essential in controlling expression of embryonic genes, cell cleavage and differentiation and maintaining correct imprinting is essential for fetal development and transmission of normal imprints to any of it subsequent offspring (De Rycke *et al.* 2002).

It has been shown that mutations in imprinting centers within the DNA (*cis*-acting centers which control imprint establishment and imprint maintenance) leads to aberrant imprinting and can cause disease (Buiting *et al.* 1995). However some disease states can be caused without mutations in these regions, in fact many imprinting defects have occurred spontaneously via aberrant imprinting without any alteration to the actual DNA sequence (Buiting *et al.* 2003). Animal studies have also shown that loss-of-function mutations to DNAmethyltransferases genes (*Dnmt3a* and *Dnmt1*) affects all imprinted domains as well as other chromosomal regions and leads to embryonic lethality (Jaenisch and Bird 2003). Also disruption of *Dnmt3L* results in azoospermia in homozygous males and heterozygous progeny of homozygous females died before midgestation (Bourc'his and Bestor 2004; Bourc'his *et al.* 2001).

DNA methyltransferases rely on S-adenosyl-methionine (SAM) as a methyl donor which is dependent on folic acid and Vitamin B. Due to the fact that folic acid and vitamin B are obtained through diet it is not surprising to find that epigenetics can be influenced by nutrition. Studies have shown that in patients with the blood disorder hyperhomocysteinemia, folate washout causes biallelic expression of *H19* (an imprinted gene) which reverses back to monoallelic when the patients are given folate (Ingrosso *et al.* 2003). It has also been demonstrated in that agouti mice (which have varying coat colour based on level of methylation of the *agouti* gene) given varying levels of methyl supplementation in their diet resulted in offspring with varying coat phenotypes indicative of alterations in DNA methylation (Cooney *et al.* 2002).

Due to the fact that important epigenetic events occur during embryo development, it is understandable that a suboptimal environment during this critical period can lead to alterations in epigenetics and gene expression. In regards to the embryo culture environment, several factors have been highlighted as impacting on epigenetics such as sub-optimal media formulation, ammonium and serum.

Inappropriate media formulations such as those lacking critical nutrients or amino acids have been linked to altered epigenetics. A study comparing media formulations found that mouse blastocysts grown in $KSOM^{AA}$ (+ amino acids) had predominately monoallelic expression of *H19* where as those grown in Whitten's medium (no amino acids) had abnormal biallelic expression (Doherty *et al.* 2000). It has also been shown that methylation patterns in 2-cell embryos are abnormally altered in embryos grown in Ham's F-10 or tissue culture media when compared to M16 simple media (Shi and Haaf 2002). The presence of toxins in the media can also impact on gene expression, with ammonium build-

up being associated with altered gene expression and imprinting of *H19* (Gardner *et al.* 2003; Lane and Gardner 2003).

The presence of serum in culture has also been demonstrated to interfere with many aspects of embryo development. Mammalian embryos are not exposed to serum *in vivo* and the inclusion of it in culture media can have serious ramifications on embryo and subsequent fetal development. Studies have shown that serum causes premature blastulation, effects embryo morphology and perturbs ultrastructure (Dorland *et al.* 1994; Gardner 1994). The presence of serum in the culture media has also been linked to large offspring syndrome (LOS) in sheep (Thompson *et al.* 1995). The mechanism behind LOS syndrome has been investigated extensively and it has been discovered that fetal overgrowth is associated with reduced fetal methylation and expression of *Igf2R* indicative of possible alteration in epigenetics and imprinted genes during *in vitro* pre-implantation development (Young *et al.* 2001).

In the mouse serum also alters expression of imprinted genes such as *H19*, *Igf2*, *Grb7* and *Grb10*, however interestingly leads to decreased fetal weight (Khosla *et al.* 2001a).

Studies have demonstrated that many imprinted genes play a key role in fetal growth and development , such as *Igf2*, and alterations in the expression of this gene can severely affect growth and morphology of the offspring (DeChiara *et al.* 1990). Interestingly children born after IVF tend to be lighter than naturally conceived children whether this is due to altered gene expression due to embryo culture remains to be elucidated (Wang *et al.* 2002).

It has also been suggested that assisted reproductive technology may be associated with increased risk of imprinting defects (Cox *et al.* 2002). In this study the authors described two children with an imprinting defect and Angelman syndrome (AS) who were conceived via intracytoplasmic sperm injection (ICSI) (Cox *et al.* 2002). A large case-control study has also recently been reported and the authors have identified a nine time greater risk of having a child born with the imprinting disorder Beckwith-Wiedemann syndrome (BWS) after IVF when compared with the general population (Halliday *et al.* 2004). Despite this increased risk however the chance of conceiving a child with BWS after ART still remains very low.

In view of the data obtained from animal models and the epidemiological evidence from human investigations, it is possible that ART is associated with an increased risk of imprinting disorders. Despite this however ART patients are a select group from the population with intertility who may possibly be genetically predisposed to loss of epigenetic control and it is still unclear whether this increased risk is due to the infertility itself, or the protocols behind ART including super ovulation, gamete manipulation such as ICSI and embryo culture (Horsthemke and Ludwig 2005). As mentioned previously, although there is evidence that *in vitro* techniques, such as embryo culture, can alter methylation patterns and gene expression, little is known about the possible genetic predisposition to loss of epigenetic control. It is possible that this predisposition could lead to increased risk of altered

programming due to epigenetic changes, which can then alter the developmental trajectory of the offspring. This hypothesis however remains untested.

1.11 Conclusions and Hypotheses

The growth and development of a conceptus from a single cell zygote all the way to a fully formed infant is a highly dynamic process and is continually influenced by the environment that surrounds it. The maternal nutritional environment can program the developing fetus and influence not only weight and glucose tolerance at birth but also significantly impact on health later in life by programming the risks for the early onset of cardiovascular and metabolic disease. It is believed that this susceptibility to disease is primarily due to a mismatch in the pre natal verses post natal environment which results in inappropriate responses to specific nutritional challenges after birth (Gluckman and Hanson 2004a; Hales and Barker 1992; Hales and Barker 2001). As these studies have clearly demonstrated the environment in-utero is very important in setting the trajectory for later life where deviations to the nutritional and metabolic balance can have dramatic events downstream.

The conceptus is not only susceptible to its environment during the later stages of gestation, it is now accepted that the first few days of development after fertilisation are just as important and programming can also be adjusted during this critical window of development.

The development during this critical window of time is highly dynamic and as has been outlined earlier, the pre and post compaction stage embryo are vastly different in structure, metabolic requirements and the ability to regulate homeostasis. It has been demonstrated that the post compaction stage mouse embryo has increased ability to regulate against pH or osmotic stress (Edwards *et al.* 1998b; Lane and Gardner 2000b). It has also been shown that embryos grown from the 2-cell to the 8-cell stage, in poor culture conditions, results in large variations in gene expression at the 8-cell stage, in contrast culture from the 8-cell to the blastocyst does not further alter gene expression levels compared to those grown in optimized conditions (Hewitt *et al.* 2003; Ho *et al.* 1994; Ho *et al.* 1995). There is currently very little known about the stages at which the mammalian pre-implantation embryo is most susceptible to stress and at what stages does stress exposure result in altered embryo programming. In regards to the culturing of human embryo, does extended culture result in cumulative trauma, or is the trauma confined to the certain stages of development, in particular the pre-compaction stage? The answer to this question is quite important for human IVF as it has been suggested that extended culture and blastocyst transfer may increase the incidence of adverse outcomes for the resultant pregnancy and offspring (DeBaun *et al.* 2003; Powell 2003; Schultz and Williams 2002). In human IVF, blastocyst culture extends the time that the embryo is in the laboratory and is exposed to the culture environment however cleavage stage transfer places the embryo prematurely back into the hyper-stimulated uterus (when it would normally be in the fallopian tube). Furthermore, evidence from animal models indicates that exposure of embryos to a hyper-stimulated

environment impacts on subsequent development and fetal growth (Ertzeid and Storeng 2001). Therefore given that an inappropriate environment can impact significantly on development and health of the offspring, it is important that we better understand exactly when embryos are susceptible to environmental stress and how stage specific stress exposure can program development, so we can try and maintain an optimal culture environment for healthy offspring.

It is therefore my hypothesis that the pre-implantation stage embryo is more susceptible to *in vitro* stress than the post compaction stage and that a short exposure to environmental stress in the early stages of development will result in similar perturbations in development and viability to embryos cultured with the stress for the whole of the pre-implantation window.

As mentioned previously, development from the zygote to the blastocyst stage is highly dynamic as the embryo changes over a five day period from a single cell with the metabolic quotient of bone to a highly organized entity with a transporting epithelium, partially differentiated cell lineages and the metabolic quotient of the heart (Leese 1991). Due to this precisely coordinated developmental trajectory, it is understandable that the embryo is heavily reliant on highly controlled and efficient metabolism to provide the energy needed to develop at such a rapid rate.

It is widely accepted that the metabolism of any given cell type is an indicator of its health and viability. The embryo is no different in this regard, with the ability of the embryo to control its metabolism being associated with its ability to implant and form a viable fetus (Lane and Gardner 1996). The mitochondria of the embryo therefore play a critical role in maintaining metabolism therefore enabling the embryo to develop and implant normally.

Due to the fact that metabolism is so important for maintaining normal embryo development, it is understandable that perturbations in metabolism are often seen after embryos are exposed to suboptimal conditions. Both *in vivo* and *in vitro* studies have demonstrated perturbations in metabolic homeostasis and mitochondrial paramaters after embryos are exposed to sub-optimal environmental conditions. In the rodent model, culture conditions that induce developmental delay or arrest result in disruptions between cytoplasmic and mitochondrial metabolism as well as premature increases in glucose metabolism coupled with a concomitmant decrease in oxidative metabolism (Gardner and Lane 1993a; Lane and Gardner 2003; Seshagiri and Bavister 1991). Additionally exposure to poor culture conditions also results in decreases in metabolic products such as ATP and ADP within the embryo (Gardner 1998a; Gardner and Lane 1997). *In vivo* models of altered nutrients availability during the peri and pre-implantation window also result in embryos with altered mitochondrial paramaters and ADP levels (Mitchell *et al.* 2009; Wakefield *et al.* 2008).

Metabolism of various nutrients, such as glucose or amino acids, is so closely related to embryo viability that they can be used as a selection tool for picking the embryo with the highest implantation potential (Brison *et al.* 2004; Gardner and Leese 1987; Houghton *et al.* 2002).

Although stress exposure is often coupled with alterations in metabolism, this does not necessarily result in embryo arrest or death. In fact the embryo is remarkably plastic and often appears to adapt to the environmental conditions to which it is exposed however often this adaptation results in altered fetal growth after implantation (Gardner *et al.* 1994; Lane and Gardner 2003; Thompson *et al.* 1995).

Although this phenomenon of adaptation is well documented, there is little known about how many different mitochondrial parameters involved in metabolism are affected and how long these perturbations exist. Also the possible mechanism behind how these stresses induce downstream programmed alterations is also unknown. Although it is probable that different environmental conditions (high oxygen, ammonium, serum, osmotic stress, lack of amino acids and even embryo culture itself) will induce perturbations in different ways, the common outcomes seen are altered blastocyst cell number, altered implantation and fetal development and often alterations to fetal or postnatal phenotype, which is possibly due to a loss in metabolic control and disrupted cellular homeostasis. Interestingly these effects are often conserved across different strains and also different species (understanding cellular disruptions).

It is therefore my hypothesis that *in vitro* stress results in a disruption to cellular and metabolic homeostasis which is mediated by alterations in mitochondrial metabolism, with mitochondria being a primary target of environmental stress. Due to the changes in mitochondrial structure and function during embryo development, it is also possible that the early stage embryo mitochondria are more susceptible to metabolic perturbation due to their immature structure so the early embryo will be the focus (Hillman and Tasca 1969).

There is growing evidence that epigenetic events in the pre-implantation embryo contribute to altered developmental potential (Khosla *et al.* 2001a; Khosla *et al.* 2001b; Lucifero *et al.* 2004). During embryogenesis, critical events occur which result in the removal, re-acquisition and maintenance of genetic imprints as well as demethylation and de-novo methylation of non-imprinted genes. Studies have shown that the embryo culture environment can alter the expression of imprinted genes such as *H19* in the resultant mouse blastocyst and exposure of sheep embryos to serum results in epigenetic changes to *Igf2R* in resultant sheep with large offspring syndrome (Doherty *et al.* 2000; Young *et al.* 2001). *In vivo* studies have shown similar results with rat embryos derived from mothers fed low-protein during pre-implantation development exhibiting reduced expression of *H19* in extra-embryonic blastocyst trophectoderm lineage (Kwong *et al.* 2003).

Due to the fact that epigenetic modification and maintenance is reliant on energy production, it is possible that alterations in metabolism will influence critical methylation events during pre-implantation development resulting in altered gene expression. It is possible then that these alterations in gene expression may then be responsible for altered embryo viability and offspring development and phenotype.

It is therefore my hypothesis that *in vitro* stress will result in alterations in critical epigenetic events during embryogenesis such as demethylation and de-novo methylation which could provide a possible explanation for the alterations seen in fetal development after exposure to altered environmental conditions.

In conclusion, pre-implantation embryo development is a window during which the conceptus is influenced by its surrounding environment. The underlying mechanisms behind the ability of the environment to control embryo development and alter the future programming of the fetus is largely unknown, however the evidence suggests that it is a multi-factorial approach with possible alterations in metabolic and epigenetic factors leading to changes in gene expression and altered fetal development. The results from this study will provide a better understanding of how the embryo responds to altered *in vitro* environmental conditions and provide insight into the mechanisms responsible for the altered fetal programming seen after exposure to environmental stress.

1.12 Specific Aims

1. To determine the consequences of exposure to environmental stress (either temporal or continual) on embryo development and viability using two model *in vitro* stresses 1) ammonium (a toxin which is known to build up in culture media due to amino acid breakdown) and 2) 5,5-Dimethyl-2,4-Oxazolidinedione (DMO: a weak non-metabolisable acid that decreases intracellular pH).
2. To assess the impact of temporal exposure to environmental stress on mitochondrial homeostasis and energy production.
3. To determine if alterations in mitochondrial homeostasis persist after the environmental stress is removed.
4. To assess the impact of exposure to environmental stress on critical DNA methylation events which occur during pre-implantation embryo development.
5. To assess the impact of exposure to environmental stress on placental gene expression and function.

2 Materials and Methods

2.1 Media preparation

2.1.1 Preparation of glassware

All glassware that was used to make media for embryo handling or culture was first washed with 7X detergent (ICN Biomedicals, Aurora, Ohio, USA) and allowed to soak in RO (reverse osmosis) water overnight. The next day, glassware was rinsed six to eight times in RO and then further soaked in RO overnight. Following this the glassware was rinsed six to eight times in Milli-Q water (18 meg Ω) and placed in an oven upside down for four hours at 110 °C until dry. The open top was then covered by blue absorbent paper, covered in aluminium foil and then sterilised at 130 °C for a further two hours.

2.1.2 Embryo culture and handling media

The composition of all culture media is shown below (Table **2-1,** Table **2-2**) The media used for embryo culture was G1.2 and G2.2, and MOPS-G1 was used as a collection and handling medium (Gardner *et al.* 2004)(Table **2-1**). A simplified version of G1.2 medium was used for quality control assessments (see section 2.2 Mouse Embryo Bioassay) (Table **2-2**). G1.2 and G2.2 is a sequential culture system, and was used as it results in high rates of embryo development and fetal outcomes (Gardner *et al.* 2000a). This sequential media also contains a di-peptide of glutamine (the most volatile amino acid), which prevents its spontaneous breakdown, which results in a negligible ammonium build up (<9μM) (Lane and Gardner 2003). For all embryo handling and manipulation, a modified version of G1.2 was used where the concentration of bicarbonate was reduced and replaced with the buffer MOPS (3-(N-morpholino) propanesulfonic acid) (Sigma Aldrich Chemical Co., St Louis, MO, USA). This MOPS-G1 handling medium was adjusted to a pH of 7.30±0.05 with NaOH. For microscopy imaging, a modified version of MOPS-G1 was used where phenol red was omitted from the formulation. All media were supplemented with serum albumin at a concentration of 5mg/mL.

2.1.3 Media preparation

Unless otherwise stated all media components were purchased from Sigma Aldrich Chemical Co. (St Louis, MO, USA) and pre-screened for ability to support embryo development with a 1-cell mouse bioassay (Gardner *et al.* 2004) (see section 2.2 Mouse Embryo Bioassay). Ammonium chloride (NH_4Cl), at a concentration of 300μM, or 5, 5-dimethyl-2, 4-oxazolidinedione (DMO), at a concentration of 2mM, were added to the medium where indicated.

For preparation of culture media, all media components were weighed out in a clean area into a sterile volumetric flask excepting $CaCl_2$-$2H_2O$ which was weighed into a plastic 14 mL tube (BD Biosciences BD Falcon ™, North Ryde, NSW, Australia). Approximately 500 mL of Milli-Q water was added to the volumetric flask and the flask swirled until all components were dissolved. Then 10 mL of Milli-Q water were added to the tube containing $CaCl_2$-$2H_2O$. The tube was agitated until the

powder was completely dissolved before the contents were added to the volumetric flask. The flask was then made up to 950 mL with Milli-Q water (this shortened volume allows for the addition of HSA prior to use). Media were sterilised by filtration through an Acrodisc 0.2µm syringe filter (Pall Corporation, Ann Arbour, MI, USA) and then stored at 4 °C in either glass bottles or plastic flasks (Nalgene Nunc International, Rochester, NY, USA).

Culture media with human serum albumin (HSA) were made up fresh every four weeks and stored in 14 mL tubes (BD Biosciences BD Falcon ™). To prepare the culture media with HSA, 9.5 mL of G1.2 medium or G2.2 medium was added to the tube and then 500µl HSA (Vitrolife, Kungsbacka, Sweden) was added to give a final concentration of HSA of 5mg/mL.

For medium containing 300µM ammonium, a 100x stock solution was prepared by the weighing of 0.01605g of NH_4Cl into a 14 mL tube. Then 10 mL of Milli-Q water were added to the tube. Once the ammonium chloride was dissolved the solution was sterile filtered through a 0.2µm syringe filter (Pall Corporation, East Hills, NY, USA). To prepare the final culture medium 100µl of the ammonium stock was added to 9.9 mL of the relevant medium.

For medium containing 2 mM DMO, a 100x stock solution was prepared by the weighing of 0.258g of DMO into a 14 mL tube. Then 10 mL of Milli-Q water were added to the tube. Once the DMO was dissolved the solution was sterile filtered through a 0.2µm syringe filter (Pall Corporation). To prepare the final culture medium 100µl of the DMO stock was added to 9.9 mL of relevant medium. The pH of the medium was reduced by the addition of DMO and therefore was then adjusted back to 7.4 by adding a small amount of NaCl dissolved in the relevant culture medium of either G1.2 or G2.2.

2.1.4 Preparation of hyaluronidase

Hyaluronidase (Bovine Testes, Sigma Aldrich Chemical Co.) at 1mg/mL was prepared in MOPS-G1 handling medium. To prepare, 30mg of hyaluronidase was added to a sterile glass beaker and dissolved in 30 mL of MOPS-G1 handling medium. This was filtered through a 0.2µm filter and frozen at –20 °C in 1 mlLaliquots for up to one month.

2.2 Mouse embryo bioassay

The majority of consumables that are required for the collection and growth of embryos are manufactured for other purposes. Research shows that it is necessary to quality control all consumable and plastic ware in order to maintain the optimal environment for embryo culture, thus ensuring normal embryo physiology and subsequent pregnancy rates (Gardner *et al.* 2005; Lane *et al.* 2008). Therefore, all plastic ware that was used for embryo collection and culture and essential chemicals for media preparation were first pre-screened using a 1-cell mouse bioassay. Mouse zygotes from prepubescent F1 hybrid female mice (C57BL/6xCBA) that were mated with F1 hybrid male mice were

grown in simple G1.2 supplemented with 5% serum albumin (HSA) from the zygote to 2-cell under test conditions (Table **2-2**). After this they were moved to simple G1.2 without albumin, under test conditions, and cultured to the blastocyst stage. This is because HSA can act as a chelator and may mask any inhibitory effects of the test condition (George *et al.* 1989). These were grown alongside control embryos; i.e. those cultured in dishes and/or media that had previously passed. Embryos were scored after 74 hours of culture on Day 4 at 4 pm and after 91 hours of culture at 9 am on Day 5 to assess time development rates. A minimum of 30 embryos were cultured under each test condition, and for consumables to pass the assay they must have >50% early blastocyst development after 74 hours of culture and >75% expanded blastocyst development after 91 hours of culture.

To test culture dishes embryos were grown in simple media on the actual test dish for 91 hours. For test tubes, media were placed in the tubes for 1 week at 4ºC; embryos were then grown in this media for 91 hours.

For testing amino acids, a differential stain was also performed to assess inner cell mass (ICM) and trophectoderm (TE) development (see section 2.8.2 Differential Cell Number Staining).

Table 2-1: Media components for culture, handling and imaging media

Component	MOPS-G1 (Collection) (mM)	G1.2 (Cleavage development) (mM)	G2.2 (Blastocyst development) (mM)	Confocal Loading Media (mM)	L-Simple G1.2 (mM)
NaCl	90.1	90.1	90.1	90.1	90.1
KCl	5.5	5.5	5.5	5.5	5.5
$NaHPO_4.2H_2O$	0.25	0.25	0.25	0.25	0.25
$MgSO_4.7H_2O$	1.0	1.0	1.0	1.0	1.0
$NaHCO_3$	5.0	25.0	25.0	5.0	25.0
$CaCl_2.2H_2O$	1.8	1.8	1.8	1.8	1.8
Glucose	0.5	0.5	3.15	0.5	
Na Lactate	10.5	10.5	5.87	10.5	5.0
Na Pyruvate	0.32	0.32	0.1	0.32	
EDTA		0.01			
Alanyl-glutamine	0.5	0.5	1.0	0.5	
Alanine	0.1	0.1	0.1	0.1	
Arginine			0.6		
Asparagine	0.1	0.1	0.1	0.1	
Aspartate	0.1	0.1	0.1	0.1	
Cystine			0.1		
Glutamate	0.1	0.1	0.1	0.1	
Glycine	0.1	0.1	0.1	0.1	
Histidine			0.2		
Isoleucine			0.4		
Leucine			0.4		
Lysine			0.4		
Methionine			0.1		
Phenylalanine			0.2		
Proline	0.1	0.1	0.1	0.1	
Serine	0.1	0.1	0.1	0.1	
Taurine					
Threonine			0.4		
Tryptophan			0.5		
Tyrosine			0.2		
Valine			0.4		
Choline chloride			0.0072		
Folic Acid			0.0023		
Inositol			0.01		
Nicotinamide			0.0082		
Pantothenate			0.0042		
Pyridoxal			0.0049		
Riboflavin			0.00027		
Thiamine			0.00296		
MOPS	20.0			20.0	
Phenol Red	0.005g/L				

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Table 2-2: Media components for Simple G1.2 (Batch Test Media)

Component	Simple G1.2 Medium (mM)
NaCl	90.1
KCl	5.5
NaH_2PO_4-H_2O	0.25
$MgSO_4$-$7H_2O$	1.0
Na Lactate (L isoform)	10.5
$NaHCO_3$	25.0
Pyruvic Acid (Na Pyruvate)	0.32
Glucose	0.5
$CaCl_2$-$2H_2O$	1.8

For the first 19 hours of culture, the Simple G1.2 medium was supplemented with 5 mg/mL HSA.

2.3 Animals and ovulation induction

2.3.1 Animals

All animals used in this study were bred at the Central Animal House, Waite Institute, Adelaide and were housed at either the Queen Elizabeth Hospital Animal House or at Laboratory Animal Services at the University of Adelaide. Animals were caged and allowed free access to food and water and maintained on a 14:10 hour light:dark cycle at 24°C. All animal experimentation was approved by The University of Adelaide Animal Ethics Committee (AEC) and for the animals housed at the Queen Elizabeth Hospital ethical approval was also obtained from the Institute of Medical and Veterinary Science (IMVS) ethics committee. All procedures were conducted in accordance with the National Institute of Health (NIH) guide for the care and use of laboratory animals as well as the 'Australian code of practice for the care and use of animals for scientific purposes – 7th Edition 2004'.

2.3.2 Induction of ovulation

Embryos were collected from pre-pubescent F1 hybrid female mice (C57BL/6xCBA) following superovulation using an intraperitoneal injection of 5 IU of PMSG (Pregnant Mares' Serum Gonadotrophin, Folligon, Intervet, Bendigo, Victoria, Australia) and by an intraperitoneal injection of 5 IU hCG (human Chorionic Gonadotropin, Pregnyl, Organon, Oss, Holland) 48 hours later. Immediately following the second injection the females were placed with Swiss males. Mating was determined the next morning by the presence of a vaginal plug.

2.3.3 Collection of pre-implantation embryos

Pregnant female mice were killed by cervical dislocation. The abdomen was sterilised with 70% ethanol and a small incision was made in the ventral midline and brought up either side of the abdomen exposing the peritoneum. Using fine scissors the peritoneum was cut to expose the visceral organs which were moved aside to expose the reproductive tract.

2.3.3.1 Zygote collection

For collection of zygotes, at 23 hours post-hCG pregnant females were cervically dislocated and the reproductive tract was isolated as mentioned above. A fine pair of forceps was used to dissect the mesentery away from the uterus and the oviduct. When the uterus was pulled taught, a cut was made between the oviduct and the ovary and a second cut was made through the utero-tubal junction. The dissected oviduct was then placed into a small vial containing warmed MOPS-G1 handling medium (37°C) and placed onto a microscope warming stage (37°C ±0.5).

Zygotes were located in the distended ampulla of the oviduct. The oviduct was grasped with a pair of fine forceps next to the swollen ampullary region and held firmly to the bottom of the dish. A second pair of forceps was used to tear the oviduct wall, releasing the zygotes and the surrounding cumulus mass into the medium. The oviduct was then discarded leaving the zygote-cumulus mass behind in MOPS-G1 handling medium.

Individual zygotes were then isolated by adding hyaluronidase (1 mg/mL) to the medium containing the expelled zygote-cumulus complexes. After one minute the cumulus cells begin to disperse and the zygotes were denuded by removing any remaining coronal cells by pipetting with a pulled pipette with an outer diameter just greater than that of the zygote (approximately 100µm). The denuded zygotes were washed three times in MOPS-G1 handling medium to remove hyaluronidase and were then washed three times in G1.2 medium before being placed into G1.2 medium culture drops.

2.3.3.2 Blastocyst collection

Collection of *in vivo* derived blastocysts was performed at 88 hours post-hCG. Both uterine horns were removed by cutting below the utero-tubal junction and a second cut through the cervix. A blunt 32-gauge hypodermic needle attached to a 1 mL syringe was inserted into one end of the uterus and blastocysts were flushed from the tract using warmed MOPS-G1 handling medium.

2.4 Surgical procedures

2.4.1 Vasectomy

Male F1 hybrid mice between six and eight weeks of age were anaesthetised with an i.p injection of 1.2% Avertin (2, 2, 2-Tribromoethanol, 2-methyl-2-butanol; 0.02 mL/g body weight (see Appendix: Section 10.1 and 10.2). Each male was placed on its back and its abdomen sterilised using 70% ethanol. A 2–3mm incision was made ventrally in the lower abdomen region (along the scrotal sac below the penis) through the body wall and peritoneum. The underlying connective tissue was dissected away to reveal the body cavity. The bursa sac containing the testes was then located and a small hole was made in the bottom of the sac near the epididymus. The epididymus was then gently pulled through the hole in the bursa. The vas deferen was located and, using a fine pair of forceps, 1–2 mm was dissected away from the testes and the blood vessel. A section of the vas deferen was then removed by making a cut near the epididymus and then another cut 10mm from the original; this was repeated on both sides. The testes were then eased back into the bursa and into the body cavity. The body wall was then stitched together with absorbable silk suture, followed by the skin wound. The mouse was then placed on a warming stage at 37 ±0.5°C to recover from the anaesthetic. The mouse was observed until he had fully recovered from the anaesthetic and was then observed every hour for the next six hours to ensure full recovery.

Males were allowed two to three weeks to recover from the surgery and were then checked for sterility. Two super-ovulated pre-pubescent F1 female mice (C57BL/6xCBA) were placed with the male, and the following morning they were checked for the presence of a vaginal plug. At 21 hours post-hCG the females were cervically dislocated and zygotes were collected and placed into culture. The following morning the embryos were checked for division as a sign of fertilisation. If none of the embryos had divided from 2 females per mouse, the male was determined to be sterile.

2.4.2 Embryo transfers

Swiss females aged 6–12 weeks were used as recipients for embryo transfers. Cycling Swiss females were placed with confirmed sterile males overnight. The following morning mating was assessed by the presence of a vaginal plug, which was then termed Day 1 of pseudo-pregnancy.

On Day 4 of pseudo-pregnancy the females were anaesthetised with an i.p injection of 1.2% Avertin (2, 2, 2-Tribromoethanol, 2-methyl-2-butanol; 0.02 mL/g body weight (see Appendix: Section 10.1 and 10.2). The female was placed on her stomach and her back was sterilised using 70% ethanol. A 5–10 mm incision was made in the midline where the middle of the incision was adjacent to the hips. The underlying connective tissue was gently dissected away using fine forceps and a 5 mm incision was made into the peritoneum directly above the fat pad. Using the fine forceps the fat pat was clasped and pulled from the body cavity bringing with it the ovary, oviduct and the uterus. The fat pad was then secured outside the cavity using an ovarian retractor. The culture dish containing the

blastocysts to be transferred was then removed from the incubator and quickly moved to a warmed microscope stage. A flame-pulled pipette, with an outer diameter just greater than that of the blastocysts to be transferred (approximately 120µm), was used to pick-up six blastocysts in a volume of media less than 1µl flanked by two air bubbles. Fine forceps grasped the utero-tubal junction and the uterus was pulled taut. A sterile 32-gauge needle was used to make a hole in the wall of the lower third of the uterus and into the uterine lumen. The pipette containing the embryos was then inserted into the hole in the uterine wall and the pipette was slid back and forth to ensure that it was in the lumen. The embryos were expelled into the uterus using the air bubbles as a guide that they had been released. After the pipette was checked to make sure that all embryos had been expelled into the tract, the reproductive tract was eased back into the body cavity, avoiding any more contact with the uterus. The entire procedure was repeated for the other horn. Once both sides had been completed the wound was closed using AutoClip 9 mm wound closing clips (Becton Dickson and Company, Sparks, Maryland, USA) and the female was placed in a recovery box on a warm stage at 37±0.5°C to recover from the anaesthetic. If multiple mice were completed they were differentiated by ear tags. The mice were observed until they had fully recovered from the anaesthetic and were then observed every hour for the next six hours to ensure full recovery.

2.4.3 Isolation of fetuses and placenta

Pregnant females were killed by cervical dislocation on either Day 15 or Day 18 of pregnancy. The abdomen was sterilised with 70% ethanol and an incision was made along the ventral midline and then take up either side of the abdomen exposing the body cavity. The internal organs were pushed aside to expose the uterine horns containing the fetuses. Both tracts were cut and removed, ensuring that the right hand side and left hand side were kept separate. Individual fetuses and their corresponding placenta were isolated by dissecting the uterine wall and separating the extra-embryonic membrane away from the fetus and placenta. The umbilical cord was cut, and any extra tissue was dissected away from the placenta which could then be weighed and measured. Absorption sites were identified by rudimentary embryonic tissue (dark protrusions) in the uterine wall. After all measures were taken, the fetus and placental tissue was placed into small plastic bags and snap frozen in liquid nitrogen.

2.5 Embryo culture

2.5.1 Manipulation of embryos

Fine glass pipettes with an inner diameter of approximately 100µm were used for manipulation of embryos. Pipettes were prepared by rotating the neck of a sterilised Pasteur pipette in a Bunsen burner flame. When the glass was softened it was removed from the flame and pulled in opposite directions to stretch the glass. Pulled pipettes were then snapped off at the desired length and checked for an even

end. A mouth pipette was used for embryo manipulation because it allows control via suction. A small piece of malleable plastic tubing was attached to a 0.2µm filter at the thick filter end. A smaller diameter tube approximately 60cm in length was attached to the smaller end of the filter. The pulled pipette was then attached to the larger piece of tubing and a 20–100µl filtered pipette tip was placed into the end of the long piece of tubing completing the mouth pipette apparatus. Introduction of a pipette into media results in initial filling due to capillary action. Therefore, in order to control the filling of the tube, capillary action was broken by alternating small volumes of medium or oil with air bubbles in the pipette by gentle suction. Thus the pipette could be used to manipulate embryos by sucking and expelling air from the mouth.

2.5.2 Preparation of culture dishes

Media for embryo culture were placed as 20µl drops under 3.5 mL light weight paraffin oil (Vitrolife, Kungsbacka, Sweden) in a 35 mm petri dish (BD Biosciences BD Falcon ™) which had passed the MEA (see section 2.2 Mouse Embryo Bioassay). Four wash drops were placed at the top of the dish and 4 culture drops were placed at the bottom of the dish. To ensure equilibration of the medium to a physiological pH of 7.3, all dishes were prepared, warmed and equilibrated at 37°C in a gas phase of 6% CO_2 in air (Sanyo CO_2 Incubator: MCO15AC) for at least four hours prior to culture.

2.5.3 Embryo culture

Embryos were collected from the female mice as stated above and were pooled and randomly allocated to each treatment in even groups. After collection embryos were washed 3 times in MOPS-G1 handling medium and twice in the respective culture medium through the wash drops in the top of the culture dish. Embryos were then placed in groups of 10–12 per 20µl drop of G1.2 medium and dishes were placed in either a modulator incubator chamber (O.N.A International, East Ivanhoe, Victoria, Australia), which was purged with 6% CO_2, 5% O_2, 89% N_2 for three minutes, sealed and incubated at 37°C, or in a tri-gas incubator (Sanyo O_2/CO_2 incubator: MCO-18M) at 5% CO_2, 6% O_2, 89% N_2 at 37°C. On Day 3 of culture (43h of culture) embryos were washed three times through G2.2 medium and moved to pre-equilibrated drops of G2.2 medium for the remaining two days of culture. To minimise stress on the embryos all collection and manipulation of embryos was performed at 37°C and the time taken for placement into culture after being in MOPS-G1 handling medium and morphological assessments was less than five minutes.

2.6 Assessment of embryo development *in vitro*

2.6.1 Assessment of embryo morphology

Embryo morphology was assessed at a magnification of 100X using a Nikon phase contrast microscope on a warm stage at 37°C. In all experiments the morphological stages of the embryos were classified using the following criteria; Zygotes (fertilised oocytes) can be identified on the basis of either the presence of two pronuclei or extrusion of the second polar body. However, as pronuclei are rarely visible at the time of collection (23 hours post-hCG), extrusion of the second polar body was primarily used to identify fertilised oocytes (Figure 2-2**, Figure 2-2**). Cleavage stage embryos were assessed by the number of clearly visible cells (2-cell to 8-cell embryos) (**Figure 2-3**, **Figure 2-4**, **Figure 2-5**). Partially compacted embryos were determined by a loss of cellular individuality; however, some cell numbers could still be determined. Compacted embryos were defined as complete loss of cell individuality (**Figure 2-6**). Embryos were classified as early blastocysts when the blastocoel cavity was 2/3 or less in volume of the entire embryo (**Figure 2-7**). Embryos were classified as expanded blastocysts when the blastocoel cavity was 2/3 or greater in volume of the entire embryo (**Figure 2-8**). Hatching blastocysts were identified by herniation of trophectoderm through the zona pellucida (**Figure 2-9**).

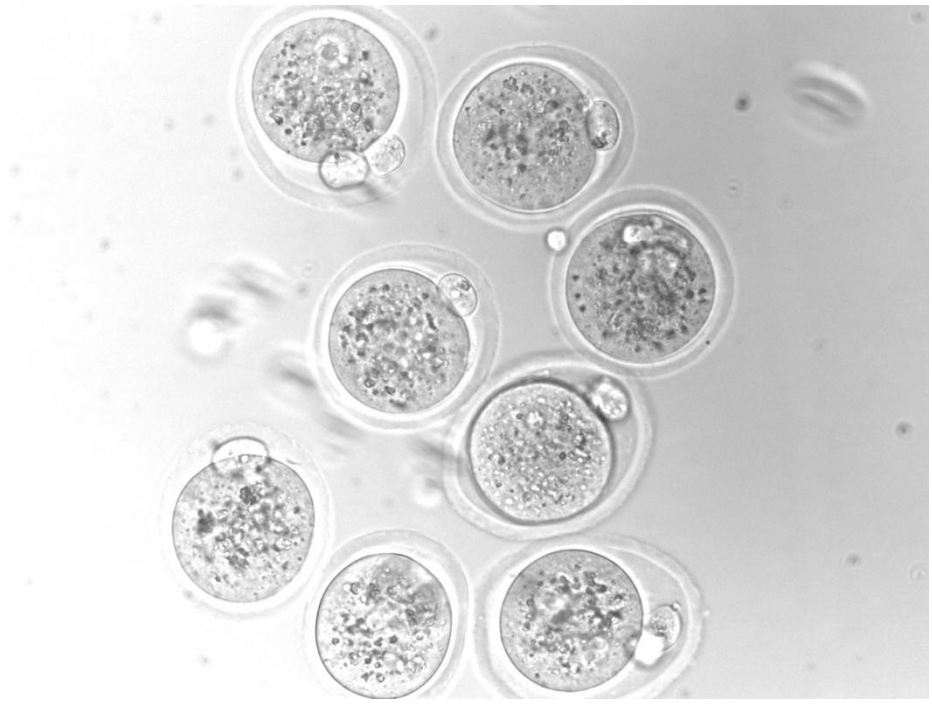


Figure 2-1: Mouse zygote at 24 hours post hCG (Day 1) at 20x objective

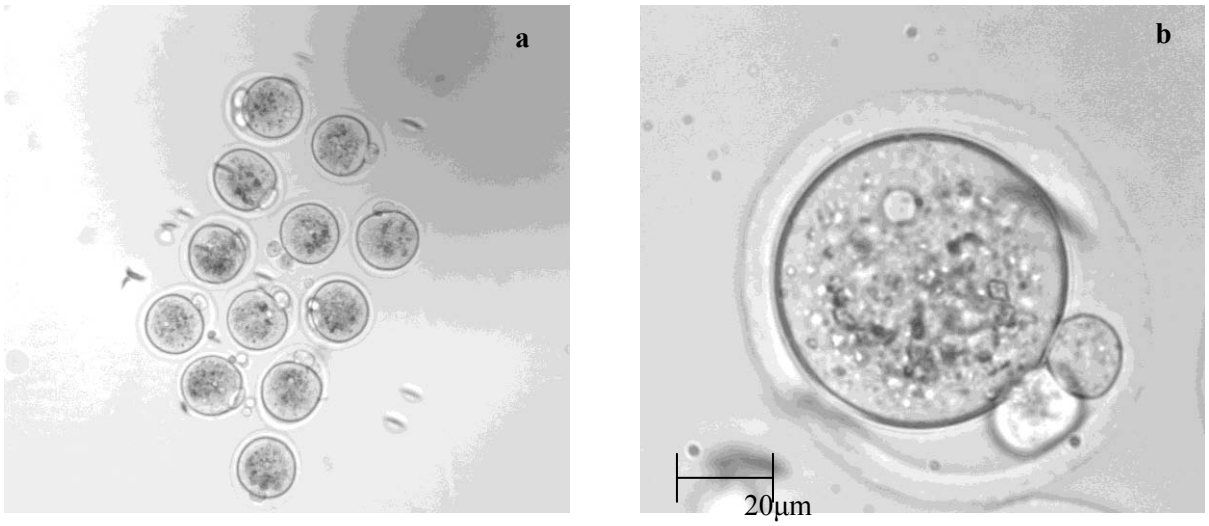


Figure 2-2: Mouse zygote at 24 hours post hCG (Day 1): a) 10x objective; b) 40x objective

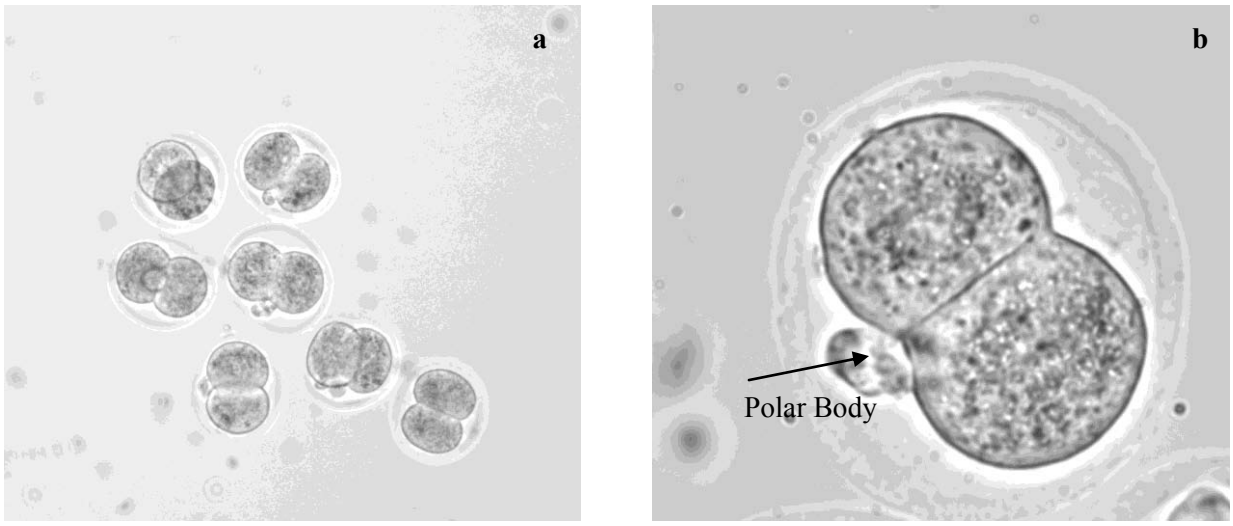


Figure 2-3: Mouse 2-cell after 19 hours culture (Day 2): a) 10x objectives; b) 40x objective

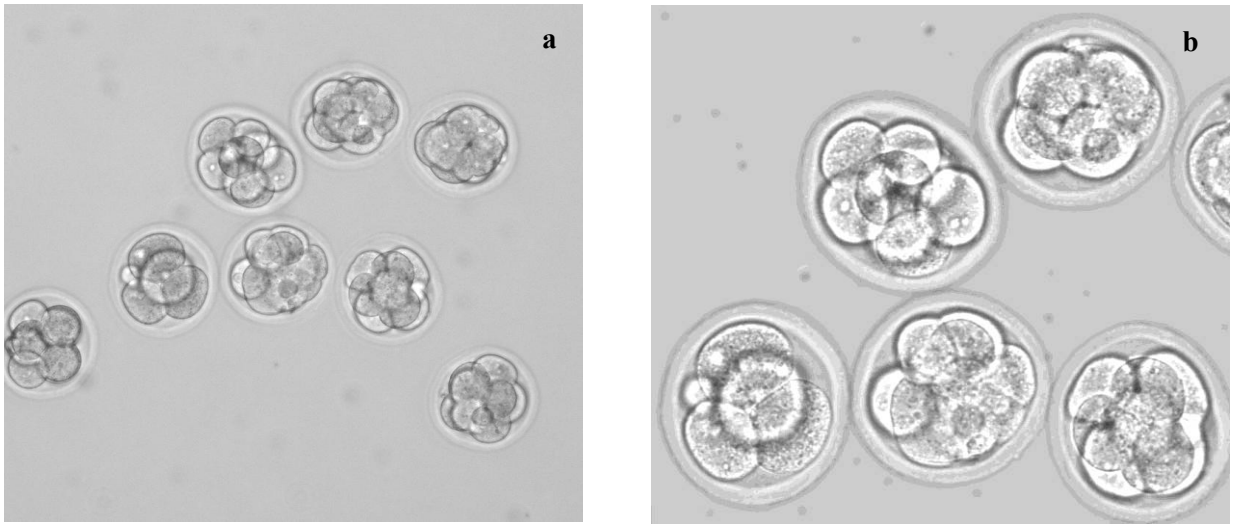


Figure 2-4: Mouse 4-cell and 8-cell embryos after 43 hours culture (Day 3): a) at 10x objective; b) at 20x objective

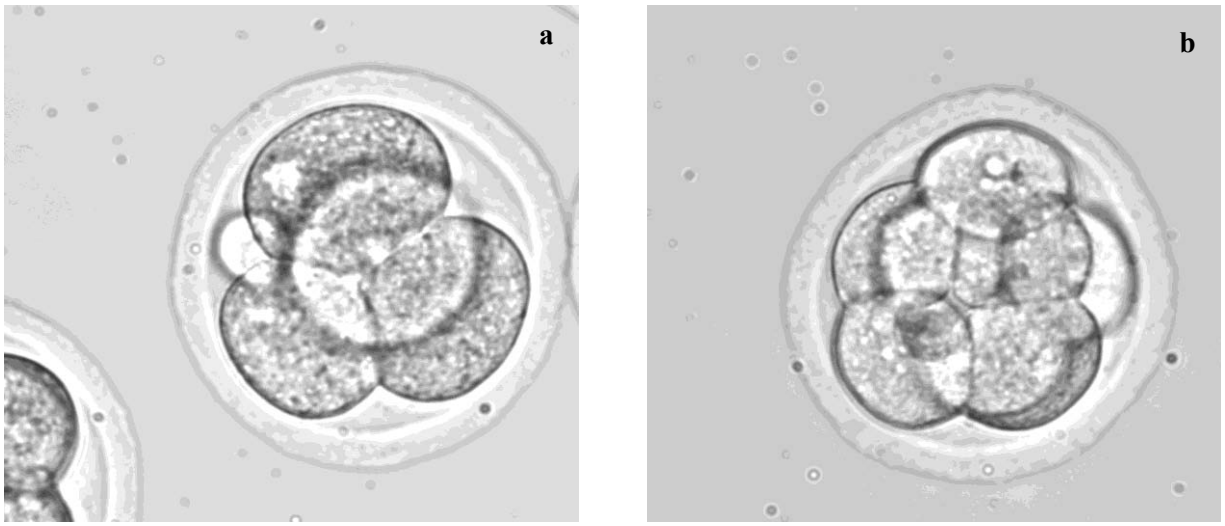


Figure 2-5: Murine 4-cell embryo: a) and 8-cell embryo b) at 40x objective (Day 3)

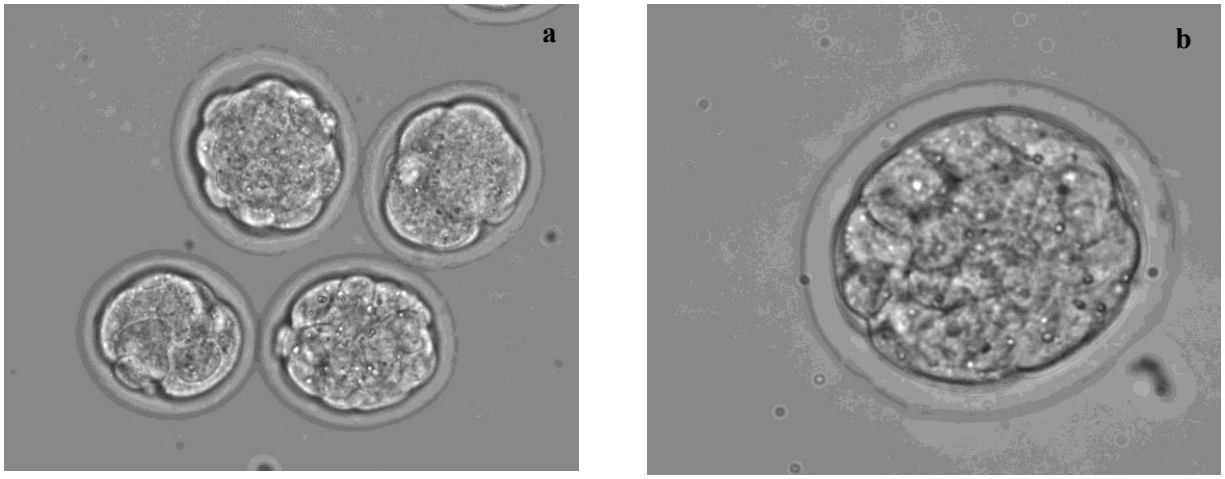


Figure 2-6: Murine morula embryos at 72 hours culture (Day 4): a) 20x objective; b) 40x objective

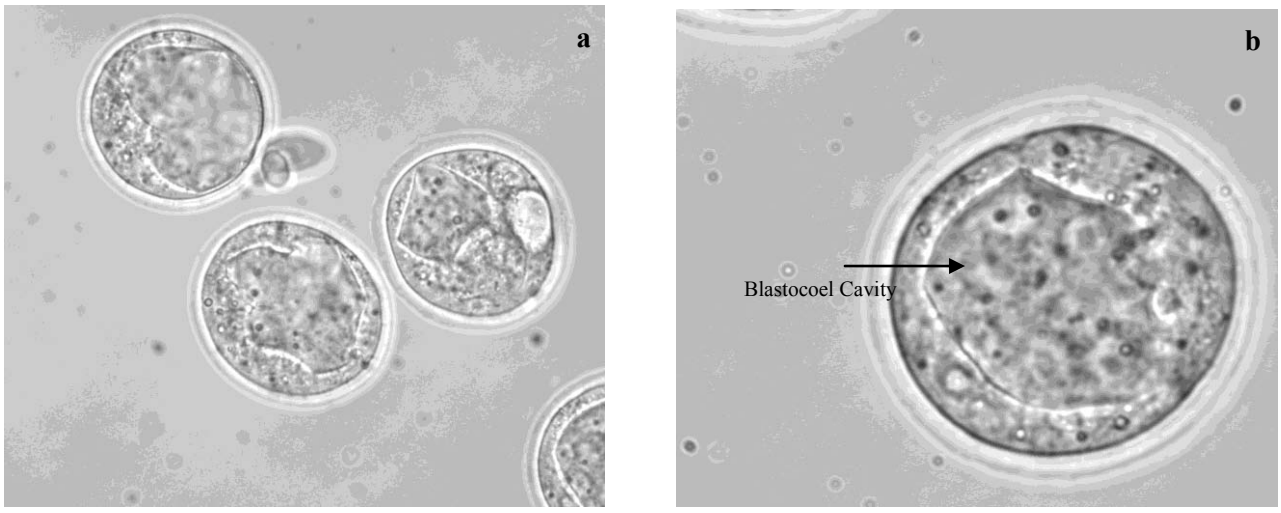


Figure 2-7: Murine early blastocysts after 72 hours culture (Day 4): a) at 20x objective; b) 40x objective

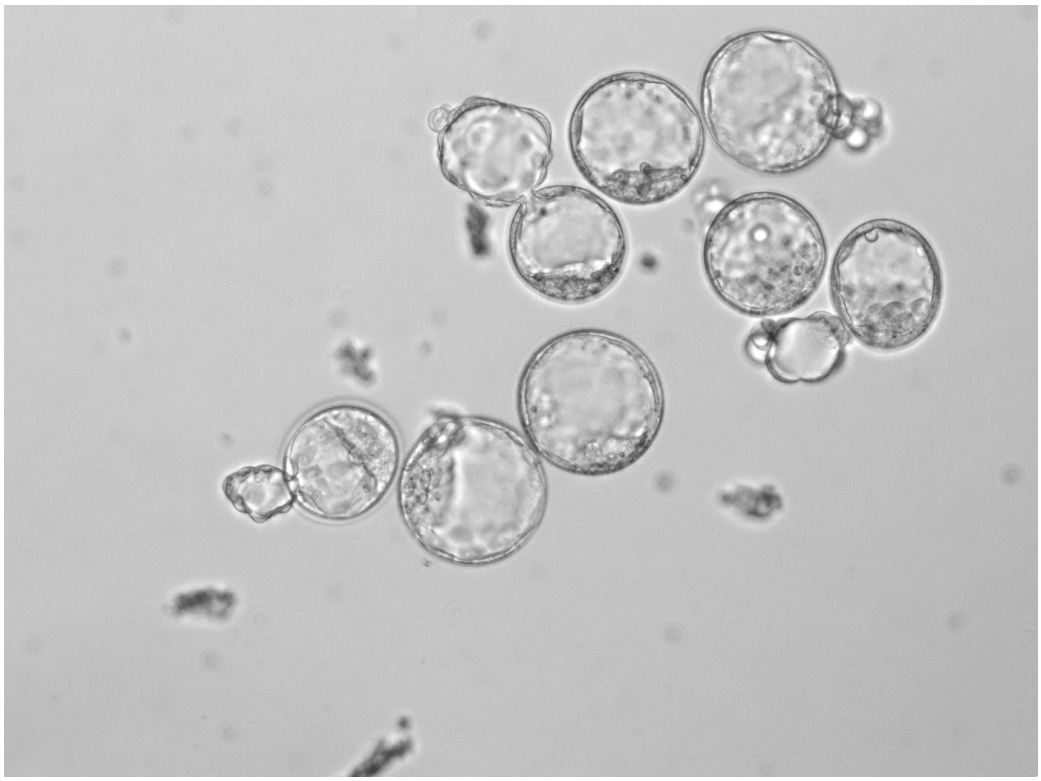


Figure 2-8: Expanded and hatching mouse blastocysts after 91 hours of culture (Day 5) at 20x objective

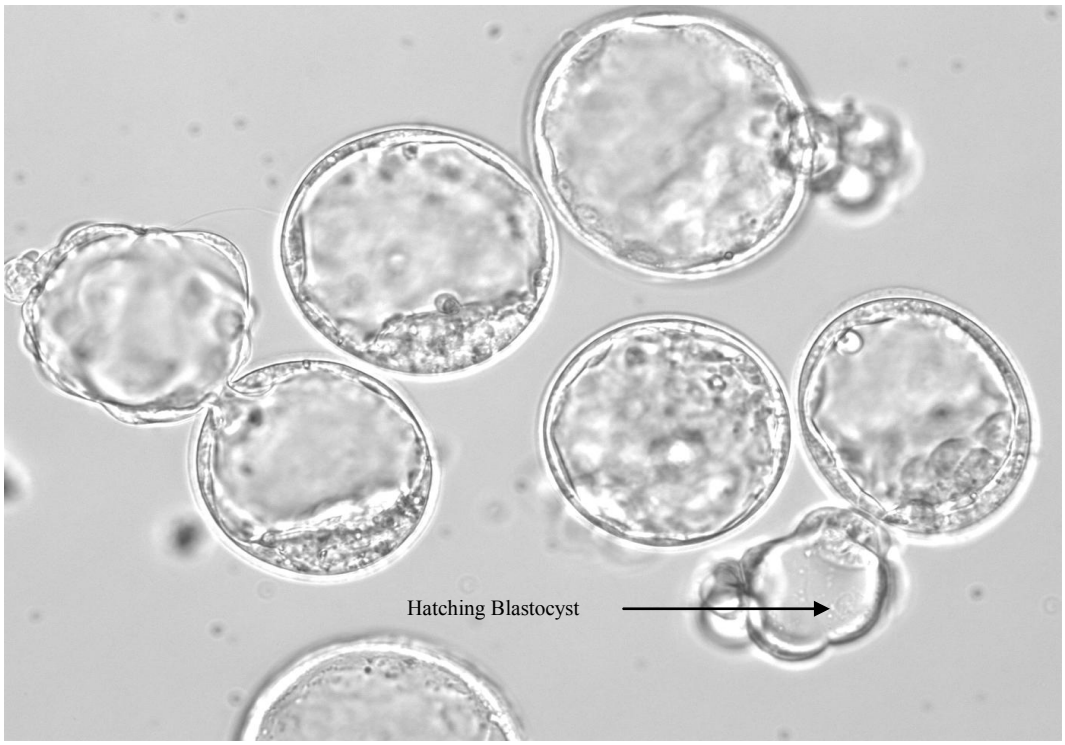


Figure 2-9: Expanded and hatching mouse blastocysts after 91 hours of culture (Day 5) at 40x objective

2.7 Selection of ammonium and DMO concentrations

Previous research has demonstrated that ammonium levels increase in a liner fashion over time when media is incubated at 37°C and contains amino acids, in particular glutamine which is highly unstable (Gardner and Lane 1993b; Lane and Gardner 2003). In media containing 1mM glutamine, ammonium levels impair development and viability after only 24 hours incubation (Gardner and Lane 1993b; Lane and Gardner 2003; Orsi and Leese 2004; Virant-Klun *et al.* 2006).

In previous experiments (on which this study was based), culture media was supplemented with ammonium chloride in the following concentrations: 0, 18.8, 37.5, 75,150, or 300 μM and embryo development and viability were assessed(Lane and Gardner 2003). In these experiments it was discovered that ammonium at all concentrations assessed did not impact on blastocyst development and morphology however blastocyst cell number was affected from 37.5μM. Interestingly increasing the concentration of ammonium in the culture media up to 150μM did not further decrease the cell number of the resultant blastocysts however 300μM ammonium resulted in further decreases in blastocyst cell number and inner cell mass number.

Culture with ammonium chloride concentrations from as low at 18.8μM significantly increased blastocyst apoptosis levels and this was further amplified as ammonium concentrations increased. In regards to metabolism, concentrations of 75μM resulted in perturbed pyruvate oxidation and 300μM ammonium perturbed blastocyst glycolysis as well. After transfer to pseudopregnant recipients, 300μM ammonium also impaired implantation rates and fetal weights. 300μM ammonium also significantly altered gene expression in the resultant blastocysts (Lane and Gardner 2003).

The purpose of the experiments in this study was to assess the impact of a model *in vitro* stress that was both physiologically relevant and would impact not only on embryo homeostasis and metabolism but also downstream fetal development. Due to this 300μM ammonium was chosen as the concentration to use in the experiments for this thesis and the same strain of mice and culture media was also used to ensure consistency.

The ability to tightly regulate intracellular pH is essential for normal cellular function. Ammonium, which is a weak acid, has been shown to decrease intracellular pH. In an effort to better understand the ammonium model of *in vitro* stress a weak non-metabolisable acid (DMO) was used to minic the decrease in intracellular pH seen after ammonium exposure. DMO has been used since the 1950's to measure intracellular pH and also decrease intracellular pH of somatic cells, gametes or embryos (Edwards *et al.* 1998b; Waddell and Butler 1959). It is a by-product of metabolism of an antiepileptic drug and is not believed to be toxic. Humans have levels as high as 1mg/ml in their blood without any toxic effects being observed. Due to this fact the observed effects of DMO exposure are believed to be entirely due to the decrease in pH_i which is causes (Waddell and Butler 1959).

A preliminary experiment was performed where DMO was titrated into culture media and intracellular pH was measured in zygotes. It was discovered that 2mM DMO induced a decrease in intracellular pH of 7.12 which is similar to the decrease in intracellular pH seen after ammonium exposure (pH 7.10) (see appendix 10.9)(Lane and Gardner 2003). Previous experiments in the literature also have used similar concentrations of DMO as a model pH stress to investigate the impact of decreasing pH on embryo development (Edwards *et al.* 1998a). It has also been shown that concentrations up to 30mM DMO do not impact on blastocyst development or morphology (Edwards *et al.* 1998a).

2.8 Assessment of cell parameters

2.8.1 Simple cell number staining

After compaction, embryo cell number was determined on Day 4 using a propidium iodide (PI) stain. Initially embryos were placed at 4°C overnight. The following day dishes were set up containing 20µl drops of 50µg/mL PI in MOPS-G1 handling medium under 3.5 mL mineral oil (Sigma Aldrich Chemical Co.) and placed in the dark. Organ wells (BD Biosciences BD Falcon ™) containing 1 mL of 0.05% Triton X-100 (Sigma Aldrich Chemical Co.) in MOPS-G1 handling medium was also prepared. Embryos were placed into the Triton solution for 30–45 seconds to permeabilise the cells. Embryos were then washed through fresh MOPS-G1 handling medium to remove any Triton solution and then placed into the PI drops in the dark for two to five minutes at 37°C. Following this, drops of glycerol were placed on a clean glass slide and two to three embryos were removed from the PI and placed in each drop. A cover slip was placed gently on top and the embryos were imaged immediately on a fluorescent microscope using the green filter (ex: 530nm em: 615nm) and a cell count was performed. The nuclei of the cells could be identified by orange fluorescent staining (**Figure 2-10**).

2.8.2 Differential cell number staining

Allocation of cells in the blastocyst to the inner cell mass (ICM) or trophectoderm (TE) was assessed using a differential staining protocol described by Gardner et al. . All procedures were carried out at 37°C in 20µl drops under mineral oil (Sigma Aldrich Chemical Co.) in dishes that were pre-warmed for at least one hour prior to the experiment. Initially embryos were placed into 0.5% pronase (Sigma Aldrich Chemical Co.) in protein free MOPS handling medium to dissolve the zona pellucida. All embryos were observed down the microscope to assess zona disintegration every two to five minutes, depending on the hatching status of the zona. Embryos were then removed and immediately placed into wash drops of protein free MOPS handling medium to remove any remaining pronase. They were then incubated in 10 mM TNBS (2.4.6-trinitrobenzenesulfonic acid, Sigma Aldrich Chemical Co.) at 4 °C in the dark for 10 minutes, washed three times until no yellow colour was present in the wash

media and placed into 0.1 mg/mL anti-DNP (anti-dinitrophenol-BSA, Sigma Aldrich Chemical Co.) at 37°C for 10 minutes. Because of the tight junctions between the TE cells, the ICM was not exposed to the anti-serum. Embryos were then incubated in complement (guinea pig serum and 50µg/µl propidium iodide (PI) (Sigma Aldrich Chemical Co.) for 5–10 minutes at 37°C. TE cells that were exposed to anti-DNP are partially lysed by the binding of complement allowing PI to enter and stain the nucleus. Embryos were then transferred to 25µg/mL bisbenzamide (Hoechst 33258, Sigma Aldrich Chemical Co.) in ethanol and incubated overnight at 4°C in the dark. As bisbenzamide stains all cells, the TE is stained with both PI and bisbenzamide and the ICM is stained with bisbenzamide only. The next day the embryos were transferred to 100% ethanol and either counted immediately or left at 4°C for up to one week before counting. To count the cells a small drop of glycerol was placed onto a siliconised glass slide. The embryos were pipetted into the glycerol transferring as little media as possible. A cover slip was placed gently on top of the slide while watching down a microscope. Small dots were made with a red permanent marker near each embryo to facilitate location under the microscope and the cover slip was compressed gently to flatten the blastocyst to allow all cells to be counted. The slides were then viewed on a fluorescent microscope at 400x using a UV filter (ex: 330-380nm) where the nuclei of the ICM cells appear blue and the nuclei of the TE cells appear pink. To assess the cell number initially the total cell number was counted by counting pink and blue nuclei using the UV filter, followed by a count of the nuclei of the TE cells only (PI) under the green filter (ex: 530nm em: 615nm) (**Figure 2-11**). The number of ICM cells was determined by subtracting the TE cell count from the total cell count. This method was deemed to be more accurate as the PI is very light sensitive and may photo bleach over time.

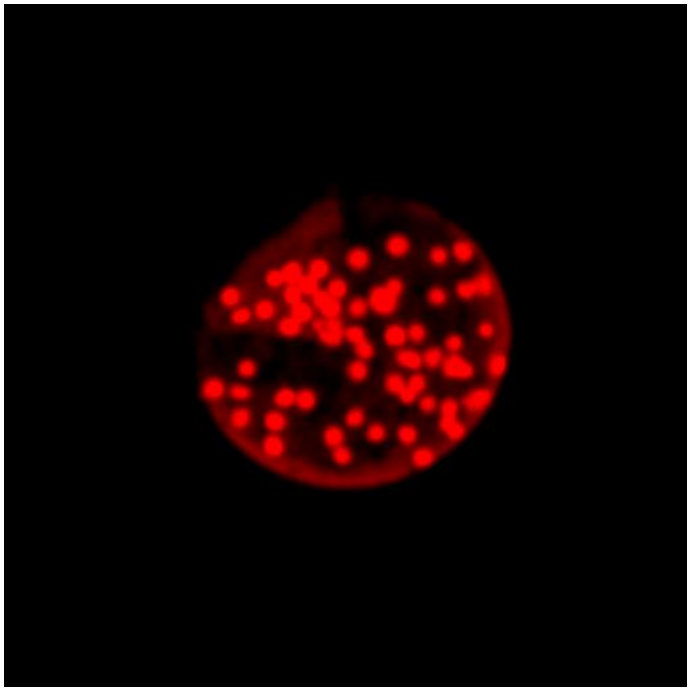


Figure 2-10: Simple cell number stained morula stage embryo. Each cell nuclei is stained orange.

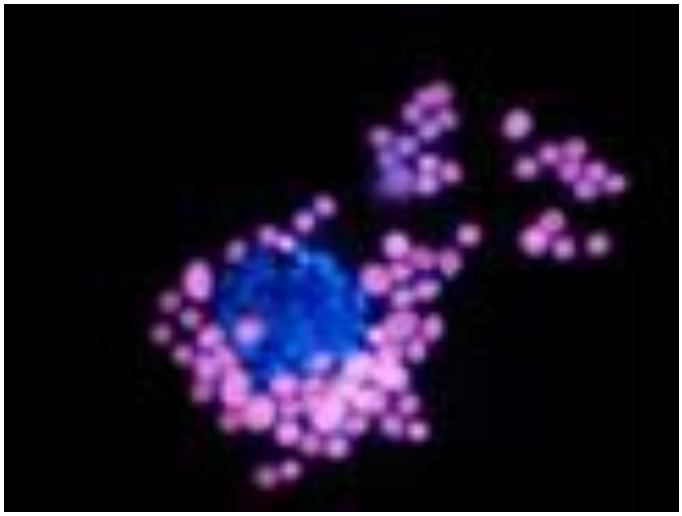


Figure 2-11: Differentially stained murine blastocyst. Pink staining indicates nuclei of TE cells and blue staining indicates nuclei of ICM cells**.**

2.8.3 Apoptosis level analysis

Blastocysts were removed from culture and washed four times through 0.3% PVP (polyvinyl-pyrrolidone) in phosphate-buffered saline (PVP/PBS). Embryos were then placed in 4% paraformaldehyde in PBS for two hours at room temperature or overnight at 4°C. After fixation in paraformaldehyde the embryos were washed twice through 0.3% PVP/PBS and then incubated in 0.5% Triton X-100 in PBS for one hour at room temperature. Embryos were then washed twice through 0.3%PVP/PBS and incubated in 10-15µl TUNEL mix (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labelling) using an In Situ Cell Death Kit (Roche Molecular Biochemical's, Indianapolis, IN) using a 1:9 ratio of TdT: TUNELbuffer. This was achieved by pipetting 10–15µl of the mix onto a 35 mm petri-dish. The embryos were pipetted into the mixture and then the embryos/TUNEL mix was immediately sucked up into a glass capillary precision bore tube with plunger (Drummond Scientific, Broomall, Pennsylvania, USA) and a small amount of oil was taken up into the capillary to prevent evaporation. These capillary tubes were then incubated for one hour, in the dark, at 37°C in a large petri-dish containing wet paper towel to prevent evaporation. After incubation the media was expelled from the capillary tube into 0.5% Triton X-100 in PBS and then washed two times through 0.3% PVP/PBS. While in the wash medium, 20µl drops of RNAse buffer (see Appendix: Section 10.3) were placed in a 35 mm dish covered by 3.5 mL mineral oil. Blastocysts were washed three times through the buffer to remove any cytoplasmic RNA and incubated in 20µl drops of PI/RNAse mix (0.05 mg/mL PI and 50µg/mL RNAse)(see Appendix; Section 10.4) under 3.5 mL mineral oil for one hour at room temperature in the dark. Embryos were washed twice through 0.5% Triton X-100 and once through 0.3%PVP/PBS before imaging. A small drop of glycerol was placed onto a glass slide and the embryos were pipetted into the drop. Using a paraffin/Vaseline spacer a cover slip was applied and the embryos were imaged using a fluorescent microscope. Apoptotic nuclei were first counted using the FITC channel (ex: 488nm em: 530nm) and then total cell number was assessed using the PI channel (ex: 530nm em: 615nm). The number of apoptotic cells was expressed as a percentage of the total cell number of each blastocyst to give the apoptotic cell index (**Figure 2-12**).

Both negative and positive controls were used where a negative control was stained using the standard protocol; however, blastocysts were incubated in 0.3%PVP/PBS instead of TUNEL mix. To perform the positive control, blastocysts were incubated in DNAse (0.005 U/µl) for 20 minutes at 37°C after the initial 0.5% Triton X-100 in PBS incubation. They were then taken through the remaining TUNEL labelling protocol as per normal (**Figure 2-12**).

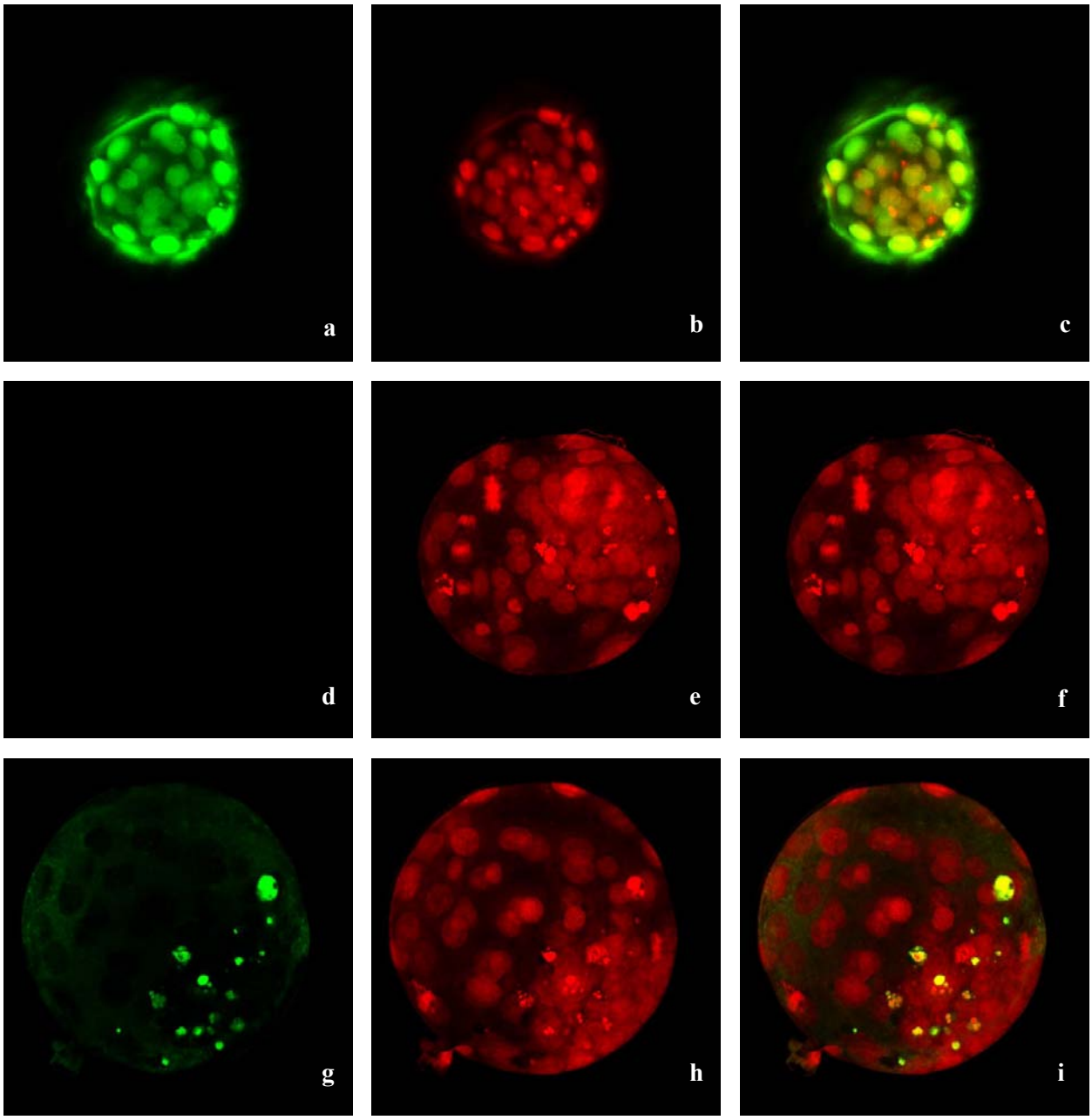


Figure 2-12: Images of blastocysts stained using TUNEL technique. a) TUNEL positive control; b) PI stain of positive control; c) merged positive control; d) TUNEL negative control; e) PI stain of negative control; f) merged negative control; g) TUNEL stain of ammonium treated blastocyst; h) PI stain of ammonium treated blastocyst; i) merged images of ammonium treated blastocyst

2.9 Glucose Uptake by blastocyst

Cultured blastocysts were incubated in 50nl drops of G2.2 media where the glucose concentration was reduced to 0.8mM (0.5mM glucose and 0.3mM of 6-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-6-deoxyglucose (6-NBDG) (Molecular Probes, Eugene, OR). 6-NBDG is a fluorescent nonhydrolyzable glucose analog that has been used to monitor glucose uptake and transport in live cells and typically displays excitation/emission maxima of ~465/540 nm (. Blastocysts were incubated for 1.5h at 37°C in 6%CO_2:5%O_2:89%N_2. At the end of the incubation period, glucose uptake was assessed by determining the levels of fluorescence in the blastocysts from the 6-NBDG using a photomultiplier with photometer attachment (Leica DMIRB, Mpvbio software) which converts levels of emission light into a numerical value. The level of fluorescence with this dye was determined to reflect glucose uptake as measured by quantitative microfluorescence (data not shown). The distribution of glucose within the blastocysts was assessed using confocal microscopy.

2.10 Assessment of mitochondrial homeostasis

2.10.1 Determination of mitochondrial distribution

Mitochondrial distribution was determined using Mitotracker green FM (Molecular Probes, Invitrogen, Eugene, OR). Mitotracker green FM is a mitochondrial-specific stain which is initially a non fluorescent aqueous solution that only becomes fluorescent when it accumulates in the lipid environment of active mitochondria. Two-cell embryos were incubated with 100nM of Mitotracker green FM in G1.2 medium for 15 minutes at 37°C in 6% CO_2, 5% O_2 and 89% N_2. After incubation the embryos were washed twice in G1.2 medium and then mounted on a large glass cover slip in a 10µl drop of confocal loading medium. A second smaller cover slip was placed on top using a paraffin/Vaseline spacer to prevent the embryos from being crushed. Images were then viewed within two minutes using confocal microscopy (Nikon, EZ-C1 software) and analysed using ImageJ software package (Version 1.31v, National Institutes of Health, USA) using a published method of analysis (Barnett *et al.* 1997). In this analysis 16 regions of pixel intensity are measured, four around each of the two nuclei and four readings in each of the two cytoplasm (**Figure 2-13**) This allows a relative distribution of mitochondria to be established and is expressed as a nuclear:cytoplasmic ratio.

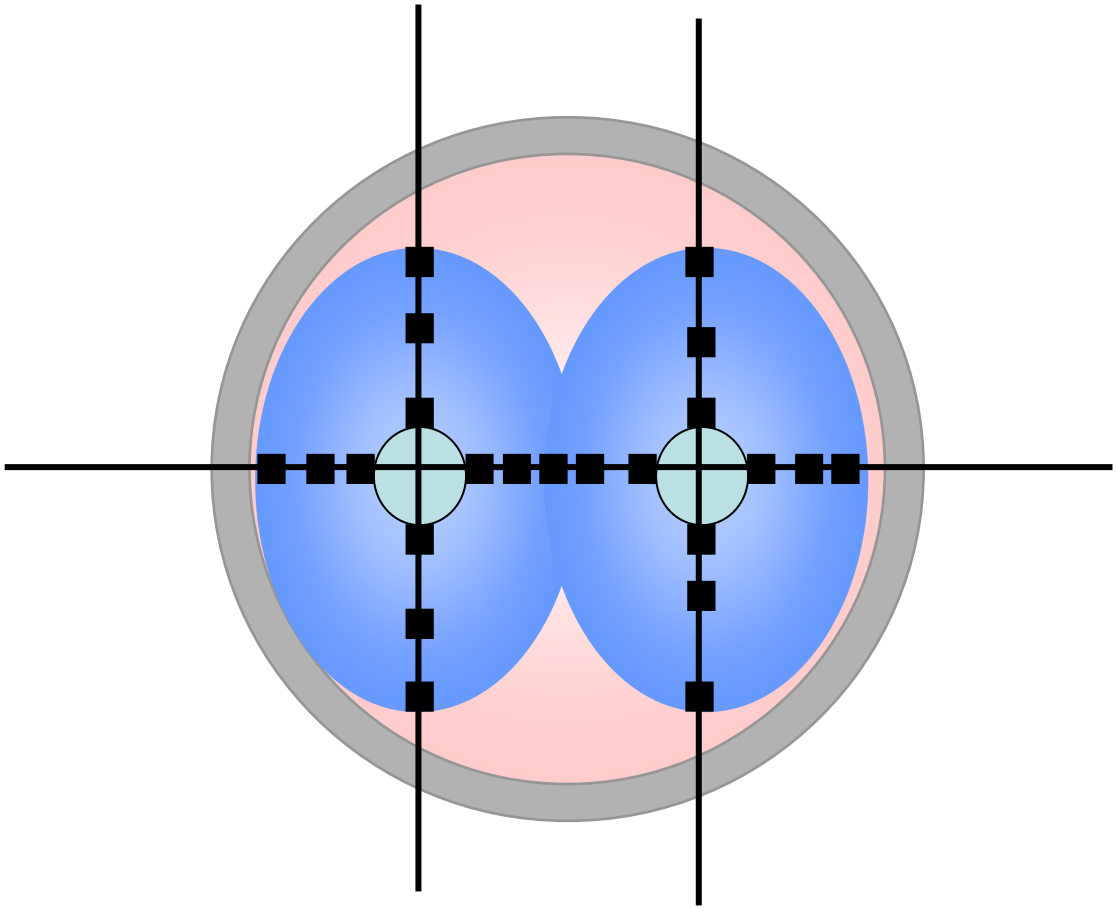


Figure 2-13: Schematic diagram of a mouse 2-cell embryo indicating the position of the regions measured. The black squares indicate the position of up to 24 pixel boxes which were drawn on each image using a transparent template overlay (4 in the peri-nuclear, 4 in the cytoplasm and 4 at the exterior of each cell) where the pixel intensity was measured.

2.10.2 Determination of mitochondrial calcium levels

Mitochondrial calcium levels were determined using the AM esther of Rhod-2 (Molecular Probes, Invitrogen, Eugene, OR). This stain binds Ca^{2+} with high affinity due to its cationic nature; it results in potential driven uptake into mitochondria and gives a punctate-staining pattern when loaded cells are viewed by fluorescence microscopy. To determine actual Ca^{2+} levels a two-point calibration was also used to detect the ability of the stain to respond to calcium (Gardner *et al.* 2004; Grynkiewicz *et al.* 1985).

Initially a Ca^{2+} free solution and a Ca^{2+} saturation solution were prepared (see Appendix: Section 10.4). Embryos were initially incubated in 5µM Rhod-2-AM stain in G1.2 medium for 15 minutes at 37°C in 6% CO_2, 5% O_2, and 89% N_2. Cells were then permeabilised using the ionophore ionomycin (Sigma Aldrich Chemical Co.), at a concentration of 10µm, and incubated, for 10 minutes, in a buffer that contains a saturating level of Ca^{2+} to determine the maximal fluorescent signal. Embryos were then mounted in confocal loading medium on a cover slip and a second smaller cover slip was placed on top using a paraffin/Vaseline spacer to prevent the embryos from being crushed. Images were then viewed within two minutes using confocal microscopy (Nikon, EZ-C1 software).

Another group of embryos were also incubated in 5µM Rhod-2-AM stain in G1.2 medium for 15 minutes at 37°C in 6% CO_2, 5% O_2, and 89% N_2 and were then incubated in a buffer that contains no Ca^{2+}; however, it does contain EDTA which is a Ca^{2+} chelator and allows minimal levels of fluorescence. Embryos were then mounted in Ca^{2+} free confocal loading medium on a cover slip and a second smaller cover slip was placed on top using a paraffin/Vaseline spacer to prevent the embryos from being crushed. Images were then viewed within two minutes using confocal microscopy (Nikon, EZ-C1 software).

Following the calibration, 2-cell embryos were incubated with 5µM Rhod-2-AM stain in G1.2 medium for 15 minutes at 37°C in 6% CO_2, 5% O_2, and 89% N_2. After incubation the embryos were washed twice in G1.2 medium and then mounted on a large glass cover slip in a 10µl drop of confocal loading medium. A second smaller cover slip was placed on top using a paraffin/Vaseline spacer to prevent the embryos from being crushed. Images were then viewed within two minutes using confocal microscopy (Nikon, EZ-C1 software) and analysed using ImageJ software package (Version 1.31v, National Institutes of Health, USA) as indicated above (**Figure 2-13**) (Barnett *et al.* 1997). In this analysis 16 regions of pixel intensity were measured, four readings in each cytoplasm and four readings around the exterior of the cell to incorporate the high level of calcium banding seen at the edge of the cell. This then allowed a relative distribution of mitochondria to be established and is expressed as cytoplasmic:exterior ratio.

2.10.3 Determination of mitochondrial membrane potential ($\Delta\psi m$)

Mitochondrial membrane potential was determined using the dual emission potential sensitive stain JC-1 (5,5,6,6-tetrachloro-1, 1,3,3 tetraethylbenzimidazolycarbocyanine iodide, Molecular Probes, Eugene, OR, USA). JC-1 is a cationic dual emission dye that exhibits potential dependent accumulation in mitochondria. Two-cell or eight-cell embryos were incubated with 1.5 μM JC-1 stain in G1.2 medium for 15 minutes at 37°C in 6% CO_2, 5% O_2, and 89% N_2. The green fluorescent JC-1 exists as a monomer at low concentrations or at low membrane potential. At higher potentials, JC-1 forms red fluorescent 'J-aggregates'. Thus, the ratio of red to green fluorescence can be used as a measure of mitochondrial membrane potential. The ability of JC-1 to detect differences in mitochondrial membrane potential has been confirmed using a K^+ diffusion method, as previously described (Reers *et al.* 1991).

After incubation the embryos were washed twice in G1.2 medium and mounted on a large glass cover slip in a 10μl drop of confocal loading medium. A second smaller cover slip was placed on top using a paraffin/Vaseline spacer to prevent the embryos from being crushed. Images were then viewed within two minutes using confocal microscopy (Nikon, EZ-C1 software). Images were analyzed using Adobe Photoshop software (Version CS2). To analyse relative membrane potential in the 2-cell embryo 24-pixel readings (12 in each cell) were taken per embryo on the green channel (525nm) and 24 readings in the exact same locations were taken on the red channel (590nm). Readings were taken in three areas: nuclear, cytoplasm and exterior (**Figure 2-13**). In the 8-cell embryo this same procedure was performed in four randomly selected cells within the embryo however due to limited cell size only 4 reading were taken per cell in the exterior region. These were then expressed per region imaged as a ratio of red: green staining, where mitochondrial depolarisation correlates to a decrease in the red/green fluorescence intensity ratio.

2.10.4 Assessment of reactive oxygen species levels

The level of reactive oxygen species (ROS) was determined using a previously validated 2',7'-dichlorodihydrofluorescein diacetate (DCDHF DA) fluorescent assay (Nasr-Esfahani *et al.* 1990b). In this assay DCDHF DA is hydrolysed by esterases present within the cell to give DCHF, which is then oxidised by reactive oxygen species (primarily H_2O_2) to yield the fluorescent dichlorofluorescein (DHF). The level of cellular uptake and hydrolysis of the DCDHF diacetate by intracellular esterases, between treatment groups, was controlled for by incubating five embryos per treatment with 1μM 5, 6, carboxyl 2',7'-dichlorodihydrofluorescein diacetate (CDCFDA) (stock dissolved in DMSO and snap frozen in liquid nitrogen and stored at –20°C) in MOPS-G1 handling medium for 20 minutes at 37°C in the dark, and measuring the level of fluorescence of CDCF using fluorescence microscopy.

Two-cell or eight-cell embryos were then incubated for 30 minutes in 1μM DCDHF DA in MOPS-G1 handling medium at 37°C in the dark (stock dissolved in DMSO and snap frozen in liquid

nitrogen and stored at –20°C) and washed in MOPS handling medium. The level of DHF was measured using fluorescence microscopy with a photometer attachment.

For both stains, embryos were mounted on glass slides in 10µl confocal loading medium and imaged using fluorescent microscopy set to the FITC channel. The level of fluorescence was determined using a photometer attachment (Leica DMIRB, Mpvbio software) which converts levels of emission light into a numerical value.

The relative fluorescence for each embryo was then determined comparative to the control embryo for the corresponding treatment, and expressed as mean fluorescence units.

2.11 Assessment of metabolic parameters

2.11.1 Assessment of malate aspartate shuttle activity

The malate-aspartate shuttle is essential for translocating electrons produced during glycolysis across the mitochondrial membrane. The conversion of lactate to pyruvate required the conversion of NAD+ to NADH. The shuttle system is required because the inner mitochondrial membrane is impermeable to NADH and its oxidised form NAD^+. As NAD+/NADH cannot cross the membrane, ions (attached to malate) cross membrane and regenerate NAD+ from NADH allowing the continuation of metabolism of lactate (**Figure 2-15**).

Activity of the malate-aspartate shuttle was determined by analysing lactate uptake in the presence or absence of aminooxyacetate (AOA), an inhibitor of shuttle activity. AOA blocks the function of the malate-aspartate shuttle and will decrease metabolism.

Initially 2-cell embryos were washed thoroughly through the L-simple G1.2 to remove any substrate from the culture media. Individual embryos were then incubated in 0.5µl drops of modified G1.2 medium (L-simple G1.2) (lactate present at 5 mM as the sole substrate) under oil (Vitrolife) at 37°C in 6% CO_2, 5% O_2, and 89% N_2 in the presence or absence of 0.5 mM AOA, for two hours as previously described (Lane and Gardner 2005a). During the incubation period a standard curve was prepared using a lactate cocktail (see Appendix: Section 10.6) and a serially diluted 1 mM, 0.5 mM, 0.25 mM, 0.125 mM and 0.0625 mM Na-Lactate standard solution (see Appendix: Section 10.6). A siliconised slide was prepared containing 5µl drops of lactate cocktail under heavy mineral oil (Sigma Aldrich Chemical Co.). The auto-fluorescence of these drops was measured using a fluorescent microscope set to the UV light channel, and the level of fluorescence was determined using a photometer attachment (Leica DMIRB, Mpvbio software) which converts levels of emission light into a numerical value. Following this, 0.2µl of each of the sequentially diluted lactate standards were added to a lactate cocktail drop and incubated for three minutes. The auto-fluorescence of each drop was then read by the photometer attachment where, in the presence of lactate, there is a linear increase in fluorescence. A standard curve was then plotted where standard curve values were $R>0.994$ (**Figure 2-14**).

Siliconised slides were once again prepared containing 5µl of lactate cocktail under heavy mineral oil (Sigma Aldrich Chemical Co.), and the auto-fluorescence of each drop was determined as mentioned above. Embryos were then removed from their culture drops and 0.2µl of media from each drop was added to a drop of lactate cocktail, incubated for three minutes and then the fluorescence was determined. Metabolism as a result of the malate-aspartate shuttle activity could be calculated from AOA specific inhibition of lactate uptake. All metabolic activity was expressed as pmol of lactate uptake/embryo/h using the standard curve.

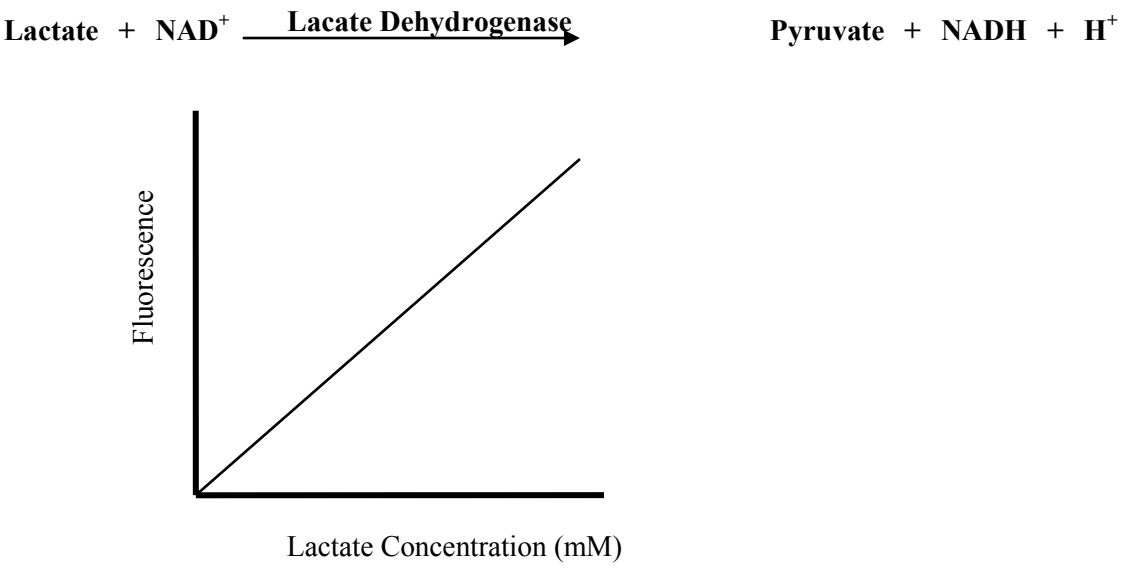


Figure 2-14: Standard curve for lactate as well as reaction equation. Levels of lactate in a sample can be assessed by a linear increase in fluorescence with a linear increase in lactate concentration.

NOTE:
This figure is included on page 111 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2-15: Malate-aspartate reducing equivalent shuttle**.** Schematic of reactions involved in the malate-aspartate shuttle, which transfers an electron across the inner mitochondrial membrane resulting in the net transfer of NADH from the cytoplasm into the mitochondria. The shuttle consists of reactions catalysed by four enzymes: cAspAT, mAspAT, cMDH, and mMDH. (Figure obtained from (Lane and Gardner 2005a)

2.12 Assessment of ATP and ADP levels

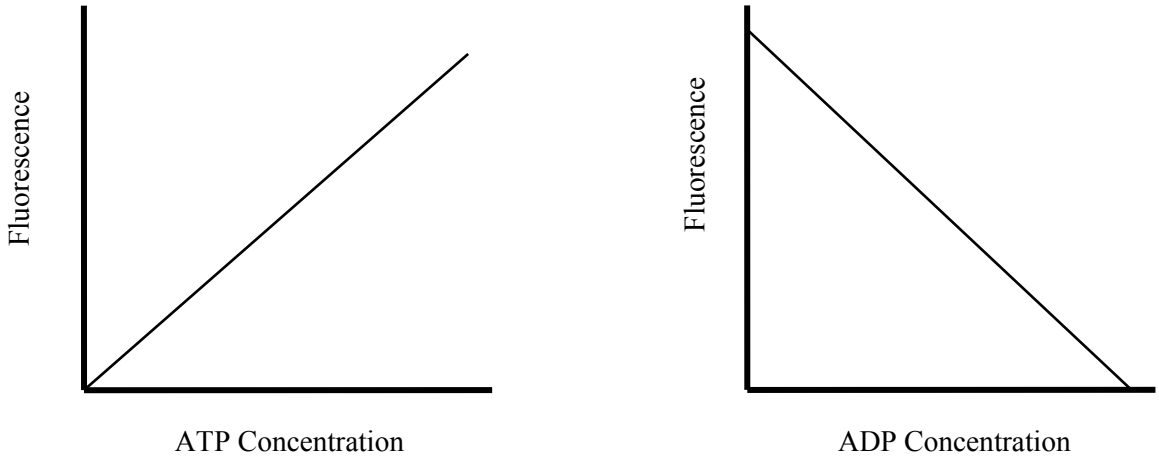
Levels of ADP and ATP of an individual 2-cell embryo were analysed using a technique described by Leese *et al.,* where each individual 2-cell was extracted initially using a 20nl droplet of MOPS-G1 and 1 mg.mL Ficoll (F8016, Sigma Aldrich Chemical Co.) under oil. Initially each individual embryo was extracted by placing each 2-cell or 8-cell embryo into an individual droplet and 4 nanolitres (nl) of 3M $HClO_4$ was added immediately using an oil-filled calibrated micropipet controlled using mouth pipetting and was then incubated for 10 minutes in the dark at 37 °C. Sixteen nl of ice-cold 2M $KHCO_3$ was then added to neutralise any extra $HClO_4$. The discreet crystals of $HClO_4$ present did not interfere with the mouth pipetting of the extracted solution. These extractions were then placed at 4 °C while standard curves were prepared with all samples being analysed the same day (Leese *et al.* 1984).

The assessment of ATP and ADP levels were performed using an assay described by Leese *et al.* (Leese *et al.* 1984). ATP levels were determined by coupled reactions catalysed by hexokinase and glucose-6-phosphate dehydrogenase leading to the formation of NADPH (**Figure 2-16**). ADP levels were determined in coupled reactions catalysed by lactate dehydrogenase and pyruvate kinase involving the conversion of NADH to NAD^+(Leese *et al.* 1984)(**Figure 2-16**). Levels of ATP and ADP are expressed as nmol/embryo and the ratio of ATP:ADP for individual embryos obtained.

Initially a standard curve for both ATP and ADP were performed using a 1 mM ATP and 1 mM ADP solution (see Appendix: Section 10.6). A siliconised slide was prepared containing 0.5µl drops of either ATP or ADP cocktail under heavy mineral oil (Sigma Aldrich Chemical Co.)(see Appendix: Sections 10.6) and the auto-fluorescence of these drops were measured using a fluorescent microscope set to the UV light channel and the level of fluorescence determined using a photometer attachment (Leica DMIRB, Mpvbio software), which converts levels of emission light into a numerical value. Following this, 0.01µl or 0.02nl of each of the sequentially diluted ATP/ADP was added to either the ATP or ADP cocktail drops, respectively, and incubated for 10 minutes. The auto-fluorescence of each drop was then read by the photometer attachment where, in the presence of ATP or ADP, there is a linear increase or decrease in fluorescence. A standard curve was then plotted where standard curve values were $R>0.994$ (**Figure 2-16**).

Siliconised slides were once again prepared containing 0.5µl of ATP cocktail under heavy mineral oil (Sigma Aldrich Chemical Co.) and the auto-fluorescence of each drop was determined as mentioned above. Afterwards, 0.01µl of extracted embryo media from each drop was added to a drop of ATP cocktail, incubated for 10 minutes and then the fluorescence was determined where the presence of ATP resulted in an increase in fluorescence. Again, siliconised slides were prepared containing 0.5µl of ADP cocktail under heavy mineral oil (Sigma Aldrich Chemical Co.) and the auto-fluorescence of each drop was determined as mentioned above. Straight after, 0.02µl of extracted embryo media from each drop was added to a drop of ADP cocktail, incubated for 10 minutes and

then the fluorescence was determined where the presence of ADP resulted in a decrease in fluorescence. The amount of ATP or ADP was then expressed as pmol/embryo.



1. Glucose + ATP $\xrightarrow{\textbf{Hexokinase}}$ Glucose-6-phosphate + ADP

Glucose-6-phosphate + $NADP^+$ $\xrightleftharpoons{\textbf{Glucose-6-phosphate}}$ 6-phosphogluconate + NADPH

2. Phosphophenolpyruvate + ADP $\xrightarrow{\textbf{Pyruvate kinase}}$ Pyruvate + ATP

Pyruvate + NADH $\xrightarrow{\textbf{Lactate dehydrogenase}}$ Lactate + NAD^+ + H^+

Figure 2-16: Standard curve for ATP and ADP as well as reaction equation for measuring 1. ATP levels 2. ADP levels. Levels of ATP in a sample can be assessed by a linear increase in fluorescence with a linear increase in ATP concentration. Levels of ADP in a sample can be assessed by a linear decrease in fluorescence with a linear decrease in ADP concentration.

2.13 Immunohistochemistry

2.13.1 5-Methylcytosine antibody staining

The level of DNA methylation was assessed by immunohistochemistry using an antibody against methylated cytosine residues (Hou *et al.* 2007; Zaitseva *et al.* 2007). Methylation was assessed at both the 2-cell and morula stage of development in either control embryos or embryos exposed to either 300μM NH_4^+ or 2mM DMO during the first cleavage division.

Initially embryos were placed into 4% paraformaldehyde in PBS for 30 minutes at room temperature to allow fixation. Embryos were then washed twice and incubated for 40 minutes in PBS with 0.25% TritonX-100 (Sigma Aldrich Chemical Co). Following this they were placed into 4M HCl in 0.1% TritonX-100 in PBS for 30 minutes and then placed immediately into 100mM Tris HCl (pH 8.5) for neutralisation for 30 minutes. Embryos were then washed twice and blocked overnight at 4°C in 1% BSA (bovine serum albumin) (ICP Bio, New Zealand) with 0.05% Tween 20 (Sigma Aldrich Chemical Co.) in PBS.

The next morning the embryos were rinsed twice in 0.05% Tween 20 in PBS and incubated in the 1°antibody 5-Methylcytosine (Eurogentec, Seraing, Belgium) for one hour at 37°C in a humidified Nunc 4-well dish (Nalgene Nunc International, Rochester, NY, USA). The primary antibody was diluted in 0.25% TritonX-100 in PBS at 1:50 dilution.

Shortly after, the embryos were washed twice in 0.05% Tween 20 in PBS and then incubated with the 2°Ab (Goat-anti mouse conjugated to FITC) (Jackson ImmunoResearch Laboratories, Westgrove, PA, USA) for two hours at room temperature in the dark. The secondary antibody was diluted in 0.25% TritonX-100 in PBS at 1:100.

The embryos were then washed twice through 0.05% Tween 20 in PBS and incubated in Propidium Iodide (1 mL/mL) for 15 minutes at room temperature in the dark. Afterwards, they were rinsed twice with 0.05% Tween 20 and mounted in a 5μl drop of Prolong (Molecular Probes)/Glycerol (Sigma Aldrich Chemical Co.)/PBS mix (see Appendix: Section 10.7) on a large glass cover slip. A paraffin/Vaseline spacer was used to prevent the embryos from being crushed. Embryos were imaged using confocal microscopy (Nikon, EZ-C1 software). Images were analyzed using Adobe Photoshop software (Version CS2) using a published method of analysis (Kim *et al.* 2002; Zaitseva *et al.* 2007). In this analysis, within 2-cell embryos, 16 regions of pixel intensity were measured, four around each of the two nuclei and four readings in each of the two cytoplasm, to obtain background fluorescence (Figure 2-13). The cytoplasmic reading gave the overall background staining level and this was then subtracted from the nuclear reading to give the final staining level.

In morula embryos an image was captured directly through the centre of the embryo and eight nuclei within the embryo were measured as mentioned above with the cytoplasm also imaged to obtain background. The cytoplasmic reading gave the overall background staining level and this was then subtracted from the nuclear reading to give the final staining level.

2.14 Measuring intracellular pH

Intracellular pH was quantitated using an in situ standard curve calibration where intracellular pH is clamped to known extracellular values using the ionophores nigercin sodium salt (Sigma Aldrich Chemical Co.) and valinomycin (Sigma Aldrich Chemical Co.).

At first, 1L of standard calibration solution was made and divided into 200 mL aliquots, and each solution was adjusted to the required pH using NaOH (pH 6.8, 7.0, 7.2, 7.4 and 7.6) (Table **2-3**). For use 10 mL of each solution was placed into a 12 mL tube (BD Biosciences BD Falcon ™) and to this 10µl of nigericin (10µg/mL) 5µl of valinomycin (5µg/mL) was added (see Appendix: Section 10.8).

The intracellular dye Seminaphthorhodafluors (SNARF-1) (Molecular Probes, Invitrogen, Eugene, OR) was then prepared in MOPS-G1, giving a final concentration of 5µM. SNARF-1 is a dual emission dye where the absorption spectrum of the carboxy SNARF-1 pH indicator undergoes a shift to longer wavelengths upon deprotonation of its phenolic substituent. Carboxy SNARF-1 also exhibits a significant pH-dependent emission shift from yellow-orange to deep red fluorescence under acidic and basic conditions, respectively.

Initially 5x 2-cell control embryos were placed into SNARF/MOPS-G1 for 0.5half an hour at 37°C in the dark. After this they were washed through MOPS-G1 and placed in a 5µl drop of confocal loading media on a glass slide. These were imaged using a confocal microscope and analysed using the time course ratiometric software (IP-Lab Advanced Image Analysis Software, BD Biosciences Bioimaging, Rockville, MD, USA).

These embryos were used to adjust the confocal and determine the filter settings to be used as well as determine gain.

The standard curve was determined by initially taking 15 control 2-cell embryos and incubating them in an organ well (BD Biosciences BD Falcon ™) containing 1 mL of SNARF/MOPS-G1 for half an hour at 37°C in the dark. They were then incubated for five minutes in the lowest pH solution containing nigericin and valinomycin. After this they were washed through MOPS-G1 and placed in a 5µl drop of confocal loading media on a glass slide and each embryo was imaged every 20 seconds for two minutes.

The embryos were removed from the slide and placed in the next pH solution and incubated for five minutes. After this they were placed in a 5µl drop of confocal loading media on a glass slide and each embryo was imaged every 20 seconds for two minutes. This protocol was then repeated for the remaining three pH solutions. The linear regression of the ratio-metric values obtained from the five different pH solutions was then calculated to give an r value of >0.99.

To calculate the actual decrease in intracellular pH of test embryos, firstly, 20 control 2-cell embryos were incubated in groups of 10 in an organ well (BD Biosciences BD Falcon ™) containing 1 mL of SNARF/MOPS-G1 for half an hour at 37 °C in the dark. After this they were washed through MOPS-G1 and placed in a 5µl drop of confocal loading media on a glass slide and each embryo was imaged every 20 seconds for two minutes.

This was repeated with embryos exposed to 2mM DMO from the zygote to 2-cell stage, and the decrease in intracellular pH was plotted using the standard curve.

Table 2-3: Composition of calibration solutions for pH

Component	Concentration (mM)
KCl	100
NaCl	17.1
Sucrose	75
Hepes (acid)	21

2.15 PCR

2.15.1 Extraction of cDNA from blastocysts

After 91h of embryo culture, blastocysts were frozen in groups of 30 in 500µl of Tri Reagent (Sigma Aldrich Chemical Co.) in irradiated sterile eppendorf tubes (Axygen Scientific, Union City, California, USA) at –80 °C.

To begin extraction, tubes were removed from –80 °C and allowed to thaw on ice. Soon after, 100µl of chloroform (ICN Biomedicals, Aurora, Ohio, U.S.A) was added to the tube and vortexed to mix. The samples were spun at 13,000 rpm for 10 minutes at 4 °C. The upper layer, containing the RNA, was transferred to a fresh tube and the bottom layer was discarded. Added to the tube was 1.5µl of glycogen (Roche Diagnostics Australia, Castle Hill, New South Wales, Australia) and 250µl of isopropanol (Sigma Aldrich Chemical Co.). The tubes were vortexed to mix and were frozen overnight at -80 °C to allow for RNA precipitation.

The following day the samples were removed from –80 °C and allowed to thaw on ice. The RNA was pelleted for 30 minutes at 13,000 rpm at 4 °C. The supernatant was removed carefully, to avoid disturbing the pellet, and 500µl of 70% ethanol was added and the tube was vortexed to wash the pellet. The sample was then spun at 13,000 rpm at 4 °C. The ethanol supernatant was removed and discarded. The tubes were then left open at room temperature to allow any remaining ethanol to evaporate. The pellet was redissolved in 10µl of Ultra Pure DNAse and RNAse free PCR-grade water (Gibco Invitrogen, Grand Island, New York, USA) and vortexed briefly.

The RNA was then DNAse treated to remove any contaminating DNA. The sample was kept on ice and 1µl of DNAse and 1µl of DNAse buffer (Ambion, Austin Texas, USA) was added. The tube was then incubated at 37 °C for 30 minutes using a PCR machine (GeneAmp PCR system 9700, Applied Biosystems, Victoria, Australia). When the tube was removed, 2µl of DNAse Inactivation

reagent was added (Ambion), the tube was incubated for two minutes at room temperature and was shaken occasionally to resuspend inactivation reagent throughout. The tube was centrifuged at 13,000 rpm for 1.5 minutes to pellet the inactivation reagent. After the supernatant containing the RNA was transferred to a fresh tube the amount was measured (usually between 11 and 13µl). PCR-grade water was added to give a final volume of 14.5µl. RNA was then stored at –80 °C or was immediately reverse transcribed.

The RNA was reversed transcribed by initially adding 1µl of 250ng/µl random primers (Invitrogen Australia, Mount Waverley, Victoria, Australia) to the sample, mixed gently and incubated at 70 °C for 10 minutes, followed by a quick chill to 4 °C in a PCR machine (GeneAmp). A master mix was prepared containing 5µl buffer, 2.5µl 0.1M DTT and 1µl 10mM dntp's (Invitrogen Australia). This 8.5µl of master mix was then added to the tube, mixed gently and incubated at 25 °C for 10 minutes, followed by 42 °C incubation for two minutes using the Geneamp PCR machine. The tubes were removed and 1µl of Superscript Π (Invitrogen, Carlsbad, California, USA) was added to each tube and mixed gently. The samples were then incubated at 42 °C for 50 minutes and then for 15 minutes at 70 °C using the Geneamp PCR machine. Samples were then stored at –20 °C.

2.15.2 Extraction of cDNA from placental tissue

Placental tissue was removed from –80 °C and placed onto a weighing tray surrounded underneath by ice. Placentas were cut in half using a scalpel, with half being snap frozen in liquid nitrogen and placed back at –80 °C. The remaining half were diced into smaller pieces, placed into a 1.7 mL irradiated sterile eppendorf tube (Axygen) and 750µl Tri Reagent (Sigma Aldrich Chemical Co.) was added. The sample was then snap-frozen in liquid nitrogen until all samples were processed to prevent RNA degradation. Once all samples had been prepared they were thawed and kept on ice. The tissue/Tri reagent solution was poured into a sterilised glass homogeniser and was homogenised on ice until tissue was completely separated. The solution was pipetted into a new 1.7 mL irradiated sterile eppendorf tube and placed on ice. The homogeniser was thoroughly cleaned by filling with 1M NaOH and rinsing up and down for 10 strokes of the homogeniser. This was then tipped out and repeated again using 1M NaOH. Afterwards, 1% SDS (Lauryl Sulfate; Sigma Aldrich Chemical Co.) was added to the homogeniser and the same procedure was repeated, as above, followed by 1% TritonX (Sigma Aldrich Chemical Co.). The homogeniser was then rinsed with sterilised water until no bubbles or froth remained, rinsed twice more and then the next tissue sample was processed. After all tissue samples had been processed the homogenised samples were spun at 12,000 rpm for 10 minutes at 4 °C. The supernatant was then transferred to a fresh tube and incubated for five minutes at room temperature to allow for dissociation of nucleoprotein complexes, and the 150µl of chloroform (ICN Biomedicals) was added and the tube was shaken for 15 seconds to mix. The sample was then incubated at room temperature for three minutes, centrifuged at 12,000 rpm for 15 minutes at 4 °C and the aqueous phase was transferred to a fresh tube. After this, 375µl of isopropanol (Sigma Aldrich

Chemical Co.) was added to the sample and the sample was frozen at –80 °C to allow RNA precipitation.

The following day the samples were thawed on ice and were centrifuged at 12,000 rpm for 30 minutes at 4 °C to pellet the RNA. The supernatant was discarded, 750µl of 70% EtOH was added and the tube was vortexed and then spun at 12,000 rpm for 30 minutes at 4 °C. The ethanol was removed and the RNA pellet was allowed to air-dry. The pellet was then re-dissolved in 40µl of PCR-grade water.

The RNA was then DNAse treated to remove any contaminating DNA by initially adding 4µl of DNAse buffer and 1µl of DNAse (Ambion) to the sample and gently mixing. The tube was then incubated at 37 °C for 30 minutes using a PCR machine (GeneAmp). The tube was removed from the machine and 5µl of DNAse inactivation reagent was added (Ambion). The tube was incubated for two minutes at room temperature and was shaken occasionally to resuspend the inactivation reagent throughout. The tube was centrifuged at 10,000 rpm for 1.5 minutes to pellet the inactivation reagent. The supernatant containing the RNA was transferred to a fresh tube and was then stored at –80 °C.

Before reverse transcription was performed the amount of RNA in each sample was measured using an RNA Spectrophotometer (NanoDrop-1000, Thermo Scientific Wilmington, DE, USA). The amount of RNA per sample was calculated to give an ng/µl concentration. The amount of RNA required for reverse transcription was 2µg in 14.5µl, so this concentration was obtained by diluting neat RNA with PCR-grade water to give a final amount of 2µg of RNA in 14.5µl.

The RNA was then reverse transcribed by initially adding 1µl of 250ng/µl random primers (Invitrogen) to the sample, mixed gently and incubated at 70 °C for 10 minutes, and was followed by a quick chill to 4 °C using a PCR machine. A master mix was prepared containing 5µl buffer, 2.5µl 0.1M DTT and 1µl 10mM dntp's (Invitrogen Australia). This 8.5µl of master mix was then added to the tube, mixed gently and incubated at 25 °C for 10 minutes, followed by 42 °C incubation for two minutes using the Geneamp PCR machine. The tube was removed and 1µl of Superscript Π (Invitrogen) was added to each tube and mixed gently. The sample was then incubated at 42 °C for 50 minutes and then for 15 minutes at 70 °C using a PCR machine. The sample was then stored at –20 °C.

2.15.3 Real-time reverse transcription PCR

Real-time reverse transcription PCR was performed on a Rotor-Gene 6000 real-time rotary analyser (Corbett Life Science, Sydney NSW, Australia). Initially cDNA was diluted to give either expression per blastocyst (a 1:3 dilution was used) or for placenta a 1:64 dilution was performed to allow for 10ng of cDNA per gene measured.

All oligonucleotide primers were designed using Primer Express (Applied Biosystems, Victoria, Australia) and synthesised by Geneworks (Adelaide, Australia) and were diluted to give a final concentration of 10pmol/µl (**Table 2–4, Table 2.5**).

To perform real-time reverse transcription PCR, the samples were diluted as above and kept on ice. The reaction was performed in 20µl volumes by using a master mix preparation containing 10µl Power SYBR® Green PCR Master Mix (Applied Biosystems), 6µl PCR-grade water, 1µl of forward primer (5' Primer) and 1µl reverse primer (3' Primer) and 2µl cDNA, giving a final volume of 20µl per reaction. This master mix was prepared for all genes to be examined except 18S, which was prepared using 10µl Power SYBR® Green PCR Master Mix, 7µl PCR-grade water, 0.5µl forward primer (5' Primer), 0.5µl reverse primer (3' Primer) and 2µl cDNA, giving a final volume of 20µl per reaction. All samples were also run in duplicate.

The thermal cycling programme used was as follows: 50 °C for two minutes, 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 15 seconds and 60 °C for one minute. Rotor Gene 6000 analysis software was used to generate cycle threshold (C_t) for each sample. Blastocyst gene expression was compared with 18S rRNA levels and was normalised against control embryos grown without ammonium or DMO using a real time quantitative PCR and the 2(-Delta Delta C[t]) method (Livak and Schmittgen 2001), and expressed as fold change.

Placental gene expression was determined relative to a standard curve of placental cDNA and corrected for 18S rRNA levels in each sample. Gene expression was then normalised against the gene expression from control placentas from embryos grown without ammonium and was also expressed as fold change.

Table 2-4: Details of primers used for the analysis of gene expression in mouse blastocysts.

Symbol	Name	Primer	Sequence
Rn 18S	18S RNA	5' Primer	AGA AAC GGC TAC CAC ATC CAA
		3' Primer	CCT GTA TTG TTA TTT TTC GTC ACT ACC T
Dnmt1	DNA Methyltransferase (cytosine-5) 1	5' Primer	GCA GGA ATT CAT GCA GTA AG
		3' Primer	GGG TCT CGT TCA GAG CTG
Dnmt3a	DNA Methyltransferase 3A	5' Primer	TCG TAC ATG TGC GGG CAT AA
		3' Primer	GGA GTC GAG AAG GCC AGT CTT
Dnmt3b	DNA Methyltransferase 3B	5' Primer	GCC AAA AGG AGG CCC ATT AG
		3' Primer	CCC AAC TCC TTG AGC ACC

Table 2-5: Details of primers used for the analysis of gene expression in mouse Day 15 placentas.

Symbol	Name	Primer	Sequence
Rn 18S	18S RNA	5' Primer	AGA AAC GGC TAC CAC ATC CAA
		3' Primer	CCT GTA TTG TTA TTT TTC GTC ACT ACC T
VEGF	Vascular endothelial growth factor	5' Primer	CCA CGT CAG AGA GCA ACA TCA
		3' Primer	CTG CTG TGC TGT AGG AAG CTC AT
H19	H19	5' Primer	GAA GAT GCT GCA ATC AGA ACC A
		3' Primer	CAC CAT CTG TTC TTT CAG CTT CAC
Igf2	Insulin-like growth factor 2	5' Primer	AAG AGT TCA GAG AGG CCA AAC G
		3' Primer	CAC TGA TGG TTG CTG GAC ATC T
Igf2R	Insulin-like growth factor 2 receptor	5' Primer	TTT TGG GCG CCT TGC AT
		3' Primer	AGG GCA AGG ATC ACC ATT CAC
Slc2a1	Solute carrier family 2 (facilitated glucose transporter) member 1 (Glut-1)	5' Primer	CCA GCT GGG AAT CGT CGT T
		3' Primer	CAA GTC TGC ATT GCC CAT GAT
Slc2a3	Solute carrier family 2 (facilitated glucose transporter) member 3 (Glut-3)	5' Primer	CTC TTC AGG TCA CCC AAC TAC GT
		3' Primer	CCG CGT CCT TGA AGA TTC C

Continued next page

Symbol	Name	Primer	Sequence
Slc38a2	Solute carrier family 38 member 3	5’ Primer	GAA GAC CGA AAT GGG AAG GTT
		3’ Primer	TTG AAG TCA CTG TTG GAG CTG TAA C
Slc38a4	Solute carrier family 38 member 4	5’ Primer	ACA GTC ACA CCG GCA TTG A
		3’ Primer	TTC GGC ATC TTC ATT AGC AAA TT
mTFAM	Transcription factor A (mitochondrial)	5’ Primer	CCA GCA TGG GTA GCT ATC CAA
		3’ Primer	TTG CAT CTG GGT GGT TAG CTT TAA
mTERF	Transcription terminator factor (mitochondrial)	5’ Primer	CAT CTT AAT TCA GAG ACA CAA ACG
		3’ Primer	TTT TTG GTA CAG TCA TTG GAA AGG
NRF1	Nuclear factor-eythroid 2-related factor 1	5’ Primer	GTC CGC ACA GAA GAG CAA AAA C
		3’ Primer	TCC TCC CGC CCA TGT TG
NRF2	Nuclear factor-eythroid 2-related factor 2	5’ Primer	TGG ATT TGA TTG ACA TCC TTT GG
		3’ Primer	TCT GGA GTT GCT CTT GTC TTT CC

2.16 Placental transport of Methyl-D-Glucose, 3-0-[Methyl-^{14}C)

2.16.1 Injection of radio-labelled substrate

This experiment was performed on Day 18 embryo transfer mice which had control embryos transferred to one horn and treatment embryos transferred to the other horn to control for any variation in the amount of substrate injected.

Initially the radiolabelled substrate (Methyl-D-Glucose, 3-0-[Methyl-^{14}C) was diluted to give 35µCi per 1 mL using 0.9% sterile saline (see Appendix: Section 10.1).

A 301/2 gauge needle was attached to a 1 mL syringe, weighed and then 100µl of diluted substrate was taken up, giving a final concentration of 3.5µCi in 100µl. The syringe was then weighed again to measure the initial volume taken up.

The pregnant mouse was then anaesthetised with an i.p injection of 1.2% Avertin (2, 2, 2-Tribromoethanol, 2-methyl-2-butanol; 0.02 mL/g body weight (see Appendix: Section 10.2) and the mouse body weight was recorded. The mouse was placed face up on a dissecting microscope and the neck area was washed down with 70% EtOH.

A small incision was made in the centre neck line and the jugular vein was exposed. Great care was taken not to damage the area, as any stress would make the vein constrict. The tip of the needle was inserted into the jugular vein and the plunger very slowly depressed while watching the fluid move down the syringe and needle. Once all the fluid had been injected, the empty syringe was weighed to assess if all the substrate was removed. Five minutes after the injection was administered, the mouse was euthanised by cervical dislocation.

An incision was made down the midline, the fetal and placental tissue was removed, and the weight and diameter of both the fetus and placenta was recorded. Fetal and placental tissue was snap frozen at –80 °C for subsequent scintillation counting.

2.16.2 Scintillation counting

Tissue was removed from –80 °C and was allowed to partially thaw before processing. Placental tissue was cut into small pieces and placed into an eppendorf tube containing 0.7 mL 1M NaOH and kept at room temperature. Fetal tissue was divided over two tubes and the sample was pooled later. Once all samples had been prepared, the tubes were placed into a water bath at 60 °C for 85 minutes, vortexing occasionally. After 85 minutes in the water bath, each tube was checked to ensure complete solubilisation had occurred. When the tubes had been cooled to room temperature they were placed in an ice-slurry. Tissue samples were then transferred to a large 15 mL conical tube (Falcon) and at this stage the fetal samples were pooled. Each sample was then neutralised by adding 2.1 mL (for placenta) and 4.2 mL (for fetus) of 6% $HClO_4$. The tubes were shaken gently to mix, immersed in an ice-slurry for 20 minutes and then spun at 4000rmp for 15 minutes at 4 °C. The centrifugation process resulted in

two distinct layers of supernatant and precipitate. The top layer was carefully removed and placed into a fresh tube and the exact volume was recorded.

Mini Poly-Q scintillation vials (Beckman Coulter Instruments Inc, Fullerton, California, USA) were prepared in duplicate by adding 1 mL of supernatant to 5 mL of Aqueous Scintillation Fluid (Ready-Safe, Beckman Coulter Australia Pty Ltd, Gladesville, NSW, Australia). Samples were then left overnight in the dark, followed by a scintillation count that was performed on an LS 6000LL scintillation counting machine (Beckman Coulter Instruments Inc.).

3 Impact of ammonium exposure on blastocyst viability, glucose uptake and fetal and placental growth

Some of the work presented in this chapter has been published in the following journal:

Zander, D.L., Thompson, J.G. and Lane, M. (2006) 'Perturbations in mouse embryo development and viability caused by ammonium are more severe after exposure at the cleavage stages'. *Biology of Reproduction* **74**(2): 288-294

Zander, D.L., Thompson, J.G. and Lane, M. (2006) Perturbations in Mouse Embryo Development and Viability Caused by Ammonium Are More Severe after Exposure at the Cleavage Stages.
Biology of Reproduction v.74 (2) pp. 288-294, February 2006

3.1 Introduction

Embryo culture systems are a vital component of assisted reproductive technology (ART) for the production of animal and human embryos. Traditionally, the *in vitro* development of the mammalian pre-implantation embryo is associated with decreased embryo viability and development, along with severe alterations in metabolism and gene expression (Gardner and Lane 1993b; Lane and Gardner 1994). However, more significantly, it has been established that the conditions that the pre-implantation stage embryo is exposed to can not only affect the ability to establish a pregnancy but also affect fetal growth and adult health in animal models (Doherty *et al.* 2000; Ecker *et al.* 2004).

The pre- and post-compaction stage embryo differs greatly in many aspects, including preferred metabolic substrate (Gardner and Leese 1988; Hardy *et al.* 1989; Leese *et al.* 1993), genome control (maternal or embryonic) (Braude *et al.* 1979), mitochondrial structure (Hillman and Tasca 1969) and level of metabolic activity (Leese 1991). The pre-compaction stage embryo also lacks efficient regulatory mechanisms for intracellular pH (Baltz *et al.* 1991a; Baltz *et al.* 1991b; Lane *et al.* 1999b; Phillips and Baltz 1999a) and reactive oxygen species (ROS) (Harvey *et al.* 1995). In contrast, following the formation of a transporting epithelium which follows compaction, there is an increase in the ability of the embryo to regulate homeostatic processes such as intracellular pH (Lane and Gardner 2000b). Furthermore the mitochondria of the early embryo are ovoid in shape and have low numbers of transverse cristae and reduced surface area (Hillman and Tasca 1969). They are also less able to regulate metabolic activity *in vitro* compared to the later stage embryo (Lane and Gardner 2000a) .

One *in vitro* stress that has been demonstrated to influence the ability of the pre-implantation embryo to develop is ammonium (Gardner and Lane 2003). Ammonium levels have been shown to increase significantly during the culture period owing to the deamination and spontaneous breakdown of amino acids, especially glutamine, in the medium (Gardner and Lane 1993b; Lane and Gardner 2003). Ammonium exposure throughout the entire pre-implantation period has been shown to decrease embryo cleavage and blastocyst development, decrease blastocyst cell number, alter metabolism and increase apoptosis (Gardner and Lane 1993b; Lane and Gardner 2003). Additionally, exposure to ammonium during the entire pre-implantation stage, from the zygote to the blastocyst, also decreases implantation rates and fetal development as well as increasing the incidence in fetal abnormalities (Lane and Gardner 1994; Lane and Gardner 1995; Lane and Gardner 2003).

Using this same model *in vitro* stress, I have previously shown that the pre-implantation stage mouse embryo is more sensitive to an ammonium stress pre-compaction compared to post compaction and that the zygote to 2-cell transition appears to be the most sensitive stage (Zander *et al.* 2006). Exposure to ammonium during this highly sensitive stage results in unchanged blastocyst morphology; however, blastocyst cell number and allocation is altered and apoptosis is increased. I have also demonstrated that exposure to ammonium for only 19 hours during the first cleavage division can subsequently alter fetal development by reducing fetal weight, maturity and crown rump length on Day 15 of development.

Although the effects of *in vitro* stress on embryo viability have been demonstrated in many models, what is currently unknown is exactly how these stress conditions can 'program' the embryo to result in an altered fetal phenotype.

The aim of this chapter is to gain increased insight into the effects that our current *in vitro* stress model, ammonium, has on embryo cell division, nutrient uptake and Day 18 of fetal development. As previously mentioned, I have demonstrated that exposure to ammonium during pre-implantation embryo development can significantly reduce fetal weight and crown rump length on Day 15. It is currently unknown if these effects will continue into later gestation when the fetus undergoes more rapid growth which is able to be assessed on Day 18 of development.

These results will help us better understand the effects that an environmental insult can have on the embryo and hopefully lead us to a possible mechanism behind the fetal programming outcomes that we have previously demonstrated.

3.2 Experimental design

3.2.1 Culture conditions

Embryos were collected from superovulated F1 hybrid female mice, after mating with Swiss males, 23 hours post-hCG at the zygote stage. All cleavage stage embryos were cultured in groups of 10–12 in 20µl drops of medium under 3.5 mL of mineral oil at 37 °C at 6% CO_2, 5% O_2, and 89% N_2. Embryos from 6–8 females were pooled and randomly allocated to each different treatment group.

The treatment groups used for this temporal assessment were:

- Treatment 1 (control group) which was cultured in sequential culture media with no ammonium exposure (total of 91 hours of culture)
- Treatment 2 which was exposed to 300µM ammonium from the zygote to the 2-cell stage (from 0 –19 hours of culture)
- Treatment 3 which was exposed to 300µM ammonium from the 2-cell to the 8-cell stage (19–43 hours of culture)
- Treatment 4 which was exposed to 300µM ammonium from the 8-cell to blastocyst stage (43–91 hours of culture)
- Treatment 5 which was exposed to 300µM ammonium for the entire culture period (total of 91 hours of culture) (**Figure 3.1**).

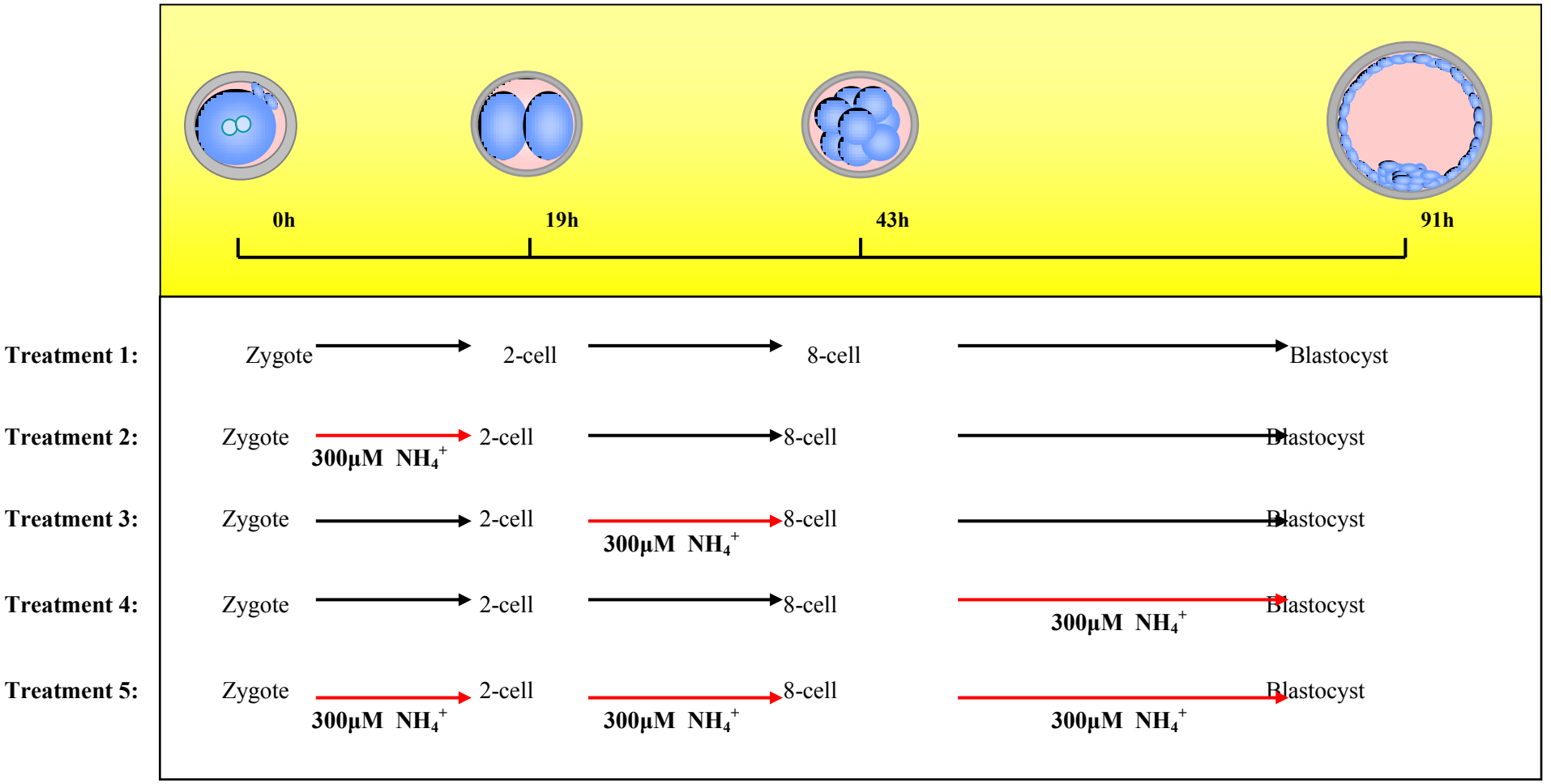


Figure 3-1: Temporal exposure of embryos to 300µM ammonium (NH_4^+).

Black arrows indicate embryos are cultured in control media either G1.2 (prior to the 8-cell stage) or G2.2 (after the 8-cell stage)

Red arrows indicate embryos are cultured in G1.2 (prior to the 8-cell stage) or G2.2 (after the 8-cell stage) with 300µM ammonium

3.3 Statistics

Early blastocyst development, morula cell number and glucose uptake were analysed using a univariate general linear model where day of experiment was treated as a covariate. Differences between treatments were assessed using the Least Significant Difference method (LSD).

Implantation data and fetal and placental data were also analysed using a univariate general linear model as LSD. Mouse number, implantations per horn, fetuses per horn, implantations per mother and fetuses per mother were treated as covariates.

3.4 Results

3.4.1 Effect of ammonium on morula cell number

I have previously shown that, although on time Day 3 development of the pre-compaction embryo culture was unchanged after temporal exposure to ammonium, at the blastocyst stage cell number and allocation was significantly different (Zander *et al.* 2006). The purpose of this investigation was to assess cell number at the morula/early blastocyst stage (when cell differentiation begins) to ascertain the stage at which cell numbers begin to change.

Exposure to 300μM ammonium at any stage did not alter on time morphological development (**Table 3-1**). Exposure to 300μM ammonium at any stage did not significantly alter morula/early blastocyst cell number after 67 hours of culture when compared to control (Figure 3-2).

Table 3-1: The effect of culture with ammonium on embryo development after 67 hours of culture

Time of exposure to ammonium	% ≤8 cell	% Compact morula	% Early blastocyst
Control	10.0	40.0	50.0
Zygote to 2-cell	10.0	40.0	50.0
2-cell to 8-cell	12.5	37.5	50.0
8-cell to blastocyst	11.2	44.4	44.4
Zygote to blastocyst	15.4	38.5	46.2

Control n=18, Zygote-2-cell n=18, 2-cell-8-cell n=16, 8-cell-blastocyst n=18, Zygote-blastocyst n=15
There was no significant difference for any stage of development between treatments.

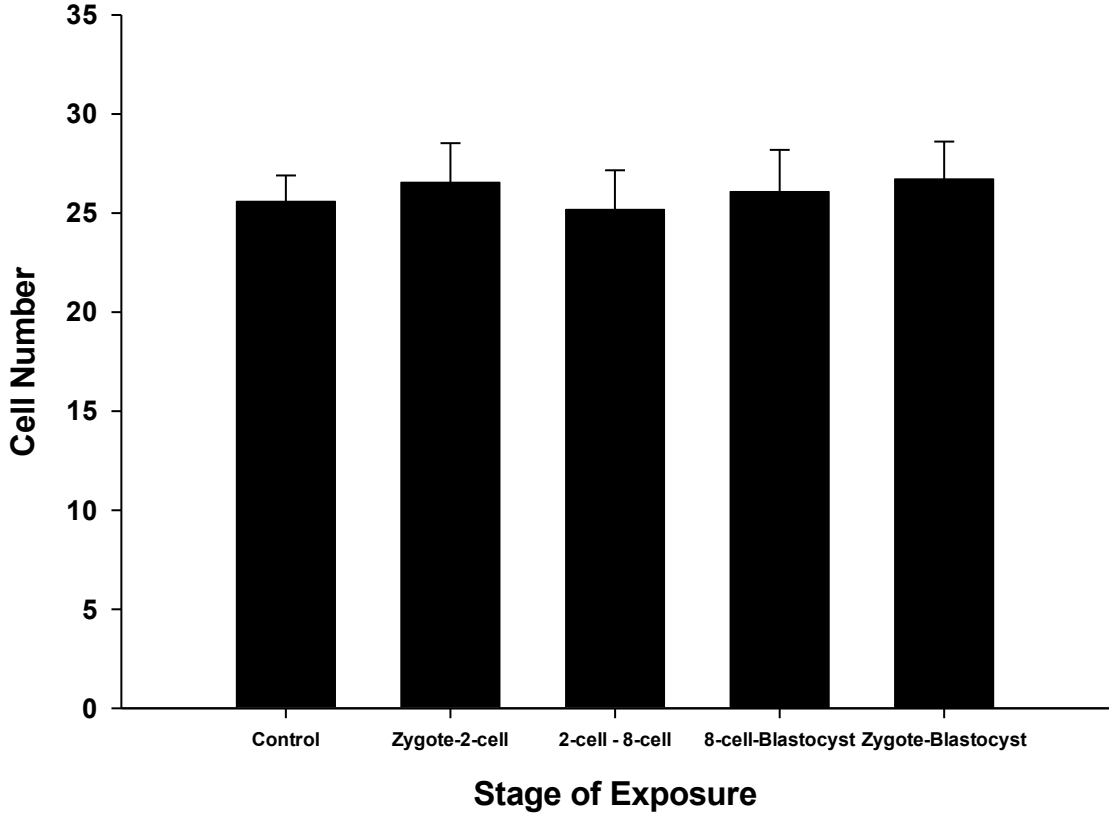


Figure 3-2: The effect of temporal ammonium exposure on morula/early blastocyst cell number after 67 hours of culture.

Control n=41, Zygote-2-cell n=35, 2-cell-8-cell n=23, 8-cell-blastocyst n=27,

Zygote-blastocyst n=35 (3 replicates)

Data is expressed as mean ± SEM

No difference in cell numbers was observed between treatments

3.4.2 Effect of ammonium on glucose uptake

The culture of embryos with ammonium at any stage of development significantly reduced the levels of glucose uptake at the blastocyst stage compared to blastocysts cultured in the absence of ammonium ($P<0.001$; **Figure 3-3**). It was observed that glucose levels appeared to be lower within the blastocoel and the inner cell mass in embryos cultured with ammonium (**Figure 3-4**).

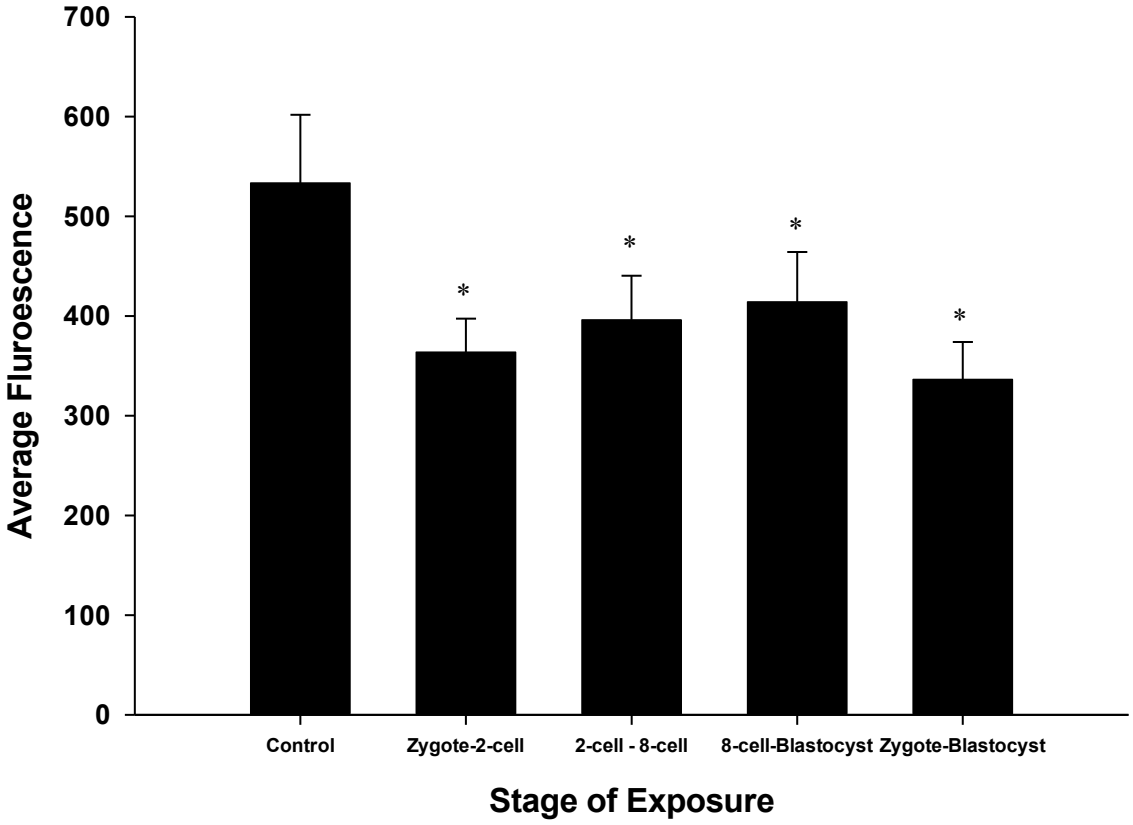


Figure 3-3: The effect of temporal ammonium exposure on glucose uptake in the resultant blastocysts. Control n=45, Zygote-2-cell n=39, 2-cell-8-cell n=43, 8-cell-blastocyst n=46, Zygote-blastocyst n=33 (3 replicates)

Data is expressed as mean fluorescence ± SEM

* indicates significant difference from control (P>0.001)

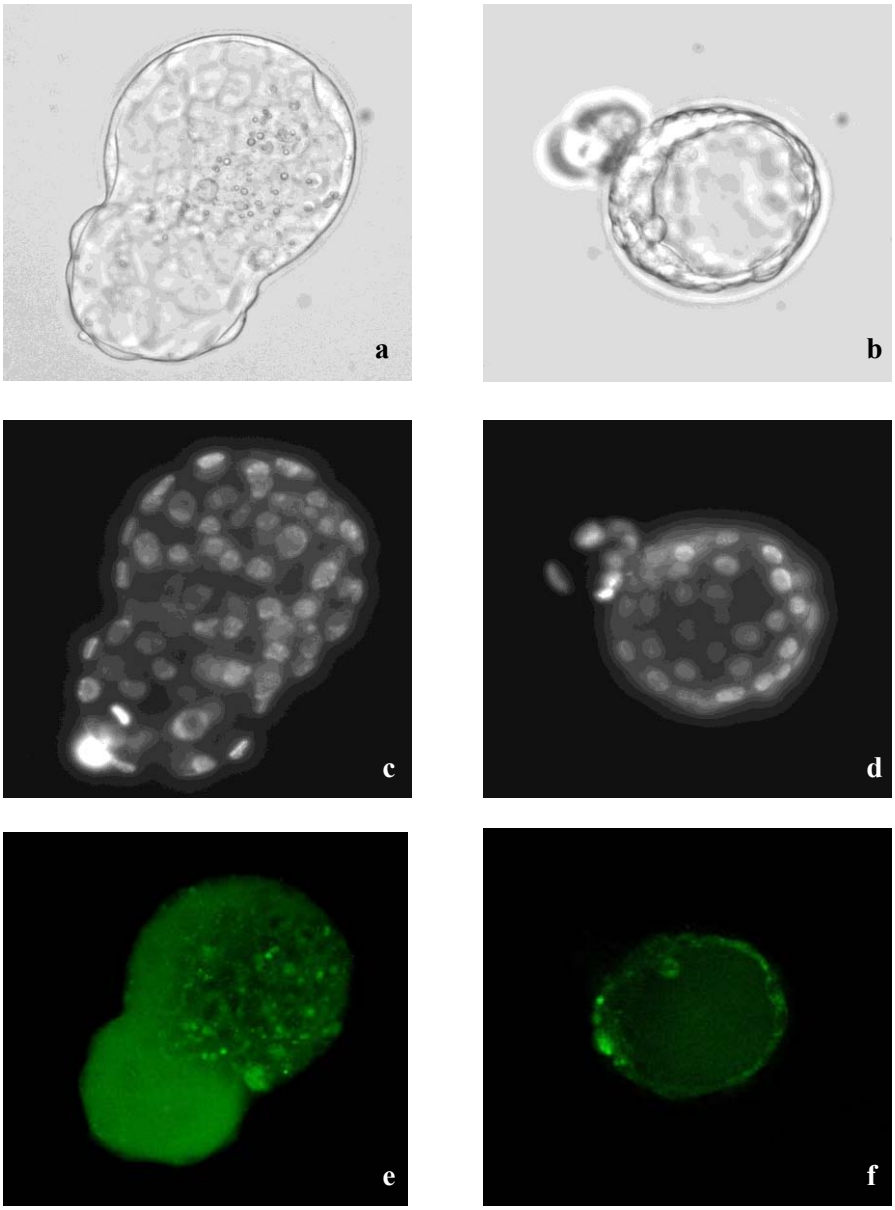


Figure 3-4: Effect of culture with ammonium on glucose distribution in cultured blastocysts. Representative blastocyst cultured in the absence of ammonium; a: brightfield, c: nuclear staining, e: distribution of 6-NBDG. Representative blastocyst cultured with ammonium from the zygote to the 2-cell stage; b: brightfield, d: nuclear staining, f: distribution of 6-NBDG.

3.4.3 Effect of ammonium exposure on blastocyst viability after transfer to pseudopregnant recipients

Culture with ammonium during pre-compaction or continually throughout development did not significantly affect implantation rates when compared to control or *in vivo* derived blastocysts on day 18 of development. Exposure to ammonium at the post-compaction stage resulted in a significant increase in implantation when compared to control ($P<0.05$; **Table 3–2**). Culture with ammonium during the 2-cell to 8-cell stage significantly reduced fetal development per embryo transferred as well as reducing fetal development per implantation ($P<0.05$; **Table 3–2**).

Culture with ammonium during the zygote to 2-cell stage, during 8-cell to blastocyst stage or continually throughout development did not significantly alter fetal weight on day 18 of development; however, exposure to ammonium during the 2-cell to 8-cell stage did trend towards a decreased fetal weight ($P=0.06$; **Table 3–3**). In contrast, *in vitro* blastocyst development resulted in a significant decrease in fetal weight when compared to *in vivo* developed blastocysts ($P<0.05$; **Table 3–3**).

Culture with ammonium at any stage did not alter placental weight; however, culture with ammonium during the zygote to 2-cell stages or continually throughout development significantly reduced the fetal:placental weight ratio indicative of larger placentas for similar fetus size.

Culture with ammonium during the pre-compaction stage or continually throughout development also significantly reduced crown rump length ($P<0.05$; **Table 3–3**).

In vivo derived blastocysts have a significantly increased fetal:placental weight ratio when compared to control and ammonium treatment groups indicative of larger fetuses, although placental size was unchanged ($P<0.05$; **Table 3–3**).

Table 3-2: Effect of culture with 300μM ammonium on blastocyst implantation and fetal development on day 18

Time of exposure to ammonium	Implantation per embryo transferred (%)	Fetal development per embryo transferred (%)	Fetal development per implantation (%)
Control	74.6[ab]	46.7[ac]	57.7[ac]
Zygote to 2-cell	64.8[ab]	32.7[a]	43.1[b]
2-cell to 8-cell	76.2[ab]	29.4[b]	33.7[b]
8-cell to blastocyst	88.0[c]	45.4[a]	48.4[abc]
Zygote to blastocyst	76.3[bc]	38.5[a]	50.6[ab]
In vivo	75.0[bc]	62.1[c]	76.3[c]

Control n=168, Zygote-2-cell n=90, 2-cell-8-cell n=66, 8-cell-blastocyst n=42, Zygote-blastocyst n=90
In vivo n=18 (7 replicates)

[a-c] Different letters within a column are significantly different ($P<0.05$)

Table 3-3: Effect of culture with 300μM ammonium on fetal and placental parameters

Time of exposure to ammonium	Fetal weight (mg)	Placental weight (mg)	Fetal:placental weight ratio	Crown rump length (mm)
Control	1007.6±15.7^{ad}	117.5±2.3^{a}	8.8±0.2^{a}	21.1±0.2^{a}
Zygote to 2-cell	1018.2±33.5^{acd}	134.6±4.2^{a}	8.1±0.4^{b}	20.6±0.2^{b}
2-cell to 8-cell	959.8±28.1^{+d}	122.8±4.8^{a}	8.3±0.4^{a}	20.1±0.4^{b}
8-cell to blastocyst	1040.1±32.4^{ac}	127.7±4.7^{a}	9.0±0.4^{a}	21.0±0.3^{a}
Zygote to blastocyst	1009.7±24.9^{d}	135.4±3.1^{a}	7.6±0.3^{b}	20.0±0.3^{b}
In vivo	1132.7±36.5^{c}	106.5±4.9^{a}	10.8±0.5^{c}	ND

Data is expressed as mean ±SEM

Control n=94, Zygote-2-cell n=40, 2-cell-8-cell n=27, 8-cell-blastocyst n=28, Zygote-blastocyst n=48 *In vivo*=11 (7 replicates)

$^{a-d}$ Different letters within a column are significantly different (P<0.05) + indicates trending (P=0.06)

ND=Not Determined

3.5 Discussion

The presence of ammonium in the embryo culture medium can have a detrimental effect on embryo development and viability. Although culture media for human embryos are now designed to limit ammonium production, levels can still increase owing to amino acid deamination and have been correlated with reduced blastocyst quality in the human (Virant-Klun *et al.* 2006). The mechanism behind the effect of ammonium is believed to be the alteration in intracellular pH (pH_i), as the presence of ammonium in culture media results in a decrease in embryonic pH_i; however, this is still remains to be elucidated (Lane and Gardner 2003). Studies in the sea urchin embryo have indicated that pH alteration can profoundly affect cellular events governing early development (Roos and Boron 1981) Similarly, in mammalian embryos alterations in intracellular pH disrupt mitochondrial distribution (Squirrell *et al.* 2001). It has been hypothesised that this dispersion disrupts efficient delivery of ATP to the nucleus, which leads to a loss of energy needed to support transcriptional activity and subsequently delayed development (Barnett *et al.* 1997). Whether the abnormal affects of ammonium on development and metabolism are mediated by changes in pH regulation are yet to be established. Alternatively ammonium may affect embryo development by having a direct effect on the ability of the embryo to produce ATP. In other cell types ammonium has been shown to inhibit mitochondrial shuttle activity as well as altering the activity of glycolytic enzymes resulting in perturbed energy production (Fitzpatrick *et al.* 1988; Hindfelt *et al.* 1977; Lai *et al.* 1989; Sugden and Newsholme 1975). Whether ammonium has these same effects on metabolic homeostasis in the embryo is as yet unknown.

The data presented in this chapter demonstrated that the cleavage stage embryo is more sensitive to the presence of ammonium in the culture media, than the post-compaction stage, and this supports previous evidence of stage specific vulnerability to *in vitro* stress, both with ammonium and also with the presence of serum (Rooke *et al.* 2007; Zander *et al.* 2006). Nevertheless, the post-compaction stage exposure to ammonium did result in decreased glucose uptake in the blastocyst, indicating that the post-compaction stage embryo may also be sensitive to stress, although the impact of this stress is reduced. As mentioned previously the pre and post compaction stage embryo vary immensely in structure and metabolic requirements and the early embryo has a reduced capacity to regulate metabolism and cellular homeostasis compared with the post-compaction stage embryo. Edwards *et al*. showed that the ability of embryos to regulate internal pH increases significantly with compaction as embryos exposed to a weak acid at the pre-compaction stage show a decrease in intracellular pH while those exposed post compaction were able to maintain pH at the physiological level (Edwards *et al.* 1998b) Similarly, the ability of the embryo to maintain normal development when exposed to osmotic stress, is significantly increased following compaction (Lane and Gardner 2001). In this study the exposure of the embryos to ammonium at the pre-compaction stage resulted in increased aberrant

outcomes compared to post compaction exposure which is likely due to the reduced ability of the pre-compaction stage embryo to regulate metabolic and cellular homeostasis.

My previous work demonstrated that exposure to 300μM ammonium during the pre-compaction stage from either the zygote to 2-cell stage or from the 2-cell to 8-cell stage or continually throughout development does not affect the ability of the embryos to form a blastocyst. Data collected in this chapter further support this observation with, similarly, no effect observed on morula/early blastocyst formation. However, one of the culture-induced effects of ammonium on the blastocyst has been a significant reduction in blastocyst cell number as well as reducing inner cell mass cell number (Zander *et al.* 2006). Interestingly, this study indicates that this reduction in blastocyst cell number must occur after compaction and as blastocoel cavity formation is initiated, as there was no significant difference in cell number after 67 hours of culture. This lack of effect on cell number was observed independent of the stage of ammonium exposure during the culture period. The reason for this slowing of cell division post-compaction is currently unknown, but it may be due to alterations in metabolism and energy production owing to decreased substrate uptake, namely glucose.

Exposure to 300μM ammonium at all stages examined also resulted in decreased glucose uptake by blastocysts. I have previously shown altered glucose transporter gene expression in blastocysts after exposure to ammonium either continually throughout development and during specific temporal time points of pre-implantation development (Zander *et al.* 2006). The results in this section support these findings as internal blastocyst glucose levels are reduced. This reduction in both glucose transporter gene expression and actual glucose uptake may result in altered metabolism because of decreased substrate availability, and could subsequently lead to decreased energy production however this remains to be shown. Previous studies have also demonstrated that glucose plays a vital role in determining blastocyst cell number as the absence of glucose resulted in decreased blastocyst cell numbers and occurrence related to a reduction in TE cell number (Sakkas *et al.* 1993).

Facilitated glucose transport is also believed to be more important after the morula cell stage when cavitation occurs, and this has demonstrated by using phloretin (a facilitated glucose transporter inhibitor) providing a possible reason as to why morula cell number is not affected, however blastocyst cell number it (Leppens-Luisier *et al.* 2001). It has also been hypothesised that facilitated glucose transporters may also have secondary consequences to the embryo which are possibly linked to the metabolism of glucose, as inhibiting the transporters resulted in decreased blastocyst development when compared to growing embryos in the absence of glucose itself (Leppens-Luisier *et al.* 2001). This secondary role however remains to be elucidated.

Previous studies into ammonium exposure have also shown alterations in nutrient uptake and consumption, where exposure to ammonium increased glycolysis and decreased pyruvate oxidation at the cleavage stage. This is possibly indicative of the crabtree effect in which elevated glycolytic activity inhibits respiratory activity and oxidative metabolism (Lane and Gardner 2003; Seshagiri and Bavister 1991). Culture with ammonium has also been shown to perturb metabolism in other tissues

by interfering with the transport of reducing equivalents between the cytoplasm and the mitochondrial via the disruption of the malate aspartate shuttle (Lai *et al.* 1989). The function of this shuttle is vital for embryo development, implantation and normal fetal growth (Lane and Gardner 2000c; Mitchell *et al.* 2008). Since glucose metabolism is regulated by the malate aspartate shuttle, a reduction in glucose uptake will possibly lead to a reduction in glucose metabolism, and a decrease in the cytoplasmic NAD^+ pool. Coupled with a possible reduction in malate aspartate shuttle activity this could lead to significantly altered metabolism within the embryo; however, this remains to be elucidated.

Tightly regulated metabolism is vital for normal embryo viability. Previous studies have shown that exposure to sub optimal environmental conditions significantly affect embryo metabolism and that embryos with arrested or delayed development often display perturbed cytoplasmic and mitochondrial metabolism as well as alterations in metabolic intermediates such as ADP and ATP levels (Gardner 1998b; Gardner and Lane 1993a; Gardner and Lane 1997; Gardner *et al.* 2000b; Lane and Gardner 2005b; Seshagiri and Bavister 1991). In addition, nutrient metabolism, such as glucose or amino acid, is often related to embryo homeostasis and can be used as a selection criterion for embryo viability (Brison *et al.* 2004; Gardner and Leese 1987; Houghton *et al.* 2002; Lane and Gardner 1996).

A study using glycolysis to determine embryo viability discovered that embryos with abnormally high levels of glycolysis and a high conversion to lactate resulted in poor fetal development. On the other hand, transfer of embryos with glycolysis levels closer to that of *in vivo* derived blastocysts resulted in high fetal development after transfer (Lane and Gardner 1996). These results demonstrate the importance of metabolic control in determining embryo viability.

The exposure of embryos to ammonium during pre-implantation embryo development has been demonstrated to significantly reduce implantation and fetal development rates, as well as decreasing fetal weight and increasing fetal abnormalities after transfer (Lane and Gardner 2003). I have previously shown similar effects with both short- and long-term exposure to ammonium on Day 15 of fetal development.

In this follow-on study, exposure to ammonium during the pre-compaction stage resulted in no alteration in implantation however, exposure during the 2-cell to 8-cell stage significantly reduced fetal development per embryo transferred and fetal development per implantation. Interestingly, on Day 15, exposure during the zygote to 2-cell stage resulted in decreased fetal weight which did not persist to Day 18, perhaps indicative of an increase in growth over these final days of gestation. Exposure to ammonium during the pre-compaction stage also resulted in significantly decreased crown rump length on Day 18 and exposure during the zygote to 2-cell stage also resulted in decreased fetal:placental weight ratio, indicating that although the fetuses are similar in weight, the placentas are larger, which is a possible reason behind the increase in growth seen from Day 15 to Day 18. Culture with ammonium continually throughout development also delivered similar results to culture with ammonium during the cleavage stage, namely decreased fetal:placental weight ratio and decreased crown rump length.

These results indicate that exposure to ammonium during pre-implantation embryo development can both significantly alter embryo homeostasis and influence subsequent fetal and placental outcomes. Similar results have been demonstrated in other studies using a variety of *in vitro* stresses such as serum, high oxygen, ammonium and *in vivo* studies using low or high protein (Feil *et al.* 2006; Kwong *et al.* 2000; Lane and Gardner 2003; Rooke *et al.* 2007).The mechanism behind how the embryo is able to be 'programmed' by an environmental stress is still currently unknown; however, alterations in metabolism may be a possible factor in determining long-term outcomes and this will be the focus of the subsequent chapters.

4 The effect of temporal intracellular pH decrease on blastocyst viability and fetal and placental outcomes

The work presented in this chapter has been published in the following journal:

Zander-Fox DL, Mitchell M, Thompson JG, Lane M. Alterations in Mouse Embryo Intracellular pH by DMO During Pre-implantation Development Impairs Pregnancy Establishment and Perturbs Fetal Growth. *RBMOnline* 2009 (In Press)

4.1 Introduction

The culture of the mammalian pre-implantation embryo has become a routine laboratory practice in a clinical setting as well as for research and agricultural breeding programs. As previously mentioned, traditionally the *in vitro* development of the pre-implantation embryo is associated with slower rates of development and decreased embryo viability, and more recently has been linked to perturbations in gene expression and metabolism in the blastocyst (Gardner and Lane 1993b; Ho *et al.* 1994; Lane and Gardner 1994). However, more significantly, it has been established that by perturbing the *in vitro* conditions that the pre-implantation stage embryo is exposed to can also affect both the ability to establish a pregnancy as well as inducing subsequent effects on fetal growth and also adult health (Lane and Gardner 2003; Rooke *et al.* 2007; Zander *et al.* 2006).

Gametes and pre-implantation embryos are susceptible to stress *in vitro* which can be induced through a variety of ways—for example sub optimal media composition, fluctuations in temperature, altered gas phase such as elevated oxygen, elevated or decreased pH, and the presence of waste build-up in the media such as ammonium (Gardner and Lane 1993b; Lane and Gardner 2005b). Interestingly, in many cases of *in vitro* stress the embryo exhibits tremendous plasticity and is able to maintain development to the blastocyst stage in culture, despite the presence of stressors, with the resultant blastocysts frequently appearing morphologically normal (Feil *et al.* 2006; Zander *et al.* 2006). However, when these embryos are transferred to recipient mothers the blastocysts result in decreased fetal development per implantation and also have perturbed fetal growth parameters (Zander *et al.* 2006).

One known *in vitro* stress which has been shown to alter fetal growth outcomes after exposure of the embryo, during the pre-implantation phase of development, is ammonium. These effects of ammonium have been particularly significant, as it is forms in any medium containing amino acids, more especially glutamine, and reaches levels that are embryo-toxic within only 48 hours of incubation (Lane and Gardner 2003). Furthermore, my studies have shown that an exposure to ammonium for only 19 hours during the first cleavage division, from the zygote to the 2-cell, can increase these effects, resulting in a decrease in blastocyst viability reduced fetal growth after transfer (Zander *et al.* 2006). The mechanism behind these perturbations of ammonium is currently unknown; however, it has been shown that exposure to ammonium induces a drop in intracellular pH_i (Lane and Gardner 2003).

Maintenance of homeostatic intracellular pH (pH_i) is essential for normal development of all cells, as it is involved in important cellular processes such as enzyme activity, protein synthesis, cell division and cell-to-cell communication (Roos and Boron 1981). Similarly, in the pre-implantation embryo there appears to be a comparable reliance on the maintenance of pH_i (Lane *et al.* 1998a). In somatic cells pH_i is regulated by two main systems: either the Na^+/H^+ antiporter, which regulates against acid loads, or the HCO_3^-/Cl^- exchange which regulates against deviations in the alkaline range

(Roos and Boron 1981) However, in the early embryo, immediately after fertilisation, there appears to be a lack of robust regulatory mechanisms to regulate pH_i against an acid challenge (Lane *et al.* 1999b). It has been previously demonstrated that immediately after fertilisation the embryo has no transport systems for pH regulation and that it is only after pronuclei formation that these systems are activated (Lane *et al.* 1998b; Phillips and Baltz 1999b). Furthermore, it has also been shown that the activity of the Na^+/H^+ antiporter is variable and is not a robust system for removing acid loads (Lane and Gardner 2005b; Steeves *et al.* 2001), especially in the 2-cell embryo where activity is virtually absent (Baltz *et al.* 1991b). Despite there being some species differences in activity of these regulatory mechanisms, it is believed that prior to compaction, the ability of an embryo to regulate pH_i is limited (Lane and Gardner 2005b). Species such as the hamster and the mouse display variation in activity of the Na^+/H^+ antiporter; however, both display perturbations in cellular homeostasis after exposure to weak acidic conditions at the pre-compaction stage, indicating that perhaps the Na^+/H^+ antiporter is not a robust system, regardless of apparent activity (Edwards *et al.* 1998b; Squirrell *et al.* 2001).

In contrast, the post-compaction stage embryo appears to have pH regulation more similar to that of a somatic cell. The pH_i of the compacted embryo remained unchanged in the presence of 5mM lactate and incubation with a weak acid did not affect the rate of glycolysis within the morula, whereas pre-compaction incubation did (Edwards *et al.* 1998b). It has also been demonstrated that HCO_3^-/Cl^- exchange for maintenance of pH_i against an alkaline load is only active in late (post compaction) stage embryos (Harvey *et al.* 1995). However, the effect of perturbations in pH_i, during pre-implantation embryo development, on the post-implantation development of the embryo, is currently unknown.

The aim of these experiments was therefore to assess the effect of decreasing pH_i on the embryo either continually throughout development from the zygote to the blastocyst stage or for defined time-points during pre-implantation development. This will assist us in understanding the effect of altered pH_i during embryo culture and will indicate specific time-points when the embryo appears to be most sensitive to disrupted pH.

4.2 Experimental design

4.2.1 Culture conditions

Embryos were collected from superovulated F1 hybrid female mice after mating with Swiss males 23 hours post-hCG at the zygote stage. All cleavage stage embryos were cultured in groups of 10–12 in 20µl drops of medium under 3.5 mL of mineral oil at 37 °C at 6% CO_2, 5% O_2, and 89% N_2. Embryos were randomly allocated to each different treatment group and exposed to 2mM DMO (5,5-dimethyl-2,4-oxazolidinedione) during a certain stage of development. 2mM DMO was chosen, as this will induce a decrease in intracellular pH similar to that seen after exposure to 300µM ammonium chloride (see Appendix: Section 10.9). The following five treatment groups were used for this temporal assessment.

The treatment groups used for this temporal assessment were:

- Treatment 1 (control group) which was cultured in sequential culture media with no DMO exposure (total of 91 hours of culture)
- Treatment 2 which was exposed to 2mM DMO from the zygote to the 2-cell stage (from 0 –19 hours of culture)
- Treatment 3 which was exposed to 2mM DMO from the 2-cell to the 8-cell stage (19–43 hours of culture)
- Treatment 4 which was exposed to 2mM DMO from the 8-cell to blastocyst stage (43–91 hours of culture)
- Treatment 5 which was exposed to 2mM DMO for the entire culture period (total of 91 hours of culture) (**Figure 4-1**).

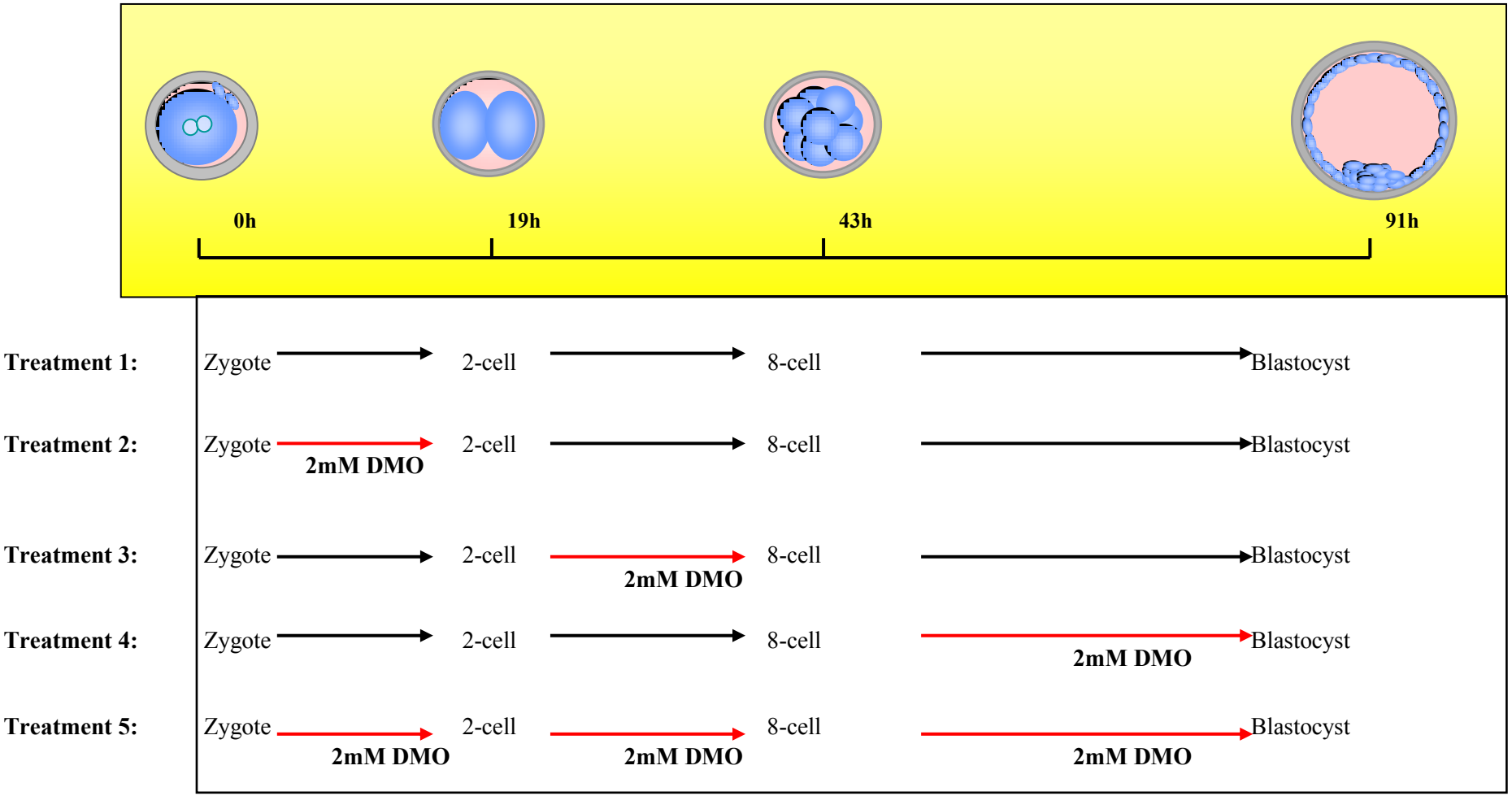


Figure 4-1 : Temporal exposure of embryos to 2mM DMO.

Black arrows indicate embryos are cultured in control media either G1.2 (prior to the 8-cell stage) or G2.2 (after the 8-cell stage)

Red arrows indicate embryos are cultured in G1.2 (prior to the 8-cell stage) or G2.2 (after the 8-cell stage) with 2mM DM

4.3 Statistics

For binomial data, each replicate was expressed as a proportion for analysis. Embryo development, cell numbers and apoptosis were analysed using a univariate general linear model. Development for each replicate experiment was expressed as a proportion, and day of replicate was treated as a co-variate. Differences between treatments were assessed using the Least Significant Difference method (LSD). Intracellular pH was assessed using a student's paired t-test. Implantation data was analysed using chi-square, and fetal and placental data was analysed using a univariate general linear model as LSD. Mouse number, implantations per horn, fetuses per horn, implantations per mother and fetuses per mother were treated as covariates.

4.4 Results

4.4.1 Measurement of intracellular pH

Exposure to 2mM DMO during the first cleavage division caused intracellular pH to decrease in the 2-cell to 7.08±0.04 when compared to control intracellular pH of 7.24±0.09 however this was not significant (p=0.07) (**Figure 4-2**). The exposure to DMO decreased intracellular pH by 0.16 units. This decrease is similar to that seen after exposure to 300µM ammonium (Lane and Gardner 2003). Exposure to 2mM DMO from the zygote to the 8-cell stage also significantly reduced intracellular pH from 7.12±0.02 units to 7.02±0.01 units. Exposure to 2mM DMO from the zygote to the morula stage significantly reduced intracellular pH from 7.44±0.05 units to 7.16±0.04 units (**Figure 4-2**).

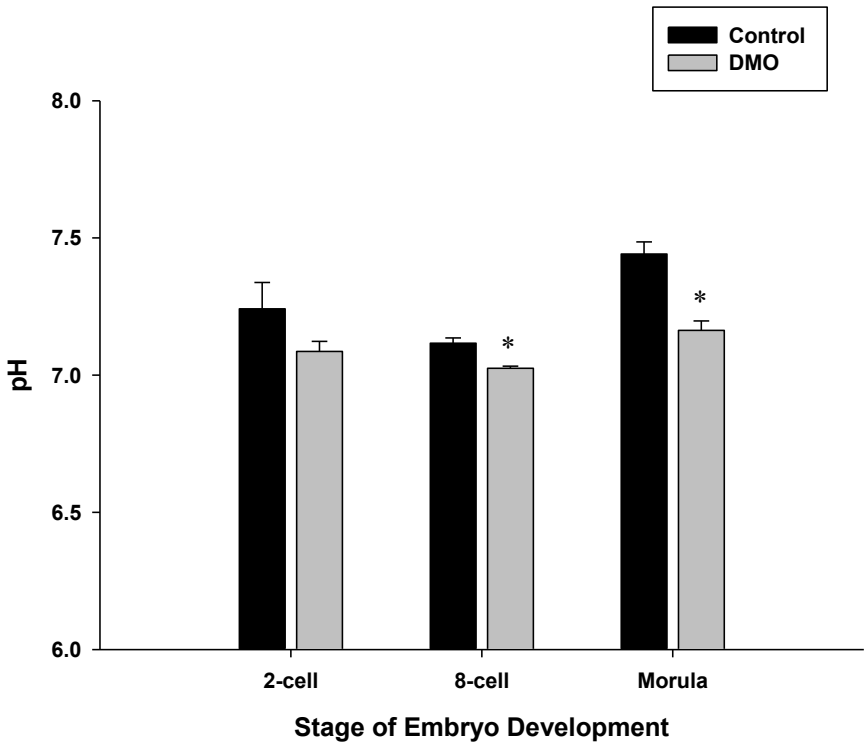


Figure 4-2: Effect of incubation with 2mM DMO on intracellular pH.

Control 2-cell n=22, 8-cell n=20, morula n=21. DMO 2-cell n= 38, 8-cell n=15, morula n=19

(2 replicates)

Data is expressed as mean ± SEM

* Indicates significantly different from control (P≤0.05)

4.4.2 Effect of DMO exposure on embryo development

Exposure of embryos to DMO did not affect development to the 2-cell stage after 19 hours of culture nor the percentage of embryos that reached the 8-cell stage after 43 hours of culture (

Table 4-1). DMO exposure also did not affect development to the morula and blastocyst stage after 74 hours nor the blastocyst stage after 91 hours of culture (**Table 4-2**). The morphology of the blastocysts from all groups cultured with DMO was similar to that of control blastocysts (**Figure 4.3**).

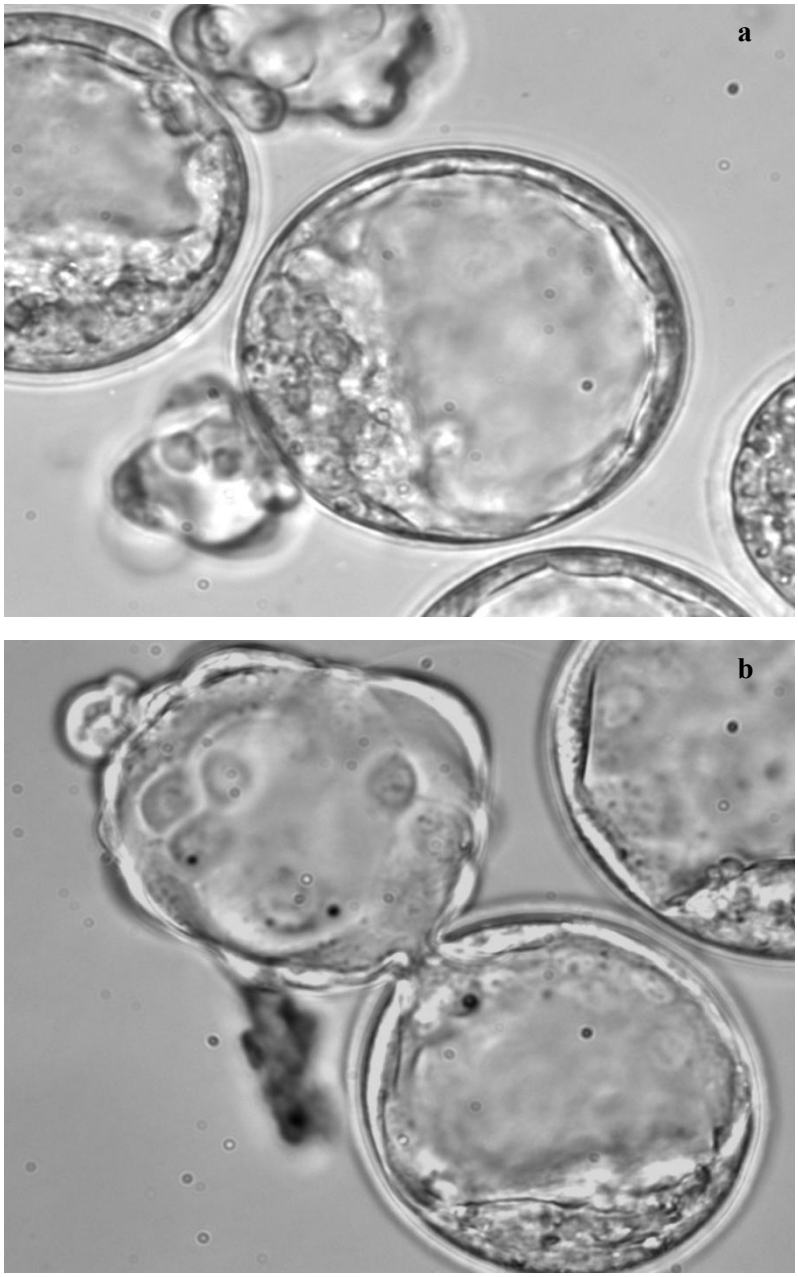


Figure 4-3: Murine blastocysts after 91 hours of culture (Day 5) at 40x objective

a) Control **b**) Exposed to 2mM DMO continually throughout development.

Table 4-1: The effect of culture with DMO on embryo development after 19, 43 and 74 hours of culture

	Day 2	Day 3	Day 4	
Time of exposure to DMO	**%2-cell**	**% ≥ 8-cell**	**% Morula**	**% ≥ Early Blastocyst**
Control	84.8±1.0	89.1±7.2	33.1±13.4	65.2±14.6
Zygote to 2-cell	85.9±2.1	79.9±7.4	21.7±15.7	77.3±15.7
2-cell to 8-cell	86.9±0.6	84.1±5.0	16.0±9.4	81.9±9.0
8-cell to blastocyst	80.4±1.8	82.1±8.9	22.9±13.4	67.9±12.9
Zygote to blastocyst	82.2±1.3	77.8±6.4	18.2±14.5	70.9±18.6

Control n=124, Zygote-2-cell n=115, 2-cell-8-cell n=112, 8-cell-blastocyst n=106, Zygote-blastocyst n=117 (6 replicates)

Data is expressed as mean ± SEM

There was no significant difference for any stage of development between treatments.

Table 4-2: The effect of culture with DMO on embryo development after 91 hours of culture

Time of exposure to DMO	**% ≤Morula**	**%Total Blastocyst**	**% Blastocyst**	**% Hatching**
Control	10.5±4.2	89.5±4.2	23.4±6.9	66.1±10.6
Zygote to 2-cell	6.7±3.3	93.3±3.3	31.2±10.9	62.0±10.7
2-cell to 8-cell	7.8±4.4	92.2±4.4	30.1±8.6	62.2±11.1
8-cell to blastocyst	13.2±5.5	86.8±5.5	23.0±9.8	63.7±14.0
Zygote to blastocyst	14.9±5.2	85.1±5.2	31.1±5.4	54.0±8.7

Control n=124, Zygote-2-cell n=115, 2-cell-8-cell n=112, 8-cell-blastocyst n=106, Zygote-blastocyst n=117 (6 replicates)

Data is expressed as mean ± SEM

There was no significant difference for any stage of development between treatments.

4.4.3 The Effect of DMO on morula cell number

The purpose of this investigation was to assess cell number at the morula stage (when cell differentiation begins) to ascertain the stage at which cell numbers begin to change.

Exposure to 2mM DMO at any stage did not alter on time morphological development (**Table 4-3**).

Exposure to 2mM DMO at any stage did not significantly alter morula cell number after 67 hours of culture when compared to control (**Figure 4-4**).

Table 4-3: The effect of culture with DMO on embryo development after 67 hours of culture

Time of exposure to DMO	% ≤8 cell	% Compact morula	% Early blastocyst
Control	10.0	40.0	50.0
Zygote to 2-cell	6.0	55.0	39.0
2-cell to 8-cell	5.0	50.0	45.0
8-cell to blastocyst	7.0	42.0	51.0
Zygote to blastocyst	10.0	50.0	40.0

Control n=18, Zygote-2-cell n=18, 2-cell-8-cell n=16, 8-cell-blastocyst n=18, Zygote-blastocyst n=18 (2 replicates)

There was no significant difference for any stage of development between treatments.

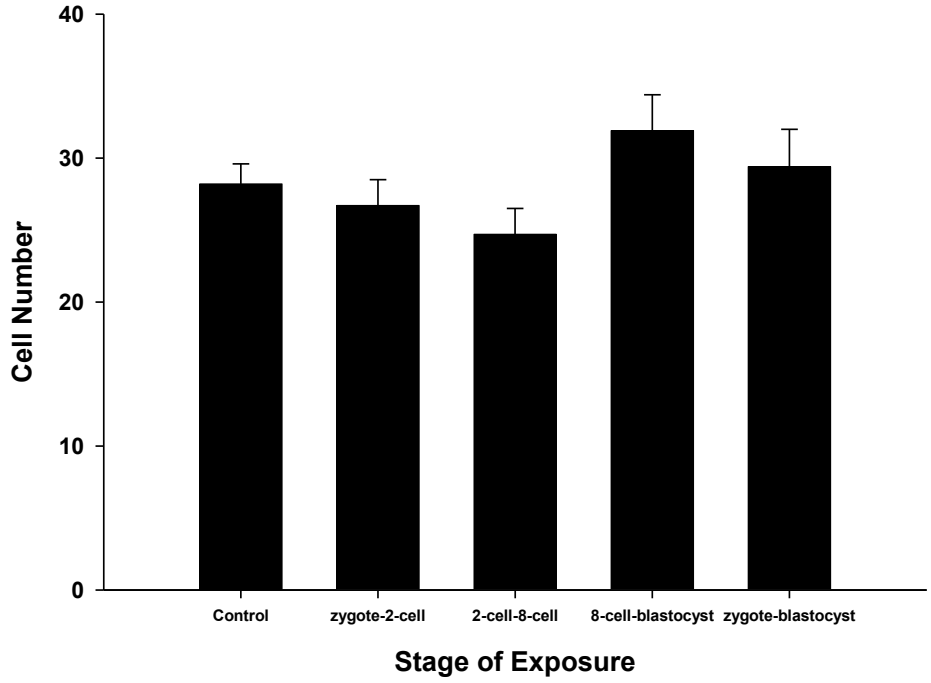


Figure 4-4: The effect of temporal DMO exposure on cell number after 67 hours of culture.

Control n=51, Zygote-2-cell n=30, 2-cell-8-cell n=30, 8-cell-blastocyst n=26, Zygote-blastocyst n=31 (3 replicates)

Data is expressed as mean ± SEM

No difference in cell numbers was observed between treatments

4.4.4 Effect of DMO on blastocyst cell allocation

In contrast to morula/blastocyst cell numbers after 67 hours of culture, blastocyst cell number after 91 hours of culture was significantly reduced after exposure to DMO aat any stage of development when compared to control ($P<0.01$; **Table 4-4**). There was also a significant reduction in trophectoderm (TE) cells in embryos exposed to DMO from the zygote to 2-cell, 2-cell to 8-cell and 8-cell to blastocyst compared to control embryos ($P<0.05$; **Table 4-4**). There was no significant difference in TE cells in the group exposed to DMO continually through culture. Calculation of the percentage of ICM cells of the overall total cell number revealed that there was a significant reduction in this value in blastocysts exposed to DMO from the 2-cell to 8-cell stage compared to those cultures in the control medium ($P<0.05$; **Table 4-4**).

Table 4-4: The effect of culture with 2mM DMO on blastocyst cell allocation after 91 hours of culture

Time of exposure to DMO	Total Cell Number	ICM Cell Number	TE Cell Number	%ICM/Total cell number
Control	83.0±6.4[a]	30.6±3.6[a]	52.4±4.9[a]	36.9±2.8[a]
Zygote to 2-cell	63.6±3.8[b]	20.2±1.8[b]	43.5±3.1[b]	32.2±2.2[ab]
2-cell to 8-cell	58.5±3.0[b]	18.0±1.2[b]	40.5±2.6[b]	31.3±1.8[b]
8-cell to blastocyst	65.4±3.7[b]	22.4±1.6[b]	43.0±3.2[b]	35.1±2.1[a]
Zygote to blastocyst	63.8±4.2[b]	21.6±2.0[b]	42.2±3.1[b]	34.1±2.0[a]

Control n=25, Zygote-2-cell n=34, 2-cell-8-cell n=37, 8-cell-blastocyst n=28 Zygote-blastocyst n=31 (3 replicates)

Data is expressed as mean ± SEM

[a-b] Different letters within a column are significantly different ($P<0.05$)

4.4.5 Effect of DMO on blastocyst apoptosis

There was a significant increase in the levels of apoptosis in blastocysts that were exposed to DMO from either the zygote to the 2-cell stage or the 2-cell to 8-cell stage ($P \leq 0.05$; **Figure 4-5**) compared to blastocysts. Similarly, blastocysts that resulted from embryos incubated with DMO continually throughout development from the zygote stage also had significantly elevated levels of apoptosis compared to control blastocysts ($P < 0.05$; **Figure 4-5**). There was no significant difference in the levels of apoptosis of blastocysts where the DMO exposure was from 8-cell to the blastocyst stage compared with control blastocysts.

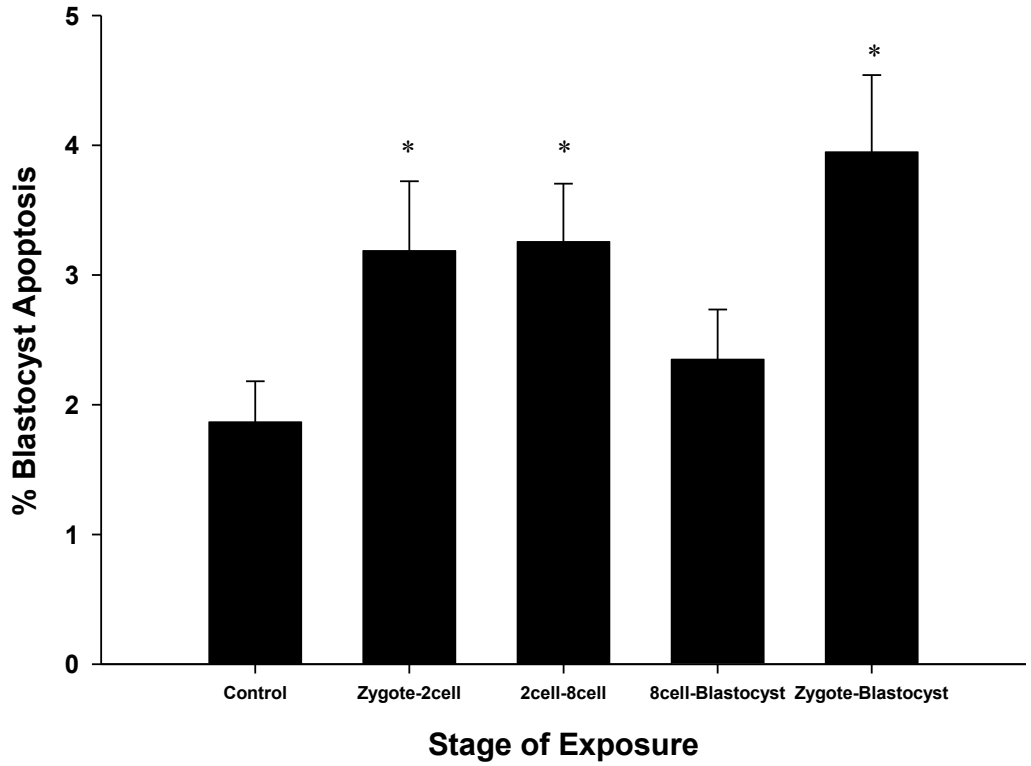


Figure 4-5: Percentage of apoptosis in blastocysts after varying stages of DMO exposure
Control n=23, Zygote-2-cell n=29, 2-cell-8-cell n=33, 8-cell-blastocyst n=25 Zygote-blastocyst n=28 (3 replicates)
Data is expressed as mean ± SEM
* Indicates significantly different from control ($P \leq 0.05$)

4.4.6 Effect of DMO on blastocyst viability

To further assess the effects of DMO on embryo viability, blastocysts cultured in control conditions or those exposed to DMO from the zygote to the 2-cell stage or through development from the zygote to the blastocyst stage were transferred to pseudo-pregnant recipients. Exposure to DMO from the zygote to the 2-cell stage or continually throughout development did not affect the ability of the resultant blastocyst to implant into a pseudo-pregnant recipient when compared to control blastocysts. On the other hand, exposure to DMO throughout development significantly decreased the percentage of fetuses that developed per embryo transferred ($P<0.001$; **Table 4-5**). Similarly there was also a reduced number of viable fetuses that formed per implantation site (with implantation site being defined by either the presence of a fetus or a resorption) however this was not significant ($P=0.06$; **Table 4-5**). Interestingly, exposure to DMO during the first cleavage division did not significantly alter the percentage of fetuses formed per embryo transferred and did not alter the resorption rate (**Table 4-5**).

In contrast, exposure to DMO during either the first cleavage division or continually throughout development significantly reduced the weight of the resultant fetuses as well as reducing fetal crown-rump length when compared to fetuses resulting from culture in control conditions. Exposure to DMO during the first cleavage division only also decreased the fetal: placental weight ratio compared to the fetuses in the control group. Exposure of cultured embryos to DMO at any stage did not alter placental weight or placental diameter (**Table 4-6**).

Table 4-5: Effect of culture with 2mM DMO on blastocyst implantation and fetal development

Time of exposure to DMO	Implantation per Embryo Transferred (%)	Fetal Development per Embryo Transferred (%)	Fetal Development per Implantation (%)
Control	90.0[a]	70.0[a]	77.8
Zygote to 2-cell	76.7[a]	63.3[a]	82.6
Zygote to blastocyst	58.3 [b]	30.5 [b]	52.4

Control n=30, Zygote-2-cell n=30, Zygote-blastocyst n=36 (5 recipients)

[a-b] Different letters within a column are significantly different ($P<0.05$)

Table 4-6: Effect of culture with 2mM DMO on fetal and placental parameters

Time of exposure to DMO	Fetal Weight (mg)	Placental Weight (mg)	Fetal:Placental Weight Ratio	Crown Rump Length (mm)	Placental Diameter (mm)
Control	1058.9±25.2[a]	122.5±4.6[a]	8.9±0.4[a]	21.9±0.4[a]	8.5±0.2[a]
Zygote to 2-cell	949.1±26.7[b]	131.3±6.9[a]	7.6±0.4[b]	20.6±0.5[b]	8.4±0.3[a]
Zygote to blastocyst	973.7±34.3[b]	122.9±6.6[a]	8.1±0.4[ab]	20.1±0.4[b]	8.5±0.5[a]

Control n=30, Zygote-2-cell n=30, Zygote-blastocyst n=36 (5 recipients)

Data is expressed as mean ±SEM

[a-b] Different letters within a column are significantly different ($P<0.05$)

One fetus in the zygote to blastocyst stage was excluded from the mean calculations as it was statistically identified as an outlier (weight: 263.5g).

4.5 Discussion

The data presented in this chapter supports the hypothesis that the pre-implantation stage embryo is sensitive to a decrease in intracellular pH (pH_i) as exposure to DMO at all stages of development altered blastocyst number and cell allocation to the ICM and TE. Interestingly, only exposure during the pre-compaction stage or continually throughout development increased blastocyst apoptosis, thus supporting the notion that the pre-compaction stage is the most sensitive to altered pH_i. The results presented here also support the concept that blastocyst development is a poor marker for embryo viability as there were no morphological differences seen between any of the treatment groups exposed to DMO despite numerous cellular and viability differences.

In somatic cells, it has been established that there are many essential roles for pH_i in development, such as cell division, differentiation, metabolism and cytoskeletal dynamics (Roos and Boron 1981). Studies have shown that very small changes in pH can result in large changes in cellular function. Many studies have shown that small changes in pH_i, as small as one-tenth of a pH unit, are sufficient to initiate large changes in cell function such as DNA synthesis (Boron 1986; Busa and Nuccitelli 1984; Roos and Boron 1981).

Previous studies have demonstrated that the pH_i of the oocyte can play a significant role in meiotic maturation, oocyte activation and fertilisation. Studies performed in the sea urchin have shown that during these early stages of development, pH_i undergoes specific and controlled shifts. These shifts are often due to alterations in intracellular Ca^{2+}, which plays a role in cytoplasmic and nuclear activities such as sperm aster formation which is essential for pronuclear movement and fusion (Hamaguchi and Hamaguchi 2001). Experiments have shown that if the alteration in pH_i is blocked, sperm aster formation does not occur and development is blocked. If the pHi is then allowed to increase, sperm aster are formed and development resumes, indicating the importance of pH_i in embryo developmental processes. (Hamaguchi and Hamaguchi 2001; Lee and Steinhardt 1981; Schatten *et al.* 1985). In the sperm, pH_i is also very important in motility, capacitation and the acrosome reaction (Carr and Acott 1989; Carr *et al.* 1985; Fraser 1993; Parrish *et al.* 1989). Studies in sea urchin sperm have demonstrated that motility is dependent on initiation of the Na^+/H^+ antiporter, which increases pH_i stimulating motility. In the bovine, sperm stored in the cauda epididymis, prior to ejaculation, is relatively immotile at a pH of 5.8. If the pH is elevated to 7.0, either by pH manipulation or by dilution, the sperm become highly motile, both of which are activities that occur during ejaculation (Acott and Carr 1984; Carr and Acott 1984). While some studies have also shown that a decrease in pH_i due to glucose metabolism by the sperm can prevent sperm capacitation, others have demonstrated that a rise in pH_i may, in turn, activate calcium channels and permit the influx of extra-cellular Ca^{2+} needed to trigger acrosomal exocytosis (Fraser 1993; Parrish *et al.* 1989). Thus it stands that alterations in the pH_i of either the oocyte or the sperm can profoundly affect the ability of these gametes to fertilise and form a viable embryo.

The embryo itself is also very sensitive to alterations in pH_i , with several studies demonstrating that exposure of the pre-compaction stage embryo to a weak acid can significantly inhibit subsequent morula and/or blastocyst development in a dose-dependent manner (5mM DMO upwards) and can significantly increase developmental arrest at the 2-cell stage in the hamster (Edwards *et al.* 1998b; Squirrell *et al.* 2001). Earlier experiments in the mouse have shown that embryos cultured in a varying range of pH from 5.87 to 7.78 were able to develop to the blastocyst stage; however, at either end of the range blastocyst development was very low at around 2-4%, which increased in a dose-dependent manner as the pH moved closer to neutral (pH 7.0) (Brinster 1965a).

Another study has also shown that the addition of increasing concentrations of D/L lactate (5mM-30mM) to the media will induce a decrease in pH_i of the mouse zygote in a dose dependent manner after only 10 minutes of culture (Edwards *et al.* 1998b). Interestingly, incubations of 60 minutes resulted in smaller alterations in pH_i, which is perhaps indicative of initiation of pH regulation. Although blastocyst development was not assessed in this study, metabolic parameters were determined, and it was shown that exposure to 5mM D/L lactate, which induces a pH drop of only approximately 0.1 pH unit, significantly reduced glycolytic activity. Exposure to 1-50mM DMO also significantly reduced glycolytic activity in the zygote. It is noteworthy that exposure of compacted morula to the same concentrations of D/L lactate or DMO did not alter glycolytic activity (Edwards *et al.* 1998b). This data supports the theory that the post-compaction stage embryo is better equipped to deal with pH stress than the pre-compaction stage embryo.

Exposure to a weak acid (20mM DMO, causing a pH_i decrease of 0.4 pH units) or a weak base (20mM TMA, increasing pHi by approximately 0.24 pH units) can also significantly disrupt cellular organisation in the hamster 2-cell by altering mitochondrial organisation. Exposure can also alter other structural components such as nuclear diameter, blastomere diameter and nuclear:blastomere ratio, as well as microfilament arrangement (Squirrell *et al.* 2001). However, in contrast to these previous studies where pH_i alteration changed development, my study did not show any alteration in morula or blastocyst development, or indeed blastocyst morphology, when embryos were exposed to the weak acid DMO when a concentration of 2mM was used. However, these previous studies were performed in the hamster and perhaps indicate that the degree of susceptibility to pH stress is species specific (Squirrell *et al.* 2001). Alternatively, the concentration of DMO used in the hamster study was significantly higher at 5mM, so perhaps the alteration in blastocyst development was due to the increased acid concentration. This may therefore suggest that there is a threshold value whereby the embryo cannot regulate pH_i, which would result in catastrophic breakdown in cellular processes and not allow development of the blastocyst to proceed. In contrast, a smaller alteration in pH_i, such as that induced in this chapter, appears to be permissive of development to the blastocyst stage, with the majority of the perturbations to development being more subtle and only evident at the sub-cellular and or cellular level. This hypothesis is supported by the observation that culture with 2mM DMO significantly inhibited cell cleavage rates, as observed by a significant reduction in blastocyst cell number. Furthermore, there were alterations in the allocation of the cells to the ICM and TE when

embryos were exposed to the perturbed pH_i induced by culture with 2mM DMO. The decrease in blastocyst cell number and alterations in cell allocation have been demonstrated in other studies for other stress exposures during *in vitro* maturation (IVM), in-vitro fertilisation (IVF) and in-vitro culture (IVC) such as poor culture media, lack of amino acids, ammonium and oxygen (Banwell *et al.* 2007; Kamjoo *et al.* 2002; Lane and Gardner 1997a; Lane and Gardner 2003; Steeves and Gardner 1999; Zander *et al.* 2006). Blastocyst cell number, cell allocation and proportion of ICM cells has been shown to play an important role in the ability of the embryo to form a viable pregnancy (Lane and Gardner 1997a). Exposure to a decreased pH at any stage of development significantly decreased blastocyst cell number and number of ICM cells; therefore this data suggests these blastocysts would have a reduced capacity to produce fetuses of normal healthy size after transfer. Interestingly, this study indicates that this reduction in blastocyst cell number must occur after compaction and as blastocoel cavity formation is initiated, as there was no significant difference in morula cell number after 67 hours of culture. This lack of effect on morula cell number was observed independent of the stage of DMO exposure during the culture period and is similar to the results seen after ammonium exposure (see chapter 3).

In addition to the reduced blastocyst cell number, blastocysts cultured with 2mM DMO at the pre-compaction stage only or continually throughout development had significantly higher rates of apoptosis compared to blastocysts cultured in control conditions. Previous studies in other stress exposures have shown an increase in the level of blastocyst apoptosis after exposure to suboptimal culture conditions (Kamjoo *et al.* 2002; Zander *et al.* 2006). The increase in apoptosis after stress exposure at the pre-compaction stage but not post-compaction has also been shown using ammonium (Zander *et al.* 2006) and supports the theory that the pre-compaction stage embryo is more sensitive to *in vitro* stress than the post-compaction stage (Edwards *et al.* 1998b; Rooke *et al.* 2007; Zander *et al.* 2006). Despite this, there is evidence that the post-compaction stage is not completely resistant to stress, with post-compaction stress causing some alterations in gene expression as well as decreasing fetal maturity after transfer (Zander *et al.* 2006). Interestingly, measurement of pH_i at the morula stage still indicated a pH perturbation was occurring. This is supported by the observed alterations to cell number and allocation in the blastocyst during post-compaction stage exposure to DMO. After the development of a transporting epithelium, pH regulation becomes more robust. Consequently, perhaps this pH perturbation seen is quickly rectified after differentiation and blastocoelic cavity formation has begun, and thus the impact is not as severe.

The combination of decreased ICM cell number and increased apoptosis after exposure to DMO at the pre-compaction stage may suggest that if these blastocysts were to be transferred, they would have a diminished capacity to form a viable pregnancy. As the early embryo from the zygote-2-cell stage has a reduced capacity to regulate pH_i, in particular against an acid load, the zygote-2-cell exposure was chosen for transfer as well as continual DMO exposure as a comparison. Continual DMO exposure from the zygote to the blastocyst stage significantly decreased the ability of blastocysts to

implant and also significantly decreased the number of implantations that went on to form a viable fetus, conversely increasing the resorption rate of these embryos. In contrast, exposure to DMO for 19 hours during the first cleavage did not alter implantation rates or fetal development rates after transfer. This observation was somewhat unexpected because a previous model of ammonium exposure during the first cleavage division similarly resulted in changes to cell number, ICM development. In that model there was also a significant effect on the number of viable fetuses that developed per implantation, which indicated that a large proportion was resorbing after implantation. Although this was observed in this study in the treatment group exposed to 2mM DMO throughout culture, this was not seen in the zygote to 2-cell treatment group. These observations taken together suggest that although ammonium exposure is decreasing pH, perhaps the NH_4^+ ion itself is interfering in other additional cellular processes. In previous studies assessing pH on mouse embryo development it was shown that decreased pH_i decreased glycolysis in mouse zygotes and in studies exposing 2-cell embryos to ammonium, which also decreases pH_i, showed an increase in glycolysis (Edwards *et al.* 1998b; Lane and Gardner 2003). These opposite alterations in metabolism may be a reflection of the different stage of measurement; however, they could also be because ammonium may induce its effects in other ways as well as decreasing pH_i.

It is worth noting, however, that although there was no effect on the numbers of viable fetuses produced after exposure during the zygote to 2-cell stage, there were still significant perturbations in fetal and placental parameters of the resultant fetuses caused by exposure to DMO. Both DMO treatments; either chronic exposure from zygote to the blastocyst stage or acute exposure for 19 hours from the zygote to the 2-cell stage significantly decreased fetal weight on Day 18 and also decreased fetal crown-rump length. As placental weights were unchanged, incubation with 2mM DMO during the first cleavage also decreased the fetal:placental weight ratio. This data suggests that the placentae from these treatments may be altered in their functional capacity to transport nutrients, and this observation requires further investigation. This result further indicates that exposure to decreased pH_i does result in embryo perturbation and that perhaps there is a threshold effect at which prolonged pH stress will induce an increased amount of damage that will decrease implantation potential. Although a small amount of damage will produce a viable blastocyst with no alteration in implantation potential, cellular alterations occurred which altered the fetal growth trajectory, perhaps suggesting alterations at a molecular level. This does pose the question, however: is this difference between the treatment groups highly significant and that the plasticity exhibited after stress treatment during pre-implantation development will come at a cost post transfer during the fetal development stage or is this plasticity a way of adapting to try and minimise fetal loss? The answer to the question remains to be elucidated.

The decrease in pH_i in these embryos exposed to DMO is similar to that seen previously with an exposure to 300μM ammonium (see Appendix: Section 10.9). Initially, I had proposed that the detrimental effects of ammonium on the embryo could be replicated by reducing pH_i to the similar level. However, although there were some similarities at a cellular level, as observed by the lack of effect on blastocyst development and also the reduction in cell number, ICM cell numbers and

apoptosis even after 19 hours exposure, there were differences in the effects on embryo viability. Therefore my hypothesis is that ammonium induces other effects on the embryo as well as decreasing pH_i. It may also be that although similar effects are seen in regards to alteration in metabolic parameters, they may be amplified as the strength of the stress increases. This difference in ammonium and DMO exposure on embryo viability still remains to be elucidated.

The significance of the observed effects of DMO on embryo physiology and viability is that it is possible to generate morphologically normal appearing blastocysts that are compromised in their subsequent fetal development at a variety of levels. The data also shows that the environment that the pre-implantation embryo is exposed to may not only affect implantation potential but can also affect fetal growth. Importantly, the data shown also demonstrates that the length of stress exposure can be reduced to the first cleavage division only and that this is enough to induce permanent alterations within the embryo resulting in perturbed fetal development.

These observations have implications not only for animal research but also for the clinical setting where human embryos are cultured for assisted reproductive technology. Embryo culture typically takes place under a controlled gas phase using bicarbonate buffered media, which is very sensitive to subtle alterations in CO_2 levels (Lane *et al.* 2008). Slight deviations such as 1% change in CO_2 levels, away from the required CO_2, can significantly change the pH of the media. This deviation may cause major changes in pregnancy outcome by exposing the embryos to acid or alkaline levels that they do not have the mechanisms to cope with (Lane *et al.* 2008). Since a pre-implantation stress exposure can significantly alter implantation potential and fetal growth, this data highlights the importance of correct media pH as well as gas phase. It also provides a valuable insight into how ammonium exposure may be affecting embryo viability. However, the mechanism as to how these effects during the first 19 hours of development are manifest in the fetus is at this point unknown and will be the focus of the subsequent chapter.

5 The effect of ammonium and DMO exposure, during the first cleavage division, on mitochondrial and metabolic parameters

5.1 Introduction

It is now well accepted that adult health and disease onset can be programmed by fetal adaptations in response to environmental conditions during pregnancy. This phenomenon was first described by David Barker, who termed the 'Barker Hypothesis', which shows that low birth-weight due to maternal under-nutrition during pregnancy is associated with an increase in heart disease, stroke, hypertension and adult-onset diabetes (Barker 1990; Barker 2004; Barker and Fall 1993). However, what is now becoming more evident (as shown in Chapters 3 and 4) is that these effects can be induced even earlier, with the environment to which the *in vitro* pre-implantation embryo is exposed also capable of influencing fetal development. Indeed it has been shown that alteration in many aspects of the culture system such as temperature, gas phase (such as oxygen concentration), pH and toxin build-up can lead to severe perturbations in embryo viability and homeostasis and can induce perturbations downstream by affecting fetal and placental development (Edwards and Hansen 1997; Edwards *et al.* 1998b; Feil *et al.* 2006; Gardner and Lane 1993b; Lane and Gardner 2003; Tseng *et al.* 2006; Zander *et al.* 2006).

Specifically, I have shown that exposure of embryos to an *in vitro* induced stress during the pre-compaction stages significantly altered cellular characteristics of the blastocysts as well as affecting ability to generate a pregnancy and also altering fetal growth trajectory. All of these alterations to physiology and viability of the embryo did not affect the ability of the embryo to develop into a morphologically normal blastocyst. A critical observation of my work is that the effects of the *in vitro* imposed stress continued even after removal of the stress and are manifest downstream in fetal development. What is not known is how these effects are mediated, and if there is a common pathway by which the embryo is programmed for altered fetal outcomes.

It has been shown in other cell types that mitochondria are a target for cell stress and have been linked to programming effects during pregnancy. Therefore mitochondrial homeostasis is essential for maintaining optimal cell health and function during pregnancy (Ogawa *et al.* 2002; Ozawa 1997; Schapira *et al.* 1998; Zhu *et al.* 2006).

Mitochondria are essential for embryo development as they are the major source of ATP production in the early embryo, particularly at the pre-compaction stages (Biggers *et al.* 1967; Gardner *et al.* 2002). During development from the zygote to the blastocyst stage, mitochondria transform from being spherical with minimal cristae and a dense matrix pre-compaction, to being elongated with increased cristae numbers by the post-compaction stages (Hillman and Tasca 1969; Stern *et al.* 1971; Van Blerkom 2004). As well as the differences in mitochondrial structure, the pre- and post-compaction stage embryo also differ in energy substrate utilisation with carboxylic acids pyruvate and lactate being preferred pre-compaction and then glucose post-compaction (Bavister 1995; Gardner and Leese 1988; Hardy *et al.* 1989; Leese *et al.* 1993).

Interestingly, a common observation associated with culture in a range of perturbing conditions is a significant and almost immediate reduction in oxidative metabolism (Gardner and Lane 1993a; Lane and Gardner 2000a; Lane and Gardner 2003; Seshagiri and Bavister 1991). This implies that there is some common perturbations within the mitochondria in response to stress. Therefore the aim of this study was to examine mitochondrial function in two model systems, ammonium (NH_4^+) and a decreased intracellular pH (pH_i), that have demonstrated programming of fetal development following pre-implantation exposure.

5.2 Experimental design

5.2.1 Culture conditions

Zygotes were collected from super-ovulated F1 hybrid female mice after mating with Swiss males at 23 hours post hCG. Denuded zygotes were cultured in groups of 10–12 in 20µl drops of G1.2 medium with or without 300µM ammonium chloride (ammonium/NH_4^+) or 2mM 5,5-dimethyl-2,4-oxazolidinedione (DMO) under 3.5 mL of mineral oil at 37 °C at 6% CO_2, 5% O_2, 89% N_2. Embryos were randomly allocated to each different treatment group. After 19 hours of culture the 2-cell stage embryos were assessed for alterations in mitochondrial homeostasis by assessing mitochondrial distribution, mitochondrial calcium levels, and mitochondrial membrane potential (**Figure 5-1**). All analysis was done using a template where two or three regions within each cell of the embryo were measured: nuclear (around the nucleus), intermediate (in the centre of the cytoplasm) and cortical (around the edge) (Figure 5-2). Metabolic homeostasis was also assessed by determining ATP and ADP levels as well as lactate uptake, malate aspartate shuttle (MAS) activity and reactive oxygen species production (**Figure 5-1**). To assess subsequent cleavage rates to the 4-cell stage, embryos were returned to culture in G1.2 for a further 24 hours at 37 °C at 6% CO_2, 5% O_2, 89% N_2.

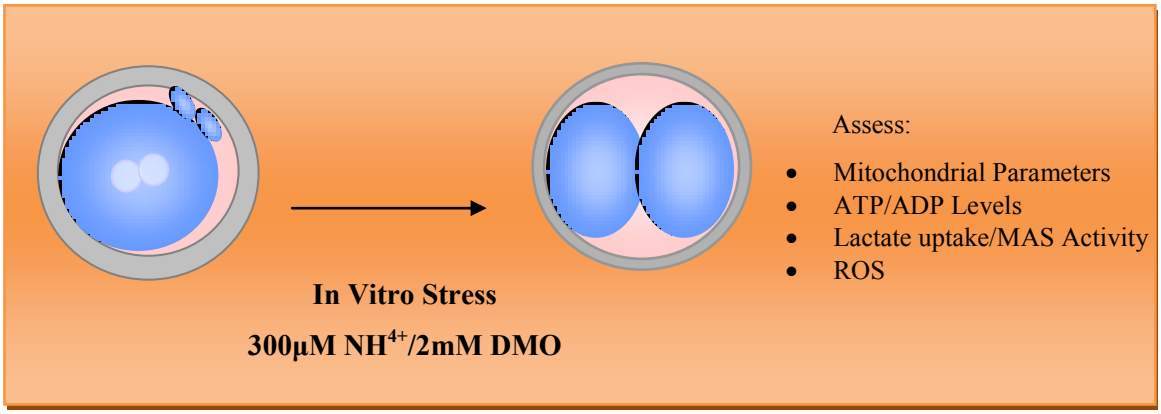


Figure 5-1: Experimental design for 2-cell stress exposure

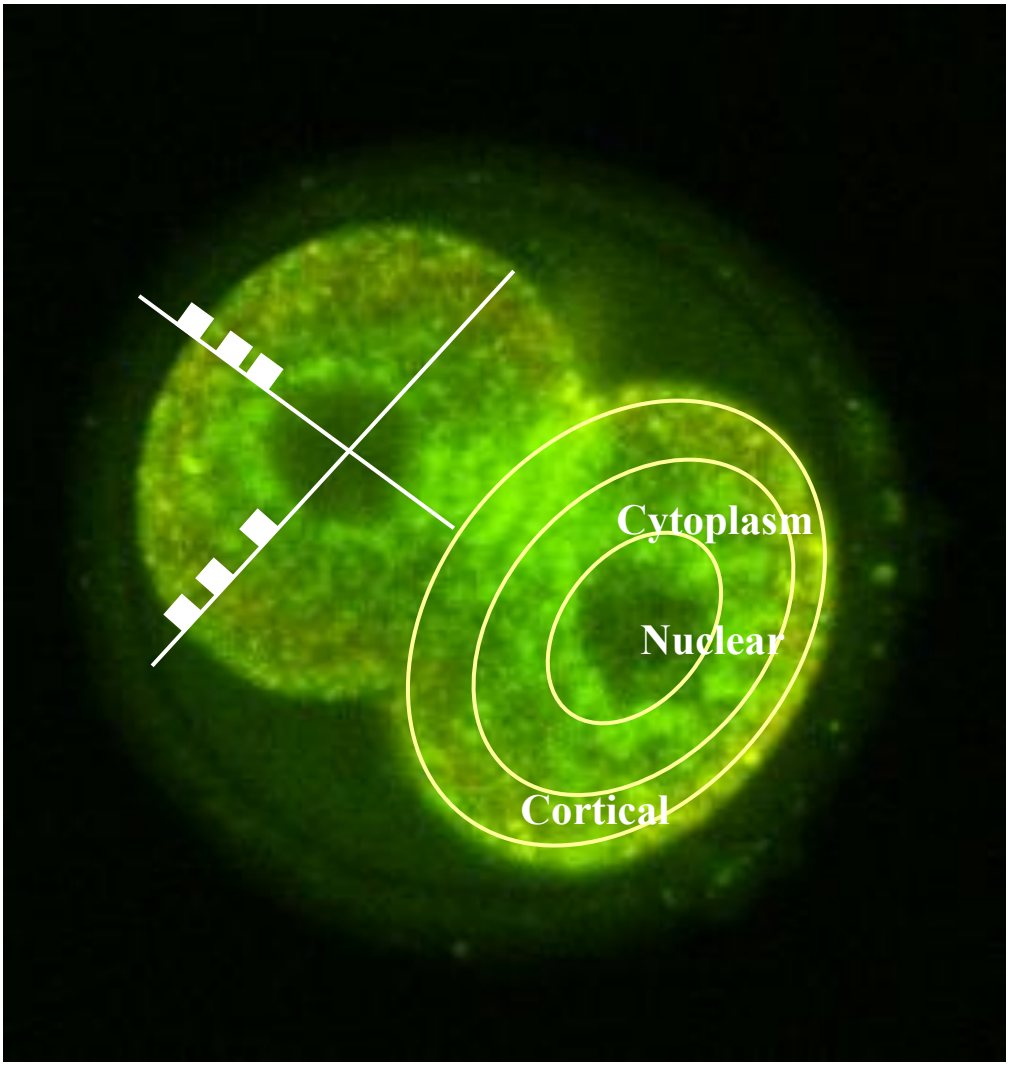


Figure 5-2: Schematic diagram indicating the position and name of the three different regions measured: nuclear, cytoplasmic, cortical, overlayed on an image of a mouse 2-cell embryo stained with JC-1. The reddish staining seen around the cortical region is indicative of higher mitochondrial membrane potential than in intermediate and nuclear region.

5.3 Statistics

Mitochondrial distribution, mitochondrial calcium levels, mitochondrial membrane potential, reactive oxygen species levels, cellular division and ATP and ADP levels and ratio were analysed using a univariate general linear model. Day of replicate was treated as a co-variate.

Differences between treatments were assessed using the Least Significant Difference method (LSD).

5.4 Results

5.4.1 The effect of ammonium and DMO on mitochondrial distribution

To determine the effects of the culture stress, mitochondrial distribution in 2-cell embryos was assessed using Mitotracker Green which stains mitochondria that maintain a membrane potential across the inner mitochondrial membrane. Control 2-cell embryos cultured in G1.2 medium, displayed a clustering of mitochondria around the nucleus (peri-nuclear) as well as having mitochondria scattered throughout the cytoplasm (intermediate region). Exposure to either ammonium or DMO during the first cleavage division from the zygote to the 2-cell stage reduced the nuclear to intermediate ratio of mitochondria when compared to control embryos indicating that the mitochondria are moving away from the nucleus and into the intermediate space ($P<0.01$; **Figure 5-4**)

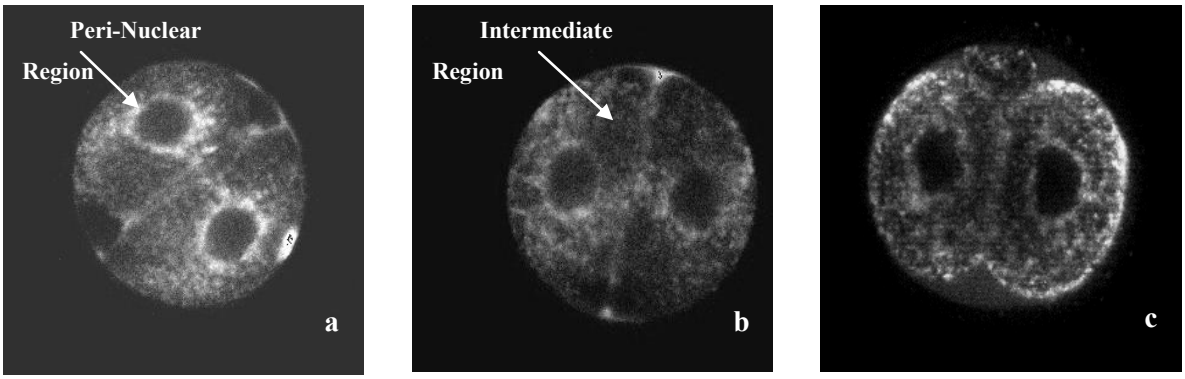


Figure 5-3: Representative images of 2-cell embryos after staining with Mitotracker Green
a) control b) ammonium c) DMO

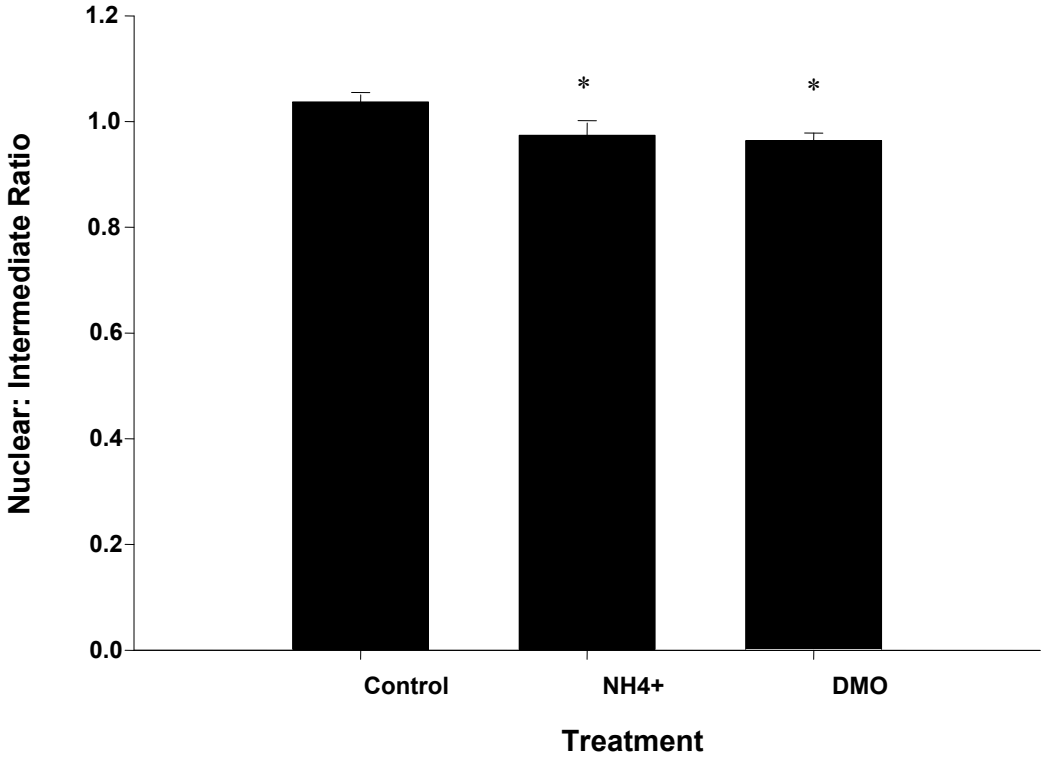


Figure 5-4: The effect of incubation from the zygote to the 2-cell stage, with either ammonium or DMO, on mitochondrial distribution.

Control n=31, NH_4^+ n= 29, DMO n= 34 (3 replicates)

Data expressed as mean ± SEM.

* indicates significant difference from control ($P<0.01$)

5.4.2 The effect of ammonium and DMO on mitochondrial calcium levels

To further determine the effects that culture treatments have on mitochondria, calcium levels were measured in 2-cell embryos using Rhod-2, a mitochondrial calcium specific stain.

All embryos maintained a significant increase in mitochondrial calcium in the cortical region when compared to the intermediate region regardless of treatment (**Figure 5-5**).

Exposure to ammonium during the zygote to 2-cell transition increased mitochondrial calcium levels both in the intermediate and cortical regions by 26% ($P<0.001$) and 20% ($P<0.01$) respectively when compared to control embryos. In contrast, incubation with DMO in 2-cell embryos did not alter mitochondrial calcium levels in either region compared to control embryos (**Figure 5-6**).

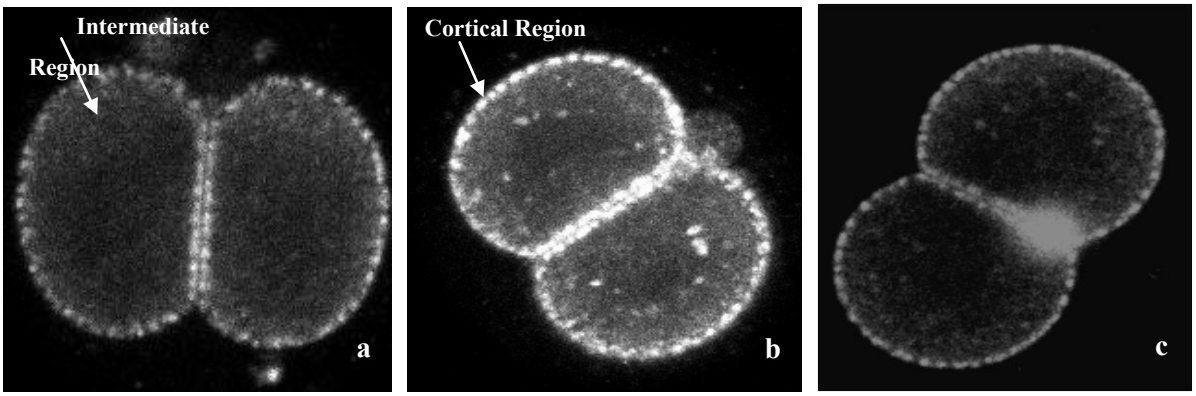


Figure 5-5: Representative images of 2-cell embryos after staining with Rhod-2-AM a) control b) ammonium c) DMO

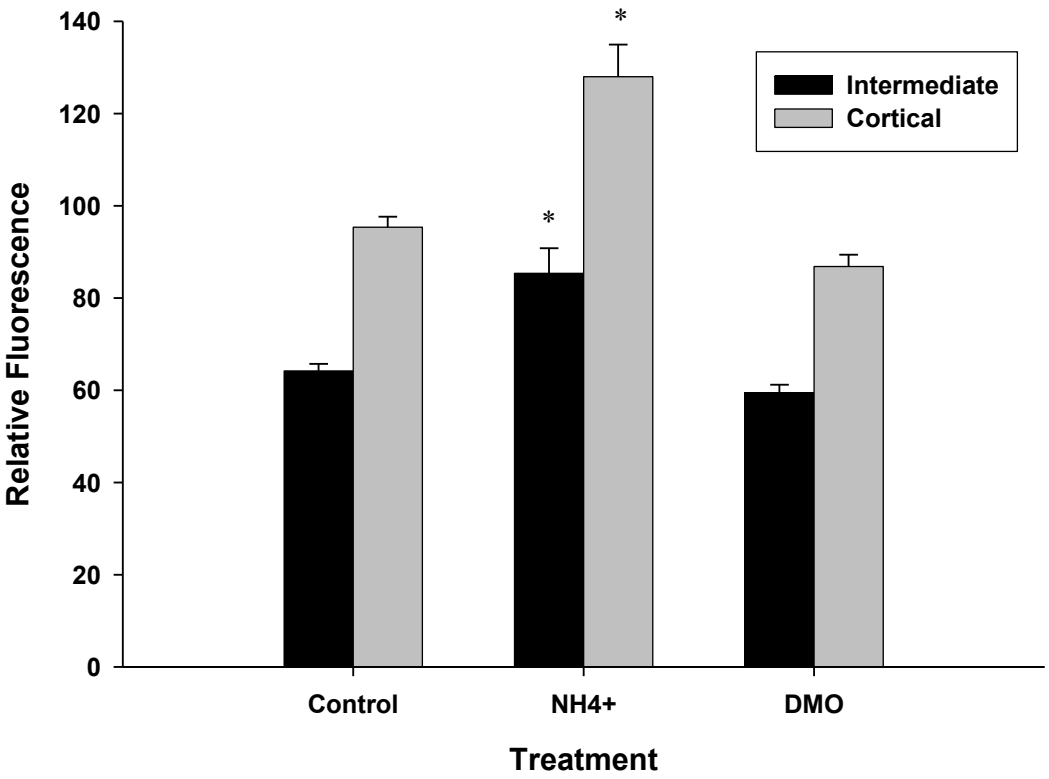


Figure 5-6: The effect of incubation with ammonium or DMO from the zygote to the 2-cell stage on mitochondrial calcium levels

Control n=30, NH_4^+ n= 28, DMO n= 39 (3 replicates)

Data expressed as mean ± SEM.

* indicates significant difference from control ($P<0.01$)

5.4.3 The effect of ammonium and DMO on mitochondrial membrane potential

Mitochondrial membrane potential in embryos was determined using the mitochondrial specific dual emission membrane potential sensitive stain JC-1. All 2-cell embryos maintained a significant gradient of mitochondrial membrane potential throughout each blastomere, with the highest membrane potential being in the cortical region (**Figure 5-2, Figure 5-7**). Incubation with ammonium significantly decreased mitochondrial membrane potential in the peri-nuclear, intermediate and cortical region of the 2-cell embryos. In contrast, incubation with DMO significantly decreased membrane potential in the cortical region only when compared to control embryos ($P<0.01$; **Figure 5-8**).

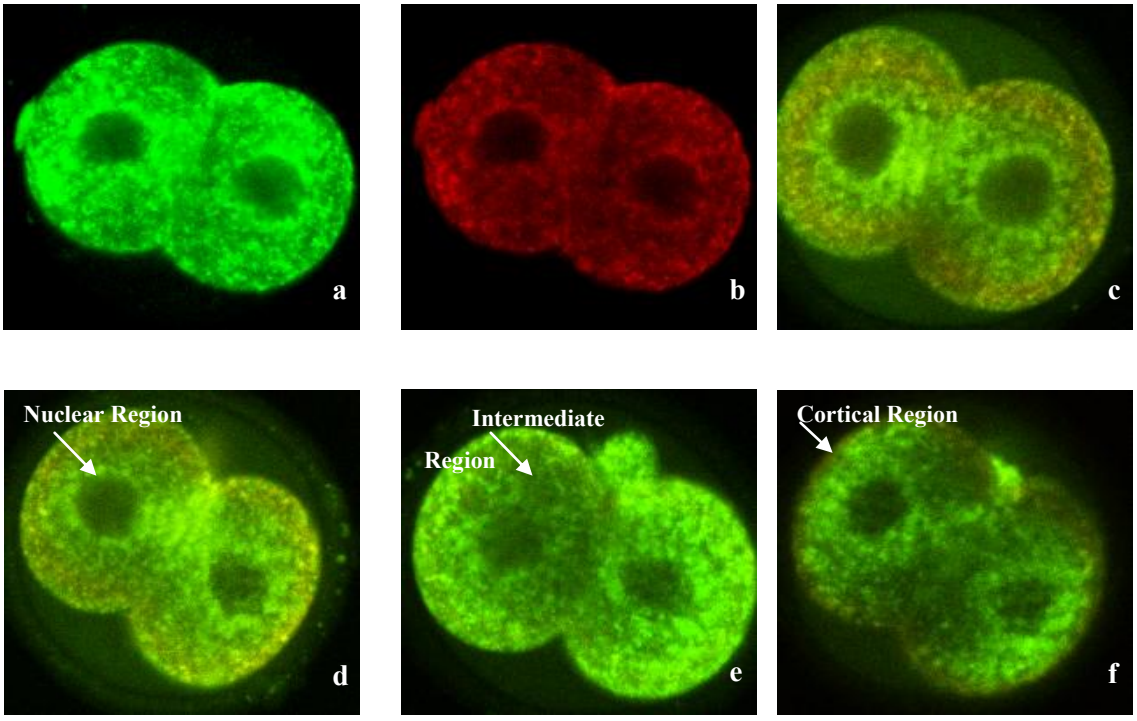


Figure 5-7: Representative images of 2-cell embryos after staining with JC-1 a) green channel b) red channel c) merged image d) control merged image e) ammonium merged image f) DMO merged image

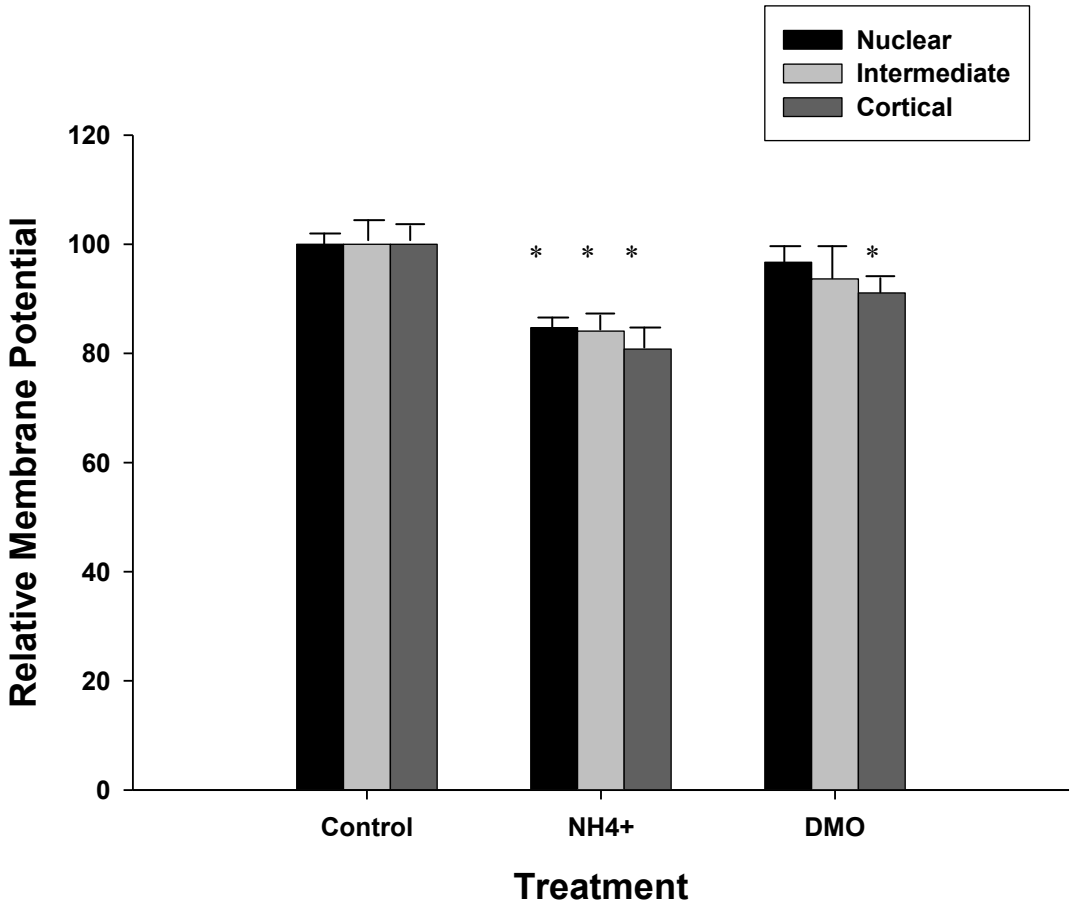


Figure 5-8: The effect of incubation with ammonium or DMO from the zygote- 2-cell on mitochondrial membrane potential.

Control n=27, NH_4^+ n= 30, DMO n= 25 (3 replicates)

Data expressed as mean ± SEM.

* indicates significant difference from control ($P<0.01$)

5.4.4 The effect of ammonium and DMO on reactive oxygen species level

Reactive oxygen species levels were measured using a fluorescent assay where the dye, DCDHF DA, is hydrolysed by esterases present within the cell and is then oxidised by reactive oxygen species to yield the fluorescent dichlorofluorescein. The level of cellular uptake and hydrolysis of the dye by intracellular esterases is controlled for using a separate fluorescent dye (CDCFDA), which directly measures esterase activity, and reactive oxygen species levels were standardised to esterase activity within each treatment.

Exposure to ammonium during the first cleavage division significantly reduced intracellular reactive oxygen species (ROS) levels by 22% when compared to control embryos ($P<0.05$; **Figure 5-9**). Exposure of embryos to DMO during the first cleavage division did not alter intracellular ROS levels when compared to control embryos (**Figure 5-9)**.

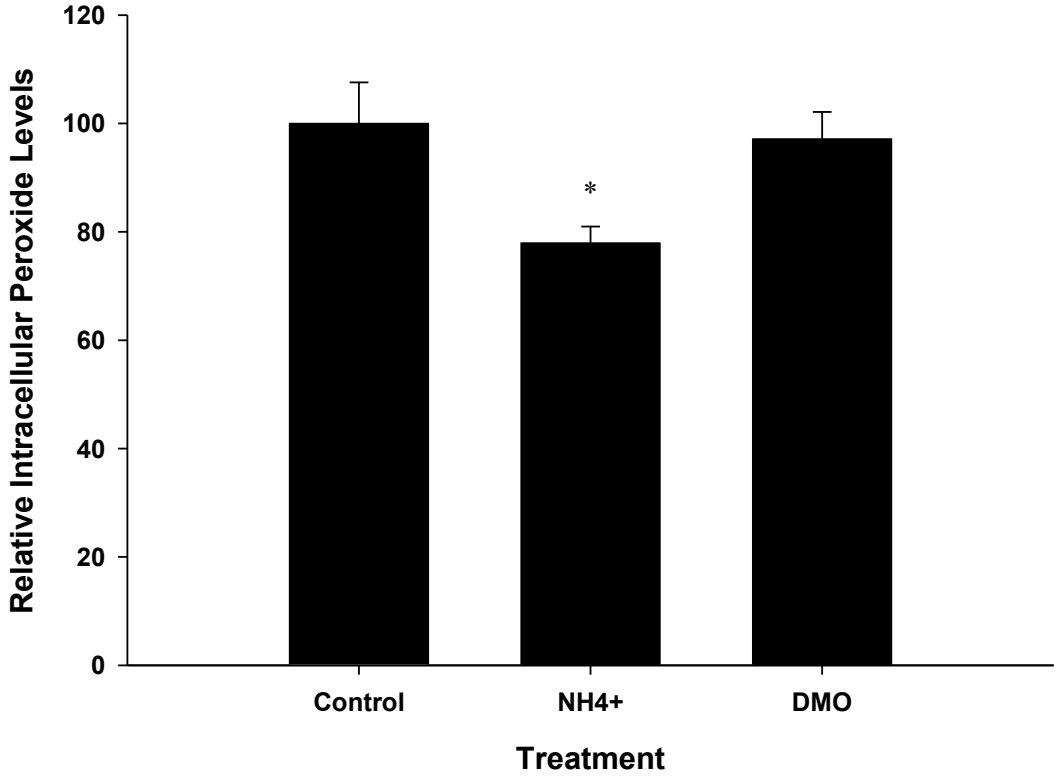


Figure 5-9: The effect of incubation with ammonium or DMO from the zygote- 2-cell on intracellular reactive oxygen species levels.

Control n=24, NH_4^+ n= 32, DMO n= 21 (2 replicates)

Data expressed as mean ± SEM.

* indicates significant difference from control ($P<0.05$)

5.4.5 The effect of ammonium and DMO exposure on early cell division

Cleavage development from the 2-cell to the 4-cell stage was assessed at half hourly intervals. Exposure to 300µM NH_4^+ or 2mM DMO did not affect cleavage stage cell division from the 2-cell to the 4-cell stage (**Figure 5-10**).

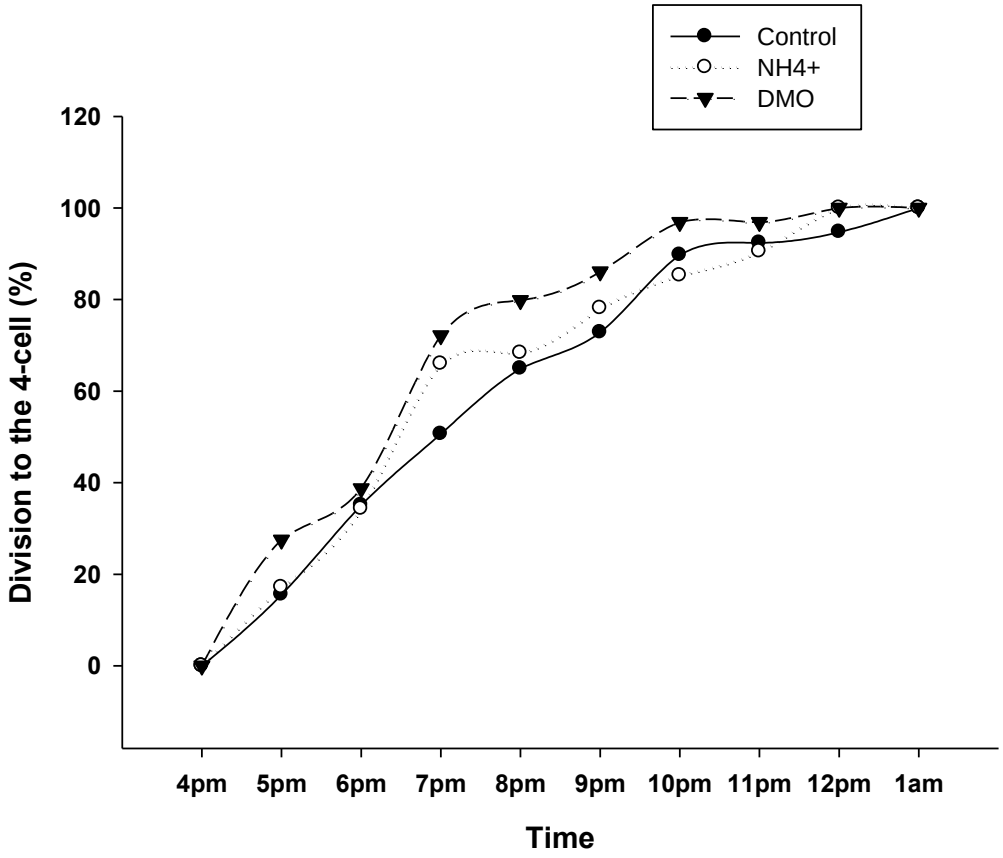


Figure 5-10: The effect of incubation with ammonium or DMO on division of embryos from the 2-cell to the 4-cell over time.

Control n=33, NH_4^+ n= 26, DMO n= 34 (2 replicates)

No significant difference between treatment groups

5.4.6 The effect of ammonium and DMO on lactate uptake and malate-aspartate shuttle activity

Exposure to ammonium during the first cleavage division did not alter lactate uptake by the 2-cell embryos. However, exposure of embryos to DMO did significantly decrease lactate uptake (P≤0.05**; Error! Reference source not found.**). After the addition of AOA (amino-oxyacetate), an inhibitor of the malate aspartate shuttle (Lane and Gardner 2000c; Mitchell *et al.* 2008), control embryos displayed an 18% reduction in lactate uptake compared to control without AOA (P=0.06; **Figure 5-11**). Embryos exposed to ammonium+AOA had a 31% reduction in lactate uptake compared to ammonium without AOA (P<0.001; **Figure 5-11**) and embryos exposed to DMO+AOA had a 46% reduction in lactate uptake compared to DMO without AOA (P<0.05;**Figure 5-11**)

The reduction in lactate uptake after ammonium+AOA exposure was not significantly different to the reduction in uptake observed in control embryos exposed to AOA (**Figure 5-11**). In contrast the reduction in lactate uptake seen in DMO+AOA embryos was significantly greater than the reduction seen in control embryos plus AOA (P<0.05; **Figure 5-11**).

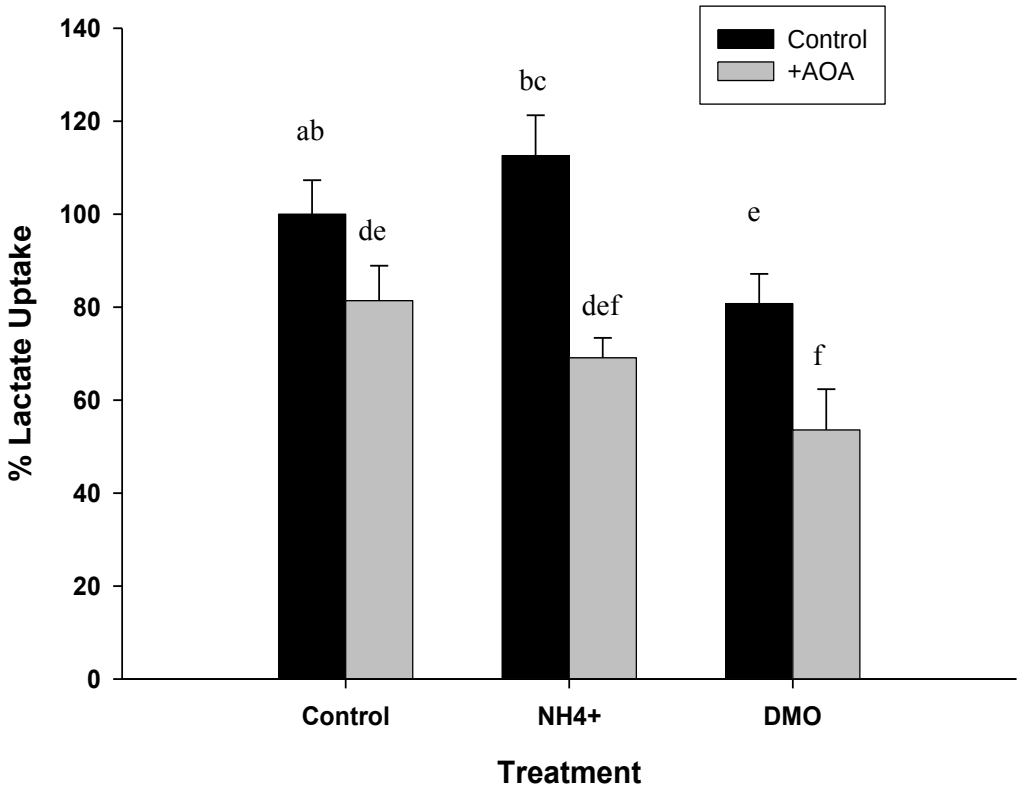


Figure 5-11: The effect of incubation with ammonium or DMO from the zygote- 2-cell on lactate uptake.

Control n=27, NH_4^+ n= 27, DMO n= 20 (2 replicates)

Data expressed as mean ±SEM.

a-f indicates significant difference (P<0.05)

5.4.7 The effect of ammonium and DMO on ADP and ATP levels and ratio

Exposure to ammonium during the first cleavage division significantly decreased ADP levels within embryos when compared to control embryos (P<0.01; **Figure 5-12**). Exposure to DMO during the first cleavage division did not significantly alter ADP levels when compared to control.

There was a decrease in ATP levels in embryos exposed to ammonium during the first cleavage division compared to control embryos however this did not reach significance (P=0.07; **Figure 5-13**). Exposure to DMO significantly reduced ATP levels when compared to control (P<0.01; **Figure 5-13**).

Exposure to ammonium or DMO did not alter the ATP:ADP ratio in 2-cell embryos when compared to control embryos (**Figure 5-14**).

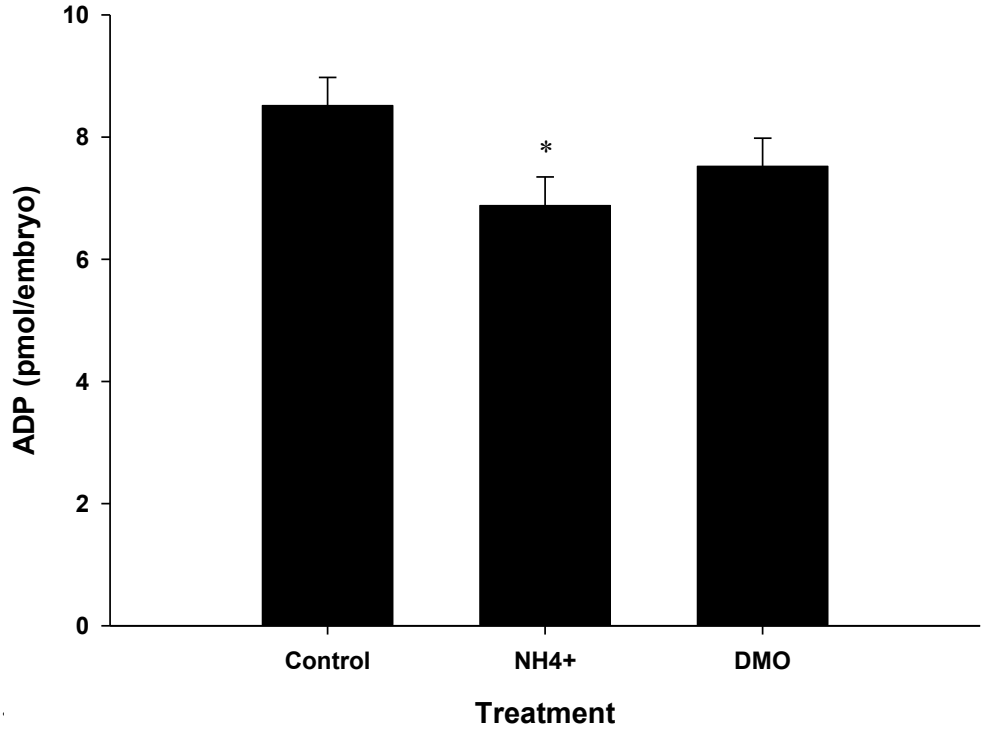


Figure 5-12: The effect of incubation with ammonium or DMO from the zygote- 2-cell on ADP levels

Control n=37, NH_4^+ n= 28, DMO n= 35 (3 replicates)

Data expressed as mean ± SEM.

* indicates significant difference from control (P<0.01)

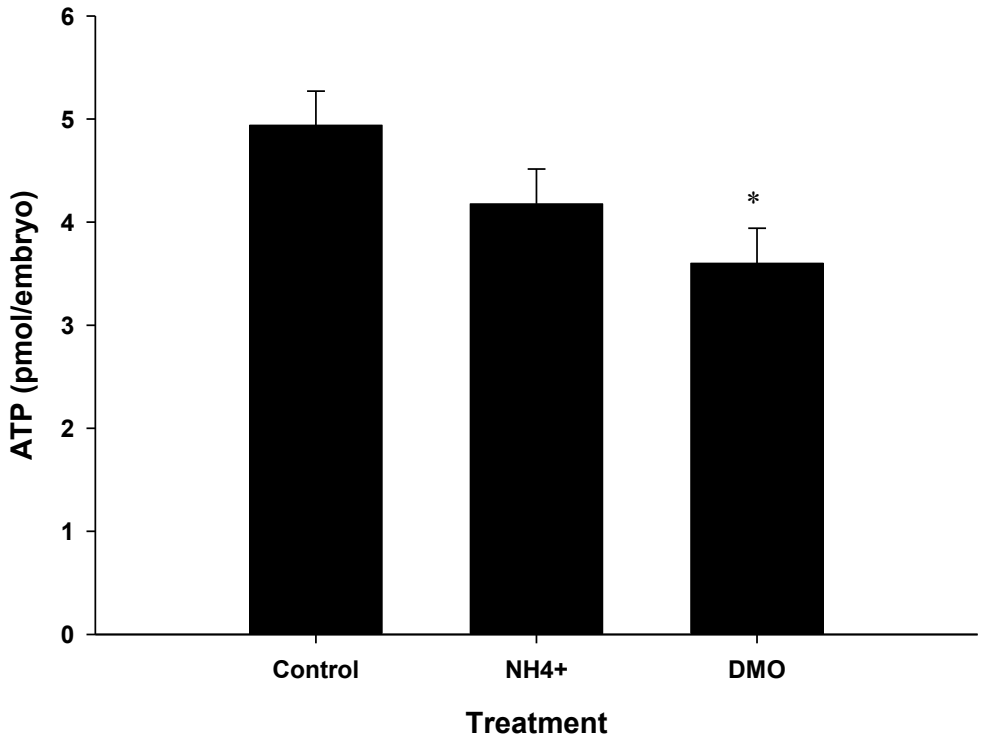


Figure 5-13: The effect of incubation with ammonium or DMO from the zygote- 2-cell on ATP levels

Control n=37, NH_4^+ n= 28, DMO n= 35 (3 replicates)

Data expressed as mean ± SEM.

* indicates significant difference from control ($P<0.01$)

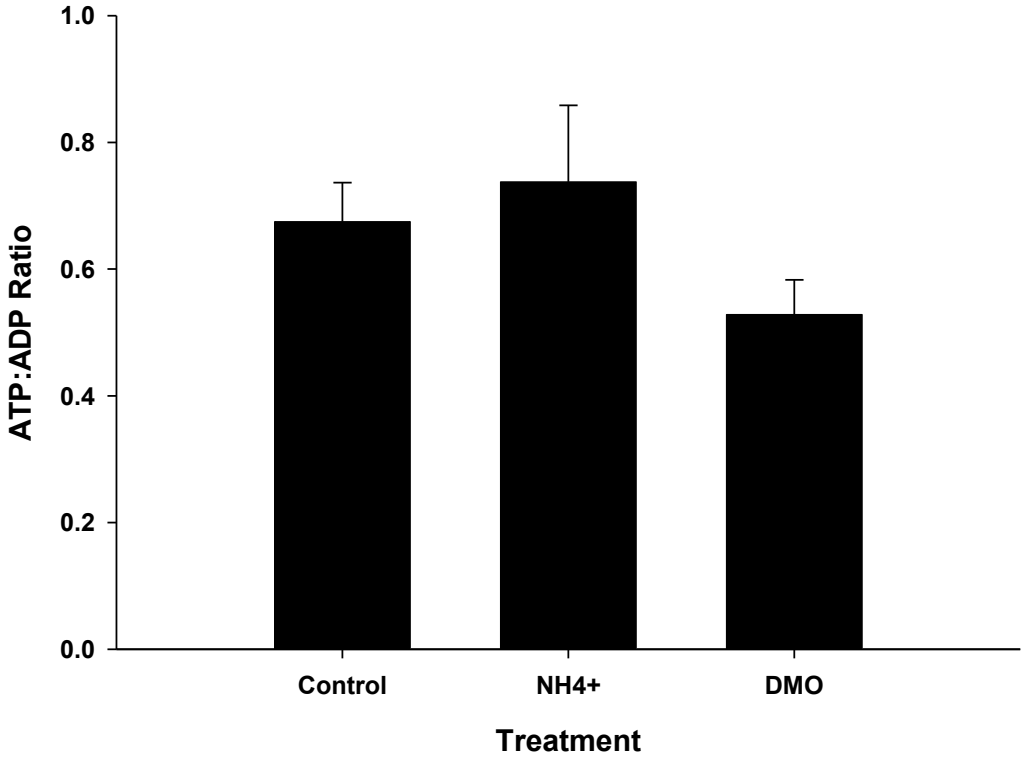


Figure 5-14: The effect of incubation with ammonium or DMO from the zygote- 2-cell on ATP:ADP ratio

Control n=37, NH_4^+ n= 28, DMO n= 35 (3 replicates)

Data expressed as mean ± SEM.

No significant difference between treatment groups

5.5 Discussion

The data presented in previous chapters has shown that a stress applied during stage-specific times during pre-implantation embryo development results in perturbed fetal and placental development; however, the reason for these alterations is currently unknown. The importance of mitochondria during pre-implantation embryo development is well documented, especially during the pre-compaction stage when the embryo relies completely on oxidative phosphorylation for the generation of energy (Biggers *et al.* 1967; Brinster 1965b; Gardner *et al.* 2002; Johnson *et al.* 2003). An adequate supply of energy is vital for the developmental competence of the pre-implantation embryo, as during the five days before implantation many important processes occur such as compaction and blastocoel formation that require large energy generation (Brinster 1974). Because this period is metabolically costly, any alterations in the metabolic profile could result in abnormal embryo development, or in extreme circumstances embryo arrest.

The data presented in this section supports the hypothesis that the mitochondria of the pre-implantation embryo are susceptible to stress and that a 19-hour stress exposure during the first cleavage division results in perturbations to multiple mitochondrial parameters as well as alteration in energy levels. Exposure to both *in vitro* stresses resulted in perturbed mitochondrial distribution. This alteration in organelle distribution has been demonstrated in other species and under other stress conditions. Studies assessing the effects of glucose and phosphate toxicity have shown that exposure of hamster embryos to these substrates, which induce a 2-cell block, results in redistribution of the mitochondria within each blastomere from the peri-nuclear region to the intermediate region (Barnett *et al.* 1997; Ludwig *et al.* 2001). Exposure to these substrates also altered the distribution of the actin microfilaments, suggesting this as a possible mechanism behind this reorganisation (Barnett *et al.* 1997). Studies on alterations in intracellular pH within hamster embryos have also demonstrated redistribution of mitochondria and alteration in cytoskeleton arrangement. Exposure to a weak acid or base resulted in movement of the mitochondria away from the peri-nuclear region and into the cytoplasm after only one hour of the addition of the pH altering compounds to the media (Squirrell *et al.* 2001). The addition of these compounds also significantly altered microfilament organisation in the hamster embryos similar to the previous study with glucose and phosphate. Other stress conditions, such as heat-shock, can also cause movement of mitochondria into the periphery of the blastomere as well as alter microfilament organisation (Rivera *et al.* 2003; Rivera *et al.* 2004). Furthermore, in other cell types stress conditions can cause similar alterations in organelle distribution in cells (Wang *et al.* 1998; Welch and Suhan 1985). The results in this chapter support these previous studies and also show that exposure to either ammonium or DMO result in a similar movement of mitochondria away from the peri-nuclear region and into the cytoplasm. Although the significance of this result is not yet fully understood, the hypothesis of Van Blerkom *et al.* is that pattern of peri-nuclear aggregation of mitochondria and microtubule organisation are related and those alterations in mitochondrial distribution can lead to uneven mitochondrial inheritance when cells divide leading to blastomeres that

contain a smaller mitochondrial population that is inadequate for supporting normal cellular function (Van Blerkom *et al.* 2000). There is also the belief that mitochondria are located in regions where ATP demand is high. If these mitochondria are then shifted to other areas there is a chance that the processes that require a large amount of energy are either suspended or the activity is reduced possibly leading to decreased embryo viability (Barnett and Bavister 1996; Barnett *et al.* 1997).

Along with alterations in mitochondrial distribution, an increase in mitochondrial calcium in both the intermediate and cortical area (ring beneath the plasma membrane) was seen after treatment with ammonium only. Intracellular calcium levels regulate many important functions such as cell division, membrane fusion, exocytosis, cell-cell communication, protein synthesis, gene transcription and metabolism as well as playing an important role in second messenger systems (Campbell 1983; Prostko *et al.* 1993; Srivastava *et al.* 1995). To maintain intracellular calcium at low or constant levels, cells involve many different mechanisms such as membrane channels, transporters and sequestration by organelles; however, during cellular injury it has been shown that there are disruptions in cellular calcium homeostasis in some cases eventually resulting in cell death (Berridge *et al.* 1998; Dormer 1985; Nicotera and Orrenius 1998). Studies have also shown that hamster embryos which develop poorly in culture have elevated intracellular free calcium and that removal of fertilised eggs from the tract before pronuclei formation causes an increase in intracellular free calcium (Lane and Bavister 1998; Lane *et al.* 1998b). These embryos also have compromised development and perturbed mitochondrial distribution as well as altered metabolic activity (Lane and Bavister 1998).

An increase in intracellular calcium often promotes sequestration of this calcium into organelles such as the mitochondria (McCormack and Denton 1980). High levels of calcium influx into the mitochondria in response to increased intracellular calcium can have detrimental effects on mitochondrial activity by causing a reduction in oxidative phosphorylation, as mitochondria calcium levels are believed to be a primary metabolic mediator for ATP production (Gunter *et al.* 2004; McCormack and Denton 1980). Therefore, the increase in mitochondrial calcium after exposure to ammonium may perturb multiple cell signalling pathways and be responsible for the decreased energy production that is observed; however, whether this increase is due to a concurrent increase in intracellular calcium is currently unknown.

Mitochondrial membrane potential can also be linked to perturbed cellular outcomes and calcium homeostasis. Perturbations in mitochondrial membrane potential have been linked to chaotic mosaicism in human embryos and differential metabolism, ATP production and altered intracellular free calcium in the mouse embryo as well as being linked to decreased developmental potential (Acton *et al.* 2004; Van Blerkom *et al.* 2003; Wilding *et al.* 2003). Exposure to either ammonium or DMO decreased mitochondrial membrane potential in the cortical region and exposure to ammonium also decreased membrane potential in the intermediate and peri-nuclear regions. This different region of mitochondrial membrane potential in the embryo has been previously described and it has been proposed that the high-polarised mitochondria in the cortical region, where there is no cell contact,

play an important role in the regulation of early developmental processes by elevating metabolism in this region (Van Blerkom *et al.* 2002). However, this has yet to be proved.

One possible outcome of the reduced membrane potential is a decreased energy production which has been observed by a reduction in ATP after DMO exposure and decreased ADP after ammonium exposure; however, in other cells high metabolic activity and high membrane potential are not synonymous, as cells that lack mitochondrial DNA and are non-functional in respiration still maintain mitochondria with high membrane potential (Diaz *et al.* 1999; Marchetti *et al.* 1996). There is some evidence in the oocyte that when oxidative phosphorylation is uncoupled using oligomycin and net ATP output is decreased, no change is seen in membrane potential; however, intracellular calcium is increased. This infers that membrane potential is not directly linked to metabolism but to ion flux. However, this study was conducted in the oocyte and this may differ in the early embryo. Whether or not this alteration in membrane potential in the 2-cell after a stress exposure is directly correlated to the alterations seen in ATP and ADP remains to be elucidated.

As well as the alterations seen in mitochondrial homeostasis a decrease in reactive oxygen species (ROS) production was also observed after exposure to ammonium. ROS are a natural by-product of cellular respiration and play an important role in many cellular events, in particular hydrogen peroxide has been proposed as a second messenger in various cell stimulation and regulation systems (Laloraya *et al.* 1989; Laloraya *et al.* 1991; Laloraya *et al.* 1988; Skoglund *et al.* 1988). Indeed studies have also shown the importance of ROS in cell proliferation where cell division is preceded by a burst of ROS production (Sundaresan *et al.* 1996). Although vital for many cellular processes, uncontrolled levels of ROS may lead to perturbations in cell cycling or cell damage by peroxidation of lipids and proteins (Nasr-Esfahani *et al.* 1990b). The decrease in ROS that is observed after ammonium exposure is not likely to be linked to the cell cycle, as there was no significant difference in cleavage rates from the 2-cell to the 4-cell stage.

While there appears to be some differences in the mitochondrial homeostatic measures investigated, in the case of both stressors there were decreases in the levels of ATP or ADP in the embryos, indicating a functional difference in the activity of the mitochondria. This would suggest that there is a reduction in mitochondrial activity as a result of the exposure to ammonium and DMO, as the early embryo is reliant on mitochondrial activity for ATP production. This reduction in ATP or ADP levels would suggest that there would be a significant loss in the ability of the embryo to maintain the normal developmental program.

As mentioned previously, metabolism is an indicator of its health and viability and metabolic perturbations are often seen after embryos are exposed to environmental stressors (Lane and Gardner 2005b). It is also clear that metabolic control is essential to maintain maximal implantation potential. It has been previously postulated that the metabolic perturbations seen after stress exposure may be part of an adaptation technique as the embryo tries to adapt and modify its metabolism and subsequent programming based on the environment it is exposed to. This has been demonstrated by exposing *in*

vitro embryos to media lacking in one particular nutrient, such as glucose. After exposure to this altered environment, the embryo alters its metabolism by increasing its uptake of pyruvate (Gardner and Leese 1988). This has also been demonstrated in human embryos (Conaghan *et al.* 1993). Evidence of embryo adaption is also apparent in experiments by Gardner *et al* who exposed mouse embryos to four different media types and then transferred them to pseudopregnant recipients which resulted in similar pregnancy rates (Gardner and Sakkas 1993). Similar results have been shown in the human where embryos are grown in a variety of media types containing different nutrient levels resulting in no difference in pregnancy rates (Monks *et al.* 1993). All of these observations, along with those in this chapter can be rationalised by the fact that the embryo has plasticity and can adapt to some environmental changes (although there appears to be a threshold effect) however the impact of these changes and perturbations on viability, fetal growth and long term health outcomes remains to be fully understood.

In conclusion, culture of embryos for 19 hours in the presence of either ammonium or DMO result in changes to mitochondrial homeostatic measures such as mitochondrial distribution, membrane potential, ROS production and mitochondrial calcium levels. Although there were differences in the effects of the two stressors on individual measures, they both reduced ATP or ADP production indicative of functional change in the mitochondria. The difference in effects of the two stressors on mitochondrial homeostasis also provides evidence that the perturbations (or adaptations?) caused by the presence of ammonium in media is not wholly due to a decrease in intracellular pH as has been previously thought (Lane and Gardner 2003). These data are suggestive that alterations in mitochondrial homeostasis and specifically energy production may be a common outcome seen after *in vitro* stress resulting in reduced viability or altered programming. However, what is unclear is whether this perturbation in mitochondrial homeostasis and reduction in ATP/ADP levels will be maintained after the stress was removed and this will be the focus of the subsequent chapter.

6 The assessment of permanent alterations on mitochondrial homeostasis and energy production after exposure to ammonium or DMO

6.1 Introduction

As described in chapter 5, the pre-implantation embryo is highly sensitive to changes in the vitro environment, with sub-optimal culture media lacking amino acids, high oxygen concentration, sub-optimal energy substrate concentrations, build-up of ammonium or alterations in temperature or intracellular pH all significantly reducing embryo viability (Edwards *et al.* 1998b; Feil *et al.* 2006; Lane and Gardner 1998; Lane and Gardner 2003). One common observation made is that these suboptimal culture conditions induce significant perturbation in embryo metabolism (Lane and Gardner 2003; Seshagiri and Bavister 1991). In particular, there appears to be a reduction in the levels of oxidative metabolism and alterations in ATP and ADP levels (Gardner 1998a; Gardner and Lane 1997; Lane and Gardner 2003). It is well understood that the ability of the embryo to control its metabolism is directly associated with its ability to form a viable pregnancy (Lane and Gardner 1996). Many studies have demonstrated that perturbations to mitochondrial and cytoplasmic metabolism result in developmental delay or cellular arrest (Lane and Gardner 1996; Seshagiri and Bavister 1991). This has led to my hypothesis that the alterations in embryo viability and pregnancy outcomes, as well as a loss in oxidative metabolism, which was observed when embryos were cultured with ammonium or DMO, may occur as a result of changes to the mitochondria.

Using a model *in vitro* culture system that expose embryos to stress during pre-implantation development, I have shown that after only a 19-hour stress exposure, during the first cleavage division, the mitochondrial homeostasis of the 2-cell embryo is significantly altered. I demonstrated in the previous chapter that mitochondrial distribution, calcium levels and membrane potential are perturbed. Reactive oxygen species levels are also changed as well as energy levels reduced. What is currently unknown is whether these changes are permanent after the stress is removed, and does exposure to stress for longer periods of time amplify the mitochondrial perturbations seen.

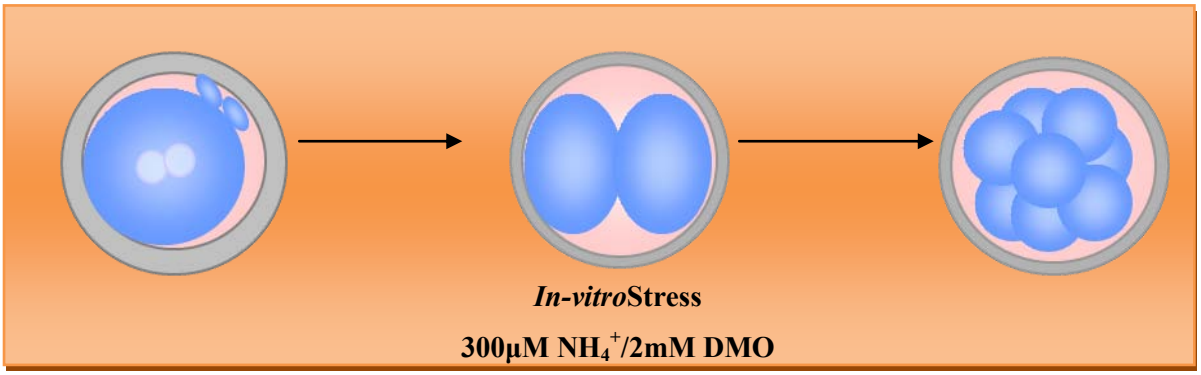
6.2 Experimental design

6.2.1 Culture conditions

Embryos were collected from super-ovulated F1 hybrid female mice after mating with Swiss males at 23 hours post-hCG at the zygote stage. All cleavage stage embryos were cultured in groups of 10–12 in 20µl drops of medium under 3.5 mL of mineral oil at 37 °C at 6% CO_2, 5% O_2, and 89% N_2.

Embryos from 3 to 5 females were pooled and then randomly allocated to either control medium or 300µM ammonium chloride or 2mM DMO (5,5-dimethyl-2,4-oxazolidinedione). After 19 hours of culture, the 2-cell stage embryos were returned to normal culture conditions or kept under stress conditions for a further 24 hours to the 8-cell stage (

Figure 6-1). Mitochondrial homeostasis was assessed in 8-cell embryos by determination of mitochondrial membrane potential, reactive oxygen species and ADP and ATP levels.



	Zygote-2-cell	2-cell – 8-cell
Control	-	-
Stress	√	√
Reversibility	√	-

Figure 6-1: Experimental design for reversibility assessment. Assessments on cellular parameters were conducted at the 8-cell stage after control conditions; exposure to the stress throughout development; or exposure during the first cleavage division followed by control conditions for the next 24 hours to the 8-cell stage.

6.3 Statistics

Mitochondrial membrane potential, reactive oxygen species levels and ATP and ADP levels and ratio were analysed using a univariate general linear model. Day of replicate was treated as a co-variate.

Differences between treatments were assessed using the Least Significant Difference method (LSD).

Levene's Test of Equality of Error Variances was checked and because mitochondrial membrane potential did not have equal variance, a Dunnett's T3 post hoc test was used.

6.4 Results

6.4.1 The effect of ammonium and DMO exposure on mitochondrial membrane potential

Exposure to ammonium during the zygote to 2-cell transition only and then placement into control medium for the remaining 24 hours of culture resulted in a significantly reduced mitochondrial membrane potential in the resultant 8-cell embryo (P<0.01; **Figure 6-2**). Exposure to ammonium continually from the zygote to 8-cell stage did not significantly alter mitochondrial membrane potential at the 8-cell stage compared to control. Exposure to DMO from the zygote to 2-cell and then returning to control medium or continually to the 8-cell stage did not result in altered mitochondrial membrane potential at the 8-cell stage (**Figure 6-2**).

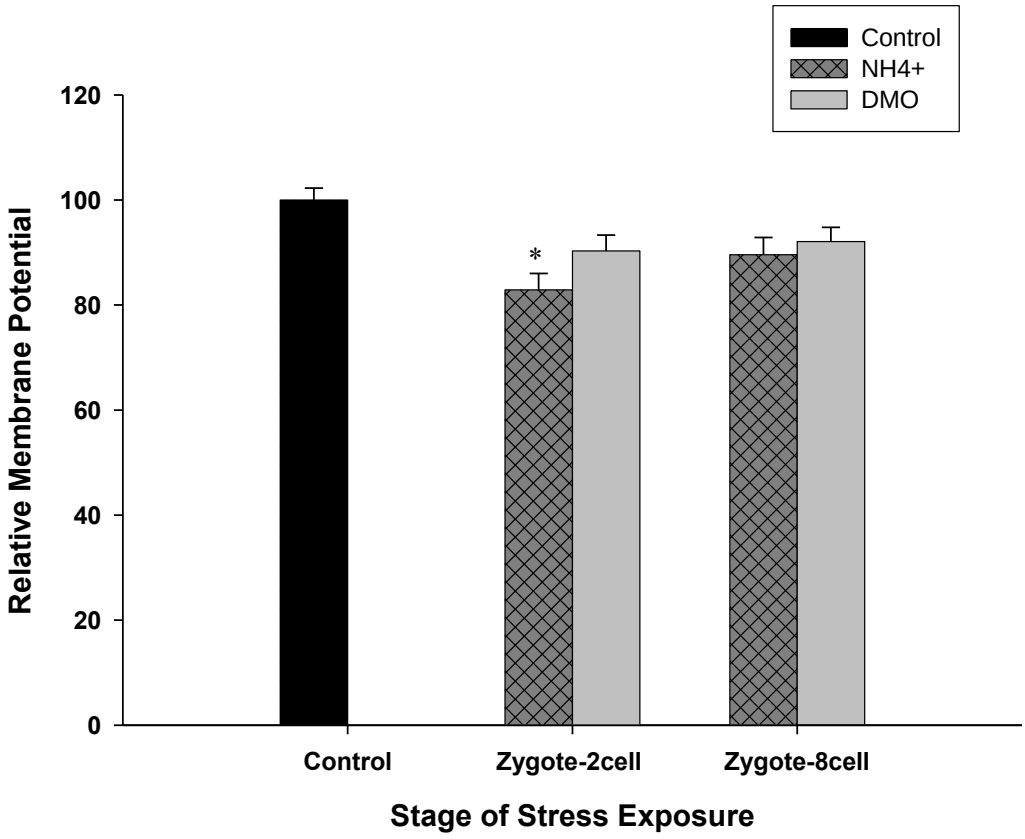


Figure 6-2: Effect of ammonium and DMO exposure for either 19 hours or 43 hours on whole embryo mitochondrial membrane potential at the 8-cell stage.

Control n=9, Zygote-2cell: NH_4^+ n=14 DMO n=12 Zygote-8cell: NH_4^+ n=12 DMO= 11 (2 replicates)

Data expressed as mean ± SEM

* indicates significantly different from control (P<0.01)

6.4.2 The effect of ammonium and DMO exposure on reactive oxygen species production at the 8-cell stage

Exposure to ammonium or DMO for 19 hours during the first cleavage division followed by return to control culture conditions resulted in significantly higher reactive oxygen species production by 117% and 86%, respectively, when compared to control embryos (P<0.001; **Figure 6-3**). Exposure to ammonium or DMO for 43 hours up until the 8-cell stage also resulted in a significantly higher reactive oxygen species production by 55% and 90%, respectively, when compared to control (P<0.001; **Figure 6-3**).

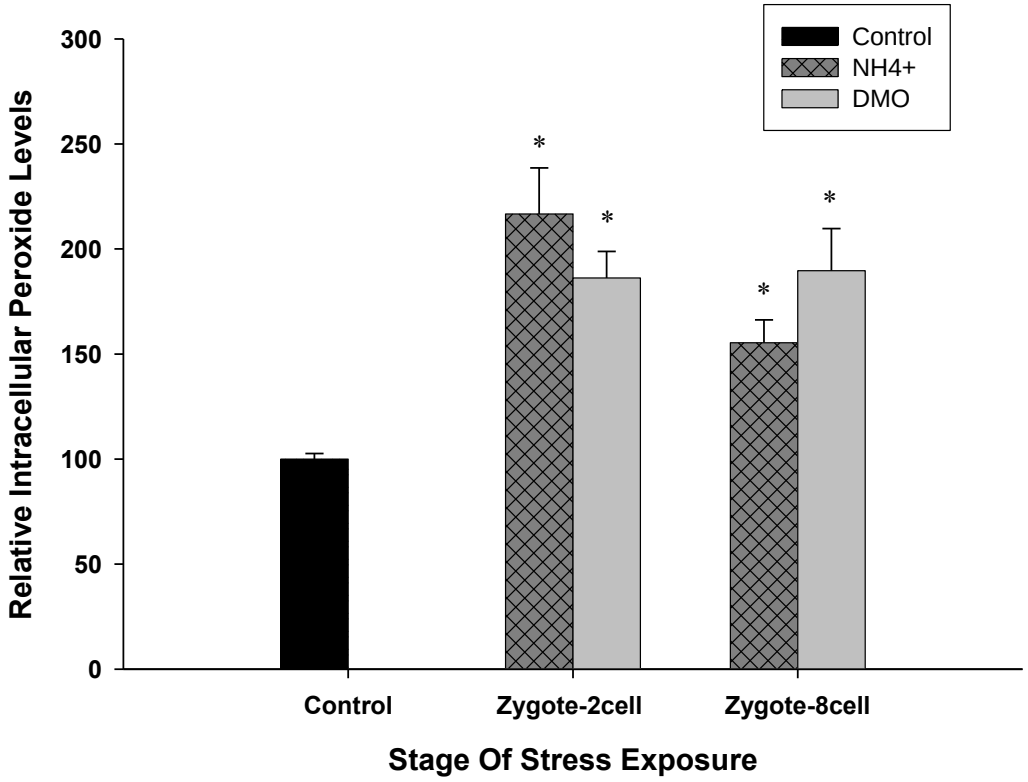


Figure 6-3: Effect of ammonium and DMO exposure for either 19 hours or 43 hours on intracellular reactive oxygen species levels at the 8-cell stage.
Control n=60, Zygote-2cell: NH_4^+ n=60 DMO n=36 Zygote-8cell: NH_4^+ n=61 DMO= 52 (3 replicates)
Data expressed as mean ± SEM
* indicates significantly different from control (P<0.001)

6.4.3 The effect of ammonium and DMO exposure on ADP and ATP levels and ratio at the 8-cell stage

Exposure to ammonium during the first cleavage division before returning to control culture conditions did not alter ADP levels in the resultant 8-cell embryos. In contrast, 8-cell embryos that were cultured in the presence of ammonium for the first 19 hours had significantly reduced ATP levels ($P<0.05$; **Figure 6-4, Figure 6-5**). However, this short-term exposure to ammonium did not alter ADP:ATP ratio (**Figure 6-6**).

Exposure to DMO during the first cleavage division before returning to control culture conditions significantly reduced ADP levels in the resultant 8-cell embryo. Interestingly, however, DMO during this first 19 hours of culture did not alter ATP levels or ADP:ATP ratio ($P<0.001$; **Figure 6-4, Figure 6-5, Figure 6-6**).

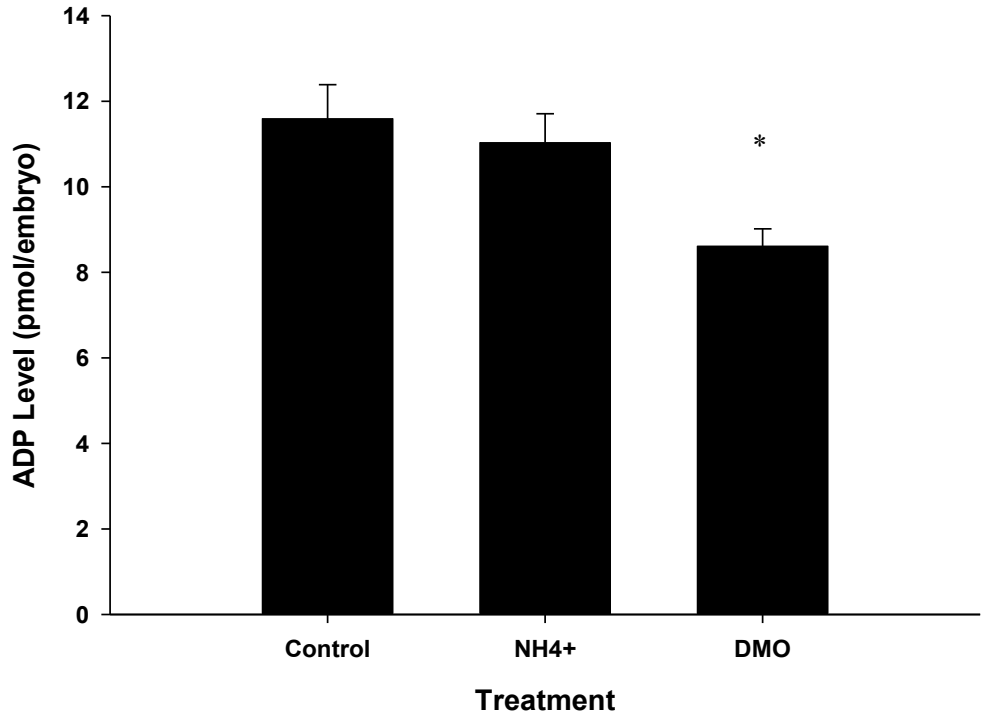


Figure 6-4: Effect of ammonium and DMO exposure for 19 hours on ADP levels at the 8-cell stage.

Control n=28, Zygote-2cell NH_4^+ n=33 Zygote-2cell DMO n=33 (3 replicates)

Data expressed as mean ±SEM

* indicates significantly different from control ($P<0.001$)

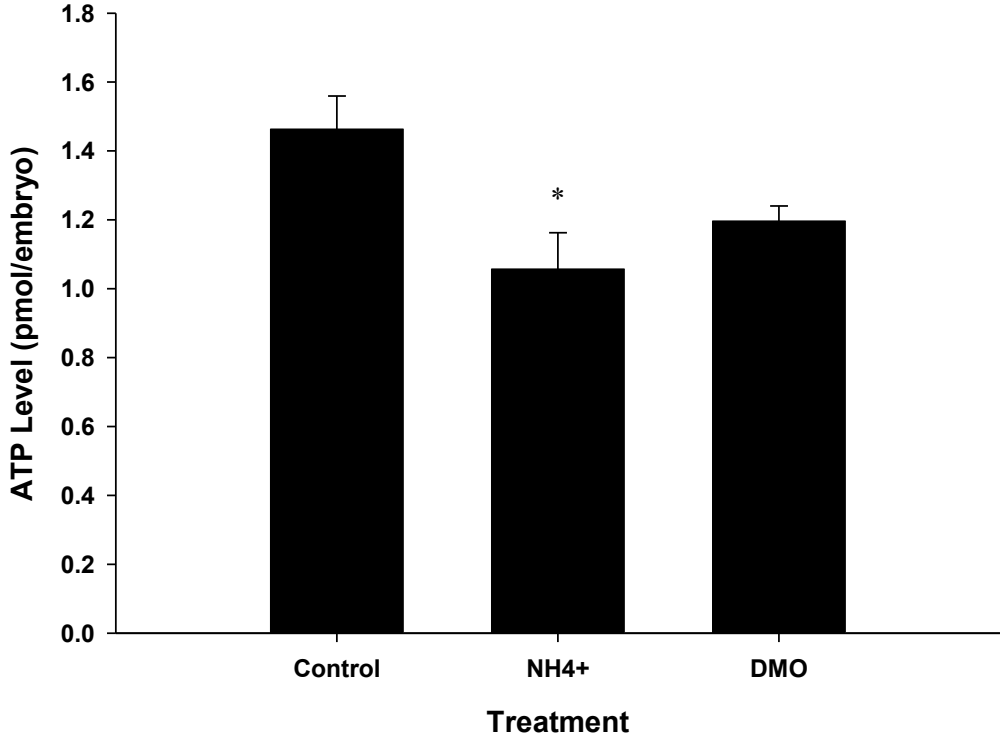


Figure 6-5: Effect of ammonium and DMO exposure for 19 hours on ATP levels at the 8-cell stage.

Control n=28, Zygote-2cell NH_4^+ n=33 Zygote-2cell DMO n=33 (3 replicates)

Data expressed as mean ± SEM

* indicates difference from control ($P<0.05$)

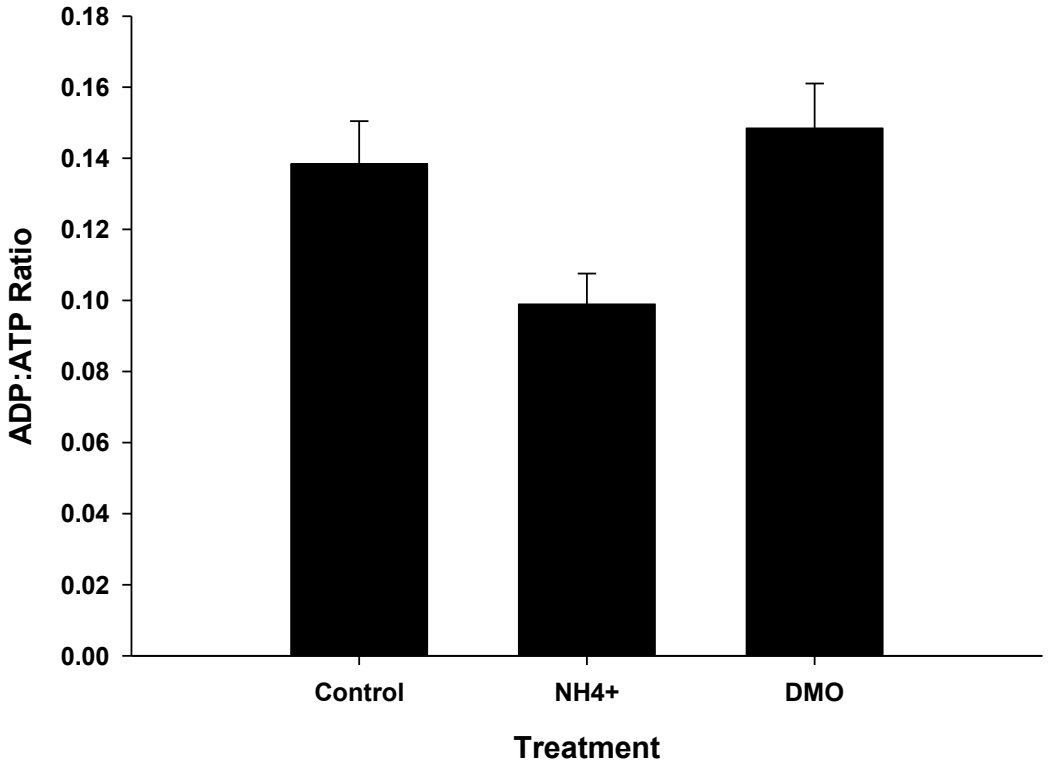


Figure 6-6: Effect of ammonium and DMO exposure for 19 hours on ADP:ATP ratio at the 8-cell stage.

Control n=28, Zygote-2cell NH_4^+ n=33 Zygote-2cell DMO n=33 (3 replicates)

Data expressed as mean ± SEM

No difference between treatment groups

6.5 Discussion

The data presented in previous chapters has shown that a stress applied during pre-implantation embryo development, for as little as 19 hours during the first cleavage division, results in perturbed fetal and placental development. This stress exposure also results in perturbed mitochondrial distribution, calcium levels and membrane potential as well as altered reactive oxygen species production, ATP and ADP levels.

The results in this section show that some of these perturbations continue after the stress is removed and, interestingly, that often prolonged exposure to the stress compared to a short temporal exposure were not significantly different.

As mentioned previously the involvement of mitochondria in cellular homeostasis is well understood and the importance of maintaining and controlling metabolism is vital to cell survival. The ability of the pre-implantation embryo to control its metabolism is directly related to its ability to implant and form a viable fetus, and alterations in the delicate metabolic balance result in compromised embryo development (Lane and Gardner 1996). Previous studies have shown that embryos grown in poor culture conditions can disrupt the balance between cytoplasmic and mitochondrial metabolism as well as altering the balance of metabolic intermediates such as ADP and ATP (Gardner and Lane 1993a; Lane and Gardner 2003; Seshagiri and Bavister 1991). After exposure to ammonium for only 19 hours during the first cleavage division, a decrease in mitochondrial membrane potential is seen (see chapter 5). This alteration in membrane potential is maintained through to the 8-cell stage even after the stress was removed.

Exposure to ammonium during the first cleavage division also perturbed reactive oxygen species levels in the 2-cell stage. However, when these embryos are grown to the 8-cell stage both ammonium and DMO exposure resulted in further large increases in reactive oxygen species levels regardless whether the stress was applied during the first cleavage division only or continually up until the 8-cell stage. This large increase in reactive oxygen species is perhaps indicative of altered mitochondrial function. Indeed, studies in other tissues have shown that exposure to increased levels of ammonium results not only in a large increase in free radical production but also reduced activity of various antioxidant enzymes (Kosenko *et al.* 1997). In tissues, the consequence of this oxidative stress was induction of the mitochondrial permeability transition, a Ca^{2+} -dependent process characterised by the opening of the permeability transition pore. The consequent result is increased mitochondrial permeability, reduction of mitochondrial membrane potential, defective oxidative phosphorylation and decreased ATP synthesis followed by increased reactive oxygen species production (Rama Rao *et al.* 2005). The results of this study in the embryo show similar outcomes, where we see irreversible alterations in membrane potential, reactive oxygen species levels and metabolic intermediates (ATP and ADP levels). Thus a similar mechanism of mitochondrial permeability transition may occur when embryos are exposed to ammonium.

Exposing embryos to DMO during the first cleavage division results in a decrease in intracellular pH similar to that seen after an exposure to ammonium (see Appendix: Section 10.9) and, as mentioned in earlier chapters, decreased intracellular pH can result in perturbed embryo viability. Both ammonium and DMO exposure at the 2-cell stage result in altered mitochondrial distribution as well as alterations in mitochondrial membrane potential, although in this instance exposure to ammonium resulted in more regions of mitochondrial membrane change than DMO exposure (see chapter 5). Ammonium exposure also resulted in increased mitochondrial calcium, which was not seen after DMO exposure. Both ammonium and DMO exposure also display different effects on 2-cell reactive oxygen species levels, lactate uptake and ADP and ATP levels (see chapter 5). These differences are also seen after the stress is removed and the embryos are assessed at the 8-cell stage, with both ammonium and DMO exposure resulting in different alterations in mitochondrial membrane potential and ATP and ADP levels, although the significant increase in reactive oxygen species was seen in both the ammonium and DMO treatments.

All of these results combined suggest that ammonium and DMO exposure may have similar mechanisms of action, in that both result in a decreased intracellular pH of the embryo; however, ammonium exposure may also have another method of action, in that some of the perturbations appear to be more amplified after ammonium exposure.

Regardless of the slight differences seen between ammonium and DMO exposure on mitochondrial homeostasis these results indicate that the repercussions of stress exposure during the first cleavage stage from the zygote to the 2-cell stage continue after the stress is removed, suggesting a possible mechanism behind the perturbed fetal and placental outcomes seen.

In earlier chapters I have shown that the pre-implantation embryo is sensitive to *in vitro* manifested stress – in particular the pre-compaction stage is vulnerable – and that stress exposure at this critical stage of development results in perturbed fetal and placental outcomes indicating that embryo 'programming' is occurring. The results of this section implicate mitochondrial perturbations as a possible mechanism behind the programming effects seen. The actual way in which mitochondria possibly 'program' the embryo is unknown; however, one possibility is via mitochondrial retrograde signalling. Mitochondrial retrograde signalling is a pathway of communication from mitochondria to the nucleus that influences many cellular and organismal activities under both normal and patho-physiological conditions. Many studies in yeast have shown that retrograde signalling is used as a sensor of mitochondrial dysfunction that initiates readjustment of carbohydrate and nitrogen metabolism (Butow and Avadhani 2004; Iurina and Odintsova 2008; Liu and Butow 2006). One possible mechanism in which mitochondria may be involved in embryo programming is by adjusting nuclear gene expression based on the alteration in metabolic intermediates (ADP and ATP). This adjustment may be made by altering DNA modifications such as methylation or histone acetylation, resulting in alterations in gene expression and re-setting the embryo trajectory; however, this remains to be elucidated and will be the focus of the subsequent chapter.

7 The effect of ammonium or DMO exposure on DNA methylation status

7.1 Introduction

DNA methylation is essential for normal mammalian development, as it plays an important role in the regulation of gene expression. After fertilisation occurs, both the paternal and maternal genomes undergo reprogramming to erase gamete epigenetic marks and reset the genome of the zygote for totipotency and then later, the establishment of the embryo's own genetic marks (Hou *et al.* 2008). Initially, the paternal genome is actively demethylated, and then, around the time of the first cleavage division, the embryo undergoes passive DNA demethylation; however, imprints, in certain 'imprinted genes', are maintained (Morgan *et al.* 2005). After cell differentiation at the late morula/early blastocyst stage, de novo methylation begins and new epigenetic marks are set with the inner cell mass (ICM) being hypermethylated compared to the trophectoderm (TE)(Morgan *et al.* 2005).

It has previously been demonstrated that the conditions that the pre-implantation embryo are exposed to can have a significant effect on subsequent development, metabolism, physiology, and viability (Gardner and Lane 1993b; Gardner and Lane 1998; Gardner and Leese 1990). It has also been demonstrated that suboptimal culture conditions can affect gene expression and imprinting of *H19* gene using allele-specific expression studies in *mus castaneus* strain (Doherty *et al.* 2000; Ho *et al.* 1994; Ho *et al.* 1995; Lane and Gardner 2003).

In vitro embryo production and cloning have a low efficiency for producing live offspring coupled with an increase in fetal abnormalities (Young and Fairburn 2000). In sheep and cattle, the perturbed outcomes are possibly related to aberrant phenotypes, resulting in increased birth-weight, altered organ growth, advanced fetal development and placental and skeletal defects, collectively called large offspring syndrome (LOS) (Young and Fairburn 2000; Young *et al.* 1998). Many of these outcomes have been linked to problems with epigenetic alterations, in particular gene imprinting and methylation.

In mice, embryo culture has been linked to alterations in embryo viability, gene expression, altered imprinting of *H19* gene (Doherty *et al.* 2000; Ho *et al.* 1994) and in cloned mice using somatic cell nuclear transfer, abnormal placenta size has been reported (Wakayama and Yanagimachi 1999). Also alterations in methylation patterns at the 2-cell stage have been linked to early developmental failure in mice (Shi and Haaf 2002). These alterations in imprinting, gene expression and methylation may in turn lead to the alterations in embryo viability and altered fetal and placental outcomes seen.

One important factor in the re-establishment of methylation, after cell differentiation, are DNA methyltransferases (*Dnmt* family), in particular *Dnmt3a* and *Dnmt3b.* These proteins are responsible for de novo methylation and are vital for normal fetal development as *Dnmt3a* null mouse fetuses are runted and die at approximately 4 weeks of age and *Dnmt3b* null mice have multiple mutations and none proceeded to birth (Okano *et al.* 1999).

In previous chapters I have demonstrated that exposure to an *in vitro* stress for as little as 19 hours, during the first cleavage division, decreased blastocyst quality, reduced viability and perturbed fetal

growth after transfer. Exposure of these embryos to *in vitro* stress during the zygote to cleavage stage also resulted in irreversible mitochondrial perturbations and altered metabolism and ATP/ADP levels. The link between the perturbations seen and the altered fetal outcomes is currently unknown. Nevertheless, it is possible that altered epigenetic regulation may play a role as the processes of epigenetic regulation such as methylation are energy dependent.

The aim of this study was to assess the effect of a stress exposure during the first cleavage development on the global methylation state at the 2-cell stage, during demethylation, as well as assessing the demethylation status over time. As cell differentiation occurs at the morula/early blastocyst, the global methylation at this point was also assessed as this is the stage when *de novo* DNA methylation begins. Expression of DNA methyltransferases, the proteins responsible for maintenance of methylation and de novo methylation, were also assessed.

7.2 Experimental design

Embryos were collected from super-ovulated F1 hybrid female mice after mating with Swiss males at 23 hours post-hCG at the zygote stage. All cleavage stage embryos were cultured in groups of 10–12 in 20µl drops of medium under 3.5 mL of mineral oil at 37 °C at 6% CO_2, 5% O_2, and 89% N_2.

Embryos from 3 to 5 females were pooled and then randomly allocated to either control medium or 300µM ammonium chloride or 2mM DMO (5,5-dimethyl-2,4-oxazolidinedione). Initially at 21 hours of culture, 2-cell stage embryos were fixed and DNA global methylation was assessed via immunohistochemistry using 5-methylcytosine, an antibody against methylated cytosine residues within DNA (Zaitseva *et al.* 2007) . To assess time point differences in demethylation 2-cell stage embryos were fixed after 16 hours, 19 hours, 22 hours or 25 hours of culture in the presence or absence of 300µM ammonium chloride or 2mM DMO and DNA global methylation was assessed via immunohistochemistry. To assess reinstatement of methylation, embryos were assessed at 67 hours of culture at the morula/early blastocyst stage via immunohistochemistry.

For assessment of blastocyst gene expression, embryos were exposed to either 300µM ammonium chloride or 2mM DMO during the first cleavage division and then returned to control culture conditions until the blastocyst stage. Control embryos were grown in standard sequential media until the blastocyst stage. At the blastocyst stage, embryos were pooled into groups of 30 and frozen in 500µl of Tri Reagent with a minimum of four replicates. cDNA was extracted and gene expression determined.

7.3 Statistics

Methylation data were analysed using a uni-variate general linear model (SPSS 15.0; SPSS Inc, Chicago, IL) where day of replicate was treated as a co-variate.

Differences between treatments were assessed using the Least Significant Difference method (LSD).

Differences in gene expression were assessed on normalised data using a paired student t-test. Levene's Test of Equality of Error Variances was checked, and when data did not have equal variance a Dunnett's T3 post hoc test was used.

7.4 Results

7.4.1 Effect of ammonium or DMO exposure on global DNA methylation at the 2-cell stage, after 21 hours of culture

Global methylation was assessed at the 2-cell stage after 21 hours of culture in either control media or media containing ammonium or DMO, via immunohistochemistry. Exposure to ammonium or DMO for the first 21 hours of culture resulted in a significant reduction in global DNA methylation when compared to control embryos (P<0.001; **Figure 7-1**).

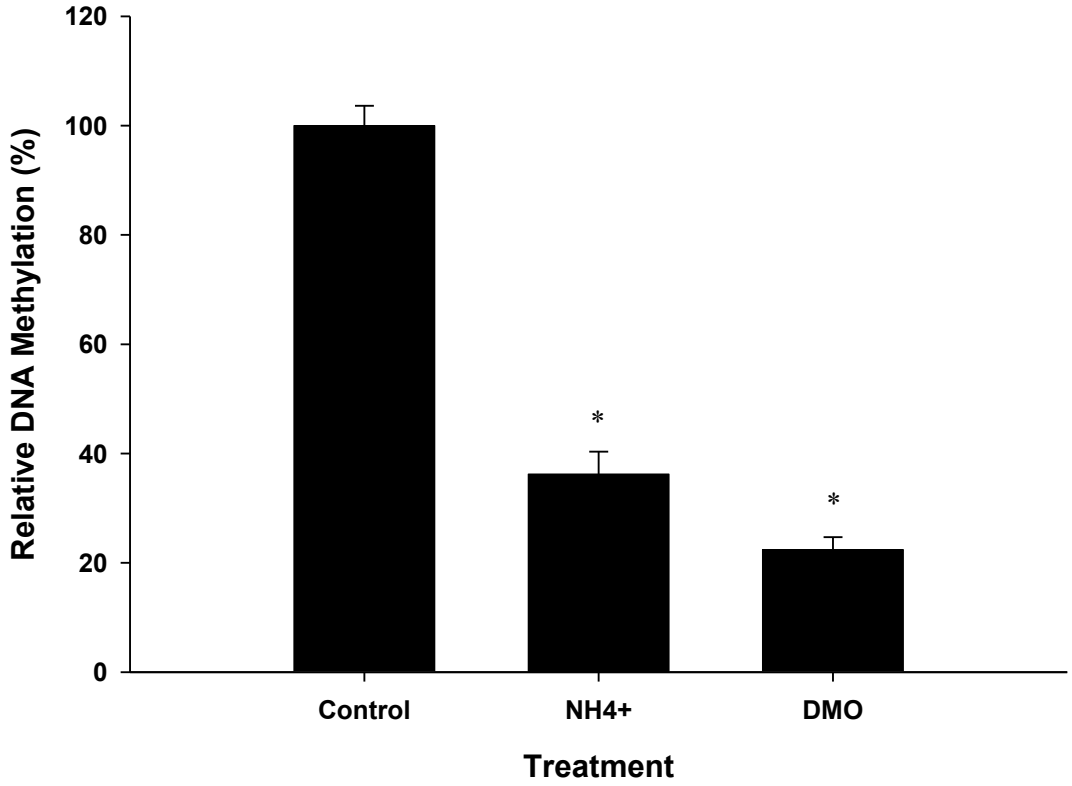


Figure 7-1: The effect of ammonium or DMO exposure after 21 hours culture on relative global DNA methylation

Control n=22, NH_4^+ n= 24, DMO n= 26 (3 replicates)

Data expressed as mean ± SEM

* indicates significant difference from control (P<0.001)

7.4.2 Effect of ammonium or DMO exposure, on global DNA methylation at the 2-cell stage, after 16h, 19h, 22h and 25h of culture

To determine if the alteration in methylation seen after 21 hours of culture was actually due to an alteration in the demethylation rate, embryos were exposed to either ammonium or DMO during the first cleavage division, and their global methylation status was assessed via immunohistochemistry at four sequential time points during the 2-cell phase. Control embryos displayed a decrease in methylation over time, indicative of a de-methylation process occurring. Both ammonium and DMO exposure resulted in an alteration in the levels of methylation at certain time points, indicative of an alteration in the rate of de-methylation.

Exposure to either ammonium or DMO, during the first cleavage division, resulted in embryos with a significant reduction in methylation after 16 hours of culture when compared to control embryos ($P<0.001$; **Figure 7-2**). Exposure to DMO only, during the first cleavage division, resulted in a significant reduction in methylation in embryos after 19 hours of culture when compared to control embryos ($P<0.001$; **Figure 7-2**). Exposure to ammonium did not alter methylation after 19 hours of culture when compared to control embryos. Exposure to both ammonium and DMO during the first cleavage division resulted in a significant reduction in methylation in embryos after 22 hours of culture when compared to control embryos ($P<0.001$; **Figure 7-2**). Exposure to ammonium during the first cleavage division resulted in a significant reduction in methylation levels in embryos after 25 hours of culture when compared to control embryos ($P<0.001$; **Figure 7-2**). Exposure to DMO did not alter methylation in embryos after 25 hours when compared to control embryos (**Figure 7-2**).

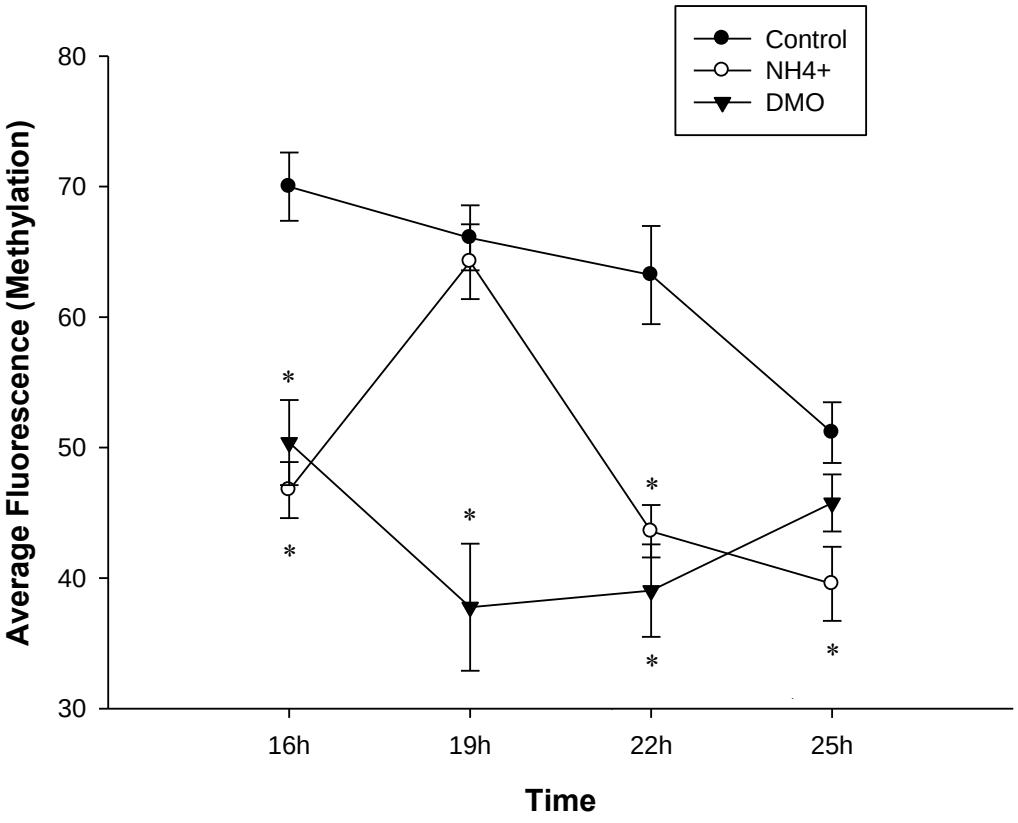


Figure 7-2: The effect of ammonium or DMO exposure on 2-cell global methylation over time. Control: 16h n= 17, 19h n=8, 22h n=12, 25h n=14 NH_4^+: 16h n= 15, 19h n=15, 22h n=15, 25h n=12 DMO: 16h n= 15, 19h n=18, 22h n=12, 25h n=15 (2 replicates)

Data expressed as mean ± SEM

* indicates significant difference from control ($P<0.001$)

7.4.3 Effect of ammonium and DMO exposure, during the first cleavage division, on global DNA methylation after 67h culture.

Global methylation was assessed via immunohistochemistry at the morula/early blastocyst stage after 19 hours of culture in either control media or media containing 300μM ammonium or 2mM DMO, followed by 48 hours culture in control media.

Exposure of embryos to DMO at any stage of pre-implantation development did not alter on-time morphological development (**Table 7-1**)

Exposure to DMO for the first 19 hours of culture significantly increased global DNA methylation, in the morula/early blastocyst, by 12% after 67 hours culture when compared to the control ($P<0.001$; **Figure 7-3**). Exposure to ammonium for the first 19 hours of culture did not alter global methylation levels at the morula/early blastocyst stage when compared to the control. Embryo cell number at this stage was also unaltered (See chapter 3 and chapter 4).

Table 7-1: The effect of culture with ammonium or DMO during the first cleavage division on embryo development after 67 hours of culture

	% ≤8cell		%Compact Morula		% Early Blastocyst	
Stage of exposure to stress	NH_4^+	DMO	NH_4^+	DMO	NH_4^+	DMO
Control	10%	7%	40.0%	53%	50.0%	40%
Zygote to 2-cell	10%	6%	40.0%	55%	50.0%	39%

Control n= 20, NH_4^+ n=22 DMO n=24 (2 replicates)

There was no significant difference for any stage of development between treatments

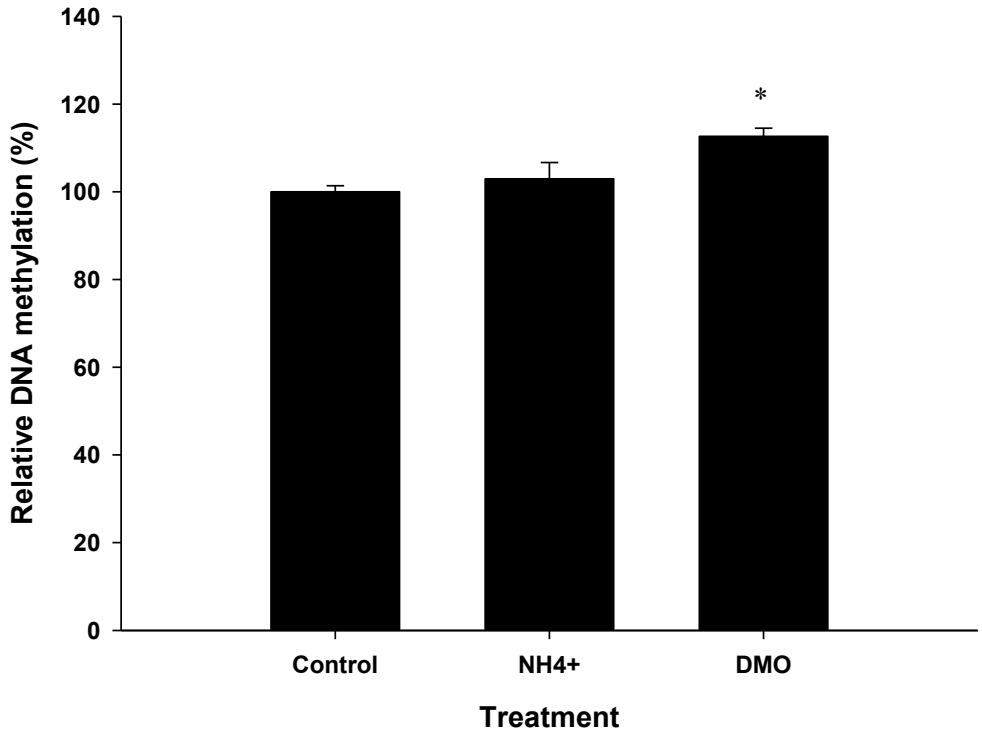


Figure 7-3: The effect of ammonium or DMO exposure, during the first cleavage division, on relative global DNA methylation in morula/early blastocyst stage embryos after 67h culture.

Control n= 18, NH_4^+ n=11 DMO n=11 (2 replicates)

Data expressed as mean ± SEM

* indicates significant difference from control ($P<0.001$)

7.4.4 Effect of ammonium or DMO exposure, during the first cleavage division, on Dnmt Family Gene Expression at the blastocyst stage.

Embryos were either cultured from the zygote to the blastocyst stage (91 hours) under control conditions, or were exposed to either ammonium or DMO for 19 hours during the first cleavage division and then cultured under control conditions for the remaining 72 hours of culture to the blastocyst stage. Blastocyst gene expression was then assessed.

Exposure to ammonium or DMO during the first cleavage division did not alter the expression of *Dnmt1* at the blastocyst stage compared to the control.

Exposure to ammonium or DMO during the first cleavage division significantly reduced gene expression levels of both *Dnmt3a* and *Dnmt3b* at the blastocyst stage when compared to the control ($P<0.05$; **Figure 7-4**).

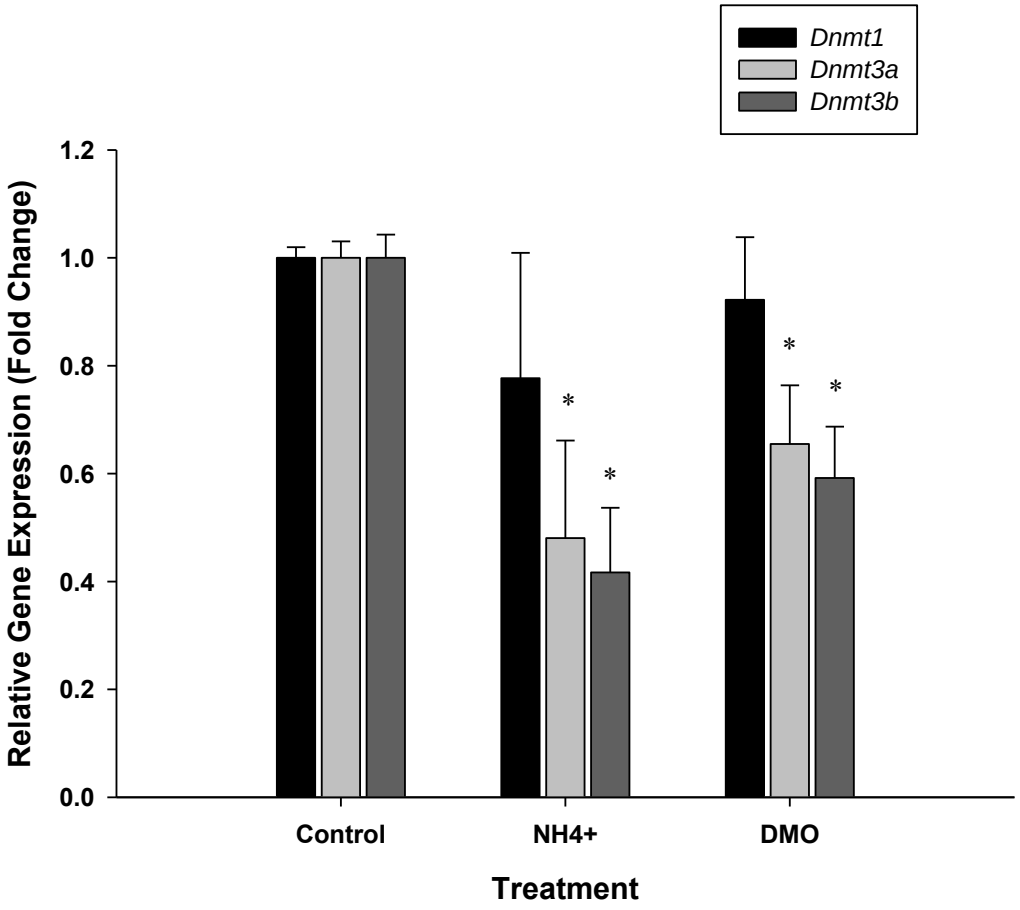


Figure 7-4: The effect of ammonium or DMO exposure, during the first cleavage division, on *Dnmt* family gene expression at the blastocyst stage.

N = 4 (representative of four replicates with 30 pooled blastocysts per replicate)

Data expressed as mean ± SEM

* indicates significant difference from control ($P<0.05$)

7.5 Discussion

Methylation of CpG dinucleotides plays an essential role in the regulation of gene expression and is required for normal mammalian development (Robertson and Wolffe 2000; Suetake *et al.* 2004). It also plays an important role in the sex-specific germline 'marking' of parental alleles that contribute to the correct expression of imprinted genes (Reik and Walter 2001). After fertilisation there is a rapid decline in methylation due to the absence of the maintenance methylase, *Dnmt1* (Carlson *et al.* 1992). During this time there is a rapid demethylation of housekeeping genes and repeat sequences throughout the genome as cell division occurs. Coincidently at the 2-cell stage, the sister chromatids are unequally methylated due to *Dnmt1* being excluded from the nucleus (Morgan *et al.* 2005). Methylation is re-established after cell differentiation at the morula/early blastocyst stage and is completed around the time of implantation and maintained thereafter. The re-establishment of methylation after cell differentiation is due to DNA methyl transferases *Dnmt3a* and *Dnmt3b* (Okano *et al.* 1999; Okano *et al.* 1998). Mice null for *Dnmt3a* or *Dnmt3b* result in embryonic lethality, demonstrating that de novo methylation is essential for mammalian development, and the presence of these proteins after gastrulation demonstrates that de novo methylation is essential, not only during embryo development but during fetal development also (Okano *et al.* 1999).

Previous studies into assisted reproductive technology have established a link between stimulation protocols, *in vitro* embryo culture and altered fetal parameters of the resultant offspring. Studies have demonstrated that children conceived from assisted reproductive technology (ART) have a lower birth weight compared to naturally conceived children (Doyle *et al.* 1992; Schieve *et al.* 2002). In addition, ART pregnancies have also been linked to an increased risk of preterm delivery (Doyle *et al.* 1992). Other studies have implicated *in vitro* culture with the increased occurrence of imprinting disorders in ART-conceived children as well as an increase in birth defects (DeBaun *et al.* 2003; Gicquel *et al.* 2003; Hansen *et al.* 2002; Maher *et al.* 2003). More recently a longer-term follow-up study was performed to assess cardio-metabolism in children conceived through IVF and compare them to spontaneous conceived controls from sub-fertile patients. It was discovered that IVF conceived children have significantly higher systolic and diastolic blood pressure as well as higher fasting glucose levels when compared to spontaneously conceived children (Ceelen *et al.* 2008).

It has been demonstrated in animal models that *in vitro* culture can affect gene expression and imprinting within the resultant embryo and that suboptimal culture conditions can also affect fetal growth after transfer and one possible mechanism behind the altered outcome seen after ART and suboptimal culture conditions is alterations in epigenetic modifications such as methylation (Doherty *et al.* 2000; Khosla *et al.* 2001a; Lane and Gardner 2003).

In previous chapters I have demonstrated that exposure of the pre-implantation embryo to *in vitro* stress results in perturbed embryo homeostasis and viability. After transfer there is a significant reduction in fetal weight, on Day 18, after exposure of pre-implantation embryos to 2mM DMO (which decreases pH_i) either during the first cleavage division or continually throughout development.

Exposure to 300μM ammonium during the first cleavage division also results in a decrease in fetal weight on Day 15 after transfer (Zander *et al.* 2006) Although this difference is not evident on Day 18 of development, crown rump length and the fetal:placental weight ratio are still reduced, indicating impaired growth rates. The mechanism behind these alterations is unknown; but it is possible that changes in methylation status during pre-implantation embryo development may be responsible for the altered outcomes seen.

Culture with either 300μM ammonium or 2mM DMO, during the first cleavage division, resulted in a significant reduction in global methylation after 21 hours of culture. DNA methylation at this stage of development is declining; while the DNA is replicating the new sister chromatid will remain unmethylated and therefore reduce the amount of global methylation by approximately half. This trend will continue during cleavage development with each cell cycle resulting in the halving of global methylation. The alteration in global methylation over time was assessed at the 2-cell stage at four time points. After 16 hours of culture, both ammonium and DMO exposure resulted in a significantly reduced global methylation status compared to control embryos. After 19 hours of culture, embryos exposed to DMO still maintained a reduction in global methylation compared to control embryos. After 22 hours of culture both ammonium and DMO has significantly reduced global methylation levels when compared to control embryos, and by 25 hours ammonium still maintained lower global methylation levels than control embryos; however, DMO embryos showed no difference. Because demethylation occurs as DNA replication is occurring, the results suggest that perhaps DNA replication is happening at a different rate. However, in chapter 5, I have shown that cell division from the 2-cell to the 3-cell and 4-cell stage is not different between the treatment groups, which indicates that DNA replication should also not be different. The reason for this alteration in the demethylation rate is therefore unknown, although the localisation of *Dnmt1* may play a role and this outcome has yet to be determined.

On Day 4 of pre-implantation embryo development the cells within the embryo undergo differentiation into two distinct lineages: inner cell mass and trophectoderm. At this stage de novo methylation occurs and the DNA of the 2-cell lineages are re-methylated (Morgan *et al.* 2005). In this study the global methylation of morula/early blastocysts was assessed after 67 hours of culture. At that point there was no significant difference in on-time morphological development; however, embryos which were exposed to DMO during the first cleavage division had a significantly higher global methylation level than control embryos and embryos exposed to ammonium. On-time cleavage development has also been assessed in these embryos. No difference was found in cell number on Day 4, indicating that this is not due to the cell cycle (see chapter 3 and chapter 4). Thus re-methylation in these embryos may begin earlier than in the control.

At the blastocyst stage, after 91 hours of culture, *Dnmt* family gene expression was assessed. Embryos exposed to both ammonium and DMO, during the zygote to 2-cell transition, had significantly reduced gene expression of *Dnmt3a* and *Dnmt3b* when compared to control blastocysts;

whereas gene expression of *Dnmt1* remained unchanged. As mentioned previously, *Dnmt3a* and *Dmnt3b* are responsible for de novo methylation after cell differentiation; the decrease in the levels of gene expression may lead to decreased protein expression and subsequently lead to alterations in de novo methylation. Interestingly, as mentioned previously, exposure to DMO during the first cleavage division resulted in a significant 12% increase in global methylation at the morula/early blastocyst stage, which indicates that *Dnmt3a* and *Dnmt3b* levels at this stage may be higher, or that gene expression is switched on earlier in these embryos; however then, 24 hours later, gene expression is lower in these blastocysts. What mechanism initiates de novo methylation is currently unknown; however, these alterations in methylation levels and methyltransferase gene expression may result in altered de novo methylation and ultimately lead to alteration in gene expression.

It is noteworthy that levels of *Dnmt1* remained unchanged at the blastocyst stage. As already mentioned, *Dnmt1* is responsible for the maintenance of methylation and is therefore also responsible for the maintenance of imprints (Hirasawa *et al.* 2008). The fact that these levels are unchanged at the blastocyst stage is indicative that both maintenance of methylation should remain unchanged and imprinting should also remain undisturbed, although this remains to be shown.

In conclusion, the data in this chapter have demonstrated that exposure to either ammonium or DMO during the first cleavage division results in altered global methylation levels as well as altered demethylation patterns at the 2-cell stage. Furthermore, exposure to DMO during the first cleavage division results in increased global methylation at the initiation of de novo methylation after cell differentiation at the morula/early blastocyst stage. Exposure to both these *in vitro* stresses during the first cleavage division also significantly reduced the gene expression of DNA methyltransferases *Dnmt3a* and *Dnmt3b* at the blastocyst stage, possibly leading to alteration in de novo methylation.

These alterations in demethylation and de novo methylation may, in turn, lead to altered epigenetic programming resulting in alterations in gene expression leading to the perturbed fetal growth seen. This study demonstrates, for the first time, a possible mechanism behind the alterations in fetal and placental parameters seen after culturing embryos in suboptimal culture conditions.

8 The effect of ammonium exposure, during embryo culture, on placental gene expression and function

8.1 Introduction

Survival and growth of the fetus is highly dependent on placental development. The placenta forms the interface between the maternal and fetal circulation and facilitates nutrient and gas exchange as well as being responsible for waste disposal (Watson and Cross 2005). Fetal development in utero and size at birth is critical in determining health in later life as well as overall life expectancy. Several epidemiological studies have shown that mothers who were undernourished during pregnancy gave birth to low birth-weight babies who had an increased prevalence of coronary heart disease (Barker 2003). Other studies have also shown the link between low birth weight, due to poor maternal nutrition, and the increased prevalence of high blood pressure, hypertension and diabetes (Barker 2005; Hales *et al.* 1991; Law and Shiell 1996). This phenomenon is known as the developmental origin of health and adult disease (DOHAD) and is indicative of 'fetal programming' in that the environment, to which the fetus is exposed to in utero, can significantly alter the health outcomes of the child in later life. Although the influence of environmental factors on fetal development has been well described, the mechanism behind the alterations seen is currently unknown.

Placental supply of nutrients to the fetus, which occurs mainly by diffusion or facilitated transport, is the major determinant of intrauterine growth (Sibley *et al.* 1997). In turn, the ability of the placenta to maintain efficient and adequate nutrient supply depends on placental size, morphology, and blood supply as well as transporter abundance.

Recently there have been a number of studies that have focused on the possibility that the 'window of sensitivity' to environmental stress can be shifted earlier, to the first five days of pre-implantation embryo growth, and that environmental perturbations applied during this stage could also alter 'fetal programming' and influence fetal outcomes. Many studies have shown that babies born as a result of assisted reproductive technology have an increased chance of prematurity, low birth weight for gestational age and an increased risk of perinatal mortality, although much of this is due to the increased rate of multiple births after ART due to transfer of multiple embryos (Doyle *et al.* 1992; Tan *et al.* 1992). Despite this it has been proposed that one mechanism by which these decreases in singleton fetal weights might occur is by alteration in epigenetics leading to alterations in placental growth.

Studies using gene knockout have shown that placental size is influenced by imprinted gene expression such as *Igf2*, with under-expression leading to significantly reduced fetal size and over expression leading to overgrowth of the fetus (Brown *et al.* 1996; DeChiara *et al.* 1990). Although some theorise that the alterations in fetal weight are caused by alterations in imprinting leading to altered gene expression resulting from ovulation induction protocols (Fortier *et al.* 2008), it has also been postulated that the five days of pre-implantation embryo development may also contribute to perturbations seen in fetal outcomes by altering gene expression and imprinting (Mann *et al.* 2004; Rivera *et al.* 2008).

Previously I have demonstrated that mitochondria are a target of cellular stress after the pre-implantation embryo is exposed to sub-optimal culture conditions. Exposure to ammonium or DMO, during the first 19 hours of embryo culture, results in perturbations to mitochondrial homeostasis, which cannot be reversed by removal of the stress. Mitochondria play an essential role in embryo growth, as they are the primary source of energy production, and I have demonstrated also that exposure to both ammonium and DMO also results in altered energy production in the embryo. After these embryos are transferred to pseudopregnant recipient, I have shown alterations in fetal and placental parameters such as weight, crown rump length and fetal to placental weight ratio. It is possible that the mitochondrial perturbations seen in the embryo may continue throughout gestation and result in continuing disturbances in energy production. It is also possible that epigenetic alterations, such as perturbed methylation status which was seen after exposure to ammonium and DMO, may lead to alterations in expression of genes involved in placental growth and nutrient transport.

The aim of this study was to assess the effect that exposure to an *in vitro* stress during pre-implantation embryo has on gene expression and placental function after embryo transfer. The focus, in particular, was on genes involved in placental establishment and nutrient transport as well as assessing mitochondrial genes involved in energy production.

8.2 Experimental design

Embryos were collected from super-ovulated F1 hybrid female mice after mating with Swiss males, 23 hours post-hCG at the zygote stage. All cleavage stage embryos were cultured in groups of 10–12 in 20µl drops of medium under 3.5 mL of mineral oil at 37 °C at 6% CO_2, 5% O_2, and 89% N_2. Embryos were randomly allocated to each different treatment group and exposed to 300µM ammonium during a certain stage of development. Blastocysts were then transferred to pseudopregnant recipients, and on Day 18 placental functions were assessed in treatment groups 1, 2 and 5. cDNA was extracted from Day 15 placentas, obtained in previous experiments, and genes expression of *VEGF*, *Igf2*, *Igf2R*, *Scl2a1*, *Scl2a3*, *H19*, *Scl38a2*, *Slc38a4*, *Tfam*, *Terf*, *Nrf1*, and *Nrf2* was assessed (Zander *et al.* 2006).

The treatment groups used for this temporal assessment follow:

- Treatment 1 (control group) was cultured in sequential culture media with no ammonium exposure (total of 91 hours of culture).
- Treatment 2 was exposed to 300µM ammonium from the zygote to the 2-cell stage (from 0 hours to 19 hours of culture).
- Treatment 3 was exposed from the 2-cell to the 8-cell stage (19 hours to 43 hours of culture).
- Treatment 4 was exposed to ammonium from the 8-cell to blastocyst stage (43 hours to 91 hours of culture).
- Treatment 5 was exposed to 300µM ammonium for the entire culture period (total of 91 hours of culture).

8.3 Statistics

Placental transport data was analysed using a univariate general linear model (SPSS 15.0; SPSS Inc, Chicago, IL). To obtain actual placental transport, the amount of fetal radiation was adjusted for actual placental weight to determine placental transport of glucose per mg of placenta. Day of replicate and mouse number were treated as co-variates.

Differences between treatments were assessed using the Least Significant Difference method (LSD).

Differences in gene expression were assessed on normalised data using a univariate general linear model and LSD. Fetal and placental weights were treated as co-variates. Levene's Test of Equality of Error Variances was checked and when data did not have equal variance a Dunnett's T3 post hoc test was used.

8.4 Results

8.4.1 The effect of ammonium exposure on Day 15 placental gene expression

Placentas from Day 15 pregnant mothers were assessed for expression of genes involved in placental establishment, nutrient transport and mitochondrial energy production. One placenta was chosen from each treatment group from each day of transfer.

There were no statistical differences between average placental weights and corresponding average fetal weights of the four placentas selected for gene expression (**Table 8–1**).

Exposure to ammonium during the 2-cell to 8-cell stage (Treatment 3) significantly reduced vascular endothelial growth factor (*VEGF*) expression in the resultant placentas when compared to control (P=0.05; **Figure 8-2**). Treatment with ammonium at any other stage did not alter *VEGF* expression. Exposure to ammonium continually throughout development (Treatment 5) significantly increased glucose transporter 1 (*Slc2a1*) and insulin-like growth factor 2 (*Igf2*) gene expression when compared to control and all other treatment groups (P<0.05; **Figure 8-2**).

.Exposure to ammonium during the first cleavage division (Treatment 2) trended towards a significant increase in nuclear factor-eythroid 2-related factor 1 (*mNRF1*) (P=0.06; **Figure 8-3**).

Exposure to ammonium at any stage did not significantly alter expression of glucose transporter 3 (*Slc3a1*), insulin-like growth factor receptor (*Igf2R*), *H19*, mitochondrial transcription factor A (*mTFAM*), mitochondrial transcription termination factor (mTERF), nuclear factor-eythroid 2-related factor 2 (*mNRF2*), and amino acid transporters *Slc38a2* and *Slc38a4* (**Figure 8-3**).

Placental gene expression was also correlated to placental weight and regression values were assessed. Both *H19* and *Slc3a1* had a high correlation with placental weight where increased placental size was highly correlated to increased gene expression. Exposure of embryos to ammonium at any stage significantly reduced this correlation for both *H19* and *Slc3a1* gene expression (**Figure 8-4 and Figure 8-5**).

Table 8–1: Average Day 15 placental and corresponding average fetal weights of placentas extracted for gene expression.

Time of exposure to Ammonium	Fetal Weight (mg)	Placental Weight (mg)	Average Placental Weight (mg) of entire day 15 cohort
Control	201.5±27.2	99.4±13.4	105.7±11.7
Zygote to 2-cell	210.0±6.8	76.3±3.9	88.9±7.4
2-cell to 8-cell	191.2±17.9	92.4±16.6	98.5±7.2
8-cell to Blastocyst	187.5±23.7	71.0±8.2	97.2±5.7
Zygote to blastocyst	207.7±11.4	86.9±12.0	95.8±5.6

N = 4 per treatment chosen and extracted

Data expressed as mean± SEM

No significant difference between any treatment groups.

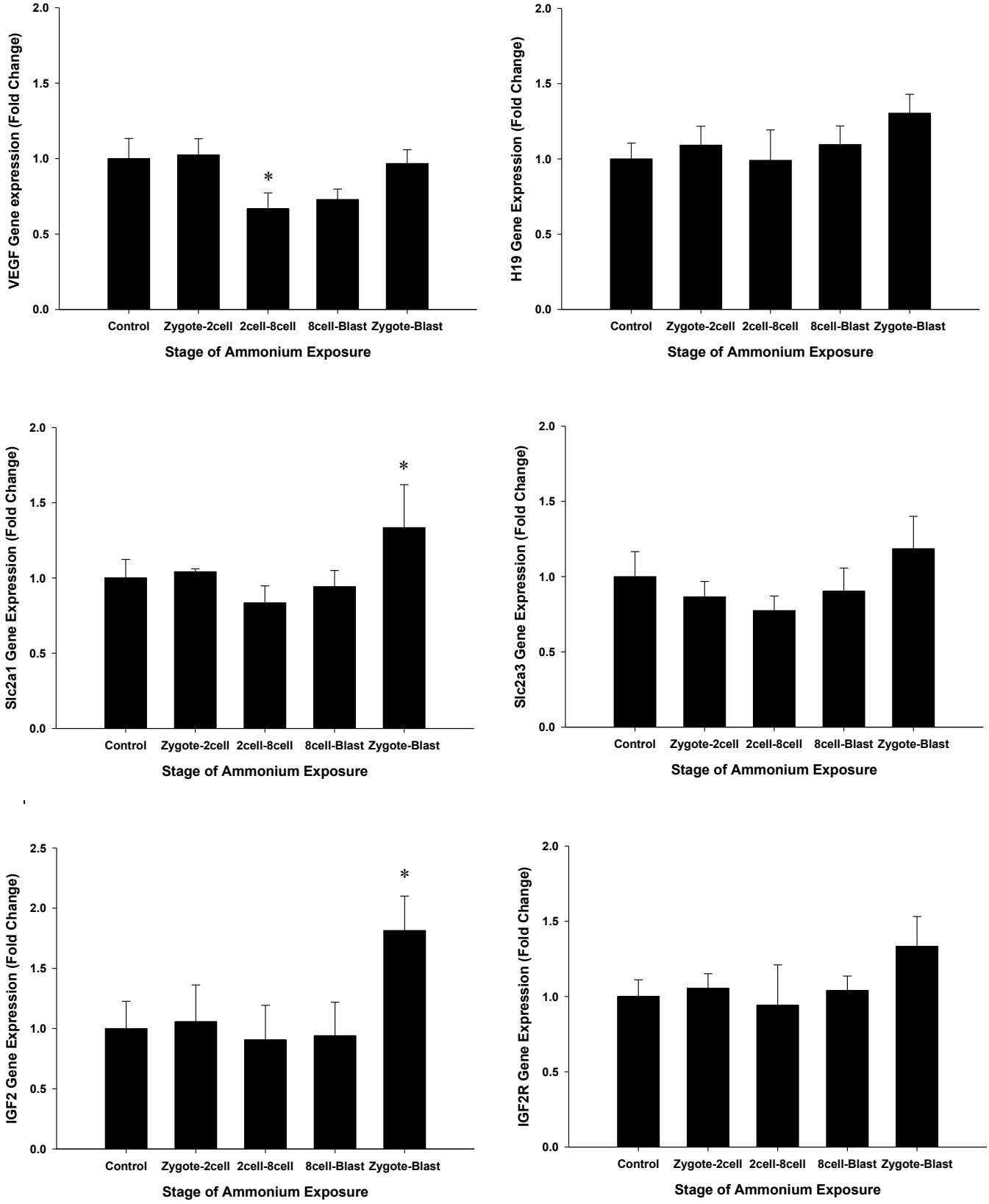


Figure 8-2: The effect of 300μm ammonium exposure at varying stages of pre-implantation embryo development on Day 15 placental gene expression. N=4 placentas extracted

Data expressed as mean ± SEM

* indicates significantly different from control ($P<0.05$)

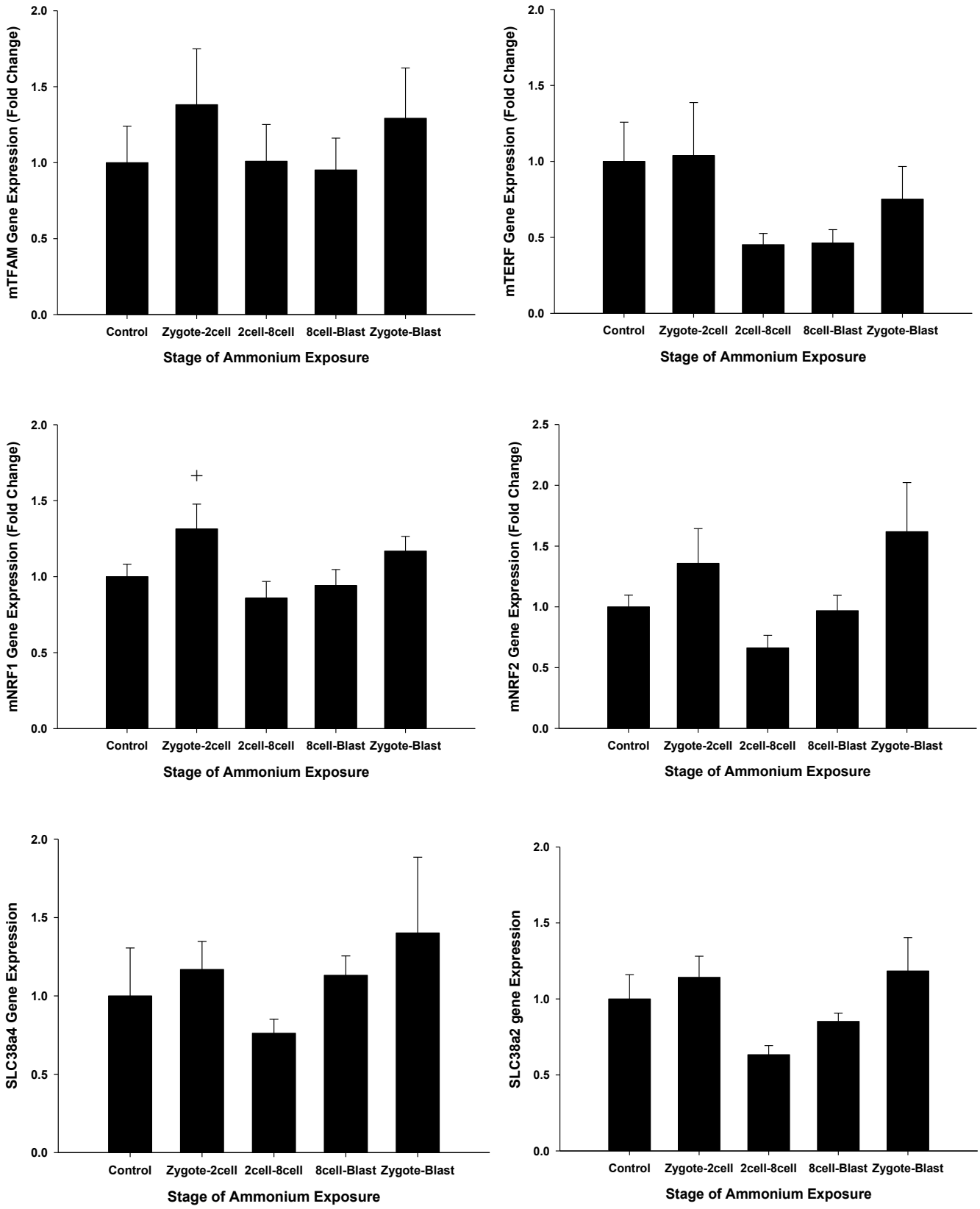


Figure 8-3: The effect of 300μm ammonium exposure at varying stages of pre-implantation embryo development on Day 15 placental gene expression. N=4 placentas extracted

Data expressed as mean ± SEM

+ indicates trending significance from control (P=0.06)

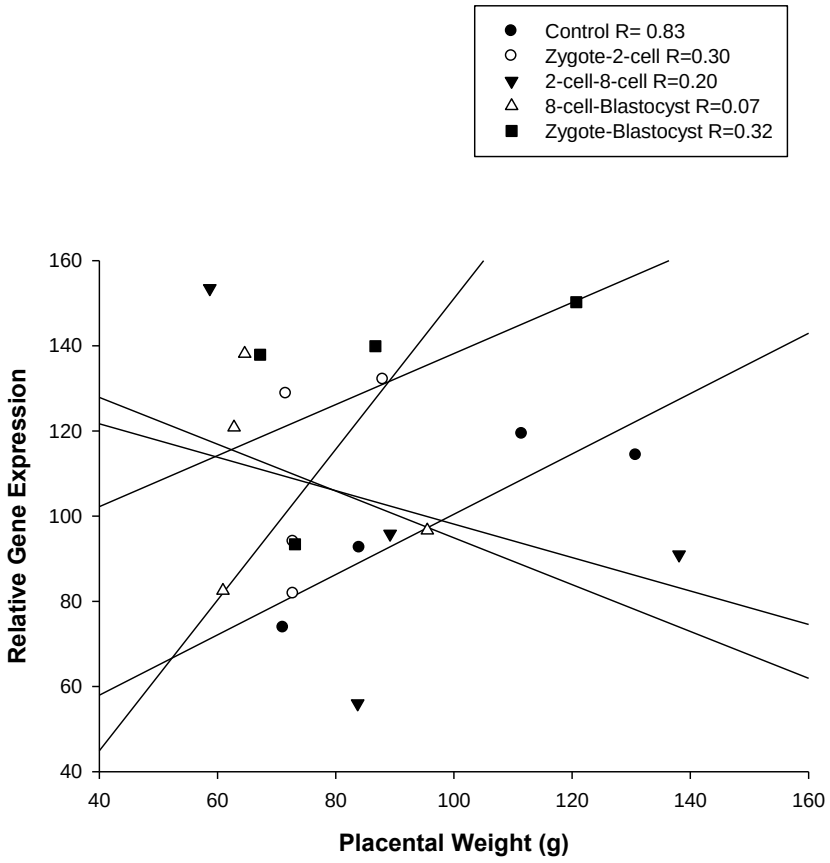


Figure 8-4: Regression analysis of *H19* placental gene expression relative to placental weight

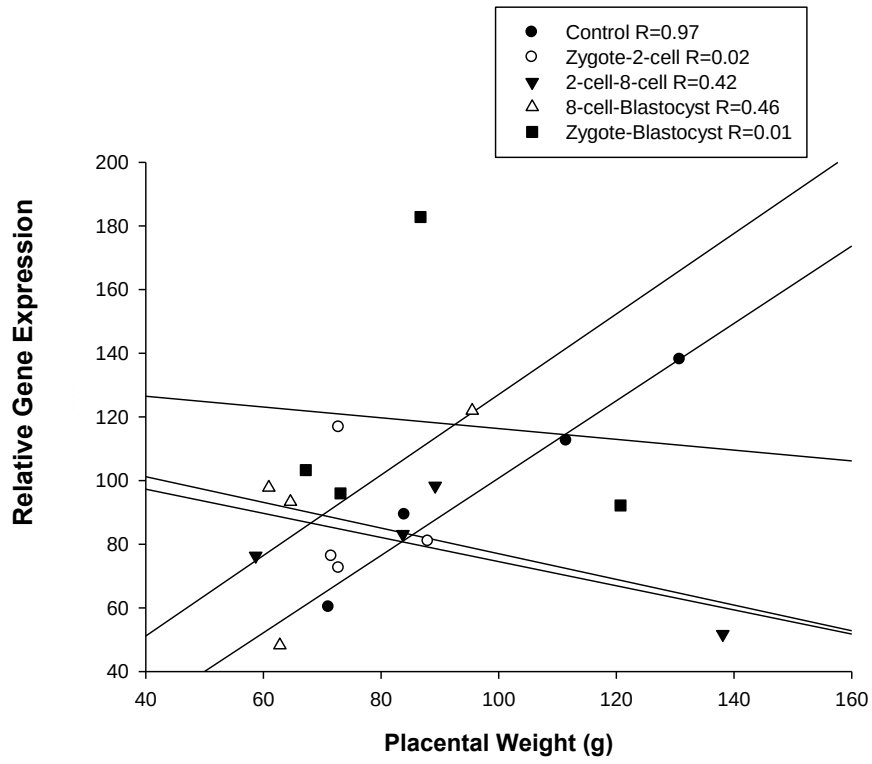


Figure 8-5: Regression analysis of *Slc2a3* placental gene expression relative to placental weight

8.4.2 The effect of ammonium exposure on placental transport

Placental glucose transport was assessed using a non-metabolisable radio-labelled form of glucose (Methyl-D-Glucose, 3-0-[Methyl-^{14}C]). This glucose analogue is transported from the placenta into the fetus at the same rate that normal glucose would be, thus it is directly indicative of actual placental glucose transport ability. The amount transported in each fetus was then calculated and divided by placental size to give relative amount transferred per gram of placenta.

Exposure to ammonium during the first cleavage division increased glucose placental transport by 25% however this was not statistically significant (**Figure 8-6**).Exposure to ammonium from the zygote to the blastocyst stage decreased placental glucose transport by 13% when compared to control, however this was also not statistically significant (**Figure 8-7**).

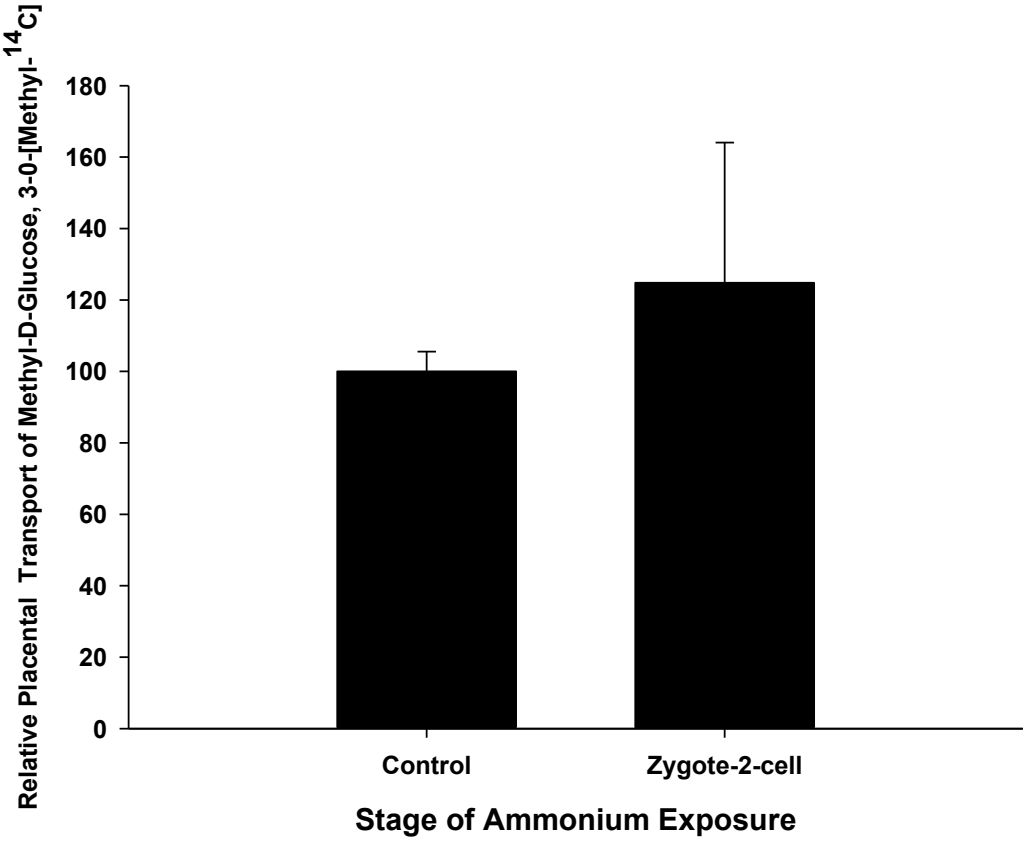


Figure 8-6: The effect of exposure to 300μM ammonium during the first cleavage division on placental glucose transport of Methyl-D-Glucose, 3-0-[Methyl-14C] on Day 15 of embryo transfer (indicative of amount of glucose transported per gram of placenta).

Control n= 4, NH_4^+ n=4

Data expressed as mean ± SEM

No significant difference between any treatment groups

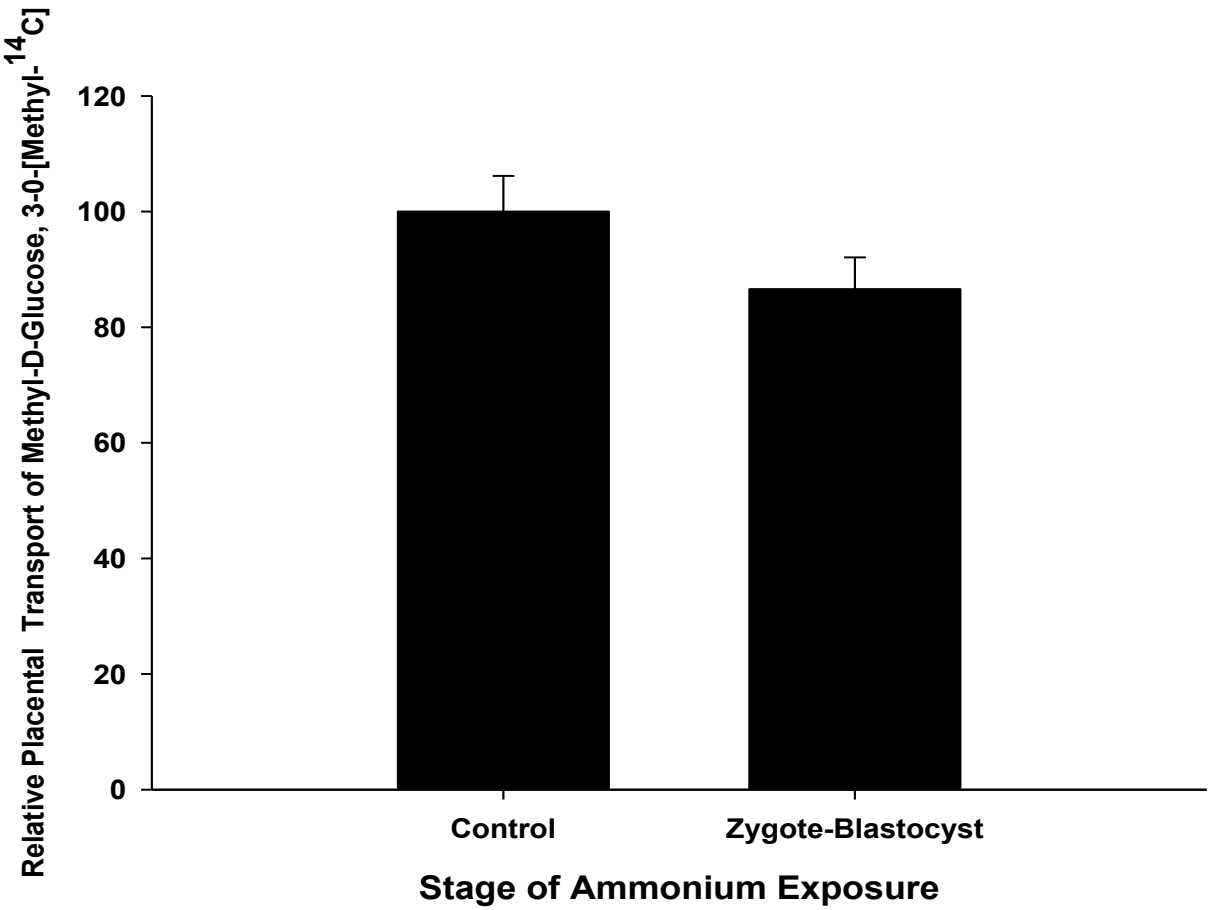


Figure 8-7: The effect of exposure to 300μM ammonium continually throughout pre-implantation embryo development on placental glucose transport of Methyl-D-Glucose, 3-0-[Methyl-14C] on Day 15 of embryo transfer (indicative of amount of glucose transported per gram of placenta).

Control n=22 NH_4^+ n= 13

Data expressed as mean ± SEM

No significant difference between any treatment groups

8.5 Discussion

Normal placental development is essential for promoting normal fetal growth and development, as the placenta is responsible for the delivery of nutrients to the fetus as well as the removal of waste. Many targeted mutations in mice exemplify how single gene mutations can affect placental development and function. A common feature among these placental mutants is the reduced ability to transport nutrients, which, in turn, results in fetal growth restriction or even embryonic death (Watson and Cross 2005).

Placental establishment requires a large number of important genes in particular for vascularisation and labyrinth establishment, both which play an important role in nutrient transport. Placental-specific knockouts of *Igf2* result in significantly reduced labyrinth area as well as altered transport systems such as amino acid transport (Constancia *et al.* 2002). Mutants for the gap junction protein conexin 26 (*Cx26*), which acts in conjunction with glucose transporter 1 (*Slc2a1*), show reduced glucose transport, reduced labyrinth development and fetal death at E11.0 (Gabriel *et al.* 1998).

Previously I have demonstrated that exposure to an *in vitro* stress (ammonium or DMO), either continually throughout pre-implantation embryo development or during specific windows of embryo development, result in multiple perturbations to embryo homeostasis and viability. After transfer there is a reduction in implantation, fetal development per implantation as well as alterations in fetal and placental parameters. In particular, exposure to 300um ammonium during the zygote to 2-cell stage significantly reduces fetal weight on Day 15 after embryo transfer (Zander *et al.* 2006); however, by Day 18 this difference had disappeared. Interestingly there was still a significant alteration in fetal to placental weight ratio as well as reduced crown rump length (see Chapter 3). Exposure to 300μM ammonium continually throughout culture resulted in similar outcomes on Day 15, with decreased fetal development per embryo transferred as well as decreased crown rump length (Zander *et al.* 2006). On Day 18 after embryo transfer, fetal to placental weight ratio and crown rump length were also reduced (see Chapter 3).

One possible mechanism behind the alterations in fetal growth is altered placental homeostasis. The alterations seen in fetal growth, maturity and crown rump length may be due to decreased placental nutrient exchange or alterations in placental growth and function.

There are many parameters of the placenta that can affect its nutrient transfer capacity. Placental size, morphology, gene expression and transporter abundance can all have an impact on placental transport efficiency. Placental vascularity is also very important, as it influences nutrient and waste exchange. The vascular endothelial growth factor family play a vital role in angiogenesis, including vascular growth, development and permeability, and the levels increase with advancing gestational age (Regnault *et al.* 2002). Decreased levels of *VEGF* are associated with impaired vascular development, decreased placental mass and impaired fetal growth. Exposure to ammonium between the 2-cell and 8-

cell stage resulted in a significant reduction in placental *VEGF* gene expression on Day 15. At this stage of development there was a tendency for reduced fetal weight on Day 18 (see Chapter 3) implicating decreased *VEGF* expression and resultant decreased nutrient transport in this observation; however, a direct link remains to be elucidated.

Exposure to ammonium continually throughout development resulted in significant increases in *Scl2a1* and *Igf2* expression. Glucose serves as one of the most important substrates for fetal growth (Takata and Hirano 1997). Glucose transporters, which are integral membrane proteins, are responsible for the transport of glucose across membranes. In the mouse and human, *Scl2a1* (previously known as *Glut1*) is considered to be the primary glucose transporter and its expression is altered by glucose concentration and hypoxic conditions and is positively regulated by *Igf2* (Baumann *et al.* 2002; Gaither *et al.* 1999; Hayashi *et al.* 2004; Ogura *et al.* 1999; Shepherd *et al.* 1992). Increased expression of Scl2a1 observed in Day 15 placentas from embryos exposed to ammonium continually throughout development may result in increased glucose transport and may be due to the increased expression of *Igf2*. Interestingly, by Day 18, glucose transport in these placentas appears to be decreased, which indicates that although gene expression is increased, perhaps actual protein expression is unchanged or actually reduced. The implications of this dichotomy between function and gene expression may suggest that there is regulation at a post-translational level. As well as glucose transporter gene expression, *Igf2* expression was also increased in the placentas resulting from blastocysts that were cultured continually in ammonium. *Igf2* is a paternally expressed imprinted gene which promotes fetal growth (Sibley *et al.* 2004). *Igf2* knockout mice display a significant reduction in fetal weight compared to wild-type as well as decreased placental transport (DeChiara *et al.* 1991; Sibley *et al.* 2004). In contrast, over-expression of the *Igf2* gene results in fetal overgrowth (Wang *et al.* 1994). However, in this study the increase in *Igf2* expression seen in Day 15 placentas does not correlate with increased fetal growth. As previously mentioned, increased gene expression does not necessarily correlate with increased protein expression. Furthermore, a limitation of this current study is that whole placentas were used to determine the levels of gene expression. However, not all genes are uniformly expressed in all cell types in the placenta. Therefore the changes in levels of gene expression need to be interpreted in this context, since whether ammonium treatment of the embryo changes the relative levels of the placental cell distribution is unknown. It is also possible that the increase seen in *Igf2* and *Scl2a1* gene expression may also be a compensatory response in order to maintain fetal and placental growth after an embryo stress exposure as there was no difference in fetal or placental weight in the group analysed.

mNRF1 is an important gene involved in redox balance and apoptosis; however, its biological function is less understood compared to *mNRF2*. Studies in fibroblast cell lines have shown that it plays an important role in oxidative stress responses (Kwong *et al.* 1999). It is also important for development as *mNRF1* knockout results in fetal lethality owing to anaemia caused by impaired liver function. Loss of *mNRF1* function also results in impaired antioxidant gene expression and increased oxidative stress (Chan *et al.* 1998; Chen *et al.* 2003). The tendency for an increase in *mNRF1* gene

expression after exposure to ammonium during the first cleavage division may indicate the presence of oxidative stress in the placenta. Exposure of pre-implantation embryos during the first cleavage division resulted in increased reactive oxygen species both at the 2-cell stage and at the 8-cell stage after the stress was removed (see chapters 5 and 6). This indicates that perhaps oxidative stress continues after implantation and is still present in the placenta by Day 15 however further placental extractions would need to be performed to increased experimental power to determine if this result is significant.

One significant issue with this experiment is the small number of fetal/placental pairs on which placental gene expression was assessed. These pairs were chosen at random from different days of transfer and due to this the significant difference seen in fetal weight between treatment groups on day 15 was not evident. It would be pertinent to expand the data set for this experiment and assess a larger group of fetal/placental pairs to determine if the differences in gene expression are maintained in a larger group where fetal weight differences are significant.

Exposure to ammonium continually throughout development produced Day 18 placentas with a 14% reduction in glucose transport. Exposure to ammonium during the zygote to 2-cell transition produced placentas with 24% increased glucose transport. Although these alterations were not statistically significant (due to small experimental numbers), the increase in glucose may be responsible for the catch-up growth seen between Day 15 and Day 18 fetal weights in embryos transferred after exposure to ammonium from the zygote to 2-cell. Once again the power of this experiment needs to be increased to assess if these differences become significant in a larger data set. It would also be of interest to assess gene expression in the day 18 placentas to see if altered transporters (such as glucose transporters or amino acid transporters) may also be partially responsible for the catch-up growth seen between day 15 and day 18.

In conclusion, the data in this chapter show that exposure to 300μM ammonium either continually throughout development or during the post compaction stage results in alterations in placental gene expression on Day 15. This indicates that environmental stress exposure during pre-implantation embryo development can possibly ‘programme’ placental function. Alterations in placental gene expression may lead to alterations in fetal growth and health not only during gestation but later in life; however, the impact of this stress exposure on fetal outcomes after birth still remain to be elucidated.

9 Concluding remarks

9.1 Introduction

The concept of 'developmental origins of adult health and disease' was originally put forward by David Barker and colleagues, who proposed that environmental factors, in particular nutrition, could have an impact on fetal development in utero and program the risks for disease in later life, especially the onset of cardiovascular and metabolic diseases (Barker 1990; Barker and Osmond 1986). From here the term 'thrifty phenotype' was coined, hypothesising that during times decreased maternal nutrition (perhaps due to inadequate food supply) during fetal development in utero, there is an adaptive response by the fetus. This response optimises the growth of key body organs, enhancing post-natal survival under such adverse conditions. It was hypothesised that these adaptations only become highly detrimental when the resultant offspring is then placed into and consumes abundant nutrition (such as 'Western' diets which contain high levels of fats and sugars). These observations lead to the concept of fetal and neonatal 'programming', where a stimulus or insult at a critical period of development can result in lasting or lifelong consequences (Barker 1990; Barker and Fall 1993; Lucas 1991; McMillen and Robinson 2005).

The experiments in this thesis have shown that alterations in programming can be induced by the presence of *in vitro* stress (such as ammonium or altered pH_i) and this 'programming' can be shifted to earlier windows of sensitivity, such as during pre-implantation embryo development. The data in this chapter also supports previous studies which have demonstrated that exposure to sub-optimal environmental conditions at crucial stages of development can also lead to altered fetal outcomes downstream (Feil *et al.* 2006; Fleming *et al.* 2004; Sinclair and Singh 2007; Zander *et al.* 2006)

The culture of the mammalian pre-implantation embryo is consistently associated with decreased embryo viability and development, along with severe alterations in metabolism and gene expression (Gardner and Lane 1993b; Lane and Gardner 1994). However, more significantly, it has been established that the conditions to which the pre-implantation stage embryo is exposed can not only affect the ability to establish a pregnancy but also affect both fetal growth and adult health in animal models (Doherty *et al.* 2000; Ecker *et al.* 2004). This is not a surprising result, as the embryo contains all the founder cell lineages for fetal and extra-embryonic tissue. Furthermore, it is understandable that a stress imposed at this critical juncture would give rise to an embryo whose cells have all been affected and these effects will carry on downstream as cell division occurs.

Studies have also demonstrated that embryo culture itself leads to increased systolic blood pressure in offspring (Watkins *et al.* 2007). Exposure of the pre-implantation embryo to *in vivo* perturbation is also associated with downstream alterations to fetal development, as well as longer-term outcome alterations such as blood pressure and glucose tolerance (Kwong *et al.* 2000). Studies using nutritional models have demonstrated that mothers who are fed a low protein diet during the pre-implantation embryo developmental window give rise to offspring that have increased weight from birth, sustained hypertension and abnormal anxiety-related behaviour (Watkins *et al.* 2008). The

combination of these results in both *in vivo* and *in vitro* models indicates that the pre-implantation embryo is highly adaptable to stress, but at a cost in terms of long term healthy development, and that the presence of sub-optimal conditions at these early stages can alter 'embryo programming', resulting in altered outcomes after birth.

As outlined in the previous chapters, the pre-implantation embryo is highly dynamic and undergoes a variety of changes over the days prior to implantation. Alterations in organelle structure and function, metabolism, substrate utilisation, genetic control, epigenetic modifications and cell differentiation all occur during pre-implantation development (Braude *et al.* 1979; Gardner and Leese 1988; Hardy *et al.* 1989; Hillman and Tasca 1969; Leese 1991). During this early developmental phase the tolerance for stress exposure also changes, with the early pre-implantation embryo appearing to lack robust mechanisms for dealing with alterations in pH (Baltz *et al.* 1990; Baltz *et al.* 1991b; Lane 2001; Zhao and Baltz 1996). In addition, the pre-compaction stage embryo also is the most vulnerable stage for exposure to ammonium and serum (Rooke *et al.* 2007; Zander *et al.* 2006).

Although the impact of stress on the embryo has been widely described, little is known about the mechanism behind the reason why a stress applied during pre-implantation embryo development can have lasting effects downstream on fetal outcomes.

9.2 Thesis discussion

The results of this study have supported the finding of many others, in that a stress applied during critical windows of pre-implantation embryo development result in alterations to subsequent fetal development. Using two well-characterised *in vitro* metabolic stress models, ammonium and DMO, in the mouse embryo model, I have shown that although the embryo is able to develop into a morphologically normal blastocyst after stress exposure, the intracellular homeostasis is perturbed, leading to alterations in implantation and fetal development after transfer. These findings are significant in that they demonstrate that the embryo is highly adaptable and has the ability to be programmed by the environmental conditions to which it is exposed prior to implantation, resulting in alterations in implantation rates and fetal growth.

This study has also been able to demonstrate that the pre-compaction stage embryo appears to be the more vulnerable to stress than the post-compaction stage. As exposure to DMO, during either the first cleavage division or from the 2-cell to the 8-cell stage, results in perturbed development—as evidenced by decreased blastocyst cell number, decreased inner cell mass number and increased blastocyst apoptosis—these results were similar to those measured if the stress was present continually throughout the entire pre-implantation developmental period. I have also shown similar outcomes using ammonium in the media as a model of *in vitro* stress, demonstrating that the early stages of embryo development are the most vulnerable to sub-optimal environmental conditions (Zander *et al.* 2006). The theory that the pre-compaction stage embryo is more sensitive to environmental stress has also been demonstrated in other *in vitro* models using serum and osmotic stress. Furthermore, in many

studies utilising pH stress it has been demonstrated that the post-compaction stage is better able to regulate intracellular pH than the pre-compaction stage (Edwards *et al.* 1998b; Lane and Gardner 2001; Rooke *et al.* 2007). Other studies have also demonstrated that exposure of the pre-compaction stage embryo to sub-optimal culture conditions results in perturbed gene expression; however, exposing the post-compaction stage embryo to similar conditions does not alter gene expression (Hewitt *et al.* 2003; Ho *et al.* 1994). These findings, as well as the outcomes of this study, are understandable considering the early embryo appears to have limited mechanisms for managing sub-optimal environmental conditions (**Figure 9-1**) (Baltz *et al.* 1990; Baltz *et al.* 1991b; Harvey *et al.* 1995; Lane and Bavister 1998; Lane and Gardner 2005b).

These results have significant implications for the clinical IVF setting, as extended embryo culture is now routinely used as a selection tool in human *in vitro* fertilisation for selecting the embryo with the highest implantation potential (Gardner 2000). The current dogma is that extended culture will result in increased stress on the embryo; however, the results of this study challenge this paradigm as it has shown that the later stage embryo is much better equipped to handle stress and it is the early stage embryo which is the highly sensitive stage.

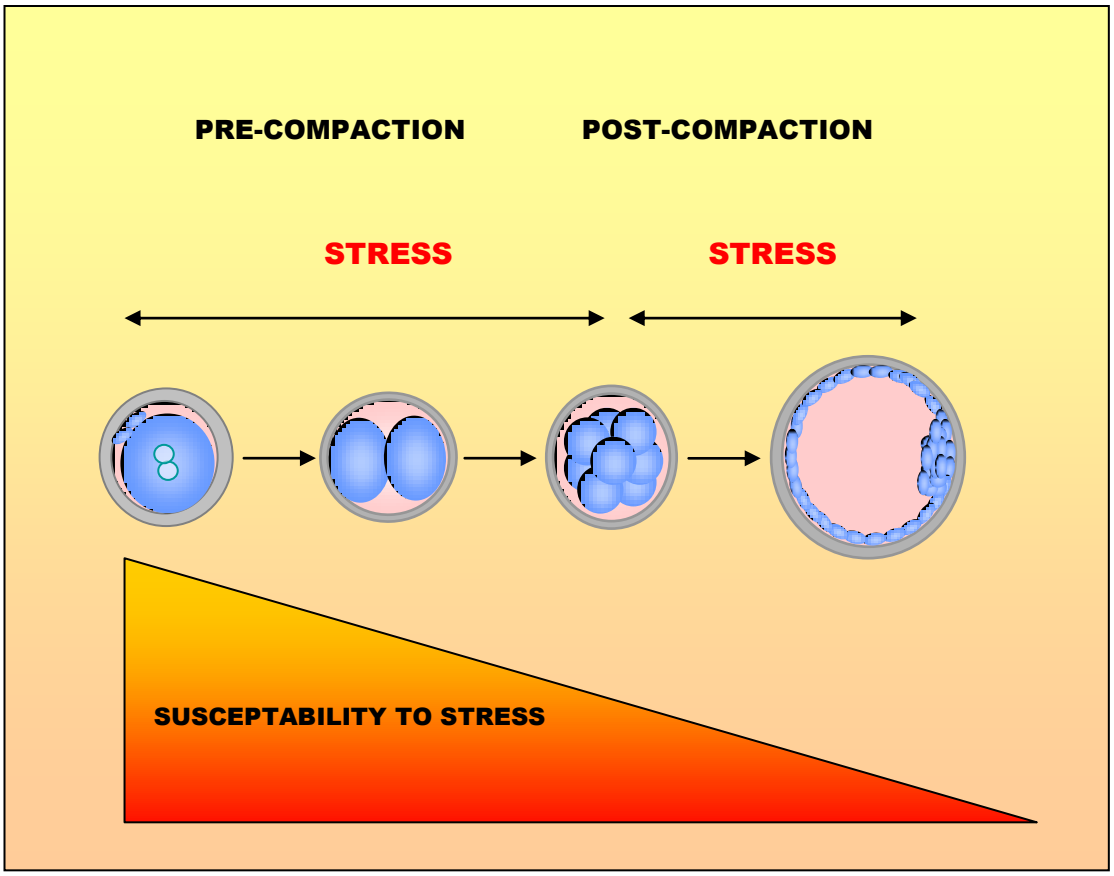


Figure 9-1: Diagram depicting the sensitivity of the pre-implantation embryo to external stress

Interestingly, although the downstream outcomes of stress exposure may vary depending on the stress used, and the alterations seen in gene expression may also vary, there appear to be some common metabolic parameters which are affected by suboptimal conditions, such as alterations in glycolytic activity. As mentioned previously, others have demonstrated that exposure to sub-optimal conditions, whether they be lack of amino acids, ammonium, high oxygen or osmotic stress, ultimately results in perturbed blastocyst development and altered blastocyst cell numbers coupled with a loss of normal metabolic activity. These perturbations to metabolism after stress seem to be conserved across animal strain and even species (Lane and Gardner 2005b). These effects have been similarly shown in my models of *in vitro* stress, as a result of treatments at the early stages of embryo development that resulted in altered blastocyst cell number coupled with changes in mitochondrial homeostasis and energy production.

Metabolism of the embryo is highly correlated to its ability to implant and form a health viable fetus (Lane and Gardner 1996). Previous studies have shown that embryos that display developmental delay or arrest also have disrupted metabolism, within both mitochondrial and cytoplasmic compartments, and studies using ammonium as an *in vitro* stress have also demonstrated alterations

between the balance of glucose metabolism and oxidative phosphorylation in embryos exposed to this *in vitro* stress (Gardner and Lane 1993a; Lane and Gardner 2003; Seshagiri and Bavister 1991). Metabolism of glucose and amino acids has been correlated to embryo viability in the mouse and human and therefore has been suggested as a non invasive marker of determining implantation potential (Gardner and Leese 1987; Houghton *et al.* 2002). My data strengthens this notion and expands it to implicate mitochondria as the primary organelle involved.

As mentioned previously, the metabolism of the pre-implantation embryo undergoes substantial change as the embryo develops, and at the early stages of development the embryo relies completely on the mitochondria for energy production via oxidative phosphorylation of pyruvate. It has been shown, in other cell types, that mitochondria are a prime target for stress and are implicated in the process of cellular damage and metabolic failure. Mitochondria are also vital for cell survival and metabolic control as well as playing a role in the cell death and apoptosis (Lane and Gardner 2005b). It can therefore be hypothesised that the metabolic perturbations seen after embryo stress exposure are mediated by the mitochondria.

The results described in this thesis have demonstrated that exposure of the pre-implantation embryo to an *in vitro* stress during the first cleavage division results in significant perturbations to mitochondrial homeostasis. Both ammonium and DMO significantly altered mitochondrial distribution and mitochondrial membrane potential both of which can influence the amount of ATP (energy) produced as well as localisation of that ATP. Exposure to ammonium also increased intracellular calcium within the 2-cell embryo and alterations in intracellular calcium have been previously associated with disrupted developmental competence (Lane and Bavister 1998). Interestingly, in these studies, increased intracellular calcium was also associated with perturbed organelle distribution and mitochondrial metabolism and supports the findings in this thesis (Lane and Bavister 1998; Ludwig *et al.* 2001).

The exposure of embryos to ammonium and DMO during the first cleavage division also significantly altered levels of ATP or ADP, which is possibly due to the alteration in mitochondrial homeostasis observed by altered calcium levels and mitochondrial membrane potential. Previous studies support this finding, as suboptimal culture conditions has also been shown to disrupt the balance of metabolic intermediates ADP:ATP (Gardner and Lane 1997; Gardner *et al.* 2000b). Exposure of embryos to ammonium also significantly reduced ROS production. As ROS are a natural by-product of oxidative phosphorylation, this is further support that the mitochondria are under stress due to ammonium treatment (**Figure 9–2**).

One of the novel findings of this thesis is the effect of *in vitro* stress on mitochondrial homeostasis after the stress has been removed. As mentioned previously, exposure of embryos to ammonium or DMO during the first cleavage division resulted in perturbations to mitochondrial homeostasis, ROS levels and ATP or ADP levels. When this stress was removed at the 2-cell cell stage and then the same

parameters assessed at the 8-cell stage disrupted mitochondrial activity, as measured by reduced mitochondrial membrane potential, altered ATP or ADP levels and increased ROS production, remained evident. These perturbations were similar to those seen in embryos that had been grown to the 8-cell stage in the continuous presence of ammonium or DMO. An alteration in ROS production has been shown in other models of embryo culture and it is likely that one of the targets of ROS is mitochondria (Nasr-Esfahani *et al.* 1990b; Nasr-Esfahani and Johnson 1991). The early embryo contains mitochondria that are classified as 'immature', as they are spherical in shape with a low surface area. Consequently, it has been hypothesised that the mitochondria of the early embryo may be more sensitive to oxidative stress. In support of this, experiments have shown that addition of components to culture media that reduce ROS production have the greatest effect at the 2-cell stage and are able to prevent developmental arrest (Nasr-Esfahani *et al.* 1990a; Nasr-Esfahani and Johnson 1992). Furthermore, high levels of ROS can also cause DNA damage and lipid peroxidation, with the latter being associated with downstream effects on cell division, metabolite transport and mitochondrial dysfunction. The '2-cell block' in mouse embryos (i.e. developmental arrest at the 2-cell stage due to suboptimal culture conditions) has also been associated with lipid peroxidation (Nasr-Esfahani *et al.* 1990b; Noda *et al.* 1991). Other effects of ROS are mitochondrial dysfunction and mitochondrial DNA (mDNA) damage (Kowaltowski and Vercesi 1999). Studies have shown that during oxidative stress, mDNA is four times more affected than nuclear DNA, and, because mDNA encodes for essential proteins involved in oxidative phosphorylation, it follows that mitochondrial metabolic perturbations may occur (Guerin *et al.* 2001). The conclusion from this body of work is that despite various metabolic stressors applied, a common outcome seen is an altered mitochondrial phenotype, indicating that this is perhaps the common mechanism behind embryo programming.

Interestingly *in utero* studies of developmental programming models have also implicated mitochondria in fetal programming and the metabolic syndrome. Administration of a high fat diet to gestating rats results in offspring with metabolic syndrome characteristics such as insulin resistence (Taylor *et al.* 2005). These offspring also display reductions in mDNA content in the kidney which has been directly linked to impaired glucose-stimulated insulin release and if indicative of a global reduction in mDNA would concur with the hypothesis that mitochondria play a vital role in fetal programming (Maechler 2003; Taylor *et al.* 2005). This study also demonstrated that mitochondrial gene expression within the aorta was altered, in particular genes involved in apoptosis and it has been hypothesised that this may lead to compromised mitochondrial function. Other studies have also demonstrated perturbations in mitochondrial structure (mitochondrial swelling and cristae disruption) in islets from diabetic fatty rats (Higa *et al.* 1999). It is proposed that theses alterations may lead to altered mitochondrial dysfunction within the cell, tissue damage and disease; however this is still under investigation (Taylor *et al.* 2005).

This thesis is also the first to show that mitochondrial perturbations, due to *in vitro* stress, persist after the cause is removed, demonstrating that the perturbation cannot be rectified by removal of the

stress, and that mitochondrial and metabolic perturbations may play a role in programming the downstream effects seen after transfer. The next step in this hypothesis will be to deliberately and specifically perturb mitochondrial function only during embryo development and assess its direct effect on metabolism and fetal outcomes. It would also be of interest to assess if perturbations occur in mDNA and due to the bottleneck theory assess if these persist throughout development and into fetal and placental tissue.

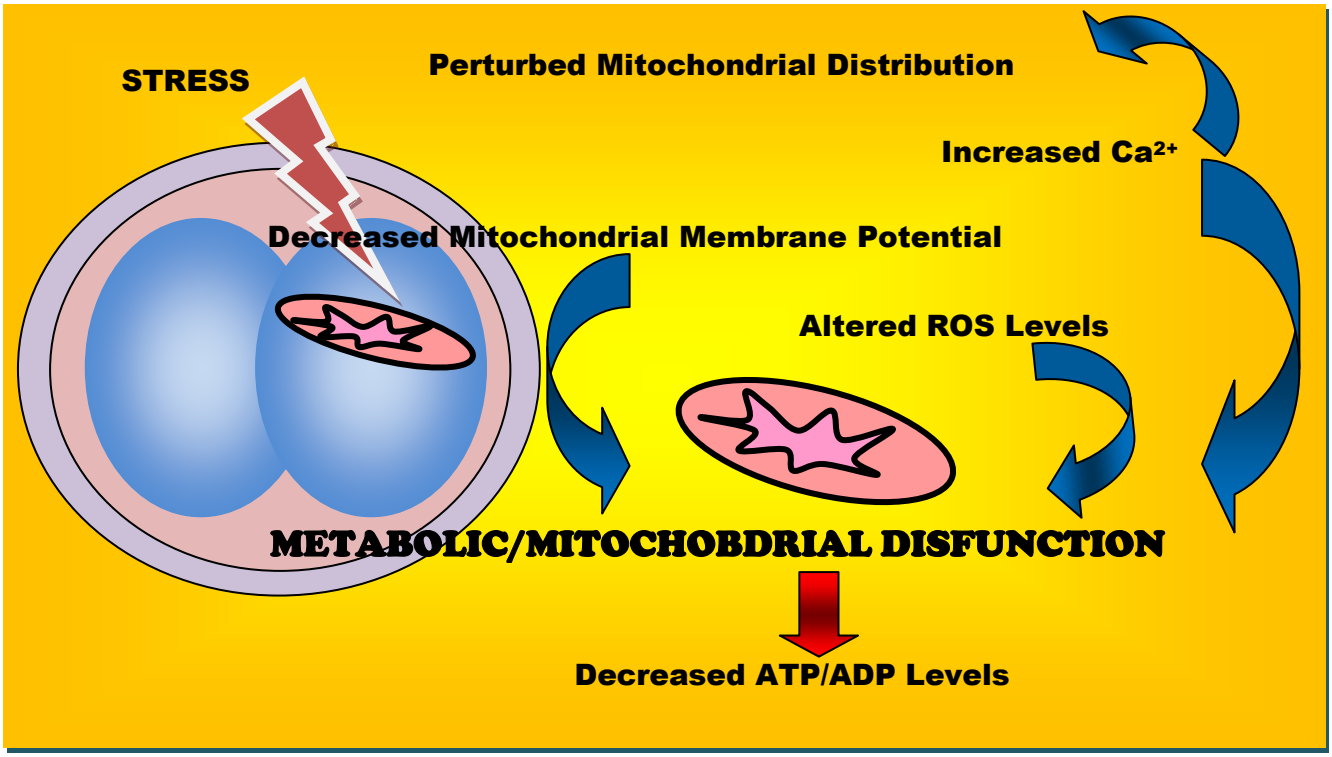


Figure 9-2: Diagram depicting the cellular and mitochondrial perturbations which result in altered metabolism and energy production.

Models of fetal programming due to exposure to altered conditions in utero, such as poor nutrition, have demonstrated that the pre-implantation embryo responds to its environment and adapts its development in direct response to it. As discussed previously, these responses allow the fetus to make adjustments to its growth and metabolism so it is compatible with the predicted nutritional availability after birth. However, these adaptations can become problematic if the post-natal environment provides the opposite of what was adapted for, such as over-nutrition. Under such circumstances discordance exists between nutrient intake and what the fetus has been programmed to expect. And this can lead to increased health risks in later life such as cardiovascular disease and diabetes (Gluckman and Hanson 2004b). The mechanism behind fetal programming is suggested to involve epigenetic alterations to the

genome further resulting in alterations to gene expression, permanently re-programming the embryo (McMillen and Robinson 2005).

Previous studies have demonstrated that sub-optimal culture conditions of the pre-implantation embryo can affect gene expression and imprinting (Doherty *et al.* 2000; Ho *et al.* 1994; Ho *et al.* 1995). Studies using ammonium as a model stress have also shown alterations in gene expression, including expression of *H19*, an imprinted gene after pre-implantation embryo exposure, and studies using serum in culture media have demonstrated fetal overgrowth in sheep due to alterations in expression of *Igf2R* (Lane and Gardner 2003; Young *et al.* 2001). In comparison, when serum is added to mouse culture, embryo development is impaired and fetal weight is significantly reduced, indicating that mammalian embryos differ in response to altered environmental conditions. This is also coupled with alterations in gene expression of *H19*, *Igf2* and *Grb7* in the resultant fetuses; although, interestingly, not all offspring exhibited this aberrant pattern of gene expression (Khosla *et al.* 2001a; Khosla *et al.* 2001b).

The pre-implantation embryo undergoes several significant epigenetic changes as development occurs. Initially after fertilisation and the first cell division there is a gradual passive loss of methylation as division occurs; however, imprints are maintained by a DNA methyltransferase *Dnmt1*. De novo methylation is initiated as cell differentiation occurs at the morula stage so that by the pre-implantation period, DNA methylation levels resemble that of a somatic cell. This re-methylation is controlled by two other DNA methyltransferases: *Dnmt3a* and *Dnmt3b* (Morgan *et al.* 2005; Okano *et al.* 1999). This thesis has demonstrated that exposure of embryos to *in vitro* stress models significantly alters the rate of passive de-methylation at the 2-cell stage, and that exposure to one of these stress models (DMO) also alters the level of do novo methylation at the morula stage. As these events are controlled by DNA methyltransferases, the levels of gene expression of this family of proteins was assessed and it was discovered that exposure of the pre-implantation embryo to ammonium or DMO during the first cleavage division resulted in significant reduction in the levels of *Dnmt3a* and *Dnmt3b* at the blastocyst stage when de-novo methylation occurs. These findings indicate that alterations in demethylation and de novo methylation and the genes that facilitate these processes may, in turn, lead to altered epigenetic programming resulting in alterations in gene expression and altered fetal growth. This data is further supported by studies in the mouse, demonstrating that alterations in methylation patterns at the 2-cell stage have been linked to early developmental failure in mice (Shi and Haaf 2002).

This thesis has demonstrated that embryo stress, which results in perturbed fetal growth, also significantly alters epigenetic events during pre-implantation development, and this may be responsible for the altered outcomes seen.

The effect of stress to embryos during the pre-implantation development on fetal outcomes has been discussed previously and has been demonstrated in a variety of models, including those used in this thesis. It is possible that the alterations seen in fetal growth are mediated by changes in placental transport of nutrients. Previous studies have shown alterations in placental gene expression after stress

exposure *in vitro* in particular imprinting was significantly perturbed (Fortier *et al.* 2008; Mann *et al.* 2004). This thesis has demonstrated that exposure to ammonium at various stages during pre-implantation development resulted in perturbations to placental genes *VEGF*, *Slc2a1*, *IGF2* and *mTFAM* on Day 15 of development. These genes are involved in vascular development, nutrient transport, placental growth and oxidative stress and indicate that the alterations in fetal growth and maturity on Day 15 (Zander et al 2006) could be due to alterations in placental growth and transport.

This idea that the environment that the early embryo is exposed to, in particular a window of 19 hours during the zygote-2-cell transition, can specifically affect the placenta is a novel finding and has implications for the developing fetus. This study has shown that the *in vitro* environment alters the programming of the early embryo and these alterations are maintained throughout development. They also maintained in the placenta as it develops, resulting in altered gene expression and possibly altered nutrient transport to the fetus giving a possible mechanism behind the altered fetal growth seen. The extent of this programming in the early embryo and the impact that this has on other forms of placental transport, such as amino acid transport as well as gas and waste exchange, remains to be elucidated.

9.3 Conclusion

This thesis has demonstrated that sub-optimal *in vitro* conditions can significantly alter blastocyst homeostasis without altering morphology. Exposure either pre-compaction or continually through pre-implantation development yields similar results in regards to decreased blastocyst cell number, decreased ICM cell number and increased apoptosis, indicating that the pre-compaction stage of development appears to be the most sensitive stage to ammonium and DMO (decreased pH_i) stress. The zygote-2-cell transition was focused on due to the significant mitochondrial, metabolic and epigenetic events that occur during this early stage. The exposure of the early embryo to both ammonium and DMO resulted in significant perturbations to mitochondrial distribution, calcium and membrane potential coupled with alterations to metabolic intermediates (ATP and ADP). These changes persisted to the 8-cell stage, regardless of whether the treatment was removed; showing for the first time that persistent mitochondrial and metabolic perturbations may be a mechanism behind embryo 'programming'. The other novel finding in this thesis is that *in vitro* stress can also alter epigenetic events that occur during embryo development and can affect the gene expression of proteins involved in methylation initiation and maintenance. This could subsequently alter gene expression in the fetal and placental tissue, and may explain the alterations seen in placental gene expression on Day 15 of development. In turn, this altered gene expression may also lead to altered placental function cumulating in the altered fetal growth seen after transfer (**Figure 9-3**).

Although this study has provided some answers, it has also raised many questions; in particular the link between metabolism and epigenetic changes has yet to be established. Recently, however, a family of genes (SNF2 family of ATPase/helicase proteins) such as ATRX has been discovered. They

have been termed ‘metabolic sensing proteins’, as they are known to be regulated by the metabolic state of the cell. These proteins respond to cellular metabolism and, in turn, modulate epigenetic changes such as chromatin methylation remodelling (Garrick *et al.* 2006; Gibbons *et al.* 2000). These proteins provide a possible link between mitochondrial metabolism and epigenetic changes; however, their contribution to embryo programming remains unclear.

Another unanswered question is: how significant are early programming events to overall health of the offspring? Many studies into *in vivo* stress during pre-implantation development have demonstrated that altered environment during this critical window results in perturbations to adult health such as altered systolic blood pressure, increased weight gain from birth and increased anxiety behaviour. One *in vitro* study has investigated the long-term effects of culture on offspring health. The study has demonstrated that embryo culture results in increased systolic blood pressure in offspring after transfer and, moreover, that enzymatic regulators of cardiovascular and metabolic physiology were also significantly elevated (Watkins *et al.* 2007). Whether similar results would be seen in the offspring derived from ammonium or DMO treated embryos remains to be seen.

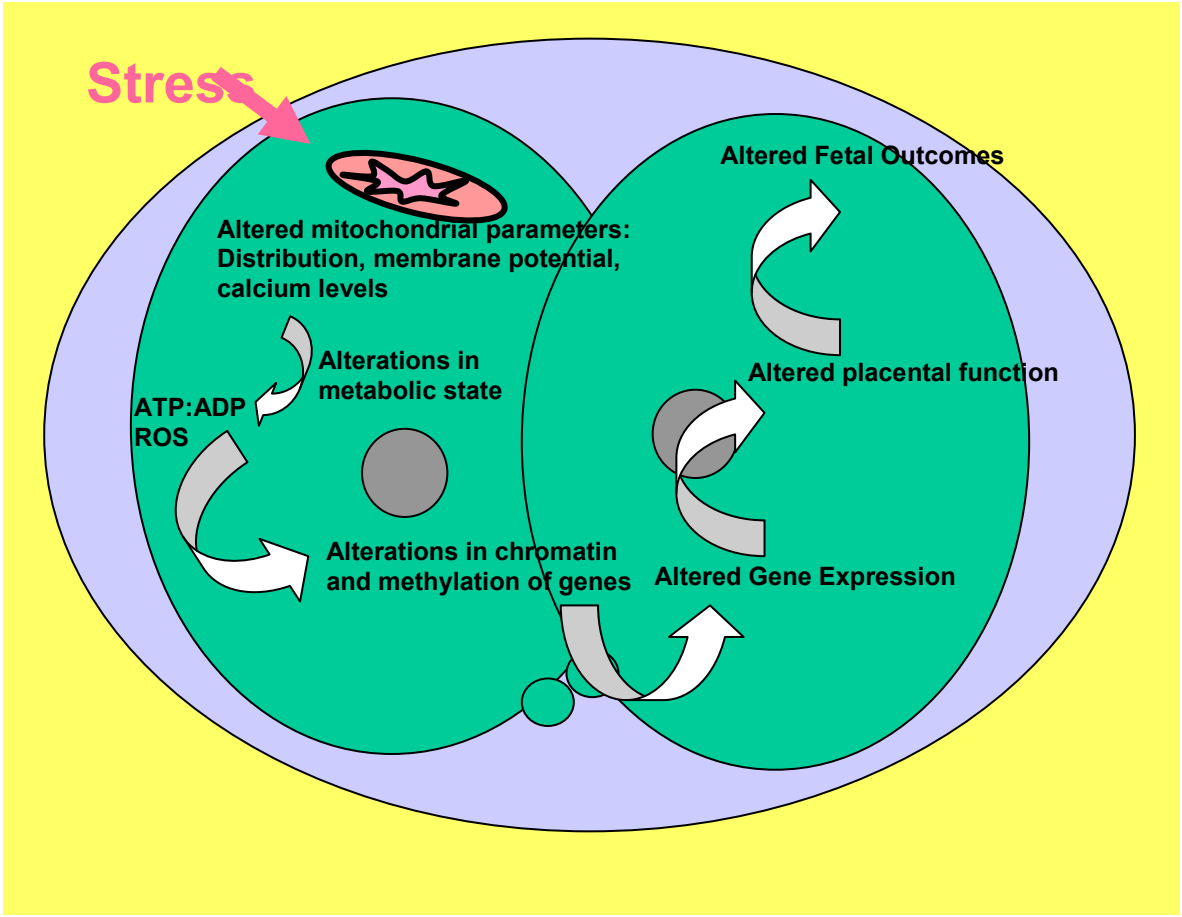


Figure 9-3: Diagram depicting the possible mechanism behind altered fetal growth after exposure to sub-optimal condition during pre-implantation embryo development

In conclusion, the evidence in this thesis supports the concept that the pre-implantation period of embryo development is a window of enhanced sensitivity to environmental conditions both *in vivo* and *in vitro*. The early embryo appears to respond to its environment, and these environmental conditions play a vital role in setting the metabolic and physiological trajectory for the developing fetus.

One possible mechanism, by which the physiology of the pre-implantation embryo is altered, in response to environmental conditions, is mitochondrial metabolism. It is possible that these alterations in metabolic control may 'program' the embryo via a cascade of altered cellular signalling and epigenetic changes within the cell, resulting in a new outcome trajectory for the resultant offspring.

10 Appendix

10.1 0.9% Saline preparation

1. 0.9g of NaCl was dissolved in 100ml MilliQ water
2. Filtered using a 0.2µm filter
3. Store at 4 ºC for 3 months

10.2 1.2% Avertin preparation

10.2.1 Stock solution (100x)

1. 0.5g 2, 2, 2-Tribromoethanol, 2-methyl-2-butan was dissolved in 0.5ml Tert-Amyl alcohol 99%
2. Store in glass at 4 ºC for 3 months

10.2.2 Working solution

1. Add 120ul of working solution to 10ml 0.9% saline
2. Store in glass at 4 ºC for 3 months

10.3 RNAse buffer preparation

1. Dissolve in 60ml of RO water
    i. 0.8764g Tris
    ii. 0.0584g NaCl
    iii. 0.057$MgCl_2$
2. Adjust pH to 8.0 and then add 39ml of RO water to give a final volume of 100ml
3. Store at 4 ºC for up to 1 year

10.4 PI/RNase A mix preparation

1. Add 500µl RNase A (0.1mg/ml stock solution) to a 1.6ml centrifuge tube
2. Add 50µl PI (1mg/ml stock solution)
3. Bring the volume up to 1ml by adding 450µl RNase buffer

10.5 Ca^{2+} calibration solutions

10.5.1 Solution A (Ca^{2+} free solution)

i. KCl 100mM (0.7455g)
ii. MOPS Acid 10mM (0.2093g)
iii. K_2H_2-EGTA 10mM (0.3804g)

The above compounds were added to 100ml Milli-Q water. The solution was titred to pH 7.2 using 2M KOH and filtered through a 0.2µm filter and stored at 4 °C.

10.5.2 Solution B (Ca^{2+} saturation solution)

i. KCl 100mM (0.7455g)
ii. K-MOPS 10mM (0.2093g)
iii. $CaCl_2$ 1mM (0.0147g)

The above compounds were added to 100ml Milli-Q water. The solution was titred to pH 7.2 using 2M KOH and filtered through a 0.2µm filter and stored at 4 °C.

10.6 Metabolic assays

10.6.1 Glycine-hydrazine buffer

i. 7.5g Glycine
ii. 5.2g Hydrazine
iii. 0.2g EDTA

Dissolve the above compounds in 49ml of Milli-Q water and add 51ml of 2M NaOH.

10.6.2 NAD+

Add 40mg of NAD+ to 1ml of Milli-Q water.

10.6.3 Lactate standard

1. Initially 1mM Na-lactate was prepared
    i. 0.0112g Na-Lacate in 100ml Milli-Q water
2. This was then serially diluted down to 0.5mM, 0.25mM, 0.125mM and 0.06mM Na-Lactate

10.6.4 Lactate cocktail

i. 450µl glycine-hydrazine buffer
ii. 25µl lactate dehydrogenase
iii. 75µl NAD+
iv. 400µl Milli-Q water

The above reagents were combined and filtered through a 0.2µm filter, alliquoted into 100µl and stored at 20 °C.

10.6.5 ATP and ADP standard

1. Initially 1mM ATP and 1mM ADP standards were prepared
    i. 4.27mg ADP in 10ml Milli-Q water
    ii. 6.1mg ATP in 10ml Milli-Q water
2. 500µl of each of the two standards was added to an eppendorf tube to give a 0.5 dilution of both
3. This was then serially diluted down to 0.25, 0.125 and 0.0625 using Milli-Q water

10.6.6 Epps buffer

1. Dissolve in 50 ml of MilliQ water
    i. 0.63075g Epps
    ii. 0.0456g MgSO4
    iii. 0.00225g Glucose
2. pH up to 8.0
3. Filtered using a 0.2µm filter
4. Store at 4 ºC for 1 year

10.6.7 ADP Cocktail

1. Add to a small glass beaker
    i. 5ml Epps Buffer
    ii. 500ul 1.45mM NADH
    iii. 250ul 5.2mM ATP
    iv. 500ul 13mM DL-Dithiothreitol (DTT)
    v. 1ml 7.1mM phosphoenol pyruvate (PEP)
    vi. 1ml 260mM $MgSO_4$
    vii. 1ml 940mM KCl

viii. 150ul Lactate Dehydrogenase (LDH)
ix. 250ul Pyruvate Kinase

2. Pipette 0.5ml aliquots into 0.6ml eppendorf tubes
3. Freeze at -80 °C

10.6.8 ATP Cocktail

1. Add to a small glass beaker
    i. 10ml Epps Buffer
    ii. 1ml 5mM DTT
    iii. 600ul 10mM NADP
    iv. 600ul Hexokinase/Glucose-6-phospahte dehydrogenase (HK/G6PD)
2. Pipette 0.5ml aliquots into 0.6ml eppendorf tubes
3. Freeze at -80 °C

10.7 Mounting media for immunohistochemistry

1. Add to a 1.7ml eppendorf tube 2 drops of Prolong Gold
2. Make up to 500µl glycerol
3. Make up to 1500µl with PBS

10.8 pH measurement

10.8.1 Valinomycin stock solution (x2000)

1. Add 10 mg of Valinomycin to 1ml of DMSO to give 10mg/ml
2. Store at 4 °C

10.8.2 Valinomycin working solution

1. Add 10µl of stock solution to 20ml of pH solution
2. Gives final working concentration of 5µg/ml

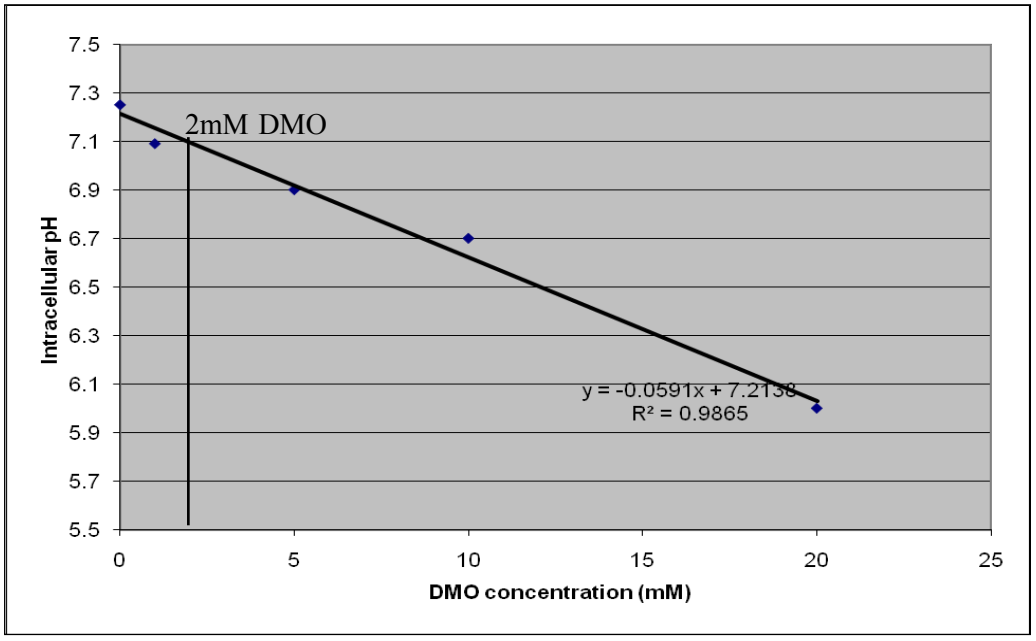
10.8.3 Nigericin stock solution (x1000)

1. Add 20mg of Nigericin to 20ml of ethanol
2. Store at -20 °C

10.8.4 Nigericin working solution

1. Add 20µl of stock solution to 20ml of pH solution
2. Gives final working concentration of 10µg/ml

10.8.5 Decrease in pH_i with increasing concentrations of DMO



The impact of increasing the concentration of DMO in the culture media on the pH_i of zygotes. Exposure to 300µm ammonium in the culture media results in a decrease in pH_i of 7.10 (Lane and Gardner 2003), 2mM of DMO resulted in a similar decrease.

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