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Investigation into the effects of oxidative stress on reproductive development

*A thesis submitted in partial fulfilment
of the requirements for the Degree
of*
Master of Science in Biology

By

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ABSTRACT

Nuclear transfer (NT), or cloning, which is the transfer of a donor nucleus to a recipient enucleated oocyte, has been successfully achieved to produce viable offspring in many species. The process is very inefficient, as reprogramming of the donor nucleus is required, and losses are high throughout development. Placentation abnormalities are a common feature amongst cloned animals. Incomplete nuclear reprogramming and erroneous epigenetic imprinting may contribute to aberrant protein transcription and DNA mutations, affecting mitochondrial metabolism and inducing cellular stress. *In vitro* produced embryos under high oxygen culture conditions may also suffer oxidative stress, with the resulting reactive oxygen species causing mitochondrial DNA mutations and cellular stress similar to clones.

In this study, expression of oxidative stress protein markers (Hsp60, SOD2, Hsp70) in NT cotyledons were compared to artificial insemination (AI) at different time points of gestation (days 50, 100, and 150). As a continuum of the oxidative stress investigation in cloned cotyledons, *in vitro* produced embryos were cultured under 20% oxygen compared to the control 7% oxygen laboratory standard culture, with oxidative stress protein markers examined between the groups at blastocyst stage (day 7) and day 15. Embryo morphology was also observed to determine apparent physiological differences between the treatment and control embryos. No previous studies to date have investigated the developmental effects of oxidative stress in day 15 bovine embryos.

The significant differences in oxidative stress proteins observed at several time points in the NT and AI groups were not repeatable, possibly due to sample freeze/thaw degradation. Morphological differences observed between embryos cultured in 20% oxygen and control groups were visually apparent, although not quantified. At day 15 manganese superoxide dismutase expression was significantly lower in the 20% group compared to control. The 20% oxygen group did not show higher heat shock protein 60 expression than control, however the same results have been observed in another study at blastocyst stage. The results of this study suggest that the effect of oxidative stress on embryonic development is evident yet inconclusive in bovine NT cotyledons, however does not appear apparent in day 15 embryos following culture in 20% oxygen.

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ABBREVIATIONS

abs	absorbance
AgR SOF	agresearch synthetic oviduct medium
AI	artificial insemination
APS	ammonium persulphate
ATP	adenosine triphosphate
AU	arbitrary unit
bp	base pair
BSA	bovine serum albumin
cDNA	complimentary deoxyribonucleic acid
cm	centremetres
COC	cumulus oocyte complexes
COX2	cyclo-oxygenase 2
DEPC	diethyl procarbonate
DNA	deoxyribonucleic acid
DNP	2, 4-dinitrophenol
dNTP	deoxyribonucleotide triphosphate
DTT	DL dithiothreitol
ECL	enhanced chemiluminescence
ESOF	early synthetic oviduct fluid
ETC	electron transport chain
FCS	fetal calf serum
GLUT1	glucose transporter I

HCL	hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid
HSOF	hepes buffered synthetic oviduct fluid
Hsp60	heat shock protein 60
Hsp70	heat shock protein 70
ICM	inner cell mass
IVF	<i>in vitro</i> fertilisation
IVP	<i>in vitro</i> produced
kDa	kilo dalton
LOS	large offspring syndrome
LSOF	late synthetic oviduct fluid
M	molar
mA	milliamps
µg	microgram
mg	milligrams
ml	millilitre
mM	millimolar
mm	millimetres
MOPS	3-(N-morpholine)-propane-sulphonic acid
MQ water	millipore Q filtered water
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial deoxyribonucleic acid
<i>n</i>	number
nDNA	nuclear deoxyribonucleic acid
nm	nanometre

NT	nuclear transfer
O ₂	oxygen
p	probability
PCR	polymerase chain reaction
PBT	phosphate buffered saline plus 0.1% Tween 20
PFA	paraformaldehyde in phosphate buffered saline
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	reverse transcription
RT PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOD2	manganese superoxide dismutase
TBE	tris borate EDTA buffer
TBS	tris buffered saline
TCM	tissue culture medium
TE	trophectoderm
TIM	triosephosphate isomerase
V	volts
(v/w)	volume over weight
(w/v)	weight over volume
ZP	zonapellucida
°C	degrees Celsius
µg	microgram
µl	microlitre

μM

micromolar

CHAPTER 1: LITERATURE REVIEW

1.1 WHAT IS NUCLEAR TRANSFER?

Nuclear transplantation has long been a topic of interest by man as a means to study developmental biology and a method of producing genetically identical animals. In the most basic terms, nuclear transfer (NT) involves transferring the nucleus from one cell to another cell which has had its own nucleus removed (Westhusin, 2001). For cloning mammals the NT process involves injecting a donor cell into a mature enucleated oocyte. The donor cell origin may be from embryonic blastomeres, cell lines, or primary cultures derived from adult biopsies (Wells, 2005). An electrical stimulus then fuses the donor cell and cytoplasm together, followed by artificial activation to initiate embryonic development (Wilmot, 2002).

1.1.1 History of Mammalian Nuclear Transfer

Embryos are grown *in vitro* to blastocyst stage before transfer to recipient females where they may grow into viable cloned animals (Wells, 2005). The basic NT (or nuclear cloning) methodology was developed in the 1950s using amphibians (Wells, 2003). The cloning of mammals began in the mid-to-late 1970s with rabbits (Solter, 2000). The first successfully cloned live mammal was in sheep using undifferentiated embryonic blastomeres (Wells, 2003) and has since been achieved using embryonic, foetal or adult cell nuclei in cattle (Forsberg *et al.*, 2002, Kato *et al.*, 2000, Oback *et al.*, 2003), goat (Keefer *et al.*, 2002, Reggio *et al.*, 2001), pig

(Boquest *et al.*, 2002), rodents (Wakayama and Yanagimachi, 2001b), a cat (Shin *et al.*, 2002), rhesus monkey (Meng *et al.*, 1997), rabbits (Chesné *et al.*, 2002), horse (Galli *et al.*, 2003), and mule (Woods *et al.*, 2003). Cloning by NT with somatic cells is commonly used to enable rapid multiplication of elite livestock, for various agricultural and biomedical purposes, although somatic cell NT cloning of cattle is most common due to large numbers of oocytes being available from abattoirs at a low cost (Westhusin, 2001).

1.2 WHAT IS A PLACENTOME?

The cow species belongs to a group called “eutherian”, which are those that have placentas. Placenta appearance varies between species. Bovine embryos have specialized cells at the blastocyst stage, trophoblast cells, which form an outer wall called the trophectoderm that develops into the placenta (Schafner *et al.*, 2000). Based on gross anatomical features, the bovine placenta is classified as cotyledonary (Igwebuike, 2005). This fetomaternal interaction site is distinguished by areas of discrete attachment, formed by interaction of patches of the chorioallantois with the endometrium in a crypt-villous interrelationship (Igwebuike, 2005, Pfarrer *et al.*, 2001). The fetal contribution is called the cotyledon, and the maternal portion of the contact site is the caruncle (referred to as the maternal cotyledon). Together they become a placentome (Igwebuike, 2005).

1.3 PREIMPLANTATION BOVINE EMBRYONIC DEVELOPMENT

Fertilization is the fusion of two specialized cell types, the sperm and oocyte. Mammalian oocytes are surrounded by an extracellular coat, the zona pellucida, which the sperm binds to upon fertilisation. The fertilised oocyte becomes a zygote where the haploid nuclei of the sperm and oocyte co-exist as separate pronuclei before remodelling to form a competent diploid genome. The two pronuclei fusing represent the first cleavage division of the zygote, which takes 32 hours for bovine (Rizos *et al.*, 2003). Cleavage is a series of mitotic divisions without cell growth whereby the enormous volume of egg cytoplasm divides into numerous increasingly smaller, nucleated cells (Figure 1.1) (Bazer *et al.*, 1987, Latham and Schultz, 2001). The second and third cleavages take 13 hours, followed by the fourth cleavage which takes 30 hours and is a critical step as it is at this stage that the embryonic genome kicks in and often a developmental block occurs (Jansen and Burton, 2004). During cleavage, cytoplasmic volume is not increased and these cleavage-stage cells are called blastomeres. A maternal reservoir of proteins and transcripts in the oocyte cytoplasm supports this process and sustains the embryo until compaction when the embryonic genome activation occurs (Jansen and Burton, 2004, Wrenzycki *et al.*, 2005).

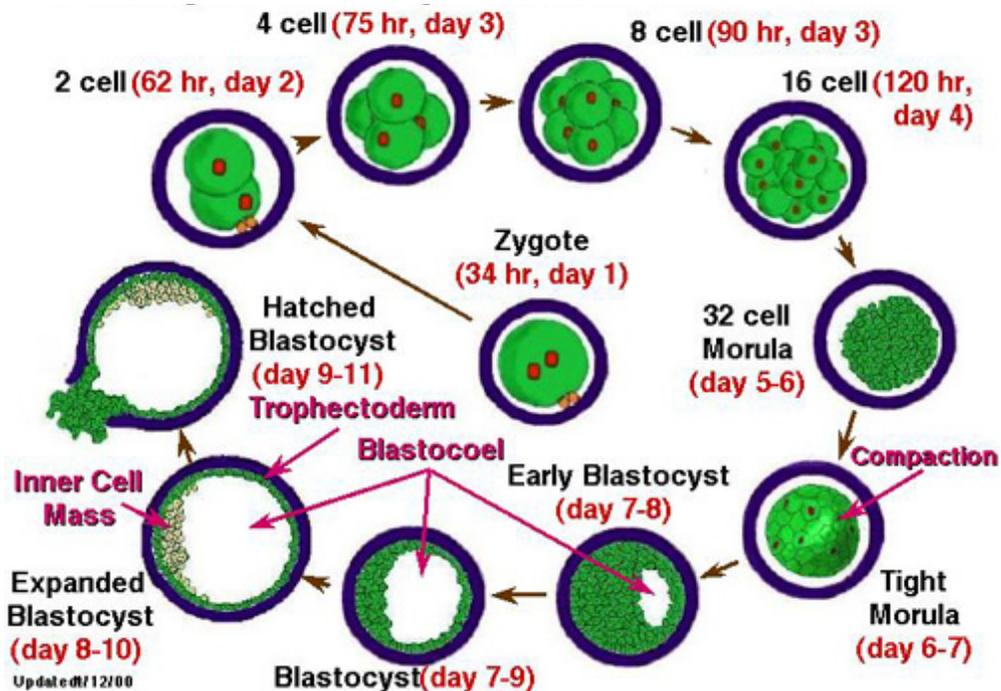


Figure 1.1 Bovine embryo development to the blastocyst stage.

Red dots represent nuclei (including pronuclei of zygote), green represents blastomeres (also yellow at inner cell mass), and blue represents zona pellucida. Time periods in brackets are typical of *in vivo* development (Source: Professor J Parrish, University of Wisconsin-Madison: http://www.wisc.edu/ansci_repro/lec/lec_18/lec18_images.html#anchor303593).

At the 8-16 cell stage the bovine embryo undergoes compaction to the morula stage where gap and tight junctions are established between the blastomeres to create a seal which is important for blastocoel formation (Abe *et al.*, 1999, Wrenzycki *et al.*, 2005). Sodium/potassium pumps form on cell surfaces and adenosine triphosphate (ATP) is required to pump sodium into the blastocoel, passively diffusing water into the cavity to complete the blastocyst stage (Harvey *et al.*, 2002). Cells are now

differentiated into inner cell mass (fetus) to establish the embryonic disc (Maddox-Hyttel *et al.*, 2003), and trophoctoderm (placenta) (Gardner, 1989).

The embryo “hatches” from the zona pellucida at around day 9 and has explosive trophoblast growth at day 14-15 (Maddox-Hyttel *et al.*, 2003). Embryonic development occurs in the oviduct lumen, is free floating, lacks blood supply, and its cellular activities are influenced by its environment (Wrenzycki *et al.*, 2005). This occurs for 25 days in bovine development before implantation with the uterine horns.

1.4 MITOCHONDRIAL STRESS

Studies on embryos produced by NT have demonstrated that gene patterns in the embryo, fetus and placenta are abnormal and suggest that it is incomplete ‘nuclear reprogramming’ that is the cause of the abnormalities observed (Campbell *et al.*, 2005, Holt *et al.*, 2004, Loi *et al.*, 2001). Detrimental effects may be not solely be due to nuclear reprogramming as similar problems are reported after culture with *in vitro* produced (IVP) embryos (Wilmut *et al.*, 2002). However, it is suggested that inappropriate epigenetic modification of imprinted genes during pre-implantation or fetal development may also influence abnormalities, which may be a result of incomplete nuclear reprogramming (Zakhartchenko *et al.*, 2001, Koo *et al.*, 2002).

1.4.1 Epigenetic Influence on Nuclear Transfer Development.

Epigenetics includes gene-regulating activity that is not directly related to the DNA sequence itself but primarily based on modifications such as the methylation of the DNA sequence, which is crucial for processes such as genetic imprinting (Wrenzycki *et al.*, 2005). Genetic imprinting is a mechanism where a proportion of genes are repressed on one of the chromosomes according to the parental origin of the gene, which is an important control during development and is thought to be especially important for regulating fetal growth (Bowles *et al.*, 2007). Disturbed imprinting has been correlated with abnormalities in NT fetal development (Wrenzycki *et al.*, 2005). NT fetuses with developmental abnormalities have displayed aberrant expression of imprinted genes known to have important roles in placental development (Wilmot *et al.*, 2002, Wrenzycki *et al.*, 2005). Failure of the placenta to develop and function correctly is a principal contributor to NT fetal loss (Koo *et al.*, 2002, Wilmot *et al.*, 2002, Wrenzycki *et al.*, 2005, Zakhartchenko *et al.*, 2001). Studies on NT compared with normal mice have resulted in placental overgrowth where NT mice displayed two- to three-fold larger placentas than control mice (Ogura *et al.*, 2002). Clones that survive to reproductive age have generally healthy normal offspring, that show no signs of impaired epigenetic imprinting (Wrenzycki *et al.*, 2005), evidence which supports aberrant growth from epigenetic modification rather than genomic.

1.4.2 Epigenetics and Mitochondrial Oxidative Stress

Epigenetic aberrations that occur due to nuclear reprogramming may result in arrested mitochondrial metabolism, creating free radicals or reactive oxygen species (ROS). During healthy embryonic development, ROS and antioxidants (e.g. superoxide dismutases) in the mitochondria remain in balance. When the balance is disrupted

towards an overabundance of ROS, oxidative stress occurs (Agarwal *et al.*, 2005). The most powerful ROS is the superoxide radical, which is usually detoxified in the mitochondria by manganese superoxide dismutase (SOD2) to produce hydrogen peroxide which is further degraded to water (Catt and Henman, 2001, Samper *et al.*, 2003).

ROS will react with DNA, proteins and lipids (Catt and Henman, 2001). When the polyunsaturated fatty acids of lipids are attacked in lipid peroxidation, numbers of the acids decrease enough to reduce membrane fluidity. Increasing the rigidity of the membranes can change the function of proteins and enzyme complexes in the membrane and also those in the electron transport chain (ETC), creating more ROS and damage, reducing the amount of ATP produced and potentially crippling cellular function (Catt and Henman, 2001, Tuma, 2001).

ROS can modify DNA bases or induce deletions in the DNA strands (Samper *et al.*, 2003). Mitochondrial DNA (MtDNA) is particularly sensitive to ROS damage as it is in close proximity to the ETC and thus first in line to ROS exposure, but mtDNA also has no protective histone proteins and minimal repair systems (Calabrese *et al.*, 2005, Catt and Henman, 2001, Heindleder and Wolf, 2003, Tuma, 2001). This can result in an increased mutation rate and hence damage to the RNA transcripts or to the mtDNA itself (Catt and Henman, 2001). Mutated DNA will synthesize abnormal proteins, including those used in the ETC, causing a further increase in ROS and exaggerating the damage.

1.4.3 Mitochondrial Stress Due to mtDNA Mutations

Aberrant gene expression due to impaired epigenetic imprinting in NT embryonic development has been described in studies (Campbell *et al.*, 2005, Vajta and Gjerris, 2006, Wells, 2003, Wilmut *et al.*, 2002). Hence it was postulated that disturbed gene expression may alter protein structure and function in the mitochondrial ETC, creating a disturbance in metabolism and excessive ROS formation. This is supported by the observation that NT IVP embryos have a darker cytoplasm due to higher lipid content than *in vivo* produced embryos (Farin *et al.*, 2001). Studies suggest higher lipid content is the result of inefficient metabolism of lipid by embryonal mitochondria (Lonergan *et al.*, 2006, Samper *et al.*, 2003).

1.4.4 Mitochondria and ATP in Bovine Embryogenesis

In mature mammalian oocytes and early stage embryos (days 1-5), mitochondria are present in low numbers and generate low levels of ATP when compared to late morula and blastocyst stage (Farin, 2001, Van Blerkom *et al.*, 1998). Dysfunctional mitochondrial respiration resulting from epigenetic modifications to the ETC, increased ROS and mtDNA mutations should be minimized at this early stage due to the minimal requirements of mitochondrial ATP production (Van Blerkom *et al.*, 1998). However, after day 5 in the bovine, ATP is required for blastulation and embryo compaction to blastocyst stage, and increasing amounts of ATP are needed due to metabolism requirements throughout gestation (Jansen and Burton, 2004, Van Blerkom *et al.*, 1998). If mtDNA mutations occur in early cleavage stage embryos, when significant replication of mitochondria and an increase in the demand of ATP are required, the mutated mtDNA will propagate the error to the next generation of cells, compounding adverse influences on mitochondrial function (Catt and Henman,

2001, Tuma, 2001, Van Blerkom *et al.*, 1998).

1.4.5 Epigenetic Errors and the Influence on Gene Expression and Growth

It has been suggested that epigenetic effects and perturbed mitochondrial metabolism may influence transcription factors (e.g. AP-1, fos, jun, myc, erg-1, SAPK and NFkB) (Calabrese *et al.*, 2005) and gene expression patterns which lead to altered fetal and placental growth (Fischer-Brown *et al.*, 2005, Harvey *et al.*, 2002, Ravelich *et al.*, 2004, Rideout *et al.*, 2001, Zakhartchenko *et al.*, 2001). Ravelich *et al.*, (2006) suggest that these growth alterations in NT pregnancies may be due to the over-expression of growth factor genes (TGF- β 1, - β 2, and - β 3) as a result of reduced anti-proliferative growth gene expression (TGF β RI, TGF β RII), ensuing an escape of growth-inhibitory signals and consequently increased growth in NT placentomes, compared to artificial insemination (AI) controls. Fischer-Brown *et al.* (2005) report that bovine fetuses that had previously been under considerable mitochondrial stress during *in vitro* production (20% oxygen (O₂) culture treatment) had 32% larger cotyledons compared to control fetuses (5% O₂ culture) at parturition, abnormalities which were related back to structural disproportionate allocations of inner cell mass (ICM) and trophoctoderm (TE) cells. TE cells consist in a single layer that differentiate into trophoblasts and contribute to the formation of placentomes (Leunda-Casi *et al.*, 2002). Koo *et al.* (2002) also suggest that the abnormal placentation observed in NT pregnancies is related to aberrant proportions of NT blastocysts having a lower ratio of TE compared to ICM cells.

1.5 MITOCHONDRIAL HETEROPLASMY

Eukaryotic cells contain two distinct genomes, DNA located in the nucleus (nDNA), and the mitochondria (mtDNA). During NT, nDNA originates from the donor somatic cell while mtDNA originates from the recipient oocyte. The mtDNA from both the donor and recipient oocyte are mixed in the reconstructed embryo (Han *et al.*, 2004). MtDNA heteroplasmy is the presence of more than one type of mitochondrial DNA within the same cell, and it has been suggested that this mtDNA heteroplasmy may contribute to the failure of NT embryos (Sutovsky *et al.*, 1999, Solter, 2000), as it is associated with numerous clinical abnormalities resulting from point mutations and mtDNA rearrangements (Van Blerkom *et al.*, 1998). Differences in mtDNA sequences from the recipient and donor during nuclear fusion may generate altered amino acid sequence and consequent protein structure, which may result in inadequate interaction between the separate subunits of the electron transport chain, resulting in deficiencies in oxidative phosphorylation and reduced capacity for energy production (Bowles *et al.*, 2007). There are mixed reports that mtDNA of cloned animals were exclusively from the recipient oocyte (Evans *et al.*, 1999, Lanza *et al.*, 2000, Loi *et al.*, 2001), and that mtDNA heteroplasmy exists in healthy cloned calves (Steinborn *et al.*, 2000, Han *et al.*, 2004).

1.6 OXIDATIVE STRESS

1.6.1 The Mitochondrion

Mitochondria are cellular organelles that operate as the cellular “powerhouse”, generating energy by the aerobic metabolism of carbohydrates and fatty acids in a form that is useful to the cell; ATP (Garrett and Grisham, 1999). Mitochondria are central to diverse cellular functions and contribute to signalling pathways and intracellular processes (Dumollard *et al.*, 2007). Not only do mitochondria produce most of the cell’s ATP but they sequester and release calcium, cytochrome *c*, and proteins (Kroemer, 2003). They also produce reactive oxygen species (ROS), intermediary metabolites and are central to cellular processes such as calcium transport and distribution and calcium signalling, the regulation of intracellular redox potential, control of apoptosis, and possibly the mediators of cellular and organismal aging (Balaban *et al.*, 2005).

1.6.2 The Electron Transport Chain and Reactive Oxygen Species

Four large enzyme complexes lie within the inner membrane of the mitochondria making up the ETC using energy substrates and oxygen in a series of reducing and oxidizing steps. The energy released when electrons move from one molecule to another is used to pump hydrogen ions across the mitochondrial membrane, finally being utilized at ATP synthase to produce ATP (Tuma, 2001). Some of the oxygen that is consumed by the ETC is not completely reduced at the end of the chain to form water, but is only partially reduced leaving unpaired electrons to form ROS, hydrogen peroxide, superoxide anion and hydroxyl radical (O’Brien *et al.*, 2004). Figure 1.2 shows the extremely reactive reducing nature of ROS unpaired electrons,

these intermediates readily react with other molecules, stripping electrons and consequently causing the donor molecules to become ROS (Harvey *et al.*, 2002), thus upsetting cellular biochemistry, including genomic integrity (Catt & Henman, 2001).

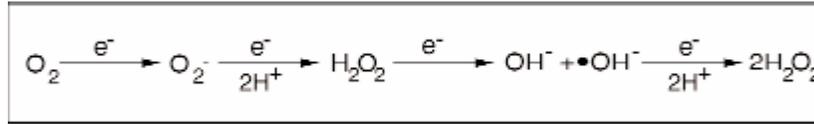


Figure 1.2 *Reduction of oxygen*

Oxygen reduction occurs one electron at a time, giving rise to unstable intermediates that can damage other cellular components. (Source: Tuma, 2001)

1.6.3 Glycolysis and Oxidative Phosphorylation

Embryo development requires energy (ATP) to fuel essential biochemical reactions, which is produced through the ETC via metabolic pathways; glycolysis, using glucose as a substrate, and oxidative phosphorylation, using pyruvate as a substrate (Harvey *et al.*, 2002). During bovine pre-compaction stages (days 1-5) approximately 90% of all ATP is derived from oxidative phosphorylation, and as the energy demand for ATP increases during compaction and blastulation there is a shift in metabolic pathways to glycolysis (Figure 1.3), which coincides with a decrease in oxygen tension as the embryo passes along the oviduct to the uterine cavity (Harvey *et al.*, 2002, Thompson *et al.*, 2000). Glycolysis is mainly inhibited pre-compaction by a block to the glycolytic enzyme phosphofructokinase. High citrate levels keep this enzyme at low levels until the 8 cell stage, and it is then that phosphofructokinase levels must increase at compaction and blastulation to allow glycolysis to produce

ATP. Compromised embryos do not increase levels of phosphofructokinase, therefore glycolysis is minimised and insufficient ATP is created for normal embryonic development (Barbehenn *et al.*, 1974). Oxidative phosphorylation occurs within the mitochondria and is regulated by environmental oxygen concentration and availability of oxygen substrate (Thompson, 2000).

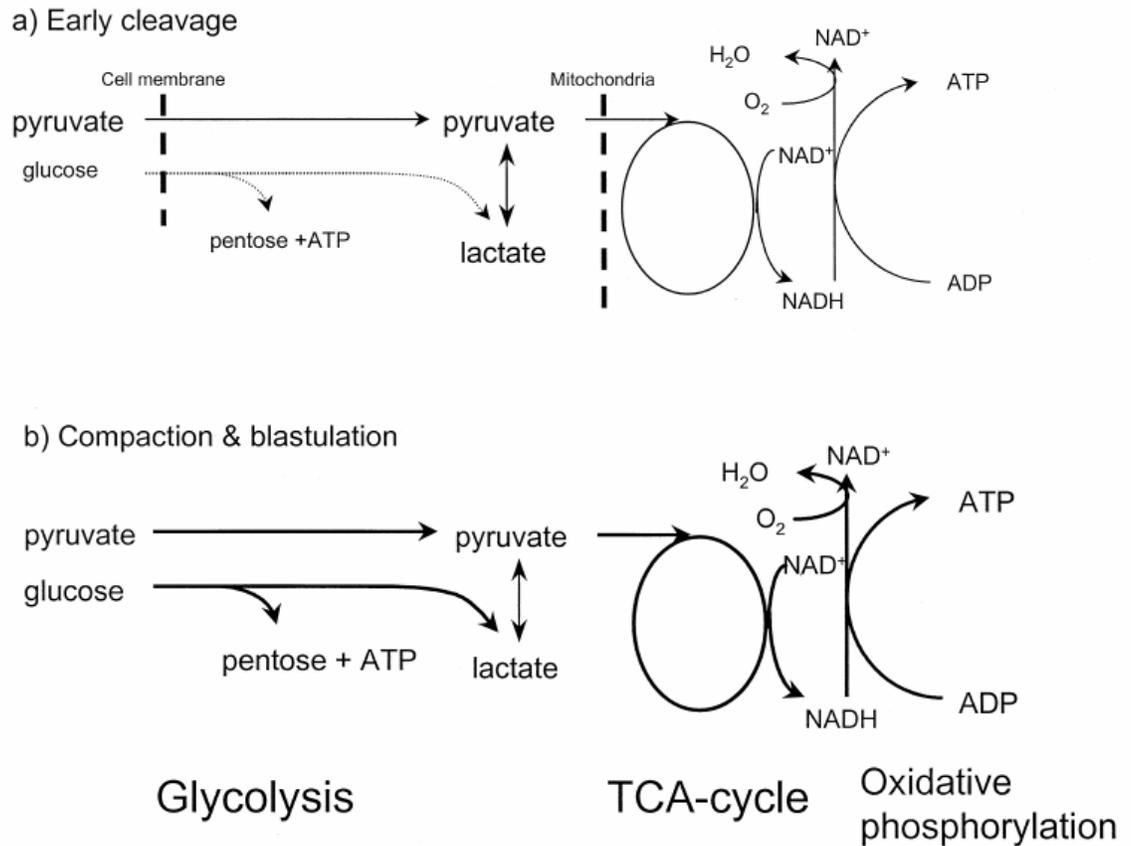


Figure 1.3 *Metabolic pathways in early cleavage, and compaction and blastulation*

a) During the early stages the uptake of glucose is low and uptake of pyruvate is high to provide the fuel for oxidative phosphorylation, with the rate of ATP production low.

b) During compaction and blastulation the uptake of both pyruvate and glucose is high to assist increased demand for ATP. (Source: Thompson, 2000)

1.6.4 The Influence of Oxygen on Oxidative Stress and Embryonic Development

Oxygen reaches the embryo by passive diffusion, which is regulated mainly by oxygen tension. Oxygen is a substrate to oxidative phosphorylation and as more oxygen is available, the integrity of the mitochondrial membrane complexes is compromised when high energy electrons are inappropriately transferred to other molecules as they are channelled along the ETC, generating ROS. Higher levels of ROS are exposed to the membrane complexes causing oxidative stress and mtDNA damage to levels that may halt embryonic development (Catt and Henman, 2001). Results have shown that decreasing the oxygen concentration within the incubation atmosphere from 7% O₂ pre-compaction to 2% O₂ during compaction and blastulation is beneficial to embryo development (Harvey *et al.*, 2002, Thompson *et al.*, 2000). Energy substrate preference can be manipulated using 2,4-Dinitrophenol (DNP), which alters metabolic pathway preference by binding hydrogen ions in the intermembrane space and transports them back to the matrix to partially uncouple oxidative phosphorylation, mimicking the inhibition of oxidative phosphorylation that occurs under reduced oxygen conditions *in vivo* (Harvey *et al.*, 2004). Thus oxidative stress and the resulting ROS are minimised, which is shown to be beneficial to bovine embryo development *in vitro* (Harvey *et al.*, 2002, Harvey *et al.*, 2004, Thompson, 2000, Thompson and Peterson, 2000, Thompson *et al.*, 2000, Rieger *et al.*, 2002).

1.6.5 Mitochondrial Response to Oxidative Stress

To cope with oxidative stress in the mitochondrion a number of protection enzymes are expressed above normal levels, including mitochondria specific SOD2, which is a major ROS scavenging antioxidant enzyme that is located within the inner mitochondrial matrix and converts unstable ROS to more stable forms (Calabrese *et al.*, 2005, Tuma, 2001, Watson, 1998). Heat shock protein 60 (Hsp60) is also mitochondria specific and is involved in cell survival as a result of oxidative stress. Hsp60 functions as a molecular chaperone by protecting stressed cells with their ability to hold, translocate or refold stress-denatured proteins, preventing irreversible aggregation with other proteins in the cell, factors that would be detrimental to cell survival (Kilmartin and Reen, 2004). Hsp60 is also viewed as an inter/intra-cellular signalling molecule and can activate key cellular activities such as the synthesis of cytokines and adhesion proteins (Ranford *et al.*, 2000). Nollen and Morimoto (2002) suggest that heat shock proteins interact with key components of signalling pathways that regulate growth and development, and the relationship of these proteins with various signalling proteins is critical for normal function of these transduction pathways. A disruption in relative levels of heat shock proteins (too high or low) may result in aberrant growth control, developmental malformations, and cell death. Nollen and Morimoto (2002) also state that the ability of a cell to properly recognize and respond to extracellular signals gives it the ability to know whether to grow, divide, differentiate or die; heat shock proteins interact with these signalling molecules. This suggests that altered levels of heat shock proteins in response to oxidative stress alters how much an organism integrates and responds to its normal physiological signals.

1.6.6 Effects of Mitochondrial Hsp60 Response

Heat shock proteins are potent immunogens, implicating Hsp60 as a mediator of immune processes (Ranford *et al.*, 2000). The antigenic properties of heat shock proteins may lead to activation of T-cells, which are involved in inflammation and autoimmunity (Kamphuis *et al.*, 2005). This antigenic response may cause an interaction with maternal epithelium cells, causing killer T-cells to prevent placental trophoblasts burying into the maternal endometrium (Tanaka *et al.*, 2001). Therefore, Hsp60 may be modulating normal placentation processes, causing pathology. The effects of epigenetic aberrations that occur due to nuclear reprogramming may result in mitochondrial stress, disruption to the ETC and consequent mitochondrial dysfunction. Hence, relative levels of mitochondrial specific stress proteins, Hsp60 and SOD2, may be appropriate markers of mitochondrial oxidative stress in NT placentomes. Higher expression of these proteins in NT placentomes compared to AI placentomes may indicate that oxidative stress plays a role in placentation abnormalities of cloned embryos.

1.7 A MODEL OF OXIDATIVE STRESS IN RELATION TO REPRODUCTIVE PROCESSES: *IN VITRO* PRODUCED EMBRYOS

1.7.1 *In Vitro* Culture Erroneous Effects

Epigenetic modification of imprinted genes is one of the main problems in cloned animals (Wilmut *et al.*, 2002, Wrenzycki *et al.*, 2005). Gutierrez-Adan *et al.* (2004) suggest that preimplantation IVP embryos produce alterations in the regulation of transcription of imprinting genes that may result in the abnormalities observed in the cloned offspring. Studies show that *in vitro* culture alters the expression of imprinted genes in murine (Doherty *et al.*, 2000), and bovine embryos (Bertolini *et al.*, 2002). Calabrese *et al.* (2005) suggest that epigenetic effects and perturbed mitochondrial metabolism may influence transcription factors due to free radical generation (ROS) and oxidative damage. The post-fertilization *in vitro* culture environment in which an embryo is grown can have dramatic effects on development (Feil *et al.*, 2006), with oxygen concentrations used during *in vitro* embryo culture cited as a major influence on embryo development, and gene expression (Harvey *et al.*, 2004). Iwata *et al.* (2000) suggest oxygen tension during *in vitro* culture to be one of the key factors involved in large offspring syndrome (LOS); LOS and placental aberrations are listed as the two main developmental dysfunctions associated with NT offspring (Campbell *et al.*, 2005, Heyman *et al.*, 2002, Holt *et al.*, 2004, Lee *et al.*, 2004, Vajta and Gjerris, 2006).

1.7.2 *In Vitro* Culture at Low Oxygen Concentrations

Many previous studies have looked at the effects of oxygen concentration on bovine embryonic development (Harvey *et al.*, 2004, Harvey *et al.*, 2007, Iwata *et al.*, 2000, Oyamada and Fukui, 2004, Yuan *et al.*, 2003). Research has focused on the developmental benefits of using low oxygen concentrations during bovine *in vitro* production, and oxidative phosphorylation uncouplers to demonstrate a significant increase in development that is not dissimilar to *in vivo* uterine tract by creating a more favourable intracellular environment with less ROS (Thompson *et al.*, 2000). However, exposure to culture with an atmosphere of 2% O₂ during post-compaction pre-implantation embryo development has adverse consequences such as reduced fetal weight and abnormal placental development in the mouse (Feil *et al.*, 2006).

1.7.3 *In Vitro* Culture at High Oxygen Concentrations

Recent studies represent embryos cultured under high (20%) and low (7, 5, or 2%) atmospheric oxygen concentrations, aimed at defining an oxygen concentration for optimal embryonic development to blastocyst stage (Harvey *et al.*, 2004, Harvey *et al.*, 2007, Iwata *et al.*, 2000, Yuan *et al.*, 2003). Embryos appear to be subjected to a decreasing oxygen concentration *in vivo* as they pass through higher oxygen tensions in the oviduct (5.3-8.7% O₂) followed by a decrease in oxygen tension at day 3 after ovulation to about 2% O₂ en route to the uterus (Harvey *et al.*, 2007, Yuan *et al.*, 2003). Studies conclude that oxygen is a significant component of the bovine embryonic environment and hyperoxic (above the normal physiological level of oxygen) conditions can influence gene expression and subsequent embryonic development, and although it is suggested that oxidative stress and ROS is associated, the exact mechanism is not known (Harvey *et al.*, 2004, Iwata *et al.*, 2000, Watson *et*

al., 1998). Catt and Henman (2001) state that oxygen reaches the embryo by passive diffusion, which is controlled by oxygen tension. Hyperoxic conditions may cause an elevated generation of ROS due to the high level of available oxygen, as increased levels of oxygen are used as a substrate in oxidative phosphorylation to generate an excess of damaging high energy ROS.

Harvey *et al.*, (2007) have investigated Hsp60 and various other cell cycle regulatory genes (e.g. glucose transporter 1, hypoxia-inducible factors 1 α and 2 α) following culture under 20% and 2% O₂ to blastocyst stage. The researchers state that these genes may have important roles during embryonic development (e.g. energy metabolism, cell cycle regulation) despite expression not being significantly altered by oxygen concentration in their study.

Fischer-Brown *et al.* (2005) demonstrated that bovine calves that were cultured under 20% versus 5% O₂ displayed individual cotyledons 32% larger in the group cultured under 20% O₂, and suggest free oxygen radicals in the culture medium may induce damaging epigenetic effects that lead to abnormal placental growth.

1.8 THIS STUDY

The aim of this research was to investigate the expression levels of Hsp60 and SOD2 as mitochondrial stress markers.

Part A of the study related to the effects of mitochondrial stress in AI, IVP, and NT placentomes. Significantly higher expression of Hsp60 and SOD2 at the protein level in NT compared to AI and IVP would indicate that mitochondrial oxidative stress may have a role in the pathology of cloned embryo placentation. Hsp60 and SOD2 expression was assessed at different time points of day 50, 100, and 150 of gestation to determine if expression levels changed significantly throughout gestation.

Part B of the study investigates the influence of *in vitro* hyperoxic (20% O₂) culture atmospheric conditions compared to control (7% and 2% O₂) without DNP culture conditions at day 7 blastocyst and day 15 time points of gestation. Significantly higher levels of expression of cellular and mitochondrial oxidative stress markers at protein and mRNA levels would suggest that ROS due to oxidative stress may induce epigenetic effects that lead to abnormal placental growth.

The hypothesis of this work was that mitochondrial oxidative stress has a role in the pathology of NT and IVP embryo placentation.

1.8.1 Objectives of Study

Section A

Objective 1 – To investigate levels of Hsp60, Hsp70 and SOD2 in NT placentomes compared to IVP and AI.

Section B

Objective 1- To produce sufficient quantities of IVP embryos under treatment (20% O₂) and control (7% and 2% O₂) without DNP conditions to enable sufficient numbers of embryos for;

- a) Transfer into recipient cows.
- b) An adequate quantity of protein to successfully complete western blot analysis.

Objective 2 – investigate the presence of various stress related markers (Hsp60, Hsp70, SOD2, COX2, TIM, GLUT1) at both protein and reverse transcriptase polymerase chain reaction (RT-PCR) levels.

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Antibodies

The antibodies and their sources are listed in Table 2.1

Table 2.1 *The antibodies used in this study*

<u>Antibody</u>	<u>Product #</u>	<u>Source</u>
Rabbit anti-Actin	MFCD00145889	Sigma-Aldrich
Rabbit anti-Hsp70 polyclonal	ab1428	Abcam
Rabbit anti-Hsp60 polyclonal	SPA-805	Stressgen
Rabbit anti-Manganese Superoxide Dismutase (SOD2) polyclonal	SOD-110	Stressgen
Goat anti-Triosephosphate Isomerase (TIM) polyclonal	sc-22031	Santa Cruz
Mouse anti-Cyclooxygenase (COX2) monoclonal	C9354	Sigma-Aldrich
Goat anti-Rabbit IgG	A6154	Sigma-Aldrich
Sheep anti-Mouse IgG	AP326P	Silenus
Rabbit anti-Goat IgG	P449	Dakopatts

2.1.2 Antigens

The antigen and its source is listed in Table 2.2

Table 2.2 *The antigen used in this study*

<u>Antigen</u>	<u>Product #</u>	<u>Source</u>
Recombinant Rat Hsp60 Protein	SPA 742	Stressgen

2.1.3 Buffers

For RNase-free solutions, the water used was treated with diethyl pyrocarbonate (DEPC; Sigma-Aldrich, USA). 0.01% DEPC was added to MQ water (Millipore Corporation, USA) and incubated at 37°C for 2-3 hours, vigorously shaken a couple of times during incubation and immediately autoclaved to inactivate the DEPC (DEPC treated water). Buffers were stored at room temperature unless otherwise stated.

CAPS Transfer Buffer

25 M tris

0.192 M glycine

20% methanol AR

RNA Loading Buffer

50% Glycerol (BDH, Poole, UK)

1 mM EDTA pH 8

0.25% Bromophenol blue

0.25% Xylene cyanol FF (BDH, Poole, UK)

RNA Electrophoresis Buffer

50 ml 20 x MOPS

178.5 ml 37% Formaldehyde

Make up to 1 litre with DEPC water.

5 x Tris-Borate-EDTA (TBE) Buffer

1 packet Tris-Borate-EDTA (TBE) Buffer mix (Sigma)

1 litre MQ water

4 x Separating Buffer

1.5 M tris (Roche ultrapure)

0.4% SDS (BioRad electrophoresis grade)

MQ water

Add concentrated hydrochloric acid (J.T.Baker) to pH 8.8.

Stored at 4°C

4 x Stacking Buffer

0.5 M tris (Roche ultrapure)

0.4% SDS (Biorad electrophoresis grade)

MQ water

Add concentrated hydrochloric acid (J.T.Baker) to pH 6.9

Stored at 4°C

10 x Tank/Running Buffer

0.025 M tris (Roche ultrapure)

0.192 M glycine (Bio-Rad #1610718)

0.1% SDS (Bio-Rad #1610302)

MQ water

3 x SDS Laemmli Sample Buffer

Original ref Laemmli UK (1970 Nature 227, 680-685)

30% glycerol

15% β -mercaptoethanol

6% SDS

0.1875 M Tris Ph6.8 (Roche ultrapure)

Add grains of bromophenol blue and stir 30 minutes until fully dissolved. The solution should be a deep blue but opaque. Store at -4°C.

6 x Sucrose DNA Loading Buffer

0.25% bromophenol blue

0.25% xylene cyanol

40% (w/v) sucrose in MQ water.

Store at 4°C

2.1.4 Detergent

10% SDS Detergent

10% (w/v) sodium lauryl sulphate (SDS; Ajax Chemicals, Australia)

Dissolved in MQ water at 68°C and adjusted to pH 7.2 with 1 M HCl (BDH, England)

Stored at room temperature

2.1.5 DNA Ladder

DNA ladder was stored at 4°C.

100 bp ladder (1 µg/ µl; Gibco BRL Life Technologies, California, USA)

2.1.6 Gels

12.5% Acrylamide Gel

Separating Gel

2.8 mls MQ water

2 mls separating buffer

3.2 mls 30% acrylamide/bis solution (Bio-Rad Laboratories),

20 µl ammonium persulphate (APS) (Sigma)

5.5 µl TEMED (Bio-Rad Laboratories)

Stacking Gel

1.2 mls MQ water

0.5 ml stacking buffer

0.27 ml 30% acrylamide/bis solution (Bio-Rad Laboratories),

15 µl ammonium persulphate (APS) (Sigma)

2 µl TEMED (Bio-Rad Laboratories)

7% Acrylamide TBS Gel (2 mini-gels)

2.32 mls 30% acrylamide/bis solution (Bio-Rad Laboratories),

1 ml 5 x TBE

6.7 ml MQ water

100 µl APS (Sigma)

10 µl TEMED (Bio-Rad Laboratories)

1.2% Agarose RNA Gel

1.2 grams high melting point (HMP) agarose

88.2 ml DEPC water

10 ml 10 x MOPS

1.8 ml Formaldehyde (BDH, Poole, UK)

2.1.7 Molecular Weight Marker

Precision Plus Protein All Blue Standards (10 µl/application; Bio-Rad Technologies)

2.1.8 PCR Primers

PCR primers and their sources are listed in Table 2.3.

Table 2.3 *The PCR primers used in this study*

Primer Name	Primer Sequence	Fragment Size (bp)	Source
Bovine glucose transporter 1 forward *	CATCGGCTCTGGCATCGTCA	443	Sigma
Genosys			
Bovine glucose transporter 1 reverse *	CGGCCTTTTGTCTCGGGA ACTT		Sigma
Heat shock protein 70 forward **	AAGGTGCTGGACAAGTGCCAG GAGGTGATT	488	Sigma
Heat shock protein 70 reverse **	ACTTGGAAGTAAACAGAAACG GGTGAAAA		Sigma

PCR Primer references

* Bovine sequence is GenBank Accession M60448. Designed by Dr Rita Lee (AgResearch Limited)

** Original reference from; Gutierrez JA and Guerriero, V. (1995) *Journal of Biochemistry*; 305:197-203, in Wrenzycki C et al., *Biology of Reproduction*, 65:309-317 (2001) Nuclear Transfer Protocol Affects Messenger RNA Expression Patterns in Cloned Bovine Blastocysts. EMBL accession number: U09861.

2.1.9 Solutions

MQ Saturated Butan-1-ol

Butan-1-ol : MQ water 1:1

Shake vigorously and stand at least 1 hour

Enhanced Chemiluminescence (ECL) (adapted from Yale University)

Solution A

5.5 mg Luminol (Sigma A8511)[5-amino-2,3-dihydro-1,4-phthalazinedione]

60 µl DMSO

Solution B

2.8 mg p-coumaric acid (Sigma #9008)

100 µl DMSO

Solutions A and B were stored at -80°C

30% hydrogen peroxide

0.1 M tris 8.6

SuperSignal® West Femto Maximum Sensitivity Substrate Kit (Pierce Biotechnology, Rockford, USA).

SuperSignal® West Femto luminol/enhancer solution

SuperSignal® West Femto stable peroxide solution

Incubate blot with 1.5 ml of each solution for 5 minutes before exposure to photographic film.

20 x MOPS (stock solution)

400 mM MOPS (Sigma, Steinheim, Germany)

100 mM Sodium Acetate (Barker, Phillipsburg, USA)

10 mM Ethylenediaminetetraacetic acid disodium salt (BDH, Poole, UK)

Make to a final volume of 100 ml. Adjust to pH 7.0. Add 10 µl DEPC to 0.5 ml ethanol and mix into solution. Leave a short while and autoclave.

10 x MOPS (working solution)

Make up with equal volumes of 20 x MOPS and MQ water.

Gel Fix Solution

50% methanol (AR grade, Biolab)

2% orthophosphoric acid (MERCK)

RO water

10 x CAPS

100mM CAPS

MQ water

Adjust to pH 11 with 10N NaOH. To make 1 x solution, (usually 4L) add 400 mls 10 x CAPS to ~ 3L MQ and then add 400mls AR methanol and make up volume to 4L.

0.3M Guanidine Hydrochloride in 95% ETOH

5.739 g guanidine hydrochloride

200 ml 96% ETOH (100% ETOH and 8 ml MQ water)

10 x Tris Buffered Saline (TBS)

1.5 M Sodium Chloride (Lab Serve)

500 mM Tris Base (J.T.Baker)

Dissolve Tris and sodium chloride in MQ water and adjust pH to 7.4-7.6 with 50-60 mls of concentrated hydrochloric acid (J.T.Baker).

TBS:Tween

100 ml 10 x TBS

1 ml 0.1% Tween-20 (BDH Laboratory Supplies)

Make up to 1 litre with MQ water.

TBS:BSA:Tween

10% 10 x TBS

0.1% bovine serum albumin (Sigma)

0.1% Tween-20 (BDH Laboratory Supplies)

2.1.10 Stains

Colloidal Coomassie Blue Stain

0.06% Coomassie Blue (Bio-Rad R or G-250)

17% ammonium sulphate (BDH)

50% methanol (AR grade, Biolab)

2% orthophosphoric acid (MERCK)

Dissolve coomassie blue powder in methanol. Add orthophosphoric acid, methanol and MQ water (previously dissolved) to volume

Ponceau S Stain

0.1% w/v Ponceau S (Sigma, St. Louis, USA)

1% acetic acid (J.T.Baker, Phillipsberg, USA)

2.1.11 IVP Embryo Media

2 x TCM 199 (500 ml)

1 x 500 ml sachet TCM 199 mix (Gibco, Auckland, NZ)

50 gm kanamycin monosulphate (Sigma)

Filter with a vacuap 0.2µm filter and store at 4°C. Lasts for one week.

HEPES Buffered TCM 199 (H199) (500ml)

250 ml 2 x TCM 199

30 ml STOCK H *(Agresearch, Hamilton, NZ)

10 ml STOCK B *(Agresearch, Hamilton, NZ)

50 ml fetal calf serum (Gibco Life Technologies, NZ)

Bring up to 500 ml with MQ water.

Bicarbonate buffered TCM 199 (B199) (500 ml)

250 ml 2 x TCM 199

50 ml STOCK B *(Agresearch, Hamilton, NZ)

3 ml STOCK C *(Agresearch, Hamilton, NZ)

50 ml fetal calf serum (Gibco Life Technologies, NZ)

Bring up to 500ml with MQ water.

* Details of composition confidential.

Aspiration Medium (100 ml)

Mix together:

2 ml 20% liquid albumin concentrate (ICPBio, Auckland, NZ)

185 µl heparin *(Agresearch, Hamilton, NZ)

Then add to:

100 ml H199

Maturation Medium

Make up 2 x follicle stimulating hormone (FSH) stock:

97.5 ml B199

17.6 mg Ovagen (ICPBio, Auckland, NZ)

Then add 50 ml 2 x FSH stock to:

50 ml B199

100 µl E217B estradiol *(Agresearch, Hamilton, NZ)

500 µl leutinizing hormone *(Agresearch, Hamilton, NZ)

HEPES Synthetic Oviduct Fluid (HSOF)

All following stock solutions are from Agresearch, Hamilton, NZ.

0.1 ml STOCK S3 *

0.02 ml STOCK B *

0.08 ml STOCK H *

0.01 ml STOCK C *

0.01 ml STOCK D *

0.01 ml STOCK KC *

0.003 (w/v) bovine serum albumin (Sigma)

Make up to 500 ml with MQ water.

IVF Medium

0.1 ml STOCK S3 *

0.1 ml STOCK B *

0.01 ml STOCK C *

0.01 ml STOCK D *

0.01 ml STOCK KC *

0.008 (w/v) bovine serum albumin (Sigma)

Make up to 500 ml with MQ water.

2.2 METHODS

2.2.1 *In Vitro* Production of Embryos

All media used during IVP of embryos was warmed and equilibrated in a humidified 5% carbon dioxide in air incubator at 38.5°C, for a minimum of two hours prior to use. Approval to undergo experimental work on animals was granted by the Ruakura Animal Ethics Committee and the University of Waikato Ethics Committee.

2.2.1.1 *Oocyte Retrieval*

Bovine ovaries were collected into a thermos flask containing sterile saline (0.9% w/v) at 30°C from the abattoir and brought back to the lab. Aspiration of oocytes from ovaries commenced within 4 hours from ovary collection. The ovaries were drained and rinsed in sterile saline (0.9% w/v) by hand agitation, strained to remove excess blood and fluid, and covered again with the warm saline. Conical tubes (15 ml) previously warmed in a 30°C incubator, and containing 2 ml of 30°C aspiration medium; HEPES-buffered TCM 199 medium (with Earle's salts; Life Technologies, N.Z.) supplemented with 10 µg/ml heparin (from pig intestinal mucosa; Sigma, St Louis, USA) and 0.4% (w/v) BSA (affinity column purified, ABRD; Immuno-Chemical Products, N.Z.). The purpose of the 2 ml of aspiration medium is to keep the oocytes as metabolically stable as possible until transfer into maturation drops under incubation environment, as described in section 2.2.1. The tubes were stopped with a rubber bung containing an 18 gauge aspiration needle that was connected to a Cook VMAR-5000 regulated vacuum pump. Follicles between 3 and 10 mm in diameter were aspirated under vacuum (45 mm Hg). Follicles were located visually

on the ovary; appearing “blister-like” with clear yellow fluid under the follicle surface. Aspiration of follicles released the oocytes from the follicles to be deposited into the tubes with the follicular fluid (Figure 2.1). Aspiration tubes containing follicular fluid, oocytes, and cellular debris were changed when $\frac{3}{4}$ full and held at 30°C until searching for oocytes (section 2.2.1.2) was completed.

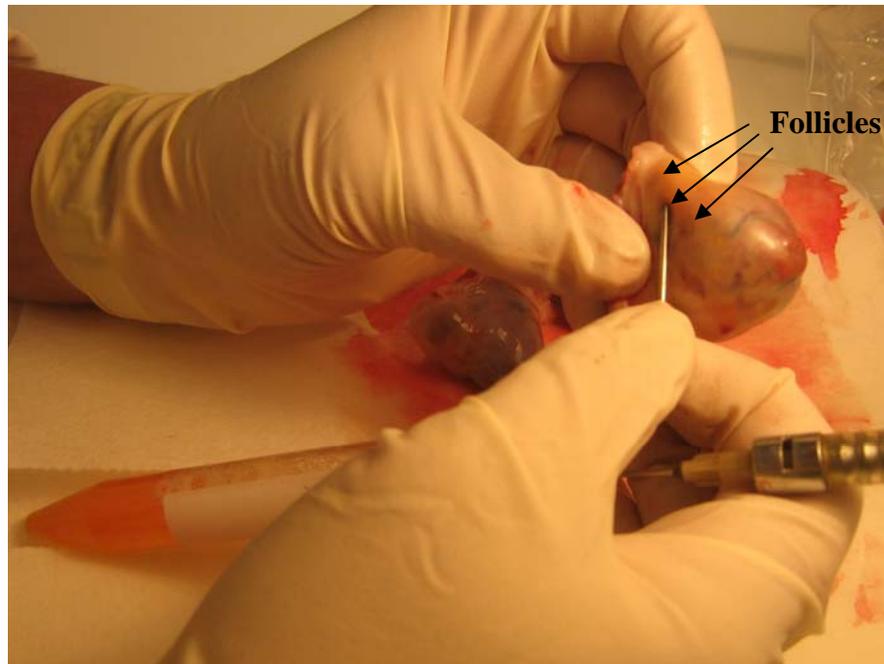


Figure 2.1 *Oocyte aspiration*

Ovaries are aspirated by insertion of a needle into follicles, which is connected to an aspiration pump. The needle is fitted into a rubber bung that is plugged into a conical tube. The oocytes are removed by the vacuum of the aspiration pump from the follicles, with fluid into the tube. The colour of the tube contents is not follicular fluid (yellow), but is due to the initial 2 ml of aspiration medium (red) that is in the tube at commencement of aspiration. Follicles appear “blister-like” on the ovary surface, as indicated by the arrows.

2.2.1.2 *In Vitro* Maturation

The granulosa cells that surround the mammalian oocyte are known as cumulus cells and are important during oocyte development as it is suggested they provide metabolic factors and cell to cell communication that are essential for oocyte maturation (Boni *et al.*, 2002). The cumulus cells and oocyte are bound together in an immature state and are termed cumulus-oocyte complexes (COCs), as shown in Figure 2.2. Successful oocyte maturation is achieved by a culture system that allows the normal progression of oocyte metabolic activity by provision of essential substrates and environmental conditions. Maturation mainly involves RNA transcription and protein synthesis, which is necessary for oocyte meiotic completion and for early embryo development.

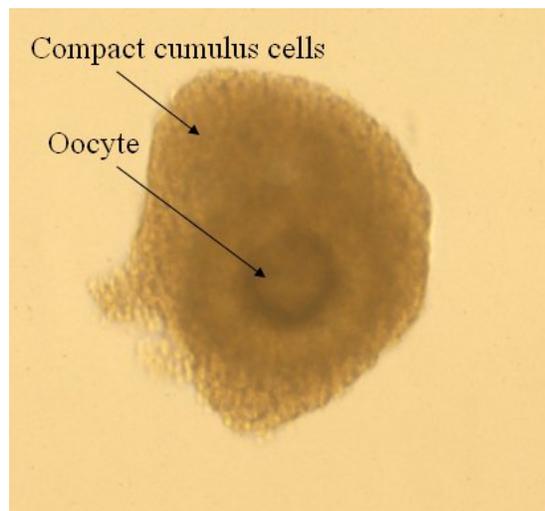


Figure 2.2 *Immature cumulus-oocyte complex (COC).*

The immature oocyte is centrally located and surrounded by cumulus cells to form a complex. (Source: <http://www.theses.ulaval.ca/2002/20201/20201-1.html#Heading209>). Permission to use Figure 2.2 granted by thesis author, Mario Mayes.

COCs were distinguished from the follicular contents using a stereomicroscope (Nikon SMZ800). COCs that appeared light in colour with even cytoplasm and completely surrounded by corona and unexpanded cumulus cells were selected using a 5-50 μ l pipettor and sterile pipette tips. COCs were transferred to a 35 mm dish containing HEPES-buffered TCM 199 + 10% (v/v) fetal calf serum (FCS; Gibco BRL Life Technologies, N.Z.) medium, washed once in a new dish of H199 + 10% (v/v) FCS, and then placed in a dish containing B199 + 10% (v/v) FCS. Ten COCs in 10 μ l of B199 + 10% (v/v) FCS were transferred into a 40 μ l drop of maturation medium under 8 ml of Roberts mineral oil (Eatontown, USA). Maturation medium was TCM 199 supplemented with 10% (v/v) FCS, 10 μ g/ml ovine FSH (Ovagen; Immuno-Chemical Products, N.Z.), 1 μ g/ml ovine lutinising hormone (Immuno-Chemical Products, N.Z.), 1 μ g/ml oestradiol (Sigma, St. Louis, USA) and 100 μ M cysteamine (Sigma, St. Louis, USA). COCs were matured in maturation media in a humidified 5% carbon dioxide in air incubator at 38.5°C for 22-24 hours, to the stage of development for optimal fertilization success (metaphase 2 of meiosis).

2.2.1.3 *In Vitro Fertilization*

Spermatozoa were prepared from frozen semen obtained from a sire that had been characterized as suitable for *in vitro* fertilization in the AgResearch laboratory. Frozen semen straws were purchased by AgResearch Limited that was commercially available from Livestock Improvement Cooperation. This semen had passed Quality Assurance criteria, which included sperm morphology and motility.

The contents of two 0.25 ml straws were thawed in 35°C water for 30 seconds, then layered upon a Percoll gradient (45%:90%). Motile spermatozoa were collected after

centrifugation (Heraeus SEPATECH Labofuge Ae) at 700 x g for 20 minutes at room temperature. The motile fraction was washed in 1 ml HEPES-buffered synthetic oviduct fluid (HSOF) medium (Tervit *et al.*, 1972), comprising of 20 mM HEPES, 5 mM NaHCO₃ and 3 mg/ml BSA (fatty acid-free; ICP Bio, Auckland, N.Z.), and centrifuged at 200 x g for 5 minutes. Following removal of the supernatant, the fraction was re-suspended in *in vitro* fertilization (IVF) medium to a final concentration of 1 million sperm per ml. IVF medium was a modified Tyrode's albumin lactate pyruvate medium (Lu *et al.*, 1987), supplemented with 0.01 mM heparin (Sigma, St. Louis, USA), 0.2 mM penicillamine (Sigma, Steinheim, Germany), and 0.1 mM hypotaurine (Sigma, St. Louis, USA) per L. Following two washes of HSOF and a final wash in IVF medium, 10 µl of IVF medium containing 5 oocytes was transferred into 30 µl IVF medium drops under 8 ml mineral oil. Sperm in 10 µl aliquots were added to each IVF drop no later than 24 hours after the start of oocyte maturation. Oocytes and sperm were co-incubated for approximately 18 hours at 38.5°C under 5% carbon dioxide in air. Fertilization was performed 22-24 hours after maturation had commenced, under the same atmospheric conditions as described for oocyte maturation (section 2.2.1.2).

2.2.1.4 *In Vitro Culture*

It is difficult to establish successful fertilization at day 1 of development without inspecting each embryo under high magnification; therefore all embryos are treated as fertilized until cleavage detection on day 5 when cell division can be visualised. Once the oocyte is fertilized it becomes a zygote. A zygote is the product of the fusion of an egg and a sperm. It contains two copies of each chromosome, one from each parent.

Presumptive zygotes were cultured *in vitro* for 7 days (day 0 = IVF) in biphasic AgResearch Synthetic Oviduct Medium (AgR-SOF). Biphasic media encompasses two phases of media for embryo culture (early SOF [ESOF], and late SOF [LSOF]). This caters to the metabolic differences in embryonic development; the early embryo (day 1-5) uses amino acids and pyruvate for energy sources, whereas morula and blastocysts (day 5-7) rely on glucose and less on amino acids and pyruvate (Thompson, 2000).

AgR-SOF is modified from a previously described formulation (Gardner *et al.*, 1994) and includes essential and non-essential amino acids (Sigma, Ayresshire, UK) and 8 mg/ml BSA (ABIVP, Immunochemical Products, Auckland, New Zealand). Presumptive zygotes were removed from IVF drops, washed twice in HEPES-buffered SOF (Tervit *et al.*, 1972) and ten zygotes were cultured under 20% oxygen, 5% carbon dioxide and 75% nitrogen in ESOF, followed by 2% oxygen, 5% carbon dioxide and 93% nitrogen when changed into LSOF drops.

Gas flow was through a dreshel bottle filled with sterile MQ water to humidify and warm the gas close to the incubation temperature of 38.5°C, enabling immediate optimal incubation conditions for the embryos. Cold gas flow would lower incubation temperature and jeopardise embryonic development. Embryos were transferred to Late SOF media on day 5 of development (day 0 = day of insemination).

DNP was left out of LSOF culture media as demonstrated by Thompson *et al.* (2000). DNP is an uncoupler of oxidative phosphorylation and therefore would interfere with the study of oxidative stress. Thompson *et al.* (2000) showed that at day 5-7 LSOF media culture, 2% O₂ without DNP gave equivalent embryonic development results as 7% O₂ with DNP.

Embryos were graded on day 7 of development using the criteria of Robertson and Nelson (1998). Grading was done visually under 50 x magnification to identify blastocysts from arrested embryos. Top grade blastocysts had a glossy sheen with a distinct membrane around the embryo and clear blastocoel. Occluded blastocysts indicated a decline in quality; more occlusion equated to less quality. Mixed grade blastocysts were washed in HSOF and transferred into a 0.5 ml eppendorf tube with a minimal amount of medium. Tubes were immediately frozen in liquid nitrogen and stored at -20°C.

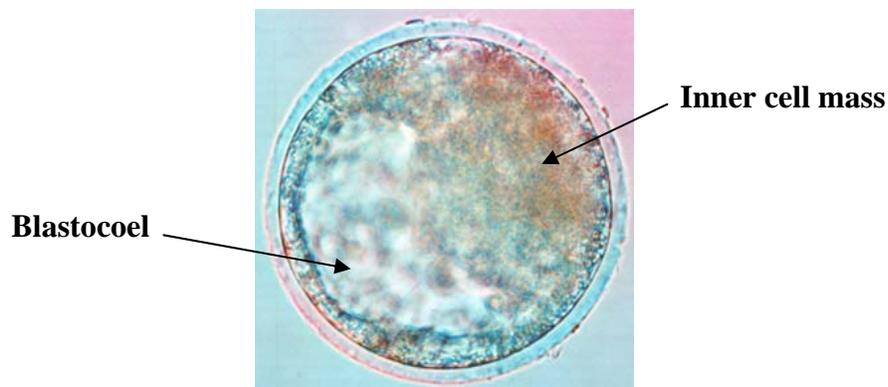


Figure 2.3 *Day 7 blastocyst*

Grade one blastocyst with prominent inner cell mass and clear blastocoel, characterised by a glossy appearance.

2.2.2 Tissue Preparation

2.2.2.1 *TRIZol RNA and Protein Extraction*

RNA and protein was extracted from IVP embryo tissue using TRIzol Reagent (Invitrogen, Carlsbad, USA).

When working with RNA, powder-free sterile latex gloves and sterile tips were used throughout procedures. All reagents and solutions were briefly vortexed (Supermixer, Lab-Line Instruments) and centrifuged in a bench centrifuge (Spectrafuge 16M, Labnet) prior to use, to thoroughly re-suspend and mix components.

Isolation was carried out under a fume hood and plastic safety glasses were worn. DEPC treated MQ water, and autoclaved RNase free eppendorf tubes were used. The procedure was carried out at room temperature, with the exception of centrifugation (Eppendorf Centrifuge 5415D), which was at 4°C.

2.2.2.2 *RNA Extraction from Day 7 Blastocysts*

Due to the very low tissue weight (< 1 mg) of the day 7 blastocysts (10 blastocysts per sample), modifications were made to the TRIzol standard RNA extraction protocol. This method was very kindly provided by Dr Craig Smith of AgResearch Limited.

100 µl of TRIzol Reagent was added to blastocysts in 0.5 ml eppendorf tubes, mixed by vortexing and left for 5 minutes. 1 µl of α -globin (5 pg/µl) and 1µl MS2 RNA

(800 µg/µl) were added to the TRIzol and blastocysts, and mixed by pipetting. After adding 20 µl of chloroform, the mixture was shaken by hand for 15 seconds before being left for 2-3 minutes and centrifuged at 12,000 x g for 15 minutes. The aqueous phase was transferred to a new 0.5 ml tube. An equal volume of isopropanol (60 µl) and 2 µl of linear acrylamide (5 µg/µl, Ambion) was added and mixed gently by hand before leaving for 10 minutes, followed by centrifugation at 12,000 x g for 30 minutes. After the supernatant was removed, a final wash of 70% ethanol was added to the small pellet and vortexed briefly before centrifugation at 12,000 x g for 10 minutes. The RNA pellet was air dried carefully as per standard TRIzol RNA extraction, before re-suspension in 8 µl DEPC water.

2.2.2.3 *Protein Extraction from Day 7 Blastocysts*

Volumes of reagents used were based on the initial TRIzol reagent administered (100 µl), due to initial tissue volume, as recommended by TRIzol reagent protocol. After phase separation, the lower red phase was aliquoted into a 1.0 ml eppendorf tube. 0.3 ml of 100% ethanol was mixed with the aliquot and left at room temperature for 3 minutes to precipitate any remaining DNA. After centrifugation at 2000 x g for 5 minutes, the supernatant was transferred to a fresh eppendorf tube. The tube was then topped up and mixed with 200 µl isopropanol to precipitate the proteins. After leaving for 10 minutes followed by centrifugation at 12,000 x g for 10 minutes, the supernatant was discarded. The remaining pellet was vortexed in 350 µl 0.3 M guanidine hydrochloride in 95% ethanol (wash solution) for 20 minutes, followed by centrifugation at 7,500 x g for 5 minutes. The wash step was repeated using 350 µl of guanidine solution two times, with vortexing for 20 minutes each wash. The samples were washed finally in 350 µl 100% ethanol by brief vortexing, and left for 20

minutes. The remaining protein pellet was air dried, then dissolved in 12 μ l 1% (w/v) SDS solution and stored at -20°C.

2.2.2.4 *RNA Extraction from samples of 50 x Day 7 Blastocysts*

The protocol for RNA extraction supplied by Dr Craig Smith is only recommended for processing small numbers of blastocysts (under 10 per sample). Therefore, the standard TRIzol RNA extraction protocol was used for the samples that equated to 50 blastocysts per sample. One modification to the standard protocol was made; the addition of glycogen to assist with precipitation of RNA.

100 μ l of TRIzol was used in the protocol from Dr Craig Smith for 10 blastocysts. Due to the small mass of 50 blastocysts, weighing the samples gave an insignificant weight to base volume calculations on; therefore the amount of TRIzol used for 10 blastocysts was used as a reference point in the decision to use 200 μ l TRIzol for 50 blastocysts.

200 μ l of TRIzol was added to each 50 x blastocysts sample in 1.0 ml eppendorf tubes before briefly shaking by hand. Samples were incubated for 5 minutes to dissociate the nucleoprotein complexes and 40 μ l of chloroform was added. Eppendorf tubes were capped securely before vigorous shaking by hand for 15 seconds and incubation for 3 minutes. Samples were centrifuged at 12,000 x g for 15 minutes. Following centrifugation the mixture was separated into a lower red, phenol-chloroform phase (protein), an interphase (DNA), and a colourless upper phase (RNA).

The aqueous phase from the initial phase separation of the embryo tissue was transferred to a fresh 1.0 ml eppendorf tube.

Due to such a low quantity of RNA available for extraction, 1 μl of 10 $\mu\text{g}/\mu\text{l}$ glycogen (Roche Diagnostics, Germany) was added with 100 μl isopropanol at the precipitation stage of RNA isolation. Glycogen is insoluble in an ethanol solution and forms a precipitate that traps target nucleic acids (Harju & Peterson, 2001). Glycogen is a high molecular weight molecule which aids with pellet formation during centrifugation, facilitating the handling of target RNA.

Samples were incubated for 10 minutes and centrifuged at 12,000 x g for 10 minutes. The supernatant was carefully removed, taking care not to disturb the gel like pellet on the bottom and side of the tube. This was followed by a wash with 300 μl of 75% ethanol by vortexing to mix and centrifugation at 7,500 x g for 5 minutes. The pellet was then briefly air-dried for 5-10 minutes, taking care not to let the pellet dry completely as this would decrease its solubility. The semi-dried pellet was then solubilised by adding DEPC water and mixing by passing the solution up and down a pipette tip, and incubating for 10 minutes in a 55°C water bath. As no initial weights were established, an estimate of 12 μl of DEPC water was added to each RNA pellet. The samples were stored at -80°C.

2.2.2.5 *Protein Extraction from Samples of 50 x Day 7 Blastocysts*

Protein extraction from the pools of 50 blastocysts per sample, were conducted using the TRIzol protein isolation protocol (section 2.2.2.3). 200 µl of TRIzol was used for 50 blastocysts and other reagent volumes were calculated according to TRIzol protocol recommendations; 60 µl 100% ethanol (DNA precipitation), 300 µl isopropyl alcohol, 400 µl 0.3 M guanidine hydrochloride in 95% ethanol, 300 µl 100% ethanol. After air-drying the remaining pellets for 5-10 minutes, the protein pellets were dissolved in 40 µl 1% (w/v) SDS according to TRIzol protocol (section 2.2.2.3). Samples were then stored at -20°C.

2.2.2.6 *RNA Extraction of Day 15 of Gestation Tissue Samples*

The modified (addition of glycogen) TRIzol protocol (section 2.2.2.4) was used for RNA extraction of day 15 of gestation tissues. The initial volume of TRIzol was adjusted according to embryo weight, which varied between 200 µl for 8 mg of embryo, to 1 ml for 110 mg of embryo. Calculations for other reagents in the procedure were based on these initial TRIzol volumes for each individual sample; 0.2 ml chloroform per 1.0 ml of TRIzol, 0.5 ml isopropanol per 1.0 ml of TRIzol.

TRIzol was added to each embryo in 1.5 ml eppendorf tubes, and tissue was homogenised using a sterile plastic rod to grind the embryo. The plastic rod was washed thoroughly between tissue samples with DEPC water. The amount of DEPC water added to the resulting RNA pellet also corresponded to the weight of the original embryo, 1 µl DEPC water per mg of embryo, which varied between each individual sample from 10-120 µl. The samples were stored at -80°C.

2.2.2.7 *Protein Extraction of Day 15 of Gestation Tissue Samples*

The lower red protein phase of the processed samples was aliquoted into a 1.5 ml eppendorf tube following dissociation of the nucleoprotein complexes. 0.3 ml of 100% ethanol was mixed with the aliquot and left at room temperature for 3 minutes to precipitate any remaining DNA. After centrifugation at 6,500 x g for 5 minutes, the supernatant was transferred to a fresh 1.5 ml eppendorf tube.

Protein extraction from the day 15 of gestation tissue samples was conducted using the standard protein isolation TRIzol protocol (section 2.2.2.3). Reagent volumes were calculated according to TRIzol protocol recommendations: 0.8 ml isopropanol, 1 ml 0.3 M guanidine hydrochloride in 95% ethanol, 0.8 ml 100% ethanol. The remaining protein pellet was air dried, then dissolved in approximately 0.5 ml 1% (w/v) SDS solution and stored at -20°C.

2.2.2.8 *Fetal and Maternal Cotyledon, and Early Gestation Protein Samples*

TRIzol extracted proteins from bovine fetal and maternal cotyledons (day 50, 100, 150 of gestation), and also bovine early gestation (days 20-31) embryos were kindly donated by AgResearch Limited. Blastocysts were made using standard AgResearch *in vitro* produced methodology, and processed using TRIzol extraction protocols, with some modification as described in this section.

2.2.2.9 *RNA Verification*

NanoDrop® was used to determine RNA quantities by measuring 1 µl of each sample. NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies) is a full-spectrum spectrophotometer that measures samples by employing surface tension to hold the sample in place. Absorbance was analysed at A280 (280nm) using the recommended reference type 1 abs = 1 ng/µl.

2.2.2.10 *Visualisation of RNA integrity*

A 1.2% agarose gel was prepared by microwaving 1.2 gram of high melting point agarose (Seakem LE Agarose, Rocklan, USA) with 88.2 ml DEPC water until melted (1-2 minutes). The agarose solution was cooled to approximately 60°C before mixing well with 1.8 ml formaldehyde (BDH, Poole, UK) and 10 ml 10 x MOPS (section 2.1.9). The gel was then poured into a Life Technologies Horizon 11.14 Horizontal Gel Electrophoresis Apparatus to set. RNA electrophoresis buffer (section 2.1.3) was added to the gel and allowed to equilibrate for 10 minutes. 1 µg RNA was mixed by pipetting with 5 µl RNA loading buffer (section 2.1.3) and samples were loaded onto a gel and run at constant 90 mA until the dye front was ½ to ¾ of the way down the gel (approximately 1 hour). The gel was then gently rocked in ethidium bromide (BDH Laboratory Supplies, UK) for 20 minutes. Product identity was confirmed by detection of ethidium bromide staining by ultraviolet light, using a BIO-RAD Gel Doc 1000 imaging densitometer. The presence of 18S and 28S bands verified RNA integrity. Sample aliquots were stored at -80°C.

2.2.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a method for separating proteins by electrophoresis using a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulphate (SDS) to denature the proteins. Protein separation by SDS-PAGE can be used to estimate relative molecular mass, to determine the relative abundance of major proteins in a sample, and to determine the distribution of proteins among fractions.

2.2.3.1 12.5% Acrylamide Gel Preparation

Preparation required casting two different layers of acrylamide gel between glass plates, using a Bio-Rad cassette system. The lower layer (separating gel) is responsible for actually separating the polypeptides by their molecular weight. The upper layer (stacking gel) includes the sample wells and concentrates the protein present in the sample volume.

Casting stands were required to prepare mini-gels. Each gel incorporated 8 x 10 cm and 6.5 x 10 cm glass plates that had been cleaned with methanol, two 0.75 mm teflon spacers and a well former comb. 12.5% acrylamide gels were used to separate polypeptides with molecular weights between 10 and 250 kDa. The separating gels contained 2.8 ml MQ water, 2 ml separating buffer (section 2.1.3), 3.2 ml 30% acrylamide/bis solution (Bio-Rad Laboratories, Hercules, USA), 20 μ l 10% (w/v) ammonium persulphate (APS) (Sigma, Steinheim, Germany), which was prepared bi-weekly, 5.5 μ l TEMED (Bio-Rad Laboratories, Hercules, USA). The gels were overlaid with 60 μ l of MQ water saturated butan-1-ol (BDH Laboratory Supplies, Poole, UK) for 20 minutes at 30°C. The butan-1-ol was rinsed off twice with MQ

water and the 1.5 cm stacking gel cavity was dried with paper towel. A 4% acrylamide stacking gel containing 1.2 mls MQ water, 0.5 ml stacking buffer (section 2.1.3), 0.27 ml 30% acrylamide/bis solution, 15 μ l 10% (w/v) APS, and 2 μ l TEMED was added to the separating gel with a 10 lane comb, and left at room temperature for 15 minutes.

2.2.3.2 *Protein Estimation*

NanoDrop was used to estimate protein levels in the samples. To estimate protein concentration, sample absorbance was analysed at A280 (280nm) using 2 μ l aliquots from each individual sample and the recommended reference type; 1 abs = 1mg/ml.

2.2.3.3 *Sample Preparation for SDS-PAGE*

Protein samples were prepared for SDS-PAGE by adding 3 x Laemmli buffer (section 2.1.3) at 2:1 protein/buffer concentration to total 150 μ l aliquots, before heating at 95°C for 5 minutes. Heating ensured proteins were denatured to obstruct tertiary and secondary structure, enabling binding of the SDS to the protein, aiding efficient polypeptide separation through the acrylamide gels. Prepared samples were cooled to room temperature and stored at -20°C.

2.2.3.4 *Protein Separation and Estimation*

After loading volumes of protein samples using protein estimation acquired from NanoDrop, which equated to 20 µg/lane into the gel lanes, samples were run by electrophoresis in a Bio-Rad Mini PROTEAN 3 Cell tank (USA) with 1 x running buffer (section 2.1.3) at 20 mA per gel for ~ 45 minutes until the dye front was almost at the end of the gel. Initially, gels were fixed in fixing solution (section 2.1.9) for one hour followed by overnight staining with colloidal Coomassie stain (section 2.1.10) to verify equal loading of the protein samples. The gel was rinsed for 15 minutes three times with MQ water. When visually satisfied that samples were of relatively even protein levels, the staining step was omitted for following gels, and proteins were transferred to a 9 x 12 cm nitrocellulose membrane sheet (PALL Life Sciences, Pensacola, USA).

2.2.4 **Western Blot Analysis**

2.2.4.1 *Protein Transfer to Nitrocellulose Membrane*

The nitrocellulose membrane was wet with MQ water for 5 minutes, and then equilibrated in 1 x CAPS transfer buffer (section 2.1.3) for 10 minutes. Two 20 x 15 cm nylon fibre pads were pre-soaked with 1 x CAPS buffer in a plastic transfer cassette before layering one fibre pad, followed by two sheets of 3mm filter paper. The pre-wetted membrane was then placed on the filter paper and the gels were carefully laid on top of the membrane before layering with two more sheets of paper. Air bubbles were removed by gently rolling a glass rod over the surface of the filter paper before placing the final fibre pad on top. After locking the cassette, transfer took place in a transfer apparatus (Bio-Rad Trans-blot Cell) with 1 x CAPS buffer.

Transfer was performed at a constant voltage: either at 20 V overnight, or 50 V for 2 hours. After transfer, the membrane was stained with ponceau S stain (section 2.1.10) for ~ 2 minutes before rinsing 3 times with MQ water and scanning of the image.

2.2.4.2 *Nitrocellulose Membrane Blocking*

The membrane was blocked with 1.2 gram of non-fat milk powder (Anchor, N.Z.) in 30 ml TBS:Tween (section 2.1.9) for either 2 hours at room temperature or overnight at 4°C, followed by 3 x 5 minute washes in TBS:Tween. Blocking destains the ponceau S from the membrane.

2.2.4.3 *Immunodetection*

Primary antibody incubation was either at room temperature for 2 hours or overnight at 4°C with an optimised primary concentration diluted in TBS:BSA:Tween (section 2.1.9). The membrane was then rinsed 3 x in TBS:Tween for 3 x 5 minute washes. The secondary antibody used was conjugated with horse radish peroxidase and was dependent on which species the primary antibody was raised in. Secondary antibody incubation was at room temperature for 1-2 hours, using a concentration optimised for the primary antibody, diluted in TBS:BSA:Tween. After incubation, the membrane was again washed in 2 x 5 minute washes of TBS:Tween, followed by 2 x 5 minute washes of TBS (section 2.1.3) alone.

2.2.4.4 *Chemiluminescence Detection*

Enhanced chemiluminescence (ECL; 24 μ l of solution A and 4 μ l of solution B in 10 mls 0.1M Tris, pH 8.6, with 3 μ l 30% hydrogen peroxide solution) detection was used to visualise the bound antibody by adding the ECL mix to the membrane in a fresh container and gently swirling the solution over the membrane for 2 minutes.

The standard method of chemiluminescence using ECL was insufficient when trying to detect a signal from the day 7 blastocyst blot. Therefore SuperSignal® West Femto Maximum Sensitivity Substrate Kit was used successfully to detect a signal. 1.5 ml each of luminol/enhancer solution and stable peroxide solution were mixed in a clean container to which the blot was added and agitated gently for 5 minutes, continuing with the standard developing procedure as follows.

The membrane was drained of ECL solution and placed between plastic sheets in a light-free X-ray cassette. In a photography darkroom, photographic film (Kodak BioMax XAR) of adequate size to cover the membrane was placed into the cassette on top of the membrane and exposed for a given amount of time. Initially the first time exposure was for 2 minutes. Subsequent exposures were increased or decreased, dependent on the degree of signal after 2 minutes, to gain an optimal signal for analyzing. The film was developed in an automatic X-ray film processor (100-Plus, All-Pro Imaging, NY, USA). After X-ray development, the membrane was rinsed briefly in TBS, dried between filter paper overnight, and kept for subsequent re-probing.

2.2.4.5 *Quantitation of Film-Based Chemiluminescent Blots*

Following chemiluminescent exposure, bands on the photographic film were scanned with a Bio-Rad GS-800 calibrated densitometer. Band intensity was assessed using Bio-Rad Quantity One software, which measures pixel density of a specified area. The background is subtracted from the pixel density within the area of the measured region, giving an arbitrary unit (AU). Proteins of interest were normalised against actin (house-keeping protein) by division with actin AU. The protein of interest AU/actin AU results represent a quantified result of the protein expression.

2.2.4.6 *Statistical Analysis*

The students t-test is the most commonly used method to evaluate the differences in means between two groups; treatment and control groups were employed to determine the significance of the levels of various oxidative stress associated proteins. Students t-test was ideal because it allowed for the uncertainty associated with estimating the variance from a small group size (<30). Variance from a small sample size is more likely to be unrepresentative of the whole population.

If the resulting students t-test was <0.05 it could be suggested that there was a significant difference between the control and treatment levels of protein expression. However, if the result was >0.05 it could be decided that there were not significant levels of protein expression between the control and treatment sample groups.

Group averages of treatment and sample populations were then plotted on bar graphs, incorporating standard error of the mean bars for a visual interpretation of the western blot analysis results.

2.2.5 Reverse Transcription-Polymerase Chain Reaction

Reverse transcription-polymerase chain reaction (RT-PCR) is a sensitive technique for mRNA detection. In the first step of RT-PCR, complementary DNA (cDNA) is made from a messenger RNA template through the process of reverse transcription. Amplification of the resulting cDNA is achieved using polymerase chain reaction.

2.2.5.1 Reverse Transcriptase and First-Strand cDNA Synthesis

Reverse transcription was carried out using a Perkin Elmer Cetus DNA Thermal Cycler. An 11 μ l reaction volume comprising of DEPC water and 1 μ g RNA was added to a nuclease-free 0.5 ml eppendorf tube, along with 1 μ l 3'RACE random primer. After vortexing to mix, the volume was heated at 70°C for 10 minutes and chilled on ice for 5 minutes, followed by brief centrifugation. 4 μ l 5 x First-Strand Buffer, 2 μ l 0.1 M DL-Dithiothreitol (DTT), and 1 μ l 10 mM Deoxyribonucleotide triphosphate (dNTP) per sample were combined, mixed and added to each sample tube. 2.5 μ l was taken from two of the sample tubes to use as a negative control. The tubes were incubated at 42°C for two minutes then 1 μ l of M-MLV reverse transcriptase was added and mixed by gentle pipetting. The samples that had the 2.5 μ l removed for the negative control only had 0.75 μ l added to compensate. After incubating for 1 hour at 42°C, the samples were then inactivated for 10 minutes at 68°C.

2.2.5.2 *Polymerase Chain Reaction*

The purpose of a polymerase chain reaction (PCR) is to make a huge number of copies of a specific section of DNA. There are three major steps in a PCR, denaturation, annealing, and extension, which are repeated for a set number of cycles. This was done on an automated cycler (Thermo Hybaid PCR Express), which heats and cools the tubes with the reaction mixture in a very short time. Per sample, PCR reaction mix consisting of 1.25 μ l 10 x PCR buffer + 15 mM magnesium chloride, 1.25 μ l 2 mM dNTP, 1.25 μ l of each forward and reverse primers (gene of interest; e.g. HSP70), 0.5 μ l of 12.5 mM magnesium chloride, 6 μ l autoclaved, distilled water, and 0.2 μ l fast-start taq polymerase (Applied Biosystems, Foster City, USA), was added to an eppendorf tube, followed by 1 μ l of cDNA reaction product. The samples were then heated at 94°C for 5 minutes to denature, followed by a pre-determined number of cycles of 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 20 seconds, and finally held at 4°C.

2.2.5.3 *DNA Gel*

Mini-gels were assembled and made using 7% acrylamide TBS gels. 2 μ l of 6 x sucrose DNA buffer (section 2.1.2) was added to the 12.5 μ l PCR samples and pipetted to mix. 5 μ l of these samples was added to the gel lanes, including 2 μ l of molecular weight marker (100bp) in one lane. Samples were run at 20 V/gel with TBS buffer until the dye-front was near the bottom of the gel, and then rocked gently in ethidium bromide (BDH Laboratory Supplies, UK) for 20 minutes. Product identity was confirmed by detection of ethidium bromide staining by ultraviolet light, using a BIO-RAD Gel Doc 1000 imaging densitometer.

2.2.6 Embryo Photographs

Several embryos were randomly chosen as representatives of their respective treatment group and fixed in 4% PFA for 10 minutes at room temperature, before placement in 400 µl drops of 0.1% Thimerosal in PBT, in a 4 well plastic dish before being photographed. Embryos were placed on glass slides and images were captured using a Leitz DMIRB inverted microscope, with a SciTec RTSE spot camera, at 100 x and 200 x magnification.

CHAPTER 3: RESULTS

SECTION A

3.1 INVESTIGATION OF HSP60 EXPRESSION BETWEEN AI, IVP AND NT FETAL AND MATERNAL COTYLEDONS

Placental abnormalities in NT cloned fetuses are common and most studies have suggested that epigenetic errors during the cloning process may be responsible for disruption cellular processes. One may speculate that one of these processes is mitochondrial metabolism, causing oxidative stress and a high expression of stress proteins.

This chapter aims to determine if unusually high levels of stress proteins are expressed in bovine cloned fetuses, which may interfere with cellular processes, altering the physiology and function of bovine NT cloned placentomes. The expression levels of proteins specific to oxidative and cellular stress will be studied at the protein level of bovine placentomes.

SDS-PAGE and western blot analysis were chosen as the technique for protein identification to compare expression levels of Hsp60, Hsp70, and SOD2, in AI, IVP, and NT placentomes at time points of day 50, day 100, and day 150 of gestation.

3.1.1 Protein Sample Preparation

Protein samples from a previous trial that were extracted using a standard TRIzol method (section 2.2.2.3) were kindly provided by AgResearch Limited, and stored at -20°C in eppendorf tubes. These samples were identified as Trial 1 for the purpose of this study.

NanoDrop was used to determine protein levels of the samples. NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies) is a full-spectrum spectrophotometer that measures 2 µl samples by employing surface tension to hold the sample in place. To determine protein concentration using NanoDrop, sample absorbance was analysed at A280 (280nm) using the recommended reference type of 1 abs = 1 mg/ml.

In preparation for this investigation, samples were thawed at room temperature before dilution at 2:1 ratio of sample to 3 x Laemmli buffer, to give a total volume of 150 µl. Aliquots were incubated at 95°C for 5 minutes for denaturation and undoing of tertiary structure to enable binding of Laemmli buffer to the protein, and then stored at -20°C.

3.1.2 Protein Separation

SDS-PAGE was employed using 12.5% acrylamide gels and 20 µg of protein per lane to check that the levels of protein did not vary between samples, and equivalent amounts of protein for each sample lane were loaded onto the SDS-PAGE gels, as shown in Figure 3.1. Using recorded NanoDrop sample measurements, calculations identified the volume of individual sample and 3 x Laemmli buffer aliquot required

for 20 µg of protein. The gels were run at 20mA/gel until the dye front was within 0.5 cm of the gel end. Gels were stained overnight with coomassie blue stain (section 2.1.10), which followed with three 15 minute rinses in MQ water. This procedure gave visual verification of even quantities of protein between each sample, as the gels visually appeared to have an even density of protein bands between sample lanes (Figure 3.1). This result indicated that NanoDrop was an accurate way of measuring protein levels in each sample. However, this accuracy is conditional upon samples being from the same tissue types. As NanoDrop estimates protein based on the absorbance at 280 nm by the aromatic amino acids in the samples, different proteins will have different proportions of these amino acids in them, which will affect absorbance. If protein samples come from the same type of tissue (e.g. placentomes), the contribution of amino acids to absorbance will be similar for all samples. The results of the SDS-PAGE gel in Figure 3.1 indicate that despite the different sample groups, the distribution of the major protein bands in each lane was similar.

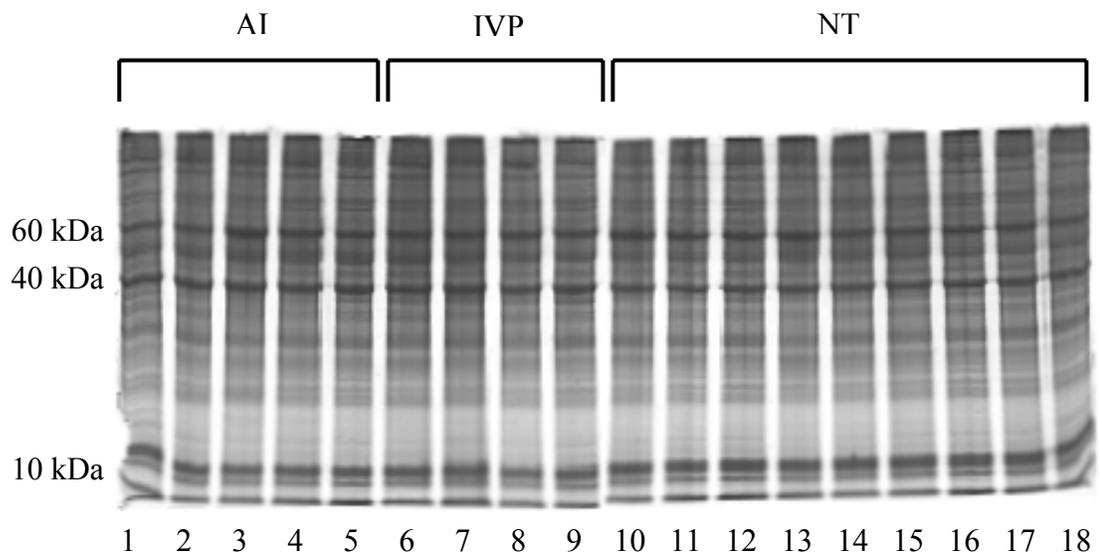


Figure 3.1 *SDS-PAGE gel verification of protein bands by Coomassie Blue staining.*

Protein samples (20 μ g) from day 150 maternal cotyledons were run on SDS-PAGE 12.5% acrylamide gels. Lanes are as follows: 1-5: AI day 150 maternal cotyledons, 6-9: IVP day 150 maternal cotyledons, 10-18: NT day 150 maternal cotyledons. Molecular weights were assigned based on previous gels with molecular weight markers. Major protein bands were present at molecular weights of 10, 40, and 60 kDa, as shown.

3.1.3 Protein Transfer

Upon satisfaction that protein concentration was even between samples (Figure 3.1), proteins separated on a 12.5% acrylamide gel were transferred onto a nitrocellulose membrane via a transfer apparatus (Bio-Rad Trans-blot Cell), as described in section 2.2.4.1. Staining with Ponceau S allowed visual verification that the proteins had been transferred to the membrane successfully (Figure 3.2). All major protein bands

were seen to be transferred onto the membrane from the gel in Figure 3.1. Again, there appears to be no distinct visual difference between the separation and density of AI, IVP, and NT bands.

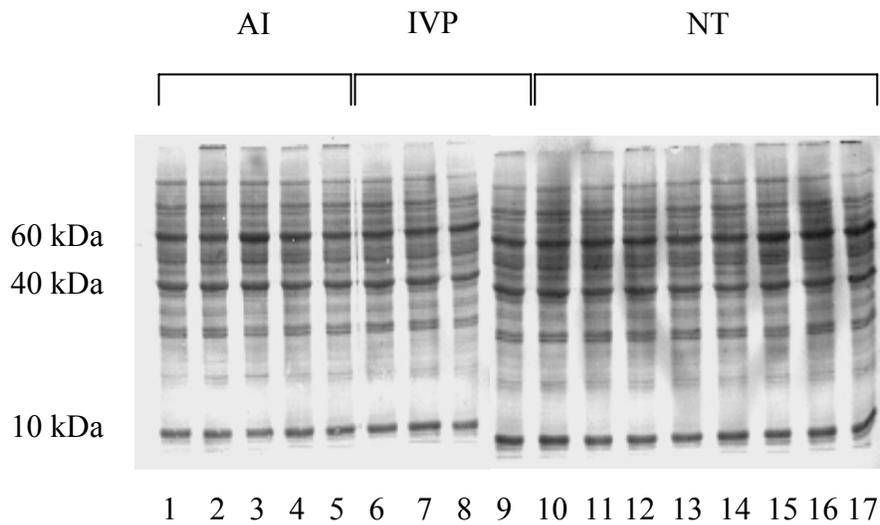


Figure 3.2 Nitrocellulose membrane stained with Ponceau S.

Ponceau S stained transfer blot of day 150 maternal cotyledons 2 hours at 50 volts. Lanes are as follows: 1-5: AI day 150 maternal cotyledons, 6-9: IVP day 150 maternal cotyledons, 10-17: NT day 150 maternal cotyledons. Major bands were seen at 10, 40, and 60 kDa.

3.1.4 Western Blot Analysis

NT day 50 fetal cotyledon proteins were transferred to a nitrocellulose membrane and blocked as described in section 2.2.4.2 for 2 hours at room temperature, prior to probing with polyclonal anti-Hsp60 (1:10,000 dilution) for 2 hours at room temperature.

The blot showed double bands at around the 60 kDa mark (Figure 3.3A), resulting in an inability to confirm which immunoreactive band was in fact Hsp60. The NT day 50 fetal cotyledon blot was repeated using the same samples and probed with fresh antibody after overnight blocking at 4°C instead of 2 hours at room temperature. 5 µl Hsp60 antigen (10 µg) was also used as a positive control to confirm Hsp60 immunoreaction. Background bands became eliminated (Figure 3.3B), suggesting that less background signalling and a clear target protein band (Hsp60) could be obtained by overnight blocking.

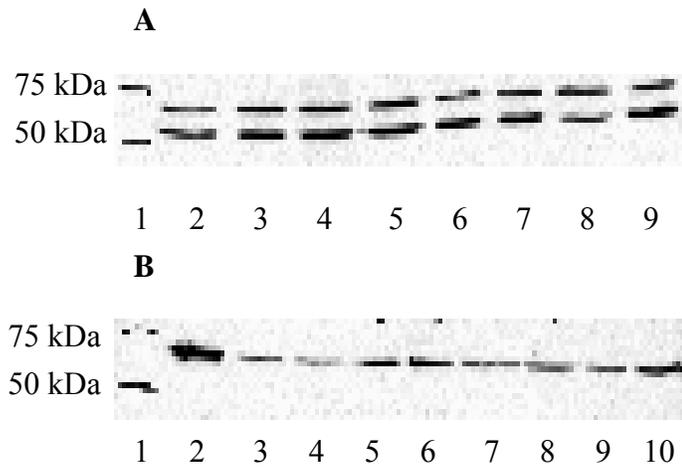


Figure 3.3 *NT fetal cotyledons at day 50 of gestation probed with anti-Hsp60.*

A NT day 50 fetal cotyledons double bands were present between molecular weight markers of 50 and 75 kDa in lanes 2-9. Lane 1: molecular weight marker. **B** lane 1: molecular weight marker, lane 2: Hsp60 antigen, lanes 3-10 NT day 50 fetal cotyledons. The single band was evident at 60 kDa, the same molecular weight as the positive control in lane 1, confirming Hsp60 identity.

3.2 INVESTIGATION OF MITOCHONDRIAL STRESS IN BOVINE FETAL AND MATERNAL COTYLEDONS.

3.2.1 Hsp60 Western Blot Analysis (Trial 1 samples)

Trial 1 protein samples were kindly provided by Agresearch Ltd and had previously been extracted using the TRizol method from fetal and maternal cotyledon tissues at time points of days 50, 100, and 150, with IVP and NT treatment groups, and AI as the control group.

SDS-PAGE 12.5% acrylamide gels were run using 20 µg of protein per sample in each lane as described in section 2.2.3. Following transfer to a nitrocellulose membrane (section 2.2.4.1) and overnight blocking with 1.2g non-fat milk powder in 30 ml TBSTween, expression of Hsp60 was determined with anti-rabbit hsp60 polyclonal antiserum (Stressgen, Victoria, Canada). Anti-rabbit IgG peroxidise-conjugated antibody was used as the secondary antibody. Chemiluminescent detection of the targeted protein was after a 2 minute incubation with ECL and the light signal being detected after exposure of the membrane for 2-4 minutes on x-ray film.

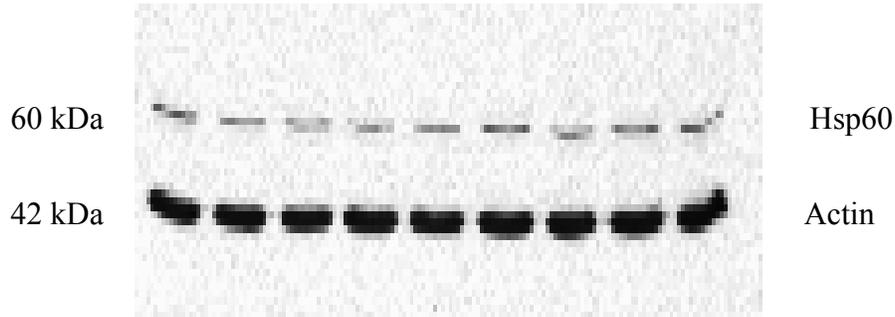


Figure 3.4 *Western Blot of maternal cotyledons at day 150.*

Blot was probed with polyclonal Hsp60 (Stressgen, Canada. 1:10,000) and Actin (Sigma, U.S.A. 1:10,000).

Chemiluminescent detection of the targeted protein (Hsp60) was captured on photographic film after 30 seconds of exposure to the membrane bound with antibodies. Actin was used as a housekeeping protein for normalisation of the level of Hsp60 on each of the blots for fetal and maternal cotyledons at the different time points (Figure 3.4). Following film exposure, Quantity One software provided comparative quantitation of the results, as described in section 2.2.4.5. This enabled statistical analysis with students t-test to compare protein expression between sample groups. Sample groups fetal cotyledons at day 50, 100, 150, and maternal cotyledons at day 50, 100, 150 are presented in Figure 3.5 after western blot and students t-test analysis.

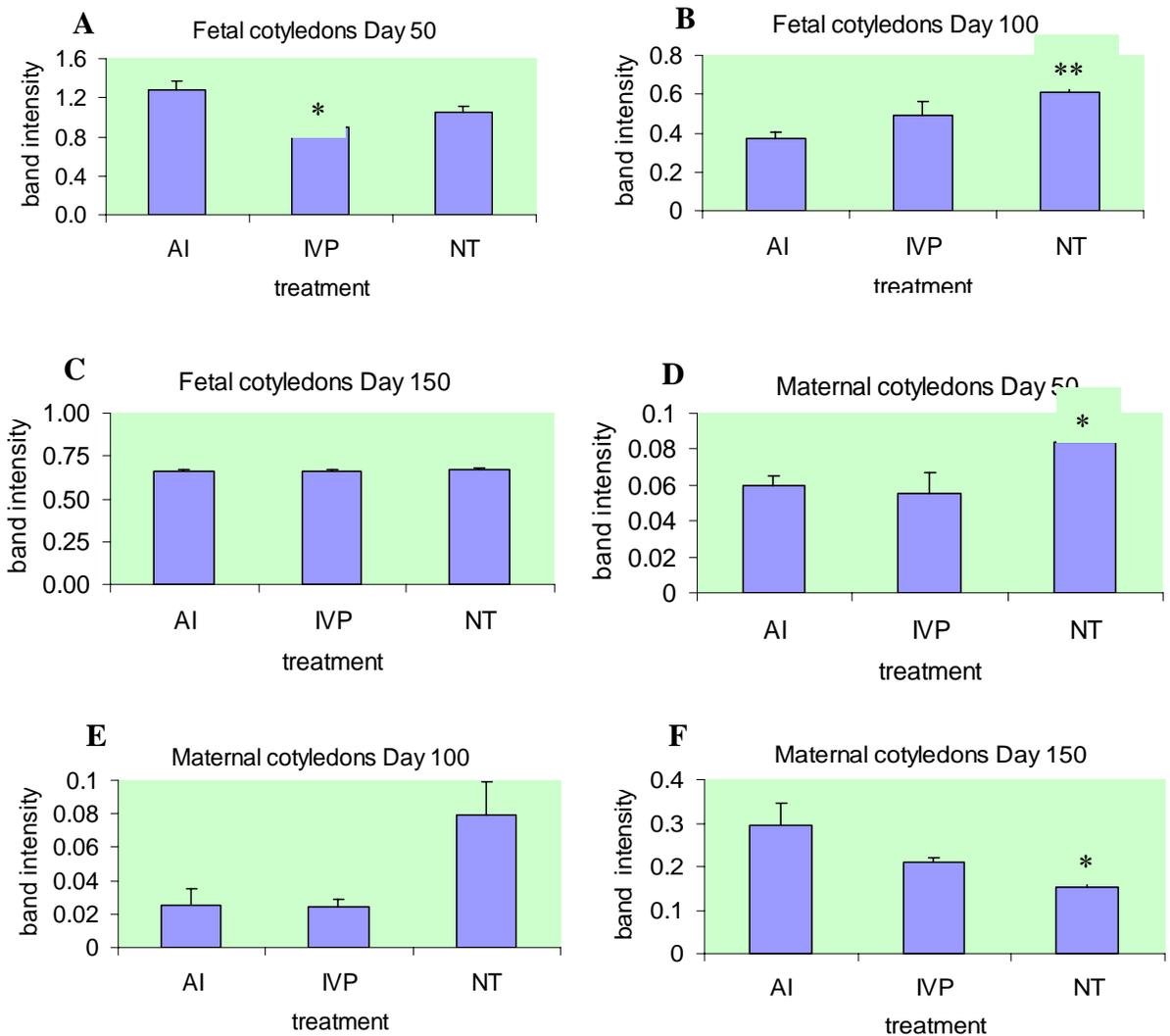


Figure 3.5 *Hsp60* expression in AI, IVP, and NT fetal and maternal cotyledons at time points of day 50, 100, 150 of gestation, from trial 1 proteins.

A AI ($n = 5$), IVP ($n = 3$), and NT ($n = 10$) fetal cotyledons at day 50. IVP was significantly lower than AI, $p = 0.03$. **B** AI ($n = 4$), IVP ($n = 4$), NT ($n = 6$) fetal cotyledons at day 100. NT was significantly higher than AI, $p = 0.009$. **C** AI ($n = 5$), IVP ($n = 4$), and NT ($n = 8$) fetal cotyledons at day 150. **D** AI ($n = 5$), IVP ($n = 3$), and NT ($n = 10$) maternal cotyledons at day 50. NT was significantly higher than AI and IVP, $p = 0.03$. **E** AI ($n = 4$), IVP ($n = 4$), and NT ($n = 6$) maternal cotyledons at day 100. **F** AI ($n = 5$), IVP ($n = 4$), and NT ($n = 8$) maternal cotyledons at day 150. NT was significantly lower than AI, $p = 0.03$. * $p \leq 0.05$, ** $p \leq 0.01$. Bars represent standard error of the mean.

Hsp60 was detected in all samples of fetal and maternal cotyledons at different time points of 50, 100, and 150 days, by western blot analysis with polyclonal anti-Hsp60. Graphs of mean expression levels are presented in Figure 3.5. Fetal cotyledons from day 150 (Figure 3.5C) showed no significant difference between IVP and NT compared to AI. Hsp60 expression was significantly lower than control (AI) in IVP at day 50 (Figure 3.5A), and significantly higher than control (AI) in NT at day 100 (Figure 3.5B). Mixed results were also obtained for maternal cotyledons at the 3 time points. At day 50 (Figure 3.5D), NT Hsp60 expression in maternal cotyledons was significantly higher than control (AI) and IVP. No significant results were obtained for maternal cotyledons at day 100 (Figure 3.5E). NT maternal cotyledons Hsp60 expression was significantly lower than control (AI) at day 150 (Figure 3.5F).

Further western blot analyses were conducted to determine if the significant results obtained with anti-Hsp60 were repeatable. The same sample groups were used under the same conditions to keep variables constant. After one repeat, the significant results obtained earlier were not repeatable, with the exception of maternal cotyledons at day 150, which displayed significant results between AI and NT. However, compared to the initial blot where AI showed significantly higher Hsp60 expression than NT (Figure 3.5F), the results were reversed with NT displaying significantly higher band densities than AI (Figure 3.7).

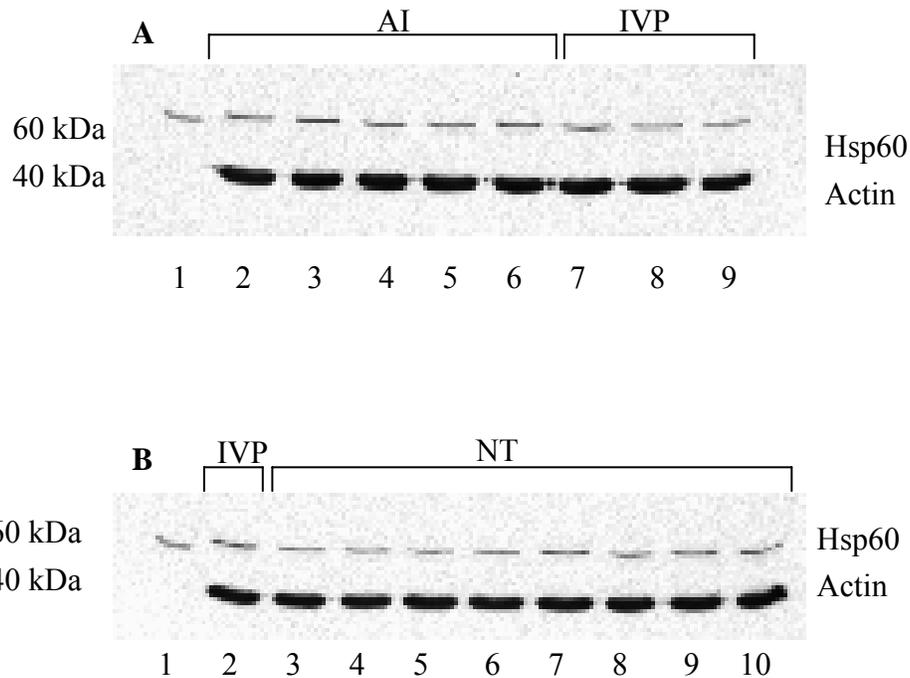


Figure 3.6 Repeat western blot of maternal cotyledons at day 150.

A lane 1: Hsp60 antigen (positive control). Lanes 2-6: AI (control) Hsp60 and actin, lanes 7-9: IVP Hsp60 and actin. **B** lane 1: Hsp60 antigen, lane 2: IVP Hsp60 and actin, lanes 3-10: NT Hsp60 and actin. 20 μ g of protein per lane was used.

The results of maternal cotyledons at day 150 repeat western blot analysis (Figure 3.6) are represented in a bar graph after a student t-test was implemented to determine a statistical difference between NT and AI (Figure 3.7).

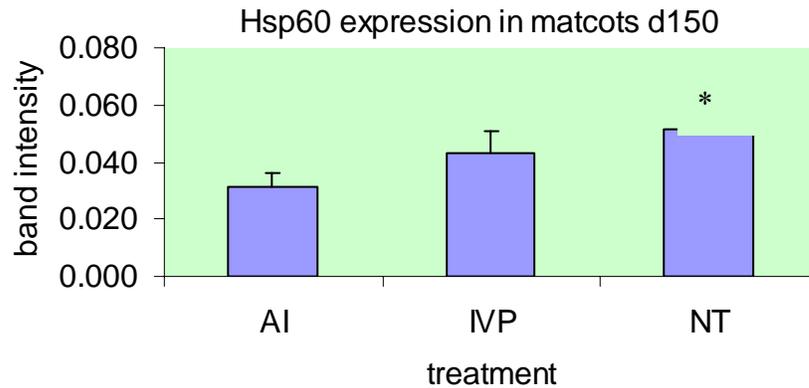


Figure 3.7 Repeat western blot analysis of Hsp60 expression in maternal cotyledons at day 150.

Hsp60 expression levels appeared to be significantly higher in the NT group of maternal cotyledons at day 150, compared to the control (AI) group when group averages were analysed with students t-test. These results differed to the original western blot analysis using the same samples under the same conditions, where NT was significantly lower than AI (Figure 3.5F). $p = 0.02$, * $p \leq 0.05$. Bars represent standard error of the mean.

By implementing visual inspections of the western blot x-ray scans, it appeared that there were minimal differences in band density when comparing between the treatment groups on each individual blot. As the Quantity One analysis software only compares samples on the same x-ray film, comparative analysis between x-ray films of different western blots may account for the inconsistent results. Example comparisons between repeated western blots are presented in Figure 3.8.

Initial Hsp60 western blot

Repeated Hsp60 western blot

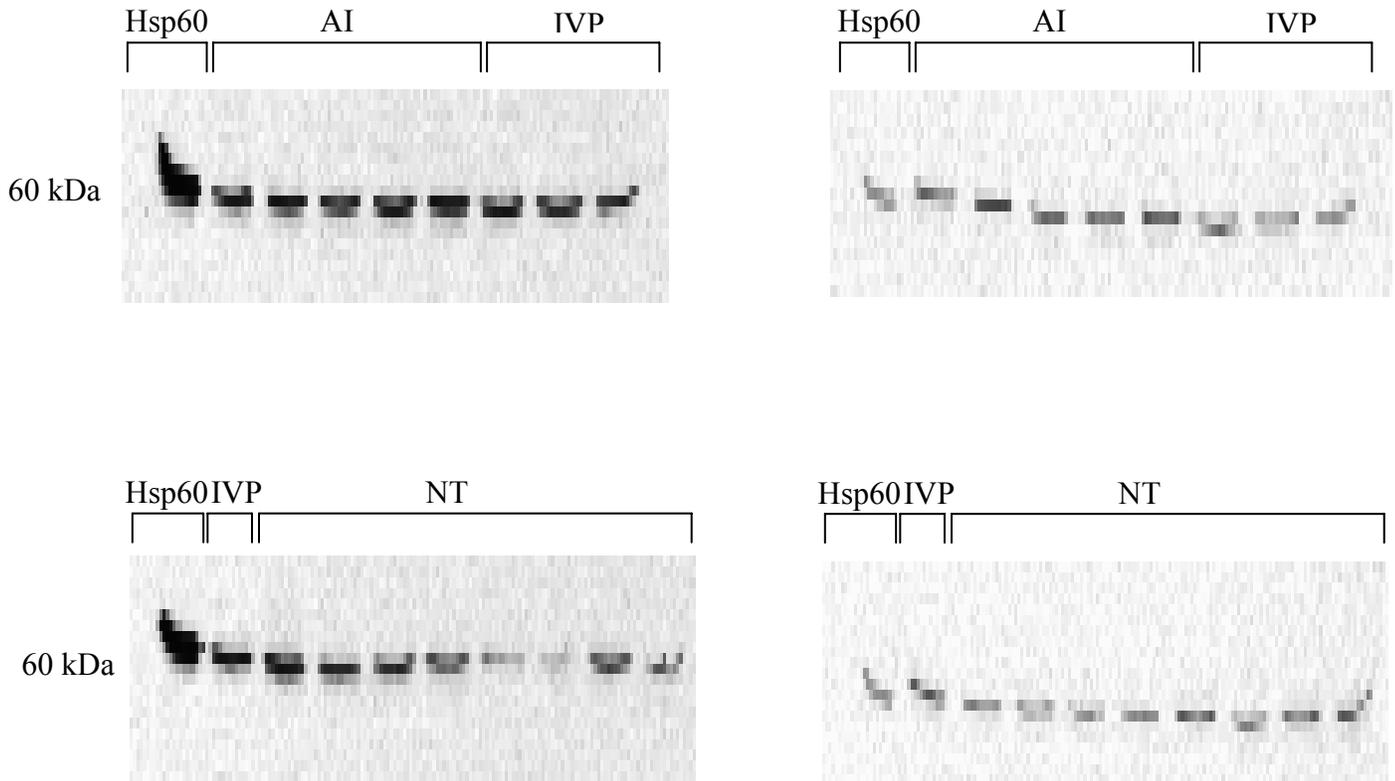


Figure 3.8 Comparison between maternal cotyledon day 150 Hsp60 initial and repeated western blots.

The same quantity of protein samples (20 μ g) were used in the each lane for the repeated blot, with an Hsp60 positive control in each blot. Visually, there is not much difference between control (AI) and treatments (IVP and NT), making any differences in band densities susceptible to errors during analysis with Quantity One software.

3.2.2 Hsp60 Western Blot Analysis (Trial 2 samples)

Previous protein samples from fetal and maternal cotyledons at time points of day 50, 100, 150, were from Trial 1. Protein samples which were extracted using TRizol were made available by Agresearch Ltd from a different trial (Trial 2) and using different time points with a treatment group of NT and AI as control. To enable a thorough investigation of Hsp60 expression between treatment and control groups by determining that the prior obtained results were not specific to Trial 1 tissue samples, protein samples from Trial 2 were probed with Hsp60, again using SDS-PAGE and western blots. Trial 2 proteins were from bovine placentomes, which differed from the protein samples of Trial 1 in that placentomes are the unseparated fetal and maternal cotyledon tissues still joined together, compared to Trial 1 fetal and maternal cotyledons, which are the separated components of the placentomes. AI served as control, with NT being the treatment group. No IVP samples were available from Trial 2. Due to the samples originating from mixed time points, they were grouped together into days 135 – 154 ($n = 16$), and days 154 – 163 ($n = 9$). Again, SDS-PAGE and western blot analysis was undertaken with polyclonal anti-Hsp60. The results of this investigation are presented in Figure 3.9.

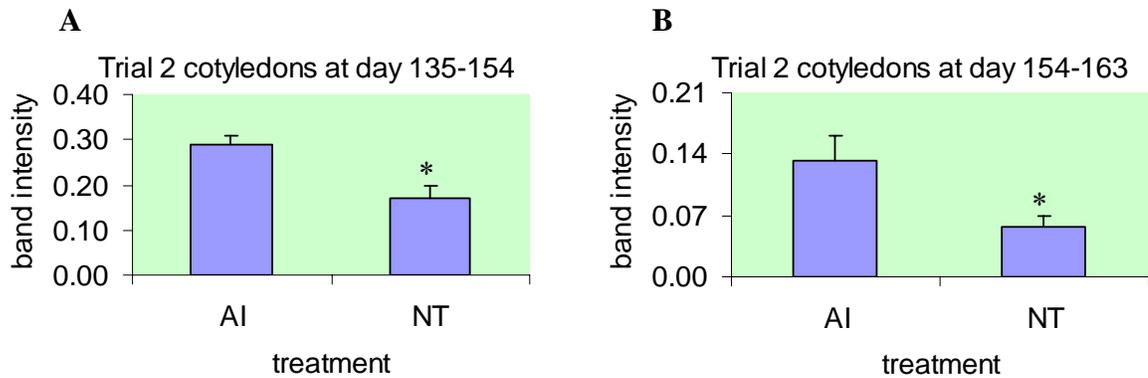


Figure 3.9 *Hsp60* expression in AI and NT placentomes at time points of day 135-163 of gestation, from Trial 2 proteins.

A AI ($n = 6$) and NT ($n = 10$) placentomes at days 135-154. NT was significantly lower than AI, $p = 0.01$. **B** AI ($n = 4$) and NT ($n = 5$) placentomes at days 135-163. When group averages were analysed using students t-test, NT was significantly lower than AI, $p = 0.03$. * $p \leq 0.05$, ** $p \leq 0.01$. Bars represent standard error of the mean.

In both groups *Hsp60* expression is significantly higher in AI compared to NT. This corresponds with maternal cotyledons at day 150 (Figure 3.5F) which had the same results at a similar time point (~ day 150).

3.2.3 Hsp70 Western Blot Analysis

Heat shock proteins are associated with cellular response to stress. They function to protect stressed cells as molecular chaperones by holding, translocating or refolding stress denatured proteins to prevent irreversible aggregation with other proteins in the cell (Oliveira *et al.*, 2005). Heat shock protein 70 (Hsp70) operates in the cell cytosol and is induced when the cell is stressed (Kawarsky & King, 2001). Hsp70 is essential for activation of nuclear hormone receptors such as glucocorticoid and progesterone receptors, hormones which are vital for embryonic/fetal development. Increased levels of Hsp70 have been shown to disrupt signalling effects on proteins involved in signalling pathways, resulting in cell growth arrest and developmental defects (Nollen & Morimoto, 2002). Hsp70 is one of the earliest embryonic genes to be expressed, has been seen to be 15 times higher in bovine 2 cell IVP embryos compared to AI, and is recognised as having an important role in fetal development by regulating apoptosis (Jansen & Burton, 2004). Altered Hsp70 transcription has also been observed in NT-derived blastocysts (Wrenzycki *et al.*, 2005) and the heart, liver, spleen, lung, kidney and brain of cloned calves (Li *et al.*, 2004)

Having looked at mitochondria specific stress protein (Hsp60) with some level of increased expression, Hsp70 expression levels may indicate general cell stress with possible involvement in embryonic/fetal development disruption.

Hsp70 proteins were detected with an anti-rabbit hsp70 polyclonal antiserum (Abcam, Cambridge, UK) in day 150 maternal cotyledons (Figure 3.10). Incubation with the primary antibody was performed for 2 hours at a 1:200 dilution. Goat anti-rabbit IgG peroxidase-conjugated antibody (1:20,000, 1 hour) was used as a

secondary antibody. After addition of ECL solution for 2 minutes the membrane was exposed on x-ray film for 20 seconds to give a strong signal.

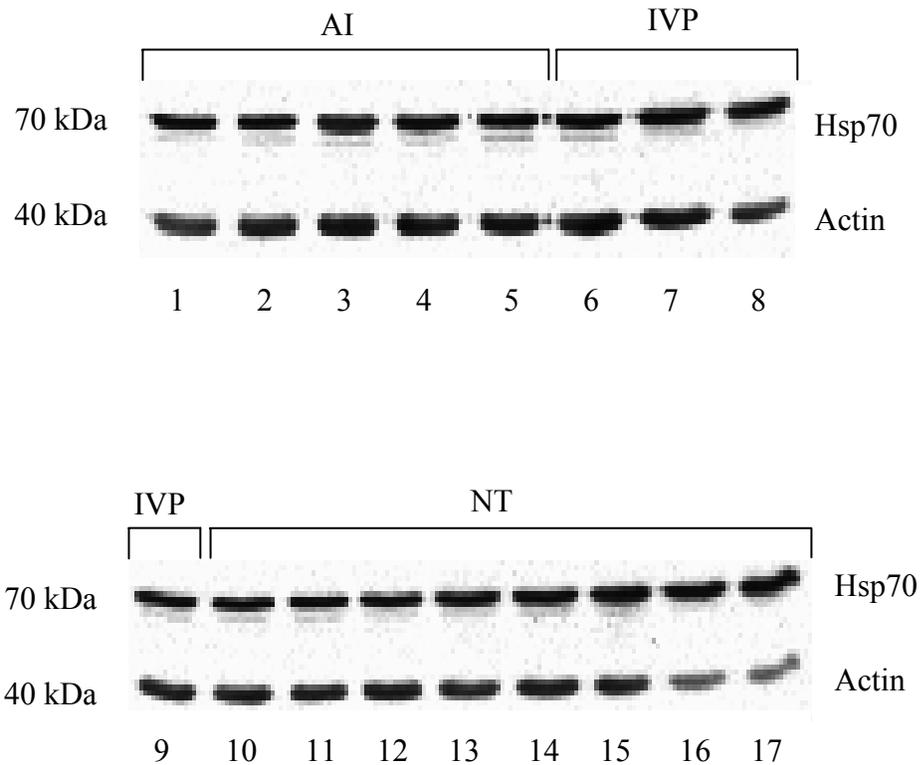


Figure 3.10 *Hsp70* western blot of maternal cotyledons at day 150.

20 μ g of protein per lane. Lanes 1-5: AI (control), lanes 6-9: IVP, lanes 10-17: NT (treatment).

20 μ g of protein per sample of day 150 maternal cotyledons were separated by SDS-PAGE (section 2.2.3) and blocked with 1.2 grams non-fat milk powder in 30 mls TBS-Tween overnight. Proteins were transferred to nitrocellulose membrane (section 2.2.4.1), prior to two hours primary incubation of anti-Hsp70 (1:200 dilution), and 2 hours secondary incubation anti-rabbit IgG peroxidase-conjugated antibody (1:20,000

dilution). Following students t-test, results were presented in a bar graph (Figure 3.11) to show that Hsp70 expression in maternal cotyledons at day 150 was significantly higher in NT (treatment) placentomes compared to AI (control), $p = 0.001$. Unfortunately further investigation into Hsp70 expression in bovine placentomes was not able to be done due to unavailability of Hsp70 antibody.

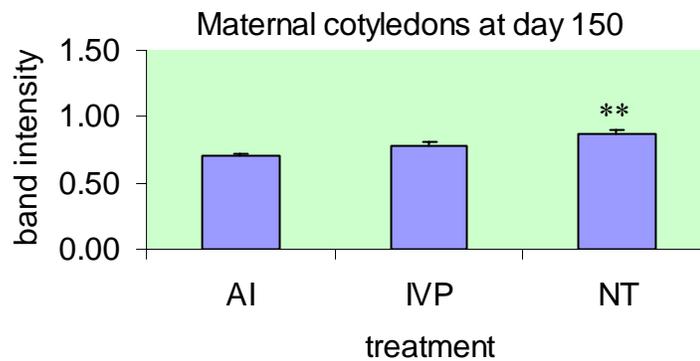


Figure 3.11 *Hsp70 expression in maternal cotyledons at day 150 of gestation*

AI ($n = 5$), IVP ($n = 4$), and NT ($n = 8$) maternal cotyledons at day 150 of gestation. Hsp70 expression was significantly higher in NT (treatment) than AI (control), $p = 0.001$. ** $p \leq 0.01$. Bars represent standard error of the mean.

3.2.4 SOD2 Western Blot Analysis

To check for oxidative stress, all six sample groups from Trial 1 protein blots were probed with SOD2 using the same western analysis method described in section 2.2.4. SOD2 is a mitochondria specific antioxidant metalloenzyme. 20 µg of protein per sample of day 150 fetal cotyledons were separated by SDS-PAGE (section 2.2.3) and blocked overnight following protein transfer, prior to two hours incubation with anti-SOD2 (1:20,000 dilution), and 2 hours incubation with anti-rabbit IgG peroxidase-conjugated antibody (1:20,000 dilution).

The only significant result was fetal cotyledons at day 150; SOD2 showed higher expression in NT than AI, $p = 0.002$ (Figure 3.12).

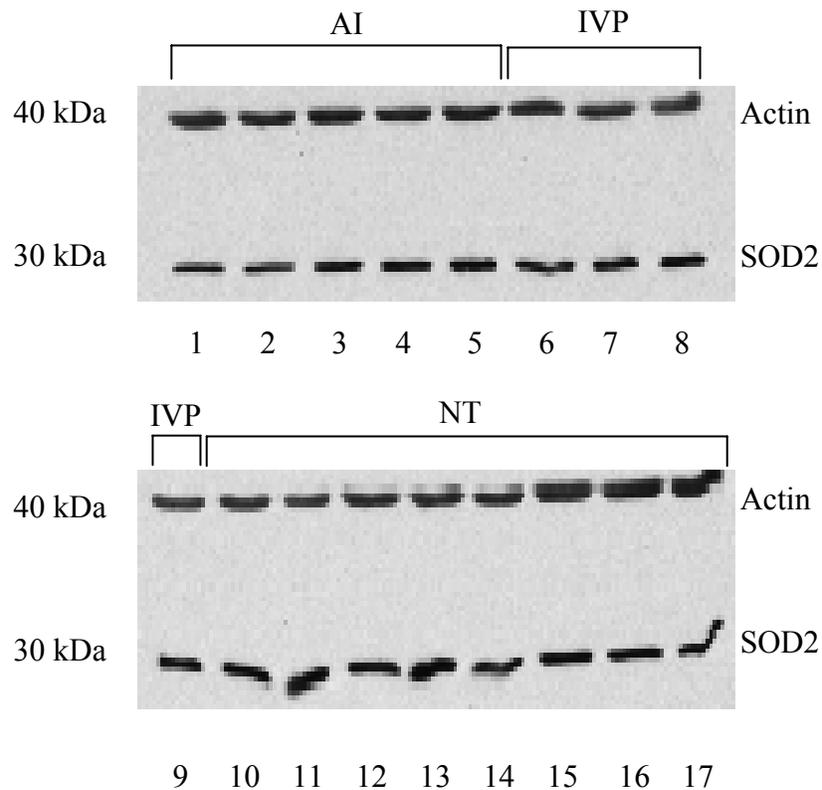


Figure 3.12 *SOD2* western blot of fetal cotyledons at day 150.

20 μ g of protein per lane. Lanes 1-5: AI ($n = 5$), lanes 6-9: IVP ($n = 3$), lanes 10-17: NT ($n = 8$).

When group averages were analysed using students t-test (Figure 3.13), SOD2 expression was significantly higher in NT (treatment) than AI (control) placentomes, $p = 0.002$. ** $p \leq 0.01$. However, when the samples from Figure 3.12 were analysed with another western blot under the same conditions with anti-SOD2, the results no longer showed any significant difference between the sample means.

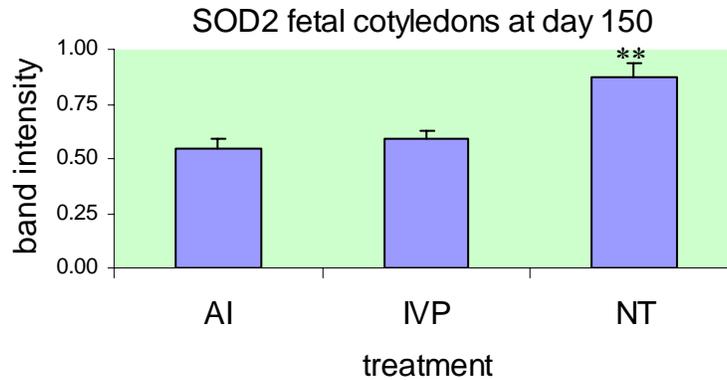


Figure 3.13 *SOD2 expression in AI, IVP, and NT fetal cotyledons at day 150*

AI ($n = 5$), IVP ($n = 4$), and NT ($n = 8$) fetal cotyledons at day 150. NT was significantly higher than AI, $p = 0.002$. ** $p \leq 0.01$. Bars represent standard error of the mean.

3.2.5 Early Gestation Pre-Trial Investigation

Western blot analyses of protein samples extracted from IVP embryonic tissue ranging from day 20-31 of gestation were completed to determine if Hsp60 was present, and a students t-test identified any statistical significance (Figure 3.14A). The samples were divided into two groups; days 20-24 (embryonic tissue) and days 26-31.

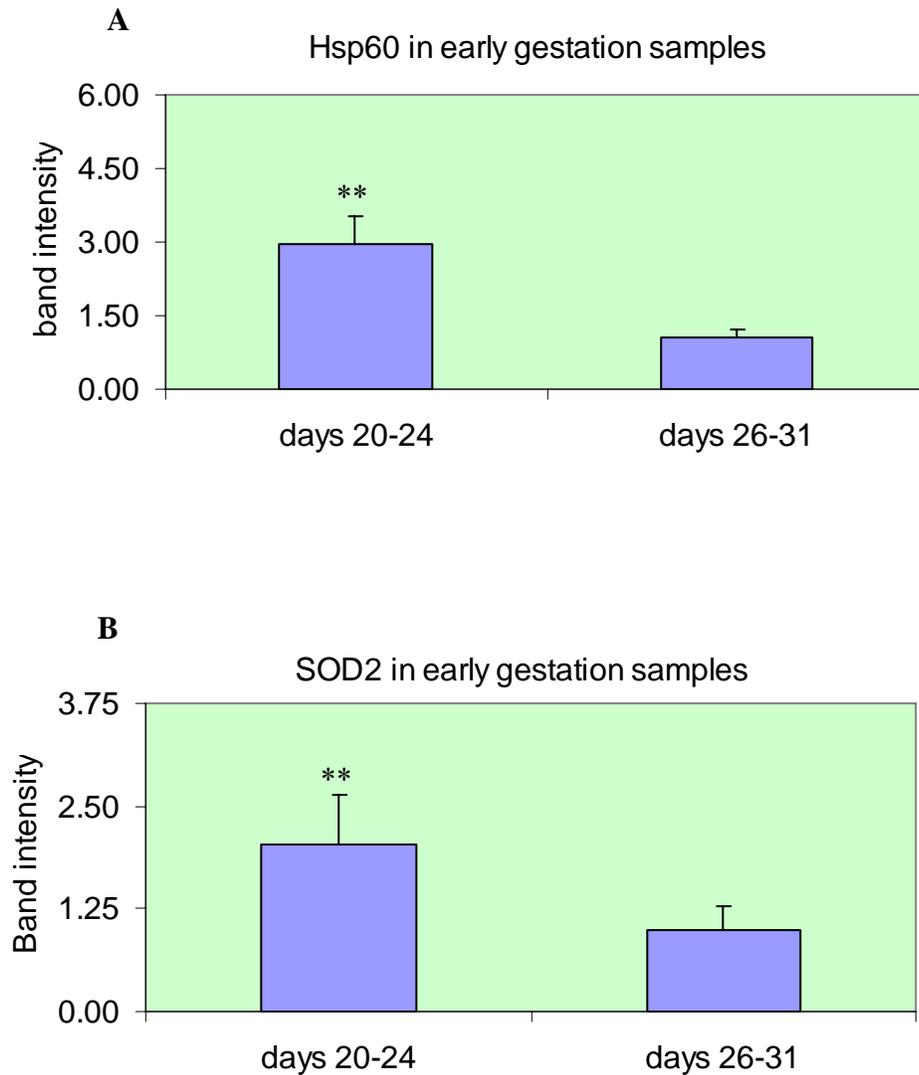


Figure 3.14 *Hsp60* and *SOD2* expression in early gestation embryonic protein samples.

A *Hsp60* expression in days 20-24 ($n = 6$) and days 26-31 ($n = 8$) of gestation. Days 20-24 *Hsp60* expression was significantly higher than days 26-31, $p = 0.006$

B *SOD2* expression in days 20-24 ($n = 6$) and day 26-31 ($n = 8$) of gestation. Days 20-24 *SOD2* expression was 2 x higher than day 26-31, $p = 0.01$. ** $p \leq 0.01$. Bars represent standard error of the mean.

Band densities of the positive controls were 0.73 (AI day 150 fetal cotyledon) and 0.56 (NT day 150 maternal cotyledon), which were low compared to the Hsp60 expression of earlier gestational time points where the median band density was 1.8, with a maximum of 5.04 at day 20. All early gestation samples were from either the whole embryo, or trophoblast tissue, as placentomes are not yet formed at this early stage.

Results from incubating the same early gestation blot with anti-SOD2 were also significant (Figure 3.14B); day 20-24 SOD2 expression was 2 x higher than day 26-31, $p = 0.01$. Again, the positive controls were also considerably lower in relative band intensity; 0.71 (AI day 150 fetal cotyledon) and 0.66 (NT day 150 maternal cotyledon) compared to mean average of 1.8 and maximum at day 20 of 5.04.

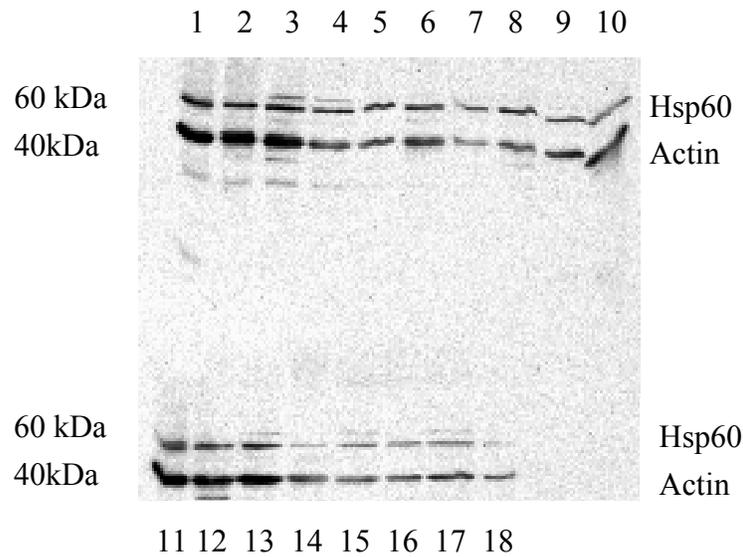


Figure 3.15 *Hsp60* western blot of early gestation embryonic protein samples.

Lane 1: NT fetal cotyledon at day 150, lane 2: day 15 embryo, lane 3: day 18 embryo, lane 4: day 20 trophoblast, lane 5: day 20 trophoblast, lane 6: day 22 embryo, lane 7: day 22 trophoblast, lane 8, 9: day 24 trophoblast, lane 10: day 26 trophoblast, lane 11: NT maternal cotyledon day 150, lane 12: day 26 trophoblast, lane 13,14: day 29 trophoblast, lane 15, 16: day 31 trophoblast, lane 17: day 27 trophoblast, lane 18: day 29 trophoblast. All samples were 20 μ g of protein per lane, with the exception of lanes 4 and 5, which had 10 μ g.

Fetal AI and maternal NT cotyledons at day 150 of gestation were used as positive controls, based on previous Hsp60 positive results from western blot analyses (Figure 3.15). The positive control samples appeared to have considerably lower band densities (4 x) than the day 20-24 sample group. Also, due to limited quantity of day 20 samples, only 10 μ g of protein was used per lane for the two day 20 samples, compared to 20 μ g in all other lanes of the SDS-PAGE gel. Therefore, results indicate that days 20-24 expression of Hsp60 was significantly higher than days 26-31, $p = 0.006$.

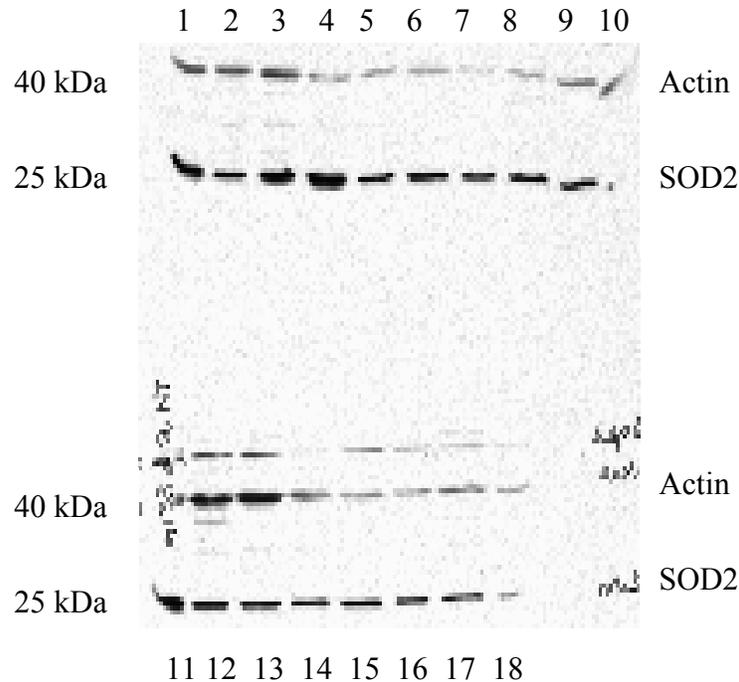


Figure 3.16 *SOD2 western blot of early gestation embryonic protein samples.*

The membrane used for this western blot was the same that was used for Figure 3.15. All sample identification and protein quantity per lane match to lane descriptions in Figure 3.15.

Expression of SOD2 was also investigated by western blot analysis and students t-test using the same blot (Figure 3.14B), SOD2 showing significantly higher expression in days 20-24 than days 26-31, $p = 0.01$.

The presence of Hsp60 in embryonic tissue at these gestational stages (days 20-31) suggests that detection of mitochondrial specific Hsp60 may be apparent in other early gestational tissues. These results suggest that Hsp60 may be present at day 7 and day 15 time points of gestation, a result that validated the procedure of a

proposed trial investigating the role of oxidative stress at gestational time points of day 7 and day 15. IVP embryos will be produced under control conditions and using varied levels of oxygen to provide stress treatment conditions on the developing embryo up to day 7, which is the optimal time point to transfer to a bovine recipient (Lonergan *et al.*, 2006). Day 15 embryos would also be investigated for indicators of stress at protein and mRNA levels, as most studies that use oxidative stress models only use day 7 of gestation (likely due to convenience of not transferring to bovine recipients).

Based on the significant results, which indicated high expression of stress proteins at around day 20 of gestation, it was decided to proceed with the proposed gestational day 15 embryo trial; investigating the role of oxidative stress *in vivo* after IVP embryonic stress treatment (see section B).

3.3 SUMMARY

Hsp70 expression was significantly higher in NT than AI in fetal cotyledons at day 150, as was SOD2 significantly higher in NT than AI in maternal cotyledons at day 150. Hsp60 expression was significantly higher in NT than AI in two of the six blots probed (fetal cotyledons at day 100, and maternal cotyledons at day 50). This suggests that there is significantly higher oxidative stress occurring in the NT groups than the AI groups, as indicated by the higher expression of stress proteins Hsp60 and Hsp70, and mitochondrial antioxidant SOD2. Interestingly, the Trial 2 placentomes at around day 150 showed significantly higher expression of Hsp60 in the AI group compared to the NT.

All placentome protein samples showed the presence of stress proteins (Hsp60, SOD2, and Hsp70) when analysed with western blots using appropriate antibodies. Significant differences of expression of these proteins were evident in many of the sample groups but unfortunately none of the results were repeatable. Several explanations for these inconsistencies may be applicable. Samples were frozen in only one aliquot that was thawed and frozen several times, resulting in possible protein degradation as displayed in Figure 3.17.

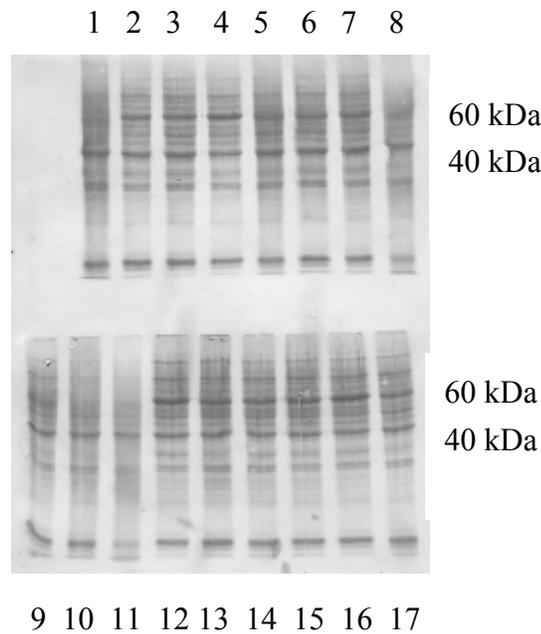


Figure 3.17 *AI, IVP, and NT Fetal cotyledons at day 150 on Ponceau S stained nitrocellulose membrane, displaying possible protein degradation.*

After transfer onto a nitrocellulose membrane, it appears that major protein bands are missing, especially in lanes 1, 5, 8, 9, 10, and 11, which may be due to protein degradation. The fetal cotyledons at day 150 aliquot had been thawed and frozen six times before the apparent degradation became obvious. Lanes 1-4: AI, 5-9: IVP, 10-17: NT.

Minimal differences between band densities were evident on scanned x-ray films prior to quantitation of band signal intensity. Analysis using Bio-Rad Quantity One software enabled a comparison of band intensity, and therefore Hsp60 expression, between samples by measuring pixel density of an area specified. Each individual band is encompassed by a rectangle using the computer mouse, which is the area that has the pixel density measured. Quantity One then adjusts the pixel density measurement by calculating in the background density and actin band densities from the same blot, to give a “normalised” measurement of each band for comparison between sample groups from the same blot. Error may occur when bands are not evenly spaced or at odd angles, making it difficult to be exact when drawing the rectangle with the computer mouse around each band, including the possibility of missing a small portion of the band or including some of the neighbouring band.

Further investigation would be required to repeat the western blot analysis on bovine AI, IVP, and NT cotyledons which has already been completed, taking care to eliminate or minimise the suggested possible errors that may be contributing to the unrepeatable results.

SECTION B

3.4 THE EFFECT OF OXIDATIVE STRESS ON EMBRYONIC DEVELOPMENT.

The investigation into bovine AI, IVP, and NT placentomes provides evidence that stress proteins are expressed at time points of day 50, 100, and 150 of gestation (results section A). In a study of bovine fetus development after treatment with varying oxygen levels during *in vitro* production of embryos by Fisher-Brown *et al.* (2005), it is suggested that free radicals due to oxidative stress may induce epigenetic effects that lead to abnormal placental growth. Western blot analysis of IVP early gestation samples (section 3.2.5) also suggest that stress proteins are expressed in bovine embryos between days 20-31. Levels of stress proteins appeared high at around days 20-24 of gestation, suggesting that significantly high levels of stress were operating at this point of gestation. Due to the difficulty of producing sufficient numbers of embryos to enable the investigation of the transcription of various stress proteins, it was decided that day 15 would be a suitable time point. This decision was based on the quantity of embryos able to be produced and transferred at this time point and the approximate survival rate from previous studies (Farin *et al.*, 2001).

Following the study of stress protein expression in bovine cloned placentomes, an investigation into the effects of oxidative stress on embryonic development was undertaken. Previous work has addressed blastocyst development and morphology at day 7 of development, and also calf physical traits and placental morphology at parturition (Fisher-Brown *et al.*, 2005, Harvey *et al.*, 2004, Yuan *et al.*, 2003).

This experiment will report on embryo development, morphology, and expression of stress proteins at day 7 and day 15 of development.

3.4.1 Conditions Used to Generate *In Vitro* Produced Embryos

Embryos were produced according to the standard operational procedure of the Agresearch Reproductive Technologies group. 60 – 80 ovaries were used per IVP run, depending on availability. The usual level of oxygen (control) during the culturing of IVP embryos is 7%. Due to this trial focussing on oxidative stress, the control group oxygen level was 7% from days 1-5, then 2% from days 5-7. This was due to elimination of DNP (section 3.4.2) from day 5-7 culture media, as dropping the oxygen level to 2% at days 5-7 without DNP gave the same results as 7% O₂ with DNP (Harvey *et al.*, 2004). Initial treatment groups of 2% and 20% O₂ were based on previous studies of differing oxygen levels during IVP embryo production (Fischer-Brown *et al.*, 2005, Harvey *et al.*, 2004, Yuan *et al.*, 2003) but due to poor developmental results to blastocyst stage in the treatment groups (refer to Table 3.1) it was decided to cancel the 2% O₂ group as there were not enough recipient cows to transfer the amount of blastocysts that would be required to successfully result in adequate day 15 conceptuses for experimental work. During initial trial runs to blastocyst stage (Table 3.1) the 2% O₂ group consistently produced lower numbers of

blastocysts than the 20% O₂ group (~ 4% lower on average), despite fertilization cleavage rates and numbers of 16 cell/morula embryos being similar across all groups. It is at the 8-16 cell stage that the embryonic genome is activated and has to support itself rather than relying on maternal RNA derived from the oocyte (Jansen and Burton, 2004, Wrenzycki *et al.*, 2005, Lonergan *et al.*, 2006). If the embryo is not able to activate the embryonic genome successfully, a typical halt in development called the “embryonic block” occurs.

3.4.2 2, 4-dinitrophenol and Oxidative Stress

The standard oxygen level used in the laboratory for IVP embryos is 7% O₂, therefore 7% O₂ was chosen as the control group. Due to the trial investigating oxidative stress, DNP was left out of the day 5-7 culture media. DNP is an uncoupler of oxidative phosphorylation and can alter the activity of metabolic pathways by binding to protons in the intermembrane space of mitochondria, providing partial inhibition of mitochondrial ATP production by dissipating the proton gradient across the mitochondrial membrane. The addition of DNP increases the demand on ATP production by glycolysis and mimics the inhibition of oxidative phosphorylation that occurs under reduced oxygen conditions *in vivo*, which aids in the development of morulae to blastocyst stage in the bovine (Harvey *et al.*, 2004). It is proposed DNP facilitates development by the stimulation of oxidative and glycolytic metabolism by increasing the uptake of pyruvate and glucose, while the partial inhibition of oxidative phosphorylation may reduce the level of intracellular reactive oxygen species (Rieger *et al.*, 2002).

3.4.3 IVP Fertilization

Fertilization rates were relatively constant with the exception of one run that had only 15% cleavage rate at the fertilization check on day 5. Possible reasons for the poor fertilization results were investigated. There may have been a problem with sperm preparation such as incorrect quantities of antioxidants added to the fertilization media. The sperm may have been added to the percoll gradient too fast, causing it to mix with the gradient and become affected by percoll toxicity. The dilution of sperm with IVF media may have been incorrect. HSOF wash may have been added to the sperm too fast during sperm preparation causing sperm to become shocked and damaged. However, these possibilities were dismissed as sperm was observed to be capacitating during the standard sperm concentration count prior to fertilization. Capacitation is associated with removal of adherent seminal plasma proteins, reorganization of plasma membrane lipids and proteins, and changes in sperm homeostasis (Moseley *et al.*, 2005). These changes make the sperm “fertilization competent”, and are identified by sperm heads binding together. Motility and sperm dilution also appeared normal when observed during check at fertilization.

Following investigation of fertilization failure it was concluded that extra attention needed to be focused on the timing of fertilization. If fertilization occurs later than 24 hours after maturation, results are poor, due to the oocyte being blocked at metaphase 2 of meiosis until sperm activation enables resumption and completion of meiosis. If fertilization does not occur the oocyte reactivates itself anytime 30-32 hours after maturation and will cleave without sperm penetration to the 4-8 cell stage, where it arrests. After careful attention to timing, the following run was back to the normal cleavage rate of 75%.

	<u>Ovaries</u>	<u>Oocytes</u>	<u>Fertilization (%)</u>	<u>Day 7 blastocysts (%)</u>
<u>Run 1</u>	60	339		
20% O2		112	85 (76)	13 (12)
2% O2		112	87 (78)	11 (10)
7 and 2% O2		113	76 (68)	29 (26)
<u>Run 2</u>	60	380		
20% O2		100	71 (71)	25 (25)
2% O2		180	136 (76)	38 (21)
7 and 2% O2		100	73 (73)	35 (35)
<u>Total Averages</u>	60	359		
20% O2		106	77 (73)	19 (18)
2% O2		146	108 (74)	22 (15)
7 and 2% O2		106	74 (70)	32 (30)

Table 3.1 *Relationship between ovaries, oocytes, fertilization rate, and blastocyst rate between treatment groups (20% O2, 2% O2) and control group (7 and 2% O2).*

The percentage figures from fertilization and blastocysts are based on the original number of oocytes. The oocyte numbers originate from the group of 60 ovaries and are split into respective treatment (20% O2, 2% O2) and control groups (7 and 2% O2).

3.4.4 Oxidative Stress IVP Embryos

Based on the results from Table 3.1, the following Trial was undertaken using the standard method of *in vitro* produced embryos as mentioned in section 2.2.1.

140 ovaries were aspirated to yield 937 COCs. The fertilization rate was 75%, resulting in 703 presumptive zygotes, which were randomly split into treatment and control groups at *in vitro* culture (24 hours post-fertilization). Due to the higher expected rate of blastocyst yield from the control group (based on results from Table 3.1), the numbers allocated were weighted to 445 oocytes in the 20% O₂ treatment group and 258 oocytes in the 7 and 2% O₂ control group. Numbers of blastocysts were calculated from original oocyte number per group that gave rise to blastocysts. The 20% O₂ group resulted in 67 blastocysts (15%) and the 7 and 2% O₂ group had 107 blastocysts (41%), as per expected. 35 treatment mixed grade blastocysts and 33 control mixed grade blastocysts were transferred into 15 recipient heifers on day 7 of development. Results are represented in Table 3.2. 3 x 10 treatment embryos and 7 x 10 control embryos were frozen in liquid nitrogen for later use.

	Ovaries	Oocytes	Fertilization (%)	Day 7 blastocysts (%)	Blastocysts transferred	Day 15 embryos retrieved (%)
Day 15 trial	140	703				
20% O2		445	338 (76)	67 (15)	35	15 (43)
7 and 2% O2		258	206 (80)	105 (41)	33	15 (45)

Table 3.2 Relationship between ovaries, oocytes, fertilization rate, and blastocyst rate between treatment groups (20% O2, 2% O2) and control group (7 and 2% O2), before and after transfer.

The percentage figures from fertilization and blastocysts are based on the original number of oocytes. The oocyte numbers originate from the group of 140 ovaries and are split into respective treatment (20% O2, $n = 445$) and control groups (7 and 2% O2, $n = 258$). The percentage of blastocysts retrieved are based on the original number of blastocysts that were transferred (20% O2, $n = 35$, 7 and 2% O2, $n = 33$)

3.4.5 Blastocyst Production

Visual differences were observed during the production of control and treatment embryos. At the day 5 check and transfer to LSOF media the treatment embryo cell masses morphologically appeared much darker than the control embryos (Figure 3.18).

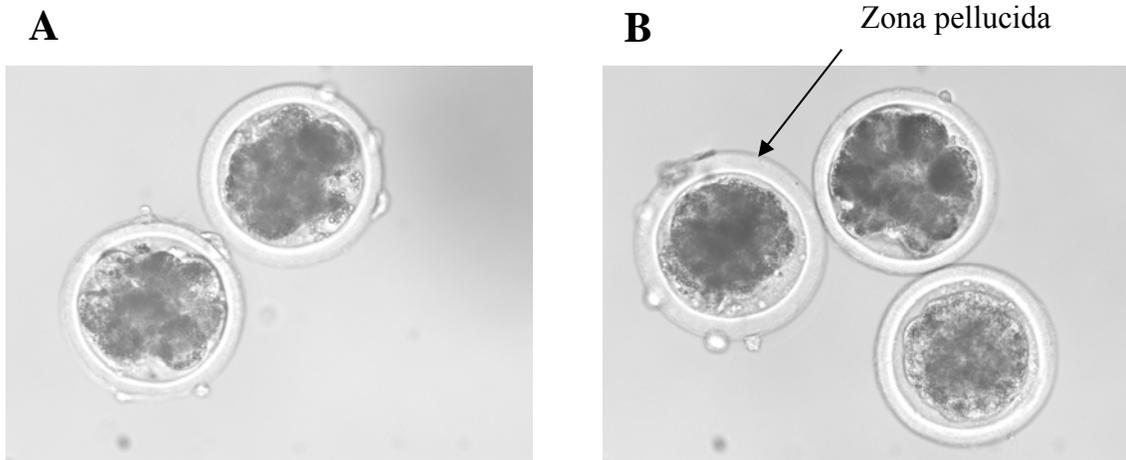


Figure 3.18 Day 5 morula embryos from stress trial (200 x magnification).

A 7 and 2% O₂ (control) day 5 morula embryos. **B** 20% O₂ (treatment) day 5 morula embryos. Blastomeres are evident as they are beginning compaction into morulas.

In Figure 3.18, the morula cells appear darker in treatment than control (A). One of the treatment embryos (B) also has slightly a thicker zona pellucida. Further into development, at day 7 blastocyst stage (Figure 3.19), the zona pellucida and perivitelline space continue to appear larger on some of the treatment embryos (Figure 3.19B). Unfortunately zona pellucida and perivitelline space morphology was not taken into account during embryo production, it was only noticed during photography of sample embryos under higher magnification than used during embryo production. It would be interesting to observe differences of zona pellucida and perivitelline space morphology in any other study similar to this one. It is at blastocyst stage (day 7) that the embryos were transferred into recipient heifers.

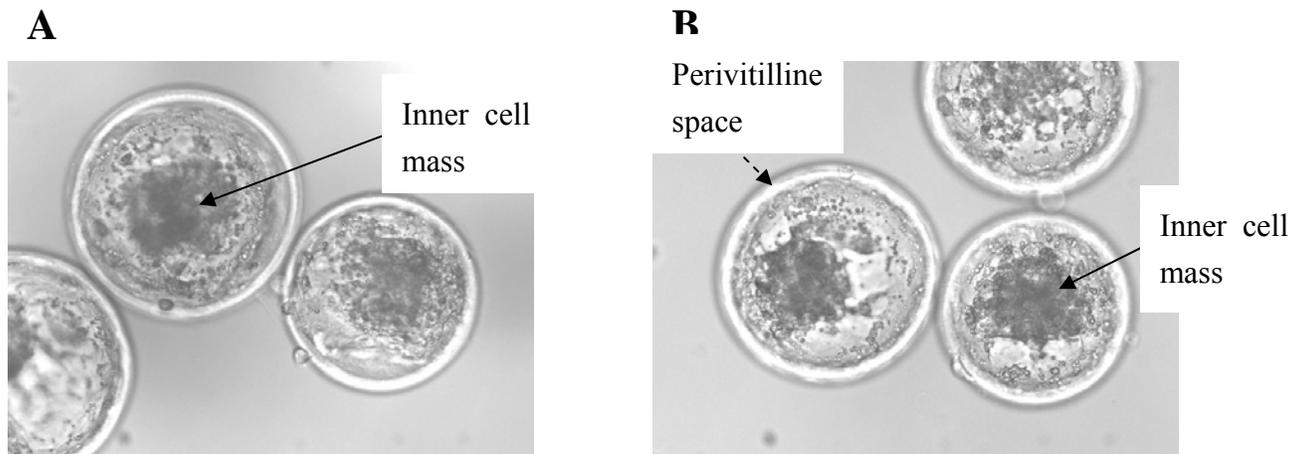


Figure 3.19 Day 7 blastocysts from oxidative stress trial (200 x magnification)

A 20% O₂ day 7 blastocysts. **B** 7 and 2% O₂ day 7 blastocysts. Solid arrows show the inner cell mass. The dashed line in B (control) shows a larger perivitelline space between the zona pellucida and trophectoderm than in A (treatment).

After 8 days *in vivo*, the day 15 embryos were removed from the recipient heifers. The uterine tracts were retrieved at the abattoir and labelled in order of slaughter. The tracts were placed in plastic bags and transported back to the sorting facilities at AgResearch where the concepti were flushed from the uterine tracts with room temperature sterile saline solution (0.9% w/v). Survival of concepti (Table 3.2) and measurements of embryo length were recorded (Figure 3.23). Embryo survival was similar between control and treatment groups, with 43% of the treatment group, and 45% of the control embryos, surviving to day 15 after transfer.

3.5 OXIDATIVE STRESS DAY 15 EMBRYOS

Photographs were taken immediately after embryo retrieval to determine any morphological differences between the treatment and control groups, as shown in Figures 3.20, 3.21, and 3.22. Embryo lengths were also recorded, with average measurements for each group presented in Figure 3.23.



Day 15 7 and 2%



Day 15 20%

Figure 3.20 *Photographs of day 15 control (7 and 2% O₂) and treatment (20% O₂) embryos after retrieval from recipient heifers.*

Photographs were taken in 90mm Petri dishes, under normal lighting conditions without magnification. At day 15, embryos appear “stocking-like”, with an embryonic disc (Figure 3.22).

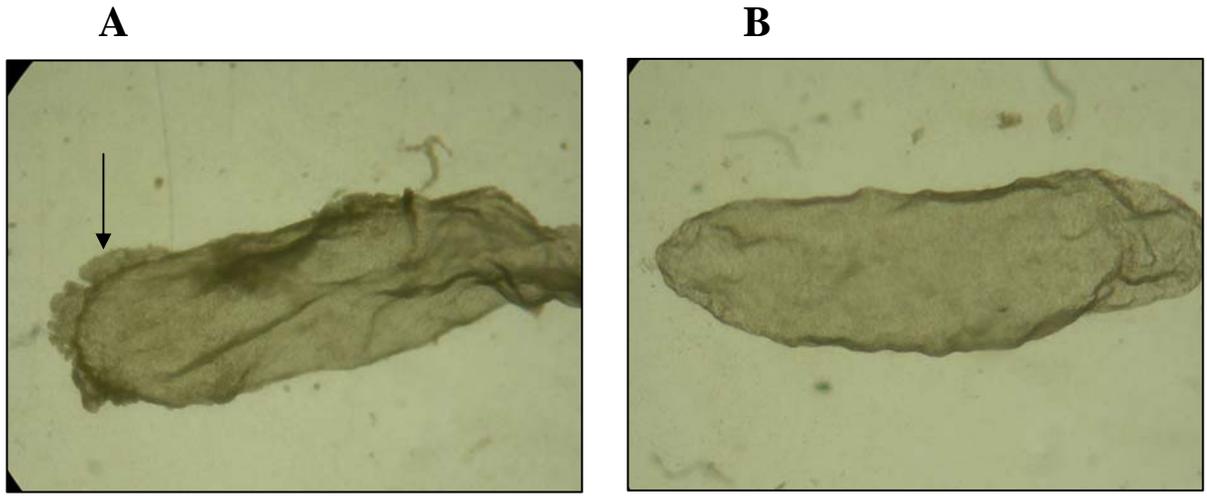


Figure 3.21 Day 15 embryos from 20% O₂ treatment group (100 x magnification).

A Day 15 20% O₂ treatment embryo. The arrow notes unusual “furry” growths on the trophoblast exterior of the embryo.

B A relatively normal looking day 15, 7 and 2% O₂ control embryo.

Figure 3.21 presents a distinct morphological difference between the treatment (A) and control (B) day 15 embryos. It is not usual to see growths on the trophoblast exterior, suggesting the treatment may have interfered with normal embryonic development. Embryonic discs were not visible in all embryos, however it is possible that they may have been difficult to detect rather than absent. Figure 3.22 depicts a normal representation of an embryonic disc.

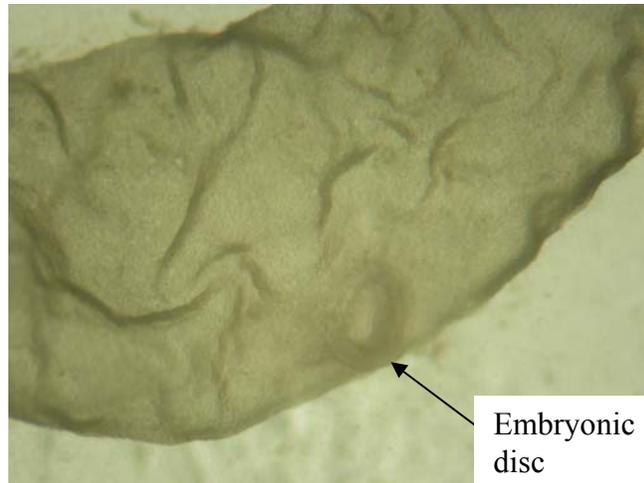


Figure 3.22 *Day 15 embryo from control group (200 x magnification).*

The solid arrow notes an embryonic disc surrounded by the trophoblast on a control group embryo, from which the fetus will form. This is a good example of normal embryonic development.

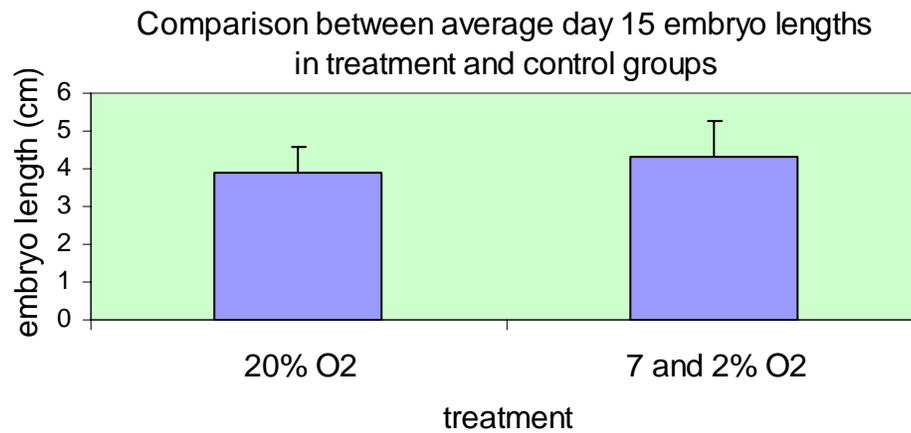


Figure 3.23 Comparison between average embryo lengths of day 15 treatment and control embryos.

The average concepti length differed by 0.6cm between treatment (20% O₂, $n = 10$) and control (7 and 2% O₂, $n = 8$) groups (Figure 3.23). Morphologically, treatment embryos appeared noticeably wider than control embryos. However measurements were not taken due to the fragility of the embryos. Bars represent standard error of the mean.

3.6 PROTEIN AND RNA EXTRACTION FROM TRIAL EMBRYOS

Day 15 embryos were processed with TRIzol to extract protein and RNA, each sample was a single embryo. Due to such low tissue amounts day 7 blastocysts were processed at 10 blastocysts per sample using the method provided by Dr Craig Smith (section 2.2.2.2).

3.6.1 Day 7 Protein and RNA Extraction

Day 7 protein samples were in 10 µl aliquots which had 5 µl 3x Laemmli buffer added and boiled for 5 minutes at 95°C. The samples were then run on a 12.5% acrylamide gel and transferred to a nitrocellulose membrane for 2 hours. The resulting ponceau S stain indicated minimal protein present (Figure 3.24). This was confirmed with an unsuccessful antibody probe (anti-hsp60), which resulted in no signal at all. Based on methods by Lequarre *et al.* (2001), it was decided that groups of 50 blastocysts would be necessary to use for immunodetection. Five groups each of treatment and control embryos were produced and stored at -20°C for later use.

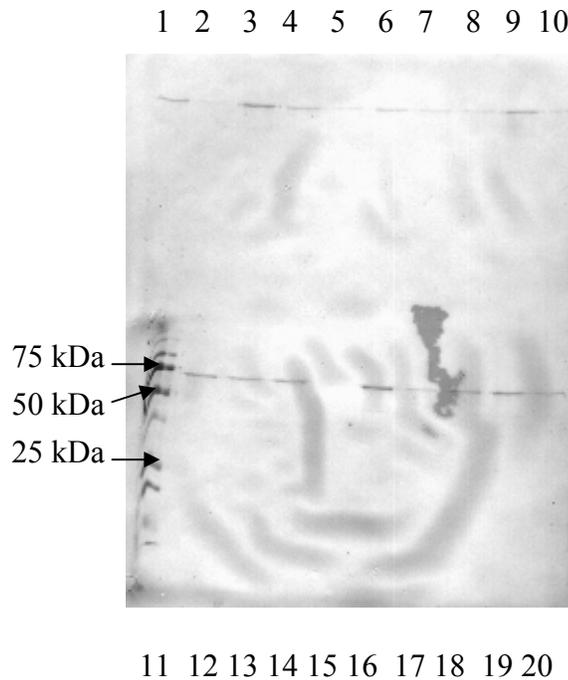


Figure 3.24 *Ponceau S stained nitrocellulose membrane of day 7 trial samples.*

Lanes 1-10: 7 and 2% O₂ control group. Lane 11: molecular weight marker. Lane 12-20: 20% O₂ treatment group. Only a single dominant protein band was transferred from 2 SDS-PAGE gels from each pool of 10 blastocysts per sample. Each lane contained the complete amount of protein extracted from the 10 blastocysts per sample, in 10µl of 1% (w/v) SDS solution. Molecular weight marker identifies this band present at ~ 70 kDa.

Harvey *et al.* (2007) extracted RNA from pools of bovine blastocysts (50 x blastocysts per pool) to obtain clear experimental results, therefore this appeared to be a more appropriate number of blastocysts to use per sample. Ten pools each of 50 x day 7 IVP blastocysts were created; five pools of the treatment and five of control (total of 500 blastocysts). The standard TRIzol method of RNA and protein extraction was used (2.2.2.4). Due to the minimal amount of RNA extracted (1 µg) samples were not run on a gel to verify RNA integrity, NanoDrop was used to

identify successful extraction. Unfortunately no protein was present. This was verified using a coomassie blue stained SDS-PAGE gel and a ponceau S stained nitrocellulose membrane stained. Both the stained gel and membrane displayed no protein bands. After careful analysis of the extraction procedure it was realised that the guanidine-HCL wash solution used contained no ethanol, therefore precipitation did not occur and the protein was discarded with the guanidine-HCL solution.

A repeat production of IVP embryos with control and treatment groups resulted in 5 samples of 50 x treatment embryos and 4 samples of 50 x control embryos. Protein and RNA was extracted successfully (verified with NanoDrop) using the standard TRIzol method (section 2.2.2.4). Each sample contained proteins from 50 embryos in minimal volume of HEPES buffered TCM, which had 5 μ l 3x laemmli buffer added and boiled for 5 minutes at 95°C prior to separation by SDS-PAGE using a 12.5% acrylamide gel. Samples contained the full amount of protein that was extracted in 20 μ l 1% SDS, combined with 10 μ l 3 x Laemmli, equated to a total volume of 30 μ l. The separated proteins were transferred to a nitrocellulose membrane for two hours at 50 volts and stained with ponceau S (Figure 3.25).

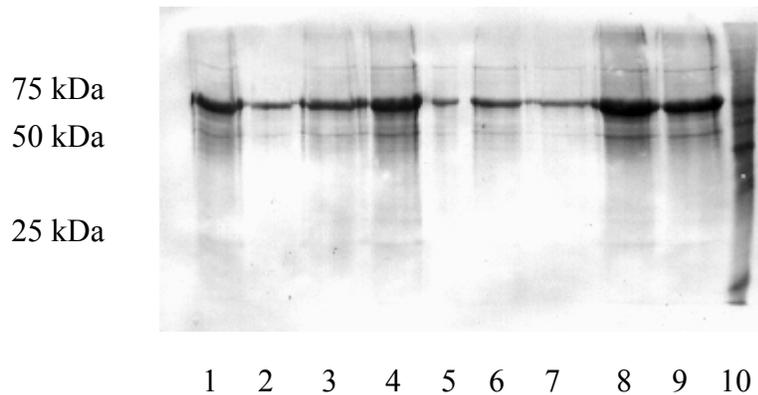


Figure 3.25 *Ponceau S stained nitrocellulose membrane of day 7 treatment and control embryos.*

Lanes 1-9: Protein from pools of 50 x day 7 embryos per lane. Lanes 1-4: control (7 and 2% O₂). Lanes 5-9: treatment (20% O₂). Lane 10: Hsp60 positive control, IVP fetal cotyledons at day 150, 20 µg protein.

Overnight incubation at 4°C of the nitrocellulose membrane with anti-Hsp60, confirmed immunoreactivity with the Hsp60 positive control sample but no signal was apparent for the day 7 blastocysts. SuperSignal®West Femto Maximum Sensitivity Substrate Kit (section 2.1.7) was employed as a supersensitive measure of Hsp60 protein presence. 1.5 ml of luminol/enhancer solution was combined with 1.5 ml stable peroxide solution and added to the nitrocellulose membrane for 5 minutes before the light signal was detected after exposure of the membrane for 4 minutes on x-ray film. Hsp60 displayed a positive signal in all lanes (Figure 3.26). However, lanes 4 and 9 had a very weak signal. Unfortunately, anti-actin did not show a strong signal in any sample except the positive control after incubation for 2 hours at room temperature (Figure 3.27), suggesting that there may not be much actin present in day 7 embryos.

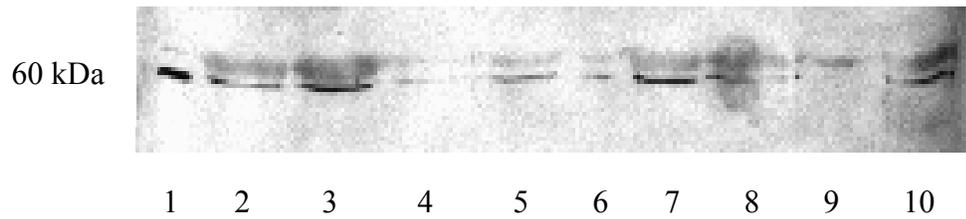


Figure 3.26 *Hsp60 western blot of day 7 blastocysts, control and treatment groups.*

Lane 1: positive control IVP fetal cotyledons at day 150, 20 μg protein. Lanes 2-6: 20% O₂ treatment group. Lanes 7-10: 7 and 2% O₂ control group.

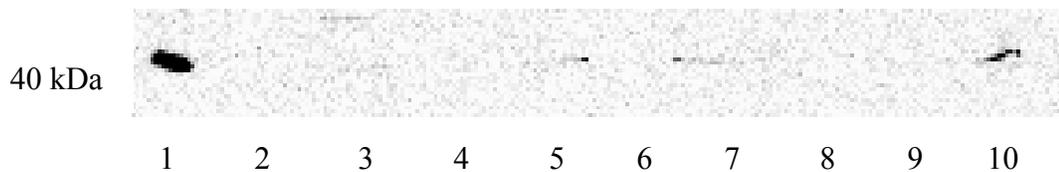


Figure 3.27 *Actin western blot of day 7 blastocysts, control and treatment groups.*

Lane 1: positive control IVP fetal cotyledons at day 150, 20 μg protein. Lanes 2-6: 20% treatment group. Lanes 7-10: 7 and 2% control group.

To determine whether there was a significant difference between the day 7 control and treatment groups, normalisation with actin using the Quantity One programme was necessary. As there was no signal from the actin immunodetection, a graph displaying the various band intensity differences between sample groups gives some indication of the differences between control and treatment groups (Figure 3.28), based on the assumption that there are equivalent amounts of protein in each sample.

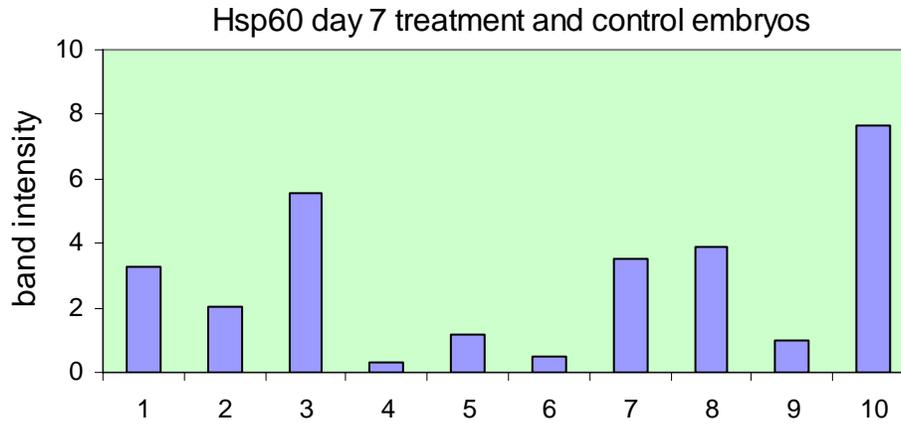


Figure 3.28 *Comparison between treatment (20% O₂) and control (7 and 2% O₂) day 7 blastocysts.*

Lane 1: positive control; fetal cotyledon day 150 of gestation, IVP. 20 µg protein. Lanes 2-6: 20% O₂ treatment group, day 7 blastocysts. Lanes 7-10: 7 and 2% O₂ control group, day 7 blastocysts. Lanes 2-10 contained all protein extracted from pools of 50 blastocysts per sample.

3.7 INVESTIGATION OF PROTEIN AND mRNA EXPRESSION ASSOCIATED WITH OXIDATIVE STRESS

Hsp60, cyclo-oxygenase 2 (COX2), Triosephosphate isomerase (TIM), and SOD2 were expression was investigated at protein level. Hsp70 and glucose transporter 1 (GLUT1) expression was investigated at mRNA level. The purpose of the investigation was to determine if oxidative stress was affecting expression levels of proteins and genes associated with stress, metabolism, and development.

3.7.1 Hsp60 Expression in Day 15 Embryo Samples

Anti-hsp60 (Stressgen, Victoria, Canada) was used for detection in Western blot analysis as described in section 2.2.4, using 1:10,000 dilution for the primary antibody, and 1:20,000 dilution for the secondary antibody (goat anti-rabbit) There was no statistical difference in Hsp60 expression between treatment and control groups as judged by band density differences when normalised with actin (Figure 3.29). Band density averages were similar; 0.40 for treatment and 0.41 for control (Figure 3.30).

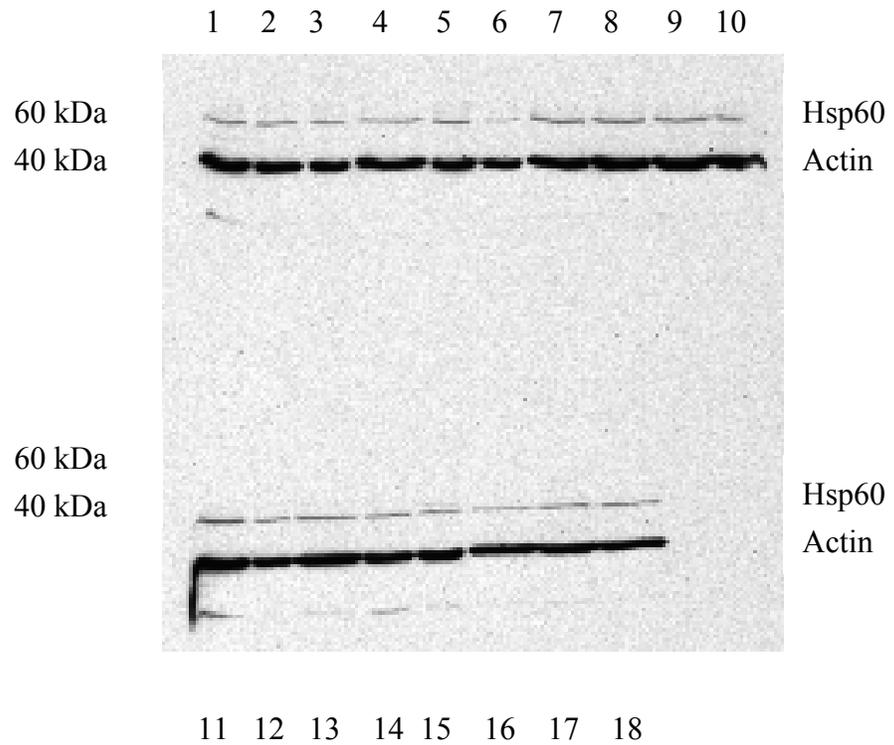


Figure 3.29 Day 15 western blot comparing Hsp60 expression between control and treatment groups.

Lanes 1-10: 20% O₂ treatment group. Lanes 11-18: 7 and 2% O₂ control group. 10 µg protein per lane.

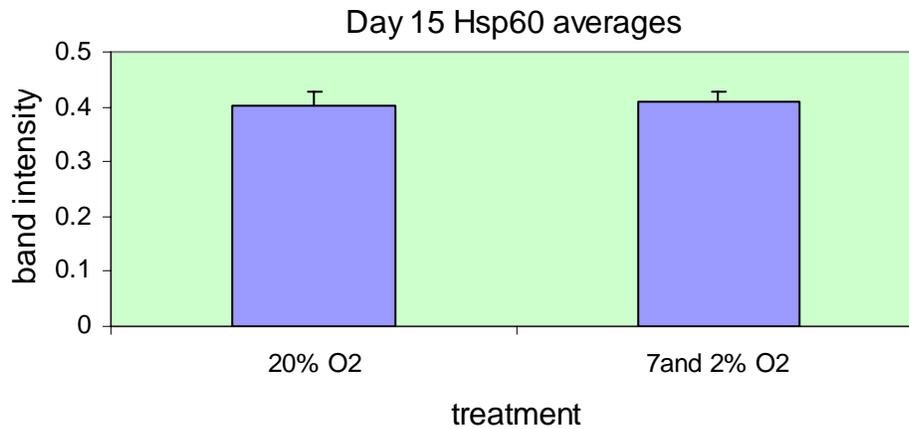


Figure 3.30 Comparison between treatment (20% O₂) and control (7 and 2% O₂).

Minimal differences were observed in average band intensities in Hsp60 expression between treatment (0.40) and control (0.41) groups. 20% O₂ $n = 10$, 7 and 2% O₂ $n = 8$. Bars represent standard error of the mean.

3.7.2 COX 2 Expression in Day 15 Embryo Samples

Due to investigation of oxidative stress, the day 15 embryo blot was probed with COX2. COX2 converts arachidonic acid to prostaglandin, and is essential for blastocyst implantation (Lim *et al.*, 1999). Prostaglandins participate in a variety of cellular functions including cell proliferation and differentiation. Probing with anti-COX2 primary antibody at a dilution of 1:2000 for 2 hours and a secondary antibody, sheep anti-mouse (1:10,000) for 2 hours gave a good chemiluminescent signal after 4 minutes of exposure to the membrane. The results after normalisation with actin were not significantly different between the control and treatment groups (Figure 3.32).

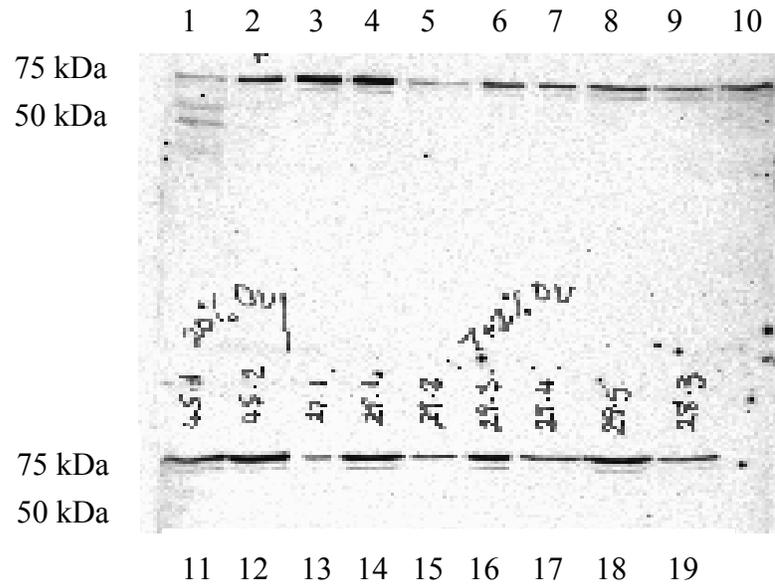


Figure 3.31 Day 15 western blot comparing COX2 expression between control and treatment groups.

Lane 1: molecular weight marker. Lane 2: positive control; day 18 embryo, AI, 10 μ g protein. Lanes 3-12: 20% O₂ treatment group. Lanes 13-19: 7 and 2% O₂ control group. 10 μ g protein per lane.

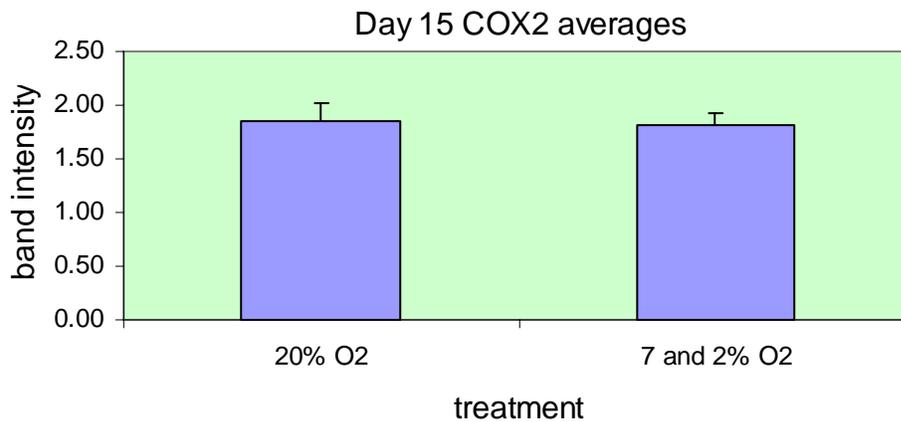


Figure 3.32 Comparison of COX2 average expression between treatment (20% O2) and control (7 and 2% O2) day 15 embryos.

Minimal differences were observed in average band intensities in COX2 expression between treatment (1.85) and control (1.81) groups. 20% O2 $n = 10$, 7 and 2% O2 $n = 8$. Bars represent standard error of the mean.

3.7.3 TIM expression in Day 15 Embryo Samples

TIM is a glycolytic enzyme that plays an important role in several metabolic pathways and is essential for efficient energy production. Larger than normal quantities of glucose are required during oxidative stress, so probing the day 15 blot with TIM antibody (Santa Cruz Biotechnology) may indicate whether more of this enzyme is transcribed in the treatment group or not. Incubation with the primary antibody was overnight at 4°C at a dilution of 1:500. Rabbit anti-goat IgG bound to the secondary antibody, with an incubation of 2 hours at a dilution of 1:10,000. The results were a good chemiluminent signal after 4 minutes exposure to the membrane (Figure 3.33). After normalisation with actin using Quantity One, there were no significant differences between control and treatment groups (Figure 3.34).

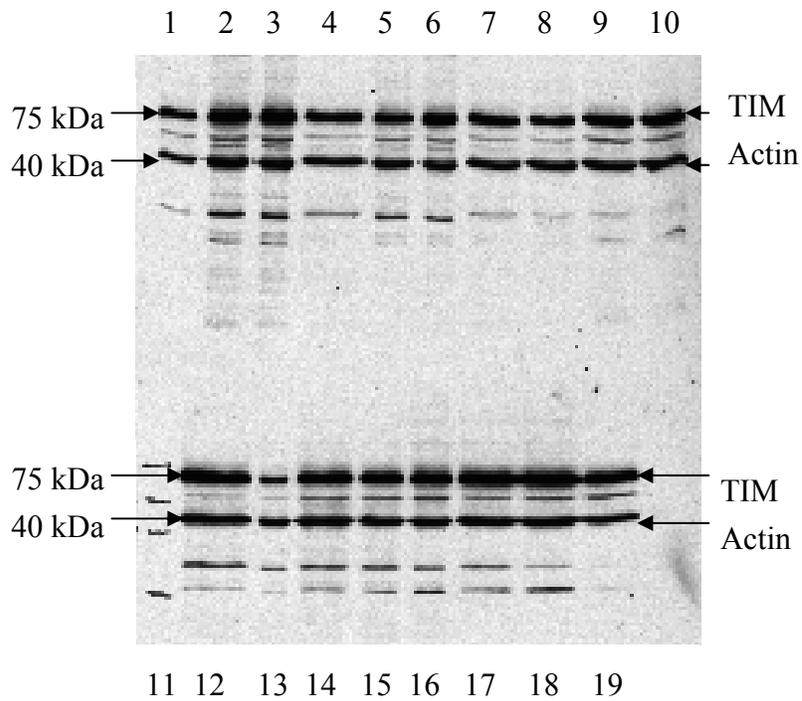


Figure 3.33 Day 15 western blot comparing TIM expression between control and treatment groups.

Lanes 1-10: 20% O₂ (treatment). Lane 11: molecular weight marker, displaying weights of 75, 50, 37, and 25 kDa. Lanes 12-19: 7 and 2% O₂ (control). 10 µg protein per lane.

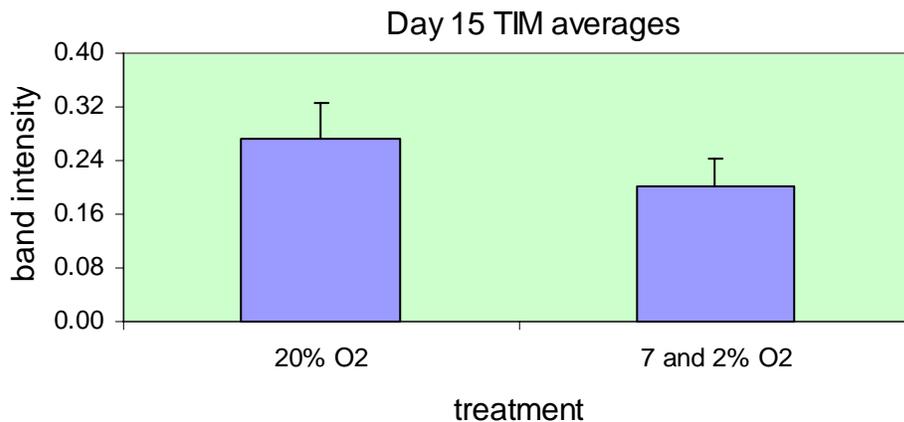


Figure 3.34 Comparison of TIM average expression between treatment (20% O₂) and control (7 and 2% O₂) day 15 embryos.

Minimal differences were observed in average band intensities in TIM expression between treatment (0.27) and control (0.20) groups. 20% O₂ $n = 10$, 7 and 2% O₂ $n = 8$. Bars represent standard error of the mean.

3.7.4 SOD2 Expression in Day 15 Embryo Samples

Continuing to check proteins related to oxidative stress, the day 15 nitrocellulose membrane was subjected to western blot analysis with anti-SOD2. The primary antibody was incubated at a dilution of 1:20,000 for 2 hours, followed by goat anti-rabbit (1:20,000) for 2 hours. The results after normalisation with actin gave a strong chemiluminescent signal after 1 minute of exposure to the membrane (Figure 3.35), with a significant result of $p = 0.009$. After normalisation with actin using Quantity One, the average expression of SOD2 was approximately 2 times higher in the control group than the treatment group (Figure 3.36).

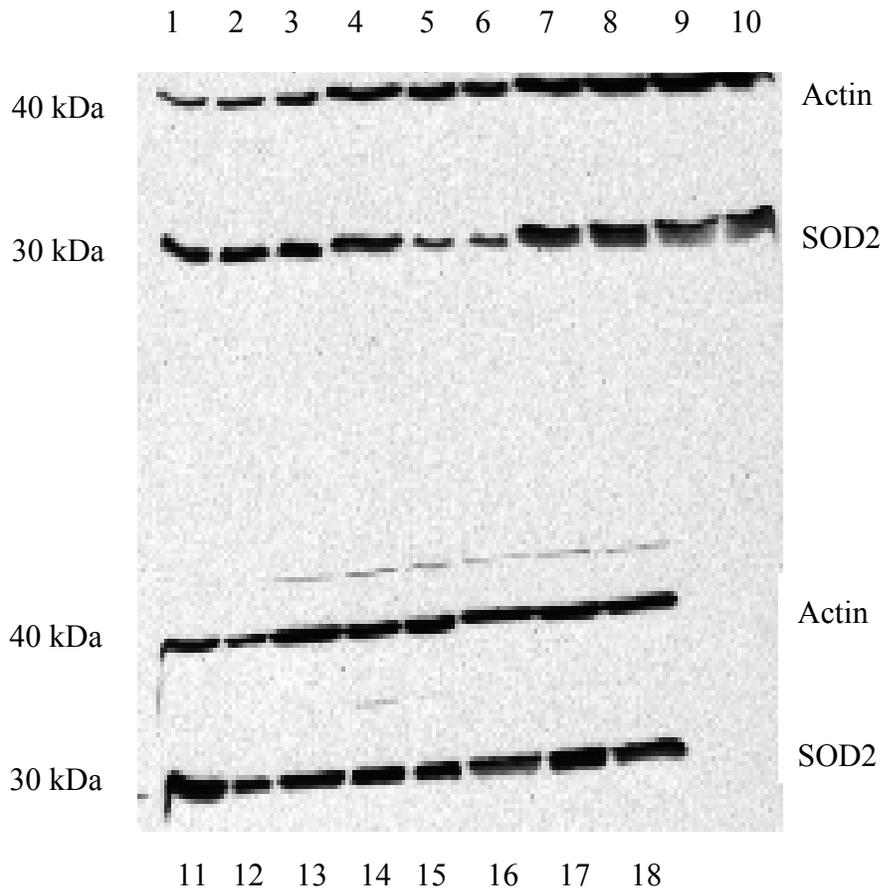


Figure 3.35 Day 15 western blot comparing SOD2 expression between control and treatment groups.

Lanes 1-10: 20% O₂ (treatment). Lanes 11-18: 7 and 2% O₂ (control). Molecular weight markers at 40 and 30 kDa bands are based on reference to previous blots. Signal obtained after one minute of exposure to x-ray film. 10 µg protein per lane.

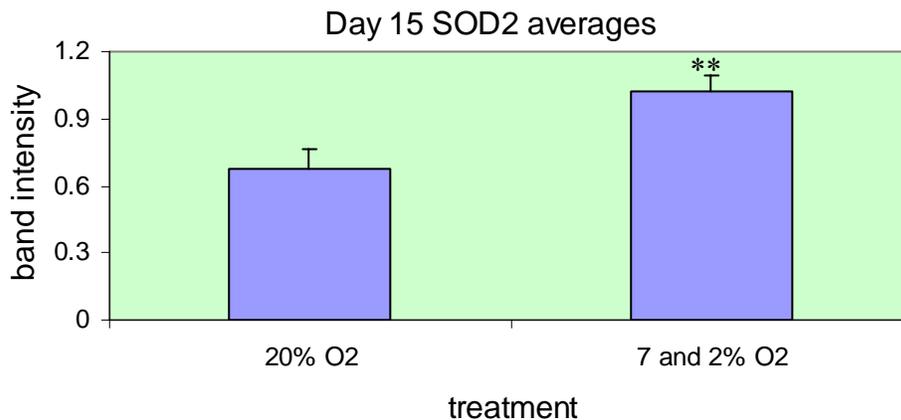


Figure 3.36 Day 15 stress trial embryos showing the average band densities between treatment (20% O₂) and control (7 and 2% O₂) groups.

The control group ($n = 8$) showed significantly higher SOD2 expression relative to the treatment group ($n = 10$). $p = 0.009$. Bars represent standard error of the mean.

3.8 RT-PCR ANALYSIS

A typical mammalian cell contains about 80-85% of ribosomal RNA, made up of subunits: 28S, 18S, and 5S (Maniatis *et al.*, 1982).

Quality of extracted mRNA from day 15 embryos was checked by running them through a 1.2% Agarose RNA gel (section 2.1.4) at 6-7 V/cm after samples were denatured at 65°C for 15 minutes and chilled for 5 minutes. Clear 28S and 18S bands were visible, indicating that the mRNA was stable and intact after extraction from embryonic tissue (Figure 3.37).

RT of the day 15 RNA was carried out as described in section 2.2.5.1, which provided cDNA transcripts for PCR amplification of selected genes. Denaturing and annealing temperatures and times are as mentioned in section 2.2.5.2, and were followed for all genes.

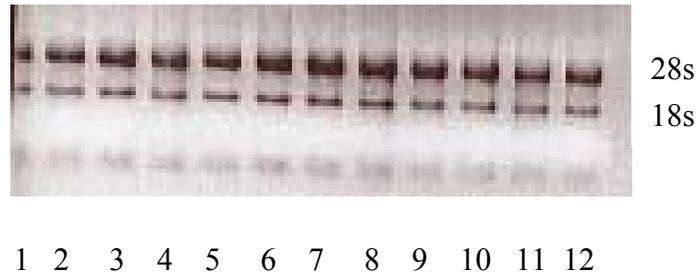


Figure 3.37 *1.2% Agarose RNA Gel providing verification of Day 15 embryo RNA integrity.*

The distinct 28s and 18s RNA bands in all lanes confirm the integrity of the RNA. 1 μg of Day 15 embryo RNA was used per lane. Lanes 1-6: day 15 control RNA. Lanes 7-12: day 15 treatment RNA.

3.8.1 GLUT1

GLUT1 is a glucose transporter. Significantly increased transcription of GLUT1 has been associated with a decrease in oxygen concentration in post-compaction bovine IVP embryos compared to *in vivo* (Harvey *et al.*, 2004).

Amplification of PCR mixture with GLUT1 forward and reverse primers (1.25 µl of each per sample) where each cycle included denaturation at 94°C for 30 seconds, reannealing primers to target sequences for 30 seconds, primer extension at 72°C for 20 seconds, was carried out for 30 cycles. After mixing 2 µl of 6x DNA gel loading dye with the 12 µl PCR product, a 100bp marker and 5 µl samples were run on a 7% acrylamide TBS gel at 20V/gel. PCR products identity were detected by ethidium bromide staining and ultraviolet light visualisation, using a BIO-RAD Gel Doc 1000 imaging densitometer. The image resulted in a very bright signal so it was decided to repeat the procedure with another PCR amplification of GLUT1, this time using only 20 cycles to see if a difference between samples could be detected. There was a visible difference but it seemed to be between samples rather than treatment and control groups.

3.8.2 Hsp70

An initial trial of RT-PCR using Hsp70 primers resulted in a very strong signal, so a repeat PCR amplification was carried out using a 1 µl sample of a 1:10 dilution of the original RT product. The dilution was centrifuged briefly and vortexed to ensure thorough mixing before pipetting. The Hsp70 PCR was run at the same cycle as mentioned for GLUT1, for 20 cycles. Following ethidium bromide staining of the gel, the samples were run on and image processing it appeared that several of the

embryo samples did not appear to express Hsp70. This is unusual as Hsp70 is known to be largely expressed in early embryonic development. To investigate whether there may have been a problem with the RT sample dilution, the Hsp70 PCR was repeated using 0.5 μ l of the original sample product instead of the 1:10 sample dilution. Results showed that Hsp70 was expressed in all embryo samples (Figure 3.28). Differences in signal could be seen between samples but not between treatment and control groups.

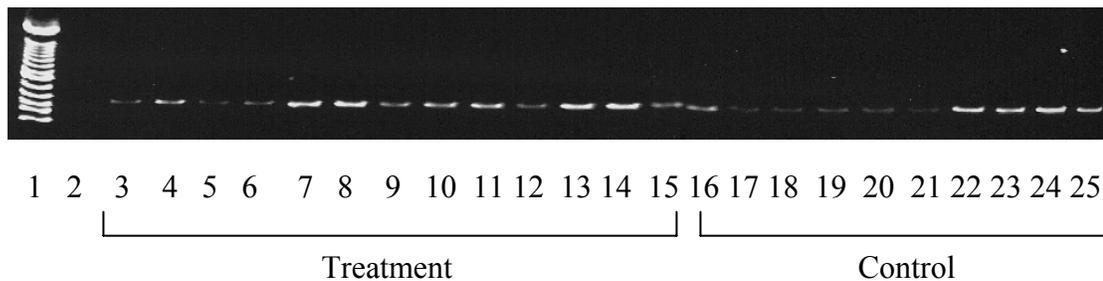


Figure 3.38 *Hsp70* gene expression on an ethidium bromide stained 7% acrylamide TBS gel

After 20 cycles expression of Hsp70 was evident in all day 15 samples. Lane 1: 100bp ladder. Lane 2: RT-negative control sample. Lanes 3-15: 20% O₂ treatment samples. Lanes 16-25: 7 and 2% O₂ control samples.

3.9 SUMMARY

Morphologically, at day 5 the treatment embryos (20% O₂) appeared darker in appearance (Figure 3.18). This darker appearance may be due to larger numbers of lipid droplets that is typical of IVP embryos, compared to *in vivo*-derived embryos (Abe *et al.*, 1999). It has been previously noted that embryos from high oxygen concentrations (20%) appear darker than those produce in less oxygen, due to oxidative stress increasing the formation of free oxygen radicals that may oxidise increased levels of fatty acids, which results in embryos with a dark appearance and less viability (Fischer-Brown *et al.*, 2005).

At day 5 the zona pellucida (ZP) in one of the treatment embryos was noticeably thicker than the control embryos (Figure 3.18). It is usual for the zona pellucida to be thinner in IVP embryos, with a thick ZP usual for *in vivo* derived embryos, which is associated with integrity of developing embryos. At day 7 the treatment embryos had a larger perivitelline space compared to control, which is also associated with *in vivo*-derived embryos (Abe *et al.*, 1999).

Similar numbers of embryos survived transfer to day 15 from both the treatment and control groups; 43% for treatment and 45% for control. Morphologically at day 15 the treatment embryos were slightly shorter and wider compared to control. One treatment embryo had strange villious growths projecting from the trophectoderm, one may speculate the possibility of interference with placental growth further into development.

COX2, TIM, SOD2, and Hsp60 were all present at protein level, although the differences were insignificant, with the exception of SOD2 which had 1.5 x the expression levels in the control group compared to the treatment group (Figure 2.23), suggesting less mitochondrial antioxidant protection when levels of oxidative free radicals may be higher in the treatment embryos due to higher oxygen and oxidative stress.

At the mRNA level, GLUT1 and Hsp70 expression was evident. On visual inspection there appeared to be no differences between treatment and control groups but there did seem to be differences between samples.

CHAPTER 4: DISCUSSION

4.1 EXPRESSION OF MITOCHONDRIAL STRESS PROTEINS IN BOVINE COTYLEDONS

Although the technology of nuclear transfer cloning still has relatively low success rates, cloning is used both within basic research and the biomedical sector; application possibilities are theoretically limitless. The next step may be to implement cloning in the agricultural production system; however cloned animal welfare and a higher efficiency of viable offspring require improvement to secure the application of cloned animals. One major developmental anomaly of cloned cattle that is linked to an unacceptably high level of losses during pregnancy is placental abnormality. Bovine cloned embryos have fewer and larger placentomes than *in vivo* counterparts. One suggestion for this phenomenon is that incomplete nuclear reprogramming leads to epigenetic errors and incorrect genomic imprinting. Mitochondrial DNA may suffer mutations which alter protein transcription, causing disruptions to the electron transport chain. This in turn may result in high levels of reactive oxygen species, inappropriate cell death via apoptosis, or transcription of mitochondrial stress proteins which can disrupt cellular signalling processes, leading to aberrant gene transcription. Significantly higher expression levels of stress proteins, Hsp60, Hsp70, and SOD2, in NT embryos compared to IVP and AI would suggest that there may be some substance to this hypothesis.

Protein studies were employed to determine if there was any differences in expression between the treatment (NT) and control (AI) bovine cotyledon groups. NT embryos were *in vitro* produced. IVP cotyledon protein samples were available for use and, hence included as an interesting comparison. NT embryos are grown in identical culture conditions to IVP embryos, therefore including an IVP sample group would eliminate explanations of any results that may be attributed to culture conditions.

4.1.1 Hsp60 Protein Analysis

Hsp60 was detected in all samples of NT, IVP, and AI fetal and maternal cotyledons at time points of day 50; at the start of placentome formation, day 100; when the placentomes were completely formed, and day 150; when hydrallantois frequently occurs. Hydrallantois is an excessive accumulation of allantoic fluid which may be a consequence of placental dysfunction (Wells *et al.*, 1999). High expression of Hsp60 was observed in NT fetal cotyledons at day 100, NT maternal cotyledons at day 50, and significantly lower expression in NT cotyledons at day 150. Results were based on comparisons with AI cotyledons. After repeating the analysis under the same experimental conditions, previous significant differences were not reproducible. One possibility is that the protein samples degraded with repeated freezing and thawing, which was demonstrated to occur in Figure 3.15. A study on the ability of proteins in frog tissue to stay unchanged after freeze/thaw procedure showed that proteins which kept their structure were only 36% of the amount detected in controls, with results differing between frog tissues (Woods *et al.*, 2006). Grange-Midroit *et al.* (2002) discovered that after diluting brain protein samples in Laemmli buffer and denaturing at 95°C for 5 minutes before freezing and storing at -80°C, that after several freeze/thaw cycles immunoreactivity of target proteins was strongly reduced. The

proteins used in this study were prepared in the same manner, and also seemed to change in immunoreactivity after freeze/thaw cycles. One may therefore speculate that protein degradation due to repeated freeze/thaw cycles may be an explanation as to why significant differences in protein expression were not repeatable.

When different protein samples from placentome tissues at day 150 were investigated, NT displayed significantly lower Hsp60 expression than AI at time points of days 135-154 and days 154-163 of gestation. Note these were protein samples were from placentome tissues, not cotyledons as previously investigated; hence a direct comparison cannot be made.

Despite the inconsistent results, it is clear that Hsp60 is present and that there may be significant differences between the NT and AI groups.

4.1.2 Hsp70 Protein Analysis

Examination of Hsp70 at protein level in day 150 maternal cotyledons showed that Hsp70 expression was significantly higher in the NT group compared to AI. A large body of evidence now suggests a correlation between oxidative stress and heat shock protein induction (Calabrese *et al.*, 2003, Calabrese *et al.*, 2005, Jansen and Burton, 2004, Kawarsky and King, 2001, Lin *et al.*, 1998, Nollen and Morimoto, 2002). With the availability of transgenic animals and gene transfer, it has become possible to over express the gene encoding Hsp70 to show that Hsp70 proteins protect cells from injury, it has been demonstrated that overproduction of Hsp70 leads to protection in several different models of nervous system injury (Kelly *et al.*, 2002, Yenari, 2002). It is recognised that Hsp70 has an important role in regulating apoptosis by

suppressing apoptotic signals after mitochondrial disruption (Stankiewicz *et al.*, 2005). The results in this study would suggest that NT cotyledon cells are responding to a higher level of stress than AI cotyledons, possibly due to oxidative stress as a result of inefficient mitochondria.

4.1.3 SOD2 Protein Analysis

Mitochondria specific stress protein, SOD2, was expressed significantly higher in NT fetal cotyledons than AI at day 150. Other studies of SOD2 have indicated conflicting results; Harvey *et al.* (1995) concluded that no SOD2 mRNAs were found in bovine blastocysts, however they were detected in mouse embryos at all stages. Lequarré *et al.* (2001) found no expression in bovine embryos of SOD2 at 9-16 cell and morula stages, but it was present at zygote, 5-8 cell, and blastocyst stages. Others have found SOD2 to be present at all developmental stages up to blastocyst (Rizos *et al.*, 2003, Gutiérrez-Adán *et al.*, 2004), and implicate oxidative stress and ROS as a probable cause of increased SOD2 transcription. Samper *et al.* (2003) state that endogenous oxidative stress in murine results in nuclear genomic instability, which may influence cell growth and embryonic lethality. This study suggests there is mitochondrial oxidative stress in NT cotyledons based on high expression of the mitochondrial stress protein marker, SOD2.

4.2 OXIDATIVE STRESS TRIAL

4.2.1 Embryo Morphology at Blastocyst Stage

Morphological differences were observed between control and treatment embryos at both morula and blastocyst stages. Morula treatment embryos appeared darker, and displayed a darker cytoplasm at blastocyst than control embryos. Several studies comparing the morphology of bovine IVP and *in vivo* embryos have observed a much darker, grainy appearance in the IVP blastocysts, which has been attributed to the presence of lipid droplets (Abe *et al.*, 1999, Farin *et al.*, 2001, Lonergan *et al.*, 2006). Both treatment and control embryos were IVP, however these morphological reports appear exaggerated in the treatment embryos, suggesting the cause of the dark appearance is extended further. Abe *et al.* (1999) found that IVP morula and blastocyst stage embryos with darker cells possessed many lipid droplets, vesicles resembling lysosomes, and fewer cellular organelles such as mitochondria, compared to *in vivo* embryos. Farin *et al.* (2001) suggest that the increase in lipid present in IVP embryos is due to lipid accumulation resulting from inefficient metabolism of lipid by embryonal mitochondria. The authors also note that during development from compact morula to blastocyst stage the cellular volume occupied by mitochondria doubled for the *in vivo* produced embryos, compared to IVP. It has also been suggested that increased lipid content of IVP embryos may result from insufficient metabolism by mitochondria, which is consistent with the observed reduction in volume density of total mitochondria in IVP embryos compared to *in vivo* (Rizos *et al.*, 2003). Thompson (2000) states that the role of intracellular lipid in mitochondrial ATP production is still unresolved within the production of bovine embryos.

Using this information to explain the darker appearance of treatment embryos compared to control, one may assume that treatment embryos have comparably more metabolically compromised mitochondria, which may lead to further developmental dysfunction. However, no difference in expression of the mitochondrial stress marker, Hsp60, was observed between the control and treatment groups.

It was interesting to note the observation that at least one treatment embryo had a noticeably thicker zona pellucida (refer to Figure 3.18). The zona pellucida maintains the integrity of early developing embryos and the absence of the zona pellucida is known to cause dispersion of the blastomeres. It has been shown that the zona pellucida is thinner in IVP embryos compared to *in vivo* (Abe *et al.*, 1999), therefore considering that the treatment group had higher stresses during production one would assume the control embryos to display a thicker zona pellucida compared to the control group, which did not occur in this study.

The perivitelline space in the control embryos was noticeably larger than the treatment embryos at the day 7 blastocyst stage in at least one instance. IVP embryos are known to have a narrower perivitelline space compared to *in vivo* (Abe *et al.*, 1999, Van Soom and de Kruif, 1992) and stressed embryos have been shown to contain higher levels of cell fragments and debris such as lipid droplets (Abe *et al.*, 1999). Therefore, based on the assumption that the treatment embryos were at a more compromised state than the control IVP embryos, it may be presumed that the perivitelline space should be larger in the control group; which was indeed the case in this study.

4.2.2 Embryo Survival at Day 15

Day 15 was chosen as a time point due to logistical reasons, to transfer as many embryos into recipients as possible. Most losses occur by day 14-15, or within a week after transfer (Farin *et al.*, 2001), however it would be of interest to observe whether treatment embryos were compromised at around day 50 when placentome formation begins (Lee *et al.*, 2004).

Embryo survival at day 15 after transfer was similar between control and treatment groups; control: 45%, treatment: 43%. Pregnancy rates following transfer of embryos produced in a variety of culture systems range from about 45% to 75% (Farin *et al.*, 2001), therefore these results were within the expected range. The minimal difference between control and treatment groups may suggest that if embryos survived to blastocyst stage they appeared relatively unaffected by treatment at day 15, possibly due to optimal *in vivo* conditions rather than different IVP culture systems.

4.2.3 Embryo Morphology at Day 15

One of the treatment embryos displayed unusual growths from the trophoblast exterior (Figure 3.21A). Although treatment embryos appeared relatively unaffected at this stage compared to control, this morphological abnormality may be an indication of pathology that will compromise embryo viability further into development. Yuan *et al.* (2003), in a study using 20% oxygen during IVP of bovine embryos to blastocyst stage state that the increased ROS levels in embryos cultured under 20% oxygen are probably implicated in retarded embryonic growth. Altered

gene expression due to increased ROS and mtDNA mutations may be a causative factor behind this unusual trophoblast growth.

4.2.4 Mitochondrial Respiration and Stress Protein Expression

4.2.4.1 Hsp60 Expression

As Hsp60 is a mitochondrial stress protein, it would be expected that the expression levels may be higher in the treatment group. However, no statistical difference in Hsp60 expression was observed between control and treatment embryos. In a study by Harvey *et al.* (2007), comparing 2%, 7%, and 20% O₂ levels during culture of IVP bovine embryos, no difference in Hsp60 expression at mRNA level was reported at blastocyst stage. In this study, at blastocyst stage Hsp60 protein was present but due to insufficient immunodetection, statistical analyses were unable to be completed.

4.2.4.2 SOD2 Expression

SOD2 is an enzyme that functions in defence against oxidative stress and is mitochondria specific (O'Brien *et al.*, 2004). The expression of SOD2 protein at day 15 was 1.5 times higher in the control group compared to the treatment group. While no studies to date have investigated SOD2 expression at day 15, others have conducted research at blastocyst level. Harvey *et al.* (1995) failed to see SOD2 transcription in bovine embryos; however Lequarre *et al.* (2001) detected SOD2 in 80% of blastocysts and suggested that culture conditions influenced expression of SOD2. Higher levels of SOD2 mRNA have been reported in *in vivo* embryos compared to IVP embryos, which was suggested to relate to higher (possibly double) cell numbers in *in vivo* embryos (Lequarre *et al.*, 2001). Due to increased ROS from oxidative stress in the treatment group of this study, apoptosis could be expected to

be higher – resulting in fewer cells (Rizos *et al.*, 2003). This may explain why the control group had significantly higher expression of SOD2 than the treatment group.

4.2.4.3 *COX2 Expression*

COX2 converts arachidonic acid to prostaglandin, and is essential for blastocyst implantation (Lim *et al.*, 1999). Prostaglandins participate in a variety of cellular functions including cell proliferation and differentiation. COX2 over-expression has also been associated with malignant cancer phenotypes, which has been linked with increased ROS (Pathak *et al.*, 2005). No statistical differences of COX2 expression were present between the control and treatment groups at day 15.

4.2.4.4 *TIM Expression*

TIM is a glycolytic enzyme that plays an important role in several metabolic pathways and is essential for efficient energy production. Higher quantities of glucose are required during oxidative stress and TIM is an enzyme involved in glycolysis. There was no statistical difference in expression between control and treatment groups, which suggests that higher amounts of TIM are not transcribed during oxidative stress. No data are presently available on increased or decreased expression of TIM during bovine embryonic development. However, one study (Kathiresan *et al.*, 2006) has investigated the expression of TIM during embryonic development of crocodiles and found that TIM activity increased with the advancement of *in vivo* embryonic development.

4.2.4.5 Hsp70 Expression

Hsp70 is one of the earliest embryonic genes to be expressed, which facilitates the refolding or degradation of damaged proteins (Calabrese *et al.*, 2005), and regulates apoptosis and embryonic development (Beere *et al.*, 2000, Saleh *et al.*, 2000). Studies have found that Hsp70 is upregulated in embryos generated in culture medium that induced cellular stress compared to control (Sagirkaya *et al.*, 2006, Wrenzycki *et al.*, 2001). Hsp70 was expressed at mRNA level in day 15 embryos; however no differences were identified between control and treatment sample groups, which suggests that the embryos were not under cellular stress in the *in vivo* environment at day 15.

4.2.4.6 GLUT1 expression

GLUT1 is a facilitative glucose transporter, which is involved in glycolysis and belongs to a family of 9 isoforms. ATP demands correlate with higher respiration rates and glucose uptake by embryos, generating an increase in the glycolytic and pentose phosphate pathways, and consequently GLUT1 transportation of glucose (Lopes *et al.*, 2007). Bovine IVP embryos have been demonstrated to respond to changes in oxygen concentrations by altering the expression of GLUT1 (Harvey *et al.*, 2004). However, other researchers (Lazzari *et al.*, 2002, Sagirkaya *et al.*, 2006) have found no difference in GLUT1 expression between the *in vivo* control and *in vitro* treatment groups; hence there is debate whether this isoform plays such a crucial role in embryonic glucose uptake from the environment as has previously been thought. In this study there was no visible difference of GLUT1 expression between treatment and control groups.

4.3 SUMMARY

Results of this study indicate that cellular stress proteins are present in cloned placental tissues and IVP embryo tissue that has been cultured under high oxygen conditions. Morphological differences were observed between control and treatment IVP embryos, which may be linked to oxidative stress. The significant differences observed between control and treatment groups in both the cloned investigation and the oxidative stress trial need to be explored further, possibly using a different quantification measurement, such as analysis with software other than Quantity One, e.g., Scion Image software or AlphaEase™ densitometry software. It has been established that mitochondrial stress proteins such as Hsp60 are involved in cellular signalling, which may result in altered gene transcription and aberrant embryonic growth. The study by Fischer-Brown *et al.* (2005) suggests a link between observational similarities in clone placental abnormalities and their study on calf placental morphology at parturition after embryonic growth in culture medium under high oxygen concentration. Their study implicates oxidative stress and increased ROS production, which may induce damaging epigenetic effects that lead to abnormal fetal and placental growth. Based on the evidence from other researchers and the results of this study, I would suggest a link between mitochondrial oxidative stress and a role in embryonic development, and would encourage further investigation in this area.

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