ROLE OF OXYGEN AND SPHINGOMYELIN METABOLISM IN ACTIONS OF HEAT SHOCK ON THE OOCYTE AND EMBRYO

By

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To my parents Maria do Rosário de Castro e Paula and João Batista de Paula Neto my brother and sisters André Felipe, Nathália and Ana Luísa de Castro e Paula my wife Camila do Amaral Brito de Castro e Paula my major advisor Dr. Peter J. Hansen

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A549	Human Lung Adenocarcinoma Cells
Acetyl-coA	Acetyl Coenzyme A
AI	Artificial Insemination
AIF	Apoptosis Inducing Factor
Apaf-1	Apoptosis Activating Factor-1
Ara-C	1-ß-D-Arabinofuranosylcytosine
aSMase	Acidic or Acid Sphingomyelinase
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
BSO	Buthionine Sulfoximine
CCCP	Carbonyl Cyanide m-Chlorophenylhydrazone
COC	Cumulus-Oocyte Complex
DISC	Death Inducing Signaling Complex
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
dUTP	Deoxy-Uridine Tri-phosphate
EFAF	Essentially Fatty-Acid Free
eNOS	Endothelial Nitric Oxide Synthase
FADH2	Reduced Flavin Adenine Dinucleotide
FOXY	Fluorometric Fiber-Optic Oxygen
FSH	Folicle-Stimulating Hormone
G6PDH	Glucose 6 Phosphate Dihydrogenase
GLM	General Linear Model

GnRH	Gonadotrophin Releasing Hormone
GSH	Glutathione
GSSG	Oxydized GSH
H_2O_2	Hydrogen Peroxide
HEK 293	Embryonic Kidney Cell Line
HeLa	Human Cervix Cancer Cell Line
HEPES	Hydroxyethylpiperazine Ethanesulfonate
HL-60 cells	Human Leukemia Cell Line
IAP	Inhibitor of Apoptosis Protein
IVF	In-Vitro Fertilization
JNK	c-Jun n-Terminal Kinase
KSOM	Potassium Simplex Optimized Medium
KSOM-BE2	Potassium Simplex Optimized Medium – Bovine Embryo 2
LH	Luteinizing Hormone
mRNA	Messenger Ribonucleic Acid
mSOF	Modified Synthetic Oviduct Fluid
mTCM	Modified Tissue Culture Medium
NADH	Nicotinamide Adenine Dinucleotide, Reduced
NADP+	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate, Reduced
nSMase	Neutral Sphingomyelinase
OCM	Oocyte Collection Medium
OMM	Oocyte Maturation Medium

OPU	Ovum Pick-Up
PBS	Phosphate Buffered Saline
PC12	Pheochromocytoma Cell Cell Line
PGF2α	Prostaglandin F-2α
PHE	0.5 mM Penicillamine, 0.25 mM Hypotaurine, and 25 μM Epinephrine in 0.9% [w/v] NaCl
PKC	Protein Kinase C
PLC	Phospholipase C
PPP	Pentose Phosphate Pathway
PVP	Polyvinylpyrrolidone
Redox	Reduction/Oxidation
RIA	Radioimmunoassay
ROS	Reactive Oxygen Species
S1P	Sphingosine-1-Phosphate
SAPK	Stress Activated Protein Kinase
SAS	Statistical Analysis System
SEK	SAPK-Kinase
SEM	Standard Error of the Mean
SH	Thiol
SMase	Sphingomyelinase
SOF	Synthetic Oviduct Fluid
TALP	Tyrode's Albumin Lactate Pyruvate
TCM-199	Tissue Culture Medium-199

TL	Tyrode's Lactate
TNF	Tumor Necrosis Factor
TNF- α	Tumor Necrosis Factor- α
TRAIL	TNF – Related Apoptosis Inducing Ligand
TUNEL	Terminal Deoxynucleotidy Transferase Mediated dUTP Nick End L Labeling
UV	Ultra Violet
UVA	Ultraviolet-A
VEGF	Vascular Endothelial Growth Factor
XIAP	Inhibitor of Apoptosis Protein
z-DEVD-fmk	N-Benzyloxycarbonyl-Asp-Glu-Val-Asp-Fluoromethyl Ketone

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Experiments described in the present dissertation were designed with the overall objective of determining potential factors contributing to the phenomenon described in the literature of decreased oocyte and embryo competence to reach the blastocyst stage and increased incidence of apoptosis caused by heat shock. Focus was given to the role of oxygen in mediating the effects of heat shock in the oocyte and embryo and to ceramide as a signal potentially involved in induction of apoptosis in embryos. The effects of heat shock in the embryo were shown to be affected by oxygen concentrations. This was demonstrated in a series of experiments where heat shock at different stages of embryo development had more severe effects in reducing development and inducing apoptosis in high oxygen when compared to heat shock in low oxygen. The antioxidant dithiothreitol (DTT) attenuated the negative effects of heat shock in high oxygen in blastocyst development and blocked heat shock-induced apoptosis, suggesting that heat shock effects involve oxidative stress. The presence of a responsive signaling mechanism involving the sphingomyelin pathway involved in induction of apoptosis was demonstrated for the first time in the preimplantation embryo. The existence of this mechanism is indicated by the fact that ceramide, but not its inactive analogue dihydroceramide, induced apoptosis in a stage-specific manner and decreased embryo development. Further support is

provided by the fact that heat shock induced activity of neutral sphingomyelinase (nSMase), an enzyme responsible for cleaving sphingomyelin to yield ceramide.

Regarding the effects of heat shock in the oocyte, it was demonstrated that heat shock effects were not dependent upon oxygen concentration as heat shock decreased oocyte competence to reach the blastocyst stage in high oxygen and at oxygen concentrations similar to the concentration found follicular fluid in vivo.

Because results have previously demonstrated that heat stress can reduce blood flow to the reproductive tract, hypoxia was considered as one possible indirect effect of heat stress in reducing fertility. A field study was designed to determine changes in oxygen concentration in the follicular fluid of dominant pre-ovulatory follicles caused by acute heat stress. Oxygen concentration in the follicular fluid was not affected by heat stress.

In summary results demonstrated that oxygen concentration determines the magnitude of deleterious effects of heat shock in embryo development and apoptosis, potentially due to oxidative stress. The demonstration of existence of a ceramide signaling mechanism in the embryo may help to further understand the mechanism by which heat shock and other stresses induce apoptosis in preimplantation embryos. In the oocyte, oxygen does not seem to influence the severity of effects of heat shock. Results from in vivo study established that follicular oxygen concentration is not affected by heat stress and therefore hypoxia is not a factor involved in decreased fertility observed during the hot months of the year.

CHAPTER 1
INTRODUCTION

Pregnancy is established when a competent oocyte is successfully fertilized and the zygote encounters a suitable environment that will nourish it and allow it to develop to birth. In a healthy and otherwise fertile female, environmental disturbances can occur at any time and compromise fertility by causing stresses that can act directly and indirectly on the developing and maturing oocyte and the developing embryo. One environmental factor that commonly affects reproductive performance is heat stress, defined as environmental conditions that make it difficult for animals to regulate their body temperature. Exposure of lactating dairy cows to an environment characterized by heat stress causes an increase in body temperature and a corresponding decrease in reproductive function that includes reduced expression of estrus (Thatcher and Collier 1986), altered follicular development (Badinga et al. 1993; Wolfenson et al. 1995; Trout et al. 1998; Wilson et al. 1998), decreased oocyte competence (Rocha et al. 1998; Roth et al. 2001a; Roth et al. 2002), reduced circulating concentrations of progesterone (Howell et al. 1994; Sartori et al. 2002a) and increased embryonic mortality (Putney et al. 1988a; Ealy et al. 1993; Sartori et al. 2002b). These actions of heat stress, which span a wide range in the reproductive life cycle, from oocyte growth and maturation to early embryo development, account for the large decrease in fertility during the hot months of the year (Elliott and Ulberg 1971; Gwazdauskas et al. 1973; Badinga et al. 1985; Cavestany et al. 1985; Wolfenson et al. 1988; Rocha et al. 1998; Al-Katanani et al. 1999). Importantly, the physiology and requirements of the oocyte and embryo change during the reproductive life cycle as does the microenvironment in which they reside. The outcome caused by heat shock is dependent, therefore, upon the interaction between the elevated temperatures to which the oocyte and embryo are exposed, the intrinsic properties of the oocyte and embryo itself (in turn affected by

stage of development) and the microenvironment of the reproductive tract (which may also be changed by heat stress).

The decreased fertility caused by elevation in body temperature can be attributed to a variety of factors involving direct and indirect effects on the oocyte and the embryo. One important factor known to modulate embryonic performance in vitro is the oxygen environment surrounding the oocyte and the developing embryo. Understanding how the oxygen environment and the reduction/oxidation (redox) state of the oocyte and embryo interact to modify responses to heat shock may contribute to overall comprehension of the pathology of decreased fertility of lactating dairy cows during summer.

Heat shock induces apoptosis in preimplantation bovine embryos in a mechanism that involves activation of caspase-9 followed by capsase-3, compatible to the mitochondrial intrinsic pathway of apoptosis. In different cell types, heat shock can cause increased production of ceramide, a second-messenger involved in early events of apoptosis. Identification of the presence of an active ceramide signaling mechanism in the preimplantation embryo could help elucidate the pathway by which different stressors (e.g. heat shock), induces apoptosis, and potentially open a new window of opportunity for developing strategies to reduce effects of heat shock and other stresses to the embryo.

Objectives of this dissertation were to 1) determine the role of oxygen environment and oxidative stress in the effects of heat shock on development and apoptosis in the preimplantation embryos, 2) demonstrate the existence of an active ceramide signaling pathway in the bovine preimplantation embryo, 3) determine the role of oxygen environment and glucose concentration in the effects of heat shock on oocyte competence and 4) demonstrate if part of effects of heat

stress in reducing fertility during the summer are due to reduction in oxygen supply to the dominant pre-ovulatory follicle in the cow.

CHAPTER 2
LITERATURE REVIEW

Effects of Heat Stress on Fertility

Factors extrinsic and intrinsic to the female can cause the environment surrounding the oocyte and the embryo to become adverse to developmental competency. Among these factors is an elevation in body temperature caused by maternal heat stress. Decreased fertility during periods of heat stress have been reported in a wide variety of species including cattle (Elliott and Ulberg 1971; Gwazdauskas et al. 1973; Badinga et al. 1985; Cavestany et al. 1985; Wolfenson et al. 1988; Rocha et al. 1998; Al-Katanani et al. 1999), pigs (Warnick et al. 1965) and mice (Matsuzuka et al. 2005a).

By virtue of its high internal heat production, the lactating dairy cow is likely the animal that is most sensitive to disruption of fertility by heat stress. The phenomenon of reduced fertility of high producing dairy cows during summer has been described in several regions around the globe (Rocha et al. 1998; Al-Katanani et al. 1999; Rutledge et al. 1999; Zeron et al. 2001). Retrospective analysis of records provided by the Dairy Herd Improvement Association regarding first service from 8124 Holstein from Florida and South Georgia showed that 90-day non-return rate decreased during the summer. This decrease was greatest in South Florida, intermediate in North Florida and least in South Georgia. Furthermore, the decrease was dependent on milk production: cows with greater milk yield experienced a more severe summer decrease in fertility than cows with lower milk yields (Al-Katanani et al. 1999). These results suggest that as milk yield increases, susceptibility to heat stress will also increase. Such an association has also been observed historically in Spain (Lopez-Gatius 2003). As a result, heat stress is not a problem only for cows in warm regions of the world. Indeed, summer depressions in days open have been seen throughout the United States (Oseni et al. 2003).

In one of the early reports on the effects of heat stress in cattle (Dunlap and Vincent 1971) beef heifers exposed continuously to heat stress from Day 1 to Day 3 after breeding had 0% conception rate whereas heifers not exposed to heat stress had a 48% conception rate. Subsequently, Putney et al. (1988) performed a study where superovulated dairy heifers were exposed to a heat stress consisting of 16 hours at 30°C and 8 hours at 42°C each day from Day 1 through Day 7 after artificial insemination (AI). Embryos collected from heat stressed heifers had decreased quality and were more retarded when compared to control heifers maintained continuously at 20°C.

Rocha et al. (1998) reported that the number of oocytes classified as normal from Holstein cows subjected to ovum pick-up (OPU) and their competence to reach the blastocyst stage, was less during the hot time of the year when compared to oocytes collected during the cool season. Similarly, the percent of non-fertilized oocytes harvested from lactating cows five days after superovulation was greater than from non-lactating animals during the summer; this difference was not observed during the winter (Sartori et al. 2002b). Furthermore, the decreased competence of oocytes associated with heat stress can continue past summer months (Roth et al. 2001a; Roth et al. 2002), indicating delayed effects of elevated temperature on oocyte competence. These delayed effects were also observed for synthesis of estradiol and androstenedione production in medium-size follicles (Roth et al. 2001b).

Although the effects of heat stress are severe early in pregnancy, data from different species are indicative that these effects become milder as pregnancy advances. Exposure of ewes to heat stress from day 1 post-breeding to Day 24 of pregnancy reduced lambing rate when compared to control ewes or ewes exposed to heat stress from Day 5 to Day 24 (Dutt 1963). Ealy et al. (1993) exposed superovulated cows to heat stress for 7 hours on Days 1, 3, 5 or 7 after

insemination. Stage of development and viability at Day 8 were compromised only for embryos derived from cows exposed to heat stress on Day 1. Similar findings were also shown in swine (Tompkins et al. 1967).

Consequences of Heat Shock for the Oocyte and Embryo

Effects of Heat Shock on Oocyte Competence in the Bovine

Experiments using cumulus-oocyte complexes (COCs) matured in vitro have been extensively used to demonstrate direct effects of heat shock on oocyte function. Heat shock at 40 or 41^{o}C during the first 12 hours of maturation caused nuclear maturation to arrest prior to initiation of meiosis II (Roth and Hansen 2005). Similar results have been described by others using bovine oocytes matured in vitro (Lenz et al. 1983; Payton et al. 2004) and mouse oocytes matured in vivo (Baumgartner and Chrisman 1987). Effects of heat shock can be mediated by alteration in spindle formation and disruption in chromosome alignment (Ju et al. 2005, Roth & Hansen 2005). Exposure of metaphase II oocytes to heat shock for 1 to 4 hours caused a 5-fold increase in spindles with altered morphology when compared to control oocytes (Ju et al. 2005). When oocytes were heat shocked at metaphase I, the majority of those that failed to reach metaphase II had spindle malformation (Roth & Hansen 2005).

Heat shock during maturation can induce oocyte apoptosis (Roth and Hansen 2004a). Furthermore, the proportion of oocytes that become blastocysts after insemination is also reduced by heat shock during maturation (Edwards and Hansen 1996; Payton et al. 2004; Roth and Hansen 2004b; Edwards et al. 2005; Ju et al. 2005). However, experiments to determine the timing of block in development induced by heat shock in the oocyte have been controversial. In one study, heat shock reduced oocyte competence to cleave and develop to blastocyst stage (Roth and Hansen 2004b). In other studies, heat shock affected the proportion of embryos that

developed to the blastocyst stage but not cleavage rate (Edwards and Hansen 1996; Payton et al. 2004; Edwards et al. 2005; Ju et al. 2005).

Effects of Heat Shock on Development of the Bovine Preimplantation Embryo

Like the oocyte, the preimplantation embryo is also susceptible to deleterious effects of heat shock. A large number of studies have aimed to understand the cellular and molecular mechanisms of responses of the embryo to elevated temperatures.

As observed in vivo (Ealy et al. 1993), cultured bovine preimplantation embryos are less susceptible to deleterious effects of heat shock at later stages of development (Ealy et al. 1995b; Ju et al. 1999; Sakatani et al. 2004). For example, Edwards and Hansen (1997) reported that the effect of exposure of embryos to 41^{o}C for 12 hours was greatest for two-cell embryos, intermediate for 4 to 8-cell embryos, and not apparent for morulae. Although embryos >16-cells are more resistant to heat shock, they are not impervious to elevated temperature. Experiments have demonstrated that the proportion of Day 4 or Day 5 bovine embryos that develop to the blastocyst stage can also be reduced by heat shock (Paula-Lopes and Hansen 2002a; Jousan and Hansen 2004).

One of the reasons that heat shock at the two-cell stage reduces development likely involves ultrastructural changes in the embryo. Heat shock at 41^{o}C caused a shift in localization of organelles located in the periphery towards the center of the cell (Rivera et al. 2003; Rivera et al. 2004c). This redistribution of organelles is caused by rearrangement of the cytoskeleton characterized by disruption of microtubules and microfilaments induced by heat shock (Rivera et al. 2004c). Heat shock can also cause an increase in the number of swollen mitochondria, compatible with membrane depolarization which can interfere with energy production by oxidative phosphorylation and potentially produce reactive oxygen species (ROS). Rivera et al. (2004a) evaluated the effect of heat shock on oxygen consumption of two-cell embryos. There

was a numerical decrease in oxygen consumption but this effect was not significant and there was no change in embryonic ATP content.

Heat Shock and Apoptosis in Preimplantation Embryos

Apoptosis is commonly observed in preimplantation embryos prior to or at the blastocyst stage (Jurisicova et al. 1996; Jurisicova et al. 1998; Long et al. 1998; Paula-Lopes and Hansen 2002b; Gjørret et al. 2003). In the bovine, TUNEL-positive cells with condensed nuclei were first observed at the 6-cell stage in vitro and at the 21-cell stage in vivo (Gjørret et al. 2003).

Recently, experiments demonstrated that heat shock can increase the incidence of TUNEL-positive cells in bovine and porcine embryos (Paula-Lopes and Hansen 2002b; Paula-Lopes and Hansen 2002a; Jousan and Hansen 2004; Isom et al. 2007; Jousan and Hansen 2007; Loureiro et al. 2007). The ability to undergo apoptosis is developmentally regulated. Embryos heat shocked at the 2 and 4-cell stages of development failed to show an increase in percent of TUNEL-positive cells or in group II caspase activity (which includes caspases-3, 7 and 2) whereas embryos $\geq$16-cells showed an increase in the percent of TUNEL-positive cells (Paula-Lopes and Hansen 2002a; Loureiro et al. 2007), caspase-9 (Loureiro et al. 2007) and group II caspase activity (Paula-Lopes and Hansen 2002b). The increase in caspase-9 and group II caspase activities and on the percent of TUNEL-positive cells was reduced by the use of the caspase inhibitors (Paula-Lopes and Hansen 2002a; Loureiro et al. 2007).

It is interesting to note that developmental changes in embryonic capacity for induced apoptosis are not restricted to heat shock. Molecules shown to trigger apoptosis in somatic cells like tumor necrosis factor-α (TNF-α) (Ding and Yin 2004) and arsenic (Florea et al. 2005) can also induce apoptosis in embryos $\geq$16-cells but not in embryos at the two-cell stage (Krininger et al. 2002; Soto et al. 2003b).

Mechanism of Apoptosis Induced by Heat Shock

General Features of Apoptosis

Throughout development, multicellular organisms undergo changes in organization and cellular distribution to achieve the requisite phenotype. At times, some cells become unnecessary or redundant and others can be damaged due to intrinsic errors or due to external stress. Apoptosis is one process of programmed cell death that causes elimination of cells during developmental processes and after cell damage. In the apoptotic process, the cell undergoes transformations that allow its elimination with little or no compromise to cells in its surroundings.

There are two main pathways that lead to apoptosis: the extrinsic or receptor-mediated pathway and the intrinsic or stress-mediated pathway. Both result in membrane blebbing, nuclear and chromatin condensation and fragmentation, shrinking of the cell, mitochondrial disintegration and formation of apoptotic bodies (budding off cellular portions). The driving force for these cellular changes is activation of a series of proteinases called caspases that cleave intracellular proteins. By 2001, 70 different caspase substrates had been identified and most of them had functions that could be correlated to phenotypical changes observed during caspase-dependent apoptosis (reviewed in Nicholson 1999).

The extrinsic pathway for apoptosis involves signaling for induction of apoptosis by extracellular regulatory ligands such as TNF-α, TRAIL, Fas and Cd95 (Nagata 1999; MacFarlane 2003; Peter and Krammer 2003; Rossi and Gaidano 2003; Ding and Yin 2004). Apoptosis is initiated by binding of these ligands to plasma membrane receptors of the TNF receptor family. Upon activation, a protein complex assembles forming the death inducing signaling complex (DISC). Adaptor proteins from the complex link the receptor to the initiator

caspases (2, 8 and/or 10). Once activated, initiator caspases interact with their substrates, the effector caspases (3, 7 and 6), which ultimately leads to cell death.

The intrinsic pathway is not receptor dependent and is a cellular response to different types of stresses like radiation, heat stress, free radicals and others. Molecules of the Bcl-2 family are responsible for one of the initial steps of the intrinsic pathway. Upon stress, a pro-apoptotic member of the Bcl-2 family localized in the cytosol, Bax, undergoes conformational change and translocates to the mitochondrial membrane and cause mitochondrial membrane depolarization and apoptosis. Whether Bax will exert its pro-apoptotic effects depends on the presence of other members the Bcl-2 family like pro-apoptotic members Bad, NOXA and PUMA and the anti-apoptotic members Bcl-2 and Bcl-X_L. From specific interactions between these molecules, the decision to progress through the apoptotic cascade is made. Therefore, at least initially, the fate of the cell is dependent on the balance between these pro- and anti-apoptotic factors affecting the mitochondrial membrane (Tsujimoto 1998; Kuwana and Newmeyer 2003; Takahashi et al. 2004; Dejean et al. 2006; Kutuk and Basaga 2006; van Delft and Huang 2006).

If pro-apoptotic factors predominate over anti-apoptotic ones, the initial signal generated as a result of stress leads to opening of permeability transition pores on the mitochondrial membrane and loss of mitochondrial membrane potential (Zamzami et al. 1996; Miyaguchi et al. 2004; Piret et al. 2004). Some proteins otherwise confined to the intermembrane space leak into the cytoplasm and nucleus where they display their pro-apoptotic characteristics. Among these proteins, cytochrome c, AIF and Smac/diablo have been thoroughly studied. AIF can target the nucleus and cause chromatin condensation and DNA cleavage (Ye et al. 2002). Cytochrome c, along with Apaf-1, interacts with procaspase 9 and ATP to form the apoptosome (Quillet-Mary et al. 1997; Ruppitsch et al. 1997; Adrain and Martin 2001). Dimerized pro-caspase-9 is then

cleaved to caspase-9 which in turn activates effector caspases-3 and 7 (Srinivasula et al. 1998; Slee et al. 1999). Smac/diablo acts by binding and neutralizing the anti-apoptotic (i.e., caspase-inhibitory) proteins of the IAP family like XIAP (Roy et al. 1997; Du et al. 2000; Verhagen et al. 2000; Ekert et al. 2001).

Although caspases have been demonstrated to be involved in cell death by apoptosis in several different models, cells have also mechanisms for inducing apoptosis in the absence of caspase activity. As mentioned above, AIF released from the mitochondria promotes apoptosis by directly targeting the DNA (Ye et al. 2002) with involvement of the co-factor cyclophilin A (Candé et al. 2004).

Induction of Apoptosis by Heat Shock

Heat shock induces apoptosis in several different cell types including female and male germ cells (Roth and Hansen 2004a; Jia et al. 2007), embryonic cells (Paula-Lopes and Hansen 2002b; Paula-Lopes and Hansen 2002a; Jousan and Hansen 2004; Jousan and Hansen 2007; Loureiro et al. 2007), somatic cells like thymocytes (Troiano et al. 1995), astrocytes (Na et al. 2001), cardiomyocytes (Qian et al. 2004) and Chinese hamster ovary cells (Bettaieb and Averill-Bates 2005), as well as cancer cell lines like human leukemia cells HL-60 (Kondo et al. 2000a), human adenocarcinoma cervical cells (Bettaieb and Averill-Bates 2005) and human leukemic monocyte lymphoma cells (Kim et al. 2005b).

The intrinsic apoptotic pathway is thought to be activated during heat shock and activation of different steps of this pathway has been demonstrated experimentally to be induced by heat shock. Upon heat shock, conformational changes in Bax drives these molecules to translocate to the mitochondrial membrane (Bettaieb and Averill-Bates 2005; Stankiewicz et al. 2005) leading to membrane permeability transition (Qian et al. 2004; Bettaieb and Averill-Bates 2005; Stankiewicz et al. 2005) and release of pro-apoptotic factor cytochrome-c (Mirkes and Little

2000; Little and Mirkes 2002; Qian et al. 2004; Bettaieb and Averill-Bates 2005; Wada et al. 2005). Cytochrome c then binds to cytoplasmic molecules Apaf-1, ATP and pro-caspase-9 (Quillet-Mary et al. 1997; Ruppitsch et al. 1997; Adrain and Martin 2001) leading to cleavage of pro-caspase-9 and formation of active caspase-9 (Enomoto et al. 2001; Zhang et al. 2003; Bettaieb and Averill-Bates 2005). Once activated, caspase-9 can activate caspase-3 (Mirkes et al. 2001; Qian et al. 2004) leading to cell death associated with the hallmarks of apoptosis.

In preimplantation bovine embryos, the Bax gene is expressed and higher expression levels were correlated with slow developmental speed (Gutiérrez-Adan et al. 2004). The anti-apoptotic molecule Bcl-2 was shown to be high in normal embryos showing little fragmentation and low in fragmented embryos (Yang and Rajamahendran 2002). Following mitochondrial depolarization, cytochrome c and Smac-diablo were released into the cytosol in fragmented murine embryos and after treatment with staurosporine (Honda et al. 2005). The direct involvement of Bcl-2 molecules and cytochrome c in heat shock-induced apoptosis in preimplantation embryos has yet to be determined. However, as mentioned in section "Heat shock and apoptosis in preimplantation embryos", the fact that heat shock-induced apoptosis causes an increase in caspase-9 activity (Loureiro et al. 2007, Brad and Hansen, unpublished) and that caspase-9 inhibitor can block apoptosis induced by heat shock (Loureiro et al. 2007) is strong evidence of the involvement of the mitochondrial intrinsic pathway in this process.

The Role of Ceramide in Heat Shock-Induced Apoptosis

While it is known that heat shock induces apoptosis via mitochondrial depolarization, the initial signal through which heat shock causes this effect on the cell is not well established. Available evidence suggests ceramide and ROS may play a crucial role in initial signaling events. Indeed, evidence exists that the sphingolipid ceramide plays a central role as a second messenger in many kinds of stress-induced apoptosis such as heat shock, ultra-violet radiation,

cytotoxic drugs, and free radicals (reviewed in Basu and Kolesnick 1998, Pettus et al. 2002, Kolesnick and Fuks 2003, Ruvolo 2003, Goldkorn et al. 2005, Taha et al. 2006). Exogenous ceramide can induce apoptosis in many different cell types such as human neuroblastoma cell line (Ito et al. 1999), pro-monocytic cells (Ji et al. 1995), lymphoma cells (Susin et al. 1997), keratinocytes (Di Nardo et al. 2000), and chicken oviduct cells (Kim et al. 2005c). Details of the involvement of ceramide in apoptosis, including its role in apoptosis induced by heat shock, are provided in this section.

Sphingomyelin, Sphingomyelinases and Ceramide Formation

There are two main pathways that lead to formation of ceramide. The first involves hydrolysis of sphingomyelin via acidic and neutral sphingomyelinases (SMases). The second involves *de novo* synthesis via sphingosine catabolism (Andrieu-Abadie and Levade 2002). Other apparently secondary pathways are involved in ceramide synthesis as well.

Sphingomyelin is composed of ceramide linked to phosphocoline via a phosphodiester bond (Fahy et al. 2005). Sphingomyelin hydrolysis is performed by several sphingomyelin-specific phospholipases C (PLCs), the SMases, which can cleave the phosphodiester bond yielding ceramide and phosphocoline. The variants of SMase differ primarily in their catalytic properties and subcellular localization, and regulation. Acidic (or acid) SMase (aSMase) is ubiquitous and localized in lysosomes and endosomes where it performs metabolic degradation of sphingomyelin at a pH optimum around 5 (Kolesnick and Fuks 2003). Deficiency of this enzyme causes the lethal Niemann-Pick hereditary disease that results in accumulation of sphingomyelin in lysosomes (Elleder 1989). aSMase was the first of the SMases to be cloned and sequenced (Quintern et al. 1989) and an aSMase knockout mouse model was developed by Otterbach and Stoffel (1995).

Because of the existence of the mouse knockout model and the Niemann-Pick disease in humans, aSMase has been the best studied of the SMases to date. A secreted form of aSMase derived from the same gene as the lysosomal aSMase has also been described (Schissel et al. 1996) but the function of this enzyme is not yet clear.

The other major SMase has basically the same function of aSMase; however, it is active under a pH optimum around 7.4 and has therefore been termed neutral SMase (nSMase). The Mg^{2+}-dependent nSMase resides in the cell membrane (Chatterjee 1993), where most of the sphingomyelin is located. A soluble nSMase Mg^{2+}-independent has also been characterized and is localized in the cell cytoplasm (Okazaki et al. 1994).

Ceramide Signaling

Several lines of evidence exist to demonstrate that ceramide is a molecule capable of inducing apoptosis. Experiments have demonstrated that exposure to ceramide is sufficient to induce apoptosis in different cell types (Obeid et al. 1993; Jarvis et al. 1994; Dbaibo et al. 1995; Ji et al. 1995; Pruschy et al. 1999; Di Nardo et al. 2000).

Different cell stressors like TNF-α, radiation, ultraviolet-A rays (UVA) and heat shock can induce ceramide generation via activation of aSMase and nSMase in non-embryonic cells. When cells from wild-type mice were exposed to UVA, an increase in aSMase activity was observed followed by ceramide generation and activation of c-Jun N-terminal kinase (JNK). In contrast, cells from aSMase-deficient mice did not undergo apoptosis induced by UVA light (Zhang et al. 2001). There was an increase in activity of nSMase and ceramide formation when human breast adenocarcinoma cells were treated with TNF-α (Luberto et al. 2002). Similar studies using different cell types showed comparable effects of TNF-α on ceramide formation (Garcia-Ruiz et al. 1997; Bulotta et al. 2001).

Heat shock can also induce ceramide formation (Chang et al. 1995; Verheij et al. 1996; Kondo et al. 2000a; Kondo et al. 2000b; Jenkins et al. 2002). In an experiment by Verheij et al. (1996), exposure of cells of the human leukemic monocyte lymphoma cell line U937 to a severe heat shock of 45 ^{o}C for 30 minutes caused a significant increase in ceramide formation. Similarly, ceramide accumulated in a temperature-dependent manner when human leukemia cells HL-60 were exposed to heat shock of 40, 42 and 44 ^{o}C for 30 minutes and this increase in ceramide was paralleled by a decrease in sphingomyelin content and increase in nSMase activity (Kondo et al. 2000a). About 20% of cells were already apoptotic 4 hours after exposure to 40^{o}C (Kondo et al. 2000a). Chung et al. (2003) found that exposure of HL-60 cells to 42^{o}C for 1 hour caused an increase in both nSMase and aSMase and further increase in temperature to 44^{o}C caused an even greater increase in aSMase.

Inhibitors of nSMase (Luberto et al. 2002; Czarny and Schnitzer 2004; Claus et al. 2005; Wu et al. 2005; Peng et al. 2006; Sakata et al. 2007) and aSMase (Sortino et al. 1999; Deigner et al. 2001; Pru et al. 2002; Hara et al. 2004b; Miyoshi et al. 2004) have been used to study the role of ceramide in cell signaling and stress-induced apoptosis. Addition of a nSMase inhibitor blocked initial accumulation of ceramide induced by TNF-α and decreased cytochrome-c release, induction of caspase-9 activity and nucleus condensation (Luberto et al. 2002). In another study, there was an increase in ceramide formation when human glioma cells were exposed to γ-radiation and this increase was suppressed by an aSMase inhibitor (Hara et al. 2004d).

The use of cells derived from patients with Niemann-Pick disease and from aSMase knockout mice has yielded further insight regarding the role of aSMase in cell signaling of apoptosis. Lymphoblasts from human patients with Niemann-Pick disease were resistant to radiation-induced apoptosis; restoration of aSMase activity by retroviral transfer of human

aSMase cDNA reversed the lack of effect of radiation on these cells (Santana et al. 1996). Likewise, mice deficient in aSMase activity were resistant to radiation-induced short term death (Garcia-Barros et al. 2003). "Spontaneous" oocyte apoptosis was also aSMase dependent (Morita et al. 2000). The proportion of fetal oocytes with characteristics of apoptosis was less for aSMase knockout mice when compared to wild-type mice (Morita et al. 2000). Additionally, oocyte apoptosis induced by cancer therapeutic drug doxorubicin was largely suppressed in mice lacking the gene for aSMase (Morita et al. 2000).

Despite the extensive data that aSMase is involved in apoptosis signaling, some experiments do not support such a role. Ségui et al. (2000) found that TNF-α was unable to trigger sphingomyelin turnover and ceramide formation in a sphingomyelin pool present in acidic lysosomal cell compartments. Furthermore, accumulation of ceramide in the lysosomes (similar to that which occurs when aSMase activity is increased) observed in cells from Farber disease patients (genetically deficient in acid ceramidase which is responsible for ceramide degradation in the lysosomes) did not show increased sensitivity to different apoptosis-inducing agents (TNF-α, daunorubicin, ionizing radiation and Fas ligand) when compared to controls. In another series of experiments, stress stimuli such as anthracyclines, ionizing radiation, and Fas ligand triggered similar apoptotic hallmarks in normal and aSMase-deficient cell lines derived from Niemann-Pick disease patients (Bezombes et al. 2001b).

The other form of ceramide accumulation possibly involved in apoptosis is via the *de novo* synthesis of ceramide. *De novo* synthesis of ceramide is catalyzed by ceramide synthase and other enzymes and was shown to increase in response to the anticancer drug daunorubicin (Boland et al. 1997) and in response to heat shock in mammalian cells (HL-60) and in the yeast Saccharomyces cerevisiae (Jenkins et al. 1997; Jenkins et al. 2002).

One of the ways in which ceramide can be metabolized is by the action of the enzyme ceramidase. The product of this enzyme, sphingosine, is further metabolized into sphingosine-1-phosphate (S1P) through phosphorylation by sphingosine kinase (Pyne and Pyne 2000). The ratio between ceramide and S1P (the "ceramide/S1P rheostat") seems to be an important determinant of cell fate (Cuvillier et al. 1996; Kester and Kolesnick 2003). An increase in the ratio of S1P:ceramide is associated with protection from several stresses. For example, S1P blocked effects of radiation in vivo on oocyte competence, as determined by increased capacity to reach the blastocyst stage after fertilization, in mice (Morita et al. 2000). Also, treatment of cultured bovine cumulus oocyte complexes (COCs) with S1P reduced effects of heat shock on apoptosis and reduced capacity for cleavage and development after fertilization (Roth and Hansen 2004b).

Activation of Downstream Apoptotic Pathways by Ceramide

Recently, ceramide and ROS have been observed to participate together in cellular responses leading to stress-induced apoptosis in processes involving mitochondria. Di Paola et al. (2000) showed that both short-chain (C(2)-ceramide) and long-chain (C(16)-ceramide) ceramides induced ROS production and cytochrome c release from isolated rat and bovine cardiac mitochondria. Similarly, very low concentrations of C(2)-ceramide (0.25 μM) induced ROS production from isolated mitochondria (Garcia-Ruiz et al. 1997). In fact, ceramide can target the mitochondrial membrane and form pores through which molecules like cytochrome c can leak out (Siskind et al. 2002; Siskind et al. 2006) and contribute to the apoptotic signaling cascade.

Ceramide can also induce ROS production in cells. Treatment of neuronally differentiated PC12 cells with exogenous C(2)-ceramide caused a significant increase in ROS production that peaked at 6 hours of exposure to the sphingolipid and caused cell death by apoptosis (France-

Lanord et al. 1997). The increase in ROS was inhibited by the mitochondrial complex I inhibitor Amytal indicating that ROS were of mitochondrial origin. Similar results regarding ceramide-induced ROS production were seen in studies from Quilet-Mary et al. (1997) and Takao et al. (2000). Additionally, inhibitors of aSMase abolished effects of TNF-α on formation of ROS and cell death in U937 human myeloid leukemia cells (Guidarelli et al. 2001). Apoptosis induced by exposure to C(2)-ceramide involved events downstream mitochondria, like activation of caspase-9 and caspase-3 (Ito et al. 1999; Movsesyan et al. 2002) with no effect in activity of caspase-8 (Movsesyan et al. 2002).

Other mechanisms of action have been proposed on how ceramide induces apoptosis. Verheij et al. (1996) showed that heat shock can induce ceramide formation, activation of the stress activated protein kinase/c-jun N-terminus kinase (SAPK/JNK), and trigger apoptosis in U937 human monoblastic leukaemia and bovine aorta endothelial cells. It was also shown that C(2)-ceramide can activate SAPK in a concentration-dependent manner and induce apoptosis (Verheij et al. 1996; Ahn et al. 1999). Zanke et al. (1996) showed that cells expressing dominant negative SEK (SAPK-kinase) mutant failed to undergo apoptosis induced by heat shock. Together, these data suggest that at least in some cells, heat shock can induce apoptosis by activating SAPK/JNK, and that ceramide might be involved in this process. In this scenario, it is possible that induced ceramide can activate SAPK/JNK which in turn can downregulate Bcl-2 allowing pro-apoptotic Bad to target mitochondria leading to apoptosis (Basu and Kolesnick 1998).

The tumor suppressor gene p53 has also been implicated in regulation of ceramide induced cell death. However the dependence upon this transcription factor varies among different cell types and stressors. p53 has been shown to be downstream (Kim et al. 2002) or upstream

(Sawada et al. 2002) of ceramide production. In addition, p53 has been shown to be necessary for ceramide-induced apoptosis (Sawada et al. 2001; Kim et al. 2002), to have no role in ceramide-induced apoptosis (as in the case of fenretinide, an anti-cancer drug that causes apoptosis by inducing ceramide production) (Corazzari et al. 2005), or even to protect cells from apoptosis induced by ceramide in γ-irradiated cells through inhibition of aSMase (Hara et al. 2004c).

ROS and Ceramide: Reciprocal Role in Cell Signaling

While ceramide can induce ROS production as described above, the reciprocal also seems to be true. Evidence is provided by experiments where antioxidants suppressed ceramide formation. N-acetyl-cysteine inhibited TNF-α-induced sphingomyelin turnover in rat primary astrocytes (Singh et al. 1998) and ceramide production in A549 human lung adenocarcinoma cells (Sultan et al. 2006). Antioxidants N-acetylcysteine and pyrrolidine dithiocarbamate prevented ceramide formation and apoptosis induced by the anti-cancer drugs daunorubicin (Mansat-de Mas et al. 1999) and 1-ß-D-arabinofuranosylcytosine (Ara-C) (Bezombes et al. 2001a) while vitamin E prevented ceramide formation and apoptosis in human keratinocytes exposed to UVA radiation (Maziere et al. 2001).

In other experiments, exposure of cells to exogenous ROS caused an increase in intracellular ceramide and apoptosis (Goldkorn et al. 1998; Lavrentiadou et al. 2001; Martín et al. 2002). Hence, whether ceramide participates in the apoptotic cascade and is localized upstream or downstream of ROS generation may depend largely on the cell type and the specific tress. Determining the presence of ceramide signaling mechanism in the preimplantation embryo would provide further understanding of the regulation of apoptosis during early development.

Importance of Oxygen Metabolism and Redox Status in Cell Viability

Oxygen and Oxidative Stress

Aerobic organisms use oxygen to produce energy via oxidative phosphorylation. The process of oxidative phosphorylation consists of series of biochemical reactions that culminates with the reduction of oxygen molecules to water (Klingenberg 1975; Babcock and Wikstrom 1992). In the presence of little or no oxygen, energy production is mainly dependent in glycolysis alone (Du and Wales 1993).

During the Krebs cycle, acetyl-coA derived from glycolysis or fatty acid oxidation is oxidized to re-generate NADH from NAD+ or $FADH_2$ (Moreno-Sanchez et al. 1990; Rustin et al. 1997). Electrons from the NADH and $FADH_2$ molecules flow through the respiratory chain in the mitochondrial inner membrane and this flow is used to drive the protons from the mitochondrial matrix to the intermembrane space (Wikstrom 1984; Moreno-Sanchez et al. 1990; Sherwood and Hirst 2006). An energy potential accumulates due to electrical and pH differential between the matrix and the intermembrane space and the protons flow back into the matrix through the ATP-producing molecule, ATP synthase (Abrahams et al. 1994; Das 2003).

The role of oxygen in this process is to function as the acceptor of the electrons that flow through the respiratory chain, which together with protons will generate water. To be fully reduced to water, each oxygen molecule must receive 4 electrons in a stepwise sequence at the complex IV of the respiratory chain (Brunori and Wilson 1995; Wikstrom et al. 2005). However, not all oxygen molecules are fully reduced to water. These partially reduced oxygen molecules are the above mentioned ROS, more chemically reactive than oxygen, which must be eliminated before reacting and damaging other molecules within the cell. Although ROS have important roles in cell signaling and other biological processes (Roth and Droge 1987; Bae et al. 1997;

Humphries et al. 2006), excessive ROS, if not neutralized by antioxidants, cause oxidative stress (Halliwell and Aruoma 1991; Ikeda et al. 1999; Lord-Fontaine and Averill-Bates 2002).

In case the cell experiences stress, the levels of ROS can increase and overwhelm the intracellular antioxidant system. Oxidative stress is characterized by several kinds of damage caused by modifications of vital molecules like DNA (Abe et al. 1995; Dizdaroglu et al. 2002; Van Remmen et al. 2003), proteins (Kishido et al. 2007), including enzymes involved in ROS detoxification (Fujii and Taniguchi 1999) and lipids (Alvarez and Storey 1985), which ultimately cause cell death. Among the ROS, superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) are the most abundant and best characterized examples and are mostly derived directly or indirectly from mitochondrial respiration (oxidative phosphorylation). Importantly, increased ROS will change the redox state of the cell to a more oxidizing state, causing modifications in the cell that can affect cell functions like energy production and signaling mechanisms without necessarily causing, for example, irreversible protein damage (Nulton-Persson and Szweda 2001; Ghezzi et al. 2005; Humphries et al. 2006).

Redox Homeostasis and Antioxidant Machinery

Redox homeostasis within the cell must be maintained by the complex array of enzymatic and non-enzymatic antioxidants which function is to transform highly reactive molecules in inert substances. Redox state is determined by the balance of ROS producing reactions and ROS removing reactions. The general concepts discussed below were based on reviews focused in the understanding of oxidative stress (Nordberg and Arner 2001; Fleury et al. 2002; Moldovan and Moldovan 2004; Agarwal et al. 2005; Le Bras et al. 2005; Halliwell 2006; Valko et al. 2007).

Superoxide is considered the primary ROS in cell biology and is formed in different reactions, either as a by-product, in mitochondrial electron transport chain, or by enzymes like cyclooxygenase, lypoxigenase and xantine oxidase when oxygen molecules are reduced by

gaining one electron. Several others ROS derive from initial formation of superoxide as this molecule is further reduced by gaining more electrons. Although ROS are necessary and play important roles in cell signaling, if ROS production exceeds ROS removal, oxidative stress takes place. These reactive molecules interact with proteins, lipids and DNA causing modifications that ultimately can lead to cell death.

The ROS removal is accomplished by the antioxidant machinery, molecules displaying specialized functions to act at specific stages of oxidative processes. These molecules can be:

- Enzymes like superoxide dismutase and superoxide reductase, which reduce superoxide to hydrogen peroxide, and catalase, peroxiredoxin and glutathione peroxidase which can all reduce hydrogen peroxide to water;
- ROS scavengers like glutathione (GSH), ascorbic acid and vitamin E that are directly oxidized to clear ROS from the cell;
- Molecules that repair actions of ROS on lipids and peptides/proteins like peroxiredoxin, glutathione peroxidase, glutaredoxin, glutathione transferase, thioredoxin and thioredoxin reductase.

One key component of the antioxidant machinery is GSH, which is an abundant tripeptide (glutamic acid, cysteine, and glycine) that features the thiol (-SH) of cysteine as its active group. It is present in the cytoplasm and in the mitochondria (Mbemba et al. 1985) and participates in different ways to maintain cell redox homeostasis. When needed, GSH works as an electron donor and is oxidized to GSSG (Verbunt et al. 1995; Enomoto et al. 2001). GSH functions to reduce proteins to form the reversible S-glutathionylated proteins (Ghezzi and Bonetto 2003), preventing further oxidation of the protein to the irreversible and inactivating formation of sulphinic and sulphonic derivates (Giustarini et al. 2004). GSH also participates as substrate in reactions catalyzed by glutathione peroxidase (De Haan et al. 2003).

To maintain redox state, GSSG must be reduced back to GSH by glutathione reductase (Smith et al. 1987; Verbunt et al. 1995) which requires NADPH reducing equivalents produced

at different steps of the pentose phosphate pathway (PPP) by utilization of glucose. In absence of glucose, restoration of GSH is delayed or impaired due to deficiency in NADPH (Kussmaul et al. 1999). Glucose-6-phosphate dehydrogenase (G6PDH) is the rate limiting enzyme of the PPP that converts glucose-6-phosphate into 6-phosphoglucolactone generating NADPH from NADP+. Deletion of the gene encoding G6PDH is lethal to the mouse embryo when exposed to oxygen (Longo et al. 2002). Furthermore, embryonic stem cells lacking G6PDH have severely reduced ability to form embryoid bodies; formation of embryoid bodies can be restored by reducing oxygen concentration in the culture environment (Longo et al. 2002). HEK 293 cells (embryonic kidney cell line) overexpressing the mitochondrial NADP+ - dependent isocitrate dehydrogenase (IDPm; enzyme necessary to produce NADPH from NADP+) had higher resistance to oxidative stress induced by heat shock (Kim et al. 2005a). These cells showed lower protein oxidation, lipid peroxidation and oxidative DNA damage as well as an increase in the NADPH pool and GSH recycling.

Oxygen, Oxidative Stress and the Developing Embryo

Oocyte Maturation and Redox Status

The COC is a large structure (300-500 μm) (Hirao et al. 2004; Sutton-McDowall et al. 2004), encompassing thousands of somatic cells surrounding the oocyte in several layers (Hashimoto et al. 1998; D'Alessandris et al. 2001). In vivo, studies have shown that glucose concentration in the follicular fluid of pre-ovulatory bovine follicles ranges between 3.9 and 4.9 mM (Iwata et al. 2004; Orsi et al. 2005) which is similar to the concentration present in the maturation medium (5.6 mM) although much greater than that present at the end of maturation (1.5 mM) after consumption by the cumulus oocyte complex (Sutton-McDowall et al. 2004). The in vivo oxygen concentration in the bovine pre-ovulatory follicle has not been determined yet.

In vitro, cumulus cells utilize glucose to produce ATP via glycolysis and oxidative phosphorylation in the presence of oxygen. Glucose is also used for synthesis of extracellular matrix and reducing agents like NADPH by the PPP (Downs et al. 1998; Cetica et al. 2002). If glucose availability is limited, several aspects of intermediary metabolism, as mentioned above, are potentially affected. In fact, when either glucose or oxygen are reduced compared to standard bovine oocyte maturation conditions (5.6 mM glucose and 20% oxygen), oocyte competence to cleave and/or reach blastocyst stage is decreased (Pinyopummintr and Bavister 1995; Hashimoto et al. 2000a). Furthermore, when 20 mM glucose was used in an oxygen environment of 20% oxygen, oocyte competence was also reduced as compared to those matured in the presence of 5.6 mM glucose and oxidative stress in the oocyte was observed (Hashimoto et al. 2000b). Therefore, a balance between energy substrate concentration and oxygen availability is necessary to allow proper oocyte maturation and embryo development, and to prevent redox imbalance caused by cellular starvation (low glucose or low oxygen) or increased production of ROS.

Embryo Culture and Redox Status

After fertilization, the putative embryo in vivo is removed from contact with the cumulus cells and must rely on the oviductal and uterine environment as well as in its own machinery to maintain energy balance and redox status compatible with survival and growth. The number of mitochondria in the preimplantation embryo is constant until the blastocyst stage (Jansen and de Boer 1998) and the existing mitochondria display a more immature phenotype which progressively change during early development to display an increased ratio of more mature over immature mitochondria at the blastocyst stage. Despite this fact, pyruvate and lactate can be readily used as energy sources by the bovine and murine embryos after fertilization (Leese and Barton 1984; Houghton et al. 1996), but not glucose, which becomes more important in supporting development only after compaction (Houghton et al. 1996; Gardner 1998; Khurana

and Niemann 2000). This is supported by findings that mitochondria are more active at early stages of development (<72 hours post-insemination), as demonstrated by increased mitochondrial membrane potential using Jc-1 dye (Tarazona et al. 2006).

A critical factor for competence of the embryo to develop in vitro is the oxygen environment. In particular, the proportion of embryos developing to the blastocyst stage is greater when embryos are cultured under low oxygen conditions (i.e., 5% oxygen) when compared to counterparts cultured under atmospheric oxygen concentration (21% oxygen) (Thompson et al. 1990; Li and Foote 1993; Liu and Foote 1995; Dumoulin et al. 1999; Lim et al. 1999). Furthermore, production of ROS in mouse and pig embryos is increased when culture is performed in 21% oxygen compared to 5% oxygen (Goto et al. 1993; Kitagawa et al. 2004). The reduced development of the cultured embryo under atmospheric oxygen concentration can be attributed at least in part to the fact that hyperoxia environment is more permissive to oxidative stress. Addition of molecules with antioxidant properties like β-mercaptoethanol (Takahashi et al. 1993; Takahashi et al. 2002), hypotaurine (Fujitani et al. 1997) and GSH precursors like cysteamine (Takahashi et al. 1993) to the culture medium improves development of embryos cultured in high oxygen.

Involvement of ROS in Effects of Heat Shock on the Oocyte and Embryo

Whether ROS play an important role in the effects of heat shock on oocyte maturation and embryonic development is controversial. In the oocyte, a role for ROS in heat shock effects was suggested by experiments from Lawrence et al. (2004). Addition of retinol during oocyte maturation decreased the negative effects of heat shock on the proportion of oocytes reaching the blastocyst stage. The protective effects of retinol could involve its actions as a free radical scavenger (Ciaccio et al. 1993), although it could also be mediated by retinol nuclear receptors found to be expressed (mRNA) in the bovine oocyte (Mohan et al. 2001).

Effects of antioxidants on embryonic responses to heat shock have been inconsistent. In early studies with mouse embryos, blocking GSH synthesis at the morula stage with buthionine-sulfoximine (BSO) prevented acquisition of induced thermotolerance, which normally occurs when embryos are exposed to mild heat shock prior to exposure to severe heat shock (Aréchiga et al. 1995). In the same study, the use of and inducer of GSH synthesis, S-adenosyl-L-methionine, decreased deleterious effects of heat shock, and supplementation with GSH increased embryo viability after heat shock. In contrast, GSH, glutathione ester and vitamin E had no thermoprotective effects on development of the bovine two-cell embryo or morula after heat shock (Ealy et al. 1995a; Paula-Lopes et al. 2003). At the blastocyst stage, however, GSH blocked the effect of heat shock on cell death (Ealy et al. 1992).

More recent data are supportive of a role for ROS in effects of heat shock on pre-implantation embryos. Murine female embryos were more resistant and produced less ROS after heat shock than male embryos (Pérez-Crespo et al. 2005). Increased resistance and lower ROS production after heat shock of the female embryos was suppressed by use of dehydroepiandrosterone, a potent inhibitor of G6PD which is an enzyme involved in antioxidant machinery and expressed in greater amounts in female embryos than in male embryos.

Bovine embryos heat shocked at two different oxygen concentrations (5 and 21%) had decreased development compared to control non-heat shocked embryos (Rivera et al. 2004a). However, further interpretation of the results indicates that the reduction in development as a percentage of control embryos was greater in embryos heat shocked in high oxygen. Sakatani et al. (2004) performed a series of experiments where bovine embryos on Days 0, 2, 4 or 6 post insemination were exposed to heat shock at 41^{o}C for 6 hours. Heat shock on Days 0 and 2 decreased the competence of embryos to reach blastocyst stage and also increased production of

ROS. Subsequently, exposure of Day 2 embryos to heat shock at 41.5°C decreased developmental competence and reduced GSH content (Sakatani et al. 2007). These effects were blocked or reduced by addition of an antioxidant extracted from purple sweet potato (*Ipomea batatas*), anthocyanin. Anthocyanin is a strong ROS-scavenger and therefore believed to exert its effects by reacting directly with ROS (Oki et al. 2002; Oki et al. 2003) and preserving endogenous GSH. Preserving GSH content may be particularly important at the 2-8 cell stages of embryo development, when GSH content is lower compared to later stages and synthesis of new GSH molecules is reduced (Lim et al. 1996b).

Heat Stress, Blood Flow and Hypoxia

While excessive oxygen has been shown to compromise cell function due to oxidative stress, reduction in oxygen levels lower than physiological range can also cause deleterious effects. In reproduction, poor blood flow and oxygenation of the follicle in humans has been correlated with decreased oocyte quality (Van Blerkom et al. 1997; Van Blerkom 1998). Chromosomal scattering was observed in oocytes from follicles that had oxygen content of less than 1% (Van Blerkom et al. 1997). Oocytes derived from follicles containing ≤1.5% oxygen had almost half the capacity of reaching 6-8 cell stage when compared to oocytes from follicles with 3-5% oxygen. Treatment of infertility of human patients with hyperbaric oxygen prior to oocyte collection resulted in more transferable embryos per patient after in vitro fertilization (Van Voorhis et al. 2005).

One of the physiological responses to increased body temperature is the redistribution of blood circulation to extremities (Choshniak et al. 1982) by vasodilatation of capillaries in skin to help dissipate heat by convection (Kenney and Musch 2004), which in Holstein cows has been estimated to account for approximately 85% of the total heat loss when ambient temperatures rise above 30°C (Maia et al. 2005b). Another physiological change involved in thermoregulation

is the increase in the frequency of respiratory cycles (Berman et al. 1985; Srikandakumar and Johnson 2004; Valtorta and Gallardo 2004; Maia et al. 2005a). This increase in frequency demands high blood flow in the muscles involved in respiratory activity and also in the upper respiratory tract to allow better blood oxygenation and efficient heat loss (Wolfenson et al. 1981; Bell et al. 1983).

Redistribution of blood flow can greatly affect the reproductive tract. In rabbits, blood flow to ovaries, cervix and oviduct decreased by 20 to 30% during heat stress while vulval blood flow increased 40% (Lublin and Wolfenson 1996). Elevation in body temperature of the laying hen by 1 to 2^{o}C caused capillary blood flow to decrease by 58% and 70 to 80% of control animals in the uterus and large ovarian follicles, respectively (Wolfenson et al. 1981). In two separate experiments, ovariectomized cows and ewes kept under no-shade during summer in Florida and injected with estradiol had 15% less increase in uterine blood flow when compared to the animals kept under shade (Roman-Ponce et al. 1978b; Roman-Ponce et al. 1978a).

Objective and Hypothesis

Several factors participate in the complex, developmentally-regulated embryonic response to heat shock. Experiments described in the present dissertation have the overall objective of determining potential factors contributing to the phenomenon described in the literature of decreased oocyte and embryo competence to reach the blastocyst stage and increased incidence of apoptosis caused by heat shock. Focus was giving to the role of oxygen and oxidative stress as a modulator of the effects of heat shock in the oocyte and embryo. The presence of a ceramide signaling mechanism linked to apoptotic pathways, which in other cells have been shown to be activated by heat shock, was also studied. The following four chapters contain a series of experiments with specific objectives and hypotheses, each contributing to the overall objective.

The specific objective in Chapter 3 was to test the hypothesis that effects of heat shock on development and apoptosis in the preimplantation embryo are dependent on oxygen environment and ROS. To test this hypothesis, embryos were exposed to heat shock under 5 or 21% oxygen atmosphere, with the latter expected to cause more severe effects to the embryo due to oxidative stress. To further address the importance of oxidative stress in effects of heat shock in embryo development and apoptosis, experiments were performed to test if effects of heat shock could be attenuated by use of the antioxidant DTT.

Because the initial steps of the apoptotic response to heat shock have been shown to depend upon ceramide generation, the objective in Chapter 4 was to test the hypothesis that ceramide signaling pathway is present in the bovine preimplantation embryo. This hypothesis was tested by evaluating actions of ceramide and its inactive analogue dihydroceramide on activation of apoptosis. Experiments were also performed to determine the activity of neutral and acid SMases in response to heat shock.

The attention in Chapter 5 was on the role of oxygen concentration on effects of heat shock in the oocyte. The hypothesis was tested that heat shock effects are more severe in oocytes matured at 21% oxygen than in those matured at 5% oxygen.

The objective for Chapter 6 was to test the hypothesis that heat stress in lactating dairy cows reduces oxygen concentration in follicular fluid of dominant pre-ovulatory follicles. The rationale is that animals under heat stress have decreased blood flow to internal organs, including the reproductive tract, and this could be an indirect effect of heat stress in fertility observed during the hot months of the year. For this experiment follicular fluid from heat-stressed cows was aspirated and concentrations of oxygen, estradiol and progesterone were determined.

CHAPTER 3
ROLE OF OXYGEN AND OXIDATIVE STRESS IN ACTIONS OF HEAT SHOCK ON DEVELOPMENT AND APOPTOSIS OF PREIMPLANTATION BOVINE EMBRYOS

Introduction

Exposure to elevated temperature reduces developmental competence of preimplantation embryos. This phenomenon has been shown in several species including mice (Aréchiga et al. 1995; Aréchiga and Hansen 1998; Ozawa et al. 2002), rabbits (Makarevich et al. 2006), and cattle (Edwards and Hansen 1997; Krininger et al. 2002; Paula-Lopes et al. 2003; Sakatani et al. 2004). The mechanism by which heat shock inhibits embryonic development is not completely understood. In the cow, exposure of two-cell embryos to elevated temperature causes a slight increase in the number of swollen mitochondria and changes in the cytoskeleton that lead to displacement of organelles away from the plasma membrane (Rivera et al. 2003; Rivera et al. 2004c). At later stages of development (after the 8-16 cell stage), apoptosis can be induced by heat shock but this phenomenon does not occur for earlier embryos (Paula-Lopes and Hansen 2002b).

Increased production of ROS may be one mechanism by which heat shock disrupts development of preimplantation embryos. Whether this is really the case is not clear from the literature and may depend on stage of development and degree of heat shock. In the mouse, effects of a heat shock of 42^{o}C for 2 hours on development were reported to be decreased by GSH (Aréchiga et al. 1995). Moreover, the phenomenon of induced thermotolerance, whereby exposure to a mild heat shock makes cells more resistant to a subsequent, severe heat shock, was dependent upon GSH synthesis in the mouse morula (Aréchiga et al. 1995). However, inhibition of GSH synthesis did not alter effects of 41^{o}C for 1 hour on development of two-cell embryos or morulae (Aréchiga and Hansen 1998). Data are inconsistent as to whether heat shock increases production of ROS. One report indicates that exposure of mouse morulae to 41^{o}C for 1 hour

tended to reduce intracellular GSH content (Aréchiga et al. 1995). In contrast, heat shock had no direct effect on redox state of mouse zygotes, as assessed by embryonic content of GSH and hydrogen peroxide (Ozawa et al. 2002).

In cattle, there was no thermoprotective effect of the antioxidants GSH, glutathione ester or vitamin E on development (Ealy et al. 1995b; Paula-Lopes et al. 2003) but administration of anthocyanins, a class of antioxidant from purple sweet potato, reduced effects of heat shock at Day 2 after insemination on development to the blastocyst stage (Sakatani et al. 2007). Likewise, GSH was effective at reducing deleterious effects of heat shock in embryos at the morula stage (Ealy et al. 1992). There are also reports that exposure of embryos at Day 0 and 2, relative to insemination, to 41^{o}C or 41.5^{o}C for 6 hours increased ROS production as determined by oxidation of 2',7'-dichlorodihydrofluorescein diacetate (Sakatani et al. 2004). Heat shock did not increase oxidation of 2',7'-dichlorodihydrofluorescein diacetate later in development at Days 4 or 6 after insemination (Sakatani et al. 2004). Exposure of Day 2 embryos to 41.5^{o}C for 6 hours also reduced GSH content (Sakatani et al. 2007) although a less severe heat (41^{o}C for 6 hours) did not reduce intracellular GSH content of two-cell embryos (Rivera et al. 2004b).

One potential approach to test the importance of ROS for mediating effects of heat shock is to modify the free radical status of embryos by manipulating the oxygen content of the atmosphere used for culture. Elevated oxygen concentration has been shown to increase hydrogen peroxide production in somatic cell culture systems (Kwak et al. 2006) as well as in culture of preimplantation embryos (Goto et al. 1993; Kitagawa et al. 2004). Under normothermia (38-39^{o}C), the proportion of embryos successfully developing in culture is increased when bovine embryos are cultured in a gas environment of 5% (v/v) oxygen as compared to culture in air (Thompson et al. 1990; Lim et al. 1999). This difference in

development is due in large part to ROS because addition of antioxidants and pro-antioxidants to culture medium of embryos cultured in air improves embryonic development and reduces the proportion of blastomeres exhibiting apoptosis (Takahashi et al. 1993; Takahashi et al. 2002).

In the present study, the role of oxidative stress in mediating deleterious effects of heat shock on the bovine preimplantation embryo was assessed. First, experiments were carried out to determine if effects of heat shock were reduced in a low oxygen environment as compared to a high oxygen environment (5% vs ~21%). Secondly, experiments were performed to determine if effects of heat shock to reduce development and induce apoptosis can be prevented by the antioxidant DTT.

Materials and Methods

Materials

The media HEPES-Tyrodes Lactate (TL), IVF-TL, and Sperm-TL were purchased from Cell and Molecular Technologies Inc. (Lavallete, NJ) and used to prepare HEPES-Tyrodes albumin lactate pyruvate (TALP), IVF-TALP, and Sperm-TALP as previously described (Parrish et al. 1986). Oocyte collection medium (OCM) was Tissue Culture Medium-199 (TCM-199) with Hank's salts without phenol red (Atlanta Biologicals, Norcross, GA) supplemented with 2% (v/v) bovine steer serum containing 2 U/ml heparin (Pel-Freez, Rogers, AR), 100 U/ml penicillin-G, 0.1 mg/ml streptomycin, and 1 mM glutamine. Oocyte maturation medium (OMM) was TCM-199 (BioWhittaker, Walkersville, MD) with Earle's salts supplemented with 10% (v/v) bovine steer serum, 2 µg/ml estradiol 17-ß, 20 µg/ml bovine follicle stimulating hormone (Folltropin-V; Vetrepharm Canada, London, ON), 22 µg/ml sodium pyruvate, 50 µg/ml gentamicin sulfate, and 1 mM glutamine. Percoll was from GE Biosciences (Uppsala, Sweden). Frozen semen from various bulls of different breeds was donated by Southeastern Semen Services (Wellborn, FL). Potassium simplex optimized medium (KSOM) that contained 1 mg/ml

bovine serum albumin (BSA) was obtained from Cell and Molecular Technologies (Lavallete, NJ) or Caisson Laboratories (Rexburgh, ID). Essentially fatty-acid free (EFAF) BSA was from Sigma (St. Louis, MO). On the day of use, KSOM was modified for bovine embryos to produce KSOM-BE2 as described elsewhere (Soto et al. 2003c).

The In Situ Cell Death Detection Kit (fluorescein or TMR red) was obtained from Roche Diagnostics Corporation (Indianapolis, IN). Hoechst 33342 was purchased from Sigma. Polyvinylpyrrolidone (PVP) was purchased from Eastman Kodak (Rochester, NY) and RQ1 RNase-free DNase was from Promega (Madison, WI). All other reagents were purchased from Sigma or Fisher Scientific (Pittsburgh, PA).

In Vitro Production of Embryos

Embryo production was performed as previously described (Jousan and Hansen 2004). Briefly, COCs were obtained by slicing 2- to 10-mm follicles on the surface of ovaries (a mixture of beef and dairy cattle) obtained from Central Beef Packing Co. (Center Hill, FL). Those COCs with at least one complete layer of compact cumulus cells were washed two times in OCM and used for subsequent steps. Groups of 10 COCs were placed in 50-µl drops of OMM overlaid with mineral oil and matured for 20–22 hours at 38.5°C in an atmosphere of 5% (v/v) CO_2 in humidified air. Matured COCs were then washed once in HEPES-TALP and transferred in groups of 30 to 4-well plates containing ~1 x 10^6 Percoll-purified spermatozoa from a pool of frozen-thawed semen from three bulls in 600 µl of IVF-TALP and 25 µl per well of a solution of PHE [0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 µM epinephrine in 0.9% (w/v) NaCl]. A different pool of three bulls was generally used for each replicate. After 10-12 hours at 38.5°C in an atmosphere of 5% CO_2 in humidified air, putative zygotes were removed from fertilization wells, denuded of cumulus cells by vortex mixing in 1 ml of 1000 U/ml hyaluronidase in HEPES-TALP, and placed in groups of 30 in 50-µl drops of KSOM-BE2. All

drops of embryos were overlaid with mineral oil and cultured as described for individual experiments. The CO_2 environment of culture was maintained at 5% (v/v) for culture at 38.5°C and was raised to 6% for cultures at 41°C to correct for reduced solubility of CO_2 and to maintain the pH of the medium at 41°C to a value similar to that for cultures at 38.5°C (pH~7.4) (Rivera and Hansen 2001). A number of ~10-20 embryos were put in each drop in any given replicate of each experiment

TUNEL and Hoescht 33342 Labeling

The TUNEL assay was used to detect DNA fragmentation associated with late stages of the apoptotic cascade. The enzyme terminal deoxynucleotidyl transferase is a DNA polymerase that catalyzes the transfer of a fluorescein isothiocyanate- or TMR red- conjugated dUTP nucleotide to a free 3' hydroxyl group characteristic of DNA strand breaks. Embryos were removed from KSOM-BE2 and washed two times in 50-µl drops of 10 mM KPO_4, pH 7.4 containing 0.9% (w/v) NaCl (PBS) and 1 mg/ml polyvinylpirrolidone (PBS-PVP) by transferring the embryos from drop to drop. Embryos were fixed in a 50-µl drop of 4% (w/v) paraformaldehyde in PBS for 25 minutes at room temperature, washed twice in PBS-PVP, and stored in 600 µl of PBS-PVP at 4°C until the time of assay. All steps of the TUNEL assay were conducted using microdrops in a humidified box.

On the day of the TUNEL assay, embryos were transferred to a 50-µl drop of PBS-PVP and then permeabilized in 0.1% (v/v) Triton X-100 containing 0.1% (w/v) sodium citrate for 10 minutes at room temperature. Controls for the TUNEL assay were incubated in 50 µl of RQ1 RNase-free DNase (50 U/ml) at 37°C in the dark for 1 hour. Positive controls and treated embryos were washed in PBS-PVP and incubated with 25 µl of TUNEL reaction mixture (containing fluorescein isothiocyanate- or TMR red- conjugated dUTP and the enzyme terminal deoxynucleotidyl transferase as prepared by following the guidelines of the manufacturer) for 45

minutes at 37°C in the dark. Negative controls were incubated in the absence of terminal deoxynucleotidyl transferase. Embryos were then washed three times in PBS-PVP and incubated in a 25-µl drop of Hoechst 33342 (1 µg/ml) for 15 minutes in the dark. Embryos were washed three times in PBS-PVP to remove excess Hoechst 33342, mounted on 10% (w/v) poly-L-lysine coated slides using 4-µl drops of antifade, and coverslips were placed on the slides. Labeling of TUNEL and Hoechst 33342 nuclei was observed using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Göttingen, Germany). Each embryo was analyzed for total cell number (blue nuclei) and TUNEL-positive blastomeres (green or red nuclei) with DAPI and FITC or rodamine filters, respectively, using a 20X objective. Digital images were acquired using AxioVision software and a high-resolution black and white Zeiss AxioCam MRm digital camera.

Experiments on Modification of Heat Shock Effects by Oxygen Environment

Effect of heat shock at the two-cell stage on development to the blastocyst stage as modified by oxygen environment throughout culture.

After insemination, putative embryos were randomly assigned to be cultured in either high (5% CO_2 in humidified air) or low oxygen (5% O_2, 5% CO_2 and N_2 in a humidified chamber). Two-cell embryos were harvested at 28 hours after insemination and randomly assigned to culture at 38.5°C or 41°C for 9 hours under high or low oxygen in a 2 x 2 factorial design. After the heat shock period, all embryos were further cultured at 38.5°C in either high or low oxygen until Day 8 when the percentage of embryos becoming blastocysts was determined. Embryos were maintained in the same oxygen environment throughout culture. Thus embryos placed in low oxygen after insemination remained in low oxygen during the heat shock period and in the post-heat shock period. The experiment was replicated 3 times with a total of 220 embryos.

Acute effect of oxygen environment on development of heat shocked two-cell embryos.

After insemination, putative embryos were cultured in high oxygen. Two-cell embryos were harvested at ~ 28 hours after insemination and randomly assigned to be cultured at either 38.5°C for 24 hours or 41°C for 9 hours followed by 38.5°C for 15 hours in either high or low oxygen. Thereafter, all embryos were cultured at 38.5°C in high oxygen until Day 8 when the percentage of embryos becoming blastocysts was determined. The experiment was replicated 9 times using a total of 672 two-cell embryos.

Acute effect of oxygen environment on development of heat shocked Day 5 embryos > 16 cells.

Putative embryos were cultured in high oxygen after insemination. Embryos ≥ 16-cells were harvested on Day 5 after insemination and were randomly assigned to be cultured at either 38.5°C for 24 hours or 41°C for 9 hours followed by 38.5°C for 15 hours in either high or low oxygen using a 2 x 2 factorial arrangement of treatments. Thereafter, embryos were cultured at 38.5°C in high oxygen until Day 8 when the percentage of embryos becoming blastocysts was determined. The experiment was replicated 7 times using a total of 447 embryos.

Heat shock effects on apoptosis of Day 5 embryos ≥ 16 cells as modified by oxygen environment throughout culture.

Putative embryos were randomly assigned to be cultured in either high or low oxygen beginning after insemination. On Day 5, embryos ≥16 cells were harvested and placed in new drops and cultured at 38.5°C for 24 hours or 41°C for 9 hours followed by 38.5°C for 15 hours. During this period, embryos were cultured in the same oxygen environment that they were exposed to earlier. Thus, the design was a 2 x 2 factorial design with two temperatures and two oxygen environments. All embryos were fixed 24 hours after initiation of the heat shock period, on Day 6 after insemination, and stored in PBS-PVP at 4°C until analysis by TUNEL and Hoechst 33342 labeling. The experiment was replicated 5 times with a total of 217 embryos.

Effects of heat shock in low oxygen on apoptosis of Day 4 and Day 5 embryos ≥ 16 cells.

Putative embryos were cultured in low oxygen after insemination. Embryos ≥ 16 cells were harvested at either Day 4 or Day 5 after insemination. Separate drops of embryos were used for selection on each day. Harvested embryos were placed in fresh drops of culture medium and randomly assigned to be cultured in low oxygen at 38.5°C for 24 hours or 41°C for 9 hours followed by 15 hours at 38.5°C. Embryos were harvested at the end of culture and stored in PBS-PVP at 4°C until analysis by TUNEL and Hoechst 33342 labeling. The experiment was replicated 3 times using a total of 239 embryos.

Experiments on Thermoprotective Actions of DTT

Protective action of DTT on development of embryos heat shocked at the two-cell stage and cultured in high oxygen continuously.

After insemination, putative embryos were cultured in high oxygen. Two-cell embryos were harvested at ~28 hours post insemination and randomly assigned to be cultured in the presence of 0, 50 or 500 µM at 38.5°C for 24 hours or 41°C for 15 hours followed by 38.5°C for 9 hours using a 3 x 2 factorial arrangement of treatments. Afterwards, embryos were washed 3 times in 100 µl drops of HEPES-TALP and cultured in fresh drops of KSOM-BE2 at 38.5°C in high oxygen. Blastocyst development was determined at Day 8. The experiment was replicated 6 times using a total of 431 embryos.

Protective action of DTT on development of embryos heat shocked at the two-cell stage in different oxygen environments and otherwise cultured in low oxygen.

After insemination, putative embryos were cultured in low oxygen. Two-cell embryos were harvested at ~28 hours after insemination and randomly assigned to one of eight treatments in a 2 x 2 x 2 factorial design with main effects of incubation temperature (38.5°C for 24 hours or 41°C for 15 hours followed by 38.5°C for 9 hours), oxygen environment (high or low oxygen) and DTT (0 or 500 µM). After 24 hours, embryos were washed 3 times in 100 µl HEPES-TALP

drops and placed in fresh drops of KSOM-BE2 and cultured in low oxygen at 38.5°C until Day 8 when blastocyst development was determined. The experiment was replicated 16 times using a total of 1264 two-cell embryos.

For 5 of the above-mentioned 16 replicates, additional treatments were included that were analyzed as a separate data set. These embryos were in high oxygen for the entire culture period. Two-cell embryos were harvested and randomly assigned to one of four treatments in a 2 x 2 factorial design with main effects of incubation temperature (38.5°C for 24 hours or 41°C for 15 hours followed by 38.5°C for 9 hours) and DTT (0 or 500 μM). A total of 184 two-cell embryos were used. After 24 hours, embryos were washed 3 times in 100 μl HEPES-TALP drops and placed in fresh drops of KSOM-BE2 and cultured in high oxygen at 38.5°C until Day 8 when blastocyst development was determined.

Protective action of DTT on development of heat-shocked embryos ≥ 16 cells cultured in high oxygen continuously.

Putative embryos were cultured in high oxygen after insemination. On Day 5, embryos ≥16 cells were harvested and randomly assigned to one of six treatments in a 2 x 3 factorial design with main effects of incubation temperature (38.5°C for 24 hours or 41°C for 15 hours followed by 38.5°C for 9 hours) and DTT (0, 50 or 500 μM). All embryos were cultured in high oxygen. Afterwards, embryos were washed 3 times in 100 μl HEPES-TALP and cultured in fresh KSOM-BE2 at 38.5°C in high oxygen until Day 8 when blastocyst development was determined. The experiment was replicated 10 times using a total of 545 embryos.

Protective action of DTT on apoptosis of heat-shocked embryos ≥ 16 cells cultured in high oxygen continuously.

Putative embryos were cultured in high oxygen after insemination. On Day 5, embryos ≥16 cells were harvested and randomly assigned to culture in the presence of 0, 50 or 500 μM DTT at 38.5°C for 24 hours or 41°C for 15 hours followed by 38.5°C for 9 hours. Afterwards, were fixed

in paraformaldehyde for TUNEL assay. The experiment was replicated 4 times using a total of 287 embryos.

Statistical Analysis

Data on percent of embryos becoming blastocysts were calculated for each replicate and analyzed by least-squares analysis of variance using the General Linear Models (GLM) procedure of SAS (SAS for Windows, Version 8, 1999–2001, Cary, NC). Replicate was considered random and other main effects were considered fixed. Effects of specific doses of DTT in protecting embryos from heat shock were calculated using contrasts. When necessary to determine the effects of one main effect within one factor of a second main effect, the first main effect was fixed using the Sort procedure from SAS. For example, to determine if there was an effect of 500 μM DTT at 41^{o}C, the main effect of temperature was fixed using the Sort procedure and effects of each DTT concentration was determined separately for embryos at 38.5 and 41^{o}C.

In some experiments, the percent reduction in blastocyst development induced by heat shock was calculated for each replicate according to the equation: 100 – [(percent development at 41°C/percent development at 38.5°C) x 100]. For apoptosis, the percent of blastomeres that were TUNEL-positive (i.e. assumed apoptotic) were determined for each embryo. Total cell number for each embryo was also analyzed. For all of these variables, data were analyzed by least-squares analysis of variance as described above.

All values reported are least-squares means ± SEM.

Results

Effect of Heat Shock at the Two-Cell Stage on Development as Modified by Oxygen Environment

In the first experiment, embryos cultured continuously in either high or low oxygen were heat-shocked by exposure to 41°C for 9 hours at the two-cell stage. As shown in Figure 3-1, a smaller proportion of two-cell embryos developed to the blastocyst stage at Day 8 after insemination when cultured in high oxygen than when cultured in low oxygen ($P < 0.05$) Heat shock decreased development of two-cell embryos to the blastocyst stage in high oxygen but not in low oxygen (temperature x oxygen, $P < 0.05$).

In the second experiment, embryos were cultured in high oxygen throughout culture except for the 24-hour period coincident with heat shock. During this time, embryos were cultured in either high or low oxygen and at either 38.5°C for 24 hours or 41°C for 9 hours followed by 38.5°C for 15 hours. Data on the proportion of embryos that became blastocysts at Day 8 after insemination are presented in Figure 3-2. The percentage of embryos becoming blastocysts did not differ between high and low oxygen at 38.5°C. Heat shock decreased the proportion of two-cell embryos becoming blastocysts in high oxygen but not in low oxygen (temperature x oxygen, $P < 0.05$).

Effect of Heat Shock on Development of Day 5 Embryos ≥ 16 Cells as Modified by Oxygen Environment

Embryos were cultured in high oxygen until Day 5 when those ≥ 16-cells were harvested and cultured at either 38.5°C for 24 hours or 41°C for 9 hours and 38.5°C for 15 hours in high or low oxygen. Thereafter, all embryos were cultured in high oxygen and blastocyst development was determined on Day 8. Results are shown in Figure 3-3. The percentage of embryos becoming blastocysts did not differ between high and low oxygen at 38.5°C. Heat shock

decreased development of embryos to the blastocyst stage when culture was in high oxygen but not when culture was in low oxygen (temperature x oxygen, $P < 0.05$)

Induction of Apoptosis by Heat Shock in Day 4 and 5 Embryos ≥ 16 Cells as Modified by Oxygen Environment

Two experiments were conducted to test whether oxygen environment altered the induction of apoptosis in Day 4 and 5 embryos. In the first study, embryos were cultured continuously in either high or low oxygen. At Day 5, embryos ≥16 cells were cultured at 38.5°C for 24 hours or 41°C for 9 hours followed by 38.5°C. As shown in Figure 3-4A, total cell number at the end of culture was lower for embryos cultured in high oxygen compared to embryos cultured in low oxygen ($P < 0.05$). Neither temperature nor temperature x oxygen affected total cell number ($P > 0.1$). Results for percent of cells undergoing apoptosis as determined by TUNEL labeling is presented in Figure 3-4B. The proportion of cells that were TUNEL-positive was higher for embryos cultured at 41°C than for embryos at 38.5°C if culture was in high oxygen but not if culture was in low oxygen (temperature x oxygen; $P < 0.05$).

In the second experiment, embryos ≥ 16 cells cultured in low oxygen were harvested at either Day 4 or 5 after insemination. Thereafter, embryos continued to be cultured in low oxygen. Embryos were cultured at 38.5°C for 24 hours or 41°C for 9 hours followed by 38.5°C for 15 hours. Embryos were then fixed and stained for TUNEL and Hoechst 33342. Heat shock increased apoptosis for embryos collected at both Days 4 and 5 (Figure 3-5; temperature, $P < 0.05$). This increase, although significant, was much lower than the increase observed in the previous experiment (compare Figures 3-4 and 3-5). As expected, Day 4 embryos had fewer cells than Day 5 embryos ($P < 0.05$) but heat shock had no effect on total cell number at either Day 4 or Day 5 ($P > 0.1$).

Protective Actions of DTT on Development of Heat-Shocked Two-Cell Embryos

Two experiments were performed to determine whether DTT blocked deleterious effects of heat shock on development of two-cell embryos. In the first experiment, embryos were cultured continuously in high oxygen. At the two-cell stage, embryos were cultured in the presence of 0, 50 or 500 μM DTT at either 38.5°C for 24 hours or at 41°C for 15 hours followed by 38.5°C for 9 hours. After the heat shock period, all embryos were further cultured at 38.5°C in fresh medium without DTT until Day 8 after insemination. The overall percentage of two-cell embryos becoming blastocysts was higher for embryos cultured in the presence of DTT ($P < 0.05$; Figure 3-6A). Heat shock reduced development of two-cell embryos to the blastocyst stage in the presence or absence of DTT (temperature; $P < 0.05$). While there was no significant DTT x temperature interaction (Figure 3-6A), the reduction in development caused by heat shock, expressed as a percentage of development for non-heat shocked embryos, was less for embryos cultured in the presence of DTT ($P < 0.05$; Figure.3-6B). Nonetheless, even at the highest dose of DTT, development was reduced 78.9% by culture at 41°C.

In the second experiment, two-cell embryos cultured in low oxygen were harvested and then cultured at either high or low oxygen in the presence or absence of 500 μM DTT and at either 38.5°C for 24 hours or 41°C for 15 hours followed by 38.5°C for 9 hours. Embryos were then washed and cultured in low oxygen at 38.5°C without DTT until Day 8 after insemination when blastocyst development was determined. As shown in Figure 3-7A, the proportion of two-cell embryos that became blastocysts was affected by the temperature x oxygen x DTT interaction ($P < 0.05$). This interaction existed because temperature reduced development in all groups except for the embryos cultured in low oxygen in the absence of DTT. Thus, unlike the previous experiment, DTT did not reduce effects of heat shock on development in high oxygen and, in fact, exacerbated the effects of heat shock in low oxygen.

To verify that the difference in response to DTT depended upon whether embryos were cultured in high oxygen (Figure 3-6) or cultured in low oxygen for most of development (Figure 3-7A), additional treatments were included in the second DTT experiment for some replicates. In particular, some embryos were cultured continuously in high oxygen but otherwise treated as for Figure 3-7A. In this subset, DTT conferred partial protection against heat shock (DTT x temperature, $P < 0.05$). The percent reduction in blastocyst development by heat shock tended ($P = 0.08$) to be lower for DTT-treated embryos (77.3 ± 6.5%) than for control embryos (95.5 ± 6.5%). The absolute magnitude of the reduction in development caused by heat shock was similar to the previous experiment conducted in high oxygen (Figure 3-6B).

Protective Actions of DTT on Development of Heat-Shocked Day 5 Embryos ≥ 16 Cells

This experiment was performed with embryos cultured continuously in high oxygen. On Day 5, embryos ≥16 cells were cultured in the presence of 0, 50 or 500 μM DTT at 38.5°C for 24 hours or 41°C for 15 hours and 38.5°C for 9 hours. Afterwards, all embryos were cultured at 38.5°C without DTT until Day 8 after insemination. As shown in Figure 3-8A, the percent of embryos becoming blastocysts was reduced by heat shock ($P < 0.01$) and increased by DTT ($P < 0.01$) and there was no significant temperature x DTT interaction. However, as shown in Figure 3-8B, the reduction in development caused by heat shock, expressed as a percentage of development for non-heat shocked embryos, was less for embryos cultured in the presence of 50 μM ($P = 0.06$) and 500 μM DTT ($P < 0.01$) than for embryos cultured without DTT.

Protective Actions of DTT on Apoptosis of Heat-Shocked Day 5 Embryos ≥ 16 Cells

This experiment was done as for the previous experiment except that embryos were analyzed for apoptosis 24 hours after initiation of heat shock using the TUNEL assay. There was no effect of temperature or DTT on total cell number (Figure 3-9A; temperature, $P > 0.1$). Overall, the proportion of cells that were apoptotic was affected by DTT (Figure 3-9B; $P < 0.01$).

Among embryos cultured at 38.5°C as well as for embryos at 41°C, DTT 500 μM decreased the percent of TUNEL positive nuclei (DTT, $P < 0.01$). Although there was no overall effect of temperature in the percent of TUNEL positive nuclei, heat shock increased the percent of TUNEL positive nuclei among embryos treated with 0 μM DTT (temperature, $P < 0.01$) but not for embryos treated with DTT (temperature, $P > 0.1$).

Discussion

The role of ROS in mediating effects of elevated temperature on function of the preimplantation embryo has been a controversial topic. As outlined in the Introduction, experiments have yielded inconsistent results as to whether heat shock increases production of ROS and whether antioxidants can protect embryos from deleterious effects of heat shock. In the present studies, use of the high oxygen vs. low oxygen model yielded clear evidence that oxygen environment is an important determinant of continued embryonic development following heat shock. Experiments using DTT have revealed that some of the actions of heat shock on development involve ROS production in a stage-specific manner and that ROS-independent actions of heat shock are also likely. Experiments also demonstrated that ROS are important triggers for induction of the apoptotic cascade during heat shock because heat-shock induced apoptosis was blocked or greatly reduced by culture in low oxygen and blocked by addition of DTT to culture medium.

In each of four experiments, deleterious effects of heat shock on continued development of embryos was only observed when embryos were in a high oxygen environment during heat shock. This was true for both two-cell embryos and Day 5 embryos $\geq$ 16 cells and was true whether embryos were cultured continuously in different oxygen environments or whether oxygen environment differed during the 24 hours after initiation of heat shock only. There are three possible explanations for this finding. One is that effects of heat shock on embryonic

development are mediated by increased ROS production and that the magnitude of this increase is greater when embryos are cultured in high oxygen. Indeed, embryos cultured in a high oxygen environment have greater production of ROS (Goto et al. 1993; Kitagawa et al. 2004). Alternatively, the deleterious effect of heat shock may not be a direct result of increased production of ROS but rather heat shock is more likely to inhibit development of embryos when their physiology is already compromised by the increased ROS production caused by culture in high oxygen. A third possibility is that high oxygen does not increase susceptibility to heat shock but that culture in low oxygen results in changes in cellular function that increase embryonic resistance to heat shock. This latter explanation is deemed less likely. There are genes in the bovine blastocyst whose expression is dependent upon oxygen concentration but the full range of oxygen-induced transcription is attenuated by the lack of hypoxia-inducible transcription factor-1 (Harvey et al. 2004b; Harvey et al. 2007a; Harvey et al. 2007b). Also, effects of oxygen were seen at the two-cell stage, a time before embryonic genome activation has occurred (Memili and First 2000).

If the effects of oxygen environment on embryonic development reflect altered ROS production, antioxidants should reduce effects of heat shock on development. DTT was used as an antioxidant in the present study because it is a strong reducing agent that contains two thiol groups for sequential thiol-disulfide exchange reactions for direct scavenging of ROS as well as regeneration of reduced GSH from GSSG (Rothwarf and Scheraga 1992). Treatment of mouse embryos with DTT blocked effects of the thiol-oxidizing agent diamide (Liu et al. 1999). In the absence of heat shock, DTT increased the proportion of two-cell embryos and Day 5 embryos $\geq$ 16 cells, maintained continuously in high oxygen, that became blastocysts. Such a result supports the previously-demonstrated idea that the deleterious effects of culture in high oxygen on

blastocyst development involve ROS (Fujitani et al. 1997; Takahashi et al. 2002). Moreover, these results demonstrate that DTT was added at concentrations sufficient to affect embryo physiology. At both stages of development tested, there was a thermoprotective effect of DTT measured as a reduction in the block to development caused by culture at 41^{o}C that was either significant or tended to be significant. The thermoprotective effect of DTT was incomplete, however, and depended upon stage of development. For the two-cell embryo exposed continuously to high oxygen, the degree of inhibition of blastocyst development caused by heat shock in the presence of the highest concentration of DTT tested (500 μM) was 77.3-78.9% compared to 95.5-100 % in the absence of DTT (Figures.3-6 and 3-7B) . When two-cell embryos were cultured in low oxygen except during heat shock, there was no protective effect of DTT (Figure 3-7). For the Day 5 embryo $\geq$ 16 cells, in contrast, the degree of inhibition of development caused by heat shock in the presence of 500 μM DTT was 26.6% compared to 70.8% of that in the absence of DTT (Figure 3-8B).

The differential effect of DTT on two-cell embryos versus embryos at Day 5 could reflect several differences in cell physiology. The two-cell embryo is more susceptible to heat shock than the Day 5 embryo and the reduced effectiveness of DTT at the two-cell stage may reflect the increased heat susceptibility of the two-cell embryo. In another experiment in which heat shock was of reduced duration (41.5^{o}C for 6 hours) compared to the study here, anthocyanins from purple sweet potato completely blocked actions of heat shock on continued development of embryos at Day 2 after fertilization (Sakatani et al. 2007). Another possible explanation for the minimal effect of DTT at the two-cell stage relates to GSH status of the embryo. Glutathione content is higher for embryos at Day 5 than at the two-cell stage (Lim et al. 1996a) and DTT

might be more effective at altering redox status later in development as a result. Effectiveness of DTT as an antioxidant is reduced in cells depleted of GSH (Zou et al. 2001).

Indeed a deficiency in GSH could be one reason why DTT did not completely reverse effects of heat shock on development. It could be postulated that DTT, which is water soluble, did not prevent oxidative damage in the lipid compartment of the cell. However, DTT can block lipid peroxidation in several models of oxidation [(see for example (Fariss et al. 1997; Zou et al. 2001)] and GSH, which is regenerated by DTT, plays an important role in preventing lipid peroxidation (Blair 2006). Another interpretation for the failure of DTT to completely block effects of heat shock is that there are effects of heat shock that are independent of ROS production. Among the possible ROS-independent effects of heat shock are protein denaturation and increased membrane fluidity.

The one unexpected result was that addition of DTT exacerbated effects of heat shock of two-cell embryos on development when embryos were cultured continuously in low oxygen (Figure 3-7). Under certain conditions, thiol reagents such as DTT can be toxic to cells as a result of their oxidation and subsequent generation of lipid peroxides mediated by the Fenton reaction (Held et al. 1996). It is possible that similar toxic effects of DTT on two-cell embryos cultured in high oxygen obscured thermoprotective effects of DTT.

In addition to being involved in mediating effects of heat shock on development, ROS were shown to be essential for induction of apoptosis by heat shock. Heat shock-induced apoptosis in preimplantation embryos is a developmentally regulated phenomenon with the acquisition of apoptosis responses first occurring for embryos greater than 8-16 cells on Day 4 after fertilization (Paula-Lopes and Hansen 2002b; Jousan and Hansen 2004; Jousan and Hansen 2007). The role of ROS in induction of apoptosis was studied in Day 5 embryos $\geq$ 16 cells

because these embryos had acquired capacity for heat-shock induced apoptosis. The increase in apoptotic cells caused by heat shock was reduced or absent when embryos were cultured in low oxygen. Furthermore, DTT decreased apoptosis in embryos cultured at 38.5^{o}C and completely blocked the increase in apoptosis induced by heat shock. In other cells as well, induction of apoptosis by heat shock is mediated by oxidative stress (Sreedhar et al. 2002; Zhao et al. 2006).

It is important to point out that the block to development caused by heat shock is not the result of the increase in apoptosis. This conclusion is based on previous observations that inhibition of apoptosis responses with the caspase inhibitor, z-DEVD-fmk, exacerbates effects of heat shock on development (Paula-Lopes and Hansen 2002a) as well as present observations that DTT completely blocked induction of apoptosis caused by heat shock but was only partially effective in blocking effects of heat shock on development.

The reduction in development caused by heat shock at the two-cell stage in the presence of high oxygen was greater if embryos were cultured in high oxygen continuously (95.5 – 100% reduction in development after 15 hours at 41^{o}C; Figure 3-6 and 3-7B) than if they were cultured in low oxygen before and after heat shock (37.6 % reduction in development after 15 hours at 41^{o}C in the same experiment; Figure 3-7A). Thus, oxygen environment before and/or after heat shock is also a determinant of embryonic survival to heat shock. One possible explanation for these effects is that ROS formation occurs beyond the 24-hour period following initiation of heat shock. Another possibility is that heat shock is more likely to arrest development when the embryo is already compromised in developmental potential. Perhaps, heat shock affected development more for embryos maintained in high oxygen continuously because competence of these embryos to become blastocysts was reduced. If there is a relationship between embryonic

capacity for development and the magnitude of heat-shock induced arrest of development, the consequences of heat shock in vivo will depend upon the characteristics of the embryo.

Oxygen content of the reproductive tract is low, ranging from 1.5 to 8.7% depending on segment of the tract and the species (Fischer and Bavister 1993). Given the importance of oxygen tension and ROS generation for heat shock-induced changes in embryonic function, the question remains as to whether an increase in temperature of the oviduct or uterus would result in reduced embryonic development and increased apoptosis. Heat stress during early embryonic development retards embryonic growth (Putney et al. 1988b; Ealy et al. 1993) but this effect could involve direct effects of heat shock on the embryo or alterations in reproductive tract environment. It is hypothesized that, despite the low oxygen concentration present in the reproductive tract, the redox state of the reproductive tract would be sufficient to allow effects of heat shock on the embryo. This is because the reproductive tract itself can generate ROS during heat shock. This interpretation is supported by results obtained in mice. In particular, maternal heat stress increased free radical production in the oviduct (Ozawa et al. 2004; Matsuzuka et al. 2005b) and administration of the antioxidant melatonin reduced the effects of maternal heat stress on embryonic development in vivo (Matsuzuka et al. 2005a). Thus, the ROS environment of the heat-shocked embryo in vivo may be greater than for the embryo heat-shocked in vitro under low oxygen conditions.

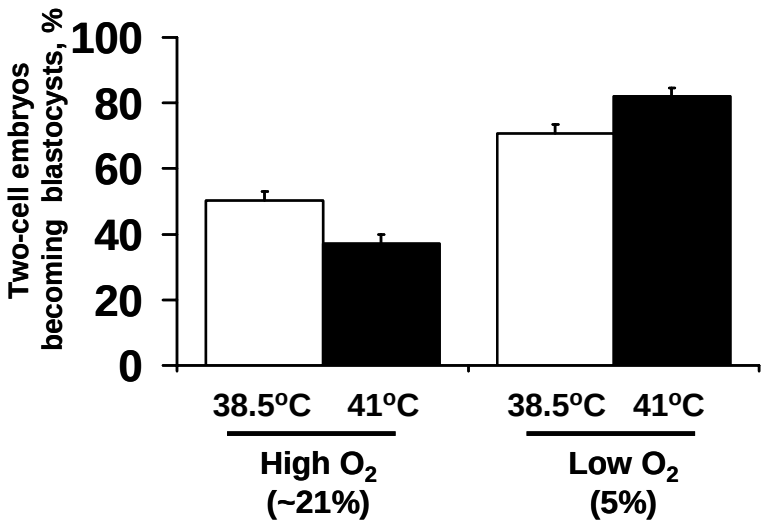

Figure 3-1. Heat shock effects on development of two-cell embryos as modified by oxygen environment throughout culture. Two-cell embryos were cultured either at 38.5°C continuously or at 41°C for 9 hours followed by culture at 38.5°C thereafter. Embryos were cultured continuously in low (5%) or high (~21%) oxygen. Heat shock reduced embryo development in high oxygen but not in low oxygen (temperature x oxygen, $P < 0.05$). Values are least-squares means ± SEM of the proportion of two-cell embryos that became blastocysts on Day 8 after insemination.

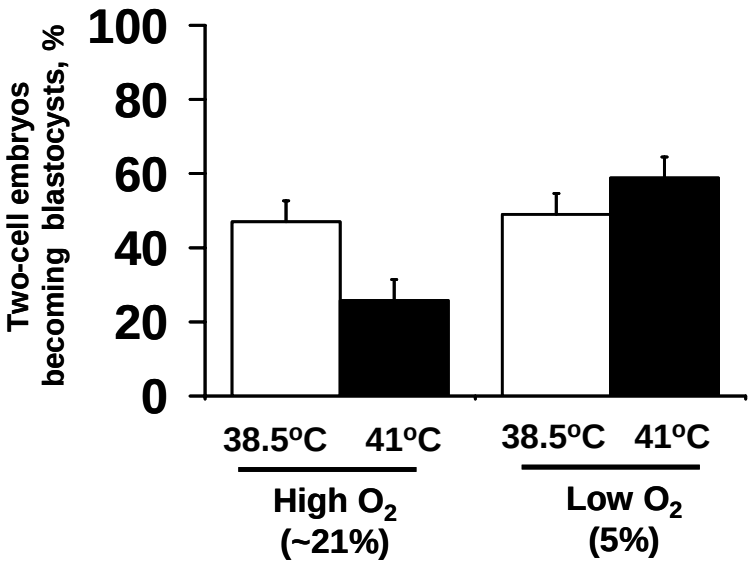

Figure 3-2. Acute effect of oxygen environment on heat shocked two-cell embryos. Embryos cultured in high oxygen were harvested at the two-cell stage. Embryos were then cultured at either 38.5°C for 24 hours or at 41°C for 9 hours and 38.5°C for 15 hours in a low (5%) or high (~21%) oxygen environment. Thereafter, all embryos were cultured at 38.5°C in high oxygen. Heat shock reduced embryo development in high oxygen but not in low oxygen (temperature x oxygen, $P < 0.05$).Values are least-squares means ± SEM of the proportion of two-cell embryos that became blastocysts on Day 8 after insemination.

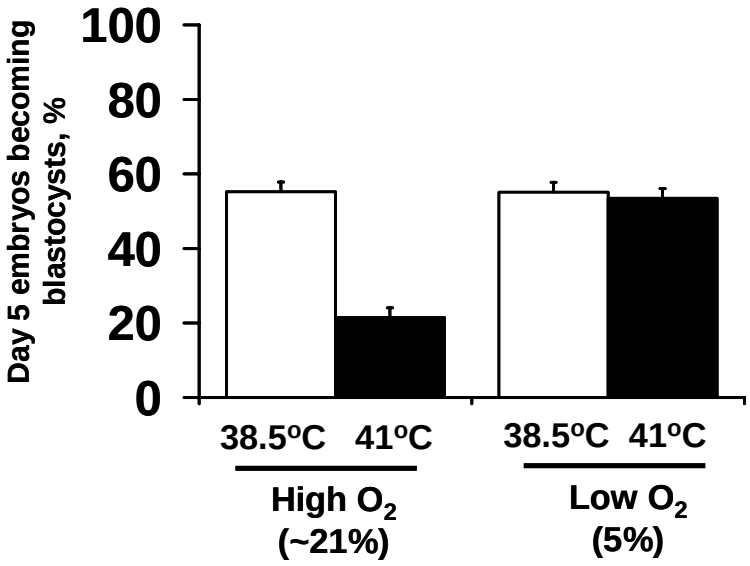

Figure 3-3. Acute effect of oxygen environment on heat shocked embryos ≥ 16 cells. Embryos were cultured in high oxygen until Day 5 when those ≥ 16 cells were harvested and cultured at 38.5°C for 24 hours or at 41°C for 9 hours and 38.5°C for 15 hours in a low (5%) or high (~21%) oxygen environment. Thereafter, all embryos were cultured at 38.5°C in high oxygen. Heat shock reduced embryo development in high oxygen but not in low oxygen (temperature x oxygen, $P < 0.05$).Values are least-squares means ± SEM of the proportion of embryos ≥ 16 cells that became blastocysts on Day 8 after insemination.

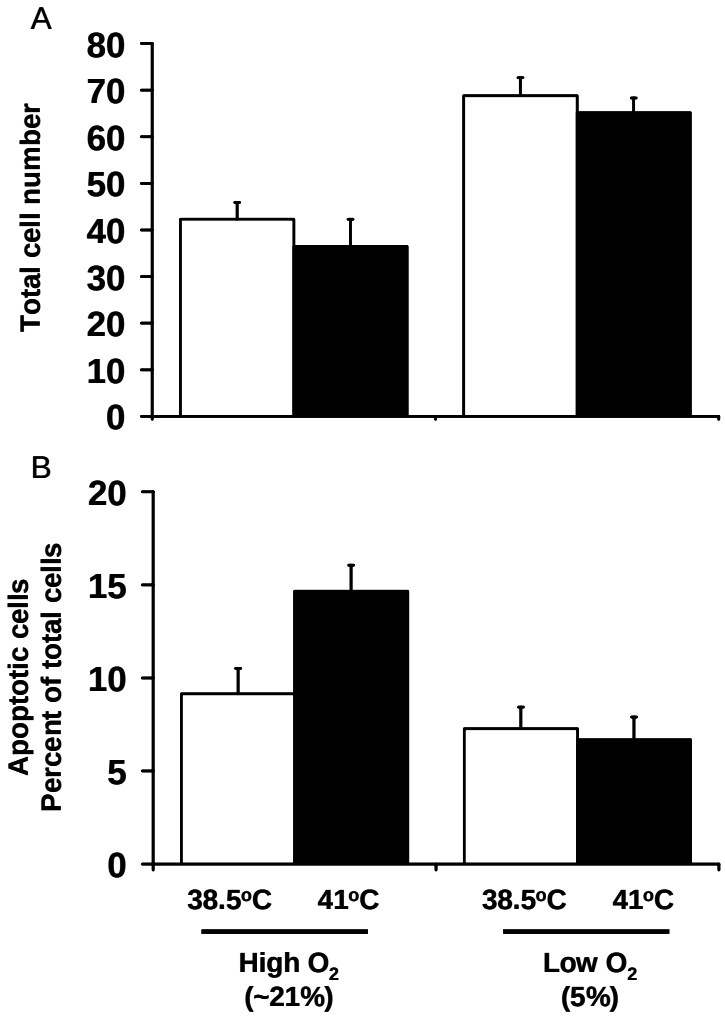

Figure 3-4. Heat shock effects on apoptosis of embryos ≥ 16 cells as modified by oxygen environment throughout culture. Embryos were cultured in high oxygen until Day 5 when those ≥ 16 cells were harvested and cultured at 38.5°C for 24 hours or at 41°C for 9 hours and 38.5°C for 15 hours in a low (5%) or high (~21%) oxygen environment. Immediately thereafter, embryos were processed for determination of total cell number and the number of blastomeres that were apoptotic (i.e. positive for the TUNEL reaction). A) Heat shock did not affect total cell number (temperature and temperature x oxygen, $P > 0.1$). B) Heat shock increased the percent of apoptotic cells (temperature x oxygen, $P < 0.05$). Values are least-squares means ± SEM.

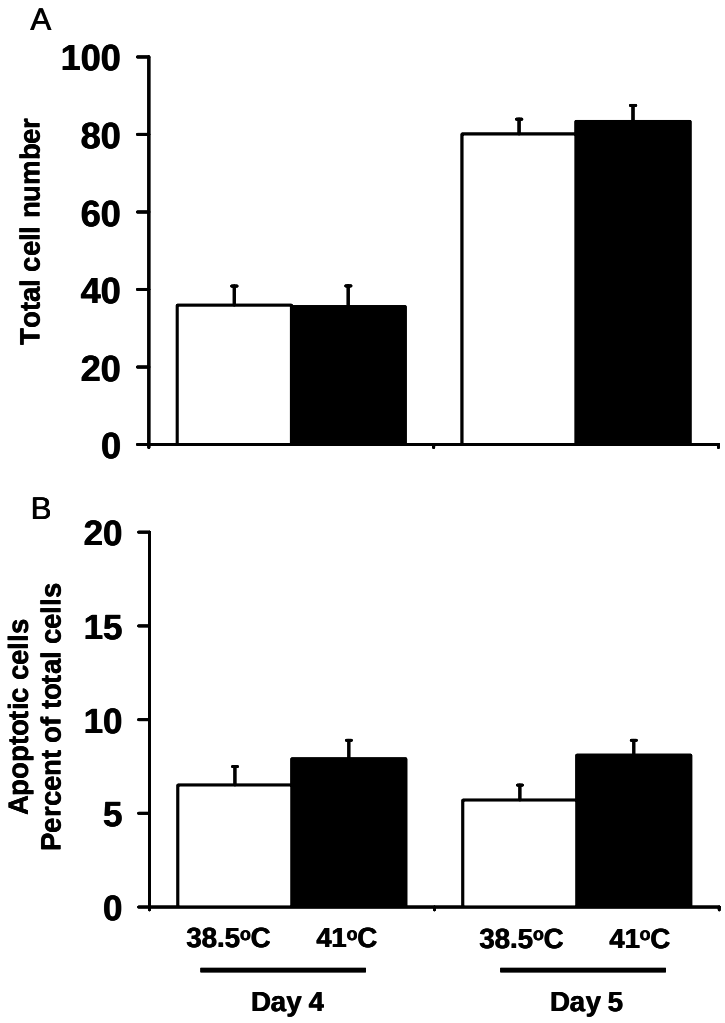

Figure 3-5. Effects of heat shock on apoptosis of Day 4 and Day 5 embryos ≥ 16 cells cultured continuously in low oxygen. Embryos ≥ 16 cells were harvested at either Day 4 or 5 after insemination and cultured at 38.5 for 24 hours or 41°C for 9 hours followed by 15 hours at 38.5°C. Immediately thereafter, embryos were processed for determination of total cell number and the percent of blastomeres that were apoptotic (i.e. positive for the TUNEL reaction). A) Heat shock did not affect total cell number (temperature and temperature x oxygen, $P > 0.1$). B) Heat shock slightly increased the percent of apoptotic cells (temperature x oxygen, $P < 0.05$). Values are least-squares means ± SEM.

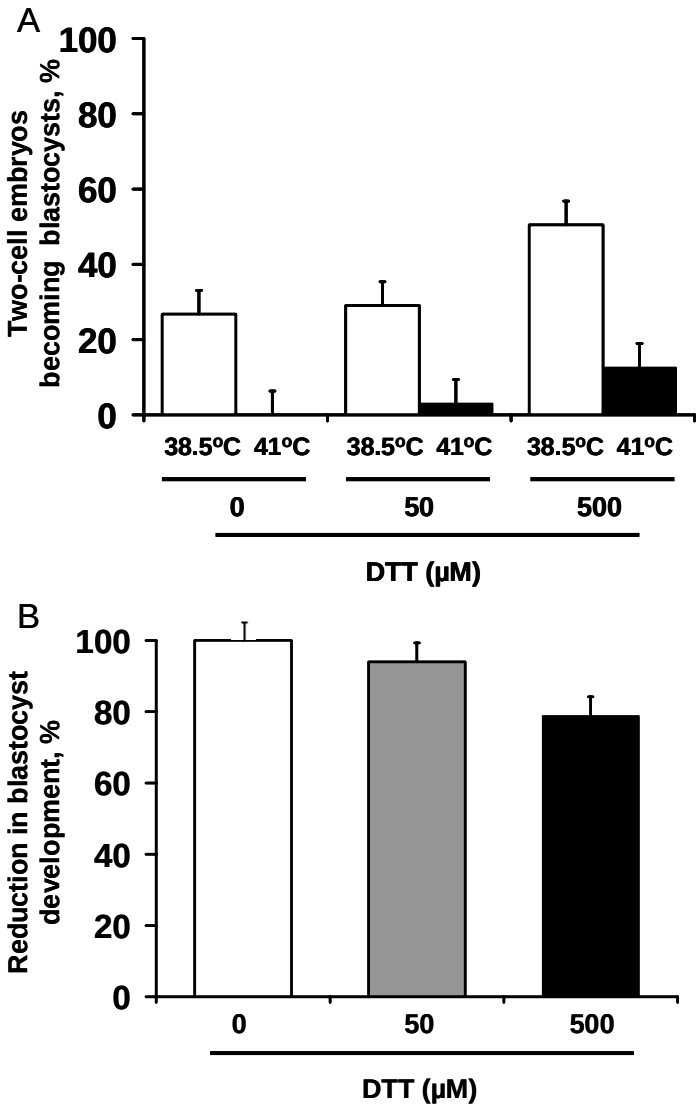

Figure 3-6. Protective action of dithiothreitol (DTT) on development of embryos heat shocked at the two-cell stage and cultured in high oxygen continuously. Embryos were harvested at the two-cell stage. Embryos were then cultured with various concentrations of DTT at either 38.5°C for 24 hours or at 41°C for 15 hours and 38.5°C for 9 hours. After washing, embryos were then cultured at 38.5°C until Day 8 after insemination without DTT. A) Percent of two-cell embryos that became blastocysts at Day 8. DTT increased ($P < 0.05$) and heat shock reduced ($P < 0.05$) the percent of embryos becoming blastocysts. B) Percent reduction in blastocyst development caused by heat shock. This variable was calculated as follows: [1 – (percent development at 41°C/percent development at 38.5°C)]*100. The percent reduction in development caused by heat shock was less for embryos treated with DTT ($P < 0.05$). Values are least-squares means ± SEM.

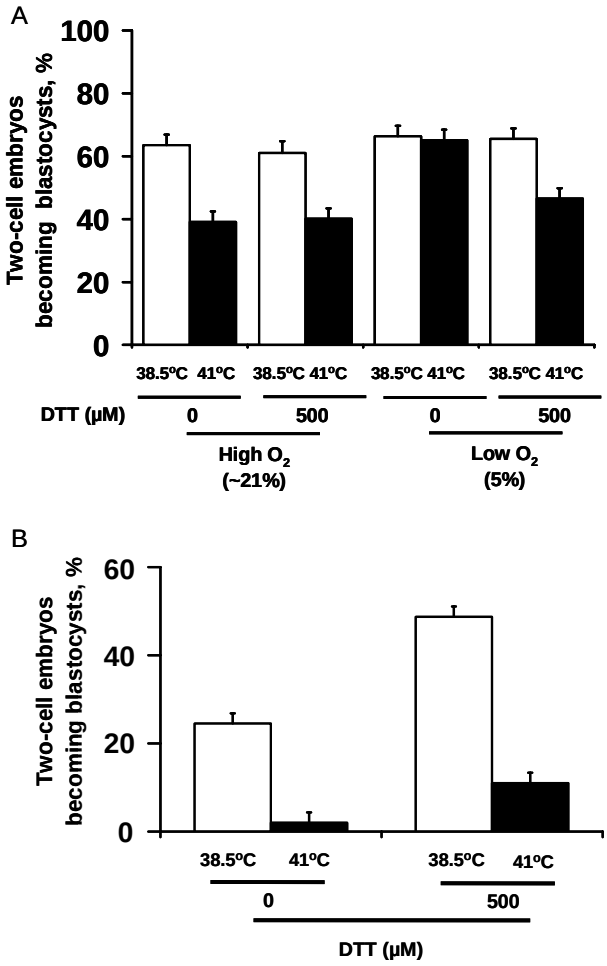

Figure 3-7. Protective action of dithiothreitol (DTT) on development of embryos heat shocked at the two-cell stage and cultured in low oxygen at the two-cell stage. A) Embryos were cultured in low oxygen after insemination. Two-cell embryos were harvested at ~28 hours after insemination and assigned to one of eight treatments in a 2 x 2 x 2 factorial design with main effects of incubation temperature (38.5°C for 24 hours or 41°C for 15 hours followed by 38.5°C for 9 hours), oxygen environment (high or low oxygen) and DTT (0 or 500 µM). After washing, embryos were placed in fresh drops of KSOM-BE2 and cultured in low oxygen at 38.5°C without DTT until Day 8 when blastocyst development was determined. Heat shock reduced development in all groups except for the embryos cultured in low oxygen in the absence of DTT (temperature x oxygen x DTT, $P < 0.05$). B) Embryos were cultured in high oxygen after fertilization. Two-cell embryos were harvested and cultured in high oxygen and assigned to one of 4 treatments in a 2 x 2 factorial design with main effects of incubation temperature (38.5°C for 24 hours or 41°C for 15 hours followed by 38.5°C for 9 hours) and DTT (0 or 500 µM). After 24 hours, embryos were washed 3 times in 100 µl HEPES-TALP drops and placed in fresh drops of KSOM-BE2 and cultured in high oxygen at 38.5°C until Day 8 when blastocyst development was determined. DTT conferred partial protection against heat shock (DTT x temperature, $P < 0.05$). Values are least-squares means ± SEM.

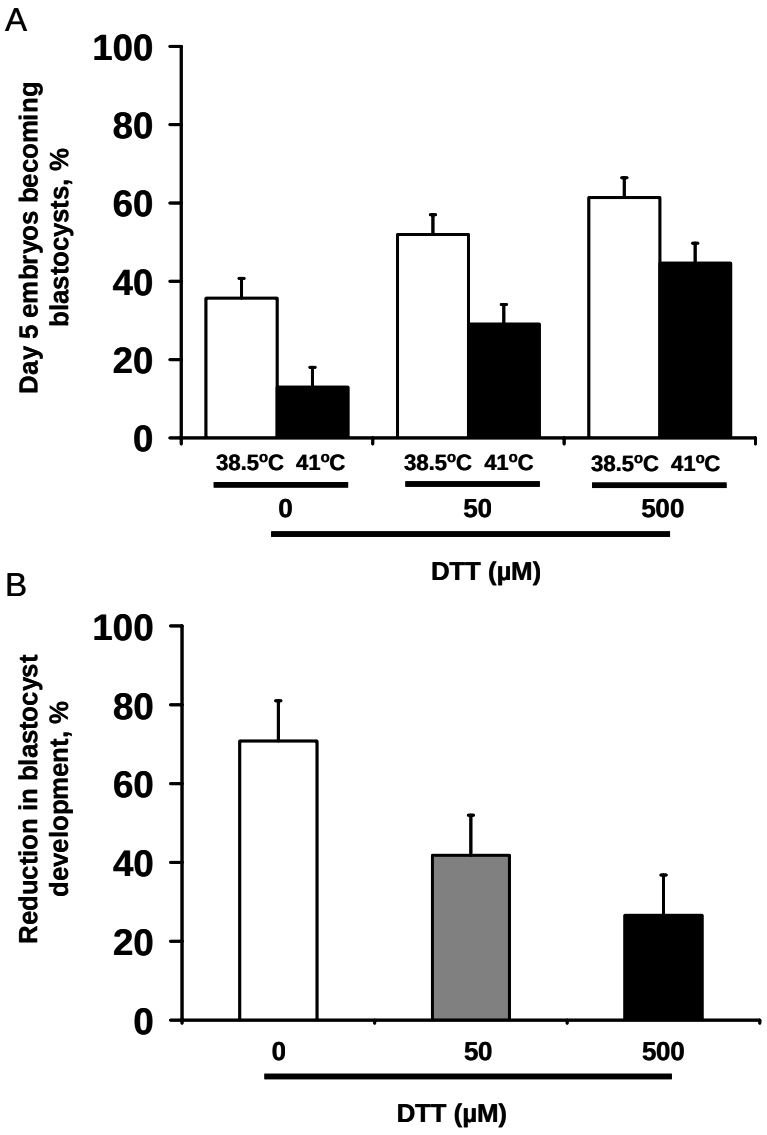

Figure 3-8. Protective action of DTT on development of heat-shocked embryos ≥16 cells cultured in high oxygen continuously. Embryos cultured in high oxygen that were ≥ 16 cells were harvested at Day 5 after insemination. Embryos were then cultured at either 38.5°C for 24 hours or at 41°C for 15 hours and 38.5°C for 9 hours in a high oxygen environment with various concentrations of DTT. After washing, embryos were then cultured at 38.5°C in a high oxygen environment until Day 8 after insemination. A) Percent of two-cell embryos that became blastocysts at Day 8. DTT increased ($P < 0.05$) and heat shock reduced ($P < 0.05$) the percent of embryos becoming blastocysts. B) Percent reduction in blastocyst development caused by heat shock expressed as a percentage of development for non-heat-shocked embryos [1 – (percent development at 41°C/percent development at 38.5°C)]*100. The percent reduction in development caused by heat shock was less for embryos treated with DTT ($P < 0.05$).Values are least-squares means ± SEM.

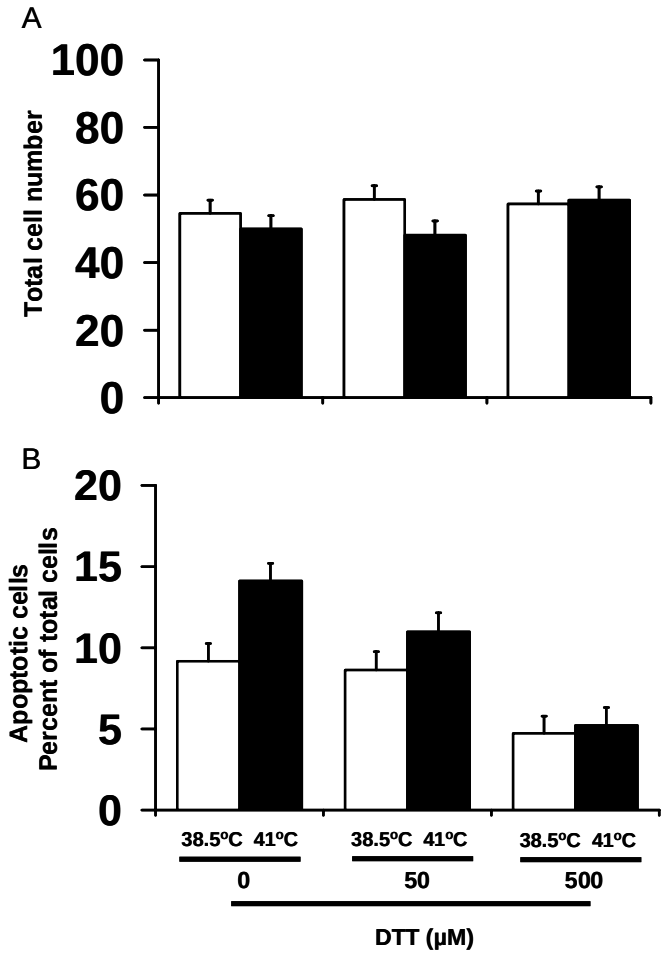

Figure 3-9. Protective action of dithiothreitol (DTT) on apoptosis of heat-shocked embryos ≥ 16 cells cultured in high oxygen continuously. Embryos were cultured in high oxygen after insemination. On Day 5, embryos ≥16 cells were harvested and assigned to culture in the presence of 0, 50 or 500 µM DTT at 38.5°C for 24 hours or 41°C for 15 hours followed by 38.5°C for 9 hours. Immediately thereafter, embryos were processed for determination of total cell number and the percent of blastomeres that were apoptotic (i.e. positive for the TUNEL reaction). A) There was no effect of temperature or DTT on total cell number ($P > 0.1$). B) Heat shock increased the percent of TUNEL positive nuclei among embryos treated with 0 µM DTT (temperature, $P < 0.01$) but not for embryos treated with DTT (temperature, $P > 0.1$). Values are least-squares means ± SEM.

CHAPTER 4
ACTIONS OF CERAMIDE TO INHIBIT DEVELOPMENT AND INDUCE APOPTOSIS IN PREIMPLANTATION BOVINE EMBRYOS

Introduction

Like other cells, preimplantation embryos can undergo apoptosis in response to a variety of stimuli. Among these stimuli are several which induce apoptosis through the so-called intrinsic pathway that involves release of mitochondrial components and activation of caspase-9 and caspase-3 (Jin and El-Deiry 2005). Examples of these stimuli include oxidative stress, heat shock, arsenic, UV light and others (Krininger et al. 2002; Yuan et al. 2003; Qian et al. 2004; Chirico et al. 2007). The capacity for apoptosis is acquired during development. In the bovine embryo, for example, induction of apoptosis by heat shock and TNF-α first occurs between the 8-cell and 16-cell stage of development (Paula-Lopes and Hansen 2002b; Soto et al. 2003a).

Ceramide is an important signaling molecule in activation of the intrinsic pathway for apoptosis by heat shock, ultraviolet radiation, cytotoxic drugs, and free radicals (Sawai and Hannun 1999; Pettus et al. 2002). Ceramide can be generated by de novo synthesis through ceramide synthase and by hydrolysis of sphingomyelin by a class of sphingomyelin-specific phospholipase C molecules called SMases that cleave the phosphodiester bond of sphingomyelin to yield ceramide and phosphocoline. There are two forms of SMase that differ in pH optimum and subcellular localization. aSMase has wide tissue distribution, is localized to lysosomes and endosomes, and has a pH optimum around 5 (Kolesnick and Fuks 2003). nSMase exists as three types that have a pH optimum around 7. The nSMase1 is ubiquitously expressed and is localized in the endoplasmic reticulum (Tomiuk et al. 1998), nSMase2 is found predominantly in the brain (Hofmann et al. 2000), and nSMase3 is more widely distributed in tissues, and is localized predominantly to the endoplasmic reticulum and mitochondria (Krut et al. 2006).

Activation of aSMase or nSMase has been shown to be essential for cell death induced by TNF-α (Guidarelli et al. 2001; Luberto et al. 2002), radiation (Hara et al. 2004a) and ultraviolet radiation (Kashkar et al. 2005). Kondo et al. (2000a) showed that heat shock of human HL-60 leukemia cells reduced cellular sphingomyelin content, increased nSMase and ceramide content, and apoptosis. There was no increase in aSMase activity following heat shock. Verheij et al. (Verheij et al. 1996) showed that heat shock caused an increase in intracellular ceramide content in human U937 monoblastic leukemia cells and bovine aortic endothelial cells. In another study with HL-60 cells, heat shock increased aSMase and not nSMase and heat-shock induced apoptosis was blocked by inhibitors of ceramide generation (Chung et al. 2003).

It is not known to what extent ceramide plays a role in apoptosis in the preimplantation embryo or whether aSMase or nSMase are involved in ceramide generation. The overall objective of the present study was to evaluate whether a functional ceramide signaling system was present in the preimplantation bovine embryo. The first goal was to determine the effect of ceramide on apoptosis and development of bovine embryos at different stages of development. In other cells, administration of ceramide, but not its inactive analogue dihydroceramide, can induce apoptosis (Obeid et al. 1993; Jarvis et al. 1994; Dbaibo et al. 1995; Ji et al. 1995; Pruschy et al. 1999; Di Nardo et al. 2000) and therefore, dihydroceramide was used as a negative control. The second goal was to determine if heat shock, an inducer of apoptosis in the preimplantation bovine embryo (Paula-Lopes and Hansen 2002b; Paula-Lopes and Hansen 2002a), induces aSMase or nSMase activity.

Materials and methods

Materials

HEPES- TL, IVF-TL, and Sperm-TL solutions were purchased from Cell and Molecular Technologies Inc. (Lavallete, NJ) and used to prepare HEPES-TALP-TALP, IVF-TALP, and

Sperm-TALP as previously described (Fischer-Brown et al. 2002). OCM was TCM-199 with Hank's salts without phenol red (Atlanta Biologicals, Norcross, GA) supplemented with 2% (v/v) bovine steer serum (Pel-Freez, Rogers, AR) containing 2 U/ml heparin, 100 U/ml penicillin-G, 0.1 mg/ml streptomycin, and 1 mM glutamine. OMM was TCM-199 (BioWhittaker, Walkersville, MD) with Earle's salts supplemented with 10% (v/ v) bovine steer serum, 2 μg/ml estradiol 17-β, 20 μg/ml bovine follicle stimulating hormone (Folltropin-V; Vetrepharm Canada, London, ON), 22 μg/ml sodium pyruvate, 50 μg/ml gentamicin sulfate, and 1 mM glutamine. Percoll was from Amersham Pharmacia Biotech (Uppsala, Sweden). Frozen semen from various bulls was donated by Southeastern Semen Services (Wellborn, FL). KSOM that contained 1 mg/ml BSA was obtained from Cell and Molecular Technologies (Lavallete, NJ). EFAF-BSA was from Sigma (St. Louis, MO). On the day of use, KSOM was modified for bovine embryos to produce KSOM-BE2 as described elsewhere (Soto et al. 2003c).

Substrates for caspase-9, 3 and 8 assays (Caspalux-9-M_1-D_2, PhiPhiLux-G_1D_2, and Caspalux- 8-L_1D_2, respectively) were obtained from Oncoimmunophilin (Gaithersburg, MD) and the assay kit for SMases (Amplex Red Sphingomyelinase Kit) was from Molecular Probes (Eugene, OR). A proteinase inhibitor cocktail was purchased from Sigma (St. Louis, MO). The In Situ Cell Death Detection Kit (fluorescein or TMR red) was obtained from Roche Diagnostics Corporation (Indianapolis, IN). Hoechst 33342 was purchased from Sigma, polyvinylpyrrolidone (PVP) from Eastman Kodak (Rochester, NY) and RQ1 RNase-free DNase from Promega (Madison, WI). All other reagents were purchased from Sigma or Fisher Scientific (Pittsburgh, PA).

In Vitro Production of Embryos

Embryo production was performed as previously described (Jousan and Hansen 2004). Briefly, COCs were obtained by slicing 2- to 10-mm follicles on the surface of ovaries (a mixture

of beef and dairy cattle) obtained from a local abattoir. Those COCs with at least one complete layer of compact cumulus cells were washed two times in OCM and used for subsequent steps. Groups of 10 COCs were placed in 50-µl drops of OMM overlaid with mineral oil and matured for 20–22 hours at 38.5°C in an atmosphere of 5% (v/v) CO_2 in humidified air. Matured COCs were then washed once in HEPES-TALP and transferred in groups of 30 to 4-well plates containing 600 µl of IVF-TALP and 25 µl of PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 µM epinephrine in 0.9% [w/v] NaCl) per well and fertilized with ~1 x 10^6 Percoll-purified spermatozoa from a pool of frozen-thawed semen from three bulls. A separate pool of bulls was used for each replicate of an experiment. After 10-12 hours at 38.5°C in an atmosphere of 5% CO_2 in humidified air, putative zygotes were removed from fertilization wells, denuded of cumulus cells by vortex mixing in 1 ml of 1000 U/ml hyaluronidase in HEPES-TALP, and placed in groups of ~30 in 50-µl drops of KSOM-BE2. All drops of embryos were overlaid with mineral oil and cultured in an atmosphere of 5% CO_2 in humidified air at 38.5°C until harvested for individual experiments.

Embryos were cultured without mineral oil during the time when ceramide and dihydroceramide were used. This practice was to prevent diffusion of test substances from culture medium. Instead, embryos were cultured in 300 µl KSOM-BE2 in 4-well plates.

TUNEL and Hoechst 33342 Labeling

The TUNEL assay was used to detect DNA fragmentation associated with late stages of the apoptotic cascade and Hoeschst 33342 labeling was used to determine cell number. The procedure was performed as described previously in Chapter 3.

Caspase Assays

Embryos were washed three times in 50-µl drops of HEPES-TALP (prewarmed at 38.5°C) and incubated in 25-µl microdrops of HEPES-TALP containing 5 µM of Caspalux-9-M_1D_2,

PhiPhiLux-G_1D_2 or Caspalux- 8-L_1D_2 for caspase-9, 3 and 8, respectively at 38.5°C for 45 minutes in the dark. Each assay included negative controls (incubated in HEPES-TALP only) and positive controls [embryos incubated for 24 hours in the presence of 100 μM of the mitochondrial uncoupling agent carbonyl cyanide m-chlorophenylhydrazone (CCCP) prior to caspase assay]. Following incubation, embryos were washed twice in 50-μl drops of HEPES-TALP and placed on two-well slides containing 100 μl of prewarmed HEPES-TALP. Embryos were viewed using a Zeiss Axioplan microscope using FITC filter. Images were acquired using AxioVision software and an AxioCam MRm digital camera. Using the computer mouse, a circular draw function was manually performed around the internal side of the zona pellucida and intensity per unit area was determined using AxioVision software.

Experiments

Induction of apoptosis in Day 5 embryos ≥ 16 cells by ceramide.

Embryos ≥ 16 cells were harvested on day 5 after insemination. Embryos were randomly assigned to one of the following treatments: 50 μM C(2)-ceramide for 9 hours followed by 15 hours in medium with ethanol vehicle [0.34% (v/v)] (C2-9 h), 50 μM C(2)-ceramide for 24 hours (C2-24 h), 50 μM C(2)-dihydroceramide for 9 hours followed by 15 hours in medium with ethanol vehicle (DC2-9 h), 50 μM C(2)-dihydroceramide for 24 hours (DC2-24 h), or 24 hours of medium with ethanol vehicle. The C(2)-dihydroceramide was used as a negative control because it is an inactive analogue of C(2) ceramide. During the treatment period, embryos were cultured in groups of 10 to 19, depending on the replicate, in 300 μl KSOM-BE2 in 4-well plates in an atmosphere of 5% CO_2 in humidified air at 38.5°C. There were similar numbers of embryos per treatment within a replicate. At 24 hours after treatment, embryos were fixed in 4% (w/v) paraformaldehyde and stored in PBS-PVP at 4°C until analysis by TUNEL and Hoechst 33342 labeling. The experiment was replicated 3 times with a total of 200 embryos.

Induction of caspase-9, 3 and 8 activity in Day 5 embryos ≥ 16 cells by ceramide.

Embryos ≥ 16 cells harvested on day 5 after fertilization were randomly assigned to one of the following treatments: 50 μM C(2)-ceramide for 24 hours (C2-24 h), 50 μM C(2)-dihydroceramide for 24 hours, and medium with ethanol vehicle [0.34% (v/v)]. Embryos were cultured in groups of 8 to 22, depending on the replicate, in 300 μl KSOM-BE2 in 4-well plates in 5% CO^2 in humidified air at 38.5°C. There were similar numbers of embryos per treatment within a replicate. After 24 hours, embryos were washed 4 times in HEPES-TALP and activity of caspase-9 (replicates = 4; total n = 167 embryos), caspase-3 (replicates = 2; total n = 76 embryos) and caspase-8 (replicates = 2; total n = 78 embryos) was measured immediately thereafter.

Effect of ceramide treatment of Day 5 embryos ≥ 16 cells on development to the blastocyst stage.

Embryos ≥ 16 cells were harvested on day 5 after insemination. Embryos were randomly assigned to one of the following treatments: 50 μM C(2)-ceramide for 9 hours followed by 15 hours in medium with ethanol vehicle [0.34% (v/v)] (C2-9 h), 50 μM C(2)-ceramide for 24 hours (C2-24 h), 50 μM C(2)-dihydroceramide for 9 hours followed by 15 hours in medium with ethanol vehicle (DC2-9 h), 50 μM C(2)-dihydroceramide for 24 hours (DC2-24 h), or 24 hours of vehicle control [ethanol, 0.34% (v/v)]. During the treatment period, embryos were cultured in groups of 21 to 30, depending on replicate, in 300 μL KSOM-BE2 in 4-well plates in 5% CO_2 in humidified air at 38.5°C. There were similar numbers of embryos per treatment within a replicate. At 24 hours after initiation of treatment, embryos were washed 3 times in HEPES-TALP, placed in 50 μL drops of KSOM-BE2 overlaid with mineral oil, and cultured in an atmosphere of 5% CO_2 in humidified air at 38.5°C. On day 8 after fertilization, the percentage of

treated embryos becoming blastocysts was determined. The experiment was replicated 3 times with a total of 336 embryos.

Apoptosis and cytokinesis in two-cell embryos treated with ceramide.

Two-cell embryos harvested 27-29 hours after insemination were randomly assigned to one of the following treatments: 50 μM C(2)-ceramide for 9 hours followed by 15 hours in medium with ethanol vehicle [0.34% (v/v)] (C2-9 h), 50 μM C(2)-ceramide for 24 hours (C2-24 h), 50 μM C(2)-dihydroceramide for 9 hours followed by 15 hours in medium with ethanol vehicle (DC2-9 h), 50 μM C(2)-dihydroceramide for 24 hours (DC2-24 h), or 24 hours of culture in medium with ethanol vehicle. During the treatment period, embryos were cultured in groups of 6 to 18, depending on the replicate, in 300 μl KSOM-BE2 in 4-well plates in 5% CO_2 in humidified air at 38.5^{o}C. There were similar numbers of embryos per treatment within a replicate. At 24 hours after initiation of treatment, embryos were fixed in 4% (w/v) paraformaldehyde and stored in PBS-PVP at 4°C until analysis by TUNEL and Hoechst 33342 labeling. Several endpoints were determined – the proportion of nuclei exhibiting TUNEL labeling, total number of nuclei, total cell number and the nuclei:cell ratio. Cell number was distinguished from nuclei number by maximizing cell auto-fluorescence digitally so that each cell could be seen individually. The experiment was replicated 5 times with a total of 260 embryos. Cell number was determined separately from nuclei number for 3 replicates only. Data for cell number and nuclei:cell ratio were obtained on 179 embryos.

Caspase-9 activity in two-cell embryos treated with ceramide.

Two-cell embryos harvested 27-29 hours after fertilization were randomly assigned to be treated with one of the following treatments: 50 μM C(2)-ceramide for 24 hours (C2-24 h), 50 μM C(2)-dihydroceramide for 24 hours (DC2-24 h), or 24 hours in vehicle [ethanol, 0.34%

(v/v)]. Embryos were washed 4 times in HEPES-TALP and caspase-9 activity was measured immediately thereafter. The experiment was replicated 3 times with a total of 170 embryos.

Acid and neutral sphingomyelinase activities induced by heat-shock in Day 5 embryos ≥ 16 cells.

Acid and neutral SMase activity was determined in embryos using the Amplex Red Sphingomyelinase Kit (Molecular Probes). In this assay, exogenous sphingomyelin is cleaved by SMase present in the sample to yield phosphocoline and ceramide. Phosphocoline is further cleaved by alkaline phosphatase to generate choline, which in turn is further oxidized by choline oxidase to generate H_2O_2. The Amplex Red reagent is converted to the fluorescent molecule resorufin in the presence of horseradish peroxidase.

Embryos ≥ 16 cells harvested on Day 5 after fertilization were randomly assigned to be cultured at 38.5^{o}C or 41^{o}C for 15 hours in 50 µL drops of KSOM-BE2 in groups of 23 to 32. After heat shock, all embryos from each treatment (32 to 96 and 50 to 86 embryos for nSMase and aSMase, respectively, depending on the replicate) were pooled in 5 µl of KSOM-BE2 and mixed with 5 µl of either nSMase lysis buffer [0.2 M Tris-HCl, pH 7.4 containing 20 mM $MgCl_2$, 2% (v/v) Triton-X and 2% (v/v)10x proteinase inhibitor cocktail] or aSMase lysis buffer [0.1 M sodium acetate buffer, pH 5.0 containing 2% (v/v) Triton-X and 2% (v/v)10x proteinase inhibitor cocktail]. Samples were immediately frozen at -80^{o}C until assayed. Prior to the assay, samples were thawed at 38.5^{o}C and frozen-thawed in liquid nitrogen three times to allow further cell membrane disruption.

Enzyme reactions were prepared using enzymes and other reagents provided with the kit. A total of 32 to 96 (nSMase) or 50 to 86 (aSMase) embryo equivalents in 10 µl of a 1:1 (v/v) mixture of KSOM-BE2 and lysis buffer were included in each enzyme reaction. For each

replicate, the number of embryos in the enzyme reaction was the same for each treatment. Additional enzyme reactions included a positive control (prepared immediately before the assay) of 0.4 U/ml bacterial SMase in 100 µl 1X reaction buffer (0.1 M Tris-HCl and 10 mM $MgCl_2$, pH 7.4) and a negative control consisting of a 1:1 mixture of KSOM-BE2 and lysis buffer.

For nSMase, each enzyme reaction consisted of 10 µl sample and 100 µl working solution (0.1 M Tris-HCl and 10 mM $MgCl_2$, pH 7.4 containing 2 U/mL horseradish peroxidase, 0.2 U/mL choline oxidase, 8 U/mL alkaline phosphatase and 0.5 mM sphingomyelin) placed in a well of a 96-well plate. Fluorescence was measured using a BioTek FLx 800 fluorometer (BioTek, Winooski VT) with excitation and emission wavelengths set to 560 nm and 590 nm, respectively, at 30 minute-intervals for 390 minutes at 37^{o}C.

For the aSMase, continuous readings were not possible because the Amplex Red reaction occurs optimally in neutral pH and aSMase activity is optimal at pH 5-5.5. Therefore, each enzyme reaction consisted of incubation of 1 µL 5 mM sphingomyelin added to the 10 µL sample for 270 minutes at 37^{o}C. Then, 100 µL working solution (0.1 M Tris-HCl and 10 mM MgCl2, pH 7.6 containing 2 U/mL horseradish peroxidase, 0.2 U/mL choline oxidase, and 8 U/mL alkaline phosphatase without sphingomyelin) was added to the reaction and fluorescence recorded after 30 minutes at 37^{o}C. For both aSMase and nSMase, relative fluorescence was subtracted from background and divided by the number of embryos present in the reaction before statistical analysis. Experiments were replicated 10 and 11 times for nSMase and aSMase, respectively.

Statistical Analysis

Data were analyzed by least-squares analysis of variance using the GLM procedure of SAS (SAS for Windows, Version 9, Cary, NC). Data calculated on a replicate basis were percent of embryos becoming blastocysts and SMase activity. Data analyzed on an embryo basis were the

percent of blastomeres that were TUNEL-positive, total embryo cell number and caspase fluorescent intensity. For all analyses, mathematical models included replicate, other main effects (fixed), and interactions. Tests of significance were made using error terms determined by calculation of expected mean squares with replicate as a random effect and other main effects as fixed effects. When necessary, mean separation tests were performed using the Tukey test. Differences between treatments at specific time-points in the enzyme assay for nSMase were determined using the slice feature of the GLM procedure of SAS.

Results

Induction of Apoptosis in Day 5 Embryos ≥ 16 Cells by Ceramide.

Quantitative analysis of total cell number and percentage of cells that were TUNEL-positive are illustrated in Figure 4-1. Total cell number was generally unaffected by treatment except that treatment with C(2)-ceramide for 24 hours reduced cell number ($P < 0.05$). Treatment with C(2)-ceramide for 24 hours caused an increase in the percentage of TUNEL positive cells compared to other treatments ($P < 0.05$), except for treatment with ceramide for 9 hours ($P = 0.08$). The inactive analog of ceramide, C(2)-dihydroceramide, did not increase the proportion of cells that were TUNEL-positive ($P > 0.1$).

Induction of Caspase-9, 3 and 8 Activity in Day 5 Embryos ≥ 16 Cells by Ceramide.

Results are shown in Figure 4-2. Treatment of Day 5 embryos with C(2)-ceramide caused a significant increase in caspase-9 activity ($P < 0.05$). There was also a numerical increase in caspase-3 activity after treatment with C(2)-ceramide but the increase was not statistically significant ($P > 0.1$). There was no effect of C(2) ceramide on caspase-8 activity and no effect of C(2)-dihydroceramide on activity of caspase-9, 3 or 8.

Effect of Ceramide Treatment of Day 5 Embryos ≥ 16 Cells on Development to the Blastocyst Stage.

The proportion of Day 5 embryos developing to the blastocyst stage was generally unaffected by treatment (Figure 4-3). The exception was for embryos treated with C(2)-ceramide for 24 hours which had lower development ($P < 0.01$).

Apoptosis, caspase-9 Activity and Cytokinesis in Two-Cell Embryos Treated with Ceramide.

As shown in Figure 4-4A, The total number of nuclei was reduced by treatment with C(2)-ceramide for 24 hours (Figure 4-4A; $P < 0.01$,) but not by other treatments. The proportion of blastomeres that were TUNEL-positive was very low (< 2%) and was not affected by treatment (Figure 4-4B; $P > 0.1$). Similarly, there was no effect of any treatment on caspase-9 activity ($P > 0.01$).

In the course of the study, it was observed that embryos treated with C(2)-ceramide, but not other treatments, often had multinucleated cells (see Figure 4-5 for representative results). To quantify this phenomenon, the number of nuclei:cell was determined for the last three replicates of the study (Table 4-1). In this subset of embryos, C(2)-ceramide treatment for 24 hours reduced total number of nuclei ($P < 0.05$), total number of cells ($P < 0.05$) and increased the nuclei:cell ratio ($P < 0.05$). Moreover, C(2)-ceramide treatment for 24 hours increased the number of nuclei per cell. Indeed, 26% (9 of 34) of the embryos treated with C(2)-ceramide for 24 hours had at least one blastomere with 3 or 4 nuclei, whereas multinucleated cells (i.e.,with 3 or 4 nuclei) were not observed for any other treatment.

Acid and Neutral Sphingomyelinase Activities Induced by Heat Shock in Day 5 Embryos ≥ 16 Cells.

There was no difference in aSMase activity between control and heat-shocked embryos (Figure 4-6A). In contrast, heat shock increased the amount of nSMase activity as determined by differences in enzyme beginning 2 hours after initiation of incubation (Figure 4-6B; $P < 0.05$).

Discussion

Experiments presented here demonstrate the existence of a functional ceramide signaling system in the preimplantation bovine embryo that regulates apoptosis, development, and cytokinesis. Two lines of evidence support this contention. First, it was shown that exposure of embryos to ceramide caused profound changes in cellular function. In particular, ceramide, but not its inactive analogue dihydroceramide, induced apoptosis in a stage-specific manner. Moreover, long-term (24 hours) exposure to ceramide reduced ability of Day 5 embryos to develop to the blastocyst stage and reduced nuclear proliferation and cytokinesis at the two-cell stage. Secondly, it was shown that the enzymatic pathway for ceramide synthesis is activated by an environmental signal that itself increases apoptosis and reduces development. In particular, heat shock increased activity of nSMase which converts sphingomyelin to ceramide.

Administration of ceramide has been shown to induce apoptosis in a variety of cell types (Ji et al. 1995; Susin et al. 1997; Ito et al. 1999; Di Nardo et al. 2000; Kim et al. 2005c) but this is the first report that ceramide can induce apoptosis in preimplantation embryos. Exposure of Day 5 embryos ≥ 16 cells to ceramide led to an increase in the percentage of nuclei that were apoptotic as determined by the TUNEL assay. Activation of TUNEL labeling appears to be mediated by the mitochondrial or intrinsic pathway (Krininger et al. 2002; Yuan et al. 2003; Qian et al. 2004; Chirico et al. 2007) for apoptosis induction because ceramide increased amounts of caspase-9 and tended to increase amounts of caspase-3. In contrast, there was no

increase in caspase-8 activity. This caspase plays a central role in the receptor-mediated or extrinsic pathway for apoptosis (Sprick and Walczak 2004).

The effect of ceramide on apoptosis of the Day 5 embryo was time dependent. Although the effect was not significant, the magnitude of the increase in apoptosis caused by 9-hour exposure to ceramide is similar to that observed for heat shock at 41^{o}C for 9 hours (Jousan and Hansen 2004; de Castro e Paula and Hansen 2005).

In contrast to the situation with the Day 5 embryo, there was no increase in TUNEL labeling or caspase-9 activity when embryos were exposed to ceramide at the two-cell stage. This finding was expected because the two-cell embryo is resistant to activation of apoptosis by a range of pro-apoptotic agents including heat shock (Paula-Lopes and Hansen 2002b), arsenic (Krininger et al. 2002) and TNF-α (Soto et al. 2003a). For heat shock, the stimulus that has been studied in most detail, embryos acquire the capacity for apoptosis between the eight and sixteen cell stage (Paula-Lopes and Hansen 2002b). The fact that ceramide-induced apoptosis is also developmentally regulated is indicative that the block to apoptosis at the two-cell stage is not simply due to reduced capacity for ceramide synthesis because exogenous ceramide did not reverse the block to apoptosis. Although not undergoing apoptosis, two-cell embryos are responsive to ceramide as indicated by the fact that exposure to ceramide for 24 hours reduced total nuclei per embryo and caused a partial block to cytokinesis.

Exposure of Day 5 embryos to ceramide also blocked development to the blastocyst stage if exposure was for 24 hours but not if exposure was for 9 hours. It is not surprising that 9-hour exposure to ceramide did not affect development even while causing a slight increase in apoptosis. The preimplantation embryo is capable of tolerating a slight amount of apoptosis and, indeed, limited apoptosis may help embryos survive exposure to cellular stress. For example,

TNF-α did not affect development of bovine embryos despite causing an increase in apoptotic cells (Soto et al. 2003b). Inhibition of apoptosis responses with caspase-3 inhibitor actually increased sensitivity of bovine embryos to the anti-developmental effects of heat shock (Paula-Lopes and Hansen 2002a). However, when the damage to the embryo is too severe and apoptosis is widespread, for example when RNA interference was used to reduce amounts of the anti-apoptotic protein survivin (Park et al. 2007), development can be compromised.

An unknown question from the current experiments is whether the inhibition to development of Day 5 embryos caused by 24-hour exposure to ceramide is the result of the large-scale induction of apoptosis or to other, apoptosis-independent events. While the former seems likely, there may also be apoptosis-independent effects as well. This contention is based on the observation that ceramide exposure for 24 hours reduced nuclei number and cytokinesis when given to the two-cell embryo that is resistant to apoptosis. The conclusion that ceramide blocks cytokinesis is based on the observation that embryos treated with ceramide contained multinucleated cells. This phenotype is compatible with the phenomenon of mitotic catastrophe (Huang et al. 2005) whereby mitosis is blocked through checkpoint activation (Molz et al. 1989; Huang et al. 2005). Whether the increase in the number of multinucleated cells induced by ceramide is a result of damage to DNA or organelles or by regulation of cell cycle regulatory molecules requires further investigation. To the authors' knowledge, this is the first report demonstrating that ceramide can cause formation of multinucleated cells.

Ceramide is an important signal for induction of apoptosis by heat shock in a variety of cells (Goldkorn et al. 1998; Mansat-de Mas et al. 1999; Chung et al. 2003). Current results are consistent with the idea that ceramide is also an important signal for the heat-shocked embryo. The magnitude of the increase in apoptosis caused by 9 hour exposure to ceramide is similar to

that observed for heat shock at 41^{o}C (Jousan and Hansen 2004; de Castro e Paula and Hansen 2005). Importantly, heat shock caused an increase in nSMase activity in Day 5 embryos. In contrast, there was no increase in aSMase activity. Heat shock of HeLa cells increased nSMase activity without increasing aSMase (Kondo et al. 2000b).

The observation that heat shock can increase nSMase activity is an important one for demonstrating a role for ceramide in the preimplantation embryo because it demonstrates the capacity for regulation of ceramide synthesis by an environmental signal known to use ceramide as signaling molecule. This result, in conjunction with findings that ceramide can trigger apoptosis, reduce development, and inhibit cytokinesis, establishes a ceramide signaling system in the bovine preimplantation embryo.

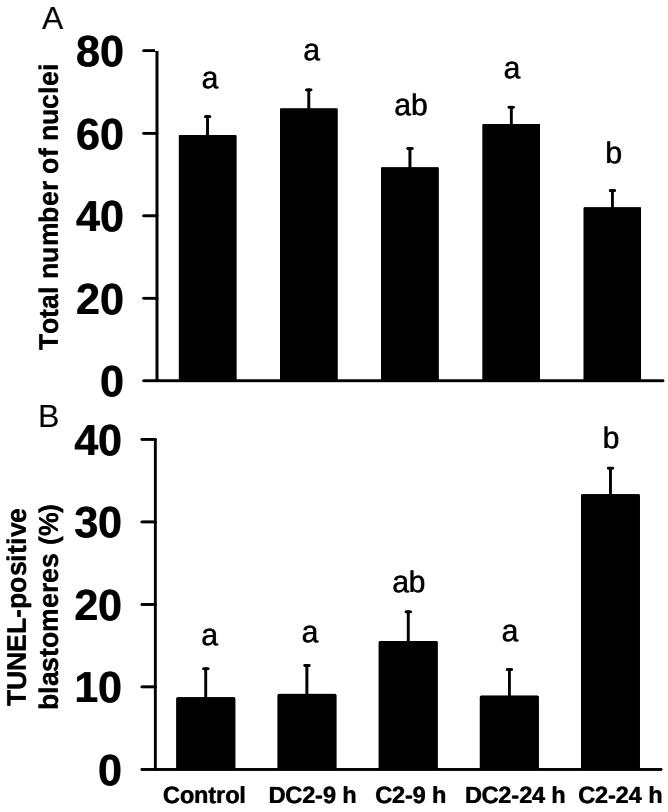

Figure 4-1. Effect of ceramide on induction of apoptosis in embryos ≥ 16 cells at Day 5 after insemination. A) Total cell number and B) percent of blastomeres that are TUNEL-positive for control embryos and embryos treated with ceramide (C2) or its inactive analogue dihydroceramide (DC2) for 9 or 24 hours. Bars with different superscripts differ ($P < 0.05$). Data are least-squares means ± SEM

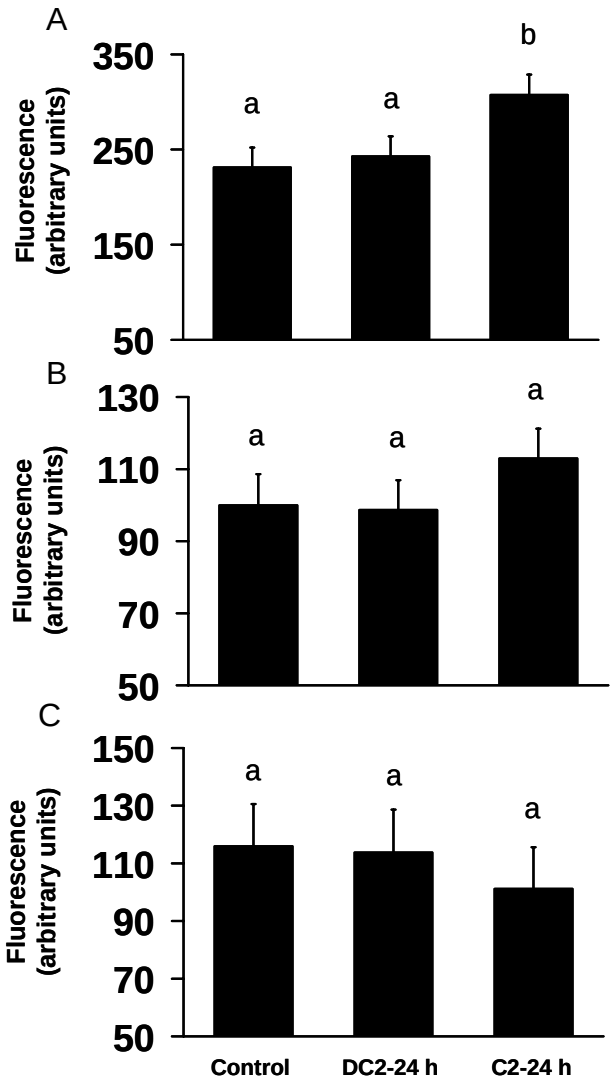

Figure 4-2. Induction of caspase-9, 3 and 8 activities by ceramide in embryos ≥ 16 cell on Day 5 after insemination. Embryos were either untreated (control) or were treated with ceramide (C2) or its inactive analogue dihydroceramide (DC2) for 24 hours. A) Caspase-9 activity. B) Caspase-3 activity. C) Caspase-8 activity. Values are least-squares means ± SEM. Bars with different superscripts differ ($P < 0.05$).

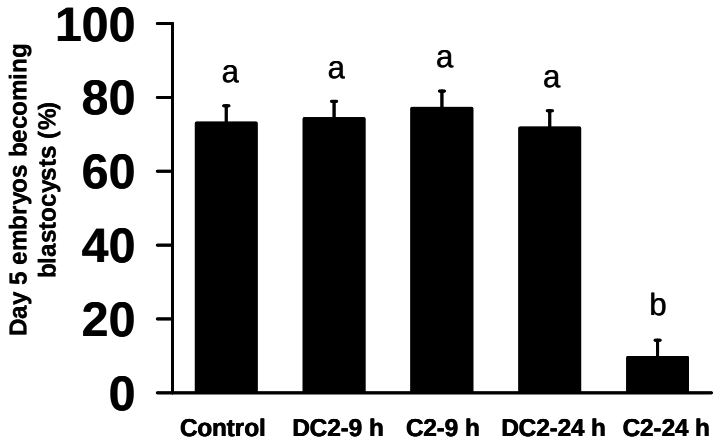

Figure 4-3. Effect of ceramide treatment on blastocyst development of embryos ≥ 16 cells at Day 5 after insemination. Data are least-squares means ± SEM of the percent of embryos that developed to the blastocyst stage at Day 8 after insemination. Embryos were either untreated (control) or were treated with ceramide (C2) or its inactive analogue dihydroceramide (DC2) for 9 or 24 hours. Bars with different superscripts differ ($P < 0.05$).

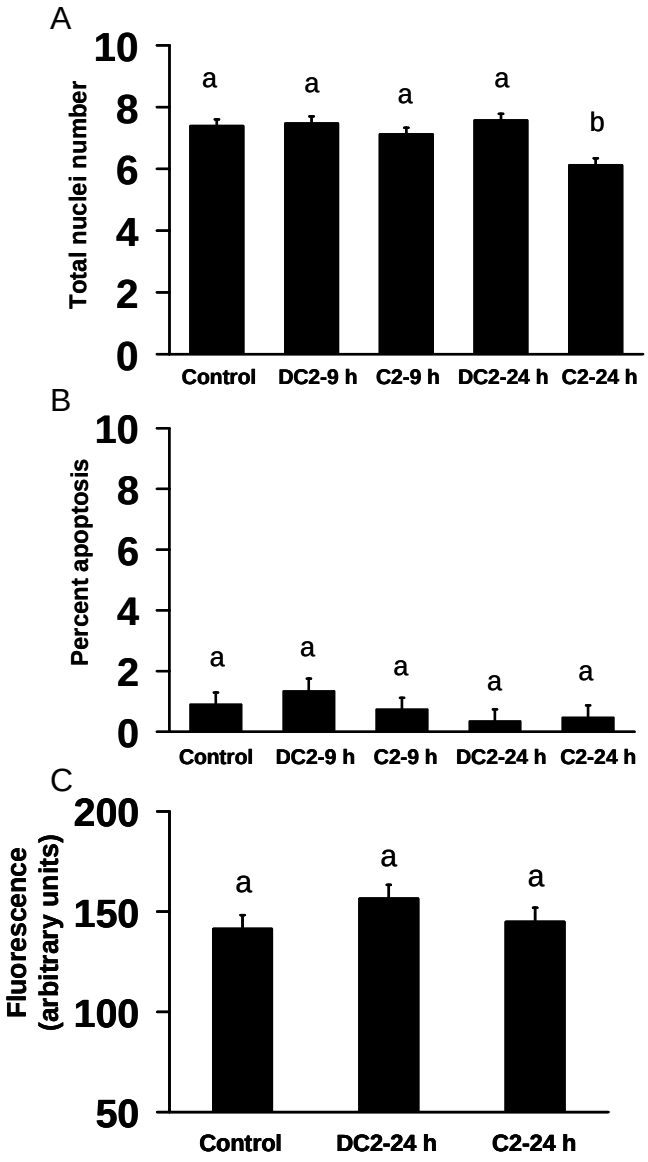

Figure 4-4. Effect of ceramide on induction of apoptosis and caspase-9 activity at the two-cell stage. Two-cell embryos were treated with ceramide (C2) or its inactive analogue dihydroceramide (DC2) for 9 or 24 hours. A) Total cell number. B) Percent apoptosis. C) Caspase-9 activity. Bars with different superscripts differ ($P < 0.05$). Data represent least-squares means ± SEM

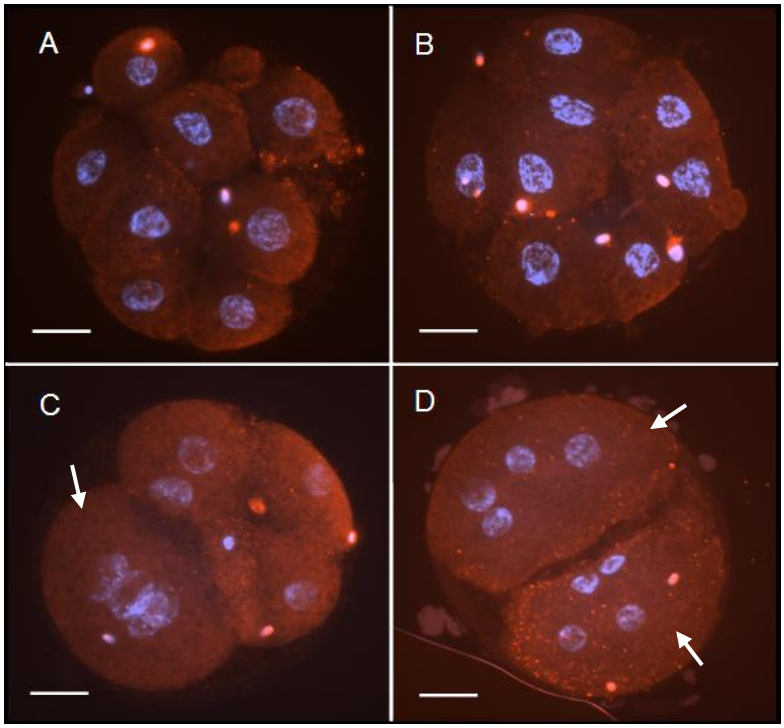

Figure 4-5. Representative images illustrating the effects of ceramide on number of nuclei per blastomere. Two-cell embryos were treated with vehicle control (A), 50 µM C(2)-dihydroceramide (B) or 50 µM C(2)-ceramide (C-D). Nucleic acid was labeled using Hoescht 33342 and individual cells (red) were identified by maximizing cell auto-fluorescence digitally. Arrows show blastomeres with 4 nuclei. Bar = 20 µM.

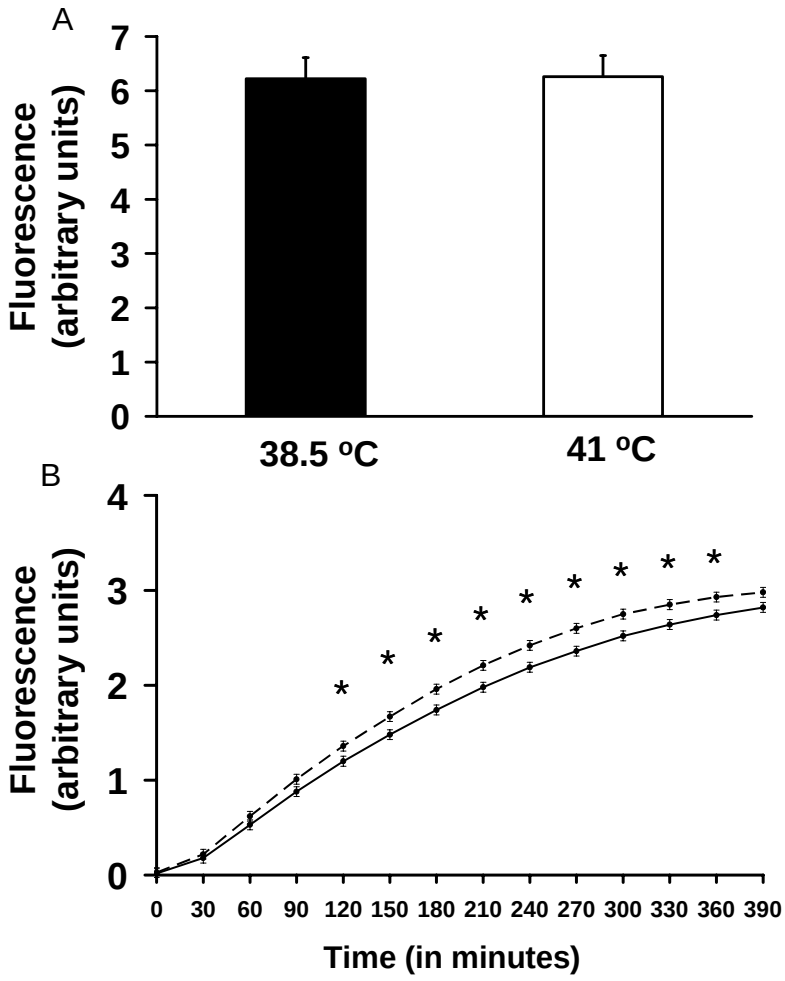

Figure 4-6. Acid and neutral sphingomyelinase (SMase) activity in Day 5 embryos ≥ 16 cells as affected by heat shock. A) Acid SMase was measured at a single time point after 4.5-hour incubation of lysed embryos with sphingomyelin as substrate. B) For neutral SMase, solid lines represent control embryos and dotted lines represent heat shocked embryos. Values are least squares means ± SEM. Asterisks represent significant differences between treatments ($P < 0.05$).

Table 4-1. Effects of ceramide on cytokinesis of two-cell embryos.

Treatment[b]	Total cell number[a]	Total nuclei number[a]	nuclei/cell ratio[a]
Control	6.6 ± 0.3[c]	7.3 ± 0.3[c]	1.1 ± 0.1[c]
DC2-9 h	6.5 ± 0.3[c]	7.3 ± 0.3[c]	1.2 ± 0.1[c]
C2-9 h	6.0 ± 0.3[c]	7.2 ± 0.3[c]	1.3 ± 0.1[c]
DC2-24 h	7.2 ± 0.3[c]	7.5 ± 0.3[c]	1.1 ± 0.1[c]
C2-24 h	3.1 ± 0.3[d]	5.9 ± 0.4[d]	2.2 ± 0.1[d]

[a] Data represent least-squares means ± SEM. [b] Treatments represent exposure to ceramide (C2) or its inactive analogue dihydroceramide (DC2) for 9 or 24 hours.[c-d] Means with different superscripts within a column differ at $P < 0.05$.

CHAPTER 5
INTERACTIONS BETWEEN OXYGEN TENSION AND GLUCOSE CONCENTRATION THAT MODULATE ACTIONS OF HEAT SHOCK ON BOVINE OOCYTES DURING IN VITRO MATURATION

Introduction

Oocyte viability is modified by the microenvironment in which the oocyte undergoes growth and maturation. One environmental determinant of oocyte viability is heat stress. Exposure of lactating dairy cows to environmental conditions leading to hyperthermia during summer can alter the temperature at which the oocyte undergoes growth and maturation. Heat stress also alters secretion of hormones involved in follicular function (e.g. steroids, LH, FSH and inhibin (Wise et al. 1988; Roth et al. 2000; Wolfenson et al. 2000). That oocytes can be compromised by heat stress is indicated by reduced capacity for fertilization in vitro (Rocha et al. 1998) and in vivo (Sartori et al. 2002b) and reduced capacity for development of embryos derived from fertilized or chemically-activated oocytes (Zeron et al. 2001; Al-Katanani et al. 2002).

Experiments evaluating restoration of follicular and oocyte function after heat stress are indicative that heat stress affects the follicle 30 to 40 days before estrus or earlier (Roth et al. 2001a; Roth et al. 2001b). The period during which the oocyte is susceptible to disruption by heat stress extends through the processes of maturation when the oocyte completes cytoplasmic and nuclear maturation. Indeed, heat stress applied for 10 hours beginning at estrus caused a reduction in the proportion of oocytes that became normal embryos later in development (Putney et al. 1989). Effects of heat stress during maturation could reflect either direct effects of elevated temperature on function of the oocyte and its supporting cumulus cells, alterations in blood flow to the ovary (as shown in the rabbit (Lublin and Wolfenson 1996)), or hormone secretion (Wolfenson et al. 2000). Direct effects of elevated temperature on oocyte maturation have been

demonstrated in vitro using experiments where COCs were matured in incubators under temperatures similar to those experienced by cows during heat stress. Heat shock during maturation also induced oocyte apoptosis (Roth and Hansen 2004b; Roth and Hansen 2004a), disrupted chromosome alignment (Ju et al. 2005; Roth and Hansen 2005), and decreased the number of oocytes reaching metaphase II (Payton et al. 2004; Roth and Hansen 2005). The proportion of oocytes that become blastocysts after insemination was also reduced by heat shock during maturation (Payton et al. 2004; Roth and Hansen 2004b; Roth and Hansen 2004a; Edwards et al. 2005; Ju et al. 2005). This reduction was associated with reduced fertilization rate in some experiments (Roth and Hansen 2004b; Roth and Hansen 2004a) and in a reduction in the proportion of cleaved embryos that develop to the blastocyst stage (Payton et al. 2004; Edwards et al. 2005; Ju et al. 2005).

One caveat with relating results of most in vitro studies of heat shock to the situation in vivo is that culture experiments are usually carried out in a gaseous environment high in oxygen (~21%, v/v). This oxygen tension is higher than encountered by the COCs in vivo, which has been estimated in follicular fluid as 5 to 9% in humans (Fischer et al. 1992; Van Blerkom et al. 1997) and from < 5 to 14% in cows (Redding et al. 2006). Effects of heat shock on preimplantation bovine embryos were greater when culture is performed in atmospheric oxygen than when performed in 5% oxygen (Chapter 3). Thus, it is possible that effects of heat shock on oocytes in vitro would not be duplicated in the lower oxygen environments in vivo.

The objective of the present study was to test the hypothesis that effects of heat shock during maturation on oocyte competence for fertilization and development would be reduced in a low oxygen environment. To test this hypothesis, it was first necessary to establish maturation conditions where oxygen concentration would have little effect on the outcome. Using a

modified Synthetic Oviduct Fluid (SOF) as maturation medium, Hashimoto et al. (Hashimoto et al. 2000a) have shown that optimal maturation of oocytes under 5% oxygen requires raising glucose concentration from 5.6 mM to 20 mM. Therefore, an initial experiment was performed to determine effects of oxygen environment during maturation as performed in two media (modified TCM-199 vs modified SOF) and under two glucose concentrations (5.6 mM vs 20 mM). Once conditions for maturation that were unaffected by oxygen concentration were determined, a second experiment was performed to determine effect of oxygen concentration on actions of heat shock during maturation. Heat shock was applied during the first 12 hours of maturation because oocytes are more sensitive to heat shock during this period than during the last 12 hours of maturation (Edwards and Hansen 1996).

Materials and Methods

Preparation of Media

OCM was TCM-199 with Hank's salts and without phenol red (Atlanta Biologicals, Norcross, GA, USA) that was supplemented with 2% (v/v) bovine steer serum (Pel-Freez, Rogers, AR, USA) containing 2 U/mL heparin. Medium was also supplemented with 100 U/mL penicillin-G, 0.1 mg/mL streptomycin, and 1 mM glutamine. Two media were used for oocyte maturation: a modified TCM-199 (mTCM-199) and a modified SOF (mSOF). The TCM-199 contained Earle's salts and was purchased from BioWhittaker (Walkersville, MD, USA). The SOF was prepared based on the formulation described by Fischer-Brown et al. (Fischer-Brown et al. 2002) by the Specialty Media Division of Millipore (Billerica, MA, USA). Both media were modified by adding 10% (v/v) bovine steer serum, 2 μg/mL estradiol 17-β, 20 μg/mL bovine FSH (Folltropin-V; Vetepharm Canada, Belleville, ON, Canada), 50 μg/mL gentamicin sulfate, and 1 mM glutamine. In addition, mSOF contained 1X Basal Medium Eagle amino acids (Sigma, St. Louis, MO, USA) as used by Hashimoto et. al. (Hashimoto et al. 2000a). The

mTCM-199 was also supplemented with 22 µg/mL sodium pyruvate. The media HEPES-TL, IVF-TL, and Sperm-TL were purchased from Specialty Media and used to prepare HEPES-TALP, IVF-TALP, and Sperm-TALP as previously described (Parrish et al. 1986). Percoll was from Amersham Pharmacia Biotech (Uppsala, Sweden). KSOM that contained 1 mg/mL bovine serum albumin was obtained from Specialty Media. EFAF-BSA was from Sigma. On the day of use, KSOM was modified for bovine embryos to produce KSOM-BE2, as described elsewhere (Soto et al. 2003c).

In Vitro Production of Embryos

COCs were obtained by slicing 2- to 10-mm follicles on the surface of ovaries (a mixture of beef and dairy cattle) obtained from Central Beef Packing Co. (Center Hill, FL, USA). Those COCs with at least two complete layers of compact cumulus cells were washed two times in OCM and used for subsequent steps. Groups of 10 COCs were placed in 50-µL drops of either mTCM-199 or mSOF, depending on the experiment, overlaid with mineral oil, and matured for 20 to 22 hours. Temperature and oxygen concentration varied between treatments and experiments and are described in detail in the next section. Matured COCs were washed once in HEPES-TALP and transferred in groups of ~30 to 4-well plates containing 600 µL of IVF-TALP and 25 µL of PHE per well and fertilized with ~1 x 10^6 Percoll-purified spermatozoa. In each replicate, the sperm represented a pool of frozen-thawed semen from three bulls (donated by Southeastern Semen Services, Wellborn, FL, USA) and a different pool was used for each replicate. After 10 to 12 hours at 38.5°C in an atmosphere of 5% CO_2 in humidified air (i.e., 21% oxygen), putative zygotes were removed from fertilization wells, denuded of cumulus cells by vortex mixing in 1 mL of 1000 U/mL hyaluronidase in HEPES-TALP, and placed in groups of up to 30 in 50-µL drops of KSOM-BE2. Within a replicate, approximately equal (±1) numbers of embryos were added for all treatments. All drops of embryos were overlaid with mineral oil and

cultured at 38.5°C in a humidified atmosphere of 5% O_2, 5% CO_2, and the balance N_2. Embryos were cultured until Day 8 after insemination (Day 0 = day of insemination).

Comparison of mTCM-199 vs mSOF as Maturation Media in High and Low Oxygen Environments

Oocytes were collected during September. The experimental design was a 2 x 2 x 2 factorial with main effects of maturation medium, glucose concentration, and oxygen environment. COCs were collected and placed in 50 uL drops of mTCM-199 or mSOF that were formulated to contain 5.6 or 20 mM glucose. Maturation was in either 5% CO_2 in humidified air (high oxygen) or a humidified environment of 5% O_2 and 5% CO_2 with the balance N_2 (low oxygen). Fertilization and embryo culture were performed as described earlier. Development was ascertained once, on Day 8 after insemination, to determine the percentage of embryos becoming blastocysts. The experiment was replicated six times, using a total of 2020 oocytes. All eight treatments were represented in each replicate.

Effect of Oxygen Concentration on Heat Shock Effects during Oocyte Maturation

Oocytes were collected during the months of October through February. The design was a 2 x 2 x 2 factorial, with main effects of temperature, glucose concentration and oxygen environment. COCs were collected and matured for 22 hours at either 38.5°C for 22 hours or at 41°C for 12 hours followed by 38.5°C for 10 hours. Maturation was performed in 50 µL drops of mSOF containing 5.6 or 20 mM glucose and in a high or low oxygen atmosphere. The CO_2 environment was maintained at 5% (v/v) for maturation at 38.5°C and was raised to 6% for maturation at 41°C to correct for reduced solubility of CO_2 and to maintain the pH of the medium at a value similar to that for maturation at 38.5°C (pH~7.4) (Rivera and Hansen 2001). Fertilization and embryo culture were performed as described earlier. Embryos were examined for development twice. Cleavage rate was recorded on Day 3 after insemination and the

percentage of oocytes and cleaved embryos becoming blastocysts was recorded on Day 8 after insemination. The experiment was replicated nine times using a total of 3215 oocytes. All eight treatments were represented in each replicate.

Statistical Analysis

Data on percentage of oocytes and cleaved embryos becoming blastocysts were calculated for all oocytes and cleaved embryos treated alike for each replicate. These percentages calculated for each replicate were analyzed by least-squares analysis of variance using the GLM procedure of SAS (SAS for Windows, Version 8, 1999–2001, Cary, NC, USA). Percentage data were analyzed without transformation and after performing arcsin transformation. Similar results were obtained for both variables; untransformed data are reported in this manuscript.

In the first experiment, main effects included medium (mTCM-199 and mSOF), glucose concentration (5.6 and 20 mM) and oxygen environment (high and low). The mathematical model for the overall analysis included the main effects and interactions. To determine interactions between two main effects within one factor of a third main effect, the third main effect was fixed using the Sort procedure from SAS. For example, to determine if there was an interaction between medium and glucose under high or low oxygen, the Sort procedure was used to determine effects of medium, glucose and their interaction for each oxygen treatment separately.

In the second experiment, main effects included temperature (38.5 and 41^{o}C), glucose concentration (5.6 and 20 mM) and oxygen environment (high and low). The mathematical model for the overall analysis included the main effects and interactions, as mentioned above. The Sort procedure was also used as described above.

All values reported are least-squares means ± SEM from non-transformed data. In the results section, it is noted whether values refer to the overall analysis or sorted analysis.

Results

Comparison of mTCM-199 vs mSOF as Maturation Media in High and Low Oxygen Environments

The proportion of oocytes that became blastocysts (Figure 5-1) was not affected by glucose concentration ($P > 0.1$) or medium ($P > 0.1$). However, there was a medium x oxygen interaction ($P < 0.05$). For oocytes matured in mTCM-199, development was greater in high oxygen than in low oxygen. For oocytes matured in mSOF, in contrast, the percentage of oocytes that became blastocysts was not affected by oxygen.

Although the overall glucose x oxygen interaction was not significant ($P > 0.1$), sorted analysis for each glucose concentration revealed that development of oocytes to the blastocyst stage was higher in high oxygen compared to low oxygen when 5.6 mM glucose was used (41.4 ± 3.3 vs 25.5 ±3.3 %; $P < 0.05$) but not when 20 mM glucose was used (30.9 ± 2.8 vs 30.5 ± 2.8%). Data were also analyzed separately for each oxygen concentration. For oocytes matured in high oxygen, those cultured in 5.6 mM glucose yielded a higher percentage of blastocysts than those cultured in 20 mM glucose (41.4 ± 3.0 vs 30.9 ± 3.0%; $p = 0.06$). The percentage of oocytes that became blastocysts was not affected by glucose concentration when oocytes were in low oxygen (25.5 ± 2.1 for 5.6 mM vs 30.5 ± 2.1% for 20 mM; $P > 0.1$).

Effect of Oxygen and Glucose Concentration on Heat Shock Effects during Oocyte Maturation

The mSOF was used for maturation in this experiment; based on the first experiment, there was no effect of oxygen on the proportion of oocytes becoming blastocysts for this medium. Overall, heat shock during maturation slightly but significantly ($P < 0.05$) reduced cleavage rate (69.1 ± 1.2 vs 65.6 ± 1.2% for 38.5 and 41^{o}C, Figure 5-2B) and the percentage of oocytes becoming blastocysts (36.7 ± 1.1 vs 32.5 ± 1.1%; $P < 0.05$; Figure 5-2A). The percentage of cleaved embryos becoming blastocysts was also reduced by heat shock, but this effect was of a

lower magnitude and only approached significance (52.9 ± 1.5 vs 48.9 ± 1.5%; $P < 0.1$; Figure 5-2C). Oocytes matured in the presence of 5.6 mM glucose had decreased development in low oxygen compared to high oxygen but development was not affected by oxygen when 20 mM glucose was used (oxygen x glucose for percentage of oocytes becoming blastocysts and percentage of cleaved embryos becoming blastocysts, $P < 0.01$).

Although the overall effect of temperature was significant and there were no interactions with temperature, there was no numerical difference in cleavage or blastocyst development between 38.5 and 41^{o}C when 20 mM glucose was used in high oxygen. To address this observation, further analysis was performed by determining how glucose concentration altered the magnitude of heat shock effects at each oxygen concentration. In high oxygen, the percentage of oocytes that cleaved was not affected by heat shock when oocytes were matured in either 5.6 or 20 mM glucose (temperature, $P > 0.1$; Figure 5-2B). The percentage of oocytes becoming blastocysts was reduced when oocytes were matured in 5.6 mM glucose, but not when oocytes were matured in 20 mM glucose (temperature x glucose, $P < 0.07$; Figure 5-2A). Results were similar for percentage of cleaved embryos becoming blastocysts (temperature x glucose, $P < 0.05$; Figure 5-2C). For oocytes matured in low oxygen, heat shock tended to decrease the percentage that cleaved ($P = 0.07$; Figure 5-2B) and decreased the percentage of oocytes that developed to the blastocyst stage ($P < 0.05$; Figure 5-2A). However, there were no significant temperature x glucose interactions for either of these variables, i.e., the effect of heat shock was independent of glucose concentration. The proportion of cleaved embryos becoming blastocysts was not affected by temperature or temperature x glucose.

Discussion

Based on the first experiment, we concluded that effectiveness of culture systems for supporting oocyte maturation depended upon interactions between medium, oxygen tension and

glucose concentration. In general, blastocyst yield was reduced if maturation was performed under a low oxygen environment, with the only exception being maturation in mSOF in 20 mM glucose. This effect of oxygen and its modulation by glucose concentration for mSOF was apparent in both experiments. The superiority of a high oxygen environment is likely related to oxygen availability to the oocyte and cumulus cells. When embryoid bodies were cultured under high oxygen, cells from the center of the bodies had about 30% less available oxygen then cells in the periphery (Gassmann et al. 1996). Therefore, when the number of cells is high and oxygen is limiting, i.e., as under the low oxygen atmosphere used here, it is likely that cumulus cells in close contact with the oocyte and the oocyte itself experience a deficit in oxygen availability. Hypoxia results in uncoupling of electron transfer and oxidative phosphorylation in the mitochondria, accumulation of pyruvate, and a shift to glycolysis (Czyzyk-Krzeska 1997; Wenger and Gassmann 1997). Because glycolysis is less efficient than oxidative phosphorylation for ATP production, it is likely that ATP production by the oocyte becomes insufficient in oxygen-restricted environments. Indeed, oocyte ATP content was reduced in oocytes matured in 5% oxygen as compared to those matured in atmospheric oxygen (Hashimoto et al. 2000a).

Under low oxygen conditions, raising glucose concentration from 5.6 mM to 20 mM increased the proportion of oocytes that became blastocysts when the maturation medium was mSOF. Indeed, there were no deleterious effects of maturing oocytes under low oxygen when the maturation medium was mSOF containing 20 mM glucose. It was also reported that increasing glucose concentration in an SOF-based maturation medium from 1.5 mM to 20 mM increased ATP content and the proportion of oocytes that reached metaphase II (Hashimoto et al. 2000a). The addition of glucose at 20 mM did not reverse the detrimental effects of maturation in low oxygen when mTCM-199 was the maturation medium. It was unclear why raising glucose

concentration did not improve oocyte competence for development for oocytes matured with mTCM-199, but it may be related to the redox status of the oocyte. Under high oxygen conditions, raising glucose concentration in maturation medium can increase production of ROS and decrease oocyte GSH content (Hashimoto et al. 2000b). Indeed, under high oxygen conditions, high glucose concentrations were detrimental to oocyte maturation as determined by subsequent blastocyst yield in the present study. Perhaps production of ROS was higher for oocytes matured with mTCM-199 than for oocytes matured with mSOF; therefore, beneficial effects of raising glucose concentrations under low oxygen on ATP synthesis were counteracted by effects on free radical metabolism.

Effects of oxygen and glucose concentrations on the magnitude of effects of heat shock were tested using mSOF, as oxygen effects were less when compared to oxygen effect using mTCM-199. Using this experimental model, previous observations that heat shock during maturation reduces oocyte competence for fertilization and development (Payton et al. 2004; Roth and Hansen 2004b; Roth and Hansen 2004a; Edwards et al. 2005; Ju et al. 2005) were confirmed. In particular, heat shock reduced the proportion of oocytes that cleaved and the proportion of cleaved embryos that became blastocysts. Furthermore, the effects of heat shock did not depend upon oxygen concentration, and glucose concentration reduced effects of heat shock only in a high oxygen environment.

That heat shock reduced oocyte competence under conditions of high or low oxygen indicated that damage to the cultured oocyte by heat shock does not require a high-oxygen environment. In contrast, in preimplantation embryos, the effects of heat shock are exacerbated when culture is in high oxygen (Chapter 3). One explanation for this difference is that the cell density is greater in cultures of maturing oocytes than for cultures of preimplantation embryos

(because of the presence of cumulus cells in the former); this increased density may make the generation of ROS by heat shock less dependent upon oxygen concentration.

One surprising observation was that effects of heat shock on oocyte competence were eliminated when oocytes matured in high oxygen (but not low oxygen) were cultured with 20 mM glucose. One should be careful in ascribing a thermoprotective effect to high glucose, however, because culture in 20 mM glucose tended to reduce oocyte competence for blastocyst development in high oxygen. Perhaps the oocyte populations being damaged by heat shock were the same ones damaged by elevated glucose concentrations so that no additive effects were seen when both stresses were combined.

Except for oocytes matured using 20 mM glucose in high oxygen, where no reduction was observed, the magnitude of reduction in blastocyst development caused by heat shock (expressed as a percentage of development at 38.5^{o}C) ranged from 14 to 17%. This magnitude of heat shock effect is less than for many studies in which a similar heat shock was applied during development. We hypothesized that the magnitude of heat shock effects during maturation on oocyte competence depends upon the suitability of the culture system for supporting development. In particular, effects of heat shock were reduced in systems where the proportion of oocytes becoming blastocysts was high. Thus, in the present study, the proportion of oocytes that developed to the blastocyst stage at 38.5^{o}C was high, ranging from 38 to 42% for all treatments but one (5.6 mM glucose in low oxygen) and the magnitude of effects of heat shock were low. Similarly, Edwards and Hansen (1997), using a similar exposure to heat shock, found that 46% of non heat-shocked oocytes became blastocysts and the reduction in development caused by heat shock was 11%. In studies in which the percentage of oocytes that became blastocysts in the absence of heat shock ranged from 20 to 30%, the reduction in development

caused by heat shock was about 40 to 65% (Payton et al. 2004; Roth and Hansen 2004b; Edwards et al. 2005). Other possible reasons for differences between studies include differences in season, breed of ovary donors, and specific modifications of the culture medium.

Based on the present study, heat shock can damage oocyte competence for development to the blastocyst stage after insemination under an oxygen tension similar to that in the follicle and oviduct. Whether such damage occurs in vivo may depend on other factors that affect embryonic capacity for development. In particular, heat stress is more likely to compromise the process of oocyte maturation when either 1) the oocyte was exposed before maturation to conditions that reduce oocyte potential for development (including heat stress and other factors such as low nutrition (Rocha et al. 1998; Snijders et al. 2000; Zeron et al. 2001; Al-Katanani et al. 2002; Sartori et al. 2002b), or 2) the subsequent embryo is exposed to conditions after fertilization that limit embryonic development.

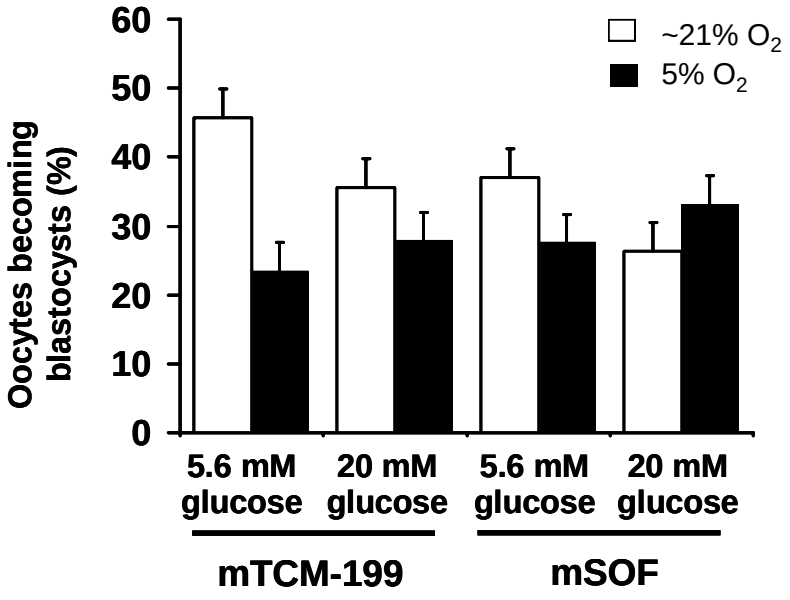

Figure 5-1. Least-squares means (± SEM) percentage of bovine oocytes developing to the blastocyst stage on Day 8 after fertilization, as determined by maturation medium,

glucose concentration and oxygen environment. Oocytes were matured for ~22 hours in modified TCM-199 or modified SOF in the presence of 5.6 or 20 mM glucose in either high (~21%) or low (5%) oxygen environment. The percentage of oocytes becoming blastocysts was affected by the medium x oxygen interaction ($P < 0.05$) because oxygen was a greater determinant of development to the blastocyst stage for mTCM-199. Also, blastocyst development was higher in high oxygen compared to low oxygen when 5.6 mM glucose was used ($P < 0.05$), but not when 20 mM glucose was used ($P > 0.10$). For oocytes matured in high oxygen, those cultured in 5.6 mM glucose yielded a higher percentage of blastocysts than those cultured in 20 mM glucose for oocytes in high oxygen ($P = 0.06$) but not for oocytes in low oxygen.

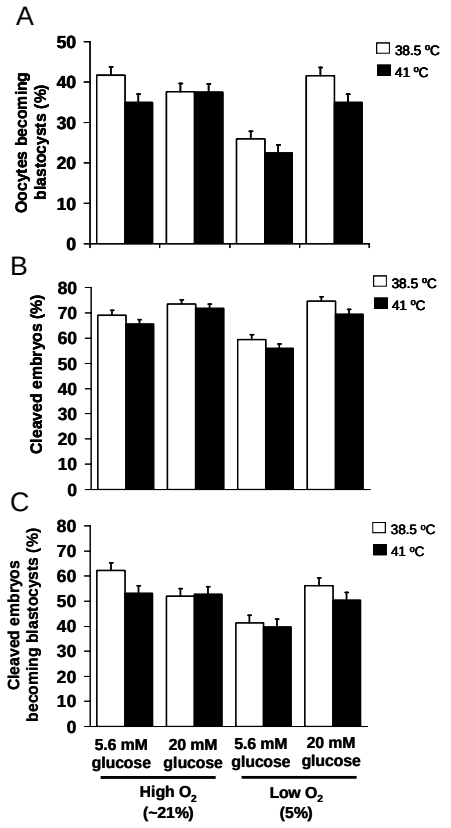

Figure 5-2. Least-squares means (± SEM) percentage of oocytes developing to the blastocyst stage on Day 8 after fertilization, percentage oocytes cleaving and percentage of cleaved embryos becoming blastocysts, as determined by incubation temperature, glucose concentration and oxygen environment. Oocytes were heat shocked or not for the first 12 hours of maturation in modified SOF in the presence of 5.6 or 20 mM glucose in either high or low oxygen environment. A) Overall, heat shock during maturation reduced ($P < 0.05$) the percentage of oocytes becoming blastocysts and B) cleavage rate. C) Heat shock also tended ($P < 0.1$) to reduce the percentage of cleaved embryos becoming blastocysts. A) Oocytes matured in the presence of 5.6 mM glucose had decreased development in low oxygen compared to high oxygen, but development was not affected by oxygen when 20 mM glucose was used [oxygen x glucose for percentage of oocytes becoming blastocysts and C) percentage of cleaved embryos becoming blastocysts ($P < 0.01$). Based on separate analyses for oocytes in high oxygen, A) the percentage of oocytes that cleaved was not affected by heat shock in either 5.6 or 20 mM glucose and B) the percentage of oocytes becoming blastocysts was reduced when oocytes were matured in 5.6 mM glucose but not when oocytes were matured in 20 mM glucose (temperature x glucose, $P < 0.07$). C) A similar result was seen for percentage of cleaved embryos becoming blastocysts (temperature x glucose, $P < 0.05$). B) For oocytes matured in low oxygen, heat shock tended to decrease ($P = 0.07$) the percentage that cleaved, decreased ($P = 0.05$) the percentage of oocytes that developed to the blastocyst stage, C) and did not significantly affect the percentage of cleaved embryos becoming blastocysts. For oocytes in low oxygen, there were no significant temperature x glucose interactions.

CHAPTER 6
EFFECT OF HEAT STRESS ON OXYGEN AND STEROID CONCENTRATIONS IN PREOVULATORY FOLLICLES OF LACTATING COWS EXPOSED TO ACUTE HEAT STRESS

Introduction

Fertility in lactating dairy cows decreases during hot months of the year (Elliott and Ulberg 1971; Gwazdauskas et al. 1973; Badinga et al. 1985; Cavestany et al. 1985; Wolfenson et al. 1988; Rocha et al. 1998; Al-Katanani et al. 1999; Morton et al. 2007) and after experimentally-induced heat stress (Putney et al. 1988a; Putney et al. 1989; Ealy et al. 1993). One cause for reduced fertility during heat stress is damage to the oocyte. For example, competence of oocytes to be fertilized and/or support development to the blastocyst stage when subjected to in vitro fertilization is reduced during the summer in warm regions of the world (Rocha et al. 1998; Roth et al. 2001a; Zeron et al. 2001; Al-Katanani et al. 2002; Roth et al. 2002). In vivo, fertilization rate after artificial insemination has been reported to be lower in lactating cows during the summer than winter (Sartori et al., 2002) and experimental application of heat stress to superovulated cows during the preovulatory period resulted in formation of embryos with reduced development (Putney et al. 1989).

One mechanism by which heat stress can result in oocyte damage involves disruption of oocyte function by elevated temperature. Indeed, culture of oocytes during maturation at elevated temperatures can reduce subsequent cleavage rate and blastocyst development rates following in vitro fertilization (Edwards & Hansen 1996, Payton *et al*. 2004, Roth & Hansen 2004a, Edwards *et al*. 2005, Ju *et al*. 2005). In addition, heat stress can compromise follicular steroid synthesis in vivo (Wolfenson et al. 1997; Roth et al. 2001b) and exposure of cultured follicular cells to elevated temperatures can reduce production of estradiol (Wolfenson et al. 1997; Bridges et al. 2005) and androstenedione and increase production of progesterone (Bridges et al. 2005).

It is also possible that alterations in follicular oxygen concentrations by heat stress could compromise oocyte function during the preovulatory period. One of the physiological responses to increased body temperature is the redistribution of blood circulation to the skin (Choshniak et al. 1982). In lactating Holstein cows, evaporative heat loss from the skin has been estimated to represent approximately 85% of the total heat loss when ambient temperatures rise above 30^{o}C (Maia et al. 2005b). Increase in respiratory cycles is also a response to heat stress (Berman et al. 1985; Srikandakumar and Johnson 2004; Valtorta and Gallardo 2004; Maia et al. 2005a) and may result in redistribution of blood flow to muscles involved in respiratory activity as occurs for other species (Wolfenson et al. 1981; Bell et al. 1983). Increased blood flow to the periphery results in reduced blood flow to the internal organs, including the reproductive tract. For example, blood flow to ovaries, cervix and oviduct from rabbits was decreased by 20 to 30% during heat stress while vulval blood flow increased 40% (Lublin and Wolfenson 1996). In another example, the blood flow to the uterus of ovariectomized cows treated with estradiol-17β was reduced by heat stress (Roman-Ponce et al. 1978b; Roman-Ponce et al. 1978a).

Studies from the human highlight the importance of follicular blood flow and oxygen content for oocyte function. In particular, increased follicular vascularity was correlated with increased pregnancy rate, perifollicular blood flow was positively correlated with follicular oxygen content (Van Blerkom et al., 1997; Huey et al., 1999) and follicular oxygen concentration was directly correlated with oocyte quality as determined by chromosomal structure, fertilization and subsequent development in vitro (Van Blerkom et al. 1997). Oocytes derived from follicles containing ≤1.5% oxygen had almost half the capacity of reaching 6-8 cell stage following insemination when compared to oocytes from follicles with 3 to 5% oxygen (Van Blerkom et al. 1997).

The objective of the present study was to test the hypothesis that acute heat stress causes a reduction in oxygen concentration in the follicular fluid of the preovulatory follicle. Since heat stress has also been reported to affect follicular steroid synthesis (Wolfenson et al. 1997; Roth et al. 2001b), an additional objective was to determine acute effects of heat stress on follicular concentrations of estradiol-17β and progesterone.

Materials and Methods

The experiment was conducted in September 2006, at the University of Florida Dairy Research Unit located near Gainesville, FL (29° 46' 45.46'' N, 82° 24' 58.53'' W). The protocol used in the experiment was approved by the University of Florida Institutional Animal Care and Use Committee.

Cows

Lactating Holstein cows (n = 31) were used in a total of two replicates initiated one week apart. Cows were fed a total mixed ration containing corn silage, corn grain and legume hay as the main ingredients and with 0.75 Mcal of NE_L/kg and 17.7% crude protein on a dry matter basis. Fresh water was available for both groups *ad libitum*. Cows were treated with Posilac® (Monsanto, St. Louis, MO, USA) according to manufacturer's directions and were milked two times per day. Except for the day of follicular aspiration, cows were housed in free-stall barns containing fans and sprinklers.

Estrous Synchronization and Identification of Cows with Preovulatory Follicles

For each replicate, estrous cycles were synchronized by 100 μg (i.m.) of GnRH (2 mL Cystorelin®; Merial (Duluth, GA, USA) on Day -9 and 25 mg (i.m.) $PGF_{2\alpha}$ (5 mL Lutalyse; Pfizer, New York, NY, USA) on Day -2. After being milked in the morning of Day 0 (the putative day of estrus), cows were subjected to transrectal ultrasonography using an Aloka 500 ultrasound unit equipped with a 5-MHz linear-array probe (Aloka, Wallingford, CT, USA) to

verify the presence of a dominant follicle and absence of a corpus luteum. Follicle diameter were measured and recorded at this time. Of the 31 cows synchronized, 3 cows did not meet the ovarian criteria listed above and were excluded from the experiment. An additional 6 were not used in any of the analyses because follicular fluid was not retrieved as a result of accidental follicle rupture during the aspiration. Thus, 22 cows were used in the experiment.

Heat Stress Treatments on Day 0

Cows with a pre-ovulatory follicle on Day 0 were blocked by days in milk [days in milk < 75 (range = 68 to 75) vs days in milk > 80 (range = 118 to 353)] and randomly assigned within block to control or heat stress treatments. Days in milk were 127.3 ± 22.9 days for control cows and 180.1 ± 37.4 days for heat-stressed cows. Beginning at 1030 hours of Day 0, cows were moved to one of two adjacent 10 x 5 m concrete-floored pens. Both pens were covered with shade cloth at a height of 3.5 m. The pen housing the control cows also contained sprinklers mounted below the shade cloth that operated continuously. In addition, each cow was soaked with a hose every 15 minutes for approximately 40 seconds. Heat-stressed cows were maintained in a similar pen except that sprinklers were turned off and cows were not soaked with a hose.

All cows were fed as a group (within treatment groups) twice during the experimental day (Day 0) at 1030 and 1500 hours. Rectal temperature was recorded at one hour intervals until follicular aspiration and immediately before follicular aspiration. Dry bulb temperature and relative humidity were recorded hourly at a height of 2.5 m.

Sampling of Follicular Fluid

Beginning at ~4 hours after initiation of heat stress treatments (1430 hours), follicular fluid was sampled using transvaginal follicular aspiration. Control and heat-stressed cows were sampled in alternating order. The entire sampling process required 2 hours so that follicular fluid

from the last cow sampled was obtained 6 hours (1630 hours) after initiation of heat stress (1030 hours).

Follicular aspiration was performed by a modification of standard follicular aspiration for ovum pick-up procedure (Bilby et al. 2006). Cows were restrained and given caudal epidural anesthesia via injection of 5 mL of 2% (w/v) lidocaine using an 18-gauge, 1.5 inch needle. Follicular fluid samples were collected using an Aloka 500 ultrasound device equipped with a needle guide and connected to a 5-MHz vaginal sector transducer probe (Figure 6-1). The 20-gauge 1½ inch needle at the end of the guide was connected to a 5 mL syringe via a 70-cm long polyetherketone tubing (outer diameter and inner diameter of 0.16 and 0.05 cm, respectively) with Teflon compression fittings in a gas tight system. After piercing the follicle with the needle, follicular fluid was aspirated manually into the 5 mL syringe.

Measurement of Follicular Oxygen Concentration

Oxygen content was measured within 1 to 2 minutes of collection of follicular fluid. Oxygen was measured using a fluorometric fiber-optic oxygen sensor system (FOXY system; Ocean Optics, Inc., Dunedin, FL, USA) that utilized a USB-2000 linear diode-array fluorometer, LS-450 blue LED excitation source, and 300 μm optical fiber probe in a 0.16 cm outer diameter stainless steel jacket. The probe was coated with a ruthenium red compound that, upon excitation, emits fluorescence. The intensity is inversely proportional to the amount of oxygen present in the sample due to quenching effects of oxygen towards the fluorescent compound.

The fluorometric fiber-optic oxygen sensor system was connected to a laptop equipped with SpectraSuite spectroscopy software. The probe tip was mounted in a low-volume (0.2 mL), flow-through chamber into which the fluid was injected via a syringe for measurement of dissolved oxygen. The oxygen sensor was kept at ambient temperature. An opaque, black

silicone coating on the optical fiber allowed a two-point calibration with gases (air and 100% N_2).

Upon collection, follicular fluid samples were injected into the flow-through chamber and the dissolved oxygen reading was monitored until it was stable (typically < 2 minutes). An additional 0.1 mL fluid was then injected to confirm that the reading was stable. The fluid was then withdrawn, placed in ice until storage at -20°C (within 2 to 4 hours). The chamber was flushed with distilled water and the calibration points checked between samples.

Measurement of Plasma Progesterone Concentrations

A blood sample was collected from each cow immediately before follicular aspiration by coccygeal venipuncture into evacuated heparinized 10-mL tubes (Becton Dickinson, Franklin Lakes, NJ, USA). After collection, blood samples were placed in ice until further processing at the laboratory (within approximately 2 to 4 hours). Blood samples were centrifuged at 2,000 x *g* for 20 minutes at 4°C. Plasma was separated and stored at – 20°C until assayed for progesterone concentration. Progesterone concentrations were determined by radioimmunoassay (RIA) using the Coat-a-Count® progesterone kit (Diagnostic Products Corp., Los Angeles, CA, USA). The intraassay sensitivity of the assay was 2.06 ng/mL and coefficient of variation was 7%.

Determination of Follicular Fluid Concentrations of Steroids

Follicular fluid concentrations of estradiol-17β were determined by radioimmunoassay as described previously (Spicer and Enright 1991). The intraassay coefficient of variation was 13% and the assay sensitivity defined as 95% of total binding was 0.05 ng/mL. Follicular fluid concentrations of progesterone were determined by RIA as described previously (Spicer and Enright 1991); the intraassay coefficient of variation was 10% and assay sensitivity, defined as 90% of total binding, was 1.7 ng/mL.

Determination of Follicle Class Based on Follicular Steroid Concentrations

Inspection of follicular concentrations of steroids revealed that cows could be classified into two groups. The more numerous group of cows (16 of 22 cows examined) had a follicle with a high estradiol-17β concentration (> 319 ng/mL) and low progesterone concentration (< 243 ng/mL) (Ireland and Roche 1983). These cows were considered to have a pre-ovulatory follicle. Another group of cows (6 of 22 cows) had a follicle with a low estradiol-17β concentration (< 102 ng/mL) and high progesterone concentration (>273 ng/ml). Cows in this group were considered characteristic of cows that had either experienced the LH surge or were cystic. For statistical analysis, cows were divided in two follicle class groups (high estradiol-17β vs low estradiol-17β) according to the steroid concentration limits described above. The days in milk at the time of follicular aspiration for cows in the high estradiol-17β class were 81.8 ± 35.8 and 172.8 ± 27.6 days for control and heat stress groups, respectively while days in milk for cows in the low estradiol-17β class were 175.7 ± 43.2 and 184 ± 61.1 for control and heat stress groups, respectively.

Statistical Analysis

Data on diameter of the largest follicle on Day 0, rectal temperature at the time of follicular aspiration, and follicular concentrations of estradiol-17β, progesterone and oxygen were analyzed by least-squares analysis of variance using the GLM procedure of SAS (SAS for Windows, Version 8, 1999–2001, Cary, NC, USA). Effects included replicate, treatment (control vs heat stress), follicle class (high vs low estradiol-17β) and treatment x follicle class interaction. In addition, subsets of cows in the high and low estradiol-17β classes were also analyzed. Data for estradiol-17β and progesterone exhibited heterogeneity of variance. Data were therefore subjected to log transformation before analysis. Probability values are based on analysis of the transformed data and results are presented as means ± SEM calculated for each subgroup.

Data on rectal temperature taken at hourly intervals at Day 0 were also analyzed by least-squares analysis of variance as described above except that time and interactions with time were included in the model. Cow(replicate x treatment) x time was used as the error for testing significance of the time effect.

Analyses of the relationship between follicle diameter and oxygen concentration were performed using the GLM procedure of SAS with treatment as a class variable, linear, quadratic and cubic effects of follicle diameter, and with interactions of treatment and follicle diameter. Based on these analyses, the final statistical analysis was performed with the linear effect of follicle diameter as the only term in the model.

Results

Characteristics of Cows at Day 0 before Initiation of Heat Stress Treatment

There was no difference in follicular diameter between control and heat-stressed cows (15.9 ± 1.2 and 16.0 ± 1.4 mm, respectively; $P > 0.1$). One heat-stressed cow from the high estradiol-17β follicle class had a plasma progesterone concentration of 1.5 ng/mL. All other cows in the analysis had a plasma progesterone concentration lower than 1 ng/mL.

Characteristics of Heat Stress Treatment: Dry Bulb Temperature, Relative Humidity and Rectal Temperature

Characteristics of the environment for control and heat-stressed cows and of the resultant rectal temperatures are presented in Figure 6-2. Dry bulb temperature increased throughout the day and relative humidity decreased during the day for both control and heat stress pens (Figure 6-2A). Temperature was higher and relative humidity was lower at all times for the heat stress pen compared to control pen. The dry-bulb temperatures and relative humidities were similar for both replictaes and are reported as averages from both replicates. Rectal temperatures were lower for control cows than for heat-stressed cows. This was true whether examining rectal temperature

at hourly intervals (Figure 6-2B; temperature x time, $P < 0.01$) or rectal temperature at the time of follicular aspiration (Figure 6-2C, $P < 0.01$). There was no significant interaction between treatment and follicle class on rectal temperature ($P > 0.1$).

Follicular Estradiol and Progesterone Concentrations

Results are shown in Table 6-1. As expected, estradiol-17β concentration was higher in the high estradiol-17β group as compared to cows in the low estradiol-17β group ($P < 0.01$). In addition, progesterone concentration was lower in the high estradiol-17β group ($P < 0.01$). There was no effect of heat stress on concentration of estradiol-17β or progesterone. This was true whether considering all cows or only those cows within the high estradiol-17β class. There was also no follicle class x treatment interaction ($P > 0.1$).

Follicular Oxygen Content

Results are presented in Table 1. Follicular oxygen concentration was affected by the follicle class x treatment interaction ($P < 0.01$). There was no effect of heat stress treatment on follicular oxygen concentration among cows in the high estradiol-17β class but oxygen content was higher for control cows than for heat-stressed cows in the low estradiol-17β class. When the subset of cows with high estradiol-17β was analyzed separately, there was also no effect of heat stress on follicular oxygen concentration ($P > 0.1$).

When all cows were analyzed, there was no relationship between follicular diameter and oxygen concentration. Among the cows in the high estradiol-17β class, however, oxygen concentration tended to be inversely correlated to follicular diameter (Figure 6-3; $P = 0.09$) ($y = -0.20x + 10.24$; $r^2 = 0.19$).

Discussion

The objective of the present study was to determine the effect of acute heat stress on follicular concentrations of oxygen, estradiol-17β and progesterone in the preovulatory follicle of

the lactating dairy cow. The main hypothesis was that the reduction in blood flow to the ovary coincident with heat stress would reduce oxygen delivery to the follicle and result in lower oxygen concentration in the follicle. In contrast to this expected result, there was no effect of heat stress on follicular oxygen concentration in cows having follicular steroid profiles characteristic of the dominant preovulatory follicle. It is believed that the degree of heat stress applied was of sufficient magnitude to cause a reduction in blood flow. The increase in average rectal temperature of ~ 1.0 ^{o}C caused by heat stress is similar to the degree of hyperthermia that reduced blood flow to the reproductive tract in the rabbit and laying hen (Wolfenson et al. 1981; Lublin and Wolfenson 1996). Thus, there are likely other reasons for the lack of effect of heat stress on follicular oxygen concentration among cows with pre-ovulatory follicles.

Perhaps, the reduction in blood flow to the ovary caused by heat stress was insufficient to cause a reduction in follicular oxygen concentration. Moreover, there may have been adjustments in the microvasculature of the follicle to ensure adequate oxygen delivery. The potential for regulation of blood flow to the follicle by oxygen is illustrated by studies showing that concentrations of oxygen in human follicles are negatively correlated with presence of the angiogenic factor, interleukin-8 (Yoshino et al. 2003) and that hypoxia stimulated vascular endothelial growth factor production in porcine cumulus cells (Basini et al. 2004). An alternative explanation for the lack of effect of heat stress is that the oxygen requirements of the follicle may be low enough that an acute reduction in flood flow would not lead to a measurable decline in follicular oxygen concentration during a heat stress period limited to several hours. Bovine cumulus cells use glycolysis extensively and it has been estimated that cumulus cells do not have a major impact on follicular oxygen content (Clark et al. 2006). Perhaps effects of heat stress on follicular metabolism further reduced oxygen requirements of the follicle.

It is possible that heat stress of a longer duration (days or longer) could compromise follicular oxygen concentration. The decision was made to focus on acute effects in the current experiment because of experimental difficulties with a chronic heat stress model, caused by possible confounding effects of heat stress on follicular growth (Badinga et al. 1993; Wolfenson et al. 1995; Trout et al. 1998; Wilson et al. 1998). It is important to note that acute heat stress during the preovulatory period (14 hours) can compromise oocyte competence (Putney et al. 1989) and that cows acutely cooled (12 hours) during summer had higher secretion of LH and FSH after administration of GnRH (Gilad et al. 1993).

Intriguingly, heat stress did reduce follicular oxygen concentration in the low estradiol 17-β class. In these cows, having a low follicular estradiol-17β concentration and a high follicular progesterone concentration, the oxygen concentration for heat-stressed cows was 4.5 to 4.6% vs 6.5 to 8.2% for control cows. Caution must be observed when interpreting these data since, despite the fact that the follicle class x treatment interaction was significant, there were only six cows in the low estradiol-17β class. It is possible, however, that local mechanisms that allow maintenance of follicular oxygen concentrations in preovulatory follicles (for example regulation of local blood flow) are lost in follicles present for cows in the low estradiol-17β group. In fact, follicles from the first follicular wave with estradiol-17β concentration higher than progesterone were shown to express more endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor (VEGF) proteins compared to follicles with estradiol-17β lower than progesterone (Grazul-Bilska et al. 2007). eNOS and VEGF are molecules responsible for increasing blood flow through vasodilation and angiogenesis, respectively (Kofidis et al. 2002; Mattson and Meister 2005; Partovian et al. 2005; Trousdale et al. 2007).

The nature of the follicles in the low estradiol-17β group is unclear. Some may be cystic follicles as there are small follicular cysts characterized by lack of granulosa cells, high amounts of progesterone and little estradiol-17β in the follicular fluid (Cairoli et al. 2002; Isobe 2007). Another possibility is that the LH surge and follicular luteinization had been initiated in some cows in the low estradiol-17β group since the LH surge is accompanied by a reduction in follicular estradiol-17β synthesis and an increase in progesterone synthesis (Dieleman et al. 1983; Fortune and Hansel 1985; Komar et al. 2001). This possibility is less likely due to the high concentrations of progesterone in the follicular fluid of the low estradiol-17β cows.

Heat stress did not affect follicular steroid concentrations. Although plasma estradiol-17β has been shown to be decreased by heat stress in some experiments (Gwazdauskas et al. 1981; Wilson et al. 1998), Rosenberg et al. (1982) found no effect of heat stress. Follicular estradiol-17β concentration was reduced in chronically heat-stressed cows (Wolfenson et al. 1997; Roth et al. 2001b) but not in acutely heat-stressed cows (Guzeloglu et al. 2001). The absence of effect in steroid concentration in the present study could be due to the fact that differences in steroid output during the experimental period were either nil or were small and not sufficient to cause a change in the pool of steroids in the follicle within the experimental period. Any effect of a reduction in steroid output on follicular fluid concentrations would be reduced if blood flow to the ovary was decreased.

To our knowledge, this is the first report describing oxygen concentrations of the bovine follicle *in situ*. The values obtained, with a mean of 6.8% and a range of 3.8 to 9.2%, are similar to results for follicular oxygen concentration in other species (Knudsen et al. 1978; Van Blerkom et al. 1997). In cows in the high estradiol-17β follicle class, having a prototypical pre-ovulatory follicle (high estradiol-17β concentration and low progesterone concentration), the follicular

oxygen concentration tended to decrease as follicle diameter increased. This is not surprising because the complex matrix of follicular vasculature is confined to the theca layer (Macchiarelli et al. 2006) and becomes more distant from the center of the follicle as the follicle expands in diameter. An increase in follicular diameter has also been shown to be accompanied by decrease in follicular oxygen concentration in humans (Fischer et al. 1992) and pigs (Basini et al., 2004).

The oocyte is surrounded by fluid containing oxygen concentrations much lower than atmospheric oxygen concentration used in most in vitro maturation systems (Pinyopummintr and Bavister 1995; de Matos et al. 1997; Hashimoto et al. 1998; Roth and Hansen 2004a). Indeed, under many conditions, oocyte maturation in vitro is more effective under atmospheric oxygen than under 5% oxygen (Pinyopummintr and Bavister 1995; Hashimoto et al. 2000a; Park et al. 2005). The greater requirement for a high oxygen concentration in vitro may reflect the static nature of the in vitro system.

In summary, acute heat stress did not cause a reduction in concentrations of oxygen, estradiol-17β or progesterone in fluid from the preovulatory follicle. Therefore, acute reductions in blood flow are unlikely to be involved in the oocyte damage caused by heat stress (Rocha et al. 1998; Roth et al. 2001a; Al-Katanani et al. 2002; Sartori et al. 2002b).

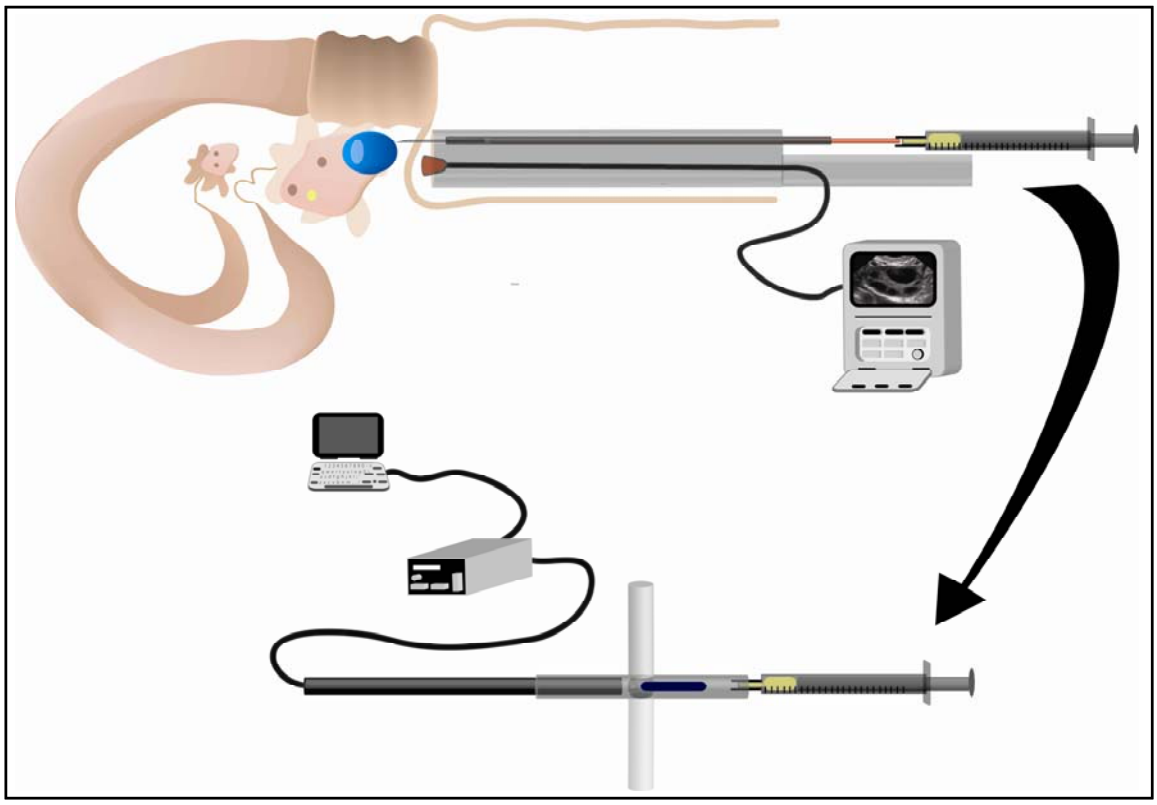

Figure 6-1. Schematic representation of the procedure for follicular fluid aspiration and oxygen measurement. Follicular fluid samples were collected using an ultrasound device equipped with a needle guide and connected to a vaginal sector transducer probe. The needle was connected to 5 mL syringe via 70 cm of tubing with Teflon compression fittings in a gas tight system. Upon collection of fluid by aspiration of the punctured follicle, fluid samples were immediately injected into a flow-through chamber connected to an oxygen measurement probe that interfaced with a computer. The reading was monitored in the computer until it was stable (typically < 2 minutes). An additional 0.1 mL fluid was then injected to confirm that the reading was stable. The chamber was flushed with distilled water and the calibration points checked between samples. Note that elements in the illustration are not drawn to scale.

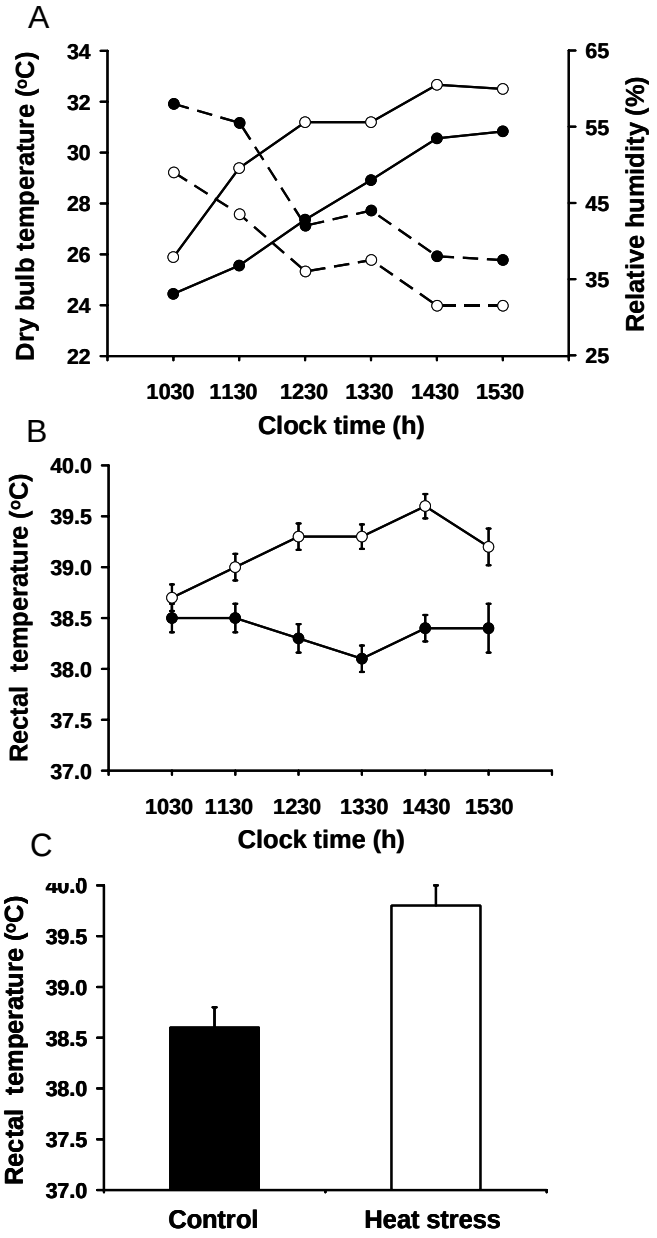

Figure 6-2. Effect of heat stress on dry bulb temperature, relative humidity and rectal temperature on the day of follicular aspiration. A) Averages of two replicates for dry bulb temperatures (solid line) and relative humidity (open lines) in pens for control (solid symbols) and heat-stressed cows (open symbols) recorded hourly beginning at the time cows were allocated to the pens (10:30 AM). B) Rectal temperatures of control (solid symbols) and heat-stressed cows (open symbols). Rectal temperatures were recorded until cows had been aspirated and therefore the number of observations per data point ranges from 3 -12. Data are least-squares means ± SEM (treatment; $P < 0.05$; treatment x time, $P < 0.01$). C) Rectal temperatures of control (n = 10) and heat-stressed cows (n = 12) at the time of follicular fluid aspiration (between 14:30 and 14:30). Data are least-squares means ± SEM (treatment; $P < 0.05$).

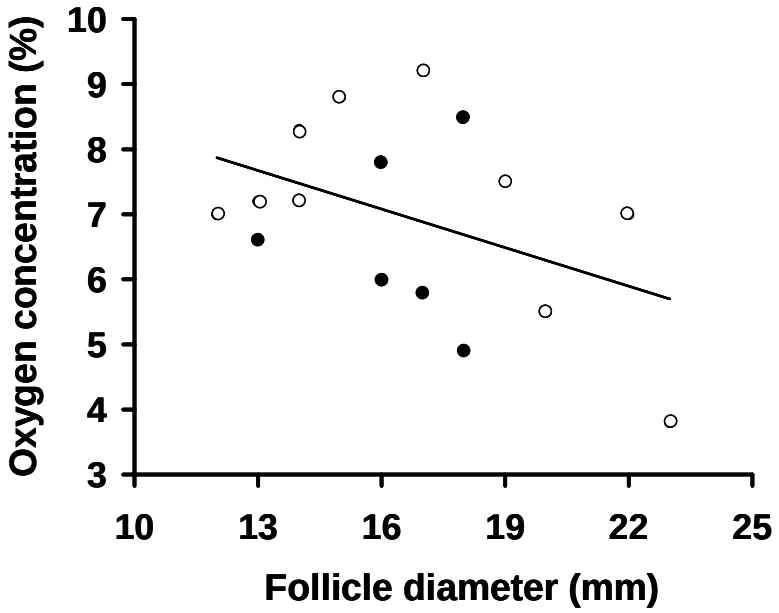

Figure 6-3. Relationship between follicular fluid oxygen concentration and follicle diameter. The line represents the predicted regression equation and circles represent values for individual cows from control (solid circles) and heat stress (open circles) groups. The regression equation describing the relationship between oxygen concentration and follicle diameter was as follows: $y = - 0.20x + 10.24$ ($r^2 = 0.19$, $P < 0.05$).

Table 6-1. Effects of heat stress and type of follicle [high estradiol-17β (> 319 ng/mL) vs low estradiol-17β (< 102 ng/mL)] on follicular concentrations of estradiol-17β, progesterone and oxygen.

Follicle class	Treatment	Estradiol-17β (ng/mL)[a]	Progesterone (ng/mL)[a]	Follicular oxygen concentration (%)[bc]
High estradiol-17β	Control (n = 6)	1264.5 ± 84.3 (562 to 1852)	142.6 ± 12.7 (62 to 243)	6.9 ± 0.4 (4.9 to 8.5)
	Heat stress (n = 10)	1707.9 ± 77.6 (319 to 2899)	103.7 ± 4.5 (43 to 194)	7.3 ± 0.3 (3.9 to 9.2)
Low estradiol-17β	Control (n = 4)	35.0 ± 4.0 (0.1 to 69)	1625.0 ± 423.5 (273 to 4102)	7.6 ± 0.5 (6.5 to 8.2)
	Heat stress (n = 2)	51.0 ± 35.7 (0.21 to 101)	714.9 ± 55.2 (636 to 793)	4.6 ± 0.7 (4.5 to 4.6)

[a] Data are means ± SEM and, in parentheses, the range of values. [b] Data are least-squares means ± SEM and, in parentheses, the range of values. [c] Follicle class x treatment; $P < 0.01$.

CHAPTER 7
GENERAL DISCUSSION

Experiments for this thesis focused on extending the current understanding of the mechanisms involved in the effects of heat shock on oocyte competence and embryo development and apoptosis. The importance of the subject is underscored by the severe reduction in fertility of lactating dairy cows due to heat stress globally (Rocha et al. 1998; Al-Katanani et al. 1999; Rutledge et al. 1999; Zeron et al. 2001). From the results presented here, we demonstrated that the effects of heat shock in the embryo are modulated by oxygen concentrations and involve oxidative stress (Chapter 3). A role for oxidative stress in effects of heat shock was based on the fact that heat shock induced apoptosis and reduced embryo development in culture under high oxygen but not under low oxygen. In addition, the use of antioxidant DTT blocked heat shock-induced apoptosis and reduced effects of heat shock in embryo development in a stage-specific manner. In the oocyte the effects of heat shock did not depend upon oxygen concentration. Oocytes had reduced competence to reach the blastocyst stage under both high and low oxygen environment (Chapter 5). It was also demonstrated for the first time in the preimplantation embryo the presence of a responsive signaling mechanism involving the sphingomyelin pathway (Chapter 4). The existence of this mechanism is indicated by the fact that ceramide, but not its inactive analogue dihydroceramide, induced apoptosis in a stage-specific manner and decreased embryo development. Furthermore, heat shock induced activity of nSMase, enzyme responsible for cleaving sphingomyelin yielding ceramide. Finally, from results from Chapter 6, acute heat stress did not cause reduction in oxygen concentration in the dominant pre-ovulatory follicle, suggesting that follicular hypoxia is unlikely to be involved in reduced oocyte competence and fertility observed during the hot months of the year. In the general discussion, implications of these findings are addressed in further detail.

As mentioned before, the resistance of the oocyte and embryo to insult changes during development and depends on the microenvironment. Heat stress can significantly alter the reproductive tract environment directly by elevation in body temperature (Gwazdauskas et al. 1981; Putney et al. 1988a; Putney et al. 1989; Ealy et al. 1993; Correa-Calderon et al. 2004; Khongdee et al. 2006) and by indirect effects such as a reduction in circulating concentrations of progesterone (Howell et al. 1994; Sartori et al. 2002a), alteration in follicular development (Badinga et al. 1993; Wolfenson et al. 1995; Trout et al. 1998; Wilson et al. 1998), reduction in blood flow to the reproductive tract (Roman-Ponce et al. 1978b; Roman-Ponce et al. 1978a; Lublin and Wolfenson 1996), and increase in production of ROS in the oviduct (Ozawa et al. 2004; Matsuzuka et al. 2005a; Matsuzuka et al. 2005b). In Chapter 3, experiments were designed to test whether effects of heat shock at two different stages of development were affected by oxygen environment. For two-cell embryos and embryos $\geq$ 16 cells, effects of heat shock on embryo survival, as determined by development to the blastocyst stage, were only apparent when embryos were heat shocked in high oxygen. Heat shock in embryos $\geq$ 16 cells induced apoptosis in high oxygen but not in low oxygen. Therefore, it was demonstrated that oxygen concentration is an important determinant of embryo survival and apoptosis.

However, oxygen concentration during heat shock was not the only determinant of embryonic fate. Heat shock in high oxygen in embryos otherwise cultured in low oxygen reduced development by 38.5% while in embryos continuously cultured in high oxygen the reduction in development was 91.8% (Figure 3-7). The oxygen concentration prior to or after heat shock was also a modifier of the outcome.

The antioxidant DTT provided partial protection against heat shock and improved overall development when culture was performed continuously in high oxygen. DTT results seem to

support the idea that 1) ROS are involved in heat shock effects and 2) the embryo develops mechanisms to respond to antioxidants as it advances in development.

The Figure 7-1 represents a tentative model, based on results in the thesis and from the literature, to explain and predict the effects of heat shock on embryonic development. In this model, the considered factors are the predicted quality of the embryo prior to heat shock, oxygen environment, redox status of the embryo and environment, and temperature. Predicted embryo quality classification is empirical with embryos produced in vivo from fertile females being considered good, embryos produced in vitro as fair and embryos whose quality has been compromised by one or more factors (heat stress, inadequate nutrition, etc) being considered bad. In panel A is plotted a hypothetical schematic representation to explain the effects of heat shock in low oxygen found in the present study. According to the model, culture in low oxygen provides the embryo to maintain its redox state by minimizing oxidative stress. Upon heat shock, oxidative stress is less due to low oxygen environment and possibly by better redox state of the embryo, preventing decrease in embryo development. Panel B represents the opposite from Panel A. According to the model in B, culture in high oxygen causes imbalance in redox state of the embryo. Upon heat shock, effects of elevated temperature and oxidative stress induced by heat shock can add up to unbalanced redox of the embryo and negatively affect embryo development. Third panel illustrates the potential role of the antioxidant DTT to mitigate effects of heat shock under high oxygen in embryo development in vitro. DTT would act by providing reducing equivalents to the embryo, improving its redox state and enabling the embryo to partially counteract some of the negative effects (oxidative stress) caused by heat shock in embryo development. A fourth model was created as an attempt to extrapolate our current findings to a situation in vivo where embryo development is compromised by maternal heat stress. According

to this model, which considers the oxygen environment in the reproductive tract to be low (Fischer and Bavister 1993), the embryo redox and competence to continue development is compromised due to a combination of direct effects of temperature and oxidative stress by increased ROS production by the oviduct and the embryo itself (Ozawa et al. 2002; Ozawa et al. 2004; Matsuzuka et al. 2005a; Matsuzuka et al. 2005b).

Under some circumstances, heat shock can affect the embryo cultured in low oxygen. Under low oxygen, heat shock can increase ROS production (Sakatani et al. 2004; Sakatani et al. 2007) and compromise embryo development (Rivera et al. 2004a; Sakatani et al. 2004; Sakatani et al. 2007). However, as mentioned above, reduction in development seems to be comparatively more severe in environment more permissive to oxidative stress like in high oxygen. A significant interaction in the effects of heat shock in high and low oxygen described by Rivera et al. (2004a) further support a role of oxygen in effects of heat shock. The difference in results from the literature highlights the fact that other factors interact with oxygen concentration and temperature to determine embryo fate. In vitro, one factor could be the permissiveness of the culture medium to oxidative stress, i.e., if embryos are not provided with appropriate energy substrates, amino acids and other nutrients, embryos may become more susceptible to stress, including oxidative stress. In mice, maternal heat stress caused increased production of ROS produced by the oviduct decreasing embryo survival (Ozawa et al. 2002; Ozawa et al. 2004; Matsuzuka et al. 2005a; Matsuzuka et al. 2005b). Decreased survival was partially counteracted by administration of antioxidant melatonin, which decreased ROS production in the oviduct (Matsuzuka et al. 2005b). Despite the origin (embryonic or maternal), there seems to be a consensus that ROS can play an important role in the effects of heat shock. Nevertheless, determining the amount of ROS production and products of oxidation in the reproductive tract

from lactating dairy cows is warranted, as means to establish a definitive model for the role of oxidative stress in heat stress effects in the cow. In the cow, the use of antioxidants to improve fertility during heat stress has generally yielded no beneficial effects (Ealy et al. 1994; Paula-Lopes et al. 2003) or slightly beneficial effects (Aréchiga et al. 1998). It is possible that the antioxidants used in these experiments (vitamin E, selenium and β-carotene), as well as dose and/or frequency of administration were insufficient to impact fertility. In mice, beneficial effects of the antioxidant melatonin described above were achieved by intensive subcutaneal injection of the drug every 2 hours during exposure to heat stress (Matsuzuka et al. 2005b).

Another objective of the thesis was to investigate the involvement of oxygen concentration and ROS in heat shock-induced apoptosis. Results here support the idea that the signaling for apoptosis by heat shock involves ROS by demonstrating that 1) apoptosis occurred in high but not low oxygen and 2) antioxidant DTT, which can block diamide-induced apoptosis in embryos by blocking ROS formation (Liu et al. 1999), completely blocked heat shock-induced apoptosis. In some circumstances, apoptosis can help protect the embryo by eliminating damaged cells. Heat shocked embryos had decreased competence to reach the blastocyst stage when apoptosis was blocked using group-II caspase inhibitors (Paula-Lopes and Hansen 2002a). However, if apoptosis is a consequence of cell oxidative damage due to ROS production and can be blocked by reducing oxidative damage, embryo should be benefited. The antioxidant DTT, despite completely blocking heat shock-induced apoptosis, improved embryonic development compared to heat shocked controls as opposed to group II caspase inhibitor (Paula-Lopes and Hansen 2002a). Hence, it is believed that part of the effects of DTT in blocking apoptosis is by reducing cell oxidative damage induced by heat shock, an early event in the apoptotic cascade.

Ceramide is a necessary signal for induction of the apoptotic cascade in many cells (Basu and Kolesnick 1998; Pettus et al. 2002; Kolesnick and Fuks 2003). In Chapter 4 we provide evidence that a ceramide signaling mechanism is present in the preimplantation embryo. Ceramide, but not its inactive analogue dihydroceramide, induced apoptosis in a stage-specific manner, as indicated by increased caspase-9 activation and TUNEL-positive labeling; long-term (24 hours) exposure to ceramide reduced ability of Day 5 embryos to develop to the blastocyst stage; long-term (24 hours) exposure to ceramide reduced nuclear proliferation and cytokinesis at the two-cell stage embryo. Presence of ceramide signaling mechanism is further supported by the fact that heat shock increased activity of nSMase which is one of the enzymes capable of cleaving sphingomyelin to yield ceramide (Luberto et al. 2002; Sakata et al. 2007). The presence of the ceramide signaling mechanism in the preimplantation embryo may prove important for understanding induced apoptosis caused by different signals including not only heat shock but also TNF-α which employs ceramide in the apoptotic cascade (Garcia-Ruiz et al. 1997; Bulotta et al. 2001; Luberto et al. 2002).

Future studies should be performed to establish the link between ceramide production and heat-shock induced apoptosis. Experiments could involve three different approaches: 1) inhibiting enzymes involved in ceramide synthesis like the SMases and ceramide synthase (Deigner et al. 2001; Luberto et al. 2002; Pru et al. 2002; Czarny and Schnitzer 2004; Miyoshi et al. 2004; Claus et al. 2005), 2) manipulating the ceramide/S1P rheostat towards an increase in S1P intracellular content (Morita et al. 2000; Roth and Hansen 2004b), and 3) use of antioxidants which can block ceramide-induced apoptosis by inhibiting ROS generation (Mansat-de Mas et al. 1999; Bezombes et al. 2001a; Maziere et al. 2001).

Like heat shock (Paula-Lopes and Hansen 2002b; Paula-Lopes and Hansen 2002a) and arsenic (Krininger et al. 2002), ceramide did not induce apoptosis at the two-cell stage. Therefore, if ceramide is involved in heat shock-induced apoptosis, it is unlikely that the block in apoptosis at the two-cell is at the level of ceramide generation. One interesting finding, also described in Chapter 4, was the partial block in cytokinesis at the two-cell stage caused by ceramide. Several different mechanisms could be involved in this phenomenon. Because apoptosis does not occur at the two-cell stage, direct damage to the DNA and/or organelles could have led to check point activation and blocked cell division. However, direct effects of ceramide or downstream molecules with cell cycle regulatory molecules cannot be excluded. Although the physiological implication of partial block in cytokinesis by ceramide is difficult to establish at this point, it demonstrates regulatory functions of ceramide that, to my knowledge, have not been observed before.

To date, effects of heat shock on the oocyte in vitro were studied exclusively using high oxygen incubators. However, results from Chapter 3 clearly demonstrated that oxygen environment can determine the magnitude of the deleterious effects to the developing embryo. In Chapter 5, the objective was to test the hypothesis that heat shock actions on the oocyte would be reduced under low oxygen. In contrast to expectations, however, heat shock decreased development regardless of whether oocytes were cultured in high or low oxygen. Reduction in oocyte competence in low oxygen, close to those observed in vivo (Chapter 6), can negatively affect the oocyte and its ability to reach the blastocyst stage. There may be a role for ROS in effects of heat shock in the oocyte cultured in low oxygen because of the large number of cumulus cells surrounding the oocyte and their potential for generating increased ROS under high oxygen. In this scenario, cumulus cells could produce ROS.

Also in Chapter 5, the overall development to blastocyst stage was high and the decrease in development caused by heat shock was low, in contrast with most studies in the literature involving effects of heat shock in the oocyte. In evaluating the results from this literature, a trend was observed that indicates that the severity of deleterious effects of heat shock tended to be more severe when overall development was low (Payton et al. 2004; Roth and Hansen 2004b; Edwards et al. 2005) and vice-versa (Edwards and Hansen 1997). This observation can be further supported by the fact that heat shock to the oocyte normally causes very little, if any, effect on cleavage rate (Edwards and Hansen 1996; Payton et al. 2004; Edwards et al. 2005; Ju et al. 2005), meaning that the oocyte is still alive past fertilization and first cleavage cycles. Maybe given suitable conditions, a higher percentage of affected oocytes/embryos may recover and develop further to at least the blastocyst stage.

Although elevated temperatures in vivo and in vitro (in high and low oxygen) reduces oocyte competence, indirect effects of heat stress to the cow can further affect oocyte competence and contribute to reduction in fertility. Among these indirect effects potentially contributing to low fertility during the hot months is increased blood flow to the periphery (Choshniak et al. 1982), reducing blood flow to the reproductive tract (Roman-Ponce et al. 1978b; Roman-Ponce et al. 1978a; Lublin and Wolfenson 1996) and potentially lowering oxygen concentration in the ovarian dominant follicle. However, oxygen concentration in the dominant preovulatory follicle from heat stressed cows was not different from non-heat stressed cows. As mentioned in Chapter 6, several factors could be responsible for preventing decrease in oxygen concentration in ovarian follicles of heat stressed cows. Experiments employing Doppler ultrasound to determine blood flow in the ovaries and follicles from heat stressed cows would provide additional information regarding local regulation of blood perfusion. Nevertheless,

decreased oxygen concentration is unlikely to play a role in reduction of fertility due to heat stress.

The negative correlation between follicular size and oxygen concentration has been reported previously in humans (Fischer et al. 1992) and pigs (Basini et al., 2004). However, this is the first report of the follicular oxygen concentration in dominant pre-ovulatory follicles in cattle. The findings mentioned above may have implications to the current protocols of in vitro maturation. The oocyte is surrounded by fluid containing oxygen concentrations much lower than atmospheric oxygen concentration used in most in vitro maturation systems (Pinyopummintr and Bavister 1995; de Matos et al. 1997; Hashimoto et al. 1998; Roth and Hansen 2004a). Under many conditions, oocyte maturation in vitro is more effective under atmospheric oxygen than under 5% oxygen (Hashimoto et al. 1998). The greater requirement for a high oxygen concentration in vitro may reflect the static nature of the in vitro system. On the other hand, glucose is usually not compensated for, and its concentration in most standard maturation media at the beginning of maturation (5.6 mM) (Hashimoto et al. 1998; Rivera and Hansen 2001; Harvey et al. 2004a) is similar to that in follicular fluid (Iwata et al. 2004; Orsi et al. 2005). This causes an imbalance in the oxygen:glucose ratio. To make matters worse, glucose concentration has been shown to drop to as low as 1.5 mM at the end of in vitro maturation (Sutton-McDowall et al. 2004), leading to an environment where the ratio oxygen:glucose is further unbalanced. Results in Chapter 5 and from Hashimoto et al. (2000a) demonstrate that decreasing oxygen to 5% in vitro (which is similar to concentration found in vivo in Chapter 6), required an increase in glucose concentration to 20 mM to allow successful oocyte maturation. Therefore, to date the variations shown to be successful in bovine oocyte maturation include

either high oxygen:glucose or high glucose:oxygen ratios, which are not similar to the ratio found in vivo.

The most commonly used protocol for bovine oocyte maturation employs 21% oxygen and 5.6 mM glucose (high oxygen:glucose ratio). I suggest that maturation in vitro could be optimized by decreasing oxygen to intermediate concentrations (e.g., 10 to 15%) and increasing glucose concentrations (e.g., 8 to 12 mM). In this scenario, neither oxygen nor glucose would be overcompensated and a similar ratio oxygen:glucose to the encountered in vivo would be achieved.

In conclusion, this thesis resulted in a new understanding of the role of oxygen and ceramide signaling in mediating effects of heat shock on oocytes and embryos. Perhaps the most significant finding was that oxygen concentration is a major determinant of the fate of embryos exposed to heat shock. Indeed, effects of heat shock on embryonic development and apoptosis was only apparent when embryos were cultured in high oxygen. Moreover, heat-shock induced apoptosis was blocked by antioxidant DTT, suggesting the necessity of ROS as an early factor in the apoptotic cascade induced by heat shock. Evidence was suggested that ceramide may be one of the signals activated by heat shock that causes apoptosis because the presence of ceramide-sensitive signaling mechanisms in the embryo were demonstrated as was the increase in nSMase by heat shock. Further experiments are warranted to further delineate the importance of this signaling pathway and its relevance to ROS production.

In the oocyte, the fact that heat shock decreased oocyte competence regardless of oxygen environment demonstrated that elevated temperature can directly affect the COC in oxygen conditions similar to encountered in vivo. Given that acute heat stress did not change oxygen

concentration in dominant pre-ovulatory follicles, it is unlikely that effects of heat stress in reducing fertility are mediated through decreased oxygen availability to the COC.

Even though heat shock blocked embryo development only in an oxygen concentration higher than present in utero, it is possible that results are physiologically relevant. Indeed, based on the results in mice that indicate that the oviduct provides a more oxidative environment during heat stress (Ozawa et al. 2002; Ozawa et al. 2004; Matsuzuka et al. 2005a; Matsuzuka et al. 2005b), effects of heat shock in vitro may be more representative of the situation encountered in vivo in the cow when embryos are cultured under high oxygen than when cultured under low oxygen.

One potentially-practical outcome of this research should be renewed focus on use of antioxidants to improve fertility of heat-stressed dairy cows. Previous attempts to improve fertility during heat stress with antioxidants have shown only limited success (Ealy et al. 1994; Aréchiga et al. 1998; Paula-Lopes et al. 2003) but it is possible that this failure reflects inadequate knowledge of how to improve redox status of the reproductive tract more than it does the lack of importance of ROS in heat-stressed induced infertility

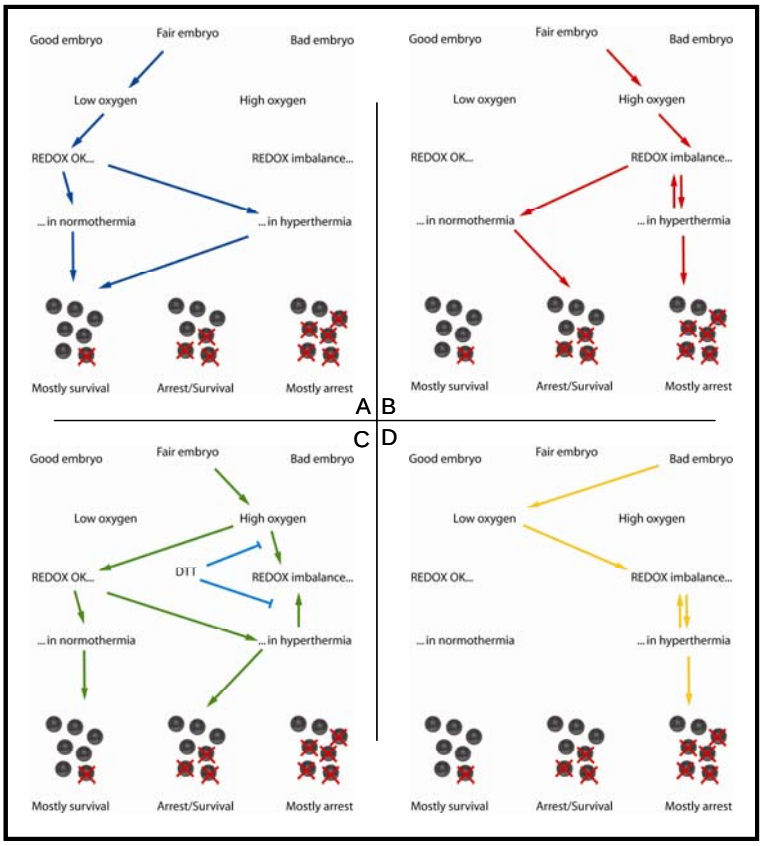

Figure 7-1. Tentative model to explain and predict the effects of heat shock on embryonic development. Considered factors are: predicted quality of the embryo prior to heat shock (good, fair and bad; see text for details), oxygen environment, redox status of the embryo and environment, and temperature. A) Hypothetical schematic representation to explain the effects of heat shock in low oxygen found in the present study. According to the model, culture in low oxygen provides the embryo to maintain its redox state by minimizing oxidative stress. Upon heat shock, oxidative stress is less due to low oxygen environment and possibly by better redox state of the embryo, preventing decrease in embryo development. B) According to the model culture in high oxygen causes imbalance in redox state of the embryo. Upon heat shock, effects of elevated temperature and oxidative stress induced by heat shock can add up to unbalanced redox of the embryo and negatively affect embryo development. C) Potential role of the antioxidant DTT to mitigate effects of heat shock under high oxygen in embryo development in vitro. DTT would act by providing reducing equivalents to the embryo, improving its redox state and enabling the embryo to counteract some of the negative effects caused by heat shock in embryo development. D) Model created as an attempt to extrapolate our current findings to a situation in vivo where embryo development is compromised by maternal heat stress. According to this model, which consider the oxygen environment in the reproductive tract to be low, the embryo redox and competence to continue development is compromised due to a combination of direct effects of temperature and oxidative stress by increased ROS production by the oviduct and the embryo it self.

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BIOGRAPHICAL SKETCH

Luiz Augusto de Castro e Paula was born in São Paulo, Brazil, to João Batista de Paula Neto and Maria do Rosário de Castro e Paula. Luiz Augusto received his veterinary degree from "Universidade Estadual de Londrina" in 1998. After receiving his degree he worked as veterinarian for a year and a half in a large beef cattle operation in the state of Mato Grosso do Sul, Brazil.

In 2000 Luiz Augusto returned to São Paulo state and enrolled in a continued education program at "Faculdade Getúlio Vargas" where he earned a degree in agribusiness. The following year he enrolled in a masters program in the department of animal reproduction at "Faculdade de Medicina Veterinaria e Zootecnia" from "Universidade de São Paulo". He received his Master of Science degree in 2003 and on the same year Luiz Augusto was married to Camila do Amaral Brito de Castro e Paula.

He and his wife moved to Gainesville, Florida, in May of 2003 where he enrolled as a Doctor of Philosophy student in the animal molecular and cellular biology program in the Department of Animal Sciences, University of Florida. He expects to graduate in August 2007 and to continue his career as a scientist in the area of animal reproduction.