Université de Montréal

OOCYTE MITOCHONDRIA: POTENTIAL MEDIATORS OF LIFE AND DEATH

par

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Faculté des études supérieures

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OOCYTE MITOCHONDRIA: POTENTIAL MEDIATORS OF LIFE AND DEATH

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Résumé

La mortalité embryonnaire avant son implantation est l'une des plus grandes causes de pertes de grossesses. Les mitochondries de l'ovocyte sont des régulateurs physiologiques des premières étapes du développement embryonnaire. Lorsque les mitochondries subissent un stress, ce stress perturbe l'ovocyte en induisant l'apoptose, ce qui affecte grandement la viabilité de l'embryon. Les objectifs de cette thèse étaient de: 1) déterminer si l'exposition d'ovocytes à un traitement de chaleur (Heat shock, HS) ou à un analogue au cyanure [carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP)] qui agit sur la dépolarisation de la membrane mitochondriale, réduit la compétence au développement de l'ovocyte, induit l'apoptose et altère l'expression de gènes apoptotique et mitochondriale. Aussi, si l'exposition d'ovocytes (HS) à la cyclosporine A (CsA), un inhibiteur des pores de la membrane imperméable mitochondriale, prévient la réduction du potentiel au développement et l'apoptose des ovocytes causé par le HS; 2) déterminer si l'apoptose des ovocytes matures observée suite au HS est en fait induite par la caspase-9 et/ou la caspase-3/7 et aussi d'évaluer si l'inhibition de cette apoptose pouvait redonner aux ovocytes leur potentiel de développement; et: 3) déterminer si les peptides anti-apoptotiques de la famille de Bcl-2 réduisent le traumatisme subit par les mitochondries des ovocytes bovins suite au traitement HS.

Des études ont été effectuées pour déterminer le rôle des mitochondries chez les ovocytes bovins devenus apoptotiques suite à un stress. L'exposition au HS ou au FCCP durant la maturation du complexe cumulus-ovocyte (COCs) a induit l'apoptose des

ovocytes et des blastocystes et était associée à la réduction de la capacité de développement. L'inhibition de l'ouverture des pores de la membrane des mitochondries par la CsA a bloqué l'apoptose habituellement induite par le HS démontrant bien que l'intégrité des mitochondries est essentielle durant les premiers stades de développement. Ces traitements ont également altérés l'expression des ARNm de gènes associés aux mitochondries et à l'apoptose chez les ovocytes matures et les blastocystes. Les résultats démontrent que les ovocytes sont sensibles au stress environnemental et pharmacologique et que leurs effets peuvent être observés au stade de blastocyste.

Nous avons établi que les effets néfastes du HS sur l'ovocyte étaient dus à l'activation des caspases. Ceci fut démontré à l'aide de plusieurs expériences où l'apoptose induite par le HS est le résultat de l'activation des caspases-9 et -3/7 dans l'ovocyte et qu'à long terme, ce stress a un effet négatif sur le blastocyste. Nous avons aussi constaté que l'ajout d'inhibiteurs de caspase (z-LEHD-fmk pour la caspase-9, z-DEVD-fmk pour la caspase-3/7 et z-VAD-fmk pour les caspases en général) élimine les effets néfastes du HS sur l'ovocyte et réduit donc la fréquence d'apoptose chez les ovocytes et chez les blastocystes. Il fut ainsi démontré pour la première fois qu'une voie intrinsèque dépendante de la caspase-9 est exprimée chez les ovocytes et activée suite à des conditions HS.

Le maintien de l'intégrité des mitochondries à l'aide de peptides anti-apoptotiques de la famille des Bcl-2 a été étudié comme alternative possible pour améliorer la fertilité chez les femelles en stress dû à une hausse de température. Les effets négatifs d'apoptose et de diminution de la capacité au développement observés sur les ovocytes en HS sont

inhibés suite à l'exposition des ovocytes à un peptide inhibiteur de Bax (BIP). Étonnement, le domaine BH4 de Bcl-xl (TAT-BH4) diminue la fréquence d'apoptose, mais n'arrive pas à rétablir le développement au stade de blastocyste. Le traitement au HS des ovocytes en combinaison avec les 2 peptides amènent une réduction du pourcentage des ovocytes et blastomères-TUNEL positifs et augmentent le développement embryonnaire. Ces données mettent en évidence la voie dépendante des domaines Bax et BH4 (Bax and BH4 dependant-pathway) sur l'induction de l'apoptose et la mortalité cellulaire chez les ovocytes traités HS. Elles semblent suggérer que l'avenir de l'ovocyte serait ultimement déterminé par le résultat final des interactions entre les membres de la famille des Bcl-2.

En résumé, nos résultats ont démontré que la dysfonction mitochondriale est un facteur déterminant dans l'amplitude des effets négatifs d'un stress sur la compétence au développement des ovocytes et l'apoptose. La démonstration de l'existence de mécanismes d'échange des signaux caspase-9, Bax et BH4 dans l'ovocyte va nous permettre de mieux comprendre les mécanismes par lesquels le HS et d'autres stress induisent l'apoptose chez l'ovocyte. De toute évidence, une meilleure compréhension de la régulation moléculaire, qui détermine l'avenir d'un ovocyte, nous donnera l'opportunité de développer des thérapies contre l'apoptose afin d'améliorer la fertilité même en condition de stress.

Mots-clés: bovin, ovocyte, apoptose, mitochondries, traitement de chaleur

Abstract

Preimplantation embryonic mortality is a major cause of pregnancy loss. Oocyte mitochondria are physiological regulators of early embryonic development and potential sites of insult that may perturb oocyte by inducing apoptosis and reducing subsequent embryonic viability. The objectives of this dissertation were to; 1) determine whether exposure of oocytes to heat shock (HS) or to a cyanid analog that reduces the mitochondrial membrane potential, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) reduce oocyte developmental competence, induce apoptosis, alter the expression of apoptotic and mitochondrial genes. Also, if HS-oocytes exposed to cyclosporin A (CsA), an inhibitor of mitochondrial permeability transition pores, reverses the compromised developmental potential and apoptosis caused by HS; 2) examine whether HS-induced apoptosis in maturing oocytes is mediated by caspase-9 and/or caspase-3/7, and whether inhibition of apoptosis in HS-oocytes rescue oocyte developmental capacity and; 3) determine whether Bcl-2 family anti-apoptotic peptides reduces HS mitochondrial injury in bovine oocytes.

Studies were performed to determine the role of mitochondrial stress-induced apoptosis in bovine oocytes. Exposure of cumulus-oocyte complexes (COCs) to HS or FCCP during maturation induced apoptosis in both oocytes and blastocysts and was associated with reduced developmental capacity. Inhibition of the opening of the mitochondrial pore by CsA blocked HS-induced apoptosis demonstrating that mitochondrial integrity is crucial for the response to stress early in development. These

treatments also altered mRNA expression of candidate mitochondrial and apoptotic related genes in matured oocytes and blastocysts. Results demonstrate that oocytes are sentive to environmental or pharmacological insults and this response to stress can be observed later at the blastocyst stage.

The deleterious effects of HS in oocytes were shown to be affected by caspase activation. This was demonstrated in a series of experiments where HS-induced apoptosis resulted in activation of caspase-9 and -3/7 in oocytes, and this stress had a negative long term effect on the resulting blastocysts by increased capase-9 and -3/7 activity. Also addition of specific caspase inhibitors (z-LEHD-fmk, a caspase-9 inhibitor and z-DEVD-fmk, a caspase-3/7 inhibitor) or a broad caspase inhibitor (z-VAD-fmk) suppressed the deleterious effects of HS on oocyte developmental capacity and reduced the frequency of apoptosis in both oocytes and blastocysts. For the first time it was demonstrated that the intrinsic (caspase-9-dependent) pathway is expressed in oocytes and activated under HS conditions.

Preservation of mitochondrial integrity by the anti-apoptotic peptides of Bcl-2 family was investigated as a possible way of improving fertility in thermal stressed females. Exposure of HS-oocytes to a Bax inhibiting peptide (BIP) abrogated the negative effect of HS on embryo developmental capacity and apoptosis. Surprisingly, the BH4 domain of Bcl-xL (TAT-BH4) did not restore development to the blastocyst stage and, in addition, reduced the frequency of apoptosis. Treating HS-oocytes with a combination of both peptides resulted in reduced percentage of TUNEL-positive oocytes and blastomeres and

increased embryonic development. These data provide evidence of Bax and BH4 domain-dependent pathway on heat-induced oocyte apoptotic cell death and suggest that the fate of oocyte is ultimately determined by the end-result of a complex interaction between Bcl-2 family members.

In summary these results demonstrate that mitochondrial dysfunction is an important factor that determines the magnitude of negative effects of stresses in oocyte developmental competence and apoptosis. The demonstration of existence of a caspase-9, Bax, and BH4 signaling mechanism in the oocyte may help to further understand the mechanism by which HS and other stresses induce apoptosis in oocytes. A better understanding of the molecular mechanisms dictating oocyte fate will in all likelihood open opportunities for development of apoptosis-based therapeutic strategies designed to improve fertility health even under stress conditions.

Keywords: Bovine, oocyte, apoptosis, mitochondria, heat shock

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List of Abbreviations

ΔΨm	Mitochondrial Membrane Potential
ADP	Adenosine Diphosphate
AIF	Apoptosis Inducing Factor
Akt	Serine/Threonine Protein Kinase B
Ant	Adenine-Nucleotide Translocase
Apaf-1	Apoptosis Activating Factor-1
Asp	Aspartic
ATP	Adenosine Triphosphate
Bad	Bcl2 antagonist of cell death
Bag	Bcl2 associated athanogene
Bak	Bax-Bcl2 Antagonist/Killer
Bax	Bcl2 Associated X-Protein
Bcl-2	B-cell Lymphoma/Leukemia 2
Bcl-w	Bcl2 like 2 protein
Bcl-x	Bcl2 like 1
Bcl-xL	Bcl2 Related Protein, Long Isoform
Bcl-xS	Bcl2 related Protein, Short Isoform
BH	Bcl-2 Homology Domains
Bid	BH3 interacting domain death agonist
Bik	Bcl2 interacting killer
Bim	Bcl2 interacting protein BIM
BIP	Bax Inhibitor Peptide
BIR	N-terminal Baculovirus-Inhibitor-of-Apoptotis-Repeat
BMP	Bone Morphogenic Protein
BSA	Bovine Serum Albumin
CAD	Caspase-Activated DNase
CARD	Caspase-Recruitment Domain
Caspases	Cysteine-dependent Aspartate-Specific Proteases
CO	Cytochrome c Oxidase
CO1	Cytochrome Oxidase 1
COCs	Cumulus-Oocyte Complexes
CREB	AMP-response-element-binding Protein
CsA	Cyclosporin A
C-terminal domain	Carboxy-Terminal Transmembrane Region
CyP-D	Cyclophilin D

Cyt c	Cytochrome c
DED	Death Effector Domain
Diablo/Smac	Second Mitochondria-derived Activator of Caspase/Direct IAP Binding Protein with Low p*I*
$DiOC_6$	3, 3' Dihexyloxacarbocyanine Iodide
DISC	Death Inducing Signaling Complex
D-loop	Displacement Loop
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
dUTP	Deoxy-Uridine Tri-phosphate
EFAF	Essentially Fatty-Acid Free
EFAF-BSA	Essentially Fatty-Acid Free Bovine Serum Albumin
EGA	Embryonic Genomic Activation
EndoG	Endonuclease G
F_1F_0–ATPase	F_1F_0–adenosine triphosphate
FADD	FAS-Associating Protein with a Death Domain
$FADH_2$	Reduced Flavin Adenine Dinucleotide
FCCP	Carbonyl Cyanide 4-(trifluoromethoxy) Phenylhydrazone
Fg	Femtograms
FSH	Follicle-Stimulating Hormone
Gapdh	Glyceraldehyde 3-Phosphate Dehydrogenase
GLM	General Linear Model
GnRH	Gonadotrophin Releasing Hormone
Group I	Caspases-1, -4, and -5
Group II	Caspases-2, -3, and -7
Group III	Caspases-6, -8, and -10
GSH	Glutathione
GV	Germinal Vesicle
GVBD	Germinal Vesicle Breakdown
H_2O_2	Hydrogen Peroxide
HeLa	Human Cervix Cancer Cell Line
HMG-box	High Motility Group-box
HS22	Heat Shock for 22 hours
HS9	Heat Shock for 9 hours
HSF-1	Transcription Factor for Heat Shock Proteins

HSP70	Heat Shock Protein 70 kDa
HSP90	Heat Shock Protein 90 kDa
HSPs	Heat Shock Proteins
H-strand	Heavy-Strand
HtrA2	High Temperature Requirement Protein A2
IAPs	Inhibitor of Apoptosis Proteins
ICAD	Inhibitor of CAD
ICM	Inner Cell Mass
IVF	In-Vitro Fertilization
JC-1	5,5',6,6'-Tetrachloro-1.1',3,3'-Tetraethyl Benzimidazolylcarbocyanide Iodide
JNK	c-Jun n-Terminal Kinase
LH	Luteinizing Hormone
L-strand	Light-Strand
MDM2	Mouse Double Minute 2
MII	Metaphase II
MOMP	Mitochondrial Outer Membrane Permeabilization
mRNA	Messenger RNA
mSOF	Modified Synthetic Oviduct Fluid
mtDNA	Mitochondrial Deoxyribonucleic Acid
mTERF	Mitochondrial Transcription Termination Factor
mtRNApol	Mitochondrial RNA Polymerase
mtTFA	Transcription Factor A
N_2	Nitrogen
NADH	Nicotinamide Adenine Dinucleotide Reduced
ND	NADH-Dehydrogenase-Ubiquinone Reductase
ND6	Mitochondrial NADH Dehydrogenase Subunit 6
Noxa	Phorbol-12-myristate-13-acetate-induced protein 1
O_2	Oxygen
O_H	Origin of Replication for H-strand
O_L	Origin of Replication for the L-strand
Omi/HrtA2	High Temperature Requirement Protein A2
OMM	Oocyte Maturation Medium
OWM	Oocyte Washing Medium
Oxphos	Oxidative Phosphorylation
p10	Small subunit

p20	Large subunit
PARP	Poly-ADP-Ribose Polymerase
PBS	Phosphate Buffered Saline
PBS-PVP	Phosphate Buffered Saline-Polyvinylpyrrolidone
PGC	Primary Germ Cells
PI3K	Phosphatidylinositol-3'-kinase
POLRMT	Mitochondrial RNA Polymerase
PT	Mitochondria Permeability Transition
Puma	Bcl2 binding component 3
PVP	Polyvinylpyrrolidone
Redox	Reduction/Oxidation
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rRNA	Ribosomal RNA
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SAPK	Stress Activated Protein Kinase
SAS	Statistical Analysis System
SMase	Shingomyelinase
TAT-BH4	BH4-domain of Bcl-xL linked to 10-amino acid HIV-TAT
TCM-199	Tissue Culture Medium-199
TE	Trophectoderm
TEM	Transmission Electron Microscopy
Tfam	Transcription Factor A
TFB1M	Mitochondrial Transcription Factor B1
TFB2M	Mitochondrial Transcription Factor B2
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
TRAIL	TNF-Related Apoptosis Inducing Ligand
tRNA	Transfer RNA
TUNEL	Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick End Labeling
Vdac	Voltage-Dependent Anion Channel
WM	Washing Medium
XAF1	Xiap-Associated Factor 1
XIAP	X-Linked Inhibitor of Apoptosis
z-DEVD-fmk	N-Benzyloxycarbonyl-Asp-Glu-Val-Asp-Fluoromethyl Ketone

z-LEHD-fmk	z-L-E-(Ome)-H-D(Ome)-fluromethyl ketone
z-VAD-fmk	N-Benzoylcarbanyl-Val-Ala-Asp-Fluoro Methylketone

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Introduction

The mitochondrial genome is maternally inherited via the oocyte cytoplasm, from one generation to the next. While oocyte mitochondria are small, round in shape, microscopically dense, and contain a few underdeveloped cristae, characteristics of cells with low metabolic rate (Van Blerkom 2004), these organelles can produce enough ATP to sustain embryo development (Van Blerkom et al. 1995; Tamassia et al. 2004). The role of mitochondria in ATP production, regulation of intracellular free Ca^{2+}, steroidogenesis, and activation of the apoptotic pathway has long be known. Recently, the notion that oocyte mitochondrial dysfunction may be crucial determinant of embryo development has emerged.

Mitochondrial defects may in part be responsible for the decline in fertility. Oocytes from older women were more likely to contain deleted mtDNA compared to oocytes from younger women (Keefe et al. 1995; Jansen and Burton 2004). Also cohorts of human oocytes with ATP content <2 pmol/oocyte have lower potential for continued embryogenesis (Van Blerkom et al. 1995). In oocytes from aged humans, an increase in volume fraction of the mitochondria was observed which might reflect subtle changes in oxidative phosphorylation capacity (Muller-Hocker et al. 1996). Higher mitochondrial membrane potential was positively correlated with the rate of development of human embryos (Wilding et al. 2001). Aberrant shifts in mitochondrial membrane potential may contribute to reduced embryonic development potential (Acton et al. 2004). Fragmented embryos generated more reactive oxygen species (Yang et al. 1998). Consequently, any environmental or pharmacological insult to oocyte mitochondria may contribute to susceptibility of the oocyte to developmental compromise.

Oocyte mitochondria are potential mediators or could even initiate apoptotic degeneration in oocytes and preimplantation embryos. It is well established that loss of oocytes from the ovarian pool involves an apoptotic process however the precise mechanisms involved are not completely understood (Morita and Tilly 1999; Reynaud and Driancourt 2000). Most, but not all, death signals converge to increase in mitochondrial membrane permeability with release of pro-apoptotic factors in the cytosol resulting in caspase activation and induction of apoptosis (Reynaud and Driancourt 2000; Lemasters 2005). Cells have also mechanisms for inducing apoptosis in the absence of caspase activation. Apoptogenic factors otherwise confined to the mitochondrial intermembrane space, such as apoptosis inducing factor (AIF) and endonuclease G (EndoG), leak into the cytoplasm and nucleus where they display their pro-apoptotic characteristics (Ye et al. 2002; Lorenzo and Susin 2004). Heat shock has been shown to decrease oocyte competence *in vivo* and *in vitro* (Putney et al. 1989; Edwards and Hansen 1997; Rocha et al. 1998; Al-Katanani et al. 2002; Roth et al. 2002; Roth and Hansen 2004), reduce oocyte protein synthesis (Edwards and Hansen 1996), and induce apoptosis (Roth and Hansen 2004). In somatic cells, heat-induced apoptosis involves the mitochondrial pathway (Mirkes and Little 2000; Qian et al. 2004; Bettaieb and Averill-Bates 2005; Wada et al. 2005). However, mitochondrial role in heat shock-induced apoptosis in the oocyte remains to be elucidated.

Identification of the mitochondrial pathway through the presence of active caspase and Bcl-2 family signaling mechanisms in the oocyte could help elucidade the pathway by

which different stressors (e.g. heat shock), induce apoptosis, and potentially open a new window of opportunity for developing strategies to reduce deleterious effects of heat shock or other stresses on the oocyte and improve fertility health under stress conditions.

CHAPTER I

LITERATURE REVIEW

The Role of Mitochondria During Early Embryonic Development

Ancient eubacterial invasions through symbiosis gave rise to mitochondria (Dyall et al. 2004). The presence of mitochondrial deoxyribonucleic acid (mtDNA) is the primary remaining evidence of their bacterial origin. Mitochondria and chloroplast are the only structures in a cell that have a deoxyribonucleic acid (DNA) distinct from the nuclear DNA. As a consequence, the control of mitochondrial biogenesis and function depends on a well orchestrated regulation between the two genomes, nuclear and mitochondrial (Garesse and Vallejo 2001). Their primary function is to provide energy in all eukaryotic cells, in form of ATP production, through oxidative phosphorylation (Oxphos) and the citric acid cycle. They also have an essential role in other biochemical pathways like calcium homeostasis and modulation of apoptosis through the release of several cell death-inducing molecules (Duchen 2004).

Mammalian mitochondrial DNA is a double stranded closed-circular molecule with approximately 16500 nucleotides that, in most cells, represents only about 0.5-1% of the total DNA content (Smith and Alcivar 1993; Fernandez-Silva et al. 2003). It is a compact gene organization, the coding sequences are contiguous or separated by a few base pairs without introns and some of the protein genes even overlap (Fernandez-Silva et al. 2003). The two strands, the heavy or H-strand and the light or L-strand, can be distinguished by their different guanine and thymine (G+T) content and different density in denaturating gradients.

The mitochondrial genome contains only 37 genes corresponding to the ribonucleic acid (RNA) components of the mitochondrial translational apparatus [two ribosomal RNA (rRNAs) called 12S and 16S and 22 transfer RNA (tRNAs) as well as messenger RNA (mRNAs) for 12 polypeptides that are subunits for Oxphos complexes (Smith et al. 2002). Seven of those polypeptides (ND1 to ND6 plus ND4L) are subunits of complex I: NADH-dehydrogenase-ubiquinone reductase; one (cytochrome b) is part of complex III: ubiquinol-cytochrome c reductase; three (COI, COII and COIII) are catalytic subunits of complex IV: cytochrome c oxidase; and ATPase 6 and 8 are subunits of complex V: ATP synthetase (Fernandez-Silva et al. 2003). These genes are asymmetrically distributed and the H-strand encodes most of the information. The L-strand encodes only eight tRNAs and one mRNA, the ND6 subunit. The rest of the Oxphos subunits as well as all the factors involved in the mtDNA metabolism are nuclear-encoded synthesized in the cytosol and transported by chaperones into the mitochondrial matrix (Garesse and Vallejo 2001). Therefore, control of mitochondrial function is complicated and involves the exchange of information between nucleus and many copies of mtDNA in each cell's cytoplasm.

Mitochondrial Genetic System

The mitochondrial genome has some features that differ from the nuclear genome. These include the phenomenon of polyploidy with respect to mtDNA where each cell contains several thousands of mitochondria and each mitochondria contains several (2-10) copies of mtDNA (Fernandez-Silva et al. 2003). Also all mtDNA in an individual is

thought to be identical (homoplasmy), although mutations can arise, maintained or amplified giving rise to condition of heteroplasmy. Mitochondria mutations higher than 60-80% are associated with diseases. The accepted dogma is that mtDNA is maternally inherited, after fertilization, the sperm carries a few paternal mitochondria which are eliminated by ubiquitin-dependent mechanism (Cummins 2001a; Fernandez-Silva et al. 2003; Cummins 2004). During oogenesis, a bottleneck phenomenon occurs where only a small number of mtDNA molecules are amplified and transmitted to the offspring. Also the evolution rate of mtDNA is almost 20 times faster than the nuclear genome (Jansen and de Boer 1998). Several reasons could explain this high rate of mutation: lack of histone proteins to protect mtDNA, physically association with the inner mitochondrial membrane where the damaged reactive oxygen species (ROS) are generated, lack of proofreading mechanism, and a less efficient DNA repair mechanism compared to the nucleus (Kowaltowski and Vercesi 1999). Accumulation of mtDNA mutations has been associated with ageing (Zeviani and Antozzi 1997; Cummins 2001b). High mutation rates (Keefe et al. 1995) and alteration in mitochondrial function (Van Blerkom et al. 1995), numerical density (Muller-Hocker et al. 1996; Steuerwald et al. 2000), reduced ATP levels (Diaz 1999), shift in mitochondrial membrane potential (Acton et al. 2004), increased ROS levels (Yang et al. 1998), and metabolic activity (Wilding et al. 2001) are also associated with reduced fertility.

The mitochondrial genome is replicated and transcribed within the organelle (Fernandez-Silva et al. 2003). In mtDNA there are two non-coding regions which contain

most of known regulatory functions. The main one is a triple-stranded structured called the displacement loop (D-loop) region, situated between the genes $tRNA^{Phe}$ and $tRNA^{Pro}$ that contains the origin of replication for H-strand (O_H) and the promoters or H- and L-strand transcription. The cis-elements are responsible for regulation of both mtDNA replication and transcription at the D-loop region. This is also the region that is most variable in sequence and size among different species. However, it contains some conserved elements with possible regulatory functions. The second non-coding region is a ~30 nucleotide-long segment that contains the origin of replication for the L-strand (O_L). All the trans-acting factors associated with mtDNA metabolism are nuclear-encoded, such as mtRNA polymerase, mtDNA polymerase (pol γ), and all factors that regulate mtDNA replication, mtDNA transcription and mtRNA processing (Garesse and Vallejo 2001).

Mitochondrial transcription starts at three different initiation points, one for L-strand (L) and two for H-strand (H_1 and H_2), all located at the D-loop region. The initiation site H_1 operates much more frequently than H_2. The H_1 is responsible for the synthesis of two ribosomal RNAs ($tRNA^{Phe}$ and $tRNA^{Val}$) and linked to transcription termination events (Fernandez-Silva et al. 2003). The H_2 initiation point directs the transcription of whole H-strand and is around 20 times less active than H_1. Also H_2 originates a polycistronic molecule that covers almost the whole H-strand. Processing of this polycistron originates mRNAs for 12 H-strand encoded polypeptides and 12 tRNAs. The L-strand originates a single polycistron from which eight tRNA and ND6 mRNA are derived (Garesse and Vallejo 2001).

The mitochondrial transcription machinery is relatively simple compared with nuclear version. It consists of a single organelle-specific RNA polymerase (mtRNApol or POLRMT) and transcription factors such as the mitochondrial transcription factor A (mtTFA or Tfam) and either mitochondrial transcription factors B1 (TFB1M) or B2 (TFB2M) for initiation and a mitochondrial transcription termination factor (mTERF) for termination of transcription (Falkenberg et al. 2002; Fernandez-Silva et al. 2003; Hyvarinen et al. 2007). Until very recently, the mtTFA was the only known transcription factor in mammals. Binding sites for mtTFA are present at the two more active transcription initiation points (H_1 and L) (Fernandez-Silva et al. 2003). However, binding and transcription activities are higher for the L promoter (LSP) compared with H_1 promoter (HSP_1). Previous data suggest that mtTFA forms a complex with DNA by its two high motility group (HMG)-box domains that induces a structural change in the promoter region of mtDNA and allows mtRNApol to initiate transcription (Shadel and Clayton 1997; Clayton 2000). In mouse embryos, mtDNA transcription starts at day 2 of development (Cummins 2002).

The replication of mtDNA is takes place at the mitochondrial matrix (Fernandez-Silva et al. 2003). The generally accepted model is that the two strands (L- and H-strand) are replicated asynchronously and asymmetrically. The synthesis starts at O_H located at the D-loop region and proceeds unidirectionally until O_L to produce a daughter H-strand circle. When H-strand replication reaches O_L, the paternal H-strand is displaced and the initiation site for L-strand exposed and synthesis proceeds in opposite direction to produce a daughter

L-strand. The mtTFA seems to be involved in replication, since it is required for L-strand transcription initiation and primer formation. In mouse embryos, transcription of replication factors are abundant at the morula and blastocyst stage but mtDNA replication occurs in the blastocyst at day 6.5 (Piko and Matsumoto 1976; Thundathil et al. 2005).

Oogenesis

In the earliest pre-migratory germ cells, probably less than 10 mitochondria are present and this number increases to around 200 in each oogonium (Cummins 2002). This phenomenon is called the "bottleneck", when the restriction of mitochondria copy number acts to maintain homoplasmy and minimize heteroplasmy followed by clonal expansion (Cummins 2001b). The bottleneck is believed to limit the effects of Muller's ratchet or the tendency for deleterious mutations to accumulate in asexually reproducing organisms (Bergstrom and Pritchard 1998). Smith et al. (2000) proposed that there are several periods when restriction of mitochondria copy number could occur including during replication and migration of primary germ cells (PGC), during oogenesis; during early embryogenesis and during the commitment of embryonic inner cell mass elements from PGC.

During preimplantation development, mitochondria undergo structural and functional differentiation. In early stages, mitochondria are spherical, have a dense matrix and a few cristae (Van Blerkom 2004). In human oocytes, mitochondria are spherical/ovoid organelles, with a few short cristae that rarely penetrate the dense matrix. Therefore, they are structurally undifferentiated and produce low concentrations of

adenosine triphosphate (ATP) (Van Blerkom and Davis 1998). This phenotype persists throughout the cleavage and late morulae stage in in vitro human embryos (Van Blerkom 2004). In most mammals at the blastoscyst stage, mitochondria are elongated with lamellar cristae that completely transverse the inner mitochondrial matrix. These features are found in mitochondria actively engaged in ATP-production by oxidative metabolism.

Mitochondria also undergo stage-specific changes in distribution during oocyte maturation and early embryo development. During maturation, mitochondria distribution shifts from random to an arrangement during leptotene, and translocates to the perinuclear region during zygotene by microtubule-mediated process (Cummins 2004; Van Blerkom 2004). After fertilization, mitochondria migrate to the perinuclear region to form a condensed aggregate surrounding the opposite nuclei (Van Blerkom 2004). A transient nuclear perinuclear accumulation is also observed in each blastomore during early cleavages. Spatial remodeling of mitochondria allows increased levels of ATP in areas of cytoplasm that have stage-specific activities with high energetic needs (Cummins 2004; Van Blerkom 2004).

Traditionally, estimation of mitochondrial numbers in oocyte and early embryos has been obtained from transmission electron microscopy (TEM) (Vander Heiden et al. 1997). In the oocyte recruited for ovulation, the increase in cytoplasmic volume is followed by an increase in mitochondria numbers (Cummins 2002). The current consensus is that each oocyte mitochondrion contains a single genome. In cattle, during the period of oocyte growth, mitochondria increase in number by more than one hundred times to reach 136 000

mitochondria in the mature preovulatory oocyte (Hauswirth and Laipis 1985) compared with the average of 92 500 mitochondria in mouse embryos (Piko and Matsumoto 1976). This may indicates that a competent oocyte requires a fixed amount of mitochondria per unit of cytoplasm (Smith and Alcivar 1993). Further, mtDNA copy number also seems to be associated with increased oocyte volume. In bovine preovulatory oocytes, mtDNA per cell increased from 0.1 pg in primordial cells to 4.5 pg or 260 000 molecules (Hauswirth and Laipis 1985).

During fetal and adult life, oocytes are eliminated by apoptosis during atresia. It is not known if this selection is based on mtDNA (Jacobson et al. 1997). Atresia might represent the mtDNA bottleneck that reduces the mtDNA genetic variability across generations and consequently restores homoplasmy. This seems to be contrary to the quiescent nature of oocyte's mitochondria. Therefore, the answer of this nuclear-mitochondria interaction at the oocyte stage is unclear. Previous studies have been shown that the oocyte can undergo apoptosis by activation of caspases or by alteration of the mitochondria membrane potential (Roth and Hansen 2004; Thouas et al. 2004). These data suggest that the apoptotic machinery is present and can be activated at the oocyte stage.

Embryogenesis

The preimplantation period begins with oocyte fertilization and ends with formation of a blastocyst ready to implant. This period is characterized by three major transitions, first embryonic genomic activation (EGA), compaction, and blastocyst formation (Zeng et

al. 2004). The matured oocyte contains sufficient maternal transcripts and proteins to support fertilization and the first two cell divisions (Devreker and Englert 2000). Activation of the embryonic genome represents the transition period from maternal to embryonic control by synthesis of embryonic mRNA and proteins. Blastomeres increase in contact with each other through intercellular gap and tight junctional complexes. Also membrane and cytoplasmic polarization occur and blastomeres develop a distinct apical and basal membrane and cytoplasmic domains (Hardy et al. 1996). The following mitosis will produce two types of cells, polar and apolar (Devreker and Englert 2000). Depending on the plane of division, the polar cells can produce two identical polar daughter cells or one polar and one apolar daughter cell. Apolar cells remain on the inside of the preimplantation embryo and are the origin of the inner cell mass (ICM) while apolar cells remain outside to form the trophectoderm (TE), the first transporting epithelium. The morula is transformed to blastocyst by the transport of fluids and accumulation resulting in cavity formation. The blastocyst is formed by a small group of ICM, a filled cavity and surrounding TE. Implantation occurs after blastocyst hatching from the zone pelucida. The ICM gives rise to embryo itself and TE gives rise to extra-embryonic membranes and placenta.

Embryo metabolism is different in the pre- and post-compaction stage (Devreker and Englert 2000). Before compaction the blastomeres are loosely attached and, in consequence, equally exposed to their environment with low level of biosynthesis, low respiratory rates and limited ability to metabolize glucose as a source of energy. In

contrast, post-compaction embryos have high rate of biosynthesis and exponential increased in energy demand, and become able to metabolizing glucose.

Mitochondria are also undergoing changes during the preimplantaion period. After fertilization, mitochondria are located close to developing pronuclei, presumably as source of ATP (Cummins 2001b). The mitochondria matrix becomes less dense, oval in shape, transcription resumes, and pyruvate become the embryo's energy substrate. Correlations between the potential for development, ATP content, and mitochondrial function are observed in bovine oocytes and embryos (Stojkovic et al. 2001). After fertilization, at the first three to four cell divisions, there is a shift in ATP production from oxidative metabolism to glycolysis which coincides with theoretical reductions in availability of oxygen (O_2), with transition from the oviduct to the uterus (Cummins 2001b). An increase in consumption of O_2 occurs during initiation of compactation and blastocyst formation because these process are energy demanding. Optimal O_2 levels during early embryonic development appear to be critical for normal development. Bovine embryos exposed to an O_2 tension of 20% displayed decreased blastocyst formation when compared with 5 or 2% O_2 environment (Yuan et al. 2003). Mitochondrial function is associated with a change in metabolic requirements in which a shift in carbohydrate substrate requirements from glucose to pyruvate occurs in the embryo moving through the first cell divisions (Cummins 2004).

This evidence emphasizes the need of fine tuned communication between nuclear and mitochondrial genes during development. A better elucidation of interactions between

mitochondrial and nuclear genomes and embryo metabolism is critical for an improvement in growth rates on cultured embryos.

Mitochondria and Apoptosis

Recently, new knowledge about mitochondria has shown that they are not only energy generators but also play a central role in the delicate process that sustains the balance between cell life and cell death.

The term "programmed cell death", originally, was used to describe a cell death process throughout animal development that occurs in a spontaneous, orchestrated and predictable manner (Lockshin and Willians 1964). After the establishment of similarities between many models of cell death and a description of conserved morphological features, this programmed cell death was named apoptosis (Kerr et al. 1972). The meaning of the word "apo" is from and "ptosis" means fall, taken together means "fall away from".

Apoptosis has been recognized as a physiological form of cell death required to control cell populations during normal tissue homeostasis or to eliminate cells damaged by stress stimuli (Haimovitz-Friedman A 1997; Jacobson et al. 1997). The programmed cell death pathway has been proposed as a major regulatory event observed during embryonic development, establishment of immune self-tolerance, immune effector cell killing and regulation of cell viability by hormones and growth factors (Zakeri and Lockshin 2002; Meirelles et al. 2004). Failure to accurately undergo apoptosis may contribute to

neurodegenerative disease, cardiovascular diseases, cancer, viral pathogenesis, and autoimmune diseases (Zimmermann et al. 2001; Van Blerkom 2004).

Apoptosis and Necrosis

The most described forms of cell death are apoptosis and necrosis. They can be differentiated based on morphological and biochemical markers. Necrosis or accidental cell death is characterized by dilatation of the endoplasmic reticulum, dissolution of ribosomes and lysosomes, swelling of the mitochondria matrix, and rupture of nuclear, organelle and plasma membranes. These rupture of membranes allows the release of intracellular content that triggers the inflammatory process (Wyllie et al. 1980).

Morphologically, in early apoptotic events, the chromatin aggregates and condenses against the nuclear membrane with appearance described as half-moon-, horse-shoe-, sickle-, lancet-, and ship-like (Majno and Joris 1995). The nucleus progressively condenses and the nuclear envelope becomes convoluted, indented and then broken up (karyohexis) which generates nuclear fragments that contains condensed chromatin (Wyllie et al. 1980). In parallel, the cytoplasm also condenses, the cell becomes round, the plasma membrane become indented, and organelles maintain intact shape and structure (Kerr et al. 1972; Kondo et al. 1997). During late apoptotic stages, condensation of the mitochondria (Kluck et al. 1999), dilatation of the endoplasmic reticulum (Ludewig et al. 1995), detachment of ribosomes from the rough endoplasmic reticulum and ribosome aggregation (Ferguson and Anderson 1981) can be observed. Compressed organelles and cytoplasm condensation

allowed formation of cytoplasmic vacuoles. The plasma membrane is preserved to allow packing of whole cell fragments into membrane-bound apoptotic bodies without leakage of toxic intracellular contents (Kerr et al. 1972). The apoptotic bodies are phagocytosed by neighbor cells or macrophages without inducing an inflammatory response or tissue damage.

The interest in describing biochemical features of apoptosis has increased. The externalization of phosphatidylserine to the outer leaflet of the plasma membrane is an early event of apoptosis which acts as a signal resulting in phagocytosis of the apoptotic cell by neighbor cells (Martin et al. 1995). The induction of mitochondrial outer membrane permeabilization (MOMP) is another early event controlled by the Bcl-2 family of proteins (Lorenzo and Susin 2004). The most well know biochemical change observed in the nucleus during cell death is mediated by caspase activation resulting in cleavage of the DNA to form internucleosomal DNA fragments (Wyllie et al. 1980). The characteristic ladder patterns of these fragments are bands of 180-200 bps in size on agarose gel electrophresis. In contrast, necrotic cells show a smear suggesting that DNA is cleaved at random.

Mitochondrial Outer Membrane Permeabilization (MOMP)

During MOMP the inner mitochondrial membrane retains proteins from the mitochondrial matrix and the outer mitochondrial membrane becomes permeable. Therefore, release of soluble intermembrane space proteins occurs. Some of these are:

cytochrome c (Cyt c), IAP binding protein with low p*I* (Diablo/Smac), high temperature requirement protein A2 (Omi/HtrA2), AIF, EndoG, and certain procaspases, including procaspase-9 (Crompton 1999; Lorenzo and Susin 2004). Leakage of mitochondrial contents through MOMP results in cytoplasmic and nuclear apoptosis.

The mechanism for outer-membrane rupture during apoptosis is not yet established. These ruptures are evident in electron micrographs of Jurkat cells undergoing apoptosis associated with matrix swelling (Vander Heiden et al. 1997). However, it is not known whether breaks in the mitochondrial outer-membrane are a result of mitochondria permeability transition (PT) pore activation. Under normal physiological conditions, the general function of components of PT pore is well known. The function of voltage-dependent anion channels (Vdac) is to allow solute access to the solute-specific transport systems of the inner membrane (Crompton 1999). The adenine-nucleotide translocase (Ant) mediates adenosine diphosphate (ADP)↔ATP exchange that is crucial for mitochondria bioenergetic function. The cyclophilin D (CyP-D) function is not established, but it is associated with catalysis of protein folding. Under pathological conditions, the PT pore consists of Vdac in the outer mitochondrial membrane, Ant in the inner mitochondrial membrane and CyP-D in the matrix (Zamzami and Kroemer 2001; Crompton et al. 2002; Halestrap 2006). One possible mechanism is the formation of Vdac-Ant-CyP-D complex leading to PT pore opening that results in a mitochondrial depolarization, uncoupling of Oxphos, mitochondrial swelling (matrix expansion), outer-membrane rupture, and release of intermembrane-space apoptogenic proteins (Crompton 1999; Gross et al. 1999).

Previous studies have shown that HeLa nuclei mixed with isolated mitochondria and exposed to various PT pore activators (atracylate, peroxides, Ca^{2+}, diamide) resulted in mitochondrial swelling, release of Cyt c and AIF, and apoptotic changes in nuclear morphology (Marchetti et al. 1996; Ellerby et al. 1997; Kantrow and Piantadosi 1997).

Another possible mechanism is through interactions between PT pore and the B-cell CLL/lymphoma 2 (Bcl-2), Bcl extra long form (Bcl-xL) and Bcl2 associated X-protein (Bax) in the outer membrane. Mitochondria isolated from Bcl-2 transfected cells demonstrated more resistant to atractylate- and peroxide-induced pore opening (Susin et al. 1996). Microinjection of fibroblasts with recombinant Bax induced mitochondria depolarization and nuclear apoptosis and these mitochondrial and nuclear effects of Bax were prevented by three prototypic inhibitors of PT pore (Marzo et al. 1998). Therefore, both pro- and anti-apoptotic Bcl-2 family proteins may interact with components of the PT pore. Co-immunoprecipitation studies have demonstrated that Bax binds to Vdac (Narita et al. 1998). Also Bcl-2 and Bcl-xL were shown to block PT pore by direct inhibition of Vdac activity (Tsujimoto et al. 2006). Bax mediated cell death was inhibited by cyclosporin A (CsA), a drug that is assumed to inhibit the pore formation by occupancy of the active site of CyP-D preventing CyP-D association with Ant (Pastorino et al. 1998; Crompton 1999). The emerging model is that Bax and, possibly, Bcl-2 and Bcl-xL may be recruited to the PT pore complex. However, a recent study has shown that Vdac is dispensable for both PT pore and Bcl-2 family member-driven cell death (Baines 2007).

The ion flow model proposes that Bcl-2 proteins have ion channel activity. The three-dimensional structure of Bcl-xL, an anti-apoptotic protein, resembles the structure of bacterial toxins that are able to insert into lipid bilayers forming channels able to conduct ions (Muchmore et al. 1996). The Bcl-xL was demonstrated to form cation-channels in lipid bilayers (Budihardjo et al. 1999). Bax also contains structural similarities to the pore-forming domains of the bacteria colicins and diphtheria toxin (Muchmore et al. 1996), and forms anion-channels in lipid bilayers (Antonsson et al. 1997). The Bax channel is large enough to admit carboxyfluorescein and interaction with Bcl-2 blocks this channel (Antonsson et al. 1997). But there is no evidence that Bax forms pores in the outer membrane sufficiently large to release fully folded apoptogenic proteins from the intermembrane space. These findings indicate that the relative proportion of pro and anti-apoptotic proteins control the ion flow. Other possibility is that Bax activation, its outer mitochondrial membrane binding and insertion followed by Bax-Bcl2 antagonist/killer (Bak) oligomerization somehow increases the local curvature stress on membranes which results in formation of lipidic pores (Kuwana and Newmeyer 2003). Basanez et al (2002) demonstrated that addition of nonlamellar lipids, or lipids that change the intrinsic curvature of the membrane, to liposomes can affect the ability of detergent-oligomerized Bax to form pores that allow the release of a fluorescent dye or Cyt c.

Bcl-2 Related Proteins

The Bcl-2 protein family is divided into two main groups according to whether they inhibit apoptosis (Bcl-2, Bcl-xL, Bcl-w, Bfl-1, Brag-1, Mcl-1, and A1) or promote apoptosis (Bax, Bak, Bcl-xs, Bad, Bid, Bik, Hrk and Bok) proteins. The Bcl-2 and Bcl-xL are the main members of this family that inhibit apoptosis, and Bax and Bad are the two best known pro-apoptotic factors (Haimovitz-Friedman A 1997). The Bcl-2 family members share one or more Bcl-2 homology domains (BH) (Kuwana and Newmeyer 2003). The pro-apoptotic family members can contain multiple BH domains (BH1, 2, 3) or only BH3. Unlike the BH1-3 domains, BH4 is conserved only among antiapoptotic Bcl-2 family members that contain all four BH1-BH4 domains (Huang et al. 1998; Petros et al. 2004). Deletion of BH4 from Bcl-2 or Bcl-xL has been shown to abrogate their antiapoptotic ability demonstrating that BH4 is crucial for this activity. The mechanisms of action of Bcl-2 proteins are not completely understood. The Bcl-2 family members interactions may involves the hydrophobic pocket formed by close arrangement of the BH1-BH3 domains (Petros et al. 2004). For example, Bax hydrophobic pocket can sequester the carboxy-terminal transmembrane region (C-terminal domain) within the same monomer (Suzuki et al. 2000). A common characteristic of Bcl-2 related proteins is the ability to produce homo- and heterodimers. The C-terminal of Bcl-xL and the hydrophobic pocket of another Bcl-xL or Bax protein can interact and form either homodimers or heterodimers (Jeong et al. 2004). The death preventing effects of Bcl-2 and the death-promoting effects of Bax depend on their ability to target and insert the membranes.

Removal of the membrane anchoring domain (C-terminal domain) decreases the efficiency of Bcl-2 and Bax as regulators of apoptosis (Hockenbery et al. 1993; Zamzami et al. 1997).

The first Bcl-2 related protein identified was Bax. In healthy cells, Bax monomer is located in the cytoplasm or loosely attached to the membranes. After the death stimulus, Bax translocates to the mitochondria and inserts into the membrane as an integral protein and forms a homodimer (Gross et al. 1998). Some studies suggest that Bax homodimers increase permeabilization of the outer mitochondrial membrane which results in release of Cyt c from the mitochondria (Rosse et al. 1998; Eskes et al. 2000). A Bax inhibiting peptide has been shown to prevent apoptosis in mouse, rat, and porcine cumulus cells induced by hormone deprivation (Yoshida et al. 2004). Whether Bax will exert its pro-apoptotic effects depends on the presence of other members of Bcl-2 family such as pro-apoptotic members Bax, NOXA, and PUMA and the anti-apoptotic members Bcl-2 and Bcl-xL (Tsujimoto 1998; Kuwana and Newmeyer 2003; Takahashi et al. 2004; Dejean et al. 2006; Kutuk and Basaga 2006; van Delft and Huang 2006). Interaction of BH3-only proteins with Bcl-2 and Bcl-xL can result in displacement of Bax/Bcl-2 or Bak/Bcl-xL binding, and therefore reactivation of Bax and Bak (Willis and Adams 2005). When Bcl-2 are in excess, Bcl2-Bcl2 homodimers and Bcl2-Bax heterodimers formation occurs and predominate to prevent apoptosis (Oltvai et al. 1993). The relative ratio of these dimers and competitive dimerization of pro- and anti-apoptotic proteins, determines that apoptotic outcome in stimulated cells.

The Bcl-2 and Bcl-xL are important inhibitors of apoptosis. There is evidence that Bcl-2 regulates calcium homeostasis (Marin et al. 1996), and prevents Cyt c release from the mitochondria (Lee et al. 2001; Sun et al. 2001). Also Bcl-xL and Bcl-w are able to prevent apoptosis following many cytotoxic stimuli. In non-stress conditions, Bcl-2 is located at the endoplasmic reticulum membrane, nuclear membrane, and outer mitochondrial membrane (Gross et al. 1999). The Bcl-xL is associated with the mitochondria or located in the cytoplasm, in some cells (Gonzalez-Garcia et al. 1994; Kaufmann et al. 2003). After an apoptotic stimulus, Bcl-xL has been shown to translocate from cytoplasm to the outer mitochondria membrane (Cuttle et al. 2001; Kaufmann et al. 2003). Both Bcl-2 and Bcl-xL preserve mitochondrial integrity, mitochondria membrane potential, outer membrane metabolite exchange, and osmotic integrity after cell death insults (Vander Heiden et al. 1997; Kowaltowski et al. 2000; Vander Heiden et al. 2001; Eliseev et al. 2003). Association of Bcl-xL with Vdac can prevent the opening of the PT pore caused by activated Bax and Bak proteins (Shimizu et al. 2000a). In addition, the BH4 domain of Bcl-2 and Bcl-xL alone has been shown to be sufficient to cause Vdac closure and prevent apoptosis (Shimizu et al. 2000a).

Activities of Bcl-2 proteins can be modulated by post-translational modifications such as phosphorylation. For example, a major identified target of the prosurvival kinase Akt is Bad that is phosphorylated by Akt at Ser-136 (Datta et al. 1997). Deprivation of survival signals results in dephosphorylation of Bad which can then bind to Bcl-xL (Yang et al. 1995; Zhou et al. 2005). Bad then inhibits the pro-survival function of Bcl-xL and

promotes cell death. Accumulating evidence suggest that some Bcl-2 family members act as molecular mediators of both apoptosis and cell cycle progression (Maddika et al. 2007). This dual function of Bcl-2 family members may be primarily governated by the multi-domain members (Zinkel et al. 2006). The Bcl-2 phosphorylation has been shown to regulate intracellular ROS levels and inhibit cell cycle progression by the delay of G1/S transition (Deng et al. 2003). In addition, Bcl-xL induce an increase of the G0 phase, enhances its arrest also a reduction in cell size and total RNA content during cell cycle arrest and entry (Janumyan et al. 2003).

Mitochondria Membrane Potential ($\Delta\Psi m$)

Mitochondria membrane potential is often used as an indicator of cellular viability. Polarity of mitochondria is a physiochemical process generated by the process of electron transport and Oxphos (Kowaltowski and Vercesi 1999; Murphy et al. 2005). Electrons, donated by nicotinamide adenine dinucleotide reduced (NADH) or reduced flavin adenine dinucleotide ($FADH_2$), are transferred down to a series of redox reactions with O_2 as the final electron acceptor. The energy from the flow of electrons is coupled to extrusion of protons from the mitochondria matrix. The extrusion of protons results in generation of $\Delta\Psi m$ and a pH gradient. Oxygen functions as the acceptor of electrons which together with protons generates water. The F_1F_0–adenosine triphosphate (ATPase) converts the proton gradient into ATP in the Oxphos pathway. The transport of electrons down the electron transport chain with extrusion of protons will cease unless the uncoupler ADP is available

as a substrate for the F_1F_0–ATPase. Lipophilic protonophores like carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) were originally classified as "uncouplers" because they could dissipate the proton gradient and maximally stimulate O_2 consumption, resulting in loss of $\Delta\Psi m$.

It remains to be elucidated whether loss of $\Delta\Psi m$ is an initiator, or an effect of apoptosis, or if it is necessary for apoptosis to occur (Ly et al. 2003). Originally, a change in $\Delta\Psi m$ was postulated to be an early event of apoptosis (Petit et al. 1995; Zamzami et al. 1995b). Lymphocytes treated with dexamethasone demonstrated a reduced uptake of 3, 3' dihexyloxacarbocyanine iodide ($DiOC_6$), a mitochondrial sensing $\Delta\Psi m$ fluoroprobe, preceding DNA fragmentation appearance (Zamzami et al. 1995b). Dexamethasone-induced thymocyte apoptosis showed both alteration on mitochondrial structure and an early decrease in $\Delta\Psi m$ measured by another $\Delta\Psi m$ fluoroprobe, 5,5',6,6'-tetrachloro-1.1',3,3'-tetraethyl benzimidazolylcarbocyanide iodide (JC-1) (Petit et al. 1995). Cell permeable BH3-peptides have also induced $\Delta\Psi m$ loss before appearance of chromatin condensation (Vieira et al. 2002). There is evidence that $\Delta\Psi m$ loss is a late and subsequent event in the apoptotic pathway. During rat thymocyte apoptosis, DNA fragmentation was detected prior any depolarization of the mitochondria of thymocytes exposed to dexamethazone (Cossarizza et al. 1994). Loss of $\Delta\Psi m$ was observed has a late event after phosphorylation of p53 and Bax translocation to the mitochondria in etoposide-induced apoptosis of L929 fibroblasts (Karpinich et al. 2002). Similarly, apoptosis induced by UV radiation in human keratinocytes showed loss of $\Delta\Psi m$ as late event in the pathway

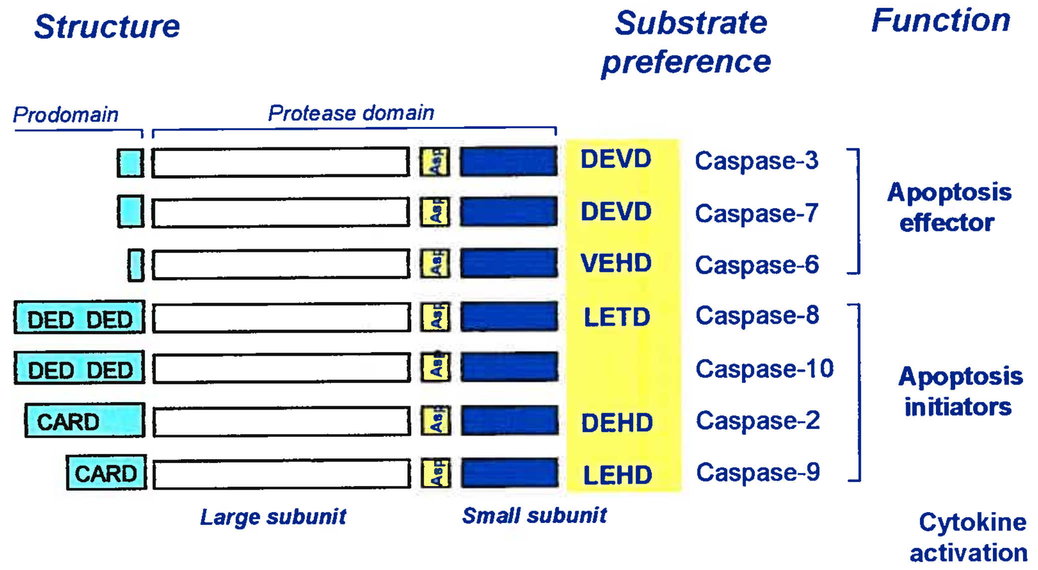
(Denning et al. 2002). In contrast, perturbation of the mitochondria or changes in $\Delta\Psi m$ was not observed during etoposide-induced apoptosis of P39 cells (Hishita et al. 2001). It is possible that loss of $\Delta\Psi m$ acts as an amplification mechanism to further augment the apoptotic process depending on cellular response to different death stimuli.

Protonophores, like FCCP, that cause loss of $\Delta\Psi m$ have been postulated as potential triggers of apoptosis. The PC12 cells treated with FCCP for 24 hours resulted in morphological changes characteristic of apoptosis, phosphatidylserine exposure, and nuclear fragmentation (Dispersyn et al. 1999). Similarly, neurons incubated with FCCP resulted in mitochondrial swelling, decreased mitochondrial motility, and DNA fragmentation (Moon et al. 2005; Safiulina et al. 2006). Reactive oxygen species are an inevitable by-product of mitochondrial respiration because not all O_2 molecules are fully reduced to water. There is also evidence that ROS causes apoptosis. Leakiness of electrons from the electron transport chain generates ROS which has been shown to induce DNA damage (Abramova et al. 2000), increase mitochondrial membrane permeability, and release of Cyt c (Skulachev 1998). Normally, Cyt c plays a role in generation of ATP via the electron transport chain (Murphy et al. 2005).

Cysteine-dependent Aspartate-specific Proteinases (Caspases)

The key apoptosis effector in mammals is a family of cysteine-dependent aspartate Asp-specific proteases with homology to the nematode (*C. elegans*) cell death gene, *ced-3*, designated as caspases (Thornberry et al. 1992). Caspases are constitutively expressed as

Figure 1. Mammalian caspase family. The caspases may be divided into three groups: the cytokines activators, the apoptotic initiators, and apoptotic executioners. The active form of caspase consists of a large (17-21 kDa) and small (10-13 kDa) subunit which contain residues that are essential for catalysis and substrate recognition. The N-terminal, also known as prodomain, is released from several caspases during activation but this is not an activating event. The cleavage between the large and small subunit generates the active protease. The N-terminal/prodomain region is not conserved between caspases, but some caspases contains structurally homologous elements required for activation. These elements are known as death effector domains (DED) and caspase recruitment domain (CARD).



Structure
Substrate preference
Function
Prodomain
Protease domain
DED DED
DED DED
CARD
CARD
DEVD
DEVD
VEHD
LETD
DEHD
LEHD
Caspase-3
Caspase-7
Caspase-6
Caspase-8
Caspase-10
Caspase-2
Caspase-9
Apoptosis effector
Apoptosis initiators
Large subunit
Small subunit
Cytokine activation

inactive proenzymes or zymogens that contain a prodomain (N-terminal prodomain), a protease domain (large subunit: ~17-21 kDa, C-terminal small subunit: ~10-13 kDa, and a short linker region between the large and small subunit) (Earnshaw et al. 1999; LeBlanc 2003). Activation of caspase occurs in a sequential manner where one caspase leads to activation of other caspases. The cascade starts with autocatalytic activation of initiator caspases that have long prodomain (such as caspase-8, -9, and -12), they cleavage and activates effector caspases with short prodomains (caspase-3, -6, and -7) (Salvesen and Dixit 1999). The main function of initiator caspases is to activate effector caspases that are responsible for dismantling cellular structures (Thornberry and Lazebnik 1998). Activation of caspases possibly involves the following two proteolytic steps at specific aspartic (Asp) acid residues (Salvesen and Dixit 1999). First cleavage occurs between the large (p20) and small subunit (p10) and removes the inter-domain linker region. Then, the prodomain is removed from the large subunit of the protease. Crystallography studies demonstrated that active caspases are heterotetramers formed by two large subunits and two small subunits (Walker et al. 1994; Rotonda et al. 1996).

Caspases can be distinguished by the requirement for an aspartate residue at the carboxy-terminal (P1 site) of the cleavage site and their substrate specificity is formed by four amino acids residues located on the amino-terminal side of the cleavage site (P4 site). The P4 site is crucial for determinant of substrate specificity (Talanian et al. 1997; Thornberry 1997). Thus, caspases are divided in three groups based on the amino acid sequence that they cleave: Group 1 have preference for WEXD motif (caspases-1, -4, and -

5); Group 2 for DEXD substrate (caspases-2, -3, and -7) and Group 3 prefer (I/L/V)EXD (caspases-6, -8, and -10), the X is any amino acid (Thornberry and Lazebnik 1998; Grutter 2000). For caspases-11, -12, -13, and -14 the specific cleavage sites are unclear. Although active caspases cleave after specific motifs recent studies have demonstrated the existence of non-consensus motifs. The caspase-7 has been shown to cleave at GELE motif of tumor necrosis factor receptor 1 (TNFR1) (Ethell et al. 2001) and caspase-3 to mediate cleavage of several non-consensus motifs within serine threonine protein kinase B (Akt) (Jahani-Asl et al. 2007). This ability to caspases to cleave at non-consensus motifs indicates that their specificity may be broader than is currently knkown.

Caspases can also be classified according with their biological function, the inflammatory caspases (Caspase-1, -4, -5, -11, and -13), initiators of apoptosis (caspases-2, -8, -9, and -10) and executioners/effectors of apoptosis (caspases-3, -6, and -7) (Stennicke and Salvesen 1998). Caspases can also be distinguished by the lengh of the prodomain (N-terminal domain). Initiators and inflammatory caspases contain long domains (up to 100 amino acids) and executioners caspases have short (less than 20 amino acids) (Earnshaw et al. 1999). There are distinct motifs within the prodomain known as death effector domain (DED) and caspase-recruitment domain (CARD) (Stennicke and Salvesen 1998). Caspases that have long prodomains such as caspase-1, -2, -4, and -9 each contain CARD (Salvesen and Dixit 1999). The CARDs of these caspases interact with CARD-containing adaptor molecules that might undergo adaptor-mediated aggregation and self activation. Caspase

with short prodomains like caspase-3, -6, and -7 depend on the upstream initiator caspases for activation.

Inhibitor of apoptosis proteins (IAPs) is a family of intracellular proteins which function as endogenous caspase inhibitors (Deveraux and Reed 1999). These proteins were first discovery in baculoviruses as suppressors of apoptosis in response to viral infection (Crook et al. 1993). Currently this family includes X-linked IAP (Xiap), human IAP-1 (Hiap-1), human IAP-2 (Hiap-2), neuronal apoptosis inhibitory protein (Niap), Survivin, Livin, and Bruce (Deveraux and Reed 1999; Kasof and Gomes 2001; Cheng et al. 2002). These proteins contain an CARD and an N-terminal baculovirus-inhibitor-of-apoptotis-repeat (BIR) motif necessary for its biological activity (Cheng et al. 2002). Apart from Naip and Survivin, these proteins also have a C-terminal RING-Zinc finger domain involved in protein-protein, protein-nucleic acid interactions and autoubiquitination. The RING zinc finger domain of Xiap and Hiap-1 is responsible for autoubiquitination and degradation of IAPs after apoptotic stimuli (Yang et al. 2000). The inhibition of caspase activity by Xiap is negatively regulated by Xiap-associated factor 1 (XAF1), Diablo/Smac, and HtrA2/Omi (Du et al. 2000; Verhagen et al. 2000; Liston et al. 2001; Suzuki et al. 2001). Overexpression of Xiap, Hiap-1, Hiap-2, Niap or Survinin has been demonstrated to block apoptosis induced by a variety of stimuli. Previous study have shown that Xiap, Hiap-1 and Hiap-2 are able to suppress apoptosis by inhibiting activation of caspase-3, -7, and -9 (Deveraux and Reed 1999). Expression of Xiap may also regulates apoptosis by involvement of the phosphatidylinositol-3'-kinase/serine threonine protein kinase B

(PI3K/Akt) survival pathway (Asselin et al. 2001). Cisplatin-induced reduction of Xiap protein levels caused activation of caspase-3, caspase-9 and cleavage of Akt resulting in activation of apoptosis in chemosensitive ovarian cancer cells. Active caspase-3 mediates the cleavage of Akt modulating the cell survival function of PI3K/Akt pathway (Jahani-Asl et al. 2007). Moreover, overexpression of Xiap increases phosphorylation of Akt which regulates its pattern of cleavage and inactivation by caspase-3 (Asselin et al. 2001; Jahani-Asl et al. 2007). The PI3/Akt pathway maintains cell survival via inhibition of apoptosis. The Akt regulates the cell survival process by phosphorylating different substrates that directly or indirectly regulate the apoptotic processes. Some of important target of Akt include phosphorylation of Bad, caspase-9, mouse double minute 2 (MDM2), and cyclic AMP response binding protein (Maddika et al. 2007). Phosphorylation by Akt of caspase-9 at Ser 196 results in conformational change that leads to inhibition of its proteolitic activity (Cardone et al. 1998). Akt also phosphorylates cyclic AMP-response-element-binding protein (CREB) that promote survival by increasing transcription of prosurvival genes like Bcl-2, Mcl-1, and Akt itself (Wang et al. 1999; Pugazhenthi et al. 2000; Reusch and Klemm 2002). Further, Akt inhibit apoptosis by increasing the degradation of p53 via phosphorylation, resulting in p53 nuclear localization and p53 binding to MDM2, a negative regulator of p53 (Mayo and Donner 2001). Recently, Akt has been shown to attenuate p53 mitochondrial accumulation and Smac, Cyt c, HtrA2/Omi release from the intermembrane space preventing apoptosis (Yang et al. 2006).

Intracellular Caspase-dependent and Caspase-independent Pathways

The mitochondrial- and receptor-mediated pathways are the two major pathways of caspase activation. Many stimuli like heat shock, oxidative stress, inhibition of protein kinases have been described to activate the mitochondrial pathway. This pathway is trigged by cellular stresses resulting in disruption of the outer mitochondrial membrane that cause release of several intermembrane space proteins (Du et al. 2000; Lorenzo and Susin 2004). Diablo/Smac and Omi/HtrA2 can facilitate caspase activation, whereas EndoG and AIF might effect DNA fragmentation even in absence of caspases (Lorenzo and Susin 2004). Once in the cytosol, Cyt c binds to the adapter protein Apaf-1 (Li et al. 1997). The binding of Cyt c/Apaf-1 results in increased affinity of Apaf-1 for dATP/ATP resulting in oligomerization of Apaf-1 and formation of a larger multimeric complex (~700 kDa) called the apoptosome (Jiang and Wang 2000; Adrain and Martin 2001). After apoptosome formation, Apaf-1 undergoes conformational change allowing the exposure of N-terminal CARD sequence of Apaf-1 to interact with the CARD sequence of procaspase-9 (Jiang and Wang 2000). This CARD-CARD interaction results in procaspase-9 autoproteolysis and self activation. Activated caspase-9 cleaves procaspase-3 leading to caspase-3 activation, the main effector caspase (Gottlieb 2000).

Diablo/Smac also remains at the mitochondrial intermembrane space until it is released at the same time as Cyt c (Du et al. 2000; Garesse and Vallejo 2001). Diablo bind and neutralize proteins of IAPs family like Xiap leading to caspase activation (Du et al. 2000; Verhagen et al. 2000; Ekert et al. 2001). The amino terminal sequences of in

Diablo/Smac bind to BIR domain of Xiap and cause its inhibition (Du et al. 2000). The XAF1 is a nuclear protein which also directly binds to Xiap (Liston et al. 2001). Xiap suppresses activation of caspases and XAF1 negatively regulate these activities. Limited activation of caspase may be required for normal cellular processes which may be controlled by XAF1. In healtly cells, XAF1 may constituvely interacts with Xiap without requirement of activation signal.

The mitochondrial intermembrane space also contains other proteins like AIF, EndoG, and Omi/HtrA2 that result in apoptosis in a caspase-independent pathway. AIF, released during apoptotic process, translocates from the mitochondria into the cytoplasm and to the nucleus where it triggers chromatin condensation as well as DNA fragmentation (Susin et al. 1999b). The EndoG is also associated with functions in DNA repair or mitochondrial DNA duplication besides its role on apoptosis. Upon apoptotic stimuli, EndoG translocates from the mitochondria to the nucleus and extensively degrades nuclear DNA into oligonucleosomal fragments, similar to caspase-effector CAD (caspase-activated DNase) (Lorenzo and Susin 2004). The Omi/HtrA2 is a nuclear-encoded mitochondrial protein with dual proapoptotic function (Suzuki et al. 2001). It is also released from the mitochondrial intermembrane space after a death signal, translocates to cytosol and binds IAPs. The binding of Omi/HtrA2 to IAPs disrupt IAP-caspase inhibitory complex which leads to caspase activation. This pathway is similar to Diablo/Smac that binds to IAPs resulting in activation of caspase-9 (Lorenzo and Susin 2004). Omi/HtrA2 also induces

apoptosis in a caspase-independent manner which is linked to its serine protease activity. This is a powerful way to ensure rapid execution of apoptosis for the mitochondria.

The tumor suppressor p53 also triggers apoptosis in response to a variety of stress stimuli (May and May 1999). The p53 can activate apoptosis by transcriptional activation of pro-apoptotic genes (e.g., Bax, Bak, Noxa, Puma), and by transcriptional repression of Bcl-2, Bcl-xL and IAPs (survivin) (May and May 1999; Bartke et al. 2001; Ryan et al. 2001; Wu et al. 2001; Hoffman et al. 2002). The p53 also induce apoptosis in a transcription-independent manner through the mitochondrial death pathway (Marchenko et al. 2000; Mihara et al. 2003). Previous study have shown that fraction of p53 protein localize to the mitochondria in tumor cells undergoing p53-dependent apoptosis (Marchenko et al. 2000). There is evidence that purified p53 target isolated mitochondria *in vitro* resulting in permeabilization of outer mitochondrial membrane and release of Cyt c (Mihara et al. 2003). Furthermore, p53 protein has also been shown to directly bind to Bcl-2 and Bcl-xL anti-apoptotic proteins (Yang et al. 2006). These anti-apoptotic proteins are known to interact and inhibit pro-apoptotic proteins Bak and Bax. It is possible that binding of p53 to Bcl-2 and Bcl-xL releases Bax and Bak allowing them to induce changes in the mitochondrial membrane, activation of caspase cascade, and promote cell death. Consistent with this proposed mechanism, excess of Bcl-xL have been reported to block p53-mediated Cyt c release (Mihara et al. 2003). Mitochondrial accumulation of p53 has been demonstrated to release Smac, Cyt c, HtrA2/Omi from mitochondria into cytoplasm inducing activation of caspases and apoptosis (Yang et al. 2006). These mitochondrial

apoptotic activities of p53 exert a rapid and direct proapoptotic role. Also amplify the transcription p53 apoptotic action which requires more time to occur.

The receptor pathway is mediated through a receptor-associated "death domain"-adaptor protein system. The binding of a ligand to the death receptors, initiates the formation of a multiprotein complex through binding of cytoplasmic proteins that contain DED motifs to the death domain receptors (Kronke 1999). For example, homotypic interactions between the DEDs of caspase-8/10 and the of an adaptor molecule, FAS-associating protein with a death domain (FADD) results in recruitment of these caspases to death receptors leading to caspase activation and subsequent cell death (Salvesen and Dixit 1999). The formation of complex is found in several receptors like tumor necrosis factor receptor-55 (TNFR-55), Fas/CD95, DR3, DR4, and DR5 (Kronke 1999; Schmitz et al. 2000).

Both pathways, mitochondrial and receptor-mediated, activate executioner caspases (caspase-3, -6, and -7) that cleave several intracellular substrates. For example, activated caspase-3 in turn activates cytosolic endonuclease caspase-activated DNAse (CAD). In physiological conditions, CAD is bound to inhibitor of CAD (ICAD) in the cytoplasm. This complex, ICAD-CAD, suppress CAD endonuclease activity. Upon apoptotic stimuli, caspase-3 cleaves ICAD from the CAD-ICAD complex, free catalytic CAD translocates to the nucleus resulting in DNA cleavage with formation of oligonucleosomal fragments (Enari et al. 1998; Sakahira et al. 1998).

Both pathways are also associated with induction of an important second messenger, ceramide. Increased intracellular levels of ceramide are induced by a variety of stress like heat stress (Verheij et al. 1996; Kondo et al. 2000), oxidative stress (Goldkorn et al. 1998), chemotherapeutic agents (Strum et al. 1994), ionizing radiation (Haimovitz-Friedman A 1997), and death receptors activation like TNFR1 (Kronke 1999). Those signals activate one or more sphingomyelinase, a sphingomyelin-specific form of phospholipase C, which hydrolyses the phosphodiester bond of sphingomyelin to generate ceramide (Haimovitz-Friedman A 1997; Sawai and Hannun 1999). The isoforms of sphingomyelinases are distinguished by optima pH. Various types of stimuli can activate both acid and neutral sphingomyelinases resulting in increased levels of ceramide within a few seconds or minutes (Chatterjee 1999).

Apoptosis and Heat Shock in Oocytes and Preimplantation Embryos

As in other cells, apoptosis in oocytes and embryos is regulated by anti- and pro-apoptotic proteins and caspases. There is evidence that the apoptotic machinery is constitutively expressed in the oocyte. The TUNEL staining associated with metaphase II chromatin was measured in 2% of mouse oocytes and 8% of human oocytes (Van Blerkom and Davis 1998). Expression and activity of the executioner caspase-3 and initiators caspase-8 and -9 have been demonstrated in mouse oocytes (Papandile et al. 2004). The mRNA expression of group I (caspase-1, -11, and -12), group II (caspase-2, -3, and -7), and group III (caspase-6, -8, and -10) caspases were identified in mouse oocytes but caspase

activity was not found (Exley et al. 1999). In addition, caspase-3 and CAD mRNA and protein were identified in mouse blastocysts (Hinck et al. 2001). Expression of anti-apoptotic members Bcl-2, Bcl-xL, and Bcl-w and pro-apoptotic Bax and Bak were detected in oocytes and embryos from zygote to blastocyst stage (Exley et al. 1999). Expression of Bcl-2 and Bcl-x mRNA was higher at the 1-cell stage and reduced levels were found as the embryo reaches the blastocyst stage (Jurisicova et al. 1998). In contrast, abundance of pro-apoptotic members Bax and Bad mRNA was reduced in zygotes and two-cell embryos and increased through the blastocyst stage (Jurisicova et al. 1998). More Bcl-2 protein than Bax protein was found in good quality 2-8 cell embryos and blastocysts whereas Bax protein levels was higher in fragmented embryos (Yang and Rajamahendran 2002).

Since mitochondria play a central role in free radical production (Thompson et al. 2000) and apoptosis (Gottlieb 2000) it is likely that it regulates oxidative-stress induced apoptosis. Reconstructed mouse zygotes were used to evaluate the role of cytoplasm in oxidative-induced apoptosis. Cytoplasm of zygotes treated with hydrogen peroxide (H_2O_2) could transfer apoptotic signals to zygotes with untreated pronuclei, whereas untreated cytoplasm could rescue H_2O_2-treated pronuclei from oxidative stress (Liu and Keefe 2000). In human, fragmented embryos demonstrated higher concentration of H_2O_2 when compared to non-fragmented embryos (Yang et al. 1998). There is evidence for the role of antioxidants in stimulating embryo development. Addition of molecules with antioxidant properties, such as β-mercaptoethanol (Takahashi et al. 2002) and hypotaurine (Fujitani et al. 1997), to culture medium increased development of embryos exposed to high oxygen.

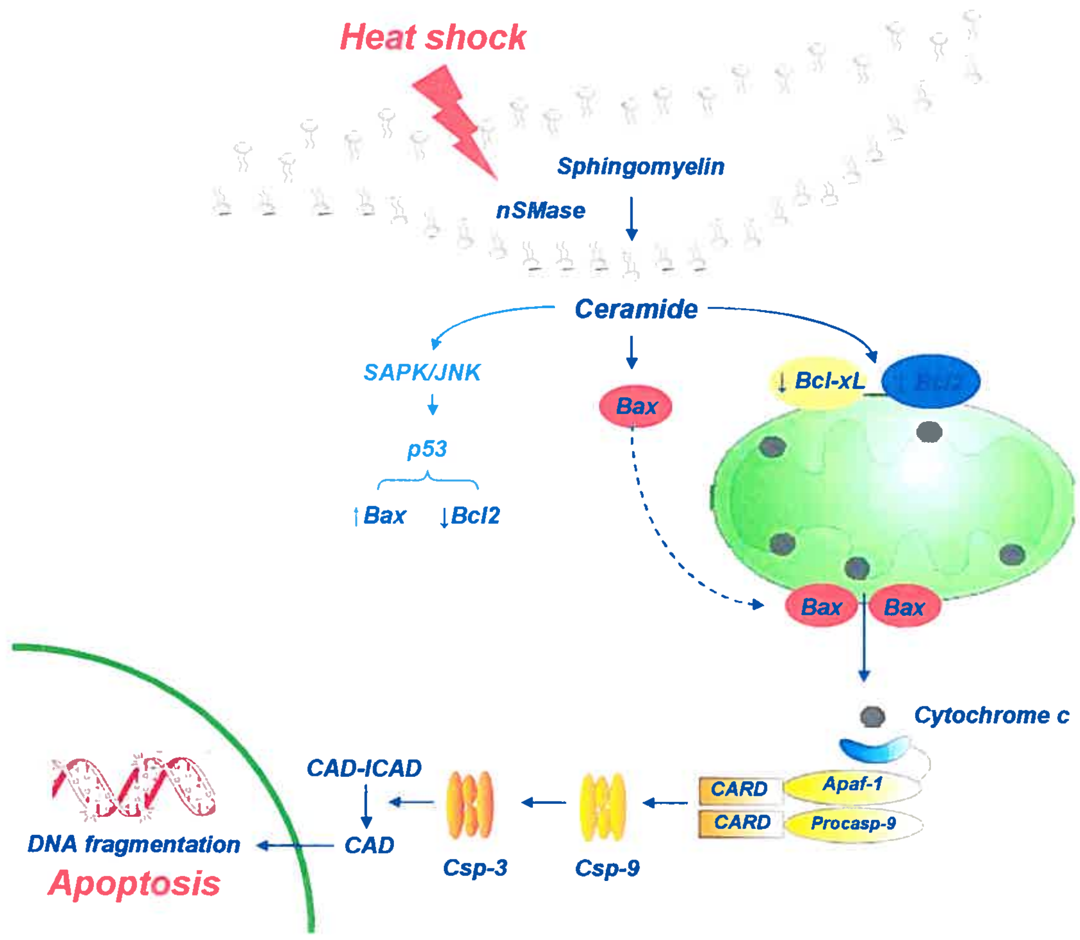
A variety of stimuli have been demonstrated to induce apoptosis in both oocytes and embryos. Hydrogen peroxide induced apoptosis resulted in Bax protein expression, DNA fragmentation, and caspase-3 activation in oocytes in which morphological apoptotic changes were observed (Chaube et al. 2005). Resting follicles isolated from human adult ovaries treated with H_2O_2 resulted in induction of apoptosis in oocytes by detection of DNA fragments, loss of $\Delta\Psi m$, and Cyt c release (Zhang et al. 2006). Cumulus oocyte-complexes exposed to heat shock during the first 12 hours of maturation resulted in increased group II caspase activity, increased proportion of TUNEL-positive cells in blastocysts, and reduced embryonic development (Roth and Hansen 2004). Cryopreserved bovine oocytes undergo apoptosis after culture through activation of caspase-3 and DNA cleavage (Men et al. 2003). Microinjection of pro-apoptotic factors such as recombinant Bax protein or Bcl-xS cRNA into isolated oocytes was capable of induction of apoptosis (Morita et al. 2000; Braun et al. 2003). Also targeting the expression of the anti-apoptotic Bcl-2 in mouse oocytes prevented apoptosis induced by doxorubicin (Morita et al. 1999). Exposure of mouse zygotes to H_2O_2 resulted in cell shrinkage, diffuse cytoplasmic Cyt c consistent with release of Cyt c from mitochondria, active caspase-3 positive staining, reduced mitochondrial membrane potential, and TUNEL-positive staining in condensed nuclei (Liu et al. 2000). The protein kinase inhibitor staurosporine induced TUNEL-positive staining on mouse embryos at 1-4 cell stage (Weil et al. 1996) and bovine embryos at 1-16 cell stage (Matwee et al. 2001). Zygotes exposed to staurosporine showed increase activity of group II caspases (Exley et al. 1999). Mouse embryos in which apoptosis was

induced by staurosporine or in fragmented embryos translocation of the Diablo/Smac from mitochondria into the cytoplasm was observed (Honda et al. 2005). Tumor necrosis factor-alpha and arsenic were able to induce apoptosis in embryos $\geq$ 16-cells but not in embryos at the two-cell stage (Krininger et al. 2002; Soto et al. 2003). Bovine and porcine embryos exposed to heat shock showed increased incidence of TUNEL-positive blastomeres (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).

Heat Induced Apoptosis

Cellular insult leads to activation of two interconnected and opposite pathways: for cellular survival by induction of heat shock proteins and for apoptosis through activation of caspases. The balance between these two pathways determines whether the cell lives or dies. Heat shock induces apoptosis in several different cell types including the female and male germ cells (Roth and Hansen 2004; 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 such as 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. 2000), human leukemic monocyte lymphoma cells (Kim et al. 2005) and adenocarcinoma cervical cells (Bettaieb and Averill-Bates 2005).

Figure 2. Heat induced apoptosis. Heat shock induces activation of neutral sphingomyelinase and hydrolysis of sphingomyelin to generate ceramide. Ceramide causes translocation of pro-apoptotic Bax from the cytoplasm to the mitochondria and reduction in anti-apoptotic Bcl-2/Bcl-xL protein in mitochondrial membranes. Once in the mitochondria Bax homodimers are formed and may cause increase in mitochondria membrane permeability allowing the release of pro-apoptotic proteins, such as cytochrome c. The interaction of cytochrome c and Apaf-1 induce autoactivation of procaspase-9. Active caspase-9 in turn cleaves procaspase-3. Activate caspase-3 can cleave several substrates like CAD-ICAD complex to generate active CAD which cleaves the DNA. Ceramide also induce activation of SAPK/JNK that in turn activates p53. Active p53 up regulates Bax and downregulates Bcl-2 transcription.


Heat shock
Sphingomyelin
nSMase
Ceramide
SAPK/JNK
p53
↑Bax ↓Bcl2
Bax
↓Bcl-xL
Bax
Bax
Cytochrome c
CARD
Apaf-1
CARD
Procasp-9
Csp-9
Csp-3
CAD-ICAD
CAD
DNA fragmentation
Apoptosis

The mechanisms of heat-induced apoptosis are associated with the second messenger ceramide and the mitochondrial pathway activation of caspases. A previous study has demonstrated that heat-shock induces activation of neutral sphingomyelinases to generate ceramide, induces mitochondrial release of Cyt c, and activation of caspases (Mirkes and Little 2000). Once ceramide intracellular levels are increased, it can activate apoptosis in different points of this cascade. Ceramide induces: Bax to translocate to the mitochondrial membrane (Kim et al. 2001; Bettaieb and Averill-Bates 2005; Stankiewicz et al. 2005), decrease amount of Bcl-2 protein (Kim et al. 2001), induce mitochondrial PT pore activation (Qian et al. 2004; Bettaieb and Averill-Bates 2005; Stankiewicz et al. 2005), decrease mitochondrial membrane potential (Ghafourifar et al. 1999), release of pro-apoptotic factor Cyt c (Ghafourifar et al. 1999; Mirkes and Little 2000; Kim et al. 2001; Little and Mirkes 2002; Qian et al. 2004; Bettaieb and Averill-Bates 2005; Wada et al. 2005), and activates the stress-activated protein kinases/c-jun N-terminal kinases (SAPK/JNK) cascade (Verheij et al. 1996; Mosser et al. 1997; Buzzard et al. 1998). Thus, SAPK/JNK phosphorylates and activates the transcription factor p53 that translocates to the nucleus upregulating Bax gene and downregulating Bcl-2 gene (May and May 1999). The role of Bax mediating ceramide-induced apoptosis has been demonstrated. Treatment of HL-60 cells with Bax antisense mRNA decreased the ceramide-induced increase in percentage of apoptotic cells, DNA-fragmentation, and release of mitochondrial Cyt c (Kim et al. 2001). Moreover, treating HL-60 cells with a broad spectrum caspase inhibitor

blocked ceramide-induced apoptosis without affect on Bax translocation to the mitochondria or Cyt c release (Kim et al. 2001).

Heat-induced apoptosis may directly activate the tumor suppressor protein p53. Heat shock has been demonstrated to induce a 24 hours cell arrest in normal human fibroblasts associated with nuclear accumulation of p53 protein (Nitta et al. 1997). In human glioblastoma cells, accumulation of p53 by heat stress was associated with heat shock proteins (HSPs) family members (Matsumoto et al. 1994). The outcome of p53 may be either cell arrest or apoptotic death. The cell cycle arrest function probably acts has a mechanism by which cells are protected from apoptosis by allowing the time for repair of damaged induced by stresses. The decision between cell arrest and apoptosis may depend on the cell type and cellular damage induced (Ko and Prives 1996). It has been proposed that the effect of thermal stress is mediated by both increased HSPs and modulation of intracellular p53 function (Chen et al. 1999).

The HSPs are major regulators of apoptosis responses induced by heat shock. Blocking HSP70 synthesis through utilization of mRNA antisense resulted in increased percentage of Molt-4 tumor cells undergoing apoptosis (Wei et al. 1995). High levels of constitutive or inducible forms of HSPs were able to protect cells from heat-induced apoptosis through prevention of SAPK/JNK, procaspase-9, and procaspase-3 activation (Mosser et al. 2000). These experiments show that HSPs are associated with protection of heat-induced apoptosis. The HSPs also plays a role on apoptosome formation. In heat treated cells, HSP70 and HSP90 have been shown to precipitate with Apaf-1 but not in

control cells (Saleh et al. 2000). The HSP70 can directly bind to Apaf-1 preventing the apoptosome formation and caspase activation (Beere et al. 2000; Saleh et al. 2000). It has been demonstrated that HSP70 interacts with the CARD sequence of Apaf-1 inhibiting oligomerization of Apaf-1 and subsequent recruitment of procaspase-9 (Saleh et al. 2000). The Apaf-1 may be not the only target of HSP70 since overexpression of HSP70 prevented serum-withdrawl induced apoptosis in Apaf-1$^{(-/-)}$ cells. The HSPs may also interact with AIF preventing chromatin condensation induced by AIF (Ravagnan et al. 2001). In addition, HSP70 protected cells undergoing AIF induced-apoptosis and antisense HSP70 that reduces expression of endogenous HSP70 made cells more susceptible to AIF (Ravagnan et al. 2001).

Effect of Heat Shock on Oocyte Competence

There is evidence that oocytes are negatively affected by heat stress. Oocytes harvested from follicles of Holstein cows during summer demonstrated decreased ability to develop to the blastocyst stage after in vitro fertilization compared to oocytes harvested during winter (Rocha et al. 1998; Al-Katanani et al. 2002). Holstein heifers exposed to heat stress between the onset of estrus and insemination increased the proportion of abnormal and retarded embryos than heifers maintained at thermoneutrality (Putney et al. 1988). The number of oocytes classified as normal and their competence to reach the blastocyst stage was decreased in oocytes collected during the hot time of the year when compared to oocytes collected during the cool season (Rocha et al. 1998). The heat-induced reduction

on oocyte competence in cows can continue past summer months demonstrating the delayed effects of elevated temperature on oocyte competence (Roth et al. 2001; Roth et al. 2002).

Direct effects of elevated temperature on oocyte competence also have been demonstrated *in vitro*. Heat shock reduced the ability of the oocyte to reach blastocyst stage (Edwards and Hansen 1997; Roth and Hansen 2004), decreased protein synthesis (Edwards and Hansen 1996), caused nuclear maturation arrest (Payton et al. 2004; Roth and Hansen 2005), and induced apoptosis (Roth and Hansen 2004). Alteration in spindle formation and disruption in chromosome alignment can mediate the negative effects of heat shock (Ju et al. 2005; Roth and Hansen 2005). Metaphase II oocytes exposed to thermal stress for 1 to 4 hours resulted in 5-fold increase in spindles with altered morphology compared to control (Ju et al. 2005). Also metaphase I oocytes exposed to heat shock showed reduced capacity to reach metaphase II and had spindle malformation (Roth and Hansen 2005). The induction of apoptosis in oocytes by heat shock has been controversial. In some studies, heat shock during maturation of bovine oocytes activated group II caspases, induced apoptosis (Roth and Hansen 2004) and reduced subsequent embryonic development (Edwards and Hansen 1996; Payton et al. 2004; Ju et al. 2005). In another study, exposure of pig oocytes to 41.5°C reduced development to the blastocyst stage but did not induce apoptosis (Tseng et al. 2006). Also, the timing of the block in development induced by heat shock in the oocyte is not well established. One study showed a reduced oocyte competence to cleave and develop to blastocyst stage (Roth and Hansen 2004) and

other studies demonstrated reduced embryonic development but not cleavage rate (Edwards and Hansen 1996; Payton et al. 2004; Ju et al. 2005).

Effect of Heat Shock on Development of Preimplantation Embryos

Like the oocyte, early embryos are also susceptible to negative effects of heat shock. Previous studies have shown that heat shock can increase the incidence of TUNEL-positive cells in bovine and porcine embryos (Paula-Lopes and Hansen 2002b; Jousan and Hansen 2004; Isom et al. 2007; Jousan and Hansen 2007; Loureiro et al. 2007). Cultured bovine embryos are less susceptible to deleterious effect of heat shock at later stages of development indicating that apoptosis is a developmentally-regulated phenomenon (Paula-Lopes and Hansen 2002b; Sakatani et al. 2004). Two-cell and 4-cell stage embryos exposed to heat shock failed to exhibit increased percentage of TUNEL-positive cells or activation of group II caspases (Paula-Lopes and Hansen 2002b). In contrast, heat stressed embryos ≥ 16-cells showed an increase in the incidence of TUNEL-positive cells, caspase-9 activity, and group II caspase activity (Paula-Lopes and Hansen 2002b; Paula-Lopes and Hansen 2002a; Loureiro et al. 2007). Caspase inhibitors reduced caspase-9, group II caspase activity, and percentage of TUNEL-positive cells (Paula-Lopes and Hansen 2002a; Loureiro et al. 2007). Two-cell stage embryos exposed to 41°C caused a shift in localization of organelles located in the periphery towards the center of the cell caused by disruption of microtubules and microfilaments (Rivera et al. 2003; Rivera et al. 2004b). Heat shock also resulted in increased number of swollen mitochondria in two-cell embryos,

compatible with membrane depolarization, reduced energy production by Oxphos and potential production of ROS (Rivera et al. 2004b). The percentage of day 4 or day 5 bovine embryos that develop to the blastocyst stage was reduced by heat shock (Paula-Lopes and Hansen 2002a; Jousan and Hansen 2004). In the majority of nuclei in blastocysts exposed to heat shock a nuclear localization of p53 protein was observed compared to control group were p53 was only detected at the cytoplasm (Matwee et al. 2001). The p53 is thought to be essential for the up-regulation of genes involved in apoptosis and cell cycle arrest and directly activates the apoptotic mitochondrial pathway (Mihara et al. 2003; Yang et al. 2006).

The HSPs are likely to protect oocytes and embryos from cell death. Microinjection of HSP70 mRNA in mouse oocytes increased the resistance to heat shock (Hendrey and Kola 1991). Culture of two-cell mouse embryos in presence of monoclonal antibodies specific for the mammalian 60 kDa (HSP60), HSP70, and 90 kDa (HSP90) heat shock proteins resulted in reduced embryo development at unique development stages (Neuer et al. 1998). Exposure of bovine embryos from day 3 to day 9 to HSP70 antibody resulted in decreased development to blastocyst stage and increased apoptosis (Matwee et al. 2001). Thus, antibodies to HSPs may make embryos more susceptible to apoptotic cell death. When bovine blastocysts were exposed to 42°C for 6 hours a reduction on the proportion of apoptotic cells was observed which may be attributed to increase in HSPs synthesis (Matwee et al. 2001).

Objective and Hypothesis

Oocyte mitochondria are potential sites of insults that may affect capacity of the oocyte to develop into a viable embryo. Experiments described in the present dissertation have the overall hypothesis of stress-induced dysfunction of oocyte mitochondria triggers apoptotic events resulting in increased embryonic loss. The following three chapters contain a series of experiments with specific objectives and hypothesis, each contributing to the overall hypothesis.

The specific objective in Chapter 2 was to test the hypothesis that stress-induced dysfunction of oocyte mitochondria activates the apoptotic machinery and compromises subsequent embryonic development. To test this hypothesis, COCs were exposed to heat shock or FCCP, both stresses expected to cause negative effects in oocyte and embryos part due to mitochondrial dysfunction. To further address the mitochondria PT pore in effects of heat-induced apoptosis of oocytes, experiments were performed to test if effects of heat shock could be attenuated by use of cyclosporin A, an inhibitor of mitochondrial PT pore opening.

Because activation of caspases plays a pivotal role in apoptotic process, the objective of Chapter 3 was to test the hypothesis that heat shock mediated apoptosis during oocyte maturation induces oocyte mitochondrial dysfunction with consequent activation of caspase-9 and caspase-3/7. This hypothesis was tested by the measurement of each activated caspase and by the use of specific and broad caspase inhibitors.

The attention on Chapter 4 was on the role of some members of Bcl-2 family on effects of heat shock in the oocyte. The hypothesis was tested that the BH4 domain of Bcl-xL (BH4-TAT) and Bax inhibitor peptide (BIP) prevent the apoptotic mitochondrial changes caused by heat shock on bovine oocytes. Experiments were performed to determine whether these antiapoptotic peptides (BH4-TAT and BIP) reduced the incidence of apoptosis in heat stressed oocytes and derived blastocysts and reestablished embryo development.

CHAPTER II

HEAT STRESS AND CARBONYL CYANIDE 4-(TRIFLUOROMETHOXY) PHENYLHYDRAZONE (FCCP) DURING OOCYTE MATURATION REDUCE DEVELOPMENT AND INDUCE APOPTOSIS BY ALTERING MITOCHONDRIAL AND APOPTOTIC GENE EXPRESSION

Short Title: Heat stress and FCCP induces apoptosis in oocytes

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Abstract

Mitochondrial dysfunction in oocytes has been associated with decreased embryonic developmental potential. Experiments were performed to examine whether heat shock (HS) and carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), a cyanide analog that reduces mitochondrial membrane potential, negatively affect preimplantation development, induce apoptosis, or alter the expression of apoptotic and mitochondrial genes. Bovine cumulus-oocytes complexes (COCs) were matured for 9 h at 41°C followed by 15 h at 39°C (HS9) or 22 h at 41°C (HS22) then fertilized and cultured at 39°C. The HS9 or HS22 treatment reduced the number of oocytes developing to the blastocyst stage and increased the percentage of TUNEL-positive nuclei of oocytes and blastocysts. In oocytes exposed to HS22, the steady state amounts of mRNA for Bax was decreased. Oocytes subjected to HS22 and treated with cyclosporin A (CsA), an inhibitor of mitochondrial permeability transition pores, had reduced frequency of oocytes that were TUNEL-positive but embryonic development was not restored. Further, COCs were matured with various concentrations of FCCP for 22 h and then fertilized and cultured without FCCP. The percentage of oocytes that developed to blastocyst stage was reduced at concentrations of 1, 10 and 100 μM. The percentage of TUNEL-positive oocytes was increased at all FCCP concentrations. In embryos, 1 and 10 μM FCCP increased the percentage of TUNEL-positive blastomeres. There was a decrease in Bcl-xL, HSP70.1, and ND6 mRNA expression in FCCP derived expanded blastocysts. In conclusion, HS and

FCCP have deleterious actions on oocyte maturation that compromises the development of the resulting embryo.

Introduction

Oocyte developmental competence depends on a well orchestrated communication between nuclear and cytoplasmic components (Gandolfi and Gandolfi 2001). Functional mitochondria are essential in determining oocyte and embryo quality, developmental potential, and reproductive performance (Smith and Alcivar 1993; Cummins 2001a; Cummins 2004). Mammalian oocytes contain thousands of nonreplicating mitochondria that are maternally inherited and act as the founding population of all daughter-cell mitochondria of developing embryo (Piko and Matsumoto 1976; Van Blerkom 2004). These semi-autonomous organelles carry their own genome (mtDNA) and are largely responsible for the generation of energy in the form of cellular ATP, for calcium signaling, metabolism, as well as controlling apoptosis by integrating numerous cell death signals (Garesse and Vallejo 2001; Chan 2006). Consequently, any condition that negatively affects mitochondrial function and differentiation during the preimplantation period is expected to have direct developmental significance for the embryo, even if only a portion of the mitochondria are affected (Van Blerkom and Davis 1998). However, little is known about the pathophysiology of oocyte mitochondria and the consequences on fertility (Thouas et al. 2004).

Apoptosis, a form of programmed cell death, is an organized cellular suicide critical for proper embryonic development and tissue homeostasis (Jacobson et al. 1997; Danial and Korsmeyer 2004). Whether apoptosis occurs depends upon the actions and interactions of at least 100 gene products that either repress or activate the process of cellular self-destruction many of which are expressed during oocyte and preimplantation embryonic development (Jurisicova and Acton 2004). Although it has been shown that programmed cell death is the mechanism underlying the loss of oocytes from ovarian pool, the process of oocyte apoptosis is not completely understood (Morita and Tilly 1999; Reynaud and Driancourt 2000). Mitochondria play a crucial role in this process. In most but not all cases, the death signals converge to bring about mitochondrial outer membrane permeabilization (MOMP) an event that releases cytochrome c and other proapoptotic factors into the cytosol, resulting in caspase activation and apoptosis (Reynaud and Driancourt 2000; Lemasters 2005). The intermembrane space of mitochondria also contains other proteins that can induce apoptosis in a caspase-independent manner (Lorenzo and Susin 2004). MOMP can be also controlled by the Bcl-2 family of proteins, which can promote (Bax, Bak, Mtd, BH3-only proteins) or suppress (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1) apoptosis (Morita and Tilly 1999).

Various pathological or pharmacological stimuli can initiate apoptosis in oocytes and embryos, including hydrogen peroxide (Zhang et al. 2006), chemotherapy (Perez et al. 1997), environmental chemicals (Matikainen et al. 2001), and heat shock (HS) (Paula-Lopes and Hansen 2002b; Roth and Hansen 2004). Exposure of cultured cumulus-oocyte

complexes (COCs) to elevated temperature during maturation decreased cleavage rate and the proportion of oocytes that became blastocysts (Edwards and Hansen 1997). In contrast, HS of 41.5°C did not induce apoptosis of pig oocytes but nonetheless reduced embryonic development (Tseng et al. 2006). Bovine COCs exposed to elevated temperature during the first 12 h of maturation demonstrated an increase in percentage of TUNEL-positive blastomeres and disruption of embryonic development (Roth and Hansen 2004). Exposure of postimplantation mouse embryos to 43°C for 15 min induced the release of cytochrome c from the mitochondria and induced caspase activation (Mirkes and Little 2000). Also, HS induced ultrastructural alterations in 2-cell bovine embryos resulting in displaced organelles and increased percentage of swollen mitochondria (Rivera et al. 2003).

Heat shock proteins (HSP) have been implicated in protection against heat-induced apoptosis (Mosser et al. 1997; Mosser et al. 2000). Microinjection of HSP70 mRNA to mouse oocytes increases the resistance to heat shock (Hendrey and Kola 1991). Bovine embryos cultured with antibody specific to HSP70 from days 3 to 9 after insemination displayed decreased development to the blastocyst stage and an increased percentage of apoptotic cells in surviving blastocysts compared to untreated controls (Matwee et al. 2001).

Little is known neither about the mechanisms of stress-induced mitochondria injury in COCs nor about its consequences for continued development. The purpose of this study was to test the hypothesis that stress-induced dysfunction of oocyte mitochondria activates the apoptotic machinery and compromises subsequent embryonic development. Objectives

were to determine whether oocyte mitochondrial dysfunction during maturation of bovine oocytes induced by either HS or FCCP 1) decreases developmental capacity of oocytes, reflected by the capacity of the oocyte to cleave and develop to blastocyst stage, increases the incidence of oocyte and blastocyst apoptosis, and alters the number of blastocyst cells 2) whether these changes are associated with altered expression of genes relevant to mitochondrial transcription function and apoptotic-related genes.

Materials and methods

Materials

Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) was purchased from Sigma (St. Louis, MO). Cyclosporin A (CsA) was from Calbiochem (Mississaga, ON). Unless otherwise mentioned the products were purchased from Gibco/Invitrogen (Burlington, ON). Oocyte washing medium (OWM) was tissue culture medium 199 with Earle's salts, with L-glutamine, with 2,200 mg/ml sodium bicarbonate, with 25 mM HEPES buffer supplemented with 10% (v/v) bovine fetal serum, 50 µg/ml gentamicin (Sigma), 0.2 mM sodium pyruvate. Oocyte maturation medium (OMM) was TCM-199 with Earle's salt , with L-glutamine, with 2,200 mg/ml sodium bicarbonate and supplemented with 10% (v/v) bovine fetal serum, 1 µl/ml estradiol 17 (Sigma), 0.5 µg/ml FSH (Follitropin-V, Vetrepharm, London, CA), 50 µg/ml LH (Intervet, Whitby, ON), 50 µg/ml gentamicin (Sigma), 0.2 mM sodium pyruvate. Essentially fatty-acid free bovine serum albumin (EFAF-BSA), pronase, and hyaluronidase were purchased from Sigma.

Percoll was from Amersham Pharmacia Biotech (Uppsala, Sweden). Frozen semen was donated from CIAQ (St-Hyacinthe, CA). Embryo culture medium (Gardner et al. 1994) (all from Sigma) was modified synthetic oviductal fluid (mSOF; 108 mM NaCl, 7.2 mM KCl, 0.5 mM $MgCl_2$m, 2.5 mM $NaHCO_3$, 1.7 mM $CaCl_2$-H_2O, 0.5 mM glucose, 0.33 mM pyruvic acid, 3 mM lactic acid, 8 mg/ml BSA, 150 µg/ml gentamicin, and 0.01% phenol red. It was supplemented with 1.4 mM glycine (Sigma), 0.4 mM alanine, 1 mM glutamine, 1% non-essential amino acids, 2% essential amino acids (Sigma).

In Situ Cell Death Detection Kit TMR red was obtained from Roche Diagnostics Corporation (Laval, QC). Hoechst 33342 and polyvinylpyrrolidone (PVP) were purchased from Sigma. Prolong Antifade Kit was obtained from Molecular Probes (Eugene, OR), RQ1 RNA-free DNase was from Promega (Madison, WI), and RNase A was from Qiagen (Mississauga, ON). And 60-well Terasaki polystyrene microtest plates were obtained from Sarstedt (Montreal, QC).

Random hexamer primers and Platinum® taq DNA polymerase high fidelity were purchased from Invitrogen. The Pico Pure RNA Isolation kit was from Arcturus (Mountain View, CA). Sensiscript RT kit, Gel Extraction Kit, QIAprep Spin Miniprep kit, and QIAquick PCR purification kit were obtained from Qiagen. The p-GEM Easy Factor System I was from Promega (Nepean, CA).

Oocyte maturation, fertilization and embryo culture

Embryos were produced based on procedures previously described (Parrish et al. 1986). Briefly, ovaries were obtained from slaughtered cows and used to obtain COCs by aspiration of 2-10 mm follicles. Groups of 10 COCs were transferred to pre-equilibrated 50 µl drops of OMM and matured for 22 hr at 39°C in an atmosphere of 5% (v/v) CO_2 in humidified air. After maturation, COCs were washed and placed in groups of 30 per 100 µl drop of Tyrode's medium (Parrish et al. 1986) supplemented with 6 mg/ml fatty-acid free bovine serum albumin (BSA; Sigma), 2 µg/ml heparin (Sigma), 10 mM pyruvic acid (Sigma) and 50 µg/ml gentamycin (Sigma). For *in vitro* fertilization (IVF) frozen semen was thawed, processed by Percoll gradient, and ~1 x 10^6 Percoll-purified spermatozoa were placed in each 100 µl drop. Fertilization was allowed to proceed for 18-20 hrs at 39°C and 5% (v/v) CO_2 in humidified air. Presumptive zygotes were then removed from fertilization drops, denuded of cumulus cells by vortexing in 50 µl of 0.2% hyaluronidase solution for 5 minutes, and cultured in pre-equilibrated 50 µl drops of mSOF at 39°C in humidified atmosphere of 5% CO_2 and 5% O_2 for 8 days. The number of cleaved oocytes and blastocysts were determined on days 3 and 8 after fertilization, respectively. Matured oocytes were harvested after 22 hr of maturation and developing blastocysts on day 8 after fertilization for further analysis.

TUNEL labeling

DNA fragmentation was determined by means of TUNEL procedure. This procedure was performed as previously described with some modifications (Soto et al. 2003; Boelhauve et al. 2005). Briefly, oocytes were denuded from their zona pellucidae by 5-10 min exposure to 0.5% pronase at room temperature. Subsequently, oocytes and embryos were fixed in 4% (w/v) paraformaldehyde and stored at 4°C until TUNEL assay. TUNEL procedure was initiated by permeabilization of oocytes or embryos with 0.5% (v/v) Triton X-100 containing 0.1% (w/v) sodium citrate for 1 hour at room temperature. Positive controls were incubated with 10 µl drop RQ1 RNase-free DNase (50 U/ml) at 37°C for 1 hour. Oocytes and embryos were washed in PBS-PVP and incubated in 20 µl drop of TUNEL reaction mixture (containing fluorescein isothiocyanate-conjugated dUTP and terminal deoxynucleotidyl transferase) for 1 hour in the dark. Negative controls were incubated in absence of terminal deoxynucleotidyltransferase. Oocytes and embryos were washed in PBS-PVP and transferred to 20 µl drops of 1 µg/ml Hoechst 33342, placed in polylysine-coated slides, and coverslips mounted using 15 µl mounting medium containing Antifade. For all 20 µl drop incubations, 60 well plates were used. TUNEL labeling was observed using a Leica MDLA microdissection microscope (Cambridge, UK) with dual filter. Images were acquired using IM50 software and Leica DC 500 digital camera. Each oocyte was analyzed for number of TUNEL-labeled pronuclei and blastocysts were analyzed for total number of cells and number of TUNEL-labeled nuclei.

RNA extraction, purification and reverse transcriptase (RT) reaction

Individual denuded oocytes and expanded blastocyst stage embryos were homogenized in extraction buffer (Arcturus) and RNA was purified using a Pico Pure RNA Isolation kit, as recommended by the manufacturer. Total RNA was eluted in 10 μl and used for RT using Sensiscript RT kit according to the manufacture's instructions.

Bovine specific cDNA cloning

Bovine specific primers for Bcl2 associated X-protein [Bax; (Lonergan et al. 2003)], Bcl2 related protein, long isoform [Bcl-xL; GenBank Accession No. AF245489], heat shock protein 70 kDa [HSP 70.1; (Lazzari et al. 2002)], mitochondrial NADH dehydrogenase subunit 6 (ND6), and cytochrome oxidase 1 (CO1, GenBank Accession No. V00654) were used (Table 1). PCR products of expected size, obtained following amplification with the primers were excised and purified using a Gel Extraction Kit then ligated into a pGEM-T Easy Factor System I according to the instructions of the manufacturer, and further transformed into competent *Escherichia coli* strain XL-1 blue. Plasmids were isolated by the use of a QIAprep Spin Miniprep kit and sequenced using a ABI PRISM 310 sequencer (Applied Biosystems, Foster City, CA), and at least 3 independent samples were sequenced for verification of identity. Bovine specific primers for glyceraldehyde 3-phosphate dehydrogenase (Gapdh) were used (GenBank Accession No. AF077815) as a control (Table 1).

Quantitative RT-PCR of oocytes and expanded blastocysts

To analyze mRNA steady state amounts of Bax, Bcl-xL, HSP 70.1, ND6, CO1, and Gapdh in mature oocytes and day 8 expanded blastocysts, specific quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays were performed as described earlier (Parrott and Skinner 1998; Arnold et al. 2006). Briefly, PCR products using gene specific primers (Table 1) were pooled and QIAquick PCR purification kit (Qiagen) was used to generate standard curve ranging from 0.001 fg/ μl to 100 fg/ μl. Five known concentrations and samples were subjected to PCR amplification in total 50 μl of PCR master mix [0.2 μM gene specific primers; 0.2 μM of dNTP's; 2 mM $MgSO_4$; 5 μl 10X PCR buffer containing 600 mM Tris-SO_4 (pH 8.9), 180 mM ammonium sulfate] using 1/20 or 2/20 of single oocyte or embryo equivalent cDNA (Table 2). After initial denaturation for 3 min at 94°C, samples were subjected to temperature cycles of 30 sec at 94°C; 40-50 sec at gene dependent anneling temperature (Table 2); and 45 sec at 68°C with final elongation at 68°C for 2 min. For each gene, optimal cycle number for amplification during the exponential phase was determined. Cycle numbers for individual oocytes samples were: Bax, 38; Bcl-xL, 40; HSP 70.1, 39; ND6, 32; CO1, 31; and Gapdh, 37. And for individual expanded blastocyst were: Bax, 34; Bcl-xL, 37; HSP 70.1, 35; ND6, 27; CO1, 27; and Gapdh, 28. PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide. Densities of the amplified fragments were calculated with a computer imaging system and analyzed with Macintosh NIH software - ImageJ (National Institute of Health, Bethesda, MD). Values for each gene were calculated by comparing

them to gene specific standard curves. Sample values were calculated using the formula: Y= A*Ln(X) + B, where Y= fg of gene, A= slope Ln(X)= natural log of the density units measured, and b= the y intercept. The results were expressed as fg of gene of interest/fg of Gapdh.

Heat shock during maturation on oocyte competence

The COCs were collected, matured for HS9 or HS22 and fertilized and cultured at 39°C. Cleavage rate was assessed at day 3 and development to blastocyst stage at day 8 postinsemination. This experiment was replicated three times using 107-169 oocytes/treatment.

Heat induced apoptosis in oocytes and blastocysts

To evaluate the effects of HS on apoptosis, COCs were matured at either 39°C or 41°C for the first 9 h or 22 h followed by 39°C. At the end of 22 h of maturation, COCs were denuded from the cumulus cells with 0.2% hyaluronidase and denuded oocytes were washed in OWM twice to remove the remaining cumulus cells. Oocytes were transferred to 20 μl drops of 0.5% pronase to remove the zona pellucida for 5-10 min at room temperature. Oocytes and blastocysts were fixed at 4% (w/v) paraformaldehyde and kept at 4°C in PBS-PVP until TUNEL assay performed. A total of 33-44 oocytes/treatment was analyzed in three replicates. In addition, a subset of blastocysts produced from oocytes

HS9 or HS22 were subjected to TUNEL analysis (29-34 embryos/treatment in three replicates).

Effect of heat shock on transcripts levels in oocytes and expanded blastocysts

The purpose was to determine the effects of heat shock on transcript abundance of apoptotic and mitochondrial related genes. COCs were matured at HS22. After 22 h maturation, a subset of oocytes were denuded from the cumulus cells by 0.2% hyaluronidase and frozen at -80°C until further analyses. Another subset of oocytes was subjected to *in vitro* fertilization and culture at 39°C. Expanded blastocysts were harvested at day 8 postinsemination and frozen at -80°C. For each treatment group, individually oocytes and expanded blastocysts were analyzed (6 oocytes or blastocyst/treatment).

Effect of cyclosporin A (CsA) on heat stressed induced-apoptosis on oocytes

The objective was to evaluate whether CsA, an inhibitor of mitochondrial permeability transition pore formation, disrupts the deleterious effects of heat shock induced apoptosis on bovine oocyte. COCs were matured at in the presence of 15 μM, 30 μM, or 60 μM CsA dissolved in ethanol and then diluted in OMM to a final concentration of 0.01% (v/v) ethanol (vehicle). Maturation was performed with varying concentrations of CsA at 41°C or vehicle at either 39°C or HS22 followed by fertilization and cultured at 39°C. Percentage of oocytes that cleaved and percentage of oocytes that developed to

blastocyst stage were recorded on days 3 and 8 after insemination, respectively. The experiment was replicated six times using 112-144 oocytes/treatment.

Effect of FCCP during maturation of bovine oocytes

This experiment determined whether addition of FCCP, to depolarize the mitochondria during maturation of bovine oocytes induces apoptosis and decreases embryonic developmental capacity. COCs were matured at 39°C in presence of 0.1, 1, 10, or 100 μM FCCP or in 0.1% (v/v) dimethyl sulfoxide (DMSO; vehicle) diluted in OMM. Fertilization and culture were performed at 39°C. Percentage of oocytes that cleaved was recorded at day 3 after insemination and development to blastocyst stage was recorded at day 8 after insemination. The experiment was replicated six times using 159-222 oocytes/treatment.

FCCP induced apoptosis on oocytes and blastocysts

To determined the effect of FCCP on apoptosis, COCs were matured in presence of 0.1, 1, 10, 100 μM FCCP or 0.1% DMSO at 39°C. At the end of 22 h maturation period, some oocytes were denuded from cumulus cells. Individual denuded oocytes were transferred to 20 μl drops of 0.5% pronase. Zona-free oocytes and blastocysts were fixed at 4% (v/v) paraformaldehyde and saved at 4°C in PBS-PVP until analysis by TUNEL. A total of 32-40 oocytes/treatment were analyzed in three replicates. In addition, blastocysts

harvested at day 8 produced from oocytes matured in the presence of 0.1, 1, 10 μM FCCP and vehicle were subjected to TUNEL assay (30-45 embryos/treatment in five replicates).

Effect of FCCP on transcript levels in oocytes and expanded blastocysts

To evaluate the effects of FCCP on transcript abundance of apoptotic and mitochondrial related genes, COCs were matured at either 0.1% DMSO or 10 μM FCCP at 39°C. After 22 h of maturation, some oocytes were denuded of cumulus cells. Denuded oocytes were frozen at -80°C until further analyses while others were inseminated and cultured at 39°C. Expanded blastocysts were harvested at Day 8 after insemination and frozen at -80°C. For each treatment group, individual matured oocytes and expanded blastocysts were analyzed (6 oocytes or blastocysts/treatment).

Statistical Analysis

Data were analyzed by least-squares analysis of variance using General Linear Model procedure of SAS (SAS Systems for Windows, Release 8.20; Cary, NC). Percentage data were analyzed after being subject to arcsin transformation. Probability values reported are based on the analysis of transformed data while least-squares means are based on untransformed analyses. Relative expression for each gene was normalized with a ratio of Gapdh (fg/fg), data is presented as 1 embryo equivalent. A mean separation procedure of SAS called pdiff was performed to determine differences between levels of individual treatments. When significant differences in groups were observed, comparisons

of means were further analyzed by Duncan's Multiple Range Test. A probability level of $P \leq 0.05$ was defined as significant.

Results

Heat shock during oocyte maturation disrupts further development

HS during maturation had no effect on the percentage of oocytes that cleaved by day 3 after insemination (Table 2). However, percentage of HS9 and HS22 oocytes that developed to blastocyst stage at day 8 postinsemination was reduced when compared to controls ($P<0.05$). Blastocysts derived from HS22 oocytes had lower ($P<0.05$) development rate than HS9.

Heat-induced apoptosis in bovine oocytes and blastocysts

Representative images illustrating analysis of oocytes and embryos for TUNEL labeling are shown in Figure 1, A and D. The proportion of TUNEL-positive oocytes at the end of maturation was greater ($P<0.05$) for HS9 or HS22 oocytes compared with control oocytes (Figure 2A). In addition, total cell number in blastocysts formed from HS22 oocytes was lower than blastocysts formed from HS9 oocytes or control oocytes (Figure 2B). The percentage of TUNEL-positive cells was increased in blastocysts derived from HS9 or HS22 oocytes compared with control oocytes (Figure 2C).

Relative abundance of apoptotic and mitochondrial related genes from HS oocytes

The quantitative RT-PCR analysis of matured oocytes and expanded blastocysts of apoptosis-related genes Bax, Bcl-xL, HSP 70.1 and mitochondrial related genes ND6 and CO1 are shown in Figure 3. Housekeeping gene, Gapdh, expression was present in all oocytes and embryos (Figure 3 and 6). Evaluation of expression patterns of two mitochondrial genes, CO1 and ND6 revealed no effects of HS during maturation on the abundance of mitochondrial transcripts. However, Bax mRNA expression was decreased ($P<0.05$) in oocytes exposed to HS22 compared to the control group (Figure 3A). Moreover, blastocyst derived from HS22 oocytes demonstrated a tendency for greater relative abundance of HSP70.1 transcript ($P=0.07$) (Figure 3B).

Addition of CsA reduces heat shock induced-apoptosis on oocytes and embryos

Addition of 60 μM CsA to oocyte maturation medium reduced ($P<0.05$) the percentage of oocytes that cleaved on day 3 after insemination when compared with vehicle group exposed at 39°C (Table 2). At all concentrations tested (15, 30, 60 μM CsA), the percentage of oocytes that developed to blastocyst stage at day 8 was reduced ($P<0.05$) (Table 2). However, the percentage of TUNEL-positive oocytes at all concentrations of CsA tested was decreased ($P<0.05$) when compared with vehicle group exposed to 41°C and no effect was observed when compared with vehicle group exposed to 39°C (Figure 4A). There was no significant effect on blastocyst cell number in blastocysts derived from

oocytes matured with CsA nor on the percentage of apoptotic (TUNEL-positive) cells compared with blastocysts derived from oocytes matured at 39°C (Figure 4B and C).

Effect of FCCP on developmental capacity of oocytes

Addition of FCCP to maturation medium at concentration of 100 µM decreased ($P<0.001$) the proportion of oocytes that cleaved (Table 2). The proportion of oocytes that developed to blastocyst stage at day 8 was reduced at concentrations of 1, 10 and 100 µM FCCP relative to control.

FCCP induced apoptosis on bovine oocytes and embryos

Representative digital images illustrating the frequency of apoptotic nuclei in bovine oocyte exposed to FCCP and blastocysts from these oocytes as determined by TUNEL analysis are shown on Figure 1, B and E. The percentage of oocytes labeling as TUNEL-positive was increased ($P<0.001$) at all FCCP concentrations (Figure 5A). There was no effect of FCCP on blastocyst cell number (Figure 5B). Note in figure 1B that these blastocysts had higher incidence of TUNEL-positive blastomeres (red signal). Indeed, the percentage of TUNEL-positive cells was higher ($P<0.001$) for concentrations of 1 and 10 µM FCCP (Figure 5C). At the 100 µM FCCP, the blastocyst development was completely blocked and TUNEL was unable to be performed.

Effect of FCCP on abundance of apoptotic and mitochondrial related genes in oocytes and expanded blastocysts

To analyse the mRNA abundance of genes involved with mitochondrial transcription (ND6 and CO1) and apoptosis (Bax, Bcl-xL, HSP 70.1) *in vitro* matured oocytes (n= 6) and expanded blastocysts (n= 6) were employed (Figure 2.6). No difference was detected in abundance of the housekeeping gene (Gapdh). Mitochondrial or apoptotic transcript abundance was not significant between FCCP treated and control groups of matured oocytes (Figure 6). However, at the blastocyst stage, Bcl-xL, HSP70.1, and ND6 mRNA levels were decreased compared to control group (Figure 6B).

Discussion

It has been demonstrated that apoptosis can be induced by *in vitro* HS in oocytes and embryos (Paula-Lopes and Hansen 2002b; Roth and Hansen 2004). This cellular stress is also associated with reduced embryonic development potential (Paula-Lopes and Hansen 2002b; Roth and Hansen 2004; Aroyo et al. 2007). In the present study, oocytes were subjected to different treatment regimes of HS (HS9 and HS22) that resulted in decreased development to blastocyst stage as well as in reduced blastocyst total cell number for the HS22 group. Moreover, the frequency of HS induced oocyte and blastomere apoptosis in HS9 and HS22 groups was higher than in the control group. Similar findings have been reported for bovine oocytes exposed to HS during the first 12 hours of maturation (Roth

and Hansen 2004). These results indicate that HS during oocyte maturation activates apoptotic machinery and results in disruption of oocyte developmental capacity.

The mechanism of cell death after exposure to HS is known to be associated with mitochondrial disruption (Mosser et al. 2000; Stankiewicz et al. 2005). There is evidence that HS induces mitochondria swelling, cytochrome c release from mitochondria, and caspase activation in embryos (Mirkes and Little 2000; Paula-Lopes and Hansen 2002b; Rivera et al. 2003). Opening of MOMP is implicated with apoptosis but it is unclear whether it is an initial or late event in this process (Kroemer 1998). Opening of the pore causes mitochondrial membrane depolarization and prevents oxidative phosphorylation (Crompton 1999). To further elucidate the role of mitochondria during HS, a potent inhibitor of pore opening in mitochondria, CsA, was employed. Cyclosporin A is known to bind mitochondrial cyclophilin and inhibit the permeability transition pore apparently independent of its immunosuppressive action (Halestrap et al. 1997). Heat shocked oocytes exposed to different concentrations of CsA demonstrated a reduced percentage of oocytes and blastomeres that were TUNEL-positive. In addition, blastocyst total cell number was similar to control group suggesting rescue from apoptosis by CsA. The percentage of HS-CsA treated oocytes that reached the blastocyst stage was reduced in all concentrations examined and cleaved rate was reduced at the CsA highest concentration. This shows that the developmental rates were compromised by heat shock and this effect was not reversed by CsA. Together, these results demonstrated the importance of MOMP during HS.

The reduced embryonic developmental competence in the presence of CsA might be associated with its non-mitochondrial effects. The immunosuppressant action of CsA is exerted by inhibition of calcineurin, a Ca2+/calmodulin-dependent protein phosphatase, by the CsA-cyclophilin complex (Liu et al. 1991). Although the downstream target of calcineurin is not known, a previous study has shown that calcineurin maintains phosphorylation of one or more kinase substrates (Weinbrenner et al. 1998). Protein kinases have been shown to modulate the maturation of bovine oocytes *in vitro* (Ali and Sirard 2005).

In the present study, we also investigated the effects of HS on the mRNA expression of genes involved in apoptosis and mitochondrial function. Steady-state amounts of mitochondrial genes (CO1 and ND6) were not affected on HS-treated oocytes. The Bcl-xL and Bax proteins are antagonists and form ion channels in mitochondria that can result in cytochrome c release and cell death (Betts and King 2001). Surprisingly, the relative abundance of proapoptotic Bax transcripts was reduced on HS-treated oocytes. This contrasts with previous observations in which expression of genes involved in cell death were elevated and expression of genes involved in cell survival were reduced in *in vitro* produced, fragmented, aged oocytes, or oocytes matured in supplemented maturation media (Jurisicova et al. 1998; Metcalfe et al. 2004; Boelhauve et al. 2005; Perez et al. 2005; Warzych et al. 2007). This reduction in the amount of mRNA of specific transcripts during maturation might be due to increase degradation of mRNA (Paynton et al. 1988; Su et al. 2007). It is well known that the balance between anti- versus pro-apoptotic members of

Bcl-2 family can be a marker of oocyte or embryo viability and a predictor of the cell fate (Jurisicova et al. 1998; Exley et al. 1999; Yang and Rajamahendran 2002; Metcalfe et al. 2004; Lobascio et al. 2007; Warzych et al. 2007). In this study, no significant change in the Bcl-xL/Bax ratio was observed between experimental groups. Further experiments to identify the role of Bax on heat-induced apoptosis in oocytes are warranted.

It is well known that disruption of mitochondrial function, caspase activation, and DNA fragmentation are major biochemical events during apoptosis. Some apoptotic events are closely associated with mitochondrial function, such as alterations in mitochondrial membrane potential ($\Delta\Psi m$) (Dispersyn et al. 1999; Lyamzaev et al. 2004; Piret et al. 2004; Moon et al. 2005), generation of reactive oxygen species (Kane et al. 1993; MacDonald et al. 1999), and release of caspase-activating proteins (Chang and Yang 2000; LeBlanc 2003). An alteration on $\Delta\Psi m$ is believed to occur after opening of permeability transition pores (PTP) that result in loss of ionic and osmotic asymmetry across inner mitochondrial membrane (Qian et al. 1997). This results in swelling of the mitochondria, release of pro-apototic proteins, uncoupling the oxygen consumption and synthesis of ATP with generation of reactive oxygen species. Protonophoric uncouplers such as FCCP are able to open the mitochondrial PTP (Scorrano et al. 1997; Susin et al. 1998). Here we explored the ability of FCCP to mimic the mitochondrial events believed to occur during apoptosis. If mitochondrial PTP plays crucial role during the apoptotic process, FCCP as a mitochondrial uncoupler might itself induce programmed cell death. It was demonstrated that addition of FCCP during maturation of oocytes correlates with reduction of percentage

of oocytes that cleaved, developed to the blastocyst stage, and increased frequency of apoptosis in both oocytes and blastocysts. Moreover, a previous study has shown that embryos with more than 16 cells exposed to the mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) demonstrated increased proportion of TUNEL-positive nuclei (Brad and Hansen 2006). To our knowledge, our study is the first to show that mitochondrial depolarization plays crucial role in inducing apoptosis in bovine oocytes, demonstrating the importance of mitochondrial integrity during early embryonic development.

The mechanisms that regulate mitochondrial function during preimplantation development have not been completely elucidated while the nuclear-mitochondrial genomic interactions are well documented (Smith and Alcivar 1993; Fernandez-Silva et al. 2003). Mammalian mitochondrial DNA is a compact gene organization comprised of a double stranded, closed-circular molecule with coding sequences that are contiguous or separated by a few base pairs without introns. Indeed, some of protein genes even overlap. The two strands, the heavy or H-strand and the light or L-strand, encode 37 genes corresponding to the RNA components of the mitochondrial translational apparatus (two ribosomal RNAs called 12S and 16S and 22 transfer RNAs) as well as mRNAs for 12 polypeptides that are subunits for oxidative phosphorylation (Smith et al. 2002). These genes are asymmetrically distributed, and the H-strand encodes most of the information (Fernandez-Silva et al. 2003). The L-strand encodes only eight transfer RNAs and one mRNA, the ND6 subunit. The rest of factors involved in the mtDNA metabolism and oxidative phosphorylation are nuclear-

encoded, synthesized in the cytosol, and transported by chaperones into the mitochondrial matrix (Garesse and Vallejo 2001). Therefore, control of mitochondria function is complicated and involves the exchange of information between nucleus and many copies of mtDNA in each cell's cytoplasm.

In the present investigation, mtDNA transcription was characterized by evaluating mRNA levels of CO1 (H-strand located) and ND6 (L-strand located) genes in oocytes and expanded blastocysts. Further, the apoptotic genes Bax, Bcl-xL, and HSP70.1 were examined. No effect of FCCP was found in oocytes for any of these gene candidates. In contrast, expanded blastocysts derived from FCCP-treated oocytes demonstrated a reduced mRNA expression of mitochondrial ND6 and anti-apoptotic Bcl-xL and HSP70.1. Decreased mitochondrial transcription levels have been previously reported in human unfertilized oocytes and arrested embryos (Hsieh et al. 2004). Interestingly, this was accompanied by a reduced Bcl-xL transcript abundance. Bcl-xL and Bcl-2 are the two major anti-apoptotic proteins of Bcl-2 family that act by heterodimerization with pro-apoptotic Bcl-2 members such as Bax and Bak (Finucane et al. 1999; Sharpe et al. 2004). It is possible that the regulatory activity of Bcl-xL was blocked by the pro-apoptotic activity of Bax. However, no significant difference was found on Bcl-xL/Bax ratio. Heat shock proteins play a protective role within a cell and also suppress procaspase-9 and -3 activation preventing stress-induced apoptosis (Mosser et al. 2000; Betts and King 2001). Their action as molecular chaperones and protection of protein degradation is well established and their expression level is also linked to response to cellular stress (Morimoto and

Santoro 1998). Previous studies have found either no difference or increased HSP70 transcript levels in blastocysts produced from oocytes matured *in vitro* or cultured in supplemented maturation medium (Wrenzycki et al. 2001; Knijn et al. 2002; Warzych et al. 2007) In contrast, we found a decrease in HSP70.1 mRNA. A possible explanation is that once the adequate amount of HSP70 is synthesized, transcription of the HSP70 gene is rapidly attenuated because high-level expression of HSP70 decreases growth rates (Morimoto 1998; Mosser et al. 2000). Our data support the notion that depolarization of oocyte mitochondria have a long-term deleterious effect on *in vitro* produced blastocysts by down-regulation of mitochondrial ND6, anti-apoptotic Bcl-xL, and HSP70.1 mRNA levels.

While not experimentally addressed here, the cumulus cells of the cumulus-oocyte complex cultured in this study might also be affected by treatments. Cumulus-oocyte interaction is a critical requirement for both follicular growth and acquisition of oocyte developmental competence (Eppig et al. 2002; Matzuk et al. 2002). A bidirectional communication between the oocyte and their surrounding cumulus cells through gap junctions has been recognized (Eppig et al. 2002; Matzuk et al. 2002; Rodriguez and Farin 2004). The growth differentiation factor-9 (GDF-9) has been proposed to be secreted only by the oocyte and determines the phenotype of the cumulus cells (Eppig et al. 1997; Li et al. 2000). Disruption of gap junctions integrity through the use of n-alkanols reversibly inhibited bovine oocyte maturation (Vozzi et al. 2001). Association of cumulus and oocyte during maturation increases the protein synthetic capacity and removal of cumulus resulted in reduction (Chian and Sirard 1995; Edwards and Hansen 1997). A previous study has

shown that ceramide was trafficked from the cumulus cells to the oocyte signaling accelerated incidence of apoptosis in oocytes of aged female mice (Perez et al. 2005). Elevated temperature or reversible mitochondrial uncoupler treatments examined here could disrupt one or more of these cumulus-oocyte interactions resulting in decreased developmental potential and activation of the apoptotic machinery.

In conclusion, elevated temperature and depolarization of mitochondria by FCCP during maturation induced apoptosis on both oocytes and blastocysts and was associated with reduced developmental capacity. Inhibiting the opening of the mitochondrial pores blocked HS-induced apoptosis showing clearly the importance of mitochondrial integrity during stress early in development. The treatments also affect mRNA expression of candidate mitochondrial and apoptosis related genes in matured oocytes and expanded blastocysts. Together these new findings suggest that oocytes are sensitive to environmental or pharmacological insults and that response to stress of the oocyte can be observed later in development, particularly at the blastocyst stage (Gardner and Lane 2005). A better understanding of the apoptosis-induced mechanisms in oocytes could help improve fertility in females.

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Table 1. Primer sequences used for RT-PCR

Gene	Sense and Antisense Primer (5'-3')	Anneal Temperature (°C)	Sequence Reference (GeneBank No.)
ND6	TAAAGCCGCAATCCCTATGG AACGGCTATGGCTACAGAC	58	V00654
CO1	ACACACGAGCCTACTTCACA GGAAATGTGCGACAACGTAG	58	V00654
Bax	TGCAGAGGATGATCGCAGCTGTG CCAATGTCCAGCCCATGATGGTC	58	Lonergan et al., 2003
Bcl-xL	AGGCAGGCGATGAGTTTGAA TCCTTGTCTACGCTTTCCAC	56	AF245489
HSP70.1	GAAGAAGGTGCTGGACAAGT CCTAATCCACCTCCTCAATG	58	Lazzari et al., 2002
Gapdh	TGTTCCAGTATGATTCCACCC TCCACCACCCTGTTGCTGTA	58	AF077815

Table 2. Effects of heat shock (HS), cyclosporin A (CsA), and carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) during maturation of cumulus-oocyte complexes on the percentage of oocytes that cleaved and developed into blastocyst on day 8 after insemination.

Temperature (°C)	Medium	n	% Cleaved oocytes	% Blastocyst Day 8
39	Control	107	87 ± 3.7	55 ± 1.7[a]
41	HS9	155	88 ± 3.7	46 ± 1.7[b]
41	HS22	169	81 ± 3.7	39 ± 1.7[c]
39	Ethanol	139	91 ± 3.9[a]	45 ± 4.1[a]
41	Ethanol	112	76 ± 3.9[b,c]	15 ± 4.1[b]
41	15 µM CsA	122	80 ± 3.9[a,b,c]	21 ± 4.6[b]
41	30 µM CsA	136	86 ± 3.9[a,b]	23 ± 4.6[b]
41	60 µM CsA	144	72 ± 3.9[c]	16 ± 4.6[b]
39	DMSO	172	89 ± 5.2[a]	52 ± 4.7[a]
39	0.1 µM FCCP	159	89 ± 5.2[a]	40 ± 4.7[a,b]
39	1 µM FCCP	181	83 ± 5.2[a]	35 ± 4.7[b]
39	10 µM FCCP	210	74 ± 5.2[a]	29 ± 4.7[b]
39	100 µM FCCP	222	12 ± 5.2[b]	2 ± 4.7[c]

[(a-c)] Data represent least-square means ± SEM. Means with different superscript within a column differ at $P < 0.05$.

Figure 1. Representative confocal images of TUNEL labeling. Shown in top panels are representative blastocysts derived from oocytes exposed to heat shock (A), oocytes matured in presence of FCCP (B), and negative control blastocyst (C) treated with DNase. Bottom panels shown matured oocytes exposed to heat shock (D) or to FCCP during maturation (E), and negative control oocytes (F) treated with DNase. Note that red/pink nuclei and pronuclei are TUNEL positive while blue represents staining of DNA with Hoechst 33342.

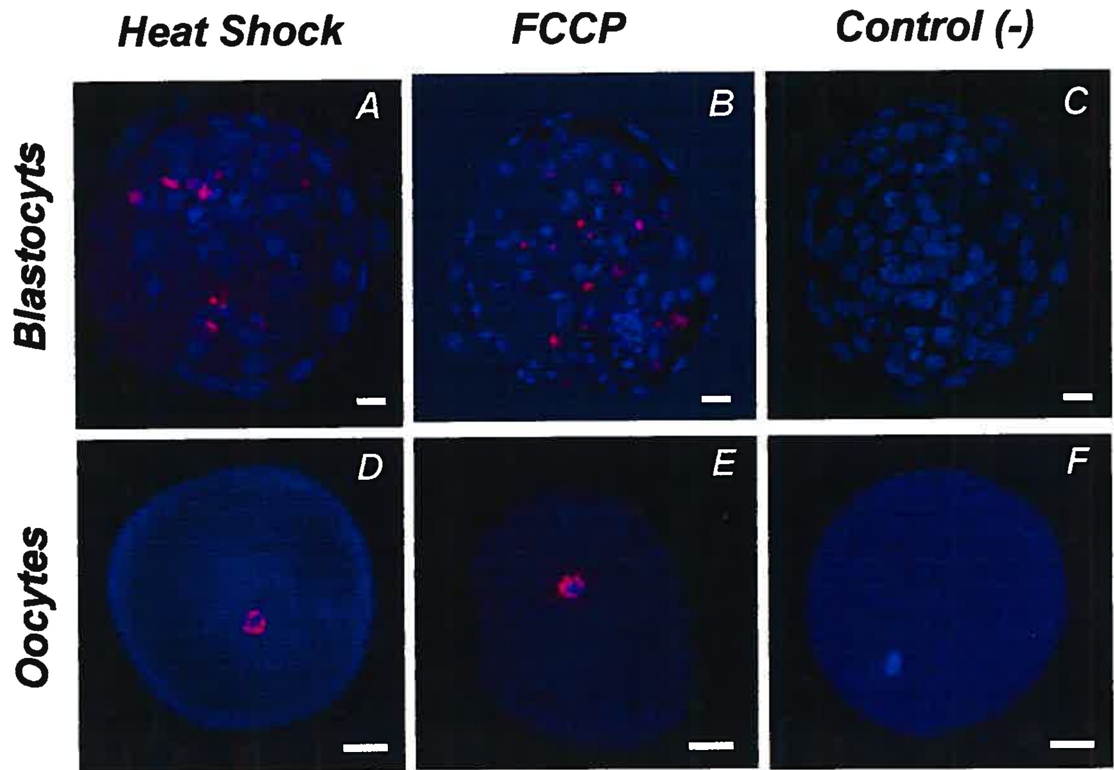

Heat Shock
FCCP
Control (-)
Blastocyts
Oocytes
A
B
C
D
E
F

Figure 2. Apoptosis after heat shock. (A) percentage of TUNEL-positive oocytes after heat shock during the first 9 hours or 22 hours of maturation (n= 33-44 oocytes/group), (B) total cell number, and (C) TUNEL-positive cells from day 8 blastocysts cultured after exposure to heat shock during maturation (n= 29-34 blastocysts/group). Open bars represent oocytes cultured at 39°C while solid bars represent oocytes cultured at 41°C. Superscripts above each bar that are different represents means that differ at $P < 0.05$.

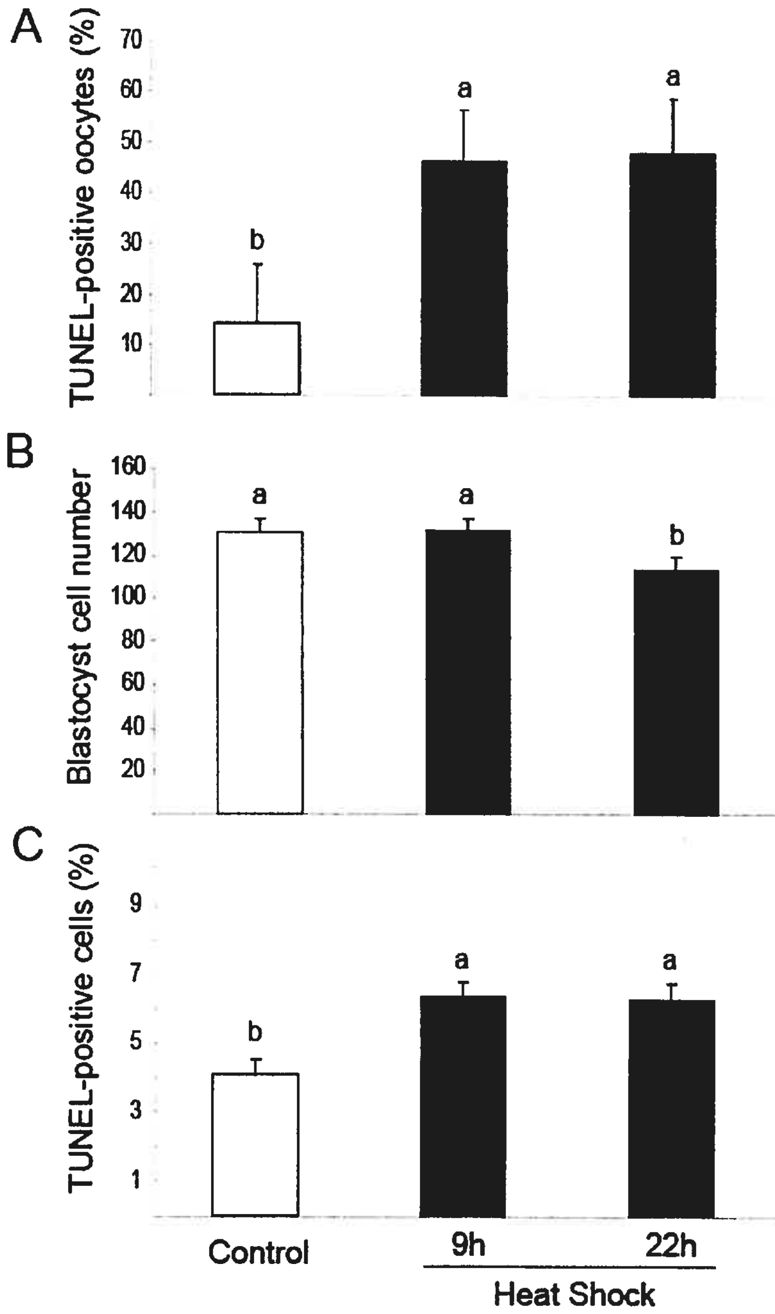
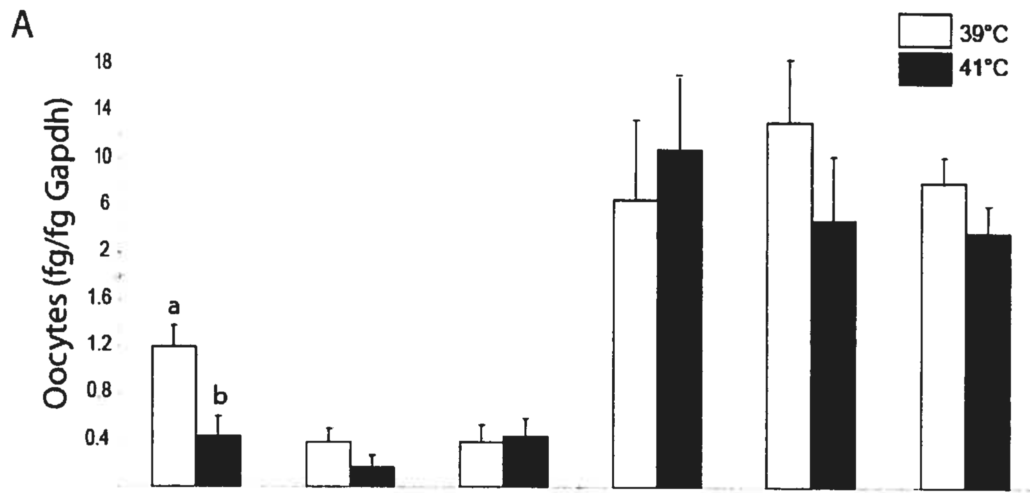

A
TUNEL-positive oocytes (%)
70
60
50
40
30
20
10
b
a
a
B
Blastocyst cell number
160
140
120
100
80
60
40
20
a
a
b
C
TUNEL-positive cells (%)
9
7
5
3
1
b
a
a
Control
9h
22h
Heat Shock

Figure 3. Abundance of mitochondrial and apoptotic transcripts after heat stress. Effect of heat stress for 22 hours of maturation (HS22) on the abundance of apoptotic (Bax, Bcl-xL, and HSP70.1) and mitochondrial (ND6 and CO1) mRNAs in matured oocytes and expanded blastocysts on day 8. Data are presented as 1 oocyte or embryo equivalent (n= 6 individual/treatment). Open bars represent oocytes cultured at 39°C while solid bar represents oocytes cultured at 41°C. Data represent least-squares means ± SEM. Superscripts above each bar represents means that differ ($P < 0.05$).


A
39°C
41°C
Oocytes (fg/fg Gapdh)
18
14
10
6
2
1.6
1.2
0.8
0.4
a
b

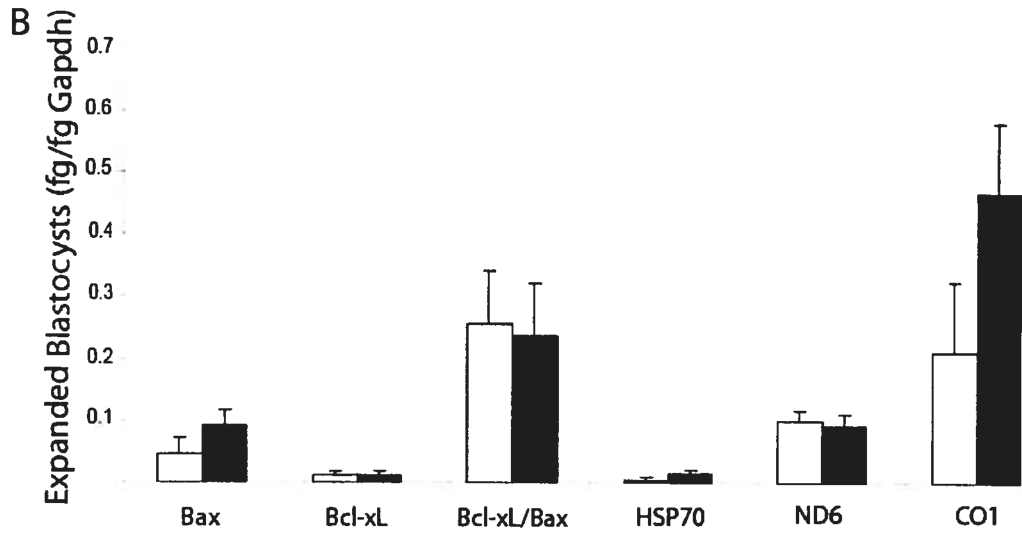
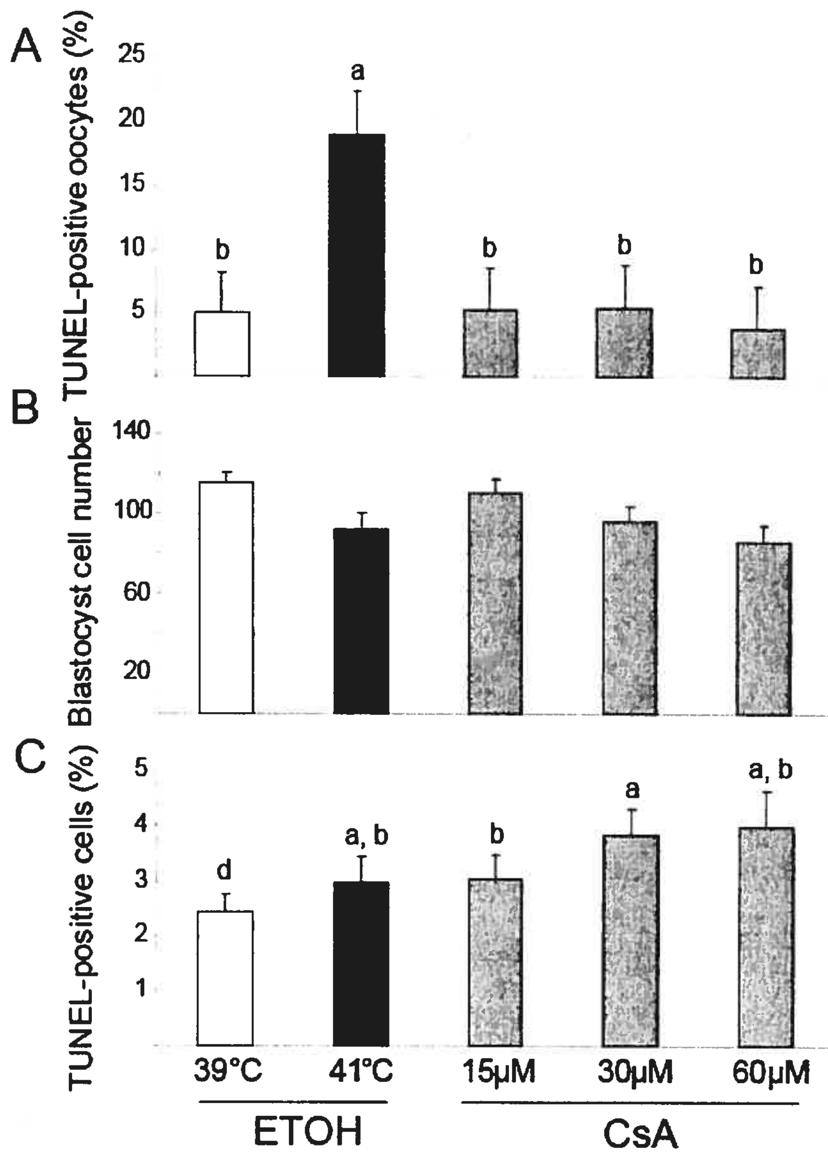

B
Expanded Blastocysts (fg/fg Gapdh)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Bax
Bcl-xL
Bcl-xL/Bax
HSP70
ND6
CO1

Figure 4. The occurrence of apoptosis after combination of cyclosporin A (CsA) and heat shock (HS) during maturation. (A) Proportion of TUNEL-positive oocytes after addition of different concentrations of CsA and 22 hours of heat shock (n= 60-67 oocyte/treatment), (B) total cell number and (C) TUNEL-positive blastomeres from HS-CsA treated-oocytes (n= 19-42 blastocysts/treatment). Open bars represent oocytes cultured at 39°C, solid bars represent oocytes cultured at 41°C while grey bars represent oocytes cultured with cyclosporin at 41°C. Data represents least-squares means ± SEM. Superscripts above each bar represent means that differ ($P < 0.05$).



A
B
C
TUNEL-positive oocytes (%)
25
20
15
10
5
a
b
b
b
b
b
Blastocyst cell number
140
100
60
20
TUNEL-positive cells (%)
5
4
3
2
1
d
a, b
b
a
a, b
39°C
41°C
15μM
30μM
60μM
ETOH
CsA

Figure 5. Apoptosis after FCCP. (A) Percentage of TUNEL-positive oocytes after FCCP exposure during maturation (n= 32-40 oocytes/treatment). (B) Total cell number and (C) TUNEL-positive blastomeres from day 8 blastocysts derived from FCCP-treated oocytes (n= 30-45 blastocysts/treatment). Open bars represent oocytes cultured with DMSO at 39°C while striped bars represents oocytes cultured with FCCP at 39°C. Superscripts above each bar that are different represent means that differ at $P < 0.05$.

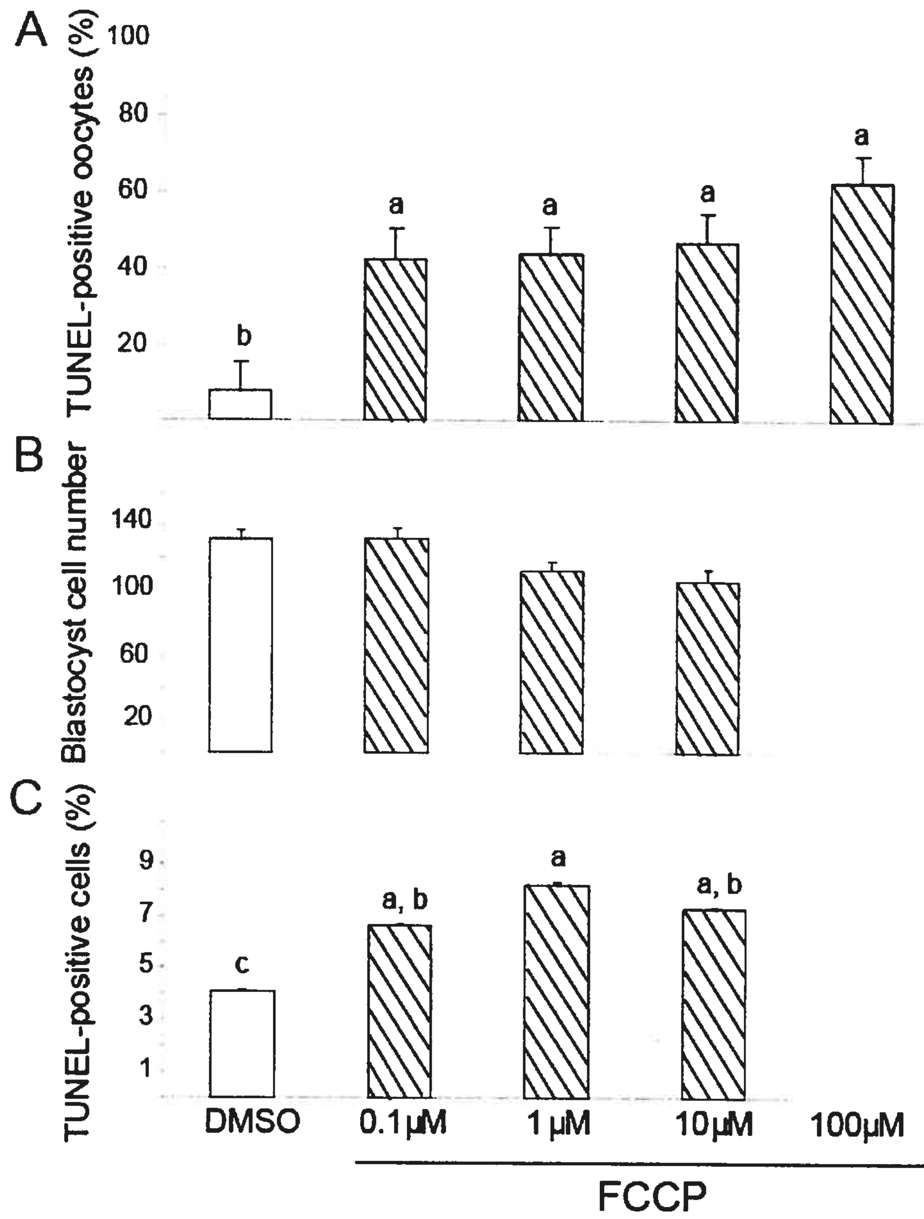
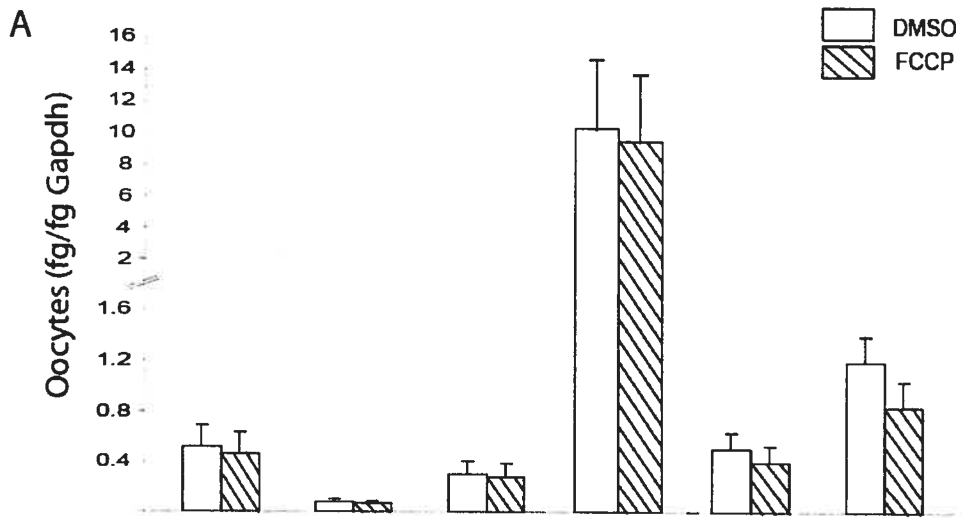
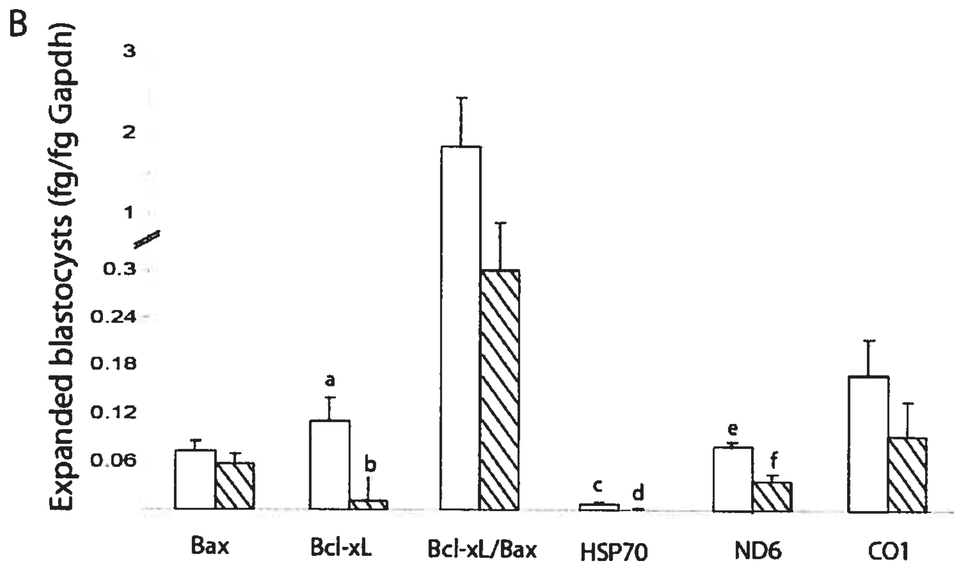

A
TUNEL-positive oocytes (%)
100
80
60
40
20
b
a
a
a
a
B
Blastocyst cell number
140
100
60
20
C
TUNEL-positive cells (%)
9
7
5
3
1
c
a, b
a
a, b
DMSO
0.1µM
1 µM
10µM
100µM
FCCP

Figure 6. Abundance of mitochondrial and apoptotic transcripts after FCCP. Effect of FCCP during maturation on the abundance of apoptotic (Bax, Bcl-xL, and HSP70.1) and mitochondrial (ND6 and CO1) mRNAs in matured oocytes and expanded blastocysts on day 8. Data are presented as 1 oocyte or embryo equivalent (n= 6 individual/treatment). Open bars represent oocytes cultured with DMSO at 39°C while striped bars represents oocytes cultured with FCCP at 39°C. Superscripts above each bar that are different represent means that differ at $P < 0.05$.


A
16
14
12
10
8
6
4
2
1.6
1.2
0.8
0.4
Oocytes (fg/fg Gapdh)
DMSO
FCCP


B
3
2
1
0.3
0.24
0.18
0.12
0.06
Expanded blastocysts (fg/fg Gapdh)
a
b
c
d
e
f
Bax
Bcl-xL
Bcl-xL/Bax
HSP70
ND6
CO1

CHAPTER III

INDUCTION OF CASPASE ACTIVATION IN OOCYTES AND BLASTOCYSTS BY HEAT SHOCK DURING MATURATION

Short title: Heat shock induces caspase activation

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Abstract

Activation of caspases is a highly recognized feature of apoptosis. Previously, we showed that exposure of bovine oocytes to heat shock (HS) during maturation induced apoptosis and reduced subsequent embryonic development. Experiments were performed to: 1) determine whether HS-induced apoptosis is mediated by caspase-9 and/or caspase-3/7, and 2) whether inhibition of caspases in the oocyte prevents interference with developmental potential caused by HS. Cumulus-oocyte complexes (COCs) were cultured at 39°C (control) or 41°C (HS) for 22 h. All COC groups were fertilized and cultured *in vitro* at 39°C for 8 days. Exposure to HS resulted in increased activity of caspase-9 and caspase-3/7 in both oocytes and blastocysts derived from oocytes exposed to HS. The COCs were then placed in maturation medium with vehicle [0.01% (v/v) DMSO] or 200 nM of caspase inhibitors: z-LEHD-fmk, an inhibitor of caspase-9 activity; z-DEVD-fmk, a caspase-3/7 inhibitor; or z-VAD-fmk, a broad caspase inhibitor. COCs were matured at 39°C or 41°C, fertilized and cultured for 8 days at 39°C. In the presence of all inhibitors, HS-treated oocytes developed to the blastocyst stage similarly to controls and showed reduced percentage of TUNEL-positive chromatin in oocytes and blastocysts. In conclusion, HS induced oocyte mitochondrial dysfunction by activation of caspase-9 and caspase-3/7, whereas caspase inhibitors rescued the capacity of oocyte to support early embryonic development.

Introduction

Apoptosis, or programmed cell death, is a physiological process of cellular self destruction that plays crucial roles throughout life, including during the very early stages of embryonic development preceding implantation (Zakeri and Lockshin 2002; Chan 2006). Moreover, preimplantation development is a dynamic process involving cell proliferation, cell differentiation, and cell death. The oocyte and embryo can be negatively affected by certain disrupters, such as heat shock (HS), that induce apoptosis and reduce embryonic developmental competence (Edwards and Hansen 1997; Paula-Lopes and Hansen 2002b; Roth and Hansen 2004). The mitochondria of oocytes are potential sites of pathological insult; indeed, induction of mitochondria dysfunction in mouse oocytes results in apoptotic degeneration (Thouas et al. 2004). For these reasons, it is possible that oocyte mitochondria mediate the apoptotic degeneration in oocytes and preimplantation embryos.

Cysteinyl aspartate-specific proteases, or caspases, are highly specific proteinases that are constitutively expressed as inactive proenzymes (Chang and Yang 2000). Their activation is fundamental to apoptotic cell death but their mode of action is not completely understood. The two major mechanisms of caspase activation are the death receptor-mediated and the mitochondrial pathways. The receptor-medicated pathway results in activation of caspase-8 (Tilly 2001; LeBlanc 2003), and the mitochondrial pathway results in activation of caspase-9 through the Bcl-2 family (Kroemer et al. 1998; Crompton 1999; Waterhouse et al. 2002). Once activated, caspases proteolytically cleave various cellular substrates, leading to morphological hallmarks of apoptosis including DNA fragmentation

(Nicholson and Thornberry 1997; LeBlanc 2003). Their substrate specificity is determined by four amino acid residues, thereby classifying caspases into three groups based on the amino acid sequences where they act. The Group I caspases (caspase-1, -4, -5, and -13) have preference for WEXD motif, while Group II (caspase-2, -3, and -7) interact with DEXD substrate, and Group III (caspase-6, -8, -9, and -10) prefer (I/L/V)EXD, the X is any amino acid (Grutter 2000).

Caspases can also be classified by their biological functions as inflammatory caspases (caspase-1, -4, -5, -11, and -13), initiators of apoptosis (caspases-2, -8, -9, and -10) and executioners of apoptosis (caspase-3, -6, and -7) (Chang and Yang 2000; LeBlanc 2003). The executioner caspases-3 and -7 are activated by mitochondrial (or intrinsic apoptotic pathway) and receptor-mediated (or extrinsic apoptotic pathway) and share similar substrate specificity (DEVD) (Grutter 2000). Although it is often assumed that caspase-7 functions like caspase-3, they have different physiological roles (Slee et al. 2001). During apoptosis, caspase-3 is the primary executioner caspase that orchestrates DNA fragmentation, nuclear condensation, and membrane blebbing by the cleavage of specific substrates (Enari et al. 1998; Coleman et al. 2001; Slee et al. 2001). In contrast, caspase-7 is involved on cleavage of poly-ADP-ribose polymerase (PARP) a protein involved in DNA repair (Slee et al. 2001). Caspase-2, -3, and -9 are released from mitochondria together with cytochrome c after disruption of mitochondrial transmembrane potential ($\Delta\Psi m$) with an uncoupler or in cell death induced by staurosporine (Samali et al.

1999; Susin et al. 1999a; Costantini et al. 2002) suggesting that mitochondrial procaspases can induce apoptosis.

Since the preimplantation embryo develops in a unique maternal environment, it is of great interest to elucidate mechanisms of cell death. Papandile et al. (2004), have demonstrated that the executioner caspase-3 and the initiators caspase-8 and -9 are expressed and active in mouse oocytes. Moreover, the mRNA of group I (caspase-1, -11, and -12), group II (caspase-2, -3, and -7), and group III caspases (caspase-6) have also been identified in mouse oocytes (Exley et al. 1999). The mRNA of caspase-1, -3, -6, -7, and -8 were found in bovine oocytes but no active caspase protein was identified (Yuan et al. 2004). However, Roth and Hansen (2004) have shown activation of caspase-3 in bovine oocyte after heat shock. Therefore it is reasonable to conclude that physiological or pharmacological induction of mitochondrial dysfunction in preimplantation embryos results in early embryonic loss. Acton et al. (2004) demonstrated an association between decreased mouse and human embryonic development and increase ratio of high-polarized mitochondria. Depolarization of mitochondrial membrane by 3-clorophenylhydrazone (CCCP) on bovine two-cell embryos increased activity of caspase-9 and group II caspase and also increased the proportion of TUNEL-positive nuclei on day 5 embryos (Brad and Hansen 2006). Photosensitization of mouse oocytes with a mitochondria-specific dye resulted in increase in permeabilization of the outer mitochondrial membrane (MOMP) and induction of apoptosis (Thouas et al. 2004).

We hypothesize that HS-mediated apoptosis during oocyte maturation induces oocyte mitochondrial dysfunction with consequent activation of caspase-9 and caspase-3/7 resulting in DNA fragmentation. The purpose of the present experiments was to examine whether HS-induced apoptosis in maturing oocytes is mediated by caspase-9 and/or caspase 3/7, and whether inhibition of apoptosis in thermal-stressed oocytes rescues oocyte developmental capacity.

Materials and Methods

Materials

Most reagents were obtained from Sigma (St. Louis, MO), including Hoechst 33342, and polyvinylpirrolidone (PVP). In Situ Cell Death Detection Kit TMR red was obtained from Roche Diagnostics Corporation (Laval, QC). Prolong Antifade Kit was obtained from Molecular Probes (Eugene, OR), RQ1 RNA-free DNase was from Promega (Madison, WI), and RNase A was from Qiagen (Mississauga, ON). The 60-well Terasaki polystyrene microtest plates were obtained from Sarstedt (Montreal, CA). Caspase-Glo 3/7 and Caspase-Glo 9 were purchased from Promega (Madison, WI) and z-L-E-(Ome)-H-D(Ome)-flurometheyl ketone (z-LEHD-fmk), N-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl Ketone (z-DEVD-fmk), N-benzoylcarbanyl-Val-Ala-Asp-fluoro methylketone (z-VAD-fmk) were obtained from R&D Systems (Minneapolis, MN).

Oocyte maturation, fertilization and embryo culture

The procedures for embryo production were performed as described earlier (Parrish et al. 1986). Briefly, ovaries were obtained from slaughtered cows and used to obtain COCs by aspiration of 2-7 mm follicles and washed in Hepes-buffered TCM199 (Gibco, Burlingtion, ON) supplemented with 10% fetal bovine serum (FBS; Gibco). Groups of 10 COCs were cultured in pre-equilibrated 50 µl drops of IVM media [bicarbonate-buffered TCM199 supplemented with 10% FBS (Gibco), 50 µg/ml of LH (Ayerst, London, ON), 0.5 µg/ml of FSH (Follitropin-V, Vetrepharm, London, ON), 1 µg/ml of estradiol-17β, 22 µg/ml pyruvate, and 50 µg/ml of gentamicin]. After 22 h in IVM, matured COCs were fertilized *in vitro* as previously described (Parrish et al. 1986). Briefly, frozen semen was thawed, processed by Percoll gradient, and fertilization was allowed to proceed for 18-20 hrs at 39°C and 5% (v/v) CO_2 in humidified air. Presumptive zygotes were then denuded of cumulus cells by vortexing in 50 µl of 0.2% hyaluronidase solution for 5 minutes, and cultured in pre-equilibrated 50 µl drops of modified synthetic oviductal fluid (mSOF; 108 mM NaCl, 7.2 mM KCl, 0.5 mM $MgCl_2$, 2.5 mM NaHCO3, 1.7 mM $CaCl_2.H_2O$, 0.5 mM glucose, 0.33 mM pyruvic acid, 3 mM lactic acid, 8 mg/ml BSA, 150 µg/ml gentamicin, and 0.01% phenol red) media plus amino acids [1.4 mM glycine, 0.4 mM alanine (Gibco), 1 mM glutamine, 2% essential amino acids, 1% non-essential amino acids (Gibco); (Gardner et al. 1994) at 39°C in humidified atmosphere of 5% CO_2 and 5% O_2 for 8 days. The number of cleaved oocytes and development to blastocyst stage were determined on

days 3 and 8 after fertilization, respectively. Oocytes were harvested after 22 h of maturation and developing blastocysts on day 8 after fertilization for further analysis.

TUNEL labeling

The TUNEL procedure was used to detect DNA fragmentation characteristics of late stages of apoptosis. Matured oocytes and embryos were removed from culture medium, washed once in 500 µl of PBS [10 mM potassium phosphate, 0.9% (w/v) NaCl, pH 7.4] containing 1 mg/ml polyvinylpyrrolidone (PBS-PVP). Oocytes were denuded from their zona pellucida by ~5 min exposure to 0.5% pronase at room temperature. Subsequently, oocytes and embryos were fixed in four-well plates containing 500 µl per well of 4% (w/v) paraformaldehyde in PBS, pH 7.4, for 1 hr at room temperature, washed once in PBS-PVP, and stored in 600 µl PBS-PVP at 4°C until TUNEL assay. The TUNEL procedure was initiated by permeabilization of oocytes or embryos in 500 µl of 0.1% (v/v) Triton X-100 containing 0.1% (w/v) sodium citrate in PBS for 1 h at room temperature. Positive and negative controls were incubated in 20 µl drop RQ1 RNase-free DNase (50 U/ml) at 37°C for 1 h. Oocytes and blastocysts were washed in PBS-PVP and incubated in 20 µl drop of TUNEL reaction mixture (containing fluorescein isothiocyanate-conjugated dUTP and terminal deoxynucleotidyl transferase) for 1 h at 37°C in the dark, according to manufacturer's instructions. For all 20 µl drop incubations, 60 well plates were used. Negative controls were incubated in absence of terminal deoxynucleotidyl transferase. Oocytes and blastocysts were then washed in PBS-PVP, transferred to 20 µl drops of 1

μg/ml Hoechst 33342 in PBS-PVP for 10 min, washed once in PBS-PVP, placed in 10% (w/v) polylysine-coated slides, and coverslips mounted using 15 μl mounting medium containing Antifade. TUNEL labeling was observed using a Leica MDLA Microdissection microscope (Cambridge, UK) with dual filter. Images were acquired using IM50 software and Leica DC 500 digital camera. Each oocyte was analyzed for number of TUNEL-labeled pronuclei and blastocysts were analyzed for total number of nuclei and number of TUNEL-labeled nuclei.

Caspase activity assay

Caspase activity was determined by means of the luminometric Caspase-Glo 3/7 assay and Caspase-Glo 9 assay. The activated caspases cleaves the substrate for luciferase, DEVD-aminoluciferin for caspase 3/7 or LEHD- aminoluciferin for caspase-9, resulting in luciferase reaction and the production of light. The signal generated is proportional to the amount of caspase activity present. To perform the procedure, single denuded oocytes or blastocysts were removed from culture medium, transferred to 100 μl of caspase reagent, and incubated for 1 hour at room temperature, according to manufacturer's instructions, followed by the measurement of luminescence using a Berthold 9501 luminometer. Blank reactions, control samples and test samples were measured. The luminescence of the blank reaction was subtracted from samples values.

Activity of caspase-9 and caspase-3/7 in oocytes and blastocysts

The purpose of these experiments was to determine whether HS-induced apoptosis is mediated by caspase-9 and/or caspase 3/7. The COCs were matured either in 39°C or 41°C for 22 hours of maturation followed by culture at 39°C, for the HS group. At the end of maturation, a subset of COCs was denuded in both groups of cumulus cells by hyaluronidase treatment. Caspase-9 and caspase-3/7 activity in oocytes were then assessed immediately. A further subset of oocytes were maintained in culture and allowed to develop to the blastocyst stage on day 8 after insemination, at which time the activity of caspase-9 and caspase-3/7 were also measured. Caspase-9 activity experiments were replicated five times using 34-41 oocytes/treatment or 17-23 blastocyst/treatment. The caspase-3/7 activity experiments were replicated four times using 33-45 oocytes/treatment or 19-20 blastocysts/treatment.

Effect of caspase inhibitors on developmental capacity and apoptosis of heat-shocked oocytes

The experiment was designed as a 2 x 2 factorial arrangement of treatments to determine whether caspase inhibitors block the deleterious effect of HS on oocyte developmental competence and inhibit heat-induced apoptosis in the oocyte and increase subsequent blastocyst development. The COCs were matured in the presence of 200 nM of caspase inhibitors reconstituted in 0.01% (v/v) dimethyl sulfoxide (DMSO) or 0.01% (v/v) DMSO alone (vehicle). COCs were cultured at either 39°C or 41°C for 22 hours during

maturation in vehicle or in the presence of: 1) z-LEHD-fmk, an inhibitor of caspase-9; 2) z-DEVD-fmk, an inhibitor of caspase-3; and 3) z-VAD-fmk, a general caspase inhibitor. A subset of oocytes was denuded from the cumulus cells, zona pellucida removed by pronase, and fixed at 4% (w/v) paraformaldehyde, and TUNEL analysis performed. In addition, zona intact blastocysts were collected on day 8 after insemination also fixed with 4% (w/v) paraformaldehyde, and DNA fragmentation determined by TUNEL. The percentage of oocytes that cleaved and development to the blastocyst stage were recorded on day 3 and day 8 after insemination, respectively. Experiments to evaluate developmental capacity for z-LEHD-fmk group was replicated five times using 131-137 oocytes/group, for z-DEVD-fmk group was five times using 100-141 oocytes per group, and z-VAD-fmk group was four times using 198-219 oocytes/group. The experiments to evaluate DNA fragmentation by TUNEL analysis for z-LEHD-fmk group was replicated five times using 40-50 oocytes/group or 23-44 blastocyst/group, z-DEVD-group was five times using 40-52 oocytes/group or 31-61 blastocyst/group, and z-VAD-fmk group was four times using 51-60 oocytes/group or 41-71 blastocyst/group.

Statistical analysis

Data were analyzed by least-squares analysis of variance using General Linear Model procedure of SAS (SAS systems for Windows, Release 8.20; Cary, NC). Independent variables were temperature, treatment, and replicate. The mathematical model included main effects and all interactions. Percentage data were analyzed after being

subjected to arcsine transformation. Probability values reported are based on the analysis of transformed data while least-squares means are based on untransformed analyses. A means separation procedure of SAS known as *pdiff* was performed to determined differences between levels of individual treatments. A probability value of $p < 0.05$ was defined as significant.

Results

Induction of caspase activation in oocytes by heat shock during maturation and in derived blastocysts

Oocytes exposed to 41°C for 22 hours of maturation demonstrated an increase in caspase-9 (Figure 1A, $p<0.05$) and caspase-3/7 (Figure 1B, $p<0.05$) activity compared with control group exposed to 39°C. Blastocysts derived from oocytes exposed to HS demonstrated an increase in caspase-9 (Figure 1A, $p<0.05$) and caspase-3/7 activity (Figure 1B, $p<0.05$).

Caspase inhibitors blocked the disruption of heat shocked-oocyte competence for cleavage, subsequent development, and apoptosis

In the presence of 200 nM of z-LEHD-fmk (a caspase-9 activity inhibitor), maturation for 22 hours at 41°C increased the proportion of oocytes that developed into blastocysts (Figure 2B, treatment x temperature, $p<0.05$), and there was no effect on the percentage of oocytes that cleaved. COCs cultured at 39°C and exposed to z-LEHD-fmk

had reduced proportion of oocytes that became blastocysts at day 8 after insemination (Figure 2B; treatment x temperature, $p<0.05$). Heat shock increased the percentage of TUNEL-positive oocytes (treatment x temperature, $p<0.05$) and blastocysts (treatment x temperature, $p<0.001$) in control group. However, there was no increase in the percentage of TUNEL-positive oocytes (Figure 3A) after HS in COCs cultured in z-LEHD-fmk nor in the percentage of TUNEL-positive blastocyst cells (Figure 3C) derived from these oocytes. Further, there was no effect on the embryo total cell number (Figure 3B).

Addition of 200 nM z-DEVD-fmk, an inhibitor of caspase 3/7, to maturation medium of COCs matured at 41°C for 22 hours resulted in increase of cleavage rate (Figure 2A; treatment x temperature, $p<0.05$) and development to blastocyst stage (Figure 2B; treatment x temperature $p<0.001$). The percentage of oocytes that became blastocysts was reduced for z-DEVD-fmk-treated oocytes matured at 39°C (Figure 2B; treatment x temperature, $p<0.001$). Exposure of control oocytes to 41°C for 22 hours increased the numbers of TUNEL-positive oocytes and consequent blastocysts (Figure 3A; treatment x temperature, $p<0.05$). Heat stressed COCs cultured in z-DEVD-fmk had a reduced percentage of TUNEL-positive oocytes (Figure 3A; treatment x temperature, $p<0.05$) and TUNEL-positive cells in blastocysts (Figure 3C; treatment x temperature, $p<0.001$) without an effect on total cell number (Figure 3B).

In the absence of 200 nM z-VAD-fmk (a broad caspase inhibitor), maturation for 22 hours at 41°C reduced the proportion of oocytes that cleaved (Figure 2A; treatment x temperature, $p<0.05$) and that developed into blastocysts (Figure 2B; treatment x

temperature, $p<0.05$) the presence of z-VAD-fmk abrogated the deleterious effect of HS. In addition, HS did not increase the percentage of TUNEL-positive oocytes cultured in z-VAD-fmk (Figure 3A, $p<0.05$) nor in blastocysts derived from HS treated oocytes (Figure 3C; treatment x temperature, $p<0.001$). There was no effect of z-VAD-fmk on total cell number (Figure 3B).

Discussion

We reported earlier that bovine oocytes exposed to HS during maturation have reduced developmental capacity due to induction of apoptosis (Soto and Smith 2006). The present experiments were performed to confirm and extend these findings by determining whether activation of caspase-9 and caspase-3/7 is involved in the apoptosis that occurs during early development.

Direct effects of elevated temperature on disruption of oocyte competence have been demonstrated *in vivo* and *in vitro*. Oocytes collected from follicles of cows during summer had decreased capacity to develop to the blastocyst stage after *in vitro* fertilization compared with oocytes collected during winter (Rocha et al. 1998; Al-Katanani et al. 2002). In addition, exposure of COCs to HS during maturation *in vitro* disrupted subsequent development of oocytes after fertilization and induced apoptotic events (Roth and Hansen 2004; Soto and Smith 2006). It is feasible that the oocyte mitochondria mediate or even initiate apoptotic degeneration in oocytes since the initiators of apoptosis, caspase-8 and caspase-9 are expressed in oocytes (Papandile et al. 2004; Yuan et al. 2004).

The present study provides clear evidence that heat shock-induced apoptosis during maturation is mediated by activation of caspase-9 and caspase-3/7 in oocytes and activation of these caspases is a crucial cause of loss of oocyte developmental capacity. The evidence for this conclusion is twofold. First, caspase-9 and caspase-3/7 activity in oocytes was increased after thermal stress, and this stress had a negative long term effect on the resulting blastocysts by increased caspase-9 and -3/7 activity. Second, addition of an inhibitor of caspase-9 (z-LEHD-fmk), an inhibitor of caspase-3/7 (z-DEVD-fmk) or a general caspase inhibitor (z-VAD-fmk) completely suppressed the detrimental effects of HS on development to the blastocyst stage and reduced the frequency of apoptosis in both oocytes and blastocysts.

A variety of stimuli have been shown to induce DNA damage and caspase activation in the oocyte. Hydrogen peroxide induces Bax protein expression, DNA fragmentation, and caspase-3 activity in oocytes that morphological apoptotic changes were observed (Chaube et al. 2005). Cryopreserved bovine oocytes degenerate via apoptosis during subsequent culture by activation of caspase-3 and DNA fragmentation (Men et al. 2003). Active caspase-3 is involved in both spontaneous and staurosporine induced apoptosis from the 8-cell stage to blastocyst stage (Gjorret et al. 2007). Caspase-3 activity was also higher in slow developing embryo compared with fast cleavers and in embryos derived from growing oocytes compared with fully grown oocytes (Vandaele et al. 2007). Heat shock has been demonstrated to induce apoptosis on bovine preimplantation embryos. Exposure of COCs to 41°C during the first 12 hours of maturation increased group II

caspase activity, while maturation in presence of an inhibitor of group II caspases, z-DEVD-fmk, and increased the percentage of oocytes that cleaved and developed to blastocyst stage (Roth and Hansen 2004). Two-cell bovine embryos exposed to HS displayed TUNEL-positive nuclei and increased group II caspase activity on day 5 embryos (Brad and Hansen 2006). Exposure of bovine embryos ≥ 16 cells on day 5 after insemination to thermal stress increased the percentage of cells undergoing apoptosis and increased group II caspase activity (Paula-Lopes and Hansen 2002b). It has been demonstrated that induced mitochondrial damage in oocytes or embryos is associated with high levels of developmental retardation or arrest and activation of apoptotic cell death (Thouas et al. 2004). Exposure of postimplantation mouse embryos to 43°C induced the release of cytochrome c from the mitochondria and induced caspase activation (Mirkes and Little 2000). Exposure of two-cell bovine embryos to 41°C for 6 hours resulted in swelling in 15% of mitochondria (Rivera et al. 2003). The present study demonstrated that the intrinsic (caspase-9-dependent) pathway is expressed in bovine oocytes and activated in oocytes by exposure to HS. We conclude that, in spite of their apparent immaturity, oocyte mitochondria play a crucial role in apoptotic events and have a negative effect further on blastocyst development.

Our further objective was to examine the capacity of caspase inhibitors to neutralize the apoptotic effects of HS on the oocyte. In this study, z-LEHD-fmk, z-DEVD-fmk, and z-VAD-fmk proved able to antagonize apoptotic actions of the oocyte of caspase-9, -3/7 or all caspases, respectively. Previous studies demonstrated that z-DEVD-fmk reduces

apoptosis of bovine COCs or embryos exposed to HS (Paula-Lopes and Hansen 2002b; Roth and Hansen 2004). Our study shows that blockage of caspase-9 or all caspases on heat shocked oocytes reduces apoptosis and increased embryonic development. These data provide evidence that initiator caspase-9 play crucial role on oocyte apoptotic cell death.

Our conclusions have potential practical applications. Present observations showed that controlling activation of caspases on heat shocked oocytes resulted in reduced apoptosis in oocytes and derived blastocysts. Indeed, rescue by z-LEHD-fmk indicates that blockage of caspase-9 or other upstream intracellular events that allow the preservation of the integrity of oocyte mitochondria indicates this treatment may be useful to reduce or eliminate HS induced apoptosis in females. Only further studies using embryo transfer could conclusively determine whether these blastocysts have normal developmental potential.

Another point that remains to be elucidated is the physiological role of caspases during oocyte maturation given that blockage of caspase-9 or -3/7 at 39°C resulted in decreased developmental potential. Previous studies using caspase-deficient mice showed that caspase-9 and caspase-3 is required for programmed cell death during embryonic brain development (Hakem et al. 1998; Kuida et al. 1998; Kuida 2000; Nakanishi et al. 2001). It also has been shown that caspases may have physiological role apart from cell death executioners including control of T-cell proliferation, cell cycle progression and even in protection of cells (Zeuner et al. 1999; Los et al. 2001; Cauwels et al. 2003; Crenshaw et al. 2007). Previous study suggested that a caspase based surveillance system recognizes and

removes most damaged, ROS-overproducing mitochondria (Fiers et al. 1999). And blocking of caspase inactivates this rescue system resulting in accumulation of high ROS producing mitochondria and more rapid demise of the cell (Vercammen et al. 1998)

In conclusion, the present results indicate that HS-induced apoptosis in bovine oocytes is mediated by activation of caspase-9 and -3/7. Inhibiting the activity of caspase-9 or preventing mitochondrial damage may be useful strategies to decrease HS induced apoptosis in oocytes and subsequently improve fertility in thermal stressed females.

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Figure 1. Heat shock during maturation induced caspase-9 and caspase-3/7 activity in both oocytes and derived blastocysts. Shown are caspase-9 activity (A, n= 31-41 oocytes/group or 17-23 blastocysts/group) and caspase-3/7 activity (B, n= 33-45 oocytes/group or 19-20 blastocysts/group) in oocytes exposed to 39°C (control, open bars) or 41°C (heat shocked, solid bars) for 22 hours and day 8 blastocysts developed from those groups. Data represents least-squares means ± SEM. Superscripts above each bar represent means that differ significantly ($p<0.05$).

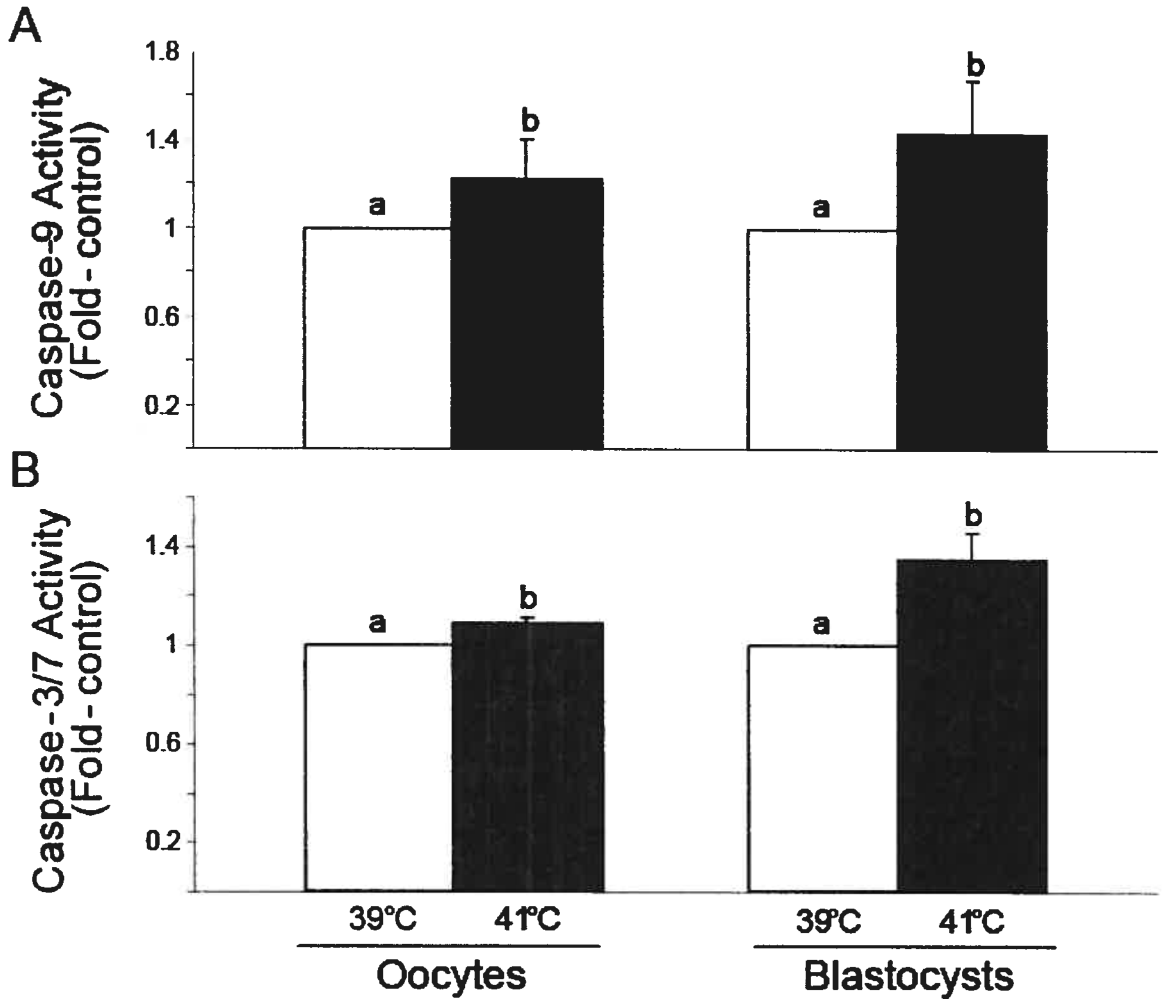

A
Caspase-9 Activity
(Fold - control)
1.8
1.4
1
0.6
0.2
a
b
a
b
B
Caspase-3/7 Activity
(Fold - control)
1.4
1
0.6
0.2
a
b
a
b
39°C
41°C
39°C
41°C
Oocytes
Blastocysts

Figure 2. Caspase inhibitors blocked the detrimental effects of heat shocked-oocyte competence. Shown are cleavage rate (A) and the percentage of oocytes that became blastocyst at day 8 (B) after insemination. Open bars represent oocytes cultured at 39°C while solid bars represent oocytes cultured at 41°C. The DMSO groups represent a pool of 468-472 oocytes/treatment. Data represents least-squares means ± SEM. Superscripts above each bar represents means that differ ($p<0.05$).

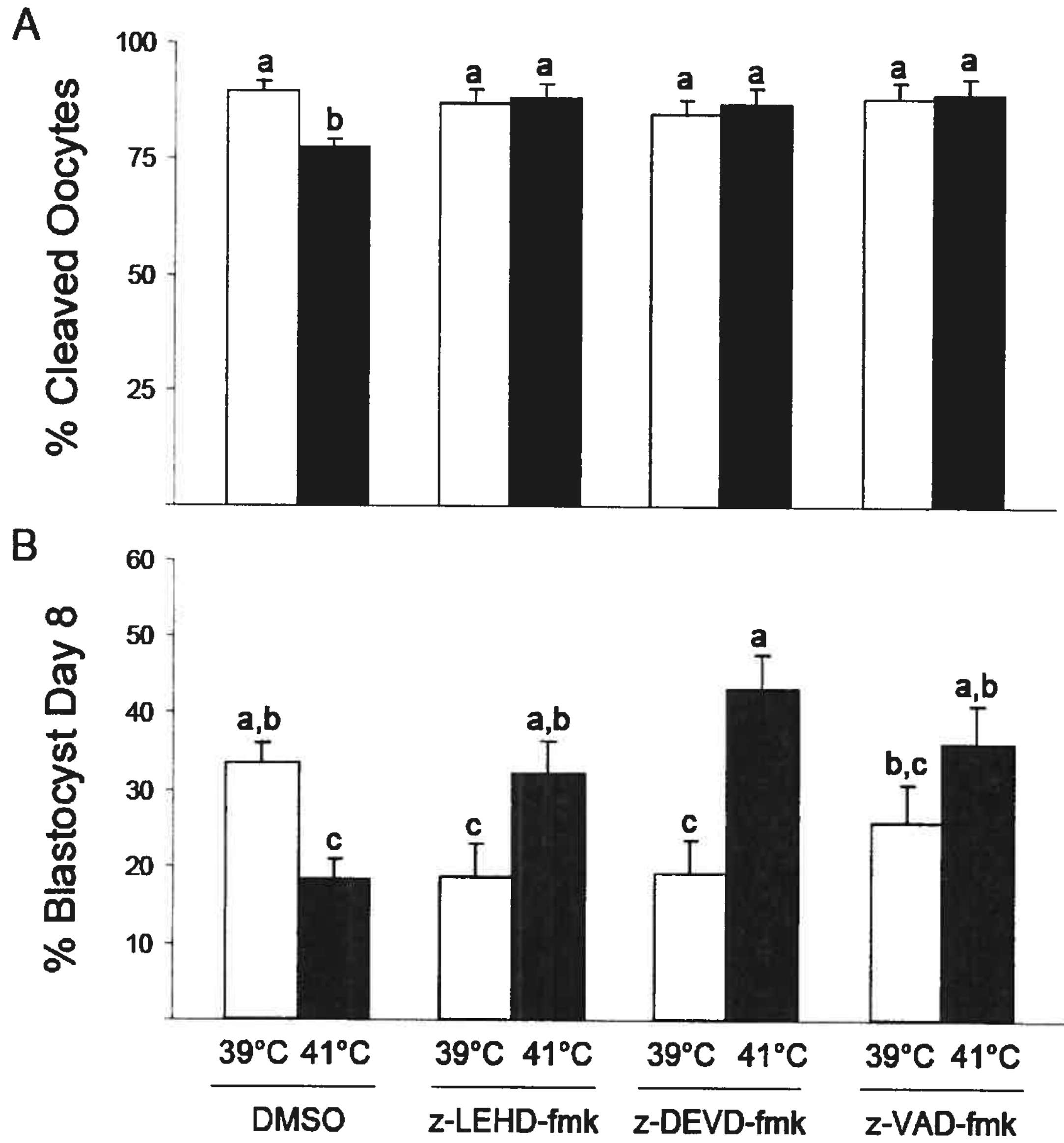
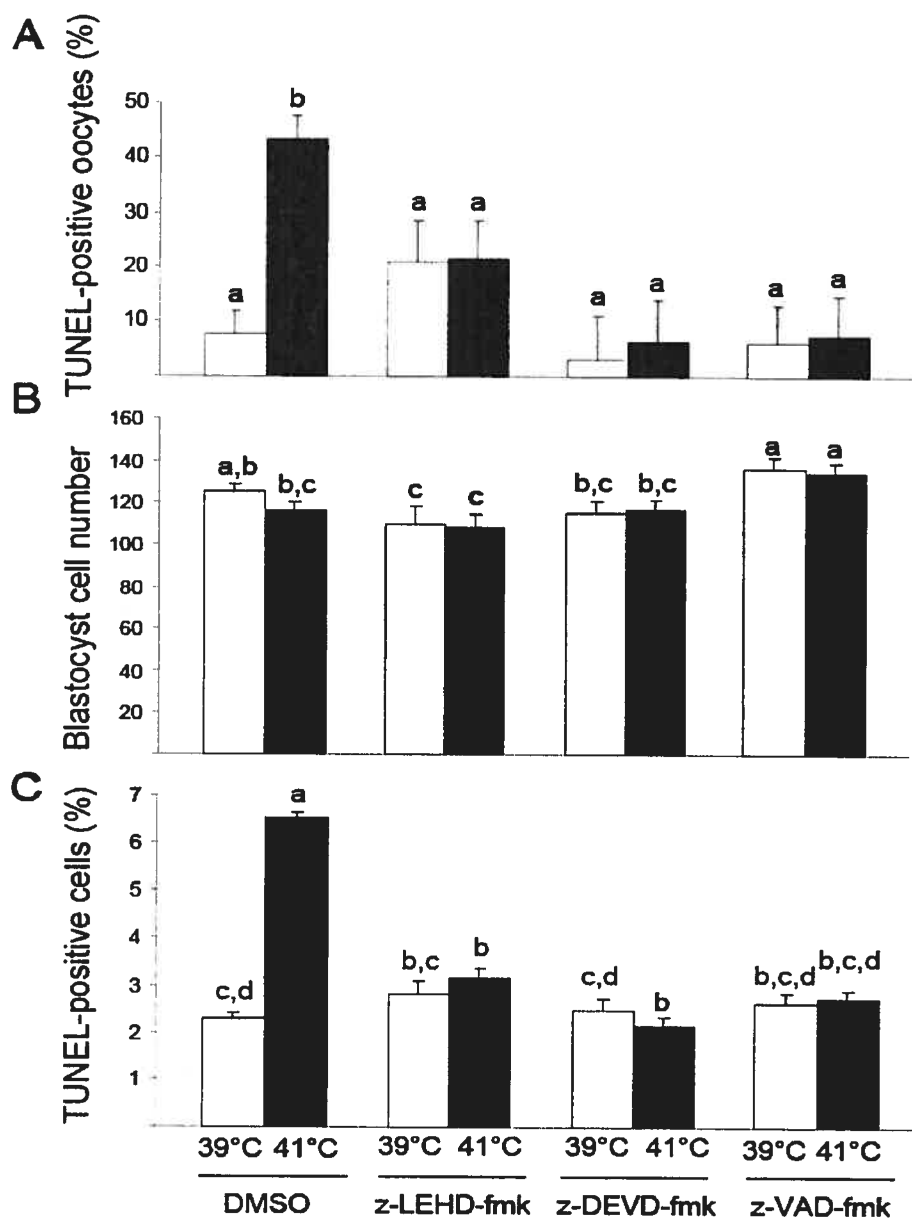

A
100
75
50
25
% Cleaved Oocytes
a
b
a
a
a
a
a
a
B
60
50
40
30
20
10
% Blastocyst Day 8
a,b
c
c
a,b
c
a
b,c
a,b
39°C 41°C
DMSO
39°C 41°C
z-LEHD-fmk
39°C 41°C
z-DEVD-fmk
39°C 41°C
z-VAD-fmk

Figure 3. Caspase inhibitors reduced the frequency of apoptosis in heat shocked oocytes and HS-derived blastocysts. Shown are percentage of TUNEL-positive oocytes (A), total cell number (B), and percentage of blastomeres that were TUNEL-positive in day 8 blastocysts (C) develop from oocytes matured at 39°C or 41°C in the presence or absence of caspase inhibitors. Open bars represent oocytes cultured at 39°C while solid bars represent oocytes cultured at 41°C. The DMSO groups represent a pool of 143-149 oocytes/treatment or 97-142 blastocysts/treatment. Superscripts above each bar that are different represents means that differ at $p<0.05$.


A
TUNEL-positive oocytes (%)
50
40
30
20
10
b
a
a a
a a
a a
B
Blastocyst cell number
160
140
120
100
80
60
40
20
a,b b,c
c c
b,c b,c
a a
C
TUNEL-positive cells (%)
7
6
5
4
3
2
1
a
c,d
b,c b
c,d b
b,c,d b,c,d
39°C 41°C
39°C 41°C
39°C 41°C
39°C 41°C
DMSO
z-LEHD-fmk
z-DEVD-fmk
z-VAD-fmk

CHAPTER IV

BH4 PEPTIDE DERIVED FROM BCL-xL AND BAX-INHIBITOR PEPTIDE SUPPRESSES APOPTOTIC MITOCHONDRIAL CHANGES IN HEAT SHOCKED BOVINE OOCYTES

Short Title: Anti-apoptotic peptides suppresses heat-induced apoptosis in oocytes

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Abstract

Mitochondria play an important role in the integration and transmission of cell death signals, activating caspases and other cell death execution events, which are mediated by Bcl-2 family proteins. Previously, we showed that heat shock (HS)-induced apoptosis in oocytes is mediated by activation of caspase-9 and caspase-9 inhibitor suppressed the detrimental effects of HS on embryo development and apoptosis. Experiments were performed to determine whether anti-apoptotic peptides BH4 domain of Bcl-xL (TAT-BH4) and Bax inhibitor peptide (BIP) suppress HS injury in bovine oocytes by reduction of apoptosis-like events and improve developmental potential. Cumulus-oocyte complexes (COCs) were matured at 39°C (control) or 41°C (HS). The COCs were then placed in maturation medium containig 0.06% water or 100 μM BIP and 0.07% dimethyl sulfoxide (DMSO) or 1 μM TAT-BH4, as well as with a combination of these vehicles (water+DMSO) and peptides (BIP+BH4). Oocytes from all COCs groups were fertilized and cultured *in vitro* at 39°C for 8 days. In the presence of BIP or BIP+BH4, HS-treated oocytes develop into blastocysts similarly to the 39°C group. Surprisingly, COCs matured with TAT-BH4 at 41°C showed reduced embryo development. In HS-treated oocytes exposed to each anti-apoptotic peptide and to a combination of both peptides there was reduced TUNEL frequency in oocytes and blastocysts cells derived from these oocytes. In conclusion, the present results show for the first time that heat-induced apoptosis in bovine oocytes involves Bax and BH4 domain-dependent pathway.

Introduction

Apoptosis is a process of programmed cell death that eliminates cells during the developmental process and after cell damage with little or no effect on surrounding cells (Jacobson et al. 1997). It has been demonstrated that heat shock (HS) induces apoptosis like events in bovine oocytes (Roth and Hansen 2004; Soto and Smith 2006). Subsequent studies have shown that caspase-9 and -3/7 activity are critical effectors of HS-induced apoptosis in oocytes (Soto and Smith 2007). Heat shocked-oocytes exposed to caspase-9 inhibitor (z-LEHD-fmk), demonstrated decreased DNA fragmentation as detected by TUNEL labeling and an increased the proportion of oocytes that become blastocysts after insemination. Together, this is strong evidence for the involvement of the mitochondrial intrinsic pathway in this process (Soto and Smith 2007). Mitochondrial dysfunction may be one of the factors that determine the magnitude of negative effects of stresses on oocyte developmental competence and apoptosis.

The mitochondria play an essential role in the simultaneous release of apoptogenic factors such as cytochrome c and other mediators (Adams and Cory 1998; Green and Reed 1998; Tsujimoto 1998). Cytochrome c in the cytosol binds to apoptosis activating factor-1 (Apaf-1) resulting in activation of one major apical caspase, caspase-9 (Li et al. 1997). Previous studies have demonstrated that caspase activity is regulated by the Bcl-2 family of proteins (Reed 1996; Adams and Cory 1998; Chao and Korsmeyer 1998; Green and Reed 1998). The Bcl-2 family can be categorized into two main groups of proteins according to whether they inhibit (Bcl-2, Bcl-xL, Bcl-w, A1, Mcl-1) or promote apoptosis (Bax, Bak,

Bad, Bim, Bcl-xS, Bok, Bik, Blk, Hrk, Bnip3, Bim) apoptosis. While anti-apoptotic members can form dimers with pro-apoptotic proteins it is unclear whether dimerization is required for activity. It is known that the ratio of these two groups of proteins, in large part determine whether the cell survives or dies after an apoptotic stimulus (Motyl 1999). Proteins of this family are characterized by four structural domains known as Bcl-2 homology domain (BH): BH1, BH2, BH3, and BH4, of which only BH4 has anti-apoptotic properties (Oltvai et al. 1993). Deletion of BH4 from Bcl-2 or Bcl-xL has been shown to abrogate their antiapoptotic ability demonstrating that BH4 is crucial for this activity (Huang et al. 1998). In response to apoptotic stimuli such as HS, Bax translocates into mitochondrial membranes (Bettaieb and Averill-Bates 2005; Stankiewicz et al. 2005) leading to membrane permeability transition pore opening (Qian et al. 2004; Bettaieb and Averill-Bates 2005; Stankiewicz et al. 2005) and release of cytochrome c (Mirkes and Little 2000; Qian et al. 2004; Bettaieb and Averill-Bates 2005; Wada et al. 2005). Heat shock can induce apoptosis in female and male germ cells (Roth and Hansen 2004; Soto and Smith 2006; Jia et al. 2007), embryonic cells (Paula-Lopes and Hansen 2002b; Loureiro et al. 2007), and various cell types. Microinjection of recombinant Bax into isolated oocytes induces apoptosis, indicating that elevation of cytoplasmic Bax levels triggers apoptosis in female germ cells (Morita et al. 2000). In preimplantion bovine embryos, higher expression levels of Bax gene were correlated with an attenuated rate of development (Gutierrez-Adan et al. 2004). Expression of Bcl-2 is elevated in good quality oocytes and embryos, low in fragmented embryos (Yang and Rajamahendran 2002). Direct

involvement of Bcl-2 molecules in heat shock-induced apoptosis in bovine oocytes has yet to be determined.

In the present study, it was hypothesized that the BH4 domain of Bcl-xL and Bax inhibitor peptide prevents apoptotic mitochondrial changes caused by heat stress in bovine oocytes. Experiments were carried out to determine whether the BH4 domain of Bcl-xL fused to HIV TAT protein (TAT-BH4) and Bax inhibitor peptide (BIP) attenuate heat shock injury in bovine oocytes by reducing the proportion of TUNEL-positive nuclei in oocytes and day 8 blastocysts, thereby restoring embryonic development.

Materials and methods

Materials

The Bcl-xL BH4 $_{4\text{-}23}$ (TAT-BH4) and Bax inhibiting peptide (BIP) were obtained from Calbiochem (Mississaga, ON). Unless otherwise mentioned, reagents were purchased from Gibco/Invitrogen (Burlington, ON). Oocyte maturation media (OMM) was hepes-buffered TCM-199 supplemented with 10% (v/v) bovine fetal serum, 1 μl/ml estradiol 17β (Sigma, St-Louis, MO), 0.5 μg/ml FSH (Follitropin-V, Vetrepharm, London, ON), 50 μg/ml LH (Intervet, Whitby, ON), 50 μg/ml gentamicin (Sigma), 0.2 mM sodium pyruvate. Essential fatty acid-free bovine serum albumin (EFAF-BSA), pronase, and hyaluronidase were purchased from Sigma. Percoll was from Amersham Pharmacia Biotech (Uppsala, Sweden). Washing medium (WM) was composed of Hepes-buffered tissue culture medium 199 supplemented with 10% (v/v) bovine fetal serum, 50 μg/ml gentamicin (Sigma), 0.2

mM sodium pyruvate. Frozen semen was donated from CIAQ (St-Hyacinthe, QC). Embryo culture medium (Gardner et al. 1994) was modified synthetic oviductal fluid (mSOF; 108 mM NaCl, 7.2 mM KCl, 0.5 mM $MgCl_2$m, 2.5 mM $NaHCO_3$, 1.7 mM $CaCl_2$-H_2O, 0.5 mM glucose, 0.33 mM pyruvic acid, 3 mM lactic acid, 8 mg/ml BSA, 150 μg/ml gentamicin, and 0.01% phenol red). It was supplemented with 1.4 mM glycine, 0.4 mM alanine, 1 mM glutamine, 1% non-essential amino acids, 2% essential amino acids (Sigma).

In Situ Cell Death Detection Kit TMR red was obtained from Roche Diagnostics Corporation (Laval, QC). Hoechst 33342 and polyvinylpyrrolidone (PVP) were purchased from Sigma. Prolong Antifade Kit was obtained from Molecular Probes (Eugene, OR), RQ1 RNA-free DNase was from Promega (Madison, WI), and RNase A was from Qiagen (Mississauga, ON). The 60-well Terasaki polystyrene microtest plates were obtained from Sarstedt (Montreal, QC).

Oocyte maturation, fertilization and embryo culture

Embryo production was performed as previously described (Parrish et al. 1986). Briefly, COCs were obtained by aspiration of 3-10 mm follicles on the surface of ovaries obtained from local abattoir. Groups of 10 COCs were transferred to pre-equilibrated 50 μl drops of OMM overlaid with mineral oil and matured for 22 hours at 39°C in an atmosphere of 5% (v/v) CO_2 in humidified air. Matured COCs were then washed once in WM and transferred in groups of 30 per 100 μl drop of Tyrode's medium (Parrish et al. 1986) supplemented with 6 mg/ml fatty acid-free bovine serum albumin (BSA; Sigma), 2

μg/ml heparin (Sigma), 10 mM pyruvic acid (Sigma) and 50 μg/ml gentamycin (Sigma) and fertilized with ~1 x 10^6 Percoll-purified spermatozoa from a frozen-thawed semen. After 18-20 hours at 39°C in an atmosphere of 5% CO_2 in humidified air, putative zygotes were removed from fertilization drops, denuded of cumulus cells by vortexing in 50 μl of 0.2% hyaluronidase solution for 5 minutes, and placed in pre-equilibrated 50 μl drops of mSOF at 39°C in humidified atmosphere of 5% CO_2 and 5% O_2 for 8 days. The number of cleaved oocytes and blastocysts was determined on days 3 and 8 after fertilization, respectively. Matured oocytes were harvested after 22 hours of maturation and developing blastocysts on day 8 after fertilization for further analysis.

TUNEL and Hoechst 33342 labeling

The TUNEL assay was used to detect DNA fragmentation associated with later stages of apoptotic cascade and Hoechst 33342 labeling was performed to determine cell number. Oocyte and embryos were removed from culture media and washed with 10 mM KPO_4, pH 7.4 containing 0.9% (w/v) NaCl (PBS) and 1 mg/ml polyvinylpirrolidone (PBS-PVP). Oocytes were denuded from their zona pellucida by ~5 min exposure to 0.5% pronase at room temperature. Oocytes and embryos were fixed in 500 μl of 4% (w/v) paraformaldehyde in PBS for 40 minutes at room temperature, washed and stored in 500 μl PBS-PVP at 4°C until the time of assay. On the day of TUNEL assay, oocytes and embryos were permeabilized in 0.1% (v/v) Triton X-100 containing 0.1 (w/v) sodium citrate for 40 minutes at room temperature. Controls for the TUNEL assay were incubated

in 10 μl of RQ1 RNase-free DNase (50 U/ml) at 37°C in the dark for 1 hour. Positive controls and treated oocyte and embryos were washed in PBS-PVP and incubated with 20 μl of TUNEL reaction mixture (containing fluorescein isothiocyanate- or TMR red-conjugated dUTP and the enzyme terminal deoxynucleotidyl transferase prepared by following manufacture's instructions) for 1 hour at 37°C in the dark. Negative controls were incubated in the absence of terminal deoxynucleotidyl transferase. Oocytes and embryos were then washed two times in PBS-PVP and incubated in 20 μl drop of Hoechst 33342 (1 μg/ml) for 10 minutes in the dark. Oocytes and embryos were washed two times in PBS-PVP to remove excess Hoechst 33342, mounted on 10% (w/v) poly-L-lysine coated slides using 10 μl drops of antifade, and coverslips were placed. Labeling of TUNEL and Hoechst 33342 nuclei was observed using a Leica MDLA microdissection microscope (Cambridge, UK) with dual filter. Images were aquired using IM50 software and Leica DC 500 digital camera. Each oocyte was analyzed for number of TUNEL-labeled pronuclei and blastocysts were analyzed for total cell number and number of TUNEL-labeled nuclei.

Experiments

Protective action of anti-apoptotic peptide on apoptosis and development of embryos

Experiments were design as 2 x 2 factorial to determine whether anti-apoptotic peptides BIP (water or 100 μM; experiment 1), TAT-BH4 (DMSO or 1μM; experiment 2) or both (experiment 3) prevent the detrimental effects of HS (39°C vs 41°C) on oocyte developmental capacity and inhibit heat-induced apoptosis in the oocytes and derived

blastocysts. In each experiment, COCs were matured with different concentrations of each peptide. The first hour maturation was performed at 39°C and, subsequently, for 21 hours at 39°C or 41°C. The first hour at 39°C was performed to allow incorporation of peptides by cells (Yoshida et al. 2004).

The COCs were matured in presence of 1 µM TAT-BH4 reconstituted in DMSO or DMSO alone (0.07% vehicle). For the BIP group, COCs were exposed during maturation to 100 µM BIP or vehicle. In addition, COCs were exposed to a combination of BIP and BH4 peptides. Maturation was performed in presence of 100 µM BIP associated with 1 µM TAT-BH4 (BIP+BH4) or vehicle (water+DMSO). A subset of oocytes was denuded from the cumulus cells, pronase was utilized to remove the zona pellucida, fixation was with 4% (w/v) paraformaldehyde for 30 minutes, and TUNEL analysis was performed. On day 8 after fertilization, zona intact blastocysts were harvested followed by fixation of embryos and TUNEL assay performed as previously described. Proportion of oocytes that cleaved and percentage of oocytes developing to blastocysts were determined on day 3 and 8, respectively. Experiments to evaluate developmental capacity for BIP were replicated five times using 111-119 oocytes per treatment, for the TAT-BH4 group, there were five replicates using 162-178 oocytes/treatment, and BIP+BH4 group was repeated six times using 140-198 oocytes/treatment. Experiments to measure DNA fragmentation by TUNEL analysis for BIP group were replicated five times using 47-64 oocytes/treatment or 17-39

blastocysts/treatment, TAT-BH4 group was five times using 54-67 oocytes/treatment or 32-56 blastocysts/treatment, and BIP+BH4, six times using 67-71 oocytes/treatment or 27-69 blastocysts/treatment.

The concentrations of peptides were based on our preliminary dose-response experiments and in the information reported by other investigators (Shimizu et al. 2000b; Futaki et al. 2001; Sawada et al. 2003; Yoshida et al. 2004; Ono et al. 2005; McConnell et al. 2007). The concentrations tested were 0.1 μM, 1μM, or 10 μM for TAT-BH4 experiments and for BIP experiments 100 μM, 200 μM, and 400 μM of BIP with respective vehicles diluted in OMM (data not shown).

Statistical analysis

Data were analyzed by least-squares analysis of variance using General Linear Model procedure of SAS (SAS systems for Windows, Release 8.20; Cary, NC). Percentage data were analyzed after being subject to arcsine transformation. Independent variables were the effects of treatment, temperature, and interaction of treatment and temperature. Probability values reported are based on the analysis of transformed data while least-squares means are based on analyses of untransformed data. A means separation procedure of SAS known as *pdiff* was performed to determined differences between levels of individual treatments. A probability value of $P<0.05$ was defined as significant.

Results

Protective actions of anti-apoptotic peptides against heat shock induced-apoptosis in oocytes and derived blastocysts

For experiment 1, HS (41°C) reduced the percentage of oocytes that cleaved when compared to control group (Figure 1A, $P<0.05$). In presence of 100 µM BIP (Bax inhibitor peptide), maturation for 21 hours at 41°C increased the percentage of oocytes that developed into blastocysts to a level equivalent to control at 39°C (Figure 1B, treatment x temperature: $P<0.001$). In contrast, COCs exposed to 39°C and 100 µM BIP displayed reduced development to blastocyst similar to control 41°C. Exposure of control oocytes to 41°C for 21 hours increased the number of TUNEL-positive oocytes and blastocyst cells (Figure 2A, $P<0.05$). Nonetheless, there was no increase in the percentage of TUNEL-positive HS-treated oocytes exposed to BIP (Figure 2A, treatment x temperature: $P<0.05$) nor in the percentage of TUNEL-positive blastomeres (Figure 2C, treatment x temperature: $P<0.001$) derived from oocytes exposed to HS. Furthermore, there was no effect of temperature or treatment on the embryo total cell number (Figure 2B).

In the experiment 2, the presence of 1 µM TAT-BH4 and maturation for 21 hours at 41°C did not increase the percentage of oocytes that developed into blastocysts (Figure 3B, treatment x temperature: $P>0.05$). Only an effect of temperature ($P<0.05$) was observed, COCs exposed to 41°C demonstrated a reduced developmental capacity for both groups (DMSO and 1 µM TAT-BH4) and similar development rate to groups exposed to 39°C. Heat stressed-COCs cultured in 1 µM TAT-BH4 had reduced percentage of TUNEL-

positive oocytes (Figure 4A, treatment x temperature: $P<0.05$) and TUNEL-positive cells in blastocysts (Figure 4C, $P<0.001$) without an effect on total cell number (Figure 4B).

And in the experiment 3, addition of combination of 100 μM BIP and 1 μM TAT-BH4 (BIP+BH4) to maturation medium of COCs matured at 41°C for 21 hours resulted in increased development to the blastocyst stage (Figure 5B, treatment x temperature: $P<0.05$) without an effect on cleavage rate (Figure 5A). In addition, HS did not increase the percentage of TUNEL-positive oocytes cultured in BIP+BH4 (Figure 6A, treatment x temperature: $P<0.05$) nor in the percentage surviving to blastocysts of HS-treated oocytes exposed to BIP+BH4 (Figure 6C, treatment x temperature: $P<0.001$). There was no effect of BIP+BH4 on total cell number (Figure 6B).

Discussion

In the present report, we showed a protective effect of members of Bcl-2 family peptides during thermal stress in oocytes. Treating HS-oocytes with BIP suppressed the deleterious effect of HS on embryo development and apoptosis. In contrast, TAT-BH4 did not reestablish the capacity of oocytes to develop to blastocyst stage despite the reduction of apoptosis frequency in both oocytes and blastocysts. Exposure of HS-oocytes to a combination of both peptides caused a decreased proportion of TUNEL-positive oocytes and blastocysts cells with increased embryo development. These results show clearly the involvement of Bax and BH4-domain pathways in cell survival and developmental potential following HS in bovine oocytes.

Apoptosis is an actively regulated process of cellular self-destruction. It compromises mitochondrial changes including the characteristic release of pro-apoptotic proteins such as cytochrome c and procaspases from the mitochondrial intermembrane space (Samali et al. 1999; Susin et al. 1999a; Costantini et al. 2002; Bettaieb and Averill-Bates 2005). Downstream in the apoptotic program, the caspase cascade activates the initiator caspase-9 and executioner caspase-3 (Enomoto et al. 2001; Zhang et al. 2003; Qian et al. 2004; Bettaieb and Averill-Bates 2005). This caspase activation is followed by cytoskeletal alterations, chromatin condensation, and DNA fragmentation, culminating with cell death (Enari et al. 1998; Sakahira et al. 1998; Slee et al. 2001). Previous experimental studies have shown that HS induces apoptosis in several different cell types including female and male germ cells (Roth and Hansen 2004; Jia et al. 2007) and embryonic cells (Paula-Lopes and Hansen 2002a; Paula-Lopes and Hansen 2002b; Jousan and Hansen 2007; Loureiro et al. 2007). Together, the facts that HS-induced apoptosis in oocytes and embryos caused an increase in caspase-9 activity (Loureiro et al. 2007; Soto and Smith 2007), and caspase-9 inhibitor can block apoptosis induced by HS (Loureiro et al. 2007; Soto and Smith 2007) and that HS caused mitochondrial swelling in two-cell embryos (Rivera et al. 2003) provide strong evidence for the involvement of the mitochondrial intrinsic pathway in this process. The direct involvement of Bcl-2 family in heat-induced apoptosis detail mechanisms remain to be elucidated.

Given the large number of publications that have, based on gene expression, implicated Bax as a key factor in the initiation of apoptosis in both oocytes and granulosa

cells, it is perhaps not surprising that HS induces apoptosis through Bax dependent pathway. Bax normally resides in the cytoplasm in a quiescent state. Previous studies in somatic cells have shown that HS induces conformational changes in Bax, resulting in translocation from the cytoplasm to the mitochondrial membrane (Bettaieb and Averill-Bates 2005; Stankiewicz et al. 2005), thereby allowing opening of mitochondrial membrane permeabilization pore (Qian et al. 2004; Bettaieb and Averill-Bates 2005; Stankiewicz et al. 2005), and release of apotogenic factors in somatic cells (Samali et al. 1999; Costantini et al. 2002; Qian et al. 2004; Bettaieb and Averill-Bates 2005; Stankiewicz et al. 2005). Bax inhibiting peptide is a membrane permeable peptide compromised of five amino acids, derived from the Bax-binding domain of Ku subunit 70 kDa (Ku70) that inhibits Bax-mediated translocation of cytochrome c and suppresses caspase activation (Sawada et al. 2003). The Ku70 also plays an important role in DNA double-strand break repair (Walker et al. 2001). And Ku70 blocks Bax-mediated apoptosis by interacting with Bax in the cytosol and preventing its mitochondrial translocation (Sawada et al. 2003). The BIP peptides demonstrate anti-apoptotic activities at range of 50-200 μM concentration and are stable and functional for at least 3 days in culture medium. Here, HS-oocytes treated with 100 μM BIP during 21 hours of maturation resulted in increased embryo development and reduced frequency of TUNEL labeling in both oocytes and derived blastocyst cells. Given that exposure of oocytes to 39°C and BIP caused reduction on development to the blastocyst stage, the physiological role of Bax during maturation remains to be elucidated.

These data indicate that HS act through Bax pathway and BIP may be used to protect oocyte mitochondria from apoptotic injury under stress condition.

The BH4 domain is well conserved in the antiapoptotic members of the Bcl-2 family (Adams and Cory 1998). Previous studies have shown that the BH4 domain is essential for Bcl-2 and Bcl-xL anti-apoptotic activities (Hanada et al. 1995; Huang et al. 1998; Shimizu et al. 2000b). The proposed mechanism of its anti-apoptotic activity is through prevention of the release of cytochrome c and the mitochondrial membrane potential loss by modulating activity of voltage-dependent anion channel (Vdac) through binding (Yang et al. 1997; Shimizu et al. 2000b). The death preventing effects of Bcl-2 and the death-promoting effects of Bax depend on their ability to insert and target the mitochondrial membranes. Here we have demonstrated that the 20 amino acid BH4 domain derived from Bcl-xL coupled to 10 amino acid HIV-TAT_{48-57} sequence (TAT-BH4) was able to provide protection in vitro against heat-induced apoptosis in bovine oocytes. Similar results have been reported in cultured cells. Isolated lymphocytes treated with TAT-BH4 were protected against radiation induced-apoptosis (McConnell et al. 2007). The TAT-BH4 peptide also inhibited endothelial cell death induced by oxidative stress (Cantara et al. 2004). The BH4 of Bcl-2/Bcl-xL is necessary and sufficient for inhibiting Vdac activity, which in turn prevents mitochondrial membrane potential loss with consequent suppression of apoptotic cell death (Shimizu et al. 2000b). This evidence strongly indicates that the BH4 domain underlies the antiapoptotic activity of Bcl-xL by preventing apoptotic mitochondrial changes.

Results from the present study also showed that HS-treated oocytes exposed to TAT-BH4 caused reduced frequency of development to blastocysts. The mitochondrial antiapoptotic activity of BH4 domain certainly does not exclude the possibility of other distinct activities. The BH4 domain has been shown to bind with other proteins including calcineurin (Shibasaki et al. 1997) and Raf-1 (Wang et al. 1996). The convergence of both protein kinase Raf-1, and a phosphatase, calcineurin onto the same BH4 domain is interesting. Interaction of BH4 domain and Raf-1 resulted in activation of Raf-1 (Wang et al. 1996), which is best known for the role it plays in growth factor mediated signal transduction pathways (Daum et al. 1994; Reed 1997). A possible explanation is that binding of BH4 domain to calcineurin may sequester active calcineurin. Previous studies demonstrated that calcineurin maintains phosphorylation of protein kinases (Weinbrenner et al. 1998) that are important modulators of maturation in bovine oocytes (Ali and Sirard 2005). The association of sequestration of calcineurin by BH4 domain with mitochondrial dysfunctions induced by HS (Mirkes and Little 2000; Rivera et al. 2003) may play a role in this reduced developmental potential.

The present study demonstrated that HS-treated oocytes exposed to a combination of both peptides (BIP+BH4) reduced the proportion of TUNEL-positive chromatin and increased embryonic development. The mechanisms of action of Bcl-2 proteins are not completely understood, a common characteristic of Bcl-2 related proteins is the ability to produce homo- and heterodimers. The C-terminal of Bcl-xL and the hydrophobic pocket of another Bcl-xL or Bax protein can interact and form either homodimers or heterodimers

(Jeong et al. 2004). However, the BH4 domain alone lacks the requisite interaction domains present in full length Bcl-xL to suppress apoptosis by this mechanism. It is therefore puzzling that oocytes cultured with BIP+BH4 combination cultured at 41°C caused embryo development rates similar to 39°C control. This is especially so since results presented in this study demonstrated that administration of BIP to COCs culture at 39°C inhibited embryonic developmental capacity. As a possible explanation, we propose inhibition of Bax prevented mitochondrial damage associated with the presence of BH4 domain, therefore allowing activation of survival pathways. Previous studies have shown that BH4 domain reversed beta-amyloide peptide-induced apoptosis by activating the survival program of serine/threonine protein kinase B (Akt) by increasing its phosphorylation (Cantara et al. 2007). Activated Akt cause cytoprotective events by phosphorylation of apoptotic factors such as Bad and caspase-9 resulting in suppression of Bad apoptotic activity and caspase-9 induced apoptosis (Datta et al. 1999; Fresno Vara et al. 2004; Lee et al. 2005).

From the data presented above, it is clear that Bcl-2 family members are fundamental components of the complex regulatory pathways responsible for controlling germ cell fate. Beside the importance of Bax in this respect, the BH4 domain has also emerged as an equally important modulator of the apoptotic process. Much more work is needed to determine the role(s) of Bcl-2 family members in controlling germ line death and life. Given the central importance of apoptosis in female germ cell loss under normal and pathological situations (Tilly et al. 1991; Tilly 2001), better understanding of the molecular

regulation dictating oocyte fate will in all likelihood open opportunities for development of apoptosis-based therapeutic strategies design to improve fertility and reproductive health even under stress conditions.

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Figure 1. Bax inhibitor peptide (100 μM BIP) blocked the detrimental effects of heat shocked-oocyte competence. Shown are cleavage rate (A) and the percentage of oocytes that became blastocyst at day 8 (B) after insemination. Open bars represent oocytes cultured at 39°C while solid bars represent oocytes cultured at 41°C (n= 111-119 oocyte/treatment). Data represents least-squares means ± SEM. Superscripts above each bar represents means that differ ($P<0.05$).

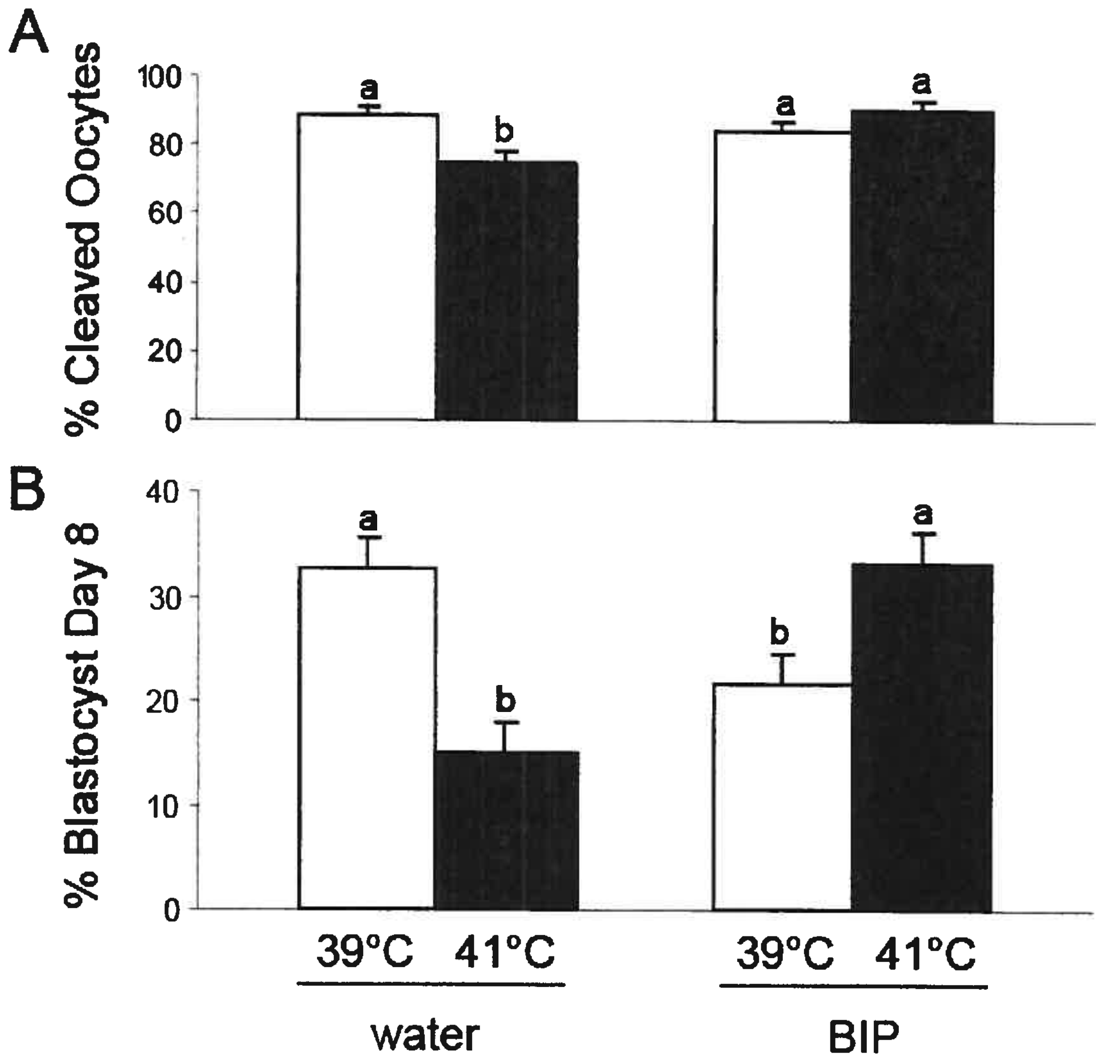
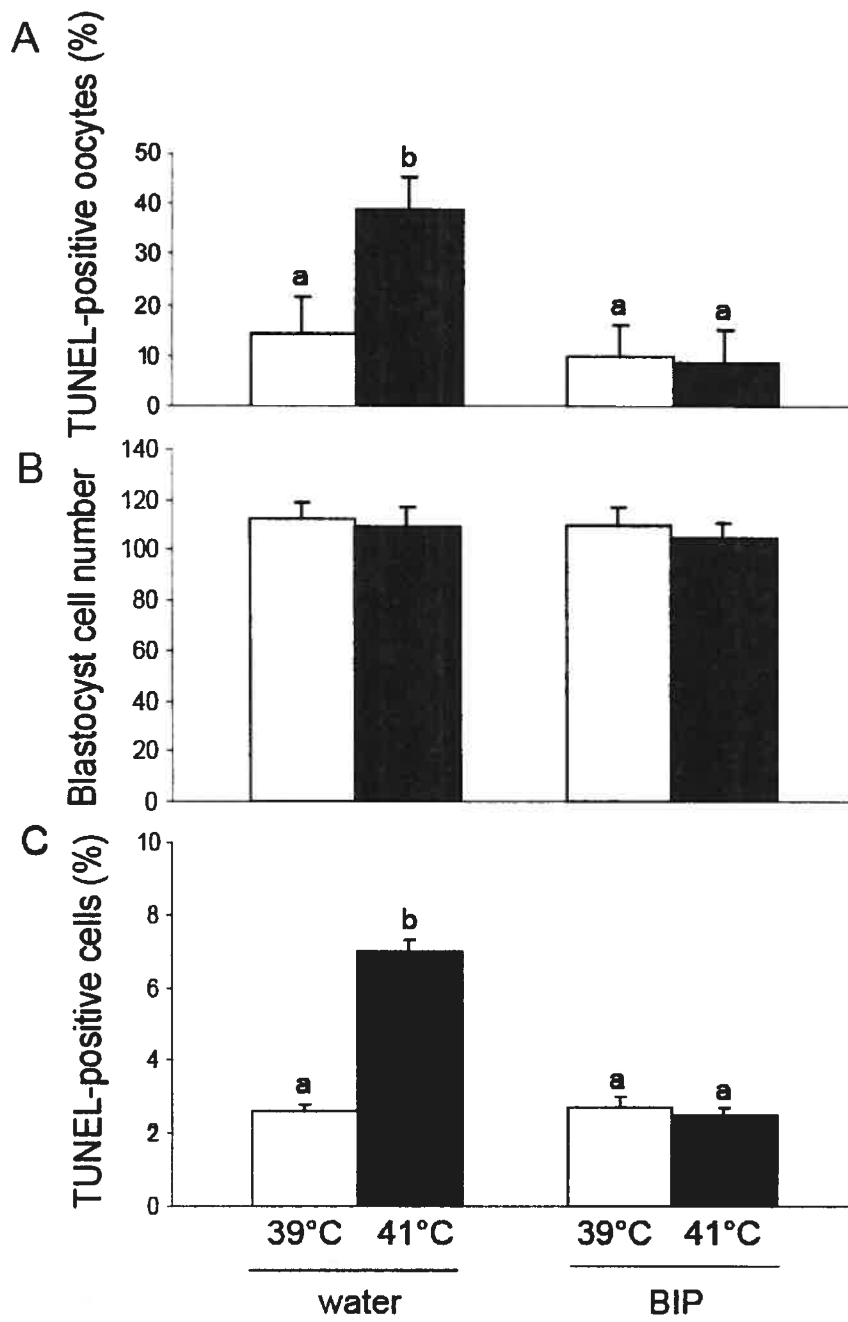

A
% Cleaved Oocytes
100
80
60
40
20
0
a
b
a
a
B
% Blastocyst Day 8
40
30
20
10
0
a
b
b
a
39°C
41°C
39°C
41°C
water
BIP

Figure 2. Effects of Bax inhibitor peptide (BIP) and heat shock (HS) during maturation on induction of apoptosis in oocytes and day 8 blastocysts. (A) Proportion of TUNEL-positive oocytes after addition of 100 μM BIP and 21 hours of heat shock (n= 47-64 oocyte/treatment), (B) total cell number and (C) TUNEL-positive blastomeres from HS-BIP treated-oocytes (n= 17-39 blastocysts/treatment). Open bars represent oocytes cultured at 39°C while solid bars represent oocytes cultured at 41°C. Data represents least-squares means ± SEM. Bar with different superscripts differ ($P<0.05$).



A
TUNEL-positive oocytes (%)
50
40
30
20
10
0
b
a
a
a
B
Blastocyst cell number
140
120
100
80
60
40
20
0
C
TUNEL-positive cells (%)
10
8
6
4
2
0
b
a
a
a
39°C
41°C
39°C
41°C
water
BIP

Figure 3. The BH4 domain of Bcl-xL (1 μM TAT-BH4) fails to block the negative effects of heat shocked-oocyte competence. The percentage of oocytes that cleaved is shown on panel A and the percentage of oocytes that became blastocyst at day 8 after insemination, on panel B. Open bars represent oocytes cultured at 39°C while solid bars represent oocytes cultured at 41°C (n= 162-178 oocyte/treatment). Data represents least-squares means ± SEM. Bar with different superscripts differ ($P<0.05$).

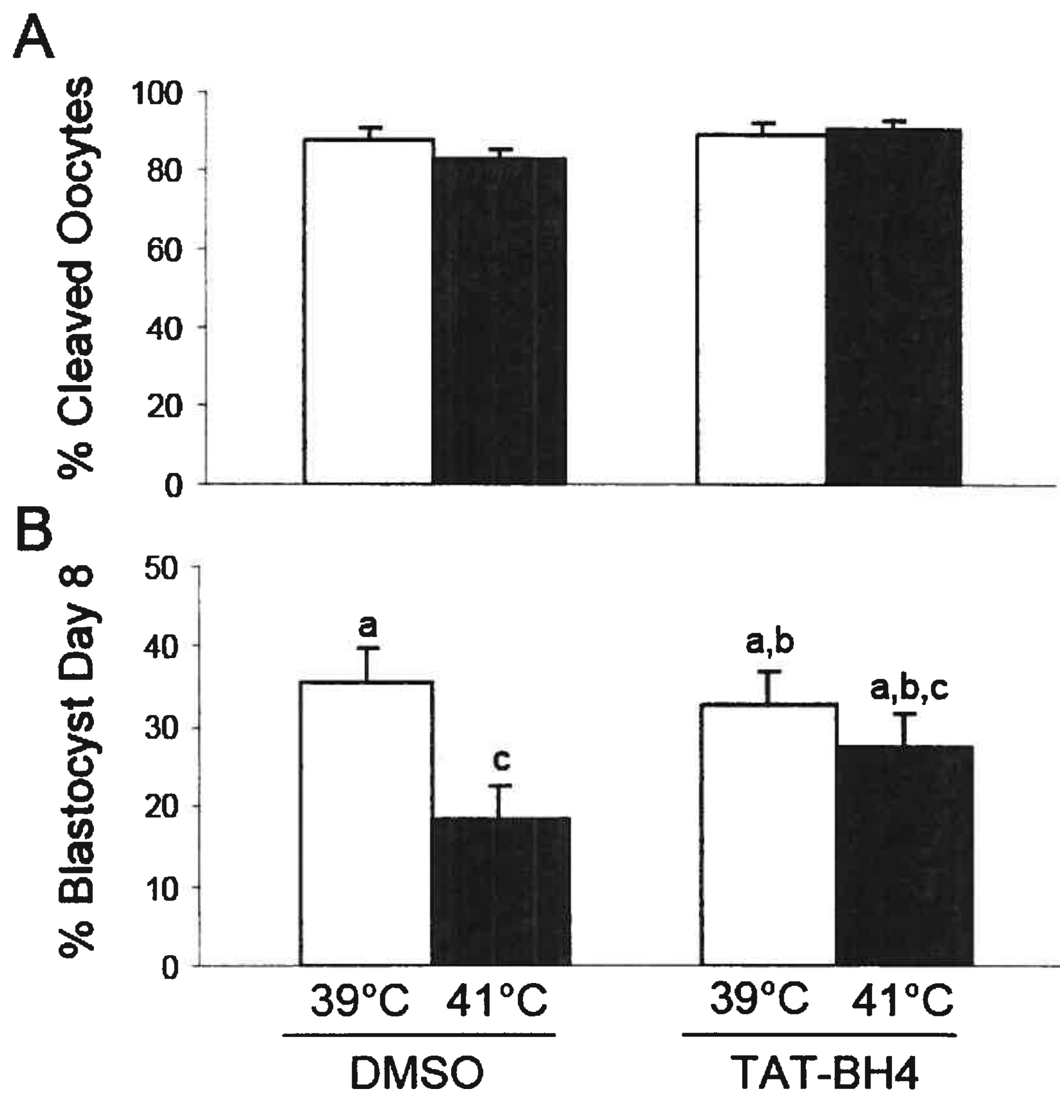

A
% Cleaved Oocytes
100
80
60
40
20
0
B
% Blastocyst Day 8
50
40
30
20
10
0
a
c
a,b
a,b,c
39°C
41°C
39°C
41°C
DMSO
TAT-BH4

Figure 4. Effects of BH4 domain of Bcl-xL (TAT-BH4) and heat shock (HS) during maturation on induction of apoptosis in oocytes and day 8 blastocysts. Shown are the frequency of TUNEL-positive oocytes after addition of 1 μM TAT-BH4 and 21 hours of heat shock (n= 54-67 oocyte/treatment), (B) total cell number and (C) TUNEL-positive blastomeres from HS-BH4 treated-oocytes (n= 32-52 blastocysts/treatment). Open bars represent oocytes cultured at 39°C while solid bars represent oocytes cultured at 41°C. Data represents least-squares means ± SEM. Bar with different superscripts differ ($P<0.05$).

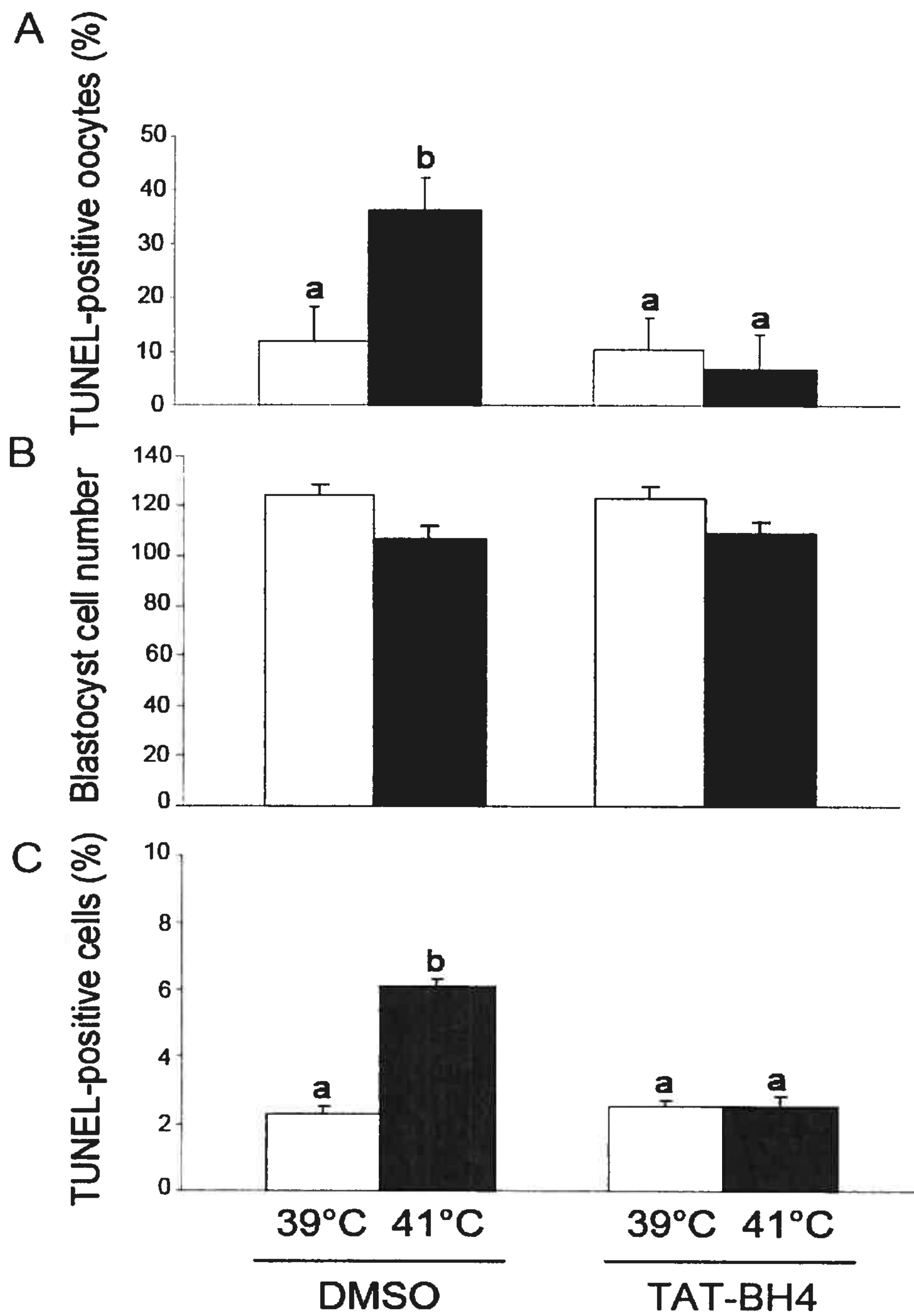

A
TUNEL-positive oocytes (%)
50
40
30
20
10
0
b
a
a
a
B
Blastocyst cell number
140
120
100
80
60
40
20
0
C
TUNEL-positive cells (%)
10
8
6
4
2
0
b
a
a
a
39°C 41°C
DMSO
39°C 41°C
TAT-BH4

Figure 5. Combination of peptides, Bax inhibitor peptide and BH4 domain of Bcl-xL (BIP+BH4), suppressed the detrimental effects of heat shocked-oocyte competence. Shown are cleavage rate (A) and the percentage of oocytes that became blastocyst at day 8 (B) after insemination. Open bars represent oocytes cultured at 39°C while solid bars represent oocytes cultured at 41°C (n= 140-198 oocyte/treatment). Data represents least-squares means ± SEM. Bars with different superscripts differ ($P<0.05$).

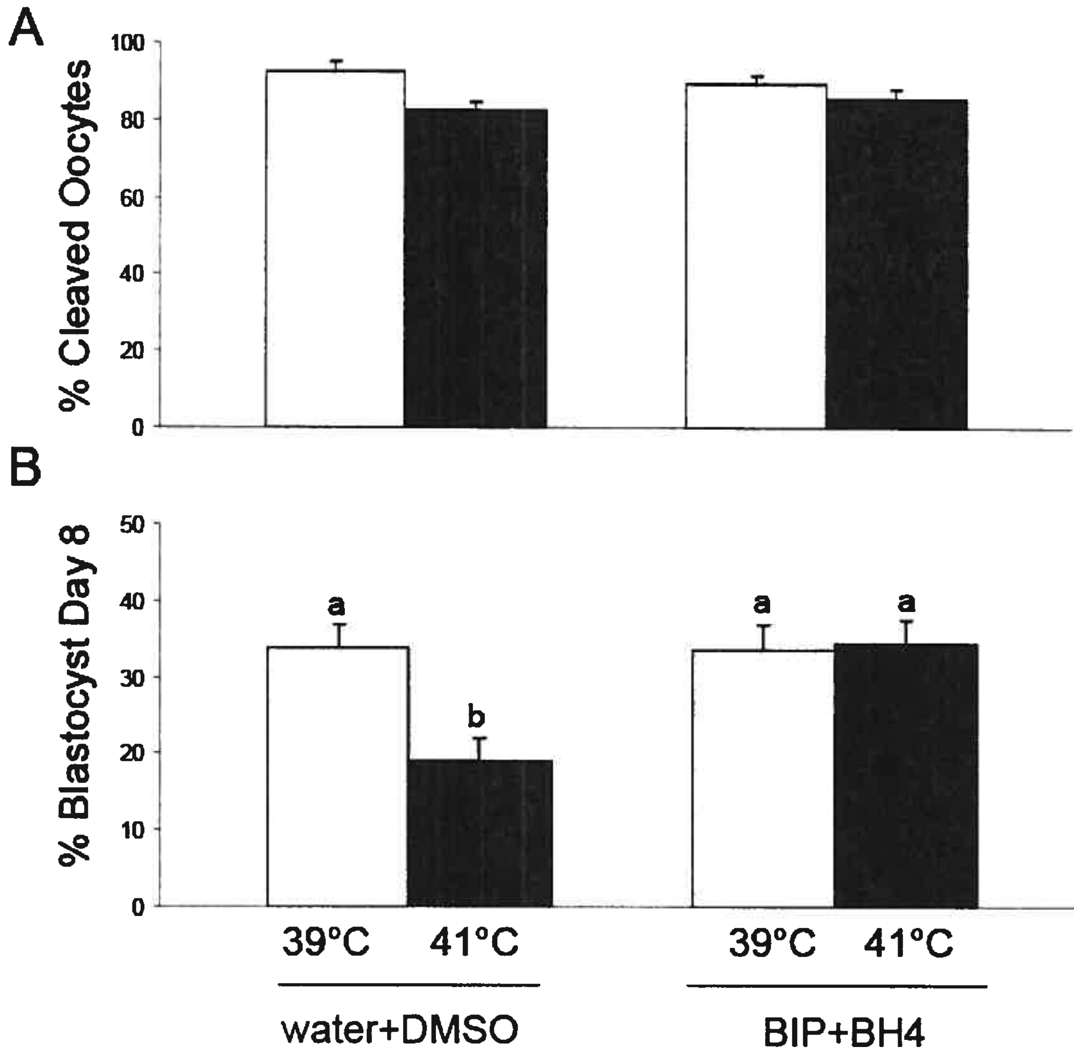
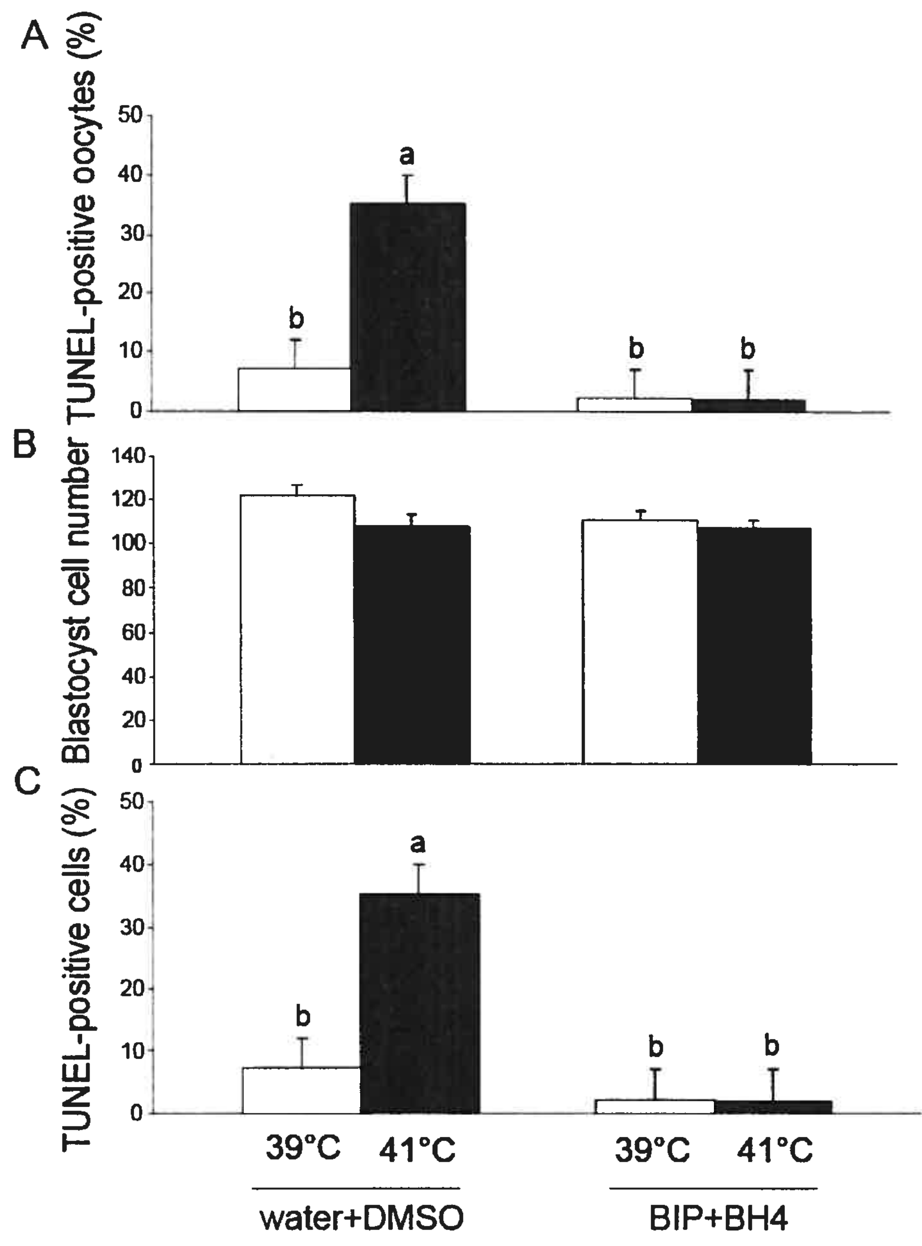

A
100
80
60
40
20
0
% Cleaved Oocytes
B
50
40
30
20
10
0
% Blastocyst Day 8
a
b
a
a
39°C
41°C
39°C
41°C
water+DMSO
BIP+BH4

Figure 6. Protective actions of combination of anti-apoptotic peptides, Bax inhibitor peptide and BH4 domain of Bcl-xL (BIP+BH4), and heat shock (HS) during maturation on induction of apoptosis in oocytes and day 8 blastocysts. Shown are the frequency of TUNEL-positive oocytes after addition of 100 μM BIP and 1 μM BH4 and 21 hours of heat shock (n= 67-71 oocyte/treatment), (B) total cell number and (C) TUNEL-positive blastomeres from HS-BH4 treated-oocytes (n= 27-69 blastocysts/treatment). Open bars represent oocytes cultured at 39°C while solid bars represent oocytes cultured at 41°C. Data represents least-squares means ± SEM. Bar with different superscripts differ ($P<0.05$).


A
TUNEL-positive oocytes (%)
50
40
30
20
10
0
a
b
b
b
B
Blastocyst cell number
140
120
100
80
60
40
20
0
C
TUNEL-positive cells (%)
50
40
30
20
10
0
a
b
b
b
39°C
41°C
39°C
41°C
water+DMSO
BIP+BH4

General Discussion

Experiments for in this thesis focused on extending the current understanding of stress-induced apoptosis in the oocyte, and more specifically the role of the mitochondrial pathway. The importance of the subject is underscored by the severe reduction in fertility associated with mitochondrial dysfunction (Keefe et al. 1995; Van Blerkom et al. 1995; Muller-Hocker et al. 1996; Acton et al. 2004; Jansen and Burton 2004; Thouas et al. 2004; Thouas et al. 2006). In the results presented here, we demonstrated that HS- and FCCP-induced apoptosis was associated with reduced embryo development and alteration of mRNA expression of candidate mitochondrial and apoptosis related genes (Chapter 2). In addition, the use of a mitochondrial membrane PT pore inhibitor, cyclosporin A, blocked heat shocked-induced apoptosis in oocytes. The selection of HS as a stress was based on its association with reduced fertility and activation of the mitochondrial apoptosis pathway in somatic cells. We demonstrated, for the first time that HS-induced apoptosis in bovine oocytes is mediated by activation of initiator caspase-9 (Chapter 3). The existence of this mechanism in oocytes indicate that HS may induce mitochondrial dysfunction by allowing the release of pro-apoptotic proteins from the mitochondrial intermembrane space resulting in activation of caspase-9 and caspase-3 (Du et al. 2000; Gottlieb 2000; Jiang and Wang 2000; Patterson et al. 2000; Adrain and Martin 2001; Lorenzo and Susin 2004). Furthermore, specific caspase-9 and -3/7 inhibitors or a general caspase inhibitor blocked HS-induced apoptosis on oocyte developmental capacity and decreased percentage of apoptosis. Finally, results from Chapter 4 showed that preservation of mitochondrial

integrity by the anti-apoptotic peptides of Bcl-2 family reestablished the oocyte capacity to develop to blastocyst stage and reduced apoptotic frequency, indicating that HS effects involves Bax and BH4-domain pathway. Implications of these findings are addressed in further detail below.

Oocyte maturation is a complex process that is initiated during a period of 24 hours from the LH surge to ovulation in most species (Dieleman et al. 2002). The oocyte undergoes both nuclear and cytoplasmic maturation. Nuclear maturation involves completion of meiosis I, germinal vesicle breakdown (GVBD), extrusion of the first polar body, and arrest of the oocyte in metaphase II (MII). Cytoplasmic maturation refers to a variety of cellular changes necessary for fertilization and subsequent development including reduction of Golgi complex, alignment of cortical granules along the oolema, redistribution of mitochondria and endoplasmic reticulum, and the ability of undergo cortical granule exocytosis (Ducibella et al. 1990; Ducibella et al. 1993) Hytell 1997). The accumulation of mRNA and proteins in the oocyte during early embryonic development is believed to be associated with the ability of an oocyte to become an embryo (Sirard 2001). However, massive degradation of transcripts occurs during oocyte maturation. In a germinal vesicle (GV) stage mouse oocyte contains an estimated 85 pg of total mRNA and this amount declines during oocyte maturation by about 50 pg; around half undergoes deadenylation and the other half undergoes degradation (Paynton et al. 1988). Little is known about which transcripts are degraded and which are stable. A recent study demonstrated that transcripts associated with meiotic arrest at the GV stage and the

progression of oocyte maturation such as oxidative phosphorylation, energy production, and protein synthesis and metabolism were dramatically degraded (Su et al. 2007). This loss of transcripts could reflect the relatively low energy demand of MII oocytes. In contrast, the stable transcripts were those participants in signaling pathways such as protein kinase pathways, essential for maintaining characteristics of MII-arrested oocyte (Su et al. 2007). Results presented in Chapter 2 showed that the relative abundance of proapoptotic Bax transcripts was reduced on HS-treated oocytes. Aberrant degradation, as observed in this study, or maintenance of certain classes of transcripts during oocyte maturation may be detrimental to oocyte quality, impacting developmental competence. Another interesting finding, also described in Chapter 2, was the down-regulation of mitochondrial ND6, anti-apoptotic Bcl-xL, and HSP70 mRNA concentrations in blastocysts derived from oocytes exposed to FCCP. This supports the notion that depolarization of oocyte mitochondria have a long term deleterious effect. The significance of these expression patterns remains to be elucidated, but it is possible that variation in detection of some transcripts may reflect differences in the health of the embryo.

Several different mechanisms may be involved in the arrest in development of embryos observed in Chapters 2, 3, and 4. As an example, failure of oocyte activation due to inability of ooplasmic mitochondria to support calcium sequestration (Dumollard et al. 2004) may have deleterious effects on oocyte maturation. Mitochondrial metabolism in oocytes is closely associated with function of endoplasmic reticulum ATP-dependent transport channels that regulate the uptake and release of cytoplasmic calcium during

fertilization. An inadequate ATP-dependent calcium sequestration and resulting ooplasmic calcium overload may have resulted in developmental demise through induction of an apoptotic process (Liu and Keefe 2000). Another possibility is the increased generation of ROS. The mitochondrial respiratory chain renders the mitochondria the major source of ROS production and accumulation (Turrens 1997). ROS may induce apoptosis as a consequence of ROS-mediated DNA damage (Abramova et al. 2000) as well as ROS-mediating increased mitochondrial membrane permeability with leakage of proapoptotic factors from the intermembrane space (Skulachev 1998). Further, inhibition of ATP-dependent and calcium-dependent cell cycle factors cannot be excluded (Eichenlaub-Ritter et al. 2004). Although it is difficult to establish the mechanisms involved at this point, it clearly demonstrates the importance of mitochondrial regulatory functions.

Although not experimentally addressed in this study, the cumulus cells that compromise the granulosa cell component of COCs might be affected by treatments. During follicular growth, the somatic cells divide to form various layers, the oocyte enlarges, and a fluid-filled antrum begins to form. During this phase, the antrum divides the granulosa cells into mural granulosa cells that form the outer layers, and the cumulus cells surround the oocyte. Cumulus-oocyte interaction is a critical requirement for both follicular growth and acquisition of oocyte competence (Eppig et al. 2002; Matzuk et al. 2002). Apoptosis can be initiated in different cellular components of antral follicles. It is common for mural granulosa cells to undergo apoptosis while cumulus cells remain healthy within the same follicle (Yang and Rajamahendran 2002). The mechanisms by which

oocytes and cumulus cells undergo apoptosis have not been completely elucidated. The cumulus and oocytes communicate via paracrine factors and by gap junctions (Albertini et al. 2001). In aged mice, ceramide has been shown to translocate from the granulosa cells into the adjacent oocyte by a gap-junction dependent communication with consequence of induction of germ cell apoptosis (Perez et al. 2005). There is evidence that oocyte secreated factors, particularly bone morphogenic protein (BMP)-6 and BMP-15, actively prevent death of cumulus cells by establishing a localized morphogenic gradient (Hussein et al. 2005). Therefore, thermal stress or treatment with reversible mitochondrial uncoupler treatments may have the effect of disruption of one or more cumulus-oocyte communications resulting in activation of apoptotic machinery.

A further objective of this thesis was to investigate the involvement of activation of caspases in heat shock-induced apoptosis. Results here support the idea that the signaling for apoptosis involves a caspase-dependent pathway by demonstrating 1) a higher activity of caspase-9 and -3/7 in HS-treated oocytes and derived blastocysts from HS-treated oocytes and 2) that specific caspase inhibitors for caspase-9 (z-LEHD-fmk) or -3/7 (z-DEVD-fmk) or general caspase inhibitor (z-VAD-fmk) completely block heat shock-induced apoptosis in both oocytes and derived blastocysts. In some circumstances, apoptosis can protect the embryo by eliminating damaged cells; however, this is not the case for oocytes. Oocytes cultured at 39°C had decreased competence to reach the blastocyst stage when apoptosis was blocked by caspase-9 or caspase-3/7 inhibitor. The involvement of both caspases (9 and 3) during embryonic brain development is well

established (Hakem et al. 1998; Kuida et al. 1998; Nakanishi et al. 2001). In addition to their well known role as cell death executioners, activated caspases also have been associated with control of T-cell proliferation, cell cycle progression and even protection of cells (Zeuner et al. 1999; Los et al. 2001; Cauwels et al. 2003; Crenshaw et al. 2007). It has been proposed that there is a caspase (or caspase-like enzyme) surveillance rescue system (Vercammen et al. 1998; Fiers et al. 1999). As mentioned before, cells produce basal levels of ROS. After an apoptotic signal, ROS production increases and many more mitochondria sustain ROS-induced damage, and these mitochondria need to be removed by a surveillance system. It is possible that, in presence of caspase inhibitor, this surveillance system is suppressed. Therefore, higher ROS-producing mitochondria accumulate resulting in demise of the cell. This scenario suggests that caspases play a crucial role in maintaining cellular homeostasis and agrees with results of this study where inhibition of caspase demonstrated to be toxic for healthy oocytes.

Future studies should be performed to establish the link between caspase inhibition, mitochondria, and increased ROS production. Experiments could be based on one or more of the following approaches: 1) measurement of ROS production as determined by oxidation of 2',7'-dichlorodihydrofluorescein diacetate, a fluorescence probe and glutathione (GSH) levels. This intracellular antioxidant is known to protect cells against ROS (Sakatani et al. 2007); 2) use of antioxidants that cause inhibition of ROS generation such as GSH itself or the antioxidant dithiothreitol (DTT) which is a strong scavenger of ROS and, as well regenerates reduced GSH; 3) measurement of mtDNA copy numbers by

either polymerase chain reaction methodology direct to specific sequences or by using probes.

One of the interesting and consistent findings of this thesis was that several molecules (z-DEVD-fmk, z-LEHD-fmk, BIP) acted as stress factors to damage the oocyte during maturation in a way that did not greatly reduce fertilization but that caused the subsequent embryo that was formed to have reduced developmental competence. Study of these molecules, as described above, and their effects on various cells and processes during oocyte maturation may lead to insights into their physiological roles and relationship between oocyte quality and embryonic development.

It is known that apoptosis results from an imbalance between anti-apoptotic and pro-apoptotic proteins. Here we analyze the possibility that BIP or TAT-BH4 can be used as a pharmacological tool to prevent HS-induced apoptosis in bovine oocytes. Results presented herein show that each peptide or a combination of two peptides caused a reduction in apoptotic-like events. However, embryonic development to the blastocyst stage was affected in some treatments. The combination of inhibition of Bax associated with the presence of BH4-domain cultured at 41°C caused embryonic development similar to 39°C control group. The BH4 domain of Bcl-xL blocked beta-amyloid peptide induced apoptosis by activation of a survival pathway, such as Akt (Cantara et al. 2007). The phosphatidylinositol-3'-kinase (PI3K) pathway it is known to recruit and activate Akt. This PI3K pathway has been reported to be an essential component of cytokine-mediated female germ cell survival (Morita et al. 1999). Activated Akt induces cytoprotective events by

phosphorylation of the apoptotic factors Bad and caspase-9 (Datta et al. 1999; Fresno Vara et al. 2004; Lee et al. 2005). This phosphorylation results in suppression of Bad apoptotic activity and suppression of caspase-9 induced apoptosis. It may be possible that the BH4 domain activates one or more survival pathways. The association of inhibition of Bax translocation allowing mitochondrial integrity with activation of survival pathways may offer a novel therapy to prevent apoptosis and improve survival of germ cells.

While not experimentally addressed in this study, survival pathways may be activated during heat-induced apoptosis in oocytes. Elevated temperature can trigger death-signaling pathways via activation of JNK (Verheij et al. 1996; Mosser et al. 1997; Buzzard et al. 1998) and survival pathways via activation of Akt (Ma et al. 2001) and extracellular signal-regulated kinases (ERK) (Woessmann et al. 1999). The fate of cells exposed to heat shock may be determined by extent of activation of these pathways. Indeed, inhibition of Akt or ERK strongly increased apoptosis and suppression of JNK blocked heat-induced apoptosis (Verheij et al. 1996; Woessmann et al. 1999; Ma et al. 2001). Besides the PI3K/Akt pathway has been demonstrated to be expressed in oocytes (Andersen et al. 1998) and embryos (Gross et al. 2005) association with environmental stress (e.g. heat shock) has not yet been elucidated. The key element of the heat-activated apoptotic cascade is mitochondrial efflux of proapoptotic factors and engagement of caspase cascade. The Diablo/Smac is one of mitochondrial proteins released into the cytoplasm that blocks the anti-apoptotic activity of IAPs inducing apoptosis. Honda et al (2005) have demonstrated the expression of Diablo/Smac mRNA and protein in mouse oocytes and preimplantation

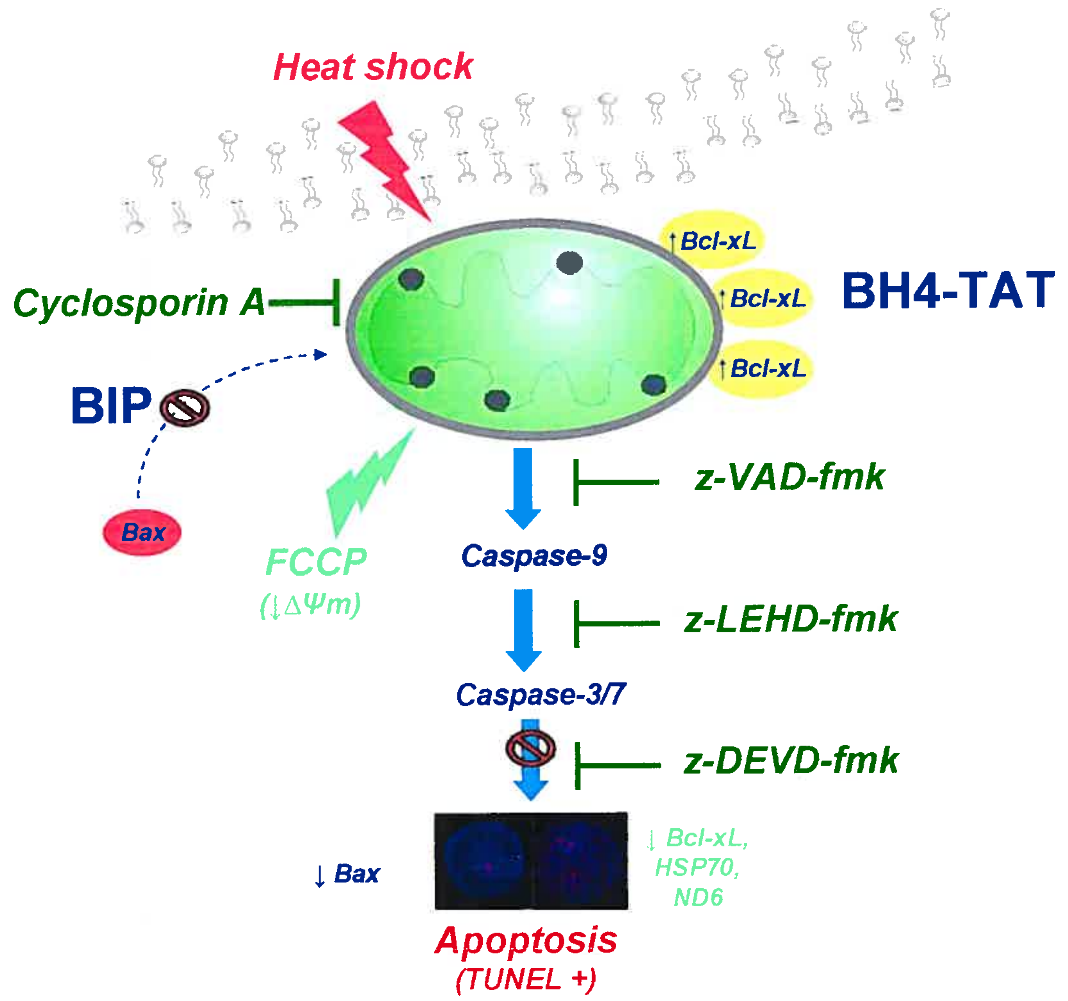
embryos also the release of Diablo/Smac protein into the cytoplasm in both staurosporine-induced apoptosis and fragmented embryos. Functioning of mitochondrial intermembrane proapoptotic proteins has not been yet studied in oocytes. The p53 has been reported to be activated during thermal stress and correlated with induction of apoptosis (Matsumoto et al. 1994; Nitta et al. 1997; Chen et al. 1999; Ota et al. 2000). Recently, it has been demonstrated the transcription independent mechanism of p53 involving its direct translocation to mitochondria followed by inhibitory interactions with Bcl-2 and Bcl-xL and liberation of proapoptotic Bax and Bak (Mihara et al. 2003). Moreover, Akt attenuated p53 mitochondrial accumulation and Smac, Cyt c, HtrA2/Omi release preventing apoptosis (Yang et al. 2006). In this study, it was demonstrated that HS-induced apoptosis in oocytes involves activation of caspases (3/7 and 9) and Bax and BH4-dependent pathway. These results clearly show the importance of mitochondrial integrity and the opportunity to elucidate these mechanisms in the oocyte.

There is a possibility that oocytes and embryos were undergoing an alternative type of death, necrosis. While detection of TUNEL-positive cells is usually indicative of apoptosis, necrosis and artifacts resulted from sample fixation can also result in TUNEL labeling (Hardy 1999). Although the mechanisms and morphologies of apoptosis and necrosis differ, there is an overlap between the two processes. For example, some factors such as a decrease in the availability of caspases and intracellular ATP can convert an ongoing apoptotic process into necrosis (Leist et al. 1997; Denecker et al. 2001). Whether a cell dies through apoptosis or necrosis is dependent in part on the nature of death signal,

cell type, and developmental stage (Elmore 2007). The present study provides strong evidence that the increase in TUNEL-positive nuclei is in fact indicative of apoptosis. This is believed to be so because HS resulted in increase caspase activity, and because caspase inhibitors, Bax inhibitor peptide and BH4 domain blocked HS-induced apoptosis by preventing increase in the number of TUNEL-positive nuclei observed after HS. From these observations we conclude that oocytes exposed to HS resulted in activation of the apoptotic machinery.

Figure 1 represents a tentative model, based on results of experiments described in this thesis to explain and predict mitochondrial changes induced by HS or FCCP on oocyte developmental capacity and apoptosis. Oocyte maturation is disrupted by both stresses in a way that reduced ability of the embryo to continue development and activate apoptosis. The transient mitochondrial uncoupler, FCCP, was explored based on its ability to reduce mitochondrial membrane potential and to open the mitochondrial PT pore (Scorrano et al. 1997; Susin et al. 1998), both events are believed to occur during apoptosis. The FCCP-induced apoptosis indicate that mitochondrial PT pore play crucial role during apoptotic process. This finding was supported by utilization of cyclosporin A, an inhibitor of mitochondrial PT pore that, by itself, caused reduction in the frequency of TUNEL-positive nuclei. It was also demonstrated that HS-induced apoptosis in oocytes is mediated by activation of caspase-9 and caspase-9 inhibitor suppressed the detrimental effects of HS on

Figure 1. Model depicting responses of bovine oocytes and early embryos to putative mediators of oocyte mitochondrial dysfunction on reproductive performance as determined by experiments in this thesis.


Heat shock
Cyclosporin A
BIP
Bax
↑Bcl-xL
↑Bcl-xL
↑Bcl-xL
BH4-TAT
FCCP
(↓ΔΨm)
z-VAD-fmk
Caspase-9
z-LEHD-fmk
Caspase-3/7
z-DEVD-fmk
↓ Bax
↓ Bcl-xL,
HSP70,
ND6
Apoptosis
(TUNEL +)

embryo development and apoptosis. The activation of the mitochondrial pathway during HS apoptotic stimuli was also supported by the presence of Bax and BH4 domain-dependent pathway and inhibition of Bax or presence of BH4 domain resulting in embryonic development and frequency of apoptosis similar to 39°C control group.

In conclusion, the findings in this thesis and their interpretation have resulted in a new understanding of the mitochondrial role in mediating apoptosis in oocytes. Perhaps the most significant finding was that mitochondrial dysfunction is one of the factors that determine the magnitude of negative effects of stresses on oocyte developmental competence and apoptosis. The present study reinforces the concepts that 1) ooplasmic mitochondrial dysfunction can induce apoptosis and influence developmental competence in a heritable manner to at least the blastocyst stage; 2) oocyte mitochondria function is sensitive to environmental and pathological stressors resulting in further depression of oocyte developmental capacity and increase in apoptotic-like events; 3) environmental stress, e.g. heat shock, activates caspase-9 dependent pathway in oocytes; 4) the fate of the oocyte might be determined by end-result of interactions between the Bcl-2 family members exemplified here by Bax and BH4 domain-dependent pathways

One potentially practical outcome of this research should be a renewed focus on integrity of oocyte mitochondria to improve fertility heath in stressed females. These results suggest it may be possible to decrease embryonic mortality and improve pregnancy rates by a better understanding of mitochondria-oocyte molecular regulation, opening opportunities for development of apoptosis-based therapeutic strategies.

General Conclusion

1. Elevated temperature and depolarization of mitochondria by FCCP during maturation induced apoptosis in both oocytes and blastocysts and was associated with reduced developmental potential. Inhibition of the opening of the mitochondria PT pore by CsA blocked heat shock-induced apoptosis showing clearly the importance of mitochondrial integrity during stress early in development. Both treatments altered mRNA expression of candidate mitochondrial (ND6) and apoptosis (Bax, Bcl-xL, HSP70.1) related genes in matured oocytes and day 8 blastocysts. Thus, these findings indicate that oocytes are sensitive to environmental or pharmacological insults and the response to stress of the oocyte can be observed later in development particularly at the blastocyst stage.

2. Heat shock-induced apoptosis in bovine oocytes is mediated by activation of caspase-9 and caspase-3/7. Addition of an inhibitor of caspase-9 (z-LEHD-fmk), an inhibitor of caspase-3/7 (z-DEVD-fmk) or a general caspase inhibitor (z-VAD-fmk) completely suppressed the detrimental effects of HS on embryonic development and reduced the frequency of apoptosis in both oocytes and blastocysts. These data provide the first evidence that the caspase-9 dependent pathway is expressed in bovine oocytes and is activated in oocytes by exposure to heat shock. Inhibiting caspase-9 activation or preventing mitochondrial damage may be useful strategies to

decrease HS-induced apoptosis in oocytes and consequently improve fertility health in thermal stressed females.

3. Heat induced apoptotic cell death in oocytes involves Bax and BH4-domain pathway. Exposure of heat shocked oocytes to a Bax inhibiting peptide blocked the negative effect of HS embryonic development and apoptosis. Surprisingly, addition of BH4 domain of Bcl-xL to HS-treated oocytes was unable to reestablish development to blastocyst stage but it did reduce the frequency of apoptosis in both oocytes and blastocysts. Treatment of HS-oocytes with a combination of both peptides resulted in increased embryo developmental capacity associated with reduced percentage of apoptosis. These data provide the first evidence of the involvement of Bax and BH4-domain pathways during HS-induced apoptosis in bovine oocytes. These results strongly suggested that, under HS conditions, the end-result from complex interactions between the Bcl-2 family members determine the fate of the oocyte.

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