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PORCINE EMBRYOGENESIS AND EFFECTS OF MYCOTOXINS ON EARLY PIG DEVELOPMENT

By

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EARLY PIG DEVELOPMENT

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The effect of culture media and mycotoxin on porcine preimplantation embryonic development has been investigated. In the first experiment, NCSU-23 significantly decreased porcine embryonic cleavage and blastocyst rates compared to the PZM-3 medium. *BAX* and *BCL2L1* transcription levels were similar in blastocysts cultured in both media. Porcine embryonic cleavage rates were significantly decreased in the presence of cycloheximide, and both α-amanitin and cycloheximide completely inhibited blastocyst formation. In the mycotoxin experiment, porcine embryonic cleavage rates decreased in 10 μM α-ZEA group, while blastocyst rates decreased in 30 μM α-ZEA group ($P<0.05$). Total cell numbers of blastocysts decreased in the 10 μM α-ZEA group ($P<0.05$). The transcription levels of *POU5F1* and *BCL2L1* were similar, while that of *BAX* and the *BAX/BCL2L1* ratio significantly increased in 3 μM and 10 μM α-ZEA groups compared to the control group.

DEDICATION

I would like to dedicate my thesis to my mother Xueqin Chen and father Haiqing Wang for their love and support.

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TABLE OF CONTENTS

Page

LIST OF TABLES

TABLE

LIST OF FIGURES

FIGURE

LIST OF ABBREVIATIONS

ΔCP	crossing point deviation
ACTB	*beta actin*
ADP	adenosine diphosphate
ANOVA	one-way analysis of variance
BAX	BCL2-associated X protein
BCL2	B-cell CLL/lymphoma 2
BCL2L1	BCL2-like 1
BECM-3	Beltsville embryo culture medium-3
BH	BCL2 homology
bp	base pair
BSA	bovine serum albumin
b.w.	body weight
cDNA	complementary DNA
Ced-3	Cell Death abnormality family member 3
Ced-9	Cell Death abnormality family member 9
CHX	cycloheximide
COCs	cumulus oocyte complexes
COW	climbing-over-a-wall
CPP32	cysteine protease protein of molecular mass = 32 kDa
Cyt C	cytochrome c
CZB	modified Chatot, Ziomek, Bavister
DISC	death-inducing signaling complex
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

DON	deoxynivalenol
dpc	days post-coitum
E	PCR efficiency
E2	17β-estradiol
EGA	embryonic genome activation
ERs	estrogen receptors
ESR1	estrogen receptor 1
ESR2	estrogen receptor 2
ET	embryo transfer
FADD	Fas-associated death domain protein
FBS	fetal bovine serum
FSH	follicle-stimulating hormone
GVBD	germinal vesicle breakdown
H4	histone 4
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hpi	hours post insemination
HSP70.1	heat shock 70kDa protein 1A
ICE	interleukin lβ converting enzyme
Ich1	caspase-2
ICM	inner cell mass
IGF	insulin-like growth factor
Igf2r	insulin-like growth factor 2 receptor
IVC	*in vitro* culture
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
IVP	*in vitro* production
LH	luteinizing hormone
M I	meiosis I
M II	meiosis II
MCF-7	oestrogen-dependent human breast cancer

mRNA	messenger RNA
mTBM	modified Tris-buffered medium
NCSU-23	North Carolina State University 23
nt	nucleotide(s)
OVF	oviductal fluid
PBS	phosphate buffered saline
PCD	programmed cell death
PCR	polymerase chain reaction
PEF	porcine fetal fibroblast
pi	post insemination
2PN	two-pronuclear
POEC	porcine oviductal epithelial cells
POU5F1	POU class 1 homeobox 1
PPN	poly-pronuclear
PVA	polyvinylalcohol
PVP	polyvinylpyrrolidone
PZM-3	porcine zygote medium 3
qRT-PCR	quantitative reverse transcription PCR
REST	relative expression software tool
RNA	ribonucleic acid
RNase H	ribonuclease H
rRNA	ribosomal RNA
rTdT	recombinant terminal deoxynucleotidyl transferase
S.E.M.	standard error mean
TBP	TATA box binding protein
TCM 199	tissue culture medium 199
TCN	total cell numbers
TE	trophectoderm
TNF	tumour necrosis factor
TRADD	TNF receptor-associated death domain

TUNEL	terminal deoxynucleotidyl transferase biotin-dUTP Nick End labeling
v/v	volume/volume
WOW	well of the well
w/v	weight/volume
ZEA	zearalenone

CHAPTER 1

INTRODUCTION

Improving quantity and quality of porcine embryos is important for biotechnological applications within the swine industry and as a model for biomedical research. The techniques of *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC) have been updated since 1980 (Nagai et al., 1984; Cheng et al., 1986; Nagai et al., 1988; Hunter, 1990; Yoshida et al., 1990; Prather et al., 1991; Yoshioka et al., 2002). Although IVF and somatic cell nuclear transfer (clone) derived piglets were born from *in vitro* maturated oocytes, the efficiency of IVF is low because of suboptimal IVC conditions (Mattioli et al., 1989; Yoshida et al., 1993; Betthauser et al., 2000; Kikuchi et al., 2002). Since culture medium has been considered responsible for the suboptimal level of IVF/IVM, other researchers have worked to optimize the embryo culture media to improve the quantity and quality of the IVC-derived porcine embryos such as modified Whitten (Beckmann and Day, 1993), NCSU-23 (Petters and Wells, 1993), modified Chatot, Ziomek, Bavister (CZB) (Pollard et al., 1995), Beltsville embryo culture medium (BECM)-3 (Dobrinsky et al., 1996), and PZM-3 (Yoshioka et al., 2002). NCSU-23 and PZM-3 are commonly used media for pig embryo culture, but PZM-3 has been reported to support development to the blastocyst stage better than NCSU-23 (Machaty et al., 1998; Yoshioka et al., 2002; Im et al., 2004).

Programmed cell death (PCD) has been observed in many kinds of cells during normal animal development since 1951 (Glucksmann, 1951). It is a spontaneous physiological process and mammalian cells can undergo PCD in the absence of serum or extracellular signaling molecules (Raff, 1992). Apoptotic regulation is important for early embryonic development also, especially to eliminate the abnormal, detrimental, or superfluous cells and control cell numbers of embryos (Fabian et al., 2005). The apoptosis during preimplantation embryonic development is caused by several factors such as, genetic regulation (reviewed in (Wyllie, 1995)), inappropriate inner cell mass (ICM) developmental potential (Handyside and Hunter, 1986), imbalance of IGF factors (Byrne et al., 2002; Fabian et al., 2004) and oxygen and heat shock (Yang et al., 1998; Paula-Lopes and Hansen, 2002). In the nematode worms, the *Ced-3* gene is required for apoptosis, which is homologous to the interleukin lβ converting enzyme (ICE) family of proteases, also named as caspases, in mammals. The expression of *Ced-9* gene can protect cells and inhibit cell death, which is homologue to *BCL2* in mammals (Wyllie, 1997). The caspases and *BCL2* families are two major genes regulating the apoptosis process in cells, and the intracellular *BAX*/*BCL2* ratio is important to determine the fate of cells (Wyllie, 1995).

Mycotoxins are produced during the inappropriate process of storing grain crops such as wheat, maize or barley. These toxicants include Aflatoxin, Ergot, Deoxynivalenol (DON), Zearalenone (ZEA), Fumonisins and Ochratoxin (Richard, 2007). The major fungal toxins are the Fusarium species (ZEA and DON), which are widely found in America, Europe, and Asia (Kuiper-Goodman et al., 1987; Rotter et al., 1996). In pigs,

fed infected grain, it has been found that both hepatic and granulosa cells can metabolize the ZEA to α-ZEA and β-ZEA. (Malekinejad et al., 2005; Malekinejad et al., 2006). The α-ZEA is the major byproduct metabolized after ZEA is absorbed by pigs, and its toxicity is higher than ZEA and β-ZEA. ZEA has a similar mode of action as 17β-estradiol (E2), and binds to estrogen receptors (ERs) in mammalian cells (Katzenellenbogen et al., 1979).

The central hypotheses were that porcine embryonic development is influenced by the culture media *in vitro* and that mycotoxin α-ZEA affects preimplantation embryogenesis. These hypotheses were tested with the following research objectives. (1) Determine culture media supporting better embryonic development and the effects of inhibition of transcription and translation. (2) Identify the effects of mycotoxin α-ZEA on porcine embryonic development *in vitro*.

CHAPTER 2

REVIEW OF PERTINENT LITERATURE

2.1 Roots of early development: Gametogenesis and Embryogenesis

2.1.1 Oogenesis

Mammalian reproduction begins with the development of spermatozoa and oocytes. Meiotic germ cells, the original oocytes, first appear at day fifty post-coitum (dpc) and in third month of gestation in pigs and humans, respectively. The number of primordial follicles is different in ovaries of different species, for example, 500,000 in pigs, 135,000 in cows, and 700,000 in humans (Baker, 1963; Erickson, 1966; Guthrie and Garrett, 2001). Approximately 99% of oocytes are arrested at the diplotene stage of meiosis I after birth.

Primordial follicles are surrounded by flat-shaped pregranulosa cells. When flattened pregranulosa cells convert to cuboidal shape granulosa cells, primordial follicles enter into the growth phase, named as primary follicles (Byskov and Hoyer, 1994). Zona pellucida forms outside of oocytes in the primary follicle stage. When granulosa cells proliferate to several layers, primary follicles convert to secondary follicles. During folliclar development, the theca interna forms an epithelial morphology and secretes steroid(s), while the theca externa remains as a spindle-shaped morphology. In the antral

follicle stage, granulosa cells develop to cumulus oophorus, antral granulosa cells, and mural granulosa cells. When antral follicles develop and become preovulatory follicles, the diameter of oocytes and follicles increase to 120 μm and 2 mm (Motlik et al., 1986). The development of primordial follicles will remain arrested until stimulated by LH surge during rupture. After LH surge, oocytes are released from meiosis I (MI) arrest. After germinal vesicle breakdown (GVBD), the first meiotic division is completed and the first polar body is excluded. Prior to ovulation, oocytes are arrested at metaphase of meiosis II (MII) stage, called oocyte maturation,. After fertilization, the second polar body is ejected, and the decondensed maternal and paternal chromosomes fuse to form the zygotes.

2.1.2 Spermatogenesis

Following the migration of primordial germ cells, these bipotential cells start to become gonocytes. Testis contains several somatic cell types, such as Sertoli cells, peritubular myoid cells, endothelial cells, fibroblasts, and Leydig cells. Pre-Sertoli cell is the first somatic cell type to differentiate in the testis, and Sertoli cells are crucial in testis differentiation. Sertoli cells are important during both of the processes of testis formation and spermatogenesis. In human, monkey and non-rodent species, Sertoli cells first proliferate in 5-18 months followed by second phase proliferation before puberty, while in rat the proliferation of these cells ceases completely after birth (Sharpe et al., 2003). The seminiferous epithelium has two basic cell types, Sertoli cell and germinal cell. The function of Sertoli cells is to nurture germ cells and the Sertoli-Sertoli cell junctions can prevent harmful blood-borne chemicals through blood-testis barrier. Spermatogenesis

involves three functionally and morphologically phases, spermatogonial (proliferation), spermatocytary (reduction-division or meiosis), and spermiogenic (differentiation) (Russell et al., 1990; Franca et al., 2005). During spermatogenesis, one germ cell undergoes meiosis I and II, and produces four small haploid cells, also called spermatids. In the differentiation stage, haploid germ cells undergo three major modifications, nucleus elongation and chromatin condensation to a dark staining species specific structure, the Golgi apparatus forming acrosome, the long tail formation with mitochondria in the proximal region and losing excess cytoplasm (Hess, San Diego: Academic Press, c1998).

2.1.3 Preimplantation Embryogenesis

After fertilization, the zygote is formed and this leads to embryogenesis. Mammalian embryos coordinate a series of cell divisions, migrations, and differentiations. Oocyte activation and development are triggered by a series of intracellular calcium oscillations, which occurs during the fusion of sperm and oocyte (Berridge and Galione, 1988; Kline and Kline, 1992). No cellular growth occurs during the first several stages of cell division, and large volume of cytoplasm divides into smaller blastomeres. In the early stages, the embryo development is controlled mostly by stored maternal mRNAs and proteins until embryonic genome activation (EGA). In the early *C. elegans* embryos, cell cleavage is asynchronous, which means blastomeres do not undergo the cleavage at the same time, and this is the reason of odd numbers of blastomeres (Gonczy and Rose, 2005). After the 8-cell stage, cell to cell adhesion is dramatically increased, and tight junctions are formed to stabilize the association among the outer flattened blastomeres

(Telford et al., 1990). This process is also named as compaction or morulae stage, which is regulated by E-cadherin, β-, and γ-catenin and other genes (Kemler, 1993; Huber et al., 1996). The characteristic of the blastocyst stage is a visible cavity formation at which time cell differentiation becomes apparent. Cells in the periphery of the blastocysts, also called trophectoderm (TE) (organized as an epithelium), remain tight junction and increase the size of the blastocoel cavity (Wiley et al., 1990). The cluster of cells is connected by gap junctions in the center, known as ICM. Trophectoderm transports fluid into the embryo, and contributes to the formation of the placenta and other extra-embryonic tissues, while the ICM will also contribute cells to the embryo development.

2.1.4 Culture medium

Although *in vitro* production (IVP) system has produced porcine, bovine, and other animals, still IVP derived embryos exhibit darker cytoplasm, swollen blastomeres, less developmental ability and lower cell numbers, which may be caused by the sub-optimal *in vitro* culture environments (Kikuchi et al., 1999; Kitagawa et al., 2004; Pomar et al., 2005). Bovine IVF-derived zygotes have been cultured with oviductal epithelial cells, cumulus cells, and amnion cells in TCM 199 (Glucksmann, 1951; Goto et al., 1988; Aoyagi et al., 1990). Various culture media have been developed for porcine embryo culture, including modified Whitten (Beckmann and Day, 1993), NCSU-23 (Petters and Wells, 1993), modified Chatot, Ziomek, Bavister (CZB) (Pollard et al., 1995), Beltsville embryo culture medium (BECM)-3 (Dobrinsky et al., 1996), and PZM-3 (Yoshioka et al., 2002). Although all these culture media support porcine embryo development to the blastocyst stage, NCSU-23 and PZM-3 are thought to be better than the other media for

porcine embryo culture (Machaty et al., 1998). PZM-3 is based on the composition of porcine oviductal fluid with addition of amino acids, while NCSU-23 is designed to meet the metabolism and nutrient needs of porcine embryos. Recently, PZM-3 has been reported to support development to the blastocyst stage better than NCSU-23 (Wang et al., 1998; Yoshioka et al., 2002; Im et al., 2004). In addition, oviduct fluid and co-culture oviductal epithelial cells have been reported to support the blastocyst formation (Smith et al., 1992; Eberhardt et al., 1994).

2.1.5 Apoptosis

Apoptosis is activated by calcium-magnesium-dependent endonucleases. Integrated DNA is digested into 185-200 base pair (bp) fragments at internucleosomal sites, which results in the characteristic DNA ladder bands in agarose gel electrophoresis (Wyllie et al., 1980; Arends et al., 1990). Apoptosis plays a critical role in elimination of defective cells, but it must also be carefully regulated to protect viable cells (Kamjoo et al., 2002). In mammalian cells, apoptosis is regulated by *BCL2* and caspases gene familes. *BCL2* family member includes promoters of apoptosis (*BAX*, *BAK*, *BAD*, *BLK*, and *BIK*) and suppresses apoptosis (*BCL-2*, *BCL-XL*, *BCL-W* and *MCL-1*). Caspase family has three subfamilies of cysteine proteases, the ICE like proteases (three members), Ich1 (also named as caspase-2) (two members), and Yama/cysteine protease protein (CPP32) (seven members) (Cerretti et al., 1992; Fernandes-Alnemri et al., 1994; Wang et al., 1994). The process of apoptosis is regulated by mitochondrial and extracellular signals. In mitochondrial regulation way, once cytochrome C (Cyt C) is released from mitochondria, it will bind to Apaf-1 and then bind to pro-caspase-9 to form the

apoptosome. This apoptosome will finally active the effector caspase-3 (Er et al., 2006; Shi, 2006). On the other hand, there are two important direct apoptotic mechanisms, tumor necrosis factor (TNF) and Fas-Fas ligand (Wajant, 2002). When TNF bind to TNF receptor1, the TNF receptor-associated death domain (TRADD) and Fas-associated death domain protein (FADD) activated, and initiate the apoptosis by activating caspases (Chen and Goeddel, 2002). When Fas receptor bind to Fas ligand, a death-inducing signaling complex (DISC) form, which contains FADD, caspase-8 and 10 (Peter and Krammer, 2003; Engels et al., 2005). The DISC may activate caspase and initiate apoptosis.

TCN and apoptotic rate of blastocysts are important factors for judging the quality of embryos, which are impacted by *in vitro* culture conditions (Pomar et al., 2005). Apoptotic pathways depend on a complex balance between expression levels of a battery of anti-apoptotic (*BCL2*, *BCL-E*, *BCL-XL*) and pro-apoptotic (*BAX*, *BAK*, *BAD*) genes (Antonsson and Martinou, 2000). In addition, the ratios between *BAX* and *BCL2* transcripts determine whether cells live or die (Oltvai et al., 1993).

2.2 Developmental control of gene expression

Oocytes accumulate large amounts of non-adenylated RNAs and proteins, which maintain development of the first few cell cycles during early embryogenesis. Untranslated regions in the 3' ends of transcripts have consensus sequences, which bind to masking proteins and prevent translation (Stebbins-Boaz and Richter, 1997).

The N-terminal tails of the histones undergo acetylation, methylation, phosphorylation, ubiquitination, and ADP-ribosylation, which regulate the chromatin compaction and gene expression by altering interactions of adjacent nucleosomes, the

positioning of nucleosomes, and contacts of histones and DNA. Histone acetylation does not change the basic structure of nucleosomes; however, it reduces the affinity of DNA binding, which permits binding of transcription factors and increases the transcription. Histone acetylation is associated with EGA during early embryonic development. In mice, if histone deacetylases are inhibited, accumulation of hyperacetylated histone H4 occur only after EGA (Dimitrov et al., 1993). In addition, changing patterns of histone H4 acetylation affect the chromatin regulated HSP70.1 protein expression in cleavage-stage mouse embryos (White et al., 1989). Histone H1 may be another transcription regulator. The onset of histone H1 synthesis with core histone deacetylation might cause the EGA to occur (Nothias et al., 1995).

Gene imprinting is an important aspect in the embryonic development, which ensures that only one allele is expressed (Surani, 1998). Embryos die at midgestation when a maternal pronucleaus is replaced by a paternal pronucleus in the early embryonic development, or in the opposite way. Two maternal pronuclei fail to form the extra-embryonic tissue, while two paternal pronuclei fail to develop the embryo portion. Methylation appears to play an important role in inheritance of imprinted genes (Wieschaus, 1996), and it requires the balanced expression between paternal and maternal alleles of imprinted genes. For example, Igf2r gene has two regions. The first region has a start region and is only methylated on the silent paternal chromosome, while the second region is inherited from the female gamete and methylated only on the expression of maternal chromosome (Stoger et al., 1993). It was shown that the level of

DNA methylation is decreased from meiotic cells to elongated spermatids during sperm development (Del Mazo et al., 1994).

At the end of spermiogenesis, protamines replace histones and bind to DNA minor groove. Spermatozoa suppress the majority of the transcription after losing most of the cytoplasm. Fusion of spermatozoan into the oocyte triggers a series of intracellular calcium oscillations, which resume the meiosis, activate oocytes and development (Pate et al., 2007). At the same time, protamines in male genome are replaced by histones (Nonchev and Tsanev, 1990). Then the maternal and paternal genomes form pronuclei, and DNA synthesis occurs within each pronuclei, and the embryonic transcription in paternal pronucleus appears more active than that in maternal pronucleus (Wiekowski et al., 1993; Peter and Krammer, 2003). However, reporter gene expression was similar in paternal and maternal pronucleus when histone deacetylases were inhibited in mouse embryos (Wiekowski et al., 1993). It is believed that the paternal DNA is more accessible to transcription complexes, after the replacement of protamines with histones. The titration of maternal histones may explain gradual activation of embryonic genome. After the depletion of maternal histones, new embryonic DNA will not bind to maternal histones, but bind to the transcription factors and EGA will occur (Wiekowski et al., 1993).

Non-equivalency of parental genome expression was detected in 1984 (Mcgrath and Solter, 1984; Surani et al., 1984). After fertilization, sperm protamines are rapidly replaced by histones from oocytes. During this stage, intensive demethylation of paternal pronuclei was detected in some species such as mouse, rat and pig (Mayer et al., 2000;

Beaujean et al., 2004; Fulka et al., 2006). It has been shown that during the pre-implantation stage human and bovine embryos only exhibited partial paternal genome demethylation (Beaujean et al., 2004; Fulka et al., 2004). Another study of human zygotes reported paternal chromatin has a lower level of methylation than maternal chromatin (Fulka et al., 2004). In *in vivo*-derived porcine embryos, parternal pronucleus were almost demethylated compared to maternal pronucleus in the one-cell stage (Fulka et al., 2006). In addition, cells from ICM have a higher level of methylation compared to the trophectodermal cells (Fulka et al., 2006). The DNA methylation pattern of porcine zygotes may relate to the quality of porcine embryos.

EGA is an essential event during embryo early development (Flach et al., 1982). High levels of transcriptional activation of genes were detected from 8-cell to 16-cell stage bovine embryos (Kopecny et al., 1989; Memili and First, 1999), 4- to 8-cell stage in the porcine and human embryos (Flach et al., 1982; Fabian et al., 2005), and at the 2-cell stage in the mouse (Flach et al., 1982). Changes in chromatin structure play an important role in EGA (Kanka, 2003; Bultman et al., 2006). During EGA embryonic RNAs are synthesized, while multiple maternal RNAs are degraded, and new proteins are synthesized. The embryonic genome becomes more active at later cell cycles. Inhibition of transcription and translation could represent a useful tool to determine the exact moment of EGA in porcine embryos and identify molecular mechanisms by which EGA is regulated.

2.3 Mycotoxins affecting mammalian embryonic development and reproduction

Improper storage of grains causes fungal growth and thus production of mycotoxins. Mycotoxins are the by-products of fungi metabolism, which have biological activity and impact animals and humans (Fink-Gremmels, 1999). There are more than 1500 existing environmental mycotoxins, and some of them may affect normal animal physiology by mimicking the hormone of estrogen. ZEA, a mycotoxin with estrogen-like properties, is produced by several *Fusarium* such as *F. graminearum, F. tricinctum, F. moniliforme* and *F. oxysporum* (Eriksen et al., 2000). In 1927, the clinical effect of ZEA was first described as causing vagina swelling and eversion (Buxton, 1927). This syndrome was described as vulvovaginitis and caused by moldy maize (Mcnutt et al., 1928). *Fusarium graminearum*, producing ZEA, was first identified in 1952 and the chemical structure was described in 1966 (Mcearlin, 1952; Urry et al., 1966). Currently, the contamination of ZEA is found primarily in maize, barley, oats, wheat, rice and sorghum (Kuiper-Goodman et al., 1987). Raw maize was the food commodity with highest level (up to 627 μg/kg) of ZEA compared to raw wheat (26 μg/kg) (Malekinejad et al., 2005). Based on the consumption of wheat, rye and oats, the average levels of ZEA daily intakes was from 0.8 to 29 ng/kg body weight (b.w.) in adults, while 6 to 55 ng/kg b.w. per day in small children (Minervini et al., 2005).

It has been reported that ZEA has a low affinity to serum hormone-binding globulins (5% *vs.* 100% of estrogen), so the high concentrations present in the blood can increase its estrogenic activity (Ahamed et al., 2001). After ZEA was hepatically and extrahepatically metabolized (Malekinejad et al., 2005), it can be metabolized into four

isomerics, α-zearalenol (α-ZEA), β-zearalenol (β-ZEA), α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL). Cell proliferation experiments using estrogen-dependent human breast cancer cells (MCF-7) indicated the estrogenic potency of ZEA and its derivatives can be ordered as following: α-ZEA > α-ZAL > ZEA > β-ZEA (Malekinejad et al., 2005), and α-ZEA is the major compound that caused hyperestrogenism in all laboratory and farm animals, especially in pigs (Malekinejad et al., 2007).

ZEA has a unique nonsteroidal resorcyclic acid lactone structure, which permits ZEA to bind to estrogen receptor (ESR) 1 and ESR2 in a number of *in vitro* and *in vivo* systems (Malekinejad et al., 2007). The binding affinity of ZEA with ESR1 and ESR2 was around 10% less than that of E2. The relative transactivation activity of ZEA was similar to E2 through binding to ESR1 (91 *vs.* 100); however, that was low when ZEA binds to ESR2 as compared to E2 (27 *vs.* 100) (Kuiper-Goodman et al., 1987). This binding action can decrease fertility, increase embryolethal absorption, reduce litter size and change the weight of adrenal and pituitary glands (Creppy, 2002).

It has also been found that estrogen was a novel regulator of microtubule dynamics and controlling cytoskeleton in cells (Kipp and Ramirez, 2003). These nongenomic effects of ZEA have been reported, which means ZEA may impact cells not only through ERs binding and activation, but also through nongenomic mechanisms (Levin, 2002).

2.4 Progress, Challenges and Promises of Porcine IVF

At present, transgenic animals are important tools for research in agriculture, pharmaceuticals, biomedicine, and biotechnology (Huguet and Esponda, 2000; Wheeler et al., 2004). Improving IVP will provide more embryos *in vitro* to be manipulated and used in transgenic research. However, two major issues are still impacting the porcine IVP: pronuclear formation and polyspermy. In addition, many factors affect the formation of blastocysts and the procedure of IVP, such as culture system, concentration of sperm, and EGA (Wheeler et al., 2004).

Special culture equipments were modified to increase the production of embryos from pig IVP. A climbing-over-a-wall (COW)-IVF method was introduced (Funahashi and Nagai, 2000). The higher motility sperm could pass the outer wall and enter into the inner wall to fertilize the oocytes. They found this COW-IVF method increased the penetration rates to 33.7%, 61.9%, and 95.1% when using 0.5, 1, and 5.0×10^5 cells/ml sperm. And the monospermic penetration was 81.7%, 49.5%, and 25.5% compared to 10.4% in the standard IVF. Another modification of culture system has been reported by using Well of the Well (WOW) system in the 4-well dishes (Vajta et al., 2000). The WOW system permits single or a small group of bovine embryos cultured in reduced volumes. Microfluidic systems have been designed to allow the unidirectional flow of oocytes, sperm, medium and supplements (Yang et al., 1998; Wheeler et al., 2004). It has been reported that these microfluidic culture systems were more similar to the *in vivo* environment of the oviduct compared with traditional microdrop systems (Wheeler et al., 2004).

The preimplantation period of mammals is one of the important stages of mammalian development (Ram and Schultz, 1993), in which zygotic/embryonic genome replaces the maternal transcripts (Kanka, 2003). Three mechanisms may be involved in this process: chromatin-mediated repression, changes in the transcriptional machinery, and transcriptional repression or abortion by rapid cell cycles (Schier, 2007). The activation of eggs can influence the pattern of gene expression and preimplantation embryonic development. In addition, highly differentiated oocytes switch into totipotent blastomeres of the early preimplantation embryos during EGA, which is necessary for the successful embryonic development (Kanka, 2003).

The development of porcine reproductive technologies is crucial because pigs are important as livestock, but also as a model animal for biomedicine. Although somatic cell cloned pigs (Betthauser et al., 2000; Polejaeva et al., 2000; Kikuchi et al., 2002) and gene knock out pigs (Im et al., 2004) have been produced, efficiency of IVP produced embryos is still very low, which may be caused in part by problems in the formation of male pronucleus and polyspermy. Since majority of oocytes in IVP come from middle size of antral follicles, more research should be done to improve the culture conditions (Funahashi and Nagai, 2000). Although cytoplasmic maturation has been improved, the problem of polyspermic penetration still needs to be resolved (Abeydeera, 2002). Global embryonic genome activation is critical for early mammalian embryogenesis. Early embryonic development is important for both pre- and postimplantation stages. Understanding this is related to understanding the events taking place in mammalian fetal development (Misirlioglu et al., 2006). In the future, research should focus on minimizing

polyspermic penetration, improving embryo quality, and understanding the dynamic genomic changes during EGA.

CHAPTER 3

EFFECTS OF CULTURE MEDIA AND INHIBITORS ON BIOLOGY OF PORCINE EARLY EMBRYONIC DEVELOPMENT *IN VITRO*

The working hypothesis was that porcine embryonic development is influenced by culture media. To test this hypothesis, the objectives were: (1) to identify the impacts of NCSU-23 and PZM-3 on porcine embryo development by embryonic developmental data, (2) to count the total cell number of blastocysts and check transcript levels of *BAX* and *BCL2L1* gene, and (3) to detect the roles of transcription and translation during porcine embryonic gene activation (EGA) by investigating the effects of α-amanitin and cycloheximide.

3.1 Abstract

In vitro-production of porcine embryos is crucial for biomedical and agricultural research, however, current culture systems for porcine embryos are sub-optimal, and the developmental potential of *in vitro*-produced embryos is not well studied. The objectives of this study were to evaluate whether PZM-3 or NCSU-23 is better support porcine embryo development and the effects of α-amanitin and cycloheximide (CHX) on porcine embryonic development. Briefly, porcine presumptive zygotes were produced after *in*

vitro maturation/fertilization (IVM/IVF) and cultured either in NCSU-23 or PZM-3 culture media. Transcript levels of *BAX* and *BCL2L1* genes from blastocysts were detected using Real-time PCR. The effects of α-amanitin and CHX were evaluated for the role of inhibiting transcription and translation during early porcine embryogenesis. Results showed that both cleavage and blastocyst rates decreased significantly in NCSU-23 group compared as PZM-3 group. However, *BAX* and *BCL2L1* transcript levels were similar in blastocysts cultured in both PZM-3 and NCSU-23 media. When porcine embryos cultured in PZM-3, cleavage rates were significantly decreased in the present of CHX, and both α-amanitin and CHX treatments completely inhibited the blastocyst formation. Results demonstrated that PZM-3 medium better supported porcine early embryonic development to the blastocyst stage than NCSU-23 medium, and the inhibition of the embryonic genome activation does not completely stop embryo cleavage but prevents development to the blastocyst stage.

3.2 Introduction

Pigs are important not only as livestock, but also as the experimental model for humans in biomedical research (Prather *et al.*, 2003). However, porcine embryos derived from *in vitro* production (IVP) systems have poor developmental ability and low cell numbers, which might be caused by the differences between *in vivo* and sub-optimal *in vitro* culture environments (Kikuchi *et al.*, 1999; Kitagawa *et al.*, 2004; Pomar *et al.*, 2005). Several culture media have been used for porcine embryonic culture, including modified Whitten (Beckmann and Day, 1993), NCSU-23 (Petters and Wells, 1993), modified Chatot, Ziomek, Bavister (CZB) (Pollard *et al.*, 1995), Beltsville embryo

culture medium (BECM)-3 (Dobrinsky *et al.*, 1996), and PZM-3 (Yoshioka *et al.*, 2002). Although all these culture media support porcine embryo development to the blastocyst stage, NCSU-23 and PZM-3 have been used more commonly for the porcine embryonic culture (Machaty *et al.*, 1998). PZM-3 is based on the composition of porcine oviductal fluid with addition of amino acids, while NCSU-23 is designed to meet the metabolism and nutrient needs of porcine embryos. Recently, PZM-3 has been reported to support embryo development to the blastocyst stage better than NCSU-23 (Yoshioka *et al.*, 2002; Im *et al.*, 2004).

The total cell number (TCN) and the apoptotic cell rates of blastocysts are important factors to judge the quality of embryos (Pomar *et al.*, 2005). Apoptosis plays a critical role in elimination of defective cells during preimplantation, but apoptosis must also be carefully regulated to protect viable cells (Kamjoo *et al.*, 2002). Apoptotic pathways depend on a complex balance between a battery of anti-apoptotic (*BCL2, BCL-W, BCL-XL*) and pro-apoptotic (*BAX*, *BAK, BAD*) genes (Antonsson and Martinou, 2000). In addition, the relative expression ratio between *BAX* and *BCL2* determine whether cells live or die (Oltvai *et al.*, 1993).

Embryonic gene activation (EGA) is an essential event during early development (Flach *et al.*, 1982). High levels of transcriptional activation of genes were detected from 8-cell to 16-cell stage in bovine embryos (Kopecny *et al.*, 1989), 4- to 8-cell stage in the porcine and human embryos (Flach et al., 1982; Fabian et al., 2005), and at the 2-cell stage in the mouse (Flach *et al.*, 1982). Changes in chromatin structure play an important role in EGA (Kanka, 2003; Bultman et al., 2006). During EGA embryonic RNAs and

proteins are synthesized, while multiple maternal RNAs are degrading gradually. The embryonic genome becomes more active at later cell cycles. The inhibition of transcription and translation could represent a useful tool to detect the importance of newly synthesized RNAs and proteins for the porcine preimplantation embryonic development.

The objectives of this study were to determine: 1) whether PZM-3 or NCSU-23 better support porcine embryonic development, 2) the transcript levels of *BAX* and *BCL2L1* genes in porcine blastocysts, and 3) the effects of inhibiting transcription and translation on porcine early embryogenesis.

3.3 Materials and Methods

3.3.1 Comparison of NCSU-23 and PZM-3 culture media

3.3.1.1 In Vitro Maturation, Fertilization and Culture (IVM/F/C)

Cumulus oocytes complexes (COCs) were aspirated from 3-6 mm follicles of porcine ovaries obtained from a local slaughterhouse. Only oocytes containing more than three layers of cumulus cells and having homogeneous cytoplasm were collected. Oocytes were washed three times in TL-HEPES-PVA and matured in Tissue Culture Medium(TCM-199, Invitrogen, Carlsbad, CA) supplemented with 0.1% polyvinylalcohol (PVA), 3.05 mM D-glucose, 20.91 mM sodium pyruvate, 0.57 mM cysteine, 25 μg/ml gentamycin, and 0.5 μg/ml LH and FSH. Fifty cumulus-oocytes complexes were matured in 500 μl maturation medium in four-well dishes under mineral oil at 39.5°C, 5% CO_2 in

100% humidified atmosphere. After 20-22 h oocytes were transferred into the same maturation medium without hormones until around 44 h.

Fresh sperm was separated by density gradient centrifugation (Parrish *et al.*, 1985) and added into fertilization drops at a final concentration of 1×10^6 sperm/ml. Fertilization was performed in 500 µl modified Tris-buffered medium (mTBM) (Abeydeera and Day, 1997) contained 2.0 mM caffeine, 2 mg/ml BSA, 0.5 µl/ml gentamycin, and 10 µl/ml penicillin-streptomycin (Invitrogen).

Five to six hours after fertilization, porcine presumptive zygotes (20) were randomly allocated to any of the two culture media, PZM-3 (Chemicon, Temecula, CA) or NCSU-23 (Chemicon, Temecula, CA) (50 µl). Cleavage rates were checked at 48 h post insemination (hpi), and 5 µl (10%) fetal bovine serum (FBS) was added into each drop on Day 4 post insemination (pi). Blastocyst rates were determined at Day 6 pi. Culturing was performed in 50 µl drops at 39.5°C, 5% CO_2 in 100% humidified atmosphere. Cleavage and blastocyst data were collected on Day 4 and 6 pi, respectively. Experiments were repeated 11 times.

3.3.1.2 Blastocyst cell number

Total cell numbers were counted after Hoechst 33342 staining (Rodriguez-Osorio et al., 2007). Briefly, blastocysts were fixed in freshly prepared 4% methanol-free formaldehyde solution for 1 h at room temperature. Then, blastocysts were incubated in a 50 µl drop of permeabilization solution [0.5% (volume/volume, v/v) Triton X-100, 0.1% (weight/volume, w/v) sodium citrate] for 15-30 min at room temperature in a humidified environment. Blastocysts were washed 3 times (2 min each) in 50 µl drop phosphate

buffered saline/polyvinylpyrrolidone (PBS/PVP), stained in 1μg/ml Hoechst 33342 (Sigma, St. Louis, MO), and washed 3 times (2 min each) in 50 μl drop PBS/PVP to remove unincorporated dye. Blastocysts were transferred onto slides and sealed by clear nail polish. The TCN of embryos was counted by evaluating each embryo under an epifluorescent microscope (Nikon, Tokyo, Japan) equipped with a 450-490 nm excitation filter, a 520 nm dichroic mirror, and a 520 nm barrier emission filter, using a 40× objective.

3.3.2 Detection of expression levels of mRNA by Real-time PCR

Total RNA was isolated from embryos cultured in NCSU-23 and PZM-3 using QIAGEN RNeasy Micro kit (Qiagen Valencia, CA) according to the manufacturer's protocol (Rodriguez-Osorio *et al.*, 2007). Briefly, three blastocysts were pooled in the RLT lysis buffer (Qiagen Valencia, CA) and frozen at -80°C until the total RNA extraction. Thelysis buffer containing blastocysts was transferred onto silica-gel membrane spin columns and washed with the RW1 buffer followed by ansther wash with 80% ethanol. Final RNA elution was conducted using 14 μl of Rnase-free water. RNA concentration was detected using a Nanodrop (ND-1000 Spectrophotometer). The integrity of RNA were assessed using a Bioanalyzer 2100 RNA 6000 Picochip kit (Agilent Technologies, Palo Alto, CA). Three independent RNA isolations were performed for the gene expression study.

Real-time quantitative PCR was performed to assess levels of transcripts for *BAX* and *BCL2L1* genes. Primers were designed to cover at least two exons by Primer Premier 5 software (Premier Biosoft International, Palo Alto, CA). Primer sequences are shown in

Table 3.1. The concentration was adjusted to 10 μM, and all primers were tested by using cDNA from *in vitro*-produced embryos. The First-Strand cDNA was synthesized by Superscript III Platinum® Two-Step qRT-PCR kit (Invitrogen) according to the manufacturer's protocol. Total RNA of each sample was normalized to 5 ng and incubated at 25°C for 10 min, 42°C for 50 min, and 85°C for 5 min. Then, 2U of *E. coli* RNase H was added and incubated at 37°C for 20 min to eliminate remaining RNA. The amount of 724.1 ng cDNA from each sample was used for quantitative Real-time PCR amplification with SYBR® GreenER™ qPCR SuperMixes for iCycler (Invitrogen) on the iCycler iQ Real-time PCR instrument (Bio-Rad). The cycling program was 50°C for 2 min, 95°C for 8 min 30 s for denaturation, 40 cycles of 15 s at 95°C and 30 s at 60°C and 30 s at 72°C for amplification and extension. The melting curve was performed starting at 55°C with 0.5°C increase for 10 s in 80 cycles. *Beta actin* (*ACTB*) was used as the endogenous internal housekeeping gene. Standard curves were generated using 10-fold serial dilutions for the endogenous control *ACTB*, and all the target genes *BAX* and *BCL2L1* by measuring the cycle number at which exponential amplification occurred. Relative expression ratios of *BAX* and *BCL2L1* genes were calculated by normalizing their values to that of the reference gene *ACTB*.

3.3.3 Inhibition of transcription and translation

The IVP-derived zygotes were randomly assigned to one of the three groups: control group without inhibitors, α-amanitin group, and CHX group. The basic culture medium was PZM-3. Alpha-amanitin and CHX were added to the culture medium 24 hpi at final concentration of 25 μg/ml and 2 μg/ml, respectively. After 60 h exposure,

embryos of all groups were transferred into new prepared culture medium without inhibitors. Cleavage rates were recorded at 48 hpi and 5 µl (10%) FBS were added into each drop on Day 4 pi. Blastocyst rates were recorded on Day 6 pi. Experiments were repeated 5 times.

3.3.4 Statistical analysis

Differences among groups were analyzed by one-way analysis of variance (ANOVA) by SAS 9.1 (SAS Institute Inc. Cary, NC), and results were expressed as means ± S.E.M. (standard error mean). Gene expression analyses were performed using the relative expression software tool (REST, 384-beta version May 2005), which is based on an efficiency corrected mathematical model for data analysis. The mathematical model used was based on the PCR efficiencies (E) and the crossing point deviation (ΔCP) between target and reference genes (Pfaffl, 2001; Pfaffl *et al.*, 2002; Sagirkaya *et al.*, 2006). Differences were considered to be significant when $P<0.05$.

3.4 Results

3.4.1 Comparison of two culture media

3.4.1.1 Effects on embryonic development

A total number of 918 and 976 porcine embryos were cultured in PZM-3 and NCSU-23, respectively. The results were summarized in Table 3.2, the NCSU-23 medium significantly decreased embryonic cleavage rates compared to the PZM-3 medium (43.9% ± 2 *vs.* 35.3% ± 1.6). Similar, blastocyst rates were significantly

decreased in NCSU-23 compared to those cultured in PZM-3 group (5.8% ± 1.6 *vs.* 20.1% ± 2.5) (Table 3.2).

3.4.1.2 Effect on total cell number of blastocysts

Total blastocyst cell number (mean ± S.E.M.) of embryos cultured in PZM-3 were not significantly different from those cultured in NCSU-23 (46 ± 12 *vs.* 36 ± 7, P=0.09) (Table 3.2).

3.4.2 Expression of BAX and BCL2L1 genes

The quality of RNA isolated from 3 replicates for both PZM-3 and NCSU-23 groups was evaluated by the Nanodrop spectrophotometer, while the integrity was assessed by bioanalyzer. Figure 3.1 shows that the total RNA profiles of 11 independent RNA, with clear bands of 28S and 18S rRNAs without any sign of degradation. Rat total RNA was the control for RNA quality.

Results of real-time PCR were analyzed using REST©, 384-beta version for data analysis. No statistical differences were found in the expression levels of *BAX* and *BCL2L1* genes in NCSU-23 group as compared to PZM-3 group (Figure 3.2).

3.4.3 Inhibition of transcription and translation

A total number of 306, 400, and 392 embryos were used for control, α-amanitin, and CHX groups, respectively. Cleavage rates in control and α-amanitin groups were similar (P>0.05), but significantly higher than those in the CHX group (41.8% ± 4.5 and 39.3% ± 3.6 *vs.* 24.5% ± 2.7). While, blastocyst rates from control group were 17.2% ±

5.3, no blastocysts were found in either of the inhibitor treatment groups ($P<0.05$). Results were summarized in Table 3.3.

3.5 Discussion

Although a number of *in vitro* culture media for porcine embryos have been produced, developmental potential of IVP embryos has not been fully studied. The *in vitro* environment may delay embryonic development and result in fewer TCN in blastocysts (Papaioannou and Ebert, 1988; Yoshioka et al., 2002). Currently, NCSU-23 and PZM-3 are thought to be better for porcine embryo culture than other media (Machaty *et al.*, 1998). NCSU-23 was designed to meet the metabolism and nutrient needs of porcine embryos, while PMZ-3 was based on the composition of porcine oviductal fluid. Because PZM-3 has been reported to support better embryo development to blastocyst stage than NCSU-23 (Yoshioka *et al.*, 2002; Im *et al.*, 2004), they were chosen to test the ability of supporting porcine preimplantation embryonic development. Results showed that PZM-3 significantly promoted porcine early embryonic development to the cleavage stage and more blastocysts were generated as compared with NCSU-23 medium (Table 3.2). These data confirmed the results of Yoshioka, *et al.* (2002) that more blastocysts and hatched blastocysts were produced when porcine embryos were cultured in PZM-3 than when embryos were cultured in NCSU-23.

Blastocysts TCN and the apoptotic cell rate are important factors to judge the quality of *in vitro* embryo (Pomar *et al.*, 2005). It has been reported that the TCN of blastocysts cultured in PZM-3 was higher than blastocysts produced in NCSU-23 (Yoshioka et al., 2002). In this study, results showed no difference for the TCN between

the two culture media (P=0.09) (Table 3.2). However, since the p value was close to significant difference, the difference of TCN might significance with the sample size increasing. *BAX* is an apoptotic regulatory gene, which involved in caspase-dependent DNA degradation (Robertson *et al.*, 2002). According to the difference of *BCL2* homology (BH) domains, *BCL2* family can be divided into three groups: anti-apoptotic proteins (BCL2, BCL2A1, BCL2L1, BCL2L2 and MCL-1), pro-apoptotic proteins (BAX, BAK1 and BOK) and proapoptotic BH3 domain-only family members (BAD, BIK, BLK, BID, BCL2L11, PMAIP1 and BBC3) (Huang and Strasser, 2000; Adams and Cory, 2001). The complex balance between the anti-apoptotic genes and pro-apoptotic genes is important for early embryonic apoptotic pathways (Antonsson and Martinou, 2000). Levels of transcripts of *BAX* and *BCL2L1* genes in blastocysts from NCSU-23 and PZM-3 culture media were not different (Figure 3.2).

Embryonic genome activation is important since zygotic/embryonic gene products replace the maternal transcripts in the early development period (Kanka, 2003). Three mechanisms regulate this transcription: chromatin-mediated repression, deficiencies in the transcription machinery, and transcriptional repression or abortion by rapid cell cycles (Schier, 2007). Several studies have shown that transcription and key nucleolar proteins could be detected by the end of the four-cell stage (Viuff et al., 2002; Fabian et al., 2005). *In vivo* fertilized porcine embryos can reach 4-cell stage at 57.41 ± 2.36 hpi when cultured *in vitro* (Mateusen *et al.*, 2005). The exposure time for inhibitors in our study was chosen from 24 to 84 hpi, to cover most of the 4-cell stage.

Alpha-amanitin can inhibit RNA polymerase II transcription (Barnes and First, 1991; Memili and First, 1999; Schramm and Bavister, 1999). RNA polymerase III also can be inhibited by α-amanitin by 400 to 1000 times of the dose required for inhibition of RNA polymerase II, while RNA polymerase I is not inhibited by α-amanitin (Barnes and First, 1991). Memili et al. (1999) found some α-amanitin-sensitive proteins by using 25 μg/ml α-amanitin in bovine embryos culture medium. The final concentration of α-amanitin was 25 μg/ml based on previous studies (Bjerregaard et al., 2004; Svarcova et al., 2007). CHX is a protein synthesis inhibitor, which can be used to block the GVBD and protein synthesis in embryos. Beux et al (2003) found that CHX could be used to block meiotic resumption in porcine oocyte culture. The CHX final concentration was 2 μg/ml as reported by other researchers (Le Beux *et al.*, 2003; Marques *et al.*, 2007). In this study, exposure time to inhibitors was chosen to cover most of the 4-cell stage, and α-amanitin was used to inhibit the embryonic genome mRNA synthesis, while CHX was used to inhibit mRNA translation. Results showed transcription was not required during the first step of cleavage of porcine embryonic development. However, mRNA translation appears critical throughout porcine embryonic development. Both transcription and translation are necessary for blastocyst formation (Table 3.3).

In summary, use of PZM-3 as a porcine embryo culture medium could better promote embryonic development than NCSU-23. No significant differences of the *BAX* and *BCL2L1* genes expression were detected in blastocysts generated from PZM-3 and NCSU-23 media. In addition, EGA might not necessary for the porcine embryos cleavage; however, mRNA translations might important for both porcine embryos cleavage and

blastocysts formation. In addition, more fundamental research on mechanisms responsible for porcine early embryonic development should be studied.

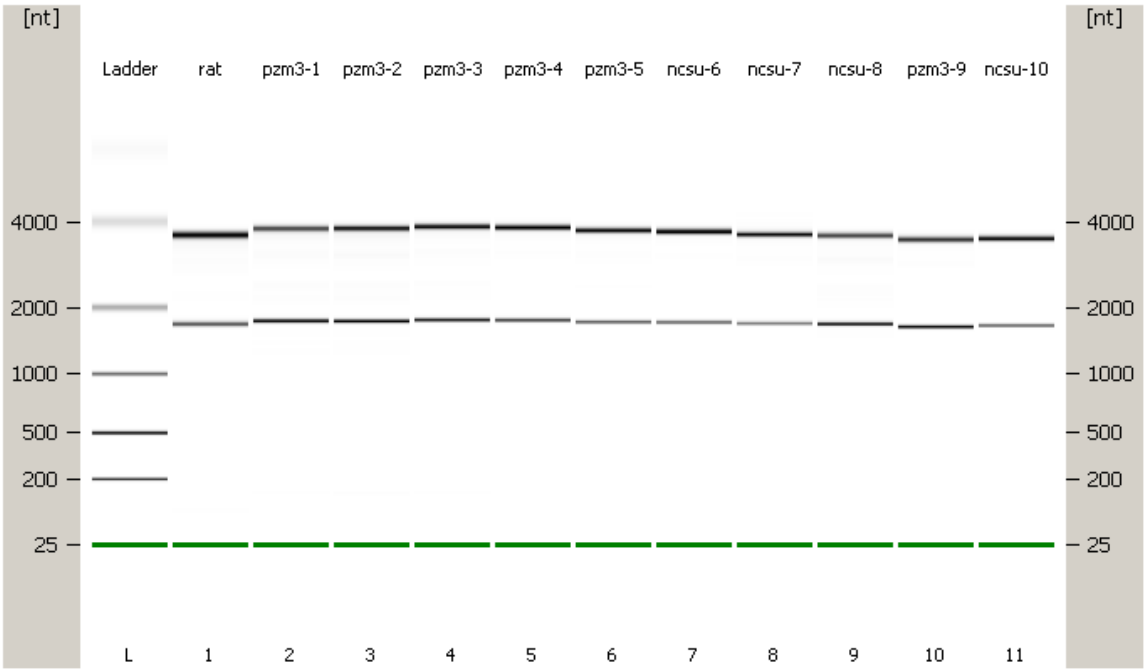

Figure 3.1 Bioanalyzer gel image of total RNA isolated from NCSU-23 and PZM-3 groups

Bioanalyzer gel image of total RNA isolated from pools of 3 blastocysts cultured in two different media. nt: size in nucleotides or bases. L: ladder. Rat: positive control. PZM3-1, 2, 3, 4, 5, and 9 were 6 repeats, NCSU-6, 7, 8, and 10 were 4 repeats. 28S rRNA was the upper band, and 18S rRNA was the lower band.

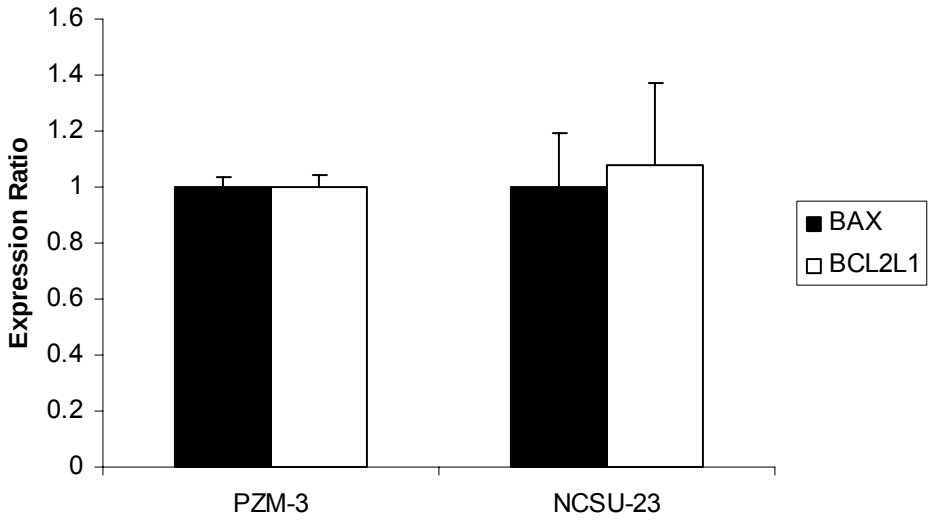

Figure 3.2 Relative gene expression ratios of *BAX* and *BCL2L1*.

Solid bar: *BAX*, open bar: *BCL2L1*. PZM-3 group was the control group. The expression levels of *BAX* and *BCL2L1* in NCSU-23 group were compared to the expression of those in PZM-3 group. No significant difference was found between PZM-3 and NCSU-23 groups.

Table 3.1 Primers used for Real-time RCR

Genes	Primer sequences and positions (5'- 3')	Fragment size (bp)	Accession Number
BAX	F: 5'TTTCTGACGGCAACTTCAACTG3' R:5'AGCCACAAAGATGGTCACTGTCT3'	236	AJ606301
BCL2L1	F:5' TGAATCAGAAGCGGAAACCC3' R:5'GCTCTAGGTGGTCATTCAGGTAAG3'	416	NM_214285
ACTB	F: 5'ACTGGCATTGTCATGGACTCTG3' R: 5'AGTTGAAGGTGGTCTCGTGGAT3'	397	U07786

Table 3.2 Effects of culture media on porcine embryonic development and the TCN in blastocysts

Culture medium	Number of oocytes	Cleavage rates (% ± S.E.M.; n)	Blastocyst rates (% ± S.E.M.; n)	Total cell numbers (mean ± S.E.M.; n)
PZM-3	918	43.9 ± 2 (403) [a]	20.1 ± 2.5 (81) [a]	46 ± 12 (11) [a]
NCSU-23	976	35.3 ± 1.6 (345) [b]	5.8 ± 1.6 (20) [b]	36 ± 7 (11) [a]

Different letters means significantly different (P<0.05).

Table 3.3 Effects of inhibitors on porcine embryonic development

Treatments	**Number of oocytes**	**Cleavage rates (% ± S.E.M.; n)**	**Blastocyst rates (% ± S.E.M.; n)**
Control	306	41.8 ± 4.5 (128) [a]	17.2 ± 5.3 (22) [a]
α-amanitin	400	39.3 ± 3.6 (157) [a]	0 ± 0 (0) [b]
CHX	392	24.5 ± 2.7 (96) [b]	0 ± 0 (0) [b]

Different letters means significantly different (P<0.05).

CHAPTER 4

MYCOTOXIN ALPHA-ZEARALENOL IMPAIRS THE QUALITY OF PREIMPLANTATION PORCINE EMBRYOS

My working hypothesis was that to mycotoxin α-ZEA disrupts porcine preimplantation embryogenesis. To test this hypothesis, my objectives were: (1) to detect the toxicity effect of α-ZEA and 17β-estradiol during porcine embryos IVC by counting the cleavage and blastocyst rates at day 4 and day 6 post insemination (pi), (2) to compare the quality of blastocysts by performing TUNEL assay, and (3) to compare the transcript levels of *POU5F1*, *BAX* and *BCL2L1* gene by conducting relative quantity Real-time PCR.

4.1 Abstract

Zearalenone (ZEA) is one of several environmental mycotoxins that impacts mammalian reproduction and development. Previous studies have shown that pigs are sensitive to the estradiol-like effects of α-ZEA. The objective of this study was to identify the direct toxicity of α-ZEA on porcine preimplantation embryonic development, embryo quality and expression of developmentally important genes. Presumptive zygotes were

cultured in porcine zygote medium (PZM)-3 in the presence of 3, 10, 30, 60 μM α-ZEA or 0.3, 3, 30, 100 μM 17β-estradiol (E2) dissolved in 0.1% Dimethyl Sulfoxide (DMSO) from 24 to 84 hours post insemination followed by determination of apoptotic cell numbers and transcript levels of *BAX*, *BCL2L1* and *POU5F1* in blastocysts. Cleavage rates on day 2 were significantly decreased in 10, 30 and 60 μM α-ZEA groups, whereas blastocyst rates on day 6 were significantly decreased in the 30 and 60 μM of α-ZEA groups. Only the 100 μM E2 group significantly decreased cleavage and blastocyst rates. Total cell numbers (TCN) in blastocysts were significantly lower in the 10 μM α-ZEA group, but no differences in apoptotic cell rates were found. The expression levels of *POU5F1* and *BCL2L1* transcripts were similar; however, levels of *BAX* transcripts and the *BAX/BCL2L1* ratio were increased in both α-ZEA groups. Since α-ZEA and E2 did not elicit similar effects, results suggest that α-ZEA might impact porcine preimplantation embryonic development through pathways other than estrogen receptor binding.

4.2 Introduction

Mammalian embryogenesis is a fascinating process critical to setting the stage for later development. Embryonic development is sensitive to environmental toxicants such as mycotoxins. There are more than 1,500 existing environmental mycotoxins such as Zearalenone (ZEA), which is a nonsteroidal estrogen. Zearalenone mimics the hormone estrogen and thereby affects early porcine embryonic development.

Zearalenone was identified in 1952 and its chemical structure was determined in 1966 (Mcearlin, 1952; Urry et al., 1966). Zearalenone is produced by several *Fusarium* species found worldwide in corn, wheat, and other cereals stored improperly in wet conditions (Eriksen et al., 2000). Once consumed in the feed, mammals metabolize ZEA into four derivatives, α-Zearalenol (α-ZEA), β-Zearalenol (β-ZEA), α-Zearalanol (α-ZAL), and β-Zearalanol (β-ZAL). Alpha-ZEA has a higher estrogenic potency than does ZEA and the other isomeric forms (Malekinejad et al., 2005). Zearalenone causes hyperestrogenism in laboratory and farm animals, especially in pigs by binding to estrogen receptors 1 and 2, where it acts as an agonist and partial antagonist to estradiol (Malekinejad et al., 2007). This binding action can decrease fertility, increase embryolethal absorption, reduce litter size, and change the weight of the adrenal and pituitary glands (Creppy, 2002). During the pig oocyte maturation period, 31.2 μM ZEA significantly decreased MII stage meiotic spindle formation and the developmental ability of oocytes (Malekinejad et al., 2007). Similarly, 15 μM ZEA significantly decreased the formation of blastocysts in pigs (Alm et al., 2002). However, the mechanism of ZEA toxicity, and its effect on porcine embryogenesis are poorly defined. There is a need for understanding how ZEA affects embryonic genome activation and developmental potential of porcine embryos.

The objective of this study was to determine the direct toxic effects of α-ZEA on porcine preimplanation embryonic development, embryo quality and expression of developmentally important genes. Findings from this study are expected to shed light

onto the molecular details of ZEA's affects on embryos at the onset of mammalian embryonic development, and how embryonic gene expression is disrupted.

4.3 Materials and Methods

4.3.1 In Vitro production of Embryos

Pig ovaries were obtained from a local slaughterhouse and transported to the laboratory in saline at 30°C. Cumulus oocyte complexes (COCs) were aspirated from 3-6 mm follicles using 18g needles. COCs with more than three layers of cumulus cells and having homogeneous cytoplasm were collected, washed three times in TL-HEPES-PVA, and matured in Tissue Culture Medium 199 (TCM-199, Invitrogen, Carlsbad, CA) supplemented with 0.1% polyvinylalcohol (PVA), 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 20 mM pyruvate stock, 25 μg/ml gentamycin, and 0.5 μg/ml LH and FSH. Fifty COCs were matured in 500 μl maturation medium under mineral oil at 39.5°C, 5% CO_2 in a 100% humidified atmosphere. These oocytes were transferred into maturation medium without hormones after 20-22 h until 40-44 h. Fertilization was performed in 500 μl modified Tris-buffered medium (mTBM) which contained 2.0 mM caffeine, 2 mg/ml BSA, 25 μg/ml gentamycin, and 10 μl/ml penicillin-streptomycin (Invitrogen). Motile sperm were prepared by by density gradient centrifugation of fresh sperm (Parrish et al., 1985) and added into fertilization drops at a final concentration of 1×10^6 sperm/ml.

4.3.2 Effect of α-ZEA on Porcine Embryonic Development

Fertilized porcine zygotes were randomly assigned to the culture media, PZM-3 (Chemicon, Temecula, CA). *In vitro* cultures were performed in 50 µl drops covered by mineral oil. Since α-ZEA and E2 were difficult to be solved in the culture medium, DMSO was used as a solvent. The final concentration of DMSO was adjusted to 0.1% in culture medium. To account for the influence of 0.1% DMSO, another group of embryos were only exposed to 0.1% DMSO in culture medium. The first experiment included control, 0.1% DMSO, 3, 10, 30, and 60 µM α-ZEA groups. The second experiment contained control, 0.1% DMSO, 0.3, 3, 30, and 100 µM E2 groups. All these chemicals were added from 24 to 84 h post insemination (hpi). Cleavage rates were counted at 48 hpi, and 10% FBS was added into each drop on day 4 post insemination (pi). Blastocyst rates were counted at day 6 pi.

4.3.3 Detection of Apoptosis in Blastocysts

Total cell number and the presence of apoptotic cells in porcine blastocysts were assessed using TUNEL (Promega, Madison, WI). Briefly, blastocysts from control, 0.1% DMSO, and 3 and 10 µM α-ZEA treatment groups were fixed in freshly prepared 4% methanol-free formaldehyde solution for 1 h at 25°C. After incubation in a 50 µl drop of permeabilization solution [0.5% (v/v) Triton X-100, 0.1% (w/v) sodium citrate] for 15-30 min at 25°C in a humidified environment, blastocysts were washed twice in 50 µl PBS/PVP drops and incubated in 50 µl drops of recombinant Terminal Deoxynucleotidyl Transferase (rTdT) incubation buffer for 1 h at 37˚C in the dark. Reactions were terminated by incubating embryos in 50 µl drops of 2× SSC solution for 15 min at 25°C.

Blastocysts were washed 3 times for 2 min in 50 µl PBS/PVP drops, stained in 1µg/ml Hoechst 33342 (Sigma, St. Louis, MO), and washed 3 times (2 min each) in 50 µl PBS/PVP drops to remove unincorporated Hoechst 33342. Blastocysts were transferred onto slides and sealed by clear nail polish. Total cell numbers and apoptotic cell numbers were counted under a 40X objective of an epifluorescent microscope equipped with a 450-490 nm excitation and 520 nm emission filter (Nikon, Tokyo, Japan).

4.3.4 RNA isolation

QIAGEN RNeasy Micro kit (Qiagen, Valencia, CA) was used to isolate total RNA from each group according to the manufacturer's protocol. Briefly, 3-5 blastocysts were pooled in RLT lysis buffer (Qiagen) and frozen at -80°C until mRNA extraction. The lysis buffer containing blastocysts was transferred onto silica-gel membrane spin columns, and washed with RW1 buffer followed by 80% ethanol washing. Final RNA elution was conducted using 14 µl of RNase-free water. RNA concentration was detected using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), and RNA integrity and quality were assessed using a Bioanalyzer 2100 RNA 6000 Picochip kit (Agilent Technologies, Palo Alto, CA).

4.3.5 Real-time PCR Analysis

Real-time quantitative PCR was performed to determine levels of relative transcripts for *BAX, BCL2L1,* and *POU5F1* genes (Rodriguez-Osorio et al. 2007). Primers were designed by Primer Premier 5 software (Premier Biosoft Int, Palo Alto, CA) (Table 4.1). Primer concentration was adjusted to 10 µM and all primers were tested

using cDNA from *in vitro*-produced embryos. A Superscript III Platinum® Two-Step qRT-PCR kit (Invitrogen) was used to synthesize the first strand of cDNA. Briefly, total RNA from each sample was normalized to 3.5 ng/µl and incubated at 25°C for 10 min, 42°C for 50 min, and 85°C for 5 min. Then, 2U of *E. coli* RNase H was added and incubated at 37°C for 20 min to eliminate RNA.

Real-time PCR reactions included 558.4ng template cDNA for each sample, primers, and SYBR® GreenER™ qPCR SuperMix (Invitrogen). The iCycler iQ Real-time PCR (Bio-Rad) machine with the following set up was used: 50°C for 2 min for uracil DNA glycosylase incubation, 95°C for 8 min 30 s for initial denaturation, 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The melting curve analysis was performed at the end, which started at 55°C and increased 0.5°C per cycle reaching to 95°C at the end of 80 cycles. *ACTB* (*β actin*) was used as the endogenous internal housekeeping gene. Standard curves were generated using 10-fold serial dilutions for the endogenous control *ACTB* and all the target genes *BAX*, *BCL2L1,* and *POU5F1,* by measuring the cycle number at which exponential amplification occurred. Relative differential mRNA expression levels of *BAX*, *BCL2L1,* and *POU5F1* genes were calculated by normalizing their values to that of the reference gene *ACTB*.

4.3.6 Statistical Analysis

Differences among experimental groups were analyzed with one-way analysis of variance (ANOVA) using SAS 9.1 software (SAS Institute Inc. Carey, NC). Gene expression analyses were performed using the relative expression software tool REST-MCS beta software v2 available at http://www.gene-quantification.info/, which is based

on an efficiency corrected mathematical model for data analysis. The mathematical model used was based on the PCR efficiencies (E) and the crossing point deviation (ΔCP) between target and reference genes (Pfaffl 2001; Pfaffl et al. 2002; Sagirkaya et al. 2006). Differences were considered to be significant when $P<0.05$.

4.4 Results

4.4.1 Alpha-ZEA impact the porcine embryonic development

A total of 300 to 500 porcine embryos from 11 replicates were used in the α-ZEA experiment. The control (non-treated) and 0.1% DMSO treated embryos had similar cleavage rates (44.3 ± 2.5 *vs*. 48.8 ± 2.6) and blastocyst rates (25.1 ± 3.6 *vs* 23.2 ± 3.6). No significant cleavage rate differences were found among control and 3 μM α-ZEA groups (44.3 ± 2.5 *vs* 43.9 ± 1.9), while the cleavage rate began to decrease in 10 μM α-ZEA group (44.3 ± 2.5 *vs* 36.5 ± 2.2, $P<0.05$). In addition, cleavage rates in the 30 μM and 60 μM α-ZEA groups (23.6±2.6 and 20.7±3.2) decreased compared to the control and 10 μM α-ZEA groups ($P<0.05$) (Table 4.2). No significant differences were found in blastocyst rates among control, 0.1% DMSO, 3 μM, and 10 μM groups. However, blastocyst development decreased in 30 μM and 60 μM α-ZEA group ($P<0.05$) (Table 4.2).

A total of 100 to 300 porcine embryos from 4 replicates were used in the E2 experiment. Differences in cleavage rates were found in the 100 μM E2 group, compared to control (19.3 ± 2.1 *vs* 30.3 ± 2.2, $P<0.05$) and other E2 groups ($P<0.05$). The

blastocyst rate in 100 μM E2 group was significantly lower than the control group (22.7 ± 5.2 *vs* 36.3 ± 3.5) (Table 4.3).

4.4.2 Detection of Apoptosis in Blastocysts

A total of 22, 23, 23, and 21 blastocysts were collected from 11 replicates in control, 0.1% DMSO, 3 μM, and 10 μM α-ZEA groups, respectively. In figure 4.1 A and 4.1 B, blue fluorescence indicates the TCN in a blastocyst, and the green-blue or teal fluorescence indicates apoptotic cells. There were no significant differences in average TCN (42.4 ± 4.3 *vs* 40.7 ± 3.9) and apoptotic cell rates (0.7 ± 0.3 *vs* 0.7 ± 0.3) between the control and 0.1% DMSO groups. No significant differences were found in the control and 3 μM α-ZEA group for average total cells and apoptotic cell numbers. The TCN in the 30 μM α-ZEA group was 26.3 ± 1.9, which was lower than other groups ($P<0.05$) (Table 4.4).

Apoptosis data were determined by dividing the number of apoptotic cells by the number of total cells in blastocysts. The percent apoptosis was increased from 0.74% in controls to 1.27% in the 10 μM α-ZEA group, but differences were not significant (Table 4.4).

4.4.3 The transcription level of BAX, BCL2L1 and POU5F1 genes

Total RNA with clear bands of 28S and 18S rRNAs without any sign of degradation were used for expression analysis (Figure 4.2). Real-time PCR expression analysis indicated that the expression level of *POU5F1* and *BCL2L1* did not change between control and treatment groups (Figure 4.3 and 4.4). Similarly, no differences were

found for the *BAX* expression between control and E2 groups. However, the expression of *BAX* and the *BAX/BCL2L1* ratio in both α-ZEA groups were increased compared to the control group ($P<0.05$) (Figure 4.5 and 4.6). There were three replicates.

4.5 Discussion

Analysis of the direct impact of α-ZEA on porcine preimplantation embryonic development and gene expression in the blastocysts stage indicated that the development of early embryos was affected by α-ZEA in a dose-dependent manner. Compared to the non-treatment control group, 10 μM α-ZEA significantly decreased cleavage rates, while 30 μM α-ZEA resulted in decreased blastocyst rates. These results were in agreement with Alm et al. (2002), where 7.5 μM α-ZEA started to significantly decrease the maturation rate of porcine oocytes as compared to the control group (55.1 ± 4.5 *vs* 81.6 ± 2.7). In addition, 15 μM α-ZEA started to decrease blastocyst rates and total number of nuclei in blastocysts compared to the control group when *in vivo*-derived zygotes were cultured *in vitro* (26.5 ± 9.2 *vs* 61.9 ± 10.0 and 15.2 ± 1.9 *vs* 48.2 ± 1.9, $P<0.05$) (Alm et al. 2002). In another study, 0.312 μM α-ZEA significantly decreased maturation of porcine oocytes, and increased the rate of aberrant nuclei of oocytes *in vitro* (Malekinejad et al. 2007).

Different concentrations of E2 (0.3, 3, 30, 100 μM) were added in the culture medium to investigate whether α-ZEA has the same effect as E2. Only 100 μM E2 significantly decreased the cleavage and blastocyst rates compared to the control group. According to Malekinejad et al. (2007), aberrant nuclei of oocytes were negatively

impacted by 0.312 μM E2, and reached the highest level by 31.2 μM E2. The author also found that the embryo cleavage and blastocyst rates were significantly decreased when these oocytes were matured in the medium containing 3.12 μM E2.

The higher toxicity of α-ZEA compared to E2 may be caused by different mechanism(s) of action of α-ZEA during early embryonic development. Estrogen can bind to the nuclear receptors ESR1 and ESR2, and this binding might cause estrogen receptors (ERs) transactivation and overexpression of the TATA box binding protein (TBP), which regulates DNA transcription (Beato et al. 1996). It was found that gene expression of ERs decreased gradually from oocytes to the 5- to 8-cell stage. The receptors of estrogen gene were not detected during the morulae stage but became detectable by the blastocyst stage (Gorski & Hou 1995). Porcine zygotes were exposed in α-ZEA at different concentrations from 24 to 84 hpi, which covers the 4-cell stage. In addition, during this stage, porcine embryos process a degrading maternal genome and activate the embryonic genome (embryonic genome activation; EGA), a critical period of mammalian early preimplantation embryonic development (Schultz et al. 1999). These results suggest that the effect of α-ZEA might be through pathways other than through binding to ERs.

Total cell numbers and apoptotic cell rates in blastocysts are important characteristics, used to evaluate for the quality of *in vitro* culture systems and *in vitro* embryos (Pomar et al. 2005). In this study, no effects on cleavage or blastocyst rates were found in the 0.1% DMSO group compared to the control group. This was similar to previous study in which 0.1% DMSO did not influence maturation and degeneration rates

of porcine oocytes (Alm et al. 2002). The results of this study showed that 10μM α-ZEA significantly decreased the TCN in blastocysts, while the apoptotic cell rates were similar. A previous study reported similar results, in which when *in vivo*-derived porcine embryos were cultured in NCSU-23, the addition of 15 μM α-ZEA initiated a decrease in the blastocyst rate and TCN in blastocysts compared to the control group (Alm et al. 2002).

Regulation between anti-apoptotic (*BCL2*, *BCL-W*, *BCL-XL*) and pro-apoptotic (*BAX*, *BAK*, *BAD*) genes plays a critical role during the embryo preimplantation stage (Antonsson & Martinou 2000). The ratio between *BAX* and *BCL2L1* determines survival or death of cells (Oltvai et al. 1993). It was found that the relative gene expression of *POU5F1* and *BCL2L1* were similar among control, α-ZEA and E2 groups. However, the expression of *BAX* and the *BAX/BCL2L1* ratio were significantly higher in 3 μM and 10 μM α-ZEA groups compared to the control group. This may represent that blastocysts were progressing towards apoptosis although their morphology in α-ZEA appeared normal. In addition, it was found that even 100 μM E2 did not increase the expression of *BAX* and the *BAX/BCL2L1* ratios compared to the control group, and this concentration was dramatically higher than 0.3 μM, which is the level in the fluid of antral follicles (Fortune & Hansel 1985).

Since α-ZEA and E2 did not elicit similar effects, these results suggest that α-ZEA might impact porcine preimplantation embryonic development through pathways other than through estrogen receptor binding and more research should be done on the mechanisms of α-ZEA effect on porcine preimplantation embryos.

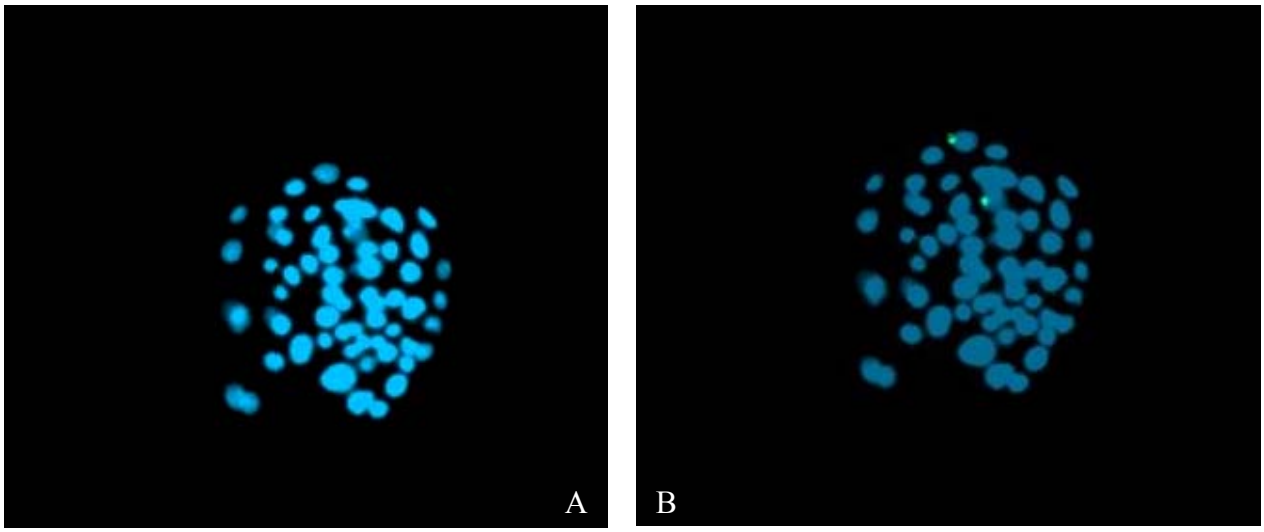

Figure 4.1 Apoptotic results from TUNEL assay

A: blue fluorescence indicates non-apoptotic cells. B: greenish-blue or teal indicates apoptotic cells.

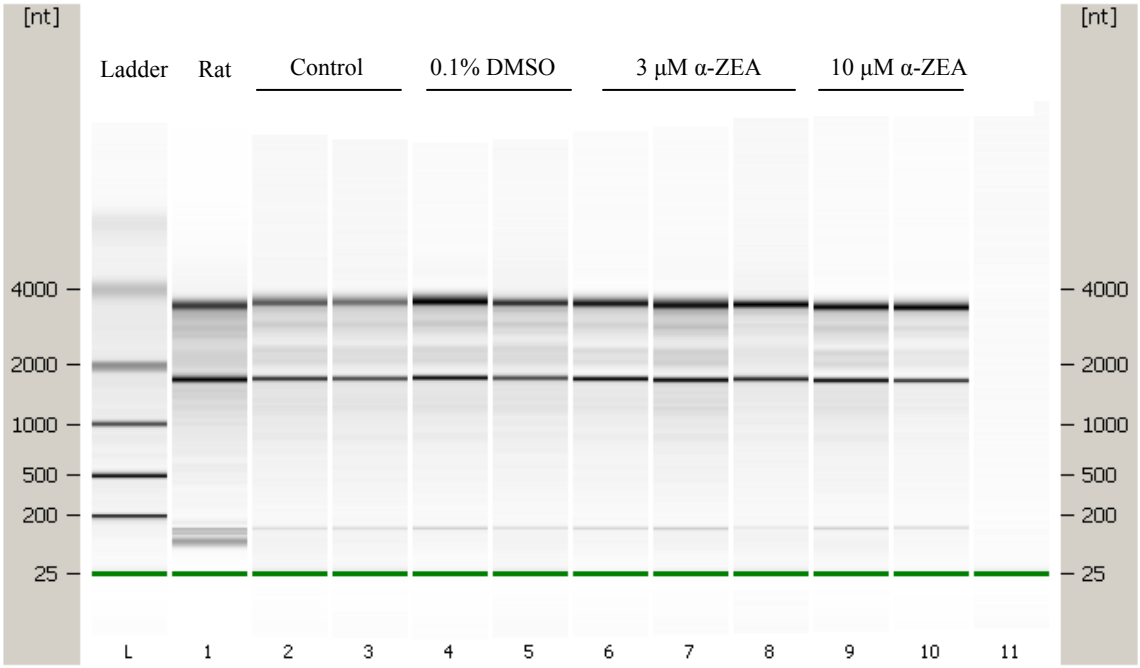

Figure 4.2 Bioanalyzer gel image of total RNA isolated from α-ZEA groups

nt: size in nucleotides or bases; L: ladder; Rat: positive control; These groups are: control, 0.1% DMSO, 3µM α-ZEA, 10µM α-ZEA groups. The upper and lower bands were 28S and 18S ribosomal RNA bands.

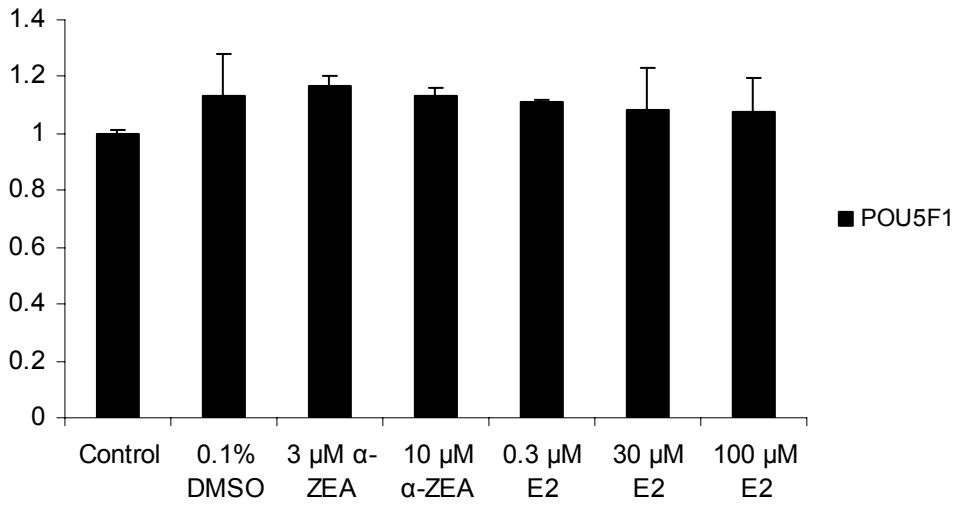

Figure 4.3 Relative gene expression ratio of *POU5F1*

Expression ratio of *POU5F1* gene in the blastocysts cultured in the PZM-3 containing 0.1% DMSO, 3 μM α-ZEA, 10 μM α-ZEA, 0.3 μM E2, 30 μM E2, and 100 μM E2 compared to the blastocysts cultured in control group. There were no significant differences of the expression for *POU5F1* among groups.

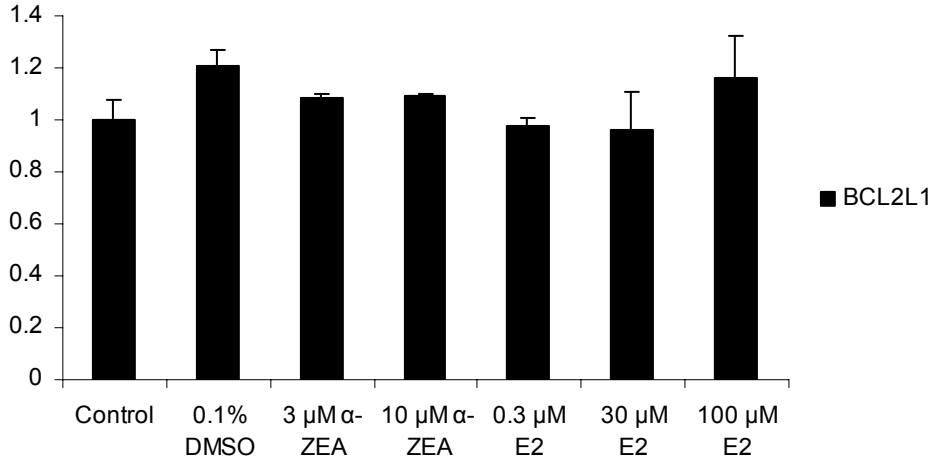

Figure 4.4 Relative gene expression ratio of *BCL2L1*

Expression ratio of *BCL2L1* gene in the blastocysts cultured in the PZM-3 containing 0.1% DMSO, 3 μM α-ZEA, 10 μM α-ZEA, 0.3 μM E2, 30 μM E2, and 100 μM E2 compared to the blastocysts cultured in control group. There were no significant differences of the expression for *BCL2L1* among groups.

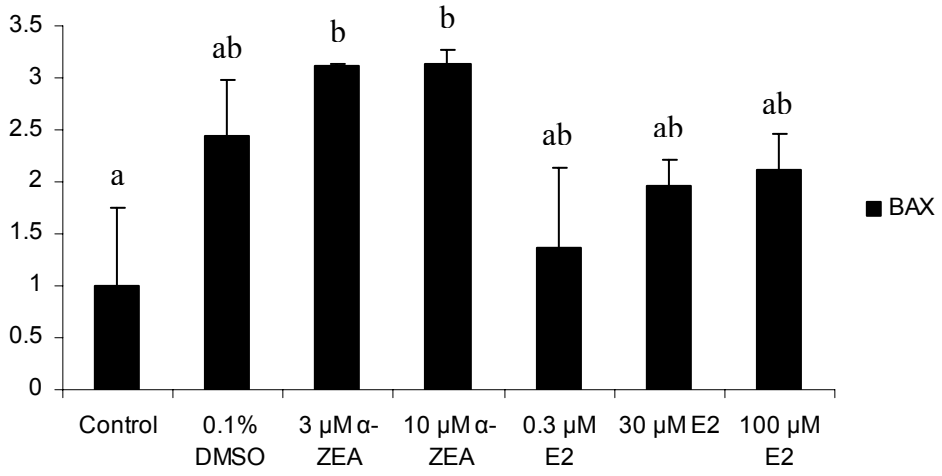

Figure 4.5 Relative gene expression ratio of *BAX*

Expression ratio of *BAX* gene in the blastocysts cultured in the PZM-3 containing 0.1% DMSO, 3 μM α-ZEA, 10 μM α-ZEA, 0.3 μM E2, 30 μM E2, and 100 μM E2 compared to the blastocysts cultured in control group. There were significant differences of the expression *BAX* between control and both α-ZEA groups. Different letters mean significantly different ($P<0.05$).

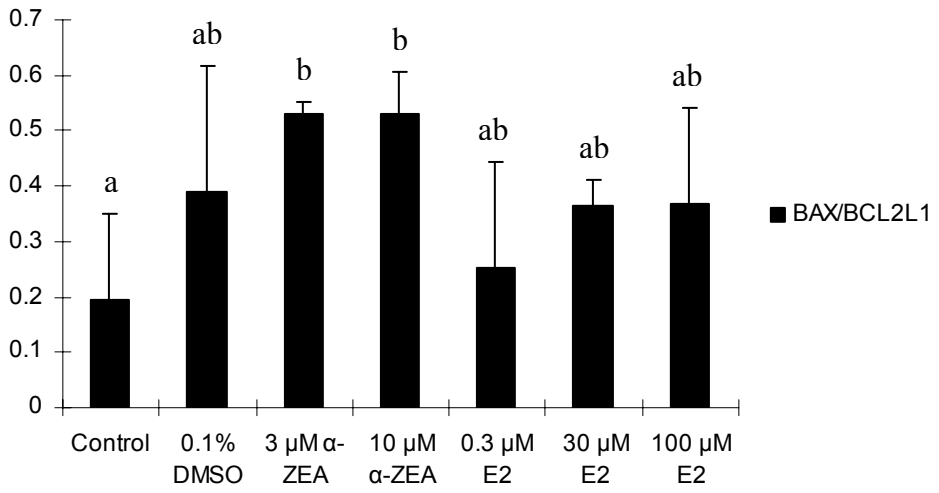

Figure 4.6 The ratio of *BAX/BCL2L1*

The ratio of *BAX/BCL2L1* in the blastocysts cultured in the PZM-3 containing 0.1% DMSO, 3 μM α-ZEA, 10 μM α-ZEA, 0.3 μM E2, 30 μM E2, and 100 μM E2 compared to the blastocysts cultured in control group. There were significant differences of the ratio of *BAX/BCL2L1* between control and both α-ZEA groups. Different letters mean significantly different (P<0.05).

Table 4.1 Primers used for Real-time RCR

Gene	Accession number	Primer sequences (5'-3')	Tm	Fragment size (bp)
BAX	AJ606301	F: TTTCTGACGGCAACTTCAACTG R: AGCCACAAAGATGGTCACTGTCT	60	236
BCL2L1	NM_214285	F: TGAATCAGAAGCGGAAACCC R: GCTCTAGGTGGTCATTCAGGTAAG	60	416
POU5F1	NM_001113060	F: AGGTGTTCAGCCAAACGACC R: GATCGTTTGCCCTTCTGGC	60	334
ACTB	U07786	F: ACTGGCATTGTCATGGACTCTG R: AGTTGAAGGTGGTCTCGTGGAT	60	397

Table 4.2 Effects of α-ZEA on porcine embryonic development at different concentration

Culture medium	Number of oocytes	Cleavage rates (% ± S.E.M.; n)	Blastocyst rates (% ± S.E.M.; n)
Control	551	44.3±2.5 (240) [a]	25.1±3.6 (59) [a]
0.1% DMSO	520	48.8±2.6 (256) [a]	23.2±3.6 (57) [a]
3μM α-ZEA	693	43.9±1.9 (303) [a]	27.2±3.3 (79) [a]
10μM α-ZEA	543	36.5±2.2 (200) [b]	21.8±3.7 (44) [a]
30μM α-ZEA	326	23.6±2.6 (78) [c]	4.4±2.5 (4) [b]
60μM α-ZEA	324	20.7±3.2 (67) [c]	2.9±2.0 (2) [b]

Different letters mean significantly different ($P<0.05$).

Table 4.3 Effects of E2 on porcine embryonic development at different concentration

Culture medium	Number of oocytes	Cleavage rate (% ± S.E.M.; n)	Blastocyst rate (% ± S.E.M.; n)
Control	248	30.3±2.2 (75) [a]	36.3±3.5 (28) [a]
0.1% DMSO	304	31.6±2.5 (96) [a]	31.5±3.8 (30) [ab]
0.3μM E2	117	35.4±9.0 (42) [a]	27.2±9.0 (13) [ab]
3μM E2	132	34.9±7.0 (47) [a]	24.9±9.2 (15) [ab]
30μM E2	316	27.1±3.3 (85) [a]	31.5±4.7 (29) [ab]
100μM E2	216	19.3±2.1 (42) [b]	22.7±5.2 (10) [b]

Different letters mean significantly different (P<0.05).

Table 4.4 The total cell number and apoptotic cell rates of porcine blastocysts

Culture medium	Total cell number	Apoptotic cells rates (% ± S.E.M.; n)
Control	42.4±4.3 (22) [a]	0.7±0.3 (22) [a]
0.1% DMSO	40.7±3.9 (23) [a]	0.7±0.3 (23) [a]
3μM α-ZEA	39.8±3.1 (23) [a]	1.0±0.4 (23) [a]
10μM α-ZEA	26.3±1.9 (21) [b]	1.3±0.9 (21) [a]

Different letters mean significantly different (P<0.05).

CHAPTER 5

CONCLUSIONS

Research to improve *in vitro* embryo production system has been studied for several years. Production of viable porcine embryos will have significantly impact on porcine reproductive management, biotechnological and biomedical fields (Wheeler et al., 2004). However, low pronuclear formation rates, high polyspermy rates, poor developmental ability and low cell numbers of blastocysts are still the major problems generated by suboptimal *in vitro* culture systems (Abeydeera and Day, 1997; Wang et al., 1998; Kikuchi et al., 1999; Pomar et al., 2005). It has been reported that poly-pronuclear (PPN) can produce lower number of ICM in the blastocyst stage compared to two-pronuclear (2PN)-derived embryos from both *in vitro* and *in vivo* culture system. In addition, both 2PN and PPN groups generated fetuses on Day 21, which means the problem of PPN may be corrected during later embryonic development (Han et al., 1999). Many IVF culture media were designed to mimic the oviduct and uterus environment during embryonic development. Oviductal fluid (OVF) has been reported to improve *in vitro*-derived porcine early embryonic development (Archibong et al., 1989). An early co-culture experiment was performed to evaluate the ability of three types of somatic cells during embryonic culture: porcine oviductal epithelial cells (POEC), orcine fetal fibroblast (PEF) monolayer, and POEC-PEF co-culture system. The results have shown

that POEC and POEC-PEF culture system generated more blastocysts than those in PEF (70%, 67% and 27%, respectively) (White et al., 1989). Currently, NCSU-23 and PZM-3 have been widely used for porcine embryo culture. In this experiment, results showed that both cleavage and blastocyst rates decreased significantly in NCSU-23 group compared as PZM-3 group. This data were similar to another report that PZM-3 promoted more blastocysts and hatched blastocysts than those in NCSU-23 (Yoshioka et al., 2002).

Embryo yield rate and embryo quality are two major parameters impacting the embryo transfer (ET). It has been reported that embryonic culture conditions determine embryo quality (Rizos et al., 2002). Apoptosis is involved in elimination abnormal and redundant cells and has been observed in blastocysts of different mammalian species (Hardy, 1997). Two kinds of cell death are known, necrosis and apoptosis. The significant characters of necrosis are cell swelling and membrane rupture, which affect other adjoining cells and cause the inflammatory response. Apoptosis is a programmed death process during which DNA is degraded into oligonucleosome-sized fragments (Wyllie et al., 1980). The cytoplasm condenses and forms fragments into apoptotic bodies covered by membrane. Apoptosis is a normal process during embryo development. Blastocyst total cell number and apoptotic rate are important factors to judge the quality of *in vitro* embryo (Pomar et al., 2005). Our results showed no difference for the TCN in blastocysts between the NCSU-23 and PZM-3 culture media group.

Gene expression is another way to reflect the quality of blastocysts. According to the difference of *BCL2* homology (BH) domains, *BCL2* family can be divided into three groups: antiapoptotic proteins (BCL2, BCL2A1, BCL2L1, BCL2L2 and MCL1), proapoptotic proteins (BAX, BAK1 and BOK) and proapoptotic BH3 domain-only family members (BAD, BIK, BLK, BID, BCL2L11, PMAIP1 and BBC3) (Huang and Strasser, 2000; Adams and Cory, 2001). The complex balance between a battery of anti-apoptotic (*BCL2*, *BCLW*, *BCL-WL*) and pro-apoptotic genes (*BAX*, *BAK*, *BAD*) is important for early embryonic apoptotic pathways (Antonsson and Martinou, 2000). It has been reported the transcripts ratio between *BAX* and *BCL2* determines cell survive or die (Oltvai et al., 1993). In this study, no significant differences were detected in gene transcription levels of the blastocysts generated from NCSU-23 and PZM-3 culture media. The results suggested that blastocysts generated from NCSU-23 medium might have similar quality compared to those from PZM-3 group, although a lower number of blastocysts were harvested from NCSU-23 group.

After fertilization, embryogenesis starts, in which maternally inherited messages are degraded and embryonic genome are activated gradually. EGA is important since zygotic/embryonic genome replaces the maternal transcripts in the early development period (Kanka, 2003). EGA has been studied in a number of mammalian species recently. In mouse, it was believed that minor gene activation occurs at the early 2-cell stage and major EGA occurs during the late 2-cell stage (Flach et al., 1982). In bovine, α-amanitin has been used to inhibit RNA polymerase II transcription during bovine embryo culture, and the results indicated that EGA was essential for bovine early embryonic development

(Barnes and First, 1991; Memili and First, 1999). In porcine, CHX, a protein synthesis inhibitor, was used to block the GVBD and protein synthesis in embryos. CHX (2 μg/ml) have been used to block meiotic resumption in porcine oocyte culture (Le Beux et al., 2003). In this study, porcine zygotes were exposed to α-amanitin and CHX from 24 to 84 hpi to cover most of the 4-cell stage. Our results showed transcription was not required during the first step of cleavage of porcine embryo development. However, mRNA translation appears critical throughout the porcine embryo development. Both transcription and translation are necessary for blastocyst formation.

Mycotoxins are found in feedstuffs and have toxic effects on livestock and humans (Fink-Gremmels, 1999). Mycotoxins are found in corn, wheat, barley and sorghum throughout of the world (Zinedine et al., 2007). ZEA, one of these mycotoxins, impact livestock by mimicking estrogen. In addition, α-ZEA, a derivative metabolized ZEA, has a higher estrogenic potency than the ZEA and the other isomers (Malekinejad et al., 2005). Alpha-ZEA causes hyperestrogenism in porcine maybe through binding to ESR1 and ESR2, where it acts as an agonist and partial antagonist to estradiol (Malekinejad et al., 2007). In our study, we determined the direct toxic effects of α-ZEA on porcine preimplantation embryonic development, embryo quality and expression of developmentally important genes. No toxicity effects of 0.1% DMSO were found in this study. Our results indicated the development of early embryogenesis was affected by α-ZEA in a dose-dependent manner. Compared to the non-treatment control group, 10 μM α-ZEA significantly decreased the cleavage rates, while 30 μM α-ZEA resulted in

decreased blastocysts rates. These results were in agreement with a previous study (Alm et al., 2002).

To detect whether α-ZEA has the same toxic impacts as E2 during early embryonic development, we found that only 100 μM E2 decreased the embryonic cleavage and blastocyst rates. Our results have shown that 10μM α-ZEA significantly decreased the total cell number in blastocysts ($P<0.05$), while the apoptotic cell rates were similar. A previous study found that when *in vivo*-derived porcine embryos cultured in NCSU-23, 15μM α-ZEA significantly decreased the blastocysts rates and the number of nuclei in the blastocysts compared to the control group (Alm et al., 2002). We found that the relative expression of *POU5F1* and *BCL2L1* were similar among groups. However, the expression of *BAX* and the ratio of *BAX/BCL2L1* were significantly higher in 3μM and 10μM α-ZEA groups compared to the control group.

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APPENDIX

COMPARISON OF NCSU-23 AND PZM-3 MEDIUM (Yoshioka et al., 2002)

Compound	NCSU-23	PZM-3
NaCI (mM)	108.73	108.00
KCL (mM)	4.78	10.00
$CaCI_2.H_2O$ (mM)	1.70	-
KH_2PO_4 (mM)	1.19	0.35
$MgSO_4$ $7H_20$ (mM)	1.19	0.40
$NaHCO_3$ (mM)	25.07	25.07
Glucose (mM)	5.55	-
Na-pyruvate (mM)	-	0.20
Ca-$(lactate)_2 \cdot 5H_2O$ (mM)	-	2.00
Glutamine (mM)	1.00	1.00
Taurine (mM)	7.00	-
Hypotaurine (mM)	5.00	5.00
Basal Medium Eagle amino acids (ml/L)	-	20.00
Minimum Essential Medium Nonessential amino acids (ml/L)	-	10.00
Gentamincin (mg/ml)	0.05	0.05
Fatty acid-free BSA (mg/ml)	4.00	3.00
Osmolarity (mOsm) [b]	291.00±2	288.00±2
PH [b]	7.30±0.02	7.30±0.02

b: Mean ± SD