Expression of the Antioxidant Enzyme and Apoptosis Genes in *In vitro* Maturation/*In vitro* Fertilization Porcine Embryos

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ABSTRACT : This study was aimed at testing the gene expression of antioxidant enzymes and apoptosis genes for *in vitro* culture in porcine embryos produced by *in vitro* maturation/*in vitro* fertilization (IVM/IVF). Pocine preimplantation embryos obtainted from IVM/IVF can be successfully culture *in vitro*, but they are delayed or stop to develop at specific developmental stage. Many factors such as reactive oxygen species and apoptosis in an IVM/IVF system followed by *in vitro* culture influence the rate of production of viable blastocysts. Porcine embryos derived from IVM/IVF were cultured in the atmosphere of 5% CO_2 and 20% O_2 at 38.5°C in NCSU23 medium. The patterns of gene expression for antioxidant enzymes and apoptosis genes during *in vitro* culture in pocine IVM/IVF embryos were examined by the modified semi-quantitative single cell reverse transcriptase-polymerase chain reaction (RT-PCR). Porcine embryos produced by *in vitro* procedures were expressed mRNAs for CuZn-SOD, GAPDH and GPX, whereas transcripts for Mn-SOD and catalase were not detected at any developmental stages. Expression of caspase-3 mRNA was detected at 2 cell, 8 cell 16 cell and blastocyst, but p53 mRNA was not detected at any stages. The fas transcripts was only detected in blastocyst stage. These results suggest that various antioxidant enzymes and apoptosis genes play crucial roles *in vitro* culture of porcine IVM/IVF embryos. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 1 : 33-38)***

Key Words : Pig, IVM/IVF Embryo, RT-PCR, Antioxidant, Apoptosis

INTRODUCTION

In vitro culture of mammalian embryos has been widely investigated in efforts to overcome the developmental retardation occurring in *in vitro* condition and to approximate efficiency provided *in vivo* condition. One of their major differences between the *in vivo* and the *in vitro* environment for the embryo is the oxygen concentration (Maas et al., 1976). A major culture-induced stress is enhanced by oxidative damage, with increased reactive oxygen species (ROS) production, including hydrogen peroxide (H_2O_2) (Goto et al., 1992) and superoxide radicals (O_2^-) (Noda et al., 1991). The destructive hydroxyl radical (OH^+) is formed by ioncatalyzed interaction of H_2O_2 and O_2^-, and reacts with extremely high rate constants with sugars, amino acid, phospholipids, nucleotides and organic acids (Halliwell and Gutteridge, 1985).

Oxygen species are regulated by superoxide dismutase (SOD) that changes superoxide into H_2O_2 or by catalase and glutathione peroxidase (GPx) which decompose H_2O_2 into H_2O. Because of their ability to degenerate ROS into nontoxic compounds, these antioxidative enzymes play roles in protecting cells from oxidative stress-induced cell death (Majima et al., 1998; Chandra et al., 2000).

When ROS overcomes the anti-apoptotic system, cells begin the process of suicide program. It is well established that up-regulation of the Fas/FasL system and release of cytochrome c are induced by ROS (Stridh et al., 1998; Suhara et al., 1998). Further nuclear translocation of p53 is known to induced by ROS (Uberti et al., 1999), and p53 induced apoptosis requires the generation of ROS (Li et al., 1999) and transcriptional activation of bax and fas (Miyashita et al., 1995; Muller et al., 1998).

There are increasing evidences that cells in mammalian preimplantation embryos undergo apoptosis (Hardy, 1997; Betts and King, 2001). Characteristic apoptotic features including fragmenting nuclei and nuclei with fragmented DNA, are rarely seen before compaction, but are commonly seen in blastocyst from a number of mammalian species including humans (Hardy, 1999; Hardy et al., 2001), mice (Handyside and Hunter, 1986; Brison and Schultz, 1997) and cows (Byrne et al., 1999; Matwee et al., 2000). Levels of apoptosis can be environmentally regulated but the role remains unknown (Hardy, 1997).

The development of technology *in vitro* embryo culture is required to the transgenesis and germ cell engineering in the field of application of porcine (Lee and Piedrahita, 2003). Our research in RT-PCR technologies of embryos will increase the efficient of transgenesis and embryo stem cell technologies

Therefore the present study was designed at testing the gene expression of antioxidant enzymes and apoptosis gene

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expression during *in vitro* culture in *in vitro* porcine embryos.

MATERIALS AND METHODS

Collection and *in vitro* maturation of oocytes

The collection and culture of oocytes were based on procedures reported by Park et al. (2001). Briefly, ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in 0.9% (w/v) NaCl at 35°C within 2 h, follicular oocytes were aspirated from antral follicles (2-5 mm diameter) using 18 gauge needle fixed to a 10 ml disposable syringe.

The oocytes-cumulus complexes were collected from the follicular contents and washed twice with Dubelco's PBS containg 1% polyvinyl alcohol (PBS-PVA) and the maturation medium I (IVM-I medium), respectively. Only oocytes possessing a compact cumulas mass and evenly granulated ooplasm were transferred to a 100 μl drop of the IVM-I medium which had been previously covered with warm mineral oil. After first maturation for 20-22 h, oocytes with an expanded cumulus cell were wash three times and then cultured in maturation medium II (IVM-II medium) for another 20- 22 h at 38.5°C, 5% CO_2 in air.

In vitro fertilization of oocytes

After maturation, all oocytes were washed twice with the insemination medium (mTBM; Wang et al., 1997). Thereafter, 20 oocytes were transferred into a 50 μl droplet of mTBM covered with mineral oil.

For IVF, 0.5 ml frozen semen straw was thawed at 37°C water bath for 30 secs. After being washed twice by centrifugation at 900 g for 5 min, spermatozoa were resuspended with mTBM containing 2 mM caffeine, to give a containing of to 2×10^6 cells ml, and 50 μl of the sperm suspension were added to 50 μl of the fertilization drops containing the oocytes. Six hours after insemination, oocytes were removed from fertilization drops, washed four times and cultured in 100 μl droplet of culture medium under mineral oil at 38.5°C in the atmosphere of 5% CO_2 and 20% O_2 in air.

Culture media

The maturation medium (IVM-I medium) for first 20-22 h consisted of NCSU23 (Wang et al., 1997) supplemented with 0.57 mM cysteine, 10 IU/ml hCG, 0.5 μg/ml FSH, 5 μg/ml LH and 10% porcine follicular fluid (pFF). For the second 20-22 h of maturation (IVM-II), the same medium was used as for IVM-I medium without hormones. The fertilization medium was used modified Tris-buffered medium (mTBM) consisted of 113.1 mM NaCl, 3.0 mM KCl, 7.5 mM $CaCl_2$, 11.0 mM Glucose, 5.0 mM Na-pyruvate, 20 mM Tris, 100 IU/ml penicillin G, 50 μg/ml streptomycin containing 2 mg/ml bovine serum albumin (BSA). *In vitro* culture medium was used NCSU23 medium supplemented 4 mg/ml BSA. All the regents were purchased from sigma except for D-PBS (Gibco BRL).

Single-cell reverse transcription (RT)

Each treatment embryos were placed in polymerase chain reaction (PCR) tubes in 2 μl of sterilized diethylprocarbonate (DEPC)-treated water. Before use, the embryos underwent thermolysis for 1 min at 100°C in order to release nucleic acids.

The reverse transcription reagents, RT buffer 10, 0.5 mM of each d NTP, 0.5 μg oligo-(dT)15, 10 IU RNase-inhibitor (Gibco-BRL) and 500 IU reverse transcriptase were mixed on ice in total volume of 20 μl, and 18 μl of the RT mix was added to each blastomere (Roche, Germany) in tubes. RT was carried out at 42°C for 60 min followed by

Table 1. Oligonucleotide nested primer pairs for antioxidant genes and GAPDH

mRNA	Type of primer	Primer sequence (5' 3')	Annealing temperature (°C)	Product size (bp)	Genbank accession number
Catalase	Forward	GAAAGGCGAAGGTGTTTGAGCA	61	814	D89812
	Outer reverse	AGGCGGTGGCGGTGAGTGTC			
	inner reverse	ATATCCGTTCATGTGCCTGTGTCC		361	
Cuzn-SOD	Forward	GCAGGGCACCATCTACTTC	61	382	AF396674
	Outer reverse	ACTTCCAGCATTTCCCGTCTTT			
	inner reverse	TCACATTGCCCAGGTCTCCA		217	
Mn-SOD	Forward	CGCGGCCTACGTGAACAACCT	61	379	X64057
	Outer reverse	CCCCAGCAGCGGAACCAGAC			
	inner reverse	AGTCGCGTTTGATGGCTTCC		208	
GPx	Forward	CCATGCCCGGAGGTCTGCTTCT	61	380	AJ243849
	Outer reverse	CCATCACGCTGTCTCCATTCTTCC			
	inner reverse	TCGGGACCAAAAATAAATACCACA			
GAPDH	Forward	ATCACCATCTTCCAGGAGCG	58		AF261085
	Reverse	GATGGCATGGACTGTGGTCA			

Table 2. Oligonucleotide nested primer pairs for apoptosis gene

mRNA	Type of primer	Primer sequence(5' 3')	Annealing temperature (℃)	Product size (bps)	Genbank accession number
p53	Forward	GCCCCTGTCGTCCTTTGTCC	63	678	AF098067
	Outer reverse	TGGCGAGGAGCTGGTGCTGG			
	inner reverse	GCCAAGTACTCGGCCCGTAA		411	
fas	Forward	CTGCCACCGCCATCTCTGAA	63	533	AF397407
	Outer reverse	CTGCTGCGGGCCCACATTTG			
	inner reverse	GCTGCCCTTCATATACTTCAC		344	
caspase-3	Forward	GAAGCAAATCAATGGACTCTGGA	63	509	AB029345
	Outer reverse	GTCTGCCTCAACTGGTATTTTCTG			
	inner reverse	GGCAGGCCTGAATTATGAAAAGTT		362	

Table 3. Timing of expression of antioxidant and apoptosis gene during porcine preimplantation development

	Stage of embryo development					
	2 cell	4 cell	8 cell	16 cell	Morula	Blastocyst
GAPDH	+	+	+	+	+	+
	(3/3)	(3/3)	(3/3)	(3/3)	(3/3)	(3/3)
Catalase	-	-	-	-	-	-
	(3/3)	(3/3)	(3/3)	(3/3)	(3/3)	(3/3)
CuZnSOD	+	-	+	+	+	-
	(3/3)	(3/3)	(3/3)	(3/3)	(3/3)	(3/3)
MnSOD	-	-	-	-	-	-
	(3/3)	(3/3)	(3/3)	(3/3)	(3/3)	(3/3)
GPx	+	+	+	+	+	+
	(3/3)	(3/3)	(3/3)	(3/3)	(3/3)	(3/3)
fas	-	-	-	-	-	+
	(3/3)	(3/3)	(3/3)	(3/3)	(3/3)	(3/3)
caspase-3	+	-	+	+	-	+
	(3/3)	(3/3)	(3/3)	(3/3)	(3/3)	(3/3)
P53	-	-	-	-	-	-
	(3/3)	(3/3)	(3/3)	(3/3)	(3/3)	(3/3)

heating to 98°C for 10 min to inactivation the reaction and storage at 4°C.

Polymerase chain reaction (PCR)

An each development stages were analyzed for presence of transcription encoding for antioxidant and apoptosis gene in each groups.

PCR analysis were carried out in 50 μl and contained cDNA (5 μl RT product), 0.2 mM each of dNTP, 0.5 mM of primer and 2 IU of Taq polymerase (IBS, Korea). After an initial denaturation stop of 5 min 95°C, 25 amplification cycles were performed.

Each cycle include denaturation at 95°C for 45 sec, annealing at each primer temperatures for 1 min and extension at 72°C for 1 min. A final extension step and extension of 5 min at 72°C was performed in order to complete the PCR reaction primer sequence used in this study are indicated in Table 1 and 2, and nested primers for 35 cycls. For the nested reaction, 5 μl of the first amplification product was added to freshly prepared PCR mix. After amplification, 20% RT-PCR products were separated by agarose (2%) gel electrophoresis stained by ethidium bromide and visualized under UV.

RESULT

To develop an mRNA phenotypic map for the expression of various antioxidant enzymes and apoptosis genes, single-cell RT-PCR analysis were carried out in porcine IVM/IVF embryos. In all instances, the assays were repeated at least three times with different embryo batches. Porcine IVM/IVF embryos were expressed mRNAs for CuZn-SOD, GAPDH and GPx, but Mn-SOD and catalase mRNA were not detected at any embryonic stages (Table 3). In apoptosis gene expression, caspas-3 mRNA was detected at specific developmental stages and Fas ligand transcripts was only detected in blastocysts, but p53 mRNA was not detected at any stages (Figure 2). Confirmation of RT-PCR products were presented in Figure 1 and 2. Transcripts for GAPDH and GPx were detected in the all of these amples. The singnals obtained for GPx weres observed from the 2 cell to the blastocyst stage. CuZn-SOD transcript were detected in 2, 8, 16 cell and morual stage, but MnSOD and catalase were not detected in all porcine IVM/IVF embryos (Figure 1). Transcription for p53 was not detected at the single embryo level during all stage of preimplantation development. However, expression of caspase-3 was

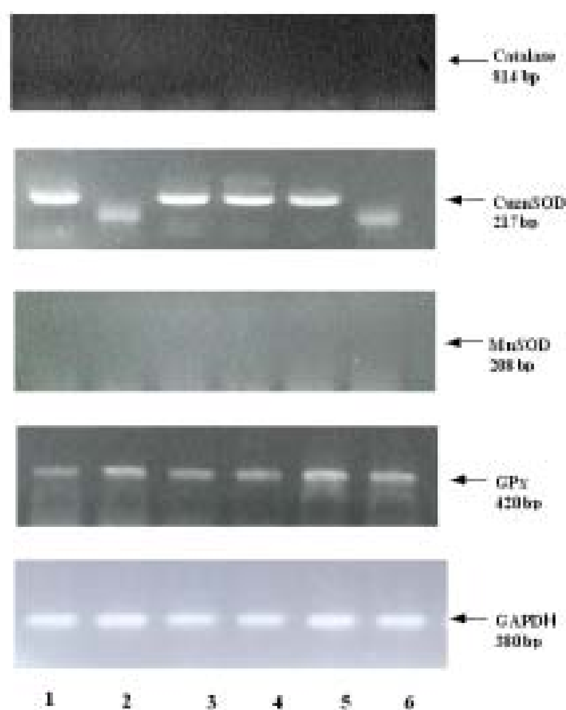

Figure 1. mRNA expression of antioxidant enzymes on porcine IVM/IVF embryos: Single cell RT-PCR were carried out to detect the CuZn-SOD, Mn-SOD, GPx and GAPDH. Porcine IVM/IVF embryo cultured under 20% CO_2, 5% O_2 at 38.5°C. Pooled data from three replicates, a representative result is shown. 1. 2 cell, 2. 4 cell, 3. 8 cell, 4. 16 cell, 5. morula, 6. blastocyst stage.

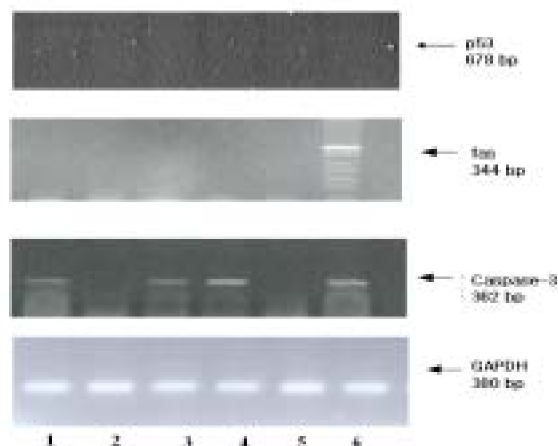

Figure 2. mRNA expression of apoptosis genes on procine IVM/IVF embryos; Single cell RT-PCR products were separated on agarose gels stained with ethidium bromide. Fas, caspase-3, p53 and GAPDH RT-PCR on in vitro produced embryos collected at successive developmental stage (2 cell-blastocyst). The experiments was repeated three times, a representative result is shown. 1. 2 cell, 2. 4 cell, 3. 8 cell, 4. 16 cell, 5. morula, 6. blastocyst stage.

detected at 2, 8, 16 cell and blastocyst stage and fas expression was detected only in blastcyst stage (Figure 2).

DISCUSSION

Preimplantation embryos of all species studied thus far display characteristic culture blocks associated with the timing of embryonic genome activation (Johnson and Nasr-Esfhani, 1994). Thus, it is at the stage when the control of development is changing from an exclusively post transcriptional level to a transcriptional level that the embryo appears to be most vulnerable to environmental insults. Recent attention has now focused on reactive oxygen species (ROS) as major causal agents for *in vitro* embryonic arrest (Johnson et al., 1994).

The ROS can alter cell conformation and activities by directly affecting kinases and transcription (Adler et al., 1999) and are probably involved in retarded embryonic development during *in vitro* culture condition (Johnson and Nasr-Esfahani, 1994). Moreover, ROS are implicated in the occurrence of apoptosis on embryo development (Parchment, 1991).

These enzymes include Mn-SOD and CuZn-SOD, which catalaze the dismutation reaction, removing O_2 species: catalase, which catalyases the decomposition of hydrogen peroxide to oxygen and water: and GPX, which utilizes glutathione as a reducing source to catalyze the removal of both hydrogen peroxide and lipid peroxides (Halliwell and Gutteridge, 1989; Johnson and Nasr-Esfahani, 1994).

The aim of the present study was to examine and contrast the expression of antioxidant enzymes and apoptosis genes in porcine IVM/IVF embryos, which develop efficiently *in vitro* in simple culture media.

In early porcine embryos, transcription for GPx was expressed in all preimplantation embryo stages CuZn-SOD transcript, were detected in 2, 8, 16 cell and morula stage, but Mn-SOD were not detected in all porcine preimplantation embryos.

In bovine embryos, the encoding catalase, CuZnSOD, and GPx were detected throughout preimplantation development, whereas no MnSOD mRNAs were detected (Harvey et al., 1995).

It is recognized that numerous additional enzymatic and nonezymatic antioxidant defenses exist within cells other than the enzymes investigated in this study.

Transcription for p53 was not detected at the single embryo level during all stage of preimplantation development. However, expression of caspase-3 was detected at the 2, 8, 16 cell and blastocyst stage and fas expression was detected in blastocyst stage.

Oxidative stress can be responsible for induction of apoptosis in mouse (Salas-Vidal et al., 1997) and bovine embryos (Yuan et al., 2003).

The present study has shown that the timing of expression of active caspases coincided with the onset of

BAD expression and also with the onset of apoptosis reported by Hardy et al. (2001). Weil et al. (1996) reported that early embryos were more resistant to induced apoptosis than were cells in the blastocyst and hypothesized that a functional apoptotic pathway may be suppressed during early development (Weil et al., 1996). The expression of apoptosis genes throughout mouse and human preimplantation development (Exley et al., 1999), certainly supports the hypothesis that although the apototic machinery is present throughout development, apoptosis is inhibited during early cleavage stages. However, the reasons for particular cells within the early stage embryo, morula, and blastocyst undergoing apoptosis are still unclear and the early signaling events that trigger and embryo cell to die are unknown.

The study of gene expression in IVM/IVF embryos could be the method of choice for evaluating embryo quality and *in vitro* culture condition in the near future. The gene expression of Mn-SOD and catalase and p53 is not detected during *in vitro* culture in porcine IVM/IVF embryos but it remain to be elucidated. Also, the precise mechanism of gene expression of antioxidant enzymes and apoptosis in preimplantation stages of porcine IVM/IVF embryos remains to be further studied.

In conclusion, various antioxidant enzyme and apoptosis gene play important roles for *in vitro* culture in porcine IVM/IVF embryos.

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