Effects of Melatonin on Gene Expression of IVM/IVF Porcine Embryos

H. Y. Jang[1, 2], H. S. Kong[1], K. D. Choi[1], G. J. Jeon[1], B. K. Yang[2], C. K. Lee[3] and H. K. Lee[1, *]

[1] Department of Genomic Engineering, Genomic Informatics Center, Hankyong National University
Ansung 456-749, Korea

ABSTRACT : The effect of melatonin on *in vitro* embryo development and the expression of antioxidant enzyme gene in preimplantation porcine embryos was determined by modified semi-quantitative single cell RT-PCR. Porcine embryos derived from *in vitro* maturation /*in vitro* fertilization were cultured in 5% CO_2 and 20% O_2 at 37°C in NCSU23 medium. Melatonin was added to medium at concentration of 1nM, 5 nM, and 10 nM. When treated with 1nM (39.0%) of melatonin, the developmental rate of embryos beyond the morula stage were higher than that of control group (31.0%) ($p<0.05$). Number of inner cell mass and tropectoderm cell in control (23.0±0.5 and 17.3±0.8), 1 nM (23.6±0.6 and 19.0±0.5), and 5 nM (23.3±1.1 and 16.3±0.8) treated with melatonin were higher than in 10 nM (20.0±0.5 and 13.3±0.8) treated with melatonin ($p<0.05$). To develop an mRNA phenotypic map for the expression of *catalase, bax* and *caspase-3*, single cell RT-PCR analysis were carried out in porcine IVM/IVF embryo. *Catalase* was detected in 0, 1 and 5 nM supplemented with melatonin, but *bax* and *caspase-3* were detected in 10 nM treated with melatonin. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 1 : 17-21)***

Key Words : Porcine Embryo, RT-PCR, *Catalase*, *Bax*, *Caspase-3*, Melatonin

INTRODUCTION

Several investigators have demonstrated the ability of porcine oocytes matured and fertilized *in vitro* to develop normally (Niwa, 1993; Nagai, 1994; Grupen et al., 1995), and the birth of piglets from embryos produced *in vitro* has been reported (Yoshida et al., 1993). Despite these achievements, the *in vitro* development of *in vitro* matured and fertilized porcine oocytes to the blastocyst stage is poor.

Oxidative stress appears to be one of the causes of impaired *in vitro* embryo development. An increased production of peroxides was measured in mouse embryos cultured *in vitro* (Nasr-Esfahani and Johnson, 1990; Goto et al., 1993) suggesting that during embryo culture the equilibrium between reactive oxygen species (ROS) production and scavenging is disrupted. Higher ROS generation has been ascribed to environmental factors such as light exposure, high oxygen tension, presence of heavy metals in culture media (reviewed by Johnson and Nasr-Esfahani, 1994) as well as to disorders in embryo developmental metabolism (Rieger, 1992).

Supplementation of culture media with ROS scavengers and metal chelators, which are normally present in the genital tract, has been shown to promote bovine embryo development (Johnson and Nasr-Esfahani, 1994; Ranina et al., 2002). Little information is available concerning antioxidative defense in preimplantation embryos. Some studies suggest that cellular enzymatic systems against oxidative injury are not fully elucidated in *in vitro* produced porcine embryos (Park et al., 1996; Jang et al., 2004) and preimplantation mouse embryos (El-Hage and Singh, 1990). The antioxidant enzymes are a part of the cellular defense against oxidative stress. Oxygen species are regulated by *superoxide dismutase (SOD)*, an emzyme 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 antioxidant emzymes play a role in protecting cells from oxidative stress induced cell death (Vega et al., 1995).

Melatonin or N-acetyl-5-methoxytryptamine is mainly synthesized in the pineal gland of all mammalian species (Klein et al., 1981). Recently it has been reported that melatonin acts as a free radical scavenger and antioxidant in mammalian cells (Lezou et al., 1996; Skaper et al., 1998; Borlongan et al., 2000). However, whether or not melatonin exerts its effects on porcine embryo development has not been determined.

The aim of the present study was to examine the effects of melatonin on the development and on antioxidant enzyme genes expression *in vitro* porcine embryos.

MATERIALS AND METHODS

Culture media

Cumulus-oocyte complexes (COCs) were washed in IVM-wash medium consisting of NCSU23 (Petters et al.,

* Corresponding Author: H. K. Lee. Tel: +82-31-670-5330, Fax: +82-31-673-2712, E-mail: lhkyu@hknu.ac.kr

[2] College of Animal Resources Science, Kangwon National University, Chuncheon 200-701, Korea.

[3] School of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Korea.

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Table 1. Oligonucleotide nested primer pairs for catalase, bax and *caspase-3*

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	
Bax	Forward	GGGTACGATAACCGGGAGAT	62	507	NM004324
	Outer reverse	GAGCCCATCTTCCAGAT			
	inner reverse	CTGTGCTGCCCAGAGGTT		250	
Caspase-3	Forward	GAAGCAAATCAATGGACTCTGGA	63	509	AB029345
	Outer reverse	GTCTGCCTCAACTGGTATTTTCTG			
	inner reverse	GGCAGGCCTGAATTATGAAAAGTT		362	

1993) supplemented with 10% porcine follicular fluid (pFF), 0.57 mM cysteine, 25 μM β-mercaptoethanol and 20 mM Hepes. The maturation medium for the first 22 h of *in vitro* maturation, designated IVM-I, was NCSU23 with 10% pFF, 0.57 mM cysteine, 25 μM β-mercaptoethanol and 20 mM Hepes, 10 IU/ml eCG (sigma) and 10 IU/ml hCG (sigma). For the second 20-22 h of maturation (IVM-II), the same medium was used as for IVM-II without eCG and hCG. The fertilization medium (IVF-medium) was modified Tris-buffered medium (mTBM, 113.1 mM NaCl, 3.0 mM KCl, 20.0 mM Tris, 11.0 mM D-glucose, 7.5 mM $CaCl_2$-$2H_2O$ and 5.0 mM Na-Pyruvate) containing 2 mM caffeine and 0.1% BSA (Sigma, USA). *In vitro* culture of the zygotes then took place in NCSU23 medium supplemented with 0.4% BSA.

Collection of cumulus oocyte complexes

Porcine ovaries obtained from a local slaughterhouse were transported at 39°C in 0.9% NaCl solution to the laboratory, where they were washed four times in sterile 0.9% NaCl solution. Ovarian follicles 2-5 mm in diameter were aspirated, and cumulus oocyte complexes (COC) containing a compact cumulus mass and even cytoplasmic pigmentation were washed three times in IVM-wash medium. Then 20-25 oocytes were cultured in 100 μl of IVM-Ⅰ medium which had peviously been covered with mineral oil and equilibrated for 2 h at 38.5°C and 5% CO_2 in air prior to use. After 22 h of maturation, the COCs were washed twice in IVM-I washing medium and placed in IVM-II medium, after which they were cultured for an additional 20-22 h.

***In vitro* fertilization and *in vitro* embryo culture**

After maturation, oocytes surrounded by expanded cumulus cell were washed twice, each in maturation medium and in IVF medium, and 20 oocytes were introduced into a 50 μl droplet of IVF medium, covered with mineral oil. Frozen semen was thawed in 37°C water for 30-40 sec. After thawing, spermatozoa were washed twice by centrifugation at 1,500 rpm for 10 min and resuspended with IVF medium to give a concentration of 2×10^6 spermatozoa/ml, and 50 μl of the sperm suspension was add to 50 μl of the fertilization drops containing oocytes.

Six hours after insemination, the spermatozoa bound to the oocytes were removed by washing four times and cultured in 100 μl of culture medium for 40-44 at 38.5°C, 5% CO_2 in air.

After 40-44 h of culture, 2-to 8-cell embryos were freed of cumulus cells by repeated pipetting and 2-to 8-cell embryos were chosen and cultured in each 100 μl drop of culture medium, covered with mineral oil in 5% CO_2 and 20% O_2 at 38.5°C.

Experimental design

In the experiment, embryos were cultured under an atmosphere of 5% CO_2 and 20% at 38.5°C with different concentration (0, 1, 5 and 10 nM) of melatonin (Sigma, USA) for development.

The culture medium was changed every 2 days and embryos were checked briefly at that time. Then embryos were examined, usually on day 6 of culture in specific treatments (7 days from fertilization), and blastocyts were examined for analysis by use of the single cell reverse transcription-polymerase chain reaction (RT-PCR).

Single-cell RT

Each treatment embryo (blastocyt stage) were placed in polymerase chain reaction (PCR) tubes in 2 μl of sterile diethylprocarbonate (DEPC)-treated water. Before use, the embryos underwent thermolysis for 1 min, 100°C in order to release nucleic acids (Kumazaki et al., 1994).

The reverse transcription reagents, RT buffer 10, 0.5 mM of each dNTP, 0.5 μg oligo (dT)15, 10 IU RNase-inhibitor (Gibco-BRL, France) and 500 IU Reverse transcriptase (Ambion, USA) were mixed on ice in total volume of 20 μl, and 18 μl of the RT mix was added to each blastomere in tubes. RT was carried out at 42°C for 60 min followed by heating to 70°C for 10 min to inactivate the reaction and storage at 4°C.

Table 2. The effect of melatonin on the development of porcine IVM/IVF embryos

Melatonin (nM)	No. of IVM/IVF embryos	No. of embryos developed to (%)			Morulae plus blastocysts (%)
		Pre-morulae	Morulae	Blastocysts	
0	45	31	7 (14.0)[a]	8 (17.0)[b]	14 (31.0)[b]
1	46	27	6 (14.0)[a]	12 (26.0)[a]	18 (39.0)[a]
5	45	30	6 (13.0)[a]	9 (20.0)[b]	15 (33.0)[b]
10	43	31	5 (11.0)[a]	7 (16.0)[b]	12 (27.0)[bc]

[a, b] Values with different superscripts within column are significantly differ. $p<0.05$.

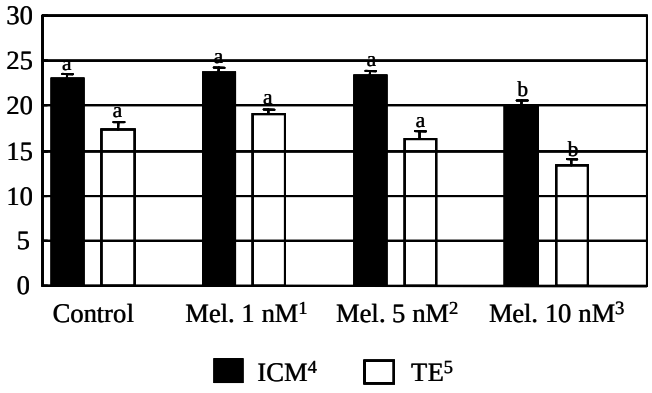

Figure 1. Number of inner cell mass and trophectoderm cell of porcine *IVM/IVF* embryos. [1] Mel. 1nM : melatonin 1nM, [2] Mel. 1 nM : melatonin 5 nM, [3] Mel. 1nM : melatonin 10 nM, [4] ICM : inner cell mass, [5] TE : trophectoderm cell. [a, b] Values with different superscripts within ICM and TE are significantly differ, $p<0.05$.

Polymerase chain reaction (PCR) and Gel electrophoresis

Each stage was analysed for presence of transcription encoding for antioxidant and apoptosis gene in each group.

PCR analyses 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, South Korea). After an initial denaturation stop of 5 min at 95°C, 25 amplification cycles were performed.

Each cycle included denaturation at 95°C, 45 sec, annealing at each primer temperature 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 sequences used in this study are indicated in Table 1 and nested primers for 35 cycles. For the nested reaction, 5°C of the first amplification product was added to freshly prepared PCR mix.

After amplification, 20% RT-PCR products were separated by 2% agarose gel electrophoresis, stained by ethidium bromide and visualized under UV.

Analysis of data

The SAS mixed linear model program was used to analyze the data. Percentage of developmental stage was based upon the number of 2- to 8- cell embryos cultured in each treatment. Treatment means were compared for differences through use of Duncan's Modified Multiple Range test.

RESULT

The developmental rates of porcine embryo

The developmental rates of porcine embryos generated in NCSU23 medium supplemented with melatonin are summarized in Table 2. Melatonin was added to medium at concentrations of 1 nM, 5 nM and 10 nM when treated with 1nM of melatonin at the developmental rate of embryos of the morula plus blatocsyts were higher than that of control group ($p<0.05$).

Number of inner cell mass and tropectoderm cell in control (23.0±0.5 and 17.3±0.8), 1 nM (23.6±0.6 and 19.0±0.5), and 5 nM (23.3±1.1 and 16.3±0.8) treated with melatonin were higher increased than in 10 nM (20.0±0.5 and 13.3±0.8) treated with melatonin ($p<0.05$)(Figure 1).

Expression patterns of *catalase*, *bax*, and *caspase-3*

To develop an mRNA phenotyic map for the expression of *catalase*, *bax* and *caspase*-3, single cell RT-PCR analysis was carried out to detect the antioxidant genes in porcine IVM/IVF embryo. In all instances, the assays were repeated at least three times with different embryo batches. *Catalase* was detected 361 bp in 0, 1 and 5 nM supplemented with melatonin, but *bax* and *caspase-3* were detected 250 bp and 362 bp in 10 nM treated with melatonin (Figure 2).

DISCUSSION

Preimplantation embryos of all species studied thus far display characteristic culture blocks associated with the timing of embryonic genome activation (Nasr-Esfhani and Johnson, 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 factors 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.,

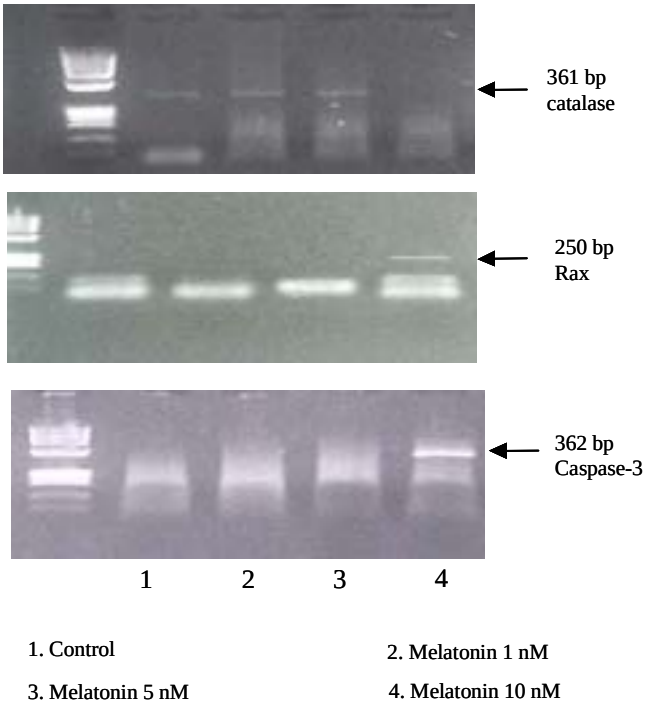

Figure 2. *mRNA* expression of antioxidant enzyme and apoptosis genes on procine *IVM/IVF* embryos; Single cell RT-PCR carried out to detect the *catalase, bax and caspase-3* on *in vitro* produced embryos collected at successive blastocyst. The experiments was repeated three times, a representative result is shown.

1999) and are probably involved in retarded embryonic development during *in vitro* culture condition (Nasr-Esfhani and Johnson, 1994). Moreover, ROS are implicated in the occurrence of apoptosis on embryo development (Parchment, 1991). Thus, recent attention has been focused on the protective biochemical function of antioxidants in biological systems, and on the mechanism of their action.

Many studies have demonstrated that the effects of melatonin may be due to its oxygen free radical scavenging property and activation of cellular antioxidant defense mechanisms (Lezoualch et al., 1996; Mayo et al., 1998; Skaper et al., 1998; Chen and Chuang, 1999; Borlongan et al., 2000). However, whether or not melatonin exerts its effects on embryo development in pigs has not been attempted. This study examined the effects of melatonin on the development and on expression of *catalase*, *bax* and *caspase-3* genes expression in *in vitro* porcine embryos. We found that melatonin improved the development rate of morula plus blastocysts when embryos were cultured in NCSU23 medium containing melatonin, and the cell numbers of blatocysts produced by IVM/IVF were significantly increased in 1 nM and 5 nM treated with melatonin.

Catalase gene is expressed in 1 nM and 5 nM treated with melatonin, whereas *bax* and *caspase-3* genes were expressed in 10 nM treated with melatonin. Amount of bax, *caspase-3* genes were higher in embryos of poor morphology at treated high concentration. These findings demonstrate that melatonin stimulates early embryo development after *in vitro* fertilization. Thus melatonin may be involved in metabolism at certain the formation of blastocysts and embryo development to the blastocysts in 1nM, 5 nM treated with melatonin groups were increased by catalase. It has been reported that melatonin is an effective ROS scavenger (Okatani et al., 1989; Poeggeler et al., 1993; Reiter et al., 1993,1995).

Thus, melatonin may support early embryo development through its ROS scavenging action. It is also possible that a melatonin receptor may be expressed in the early embryos and may mediate its effects on embryos.

In conclusion, the present study demonstrates that addition of melatonin in culture medium to early pre-implantation porcine embryos enhances embryonic development. Although our data from porcine embryo development can not be directly applied to human fertilization and embryo transfer *in vitro*, melatonin might improve the culture conditions for such programs.

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