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).

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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 enzyme that changes superoxide into H2O2 or by catalase and glutathione peroxidase (GPs), which decompose H2O2 into H2O. Because of their ability to degenerate ROS into nontoxic compounds, these antioxidant enzymes 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.,
supplemented with 10% porcine follicular fluid (pFF), 0.57 mM cysteine, 25 \( \mu \)M \( \beta \)-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 \( \mu \)M \( \beta \)-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\(_2\)O 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 \( \mu \)l of IVM-1 medium which had previously been covered with mineral oil and equilibrated for 2 h at 38.5°C and 5% CO\(_2\) in air. 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 \( \mu \)l 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 \( \mu \)l 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 \( \mu \)l 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 \( \mu \)l 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 blastocysts were examined for analysis by use of the single cell reverse transcription-polymerase chain reaction (RT-PCR).

Single-cell RT
Each treatment embryo (blastocyst stage) were placed in polymerase chain reaction (PCR) tubes in 2 \( \mu \)l 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 \( \mu \)g 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 \( \mu \)l and 18 \( \mu \)l of the RT mix was added to each blastomere in tubes. RT was carried out at 42°C for 60 min followed by heating by 70°C for 10 min to inactivate the reaction and storage at 4°C.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Type of primer</th>
<th>Primer sequence (5’ 3’)</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
<th>Genbank accession number</th>
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<td>D89812</td>
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<tr>
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<tr>
<td>Bax</td>
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<tr>
<td>Caspase-3</td>
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<td>GCCAGGCCTGAATTATGAAAGTT</td>
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<td>362</td>
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</table>
EFFECTS OF MELATONIN ON IVM/IVF PORCINE EMBRYOS

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 blastocysts were higher than that of control group (p<0.05).

Number of inner cell mass and trophectoderm 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 phenotypic 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 (p<0.05)(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.,

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

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

a, b Values with different superscripts within column are significantly differ. p<0.05.
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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 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 blastocysts 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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REFERENCES


Jang, H. Y., H. S. Kong, S. S. Lee, K. D. Choi, G. J. Jeon, B. K.


