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Melatonin Attenuates Nitric Oxide Induced Oxidative Stress on Viability and Gene Expression in Bovine Oviduct Epithelial Cells, and Subsequently Increases Development of Bovine IVM/IVF Embryos

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ABSTRACT : The objective of the present study was to elucidate the fundamental mechanism of bovine oviduct epithelial cell (BOEC) co-culture on developmental capacity of bovine IVM/IVF embryos and to determine whether or not melatonin acts as an antioxidant in BOEC culture and subsequent embryo development. These studies examined the effects of melatonin against NO-induced oxidative stress on cell viability, lipid peroxidation (LPO) and the expression of antioxidant genes (*CuZnSOD*, *MnSOD* and *Catalase*) or apoptosis genes (*Bcl-2, Caspase-3* and *Bax*) during BOECs culture. We also evaluated the developmental rates of bovine IVM/IVF embryos with BOEC co-culture, which were pre-treated with melatonin (1,000 μM) in the presence or absence of sodium nitroprusside (SNP, 1,000 μM) for 24 h. Cell viability in BOECs treated with SNP (50-2,000 μM) decreased while melatonin addition (1-1,000 μM) increased viability in a dose-dependent manner. Cell viability in melatonin plus SNP (1,000 μM) gradually recovered according to increasing melatonin addition (1-1,000 μM). The LPO products were measured by thiobarbituric acid (TBA) reaction for malondialdehyde (MDA). Addition of melatonin in BOEC culture indicated a dose-dependent decrease of MDA, and in the SNP group among BOECs treated with SNP or melatonin plus SNP groups MDA was significantly increased compared with SNP plus melatonin groups ($p<0.05$). In expression of apoptosis or antioxidant genes detected by RT-PCR, *Bcl-2* and antioxidant genes were detected in melatonin or melatonin plus SNP groups, while *Caspase-3* and *Bax* genes were only found in the SNP group. When bovine IVM/IVF embryos were cultured for 6-7 days under the BOEC co-culture system pre-treated with melatonin in the presence or absence of SNP, the highest developmental ability to blastocysts was obtained in the 1,000 μM melatonin group. These results suggest that melatonin has an anti-oxidative effect against NO-induced oxidative stress on cell viability of BOECs and on the developmental competence of bovine IVM/IVF embryo co-culture with BOEC. (**Key Words :** Melatonin, Bovine Oviduct Epithelial Cell, Bovine IVM/IVF Embryos, Nitric Oxide, Antioxidant)

INTRODUCTION

Co-culture with somatic cells have a positive effect on the development rates of *in vitro* produced (IVP) embryos and particularly BOECs are beneficial for achieving increased developmental rates beyond the morula stage in bovine embryo culture (Rief et al., 2002). Beneficial effects of oviduct epithelial cells on co-cultured embryos have been used to overcome the developmental block at the 8-16 cell stage in ruminants, and to improve pregnancy rates and outcomes with embryos generated *in vitro* (Galli et al., 2003). The exact mechanism of BOEC co-culture is not yet clearly understood. BOECs used for embryo culture may produce unknown substances, making it difficult to determine the exact requirement for embryo development and preventing a clear understanding of embryo metabolism. However, the bovine embryo co-culture system using somatic cells may include the secretion of embryotrophic factors and inactivation of embryotoxic agents such as free radicals, heavy metals and other substances (Flood and Shirtey, 1991; Gardner et al., 1994).

The major embryotoxic factors in an *in vitro* culture system may be the reactive oxygen species (ROS) and deleterious heavy metal ions from the culture medium. Among them, ROS is one of the major factors damaging and/or retarding embryo development *in vitro* (Ashok et al., 2003; de Lamirane and O'Flaherty, 2008). Therefore, recent

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attention has focused on ROS produced by *in vitro* culture media and on high level oxygen tension as a major problem for *in vitro* embryonic developmental arrest and cell death (Tanaka et al., 2002). The critical concentration of ROS improved oocyte maturation and embryo development, and sperm function such as capacitation, hyperactivation and acrosome reaction (Viana et al., 2007; de Lamirande and O'Flaherty, 2008), but the high level of ROS in the *in vitro* culture system might induce apoptosis, also described as programmed cell death. ROS are regulated by enzymatic or non-enzymatic antioxidants and the antioxidant enzymes play an important role in protective action from oxidative stress-induced cell damage and/or death, but the exact antioxidant mechanisms in *in vitro* embryo culture are still unclear.

Melatonin (N-acetyl-5-methoxytryptamine), a derivative of tryptophan, is mainly synthesized and secreted in the pineal gland, and also found in many other tissues including the ovary, testes, bone marrow, retina and lens in mammalian species (Reiter et al., 2000; Tranguch et al., 2003; Siu et al., 2006). Melatonin has several important physiological functions such as the control of circadian rhythms, sleep induction, regulation of seasonal reproduction, immune enhancement and antioxidant action. One of the most basic functions of melatonin is speculated to be its antioxidant function, which protects cells or organisms from oxidative damage. Melatonin acts as a potent free radical scavenger, including peroxide nitric oxide and hydroxyl radicals which can initiate lipid peroxidation (Reiter et al., 2000; Allegra et al., 2003; Juknat et al., 2005).

This study was designed to elucidate the evolution of the regulatory mechanism of melatonin against nitric oxide-mediated oxidative stress during BOEC culture and subsequently the developmental competence of bovine embryos in a BOEC co-culture system pre-treated with melatonin in the presence or absence of sodium nitroprusside (SNP).

MATERIAL AND METHODS

Manipulation of BOECs

Bovine oviducts were carefully trimmed on ice to remove the excess fat and tissue debris and washed with Dulbecco′s phosphate buffered saline (D-PBS; Gibco, USA) containing 1% antibiotics and antimyotic solution (Gibco), and were dispersed two times by forcing them through an 18-gauge needle attached to a 10 ml syringe. The cell suspension was transferred into 50 ml of low-glucose Dulbecco's modified eagle medium (DMEM; Gibco) with 10% fetal bovine serum (FBS, Gibco) and washed twice by centrifugation at 200×g at 4°C. According to the experimental purpose, BOEC pellets were suspended and cultured in DMEM containing 10% FBS for 2-3 days on 4-well plates (a density of 1×10^5 cells per well), 6-well plates (a density of 2×10^5cells per well) and 96-well plates (a density of 1×10^4 cells per well) in DMEM containing 10% FBS at 37°C in 5% CO_2 in air. Each monolayer was treated with a different concentration of SNP (0, 50, 100, 1,000 and 2,000 μM), melatonin (0, 1, 10, 100 and 1,000 μM) alone or melatonin (1, 10, 100 and 1,000 μM) in the presence or absence of SNP (1,000 μM) and cultured for 24 h. The BOECs, or monolayered cells, in 4-well, 6-well and 96-well plates were used for cell viability and LPO, antioxidant or apoptosis gene detection and embryo culture, respectively. Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich and were analytical grade.

Evaluation of BOECs

Cell viability : MTT [3-4, 5-dimethylthiazol-2-yl-2, 5-diphenyltetrazolium-bromide] assay, an index of cell viability and cell growth, is based on the ability of viable cells to reduce MTT from a yellow water-soluble dye to a dark blue insoluble formazan product. The BOECs treated with SNP (50-2,000 μM), melatonin (1-1,000 μM) or melatonin (1-1,000 μM) in the presence or absence of SNP (1,000 μM) were seeded in multi-well plates at a density of 1×10^4 per well, mixed with 20 μM MTT dye, and then incubated for 4 h in the dark. The optical density (OD) values were measured at 570 nm by microtiter plate reader (Bio-Tek, USA). For determination of cell viability, relative cell viability (%) was calculated as OD of treated sample/control OD×100.

Quantification of lipid peroxidation (LPO) : LPO was measured using the thiobarbituric acid (TBA) reaction for malondialdehyde (MDA). Each experimental group was treated with 0.01 M sodium phosphate buffer (pH 7.4) containing 1.15% (w:v) KCl and adjusted in Ca^{2+} and Mg^{2+} free D-PBS (Gibco) to give a concentration of 3×10^5 cell/ml. For the stimulation of lipid peroxide to MDA, LPO was promoted using a combination of ferrous sulfate and sodium ascobate. Cell suspension (1 ml) was mixed with 10 ml of 1 mM ferrous sulfate and 10 ml of 5 mM sodium ascorbate, and then incubated for 1 h at 37°C. The reaction mixture was added to 250 μl of 40% trichloroacetic acid, held for 10 min at 0°C, and centrifuged (2,500×g) for 10 min. Supernatant (1 ml) mixed with TBA was boiled in hot water for 10 min. The amount of MDA produced was quantified against a standard curve at 532 nm wavelength in a spectrophotometer.

Gene detection of BOECs :

i) RNA isolation of BOECs

Total RNA was extracted from BOECs treated with SNP

and melatonin alone or melatonin in the presence or absence of SNP by the guanidium isothiocyanate method (Chomczynski and Sacchi, 1987). Oviductal epithelial cells were placed into extraction solution (0.025 M sodium citrate, 0.5% sarcosyl, 4 M guanidiumthiocyanate, pH 7.0), vortexed with phenol, chloroform:isoamyl alcohol (24:1) and 2 M NaOAC, centrifuged at 20,000×g at 4°C for 20 min, and the supernatant transferred to a new tube. After addition of isopropanol and gentle shaking, it was then precipitated with isopropanol at -20°C overnight. The samples were then centrifuged, washed with 70% ethanol, re-centrifuged and air-dried before being dissolved in diethyl procarbonate (DEPC)-treated distilled water. The yield of extracted total RNA for each sample was determined by UV spectrophotometer at 260 nm.

ii) RT-PCR analysis for gene detection

Each mRNA collected in the total RNA sample was analyzed to detect the genes by RT-PCR. Reverse-transcriptase generation of cDNA was performed on 2 μg of total RNA, a final volume of 2 μl, the reverse transcription reagents (RT mixture), RT buffer, 0.5 mM of each dNTP, 0.5 μg oligo (dT), 10 IU RNase-inhibitor (Gibco) and 500 IU reverse transcriptase (Ambion, USA) carried out at 42°C for 60 min followed by denaturation for 10 min at 98°C and the remaining RT products were stored at 4°C. Subsequent PCR analysis was performed on 2 μl of the cDNA in a final volume of 20 μl, the PCR mixtures consisted of PCR buffer, 0.2 mM dNTP, 10 μM primer and 2 IU Taq polymerase. Each primer sequence is shown in Table 1. PCR was then carried out at 94°C for 5 min, followed by 35 amplification cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 58-60°C for 40 sec, and extension at 72°C for 1 min, followed by an additional extension step at the end of the procedure at 72°C for 10 min. In each sample, PCR amplification products were analyzed on 2.0% agarose gel stained with 0.5 μg/ml ethidium bromide, visualized under UV light and photographed. Standard DNA markers (1 kb DNA ladder) were also used to determine the size of amplified products.

Culture of bovine IVM/IVF embryos

The cumulus-oocyte complexes aspirated from the ovaries obtained from the slaughterhouse were matured in TCM 199 (Gibco) supplemented with 10% FBS, 1 μg/ml estradiol-17β, 5 μg/ml LH, 0.5 μg/ml FSH for 20-22 h at 38.5°C in humidified 5% CO_2 in air. Following the maturation, 10-15 matured oocytes were transferred in 50 μl of fertilization drops (BO medium). Frozen/thawed Hanwoo semen was washed in BO medium containing 10 mM caffeine and 50 μl sperm suspension was introduced to each fertilization drop. Presumptive zygotes produced at 6-8 h after insemination were cultured in CR_1aa medium for 34-36 h and then 2-and 8-cell embryos were randomly allocated into each experimental groups. After 6-7 days of culture, morphological normality and developmental stages of IVM/IVF embryos were evaluated at ×100 magnification under a phase contrast microscope (Nikon, Japan). The total cell numbers of blastocysts were evaluated by Hoechst 33342 staining (Yang et al., 1993).

Statistical analysis

Statistical analysis of replicated experimental results were used for treatment comparisons and were carried out by one-way analysis of variance (ANOVA) using the SAS program. Mean values in treatments were compared for difference using Duncan's modified multiple range tests. Repeated measures ANOVA was used to test the main effects of melatonin and SNP on cell viability and LPO, and subsequent development rates of bovine IVM/IVF embryo with BOEC co-culture. A p-value below 0.05 was considered significant.

Table 1. Oligonucleotide primer sequences for antioxidant and apoptosis genes

mRNA	Type of primer	Primer sequence (5' 3')	Annealing temperature (°C)	Product size (bp)	Genebank accession number
Catalase	Forward	GAAAGGCGAAGGTGTTTGAGCA	61	814	D89812
	Reverse	AGGCGGTGGCGGTGSGTGTC			
CuZnSOD	Forward	GCAGGGCACCATCTACTTC	61	382	AF396674
	Reverse	ACTTCCAGCATTTCCCGTCTTT			
MnSOD	Forward	CGCGGCCTACGTGAACAACCT	61	379	X64057
	Reverse	CCCCAGCAGCGGAACCAGAC			
Bax	Forward	GCCCCTGTCGTCGTCCTTTGTCC	63	678	AF098067
	Reverse	TGGCGAGGAGCTGGTGCTGG			
Caspase-3	Forward	GAAGCAAATCAATGGACTCTGGA	63	509	AB029345
	Reverse	GTCTGCCTCAACTGGTATTTTCTG			
Bcl-2	Forward	CGACTTTGCAGAGATGTCCA	63	274	L14680
	Reverse	TAGTTCCACAAAGGCATCCC			
GADPH	Forward	CAGCAATGCATCCTGCAC	60	429	X02231
	Reverse	GAGTTGCTGTTGAAGTCACAGG			

RESULTS

The antioxidative effects of melatonin against NO-induced oxidative stress on cell viability and LPO during *in vitro* culture of BOECs are shown in Figure 1 and 2.

To determine whether melatonin or SNP affects cell viability during *in vitro* culture of BOECs, we investigated the antioxidative effects on cell viability of BOECs following cellular exposure to SNP and SNP plus melatonin. The cell viability of BOECs treated with SNP decreased in a dose-dependent manner (50 μM, 81.7%±3.6; 100 μM, 86.0%±9.1; 1,000 μM, 37.2%±3.9 and 2,000 μM, 21.3%± 1.3). The cell viability of BOEC in 50 μM and 100 μM SNP groups increased significantly compared with 1,000 μM and 2,000 μM SNP groups ($p<0.05$). When BOEC were cultured with different concentrations of melatonin (1-1,000 μM), cell viability slightly increased from 1 μM to 1,000 μM melatonin groups (100.6%±4.2 in 1 μM, 104.5%±2.1 in 10 μM, 114.3%±0.9 in 100 μM and 123.5±4.7 in 1,000 μM).

The viability in the SNP 1,000 μM (49.8%±5.6) group was not greatly different to SNP 1,000 μM plus melatonin 1 μM (52.5%±3.6) and SNP 1,000 μM plus melatonin 10 μM (58.4%±5.4), but lower than SNP 1,000 μM plus melatonin 100 μM (72.7%±9.0) and SNP 1,000 μM plus melatonin 1,000 μM (92.6%±11).

The LPO measured by MDA was similar in the control and 50-100 μM melatonin groups, but in the 1,000 μM melatonin group was significantly lower than in the control and 1-10 μM melatonin groups. The MDA levels of BOECs in the SNP group (145.9±0.7 nmol/L×10^6) were significantly increased ($p<0.05$) when compared to SNP plus melatonin groups (134.0±1.5 nmol/L×10^6 in SNP 1,000 μM plus melatonin 1 μM; 116.3±2.1 nmol/L×10^6 in SNP 1,000 μM plus melatonin 10 μM; 104.2±1.5 nmol/L×10^6 in SNP 1,000 μM plus melatonin 100 μM and 93.3±1.7 nmol /L×10^6 in SNP 1,000 μM plus melatonin 1,000 μM).

The cell viability, LPO and expression of apoptosis and antioxidant genes in BOEC pre-treated with melatonin (1,000 μM) in the presence or absence of SNP (1,000 μM) are indicated in Figure 3.

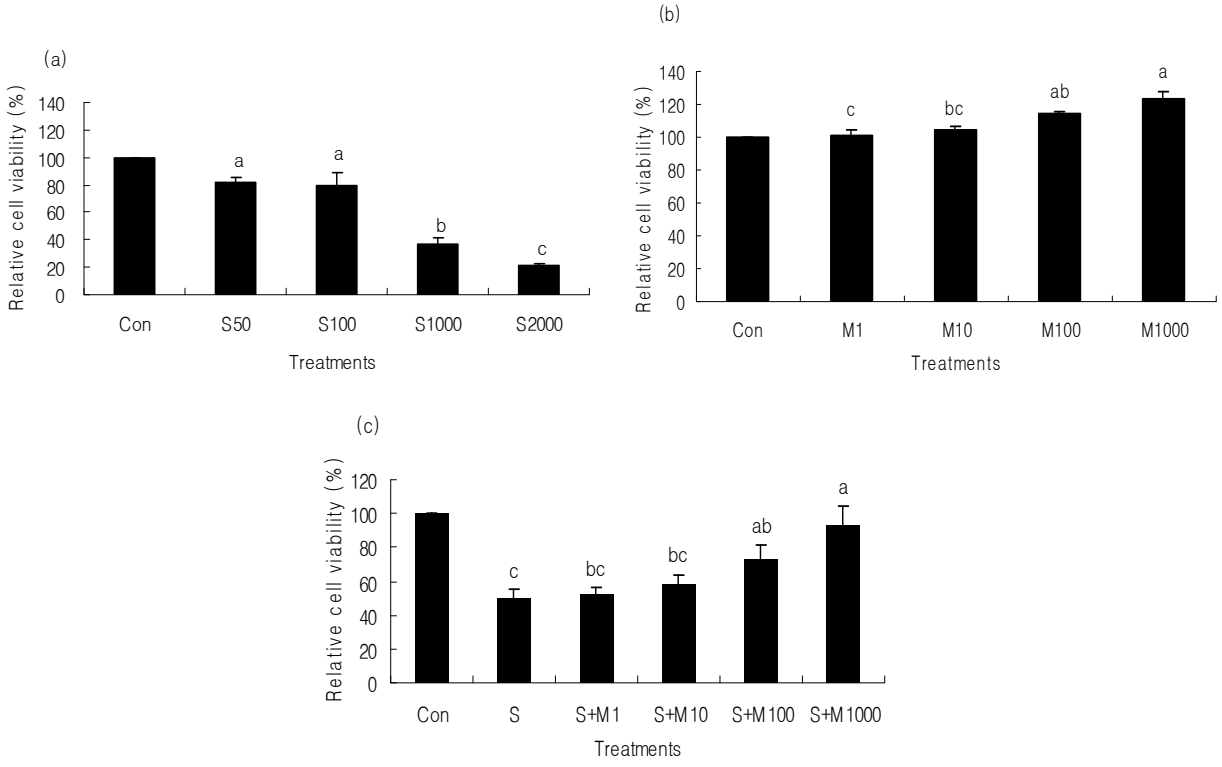

Figure 1. Effects of melatonin against SNP on cell viability in BOEC culture. BOECs were cultured for 2 days in DMEM plus 10% FBS following the adjustment of cells to give 1×104 cell/ml and each monolayer was treated with different concentrations of (a) SNP (50-2,000 μM), (b) melatonin (1-1,000 μM) and (c) SNP (1,000 μM) plus melatonin (1-1,000 μM) for 24 hrs. After 24 hrs, the cell viability was measured by MTT assay. Cell viability is expressed as the ratio (%) of optical density values of treated cells to control cells. Con; control, S; SNP, S 50; SNP 50 μM , S100; SNP 100 μM , S 1000; SNP 1,000 μM , S 2,000; SNP 2,000 μM, M1; melatonin 1 μM, M10; melatonin 10 μM, M100; melatonin 100 μM, M1000; melatonin 1,000 μM , S+M1; SNP 1,000 μM+melatonin 1 μM, S+M10; SNP 1,000 μM+melatonin 10 μM, S+M100; SNP 1,000 μM6 melatonin 100 μM, S+M1,000; SNP 1,000 μM+melatonin 1,000 μM. [a,b,c] Mean values with different superscripts are significantly different, $p<0.05$. Values presented are the mean±SEM of three experiments.

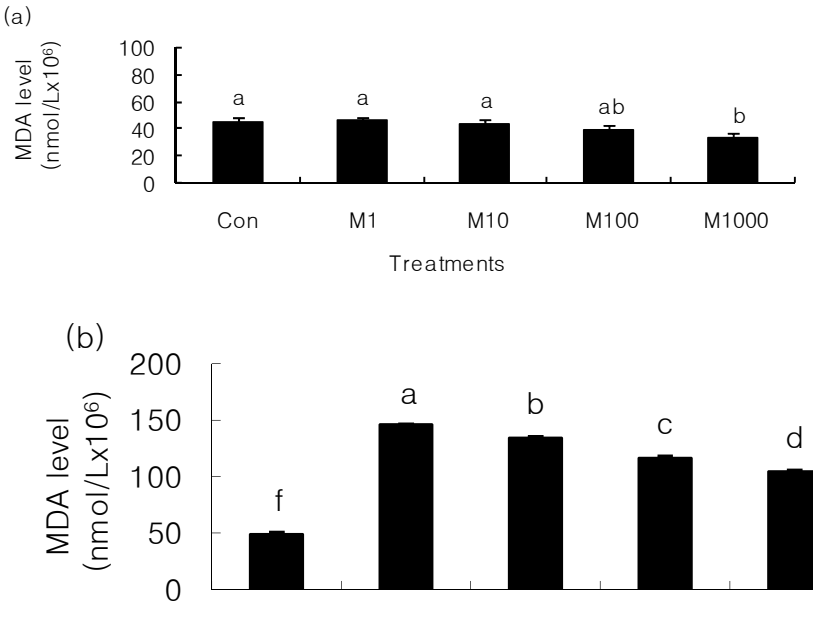

Figure 2. Effects of melatonin against SNP on lipid peroxidation in BOEC culture. BOECs were cultured for 2 days in DMEM plus 10% FBS following the adjustment of cells to give 3×10^5 cell/ml and each monolayer was treated with different concentrations of (a) melatonin (1-1,000 μM) and (b) SNP (1,000 μM) plus melatonin (1-1,000 μM) for 24 h. After 24 h, LPO was analyzed by TBA reaction for MDA. [a,b,c, d, e] Mean values with different superscripts are significantly different, $p<0.05$. Values presented are the mean±SEM of three experiments.

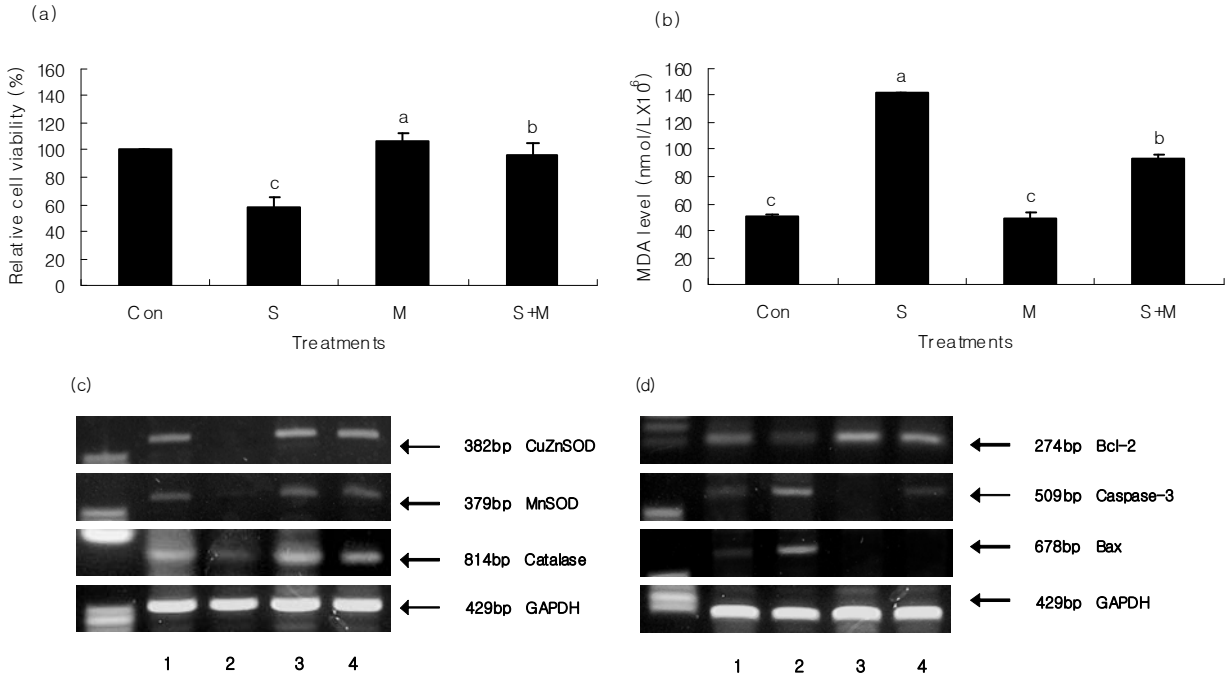

Figure 3. Effects of melatonin against SNP-induced oxidative stress on cell viability, LPO and gene expression during BOECs culture. BOECs were cultured for 2 days in DMEM plus 10% FBS and each monolayer was treated with SNP (1,000 μM), melatonin (1,000 μM) alone and melatonin (1,000 μM) in the presence or absence of SNP (1,000 μM) for 24 h; (a) Cell viability by MTT assay, (b) LPO by TBA reaction for MDA, (c) transcripts for apoptosis genes and (d) transcripts for antioxidant genes by RT-PCR. 1 = control, 2 = SNP 1,000 μM, 3 = melatonin 1,000 μM, 4 = SNP 1,000 μM+melatonin 1,000 μM. [a,b,c] Mean values with different superscripts are significantly different, $p<0.05$. Values presented are the mean±SEM of three experiments.

Table 2. Effects of melatonin against SNP on developmental ability of bovine IVM/IVF embryos co-cultured with BOEC

BOEC treated with		No. of IVM/IVF embryos	No. of embryos developed to (%)			Cell no. of blastocysts
SNP (μM)	Melatonin (μM)		Pre-morula	Morulae	Blastocysts	
0	-	63	26 (41.3^{b}±3.3)	19 (30.2^{a}±8.0)	18 (28.5^{bc}±5.3)	106.3±2.9
0	1,000	65	15 (21.3^{c}±0.7)	21 (30.7^{a}±4.2)	29 (48.0^{a}±3.4)	108.3±1.5
1,000	-	62	36 (57.9^{a}±4.8)	15 (24.2^{a}±2.8)	11 (17.9^{c}±0.6)	100.3±1.5
1,000	1,000	66	22 (33.2^{bc}±3.4)	20 (30.4^{a}±3.1)	24 (36.4^{ab}±1.0)	107.7±1.5

^{a,b} Different superscripts within columns are significantly different, $p<0.05$. Data are expressed as mean±SEM of three experiments.

The cell viability in the SNP group was significantly lower than in melatonin or melatonin plus SNP groups ($p<0.05$), and the LPO showed adverse effects on cell viability. Transcriptions for *Caspase-3* and *Bax* were observed in control and SNP 1,000 μM groups, while the *Bcl-2* gene was detected in control, melatonin (1,000 μM) and SNP (1,000 μM) plus melatonin (1,000 μM) groups. Expressions of *CuZnSOD, MnSOD* and *Catalase* gene were detected in control, melatonin and SNP plus melatonin groups, but these genes were not expressed in the SNP 1,000 μM group. Transcription for GAPDH as an interval control was detected in all of these samples.

The results of morula development were not significantly different in all treatment groups ($p>0.05$). Developmental rate to blastocyst stage in the BOEC co-culture system, which was pre-treated with SNP, melatonin alone or melatonin plus SNP, was 28.5% in control, 48.0% in 1,000 μM melatonin, 17.9% in 1,000 μM SNP and 36.4% in SNP plus melatonin groups, respectively. There were no significant differences in blastocyst cell numbers.

DISCUSSION

In the present study, the effects of BOEC co-culture on bovine IVP embryo development were conducted to elucidate its efficiency as a suitable model for studying the embryo culture system *in vitro.*

The developmental rates of IVP embryos are related to various factors, including the oocyte itself, protein source, somatic cells, culture media, oxygen tension and energy substrates, which might affect pre-implantation embryo development *in vitro* (Bavister, 1995). Among the factors detrimental to embryo development during *in vitro* culture, ROS have been implicated as a major cause of embryonic arrest, delayed and/or cell death (Ashok et al., 2003). ROS are highly reactive with complex cellular molecules such as proteins, lipids and DNA, and cause serious dysfunction such as enzyme inactivation, mitochondrial abnormality or DNA fragmentation (Guerin et al., 2001). The superoxide anion, hydrogen peroxide and nitric oxide that are derived from the metabolism of molecular oxygen are considered the major ROS that participate in redox reactions in diverse biological processes (Hancok et al., 2001). ROS induce membrane lipid peroxidation (LPO) in cells which leads to loss of membrane integrity causing increased cell permeability to electrolytes, and the toxicity of generated fatty acid peroxides is an important cause of decreased cell function (Griveau et al., 1995; Halliwell, 2000).

NO, a free radical, is considered to be unique among the regulators of apoptosis in that it can function as either a pro- or an anti-apoptotic agent. The critical concentration of NO in pre-implantation embryo culture enhances the developmental rate which regulates mitotic division in these embryos. It also stimulates sperm capacitation and acrosome reaction at a physiological concentration in mammals. However, excess NO leads to developmental arrest and/or apoptosis of the embryos (Tranguch et al., 2003; de Lamirande and O'Flaherty, 2008).

Melatonin is a neuroendocrine hormone secreted nightly by the pineal gland which regulates biological rhythms and acts as a circadian rhythm transducer (Siu et al., 2006; Tamura et al., 2008). Many studies have demonstrated that melatonin has a non-enzymatic antioxidant property due to its free oxygen radical scavenging activity, activation of cellular antioxidant defense mechanisms, and potent anti-apoptotic effect on different cell types (Reiter et al., 2000; Allegra et al., 2003; Juknat et al., 2005).

Therefore, this study was designed to elucidate the anti-oxidative effects of melatonin against NO-induced oxidative stress on cell viability and antioxidant or apoptosis gene expression in BOECs and subsequently the developmental capacity of bovine IVM/IVF under the BOEC co-culture system.

This present study indicated that when BOEC were cultured in DMEM supplemented with different concentrations of SNP (50-2,000 μM), SNP groups showed decreased cell viability in a dose-dependent manner. Also, with supplementation of different concentration of melatonin (1-1,000 μM), relative cell viability in the 1,000 μM melatonin group was significantly increased when compared with other groups ($p<0.05$). This result indicated that high concentration of NO (>1,000 μM) has a negative effect, while high concentration of melatonin (1,000 μM) has a positive effect on cell viability in BOEC culture. The

viability of BOECs treated with SNP (1,000 μM) plus melatonin (>1 μM) was significantly increased when compared to the SNP (1,000 μM) group ($p<0.05$), and LPO of BOECs treated with SNP (1,000 μM) plus melatonin (>1 μM) significantly decreased as compared to the SNP (1,000 μM) group ($p<0.05$). Results of the present study were in accordance with many researchers (Papis et al., 2007; Rodriguez-Osorio et al., 2007; Sonmez et al., 2007) who reported that melatonin has an anti-oxidative feature due to its free oxygen radical scavenging property and activation of cellular antioxidant defense mechanisms. This study is also in agreement with Kim and Kwon (1999) and Liu et al. (1998) who reported that antioxidant supplement inhibited the LPO of cells *in vitro* and improved the development of bovine embryos, possibly by releasing embryotrophic factors and removing or alleviating embryotoxic substances during culture.

Apoptosis is considered a self-programmed cell death controlled by a molecular genetic mechanism that characteristically affects single cells and can be induced by a variety of external and internal stimuli (Hardy, 1999). Apoptosis also plays an essential role in the processes of gamete maturation and embryogenesis, contributing to the appropriate formation of various organs and structures (Tranguch et al., 2003). Apoptosis needs *caspase* and *caspase* mRNAs, which were detected in cells and preimplantation embryos. The *Bcl-2* gene family is located in the intracellular membranes and is mainly involved in either triggering or regulating apoptosis through cell specific enzymes. *Bcl-2* and *Bax* are the prototype oncoproteins whose functions include the regulation of apoptosis. *Bcl-2* prevents apoptosis by maintaining cell survival through the release of *cytochrome c* from mitochondria rather than by altering proliferation. The *Bax* gene is the first pro-apoptotic member that, when over-expressed, initiates the cellular apoptotic pathway (Haunstetter and Izumo, 1998; Exley et al., 1999; Yang and Rajamahendran, 2002). We detected different patterns of mRNA expression for antioxidant and apoptosis genes on treatment of antioxidant (melatonin) and NO donor (SNP) in BOECs culture. Expressions of *CuZnSOD*, *MnSOD* and *Catalase* genes were detected in control, melatonin (1,000 μM) and SNP (1,000 μM) plus melatonin (1,000 μM) groups, while these genes were not expressed in the SNP 1,000 μM group. On the other hand, apoptosis genes such as *Caspase-3* and *Bax* were observed in control and SNP alone groups, whereas expression of the *Bcl-2* gene was detected in the control, melatonin and SNP plus melatonin groups. This study indicated that BOECs treated with SNP had increased apoptotic genes, while BOECs treated with melatonin alone or melatonin plus SNP showed anti-apoptotic genes.

This study evaluated the effects of melatonin (1,000 μM) against SNP (1,000 μM) on the development of bovine IVM/IVF embryos co-cultured with BOECs pre-treated with melatonin or SNP alone and a combination of melatonin and SNP. Development rates of blastocysts with BOEC co-culture pre-treated with SNP (1,000 μM) plus melatonin (1,000 μM) were significantly higher when compared to SNP and control groups ($p<0.05$), but the number of blastocysts was not significantly different in all groups. Bovine embryos co-cultured with BOECs treated with antioxidant can enhance the *in vitro* developmental competence (Lawlor and O'Brien, 1995; Jeon et al., 2008). Melatonin supplementation at 10^{-6} to 10^{-8} concentration increased the *in vitro* development of mouse embryos (Ishizuka et al., 2000) and melatonin at a concentration of 10^{-6} to 10^{-9} had positive effects on cleavage rates and developmental rate in porcine pre-implantation embryos (Rodriguez-Osorio et al., 2007). This result indicated that increased blastocyst development may be due to embryotropic factors provided by the epithelial cell and the scavenging action of nitric oxide radicals by melatonin supplementation. However, the specific mechanism is still unclear and needs to be elucidated by further studies.

In conclusion, our results demonstrate that melatonin supplementation improved the cell viability and LPO during BOEC culture and embryo development *in vitro*, suggesting that melatonin has an anti-oxidative feature and its effects may be associated with the expression of antioxidant genes and the suppression of apoptosis genes.

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