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Effects of Cell Status of Bovine Oviduct Epithelial Cell (BOEC) on the Development of Bovine *IVM/IVF* Embryos and Gene Expression in the BOEC Used or Not Used for the Embryo Culture

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ABSTRACT : The objective of this study was to investigate the effects of cell status of BOEC on development of bovine *IVM/IVF* embryos and gene expression in BOEC before or after culturing of embryos. The developmental rates beyond morula stage in the BOEC co-culture group was significantly higher than in the control group ($p<0.05$). In particular, blastocyst production in the BOEC co-culture group (28.3%) was dramatically increased compared with the control group (7.2%). In the *in vitro* development of bovine *IVM/IVF* embryos according to cell status, the developmental rates beyond morula stage in the primary culture cell (PCC) co-culture group were the highest of all experimental groups. Expression of genes related to growth (*TGF-β, EGF* and *IGFBP*), apoptosis (*Bax*, *Caspase-3* and *p53*) and antioxidation (*CuZnSOD*, *MnSOD*, *Catalase* and *GPx*) in different status cells of BOEC for embryo culture was detected by RT-PCR. While *EGF* gene was detected in isolated fresh cells (IFC) and PCC, *TGF-β* and *IGFBP* were found in IFC or PCC after use in the embryo culture, respectively. *Caspase-3* and *Bax* genes were detected in all experimental groups regardless of whether the BOEC was used or not used in the embryo culture. However, *p53* gene was found in IFC of both conditions for embryo culture and in frozen/thawed culture cells (FPCC) after use in the embryo culture. Although antioxidant genes examined were detected in all experimental groups before using for the embryo culture, these genes were not detected after use. This study indicated that the BOEC co-culture system used for *in vitro* culture of bovine *IVF* embryos can increase the developmental rates, and cell generations and status of BOEC might affect the *in vitro* development of bovine embryos. The BOEC monolayer used in the embryo culture did not express the growth factors (*TGF-β* and *EGF)* and enzymatic antioxidant genes, thereby improving embryo development *in vitro*. (**Key Words :** Bovine Oviduct Epithelial Cell, Bovine *IVM/IVF* Embryos, Growth Factor Gene, Apoptosis Gene, Antioxidant Gene)

INTRODUCTION

Since the birth of the first calf derived from *in vitro* fertilization (Brackett et al., 1982), considerable progress has been made in the development of techniques for *in vitro* production (IVP) of bovine embryos for both research and commercial purposes. In particular, *in vitro* culture systems using somatic cells were paid attention by many investigators for the IVP system in bovine embryos. The techniques for IVP of bovine embryos for breeding or research purposes require that the culture system supports high embryo development with repeatable results (Kim et al., 2007). However, developmental rates to blastocysts for *in vitro* culture systems remain low and range between 20-30 percent (Carolan et al., 1996; Kreysing et al., 1997).

Several types of somatic cell have been used for *in vitro* culture of bovine embryos including bovine oviduct epithelial cell (BOEC), granulosa cell, uterine fibroblasts, trophoblastic vesicles and buffalo rat liver cells (Orsi and Reischl, 2006). The co-culture system with somatic cells is an effective and useful method for *in vitro* culture of bovine *IVF* embryos, affecting the success of *IVP* systems which may stimulate embryo development through amelioration of embryotoxic substance or secretion of embryotropic factor (Carolan et al., 1995; Donnay et al., 1997). Antioxidant enzymes (MnSOD, CuZnSOD, Gpx, glutamylcysteine synthetase (GCS) and catalase) as an embryotropic factor were constituently expressed in both bovine oviduct cell monolayers and nonattached epithelial cell vesicle cultures during an 8-day culture period (Harvey et al., 1995; Jang et al., 2005). The somatic cells used to increase development

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in the embryo co-culture system may secrete various growth factors, including IGF, LIF and TGF (Rief et al., 2002). BOEC are the most popular and notable somatic cells in bovine embryo culture *in vitro*. The BOEC co-culture system is an excellent method for overcoming the *in vitro* developmental block of bovine *IVM/IVF* embryos. However, BOEC may produce unknown substances, embryotropic factors and/or embryotoxic factors, making in difficult to determine the exact requirements for embryo development and preventing a clear understanding of embryo metabolism. Moreover, the effects of status (more than 1-passage and fresh or frozen/thawed) of BOEC on *in vitro* development of embryo culture has not been investigated and also the specific mechanism of their action is still unclear. Therefore, more complete analysis of the somatic cell co-culture system supporting high developmental rates is needed, not only to obtain consistent results but also to examine the exact requirements of embryo culture *in vitro*. Furthermore, cell lines that retain the ability over many passages to provide the conditions favorable for embryo development through the transition stage should be established.

The goal of the present study was to investigate the factors affecting increased *in vitro* developmental rates when Hanwoo IVM/IVF embryos were co-cultured with BOEC. The present study was designed to determine 1) the effects of the BOEC co-culture system on *in vitro* development rate of bovine *IVM/IVF* embryos, 2) the effect of status of BOEC on *in vitro* development of bovine embryos, and 3) detection of the temporal patterns of expression of growth factor genes (*TGF*, *EGF* and *IGFBP*), antioxidant genes (*Cu/Zn-SOD, Mn-SOD, Catalase* and *GPx*) and apoptosis genes (*Bax, p53* and *Caspase-3*) in different types of BOEC co-cultured with or without bovine *IVM/IVF* embryos.

MATERIALS AND METHODS

In vitro production of bovine IVM/IVF embryos

The cumulus-oocyte complexes (COCs) aspirated from the ovaries obtained from an abattoir were matured in TCM 199 (Gibco, USA) that was supplemented with 10% fetal bovine serum (FBS, Gibco), 1 μg/ml estradiol-17β, 5 μg/ml luteinizing hormone and 0.5 μg/ml follicle stimulating hormone at 38.5°C in humidified 5% CO_2 in air. Following maturation, 10-15 matured oocytes were transferred into a 50 μl fertilization drop (BO medium; Brackett and Oliphant, 1975). Frozen/thawed Hanwoo semen was washed in BO medium containing 10 mM caffeine and 50 μl sperm suspension was introduced into each fertilization drop. Presumptive zygotes produced at 6-8 h after insemination were cultured for 34-36 h in CR_1aa medium and 2-and 8-cell embryos were randomly allocated into each experimental group. Embryos were cultured for 5-6 days in 5% CO_2 in humidified air at 38.5°C and were examined for appearance, stage of development and cell number in blastocysts following Hoechst 33342 staining. All chemicals used in this study were purchased from Sigma-Aldrich (USA) unless otherwise stated.

Isolation, primary and secondary culture of bovine oviduct epithelial cell (BOEC)

Bovine oviducts were transported on ice from the slaughterhouse to the laboratory within 2 h. The oviducts were carefully trimmed on ice and rinsed with phosphate-buffered saline containing 1% antibiotic and antimycotic solution (Gibco). The epithelial cells were dispersed by forcing medium through an 18-gauge needle attached to a 10ml syringe and then the samples were transferred into 50 ml conical tubes containing 25 ml of Dulbecco's modified eagle medium (DMEM, Gibco) containing 10% FBS for washing twice by centrifugation (200×g) at 4°C. Cells were adjusted to 3×10^6 cell/ml with DMEM containing 10% FBS. The cell pellet was then equally dispensed as 1 ml volumes into a 4-well tissue culture dish and cultured at 38.5°C. In 5% CO_2 in humidified air, and the fresh medium changed every two days. Formation of a confluent monolayer with some ciliary activity within 2-3 days was affirmed and cultured with or without embryos for 6-7 days, and the other one was used for secondary-culture or cryopreservation.

Freezing and thawing methods of BOEC

BOEC monolayer was dissolved with Trypsin-EDTA (Gibco). After washing of the cells by centrifugation (500×g, 10 min), the supernatants were removed and cell pellets were frozen for cryopreservation. Basic freezing medium was used with DMEM containing 20% FBS. Dimethyl sulfoxide (DMSO) was then slowly added to reach a final concentration of 10% of the total volume. The cell suspension was dispensed into freezing vials and then placed in a -70°C freezer overnight. Freezing vials were plunged directly into liquid nitrogen on the next day and stored until use. Frozen BOEC was thawed at 37°C for 30 sec and used either for embryo culture or gene detection.

Morphological analysis

Following the different cell status, the morphology of BOEC was evaluated by visual examination under phase-contrast microscopy. Another morphological examination using a fluorescence staining procedure with 4', 6'-diamidino-2'-phenylindole (DAPI) was performed as described previously (Yim et al., 2000). Briefly, the BOEC was cultured in 1-chamber slides at a density of 2×10^4 cell per chamber. The cells were washed twice with PBS and fixed by incubation in 4% paraformaldehyde for 30 min. The fixed cells were again rinsed twice with PBS and then

Table 1. Oligonucleotide primer sequence for growth factors, antioxidant and apoptosis genes

mRNA	Type of primer	Primer sequence (5' 3')	Annealing temperature (°C)	Product size (bp)	Genbank accession number
Catalase	Forward	GAAAGGCGAAGGTGTTTGAGCA	61	814	D89812
	Reverse	AGGCGGTGGCGGTGAGTGTC			
CuznSOD	Forward	GCAGGGCACCATCTACTTC	61	382	AF396674
	Reverse	ACTTCCAGCATTTCCCGTCTTT			
MnSOD	Forward	CGCGGCCTACGTGAACAACCT	61	379	X64057
	Reverse	CCCCAGCAGCGGAACCAGAC			
GPx	Forward	CCATGCCCGGAGGTCTGCTTCT	61	380	AJ243849
	Reverse	CCATCACGCTGTCTCCATTCTTCC			
TGF-β	Forward	GCGAGCGCAGCGAGGAATACT	66	687	L08375
	Reverse	GGCACGCCCCGGCACAGAAG			
EGF	Forward	GCACCCATGGCAGAAGGAGA	55	718	X81380
	Reverse	TTTTTGCAGGAACATTTACACG			
IGFBP	Forward	CCGAGGAGGACCGCAGTGTAG	58	455	AF085482
	Reverse	ATCGTGTCCTTGGCAGTCTTTTGT			
Bax	Forward	GCCCCTGTCGTCCTTTGTCC	63	678	AF098067
	Reverse	TGGCGAGGAGCTGGTGCTGG			
P53	Forward	CTGCCACCGCCATCTCTGAA	63	533	AF397407
	Reverse	CTGCTGCGGGCCCACATTTG			
Caspase-3	Forward	GAAGCAAATCAATGGACTCTGGA	63	509	AB029345
	Reverse	GTCTGCCTCAACTGGTATTTTCTG			
B-actin	Forward	ATCACCATCTTCCAGGAGCG	58	280	AF261085
	Reverse	GATGGCATGGACTGTGGTCA			

incubated in 1 μg/ml DAPI solution for 30 min. The apoptotic cells were observed under epifluorescence microscopy equipped with an excitation/barrier filter of 280-365 nm and a digital camera.

RNA isolation of BOEC

Total RNA was also extracted by the guanidium isothiocyanate method (Chomczynski and Sacchi, 1987) from different status of BOEC or from BOEC monolayer which had been used or not used for embryo culture. Oviduct cells were placed into extraction solution (0.025 M sodium citrate, 0.5% sarcosyl, 4 M guanidiumthiocyanate, pH 7.0) and were vortexed with phenol, chloroform:isoamyl alcohol (24:1) and 2M 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 gently shaking, the supernatant was then precipitated with isopropanol at -20°C overnight. The samples were then centrifuged, washed with 70% ethanol, recentrifuged and air-dried before being dissolved in diethylprocarbonate (DEPC)-treated distilled water. The yield of extracted total RNA for each sample was determined by UV spectrophotometer at 260 nm.

Reverse-transcription polymerase chain reaction (RT-PCR) analysis

Each mRNA collected in the total RNA sample was analyzed by RT-PCR. Reverse-transcriptase generation of cDNA was performed on 2 μg of total RNA in a final volume of 2 μl with reverse transcription reagents (RT mixture), RT buffer, 0.5 mM of each dNTP, 0.5 μg oligo (dT), 10 IU RNase-inhibitor (Gibco) and 500IU reverse transcriptase (Ambion). The reaction was carried out at 42°C.for 60min 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. PCR was carried out for 3 min at 95°C, 50 sec at 60°C, 1 min at 72°C and 30 cycles. Primer sequences used in this study are indicated in Table 1. For 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.

Statistical analysis

The SAS mixed linear model program was used to analyze the data. Percentage of developmental stages in each treatment was compared for differences through use of Duncan's modified multiple range tests. Values at 5% level or less ($p<0.05$) were considered statistically significant.

RESULTS

Effects of BOEC co-culture system on bovine *IVM/IVF* embryos

The effects of different status of the BOEC co-culture

Table 2. Effect of BOEC co-culture system on the development of bovine *IVM/IVF* embryos

BOEC	No. of *IVM/IVF* embryos	No. of embryos developed to (Means, %±SD)			Cell no. of blastocysts (Mean±SD)
		Pre-morulae	Morulae	Blastocysts	
-	42	25 (59.5±2.0)[a]	14 (33.3±5.5)[a]	3 (7.2±1.3)[b]	79.0±4.6
+	46	16 (34.8±3.6)[b]	17 (37.0±2.7)[a]	13 (28.3±0.7)[a]	79.9±4.9

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

Table 3. Effects of the generation and status of BOEC on development of bovine *IVM/IVF* embryos

Cell status	No. of *IVM/IVF* embryos	No. of embryos developed to ((Mean, %)±SD)			Cell no. of blastocysts (Mean±SD)
		Pre-morulae	Morulae	Blastocysts	
-	51	30 (59.7±9.6)[ab]	11 (19.4±6.6)[a]	10 (20.9±2.9)[bc]	76.6±6.7
Primary-culture	68	26 (39.0±8.5)[b]	14 (20.5±0.5)[a]	28 (40.6±5.0)[a]	80.5±5.7
Secondary-culture	68	43 (63.1±2.8)[a]	4 (6.1±5.4)[b]	21 (30.8±6.2)[ab]	75.9±5.6
Frozen/thawed-culture	70	40 (58.3±11.9)[ab]	20 (27.0±5.0)[a]	10 (14.7±4.6)[c]	77.6±3.4

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

system on development of bovine *IVM/IVF* embryos were examined. The difference of developmental rates in Table 2 and 3 was considered from results of independent experiments. The developmental rate of blastocysts in the BOEC co-culture group (28.3%±0.7) was almost three times than that of the control group (7.2%±1.3, $p<0.05$). There was no difference in mean cell number of blastocysts between groups (Table 2). In the bovine *IVM/IVF* embryos cultured with different BOE cell generations and status, the developmental rates to blastocysts in PCC (40.6%±5.0) and secondary culture cell (SCC, 30.8%±6.2) were higher than in the control (20.9%±2.9) and FPCC groups (14.7%±4.6). The FPCC group gave rise to fewer blastocysts among the experimental groups, but mean cell number of blastocysts was not different among the experimental groups.

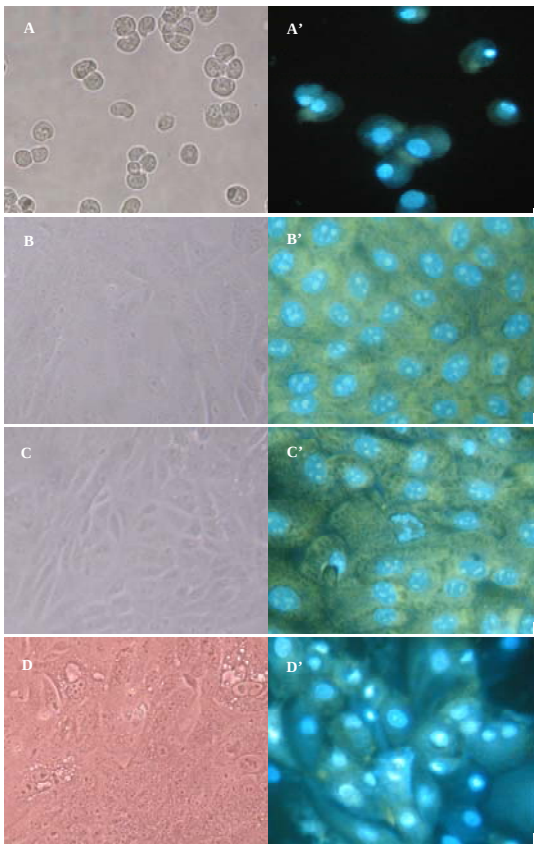

Figure 1. Photomicrograph of different BOEC status (A-D) and fluorescently stained BOEC with DAPI (A'-D'). A, A', Isolated fresh cell; B, B', Primary culture cell; C, C', Secondary-culture cell; D, D', Frozen/thawed primary culture cell.

Morphology and gene expression in different status of BOEC used or not used in embryo culture

The influence of different cell status on cell morphology was evaluated from visual examination by phase-contrast microscopy and DAPI staining under eqifluorescence microscopy. In Figure 1, FPCC showed retraction of membranes, cytoplasmic condensation and irregularity in shape when compare to PCC and some nuclei of the FPCC exhibited typical features of apoptosis, such as nuclear condensation and nuclear fragmentation, however, the SCC lessened these typical features of apoptosis.

To establish an mRNA phenotypic map for the expression of various growth factors, apoptosis and antioxidant genes, BOEC used or not used in embryo culture were analyzed according to different cell status by RT-PCR. The expression patterns for growth factor genes in BOEC with different status which was used or not used in embryo culture are shown in Figure 2. *TGF-β* and *EGF* genes were expressed in IFC, whereas another *EGF* gene was only expressed in PCC. Transcription for *IGFBP* was only expressed in PCC after use in the embryo culture.

The expression patterns of apoptosis genes (*Bax*, *Caspase-3*, and *p53*) in BOEC with different cell status, which was used or not used in embryo culture, were examined (Figure 3). Expression of *p53* was detected in IFC regardless of embryo culture and was only detected in FPCC after use in the embryo culture. On the other hand, *Bax* and *Caspase-3* genes were expressed in all treatment groups regardless of embryo culture.

The transcripts for *CuZnSOD*, *MnSOD*, *Catalase*, and *GPx* were detected in BOEC used or not used in embryo culture according to different cell generation and status

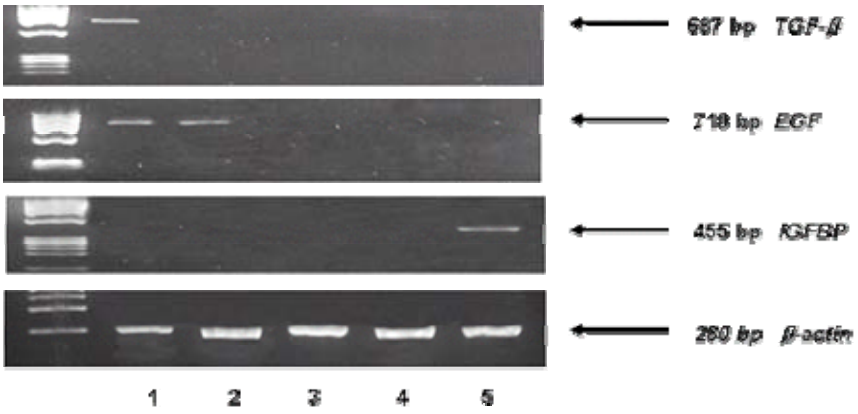

Figure 2. mRNA expression of growth factor genes in different cell status of BOEC used or not used in embryo culture (1, Isolated fresh cell; 2, Primary culture cell; 3, Secondary-culture cell; 4, Frozen/thawed primary culture cell; 5, Primary culture cell after use in embryo culture).

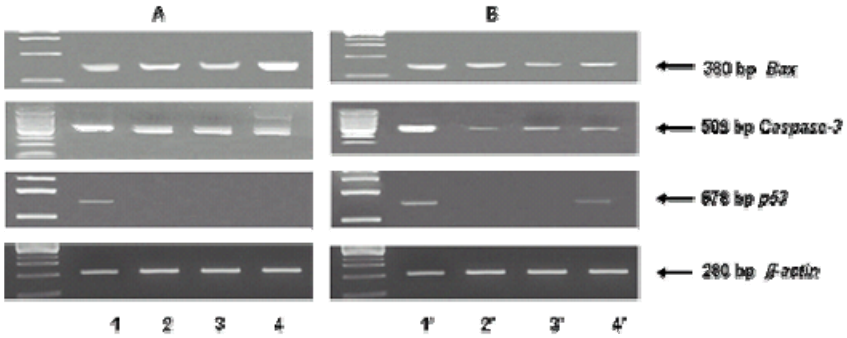

Figure 3. mRNA expression of apoptosis genes in different cell status of BOEC used or not used in embryo culture (A: Before use in embryo culture, B: After use in embryo culture; 1, 1', Isolated fresh cell; 2, 2', Primary culture cell; 3, 3', Secondary-culture cell; 4, 4', Frozen/thawed primary culture cell).

(Figure 4). All antioxidant genes were observed in BOEC groups that were not used in embryo culture. In contrast, the antioxidant genes were not expressed in BOEC groups after use in the embryo culture.

DISCUSSION

Mammalian preimplantation embryos produced or cultured *in vitro* commonly exhibit both short- and long-term retarded development (Kim et al., 2004), reduced viability (Gardner and Lane, 2005) and developmental arrest (Jurisicova and Acton, 2004). Mammalian preimplantation embryos for *in vitro* culture inevitably face the deleterious conditions that retard or arrest the development of embryos *in vitro*. The deleterious conditions for *in vitro* culture of implantation embryos are the exposure of ROS by high oxygen tension, heavy metal ions, unknown embryotoxic substances and others.

Recent attention has focused on ROS as major factors of *in vitro* embryonic arrest and cell death on *in vitro* development (de Lamirane and Gagnon, 1992; Guerin et al., 2001). The generation of ROS is an essential prerequisite for the normal function of many cells, however, excessive formation can lead to cellular damage and pathology (Halliwell and Aruoma, 1991). ROS exert a powerful oxidizing potential and have a pronounced effect on DNA, RNA and protein synthetic activities (Nasr-Esfahani et al., 1992; Johnson and Nasr-Esfahani, 1994). The deleterious effects of ROS are counter-balanced by antioxidant that neutralizes the activated oxygen species.

To overcome the *in vitro* block events or deleterious action of ROS, preimplantation embryos produced either *in vitro* or *in vivo* were cultured in a somatic cell co-culture system, with supplementation of specific compounds such as thiol compound, macro-molecules, growth factors and antioxidants. The somatic cell co-culture system has been used by many researchers to enhance *in vitro* development, to overcome cell block events and to delay apoptosis (Harvey et al., 1995; Vanroose et al., 2001). The specific antioxidant enzymes are produced by cells and are able to detoxify O_2, H_2O_2, and organic peroxides. These enzymes include MnSOD and CuZnSOD, which catalyze the dismutation reaction, removing O_2 species; catalase, which catalyses the decompositon of hydrogen peroxide to oxygen and water; and Gpx, which utlilzes glutathione as a reducing source to catalyze the removal of both hydrogen

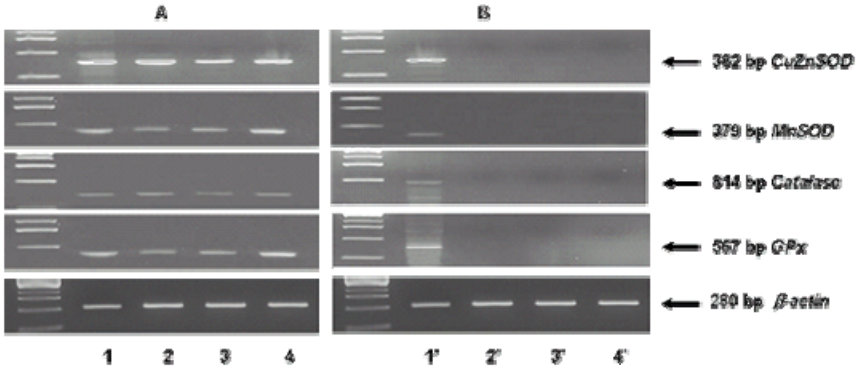

Figure 4. mRNA expression of antioxidant genes in different cell status of BOEC used or not used in embryo culture (A: Before use in embryo culture, B: After use in embryo culture; 1, 1', Isolated fresh cell; 2, 2', Primary culture cell; 3, 3', Secondary-culture cell; 4, 4', Frozen/thawed primary culture cell).

peroxide and lipid peroxides (Johnson and Nasr-Esjahani, 1994). However, the exact mechanism of somatic cell co-culture system on development of preimplantation embryos *in vitro* is still unclear.

This study examined the effects of BOEC co-culture system on the development of bovine *IVM/IVF* embryos. We found that BOEC co-culture system improved significantly the development rates beyond morula stages when embryos were cultured in PCC. Bavister (1992) reported that the somatic cell co-culture system could increase the developmental rate of embryos *in vitro*, including overcoming the developmental block, increasing the number of embryos reaching the blastocyst stage and improving the viability of embryos following transfer. Our results were consistent with the previous findings that co-culture with somatic cells could enhance *in vitro* development of embryos (Bavister, 1992; Harvey et al., 1995; Liu et al., 1998). In addition, we examined whether the different cell status of BOEC co-culture system affected *in vitro* development of bovine *IVM/IVF* embryos. Studies evaluating the use of fresh or sub-passaged monolayer indicated that previously sub-passaged cell monolayer might provide a more uniform and consistent co-culture system for mammalian embryos (Ouhibi et al., 1990). Furthermore, other researchers have evaluated *in vitro* development of ovine embryo after using either primary or frozen/thawed oviduct cells for co-culture (Rexroad and Powell, 1988). They reported that there was no significant difference between the two sources of oviduct cells in their ability to support *in vitro* development of one-cell ovine eggs. However, developmental rates from our results were significantly higher when embryos were cultured in PCC than those of control, SCC and FPCC groups ($p<0.05$). Our results agreed with the results of Ouhibi et al. (1990), but disagreed with those of Rexroad and Powell (1988) considering the difference in embryo source and methodology used by different laboratories. Our results indicated that the status of BOEC largely affected the development of bovine *IVM/IVF* embryos. It is suggested that fresh oviduct cell monolayers in the embryo co-culture system may indirectly provide evidence indicating that the embryotropic factors secreted by somatic cells can enhance embryo development *in vitro*.

The somatic cells used to increase the *in vitro* development of preimplantation embryos during *in vitro* culture may secrete embryotropic factors, which may include specific proteins such as glycoprotein 85-97 and various growth factors, including *IGF, LIF, TGF,* and some kinds of antioxidants (Rief et al., 2002). The antioxidant enzymes that regulate the ROS generated during *in vitro* culture of preimplantation embryos play a critical role in protection of somatic cells from oxidative stress-induced cell death. However, when embryo and somatic cells during *in vitro* culture overcome the block system and the anti-apoptotic system, cells begin programmed cell death, namely apoptosis. Genes of the caspase family, *p53, Bax* and cytochrome c, are relative to apoptosis in cell life. Nuclear translocation of *p53* and transcriptional activation of *Bax* and *Fas* (Muller et al., 1998; Lee et al., 2007) are known to be induced by H_2O_2 (Uberti et al., 1999) and *p53*-induced apoptosis requires the generation of ROS (Li et al., 1999). We detected different patterns of mRNA expression for growth factors, antioxidants and apoptosis genes on different status of BOEC used or not used in embryo culture. Expression of growth factor genes and *TGF-β* gene was detected in IFC and *EGF* gene was detected in IFC and PCC before use in embryo culture. Furthermore, transcripts for *IGFBP* were only expressed in PCC cultured with bovine *IVM/IVF* embryos. Apoptosis genes such as *Bax* and *Caspase-3* were observed in all experimental groups irrespective of the embryo culture. On the other hand, transcription for *p53* was only detected in IFC used or not used in embryo culture and FPCC after use in the embryo culture. Expression of antioxidant genes was observed in all

groups of BOEC before use in embryo culture, while this expression was not detected in BOEC after use in embryo culture. This study indicated that growth factors and antioxidants might influence the development of bovine embryos *in vitro*, indicating that both genes were only detected in fresh cells or fresh and frozen/thawed BOEC monolayer before use in embryo culture.

Therefore, our results demonstrated that the co-culture system of bovine oviduct epithelial cell according to cell status could increase the developmental rates of bovine *IVM/IVF* embryos through its antioxidant activity and the secretion of embryotropic factors.

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