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Co–Culture with Amniotic Membrane Stem Cells for Oocyte Maturation and Early Embryonic Development in Bovine

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To improve early embryonic development, many strategies have been studied in various species including bovine. The co–culture of embryos with somatic cells has been known to improve embryo quality during in vitro culture and pregnancy rates compared with conventional culture systems. The co–culture of embryos with oviductal cells which have been used for blastocyst expansion appears to shorten the time needed for synthesis of hCG, early embryo development, and hatching blastocysts. The aim of this study is to investigate whether the co–culture system with amniotic membrane stem cells (bAMS) improves bovine embryonic development competence.

In order to investigate the influence of bAMS on early embryo development, bAMS originated from fetal bovine amniotic membranes were used as the feeder layer during *in vitro* maturation. The rates of matured oocytes, cleavage division, and blastocysts formation were increased ($p<0.05$) after co–culture with the bAMS compared to the control group. Moreover the rates of oocyte maturation and embryo development were significantly higher with using bAMS below 5 passages. In addition, the GSH level and the total cell number of blastocysts were significantly higher in co–culture group than in those of control group. Apoptotic cells were found more in the control group, and the expression of anti–apoptosis related genes such as Mcl–1 and Bcl–2 were increased after co–culture with bAMS.

In conclusion, the present study demonstrated that co–culture with bAMS cells enhances oocyte maturation and embryo developmental competence by increasing GSH level and expression of anti–apoptotic genes with reducing apoptotic cells in bovine embryos. We also found that it is more suitable to use bAMS below 5 passages.

Key words: amniotic stem cells, bovine embryo, co–culture, Hanwoo

INTRODUCTION

Co–culture system has been used in *in vitro* culture (IVC) of embryos. It has been reported that using a co–culture system with various somatic cells improves embryo quality and pregnancy rates (Dominguez *et al.*, 2010). The co–culture system has been also known to reduce the time of embryo development and hatching blastocyst with increasing the synthesis of hCG (Menzo *et al.*, 1990; Fanchin *et al.*, 2001). The cells used as the feeder layer release antioxidants such as hypotaurine which is one of the natural free radical scavengers in the embryo environment (Cassuto *et al.*, 2003; Guerin *et al.*, 1995). Many researches have been conducted to improve the co–culture conditions by using various types of cells. However, how co–culture system provides beneficial environment to embryos during IVC is not definitely known.

Several studies have been trying and developing successful techniques to obtain high quality of oocytes. For example, ultrasound–guided ovum pick up (OPU) technology is a method that allows obtaining a large number of oocytes from live donors with high genetic advantages (Kruip *et al.*, 1994). OPU is possible to be conducted every 3~5 days intervals without hormone treatment and able to use same live donor animals continuously. The technique has been known to be useful to produce numerous oocytes compared to the multiple ovulation/embryo transfer (MOET) method (Pontes *et al.*, 2011). However, OPU system is still in need to be optimized (Goovaertsa *et al.*, 2010). OPU is able to collect approximately only 5~10 oocytes per animal at a time which requires to culture oocytes in a small number of a group (Petyim *et al.*, 2003; Machado *et al.*, 2006). It has been accepted that *in vitro* culture system in a large number improves the maturation of oocytes by secreting several growth factors (Lim *et al.*, 1996; O'Neill *et al.*, 1997). Moreover oocytes collected by OPU include different degrees of quality. A poor quality of cumulus–oocyte–complex (COC) reduces not only the number of oocytes to be cultured at a time but also the subsequent embryo development compared to the oocytes with multi–layers and compact COC (Ferry *et al.*, 1994). Therefore it would be important to develop advanced IVM system for small number of immature oocytes including a poor quality of COC that allow obtaining more matured oocytes and reducing the number of oocytes needed.

Oocyte quality affects on embryo development

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(Hardy *et al.*, 2000), and it has been noticeably recognized in bovine oocytes among those of any other species (Sirard *et al.*, 2006). Oocytes obtain the ability to resume meiosis during *in vitro* maturation (Richard *et al.*, 1996) prior to be fertilized, cleaved and develop (Ware *et al.*, 1989, Dinnyes *et al.*, 2000) that achieves pregnancy (Peterson *et al.*, 2003). With that, the present study aims that co–culture with bAMS provides a beneficial environment for the maturation of bovine oocytes including further embryo development. In order to confirm the influence of co–culture with bAMS, we investigated the level of GSH, apoptosis, and apoptosis related gene expression.

MATERIALS AND METHODS

Chemicals and Animals

All chemicals and reagents were purchased from Sigma–Aldrich Chemical Co. (St. Louis. MO. USA) unless otherwise stated. Three Hanwoo were used as oocyte donors for OPU. All animals are healthy, non–pregnant, non–lactating, and cycling. The average age of Hanwoos was around 5 years and they were weighing 400±35 kg. The animals belonged to Chungnam livestock Research Institute. All animal studies were performed in accordance with recommendations described in "The Guide for the Care and Use of Laboratory Animals" published by Institutional Animal Care and Use Committee of Chungnam livestock Research Institute.

Oocytes Collection by OPU

Follicular aspiration was performed as described by the previous report (Seneda *et al.*, 2008). Briefly, each follicle of ≥ 2 mm in diameter was aspirated using ultrasound scanner (Mylab30Vet GOLD, Italy). A 7.5 MHz convex array transducer was fitted into the intravaginal device and guided by a stainless steel. Follicular puncture was performed by using a disposable 19–gauge × 60 mm needle (JEIL TECH CO, Korea) connected to a 50 ml conical tube (BD Falcon, USA) via a silicon tube. Aspiration was performed by using a vacuum pump (Gast, USA) with a negative pressure of 10 to 12 ml of water/min. The aspirated sediments were immediately filtered through a con filter. Oocytes were collected through the needle, transferred into 0.25 ml sterile straw and immediately transported to the laboratory by a portable 36°C incubator.

COCs were classified into four grades based on the presence and morphology of cumulus cell layers: (A) Complete and compact > 1~3 COC layer, (B) 1~3 compact layer, (C) partial layer and (D) denuded oocytes and expanded/loosened cumulus cells.

Isolation and In Vitro Culture of bAMS

Bovine amniotic membranes were obtained from a local slaughterhouse. The samples were transferred to the laboratory in a short time. The collected membranes were washed with phosphate–buffered saline (PBS; Gibco, USA) containing 0.1% penicillin–streptomycin (500 U/ml penicillin–5 mg/ml streptomycin; Gibco, USA) and minced in washing medium supplemented with 5% fetal bovine serum (FBS) (Gibco, USA). After minced, the pellets were washed with washing medium and centrifuged at 2,000 rpm for 5 min twice. The bovine amniotic membrane cells were grown in low–glucose Dulbecco modified Eagle medium (L–DMEM) (Sigma–Alorich, USA) supplemented with 10% FBS, 0.1% penicillin/streptomycin, 5 ng basic fibroblast growth factor (bFGF) (Sigma–Alorich, USA) and 10 ng epidermal growth factor (EGF) (Sigma–Alorich, USA) at 39°C with 5% CO_2 and 5% O_2.

Oocytes Collection from Slaughterhouse–Derived Ovaries

Ovaries were collected from animals shortly after slaughtering at a local slaughterhouse and transported to the laboratory within 3 h in physiological saline containing penicillin/streptomycin at 30 to 35°C. Ovaries were then washed three times in fresh warmed saline. The contents of antral follicles of 2 to 8 mm in diameter were aspirated using an 18–gauge needle attached to a 10 ml disposable syringe. Follicular fluid with oocytes was collected in 15 ml centrifuge tubes and allowed to settle for 10 to 15 min. The sedimentation was washed two times in HEPES–buffered Tissue Culture Medium 199 supplemented with 0.5% (w/v) bovine serum albumin (BSA fatty acid free, Gibco.), 2 mM sodium bicarbonate and 10 mM HEPES (Sigma Co.). COCs surrounded by cumulus cells and homogeneous cytoplasm were selected for further experiments.

In Vitro Maturation, In Vitro Fertilization and In Vitro Culture

The bAMS were cultured in 4–well dish in L–DMEM containing 10% FBS and 0.1% penicillin/streptomycin. After 24 h, the culture medium of bAMS was changed to oocyte maturation medium (TCM199 Supplemented with 10% FBS).

The COCs of co–culture group were cultured in the 4–well dish which contains pre–cultured feeder layer in 500 μl TCM199–culture medium supplemented with 10% FBS, 1 mg/ml FSH (folltropin–V®, Bioniche Co., Belleville, Canada), 1 μg/ml estradiol–17β, 0.2 mM sodium pyruvate, and 1 μg/ml epidermal growth factor. The COCs of control group were matured without feeder layers *in vitro* in incubator at 38.5°C in 5% CO_2 with saturated humidity for 22~24 h.

Fertilization was accomplished by frozen/thawed semen of fertile Hanwoo. Straws were thawed for 1 min in a water bath at 36°C. Sperms were washed in 4 ml of Tyrode's albumin–lactate pyruvate (TALP)–capacitation medium followed by centrifugation at 800 × g for 6 min twice, and was capacitated in TALP medium supplemented with heparin (20 μg/ml). Sperm concentration was adjusted to 1×10^7 sperm/ml in TALP–IVF medium. After maturation, COCs were washed three times in TCM–washing medium and inseminated in TALP–IVF medium containing sperms in 50 μl droplets covered by mineral oil. The droplets (10 COCs/droplet) were incubated under the same gas atmosphere as for IVM.

After 18~20 h, sperms and cumulus cells around pre-

sumptive zygotes were removed by gentle pipetting in a TCM–washing medium. After then, the presumptive zygotes were transferred to $50\,\mu$l droplets of culture medium consisting of modified synthetic oviduct fluid medium with essential and non–essential amino acids and bovine serum albumin (BSA) in modular chamber with a gas atmosphere of 5% CO_2 for 7 days.

Measurement of GSH

The level of GSH was measured by using 4–chloromethyl–6.8–difluoro–7–hydroxycoumarin (CellTracker Blue; CMF2HC; Invitrogen Corporation). Briefly, matured oocytes were washed in DPBS supplement with 0.10 g/L $CaCl_2$ and 0.10 g/L $MgCl_2·6H_2O$. After washing, oocytes were transferred into bovine zygote medium mSOF containing $100\,\mu$M TBHP at 39°C in 5% CO_2 for 60–90 min. After 60–90 min of incubation, oocytes were transferred into DPBS containing $25\,\mu$M carboxy–H2DCFDA and $25\,\mu$M Cell Tracker Blue, and incubated for 30 min at 39°C in 5% CO_2 protected from light. After incubating for 30 min, oocytes were washed in DPBS. The fluorescent emissions from the oocytes were recorded using the camera attached to a fluorescence microscope with UV filters (370 nm for GSH). The recorded fluorescent images were analyzed by Image J software (Version 1.46r; National Institutes of Health, Bethesda, MD, USA) and normalized to those of control oocytes.

TUNEL Assay

TUNEL assay was performed to assess the presence of apoptotic cells (In Situ Cell death Detection Kit, TMR red) in blastocysts. At day 7 blastocysts were washed 3 times for 5 min in PBS–PVA (containing 0.1% PVA). Embryos were fixed in 4% paraformaldehyde in PBS–PVA for 1 h and permeabilized in PBS–PVA with 1.0% Trition X–100 for 30 min at room temperature. The embryos were washed in PBS–PVA for 3 times and incubated in TUNEL reaction medium (Enzyme solution: Label solutions = 1 : 9) for 1 h at 37°C in dark. After the reaction completed, the embryos were washed and stained with hoechest 33342 and mounted in mounting medium (Vectashield, Vector Laboratories, Inc, CA) at room temperature in dark. The number of apoptotic nuclei and fragments were subsequently subjected to confocal laser scanning microscopy.

RNA Isolation and RT–PCR

mRNA was isolated from triplicate pools of 10 blastocysts using oligo (dT) 25–attached magnet beads (Dynalbead, Invitrogen) according to the manufacturer's instructions. Briefly, embryos in lysis buffer were mixed with $40\,\mu$l of binding buffer (20 mM Tris–HCl, pH 7.5, 1 M LiCl, 2 mM EDTA, pH 8.0), and incubated at 65°C for 5 min to facilitate the lysis of the embryo and release of RNA. Oligo (dT) magnetic beads were washed three times with washing buffer (10 mM Tris–HCl, pH 7.5, 0.15 LiCl, 1 mM EDTA, pH 8.0). Finally, mRNA samples were eluted in $10\,\mu$l of double–distilled diethylpyrocarbonate C–treated water and reverse transcribed using oligo (dT) primer and iScript reverse transcriptase (BIO–RAD,

US).

Synthesized cDNA was amplified by PCR using specific primers for anti– apoptosis genes (Mcl–1 and Bcl–w). PCR reaction was consisted of 5 min denaturation at 94°C, followed by 35 cycles at 94°C for 30 sec, 62°C for 30 sec and 72°C for 30 sec. A final extension for 5 min was performed at 72°C. Amplified target genes by RT–PCR were confirmed by DNA sequencing, and RT–PCR results were evaluated by Image J software.

Statistical Analysis

All data were analyzed using one way ANOVA software program (SPSS statistics Inc. ver 18.0.0). A p–value < 0.05 was considered to be statistically significant.

RESULTS

The grades of OPU–derived oocytes

The grades of recovered oocytes from aspirated follicles by OPU are shown in Table. 1. Oocytes with complete and compact >3 layers of cumulus cells (A grade) and oocytes with 1~3 layers of cumulus cells (B grade) were consisted of 57.9% of total recovered oocytes from aspirated follicles by OPU. 16.3% of oocytes have no cumulus cell layer that is considered as poor quality cumulus–oocyte complexes.

Table 1. Evaluation of oocytes recovered by OPU

Total no. of recovered oocytes	Grades of oocytes (%)			
	A	B	C	D
252	62 (24.6%)	84 (33.3%)	57 (22.6%)	49 (16.3%)

(A) Complete and compact 1~3 > multilayer, (B) 1~3 compact layer, (C) partial layer, (D) no cumulus cell or expanded cumulus cells. (N of Hanwoo = 3)

The effect of co–culture with bAMS on oocyte maturation and early embryonic development

Feeder layers of bAMS were used during IVM for immature oocytes. In comparison with the immature oocytes cultured without bAMS feeder layers during IVM there were increased matured oocytes significantly (75.6%). In addition, the rates of embryo cleavage and blastocyst formation were also increased (63.8%, 29.2%) (p<0.05) (Fig. 1.a).

The effect of bAMS senescence on oocyte maturation and early embryonic development in co–culture system

The effect of the bAMS senescence was investigated in co–culture system based on the rates of oocyte maturation and embryo development. When bAMS were over passage 6 there was no significant difference in the rates of oocyte maturation and embryo development ($p<0.05$) compared to the control group (Fig 1.b). However, the rates of oocyte maturation and blastocyst formation in the group of co–culture with below 5 passages of bAMS
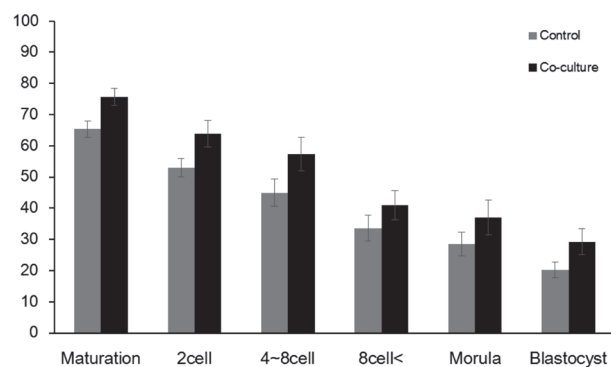
Fig. 1. a. Rates of oocyte maturation and embryo development after co–culture with bAMS.
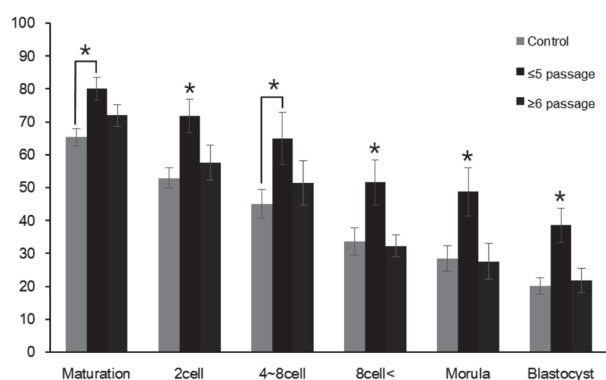

Fig. 2. The expression level of GSH in *in vitro* matured bovine oocytes by co–culture with bAMS. * $P < 0.05$

Fig. 1. b. The effect of bAMS senescence on the rates of oocyte maturation and embryo development. * $P < 0.05$.

Table 2. The number of apoptotic cells in blastocysts after 7 days *in vitro* culture

	Blastocyst	Total cells (mean+SEM)	Apoptotic cells (mean+SEM)
Control	5	411 (82.2±13)[a]	16 (3.2±0.66)
Co–culture	5	525 (105±2.92)[b]	7 (1.4±0.51)

[a–b]Values with different superscripts are significantly different ($P < 0.05$).
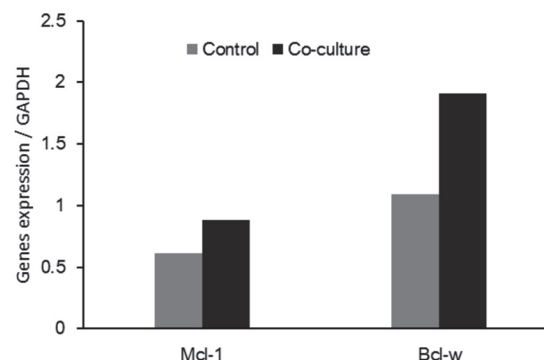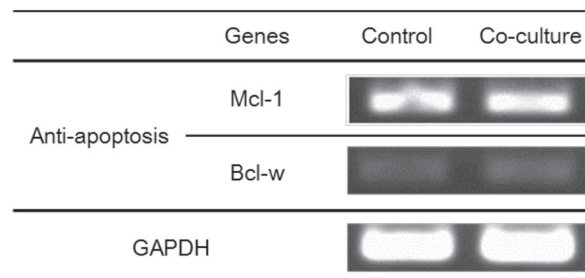
Fig. 3. Expressions of anti–apoptosis related genes in blastocysts were confirmed by RT–PCR. RT–PCR results were analyzed by using Image J software

were significantly higher than the control group ($p<0.05$) (Fig 1.b).

Measurement of GSH in matured oocytes

Matured oocytes were selected based on the presence of first polar body and the level of oocyte maturation and anti–oxidant activity was measured by GSH expression. GSH was detected by using Blue CMF2HC (Fig. 2). The relative expression level of GSH was significantly higher in the oocytes from the co–culture group than those of the control group.

Apoptosis

To investigate the effect of co–culture on cell death in blastocysts TUNEL staining was performed with blastocysts at 7 day from both control and co–culture groups. Total cell numbers of the blastocysts in co–culture group were significantly higher (105.0±2.92) than control group (82.2±8.13). Apoptotic cells of blastocyst in the control group (3.2±0.66) were higher than co–culture group (1.4±0.51). However, there was no significant difference (Table 2).

Expression of anti–apoptotic genes

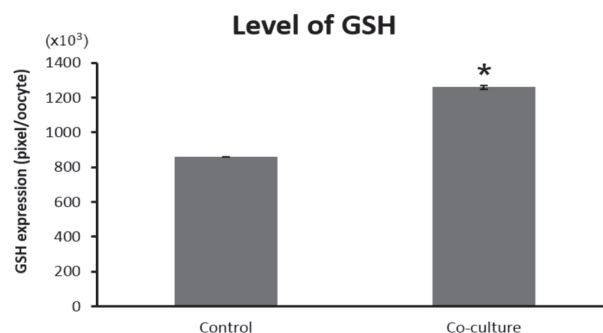To determine whether the bAMS feeder layers affect on apoposis in bovine embryos development, the expression of anti–apoptotic genes was detected by RT–PCR (Fig. 3). The results indicated that the expression of anti–apoptotic genes such as Mcl–1, Bcl–w in bovine embryos was increased (Fig. 3), and apoptotic genes were not expressed both control and co–culture groups (Data not shown).

DISCUSSION

The amniotic sac contains fetus and amniotic fluid that provides essential environment for developing fetus. The amnion is a thin transparent membrane surrounding fetus which is comprised of three major layers: an epithelial monolayer, a thick basement membrane, and an avascular stroma (Niknejad *et al.*, 2008). The amniotic membrane is basically to protect the embryo and secrets cytokines that maintain the crucial environment to develop fetus. It has been shown that native amnion contains higher levels of EGF, KGF, HGF, bFGF, TNF–α, NGF, BDNF and activin (Koizumi *et al.*, 2000, Uchida *et al.*, 2000). The basement membrane of the amnion involves collagen, fibronectin, laminin and other proteoglycans that have important roles in cell adhesion and growth (Niknejad *et al.*, 2008). The epithelium of amniotic innermost layer produces prostaglandins and prostaglandin E2 (PGE$_2$) (Okazaki *et al.*, 1981), also expresses prostaglandin biosynthesis enzymes (Bryant–Greenwood *et al.*, 1987; Toda *et al.*, 2007). PGE$_2$ may induce the indirect stimulation of cumulus expansion (Eppig, 1981). Expansion of cumulus has important roles for maturation, fertilization, and embryonic development (Downs *et al.*, 1988; Hizaki *et al.*, 1999). PGE$_2$ has an ability of stimulating cell cycle progress during oocyte maturation and early embryo development. (Nuttinck *et al.*, 2011). Recently, the amnion has been also applied clinically, and stem cells derived from amniotic membrane have been studied (Parolini *et al.*, 2008 ; Yu *et al.*, 2009).

In the present study, co–culture with bAMS induced significantly increased oocyte maturation and embryo developmental competence. It is probably due to the several growth factors and cytoprotective cytokines secreted from bAMS. In addition co–culture of bAMS below 5 passage improved oocyte maturation and embryo development competence more than those of bAMS cells over 6 passage. Therefore the early passage of bAMS promotes oocyte maturation and embryo development, and the later passage of bAMS would attenuate the benefit of the co–culture with bAMS.

In this study, the oocytes matured on the bAMS feeder layers expressed higher GSH level, and blastocysts after co–culture with bAMS had significantly the increased total cell number with decreased apoptosis. Moreover, anti–apoptotic gene expressions were increased in blastocysts after co–culture with bAMS. GSH maintains the redox state and protects the detrimental effect of oxidative damage (Lafleur *et al.*, 1994 ; Pastore *et al.*, 2003). Embryo quality and developmental competence were improved when GSH synthesis was increased during *in vitro* maturation (de Matos *et al.*, 2000). Apoptosis is an active physiological process which triggers chromatin condensation, reducing cell volume, resulting in fragments and removal of unnecessary, damaged and risky individual cells (Schwartzman and Cidlowski 1993). In addition, the correlation of the embryo development and apoptosis in arrested bovine embryos was reported (Antunes *et al.*, 2010). Apoptosis is controlled by the activity of pro– and anti–apoptotic genes, during pre–implantation development (Bergeron *et al.*, 1998).

In conclusion, the present study demonstrated that co–culture system with bAMS during *in vitro* maturation improved oocyte maturation and embryo development by increasing GSH level, increasing anti–apoptotic gene expression and reducing DNA fragmentation in embryos. Therefore co–culture with bAMS would be an optimal *in vitro* culture system to improve the efficiency of oocyte maturation and embryo development that allow obtaining high quality of oocytes.

ACKNOWLEDGMENTS

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