Toxicity evaluation of ethanol treatment during *in vitro* maturation of porcine oocytes and subsequent embryonic development following parthenogenetic activation and *in vitro* fertilization

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Abstract. Ethanol is frequently used as a solvent in several techniques for *in vitro* production (IVP). It is also used for the parthenogenetic activation (PA) of oocytes. Although a number of studies have suggested that ethanol has detrimental effects on fibroblasts and neuronal cells, little attention has been paid to the effects of ethanol on porcine oocytes. Thus, the aim of this study was to evaluate the effects of the addition of ethanol to *in vitro* maturation (IVM) medium. We investigated the effects of ethanol (0, 1 and 3%) on the following parameters: nuclear maturation, intracellular glutathione (GSH) and reactive oxygen species (ROS) levels, and subsequent embryonic development following PA and *in vitro* fertilization (IVF). After 44 h of IVM, the 3% group showed a significant (P<0.05) decrease in nuclear maturation (34.0%) compared with the control group (70.3%). The 1 and 3% groups exhibited a significant (P<0.05) decrease in GSH levels and an increase in ROS levels compared with the control group. Compared with the control group, the 3% group had significantly (P<0.05) lower cleavage rates following PA (51.6 vs. 86.9%) and IVF (53.2 vs. 70.6%), as well as lower blastocyst formation rates and decreased total cell numbers following PA (11.3% and 31.8 vs. 53.6% and 65.4, respectively) and IVF (4.1% and 22.0 vs. 36.1% and 70.3, respectively). We evaluated the mRNA expression levels of DNA repair-related and apoptosis-related genes in the cumulus oocyte complexes (COCs). The 1% ethanol group showed significantly (P<0.05) higher mRNA expression levels of poly(ADP-ribose) polymerase-1 (PARP-1), Bax, Bak and caspase-3, and the 3% ethanol group had significantly (P<0.05) increased PARP-1, Bax and caspase-3 mRNA expression levels compared with the control group. Our results suggest that treatment with >1% ethanol during IVM exerts a toxic effect on the developmental potential of PA and IVF porcine embryos by decreasing the intracellular GSH level, thereby increasing the intracellular ROS level and upregulating the expression of apoptosis-related genes.

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Introduction

The *in vitro* production (IVP) of embryos has been widely used in reproduction technology. Pigs have been used in biomedical research for decades as disease models, disease-resistance animals and genetically-defined models for surgery and xenotransplantation as their anatomy and physiology are similar to those of humans (1,2). However, the potential of porcine embryos to develop *in vitro* is still inferior to that of embryos obtained *in vivo*. One of the reasons of this inferiority is thought to be improper *in vitro* maturation (IVM) systems. Several researchers have made tremendous efforts to improve IVM conditions by the addition of pharmacological compounds (3,4). The addition of pharmacological compounds in IVM medium requires the use of solvents in order to dissolve these compounds.

Ethanol, which is known as a cryoprotectant (5,6) and an inducer of the artificial parthenogenetic activation (PA) of oocytes (7-10), is frequently used in a number of techniques for IVP as one of the solvents for pharmacological compounds that are not dissolved in water. For example, in a previous study on the effect of lanosterol supplementation to porcine IVM medium, an ethanol concentration of 1.68% was used for the lanosterol solvent (3). Moreover, ethanol has been used for the addition of meiosis-activating sterols (MAS) to IVM medium to improve the conditions of IVM (4). However, although a number of studies have suggested that high concentrations of ethanol have detrimental effects on several cell types (11-13),

little attention has been paid to the effects of low concentrations ethanol, which are often used as a solvent in the IVM of porcine oocytes.

Previous studies have demonstrated that ethanol promotes apoptotic cell death in cultured neurons (14). There are multiple apoptotic pathways activated in cells by ethanol, including the caspase-dependent pathway and caspase-independent apoptotic pathway known as the poly(ADP-ribose) polymerase-1 (PARP-1)-related pathway. Caspases have been thought to be the central executioners of the apoptotic pathway (15). Apoptosis can be directed by the activation of caspases, which cleave specific substrates and trigger cell death. PARP-1 is a zinc (Zn) finger nuclear protein activated by DNA strand-breaks and utilizes β-nicotinamide adenine dinucleotide (β-NAD) as a substrate to catalyze the synthesis of ADP-ribose polymers on nuclear proteins, including PARP-1 itself (16). Thus, PARP-1 plays a key role in caspase-independent cell death and survival under multiple stress conditions (17,18).

Considering how ethanol influences cells, the potential effects of ethanol on IVM may be related to apoptosis. Therefore, the aim of this study was to evaluate the toxic effects of the addition of low concentrations of ethanol to IVM medium for the culture of immature porcine oocytes. For this purpose, various parameters were compared in mature porcine oocytes, including nuclear maturation, intracellular levels of glutathione (GSH), intracellular levels of reactive oxygen species (ROS), mRNA expression levels of DNA repair- and apoptosis-related genes [proliferating cell nuclear (PCNA), PARP-1, Bax, Bak and caspase-3] in the cumulus oocyte complexes (COCs) at the end of the maturation period and embryo developmental competence following PA and *in vitro* fertilization (IVF).

Materials and methods

Chemicals. All chemicals and reagents used in this study were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), unless otherwise stated.

Oocyte collection and IVM. Ovaries of prepubertal gilts were collected at a local abattoir and transported to the laboratory within 2 h in physiological saline supplemented with 100 IU/l penicillin G and 100 mg/ml streptomycin sulfate at 32-35°C. The COCs in the ovaries were aspirated from 3- to 6- mm diameter superficial follicles using an 18-gauge needle attached to a 10 ml disposable syringe and allowed to settle down as sediment in 15 ml conical tubes at 37˚C for 5 min. The supernatant was discarded and the precipitate was resuspended with HEPES-buffered Tyrode's medium (TLH) containing 0.05% (wt/vol) polyvinyl alcohol (PVA) (TLH-PVA). Subsequently, it was observed under a stereomicroscope for recovering the COCs. Only COCs having ≥3 uniform layers of compact cumulus cells and a homogeneous cytoplasm were selected and washed 3 times in TLH-PVA. Approximately 60 COCs were placed into each well of a 4-well Nunc dish (Nunc, Roskilde, Denmark) containing 500 μl of culture medium (TCM199; Invitrogen Corp., Carlsbad, CA, USA) which was supplemented with 0.6 mM cysteine, 0.91 mM sodium pyruvate, 10 ng/ml epidermal growth factor, 75 μg/ml kanamycin, 1 μg/ml insulin, 10% (vol/vol) porcine follicular fluid (pFF), 10 IU/ml equine chronic gonadotropin (eCG) and 10 IU/ml hCG (Intervet, Inc., Boxmeer, the Netherlands). The selected COCs were incubated at 39˚C with 5% CO_2 in 95% humidified air for IVM. Following 21-22 h of maturation with hormones, the COCs were washed twice in fresh hormone-free IVM medium and then cultured in hormone-free IVM medium for an additional 21-22 h. The COCs during IVM were treated with or without low concentrations (0, 1 and 3%) of ethanol according to the experimental design.

Evaluation of nuclear maturation. The oocytes at the metaphase II (MII) stage, 42-44 h after IVM, were sampled to analyze nuclear maturation. Samples of oocytes (a total of 509 oocytes was used for the analysis of nuclear maturation) were by gently pipetting with 0.1% hyaluronidase in IVM medium and washed in TLH-PVA. The denuded oocytes were fixed with fixative solution containing 2% formaldehyde and 0.25% glutaraldehyde, and stained with TLH-PVA containing 5 μg/ml Hoechst 33342 for at least 5 min. The stained oocytes were evaluated using a fluorescence microscope (Nikon Corp., Tokyo, Japan) and classified as germinal vesicle (GV), metaphase I (MI), anaphase-telophase I (AT-I), or MII according to the meiotic maturation stage. The oocytes at MII were considered to have matured.

Measurement of intracellular GSH and ROS levels. The oocytes at 42-44 h after IVM were sampled to determine intracellular GSH and ROS levels. The measurement of GSH and ROS levels was carried out according to previously described methods (19,20). In brief, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Invitrogen Corp.oration, Paris, France) and 4-chloromethyl-6.8-difluoro-7-hydroxycoumarin (CellTracker Blue; CMF2HC; Invitrogen Corp.) were used to detect intracellular ROS levels as green fluorescence and GSH levels as blue fluorescence, respectively. From each treatment group, 10 oocytes were incubated (in the dark) for 30 min in TLH-PVA supplemented with 10 μM H_2DCFDA and 10 μM CellTracker Blue. Following incubation, the oocytes were washed with Dulbecco's phosphate buffered saline (DPBS) (Invitrogen Corp.) containing 0.1% (wt/vol) PVA, placed into 10-μl droplets, and fluorescence was evaluated under an epifluorescence microscope (TE300; Nikon Corp.) with UV filters (460 nm for ROS and 370 nm for GSH). These fluorescence images were saved as graphic files in TIFF format. The experiment was replicated 3 times with a total of examined oocytes (GSH samples, n=28; ROS samples, n=28).

Each intracellular GSH level was quantified according to the method previously described by Baker *et al* (21) with some modifications. Briefly, MII oocytes from each experimental group were washed 3 times in 0.2 M sodium phosphate buffer (Na_2HPO_4, NaH_2PO_4, and 10 mM EDTA-2Na, pH 7.2) and groups of 50-60 oocytes (per sample) in 10 μl sodium phosphate buffer were transferred to 1.7 ml microfuge tubes; 10 μl of 1.25 mM phosphoric acid (final concentration of 0.625 M H_3PO_4) in distilled water was added to each sample. Tubes containing the samples were frozen at -80˚C until analysis. GSH concentrations in the oocytes were measured using a 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)-GSH reductase (GSSG) recycling assay. The frozen samples were thawed at room-temperature, vortexed, centrifuged and microscopically

evaluated to ensure complete lysis of the oocytes prior to the assay. The supernatants were transferred to a 96-well microtiter plate, and for each sample, 700 μl of 0.33 mg/ml nicotinamide adenine dinucleotide phosphate (NADPH) in 0.2 M assay buffer containing 10 mM EDTA (stock buffer, pH 7.2), 100 μl of 6 mM DTNB in the stock buffer and 180 μl of distille water and 1 U per sample of DTNB-GSH reductase (441 U/ml) were added to a conical tube, mixed and immediately added to the sample. The plate was immediately placed in a microtiter plate reader, and optical density was measured with a 405 nm filter (EMax; Molecular Devices, Sunnyvale, CA, USA). The formation of 5-thio-2 nitrobenzoic acid was monitored every 30 sec for 3 min. Standard curves were prepared for each assay and the GSH content per sample was determined by a standard curve. The GSH concentrations (pmol/oocyte) were calculated by dividing the total concentration per sample by the total number of oocytes present in the sample. The experiment was replicated 3 times.

PA of oocytes. For PA, the COCs were denuded at 42-44 h following IVM by gently pipetting with 0.1% hyaluronidase, washed 3 times in TLH-PVA and then rinsed twice in activation medium (280 mM mannitol solution containing 0.01 mM $CaCl_2$ and 0.05 mM $MgCl_2$). For activation, the matured oocytes at the MII stage were placed between electrodes covered with activation medium in a chamber connected to an electrical pulsing machine (LF101; Nepa Gene, Co., Ltd., Ichikawa, Japan). Oocytes were activated with 2 direct-current (DC) pulses of 120 V/mm for 60 μsec. Following electrical activation, the oocytes were immediately placed into IVC medium supplemented with 5 μg/ml of cytochalasin B for 6 h. The PA embryos were washed 3 times in fresh IVC medium, placed into 30 μl IVC droplets (10 gametes per drop) covered with pre-warmed mineral oil, and then cultured at 39˚C in a humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2 for 7 days.

IVF and culture. For IVF, the COCs were denuded at 42-44 h following IVM by gently pipetting with 0.1% hyaluronidase and washed 3 times in TLH-PVA. Groups of 15 matured oocytes at the MII stage were randomly placed into 40 μl droplets of modified Tris-buffered medium (mTBM) in a 35x10 mm Petri dish (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA) covered with pre-warmed mineral oil. Subsequently, liquid semen supplied weekly from the Veterinary Service Laboratory (Department of Livestock Research, Yong-in, Korea) was kept at 17˚C for 5 days prior to use. The semen sample was washed twice with DPBS supplemented with 0.1% BSA by centrifuging at 2,000 x g for 2 min. After washing, the sperm pellet was resuspended in mTBM, as previously described (22), which had been pre-equilibrated for 18 h at 39˚C at 5% CO_2. After appropriate dilution, 5 μl of the sperm suspension were added to a 40 μl drop of fertilization medium (mTBM) to set a final sperm concentration of $1x10^6$ sperm/ml. Immediately before fertilization, sperm motility was assessed and >80% motile sperm was used in each experiment. To use stored liquid semen, a modified two-step culture system (23) was used. The oocytes were co-incubated with sperm for 20 min at 39˚C in a humidified atmosphere of 5% CO_2 and 95% air. After 20 min of co-incubation with sperm, the loosely attached sperm was removed from the zona pellucida (ZP) by gentle pipetting. Subsequently, the oocytes were washed 3 times in mTBM and incubated in mTBM without sperm for 5-6 h at 39˚C in a humidified atmosphere of 5% CO_2 and 95% air. Thereafter, the gametes were washed 3 times in embryo culture medium and cultured in 25 μl microdrops (10 gametes/drop) of porcine zygote medium 3 (PZM3), as previously described (24) with pre-warmed mineral oil. The embryos with cultured drops were incubated at 39˚C for 168 h under a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2. In all the experiments, the culture medium was renewed at 48 h (day 2) and 96 h (day 4) following IVF.

Embryo evaluation and total cell count of blastocysts. The day on which PA or IVF was performed was considered day 0. The embryos were evaluated under a stereomicroscope for cleavage on day 2 (48 h). Evenly cleaved embryos were classified into 3 groups (2-3, 4-5 and 6-8 cells). Blastocyst formation was assessed on day 7 (168 h) following IVF, and blastocysts were classified according to the degree of expansion and hatching status, as previously described (25): early blastocyst (small blastocyst with a blastocoel equal to or less than half of the embryo volume), expanded blastocyst (a large blastocyst with a blastocoel greater than half of the embryo volume or blastocyst with a blastocoel completely filling the embryo) and hatched blastocyst (hatching or already hatched blastocyst). To count the total cell number of blastocysts, on day 7, the blastocysts were collected and washed in 1% (wt/vol) PBS-BSA and stained with 5 μg/ml Hoechst 33342 (bisbenzimide) for 5 min. After a final wash in PBS-BSA, the embryos were briefly fixed in 4% paraformaldehyde in PBS. Subsequently, the blastocysts were mounted on glass slides in a drop of 100% glycerol, squashed gently with a cover slip and observed under a fluorescence microscope (Nickon Corp., Tokyo, Japan) at x400 magnification.

Gene expression analysis by quantitative reverse transcription polymerase chain reaction (RT-qPCR). For the gene expression analysis, groups (control, 1 and 3% ethanol) of 140-150 mature COCs were separately sampled under a stereomicroscope. All samples were stored at 80˚C until analysis. The mRNA expression of PCNA, PARP-1, Bak, Bax and caspase-3 in the COCs was analyzed by RT-PCR. Total RNA was extracted using TRIzol reagent (Invitrogen Corp.), according to the manufacturer's instructions, and the total RNA concentration was determined by measuring the absorbance at 260 nm. First-strand complementary DNA (cDNA) was prepared by subjecting 1 μg of total RNA to reverse transcription using Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen Corp.) and random primers (9-mers; Takara Bio, Inc., Otsu, Shiga, Japan). To determine the conditions for the logarithmic phase PCR amplification of target mRNA, 1-μg aliquots were amplified using differing numbers of cycles. The housekeeping gene, GAPDH, was PCR-amplified to rule out the possibility of RNA degradation and to control for the variation in mRNA concentrations in the reverse transcription (RT) reaction. A linear relationship between the PCR product band visibility and the number of amplification cycles was observed for the target mRNAs. The GAPDH and target genes were quantified using 40 cycles. The

Table I. Primer sequences used for gene expression analysis.

mRNA	Primer sequences	Product size (bp)	GenBank accession no.
GAPDH	F: 5'-GTCGGTTGTGGATCTGACCT-3' R: 5'-TTGACGAAGTGGTCGTTGAG-3'	207	NM_001206359.1
PCNA	F: 5'-CCTGTGCAAAAGATGGAGTG-3' R: 5'-GGAGAGAGTGGAGTGGCTTTT-3'	187	XM_003359883
PARP-1	F: 5'-ACTTTAAGACGTCCCTGTGG-3' R: 5'-GATTGGAGAAGTTGGGAAAA-3'	202	XM_003357641.1
Bax	F: 5'-TGCCTCAGGATGCATCTACC-3' R: 5'-AAGTAGAAAAGCGCGACCAC-3'	199	XM_003127290
Bak	F: 5'-GCGGAAAACGCCTATGAGTA-3' R: 3'-GCAGTGATGCAGCATGAAGT-5'	189	XM_001928147
Caspase-3	F: 5'-CGTGCTTCTAAGCCATGGTG-3' R: 5'-GTCCCACTGTCCGTCTCAAT-3'	186	NM_214131

cDNA was amplified in a 20 μl PCR reaction, which contained 1 U Taq polymerase (Intron Biotechnology, Inc., Seongnam, Korea), 2 mM deoxyribonucleoside triphosphate (dNTP) mix and 10 pM of each gene-specific primer. Quantitative PCR was performed with 1 μl cDNA template added to 10 μl 2X SYBR Premix Ex Taq (Takara Bio, Inc.) containing specific primers at a concentration of 10 pM each. The reactions were carried out for 40 cycles and the cycling parameters were as follows: denaturation at 95˚C for 30 sec, annealing at 55˚C for 30 sec and extension at 72˚C for 30 sec. All oligonucleotide primer sequences are presented in Table I. The fluorescence intensity was measured at the end of the extension phase of each cycle. The threshold value for the fluorescence intensity of all samples was set manually. The reaction cycle at which the PCR products exceeded this fluorescence intensity threshold was deemed the threshold cycle (Ct) in the exponential phase of the PCR amplification. The expression of each target gene was quantified relative to that of the internal control gene (GAPDH). The relative quantification was based on a comparison of Ct values at a constant fluorescence intensity. The amount of transcript present was inversely related to the observed Ct and, for each 2-fold dilution in the amount of transcript, Ct was expected to increase by one. The relative expression (R) was calculated using the following equation: $R = 2^{-[\Delta Ct\ sample - \Delta Ct\ control]}$. To determine a normalized arbitrary value for each gene, each obtained value was normalized to that of GAPDH. The experiments were repeated at least 4 times.

Experimental design. In experiment 1, the effects of various concentrations (0, 1 and 3%) of ethanol treatment during IVM on nuclear maturation were examined. In experiment 2, the effects of ethanol treatment during IVM on intracellular levels of GSH and ROS were investigated. In experiment 3, the effects of ethanol treatment during IVM on subsequent embryonic development of PA and IVF embryos were examined. In experiment 4, the effects of ethanol treatment on the mRNA expression of PCNA, PARP-1, Bak, Bax and caspase-3 in the COCs were analyzed.

Statistical analysis. Statistical analyses were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Percentage data (e.g., rates of maturation, cleavage, blastocyst formation and number of nuclei) were compared by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test. All results are expressed as the means ± SEM. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of ethanol on nuclear maturation during IVM. There was no significant difference in maturation (MII stage) between the control group (70.3%) and the 1% ethanol group (59.8%) (Table II). However, the 3% ethanol group (34.0%) showed a significantly decreased ($P<0.05$) number of MII stage oocytes compared with the control group. In addition, the 3% group had a significantly increased number of MI oocytes (19.3 vs. 11.0%) ($P<0.05$), as well as AT-I oocytes (44.3 vs. 17.5%) compared with the control group.

Effects of ethanol on intracellular GSH and ROS levels during IVM. Ethanol treatment decreased the intracellular GSH levels ($P<0.05$) and increased ROS generation ($P<0.05$) in MII oocytes derived from the maturation medium supplemented with 1 and 3% ethanol (Fig. 1).

Effects of the addition of ethanol to IVM medium on subsequent embryonic development following PA and IVF. Mature oocytes from each IVM group were subjected to PA. The cleavage rate was significantly lower ($P<0.05$) in the 3% ethanol group (51.6%) than the control group (86.9%) and the 1% group (78.8%) (Table III). For the cleavage pattern, there were significantly fewer 4- to 5-cell PA embryos in the 3% ethanol group than in the other groups (Fig. 2A). However, no significant differences were observed in the cleavage pattern of 2- to 3-cell PA embryos and 6- to 8-cell PA embryos. The 3% ethanol group had the lowest blastocyst

Table II. Effects of ethanol treatment during porcine IVM on nuclear maturation.

Ethanol concentration (%)	No. of oocytes cultured for maturation[a]	No. of oocytes at different stages of maturation			
		Germinal vesicle (%)	Metaphase I (%)	Anaphase-telophase I (%)	Metaphase II (%)
0 (control)	172	2 (1.2±1.2)	19 (11.0±0.4)[b]	30 (17.5±1.4)[b]	121 (70.3±1.5)[b]
1	167	2 (1.2±0.6)	20 (12.0±1.7)[b]	45 (27.0±3.9)[b]	100 (59.8±5.6)[b]
3	170	4 (2.3±1.5)	33 (19.3±2.3)[c]	75 (44.3±4.4)[c]	58 (34.0±2.2)[c]

[a]Experiments repeated 3 times; [b,c]Values with different letters in superscript within a column indicate a statistically significant difference (P<0.05). IVM, *in vitro* maturation.

Table III. Effects of ethanol treatment during IVM on embryonic development in porcine PA embryos.

Ethanol concentration (%)	No. of embryos cultured	No. of embryos developed into (%)		Total cell numbe in blastocyst (N)[a]
		≥2-cell embryos	Blastocysts	
0 (control)	130	113 (86.9±2.4)[b]	67 (51.5±2.7)[b]	65.0±5.1[b] (12)
1	118	93 (78.8±2.4)[b]	43 (36.4±5.0)[c]	52.3±3.8[c] (12)
3	97	50 (51.6±3.9)[c]	11 (11.3±0.3)[d]	30.6±3.1[d] (11)

The experiment was repeated 3 times. The data represent the ± SEM. [a]Number of examined blastocysts; [b-d]Values with different letters in superscript within a column indicate a statistically significant difference (P<0.05). IVM, *in vitro* maturation; PA, parthenogenetic activation.

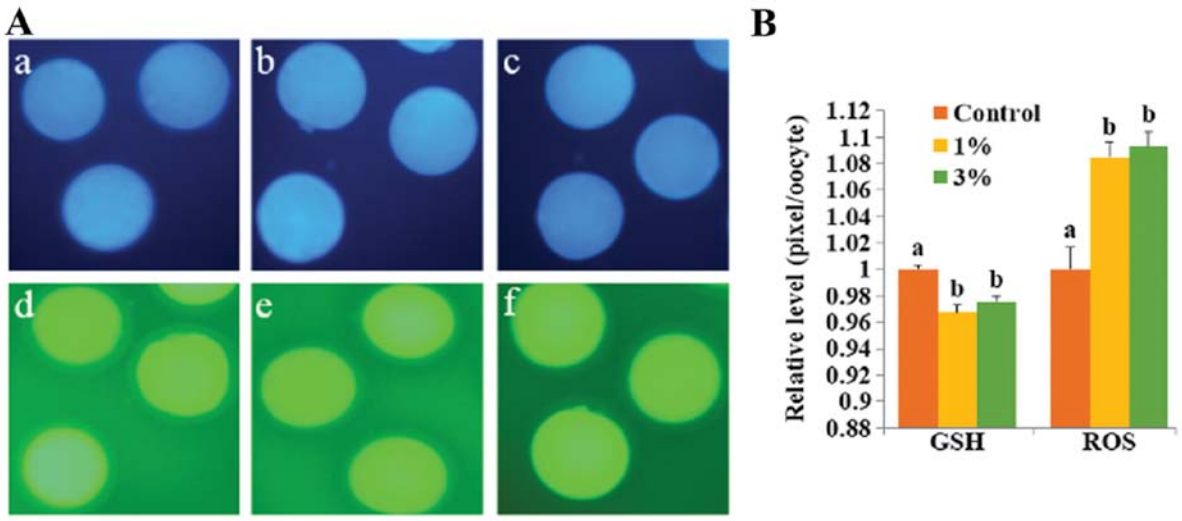

Figure 1. Epifluorescence photomicrographic images of *in vitro* matured porcine oocytes. (A) Oocytes were stained with (a-c) Cell Tracker Blue and (d-f) 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) to detect intracellular levels of glutathione (GSH) and reactive oxygen species (ROS), respectively. Metaphase II (MII) oocytes derived from the maturation medium supplemented with (a and d) 1% ethanol, (b and e) 3% ethanol, and (c and f) without ethanol. (B) Relative levels of intracellular GSH and ROS in *in vitro* matured porcine oocytes between the 3 groups (1, 3% and without ethanol treatment). For each analysis (GSH and ROS levels), bars with different letters (a and b) indicate a statistically significant difference between groups (P<0.05). Total no. of examined oocytes: GSH samples, n=28; ROS samples, nN=28. The experiment was replicated 3 times.

formation rates (P<0.05) and total cell numbers (11.3% and 30.6, respectively) compared with the other groups (Table III). The blastocyst formation rates and total cell number of the 1% ethanol group (36.4 and 52.3%, respectively) were significantly lower (P<0.05) than those of the control group (51.5 and 65.0%, respectively). The blastocyst formation rates and total cell number of blastocyts tended to decrease as the ethanol concentration increased (Table III). When comparing blastocyst formation patterns on day 7, early, expanded and hatched PA blastocyst numbers were significantly (P<0.05) lower in the 3% ethanol group than in the other groups (Fig. 2B). However, there were no statistically significant differences in

Table IV. Effects of ethanol treatment during IVM on embryonic development following IVF.

Ethanol concentration (%)	No. of embryos cultured	No. of embryos developed into (%)		Total cell number in blastocyst (N)[a]
		≥2-cell embryos	Blastocysts	
0 (control)	201	142 (70.6±0.7)[b]	57 (28.4±3.3)[b]	64.8±4.5[b] (12)
1	183	121 (65.6±1.3)[c]	40 (21.9±2.0)[c]	50.2±2.5[c] (13)
3	137	73 (53.3±1.5)[d]	4 (2.9±1.4)[d]	27.3±4.7[d] (4)

The experiment was repeated 5 times. The data represent the means ± SEM. [a]Number of examined blastocysts; [b-d]Values with different letters in superscript within a column indicate a statistically significant difference (P<0.05). IVM, *in vitro* maturation; IVF, *in vitro* fertilization.

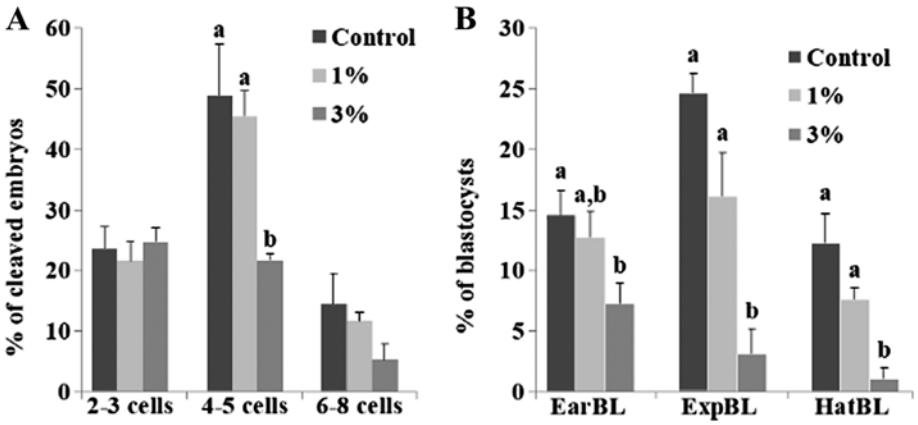

Figure 2. (A) Effects of various concentrations of ethanol during *in vitro* maturation (IVM) on the cleavage pattern of parthenogenetic activation (PA) embryos on day 2 and (B) the percentage of PA embryos that developed into blastocysts on day 7. For each endpoint, bars with different letters (a and b) indicate a statistically significant difference between groups (P<0.05) for different concentrations of ethanol. EarBL, early blastocyst; ExpBL, expanded blastocyst; HatBL, hatched blastocyst. The experiment was repeated 3 times.

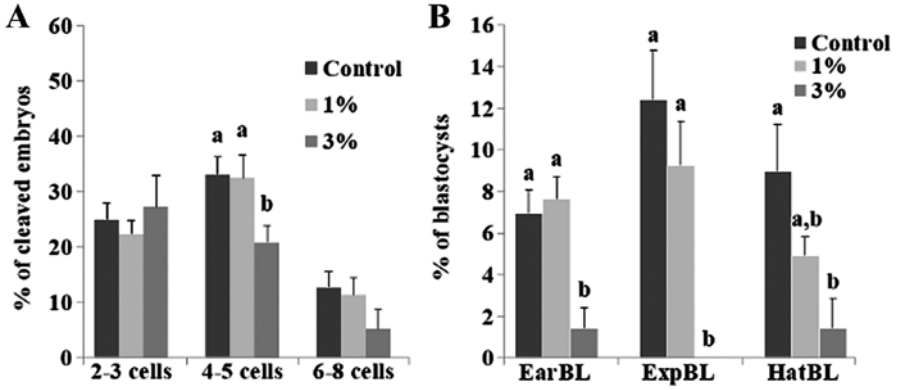

Figure 3. (A) Effects of various concentrations of ethanol during *in vitro* maturation (IVM) on the cleavage pattern of *in vitro* fertilization (IVF) embryos on day 2 and (B) the percentage of IVF embryos that developed into blastocysts on day 7. For each endpoint, bars with different letters (a and b) indicate a statistically significant difference (P<0.05) for different concentrations of ethanol. EarBL, early blastocyst; ExpBL, expanded blastocyst; HatBL, hatched blastocyst. The experiment was repeated 5 times.

the blastocyst formation patterns between the control and the 1% ethanol groups.

As shown in Table IV, *in vitro* fertilized embryos from the 3% ethanol group displayed the lowest cleavage rates (P<0.05), blastocyst formation rates and total cell numbers (53.3, 2.9 and 27.3%, respectively) as compared with the other groups. The cleavage rates, blastocyst formation rates and total cell numbers were significantly lower (P<0.05) in the 1% ethanol group (65.6, 21.9 and 50.2%, respectively) than the control group (70.6, 28.% and 64.8%, respectively). On day 2, there were significantly fewer 4- to 5-cell IVF embryos in the 3% ethanol group compared with the other groups, and no significant difference was observed in the cleavage patterns of 2- to 3-cell IVF embryos and 6- to 8-cell IVF embryos (Fig. 3A). The early, expanded and hatched IVF blastocyst formation rates on day 7 were significantly (P<0.05) lower in the 3% ethanol group

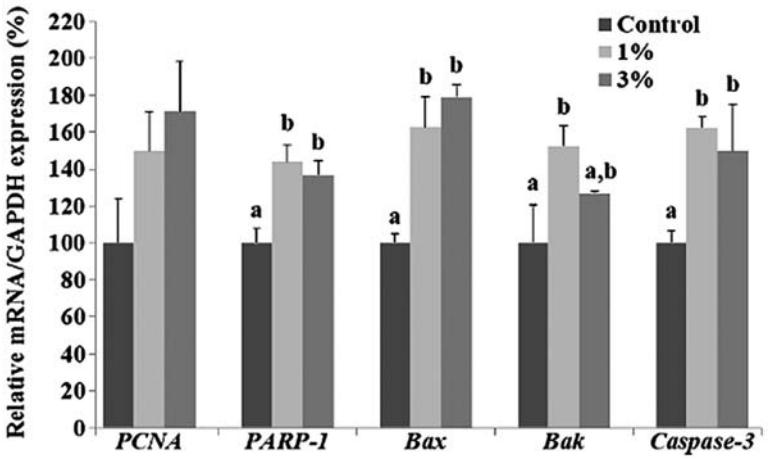

Figure 4. mRNA expression levels (mean ± SEM) of proliferating cell nuclear (PCNA), poly(ADP-ribose) polymerase-1 (PARP-1), Bax, Bak, and caspase-3 in cumulus oocyte complexes (COCs) treated with ethanol during *in vitro* maturation (IVM). Within the same target mRNA, bars with different letters indicate a statistically significant difference between groups (P<0.05). The experiment was replicated 3 times.

than in the other groups (Fig. 3B). However, no statistically significant difference was observed between the control and 1% ethanol groups.

Effects of ethanol treatment during IVM on gene expression in the COCs. To examine the expression of DNA repair-related and apoptosis-related genes, we evaluated the mRNA expression levels of PCNA, PARP-1, Bax, Bak and caspase-3 in the COCs of each group (Fig. 4). The COCs derived from the the 1% ethanol group showed significantly higher (P<0.05) mRNA expression levels of PARP-1, Bax, Bak and caspase-3 compared to those from the control group. The 3% ethanol group had significantly increased (P<0.05) PARP-1, Bax, Bak and caspase-3 mRNA expression levels in the COCs compared with the control group. No significant difference in PCNA transcript levels was observed in the 1 or 3% ethanol groups compared with the control group.

Discussion

Assisted reproduction technology (ART) strategies, including IVP of embryos, have been developed and used over several decades, and their application in porcine reproduction is well established worldwide (26). However, there are still many unresolved issues in current IVM-IVF systems, which are inefficient compared with *in vivo* systems.

The pig is a particularly difficult species in which to obtain high rates of fertilization and subsequent blastocyst development *in vitro* (27). The major obstacles that still need to be overcome are *in vitro* oocyte cytoplasmic maturation and the high incidence of polyspermy following IVF, as well as a low development rate and poor quality of blastocysts at the end of culture (28,29). Therefore, the addition of various pharmacological compounds has been examined in an attempt to improve the quality of *in vitro*-produced porcine embryos. During these efforts, ethanol has been frequently used as a solvent for some compounds, such as lanosterol and MAS (3,4). However, studies on the effects of ethanol treatment during IVM are limited. In the present study, we demonstrated that treatment with a low ethanol concentration during IVM had detrimental effects on oocyte maturation and the subsequent embryonic development of PA and IVF embryos.

Oocyte maturation includes both nuclear and cytoplasmic maturation. First, in terms of nuclear maturation, IVM medium with 3% ethanol significantly decreased the maturation rate. This finding therefore suggests that 3% ethanol-treated oocytes fail to progress beyond the AT-I stage. We also investigated intracellular GSH and ROS levels to examine cytoplasmic maturation. Intracellular levels of GSH and ROS are critical factors that influence oocyte IVM and oocyte developmental potential following PA and IVF (19,30,31). Intracellular GSH is a molecular marker in mature oocytes that predicts cytoplasmic maturation in porcine oocytes (32), and low intracellular GSH concentrations are responsible for lower developmental competence in porcine oocytes (33). Moreover, intracellular GSH plays a pivotal role protecting cells against the destructive effects of reactive oxygen intermediates and free radicals (34). In our study, ethanol treatment (1 and 3%) during IVM significantly increased intracellular ROS levels and decreased intracellular GSH levels. We demonstrated that ethanol treatment at levels >1% had detrimental effects on the cytoplasmic maturation of porcine oocytes.

Embryonic development and blastocyst viability following PA and IVF also decreased by 1 and 3% ethanol treatment due to the detrimental effects of ethanol on cytoplasmic maturation. A previous study that examined the effects of ethanol on bovine embryonic development demonstrated that the cleavage rate was not significantly affected by 1% ethanol, but that the blastocyst formation rate of IVF was significantly decreased by 1% ethanol (35). In comparison with bovine, our results indicated that 1% ethanol significantly decreased porcine blastocyst formation rates and also decreased the cleavage rate of IVF porcine embryos. This difference may be due to the different species used or to the fact that porcine COCs are more sensitive to ethanol.

Modifications in culture environment modulate gene expression in mammalian cells and embryos (36,37). For example, mouse blastocyst formation depends on oocyte transcripts

generated before fertilization (38). The presence of surrounding cumulus cells is responsible for appropriate oocyte maturation and the developmental competence of oocytes. Cumulus cells participate in oocyte development during IVM by secreting soluble factors, which induce developmental competence (39). Moreover, apoptosis plays a critical role in development and differentiation. Environmental stressors, such as those created by *in vitro* culture, can cause unplanned apoptosis in cultured embryos, which may lead to embryonic arrest or abnormal development and lower embryo viability (40,41). Therefore, we examined the mRNA expression levels of DNA repair-related and apoptosis-related genes in the COCs. PCNA is a necessary component of DNA repair machinery, and a PCNA signal suggests the activation of the DNA repair process (42,43). In this study, no significant differences in PCNA transcript levels were observed in the 1 or 3% ethanol groups compared with the control group. However, considering that the PCNA transcript tended to increase as ethanol concentration increased, this result appears to be due to DNA damage induced by ethanol. PARP-1 is emerging as an important activator of caspase-independent cell death (18). Among multiple apoptotic pathways activated in neurons by ethanol, PARP-1 pathways also play a role in the subsequent apoptotic death (14). In the present study, the 1 and 3% ethanol groups had significantly increased PARP-1 transcript levels in the COCs compared to the control group. These results indicate that the PARP-1-related apoptotic pathway may be induced by ethanol treatment during IVM in the COCs. The expression of PARP-1 may be useful as another indicator of apoptosis in porcine oocytes. Bax, Bak, and caspase-3 are also associated with apoptosis initiated by the mitochondrial release of apoptogenic factors (44). In this study, the 1% ethanol group had increased Bax, Bak and caspase-3 transcript levels in the COCs, and the 3% ethanol group had increased Bax and caspase-3 transcript levels in the COCs, thereby suggesting that ethanol promoted the expression of pro-apoptotic genes in the intrinsic apoptotic pathway in oocytes and cumulus cells. Fine modulation of gene expression plays a key role in the proper alterations and transitions that occur during oocyte maturation and development. These processes seem to be largely under the control of post-transcriptional regulatory mechanisms and mostly driven by cytoplasmic components (45). In this regard, changes in gene expression levels are likely related to the observed decrease in the blastocyst formation rate and blastocyst quality (less expanded and hatched blastocysts in the 1 and 3% ethanol-treated groups), which may have also affected the number of nuclei in the blastocysts. The decreased cell number in blastocysts derived from ethanol-treated mature oocytes may be due to promoted apoptosis. These results suggest that ethanol supplementation to the maturation medium significantly affects the expression of apoptosis-related genes in mature oocytes and cumulus cells, thereby leading to an overall increase in their susceptibility to apoptosis.

The molecular mechanisms through which ethanol exerts its pro-apoptotic effects on oocyte maturation and subsequent embryonic development are unclear. In previous studies that examined ethanol-induced oxidative stress on fetal cortical neurons, it was shown that multiple apoptotic pathways were activated in neurons by ethanol, including the well-documented intrinsic components associated with caspase (caspase-3) activation and DNA damage (46,47). In cultured fetal cortical rat neurons, ethanol elicits an increase in ROS and, subsequently, a decrease in cellular GSH (46). DNA damage caused by ROS then activates PARP-1-related DNA repair processes. However, with longer term ethanol exposure, the hyperactivation of PARP-1 occurs, which may cause pro-apoptotic activities (14). Yu *et al* (48,49) demonstrated that PARP-1 activation signals cause the translocation of apoptosis-inducing factor (AIF), a 67 kDa flavoprotein, from the mitochondria to the nucleus, resulting in a caspase-independent pathway of programmed cell death. In turn, the upregulation of PARP-1 expression and poly(ADP-ribose) (PAR) formation cause apoptotic cell death (14, 50).

In this study, we demonstrated that ethanol promoted the expression of both pro-apoptotic genes in the intrinsic apoptotic pathway and PARP-1 in oocytes and cumulus cells. It is therefore suggested, that ethanol increases intracellular ROS levels, which is strongly related to apoptosis, thus resulting in two possible mechanisms performed by its cascade of caspase-dependent apoptotic events and PARP-1-related apoptotic pathways (caspase-independent).

In conclusion, ethanol treatment during IVM was detrimental for the cytoplasmic maturation of porcine oocytes by increasing intracellular ROS levels, thereby decreasing GSH concentrations and upregulating apoptosis-related genes. Furthermore, porcine oocytes treated with >1% ethanol may have reduced developmental competence, which would greatly decrease blastocyst formation and the total cell number of blastocysts in PA- and IVF-derived embryos. Therefore, our results suggest that ethanol diminishes the quality of porcine oocytes and the subsequent *in vitro* development when IVM medium is supplemented with >1% ethanol.

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