Autophagy Inhibitor, 3-Methyladenine, Reduces Preimplantation Development and Blastocyst Qualities in Pigs

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ABSTRACT

Autophagy is a process of intracellular bulk protein degradation, in which the accumulated proteins and cytoplasmic organelles are degraded. It plays important roles in cellular homeostasis, apoptosis, and development, but its role during early embryo development remains contentious. Therefore, in the present study, we investigated the effects of 3-methyladenine (3-MA) on early embryonic development in pigs. we also investigated several indicators of developmental potential, including mitochondrial distribution, genes expressions (autophagy-, apoptosis- related genes), apoptosis and ER-stress, which are affected by 3-MA. After *in vitro* maturation and fertilization, presumptive pig embryos were cultured in PZM-3 medium supplemented with 3-MA for 2 days at 39℃, 5% CO_2 in air. Developmental competence to the blastocyst stage in the presence of 3-MA was gradually decreased according to increasing concentration. Thus, all further experiments were performed using 2 mM 3-MA. Blastocysts that developed in the 3-MA treated group decreased LC3-II intensity and expressions of autophagy related genes than those of the untreated control, resulting in down-regulates the autophagy. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) showed that the number of containing fragmented DNA at the blastocyst stage increased in the 3-MA treated group compared with control (6.0±1.0 vs 3.3±0.6, $p<0.05$). Also, the expression of the pro-apoptotic gene Bax increased in 3-MA treated group, whereas expression of the anti-apoptotic gene Bcl-XL decreased. Mito Tracker Green FM staining showed that blastocysts derived from the 3-MA treated group had lower mitochondrial integrity than that of the untreated control, resulting in decrease the embryonic qualities of preimplantation porcine blastocysts. Then, the expression of the spliced form of pXBP-1 product (pXBP-1s) increased in 3-MA treated group, resulting increase of ER-stress. Taken together, these results indicate that inhibition of autophagy by 3-MA is closely associated with apoptosis and ER-stress during preimplantation periods of porcine embryos.

(Key words : Autophagy, 3-Methyladenine, Apoptosis, Embryo development, Pig)

INTRODUCTION

Autophagy is an intracellular bulk protein degradation process and recycling of cellular constituents, in which the most long-lived proteins, entire organelles for cell survival and cellular maintenance are degraded (Gozuacik and Kimchi, 2004). This process is ubiquitously observed in eukaryotes from yeast to mammals (Klionsky *et al.*, 2007). Autophagy has been implicated in various physiological processes, including turnover of cytoplasmic components, cellular differentiation, nutrient starvation, and pathogenic infections (Gozuacik and Kimchi, 2004; de Bruin and Medema, 2008). In the autophagic process, double-membrane vesicles termed autophagosomes are formed, which subsequently fused with lysosomes, where their contents are degraded (Klionsky *et al.*, 2007; Ren *et al.*, 2009). Therefore, autophagy plays an important role in survival mechanism in conditions of stress. Autophagy can also triggers a form of cell death known as autophagic cell death or programmed cell death. However, its role in the preimplantation development of porcine embryos has not been studied adequately.

Mitochondria play a role in the oocytes and preimplantation embryos by supplying adenosine triphosphate (ATP) for fertilization and preimplantation embryo development (Jeong *et al.*, 2009; Wang *et al.*, 2009) and have a role in the regulation of apoptosis by controlling the release of mitochondrial proteins such as cyto-

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chrome C and other pro-apoptotic factors. Subsequently, the release of cytochrome C which activates caspase cascade pathway induces cellular degradation (Thouas *et al.*, 2004). Researchers reported that inadequate distribution of mitochondria may result in poor oocyte fertilization and embryo development (Wang *et al.*, 2009; Thouas *et al.*, 2004). Consequently, we hypothesized that inhibition of autophagy may affect embryonic quality, including mitochondrial distribution and apoptosis.

Another important organelle is endoplasmic reticulum(ER). The ER is the initial compartment in the secretory pathway. It is factory responsible for the synthesis, post-translational modification, proper folding, delivery of proteins to their proper target sites, as well as a regulator of intracellular calcium homeostasis (Huang *et al.*, 2009; Lee *et al.*, 2010). The ER is vulnerable to various forms of stress, including expression of mutant proteins, viral infection, nutrient deprivation, extreme environmental conditions, causing the accumulation of unfolded and misfolded proteins (Wu and Kaufman, 2006). UPR includes: (1) transcriptional up-regulation of ER chaperones and folding enzymes to increase the ability of ER in processing unfolded proteins, (2) translational attenuation to limit further accumulation of misfolded proteins, and (3) ER-associated degradation (ER-AD) which eliminates unfolded proteins from the ER (Huang *et al.*, 2009; Schroder and Kaufman, 2005). Thus, excessive stresses lead to cells apoptosis (Lee *et al.*, 2010). Several studies also have reported a linkage between autophagy and ER stress (Kawakami *et al.*, 2009).

Autophagy plays an important role in various cellular processes. However, the role of autophagy in porcine embryos has not yet been reported. Therefore, this study investigated the role of autophagy in preimplantation development and qualities of porcine embryos focusing on apoptosis, mitochondrial distribution, and ER stress.

MATERIALS AND METHODS

Chemicals

Unless noted otherwise, all chemicals used in the present study were purchased from Sigma Aldrich Korea (Yongin, Korea).

In Vitro Maturation (IVM)

Porcine ovaries were collected from a local slaughterhouse and transported to the laboratory at 30~35℃ in 0.9% saline. Cumulus oocyte complexes (COCs) were collected from follicles 3~6 mm in diameter by aspiration through a 10 ml with an 18-gauge needle. After washing three times with TL-Hepes medium (Funahashi *et al.*, 1994), approximately 50 oocytes were matured in 500 μl of *in vitro* maturation medium in a four-well multi-dish (Nunc, Roskilde, Denmark) at 39℃, 5% CO_2 in air. North Caorlina State University-23 (NCSU-23) medium (Petters and Wells, 1993) was used for oocyte maturation with 10% follicular fluid, 0.57 mM cysteine, 10 ng/ml β-mercaptoethanol, 10 ng/ml EGF, 10 IU/ml pregnant mare's serum gonadotropin (PMSG), and 10 IU /ml human chorionic gonadotropin (hCG). After 22 h culture, oocytes further cultured for another 22 h in maturation medium without PMSG and hCG.

In Vitro fertilization (IVF)

After IVM stage, the oocytes were subjected to IVF as described by Abeydeera and Day (1997). IVF was performed in modified Tris-buffered medium (mTBM), consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris (crystallized free base; Fisher Scientific, Fair Lawn, NJ, USA), 11 mM glucose, 5 mM sodium pyruvate, and no antibiotics. Ejaculated fresh semen was washed three times by centrifugation (100 g for 3 min at room temperature) with Dulbecco's phosphate buffered saline (DPBS; Gibco-BRL, Grand Island, NY, USA) supplemented with 1 mg/ml bovine serum albumin (BSA; Fraction V; Sigma), 100 mg/ml penicillin G and 75 mg/ml streptomycin sulfate. After washing, the spermatozoa were suspended in mTBM at pH 7.8. Oocytes were washed three times in mTBM with 2.5 mM caffeine sodium benzoate and 4 mg/ml BSA (fatty acid free) and placed into 48 μl of mTBM under paraffin oil. Diluted spermatozoa (2 μl) were added to 48 μl of mTBM containing 15~25 oocytes to give a final concentration of 1.5×10^5 sperms/ml. The oocytes were co-incubated with the spermatozoa for 6 h at 39 ℃ in an atmosphere of 5% CO_2 in air.

In Vitro Culture (IVC) and Assessment of Embryo Quality

After IVF, the embryos were cultured in 50 μl drops of PZM-3 supplemented with 3 mg/ml BSA at 39 ℃ in an atmosphere of 5% CO_2 in air. At this stage, 3-methlyadenine (1, 2 and 5 mM) was added to the culture medium. After 2 days of culture, the cleaved embryos were transferred into fresh culture medium: PZM-3 without 3-methlyadenine. The cleaved embryos were cultured for an additional 4 days to evaluate their ability to develop to the blastocyst stages.

Immunofluorescence Staining

To investigate the expression of autophagy related protein (LC3-II), blastocysts (Day 6) were washed three times with 0.1% polyvinylpyrrolidone (PVP) in DPBS and fixed for 1 hr in 4% (v/v) paraformaldehyde diluted in DPBS at 4℃. For membrane permeabilization, the fixed embryos were incubated in DPBS containing 2% (v/v) Triton X-100 for 1 hr at room temperature.

The fixed embryos were incubated primary antibody, against LC3-II (Cell Signaling, Danvers, MA, USA) for overnight at 4℃ and then with FITC-labeled secondary antibody (Invitrogen, Carsbad, CA, USA). Hoechst 33342 was used to stain the nuclei. Whole-mount embryos were examined under a laser-scanning confocal microscope (Carl Zwiss Meditec, Oberkochen, Germany). Intensity of LC3-II was measured with the Image J 1.38x program (NIH- http://rsb.info.nih.gov/ij/).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-end Labeling

Apoptotic cells in blastocysts were detected using the In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany). On day 6, blastocysts were washed three times with 0.1% PVA in DPBS and fixed in 4% (v/v) formaldehyde diluted in DPBS solution for 1 hour at room temperature. For membrane permeabilization, the fixed embryos were incubated in DPBS containing 0.1% (v/v) Triton X-100 for 1 hr at 4℃. The fixed embryos were incubated in Terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end-labelling (TUNEL) reaction medium for 1 h at 39℃ in the dark, and then washed and then transferred into 2 mg/ml of DAPI and mounted on slides. After TUNEL and DAPI staining, whole-mount embryos were examined under an epifluorescence microscope (Olympus, Tokyo, Japan) to determine the number of apoptotic nuclei and the total number of nuclei.

Mitochondrial Staining and Image Analysis

To evaluate the distribution of mitochondria, selected porcine blastocysts were washed three times with 0.1% PVA in DPBS and stained with 100 nM Mito Tracker Green FM in DPBS with 0.1% PVA. For fixation, the blastocysts were fixed in 4% (v/v) formaldehyde diluted in DPBS solution for 1 hr at room temperature. After fixation, the nuclei were stained with Hoechst 33342. The blastocysts were washed three times with 0.1% PVA in DPBS and then transferred into 2 mg/ml of DAPI and mounted on slides. After staining, whole-mount embryos were examined under a laser-scanning confocal microscope (Carl Zwiss Meditec, Oberkochen, Germany). Intensity of Mito Tracker Green FM was analyzed using Image J program (NIH- http://rsb.info.nih.gov/ij/).

Total RNA Isolation and cDNA Synthesis

The cleaved embryos and *in vitro* produced blastocysts were transferred into 0.1% PVA with DPBS. Then groups of 40 embryos and 10 blastocysts were washed three times and transferred into 1.7 ml microtube with a minimum amount of DPBS. Following the transfer into centrifuge tubes, samples were snap frozen and kept at −80℃ until the use for the RNA isolation. Total RNA was isolated using the Dynabeads mRNA direct kit (DYNAL; Invitrogen, Carsbad, CA, USA) and Rne asyplus micro kit (Qiagen, Chatsworth, CA, USA) according to the manufacture's instructions. The RNA was reversed transcribed in a 20 μl reaction mixture containing 8 μl RNA, 50 ng/μl random hexamers, 10 mM dNTP mix, 10X RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, RNase OUT 40 U/μl, and superscript III RT 200 U/μl. The reaction was carried out as follows: 65℃ for 5 min, 50℃ for 50 min, 85℃ for 5 min and chill to 4℃. Following inactivation of cDNA, that can be used as a template for amplification PCR.

Real-time Reverse Transcription-Polymerase Chain Reaction

Real-time quantitative PCR was performed using an Applied Stratagene mx 3000 p QPCR System (San Diego, CA, USA) in a final reaction volume of 20 μl with SYBR Green (Applied Biosystems, Foster City, CA, USA), a double-strand DNA-specific fluorescent dye. For each the quantification, a 2 μl aliquot of the reverse transcribed reaction was used. All samples were quantified simultaneously during the same run with the housekeeping gene GAPDH. The primers used for real-time RT-PCR are shown in Table 1. The amplification process consisted of pre-incubation for fast-start polymerase activation at 95℃ for 10 min, followed by 45 amplification cycles of denaturation at 95℃ for 40 sec, annealing at 58~60℃ for 40 sec, and elongation at 72℃ for 45 sec, and then acquisition of fluorescence. After the end of the last cycle, the melting curve was generated by starting fluorescence acquisition at 72℃ and taking measurements every 0.1℃ until 95℃ was reached. After completion of quantitative PCR analysis, the PCR products were electophoresed on a 2% agarose gel, stained with ethidium bromide and visualized by exposure to ultraviolet light. Images were obtained using a Gel Doc apparatus (Cell Biosciences, Santa Clara, CA, USA).

Statistical Analysis

Each experimental were repeated more than three times. All percentage data were subjected to arcsine transformation. All percentage data and datasets obtained in the present study are presented as the mean±standard deviation (SD) throughout the text. Maturation and fertilization rates of porcine oocyte development and cell numbers in blastocysts were analyzed by ANOVA and Student's *t*-test. A probability of $p<0.05$ was considered significant.

RESULTS

Effect of 3-MA Treatment on Preimplantation Development of Porcine Embryos

Table 1. Primer sequences for RT-PCR or real-time RT-PCR

Genes (GenBank accession number)	Primer sequences	Annealing temperature(℃)	Product size (bp)
Map1lc3b (NM_001190290)	F:CCGAACCTTCGAACAGAGAG R:AGGCTTGGTTAGCATTGAGC	60	206
Lamp2 (NM_001190290)	F:CACCCACTCCAAAGGAAAAA R:GGTTGTCGTTTTTCACAGCA	60	246
Bax (AJ606301)	F:AACGCCATTGGAGATGAACT R:CGATCTCGAAGGAAGTCCAG	60	183
Bcl-XL (AF213205)	F:AGGGCATTCAGTGACCTGAC R:TGGATCCAAGGCTCTAGGTG	60	183
sXBP1 (FJ213449.1)	F:GCAGAGACCAAGGGGAATGG R:CTGGGTCGACTTCTGGGAGC	58	485 (unspliced) 463 (spliced)
GAPDH (U07786)	F:GGGCATGAACCATGAGAAGT R:AAGCAGGGATGATGTTCTGG	60	104

There are no previous reports regarding the concentration and effects of 3-MA to use in porcine embryo culture experiments. In the present study, we investigated the effect of 3-MA on preimplantation development of pig embryos. After completion of IVF, presumptive porcine embryos were cultured in PZM-3 medium containing 0.3% BSA supplemented with or without 3-MA at different concentration (0, 1, 2 and 5 mM). As shown in Table 2, developmental competence to the blastocyst stage was significantly decreased in groups treated with 3-MA compared with untreated control group (37.0±9.4 vs 25.7±9, 21.0±5.7, and 10.5±4.6; $p<0.05$). Namely, the rates of blastocyst formation were decreased with increasing 3-MA concentration. Then, all further experiments were performed using 2 mM 3-MA.

Effect of 3-MA Treatment on the LC3-II Expression in Blastocyst Stage Porcine Embryos

In general, LC3-II has been used as indicator of autophagy. Thus, we also examined the effect of 3-MA on the expression level of LC3-II. To determine the fluorescence intensity of LC3-II expression, immunostaining was performed in blastocyst stage porcine embryos derived from 3-MA treated and untreated control groups. Also, level of autophagy-related mRNA expression was evaluated in blastocyst stage porcine embryos by using semi-quantitative RT-PCR. Results showed that the fluorescence intensity of LC3-II protein in the 3-MA treated group was significantly lower than that of control group (1.5±0.1 vs 0.5±0.1, $p<0.05$; Fig. 1, Table 3). Also, the mRNA expression levels of Map1lc3b and Lamp2 decreased compared with those of the control group (Fig. 2). This result indicates that 3-MA inhibits the role of autophagy during preimplantation porcine embryos.

Table 2. Effect of various concentrations of 3-MA on developmental ability of porcine embryos *in vitro*

Concentration	No. of oocytes examined	No. (%) of embryos cleaved	No. (%) of blastocysts
Control	189	151 (81.1±13.1)	68 (37.0±9.4)[a]
1 mM	189	157 (84.5±10.8)	48 (25.7±9.0)[b]
2 mM	191	157 (82.0±11.1)	40 (21.0±5.7)[b]
5 mM	203	143 (69.1±16.6)	23 (10.5±4.6)[c]

Embryos were treated with various concentrations of 3-MA for 48 h. Data are representative results in six independent experiments, and expressed as mean±SD. Values with different superscripts within a column differ significantly ($p<0.05$).

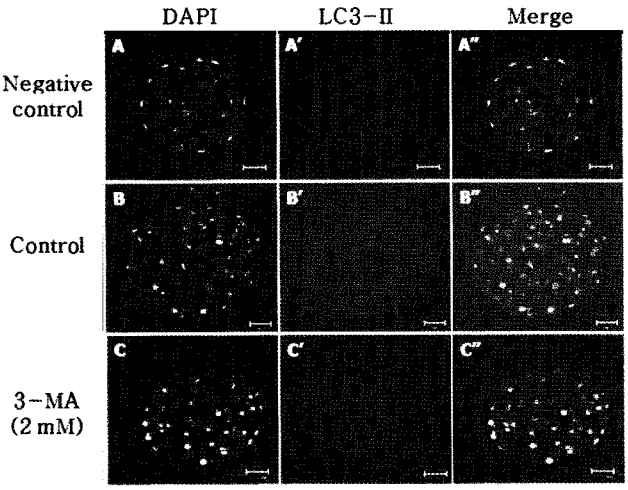

Fig. 1. Comparison of LC3-II expression patterns in porcine blastocysts after immunostaining. Negative control (A-A"), control (B-B"), and 3-MA (C-C") treated blastocyst. Scale bars=50 μm.

Table 3. Effect of 3-MA on LC3-II expression level in porcine blastocysts

Group	No. of blastocysts	Degree of intensity (mean±SD)
Control	10	1.5±0.1[a]
3-MA (2 mM)	11	0.5±0.1[b]

This experiment was replicated three times. Data are the mean± SD. Values with different superscripts within a column differ significantly ($p<0.05$).

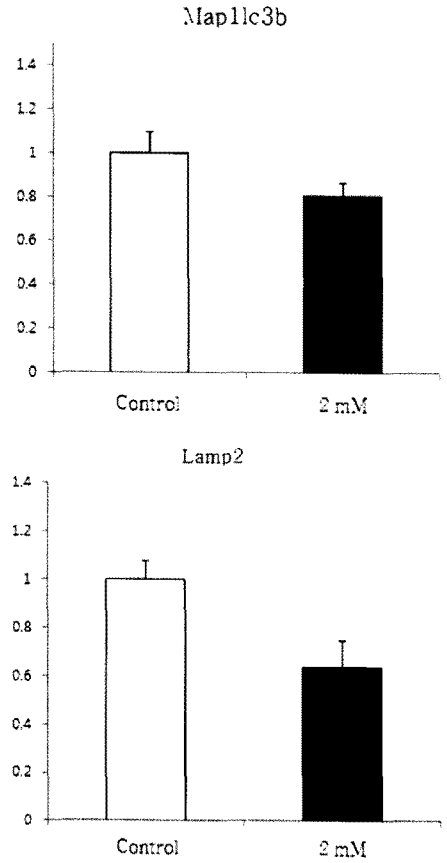

Fig. 2. Relative mRNA expression levels of autophagy-related genes in the blastocyst stage embryos by using real-time RT-PCR.

Apoptosis and Gene Expression in Blastocyst Stage Embryos Derived from 3-MA Treated Embryos

Susceptibility to apoptosis was assessed in bastocyst stage embryos derived from 3-MA treated and untreated embryos. Apoptosis was measured using the TUNEL assay, which allows for the identification of fragmented DNA. The number of TUNEL-positive nuclei increased significantly in blastocysts derived from 3-MA treated embryos compared with untreated control (6.0± 1.0 vs 3.3±0.6, $p<0.05$; Fig. 3, Table 4). Then, to investigate the expression of apoptosis-related genes, we used real-time RT-PCR to determine the relative abundance

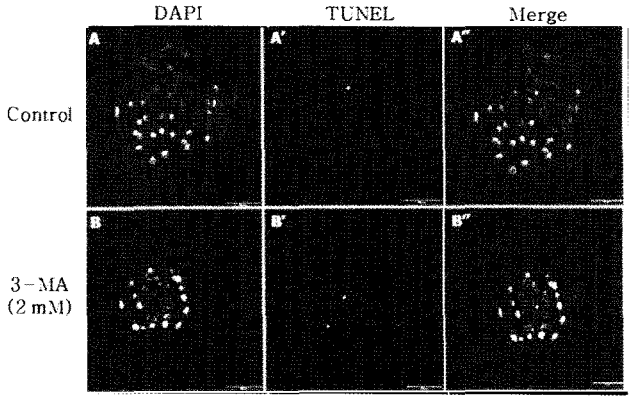

Fig. 3. Epifluorescent images of porcine blastocysts undergoing apoptosis *in vitro*. The chromatin content is stained by DAPI (blue; A, B), fragmented DNA is labeled by the TUNEL reaction (green; A', B'), and colocalization with DAPI appears sky-blue (A", B"). Scale bars = 100 μm.

Table 4. Apoptotic patterns in porcine blastocysts derived from 3-MA treated and untreated control

Group	No. of blastocysts	Apoptosis (nuclei)	
		DAPI	TUNEL
Control	18	50.4±6.2[a]	3.3±0.6[a]
3-MA (2 mM)	15	39.3±4.5[b]	6.0±1.0[b]

This experiment was replicated three times. Data are the mean ±SD. Values with different superscripts within a column differ significantly ($p<0.05$).

of Bax and Bcl-XL transcripts in blastocyst stage embryos. Results showed that the expression of pro-apoptotic gene (Bax) in a 3-MA treated group was higher than that of untreated control. However, the mRNA expression of the anti-apoptotic gene (Bcl-XL) in 3-MA treated group was decreased (Fig. 4).

Effect of 3-MA on Mitochondrial Distribution in the Blastocyst Stage Embryos

To examine the effect of 3-MA on mitochondrial distribution, we detected the fluorescence intensity of mitochondria in the porcine blastocysts stained by Mito Tracker, and data were analyzed by using confocal microscopy and Image J program. Results showed that mitochondrial fluorescence intensity of blastocysts derived from untreated control group was higher than that of 3-MA treated group (1.3±0.1 vs 0.9±0.3; Fig. 5, Table 5). This result indicates that 3-MA affects mitochondrial distribution in porcine embryonic cells.

ER Stress-related Gene (XBP-1) Expression in Cleavage Stage Embryos Derived from 3-MA Treated Embryos

ER-stress induced by inhibitor of autophagy is kn-

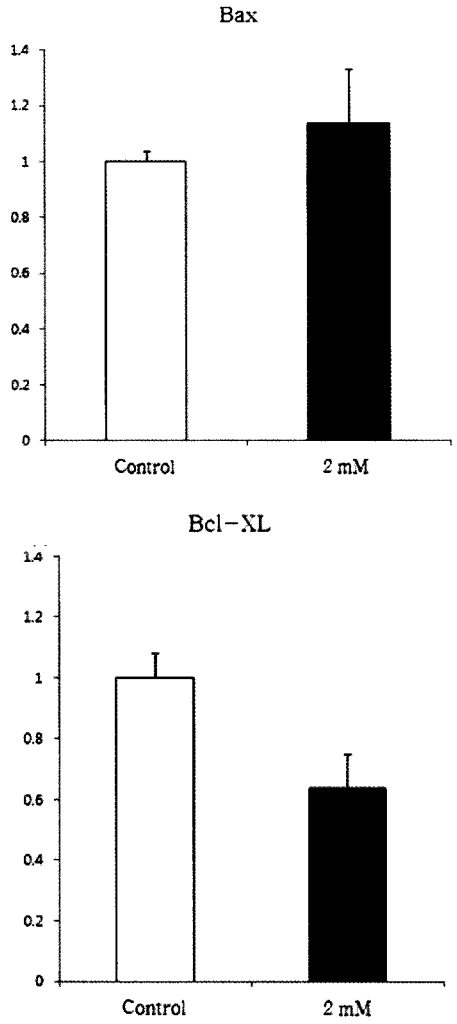

Fig. 4. Relative mRNA expression levels of apoptosis-related genes in the blastocyst stage embryos by using real-time RT-PCR.

Table 5. Effect of 3-MA on mitochondrial distribution in porcine blastocysts

Group	No. of blastocysts	Degree of intensity (mean±SD)
Control	13	1.3±0.1
3-MA (2 mM)	13	0.9±0.3

This experiment was replicated three times. Data are the mean ±SD.

own to regulate cell survival pathway in many cell types. Then, susceptibility to ER-stress was assessed in cleavage stage embryos (4-cell) derived from 3-MA treated and untreated embryos. As shown in Fig. 6, the spliced form of pXBP-1 was detected in the 3-MA treated groups (2 mM and 5 mM), but it is not detected in 3-MA treated group of 1 mM and control group.

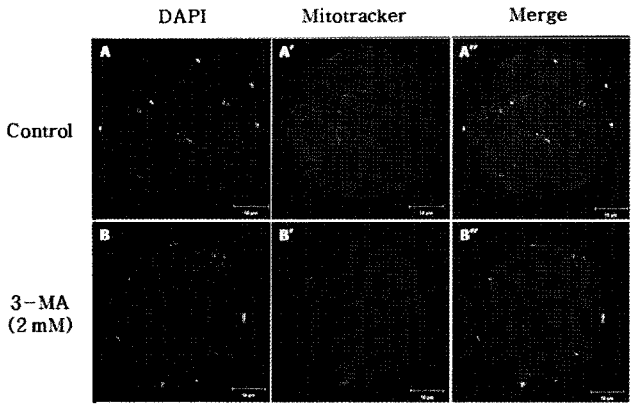

Fig. 5. Comparison of mitochondrial distribution patterns in porcine blastocysts derived from untreated control (A-A") and 3-MA treated embryos (B-B"). Scale bars=50 μm.

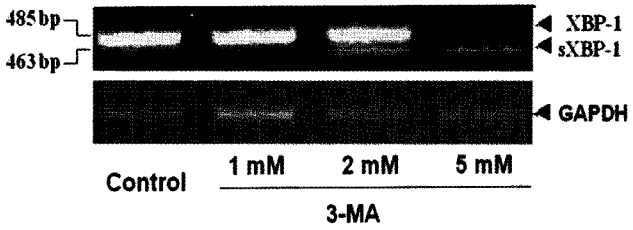

Fig. 6. The mRNA expression patterns of XBP-1 gene in cleaved porcine embryos (4-cell).

DISUSSION

In the present study, we demonstrated that 3-MA affects blastocyst development and cell number of blastocyst during preimplantation embryo development in pigs. Additionally, we verified 3-MA treatment affects on reducing preimplantation development and structural integrity of blastocysts, and enhancing apoptosis. Inhibitor of autophagy also reduced the mitochondrial intensity, thereby affecting preimplantation development in porcine embryos. Finally, we found that retardation of preimplantation development and reduction of embryonic viability by 3-MA treatment was associated with the expression patterns of the autophagy-, apoptosis-, and ER stress-related genes in early embryos in pigs.

Autophagy is an intracellular protein degradation system. Its system conserved throughout eukaryotes, which plays an important role in a variety of biological processes, including cell death, development defense against infection and nutrient starvation (Gozuacik and Kimchi, 2004; Maiko *et al.*, 2006). To investigate the effect of autophagy on blastocyst development and embryonic quality, 3-MA was added to the culture medium for 48 h. As indicated in Table 2, the rate of development to the blastocyst stage in porcine embryos treated with 3-MA was significantly lower than that of untreated con-

trol. Thus, we confirmed that 3-MA reduced the developmental competence of porcine embryos *in vitro*. This finding indicates that inhibition of autophagy affects embryonic developmental retardation in pigs. Based on this result, we suggest that 3-MA treatment disturbs degradation of unnecessary proteins in mammalian preimplantation embryos.

LC3-II are autophagy-related protein, exists in two forms. LC3-I are localized in the cytoplasm, LC3-II are localized the autophagosomal membranes. Therefore, LC3-II can be used to investigate the abundance of autophagosomes before they are degraded through the lysosomes (Maiko *et al.*, 2006; Yan *et al.*, 2011). The amount of LC3-II is closely related with the number of autophagosomes and as an indicator of autophagy (Palacios *et al.*, 2010). Thus, we confirmed that 3-MA reduced the expression of LC3-II, indicating decrease of autophagy in the pig embryos. As indicated in Fig. 1 and Table 3, the density of LC3-II in porcine embryos treated with 3-MA was significantly lower than that of untreated control. Also, autophagy-related genes (Map1lc-3b, Lamp2) were lower than that of untreated control (Fig. 2). This finding indicates that Map1lc3b and Lamp2 are closely correlated with the number of autophagosomes and lysosomes, indicating indicator of autophagolysosome formation. Therefore, this result suggests that the reduction of autophagy may be induces the result of poor embryonic quality.

Apoptosis in embryonic cells has received increasing attention, mostly because of its potential role in the cellular response to suboptimal developmental conditions and stress (Betts and King, 2001). Increased incidence of cell death is an important indicator of inadequate *in vitro* environments for embryos. Thus, the apoptotic pattern of an embryo should be considered. As a result of TUNEL assay, we found that the number of apoptotic nuclei in blastocysts derived from the 3-MA treated group was higher than that of control group (Fig. 3, Table 4). Similarly, the incidence of cell death has been reported to be correlated with embryo quality in other mammalian blastocysts (Hao *et al.*, 2004; Levy *et al.*, 2001). In addition, we found in the present study that the expression of apoptosis related gene (Bax) increased in the 3-MA treated group, whereas expression of the anti-apoptotic gene (Bcl-XL) decreased (Fig. 4). These results are likely related to the observed decrease in blastocyst quality. These findings suggest that the inhibition of autophagy significantly affects the expression of apoptosis-related genes in blastocyst stage embryos, leading to an overall increase in their susceptibility to apoptosis.

Then, to more investigate the effect of 3-MA in the pig blastocysts, the mitochondrial density were measured by using Mito Tracker Green Staining. Mitochondria are energy supplying organelles, whose functional integrality is essential for survival and development, can provide adenosine triphosphate (ATP) for fertilization and preimplantation embryo development. Also, high levels of mitochondrial activity appear to be necessary for further maturation events that are dependent on maturation of the nucleus and accumulation of the mRNAs necessary for early embryo development (Jeong *et al.*, 2009; Wang *et al.*, 2009). Therefore, mitochondria are deeply associated with apoptosis. Based on the mitochondria staining, we found that the mitochondrial density in blastocysts derived from the 3-MA treated group was lower than that of blastocysts derived from the control group (Fig. 5, Table 5). Moreover, the incidence of cell death in mammalian blastocysts seems to be associated with cell number and embryo quality (Hao *et al.*, 2004), in agreement with this result.

ER stress activates CHOP, which induces down-regulation of Bcl-2. Then, release of cytochrome C was increased in mitochondria (de Bruin and Medema, 2008). And the generated splice variant (XBP-1s) encodes a stable, active transcription factor (Yoshida *et al.*, 2001). XBP-1s translocates into the nucleus and binds to the unfolded protein response element triggering the transcription of numberous genes involved in the ER secretory machinery including the ER-assosiated degradation system (Patterson *et al.*, 2008). In general, ER stress is also related to apoptosis and embryo developmental competence. Therefore, we confirm the expression pattern of ER stress-related gene (XBP-1) in 3-MA treated groups, detecting the spliced form of XBP-1 in the 3-MA treated groups as dose dependent manner (Fig. 6). Therefore, this result suggests that autophagy inhibitor induces ER stress and developmental defects in pig embryos.

In conclusions, the findings of the present study indicate that the inhibition of autophagy by 3-MA treatment affects preimplantation embryo development in pigs. Also, culture conditions (i.e. the presence or absence of 3-MA) affect both the developmental potential and embryonic qualities, including structural integrity and apoptosis, of porcine IVF embryos. Reduction of autophagy and mitochondrial distribution caused by 3-MA induced the apoptosis, resulting in up-regulation of Bax gene and down-regulation of Bcl-XL gene. Reduction of autophagy also increased the expression patterns of spliced XBP-1 as a UPR marker, indicating increase of ER stress. Taken together, autophagy appears to play a direct role in early embryo development in pigs.

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