Hsp90 Inhibitor Induces Cell Cycle Arrest and Apoptosis of Early Embryos and Primary Cells in Pigs

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ABSTRACT

Heat shock protein 90 (Hsp90) is ATPase-directed molecular chaperon and affects survival of cancer cell. Inhibitory effect of Hsp90 by inducing cell cycle arrest and apoptosis in the cancer cell was reported. However, its role during oocyte maturation and early embryo development is very insufficient. In this study, we traced the effects of Hsp90 inhibitor, 17-allylamino-17-demethoxygeldanamycin (17-AAG), on meiotic maturation and early embryonic development in pigs. We also investigated several indicators of developmental potential, including structural integrity, gene expression (Hsp90-, cell cycle-, and apoptosis-related genes), and apoptosis, which are affected by 17-AAG. Then, we examined the roles of Hsp90 inhibitor on viability of primary cells in pigs. Porcine oocytes were cultured in the NCSU-23 medium with or without 17-AAG for 44 h. The proportion of GV arrested oocytes was significantly different between the 17-AAG treated and untreated group (78.2 vs 34.8%, $p<0.05$). After completion of meiotic maturation, the proportion of MII oocytes was lower in the 17-AAG treated group than in the control group (27.9 vs 71.0%, $p<0.05$). After IVF, the percentage of penetrated oocytes was significantly lower in the 17-AAG treated group (25.2%), resulting in lower normal pronucleus formation (2PN of 14.6%). Therefore, the inhibition of meiotic progression by Hsp90 inhibitor played a critical role in fertilization status. Porcine embryo were cultured in the PZM-3 medium with or without 17-AAG for 6 days. In result, significant differences in developmental potential were detected between the embryos that were cultured with or without 17-AAG. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) showed that the number of containing fragmented DNA at the blastocyst stage increased in the 17-AAG treated group compared with control (7.5 vs 4.4, respectively). Blastocysts that developed in the 17-AAG treated group had low structural integrity and high apoptotic nuclei than those of the untreated control, resulting in decrease the embryonic qualities of preimplantation porcine blastocysts. The mRNA expressions of cell cycle-related genes were down-regulated in the 17-AAG treated group compared with control. Also, the expression of the pro-apoptotic gene Bax increased in 17-AAG treated group, whereas expression of the anti-apoptotic gene Bcl-XL decreased. However, the expression of ER stress-related genes did not changed by 17-AAG. Cultured pESF cells were treated with or without 17-AAG and used for MTT assay. The results showed that viability of pESF cells were decreased by treatment of 17-AAG (2 μM) for 24 hr. These results indicated that 17-AAG decreased cell proliferation and increased cell death. Expression patterns Hsp90 complex genes (Hsp70 and p23), cell cycle-related genes (cdc2 and cdc25c) and apoptosis-related genes (Bax and Bcl-XL) were significantly changed by using RT-PCR analysis. The spliced form of pXbp-1 product (pXbp-1s) was detected in the tunicamycin (TM) treated cells, but it is not detected in 17-AAG treated cells. In conclusion, Hsp90 appears to play a direct role in porcine early embryo developmental competence including structural integrity of blastocysts. Also, these results indicate that Hsp90 is closely associated with cell cycle- and apoptosis-related genes expression in developing porcine embryos.

(Key words : Hsp90, 17-AAG, Gene expression, Developmental competence, Porcine embryo)

INTRODUCTION

The heat shock protein (Hsp) family functions as molecular chaperones and guide the folding, intracellular localization and proteolytic turnover of key regulators of cell growth, differentiation and survival (Ferrario and Gomer, 2010). The 90 kDa heat shock protein (Hsp90) is one of the most abundant cytosolic molecular chaperones, comprising 1~2% of the total cellular protein

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under non-stressed conditions (Taiyab *et al.*, 2009). A purified system consisting of 5 proteins (ie, Hsp90, Hsp-70, Hsp40, Hop, and p23) has been studied for understanding of chaperone function (Kosano *et al.*, 1998). Especially, Hsp90 simultaneously regulates the transcriptional regulation, signal transduction, cell cycle control of a wide range of client protein. Also, Hsp90 is involved in the folding, assembly, maturation, and stabilization of specific target protein (Kamal *et al.*, 2004).

The geldanamycin (GA) derivative 17-allylamino-17-demethoxygeldanamycin (17-AAG) has shown antitumor activity in several human xenograft models (Basso *et al.*, 2002; Solit *et al.*, 2002). The 17-AAG binds to the N-terminal ATP binding pocket of Hsp90 and block the ability of this chaperone to properly fold or stabilize client proteins (Neckers and Ivy, 2003). It is the first Hsp90 inhibitor to be extensively studied, inducing cell cycle arrest and apoptosis in various tumors (Yao *et al.*, 2010). Hsp90 is a chaperone protein that participates in the regulation of the intracellular disposition of diverse cellular proteins, including the components of signaling pathways that are exploited by cancer cells for survival and proliferation (Schumacher *et al.*, 2007). Thus, 17-AAG had an additive effect on cell growth inhibition and reduction of cdc25c and cdc2 protein levels (Garcia-Morales *et al.*, 2007; Senju *et al.*, 2006). Also, Cdc25c is a client protein of Hsp90, since Hsp90 bound to cdc25c, and treatment with 17-AAG induced degradation of cdc25c by proteasome pathway (Senju *et al.*, 2006). Previous report suggested that 17-AAG-mediated inhibition of Hsp90 abrogates cancer cell growth through cdc2-mediated G2/M cell cycle arrest and apoptosis (Watanabe *et al.*, 2009). Full grown oocytes are arrested at the first meiotic prophase stage (germinal vesicle (GV) stage) in mammals. During porcine oocyte maturation, cyclin B proteins exist initially at very low levels in GV stage oocytes, and later both cyclin B synthesis are activated during germinal vesicle breakdown (GVBD) (Kuroda *et al.*, 2004), which suggests the importance of pig oocytes de novo protein translation for meiotic resumption. Activity of M-phase promoting factor (MPF) is regulated by cyclin B binding to cdc2 (Zhang *et al.*, 2010). Thus, Hsp90 appears to be an important factor cell growth and survival, but its role in the maturation and development of porcine eggs has not been studied adequately.

The endoplasmic reticulum (ER) is an essential intracellular organelle for the synthesis and maturation of cell surface and secretory proteins and maintenance of Ca^{2+} homeostasis (Li *et al.*, 2008). If the homeostasis of the ER lumen is disturbed, it will affect proper protein folding and lead to accumulation and aggregation of unfolded and misfolded protein in the ER. This phenomenon is defined as ER stress (Kaufman, 1999). ER stress transducers play important roles in signal transduction of the unfolded protein response (UPR). Prolonged UPR signaling and ER stress lead to apoptosis (Masud *et al.*, 2007). Especially, embryonic apoptosis has received increasing attention for its proposed role in the cellular response to suboptimal developmental conditions (Betts and King, 2001). Increased apoptosis is an important indicator of inadequate conditions for mammalian eggs.

According to previous research, Hsp90 is ATPase-directed chaperon and affects survival of cancer cell lines. Especially, inhibitory effect of Hsp90 by inducing cell cycle arrest and apoptosis in the lung cancer cell lines was found (Senju *et al.*, 2006). Hsp90 inhibitor was used as novel therapeutic strategies that disrupt folding of immunoglobulin in the ER. However, it is very insufficient about Hsp90 to study regarding in the mammalian embryos. This study investigated the role of Hsp90 in the preimplantation development of porcine embryos and primary cells focusing on cell cycles arrest, ER stress, and apoptosis.

MATERIALS AND METHODS

Chemicals

Unless otherwise mentioned all chemicals used in this study were purchased from Sigma Chemical Co. (St Louis, MO, USA).

In Vitro maturation (IVM)

Porcine follicular fluid was collected from follicles of prepubertal gilts with a diameter of above 5 mm in 50 ml tubes. Cellular debris was allowed to settle for 20 minutes, and tubes were centrifuged for 30 min at 8,000 rpm at 4℃. The supernatant was aspirated and filtered through 0.45 μm membrane (Sterilin, Milan, Italy). Aliquots of 1.2 ml pFF were stored at −20℃ until use. Porcine ovaries were collected from a local slaughterhouse and transported to the laboratory at 25~30℃ in 0.9% saline supplement with 75 μg/ml potassium penicillin G. Cumulus oocyte complexes (COC) were aspirated through an 18 gauge needle into a disposable 10 ml syringe from follicles of 3 to 6 mm in diameter (Funahashi *et al.*, 1994). After washing three times with TL-HEPES medium, approximately 50 COC were matured in 500 μl of the *in vitro* maturation medium in a 4 well multidish (Nunc, Roskilde, Denmark) at 38.5 ℃, 5% CO_2 in air. The medium used for oocyte maturation was BSA free North Carolina State University (NCSU) 23 medium supplemented 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) (Petters and Wells, 1993). At 22 h of culture, oocytes were washed three times and then further cultured in maturation medium without hormones

supplement (PMSG and hCG) for 22 h. At this stage, 17-AAG (2 μM) was added to the maturation medium. After the completion of IVM, the oocytes were subjected to *in vitro* fertilization.

In Vitro Fertilization (IVF)

In vitro fertilization of porcine oocytes was performed as described by Abeydeera and Day (Abeydeera and Day, 1997). This medium, designated as modified Tris-buffered medium (mTBM). Fresh semen was kindly supplied once a week by AI company (Darby Pig AI Center, Anseong, Korea), and kept at 17℃ for 5 days. Semen was washed three times by centrifugation with Dulbecco's phosphate buffered saline (DBPS, Gibco BRL, Grand Island, NY) supplemented with 1 mg/ml BSA (Fraction V, Sigma), 100 mg/ml penicillin G, and 75 mg/ml streptomycin sulfate. At the end of washing, the spermatozoa were resuspended in mTBM at pH 7.8. Oocytes were washed three times in mTBM with 2.5 mM caffeine sodium benzoate and 1 mg/ml BSA (fatty acid free) and placed into 48 μl of mTBM under paraffin oil. Then 2 μl of diluted spermatozoa was added to a 48 μl drop of the medium containing 15~20 oocytes to be a final concentration of 1.5×10^5 sperms/ml. The oocytes were coincubated with spermatozoa for 6 h at 38.5℃ in an atmosphere of 5% CO_2 in air.

In Vitro Culture (IVC)

In all experiments, the embryos were cultured in 50 μl drops of PZM-3 medium with 3 mg/ml BSA at 38.5 ℃, 5% CO_2 in air. After 48 hr of culture, 25~30 cleaved embryos were further cultured in 50 μl drops of PZM-3 medium supplemented with 3 mg/ml BSA at 38.5℃, 5% CO_2 in air for 4 days. Porcine embryos were also treated with 17-AAG (2 μM) during full culture periods. Blastocyst formation was evaluated after 6 days of culture.

Assessment of Meiotic Maturation

At the end of each IVM experiment, a representative sample of oocytes (approximately 50% of the total 834 oocytes used for the developmental studies) was denuded by gently pipetting in NCSU-23 medium containing 0.1% hyaluronidase, washed in medium PVA-PBS and mounted on microscope slides. The samples were fixed at 3 days in acetic acid : ethanol (1:3, v/v) and stained with acetic orcein 0.1% (v/v) for 5 min. The samples were destained in glycerol : acetic acid : water (1:1:3, v/v/v) and the meiotic stage was evaluated under a microscope at 200× and 400× magnification (Leica, Solms, Germany).

Confirmation of Pronucleus Formation

Pronucleus formation patterns of presumptive zygotes derived from IVF were examined. A representative sample of oocytes was denuded by gently pipetting in TL-HEPES medium containing 0.1% hyaluronidase, washed in medium PVA-PBS and mounted on microscope slides. The samples were fixed at 3 days in acetic acid : ethanol (1:3, v/v) and stained with acetic orcein 0.1% (v/v) for 5 min. The samples were destained in glycerol : acetic acid : water (1:1:3, v/v/v) and the meiotic stage was evaluated under a microscope at 200× and 400× magnification (Leica).

Differential Staining

Differential staining of ICM and TE cells of blastocysts (Day 6) was performed using the technique described by (Machaty *et al.*, 1998). Briefly, the zona pellucida of blastocysts was removed by 1 min incubation in 0.5% pronase solution. After rinsing in TL-HEPES medium containing 1 mg/ml PVA, zona pellucida-free embryos were exposed to a 1:5 dilution of rabbit anti-pig whole serum for 1 h. They were then rinsed three times for 5 min in TL-HEPES and placed into a 1:10 dilution of guinea pig complement containing 10 mg/ml propidium iodide and 10 mg/ml bisbenzimide for 1 h. After brief rinsing in TL-HEPES, the stained embryos were mounted on slides under cover glass and examined under UV light using an epifluorescent microscope (Olympus, Tokyo, Japan). Blue and red colors were counted as ICM and TE cell, respectively.

TUNEL (Terminal Deoxynucleotidyl Transferase-mediated dUDP Nick-end Laveling) Assay

Apoptotic cells in the blastocysts were detected using the *In Situ* Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany). The blastocysts at Day 7 from IVF were washed three times with 0.1% PVA-PBS and fixed in 4% (v/v) paraformaldehyde/PBS solution for 1 h at room temperature. For membrane permeabilization, the fixed embryos were incubated in PBS containing 0.1% (v/v) Triton X-100 for 30 min at 4 ℃. The fixed embryos were incubated in TUNEL reaction medium for 1 h at 38.5℃ in the dark and then washed and transferred in to 2 mg/ml of DAPI and mounted on slides. Whole-mount embryos were examined under an epifuorescence microscope (Olympus) using the TUNEL assay and DAPI. The numbers of apoptotic nuclei and total number of nuclei were determined.

Cell Viability Analysis (MTT Assay)

Porcine ear skin fibroblasts (pESF) cells were seeded in 96 well plates containing 200 μl of growth medium. Following a 24 h attachment period, the cells were exposed to various concentrations of 17-AAG (0.5, 1, 2 and 4 μM). All studies were performed at least three times independently. After 24 h, 50 μl of a 5 mg/ml stock solution of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphe-

nyltetrazolium bromide (MTT) was added to each well, and cells were incubated at 37℃ for 4 h. 100 μl DMSO was added to dissolve the dark blue formazan crystals that were formed by the living cells. The absorbance of reaction mixtures were then measured at 540 nM(A_{540}) using a plate spectrophotometer (original Multiskan EX, Type 355, Thermo Electron Corporation, Shanghai, China). The background A_{540} of the wells that did not contain cells was subtracted before the percentage of viable cells was calculated [(A_{540} of 17-AAG treated sample/A_{540} untreated cells) × 100%].

Cell Culture and 17-AAG Treatment

Porcine ear skin fibroblasts of passages 4~6 were used in this study and cultured in growth media containing Dulcecco's modified Eagle's medium (WelGENE, Daegu, South Korea) supplemented with 10% FBS, 1% non-essential amino acid (Gibco BRL) and 500 μg /ml penicillin/streptomycin (Hyclone, Thermo scientific, MA, USA) at 37℃, 5% CO_2 in air. The pESF cells were seeded 0.8×10^6 cells on a 60 mm culture dish or 2.2×10^6 cells on a 100 mm culture dish, and treated with 17-AAG of 2 μM for 24 h.

RNA Synthesis and Real-time RT-PCR

Total RNA from embryos and primary cells was isolated using the Dynabeads mRNA direct kit (DYNAL; Invitrogen, Carsbad, CA, USA) and Rneasy plus micro kit (Qiagen, Chatsworth, CA, USA) according to the manufacture's protocol. 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 RT-PCR was performed using an Applied Stratagene mx 3000p 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 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 β-actin. The primers used for real-time RT-PCR are shown in Table 1. The amplification program was as follows: 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,

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

Gene (GenBank accession number)	Primer sequence	Annealing temperature (℃)	Expected PCR size (bps)
HSP90 (NM213973)	F: ACCAGAATGAAGGAGAACCA R: ACACAACCACCTTTTCGACT	58	316
HSP70 (X68213)	F: ATGTCCGCTGCAAGAGAAGT R: GGCGTCAAACACGGTATTCT	58	216
P23 (AY574050)	F: ACGCTGGCCCCCTACTAC R: ATGCACCTCCACATGTTCG	60	156
Cdc25c (x78317)	F: GGAAACTTGGTGGAAGGTGA R: AAGCCCTTCCTGAGCTCTTC	58	311
Cdc2 (GQ184633)	F: TTGGTCTTGCCAGAGCTTTT R: AAGTTTTTGACGTGGGATGC	58	328
Bax (AJ606301)	F: AAGCGCATTGGAGATGAACT R: CGATCTCGAAGGAAGTCCAG	60	251
Bcl-xL (AF213205)	F: AGGGCATTCAGTGACCTGAC R: TGGATCCAAGGCTCTAGGTG	60	242
sXBP1 (FJ213449)	F: AAACAGAGTAGCAGCTCAGACTGC R: TCCTTCTGGGTAGACCTCTGGGAG	58	485 (unspliced) 463 (spliced)
GRP78 (X92446)	F: TGGACCTTTTCCGTTCTACC R: AGCCTCATCTGGGTTTATGC	59	185
β-actin (U07786)	F: CTCTTCCAGCCCTCCTTCCT R: GGCGTAGAGGTCCTTCCTGAT	60	104

and then acquisition of fluorescence. After the end of the last cycle, a melting curve was generated by initiating fluorescence acquisition at 72℃ and taking measurements every 0.1℃ until 95℃ was reached. After the completion of the quantitative PCR analysis, PCR products were electrophoresed on a 2% agarose gel stained with ethidium bromide and visualized by exposure to ultraviolet light. The images were obtained using a gel doc apparatus (Cell Biosciences, Santa Clara, CA, USA).

Statistical Analysis

All experiments were repeated more than three times. All percentage data were subjected to arcsine transformation. Some percentage data and data sets obtained in this study are presented as the mean±SD. throughout the text. Maturation and fertilization rates of porcine oocyte were analyzed by Student's *t*-test. Preimplantation development, the cell number of blastocyst, and mRNA expressions in the experiments were analyzed by one-way ANOVA and Duncan's multiple range test using the General Linear Models procedure in the Statistical Analysis System (SAS, Cary, NC, USA). A probability of $p<0.05$ was considered significant.

RESULTS

Effects of Hsp90 Inhibitor, 17-AAG, Treatment during Meiotic Maturation

There are no previous reports regarding the concentration of 17-AAG to use in porcine oocytes or embryo culture experiments. In the present study, we determined the proper concentration of 17-AAG in terms of its developmental effects by evaluating oocytes maturation. To test the effect of 17-AAG on preimplantation development, pig oocytes were cultured in maturation medium supplemented with various concentrations (2 or 4 μM) of 17-AAG for 22 h or 44 h. After completion of IVM and IVF, blastocyst formation was evaluated in the groups exposed to different concentrations of 17-AAG. As indicated in Table 2, developmental competence to the blastocyst stage was significantly lower for oocytes treated with 2 μM 17-AAG compared with the control tested ($p<0.05$). Especially, no blastocyst was found in group of 17-AAG treated with high concentration (4 μM). Therefore, all further experiments were performed using 2 μM 17-AAG.

Porcine oocytes released from follicles at the GV stage initiated GVBD at 22 h, most of the oocytes reached MI. Following this phase, the oocytes progressed through anaphase I/telophase I stage, and by 44 h, most reached the MII stage with the extruded typical first polar body. Thus, meiotic maturation stages of the pig oocytes were confirmed using acetic orcein staining method. To define the effect of 17-AAG on porcine oocyte maturation, the nuclear morphologies were investigated in porcine oocyte that were treated with 17-AAG for 44 h throughout full maturation period (Table 3). The proportion of GV arrested oocytes was significantly different between the 17-AAG treated and untreated group. Most oocytes in the 17-AAG treated group remained at the GV stage (78.3±9.1 vs 34.8±5.7%, $p<0.05$). By the end of the maturation period (44 h), the proportion of MII oocytes was lower in the 17-AAG treated group than in the control group (27.9±7.0 vs 71.0 ±3.1%, $p<0.05$). Moreover, there were more MI oocytes in the 17-AAG treated group than in the control group (69.1±6.9 vs 25.6±2.4%, $p<0.05$). This result indicates that 17-AAG has negative effect on nuclear maturation of porcine oocytes.

Effect of 17-AAG on Fertilization Parameters

To investigate the effect of 17-AAG treatment on fertilization, pig oocytes were matured in the absence or presence of 2 μM 17-AAG for the 44 h maturation period, and then were co-incubated with spermatozoa for 6 h. After 12 h after fertilization, presumptive zygotes were stained by acetic orcein staining to confirm the pronucleus formation patterns. As shown in Table 4, the percentage of penetrated oocytes was significantly lower in the 17-AAG treated group (25.2%). Thus, the proportion of normal fertilized oocytes (2PN) decreased in the experimental versus the control group (16.4±1.0 vs 32.8±2.1%, $p<0.05$). However, the percentage

Table 2. Effect of 17-AAG treatment during IVM on preimplantation development of pig embryos

Concentration	No. of oocytes examined	% of embryos cleaved (n)	% of blastocysts (n)
Control	91	81.1±1.7^a (74)	43.7±1.9^a (40)
2 μM	90	59.6±14.1^b (55)	3.2±3.3^b (3)
4 μM	93	13.0±8.8^c (12)	-

This experiment was repeated three times. Data are the mean±SD. Different superscripts denote a significant difference compared with other groups ($p<0.05$).

Table 3. Effects of 17-AAG (2 μM) on nuclear maturation in porcine oocytes

Time (h)	Group	No. of oocytes examined	% of oocytes (n)			
			GV	GVBD	M I	M II
22	Control	221	34.8±5.7[a] (77)	19.2±2.6 (43)	45.9±4.8[a] (101)	-
	17-AAG	238	78.3±9.1[b] (187)	19.46±8.3 (46)	2.2±1.3[b] (5)	-
44	Control	173	2.9±2.6 (5)	0.5±1.0 (1)	25.6±2.4[a] (44)	71.0±3.1[a] (123)
	17-AAG	202	1.9±1.5 (4)	1.0±1.1 (2)	69.1±6.9[b] (140)	27.9±7.0[b] (56)

This experiment was repeated four times. Data are the mean±SD. Values with different superscript letters within a column differ significantly ($p<0.05$). GV, germinal vesicle; GVBD, GV breakdown; MI, Metaphase I; MII, Metaphase II.

Table 4. Effects of maturation conditions on fertilization variables in pig oocytes

Group	No. of oocytes examined	% of pronuclear formation (n)					% of oocytes	
		≤ M I	M II	1 PN	2 PN	≥ 3 PN	Penetrated	Polyspermic*
Control	122	4.9±1.1 (6)	18.9±1.1 (23)	9±0.8 (11)	32.8±2.1 (42)	34.4±2.1 (42)	76.2±3.4[a]	45.2±2.1[a]
17-AAG	151	60.3±2.3 (91)	14.6±1.8 (22)	5.3±1.2 (8)	14.6±1.0 (22)	5.3±0.9 (8)	25.2±1.3[b]	21.1±0.9[b]

Data shown are representative of at four independent experiments. Data are the mean±SD Different superscripts denote a significant difference compared with other groups ($p<0.05$).
* Percentage of the number of polyspermic oocytes/total of penetrated oocytes.

of polyspermic oocytes decreased significantly in the treated compared with the untreated group (21.1±0.9 vs 45.2±2.1%, $p<0.05$). In particular, more oocytes in the 17-AAG treated group remained in the MI stage (60.3 %). This phenomenon can be explained by the failure of meiotic progression, although some oocytes were normally fertilized in 17-AAG treatment group.

Effects of 17-AAG Treatment on Preimplantation Development and Structural Integrity of Porcine Embryos

After fertilization, presumptive porcine zygotes were cultured in IVC medium supplemented with 0, 2, or 4 μM 17-AAG for 6 days. Developmental competence to the blastocyst stage in the presence of 17-AAG was significantly lower than that for untreated control (Table 5). Also, there was a significant difference in cleavage rate among different 17-AAG treated groups. Therefore, further experiments were performed using 2 μM 17-AAG. Then, the structural integrity of blastocysts was examined using differential staining (Table 6). The 17-AAG treated blastocysts had lower cells on average than untreated blastocysts (38.9±1.3 vs 49.9±2.7, $p<0.05$). Furthermore, both lower ICM and TE cells were observed in blastocysts with 17-AAG (3.5±0.8 and 32.3±1.9)

Table 5. Effect of various concentrations of 17-AAG on developmental ability of pig embryos *in vitro*

Concentration	No. of embryos cultured	% of embryos cleaved (n)	% of blastocysts (n)
Control	92	86.4±4.8[a] (79)	27.6±6.4[a] (26)
2 μM	95	76.3±5.3[b] (73)	13.0±2.9[b] (12)
4 μM	96	59.4±7.7[c] (57)	4.2±5.2[c] (4)

This experiment was replicated three times. Data are the mean±SD. Different superscripts denote a significant difference compared with other groups ($p<0.05$).

Table 6. Effect of 17-AAG on blastocysts formation and quality in porcine embryos

Group	No. of embryos cultured	% of blastocysts (n)	No. of nuclei			ICM/total cells (%)
			ICM	TE	Total	
Control	174	26.1±6.8[a] (46)	6.8±0.6[a]	43.1±1.1[a]	49.9±2.7[a]	13.8±0.6
17-AAG	289	11.4±0.2[b] (33)	3.5±0.8[b]	32.3±1.9[b]	38.9±1.3[b]	9.7±3.6

This experiment was replicated four times. Data are the mean±SD. Different superscripts denote a significant difference compared with other groups ($p<0.05$).

than without 17-AAG (6.8±0.6 and 43.1±1.1), resulting in deterioration of embryonic quality in the pig.

Effect of 17-AAG on the mRNA Expression of Hsp90 Complex and Cell Cycle Related Genes in the Cleaved Embryos and Blastocyst Stage Embryos

To identify mRNA expressions of Hsp90 complex genes, we determined the relative abundance of Hsp90, Hsp70 and p23 transcripts in cleaved embryos and blastocysts derived from 17-AAG treated and untreated groups using real-time RT-PCR (Fig. 1). After 48 h of continuous 17-AAG stimulation, the expression of Hsp90 transcripts was similar to that in the untreated controls. However, significantly more Hsp90 mRNA was detected at blastocyst stage with 17-AAG than without. In case of Hsp70 transcripts, significant differences were observed in mRNA expression levels between groups treated with and without 17-AAG in both cleaved and blastocyst stage embryos. However, expression of p23

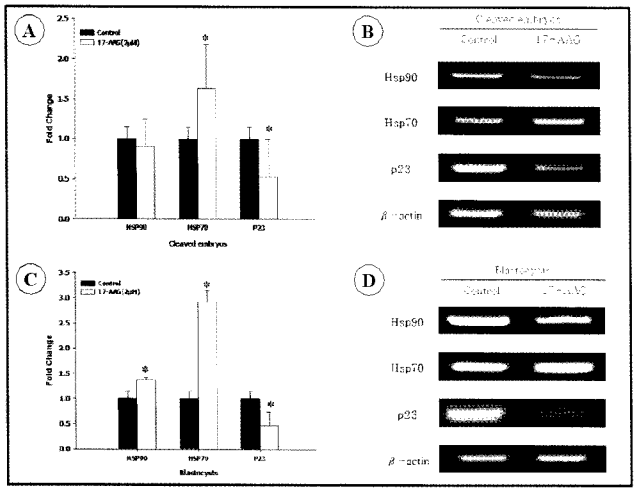

Fig. 1. The mRNA expressions of Hsp90-related genes in the early developmental stage pig embryos *in vitro*. Relative mRNA expression levels of Hsp90-related genes in the cleaved embryos (A, B; 30 cleaved embryos per batch) and blastocyst stage embryos (C, D; 10 blastocysts per batch) were presented by using real-time RT-PCR. β-actin was used as an internal standard. Data are the mean±SD. * $p<0.05$ compared with control. Data represent means of three independent experiments.

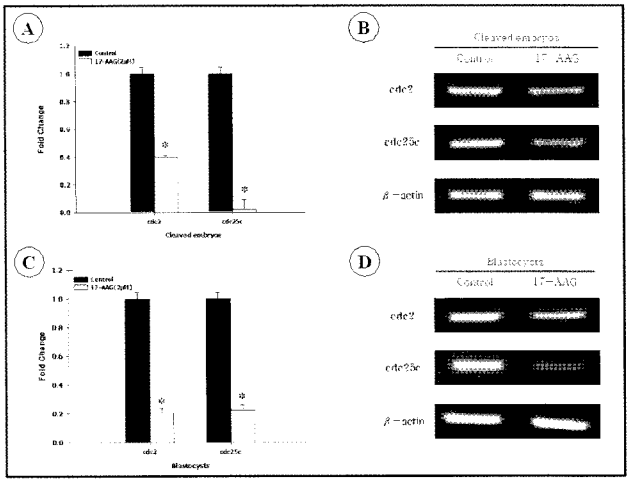

Fig. 2. The mRNA expressions of cell cycle-related genes in the early developmental pig embryos *in vitro*. Relative mRNA expression levels of cell cycle-related genes in the cleaved embryos (A, B; 30 cleaved embryos per batch) and blastocyst stage embryos (C, D; 10 blastocysts per batch) were presented by using real-time RT-PCR. β-actin was used as an internal standard. Data are the mean±SD. * $p<0.05$ compared with control. Data represent means of three independent experiments.

was significantly decreased after 17-AAG treatment in cleaved and blastocyst stage embryos.

In general, cdc2 and cdc25c genes were involved in the G2/M transition during cell cycle. Thus, we also examined the effect of 17-AAG on the expression of cell cycle related genes after culture with 17-AAG using real-time RT-PCR. There was decrease in the expression of the two cell cycle related genes in the cleaved and blastocyst stage embryos treated 17-AAG compared to untreated control (Fig. 2). This result suggests that the reduction of 17-AAG treated embryos to develop into blastocysts was due to the down-regulation of cell cycle related genes transcription during preimplantation periods.

Apoptosis and Gene Expression in Preimplantation Stage Embryos Derived from 17-AAG Treated Embryos

Susceptibility to apoptosis was assessed in cleaved and blastocyst stage embryos derived from 17-AAG treated and untreated embryos. Apoptosis was measured

Table 7. Apoptotic patterns in porcine blastocysts derived from 17-AAG-treated and non-treated groups

Group	No. of blastocysts	Apoptosis (nuclei)	
		DAPI	TUNEL
Control	20	50.2±1.9[a]	4.4±0.8[a]
17-AAG	19	44.9±4.7[b]	7.5±1.3[b]

This experiment was replicated three times. Data are the mean±SD. Different superscripts denote a significant difference compared with other groups ($p<0.05$).

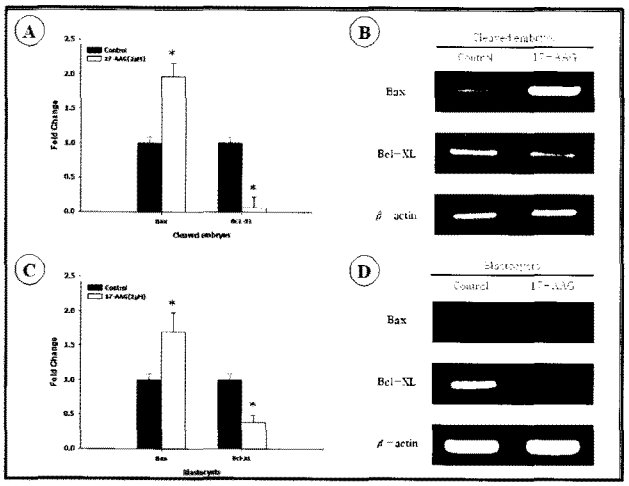

Fig. 3. The mRNA expressions of apoptosis-related genes in the early developmental pig embryos *in vitro*. Relative mRNA expression levels of apoptosis-related genes in the cleaved embryos (A, B; 30 cleaved embryos per batch) and blastocyst stage embryos (C, D; 10 blastocysts per batch) were presented by using real-time RT-PCR. β-actin was used as an internal standard. Data are the mean±SD. * $p<0.05$ compared with control. Data represent means of three independent experiments.

using the TUNEL assay, which allows for the identification of fragmented DNA. The number of TUNEL-positive nuclei increased significantly in blastocysts derived from 17-AAG treated embryos compared with control (7.5±1.3 vs 4.4±0.8, $p<0.05$; Table 7). To examine the expression of apoptosis-related genes, we used real-time RT-PCR to determine the relative abundance of Bax and Bcl-XL transcripts in cleaved embryos and blastocyst stage embryos derived from 17-AAG treated and untreated embryos. Significantly more Bax mRNA was detected in the 17-AAG treated group compared with control, however, Bcl-XL mRNA expression was significantly less in the 17-AAG treated group (Fig. 3; $p<0.05$).

ER Stress-related Genes Expressions in Cleaved and Blastocyst Stage Embryos Derived from 17-AAG Treated Embryos

Firstly, to investigate the developmental competence of 17-AAG and/or TM treated embryos, porcine embryos derived from IVF were cultured in PZM3 medium containing 17-AAG and/or TM for 6 days and blastocyst formation was monitored. The rate of development to the blastocyst stage in IVF-derived embryos was 25.1±3.6% in the control, 11.9±2.5% in the presence of 17-AAG and 5.4±4.7% in the presence of TM (Table 8). Then, susceptibility to ER stress was assessed in cleaved and blastocyst stage embryos derived from 17-AAG treated and untreated embryos. We found that GRP78 mRNA was decreased in cleaved and blastocyst stage embryos. However, the expression of XBP-1 mRNA did not change in both embryos (Fig. 4). Moreover, to investigate the mRNA levels of spliced XBP-1, RT-PCR analysis were performed on the cleaved embryos treated with 17-AAG and TM alone or combination. Induction of pXBP-1s by 17-AAG and/or TM was investigated (Fig. 5). Interestingly, 17-AAG alone did not induce ER stress in cleaved embryos, but treatment with TM alone/combination resulted in induction of ER stress in cleaved embryos. Previous reports have suggested that XBP-1 as a UPR marker in involved in ER stress-mediated cell death in the porcine system. However, the expression of ER stress-related gene, XBP-1s, did not change by 17-AAG.

Effect of 17-AAG on Viability of Pig Primary Cells

We investigated that the effect of 17-AAG on the growth and survival of primary cells (pESF) using MTT

Table 8. Effect of tunicamycin and/or 17-AAG treatment on blastocyst formation of porcine embryos *in vitro*

Concentration	No. of embryos cultured	% of embryos cleaved (n)	% of blastocysts (n)
Control	187	80.2±5.1[a] (150)	25.1±3.6[a] (47)
17-AAG	193	73.1±5.3[b] (141)	11.9±2.5[b] (23)
Tunicamycin	188	58.2±0.7[c] (109)	5.4±4.7[c] (7)
17-AAG + tunicamycin	186	63.9±0.6[d] (119)	-

This experiment was replicated three times. Data are the mean±SD. Different superscripts denote a significant difference compared with other groups ($p<0.05$).

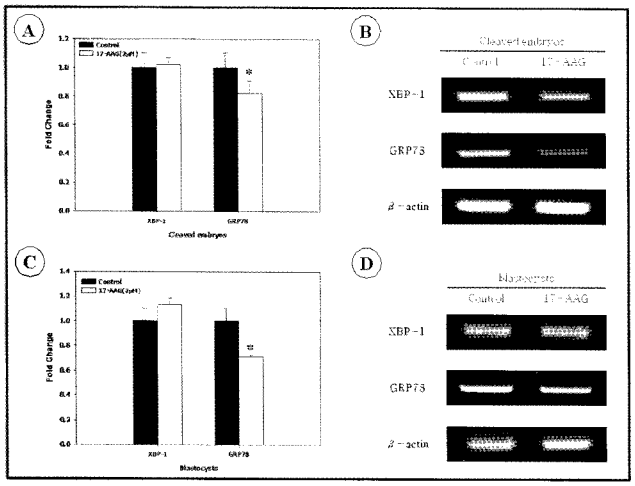

Fig. 4. The mRNA expressions of ER stress-related genes in the early developmental pig embryos *in vitro*. Relative mRNA expression levels of ER stress-related genes in the cleaved embryos (A, B; 30 cleaved embryos per batch) and blastocyst stage embryos (C, D; 10 blastocysts per batch) were presented by using real-time RT-PCR. β-actin was used as an internal standard. Data are the mean±SD. * $p<0.05$ compared with control. Data represent means of three independent experiments.

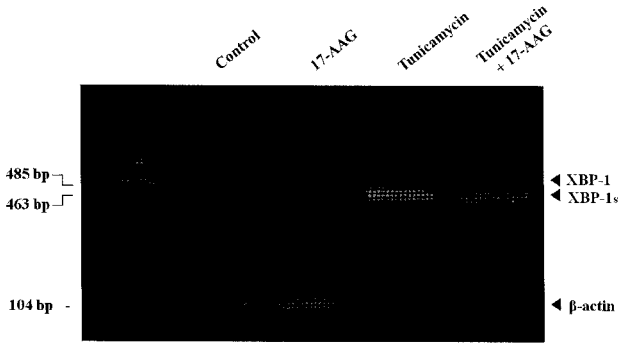

Fig. 5. The mRNA expression patterns of XBP-1 gene in the cleaved early 4-cell stage embryos treated with tunicamycin and/or 17-AAG.

assay. MTT assay showed that the viability of pESF cells decreased with treatment of 17-AAG from 0.5 to 4 μM for 48 h, suggesting that Hsp90 inhibition decreases primary growth. In addition, 17-AAG induced cell death at all time points in a time- and dose-dependent manner (Fig. 6). The apparent IC_{50} values of 17-AAG for pESF cells at 24 h were 2 and 4 μM. Therefore, further experiments were performed using 2 μM for pESF cells.

Expression of Hsp90-, Cell Cycle- and Apoptosis-related Genes in 17-AAG Treated Primary Pig Cells

The biological activity of 17-AAG was first evaluated by examining the expression profiles of the Hsp90, Hsp70, and p23 in pESF cells. Expressions of Hsp70 and Hsp90 were increased after 17-AAG treatment. However, the expression of p23 was decreased in 17-AAG

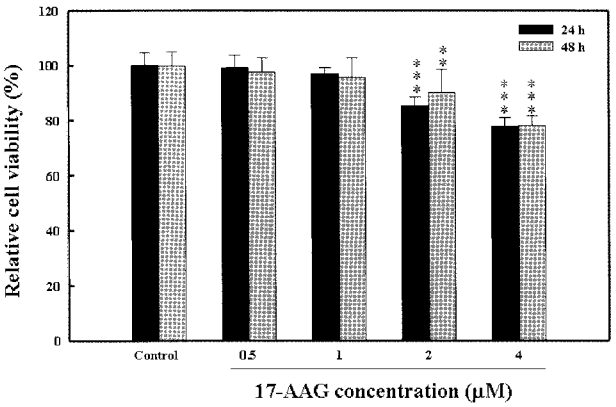

Fig. 6. Effects of 17-AAG on viability of cultured pESF cells. The pESF cells were treated with various concentrations of 17-AAG for 24 or 48 h. Data are representative results in three independent experiments, and expressed as mean±SD. ** and *** indicate $p<0.01$ and $p<0.001$, respectively, compared with negative controls.

treated cells (Fig. 7A). Also, cell cycle-related genes (cdc-2 and cdc25c) involved in G2/M transition were analyzed by RT-PCR. Since substantial G2/M arrest occurred within 24 h of 17-AAG treatment, we examined changes in these genes at this time point. The result of RT-PCR showed decreased total mRNA of cdc2 and cdc25c at 24 h (Fig. 7B). This result suggests that 17-AAG-mediated G2/M arrest is associated with decreased cdc2 and cdc25c levels. To further investigate the apoptosis related gene expression, the expression levels of Bax and Bcl-XL gene were evaluated in 17-AAG treated pESF cells by using RT-PCR. The expression of the pro-apoptotic gene Bax increased in 17-AAG treated

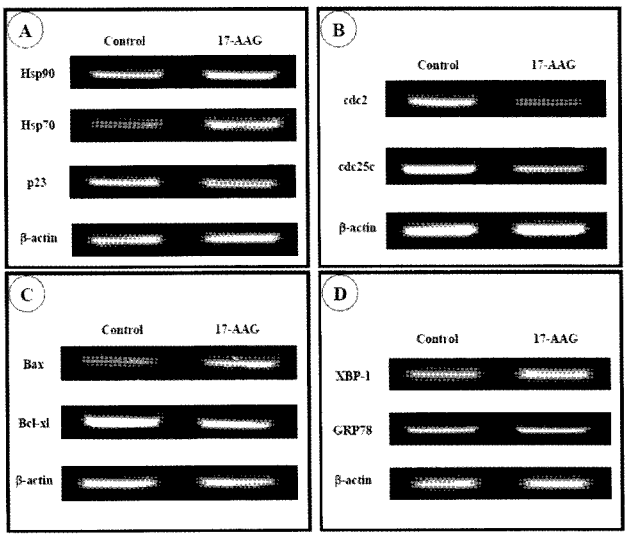

Fig. 7. The mRNA expressions of Hsp90-, cell cycle-, apoptosis-, and ER-stress-related genes in the cultured pESF cells. Relative mRNA expression levels of Hsp90- (A), cell cycle- (B), apoptosis- (C), and ER stress-related (D) genes in the cultured pESF cells were presented. β-actin was used as an internal standard.

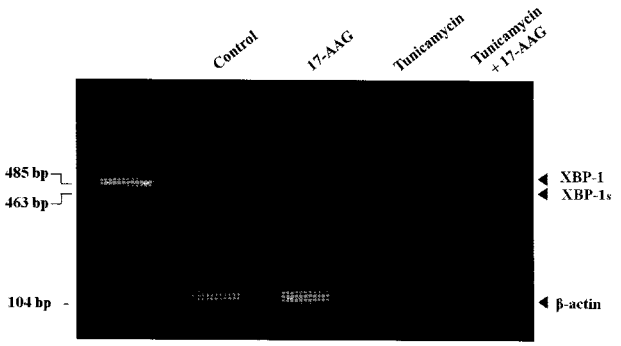

Fig. 8. The mRNA expression patterns of XBP-1 gene in the pESF cells treated with tunicamycin and/or 17-AAG.

cells, whereas expression of the anti-apoptotic gene Bcl-XL decreased (Fig. 7C). This result was in agreement with of embryo data. Therefore, these results indicate that 17-AAG increased cell death and decreased cell proliferation.

Expression of ER Stress-related Genes in 17-AAG Treated Primary Pig Cells

Susceptibility to ER stress was assessed in 17-AAG treated pESF cells. XBP-1 and GRP78 was also found to not change in pESF cells (Fig. 7D). Activated IRE1 is one of sensors of UPR and induces the XBP-1s mRNA during ER stress. ER stress induced by TM is known to regulate cell survival pathway in many cell types. Thus, RT-PCR analysis was performed on the pESF cells treated with 17-AAG and TM in combination or alone. The spliced form of pXBP-1 was detected in the TM-treated cells, but it is not detected in 17-AAG treated cells (Fig. 8). However, the expressions of ER stress-related genes did not changed by 17-AAG.

DISCUSSION

The results of the present study demonstrate that Hsp90 inhibitor (17-AAG) treatment reduces meiotic progression during IVM, arresting maturation to the MII stage. We also showed that penetration rate was reduced in 17-AAG treated oocytes compared with untreated control oocytes. Hsp90 inhibitor treatment also reduced the frequency of normal pronucleus formation, thereby affecting preimplantation development in pig embryos. Additionally, we verified 17-AAG treatment during culture periods affects on reducing preimplantation development and structural integrity of blastocysts, and enhancing apoptosis. Finally, we found that retardation of preimplantation development and reduction of cell viability by 17-AAG treatment was associated with the expression patterns of the cell cycle-, apoptosis-related genes in early embryos and primary cells in pigs.

Hsp90 is an abundant cytosolic molecular chaperone which is involved in maturation and stability of several proteins, most of which are involved in cell proliferation and survival (Taiyab *et al.*, 2009). Hsp90 inhibitor, 17-AAG, binds to the ATP-binding pocket of Hsp90 and prevents the interaction between the chaperone and its target (Francis *et al.*, 2006). Inhibition of Hsp90 has been reported to induce cell cycle arrest and apoptosis in various cancer cells as dose- and time-dependent manner (Garcia-Morales *et al.*, 2007; Georgakis *et al.*, 2006; Karkoulis *et al.*, 2010). Therefore, the resent study was performed whether inhibiting Hsp90 will lead to inhibition of oocyte maturation and preimplantation embryo development in pigs, as well on the structural integrity, gene expression and apoptosis in blastocysts.

Meiotic maturation of mammalian oocytes is controlled by the maturation/M-phase promotion factor (MPF), a complex of cdc2 kinase and cyclin B protein (Zhang *et al.*, 2010). The proteins such as cdc2 and cdc25c were involved in the G2/M checkpoint. However, 17-AAG had an additive effect on cell growth inhibition and reduction of cdc2 and cdc25c protein levels (Garcia-Morales *et al.*, 2007). Therefore, this result demonstrated that meiotic progression from GV to the MII stage was significantly reduced in 17-AAG treated porcine oocytes during IVM; this confirmed that exposure COCs to 17-AAG during IVM arrested oocytes in the GV (at 22 h) or MI (at 44 h) stage and reduced meiotic maturation (MII stage, Table 3). This finding indicates that Hsp90 play a role in the regulation of meiotic progression in porcine oocytes. Based on this result, we suggest that 17-AAG treatment arrests meiotic progression during IVM, resulting in a lower maturation rate and a reduced frequency of penetrated oocytes after IVF (Table 4). The observed decrease in maturation rate (MII) may be the result of reduced monospermic fertilization.

As indicated in Table 5, the rate of development to the blastocyst stage in porcine embryos treated with 17-AAG was significantly lower than that of untreated control. Thus, we confirmed that 17-AAG reduced the developmental competence of porcine embryos *in vitro*. Moreover, blastocysts that developed in the 17-AAG treated group had smaller numbers of ICM, TE, and total cells than those of the untreated control (Table 6). Fewer ICM and TE cells in the groups that were 17-AAG treatment during early embryo development might have been due to retardation of preimplantation development and enhanced apoptosis. Thus, embryo quality may greatly affect the structural integrity of blastocyst in pig.

Hsp90 participates in two multichaperoning complexes with opposing activities. This chaperoning depends on Hsp90's association with a number of cochaperones and is intimately associated with and dependent on the folding activity of Hsp70 and its cofactor (Felts

et al., 2007). When ATP is bound, the complex, which includes p23 and $P50^{cdc37}$, binds to and stabilizes Hsp90 client protein. The p23 was first identified as a protein associated with unactivated progesterone receptor multi-subunit complexes (Smith *et al.* 1990). Then, p23 has been shown to bind directly to domains of Hsp90 encompassing both N- and C-terminal regions in an ATP-dependent fashion (Chadli *et al.*, 2000; Johnson and Toft, 1995), and to act as an important modulator for Hsp90 chaperoning pathway (McLaughlin *et al.*, 2006; Sullivan *et al.*, 2002). Thus, in the present study I investigated the expression patterns of Hsp90 complex genes by 17-AAG treatment in cleaved and blastocyst stage embryos using real-time RT-PCR. I found that the expression of Hsp70 increased in the 17-AAG treated embryos, whereas expression of p23 decreased (Fig. 1). This observation indicates the changes of the Hsp90-associated cochaperone Hsp70 and p23 in response to 17-AAG in early porcine embryos.

The molecular mechanisms underlying 17-AAG induce cell cycle arrest and apoptosis were examined. In accordance with polyadenylation status, the cdc2 level diminished in M II oocytes and after activation due to mRNA degradation (Zhang *et al.*, 2010). In addition, cdc25c possibly interacted with Hsp90 and 17-AAG to induce the degradation of cdc25c in the proteasome pathway. The activity of cdc25c, a member of the cdc25 family of protein phosphatases involved in G2/M progression (Senju *et al.*, 2006). In 17-AAG treated group, both transcription levels of cdc2 and cdc25c were significantly reduced in the cleavage and blastocyst stage embryos (Fig. 2). This result suggests that cell cycle arrest was induced by 17-AAG treatment during early embryonic development. Apoptosis in embryonic cells has received increasing attention, mostly due to 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 17-AAG-treated group was higher than that of control group (Table 7). 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 the pro-apoptotic gene Bax increased in the 17-AAG treated group, whereas expression of the anti-apoptotic gene Bcl-XL decreased (Fig. 3). These results are likely related to the observed decrease in blastocyst quality. Therefore, these findings suggest that the inhibition of the Hsp90 function significantly affects the expression of apoptosis-related genes in cleaved and blastocyst stage embryos, leading to an overall increase in their susceptibility to apoptosis.

IRE1 is a dual-activity enzyme, having a serine-threonine kinase domain and an endoribonuclease domain. On activation, the endonuclease activity of IRE1 removes a 26-nucleotide intron from the XBP-1 mRNA, previously induced by ATF6 (Szegezdi *et al.*, 2006). 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 induced by TM is known to regulate cell survival pathway in several cell types. The previous report shows that TM, a naturally occurring antibiotic that induce ER stress by inhibition of glycosylation (Jiang *et al.*, 2007). Therefore, to analyze the splicing of pXBP-1 during ER stress, 17-AAG and TM were used to induce ER stress in porcine embryos. This result showed that XBP-1 was not spliced with 17-AAG. However, XBP-1 mRNA was spliced by TM (Fig. 5). This result indicated there were no differences in inducing these sensors in ER-stress with 17-AAG.

In previous reports, inhibition of Hsp90 function by 17-AAG caused downregulation of cdc2 and cdc25c and G2/M arrest in glioblastoma and lung cancer cell lines (Garcia-Morales *et al.*, 2007; Senju *et al.*, 2006). Therefore, we investigated the cell viability and genes expression (cell cycle-, apoptosis- and ER-stress-related genes) in 17-AAG treated pig primary cells. MTT assay showed that viability of pESF cells were decreased by treatment of 17-AAG (Fig. 6). This result indicated that 17-AAG decreased cell proliferation and increased cell death. Also, the present result suggests that downregulation of both cdc2 and cdc25c mRNA levels in 17-AAG treated pESF cells is associated with inactivation of cdc2 kinase activity (Garcia-Morales *et al.*, 2007). The expression of the pro-apoptotic gene Bax increased in 17-AAG treated pESF cells, whereas expression of the anti-apoptotic gene Bcl-XL decreased (Fig. 7C). This result was in agreement with that of embryo data. To confirm the expression pattern of ER-stress related gene (pXBP-1) in 17-AAG treated cells, we investigated the expression of pXBP-1s using RT-PCR. The spliced form of pXBP-1 product (pXBP-1s) was detected in the TM-treated cells, but it was not detected in 17-AAG treated cells (Fig. 8).

In conclusion, the findings of the present study suggest that meiotic maturation decreased in 17-AAG treated oocytes, which affects fertilization status and subsequent preimplantation development. Also, culture conditions (i.e. the presence or absence of 17-AAG) affect both the developmental potential and embryonic qualities, including structural integrity and apoptotic pattern, of porcine IVF embryos. The decreased develop-

mental ability and cell viability caused by 17-AAG induced the cell cycle arrest in porcine embryos and primary cells, resulting in downregulation of cdc2 and cdc25c genes. The inhibition of the Hsp90 expression also affects the expression of apoptosis-related genes in preimplantation stage embryos and primary cells. In general, Xbp-1 is involved in ER stress-mediated cell death in pig cells. However, Hsp90 inhibitor, 17-AAG, did not associated with the expression patterns of XBP-1 as a UPR marker. Taken together, Hsp90 appears to play a direct role in early embryo development and primary cell survival in the porcine system.

REFERENCES

1. Abeydeera LR, Day BN (1997): Fertilization and subsequent development *in vitro* of pig oocytes inseminated in a modified tris-buffered medium with frozen-thawed ejaculated spermatozoa. Biol Reprod 57: 729-734.
2. Basso AD, Solit DB, Munster PN, Rosen N (2002): Ansamycin antibiotics inhibit Akt activation and cyclin D expression in breast cancer cells that overexpress HER2. Oncogene 21:1159-1166.
3. Betts DH, King WA (2001): Genetic regulation of embryo death and senescence. Theriogenology 55: 171-191.
4. Chadli A, Bouhouche I, Sullivan W, Stensgard B, McMahon N, Catelli MG, Toft DO (2000): Dimerization and N-terminal domain proximity underlie the function of the molecular chaperone heat shock protein 90. Proc Natl Acad Sci USA 97:12524-12529.
5. Felts SJ, Karnitz LM, Toft DO (2007): Functioning of the Hsp90 machine in chaperoning checkpoint kinase I (Chk1) and the progesterone receptor (PR). Cell Stress Chaperones 12:353-363.
6. Ferrario A, Gomer CJ (2010): Targeting the 90 kDa heat shock protein improves photodynamic therapy. Cancer Lett 289:188-194.
7. Francis LK, Alsayed Y, Leleu X, Jia X, Singha UK, Anderson J, Timm M, Ngo H, Lu G, Huston A, Ehrlich LA, Dimmock E, Lentzsch S, Hideshima T, Roodman GD, Anderson KC, Ghobrial IM (2006): Combination mammalian target of rapamycin inhibitor rapamycin and HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin has synergistic activity in multiple myeloma. Clin Cancer Res 12:6826-6835.
8. Funahashi H, Cantley TC, Stumpf TT, Terlouw SL, Day BN (1994): *In vitro* development of *in vitro*-matured porcine oocytes following chemical activation or *in vitro* fertilization. Biol Reprod 50:1072-1077.
9. Garcia-Morales P, Carrasco-Garcia E, Ruiz-Rico P, Martinez-Mira R, Menendez-Gutierrez MP, Ferragut JA, Saceda M, Martinez-Lacaci I (2007): Inhibition of Hsp-90 function by ansamycins causes downregulation of cdc2 and cdc25c and G(2)/M arrest in glioblastoma cell lines. Oncogene 26:7185-7193.
10. Georgakis GV, Li Y, Younes A (2006): The heat shock protein 90 inhibitor 17-AAG induces cell cycle arrest and apoptosis in mantle cell lymphoma cell lines by depleting cyclin D1, Akt, Bid and activating caspase 9. Br J Haematol 135:68-71.
11. Hao Y, Lai L, Mao J, Im GS, Bonk A, Prather RS (2004): Apoptosis in parthenogenetic preimplantation porcine embryos. Biol Reprod 70:1644-1649.
12. Jiang CC, Chen LH, Gillespie S, Kiejda KA, Mhaidat N, Wang YF, Thorne R, Zhang XD, Hersey P (2007): Tunicamycin sensitizes human melanoma cells to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by up-regulation of TRAIL-R2 via the unfolded protein response. Cancer Res 67:5880-5888.
13. Johnson JL, Toft DO (1995): Binding of p23 and hsp90 during assembly with the progesterone receptor. Mol Endocrinol 9:670-678.
14. Kamal A, Boehm MF, Burrows FJ (2004): Therapeutic and diagnostic implications of Hsp90 activation. Trends Mol Med 10:283-290.
15. Karkoulis PK, Stravopodis DJ, Margaritis LH, Voutsinas GE (2010): 17-Allylamino-17-demethoxygeldanamycin induces downregulation of critical Hsp90 protein clients and results in cell cycle arrest and apoptosis of human urinary bladder cancer cells. BMC Cancer 10:481.
16. Kaufman RJ (1999): Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev 13:1211-1233.
17. Kosano H, Stensgard B, Charlesworth MC, McMahon N, Toft D (1998): The assembly of progesterone receptor-hsp90 complexes using purified proteins. J Biol Chem 273(49):32973-32979.
18. Kuroda T, Naito K, Sugiura K, Yamashita M, Takakura I, Tojo H (2004): Analysis of the roles of cyclin B1 and cyclin B2 in porcine oocyte maturation by inhibiting synthesis with antisense RNA injection. Biol Reprod 70:154-159.
19. Levy RR, Cordonier H, Czyba JC, Guerin JF (2001): Apoptosis in preimplantation mammalian embryo and genetics. Ital J Anat Embryol 106:101-108.
20. Li J, Ni M, Lee B, Barron E, Hinton DR, Lee AS (2008): The unfolded protein response regulator GRP-78/BiP is required for endoplasmic reticulum integrity and stress-induced autophagy in mammalian cells. Cell Death Differ 15:1460-1471.
21. Machaty Z, Day BN, Prather RS (1998): Development of early porcine embryos *in vitro* and *in vivo*. Biol Reprod 59:451-455.
22. Masud A, Mohapatra A, Lakhani SA, Ferrandino A, Hakem R, Flavell RA (2007): Endoplasmic reticulum

stress-induced death of mouse embryonic fibroblasts requires the intrinsic pathway of apoptosis. J Biol Chem 282:14132-14139.
23. McLaughlin SH, Sobott F, Yao ZP, Zhang W, Nielsen PR, Grossmann JG, Laue ED, Robinson CV, Jackson SE (2006): The co-chaperone p23 arrests the Hsp90 ATPase cycle to trap client proteins. J Mol Biol 356: 746-758.
24. Neckers L, Ivy SP (2003): Heat shock protein 90. Curr Opin Oncol 15:419-424.
25. Patterson J, Palombella VJ, Fritz C, Normant E (2008): IPI-504, a novel and soluble HSP-90 inhibitor, blocks the unfolded protein response in multiple myeloma cells. Cancer Chemother Pharmacol 61:923-932.
26. Petters RM, Wells KD (1993): Culture of pig embryos. J Reprod Fertil Suppl 48:61-73.
27. Schumacher JA, Crockett DK, Elenitoba-Johnson KS, Lim MS (2007) Proteome-wide changes induced by the Hsp90 inhibitor, geldanamycin in anaplastic large cell lymphoma cells. Proteomics 7:2603-2616.
28. Senju M, Sueoka N, Sato A, Iwanaga K, Sakao Y, Tomimitsu S, Tominaga M, Irie K, Hayashi S, Sueoka E (2006): Hsp90 inhibitors cause G2/M arrest associated with the reduction of Cdc25C and Cdc2 in lung cancer cell lines. J Cancer Res Clin Oncol 132:150-158.
29. Smith DF, Faber LE, Toft DO (1990): Purification of unactivated progesterone receptor and identification of novel receptor-associated proteins. J Biol Chem 265:3996-4003.
30. Solit DB, Zheng FF, Drobnjak M, Munster PN, Higgins B, Verbel D, Heller G, Tong W, Cordon-Cardo C, Agus DB, Scher HI, Rosen N (2002): 17-Allylamino-17-demethoxygeldanamycin induces the degradation of androgen receptor and HER-2/neu and inhibits the growth of prostate cancer xenografts. Clin Cancer Res 8:986-993.
31. Sullivan WP, Owen BA, Toft DO (2002): The influence of ATP and p23 on the conformation of hsp90. J Biol Chem 277:45942-45948.
32. Szegezdi E, Logue SE, Gorman AM, Samali A (2006): Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO Rep 7:880-885.
33. Taiyab A, Sreedhar AS, Rao CM (2009): Hsp90 inhibitors, GA and 17AAG, lead to ER stress-induced apoptosis in rat histiocytoma. Biochem Pharmacol 78: 142-152.
34. Watanabe G, Behrns KE, Kim JS, Kim RD (2009): Heat shock protein 90 inhibition abrogates hepatocellular cancer growth through cdc2-mediated G2/M cell cycle arrest and apoptosis. Cancer Chemother Pharmacol 64:433-443.
35. Yao JQ, Liu QH, Chen X, Yang Q, Xu ZY, Hu F, Wang L, Li JM (2010): Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin inhibits the proliferation of ARPE-19 cells. J Biomed Sci 17:30.
36. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K (2001): XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 107:881-891.
37. Zhang DX, Cui XS, Kim NH (2010) Molecular characterization and polyadenylation-regulated expression of cyclin B1 and Cdc2 in porcine oocytes and early parthenotes. Mol Reprod Dev 77:38-50.

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