Master's Thesis

Treatment of Allicin Improves Maturation of Immature Oocytes and Subsequent Developmental Ability of Preimplantation Embryos

Sang-Gi Jeong

Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

May, 2018

알리신 처리를 받은 미성숙 난자의 성숙과 착상 전 배아 발달 능력 제고

지도교수 박세필

정 상 기

이 논문을 이학 석사학위 논문으로 제출함

2018 년 5 월

정상기의 이학 석사학위 논문을 인준함

심사위원장 _____

위 원 _____

위 원 _____

제주대학교 대학원

2018 년 5 월

Treatment of allicin improves maturation of immature oocytes and subsequent developmental ability of preimplantation embryos

Sang-Gi Jeong

(Supervised by Professor Se-Pill Park)

A thesis submitted in partial fulfillment of the requirement

For the degree of Master of Science

May, 2018

This thesis has been examined and approved.

Key Zung Riu

Chairperson of the supervising committee

Professor Key-Zung Riu, Ph.D., College of Applied Life Sciences,Jeju National University

Eun Young Kim

Professor Eun-Young Kim, Ph.D., College of Applied Life Sciences,Jeju National University

Se Pill Park

Professor Se-Pill Park, Ph.D., College of Applied Life Sciences,Jeju National University

Department of Biotechnology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Allicin (AL) regulates the cellular redox, proliferation, viability, and cell cycle of different cells against extracellular-derived stress. This study investigated the effects of allicin treatment on porcine oocyte maturation and developmental competence. Porcine oocytes were cultured in media supplemented with 0 (control), 0.01, 0.1, 1, 10, and 100 μM AL, respectively, during *in vitro* maturation (IVM). The rate of polar body emission was higher in the 0.1 AL-treated group (74.5% ± 2.3%) than in the control (68.0% ± 2.6%) ($p < 0.1$). After parthenogenetic activation, the rates of cleavage and blastocyst formation were significantly higher in the 0.1 AL-treated group than in the control ($p < 0.05$). The reactive oxygen species level at metaphase II did not significantly differ among all groups. In matured oocytes, the expression of both *BAK* and *CASP3*, and *BIRC5* were significantly lower and higher, respectively, in the 0.1 AL-treated group than in the control. Similarly, the expression of *BMP15* and *CCNB1*, and the activity of phospho-p44/42 MAPK, significantly increased. These results indicate that supplementation of oocyte maturation medium with allicin during IVM improves the maturation of oocytes and the subsequent developmental competence of porcine oocytes.

Key words: *Allicin, in vitro maturation, oocyte maturation, embryo development, pig*

1. INTRODUCTION

The efficiency of the *in vitro* production (IVP) of oocytes is significantly lower than that of *in vivo* culture (IVC). It is important to optimize conditions during *in vitro* maturation (IVM) and IVC, because they influence both maturational (cytoplasmic and nuclear) and developmental (fertilization, pronuclei formation, and cleavage) competencies (Kere *et al.*, 2013; Phongnimitr *et al.*, 2013). Different culture conditions, such as temperature, atmosphere, media supplements, and other components, can generate reactive oxygen species (ROS) that directly or indirectly affect mitochondrial function (Zhang and Liu, 2002), cytoskeletal dynamics (Albarracin *et al.*, 2005), and the functions of other organelles within oocytes and embryos (Tang *et al.*, 2013). A high concentration of extracellular-derived oxygen in the intracellular environment has a multitude of serious effects that ultimately affect the development rate (Jeon *et al.*, 2013). Other recent studies showed that a negative energy balance during IVM can lead to the loss of imprinted gene methylation in bovine oocytes (O'Doherty *et al.*, 2014). Imprinting is crucial for the function of the placenta and the regulation of fetal growth. Therefore, a failure to establish and maintain imprints during oocyte growth may contribute to early embryonic loss. For example, changing *in vitro* or *in vivo* maturation conditions by including/omitting hormonal supplements and altering exposure times can influence the morphogenesis of metaphase II (MII) spindles in porcine oocytes (Ueno *et al.*, 2005). Therefore, many researchers are striving to define the intracellular environment and developmental competence of cells, oocytes, and embryos.

Allicin is the most biologically active substance found in garlic, and it can be easily extracted and synthesized in the laboratory. It is a natural sulfur-containing compound with many biological properties and is responsible for the strong smell and flavor of garlic. Allicin has anticancer, antiviral, and antioxidant activities. The mechanism by which allicin

affects cancer cells has been examined at the molecular level. The induction of apoptosis is crucial for the anticancer effects of allicin (Borlinghaus *et al.*, 2014). For example, allicin inhibits lymphangiogenesis, one of the critical cellular events of tumor metastasis (Wang *et al.*, 2016). Allicin exhibits anticancer activity by suppressing the phosphorylation of vascular endothelial growth factor receptor 2 and focal adhesion kinase. In addition, other studies illustrate that allicin induces p53-mediated autophagy and reduces the viability of human hepatocellular carcinoma cell lines (Chu *et al.*, 2012). On the other hand, allicin protects human umbilical vein endothelial cells from apoptosis, which is mediated via a mechanism involving protection from ROS-mediated oxidative stress (Chen *et al.*, 2014). Allicin can also be used to decrease the doses of antifungal agents required to inhibit *C. albicans* growth (Kim *et al.*, 2012). Although treatment with allicin alone did not show any positive effects, the combination of allicin with an antifungal drug significantly enhanced the antifungal activity of the drug.

Culture conditions represent the most important factor influencing the developmental potential of embryos produced *in vitro*, and they affect both oocyte maturation and embryo development. Therefore, we investigated whether allicin affects the maturation and developmental competence of porcine oocytes during IVM. We also established a novel IVC system that improves the efficiency of IVM. Based on the results of this study, we expect the production efficiency of porcine embryos to significantly increase in the future.

2. MATERIALS & METHODS

2.1. Chemicals and reagents

All chemicals and reagents were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise.

2.2. Oocyte collection and IVM

Pre-pubertal porcine ovaries were collected from a local slaughterhouse and transported to the laboratory in saline supplemented with 75 μg/mL penicillin G and 50 μg/mL streptomycin sulfate within 2 h at 30–33℃. Cumulus-oocyte complexes (COCs) were aspirated from follicles with a diameter of 2–8 mm using an 18-gauge needle and a disposable 10 mL syringe. COCs were washed three times in tissue culture medium (TCM)-199-HEPES containing 0.1% (w/v) bovine serum albumin (BSA). Thereafter, COCs were matured in groups of 50 in 500 μL TCM-199 (Gibco, Grand Island, NY, USA) containing Earle's salts, 0.57 mM cysteine, 10 ng/mL epidermal growth factor, 0.5 μg/mL follicle-stimulating hormone, 0.5 μg/mL luteinizing hormone, and 10% (v/v) porcine follicular fluid under mineral oil for 44 h at 38.8℃ in 5% CO_2 and 95% air. COCs were cultured in IVM medium, containing 0, 0.01, 0.1, 1, 10, and 100 μM allicin (Sigma, St. Louis, MO, USA) for 44 h. The experiment was independently repeated nine times, with 50 oocytes per experiment.

2.3. Parthenogenetic activation (PA) and embryo culture

Following maturation, cumulus cells were removed from COCs by pipetting in the

presence of 0.1% (w/v) hyaluronidase. Denuded oocytes were parthenogenetically activated by treatment with 5 μM Ca^{2+}-ionomycin for 5 min. Activated oocytes were cultured in porcine zygote medium (PZM)-5 supplemented with 7.5 μg/mL cytochalasin B for 3 h, transferred to PZM-5 containing 0.4% (w/v) BSA, and cultured for 7 days at 38.8℃ in a humidified atmosphere of 5% CO_2 and 95% air.

2.4. Measurement of intracellular ROS levels

Intracellular ROS activity in oocytes and embryos was measured using a 2, 7-dichlorofluorescein assay, as previously described (Gupta et al., 2010). In brief, oocytes and embryos were incubated with 100 μM 2, 7-dichlorodihydrofluorescein diacetate (DCHFDA) for 20 min at 38.8℃, washed three times in PZM-5 to remove excess dye, and immediately analyzed under an epifluorescent microscope (Olympus, Tokyo, Japan) using excitation and emission wavelengths of 450 to 490 nm and 515 to 565 nm, respectively. Grayscale images were acquired with a digital camera (Nikon, Tokyo, Japan) attached to the microscope, and the mean grayscale values were measured with ImageJ software (NIH, Bethesda, MD, USA). Background fluorescence values were subtracted from the final values before statistical analysis. The experiment was independently repeated four times, with 25 to 30 oocytes per experiment.

2.5. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

To detect fragmented DNA, blastocysts were fixed overnight at 4℃ with 4.0% (w/v) paraformaldehyde prepared in phosphate-buffered saline (PBS) and then incubated with 0.1% Triton X-100 at 38.8℃ for 30 min. Blastocysts were incubated with fluorescein-conjugated

dUTP and terminal deoxynucleotidyl transferase (In Situ Cell Death Detection Kit, Roche, Manheim, Germany) in the dark for 1 h at 38.8℃. Mitotic and apoptotic cells were scored. Nuclei were stained with Hoechst 33342 (1 μg/mL) for 30 min, and embryos were washed with PBS containing 0.1% BSA. Blastocysts were mounted onto glass slides and examined under an inverted Olympus IX-71 fluorescent microscope. The experiment was independently repeated three times, and at least ten blastocysts were examined per group.

2.6. Hoechst staining

Blastocysts were cultured for 7 days after PA, fixed overnight at 4℃ in 4.0% (w/v) paraformaldehyde prepared in phosphate-buffered saline (PBS), washed more than three times with PBS containing 0.1% BSA, and incubated with Hoechst 33342 (1 μg/mL) at 38.8℃ for 30 min. Thereafter, blastocysts were washed with PBS containing 0.1% BSA, mounted onto glass slides, and examined under an epifluorescence microscope. The experiment was independently repeated three times, and at least ten blastocysts were examined per group.

2.7. mRNA extraction and cDNA synthesis

The mRNA was isolated from more than three biological replicates, with 30–40 oocytes per replicate, using the Dynabeads mRNA Direct kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. mRNA from each sample was collected in 10 μL elution buffer from the kit. Eluted RNA was reverse-transcribed into cDNA using an oligo (dT)20 primer and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

2.8. Real-time reverse transcription (RT)-PCR

The protocol used was basically the same as the one described previously (Lee et al., 2014). Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using the primer sets listed in Table 1 in a Step One Plus Real-time PCR System (Applied Biosystems, Warrington, UK) with a final reaction volume of 20 μL containing SYBR Green PCR Master Mix (Applied Biosystems). The PCR conditions were as follows: 10 min at 95℃, followed by 40 cycles of 15 sec at 95℃ and 60 sec at 55℃ or 60℃. Samples were then cooled to 12℃. The relative gene expression was analyzed by the 2-ΔΔCt method (Livak and Schmittgen, 2001) after normalization against the GAPDH mRNA level. The experiment was independently repeated three times.

Table 1. Primers used for real-time PCR

Gene	GenBank accession no.	Primer sequence	Annealing temperature (℃)	Product size (bp)
GAPDH	AF017079	F: GGGCATGAACCATGAGAAGT R: AAGCAGGGATGATGTTCTGG	60	230
BMP15	NM_001005155	F:CCCTCGGGTACTACACTATG R:GGCTGGGCAATCATATCC	60	192
CCNB1	NM_001170768.1	F:CCAACTGGTTGGTGTCACTG R:GCTCTCCGAAGAAAATGCAG	60	195
BCL2L1	NM_214285	F: GAAACCCCTAGTGCCATCAA R: GGGACGTCAGGTCACTGAAT	60	196

BIRC5	NM_214141	F: CCTGGCAGCTCTACCTCAAG R: GAAAGCACAACCGGATGAAT	60	233
BAK	XM_001928147	F: GTACGCAGATTCTTCAGGTC R: AAAGTCCATAAAGGGGTCTC	60	70
CASP3	NM_214131	F: GAGGCAGACTTCTTGTATGC R: CATGGACACAATACATGGAA	55	236

*F, forward; R, reverse.

2.9. Western blot analysis

The protocol was basically the same as that described previously (Lee et al., 2015). In brief, oocytes (40 per sample) were solubilized in 20 μL of 1× sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, 50 μM dithiothreitol, and 0.01% (w/v) bromophenol blue or phenol red) and heated for 5 min at 95℃. Proteins were resolved on 5–12% Tris SDS-PAGE gels for 1.5 h at 80–100 V. Samples were then transferred to Hybond-ECL nitrocellulose membranes (Amersham, Buckinghamshire, UK) at 300 mA for 2 h in transfer buffer (25 mM Tris, pH 8.5, containing 200 mM glycine and 20% (v/v) methanol). After blocking with 5% (w/v) nonfat milk prepared in PBS for 1 h, the membranes were incubated for at least 2 h with an anti-p44/42 mitogen-activated protein kinase (MAPK) or anti-phospho-p44/42 MAPK antibody diluted 1:500 in blocking solution (1× Tris-buffered saline, pH 7.5, containing 0.1% (v/v) Tween-20 and 5% (w/v) nonfat milk). Thereafter, the membranes were washed three times in Tris-buffered saline containing Tween-20 (TBST; 20 mM Tris-HCl, pH 7.5, containing 250 mM NaCl and 0.1% (v/v) Tween-20) and incubated for 1 h with anti-rabbit IgG-horseradish peroxidase diluted 1:2,000 in blocking solution. After three washes with TBST, immunoreactive protein bands were visualized with a chemiluminescent reagent (Invitrogen).

The experiment was independently repeated three times.

2.10. Statistical analysis

The general linear model procedure within the Statistical Analysis System (SAS User's Guide, 1985, Statistical Analysis System Inc., Cary, NC, USA) was used to analyze data from all experiments. The paired Student's t-test was used to compare relative gene expression. *P*-values of < 0.05 were considered significant.

3. RESULTS

3.1. Allicin treatment improves porcine oocyte maturation and subsequent development

The effect of allicin treatment on the IVM of porcine oocytes was evaluated by determining the survival rate (Table 2). The nuclear maturation of porcine oocytes was determined by quantifying the polar body emission rate of matured oocytes at the MII stage. Although the oocyte survival rate did not significantly differ between the control and allicin-treated groups (control, 88.6% ± 2.0%; 0.01 AL, 88.3% ± 3.4%; 0.1 AL, 84.7% ± 2.1%; 1 AL, 83.3% ± 3.5%; 10 AL, 88.8% ± 2.7%; and 100 AL, 88.0% ± 4.4%), the rate of polar body emission was tended to be higher in the 0.1 AL-treated group than in the control group (control, 68.0% ± 2.6%; and 0.1 AL, 74.5% ± 2.3%, $p < 0.1$). But, the rate of polar body emission was not higher in other AL-treated groups than in control (control, 68.0% ± 2.6%; 0.01 AL, 69.6% ± 2.8%; 1 AL, 69.2% ± 2.8%; 10 AL, 72.5% ± 5.2%; and 100 AL, 70.7% ± 3.3%).

After the parthenogenetic activation of oocytes, we examined the effects of allicin treatment on development and embryo quality during IVC (Table 3). The cleavage rate was significantly higher in the 0.01, 0.1, and 1 (control, 63.7% ± 3.9%; 0.01 AL, 72.3% ± 2.3%; 0.1 AL, 74.3% ± 3.3%; and 1 AL, 74.2% ± 2.6%, $p < 0.05$) AL-treated groups, and tended to be higher in 10 AL-treated groups (10 AL, 72.4% ± 3.2%, $p < 0.1$) than in the control. However, the cleavage rate was not higher in 100 AL-treated groups than in the control (100 AL, 71.8% ± 4.8%). Furthermore, the rate of blastocyst formation was significantly higher in the 0.1 AL-treated group (43.0% ± 1.8%) than in the control (29.9% ± 6.6%, $p < 0.05$) and the other allicin-treated groups (0.01 AL, 37.5% ± 3.1%; 1 AL, 30.1% ± 3.7%; 10 AL, 40.2% ± 3.6%; 100 AL, 35.5% ± 3.0%).

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed to evaluate embryo quality. The total number of cells did not markedly differ between the control and 0.1 AL-treated groups (control, 67.4 ± 7.7; 0.01 AL, 61.9 ± 5.9; 0.1 AL, 72.6 ± 5.7; 1 AL, 72.5 ± 6.6; 10 AL, 71.9 ± 6.3; and 100 AL, 74.1 ± 6.5). The percentage of apoptotic cells was slightly lower in the AL-treated groups than in the control. However, these differences were not statistically significant (control, 3.2% ± 0.3%; 0.01 AL, 2.5% ± 0.3%; 0.1 AL, 2.8% ± 0.3%; 1 AL, 2.9% ± 0.5%; 10 AL, 2.9% ± 0.3%; and 100 AL, 1.9% ± 0.2%).

Table 2. Effects of allicin on porcine oocyte survival and polar body emission following IVM

Concentration of AL (μM)	No. of GV	No. (%) of	
		oocyte survival	polar body emission
0	430	372 (86.6 ± 2.0)	253 (68.0 ± 2.6)
0.01	420	371 (88.3 ± 3.4)	258 (69.6 ± 2.8)
0.1	420	355 (84.7 ± 2.1)	264 (74.5 ± 2.3)*
1	420	348 (83.3 ± 3.5)	241 (69.2 ± 2.8)
10	420	373 (88.8 ± 2.7)	270 (72.5 ± 5.2)
100	420	369 (88.0 ± 4.4)	261 (70.7 ± 3.3)

Values are means ± standard error of the mean of independent experiments. *Value is considered different from the control at $p < 0.1$. AL, allicin.

Table 3. Effect of allicin treatment on development of porcine embryos during IVM (r = 7)

Concentration of AL (μM)	No. (%) of		Total no. of cells	Rate (%) of apoptotic cells
	cleaved oocytes at day 2	blastocysts at day 7		
0	237 (63.7 ± 3.9)	70 (29.9 ± 6.6)	67.4 ± 7.7	3.2 ± 0.3
0.01	268 (72.3 ± 2.3)**	100 (37.5 ± 3.1)	61.9 ± 5.9	2.5 ± 0.3
0.1	263 (74.3 ± 3.3)**	114 (43.4 ± 1.8)**	72.6 ± 5.7	2.8 ± 0.3
1	262 (74.2 ± 2.6)**	74 (30.1 ± 3.7)	72.5 ± 6.6	2.9 ± 0.5
10	270 (72.4 ± 3.2)*	108 (40.2 ± 3.6)	71.9 ± 6.3	2.9 ± 0.3
100	265 (71.8 ± 4.8)	94 (35.5 ± 3.0)	74.1 ± 6.5	1.9 ± 0.2

Values are means ± standard error of the mean of independent experiments. *$p < 0.1$, **$p < 0.05$ are considered as the tendency and significant difference, respectively.

3.2. Allicin treatment does not affect the reactive oxygen species level in matured oocytes

To determine whether the effects of allicin were dose dependent, oocytes were cultured in IVM media supplemented with 0, 0.01, 0.1, 1, 10, and 100 AL, and the ROS level was measured in MII oocytes (Fig. 1). The ROS level did not significantly differ between the control and the allicin-treated groups (control, 47.4 ± 1.4; 0.01 AL, 46.8 ± 2.2; 0.1 AL, 46.0 ± 2.1; 1 AL, 45.0 ± 1.5; 10 AL, 44.8 ± 1.7; and 100 AL, 45.4 ± 1.2; Fig. 1B).

Because the rates of cleavage and blastocyst formation were significantly higher in the 0.1 AL-treated group than in the control, we set the optimal concentration of allicin to 0.1 μM (Tables 2 and 3). Next, we examined whether the antioxidant activity of 0.1 AL could affect the cellular ROS level at each nuclear stage, germinal vesicle breakdown (GVBD, 26 h), MI (32 h), and MII (44 h). Although the ROS level from GVBD to MII did not significantly differ between control and 0.1 AL-treated oocytes, it was slightly higher in the latter group than in the former group (control, 47.3 ± 5.6; 0.01 AL, 46.8 ± 2.2; 0.1 AL, 46.0 ± 2.1; 1 AL, 45.0 ± 1.5; 10 AL, 44.8 ± 1.7; and 100 AL, 45.4 ± 1.2; Fig. 1C).

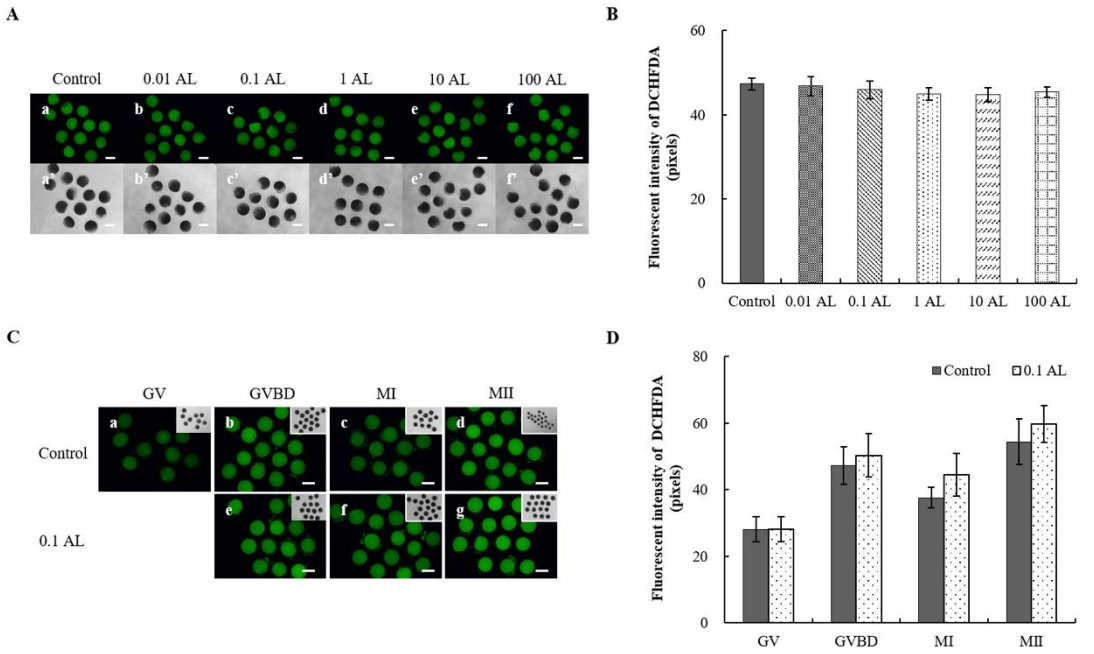

Fig. 1. Effects of different concentrations of allicin on the ROS level in porcine oocytes during IVM. Oocytes from the control (C-a to -d) and 0.1 AL-treated (C-e to -g) groups were stained with DCHFDA following maturation (A) and at GVBD (26 h), MI (32 h), and MII (44 h) (C). The ROS level was determined by quantifying the fluorescent intensity of MII porcine oocytes in each group (B and D). Values are means ± standard error of the mean of independent experiments. Scale bars = 100 μm. Original magnification, ×160.

3.3. Allicin affects the expression of apoptosis-related genes in matured porcine oocytes

To determine whether allicin treatment during IVM can affect the expression levels of apoptosis-related genes, we performed real-time PCR analyses (Fig. 2). The expression of baculoviral IAP repeat-containing 5 (*BIRC5*), an anti-apoptotic gene, was significantly higher in the 0.1 AL-treated group than in the control. By contrast, the expression of another anti-apoptotic gene, B-cell lymphoma 2-like protein 1 (*BCL2L1*), did not markedly differ between the control and 0.1 AL-treated groups. The expression of Bcl-2 homologous antagonist killer (*BAK*) and caspase 3 (*CASP3*), both of which are pro-apoptotic genes, was significantly lower in the 0.1 AL-treated group than in the control ($p < 0.05$).

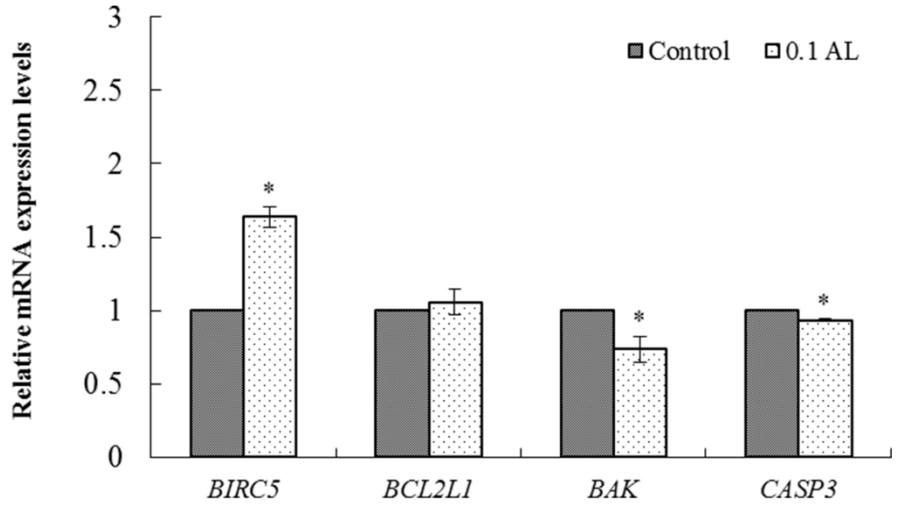

Fig. 2. Relative expression levels of pro-apoptotic and anti-apoptotic genes

Relative expression levels of pro-apoptotic (BAK and CASP3) and anti-apoptotic (BCL2L1 and BIRC5) genes in mature oocytes from the control and 0.1 AL-treated groups Significant differences from the control are indicated (*$p < 0.05$). Values are means ± standard error of the mean of independent experiments.

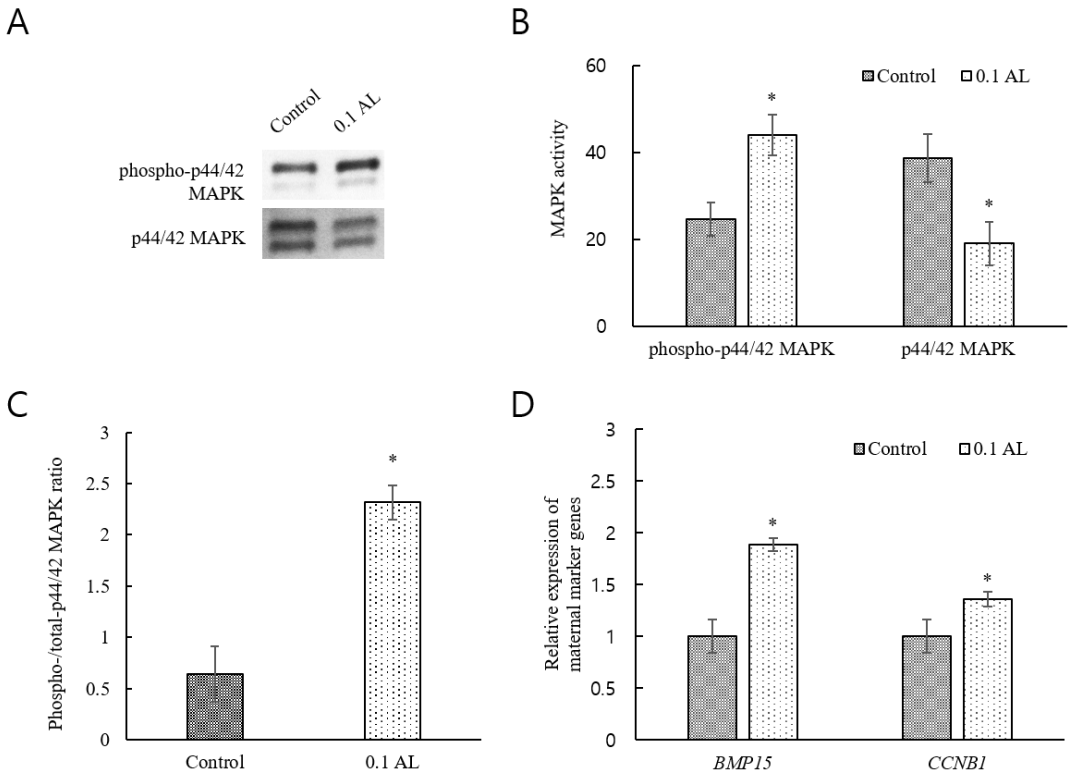

Fig. 3. Effects of AL treatment on the cytoplasmic maturation of porcine oocytes during IVM. Phosphorylated MAPK activity was confirmed by western blotting (A), and phosphorylated MAPK activity was evaluated by quantifying the fluorescent intensity (B). Ratio of active phospho-p44/42 MAPK to p44/42 MAPK (C). Expression of maternal marker genes in MII porcine oocytes was confirmed by real-time RT-PCR (D) Significant differences from the control are indicated (*$p < 0.05$). Values are means ± standard error of the mean of independent experiments.

3.4. Allicin increases molecular maturation factors in porcine oocytes in vitro

To examine the effect of allicin treatment on the cytoplasmic maturation of oocytes, we measured p44/42 MAPK activity and maternal gene expression following IVM (Fig. 3). The phosphorylated p44/42 MAPK level in maturing porcine oocyte lysates was analyzed by western blotting (Figs. 3A and B). The level of phosphorylated p44/42 MAPK (phospho-p44/42 MAPK) was >2-fold higher in the 0.1 AL-treated group (44.0 ± 2.9) than in the control (19.0 ± 3.2, $p < 0.05$). The ratio of phosphorylated p44/42 MAPK to p44/42 MAPK was ~4-fold higher in the 0.1 AL-treated group (2.3 ± 0.2) than in the control (0.6 ± 0.3, $p < 0.05$, Fig. 3C). The expression of the maternal marker genes *BMP15* and *CCNB1* was analyzed at MII during IVM by real-time RT-PCR (Fig. 3D). There was no difference in the expression of *BMP15* and *CCNB1* from the GV stage to the MI stage between the control and the AL-treated groups (Data not shown). The expression of *BMP15* and *CCNB1* was 2- and 1.5-fold higher in the AL-treated group than in the control at MII, respectively ($p < 0.05$).

4. DISSCUSTION

In this study, we investigated whether allicin affects the maturation and developmental competence of porcine oocytes, as well as the ROS level and the apoptotic rate. The addition of 0.1 μM allicin to IVM media improved not only the rates of polar body emission, cleavage, and blastocyst formation, but also the expression of anti-apoptotic and maternal marker genes, and MAPK activity in matured oocytes.

The survival and polar body extrusion rates were estimated by stereomicroscopy. Although the survival rate did not differ among the groups, the polar body emission rate was tended to higher in the 0.1 AL-treated group than in the control ($p < 0.1$, Table 2). Polar body emission associates with meiosis, and it occurs at MII (Choi *et al.*, 2013; Ogawa *et al.*, 2010). The cleavage rate was significantly higher in the 0.01, 0.1, and 1 AL-treated groups than in the control, while the blastocyst formation rate was significantly higher in the 0.1 AL-treated group (Table 3). However, the total number of cells and the percentage of apoptotic cells did not differ among all groups. Another study demonstrates that the increased rate of MII oocytes is indicative of nuclear maturation. All-*trans* retinoic acid significantly improved goat nuclear oocyte maturation via increased polar body formation following IVM (Pu *et al.*, 2014). Effect of melatonin melatonin supplementation during porcine IVM resulted in a greater proportion of oocytes extruding the polar body and beneficial effects of melatonin were shown on oocyte maturation (Kang *et al.*, 2009). Caffeine supplementation during IVM can also improve nuclear maturation and subsequent preimplantation development of dromedary camel oocytes (Fathi *et al.*, 2014). In light of these results, allicin treatment may regulate meiosis by promoting oocyte maturation to MII, which led us to conclude that allicin enhanced the developmental rate of embryos by improving the oocyte maturation rate. Therefore, we set the allicin concentration at 0.1 μM.

We stained *in vitro*-matured oocytes with DCHFDA to determine whether the ROS level was altered by the antioxidant activity of allicin (Fig. 1). Oocytes were treated with different concentrations of allicin (0, 0.01, 0.1, 1, 10, and 100 AL) during IVM (44 h). Allicin can protect cells from oxidative stress by triggering the production of antioxidants, thereby reducing the levels of cytotoxic substances and scavenging free radicals (Chan *et al.*, 2013). However, the ROS level did not differ between the control and allicin-treated groups. To further investigate the antioxidant activity of allicin, control and 0.1 AL-treated oocytes were evaluated at GVBD (26 h), MI (32 h), and MII (44 h) (Figs. 1C and D). Similar to the results shown in Figure 1A and B, there was no difference in the ROS level among the different stages. Although most studies have reported changes in the oxidant–antioxidant balance after the addition of H_2O_2 into IVM media (Chen *et al.*, 2014; Tu *et al.*, 2016), extracellular-derived oxidative stress was not a factor in the present study. Taken together, these results indicate that allicin does not possess antioxidant activity under normal culture conditions. Other studies have reported that allicin, when combined with other compounds, may possess antioxidant activity (Cai *et al.*, 2007; Kim *et al.*, 2012), although further studies are necessary to confirm the effects of allicin in combination with other substances.

Apoptosis influences oocyte quality and fertility by increasing the percentage of GV-stage oocytes, damaging the cytoskeleton (an increased percentage of abnormal spindles), and triggering epigenetic modifications in oocytes (Duan *et al.*, 2015). Apoptosis is also involved in normal ovarian development and function such as prenatal germ cell death, granulosa cell death during postnatal follicular atresia, and ovarian surface epithelial cell death (Liu *et al.*, 2015). *BIRC5* is involved in the regulation of the cell cycle, especially the G2/M stage. It also plays roles in cell division and cell function, which are essential for reproduction, and inhibits apoptosis (Siffroi-Fernandez *et al.*, 2014). *BCL2L1* encodes an anti-apoptotic protein that inhibits the pro-apoptotic proteins *BAX* and *BAK*, which form

pores in the outer mitochondrial membrane and induce the release of mitochondrial cytochrome c into the cytoplasm. Another pro-apoptotic gene, *CASP3*, functions in the final stage of apoptosis. As a result of the *BAK*/*BAX*-mediated pore formation, cytochrome c present in the mitochondrial matrix is released into the cytoplasm. Released cytochrome c binds to apoptotic protease-activating factor-1 and caspase 9 present in the cytoplasm of oocytes, leading to the formation of the apoptosome, which induces apoptosis (Shamas-Din *et al.*, 2013). Although *BCL2L1* expression did not differ between the control and 0.1 AL-treated groups, *BIRC5* expression was significantly higher in the 0.1 AL-treated group than in the control at MII (Fig. 2). By contrast, *BAK* and *CASP3* expression was significantly lower in the 0.1 AL-treated group than in the control. Other studies reported that allicin reduces the activities of CASP3 and poly (ADP-ribose) polymerase, which is consistent with the role of allicin in preventing apoptosis (Chen *et al.*, 2014). These results indicate that allicin influences MII oocytes via an anti-apoptotic mechanism by increasing the expression of anti-apoptotic genes and decreasing the expression of pro-apoptotic genes.

The present study showed that 0.1 AL-treated oocytes exhibited a >2-fold increase in phosphorylated p44/42 MAPK activity than in the control (Figs. 3A and B). The ratio of phospho-p44/42 MAPK to p44/42 MAPK was ~4-fold higher in the 0.1 AL-treated group than in the control (Fig. 3C). MAPK and MPF are important that needed to regulate microtubule and actin filaments. MAPK regulates cell cycle progression (Yan *et al.*, 2012), and MAPK activity is required for both the resumption of meiosis and maintenance of meiotic arrest at MII (Dedieu *et al.*, 1996; Lee *et al.*, 2014). MAPK controls spindle stability during MII arrest and microtubule organization of MII oocytes in mice (Terret *et al.*, 2003; Sun *et al.*, 2008). In addition, the expression of *BMP15* and *CCNB1*, maternal marker genes, was higher in 0.1 AL-treated oocytes at MII than in the control (Fig. 3D). *BMP15* is an important maternal gene that regulates cumulus cell proliferation, expansion, and oocyte

development (Hussein *et al.*, 2006). Another study reported that *CCNB1* synthesized from mRNA stored in the cytoplasm of oocytes can affect MAPK and the MPF pathway (Liang *et al.*, 2007; Sanchez and Smitz, 2012). Taken collectively, these results indicate that allicin enhances cytoplasmic maturation by increasing MAPK activity in matured oocytes. Maternal gene expression may also be involved, because we showed that increased expression of maternal genes influenced the polar body emission rate. Nevertheless, allicin affects the cytoplasmic maturation of porcine oocytes and the developmental competence of embryos.

In conclusion, allicin had positive effects on porcine oocyte maturation and developmental competence *in vitro*. We hypothesized that allicin may regulate the extracellular-derived oxygen concentration and metabolic events in porcine oocytes during IVM. But, the effects of allicin were mediated by an anti-apoptotic mechanism that involved an increase in MAPK activity. Although allicin may also control other aspects of embryonic development, we conclude that allicin affected the developmental competence of oocytes by regulating apoptosis and the expression of maturation factors. For these reasons, allicin should be included in IVM medium, which should significantly increase the production efficiency of porcine embryos *in vitro*.

REFERENCE

Albarracin, J. L., Morato, R., Izquierdo, D. and Mogas, T. (2005) Vitrification of calf oocytes: effects of maturation stage and prematuration treatment on the nuclear and cytoskeletal components of oocytes and their subsequent development. Molecular reproduction and development, 72, 239-249.

Borlinghaus, J., Albrecht, F., Gruhlke, M. C., Nwachukwu, I. D. and Slusarenko, A. J. (2014) Allicin: chemistry and biological properties. Molecules, 19, 12591-12618.

Cai, Y., Wang, R., Pei, F. and Liang, B. B. (2007) Antibacterial activity of allicin alone and in combination with beta-lactams against Staphylococcus spp. and Pseudomonas aeruginosa. The Journal of antibiotics, 60, 335-338.

Chan, J. Y., Yuen, A. C., Chan, R. Y. and Chan, S. W. (2013) A review of the cardiovascular benefits and antioxidant properties of allicin. Phytotherapy research : PTR, 27, 637-646.

Chen, S., Tang, Y., Qian, Y., Chen, R., Zhang, L., Wo, L. and Chai, H. (2014) Allicin prevents H(2)O(2)-induced apoptosis of HUVECs by inhibiting an oxidative stress pathway. BMC complementary and alternative medicine, 14, 321.

Choi, J. Y., Kang, J. T., Park, S. J., Kim, S. J., Moon, J. H., Saadeldin, I. M., Jang, G. and Lee, B. C. (2013) Effect of 7,8-dihydroxyflavone as an antioxidant on in vitro maturation of oocytes and development of parthenogenetic embryos in pigs. The Journal of reproduction and development, 59, 450-456.

Chu, Y. L., Ho, C. T., Chung, J. G., Rajasekaran, R. and Sheen, L. Y. (2012) Allicin induces p53-mediated autophagy in Hep G2 human liver cancer cells. Journal of agricultural and food chemistry, 60, 8363-8371.

Craig, J., Zhu, H., Dyce, P. W., Petrik, J. and Li, J. (2004) Leptin enhances oocyte nuclear and cytoplasmic maturation via the mitogen-activated protein kinase pathway.

Endocrinology, 145, 5355-5363.

Dedieu, T., Gall, L., Crozet, N., Sevellec, C. and Ruffini, S. (1996) Mitogen-activated protein kinase activity during goat oocyte maturation and the acquisition of meiotic competence. Molecular reproduction and development, 45, 351-358.

Duan, X., Wang, Q. C., Chen, K. L., Zhu, C. C., Liu, J. and Sun, S. C. (2015) Acrylamide toxic effects on mouse oocyte quality and fertility in vivo. Scientific reports, 5, 11562.

Fathi, M., Seida, A. A., Sobhy, R. R., Darwish, G. M., Badr, M. R. and Moawad, A. R. (2014) Caffeine supplementation during IVM improves frequencies of nuclear maturation and preimplantation development of dromedary camel oocytes following IVF. Theriogenology, 81, 1286-1292.

Gupta, M. K., Uhm, S. J. and Lee, H. T. (2010) Effect of vitrification and beta-mercaptoethanol on reactive oxygen species activity and in vitro development of oocytes vitrified before or after in vitro fertilization. Fertility and sterility, 93, 2602-2607.

Hussein, T. S., Thompson, J. G. and Gilchrist, R. B. (2006) Oocyte-secreted factors enhance oocyte developmental competence. Developmental biology, 296, 514-521.

Jeon, Y., Kwak, S. S., Cheong, S. A., Seong, Y. H. and Hyun, S. H. (2013) Effect of trans-epsilon-viniferin on in vitro porcine oocyte maturation and subsequent developmental competence in preimplantation embryos. The Journal of veterinary medical science / the Japanese Society of Veterinary Science, 75, 1277-1286.

Kang, J. T., Koo, O. J., Kwon, D. K., Park, H. J., Jang, G., Kang, S. K. and Lee, B. C. (2009) Effects of melatonin on in vitro maturation of porcine oocyte and expression of melatonin receptor RNA in cumulus and granulosa cells. Journal of pineal research, 46, 22-28.

Kere, M., Siriboon, C., Lo, N. W., Nguyen, N. T. and Ju, J. C. (2013) Ascorbic acid improves the developmental competence of porcine oocytes after parthenogenetic activation and somatic cell nuclear transplantation. The Journal of reproduction and development, 59, 78-84.

Kim, Y. S., Kim, K. S., Han, I., Kim, M. H., Jung, M. H. and Park, H. K. (2012) Quantitative and qualitative analysis of the antifungal activity of allicin alone and in combination with antifungal drugs. PloS one, 7, e38242.

Lee, S. E., Kim, E. Y., Choi, H. Y., Moon, J. J., Park, M. J., Lee, J. B., Jeong, C. J. and Park, S. P. (2014) Rapamycin rescues the poor developmental capacity of aged porcine oocytes. Asian-Australasian journal of animal sciences, 27, 635-647.

Liang, C. G., Su, Y. Q., Fan, H. Y., Schatten, H. and Sun, Q. Y. (2007) Mechanisms regulating oocyte meiotic resumption: roles of mitogen-activated protein kinase. Molecular endocrinology, 21, 2037-2055.

Liu, S., Jiang, L., Zhong, T., Kong, S., Zheng, R., Kong, F., Zhang, C., Zhang, L. and An, L. (2015) Effect of Acrylamide on Oocyte Nuclear Maturation and Cumulus Cells Apoptosis in Mouse In Vitro. PloS one, 10, e0135818.

Livak, K. J. and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 25, 402-408.

O'Doherty, A. M., O'Gorman, A., al Naib, A., Brennan, L., Daly, E., Duffy, P. and Fair, T. (2014) Negative energy balance affects imprint stability in oocytes recovered from postpartum dairy cows. Genomics, 104, 177-185.

Ogawa, B., Ueno, S., Nakayama, N., Matsunari, H., Nakano, K., Fujiwara, T., Ikezawa, Y. and Nagashima, H. (2010) Developmental ability of porcine in vitro matured oocytes at the meiosis II stage after vitrification. The Journal of reproduction and

development, 56, 356-361.

Phongnimitr, T., Liang, Y., Srirattana, K., Panyawai, K., Sripunya, N., Treetampinich, C. and Parnpai, R. (2013) Effect of L-carnitine on maturation, cryo-tolerance and embryo developmental competence of bovine oocytes. Animal science journal = Nihon chikusan Gakkaiho, 84, 719-725.

Pu, Y., Wang, Z., Bian, Y., Zhang, F., Yang, P., Li, Y., Zhang, Y., Liu, Y., Fang, F., Cao, H. and Zhang, X. (2014) All-trans retinoic acid improves goat oocyte nuclear maturation and reduces apoptotic cumulus cells during in vitro maturation. Animal science journal = Nihon chikusan Gakkaiho, 85, 833-839.

Sanchez, F. and Smitz, J. (2012) Molecular control of oogenesis. Biochimica et biophysica acta, 1822, 1896-1912.

Shamas-Din, A., Kale, J., Leber, B. and Andrews, D. W. (2013) Mechanisms of action of Bcl-2 family proteins. Cold Spring Harbor perspectives in biology, 5, a008714.

Siffroi-Fernandez, S., Dulong, S., Li, X. M., Filipski, E., Grechez-Cassiau, A., Peteri-Brunback, B., Meijer, L., Levi, F., Teboul, M. and Delaunay, F. (2014) Functional genomics identify Birc5/survivin as a candidate gene involved in the chronotoxicity of cyclin-dependent kinase inhibitors. Cell cycle, 13, 984-991.

Sun, S. C., Xiong, B., Lu, S. S. and Sun, Q. Y. (2008) MEK1/2 is a critical regulator of microtubule assembly and spindle organization during rat oocyte meiotic maturation. Molecular reproduction and development, 75, 1542-1548.

Tang, D. W., Fang, Y., Liu, Z. X., Wu, Y., Wang, X. L., Zhao, S., Han, G. C. and Zeng, S. M. (2013) The disturbances of endoplasmic reticulum calcium homeostasis caused by increased intracellular reactive oxygen species contributes to fragmentation in aged porcine oocytes. Biology of reproduction, 89, 124.

Terret, M. E., Lefebvre, C., Djiane, A., Rassinier, P., Moreau, J., Maro, B. and Verlhac, M. H.

(2003) DOC1R: a MAP kinase substrate that control microtubule organization of metaphase II mouse oocytes. Development, 130, 5169-5177.

Tu, G., Zhang, Y. F., Wei, W., Li, L., Zhang, Y., Yang, J. and Xing, Y. (2016) Allicin attenuates H(2)O(2)-induced cytotoxicity in retinal pigmented epithelial cells by regulating the levels of reactive oxygen species. Molecular medicine reports, 13, 2320-2326.

Ueno, S., Kurome, M., Ueda, H., Tomii, R., Hiruma, K. and Nagashima, H. (2005) Effects of maturation conditions on spindle morphology in porcine MII oocytes. The Journal of reproduction and development, 51, 405-410.

Wang, W., Du, Z., Nimiya, Y., Sukamtoh, E., Kim, D. and Zhang, G. (2016) Allicin inhibits lymphangiogenesis through suppressing activation of vascular endothelial growth factor (VEGF) receptor. The Journal of nutritional biochemistry, 29, 83-89.

Yan, L., Luo, H., Gao, X., Liu, K. and Zhang, Y. (2012) Vascular endothelial growth factor-induced expression of its receptors and activation of the MAPK signaling pathway during ovine oocyte maturation in vitro. Theriogenology, 78, 1350-1360.

Zhang, W. and Liu, H. T. (2002) MAPK signal pathways in the regulation of cell proliferation in mammalian cells. Cell research, 12, 9-18.

ABSTRACT IN KOREAN

알리신은 세포 외부 유래 스트레스로부터 세포 내의 산화 환원, 증식, 생존력 및 세포 주기를 조절한다. 본 연구에서는 돼지 난자의 체외 성숙 과정과 이들 난자로부터 만들어진 체외생산 배아의 발달에 알리신이 미치는 영향을 조사하였다. 돼지의 난자는 각각 0, 0.01, 0.1, 1, 10, 100 μM AL 의 알리신을 포함하는 체외성숙용 배양액에서 44 시간 동안 체외성숙을 유도하였다. 극체 방출률은 0.1 AL-처리군에서 대조군에 비해 증가하였다. 단위발생 후 난할율과 배반포 생성률은 0.1 AL 처리군에서 대조군에 비해 유의적으로 높게 나타났다. 성숙된 난자에서 활성산소 수준은 대조군과 0.1 AL 처리군 사이에 유의적인 차이를 보이지 않았다. 성숙된 난자에서 BCL2 antagonist/killer 1 (*BAK*)과 카스파제 3 (*CASP3*)의 발현은 0.1 AL 처리군에서 대조군에 비해 유의적으로 낮아지고, 그리고 Baculoviral inhibitor of apoptosis repeat-containing 5 (*BIRC5*)의 발현이 0.1 AL 처리군에서 대조군에 비해 유의적으로 증가되었다. 유사하게, 모계 유전자 (*BMP15* 와 *CCNB*)의 발현과 인산화-p44/42 미토겐 활성 단백질 인산호소 (phospho-p44/42 MAPK)의 활성이 0.1 AL 처리군에서 대조군에 비해 유의적으로 증가하는 것을 확인했다. 이러한 결과들은 체외성숙 기간 동안 성숙용 배양액에 알리신의 첨가가 돼지 난자의 체외성숙과 돼지 발달능력을 향상시킬 수 있다는 것을 나타낸다.

ACKNOWLEDGEMENT

3 년의 시간 동안 실험실 생활을 하면서 도움을 주신 모든 분들께 감사의 말씀을 드립니다. 부족함 없는 좋은 실험 환경을 제공해 주심은 물론 학생들을 위한 지원을 아끼지 않으신 박세필 교수님, 서울부설연구소에서 개인지도와 더불어 많은 새로운 실험을 할 수 있게 지원하고 지도해주신 김은영 소장님, 바쁘신 와중에도 끝까지 실험에 대한 관심과 열정으로 지도를 병행하여 실험실 생활에 큰 도움을 주신 이승은 박사님께 감사의 말씀을 드립니다. 그 밖에도 3 년의 기간 동안 옆에서 실험실 생활과 실험에 도움과 지원을 거리낌없이 해준 대학원 또는 학과 선·후배, 박민지 박사님, 박윤귀 연구원, 김원재, 윤재욱, 박찬오, 박효진, 홍동용 학생들에게도 감사의 말씀을 드리며 실험을 수행하기 위한 난소 확보를 위하여 새벽부터 고생하시는 박철희 부장님께도 감사의 말씀을 전합니다. 또한 석사과정 생활을 하는 동안 함께 해주신 문성호 교수님, 오창언 선생님, 김연옥 선생님, 오경돈 팀장님, 강상경 선생님, 오효진 선생님들께 감사의 말씀을 드립니다.