

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

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Summary

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 medium supplemented with 0 (control), 0.01, 0.1, 1, 10 or 100 μM AL, respectively, during *in vitro* maturation (IVM). The rate of polar body emission was higher in the 0.1 AL-treated group ($74.5\% \pm 2.3\%$) than in the control ($68.0\% \pm 2.6\%$) ($P < 0.1$). 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* was 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-44/42 mitogen-activated protein kinase (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.

Keywords: Allicin, Embryo development, *In vitro* maturation, Oocyte maturation, Pig

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, medium supplements, and other components, can generate reactive oxygen species (ROS) that directly or indirectly affect mitochondrial function (Zhang & Liu, 2002), cytoskeletal dynamics (Albarraquin *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 have shown 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

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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 flavour 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 tumour 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). Conversely, 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.

Materials and Methods

Chemicals and reagents

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

Oocyte collection and *in vitro* maturation

Prepubertal 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 32 to 35°C. Cumulus–oocyte complexes (COCs) were aspirated from follicles with a diameter of 2 to 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). Groups of 50 COCs were matured in 500 µl of 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°C in a humidified atmosphere of 5% CO₂ in air.

COCs were cultured in IVM medium, containing 0, 0.01, 0.1, 1, 10 or 100 µM allicin (0, 0.01, 0.1, 1, 10 or 100 AL, respectively) for 44 h. The experiment was independently repeated nine times, with 50 oocytes per experiment.

Parthenogenetic activation and embryo culture

Following maturation, cumulus cells were removed by pipetting in the presence of 1 mg/ml hyaluronidase for 2 to 3 min. Oocytes were parthenogenetically activated with 5 µM Ca²⁺ ionomycin (Sigma) for 5 min. After 3 h of culture in porcine zygote medium (PZM)-5 containing 7.5 µg/ml cytochalasin B (Sigma), embryos were washed three times in PZM-5 containing 0.4% (w/v) BSA and cultured in the same medium for 7 days at 38.8°C in a humidified atmosphere of 5% CO₂ in air. The oocytes and embryos were washed in Dulbecco's Phosphate-Buffered Saline (DPBS), and, depending on the experiment, either fixed in 4.0% (w/v) paraformaldehyde for 20 min and stored at 4°C or snap-frozen in liquid nitrogen and stored at –70°C.

Measurement of the intracellular ROS level

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°C, washed three times in PZM-5 to remove excess dye, and immediately analysed under an epifluorescence 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.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

To detect fragmented DNA, blastocysts were fixed overnight at 4°C with 4.0% (w/v) paraformaldehyde prepared in phosphate-buffered saline (PBS) and then incubated with 0.1% Triton X-100 at 38.8°C for 30 min. Blastocysts were incubated with fluorescein-conjugated dUTP and terminal deoxynucleotidyl transferase (In Situ Cell Death Detection Kit, Roche, Mannheim, Germany) in the dark for 1 h at 38.8°C. 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 fluorescence microscope. The experiment was independently repeated three times, and at least ten blastocysts were examined per group.

Real-time reverse transcription-polymerase chain reaction with SYBR green

The protocol used was basically the same as the one described previously (Lee *et al.*, 2014). mRNA was isolated from groups of 20 *in vitro*-matured oocytes using the Dynabeads mRNA Direct Kit (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using an oligo(dT)₂₀ primer and SuperScript III reverse transcriptase (Invitrogen). 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°C, followed by 40 cycles of 15 s at 95°C and 60 s at 55°C or 60°C. Samples were then cooled to 12°C. The relative gene expression was analysed by the 2^{-ΔΔCt} method (Livak & Schmittgen, 2001) after normalization against the *GAPDH* mRNA level. The experiment was independently repeated three times.

Western blot analysis

The protocol was basically the same as the one described previously (Lee *et al.*, 2014). In brief, oocytes (40 oocytes 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°C. For western blotting, proteins were resolved on 5% to 12% Tris-SDS-PAGE gels for 1.5 h at 80 to 100 V. Samples were then transferred onto 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) non-fat milk 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× TBS, pH 7.5, containing 0.1% (v/v) Tween-20 and 5% (w/v) non-fat 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.

Statistical analysis

The general linear model procedure within Statistical Analysis System software (SAS User's Guide 2013, Statistical Analysis System Inc., Cary, NC, USA) was used to analyse data from all experiments. The paired Student's *t*-test and Tukey's multiple range test were used to determine significant differences. *P*-values of < 0.05 were considered significant.

Results

Allucin treatment improves porcine oocyte maturation and subsequent development

The effect of allucin 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 allucin-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

Table 1 Details of the primers used for real-time RT-PCR

Gene	GenBank accession no.	Primer sequence	Annealing temperature (°C)	Product size (bp)
<i>GAPDH</i>	AF017079	F: GGGCATGAACCATGAGAAGT; R: AAGCAGGGATGATGTTCTGG	60	230
<i>BMP15</i>	NM_001005155	F:CCCTCGGGTACTACACTATG; R:GGCTGGGCAATCATATCC	60	192
<i>CCNB1</i>	NM_001170768.1	F:CCAACCTGGTTGGTGTCACTG; R:GCTCTCCGAAGAAAATGCAG	60	195
<i>BCL2L1</i>	NM_214285	F: GAAACCCCTAGTGCCATCAA; R: GGGACGTCAGGTCACTGAAT	60	196
<i>BIRC5</i>	NM_214141	F: CCTGGCAGCTCTACCTCAAG; R: GAAAGCACAACCGGATGAAT	60	233
<i>BAK</i>	XM_001928147	F: GTACGCAGATTCTTCAGGTC; R: AAAGTCCATAAAGGGGTCTC	60	70
<i>CASP3</i>	NM_214131	F: GAGGCAGACTTCTTGTATGC; R: CATGGACACAATACATGGAA	55	236

F, forward; R, reverse.

Table 2 Effects of AL on porcine oocyte survival and polar body emission following IVM ($r = 7$)

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

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

AL-treated groups than in control (control, 68.0% \pm 2.6%; 0.01 AL, 69.6% \pm 2.8%; 1 AL, 69.2% \pm 2.8%; 10 AL, 72.5% \pm 5.2%; and 100 AL, 70.7% \pm 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% \pm 3.9%; 0.01 AL, 72.3% \pm 2.3%; 0.1 AL, 74.3% \pm 3.3%; and 1 AL, 74.2% \pm 2.6%, $P < 0.05$) AL-treated groups, and tended to be higher in 10 AL-treated groups (10 AL, 72.4% \pm 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% \pm 4.8%). Furthermore, the rate of blastocyst formation was significantly higher in the 0.1 AL-treated group (43.0% \pm 1.8%) than in the control (29.9% \pm 6.6%, $P < 0.05$) and the other allicin-treated groups (0.01 AL, 37.5% \pm 3.1%; 1 AL, 30.1% \pm 3.7%; 10 AL, 40.2% \pm 3.6%; 100 AL, 35.5% \pm 3.0%).

The terminal deoxynucleotidyl 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 \pm 7.7; 0.01 AL, 61.9 \pm 5.9; 0.1 AL, 72.6 \pm 5.7; 1 AL, 72.5 \pm 6.6; 10 AL, 71.9 \pm 6.3; and 100 AL, 74.1 \pm 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% \pm 0.3%; 0.01 AL, 2.5% \pm 0.3%; 0.1 AL, 2.8% \pm 0.3%; 1 AL, 2.9% \pm 0.5%; 10 AL, 2.9% \pm 0.3%; and 100 AL, 1.9% \pm 0.2%).

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 medium supplemented with 0, 0.01, 0.1, 1, 10 or 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 \pm 1.4; 0.01 AL, 46.8 \pm 2.2; 0.1 AL, 46.0 \pm 2.1; 1 AL, 45.0 \pm 1.5; 10 AL, 44.8 \pm 1.7; and 100 AL, 45.4 \pm 1.2; Fig. 1B).

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

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

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

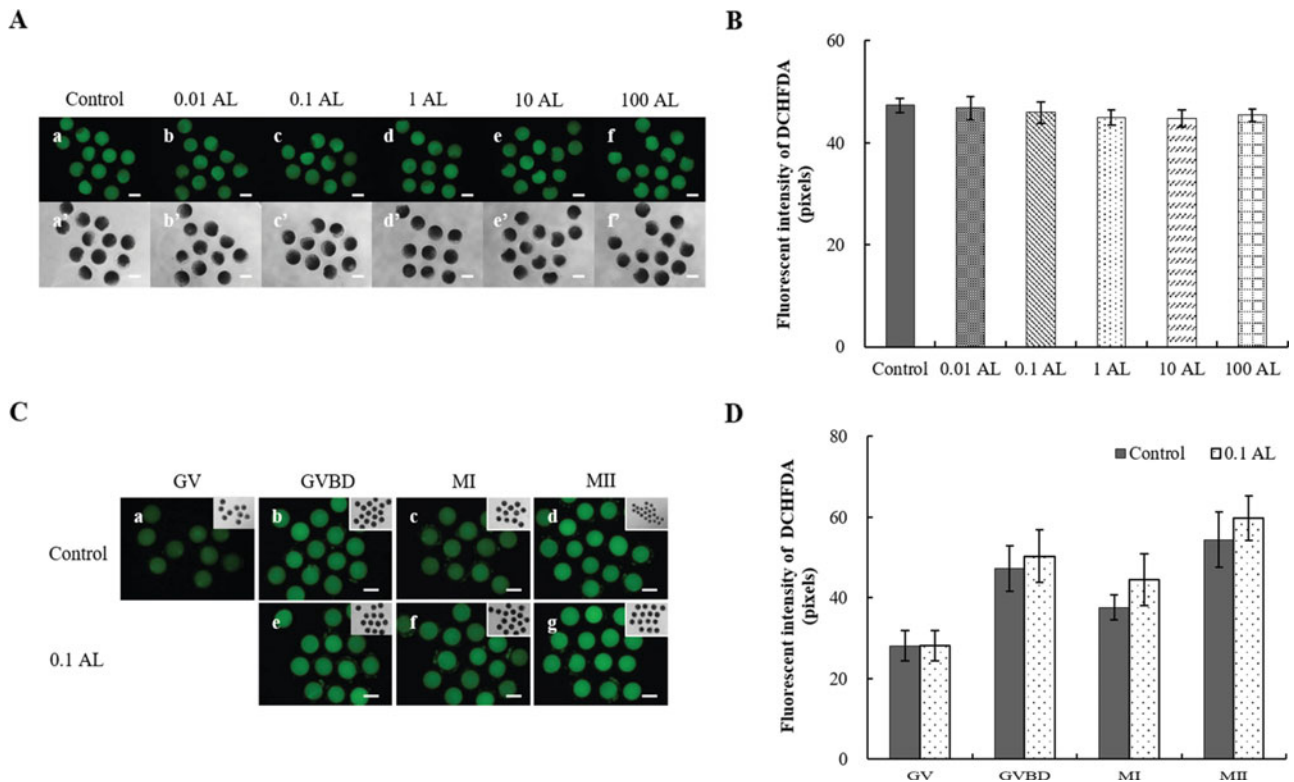


Figure 1 Effects of different concentrations of AL on the ROS level in porcine oocytes during IVM. Oocytes from the control (C-a to C-d) and 0.1 AL-treated (C-e to C-g) groups were stained with DCHFDA following maturation (A) and at GVBD (26 h), MI (32 h), and MII (44 h) (C). The ROS level was determined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

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 \pm 5.6; 0.01 AL, 46.8 \pm 2.2; 0.1 AL, 46.0 \pm 2.1; 1 AL, 45.0 \pm 1.5; 10 AL, 44.8 \pm 1.7; and 100 AL, 45.4 \pm 1.2; Fig. 1C).

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

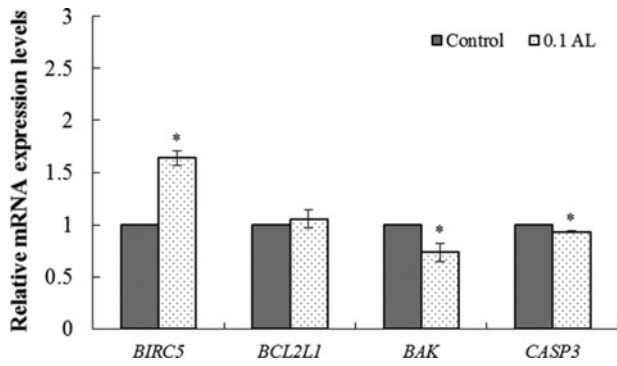


Figure 2 Relative expression levels of pro-apoptotic (*BAK* and *CASP3*) and anti-apoptotic (*BCL2L1* and *BIRC5*) genes in mature oocytes from the control and 0.1 AL-treated groups. Significant differences from the control are indicated (* $P < 0.05$). Values are means \pm standard error of the mean of independent experiments.

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 ($P < 0.05$).

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 analysed 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 analysed at MII during IVM by real-time RT-PCR (Fig. 3D). There was no difference in the expression of *BMP15* and *CCNB1* from the germinal vesicle (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-fold and 1.5-fold higher in the AL-treated group than in the control at MII, respectively ($P < 0.05$).

Discussion

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 μ M allicin to IVM medium 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 (Ogawa *et al.*, 2010; Choi *et al.*, 2013). 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 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 μ M.

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 or 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) (Fig. 1C, D). Similar to the results shown in Fig. 1(A, B), there was no difference in the ROS level among the different stages. Although most studies

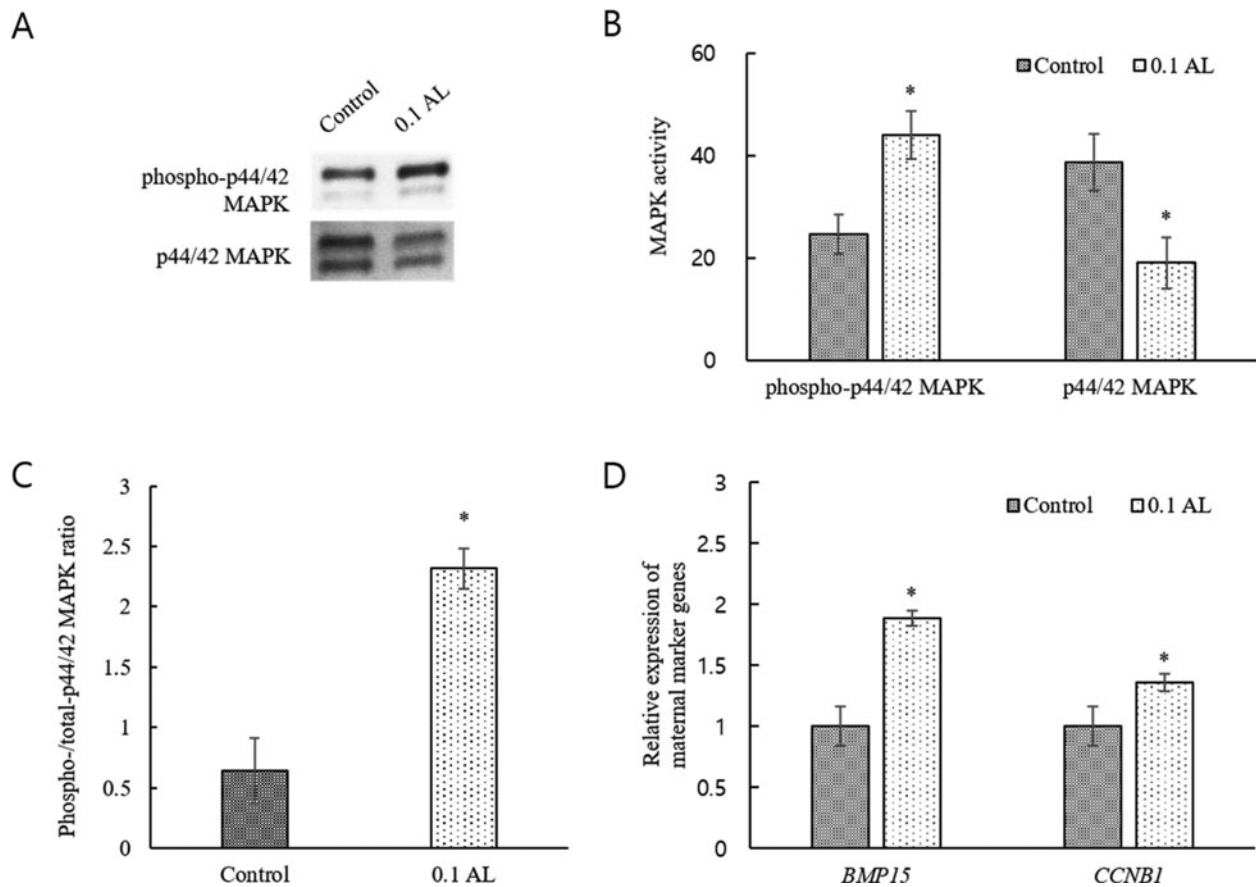


Figure 3 Effects of AL treatment on the cytoplasmic maturation of porcine oocytes during IVM. Phosphorylated MAPK activity was confirmed by western blotting (A), and phosphorylated MAPK activity was evaluated by quantifying the fluorescence intensity (B). Ratio of active phospho-p44/42 MAPK to p44/42 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 \pm standard error of the mean of independent experiments.

have reported changes in the oxidant–antioxidant balance after the addition of H_2O_2 into IVM medium (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 (Sifroi-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 (Fig. 3A, 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 & Smits, 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*.

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Conflict of interest

The authors do not have any conflicts of interest to declare.

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