JAST (Journal of Animal Science and Technology) TITLE PAGE

Upload this completed form to website with submission

ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title (within 20 words without abbreviations)	Rapamycin Treatment During Prolonged *In Vitro* Maturation Enhances the Developmental Competence of Immature Porcine Oocytes
Running Title (within 10 words)	Rapamycin Treatment During Prolonged IVM Improves Development of Porcine Oocytes
Author	Seung-Eun Lee[1,2,a], Han-Bi Lee[1,2,a], Jae-Wook Yoon[1], Hyo-Jin Park[1], So-Hee Kim[1], Dong-Hun Han[1,2], Eun-Seo Lim[1,2], Eun-Young Kim[1,2,3], and Se-Pill Park[1,2,4*]
Affiliation	[1]Stem Cell Research Center, Jeju National University, 102 Jejudaehak-ro, Jeju-si, Jeju-do, 63243, Republic of Korea [2]Faculty of Biotechnology, College of Applied Life Sciences, Jeju National University, 102 Jejudaehak-ro, Jeju-si, Jeju-do, 63243, Republic of Korea [3]Mirae Cell Bio, 1502 Isbiz-tower 147, Seongsui-ro, Seongdong-gu, Seoul, 04795, Republic of Korea [4]Department of Bio Medical Informatic, College of Applied Life Sciences, Jeju National University, 102 Jejudaehak-ro, Jeju-si, Jeju-do, 63243, Republic of Korea
ORCID (for more information, please visit https://orcid.org)	Seung-Eun Lee (https://orcid.org/0000-0001-6128-8560) Han-Bi Lee (https://orcid.org/0009-0004-5165-1513) Jae-Wook Yoon (https://orcid.org/0000-0001-7417-6963) Hyo-Jin Park (https://orcid.org/0000-0002-1778-5866) So-Hee Kim (https://orcid.org/0000-0003-3361-1361) Dong-Hun Han (https://orcid.org/0009-0003-2510-1764) Eun-Seo Lim (https://orcid.org/0009-0005-0575-4092) Eun-Young Kim (https://orcid.org/0000-0001-6299-6876) Se-Pill Park (https://orcid.org/0000-0002-6965-7831)
Competing interests	The authors declare that the research was conducted in the absence of any commercial of financial relationships that could be construed as a potential conflict of interest.
Funding sources State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available.	This work was supported by a grant (RS-2023-00254212) from Technology Commercialization Support Program, Ministry of Agriculture, Food and Rural Affairs, Republic of Korea.
Acknowledgements	Technology Commercialization Support Program, Ministry of Agriculture, Food and Rural Affairs, Republic of Korea (Grant No. RS-2023-00254212).
Availability of data and material	Upon reasonable request, the datasets of this study can be available from the corresponding author.
Authors' contributions Please specify the authors' role using this form.	Conceptualization: Lee SE, Park SP Data curation: Lee SE, Lee HB Formal analysis: Yoon JW, Park HJ, Kim SH, Kim EY Methodology: Lee SE, Lee HB Software: Han DH, Lim ES Validation: Park SP Investigation: Lee SE, Kim EY Writing – original draft: Lee SE, Lee HB Writing – review & editing: Lee SE, Lee HB, Yoon JW, Park HJ, Kim SH, Han DH, Lim ES, Kim EY, Park SP
Ethics approval and consent to participate	This article does not require IRB/IACUC approval because there are no human and animal experiments.

CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	Fill in information in each box below
First name, middle initial, last name	Se-Pill Park
Email address – this is where your proofs will be sent	sppark@jejunu.ac.kr
Secondary Email address	
Address	Department of Bio Medical Informatic, College of Applied Life Sciences, Jeju National University, 102 Jejudaehak-ro, Jeju-si, Jeju Special Self-Governing Province, 63243, Korea
Cell phone number	
Office phone number	82-64-754-4650
Fax number	0303-3130-4650

Rapamycin Treatment During Prolonged *In Vitro* Maturation Enhances the Developmental Competence of Immature Porcine Oocytes

Running head: Rapamycin Treatment During Prolonged IVM Improves Development of Porcine Oocytes

Seung-Eun *Lee*[1,2,a], Han-Bi *Lee*[1,2,a], Jae-Wook *Yoon*[1], Hyo-Jin *Park*[1], So-Hee *Kim*[1], Dong-Hun *Han*[1,2], Eun-Seo *Lim*[1,2], Eun-Young *Kim*[1,2,3], and Se-Pill *Park*[1,2,4*]

[1]Stem Cell Research Center, Jeju National University, 102 Jejudaehak-ro, Jeju-si, Jeju-do, 63243, Republic of Korea

[2]Faculty of Biotechnology, College of Applied Life Sciences, Jeju National University, 102 Jejudaehak-ro, Jeju-si, Jeju-do, 63243, Republic of Korea

[3]Mirae Cell Bio, 1502 Isbiz-tower 147, Seongsui-ro, Seongdong-gu, Seoul, 04795, Republic of Korea

[4]Department of Bio Medical Informatic, College of Applied Life Sciences, Jeju National University, 102 Jejudaehak-ro, Jeju-si, Jeju-do, 63243, Republic of Korea

***Corresponding Author:** Se-Pill Park, Ph.D., Department of Bio Medical Informatic, College of Applied Life Sciences, Jeju National University, 102 Jejudaehak-ro, Jeju-si, Jeju-do, 63243, Republic of Korea. Tel: 82-64-754-4650, E-mail: sppark@jejunu.ac.kr

[a]These authors contributed equally to this work.

Abstract

Porcine oocytes undergo *in vitro* maturation (IVM) for 42–44 h. During this period, most oocytes proceed to metaphase and then to pro-metaphase if the nucleus has sufficiently matured. Forty-four hours is sufficient for oocyte nuclear maturation but not for full maturation of the oocyte cytoplasm. This study investigated the influences of extension of the IVM duration with rapamycin treatment on molecular maturation factors. The phospho-p44/42 mitogen-activated protein kinase (MAPK) level was enhanced in comparison with the total p44/42 MAPK level after 52 h of IVM. Oocytes were treated with and without 10 μM rapamycin (10 R and 0 R, respectively) and examined after 52 h of IVM, whereas control oocytes were examined after 44 h of IVM. Phospho-p44/42 MAPK activity was upregulated the 10 R and 0 R oocytes than in control oocytes. The expression levels of maternal genes were highest in 10 R oocytes and were higher in 0 R oocytes than in control oocytes. Reactive oxygen species (ROS) activity was dramatically increased in 0 R oocytes but was similar in 10 R and control oocytes. The 10 R group exhibited an increased embryo development rate, a higher total cell number per blastocyst, and decreased DNA fragmentation. The mRNA level of development-related (*POU5F1* and *NANOG)* mRNA, oocyte-apoptotic (*BCL2L1)* genes were highest in 10 R blastocysts. These results suggest that prolonged IVM duration with rapamycin treatment represses ROS production and increases expression of molecular maturation factors. Therefore, this is a good strategy to enhance the developmental capacity in porcine oocytes.

Key words: IVM extension; Rapamycin; Molecular maturation factors; ROS; Porcine oocyte

Introduction

In vitro maturation (IVM) is essential for better understanding of process of oocyte development and maturation in various species, including pigs [1]**.** IVM induces meiotic maturation from prophase I to metaphase II (MII) *in vitro*. Oocytes generated by IVM are used to produce high-quality embryos upon *in vitro* fertilization (IVF) and somatic cell nuclear transfer (SCNT). Modulation of follicle size [2, 3], medium composition [4, 5], culture temperature [6, 7], antioxidants [8, 9] and cumulus features [10] can enhance the quality of porcine oocytes in IVM. Specifically, supplementation of antioxidant to IVM medium improved embryo quality, reduced early apoptosis levels, increased GSH content and reduced ROS accumulation, promoting embryonic development [11]. However, further research is needed to improve IVM of porcine oocytes.

Porcine oocytes, IVF embryos, and parthenotes are matured for 42–44 h and SCNT oocytes are matured for 36–38 h *in vitro*. During this period, most oocytes proceed to metaphase and then to pro-metaphase if the nucleus has sufficiently matured [12, 13]. While in meiotic arrest, the nuclear status and structural morphology of matured oocytes remain unchanged. This amount of time is sufficient for oocyte nuclear maturation but not for full maturation of the oocyte cytoplasm [14]. Many studies have been conducted on different maturation starts to acquire oocyte capacitation, which plays an important role in oocytes reaching MII [15-17]. Lin *et al.* extended the duration of IVM to 52 h in order to increase the poor-quality oocytes and performed treatment with melatonin to inhibit reactive oxygen species (ROS) production, apoptosis, and DNA damage [15]. However, oocyte maturation for an excessive amount of time causes cytoplasmic changes that negatively affect oocyte quality and increase the risk of spontaneous oocyte activation [18, 19] and subsequent aberrant cleavage characterized by unequally sized blastomeres [19, 20]. It is possible to prevent, delay, or reverse these cellular and molecular abnormalities [21].

Rapamycin has antifungal and immunosuppressant properties [22] and binds to FK506-binding protein 12 to form a complex that prevents the kinase activity and function of mTOR [23]. mTOR activity inhibition by rapamycin affects establishment of the cortical granule-free zone and actin cap, and disrupts alignment of the surrounding spindle and division during oocyte meiotic maturation [24]. Administration of rapamycin during IVM of porcine oocytes dose-dependently enhances cytoplasmic and nuclear maturation by inducing autophagy [25]. Aged porcine oocytes treated with 10 μM rapamycin enhances blastocyst quality by regulating the mitochondrial distribution, autophagy, apoptotic cells, and mTOR signaling [26]. Supplementation of tissue culture medium (TCM)-199 with 0.5 μM

rapamycin increases expression of matrix metallopeptidase in the trophoblast and inner cell mass (ICM), while it inhibits apoptosis [27]. Therefore, in our study, we extended the IVM duration to fully mature porcine oocytes and performed rapamycin treatment to reduce apoptosis, ROS production, and oocyte aging. This study investigated the impacts of rapamycin treatment during prolonged IVM on molecular maturation factors and the developmental capacity of porcine oocytes *in vitro*. Activated mitogen-activated protein kinase (MAPK) and ROS levels were modulated upon extension of the IVM duration with rapamycin treatment. The *in vitro* development rate, total cell numbers, and level of apoptosis were determined using blastocysts generated from these oocytes.

Materials and Methods

Chemicals and reagents

Unless otherwise specified, all chemicals and reagents utilized in this study were procured from Merck (St. Louis, MO, USA). The oocytes and embryos were maintained in CO_2 incubator at 38.8°C in a humidified atmosphere containing 5% CO_2 and 95% air.

IVM of rapamycin-treated porcine oocytes

Porcine ovaries from pre-pubertal sows were obtained from a provincial slaughterhouse. We used an 18-gauge needle attached to 10mL syringe to aspirate follicles measuring 2-8mm from the follicles, and selected cumulus-oocyte-complexes (COCs). After washing in TCM-199-HEPES supplemented with 0.1% (w/v) bovine serum albumin (BSA), a group of approximately 50 selected COCs were cultured in 500 µL of TCM-199 (M-199; Gibco, Grand Island, NY, USA) containing Earle's salts, 0.57 mM cysteine, 10% (v/v) porcine follicular fluid, 10 ng/mL epidermal growth (E-9644), 0.5 µg/mL follicle-stimulating hormone (F-2293), and 0.5 µg/mL luteinizing hormone (L-5269). The maturation process was conducted for 44 h, with the COCs placed beneath a layer of mineral oil. Thereafter, MII oocytes were transferred into TCM-199 supplemented with 0 or 10 µM rapamycin (R-8781) and incubated for 8 h (total IVM duration of 52 h). According to our previous studies, the concentration of rapamycin was set at the 10 µM [26].

Parthenogenetic activation and embryo culture

Subsequent IVM, adherent cumulus cells were eliminated using 0.1% (w/v) hyaluronidase. Collected oocytes were parthenogenetically activated by incubation in 5 µM Ca^{2+} ionomycin (Merck) for 5 min. Following culture in porcine zygote medium-5 (PZM-5) with 7.5 µg/mL cytochalasin B (Merck) for 4 h, the oocytes were washed with PZM-5 containing 0.4% (w/v) BSA. Following 6 days of incubation in the same medium, the oocytes were washed with Dulbecco's phosphate-buffered saline (PBS). Finally, oocytes and embryos were fixed in 3.7% (w/v) paraformaldehyde for 20 min at 4℃ or they were snap freezing using liquid nitrogen and subsequently stored at −70°C.

Detection of intracellular ROS activity

Intracellular ROS activity in denuded oocytes were quantified using the 2,7-dichlorofluorescein protocol described previously [26]. Concisely, oocytes (25–30 oocytes per sample, four replicates) were cultured in 100 µM 2',7'-dichlorodihydrofluorescein-diacetate (DCHF-DA) for 20 min, washed five times with PZM-5, and promptly observed under epifluorescence microscopy (Olympus, Tokyo, Japan) with an ultraviolet filter (450–490 nm and 515–565 nm). A microscope-mounted digital camera (Nikon, Tokyo, Japan) was used to capture grayscale images, and ImageJ software (NIH, Bethesda, MD, USA) was employed to acquire mean grayscale values. Image analysis was conducted with the Adobe Photoshop CS6 software package (version 13, Adobe Systems Inc.) by quantitating the average pixel intensities in various regions of the raw image. Before statistical analysis, background fluorescence values were calculated by subtracting them from the final values.

TUNEL assay

Blastocysts on day 6 were washed with PBS (pH 7.4) with 1 mg/mL polyvinylpyrrolidone (PBS/PVP). After fixing with 3.7% formaldehyde prepared with PBS overnight at 4℃. Following washing with PBS/PVP, the oocytes were subsequently permeabilized by culture in the dark for 1 h with 0.3% Triton X-100 at room temperature. After washing with PBS/PVP, the blastocysts were cultured at 37℃ for 1 h in fluorescein-conjugated dUTP and terminal deoxynucleotidyl transferase using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany). Following counterstaining with Hoechst 33342 in the dark for 30 min at 37℃ to label the nuclei, the samples were washed with PBS/PVP and then carefully set on glass slides. The experiment was independently repeated three times.

Real-time quantitative PCR

mRNA extraction from oocytes at the MII stage and *in vitro*-cultured embryos at day 6 (20 embryos per sample, three replicates) by a Dynabeads mRNA Direct Kit (DynalAsa, Oslo, Norway). SuperScript™ III reverse transcriptase (Invitrogen, Grand Island, NY, USA) and oligo $(dT)_{12–18}$ primers were used to synthesize first-strand cDNA. Real-time quantitative PCR was conducted on a Step One Plus Real-time PCR system (Applied Biosystems, Warrington, UK) using the primers specified in Table 1. The total reaction volume for the final PCR consisted of 20 μL, including SYBR Green PCR Master Mix (Applied Biosystems). As follows were the amplification conditions: 10 min at 94℃, followed by 39 cycles of denaturation for 30 sec at 94℃, annealing for 30 sec at 55℃, and extension for 55 sec at 72℃, and a final extension for 5 min at 72℃. Relative mRNA expression levels were determined according to the $2^{-\Delta\Delta Ct}$ protocol [28] by normalization to *GAPDH*.

Western blot analysis

The experimental assay followed a previously described protocol [24]. For protein extraction, 20 μL of 1× SDS sample buffer containing 5 mM Tris-HCl, pH 6.8 at 25℃, 2% (w/v) SDS, 10% (v/v) glycerol, 50 mM DTT, and 0.01% (w/v) bromophenol blue or phenol red was added to oocytes (20 oocytes per sample, three replicates), and the samples were boiled for 5 min at 95℃. Subsequently, proteins were separated by electrophoresis on a 5–12% Tris-SDS-PAGE gel for 1.5 h at 80–100 V. Thereafter, proteins were electrophoretically transferred to a nitrocellulose membrane (Hybond-ECL; Amersham, Buckinghamshire, UK) at 300 mA for 2 h using transfer buffer (25 mM Tris, 200 mM glycine, and 20% methanol, pH 8.5). After being blocked with 5% (w/v) skim milk prepared in PBS for 1 h, the membranes were cultured with an anti-Cdc2 p34, anti-p44/42 MAPK, or anti-phospho-p44/42 MAPK antibody (1:500; Cell Signaling Technology, Danvers, MA, USA) in blocking solution (1× Tris-buffered saline containing 0.1% (v/v) Tween® 20 and 5% (w/v) skim milk) for at least 2 h. After washing with TBST (20 mM Tris-HCl, pH 7.5, 250 mM NaCl, and 0.1% (v/v) Tween® 20), the membranes were cultured with anti-rabbit IgG-horseradish peroxidase (1:2000, Cell Signaling Technology) with blocking solution for 1 h. Following washing with TBST, binding of antibody was analyzed with chemiluminescence luminol reagent (Invitrogen).

Statistical analysis

The Statistical Analysis System (SAS User's Guide, 1985; Statistical Analysis System Inc., Cary, NC, USA) was employed to analyze data using the general linear model procedure. Significant differences were determined using

Tukey's multiple range test. Relative gene expression levels were compared by the Student's *t*-test. Differences were regarded as biologically significant at $p < 0.05$ and $p < 0.01$.

Results

Rapamycin treatment increases the levels of maturation factors in porcine oocytes

To explore the influence of extension of the IVM duration on molecular maturation of porcine oocytes, we examined expression of maturation/M phase-promoting factor (MPF), activated MAPK, and maternal genes (Fig. 1 and 2). We monitored total and phospho-p44/42 MAPK levels at 2 h intervals after 42–52 h of IVM (Fig. 1A). The phospho-p44/42 MAPK level was normalized against the total MAPK level at 44 h (set to 1). The phospho-p44/42 MAPK level was upregulated in comparison with the total MAPK level at 52 h (42 h, 1.02 ± 0.09; 46 h, 0.94 ± 0.07; 48 h, 0.82 ± 0.07; 50 h, 0.90 ± 0.11; and 52 h, 1.36 ± 0.20; $p < 0.05$ and $p < 0.01$; Fig. 1B). The phospho-p44/42 MAPK level was higher at 52 h than at the other time points (Fig. 1A and B).

Oocytes were supplemented with and without 10 μM rapamycin and examined after 52 h of IVM (10 R and 0 R, respectively), whereas control oocytes were examined subsequent 44 h of IVM. The mRNA level of the maternal genes *BMP15*, *GDF9*, and *MOS* was analyzed using real-time quantitative PCR (Fig. 2A). The mRNA level of these genes was upregulated in 10 R oocytes compared to control and 0 R oocytes ($p < 0.05$). There was an increase ($p < 0.01$) in the levels of *BMP15* and *GDF9* in 10 R oocytes compared with control oocytes. We monitored the levels of Cdc2 p34 and phospho-p44/42 MAPK (Fig. 2B). Expression of Cdc p34 and phospho-p44/42 MAPK was normalized to the total MAPK in each group (set to 1, Fig. 2C and D). The levels of Cdc2 p34 and phospho-p44/42 MAPK were higher in 10 R oocytes ($p < 0.01$) than in control and 0 R oocytes (0 R, 1.05 ± 0.11 and 1.14 ± 0.01, respectively; and 10 R, 1.21 ± 0.02 and 1.29 ± 0.06, respectively; Fig. 2C and D).

Rapamycin treatment reduces ROS activity in porcine oocytes

ROS activity was analyzed in control, 0 R, and 10 R groups using DCHFDA. ROS activity was lower in 10 R group ($p < 0.01$) compared to 0 R oocytes and was similar to that in control oocytes (control, 32.3±3.6; 0 R, 65.1±5.9; and 10 R, 28.9±7.6; Fig. 3A and B). Treatment with 10 μM rapamycin enhanced the developmental capacity of oocytes aged for 8 h by inhibiting ROS activity.

Rapamycin treatment enhances the developmental capacity of porcine oocytes

Control (n=400) and 0 R (n=345) and 10 R (n=310) groups were matured for 44 and 52 h, respectively. The percentage of matured oocytes was no differences among the control, 0 R, and 10 R groups (86.40% ± 1.92%, 90.30% ± 2.03%, and 91.77% ± 2.86%, respectively; Fig. 4B). After IVM, control (*n*=345), 0 R (*n*=312), and 10 R (*n*=284) groups were parthenogenetically activated. The morphology and percentage of embryos reaching the 2–4-cell stage showed no significant difference among the control, 0 R, and 10 R groups (68.73% ± 5.57%, 56.58% ± 5.10%, and 57.61% ± 4.60%, respectively; Fig. 4A and B). The percentage of blastocyst at day 6 was highest in the 10 R group (*n*=75, 48.36% ± 7.06%) and lowest in the 0 R group (*n*=38, 22.81% ± 4.12%; $p < 0.05$ or $p < 0.01$; Fig. 4A and B).

Confirming the influence of rapamycin treatment on blastocyst quality, blastocysts at day 6 in the various groups were stained (Fig. 4C). The cell number per blastocyst at day 6 was highest in the 10 R group (control, 50 ± 6.92; 0 R, 54 ± 9.29; and 10 R, 76 ± 12.49; $p < 0.05$; Fig. 4C). Reprogramming-related transcription factor genes *POU5F1*, *SOX2*, *NANOG*, and *CDX2* were examined for their expression levels by real-time RT-PCR (Fig. 4D). The 10 R group was upregulated ($p < 0.05$) in *POU5F1* and *NANOG* expression, whereas it did not significant difference among the control and 0 R groups. *SOX2* expression tended to show a similar pattern, although there were not significantly differences among the groups. Expression of *CDX2* showed no significant difference among the groups.

Rapamycin treatment of porcine oocytes decreases apoptosis in resultant blastocysts

Using the TUNEL assay, individual embryos were assessed for genomic DNA fragmentation (an indicator of apoptosis). The percentage of fragmented DNA in the 10 R group (7.08% ± 0.50%) was significantly downregulated ($p < 0.05$) compared with the 0 R group (13.06% ± 2.24%) and was similar to the control group (7.07% ± 1.37%, Fig. 5B). The 10 R group showed significantly higher levels of *BCL2L1*, the anti-apoptotic gene, compared to the 0 R group ($p < 0.05$), while the control group exhibited the highest expression ($p < 0.05$) (Fig. 5C). The mRNA level of the *FAS* and *CASP3*, pro-apoptotic genes, tended to be downregulated in the 10 R group, although these differences were not significant.

Discussion

Oocyte maturation is a multifaceted process involving both nucleus and cytoplasmic changes. An IVM duration of 42 to 44 h is sufficient for nuclear maturation through a mechanism such as oocyte capacity, but not

sufficient for complete maturation of the cytoplasm [14]. However, excessive maturation duration exposes oocytes to increased oxidative stress [29, 30]. ROS-induced oocyte damage reduces the ability and quality of subsequent embryonic development [31]. This study investigated the influences of extension of the IVM duration with rapamycin treatment on molecular maturation factors and embryonic development of porcine oocytes.

After 52 h of IVM, the phospho-p44/42 MAPK level was high in comparison with the total MAPK level (Fig. 1A and B). Our previous study demonstrated that 10 μM rapamycin increases blastocyst quality by affecting developmental rate and total cell number and reducing mitochondrial distribution, apoptosis, autophagy and ROS activity that regulates mTOR signaling [26]. The maternal genes expression levels of *BMP15*, *GDF9*, and *MOS* was higher in 0 R and 10 R oocytes than in control oocytes (Fig. 2A). Confirming the effect of rapamycin, levels of Cdc2 p34 and phospho-p44/42 MAPK were higher in 10 R oocytes than in control and 0 R oocytes (Fig. 2B–D). MAPK and MPF are critical for meiotic molecular maturation of oocytes. Protein phosphorylation and dephosphorylation are essential for the meiotic cell cycle of oocytes. MPF and MAPK, which are integral components of the key regulatory pathways involved in activation of extracellular signal-regulated kinase 1 and 2, are serine/threonine kinases that are phosphorylated and subsequent activated by MAPK kinase [32, 33]. MPF consists of the catalytic subunit p34/Cdc2, which possesses serine/threonine kinase activity, and the regulatory subunit cyclin B. Activation of MPF is regulated by various mechanisms, including binding of cyclin B to Cdc2, phosphorylation of threonine 161, and dephosphorylation of tyrosine 15 and threonine 14 [34]. MAPK activity usually peaks in porcine oocytes during IVM for 42–44 h. Many researchers have extended the IVM duration to improve oocyte maturity due to supply of insufficient maturation factors. The developmental rate reportedly increases when the IVM duration is extended (48–72 h). Specifically, the developmental rate is increased at 56 h due to maintenance of high MPF expression, but cytoplasmic senescence at 72 h decreases the developmental rate and suppresses MPF expression [35]. Another study confirmed that the developmental rate is improved by extending the IVM duration from 44 h, which is normally used. In the extended culture duration of 24 to 52 h, the cleavage rate of porcine oocytes was highest at 48 h as determined through the number of cumulus cell layers. [36]. Although that study did not reveal whether this approach affects a molecular factor, it showed that long-term culture is more effective for oocyte maturation [36]. To induce resumption of meiosis, porcine COCs at the pre-IVM stage were preincubated for 12 h [17]. Extension of the total IVM duration to 52 h increases the development rate of IVF embryos at the blastocyst stage [17]. Excessive prolongation of oocyte maturation leads to aging, and reduced MAPK activity decreases the quality of oocytes [37, 38]. The IVM duration of poor-quality oocytes was previously

extended to 52 h, and treatment with the antioxidant melatonin improved the IVM rate and expression levels of maturation factors [15]. We previously showed that treatment of aged oocytes with rapamycin increases phosphorylated p44/42 MAPK activity and mRNA levels of maternal genes compared with untreated aged oocytes [26]. Rapamycin activates MPF and MAPK, which reduces oocyte activation susceptibility via inhibition of protein kinase A [39]. Therefore, we suggest that rapamycin treatment facilitates molecular maturation by preserving the ooplasm of MII oocytes, leading to enhanced transcription of maternal genes. This study indicates that extension of the IVM duration with rapamycin treatment, which maintains expression of molecular maturation factors, does not negatively affect porcine oocytes.

ROS function are crucial signaling molecules in diverse physiological processes, including resumption of the meiotic cell cycle, and contribute to pathological processes such as apoptosis and senescence [40, 41]. ROS were proposed to participate in oocyte meiotic arrest [42-44]. Oxidative stress perturbs bovine embryonic development after fertilization [45]. Furthermore, a change in the redox status of human oocytes during *in vitro* culture is related to an increased occurrence of apoptosis in gametes [46]. Porcine gametes can incur DNA damage and undergo apoptosis during IVM [47-49]. Treatment with rapamycin effectively reduces intracellular ROS levels and improves mitochondrial localization [26]. Extension of the IVM duration with rapamycin treatment demonstrated a significant reduction of levels of ROS (Fig. 3A and B). This finding suggests that this approach improves *in vitro* oocyte culture and maintains oocyte health.

We investigated the influence of extension of the IVM duration with rapamycin treatment on porcine embryonic development. The percentage of blastocyst formation at day 6 and total cell number per blastocyst were increased when the IVM duration was extended with rapamycin treatment (Fig. 4A–C). Gene expression is a major contributor to embryonic development, and any disruption in gene expression during culture of embryos *in vitro* can potentially hinder embryo production [50, 51]. We examined mRNA levels of *POU5F1*, *SOX2*, *NANOG*, and *CDX2* in the 0 R, 10 R, and control groups. Transcription factor genes such as these play essential roles in early development and are indispensable for proliferation of undifferentiated embryonic stem (ES) cells in culture. mRNA and protein expression of *POU5F1* has been detected in various cellular components, including blastomeres of preimplantation embryos, the ICM of blastocysts, epiblasts, primordial germ cells, and the majority of germ cells [52-54]. *SOX2* and *NANOG* form interactions with *POU5F1* to regulate the transcriptional hierarchy that specifies ES cell identity [55-57]. The transcription factors *POU5F1* and *SOX2* are expressed in both the ICM and

trophectoderm (TE) of porcine blastocysts. *CDX2* expression is essential for TE formation [58]. *POU5F1* and *NANOG* exhibited significant upregulated ($p < 0.05$) in 10 R blastocysts (Fig. 4D). However, *CDX2* expression was not significantly affected. These data show that extension of the IVM duration with rapamycin treatment upregulates specific transcription factors related to the ICM, including *POU5F1* and *NANOG*, but does not affect expression of the TE-related factor *CDX2* in blastocysts. Taken together, these observations demonstrate that extension of the IVM duration with rapamycin treatment significantly influences porcine embryonic development *in vitro*.

We explored the effect of extension of the IVM duration with rapamycin treatment on apoptotic cell death in porcine embryos. DNA fragmentation was significantly decreased in 10 R blastocysts extended duration of *in vitro* maturation (IVM) by rapamycin treatment (Fig. 5B). We also examined the apoptosis expression levels of *BCL2L1*, *FAS*, and *CASP3* (Fig. 5C). Although the pro-apoptotic expression levels of *FAS* and *CASP3* was not different in all groups, the anti-apoptotic expression level of *BCL2L1* in blastocysts was increased in the 10 R group. *BCL2L1* encodes the protein BCL-xL, an anti-apoptotic protein [59]. BCL-xL prevents cell death by inhibiting cytochrome c release from mitochondria, a key step in the cell death pathway [60, 61]. These observations suggest that extension of the IVM duration with rapamycin treatment facilitates embryonic development by suppressing apoptosis at the molecular level during preimplantation stages.

Our results indicate that extension of the IVM duration with rapamycin treatment enhances molecular maturation of porcine oocytes by repressing ROS production and improves porcine embryonic development. This study demonstrates the combination of extension of the IVM duration and treatment with rapamycin enhances maturation of porcine oocytes. These results should be further applied to assisted reproductive technology to produce high-quality embryos.

References

1. Hatırnaz Ş, Ata B, Hatırnaz ES, Dahan MH, Tannus S, Tan J, et al. Oocyte in vitro maturation: A sytematic review. Turkish journal of obstetrics and gynecology. 2018;15(2):112.

2. Lee JB, Lee MG, Lin T, Shin HY, Lee JE, Kang JW, et al. Effect of oocyte chromatin status in porcine follicles on the embryo development in vitro. Asian-Australasian Journal of Animal Sciences. 2019;32(7):956.

3. Motlik J, Crozet N, Fulka J. Meiotic competence in vitro of pig oocytes isolated from early antral follicles. Reproduction. 1984;72(2):323-8.

4. Pawlak P, Warzych E, Cieslak A, Malyszka N, Maciejewska E, Madeja ZE, et al. The consequences of porcine IVM medium supplementation with follicular fluid become reflected in embryo quality, yield and gene expression patterns. Scientific reports. 2018;8(1):1-12.

5. Wang Q-L, Zhao M-H, Jin Y-X, Kim N-H, Cui X-S. Gonadotropins improve porcine oocyte maturation and embryo development through regulation of maternal gene expression. Journal of Embryo Transfer. 2013;28(4):361-71.

6. Ye J, Coleman J, Hunter M, Craigon J, Campbell K, Luck M. Physiological temperature variants and culture media modify meiotic progression and developmental potential of pig oocytes in vitro. Reproduction. 2007;133(5):877-86.

7. Abeydeera LR, Wang W-H, Prather RS, Day BN. Effect of incubation temperature on in vitro maturation of porcine oocytes: nuclear maturation, fertilisation and developmental competence. Zygote. 2001;9(4):331-7.

8. Nie J, Yan K, Sui L, Zhang H, Zhang H, Yang X, et al. Mogroside V improves porcine oocyte in vitro maturation and subsequent embryonic development. Theriogenology. 2020;141:35-40.

9. Almubarak AM, Kim E, Yu I-J, Jeon Y. Supplementation with Niacin during in vitro maturation improves the quality of porcine embryos. Theriogenology. 2021;169:36-46.

10. Alvarez GM, Dalvit GC, Achi MV, Miguez MS, Cetica PD. Immature oocyte quality and maturational competence of porcine cumulus-oocyte complexes subpopulations. Biocell. 2009;33(3):167-77.

11. Chen Q, Gao L, Li J, Yuan Y, Wang R, Tian Y, et al. α-Ketoglutarate improves meiotic maturation of porcine oocytes and promotes the development of pa embryos, potentially by reducing oxidative stress through the Nrf2 pathway. Oxidative Medicine and Cellular Longevity. 2022;2022.

12. Suzuki H, Jeong B-S, Yang X. Dynamic changes of cumulus-oocyte cell communication during in vitro maturation of porcine oocytes. Biology of reproduction. 2000;63(3):723-9.

13. Wongsrikeao P, Kaneshige Y, Ooki R, Taniguchi M, Agung B, Nii M, et al. Effect of the removal of cumulus cells on the nuclear maturation, fertilization and development of porcine oocytes. Reproduction in Domestic Animals.

2005;40(2):166-70.

14. Baruffi R, Avelino K, Petersen C, Mauri A, Garcia J, Franco J. Nuclear and cytoplasmic maturation of human oocytes cultured in vitro. Fertility and Sterility. 2004;82:S265-S6.

15. Lin T, Lee JE, Kang JW, Oqani RK, Cho ES, Kim SB, et al. Melatonin supplementation during prolonged in vitro maturation improves the quality and development of poor-quality porcine oocytes via anti-oxidative and anti-apoptotic effects. Molecular Reproduction and Development. 2018;85(8-9):665-81.

16. Van NTT, Van Thuan N, Bui H-T. Improve the developmental competence of porcine oocytes from small antral follicles by pre-maturation culture method. Theriogenology. 2020;149:139-48.

17. Funahashi H, Cantley T, Day B. Preincubation of cumulus-oocyte complexes before exposure to gonadotropins improves the developmental competence of porcine embryos matured and fertilized in vitro. Theriogenology. 1997;47(3):679-86.

18. Chian R, Nakahara H, Niwa K, Funahashi H. Fertilization and early cleavage in vitro of ageing bovine oocytes after maturation in culture. Theriogenology. 1992;37(3):665-72.

19. Kikuchi K, Izaike Y, Noguchi J, Furukawa T, Daen F, Naito K, et al. Decrease of histone H1 kinase activity in relation to parthenogenetic activation of pig follicular oocytes matured and aged in vitro. Reproduction. 1995;105(2):325-30.

20. Nagai T. Parthenogenetic activation of cattle follicular oocytes in vitro with ethanol. Gamete Research. 1987;16(3):243-9.

21. Miao Y-L, Kikuchi K, Sun Q-Y, Schatten H. Oocyte aging: cellular and molecular changes, developmental potential and reversal possibility. Human reproduction update. 2009;15(5):573-85.

22. Dumont FJ, Melino M, Staruch M, Koprak S, Fischer P, Sigal N. The immunosuppressive macrolides FK-506 and rapamycin act as reciprocal antagonists in murine T cells. The Journal of Immunology. 1990;144(4):1418-24.

23. Guertin DA, Sabatini DM. Defining the role of mTOR in cancer. Cancer cell. 2007;12(1):9-22.

24. Lee SE, Sun SC, Choi HY, Uhm SJ, Kim NH. mTOR is required for asymmetric division through small GTPases in mouse oocytes. Molecular reproduction and development. 2012;79(5):356-66.

25. Song B-S, Kim J-S, Kim Y-H, Sim B-W, Yoon S-B, Cha J-J, et al. Induction of autophagy during in vitro maturation improves the nuclear and cytoplasmic maturation of porcine oocytes. Reproduction, Fertility and Development. 2014;26(7):974-81.

26. Lee SE, Kim EY, Choi HY, Moon JJ, Park MJ, Lee JB, et al. Rapamycin rescues the poor developmental capacity of aged porcine oocytes. Asian-Australasian journal of animal sciences. 2014;27(5):635-47.

27. Kim DS, Kim SH, Yoon JT. Regulatory effect of apoptosis on morphological changes in cell mass of porcine blastocyst through supplementation of rapamycin during in vitro culture. Journal of Advanced Veterinary and Animal Research. 2020;7(4):614.

28. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. methods. 2001;25(4):402-8.

29. Jia B-Y, Xiang D-C, Shao Q-Y, Zhang B, Liu S-N, Hong Q-H, et al. Inhibitory effects of astaxanthin on postovulatory porcine oocyte aging in vitro. Scientific Reports. 2020;10(1):20217.

30. Jiao Y, Wang Y, Jiang T, Wen K, Cong P, Chen Y, et al. Quercetin protects porcine oocytes from in vitro aging by reducing oxidative stress and maintaining the mitochondrial functions. Frontiers in Cell and Developmental Biology. 2022;10:915898.

31. Pyeon DB, Lee SE, Yoon JW, Park HJ, Park CO, Kim SH, et al. The antioxidant dieckol reduces damage of oxidative stress-exposed porcine oocytes and enhances subsequent parthenotes embryo development. Molecular Reproduction and Development. 2021;88(5):349-61.

32. Fan H-Y, Sun Q-Y. Involvement of mitogen-activated protein kinase cascade during oocyte maturation and fertilization in mammals. Biology of reproduction. 2004;70(3):535-47.

33. Liang C-G, Su Y-Q, Fan H-Y, Schatten H, Sun Q-Y. Mechanisms regulating oocyte meiotic resumption: roles of mitogen-activated protein kinase. Molecular endocrinology. 2007;21(9):2037-55.

34. Wehrend A, Meinecke B. Kinetics of meiotic progression, M-phase promoting factor (MPF) and mitogen-activated protein kinase (MAP kinase) activities during in vitro maturation of porcine and bovine oocytes: species specific differences in the length of the meiotic stages. Animal Reproduction Science. 2001;66(3-4):175-84.

35. Kim H, Choi S, Han M, Son D, Ryu I, Kim I, et al. Effects of maturation duration and activation treatments on activation and development of porcine follicular oocytes. Journal of Embryo Transfer. 2005;20(1):25-33.

36. Hua Z, Zheng X, Wei Q, Xu H, Wang Y, Liu X, et al. Effect of cumulus-oocyte complexes (COCs) culture duration on in vitro maturation and parthenogenetic development of pig oocyte. African Journal of Biotechnology. 2011;10(5):867-73.

37. Ma W, Zhang D, Hou Y, Li Y-H, Sun Q-Y, Sun X-F, et al. Reduced expression of MAD2, BCL2, and MAP kinase activity in pig oocytes after in vitro aging are associated with defects in sister chromatid segregation during meiosis II and embryo fragmentation after activation. Biology of Reproduction. 2005;72(2):373-83.

38. Steuerwald NM, Steuerwald MD, Mailhes JB. Post-ovulatory aging of mouse oocytes leads to decreased MAD2 transcripts and increased frequencies of premature centromere separation and anaphase. Molecular human reproduction. 2005;11(9):623-30.

39. Lin F-H, Zhang W-L, Li H, Tian X-D, Zhang J, Li X, et al. Role of autophagy in modulating post-maturation aging of mouse oocytes. Cell Death & Disease. 2018;9(3):1-12.

40. Agarwal A, Gupta S, Sharma R. Oxidative stress and its implications in female infertility–a clinician's perspective. Reproductive biomedicine online. 2005;11(5):641-50.

41. Tripathi A, Khatun S, Pandey A, Mishra S, Chaube R, Shrivastav T, et al. Intracellular levels of hydrogen peroxide and nitric oxide in oocytes at various stages of meiotic cell cycle and apoptosis. Free radical research. 2009;43(3):287-94.

42. Choi W-J, Banerjee J, Falcone T, Bena J, Agarwal A, Sharma RK. Oxidative stress and tumor necrosis factor–α–induced alterations in metaphase II mouse oocyte spindle structure. Fertility and sterility. 2007;88(4):1220-31.

43. Downs SM, Mastropolo AM. The participation of energy substrates in the control of meiotic maturation in murine oocytes. Developmental biology. 1994;162(1):154-68.

44. Zhang X, Wu XQ, Lu S, Guo YL, Ma X. Deficit of mitochondria-derived ATP during oxidative stress impairs mouse MII oocyte spindles. Cell research. 2006;16(10):841-50.

45. Fatehi AN, Roelen BA, Colenbrander B, Schoevers EJ, Gadella BM, Bevers MM, et al. Presence of cumulus cells during in vitro fertilization protects the bovine oocyte against oxidative stress and improves first cleavage but does not affect further development. Zygote. 2005;13(2):177-85.

46. Zhang X, Li X-H, Ma X, Wang Z-H, Lu S, Guo Y-L. Redox-induced apoptosis of human oocytes in resting follicles in vitro. Journal of the Society for Gynecologic Investigation. 2006;13(6):451-8.

47. Tatemoto H, Sakurai N, Muto N. Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during in vitro maturation: role of cumulus cells. Biology of reproduction. 2000;63(3):805-10.

48. Tatemoto H, Ootaki K, Shigeta K, Muto N. Enhancement of developmental competence after in vitro fertilization of porcine oocytes by treatment with ascorbic acid 2-O-α-glucoside during in vitro maturation. Biology of reproduction. 2001;65(6):1800-6.

49. Tatemoto H, Muto N, Sunagawa I, Shinjo A, Nakada T. Protection of porcine oocytes against cell damage caused by oxidative stress during in vitro maturation: role of superoxide dismutase activity in porcine follicular fluid. Biology of reproduction. 2004;71(4):1150-7.

50. Kikuchi K, Onishi A, Kashiwazaki N, Iwamoto M, Noguchi J, Kaneko H, et al. Successful piglet production after transfer of blastocysts produced by a modified in vitro system. Biology of Reproduction. 2002;66(4):1033-41.

51. Pomar FR, Teerds K, Kidson A, Colenbrander B, Tharasanit T, Aguilar B, et al. Differences in the incidence of apoptosis between in vivo and in vitro produced blastocysts of farm animal species: a comparative study. Theriogenology. 2005;63(8):2254-68.

52. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature. 2001;409(6818):363-6.

53. Schöler H, Dressler GR, Balling R, Rohdewohld H, Gruss P. Oct-4: a germline-specific transcription factor mapping to the mouse t-complex. The EMBO journal. 1990;9(7):2185-95.

54. Rosner MH, Vigano MA, Ozato K, Timmons PM, Poirie F, Rigby PW, et al. A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. Nature. 1990;345(6277):686-92.

55. Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell. 1998;95(3):379-91.

56. Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, et al. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell. 2003;113(5):643-55.

57. Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, et al. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell. 2003;113(5):631-42.

58. Niwa H, Toyooka Y, Shimosato D, Strumpf D, Takahashi K, Yagi R, et al. Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. Cell. 2005;123(5):917-29.

59. Wei Y, Zhang L, Wang C, Li Z, Luo M, Xie G, et al. Anti-apoptotic protein BCL-XL as a therapeutic vulnerability in gastric cancer. Animal Models and Experimental Medicine. 2023.

60. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, et al. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science. 1997;275(5303):1129-32.

61. Garrido C, Galluzzi L, Brunet M, Puig P, Didelot C, Kroemer G. Mechanisms of cytochrome c release from mitochondria. Cell Death & Differentiation. 2006;13(9):1423-33.

Figure legends

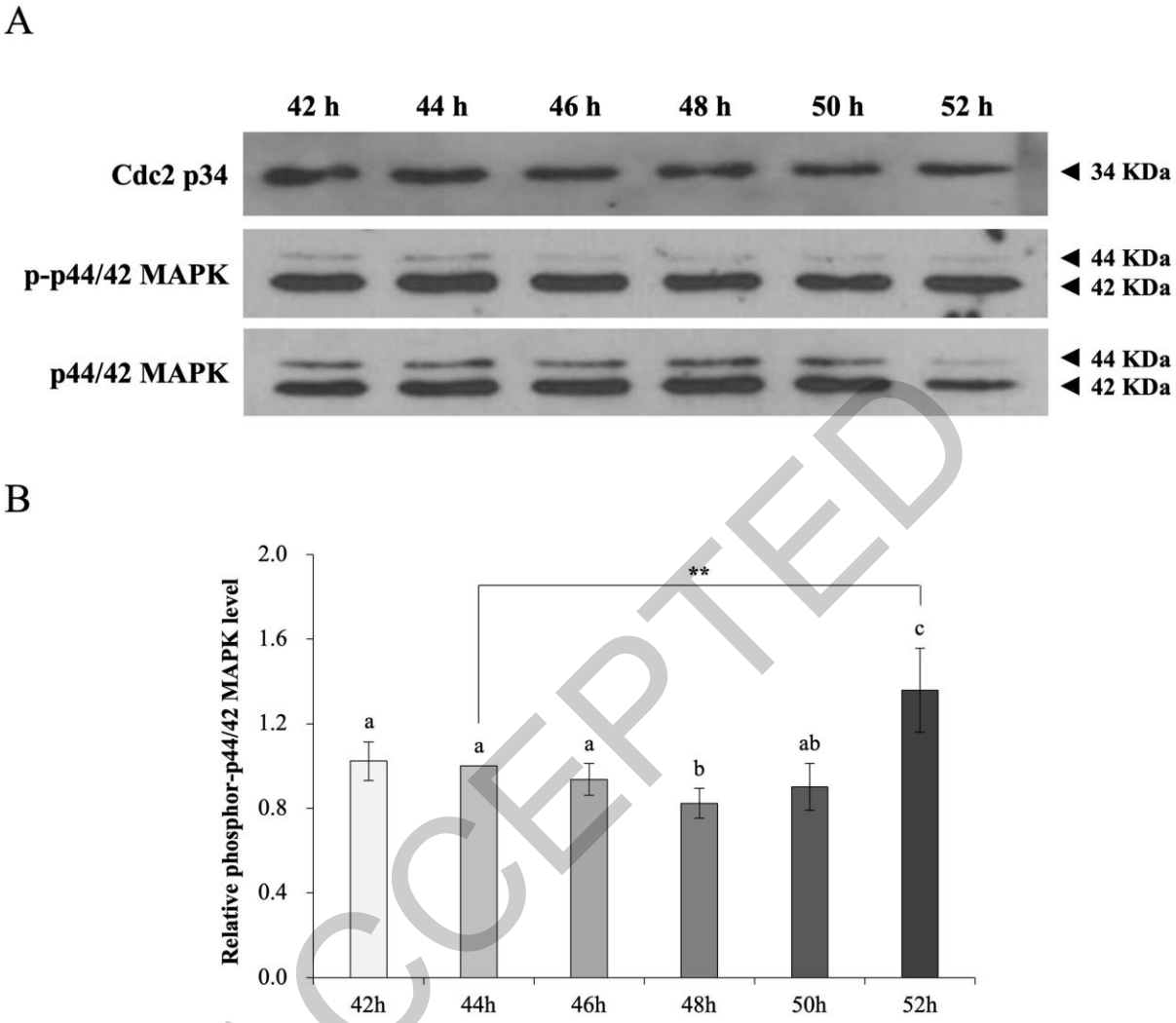

Figure 1. Levels of maturation factors during the extended *in vitro* maturation (IVM) of porcine oocytes. Western blot analysis of Cdc2 p34, p44/42 mitogen-activated protein kinase (MAPK), and phospho-p44/42 MAPK (A) and band intensities (B) of Cdc2 p34, p44/42 MAPK, and phospho-p44/42 MAPK in porcine oocytes were examined every 2 h after 42–52 h of IVM. The experiment was independently repeated three times. Significant differences compared with control oocytes are indicated ($^{a-c}p < 0.05$ and $^{**}p < 0.01$). Values represent means ± standard error of the mean of independent experiments.

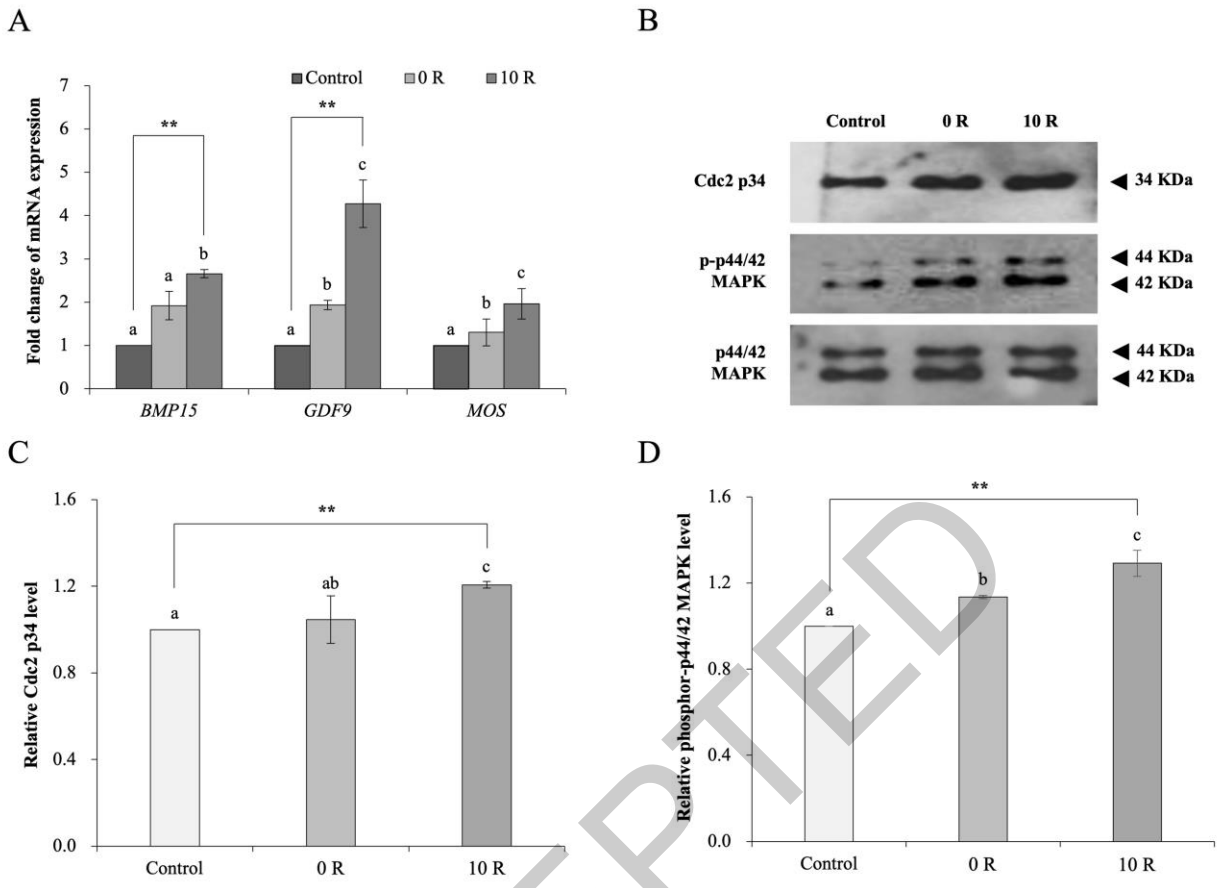

Figure 2. Effect of rapamycin treatment on molecular maturation factors in porcine oocytes. Maternal gene expression (A) and levels of Cdc2 p34, p44/42 MAPK, and phospho-p44/42 MAPK (B) were examined in control, 0 R, and 10 R oocytes. Relative levels of Cdc2 p34 (C) and phospho-p44/42 MAPK (D) were determined. *GAPDH* was used as an internal standard. The experiment was independently repeated three times. Significant differences compared with control oocytes are indicated ($^{a-b}p < 0.05$ and $^{**}p < 0.01$). Values represent means ± standard error of the mean of independent experiments.

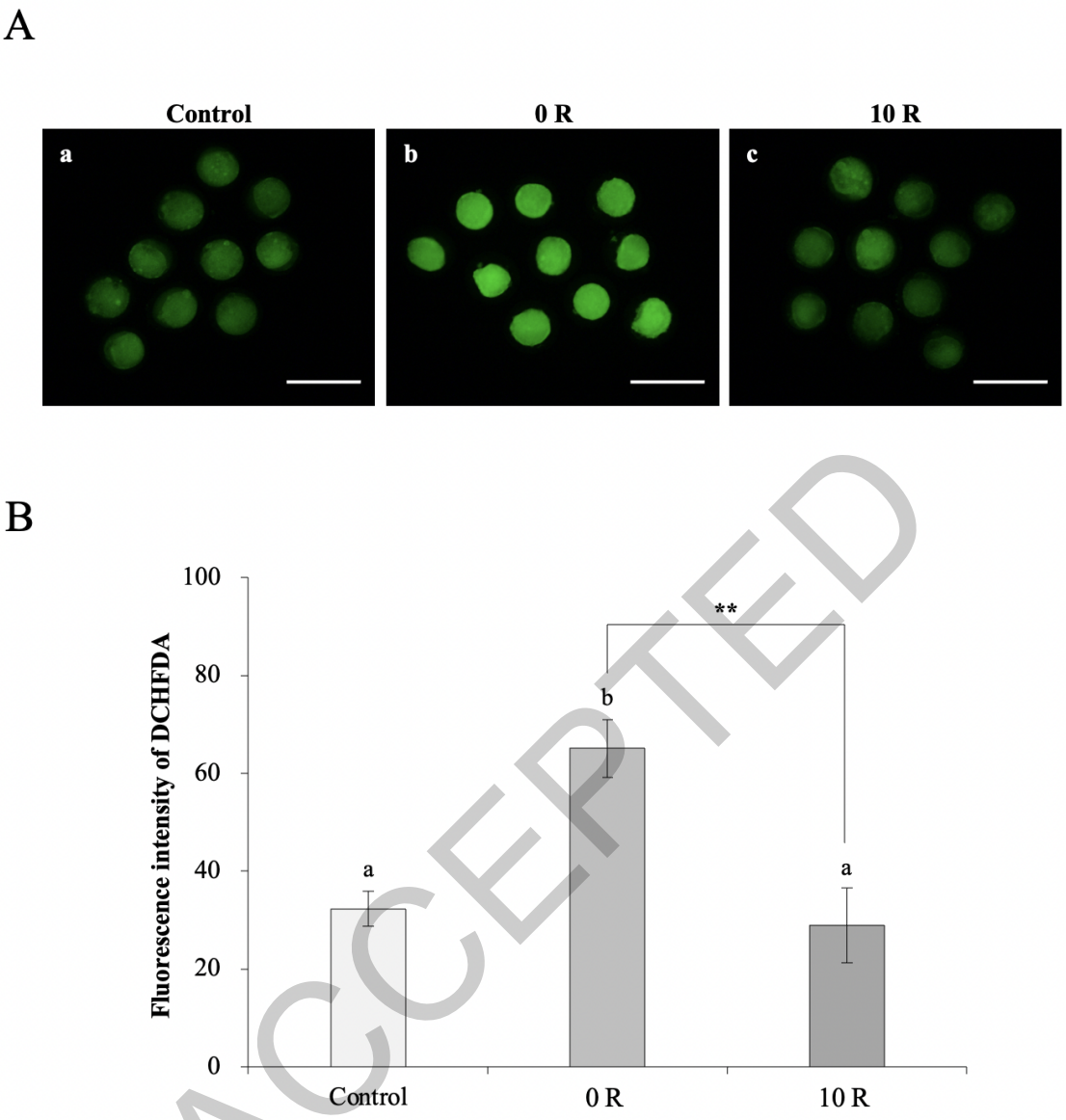

Figure 3. Antioxidant effect of rapamycin treatment during IVM. Images of oocytes stained with DCHFDA in rapamycin-untreated 44 h IVM (control), rapamycin-untreated 52 h IVM (0 R), and 10 μM-rapamycin treated 52 h IVM (10 R) (A) and the fluorescence intensity of DCHFDA (B) were evaluated in metaphase II oocytes in the control, 0 R, and 10 R groups. The experiment was independently repeated four times. Significant differences compared with control group are indicated ($^{a\text{-}b}p < 0.05$ and $^{**}p < 0.01$). Values represent means ± standard error of the mean of independent experiments. Bar = 200 μm.

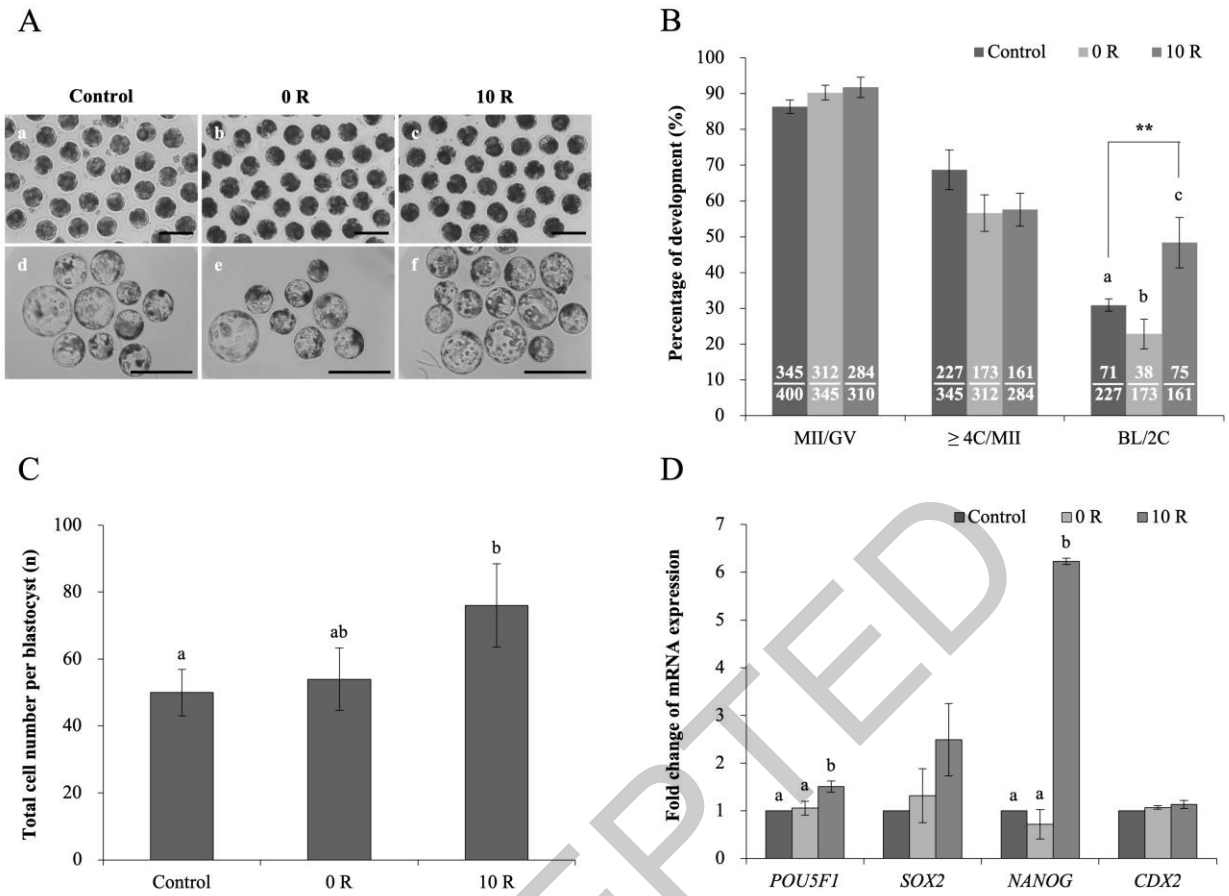

Figure 4. Effect of rapamycin treatment on the developmental capacity of porcine oocytes. (A) Morphology of embryos at day 2 (a–c) and blastocysts at day 6 (d–f) were examined in the control (a and d), 0 R (b and e), and 10 R (c and f) groups. The *in vitro* development rate (B), total cell number per blastocyst at day 6 (C), and relative mRNA expression of the development-related genes *POU5F1*, *SOX2*, *NANOG*, and *CDX2* (D) were examined in blastocysts in the control, 0 R, and 10 R groups. MII, Metaphase II; GV, Germinal vesicle; BL, Blastocyst. The experiment was independently repeated five times (A and B) and three times (C and D). Significant differences compared with the control group are indicated ($^{a-c}p < 0.05$ and $^{**}p < 0.01$). Values represent the means ± standard error of the mean of independent experiments. Bar = 200 µm.

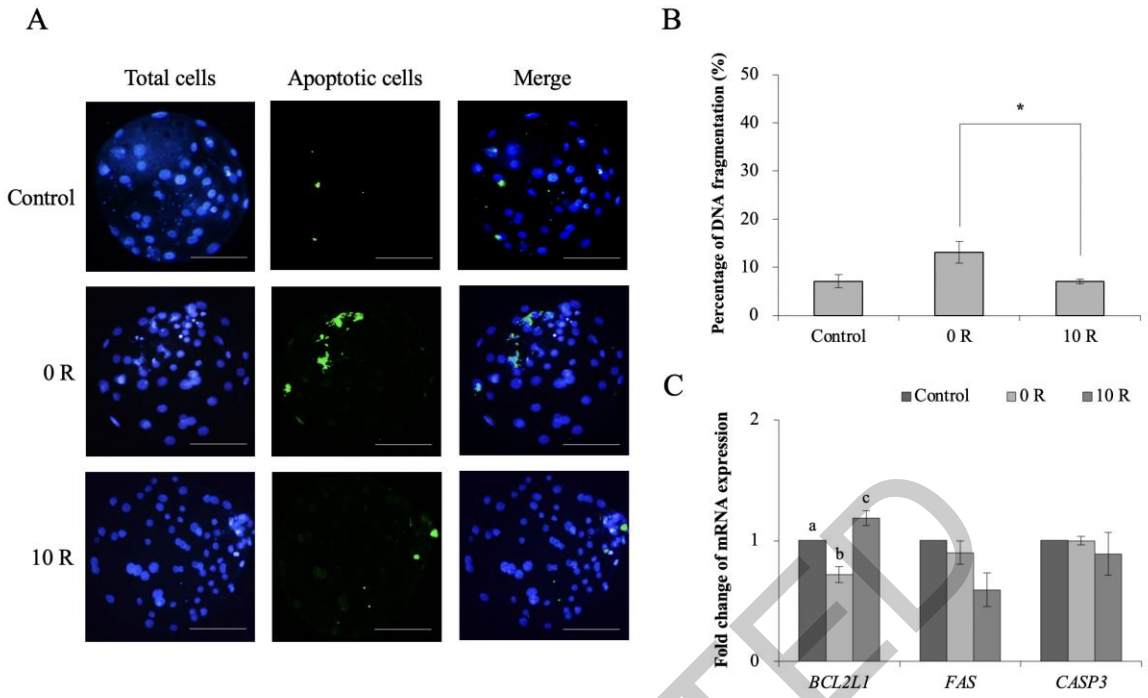

Figure 5. Effect of treatment of porcine oocytes with rapamycin on the level of apoptosis in blastocysts. Morphology of blastocyst total cell and apoptotic cells (A), DNA fragmentation (B) and relative mRNA expression of the apoptosis-related genes *BCL2L1*, *FAS*, and *CASP3* (C) were examined in blastocysts in the control, 0 R, and 10 R groups. The experiment was independently repeated three times. Significant differences compared with the control group are indicated (*$p < 0.05$). Values represent means ± standard error of the mean of independent experiments. Bar = 50 µm.

472 **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
GDF9	AY_626786	F: GAGCTCAGGACACTCTAAGCT R: CTTCTCGTGGATGATGTTCTG	60	272
MOS	NM_001113219	F: TGGGAAGAAACTGGAGGACA R: TTCGGGTCAGCCCAGGTTCA	60	121
POU5F1	NM_001113060	F: AGTGAGAGGCAACCTGGAGA R: TCGTTGCGAATAGTCACTGC	60	166
NANOG	DQ447201.1	F: GAACTTTCCAACATCCTGAA R: TTTCTGCCACCTCTTACATT	55	87
SOX2	EU503117	F: GCCCTGCAGTACAACTCCAT R: GCTGATCATGTCCCGTAGGT	60	216
CDX2	AM778830	F: AGCCAAGTGAAAACCAGGAC R: TGCGGTTCTGAAACCAGATT	60	178
BCL2L1	AF216205	F: ACTGAATCAGAAGCGGAAAC R: AAAGCTCTGATACGCTGTCC	60	249
FAS	AJ001202	F: AAGTTCCCAAGCAAGGGATT R: AATTTCCCATTGTGGAGCAG	60	207
CASP3	NM_214131.1	F: GAGGCAGACTTCTTGTATGC R: ACAAAGTGACTGGATGAACC	55	93

473 *F, forward; R, reverse.