

Research Article

Gentisic Acid Enhances Porcine Oocyte Maturation Quality and Developmental Competence of Somatic Cell Nuclear Transfer by Decreasing Oxidative Stress

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- Somatic Cell Nuclear Transfer

Abstract

Gentisic acid (GA) is a phenolic compound found in citrus fruits (*Citrus spp.*), grapes (*Vitis vinifera*), and gentians (*Gentiana spp.*), known for its antioxidant activity and ability to scavenge free radicals. To examine a potential antioxidant role of GA during oocyte *in-vitro* maturation (IVM), porcine cumulus-oocyte complexes were cultured in IVM medium, supplemented with various GA concentrations to identify its optimal concentration. Supplementation with 100 μ M GA (100 GA) during porcine oocyte IVM enhanced the developmental capacity and resulting blastocyst quality of parthenogenetically activated embryos. The 100 GA group was significantly decreased levels of reactive oxygen species, and the glutathione and mRNA expression levels of antioxidant-related genes (*CAT*, *HO-1*, *NFE2L2*, *SOD1*, and *SOD2*) were significantly increased compared with the control group. Also, both normal spindle formation and chromosomal alignment occurred significantly more frequently in the 100 GA group. Blastocysts resulting from 100 GA-treated oocytes showed significantly increased expression of developmental (*CDX*, *SOX2*, *NANOG*, and *POU5F1*) and anti-apoptotic (*BCL2L1*, and *BIRC5*) genes, while pro-apoptotic gene expression (*BAK*, and *CASP3*) decreased significantly. Somatic cell nuclear transfer using normal or transgenic somatic cells improved the developmental capacity and quality of the 100 GA group blastocysts. These results indicate that supplementation with 100 GA enhances oocyte maturation and subsequent developmental competence of porcine embryos. These also suggest that GA supplementation improves *in-vitro* production in assisted reproductive technologies and biotechnology.

ABBREVIATIONS

AD: Alzheimer's Disease; ART: Assisted Reproductive Technology; BAK: Bcl-2 Antagonist/Killer; BCL2L1: BCL2-Like 1; BIRC5: 20 Baculoviral IAP Repeat-Containing 5; BL: Blastocyst; BSA: Bovine Serum Albumin; CASP3: Cysteine21 Aspartic Acid Protease 3; CAT: Catalase; CDX2: Caudal Type Homeobox Protein 2; CMF2HC: 4- 22 Chloromethyl-6,8-Difluoro-7-Hydroxycoumarin; COC: Cumulus-Oocyte Complex; DCFHDA: 2,3 Dichlorohydrofluorescein Diacetate; DPBS: Dulbecco's Phosphate-Buffered Saline; DPPH: 2,2-Diphenyl-1-Picrylhydrazyl; FAS: Fas Cell Surface Death Receptor; GSH: Glutathione; HO-1: Heme Oxygenase 1; IVC: *In Vitro* Culture; IVM: *In Vitro*

Maturation; MAPK: 2 Mitogen-Activated Protein Kinase; MII: Metaphase II; Mrna: Messenger RNA; NANOG: Nanog 3 Homeobox; *NFE2L2*: Nuclear Factor Erythroid 2-Like 2; OS: Oxidative Stress; PB: Polar Body; PBS: Phosphate-Buffered 4 Saline; PIP: Piperine; POU5F1: POU Domain, Class 5, Transcription Factor 1; PZM: Porcine Zygote 5 Medium; ROS: Reactive Oxygen Species; RT-PCR: Reverse Transcription Polymerase Chain Reaction; 6 SCNT: Somatic Cell Nuclear Transfer; SDS: Sodium Dodecyl Sulfate; SOD1: Superoxide Dismutase 1; 7 SOD2: Superoxide Dismutase 2; SOX2: Sex-Determining Region Y-Box Transcription Factor 2; TCM: 8 Tissue Culture Medium; TE: Trophectoderm; TF: Transcription Factor; Tg: Transgenic

INTRODUCTION

Somatic cell nuclear transfer (SCNT) deploys electric pulses to fuse donor cells with enucleated oocytes and can induce reprogramming of terminally differentiated towards totipotent cells [1]. SCNT constitutes assisted reproductive technology (ART), which aims to preserve genetically valuable samples, generate animal models of human disease, such as cardiovascular disease, cancer, and Alzheimer's disease (AD), and produce animal models (e.g., pigs) that bear anatomical and physiological characteristics similar to humans for interspecies transplantation [2-5]. However, the success rate of SCNT-generated recombinant embryos is relatively low, and there is a high risk of miscarriage and malformations [6,7], which is mostly attributed to the low developmental competence of porcine oocytes during *in-vitro* maturation (IVM), in contrast to maturation *in vivo* [8,9]. The low developmental competence of porcine oocytes undergoing IVM is caused by abnormal spindle formation and cytoplasmic maturation despite a complete nuclear maturation [10,11]. It follows that optimizing culture systems is crucial for successful IVM as it significantly influences embryo production [12,13]. Many studies have sought to improve IVM efficiency by changing the culture medium composition, altering the atmospheric composition, or supplying hormones or antioxidants [14-16].

Gentisic acid (GA), also known as phenolic acid, is found in natural sources including citrus fruits (*Citrus spp.*), grapes (*Vitis vinifera*), and gentians (*Gentiana spp.*) and is an aspirin metabolite [17,18]. Previous studies have reported various beneficial effects of GA supplementation including anti-inflammatory, antimicrobial, anticancer, and antioxidant activities [19-21]. GA supplementation attenuated prostaglandin formation in response to lipopolysaccharides by suppressing COX-2 activity in RAW 264.7 cells, oxidized low-density lipoprotein in THP-1 cells, and formation of lipid hydroperoxides in endothelial cells [22-23]. Also, GA supplementation enhances keratinocyte proliferation by phosphorylating ERK1/2, as well as apoptosis of human colorectal carcinoma-116 cells through heme-ferritin interactions upon oxidative stress (OS) [19-24].

Nevertheless, how IVM medium supplementation with GA affects porcine oocytes remains unknown. Here, we demonstrated that GA functions as a beneficial natural compound during IVM, by improving the quality of porcine parthenogenetic activation (PA) and SCNT embryos. We also investigated the reactive oxygen species (ROS) and glutathione (GSH) levels, as well as meiotic

spindle formation, cytoplasmic maturation, and mRNA expression of antioxidant, developmental, and apoptotic genes. Based on these experiments, we predicted that GA supplementation in IVM improves transgenic (Tg) animal cloning.

MATERIALS AND METHODS

Chemicals and Reagents

All reagents were purchased from Merck Millipore (Darmstadt, Germany) unless otherwise stated. Oocytes, embryos, and donor fibroblasts were cultured in a CO₂ incubator (38.8°C, 5% CO₂, and 95% humidity).

Collection of Porcine Oocytes and IVM

Prepubertal porcine ovaries were obtained from a local abattoir and promptly transported to the laboratory in saline solution supplemented with 75 µg/mL penicillin G (DUCHEFA, P0142) and 50 µg/mL streptomycin sulfate (DUCHEFA, S0148). The transit time was kept within 2 h, and a temperature range of 30–33°C. Cumulus-oocyte complexes (COCs) with 2–8 mm diameters were collected by aspirating ovarian follicles using an 18-gauge needle connected to a disposable 10-mL syringe. The COCs were washed in tissue culture medium (TCM)-199-HEPES, supplemented with 0.1% (w/v) bovine serum albumin (BSA). Subsequently, groups of 44–50 COCs were washed for maturation in 50 µL TCM-199 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, 10% (v/v) porcine follicular fluid, and various GA concentrations in 1% dimethyl sulfoxide (DMSO): 1 µM, 10 µM, 100 µM, 1 mM, or 10 mM. COCs were cultured in the same medium for 44 h in a CO₂ incubator, covered with mineral oil.

PA and In Vitro Culture (IVC)

Following 44 h of maturation, cumulus cells (CCs) were removed by gently pipetting with HEPES supplemented with 1 mg/mL hyaluronidase for 2–3 min. Denuded oocytes were parthenogenetically activated with 0.4% (w/v) BSA and 5 µM Ca²⁺ ionomycin for 5 min. After 4h of culture in porcine zygote medium (PZM)-5 containing 0.4% (w/v) BSA and 7.5 µg/mL cytochalasin B (CB), zygotes were washed at least three times with IVC medium containing with PZM-5 and 0.4% (w/v) BSA and cultured in the same medium for 7 days in a CO₂ incubator, covered with mineral oil. Oocytes and embryos were washed at least three times with 0.1% BSA-PBS (B-PBS) containing Dulbecco's phosphate-buffered saline (DPBS) and 0.1% (w/v) BSA. Subsequently, they were either fixed in 4.0%

(w/v) paraformaldehyde (PFA) for 20 min and stored at 4°C or snap-frozen with liquid nitrogen and stored at -80°C, depending on the experiment. Transfer of the activated or reconstructed embryos to the IVC medium was designated as day 0. The percentages of embryos undergoing cleavage and blastocyst (BL) formation were recorded on days 2 and 7, respectively. Each experiment was repeated seven independent times.

Cell Culture

Donor fibroblasts for SCNT originated from Jeju and AD Tg Jeju black pigs as previously described [5]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (HyClone, US, SH30071.03), 0.1 mM β -mercaptoethanol, and 1% penicillin/streptomycin. Cells at passage 5–10 were cultured for 2–3 days until they reached confluency.

SCNT and IVC

Following a 34h maturation period, CCs were removed by gently pipetting with HEPES buffer, supplemented with 1 mg/mL hyaluronidase for 2–3 min. The first polar body (PB) and nucleosome were removed in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer containing 0.3% (w/v) BSA and 7.5 μ g/mL CB using a 20- μ m glass pipette under an Oosight Imaging System (Cambridge Research & Instrumentation, Inc.). The donor cells were inserted into the perivitelline space of each oocyte, which surrounds its cytoplasm. Karyoplast–cytoplasm complexes were fused in fusion medium containing 0.3 M D-mannitol, 0.5 mM HEPES, 0.05% (w/v) fatty acid-free BSA, 0.05 mM CaCl_2 , and 0.1 mM MgSO_4 . Oocyte–cell couplets were aligned to the negative electrode in a fusion chamber (Nepa Gene) and exposed to a direct current of 105 V/cm for 60 μ s. After fusion, SCNT embryos were activated in the presence of PZM-5 containing 0.4% (w/v) BSA and 7.5 μ g/mL CB for 4 h in a CO_2 incubator and then transferred to PZM-5 supplemented with 0.4% (w/v) BSA. SCNT embryo transfer to the IVC medium was designated as day 0. The percentages of embryos undergoing cleavage and BL formation were recorded on days 2 and 7, respectively. This experiment was conducted with three groups (normal, control, and 100 GA). In the normal group, normal pig somatic cells were injected into oocytes, matured in IVM medium, supplemented with 1% DMSO. In the control and 100 GA groups, AD Tg model pig somatic cells were injected into oocytes, matured in IVM medium, supplemented with 1% DMSO or 100 μ M GA. Each experiment was repeated six independent times.

Terminal Deoxynucleotidyl Transferase dUTP Nick-

End Labeling Assay and Hoechst Staining

At 7 days after PA or SCNT, BLs were fixed overnight at 4°C with 4% (w/v) PFA. They were washed at least three times with 0.1% B-PBS, and permeabilized with 0.1% TritonX-100 for 30 min in a CO_2 incubator, covered with foil. The BLs were then incubated with fluorescein-conjugated dUTP and terminal deoxynucleotidyl transferase (In Situ Cell Death Detection Kit; Roche, Mannheim, Germany) for 1 h in a CO_2 incubator, covered with foil. They were subsequently washed at least three times with 0.1% B-PBS. Nuclei were stained with Hoechst 33342 (1 μ g/mL) for 30 min in a CO_2 incubator, covered with foil. The BLs were then washed at least three times with 0.1% B-PBS, mounted on glass slides, and examined under an inverted Olympus IX-71 fluorescence microscope. Total and apoptotic cells were scored. The experiment was repeated seven times independently, with 5–18 BLs being analyzed per experiment.

Measurement of Intracellular ROS and GSH Levels

Dichlorohydrofluorescein diacetate (DCHFDA) was utilized to quantify intracellular ROS levels, and CellTracker™ Blue 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (CMF2HC) was used to determine reduced intracellular GSH levels. After 44 h, CCs were removed by gently pipetting with HEPES buffer, supplemented with 1 mg/mL hyaluronidase for 2–3 min. Denuded oocytes were washed at least three times with 0.1% B-PBS and incubated in 0.1% B-PBS containing with 50 μ M DCHFDA or 100 μ M Blue CMF2HC for 20 min in a CO_2 incubator, covered with foil. To ensure complete removal of excess dye, the oocytes were washed at least five times with 0.1% B-PBS. Subsequently, they were analyzed by epifluorescence microscopy (Olympus, Tokyo, Japan). ROS levels were assessed using excitation and emission wavelengths of 450–490 nm and 515–565 nm, respectively. For CMF2HC, the excitation and emission wavelengths were 371 nm and 464 nm, respectively. Grayscale images were captured using a Nikon digital camera attached to the microscope. Mean grayscale values were calculated using ImageJ software (National Institute of Health, Bethesda, MD, USA). Before conducting statistical analyses, background fluorescence was subtracted from the final values. The experiment was repeated six times independently, with 10–12 oocytes being analyzed per experiment.

mRNA Extraction and cDNA Synthesis

mRNA was isolated from at least three biological replicates, with 20–30 oocytes or 15–28 BLs per replicate,

using the Dynabeads mRNA Direct Kit (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. The isolated mRNA was collected in 10 µL elution buffer provided with the kit. The eluted RNA was reverse-transcribed to cDNA using an oligo (dT) 20-base primer and SuperScript™ III reverse transcriptase (Invitrogen, 18080093), following the manufacturer's instructions.

Real-Time Reverse Transcription Polymerase Chain Reaction

The experimental protocol of this study closely followed previously described methodology [25]. Real-Time reverse transcription polymerase chain reaction was carried out using the primer sets specified (Table 1). The reactions were conducted using a StepOnePlus Real-time PCR System (Applied Biosystems, Warrington, UK) in a final reaction volume of 20 µL, which included SYBR Green PCR Master Mix (Applied Biosystems). We used the following PCR cycling conditions: initial denaturation at 95°C for 10 min, 39 cycles of denaturation at 95°C for 15 s, annealing/extension at 54°C or 60°C for 60 s, and final cooling at 12°C. Relative gene expression levels were assessed using

the $2^{-\Delta\Delta Ct}$ method [26], with data normalization against the expression level of a housekeeping gene (ACTB). The experiment was repeated independently six times.

Western Blot Analysis

A previously described protocol was followed [25]. About 40–45 oocytes per sample were dissolved in 20 µL of 1×sodium dodecyl sulfate (SDS) sample buffer (consisting of 62.5 mM Tris-HCl pH 6.8, 2% [w/v] SDS, 10% [v/v] glycerol, 50 µM dithiothreitol, and 0.01% [w/v] bromophenol blue or phenol red) and subjected to heating at 95°C for 5 min. Proteins were resolved on 5–12% Tris SDS-polyacrylamide gel electrophoresis gels for 1.5 h at 80–120 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, 200 mM glycine, and 20% [v/v] methanol). After blocking with 5% (w/v) skimmed milk in PBS for 2 h, the membranes were incubated overnight at 4°C with a rabbit anti-p44/42 mitogen-activated protein kinase (MAPK) or an anti-phospho-p44/42 MAPK antibody (Cell Signaling Technology) at room temperature (RT) (diluted

Table 1: Primers used for quantitative PCR

Gene	GenBank Accession no.	Primer sequences	Annealing temperature (°C)	Product size (bp)
ACTB	AY550069.1	F: AGATCATGTTTCGAGACCTTC	54	220
		G: GTCAGGATCTTCATGAGGTAGT		
CAT	NM_214301.2	F: AGGGAGAGGCGGTTTATTC	59	117
		R: GGACTCGTTGGTGAAGCTCA		
HO-1	NM001004027.1	F: ACCCAGGACACTAAGGACCA	54	227
		R: CGGTTGCATTACAGGGTTG		
NFE2L2	XM_005671981.2	F: ACAACTCAGCACCTTGTACC	54	81
		R: CCTTACTCTCCAAGTGAGTACTC		
SOD1	GU9444822.1	F: GCCACTGTGTACATCGAAGAT	54	173
		R: GTGATCCCAATTACACCACAG		
SOD2	NM_214127.2	F: AGACCTGATTACCTGAAAGC	54	110
		R: CTTGATGTACTCGGTGTGAG		
CDX2	AM778830	F: AGCCAAGTGAAAACAGGAC	60	178
		R: TGCGGTTCTGAAACCAGATT		
SOX2	EU503117	F: GCCCTGCAGTACAACCTCAT	60	216
		R: GCTGATCATGTCCGTAGGT		
NANOG	DQ447201	F: TTCCTTCCTCCATGGATCTG	60	214
		R: ATCTGCTGGAGGCTGAGGTA		
POU5F1	NM_001113060	F: AGTGAGAGGCAACCTGGAGA	60	166
		R: TCGTTGCGAATAGTCACTGC		
BIRC5	NM_214141.1	F: CTTCTGCTTCAAAGAGCTG	54	154
		R: GGCTCTTTCTTTGTCCAGT		
BCL2L1	NM_214285.1	F: GGTGACTTTCTCTCTACA	54	118
		R: CTCAGTTCTGTTCTTCCA		
BAK	AJ001204	F: CTAGAACCTAGCAGCACCAT	60	151
		R: CGATCTTGGTGAAGTACTC		
FAS	AJ001202.1	F: GAGAGACAGAGGAAGACGAG	54	194
		R: CTGTTCACTGTATCTTTGG		
CASP3	NM_214131	F: GAGGCAGACTTCTGTATGC	55	236
		R: CATGGACACAATACATGGAA		

F: forward; R: reverse.

1:300 in blocking solution [1×Tris-buffered saline pH 7.5, 0.1% (v/v) Tween-20, and 5% (w/v) skimmed milk]). Thereafter, the membranes were washed three times in TBST (20 mM Tris-HCl pH 7.5, 250 mM NaCl, and 0.2% [v/v] Tween-20) and incubated for 1h with mouse anti-rabbit IgG-horseradish peroxidase (Santa Cruz Biotechnology) at RT (diluted 1:2000 in blocking solution). After three washes with TBST, immune-reactive protein bands were visualized with a chemiluminescent reagent (Invitrogen). The experiment was repeated six independent times.

Statistical Analysis

All experimental data were analyzed using the general linear model procedure within the Statistical Analysis System (SAS) software (SAS User's Guide 1985, Statistical Analysis System Inc., Cary, NC, USA). To determine significant differences, the paired Tukey's multiple range test was employed. P-values of <0.05 were considered to denote statistical significance.

RESULTS

Effect of Various Concentrations of GA on Porcine PA Embryonic Development

To investigate the optimal GA concentration for porcine oocyte IVM, we assessed their maturation efficiency. Oocytes were incubated in IVM medium containing 1% DMSO at various GA concentrations (1 μ M, 10 μ M, 100

μ M, 1 mM, or 10 mM). The PB emission percentage at the metaphase-II (MII) stage was significantly higher in the 100 GA but did not differ significantly in other GA groups compared with the control group (control, 72.9±0.7%; 1 μ M GA, 73.9±1.5%; 10 μ M GA, 74.5±0.8%; 100 μ M GA, 75.2±0.8%; 1 mM GA, 73.2±0.5%; 10 mM GA, 73.2±1.1%; p <0.05; Figure 1A). After PA, the cleavage percentage was significantly higher in the 100 GA but did not in other GA groups compared with the control group (control, 71.7±0.6%; 1 μ M GA, 73.4±3.0%; 10 μ M GA, 73.6±3.3%; 100 μ M GA, 74.2±1.0%; 1 mM GA, 73.6±2.2%; 10 mM GA, 72.7±1.4%; p <0.05; Figure 1A). The BL-formation percentage was significantly higher in the 100 GA group, but not in other GA groups, compared with the control group (control, 28.4±1.4%; 1 μ M GA, 29.2±0.8%; 10 μ M GA, 30.0±1.0%; 100 μ M GA, 31.8±0.9%; 1 mM GA, 29.3±1.0%; 10 mM GA, 28.5±1.6%; p <0.05; Figure 1A).

We also evaluated the PA embryo quality upon GA supplementation during porcine oocyte IVM. The total cell number per BLs was significantly higher in the 100 GA group but not in other GA groups compared with the control group (control, 83.7±2.0; 1 μ M GA, 84.0±2.2; 10 μ M GA, 86.1±2.1; 100 μ M GA, 89.3±2.1; 1 mM GA, 87.9±2.2; 10 mM GA, 84.7±2.1; p <0.05; Figure 1B). The apoptotic cell percentage was significantly higher in the control than in the 100- μ M and 1-mM GA groups, but did not differ significantly in other GA groups (control, 1.98±0.2%; 1 μ M GA, 1.79±0.2; 10 μ M GA, 1.70±0.1; 100 μ M GA, 1.51±0.1%;

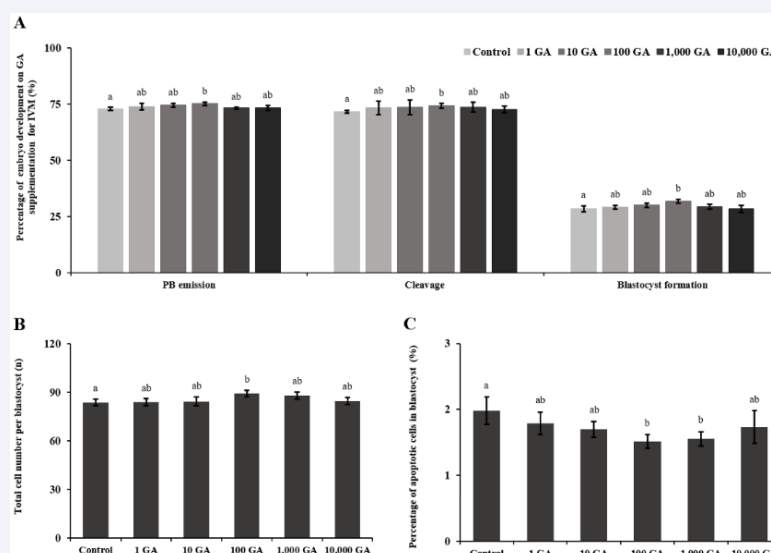


Figure 1 Effect of various concentrations of GA supplementation on the developmental capacity of parthenogenetically activated (PA) embryos. A) Polar body (PB) emission rate of MII oocytes on day 0, cleavage rate of porcine parthenogenetic embryos on day 2, and blastocyst (BL) formation rate of PA porcine embryos on day 7. B) Total BL cell numbers on day 7. C) Percentage of BL apoptotic cells on day 7. Values are presented as means ± standard errors of the mean of independent experiments ($a-bp < 0.05$).

1 mM GA, 1.55 ± 0.1 ; 10 mM GA, 1.73 ± 0.2 ; $p < 0.05$; Figure 1C). Based on these results, 100 μ M GA were selected for all subsequent experiments.

Effect of GA Supplementation on the Oxidation Resistance of Porcine Oocytes Undergoing IVM

To investigate the antioxidative activity of GA supplementation during the IVM of porcine PA embryos, we measured their ROS and GSH levels and demonstrated their corresponding ROS and GSH morphologies (Figure 2A). DCFH-DA fluorescence intensity was significantly higher in the control than in the 100 GA group (control, 54.6 ± 2.0 pixels/oocyte; 100 GA, 45.5 ± 1.9 pixels/oocyte; $p < 0.01$; Figure 2B). Similarly, CMF₂HC fluorescence intensity was significantly higher in the 100 GA group than in the control (control, 80.0 ± 2.1 pixels/oocyte; 100 GA, 87.6 ± 3.2 pixels/oocyte; $p < 0.05$; Figure 2C). In addition, we investigated the effects of these antioxidants on the expression of the antioxidant genes, *catalase (CAT)*, *Heme Oxygenase-1 (HO-1)*, *nuclear factor erythroid 2-like 2 (NFE2L2)*, *superoxide dismutase 1 (SOD1)*, and *superoxide dismutase 2 (SOD2)*, whose mRNA levels were normalized against those in the control group. *NFE2L2*, *CAT*, *HO-1*, *SOD1*, and *SOD2* expression was significantly higher in the 100 GA group than in the control ($p < 0.05$; Figure 2D).

Effect of GA Supplementation during IVM on Meiotic Spindle Morphology and Cytoplasmic Maturation of Porcine Oocytes

To investigate the protective effect of GA on meiotic spindle morphology of porcine oocytes, we measured chromosome alignment and spindle organization. Meiotic spindle morphology and organization have been previously studied in oocytes [27], and normal and abnormal morphologies have been characterized (Figure 3-4A). A percentage of normal chromosome alignment and meiotic spindle formation oocyte was significantly higher in the 100 GA than in the control group (control, $58.8 \pm 2.1\%$; GA, $68.8 \pm 2.7\%$; $p < 0.05$; Figure 3B). To investigate the protective effect of GA supplementation on porcine oocyte cytoplasm maturation, we measured the p44/42 MAPK activity. Western blot analysis revealed that phospho-p44/42 MAPK, the active form of this kinase, migrated as a doublet in matured porcine oocyte lysates (Figure 3C). The ratio of phospho-p44/42 to p44/42 MAPK was normalized against that in the normal group and found to be significantly higher in the 100 GA group than in the control (control, $1.0 \pm 0.0\%$; GA, 1.1 ± 0.08 ; $p < 0.05$; Figure 4D).

Effect of GA Supplementation during IVM on the

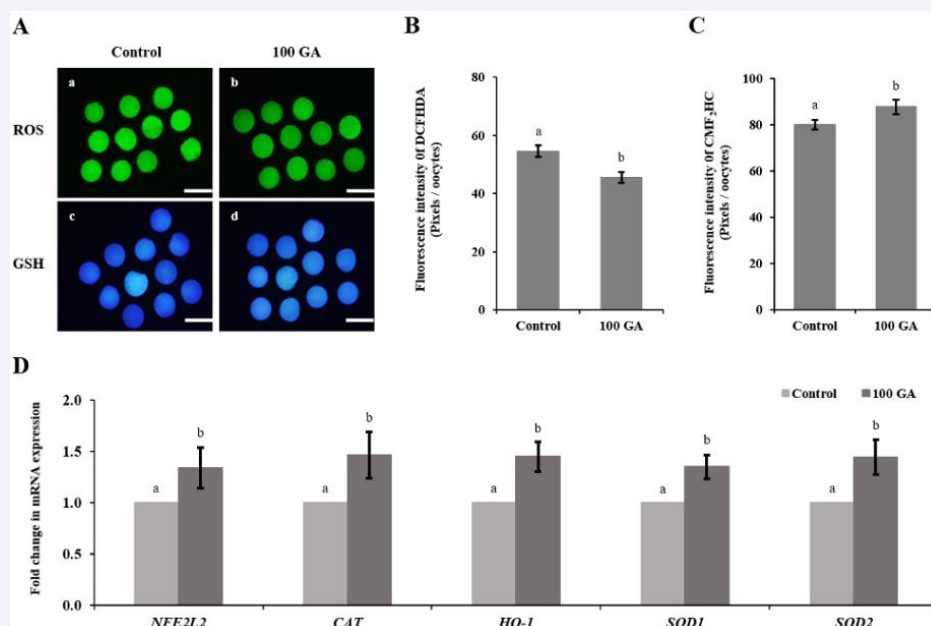


Figure 2 Effect of GA supplementation on the oxidation resistance of porcine oocytes during IVM. A) Images of oocytes stained with DCFH-DA (green) and CMF₂HC (blue). a and b, reactive oxygen species (ROS) staining; c and d, glutathione (GSH) staining; a and c, control group; b and d, 100 GA group. B) Fluorescence intensity quantification of DCFH-DA. C) Fluorescence intensity quantification of CMF₂HC. D) Relative expression of antioxidant genes (*CAT*, *HO-1*, *NFE2L2*, *SOD1*, and *SOD2*) at MII stage. *ACTB* expression served as internal control. Oocytes were analyzed at MII stage. Values are presented as means $\pm$ standard errors of the mean of independent experiments ($^{a,b}p < 0.05$). Scale bar, 100 μ m.

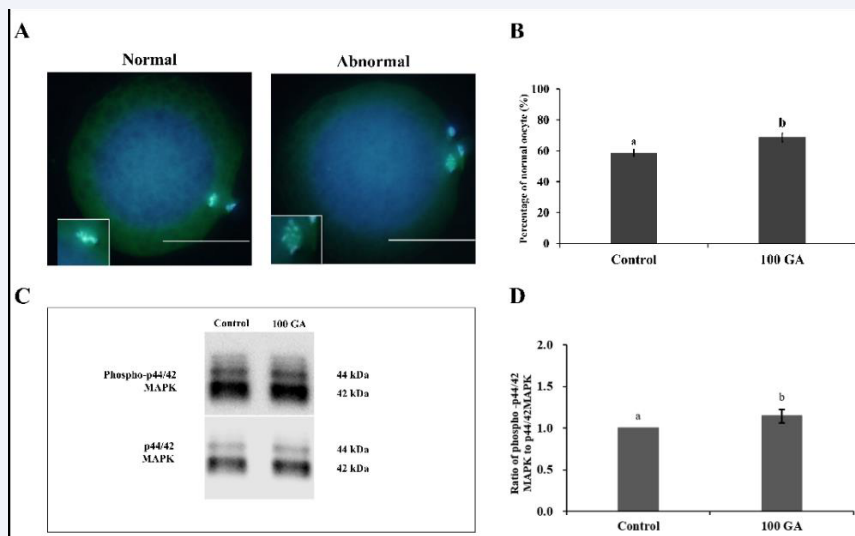


Figure 3 Effect of GA supplementation on meiotic spindle morphology and MAPK activity of porcine oocytes *in vitro*. A) Normal and abnormal chromosomal alignment and meiotic spindle formation in oocytes. B) Percentage of oocytes in which the chromosome morphologies and meiotic spindles were normal. C) MAPK activity determined by western blot analysis and the ratio of phospho-p44/42 to p44/42 MAPK. D) Ratio of phospho-p44/42 to p44/42 MAPK. Data were derived from 6 independent replicates per group. Data are presented as means $\pm$ standard errors of the mean ($^{a-b}p<0.05$). Scale bar, 50 μ m

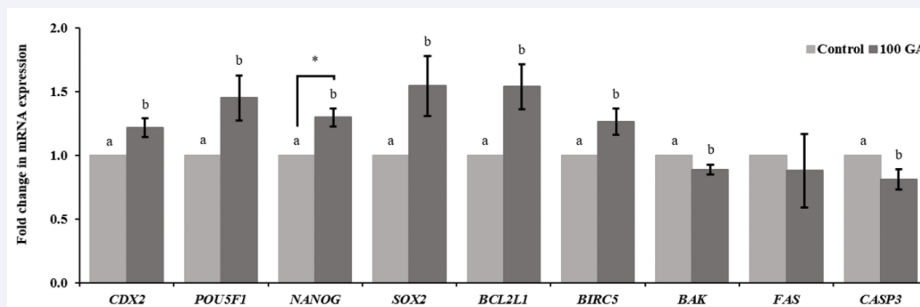


Figure 4 Effect of GA supplementation on subsequent porcine blastocyst (BL) quality during IVM. Relative expression of developmental (*CDX2*, *SOX2*, *NANOG*, and *POU5F1*) and apoptotic (*BIRC5*, *BCL2L1*, *BAK*, *FAS*, and *CASP3*) genes in porcine embryos at BL stage. BLs were analyzed at day 7. Values are presented as means $\pm$ standard errors of the mean of independent experiments ($^{a-b}p<0.05$, $^{*}p<0.01$).

Quality of Porcine PA BLs

To determine whether GA supplementation during IVM affects the expression of development-related genes, we investigated the expression of developmental (*caudal-related homeobox protein 2*, *CDX2*; *SRY* [*sex-determining region Y*]-box 2, *SOX2*; *Nanog homeobox*, *NANOG*; *POU* [*Pit-Oct-Unc*] class 5 homeobox 1; *POU5F1*), anti-apoptotic (*Baculoviral IAP Repeat Containing 5*, *BIRC5*; *B-cell lymphoma 2* [*Bcl-2*]-like 1, *BCL2L1*), and pro-apoptotic (*BCL2 Antagonist/Killer 1*, *BAK*; *Fas Cell Surface Death Receptor*, *FAS*; *Caspase 3*, *CASP3*) genes in the BL. Expression of all aforementioned developmental and anti-

apoptotic genes was significantly higher in the GA 100 group than in the control ($p<0.05$; Figure 5). Additionally, among the pro-apoptotic genes examined, *BAK*, *FAS*, and *CASP3* were significantly upregulated in the 100 GA group compared with the control ($p<0.05$; Figure 4).

Effect of GA Supplementation During IVM on Porcine SCNT Embryo Developmental Competence

To investigate whether GA supplementation of porcine oocyte IVM improves the development competence of SCNT^{Tg} embryos, we assessed oocyte fusion, embryo cleavage, and BL-formation rates after SCNT. We observed

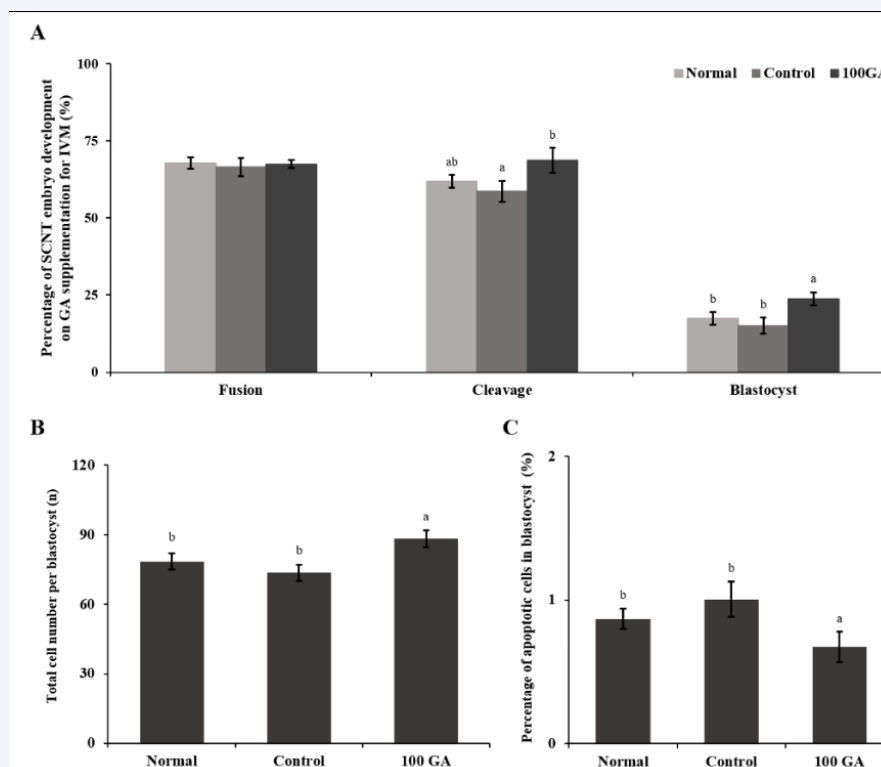


Figure 5 Effect of GA supplementation on the developmental competence of porcine SCNT embryos during IVM. A) Percentage of developed SCNT embryos upon IVM supplementation with GA. Percentages of oocytes with fusion, cleavage on day 2, and blastocyst (BL) formation on day 7 after SCNT. B) Total BL cell numbers on day 7. C) Percentage of apoptotic cells in BLs on day 7. Values are presented as means $\pm$ standard errors of the mean of independent experiments ($^{a-b}p < 0.05$).

that the percentage of fused oocytes was lower in control group than in the normal and 100 GA groups but did not significantly differ in other groups (normal, $67.8 \pm 1.9\%$; control, $66.6 \pm 2.9\%$; 100 GA, $67.5 \pm 1.4\%$; $p < 0.05$; Figure 5A). The cleavage percentage was significantly higher in 100 GA group than in the normal and control groups (normal, $61.9 \pm 2.1\%$; control, $58.7 \pm 3.4\%$; 100 GA, $68.6 \pm 4.1\%$; $p < 0.05$; Figure 5A). The BL-formation percentage was significantly higher in 100 GA group than in the normal and control groups (normal, $17.4 \pm 2.0\%$; control, $15.1 \pm 2.6\%$; 100 GA, $23.8 \pm 2.1\%$; $p < 0.05$; Figure 5A). In addition, we evaluated the SCNT BL quality of GA supplementation of porcine oocyte IVM. The total cell number per BL was significantly higher in the 100 GA group than in the normal and control groups (normal, 78.4 ± 3.5 ; control, 73.5 ± 3.6 ; 100 GA, 88.3 ± 3.7 ; $p < 0.05$; Figure 5B), and the apoptotic cell percentage was significantly higher in the control than in the GA and normal groups (normal, $0.87 \pm 0.1\%$; control, $1.01 \pm 0.1\%$; 100 GA, $0.67 \pm 0.1\%$; $p < 0.05$; Figure 5C).

DISCUSSION

In mammals, oocyte quality in optimized IVM systems

has improved significantly owing to the success of ARTs, which have enhanced the development of the resulting embryos, derived from PA and SCNT cloning or Tg animals [28,29]. Therefore, recent studies have reported that supplementation of IVM with antioxidants such as melatonin, leonurine, and procyanidin B2 has efficiently reduced oocyte OS, thereby promoting its maturation and enhancing its developmental capacity [30-32]. To explore novel roles of other antioxidants on IVM, we investigated whether supplementing IVM with GA improves porcine oocyte quality and the developmental competence of the resulting embryo. We confirmed that GA acts as an antioxidant, as previously demonstrated, and by measuring a reduction in absorbance, we found that it acts by directly reducing free 2,2-diphenyl-1-picrylhydrazyl radicals. This indicates that GA can neutralize free radicals and inhibit their damaging effects. By donating an electron, GA effectively stabilizes these free radicals, thereby preventing them from causing oxidative damage to crucial cellular components such as DNA, proteins, and lipids [33-35].

This study demonstrated that PB emission rates,

cleavage, and BL formation were significantly higher in the 100 GA group than in the control group (Figure 1). Previous studies have shown that GA supplementation provides effective radioprotection at 10- μ M and 100- μ M concentrations in lipid and aqueous phases, respectively [33]. Our results also indicated that supplementation with 100 μ M GA had antioxidative effects which improved the BL-formation rate by promoting oocyte maturation. This suggests that 100 μ M GA enhances the embryonic developmental competence and quality by ameliorating OS during IVM.

Several studies have indicated that early-cleavage embryos tend to develop into high-quality BLs, leading to significantly elevated pregnancy rates, implantation, and live births. Additionally, how efficiently these embryos reach the morula or BL stage correlates with early variations in the timing of their first zygotic cleavage towards the two-cell stage [36,37]. Similarly previous studies, a high percentage of cleavage correlated with high BL percentages. Therefore, 100 μ M GA improved the embryonic developmental capacity and quality by reducing OS during IVM.

To assess whether GA improves embryonic development through antioxidant effects, we quantified intracellular ROS and GSH levels. The balance between intracellular oxidative and antioxidative is primarily determined by ROS and GSH activity. Controlled ROS levels promote meiotic resumption by activating the maturation factor, extruding the first polar corpuscle, and rupturing the follicular wall, thereby allowing the release of oocytes during ovulation [38,39]. Therefore, they play essential signaling roles in reproduction. Oocytes are equipped with antioxidant molecules to eliminate ROS resulting from cellular metabolism [6,40]; however, excessive ROS due to intracellular antioxidant regulatory system imbalance are a main cause of low oocyte competence [41,42]. GSH can protect oocytes from OS and is essential for cytoplasmic maturation [43]. Our data indicated that the intracellular ROS levels were significantly decreased and those of GSH were significantly increased in the 100 GA group (Figure 2B and C).

Based on this result, we demonstrated that the expression of antioxidant genes (*NFE2L2*, *HO-1*, *CAT*, *SOD1*, and *SOD2*) was significantly increased in the 100 GA group. The *NFE2L2* transcription factor (TF) is a master regulator of cellular responses to oxidative and electrophilic stresses [44]. It controls multiple antioxidant defense mechanisms and maintains the cellular ROS balance. *NFE2L2* target genes contain antioxidant response elements (AREs) that help regulate intracellular oxidant levels [45,46].

When ROS levels increase and OS occurs, antioxidant mechanisms get activated to counteract the damage. The *Keap1-NFE2L2* signaling pathway is crucial for connecting cellular antioxidant response with aerobic metabolism. It regulates the cellular response to OS and redox balance by controlling *NFE2L2* activation and antioxidant gene expression [47,48]. The *NFE2L2/HO-1* signaling pathway activates cytoprotective genes including *HO-1* against the ROS destructive effects [49]. *HO-1* is a known oxidation scavenger and plays a crucial role in repairing damage caused by ROS [50]. It catalyzes the initial and rate-limiting reaction of cellular heme breakdown, thus generating an important defense product against OS [51]. Therefore, *HO-1* plays a critical role in embryonic survival [52]. Free-radical scavenger enzymes such *CAT* and *SOD* have been shown to improve porcine embryo development by scavenging ROS and dampening OS [53]. *SOD1* catalyzes the conversion of superoxide radicals into molecular oxygen and hydrogen peroxide [54], and is the first line of defense against the superoxide, produced as a byproduct of oxidative phosphorylation [55]. Similar to our findings, previous studies showed that GA activates *NFE2L2*, which triggers antioxidant enzyme synthesis. *NFE2L2* binds to target-gene AREs, increasing the *NFE2L2/HO-1* antioxidative pathway enzymes, *CAT* and *SOD* [56,57]. To summarize, GA improves embryonic development by inducing antioxidant effects, which are associated with decreased ROS levels and antioxidant gene expression, as well as increased GSH levels.

To assess whether GA improves embryonic development by regulating meiotic maturation, we investigated oocyte nuclear maturation. Oocyte maturation involves both nuclear and cytoplasmic maturation and is crucial for its subsequent development [58]. The regulated alignment of chromosomes and formation of a normal spindle are important for oocyte maturation [59]. OS induced the formation of an abnormal spindle, chromosome misalignment in regions where spindle-related proteins were disrupted, acetylation of α -tubulin which resulted in DNA damage, and pronounced spindle defects during the MII stage. Therefore, antioxidant supplementation could prevent the occurrence of such abnormalities during the MII stage by inhibiting OS [60,61]. We found that GA supplementation during IVM enhanced nuclear maturation by protecting the spindle and ensuring normal chromosome alignment in MII oocytes.

MAPK regulates cell cycle progression by controlling the formation of microtubules and actin filaments. It is activated upon breakdown of the germinal vesicle during oocyte maturation, and it plays a pivotal role in maintaining meiotic arrest and allowing it to resume [62].

MAPK is activated by the extracellular signal-regulated kinase (ERK) signaling pathway and participates in a crucial signal transduction cascade of extracellular signals to the nucleus [63]. Extracellular signals are transmitted to the nucleus by ERK 1/2 and induce specific transcriptional responses [64]. The phospho-p44/42 to p44/42 MAPK ratio was significantly increased in the 100 GA group, suggesting that GA supplementation during IVM improves porcine oocyte nuclear maturation.

To assess how supplementing IVM with GA affects the embryonic developmental potential, we investigated the expression of genes related to development and apoptosis. Developmental genes (*CDX2*, *POU5F1*, *NANOG*, and *SOX2*) were significantly upregulated in the 100 GA group. *CDX2*, a caudal-type homeodomain TF, is known to be specifically expressed in the BL trophoblast, and its expression is sustained within the actively proliferating extra-embryonic ectoderm (ExE) [65]. *POU5F1* plays a crucial role to differentiate pluripotently in the inner cell mass during early embryonic development and has contributed to our understanding of how gene regulation orchestrates cellular pluripotency and facilitates progression to the BL stage [66]. *NANOG*, a core pioneer TF of pluripotent cells, is a strong transcriptional activator of *POU5F1* expression [67,68]. *SOX2* functions with *POU5F1* to control the expression of target genes required for sustaining pluripotency. Its expression extends beyond pluripotent cells to regulate the ExE, trophoblast stem cells, and neural stem cells [69].

Apoptosis (programmed cell death) constitutes a specific response to OS; however, excessive apoptosis may cause early embryonic death or fetal deformities [70,71]. In the 100 GA group, anti-apoptotic genes (*BCL2L* and *BIRC5*) were significantly upregulated and pro-apoptotic genes (*BAK* and *CASP3*) were significantly downregulated, respectively. *BCL2L1* supports cell survival by forming heterodimers that inhibit mitochondrial outer membrane disruption, consequently preventing the release of cytochrome c to the cytoplasm [72]. Cytochrome c, a key player in the mitochondria-dependent apoptotic pathway, activates the caspase cascade, including caspase-9 and caspase-3, which lead to apoptosis [73]. *BIRC5* has been shown to downregulate programmed cell death by inhibiting caspase activation [74]. Also, *BCL2L1* and *BIRC5* can inhibit apoptosis in mitochondria [75]. By contrast, *BAK* triggers mitochondrial outer membrane permeabilization, leading to the release of cytochrome c into the cytoplasm and subsequent activation of the apoptotic pathway [76]. *CASP3 facilitates the degradation of essential matrix proteins and initiates morphological and biochemical alterations that are characteristic of apoptosis.*

These changes include DNA fragmentation and chromosome condensation [76,77]. This suggests that supplementation of IVM with GA may exert positive effects on maintaining embryonic pluripotency, normal embryonic development, and the inhibition of apoptosis.

Based on previous experiments showing that GA enhances oocyte maturation and subsequent embryonic developmental rates by means of its antioxidant activity, we investigated whether it also enhances subsequent SCNT embryonic development using normal and transduction donor cells. The developmental capacity of SCNT embryos using Tg cells was similar to that using non-Tg cells [78]. Also, SCNT embryos showed low developmental competence compared with PA embryos [79,80]. This may be related to the SCNT technique, genetic background, or incorrect epigenetic reprogramming of the donor cell nuclei [81,82]. Nonetheless, the 100 GA supplementation enhanced the development and BL quality of SCNT embryos compared with the normal and control groups. Improving the recipient oocyte quality is important for efficient epigenetic reprogramming of donor cell nuclei [83]. Previous studies have demonstrated that the recipient cytoplasm influences both BL formation and fetal survival [84]. These results suggest that the GA supplementation improves the developmental capacity and quality of porcine embryos by protecting OS during IVM.

FUNDING

This study demonstrated that supplementation with 100 μ M GA enhanced the oocyte maturation and subsequent developmental capacity of porcine PA and SCNT embryos. These findings suggest that GA supplementation may help improve IVM in ART and biotechnology, as well as increase its potential for clinical

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