Transient meiotic arrest maintained by DON (6-diazo-5-oxo-L-norleucine) enhances nuclear/cytoplasmic maturation of porcine oocytes

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Abstract

The developmental competence of *in vitro*-matured oocytes is still lower than that of the *in vivo*-matured oocytes due to precocious meiotic resumption and inappropriate cytoplasmic maturation. Although numerous efforts have been attempted to accomplish better *in vitro* maturation (IVM) condition, only limited progress has been achieved. Thus, a current study was conducted to examine the effects of 6-diazo-5-oxo-L-norleucine (DON, an inhibitor of hyaluronan synthesis) during the first half period of IVM on nuclear/cytoplasmic maturation of porcine oocytes and subsequent embryonic development. Based on the observation of the nucleus pattern, metaphase II (MII) oocyte production rate in 1 μ M DON group was significantly higher than other groups at 44 h of IVM. The 1 μ M of DON was suggested to be optimal for porcine IVM and was therefore used for further investigation. Meiotic arrest effect of DON was maximal at 6 h of IVM, which was supported by the maintenance of significantly higher intra-oocyte cAMP level. In addition, increased pERK1/2 levels and clear rearrangement of cortical granules in membrane of MII oocytes matured with DON provided the evidence for balanced meiosis progression between nuclear and cytoplasmic maturation. Subsequently, DON significantly improved blastocyst formation rate, total cell numbers, and cellular survival in blastocysts after parthenogenetic activation, *in vitro* fertilization, and somatic cell nuclear transfer. Altogether, our results showed for the first time that 1 μ M DON can be used to increase the yield of developmentally competent MII oocytes by synchronizing nuclear/cytoplasmic maturation, and it subsequently improves embryo developmental competence.

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Introduction

Pigs have been widely used in biomedical research, as a model for studying human diseases and xenotransplantation (Prather *et al.* 2003, Hall *et al.* 2013); this is due to the similarities between pigs and humans: their anatomy and physiology (Aigner *et al.* 2010). Several techniques for *in vitro* production (IVP) of preimplantation porcine embryos, including *in vitro* maturation (IVM), *in vitro* fertilization (IVF), *in vitro* culture (IVC), intracytoplasmic sperm injection (ICSI) and somatic cell nuclear transfer (SCNT), have been

used (Betthausen *et al.* 2000, Nagashima *et al.* 2003) to produce pig models for specific purposes. However, the defects in early development of *in vitro*-produced porcine embryos are major obstacles for the generation of these pig models.

IVP of mammalian early embryos is still in need of further improvement by elucidating the developmental events or mechanisms for numerous developmental problems (Galli *et al.* 2012, Luo *et al.* 2012). Considering that the early development of porcine embryos is highly dependent on the quality of the oocytes (Krisner 2004), incomplete maturation of porcine oocytes via

spontaneous meiotic resumption is one of the main reasons for improper development of *in vitro*-produced porcine embryos. When cumulus oocyte complexes (COCs) are removed from antral follicles using a disposable syringe or vacuum pump, oocytes undergo spontaneous meiotic resumption (Dieci *et al.* 2013). Therefore, to improve oocyte quality, a variety of reports suggest that transient meiotic arrest by preventing degradation of intra-oocyte cAMP is required to synchronize nuclear/cytoplasmic maturation and ultimately to improve the oocyte competence (Luciano *et al.* 1999, 2004, Ponderato *et al.* 2002). Temporary blockage of spontaneous meiotic resumption can be achieved through supplementing phosphodiesterases (PDEs) inhibitors (Mayes & Sirard 2002, Lodde *et al.* 2013) or dibutyryl cAMP (Funahashi *et al.* 1997) into IVM media. In particular, various PDEs inhibitors such as milrinone (Gruppen *et al.* 2006), cilostazole (Elahi *et al.* 2016), cilostamide, and rolipram (Laforest *et al.* 2005) have been used to improve porcine IVM, as they prevent the hydrolyze of cAMP and cGMP in oocytes.

Another way to retain high levels of cAMP in oocyte is to lower the level of hyaluronan by treating 6-diazo-5-oxo-L-norleucine (DON; an inhibitor of hyaluronan synthesis). Hyaluronan is a linear glycosaminoglycan with a high-molecular-weight polymer and repeating disaccharides linked by 1-3 and α 1-4 glycosidic bonds, which functions as a major component governing cumulus expansion (Yokoo *et al.* 2010). DON binds with and inactivates the aminotransferase that transfers an amino group from glutamine to fructose-6-phosphate, thus inhibiting the formation of glucosamine-6-phosphate (Bates *et al.* 1966). Glucosamine availability appears to be rate-limiting for the biosynthesis of hyaluronan (McCarty 1996) and inhibition of glucosamine synthesis is a molecular mechanism how DON inhibits hyaluronan synthesis. Since gap junctional communication between the oocyte and cumulus cells can be shut down by expanding cumulus cells under hormone stimulation (Chen *et al.* 1990), inhibition of cumulus expansion by DON treatment during initiation of maturation can keep the gap junction open, which leads to a continuous introduction of cumulus cell-origin cAMP into oocytes.

A previous study demonstrated that a majority of COCs cultured in IVM medium supplemented with DON were arrested at germinal vesicle (GV) stage, and there was tight association with cumulus cells (Yokoo *et al.* 2010). However, the effects of DON on cytoplasmic maturation of oocytes for subsequent embryo development and underlying mechanism(s) are largely unknown. In the current study, we investigated the effects of DON supplementation during initiation of IVM on quality of porcine oocytes through monitoring maturation and subsequent embryo development. To understand the relationship between these effects and meiotic arrest activity of DON, cAMP levels, ERK phosphorylation kinetics, cortical granule rearrangement, and expression

of COCs-derived intrinsic factors (proteins and genes) regulating IVM were also investigated.

Materials and methods

Chemicals

All chemicals and reagents were purchased from Sigma-Aldrich Chemical Company, unless otherwise indicated.

Oocyte recovery and *in vitro* maturation

Porcine ovaries were collected from a local slaughterhouse and transported to the laboratory in 0.9% saline solution supplemented with 75 μ g/mL potassium penicillin G and 50 μ g/mL streptomycin sulfate, which was maintained at 25–30°C. COCs were separated from follicles (3–6 mm in diameter) by aspiration through an 18-gauge needle into a disposable 10 mL syringe. After washing COCs three times in TL-HEPES medium (1 mM/mL polyvinyl alcohol (PVA) in low-carbonate Tyrode albumin lactate pyruvate (TALP) medium), approximately 50 COCs were matured in a 500 μ L IVM medium in a four-well multi-dish (Nunc, Roskilde, Denmark) at 38.5°C and under 5% CO₂ in air. The medium used for oocyte maturation was tissue culture medium 199 (TCM-199), supplemented with 10% porcine follicular fluid, 0.57 mM cysteine, 25 μ M β -mercaptoethanol, 10 ng/mL epidermal growth factor (EGF), 10 IU/mL pregnant mare serum gonadotropin (PMSG), and 10 IU/mL human chorionic gonadotropin (hCG). During the first half period of IVM (0–22 h), 0, 1, 5, and 10 μ M DON was supplemented into the IVM medium. After 22 h of culture, COCs were washed three times and then cultured in fresh hormone- and DON-free IVM medium for an additional 20–22 h.

Assessment of nuclear maturation

After culture for 0, 6, 22, and 44 h, COCs were denuded by gently pipetting in TL-HEPES medium containing 0.1% hyaluronidase to remove cumulus cells. Then, the denuded oocytes were washed three times in phosphate-buffered saline (PBS)-polyvinyl alcohol (PVA) (0.1%) medium and fixed with 4% paraformaldehyde at 4°C overnight. The oocytes were washed three times in PBS-PVA (0.1%) and mounted on glass slides in a drop of DAPI (4'-6-diaminoin-2-fenylindol). The meiotic stage was evaluated under ultraviolet (UV) light using an epifluorescence microscope (Olympus, Tokyo, Japan) at 200 $\times$ magnification.

Measurement of intra-oocytes cAMP

After culture for 0, 6, 22, and 44 h, the COCs were denuded by gently pipetting in TL-HEPES medium containing 0.1% hyaluronidase and washed three times in PBS-PVA (0.1%) medium. Forty of denuded oocytes were transferred into microtubes and stored at –80°C until they were assayed. The cAMP levels in oocytes were measured using the cAMP assay kit (Cayman Chemical Co.), following the manufacturer's protocol. In brief, the 40 μ L of 5% trichloroacetic acid in

water was added to the sample tube, and the sample was homogenized on ice using a Polytron-type homogenizer. After centrifugation at maximum speed for 10 min, the precipitate was removed, and the supernatant was carefully transferred to a clean test tube. Trichloroacetic acid was extracted from the sample, using water-saturated ether. Five volumes of ether to one volume of supernatant was added and mixed for 10 s, and the organic and aqueous phases were separated. Then, the top ether layer was carefully discarded. The residual ether from the aqueous layer was removed by heating the sample to 70°C for 5 min. Each sample was transferred to a plate immediately, and the reagents were added, according to the manufacturer's protocol. Finally, the plate was read at a wavelength between 405 and 420 nm, and the cAMP levels were calculated, in accordance with the instructions of the manufacturer.

Western blot analysis

Twenty oocytes of each group and cumulus cells separated from 20 oocytes of each group were washed twice with PBS-PVA and lysed in 20 µL of lysis buffer (20 mM HEPES, 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 20 mM glycerol phosphate, 1% Triton X-100, and 10% glycerol) containing a protease inhibitor cocktail for 2 h, and subsequently boiled at 100°C for 10 min. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes (Millipore), and detected by immunoblotting. Membranes were blotted with primary antibodies against pERK (1:1000 dilution; #4370L; Cell Signaling Technology), ERK (1:2000 dilution; #9102S; Cell Signaling Technology), pAKT Ser473 (1:1000 dilution; #4060L; Cell Signaling Technology), AKT (1:1000 dilution; #9272S; Cell Signaling Technology), CCNB1 (1:1000 dilution; #ADI-KAM-CC195; Enzo Life Sciences, NY, USA), CDK1 (1:1000 dilution; #sc-54; Santa Cruz Biotechnology), PRKAR1A (1:1000 dilution; #ab230714; Abcam), PRKACA (1:1000 dilution; #ab26322, Abcam), and GAPDH (1:2000 dilution; LF-PA0212; ABfrontier, Seoul, South Korea) at 4°C overnight. The following day, membranes were washed three times with Tris-buffered saline with Tween-20 (TBST) buffer (10 mM Tris-HCl Tris-HCl (pH 7.5), 150 mM NaCl and 0.2% Tween-20) and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG at 4°C overnight. The membranes were visualized using enhanced chemiluminescence detection reagent (ELPIS Biotech, Daejeon, South Korea), according to manufacturer's instructions. The band intensities were quantified using TINA20 software (Raytest GmbH, Straubenhardt, Germany).

Staining of cortical granules

Cumulus cell-free oocytes were fixed with 4% (w/v) paraformaldehyde in PBS for 30 min, washed three times with PBS containing 0.3% BSA (Fraction V) and 100 mM glycine for 5 min each, and treated with 0.1% Triton X-100 in PBS for 5 min. The oocytes were washed twice in PBS for 5 min each and incubated with FITC-labeled peanut agglutinin (100 mg/mL) in PBS for 30 min under dark condition for cortical granule staining. The oocytes were then washed three times in PBS containing 0.3% BSA and 0.01% Triton X-100. Finally, oocytes were mounted on clean glass slides and observed

under a laser-scanning confocal fluorescent microscope (Zeiss LSM700).

Real-time quantitative PCR (qPCR)

Poly(A) mRNAs were extracted from 20 oocytes or embryos using the Dynabeads mRNA Direct Kit (Invitrogen), according to the manufacturer's protocol. Briefly, after thawing, samples were lysed in 300 µL of lysis/binding buffer (Invitrogen) at room temperature for 10 min, and 10 µL of Dynabeads oligo(dT)25 was added to each sample. The beads were hybridized for 5 min and then separated from the binding buffer using a Dyna magnetic bar (Invitrogen). Bound poly (A) mRNAs and beads were washed with buffers A and B (Invitrogen) and separated by addition of 10 µL of Tris buffer. The resulting poly (A) mRNAs were reverse transcribed in 20 µL reactions containing oligo (dT) 20, 5× RT buffer (containing 25 mM Mg²⁺+, 10 U of the RNase inhibitor ReverTra Ace (Toyobo), and a 10 mM mixture of dNTPs. Secondary RNA structure was denatured by incubating at 42°C for 20 min in order to facilitate cDNA production. The reaction was terminated by incubating at 99°C for 5 min. The resulting cDNA was used as a template for PCR amplification. The following PCR conditions were used: 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by extension at 72°C for 5 min. The Mx3000P QPCR system (Agilent) and Brilliant III Ultra-fast SYBR Green QPCR Master Mix (Agilent) were used for qPCR. All oligonucleotide primer sequences are presented in Table 1. GAPDH was used as an internal standard for each group. Porcine primers were designed using Primer3 (<http://bioinfo.ebc.ee/mprimer3>).

Parthenogenetic activation

Cumulus cell-free MII oocyte were placed on a 1-mm gab wire chamber (CUY5000P1, NEPAGENE) overlaid with 10 µL of 280 mM mannitol solution containing 0.1 mM MgSO₄·7H₂O, 0.1 mM CaCl₂·2H₂O, 0.5 mM HEPES and 0.01% PVA, as described previously (Beebe et al. 2009). Oocytes were immediately activated with 1.1 kV/cm direct current (DC) for 50 µs using an electro cell fusion generator (LF101, NEPAGENE Co., China, Japan). The activated oocytes were placed into 40 µL IVC medium supplemented with 5 µg/mL cytochalasin B (CB) and 2 mM 6-dimethylaminopurine (6-DMAP) for 4 h at 38.5°C. After 4 h, oocytes were washed in IVC medium and transferred into 40 µL IVC medium at 38.5°C in an atmosphere of 5% CO₂ in air. Cleavage and blastocyst formation were evaluated on day 2 and day 6, respectively.

In vitro fertilization

After IVM, oocytes were subjected to IVF as described previously (Kim et al. 2010). IVF was performed in modified Tris-buffered medium (mTBM), consisting of 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl₂·2H₂O, 20 mM Tris (crystallized free base; Fisher Scientific, Fair Lawn, NJ, USA), 11 mM glucose, 5 mM sodium pyruvate and no antibiotics. Fresh ejaculated semen was washed three times by centrifugation (100 g for 3 min at room temperature) in Dulbecco's PBS (DPBS; Gibco-BRL) supplemented with 1 mg/mL BSA, 100 µg/mL

Table 1 PCR sequences used for real-time PCR.

Genes	Primer sequences		Accession number	Product size (bp)
	Forward	Reverse		
<i>CCNB1</i>	AAGATGGAGGGGATCCAAAC	TGGCTCTCATGTTTCCAGTG	NM_001170768.1	135
<i>CDK1</i>	CGCGGATAATAAGCTGGGA	CATGGCTACCACTTGACCTGT	NM_001159304.2	139
<i>HAS1</i>	CTCTGGACTCTTGGGTGAGC	CAGACCTAGGGGACCACTGA	NM_001136212.1	119
<i>HAS2</i>	TTAGTGGACCTCTGGGGATG	AGACTCAGCACTCGGTTCGT	NM_214053.1	133
<i>PRKARIA</i>	CGTTGGAACCACTCCAGTTT	ATCAGAAGGCCCAATCTTC	NM_214026.1	158
<i>PRKACA</i>	CCCTGGGGGTTCTCATCTAT	ATGGCAATCCAGTCAGTCGT	XM_003354103.2	247

penicillin G and 75 µg/mL streptomycin sulfate. After washing, the sperm pellet was resuspended in mTBM. The oocytes were washed three times in mTBM with 2.5 mM caffeine sodium benzoate and 4 mg/mL BSA (fatty acid free) and placed into 48 µL mTBM under mineral oil. Diluted spermatozoa (2 µL) were added to 48 µL mTBM containing 15–25 oocytes, giving a final concentration of 1.5×10^5 spermatozoa/mL. The oocytes were co-incubated with the spermatozoa for 6 h at 38.5°C in an atmosphere of 5% CO₂ in air. After 6 h, the oocytes were denuded by gently pipetting to remove spermatozoa, and transferred to IVC medium (PZM-3 containing 0.4% BSA). To count pronuclei, fertilized embryos at 10 h after insemination were washed three times with PBS-PVA (0.1%) and fixed with 4% paraformaldehyde overnight at 4°C. Then, they were washed three times in PVA-PBS (0.1%) and mounted on glass slides in a drop of DAPI. Pronuclei were observed under UV light using an epifluorescence microscope at 200× magnification. The other embryos were cultured in 40 µL drops of IVC medium at 38.5°C in an atmosphere of 5% CO₂ in air. The number of embryos cleaved and developed to blastocysts stage was scored at 2 and 6 days of IVC, respectively.

Somatic cell nuclear transfer

After IVM, matured oocytes with a visible first polar body were selected for SCNT. Cumulus cell-free oocytes in PB1 (DPBS supplemented with 4 mg/mL BSA, 75 µg/mL penicillin G, 50 µg/mL streptomycin sulfate) containing 7.5 µg/mL CB were enucleated by sharp pipette under an automated inverted microscope (DMI 4000B, LEICA) equipped with a micromanipulator (NT-88-V3, NIKON NARISHIGE, Japan). Porcine kidney cells were selected as donor cell to construct SCNT embryos, as they exhibited a higher proliferation rate and blastocyst formation rate after SCNT compared to porcine fetal or ear fibroblasts (Richter *et al.* 2012). Porcine kidney cells (at passages 4–6) that were round, had a 15–20 µm diameter, and good refractivity were selected and placed into the perivitelline space through the near slit in the zona pellucida that had been made for enucleation. For electrical fusion, a single cell-oocyte couplet was sandwiched between two parallel electrodes (CUY 5100-100, NEPA GENE) approximately 100 µm apart attached to micromanipulator. The contact surface between the cytoplasm and the donor cell was parallel to the electrodes, and fused by one direct current pulse of 0.23 kV/cm for 50-µs using an Electro Cell Fusion generator in fusion medium consisting of 280 mM mannitol containing 0.1 mM MgSO₄·7H₂O, 0.01% PVA (Beebe *et al.* 2009), and incubated at 38.5°C in 5% CO₂ in air. After 2 h, oocyte-cell couplets that were found to be completely fused

under an automated inverted microscope were selected, then transferred to a 1 mm gap wire chamber overlaid with 10 µL 280 mM mannitol solution containing 0.1 mM MgSO₄·7H₂O, 0.1 mM CaCl₂·2H₂O, 0.5 mM HEPES, and 0.01% PVA and activated with 110V DC for 50 µsec using an electro cell fusion generator. The electro-activated oocytes were transferred into the chemically assisted activation medium supplemented with 50 nM trichostatin A (TSA) for 4 h at 38.5°C in 5% CO₂ in air. After 4 h, activated embryos were transferred in IVC medium supplemented with 50 nM TSA and cultured until 20 h. After 20 h, oocytes were washed in IVC medium without TSA and transferred to IVC medium without TSA at 38.5°C in 5% CO₂ in air. Cleavage and blastocyst formation were evaluated on day 2 and day 6, respectively.

Differential staining

After 6 days in culture, blastocyst formation was observed and the number of inner cell mass (ICM) and trophectoderm (TE) was determined by differential staining, as described previously (Kim *et al.* 2010). The zona pellucida of the blastocysts was removed by 1 min incubation in 0.5% pronase solution. After washed in PBS-PVA (0.1%), the zona pellucida-free blastocysts were exposed to a 1:5 dilution of rabbit anti-pig whole serum for 1 h. Then, they were washed three times for 5 min each time in TL-HEPES and placed in a 1:10 dilution of guinea-pig complement containing 10 µg/mL propidium iodide (PI) and 10 µg/mL Hoechst 33342 (bisbenzimidazole) for 1 h. After a brief wash, the zona pellucida-free blastocysts were examined under UV light using an epifluorescence microscope (Olympus).

TUNEL assay

To apoptotic cells in the blastocysts, the terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end-labeling (TUNEL) assay was performed using an *In Situ* Cell Death Detection Kit (Roche, Basel, Swiss). Blastocysts were harvested at day 6, washed three times in DPBS containing 1 mg/mL PVA, and fixed in 4% paraformaldehyde overnight at 4°C. Fixed blastocysts were permeabilized in DPBS containing 0.5% (v/v) Triton X-100 at room temperature for 30 min. Nonspecific binding sites were blocked by incubation with PBS containing 10 mg/mL BSA for 1 h. Subsequently, blastocysts were washed three times in PBS-PVA and stained with fluorescein conjugated dUTP and terminal deoxynucleotidyl transferase for 1 h at 38.5°C. As a positive control for the TUNEL reaction, a group of blastocysts was incubated in 1000 IU/mL deoxyribonuclease at 38.5°C

for 10 min, followed by TUNEL staining. Another group of blastocysts was incubated in fluoresceind UTP in the absence of terminal deoxynucleotidyl transferase as a negative control. Subsequently, the blastocysts were washed three times in PBS-PVA and mounted on slides with mounting solution containing 1.5 µg/mL DAPI. DAPI-labeled or TUNEL positive nuclei were observed with a fluorescence microscope.

Confocal microscopy of hyaluronan content

After culture for 0, 6, 22, and 44 h, the COCs were fixed in 4% paraformaldehyde. The fixed COCs were washed three times in PBS-PVA and then incubated in blocking solution (10% normal goat serum in PBS-PVA) for 1 h at room temperature. The COCs were stained with biotinylated hyaluronan binding protein (1:100; #AMS.HKD-BC41, Amsbio, Abingdon, UK) in blocking solution 4°C for 4 h. Fluorophore conjugated Streptavidin (2 ng/ml; #016-540-084, Jackson immuno research laboratories, PA, USA) was used as a secondary conjugate in blocking solution at room temperature for 2 h. After the final three times wash with PBS-PVA, the COCs were mounted with Vectashield containing DAPI (Vector Laboratories, CA, USA) on clean glass slides and observed under a laser-scanning confocal fluorescent microscope (Zeiss LSM700, Germany).

Measurement of cumulus expansion

After 0, 6, 22, and 44 h of IVM, morphology of COCs was observed using inverted microscope (LEICA, Germany). Cumulus area from COCs was measured using Image J software (National Institutes of Health, Bethesda, MD, USA) and normalized to control group.

Confocal microscopy of active mitochondria

To assess the distribution of active mitochondria, oocytes were stained after 44 h of IVM. A stock solution of the dye at a concentration of 1 mM was prepared in dimethyl sulfoxide and stored at -20°C. Oocytes were stained with 0.5 µM MitoTracker Deep Red FM (M22426, Invitrogen) in IVM medium for 30 min at 39°C in 5% CO₂ in air. Labeled oocytes were then washed in PBS-PVA, three times for 15 min each. After washing, samples were fixed in 4% paraformaldehyde for 2 h at room temperature. After fixed samples were washed three times for 15 min each in PBS-PVA, oocytes were mounted on slide glasses in Vectashield containing DAPI (Vector Laboratories) and observed under a laser-scanning confocal fluorescent microscope (Zeiss LSM700, Germany).

Confocal microscopy of actin filaments

To assess the distribution of the membrane actin, oocytes were stained after 44 h of IVM. After three washes with PBS-PVA, oocytes were fixed in 4% paraformaldehyde overnight at 4°C. The fixed oocytes were washed three times in PBS-PVA and then incubated in blocking solution (PBS-PVA supplemented with 2% BSA) for 1 h at room temperature. Then, oocytes were stained with 10 µg/ml phalloidin-tetramethylrhodamine B isothiocyanate (Phalloidin-TRITC, P1951) for 2 h at room

temperature. After the final three times wash with PBS-PVA, oocytes were mounted with Vectashield containing DAPI (Vector Laboratories) on clean glass slides and observed under a laser-scanning confocal fluorescent microscope (Zeiss LSM700).

Statistical analysis

All experiments were repeated at least three times. Data are presented as the means ± SEM. The results were compared by one-way ANOVA, followed by Tukey's multiple range test or Student's *t*-test using SigmaStat software (SPSS, Inc.). *P* values less than 0.05 were considered to be statistically significant.

Results

Effect of various concentrations of DON supplementation during first half period of IVM on nuclear maturation

To examine the effect of transient meiotic arrest activity of DON on the nuclear maturation of porcine oocytes during first half period of IVM, COCs were cultured in 0, 1, 5 and 10 µM DON for 22 h and subjected to nucleus staining analysis using DAPI for determination of meiotic developmental stages. Compared to control, administration of DON led to a significant increase of proportion of GV stage oocytes in a dose-dependent manner (10.1 ± 1.5 vs 24.2 ± 1.5, 30.2 ± 3.3 and 46.4 ± 4.5%, respectively, *P* < 0.05) and highest (*P* < 0.05) proportion was found in 10 µM DON treatment group. In terms of germinal vesicle breakdown (GVBD) stage, 1 and 5 µM DON showed significant higher GVBD proportion compared to the control (57.6 ± 1.2 and 58.8 ± 3.4 vs 45.9 ± 1.8%, *P* < 0.05). In addition, the proportion of metaphases I (MI) stage was significantly decreased in DON treatment groups compared to control (18.2 ± 1.2, 10.9 ± 1.0, 3.3 ± 1.3 vs 44.0 ± 2.4%, *P* < 0.05; Fig. 1B). At the other half period of IVM culture without DON supplementation, metaphase II (MII) stage was significantly increased in only 1 µM group compared to control (83.7 ± 0.8 vs 75.5 ± 1.1%, *P* < 0.05; Fig. 1C). Based on nuclear maturation rate at the end of IVM, 1 µM DON was suggested to be optimal for porcine IVM. Then, to clarify meiotic arrest activity of DON, meiosis progression was assessed after 3, 6, and 9 h of IVM. No differences in GV stage arrest were found after 3 h and 9 h of IVM (82.5 ± 2.5 vs 80.0 ± 2.3% and 54.4 ± 2.2 vs 50.0 ± 2.3%, respectively; Fig. 1D). However, at 6 h of IVM, oocytes matured with 1 µM DON showed a significant increase in proportion of GV stage compared to the control (75.6 ± 3.2 vs 63.8 ± 2.3%, *P* < 0.05; Fig. 1D). To investigate how DON exerted meiotic arrest activity, the concentration of cAMP was assessed after 6, 22, and 44 h of IVM. At 6 h of IVM, the concentration of cAMP in 1 µM DON group was significantly higher than control group (1.27 ± 0.05 pmol/oocyte vs 0.53 ± 0.05 pmol/oocyte, *P* < 0.05; Fig. 1E). Additionally, the effects

Figure 1 Effect of DON supplementation during the first half period of IVM on the meiotic progression of porcine oocytes. (A) Representative images for determination of oocyte nucleus pattern after DAPI staining (dotted circle). GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, Metaphase II. Bar = 50 μm. (B) Effect of various concentrations of DON supplementation during the first half period of IVM on oocyte nucleus pattern after 22 h of IVM. In total, 402 oocytes were used. (C) Effect of various concentrations of DON supplementation during the first half period of IVM on oocyte nucleus pattern after 44 h of IVM. In total, 433 oocytes were used. (D) Effect of 1 μM DON supplementation during first half period of IVM on oocyte nucleus pattern after 3, 6 and 9 h of IVM. In total, 960 oocytes were used. (E) Effect of 1 μM DON supplementation during first half period of IVM on intra-oocyte cAMP concentration after 6, 22 and 44 h of IVM. In total, 1200 oocytes were used. The data are from at least three independent experiments and the values represent the means $\pm$ S.E.M. (* $P < 0.05$).

of DON supplementation during first half period of IVM on hyaluronan contents and area of cumulus expansion were investigated. Hyaluronan contents were decreased in 1 μM DON treatment group compared to control at 6, 22 and 44 h of IVM (Supplementary Fig. 1A, see section on [supplementary materials](#) given at the end of this article). In terms of area of cumulus expansion (Supplementary Fig. 1B and C), there was no significant difference in area of cumulus expansion between control and DON treatment groups at 0 and 6 h of IVM. However, at 22 and 44 h of IVM, COCs of DON treatment group showed significantly ($P < 0.05$) decreased area of cumulus expansion (Supplementary Fig. 1C).

Effect of DON supplementation during first half period of IVM on cytoplasmic maturation

The effect of DON supplementation during first half period of IVM on pAKT and pERK1/2 expression level was investigated. The pAKT protein level was significantly ($P < 0.05$) increased in 1 μM DON treatment group compared to control at 6 h of IVM (Fig. 2A and B). However, there was no significant difference at 22 h and 44 h of IVM. In addition, protein expression level of pERK1/2 was significantly ($P < 0.05$) increased by 1 μM DON supplementation at 44 h of IVM. However, expression level of pERK1 at 6 and 22 h of IVM and

pERK2 at 22 h of IVM was significantly ($P < 0.05$) decreased (Fig. 2A and B). To investigate rearrangement of cortical granule, MII oocytes matured with or without 1 μM DON were labeled with FITC-labelled peanut agglutinin. MII oocytes treated with 1 μM DON showed a more sharp and tight form of cortical granule staining and significant decreased left and right lectin thicknesses (5.5 ± 0.3 vs 11.4 ± 1.1 and 5.1 ± 0.3 vs 11.1 ± 1.6 μm, respectively, $P < 0.05$; Fig. 2C and D). Furthermore, distributions of mitochondria and actin filaments in MII oocytes matured with or without 1 μM DON were investigated. DON treatment group showed significantly ($P < 0.05$) higher diffuse mitochondrial distribution and lower semi-peripheral mitochondrial distribution compared to the control group (Supplementary Fig. 2A and B). However, in actin status, there was no significant difference in normal and abnormal distribution between control and DON treatment groups (Supplementary Fig. 2C and D). Then, expression levels of transcripts related to maturation promoting factor (MPF) and protein kinase A (PKA) in oocytes and cumulus expansion in cumulus cells were investigated at 6 h of IVM. MPF-related genes (*CCNB1* and *CDK1*) in oocytes and cumulus expansion-related genes (*HAS1* and *HAS2*) were significantly ($P < 0.05$) decreased in DON-treated oocytes and cumulus cells at 6 h of IVM, respectively (Fig. 2E). In terms of PKA transcripts expression level,

Figure 2 Effect of DON supplementation during the first half period of IVM on cytoplasmic maturation of porcine oocytes. (A) Western blot analysis of pAKT, AKT, pERK1/2 and ERK1/2 in oocytes matured in the presence or absence of 1 μ M DON at 0, 6, 22 and 44 h of IVM. (B) The band intensities of pAKT against AKT and pERK1/2 against ERK were densitometrically analyzed. In total, 840 oocytes were used. (C) Representative images of staining of cortical granules in MII oocytes matured in the presence or absence of 1 μ M DON at 44 h of IVM. Bar = 50 μ m. (D) Measurement of a thickness (D, left) and intensity (D, right) of peanut agglutinin signals in MII oocytes matured in the presence or absence of 1 μ M DON at 44 h of IVM. In total, 44 oocytes were used. (E) The qPCR analysis for measurement of maturation promoting factor (MPF)-, protein kinase A (PKA)- and cumulus expansion-associated transcripts levels in oocytes or cumulus cells matured in the presence or absence of 1 μ M DON at 6 h of IVM. In total, 120 oocytes were used. The data are from at least three independent experiments and the values represent the means $\pm$ S.E.M. (* P < 0.05).

1 μ M DON supplementation significantly (P < 0.05) increased *PRKAR1A* and *PRKACA* expression levels in oocytes (Fig. 2E). Additionally, the effect of DON supplementation during first half period of IVM on the protein expression levels of *CCNB1*, *CDK1*, *PRKAR1A* and *PRKACA* in oocytes and pERK1/2 in cumulus cells at 6, 22 and 44 h of IVM were investigated (Supplementary Fig. 3). As shown in Supplementary Fig. 3A and B, DON supplementation during first half period of IVM significantly (P < 0.05) increased the protein expression of *CCNB1*, *CDK1*, *PRKAR1A* and *PRKACA* in oocytes at 6, 22 and 44 h of IVM compared to the control. There was no significant difference in pERK1/2 level between control and DON treatment groups in cumulus cells at 6, 22 and 44 h of IVM (Supplementary Fig. 3C and D).

Effect of DON-assisted IVM on subsequent embryo development after PA and IVF

The 1 μ M DON-treated group was selected as optimal condition for IVM, because MII oocyte rate was significantly (P < 0.05) increased at the end of IVM. We investigated subsequent embryo development after PA and IVF as an indirect cytoplasmic maturation parameter, including blastocyst formation rate, number of ICM/TE cells and monospermy. After PA, 1 μ M DON significantly (P < 0.05) increased the rate of blastocyst formation (51.3 ± 3.2 vs $37.5 \pm 3.6\%$, Fig. 3A and B) and total cell number (32.7 ± 0.1 vs 28.1 ± 0.4) including ICM/TE cell number (10.0 ± 0.2 vs 8.3 ± 0.1 and 22.7 ± 0.3 vs 19.7 ± 0.5 , P < 0.05; Fig. 3C) and decreased the number and percentage of apoptotic cells in blastocysts compared to the control (2.2 ± 0.1 vs

3.3 ± 0.1 and 6.2 ± 0.4 vs $11.3 \pm 0.5\%$, P < 0.05; Fig. 3D). After IVF, DON at 1 μ M significantly (P < 0.05) increased the rate of monospermy (51.8 ± 0.6 vs $34.1 \pm 2.7\%$, Fig. 4B) and decreased the rate of polyspermy post IVF 10 h (27.8 ± 3.0 vs $56.5 \pm 1.9\%$, Fig. 4B). Furthermore, the rate of blastocyst formation (39.3 ± 0.9 vs $29.3 \pm 1.5\%$, Fig. 4D) and total cell number (39.0 ± 0.6 vs 31.6 ± 1.2) including ICM/TE cell number (9.3 ± 0.3 vs 7.9 ± 0.3 and 29.3 ± 0.8 vs 23.8 ± 0.8 , Fig. 4E) were significantly (P < 0.05) increased and the percentage of apoptotic cell in blastocysts was significantly (P < 0.05) decreased in 1 μ M DON treatment group (8.3 ± 0.1 vs $5.7 \pm 0.1\%$, Fig. 4F).

Effect of DON-assisted IVM on subsequent embryo development after SCNT

Finally, we examined whether DON-assisted IVM could improve preimplantation embryo development of SCNT embryos. After SCNT, the rate of cell-oocyte fusion was significantly increased in 1 μ M DON treatment group compared to control (79.7 ± 0.9 vs $72.5 \pm 1.0\%$, P < 0.05; Fig. 5B). Moreover, the blastocysts formation rate was significantly increased in 1 μ M DON-treated group compared to control (41.8 ± 2.1 vs $30.1 \pm 1.1\%$, P < 0.05; Fig. 5A and B). The quality of blastocysts was assessed by counting the total cell number including ICM/TE cell number and apoptotic cell. Supplementation of 1 μ M DON significantly increased the cell number of blastocysts (40.7 ± 0.7 vs $28.3 \pm 1.1\%$, P < 0.05; Fig. 5C) with a significantly increased number of ICM cell (8.0 ± 0.4 vs $6.4 \pm 0.3\%$, P < 0.05; Fig. 5C) and TE cell of blastocyst (32.7 ± 1.0 vs $21.9 \pm 0.9\%$, P < 0.05; Fig. 5C).

Figure 3 Effect of DON-assisted IVM on the subsequent development of parthenogenetic embryos. (A, top) Representative images of blastocysts developed from oocytes matured in the presence or absence of 1 μ M DON. Bar = 100 μ m. (A, middle) Differential staining of ICM and TE cells in blastocysts in the indicated groups. The nuclei of ICM and TE cells were stained with Hoechst (blue) and PI (red) dye, respectively. Bar = 50 μ m. (A, bottom) Apoptosis detection analysis using blastocysts of the indicated groups. Merged images between TUNEL (green, white arrow) and DAPI (blue) signals are shown. Bar = 50 μ m. (B) The rate of cleavage and blastocyst formation. In total, 210 embryos were used. (C) Quantification of total cell numbers in the blastocysts including ICM and TE cells of the indicated groups. In total, 63 blastocysts were used. (D) Quantification of the numbers and proportions of apoptotic cells in the indicated groups. In total, 63 blastocysts were used. The data are from at least three independent experiments and the values represent the means $\pm$ S.E.M. (* P < 0.05).

Figure 4 Effect of DON-assisted IVM on the subsequent development of *in vitro*-fertilized embryos. (A) Representative images of various pronuclear formation (white arrow). Bar = 50 μ m. (B) The rate of monospermy, polyspermy and fertilization after IVF 10 h. In total, 191 embryos were used. (C, top) Representative images of blastocysts developed from oocytes matured in the presence or absence of 1 μ M DON. Bar = 100 μ m. (C, middle) Differential staining of ICM and TE cells in blastocysts in the indicated groups. The nuclei of ICM and TE cells were stained with Hoechst (blue) and PI (red) dye, respectively. Bar = 50 μ m. (C, bottom) Apoptosis detection analysis using blastocysts of the indicated groups. Merged images between TUNEL (green, white arrow) and DAPI (blue) signals are shown. Bar = 50 μ m. (D) The rate of cleavage and blastocyst formation. In total, 191 embryos were used. (E) Quantification of total cell numbers in the blastocysts including ICM and TE cells of the indicated groups. In total, 50 blastocysts were used. (F) Quantification of the numbers and proportions of apoptotic cells in the indicated groups. In total, 109 blastocysts were used. The data are from at least three independent experiments and the values represent the means $\pm$ S.E.M. (* P < 0.05).

Figure 5 Effect of DON-assisted IVM on the subsequent development of somatic cell nuclear transferred embryos. (A, top) Representative images of blastocysts developed from oocytes matured in the presence or absence of 1 μ M DON. Bar = 100 μ m. (A, middle) Differential staining of ICM and TE cells in blastocysts in the indicated groups. The nuclei of ICM and TE cells were stained with Hoechst (blue) and PI (red) dye, respectively. Bar = 50 μ m. (A, bottom) Apoptosis detection analysis using blastocysts of the indicated groups. Merged images between TUNEL (green, white arrow) and DAPI (blue) signals are shown. Bar = 50 μ m. (B) The rate of fusion, cleavage and blastocyst formation. In total, 664 embryos were used. (C) Quantification of total cell numbers in the blastocysts including ICM and TE cells of the indicated groups. In total, 64 blastocysts were used. (D) Quantification of the numbers and proportions of apoptotic cells in the indicated groups. In total, 64 blastocysts were used. The data are from at least three independent experiments and the values represent the means $\pm$ S.E.M. (* P < 0.05).

In addition, the number and percentage of apoptotic cell in blastocysts was significantly decreased in 1 μ M DON-treated group (2.1 ± 0.1 vs 3.2 ± 0.3 and 4.3 ± 0.5 vs $8.7 \pm 0.5\%$, P < 0.05; Fig. 5D).

Discussion

In the present study, for the first time, we demonstrated that DON supplementation during the first half period of IVM produced large numbers of developmentally competent MII oocytes by transiently arresting meiosis of immature oocytes. DON (at 1 μ M) effectively synchronized nuclear and cytoplasmic maturation of oocytes by maintaining higher intra-oocyte cAMP levels and supported ERK phosphorylation and clear rearrangement of cortical granules in membrane of MII oocytes, showing balanced meiosis progression between nuclear and cytoplasmic maturation. Subsequently, DON improved the developmental competence of embryos after PA, IVF and SCNT. Therefore, 1 μ M DON can be used to increase the yield of competent MII oocytes by synchronizing nuclear/cytoplasmic maturation.

Growth of oocytes and the regulation of oocyte meiotic maturation are affected by follicular somatic cell compartments such as the granulosa and cumulus cells surrounding the oocyte (Gilchrist *et al.* 2004). Specifically, cumulus cells and oocyte are connected and communicate with each other by cell-cell communication via gap junctions (Dekel 1988). Small ions, peptides, and other factors could be migrated between oocyte and cumulus cells through gap junction (Funahashi & Day 1997). Generally, it was known that EGF-like peptides (Downs & Chen 2008), amino acids (Colonna & Mangia

1983), glucose metabolites, purines/pyrimidines and cAMP (Sutton *et al.* 2003) are transferred from cumulus cells to oocytes via gap-junctional communication. Therefore, cumulus cell-oocyte gap junction coupling is important for oocyte maturation and a key component of oocyte developmental competence (Gilchrist *et al.* 2004). Among the many factors, cAMP has the important role in the regulation of meiosis of oocytes. Maintaining the relatively greater concentrations of cAMP in the oocyte is required for sustaining oocytes in the meiotic-arrested state for proper maturation of oocytes (Albuz *et al.* 2010), as precocious meiotic resumption could be begun by a decrease in cAMP concentration (Ramos Leal *et al.* 2018). Naturally, pre-ovulatory LH surge induces a decrease in intra-oocyte cAMP by the breakdown of gap junctions between the oocyte and granulosa cells, and it allows for the resumption of meiosis (Eppig 1991). Therefore, many researchers have tried to improve the quality of MII oocytes by adding the cAMP regulators, such as PDE inhibitors or dbcAMP, into collection or IVM medium to prevent spontaneous meiosis activation (Gilchrist *et al.* 2016). Various types of PDE inhibitors have been used to improve porcine IVM, including nonspecific (IBMX) (Kawashima *et al.* 2008), PDE3-specific (cilostamide) (Dieci *et al.* 2013) or PDE4-specific inhibitors (Rolipram) (Laforest *et al.* 2005). In addition, supplementation of IBMX into porcine COC collection medium has been tried (Appeltant *et al.* 2015) to prevent spontaneous meiotic resumption by physical stimulation (Funahashi & Day 1997).

Another way to sustain higher concentrations of intra-oocyte cAMP might be the use of an inhibitor of hyaluronan synthesis, DON. Yokoo and Sato (2004)

suggested that the haluronan-CD44 interaction during cumulus expansion induces disruption of the Cx43 gap junction in the COCs, which inhibits the transport of cAMP from cumulus cells into oocytes and thereby leads to precocious meiotic resumption of oocytes. Therefore, transient inhibition of cumulus expansion by DON might be helpful for keeping the gap junction open and leading to continuously introducing cumulus cell-origin cAMP into oocytes. Previously, it was reported that no evidence of porcine cumulus expansion was shown, when DON was supplemented during the culture period of IVM (Yokoo *et al.* 2010). Based on this scheme, we assumed that the developmental competence of oocyte matured *in vitro* could be enhanced by mimicking active communication between the oocyte and follicular cells, which is induced by DON supplementation during initiation of maturation. Therefore, we investigated the effects of DON supplementation during the first half period of IVM on oocyte nuclear and cytoplasmic maturation and subsequent embryo development.

In the present study, although oocytes matured with 10 μ M DON showed significant higher proportions of GV stage after 22 h of IVM, only 1 μ M DON group showed significantly increased MII rate compared to other groups at the end of IVM culture. Based on nuclear maturation rate at the end of IVM, 1 μ M DON supplementation during the first half period of IVM was suggested to be optimal for porcine IVM and used for further investigation. To clarify meiotic arrest activity of DON, meiosis progression was assessed after 3, 6, and 9 h of IVM. No differences in GV stage arrest were found after 3 h and 9 h of IVM. However, at 6 h of IVM, oocytes matured with DON showed significant increase in GV stage. This result suggested that activity of DON might be closely associated with GV to GVBD transition, particularly at 6 h of IVM. To investigate how DON exerted meiotic arrest activity, the concentration of cAMP was assessed after 6, 22, and 44 h of IVM, as maintaining higher cAMP concentration is required for meiotic arrest (Bilodeau-Goeseels 2011). At 6 h of IVM, the concentration of cAMP in DON treatment group was significantly higher than control group, supporting meiotic arrest activity of DON found at 6 h. Furthermore, these changes could be supported by increased pAKT/AKT ratio and PKA-associated transcripts expression (*PRKAR1A* and *PRKACA*) in oocytes matured with DON. In porcine oocyte, it was reported that AKT signaling is constantly detected in oocytes during IVM and involved in meiotic progression (Kalous *et al.* 2009). PKA, cAMP-dependent protein kinase, is the main effector of cAMP signaling in all tissues (Berthon *et al.* 2015) and composed of the regulatory (*PRKAR1A*) and catalytic (*PRKACA*) subunits (Tseng *et al.* 2017). AKT- and PKA-mediated phosphorylation of PDE3A is required for resumption of meiosis by hydrolyzing cAMP (Han *et al.* 2006). Therefore, the increase in pAKT/AKT ratio and PKA-associated transcripts (*PRKAR1A* and *PRKACA*)

might be due to demand for high activity of PDE3A to control intra-oocyte cAMP levels increased by DON.

In terms of cytoplasmic maturation of oocytes, several direct parameters, including ERK phosphorylation and cortical granule rearrangement, were reported. ERK phosphorylation at MII stage is characterized as a parameter of cytoplasmic maturation (Popelkova *et al.* 2006). No ERK phosphorylation was observed at the GV stage, whereas it gradually increased until MI and MII stages (Song *et al.* 2018). Treatment of ERK inhibitors, such as PD98059 and U0126, during IVM prevented meiosis progression in COCs (Fan *et al.* 2003, Meinecke & Kirschek 2003) and caused abnormal spindle formation (Lee *et al.* 2007), suggesting that ERK phosphorylation kinetics may be involved in dynamics of cytoskeleton proteins. The results in the present study revealed that ERK1/2 phosphorylation was retarded with DON treatment until 22 h of IVM, whereas phosphorylation of ERK1/2 in MII oocytes was significantly increased at 44 h of IVM, suggesting DON improved cytoplasmic maturation at the end of IVM. In addition, another criterion for cytoplasmic maturation is cortical granule rearrangement (Liu *et al.* 2005). The peanut agglutinin was reported to be a reliable molecular probe for microscopic visualization of cortical granules in pig oocytes (Yoshida *et al.* 1993), as peanut agglutinin lectin specifically binds with sugars on mammalian cortical granules (Ducibella *et al.* 1990, Long *et al.* 1994). The present study showed that both left and right thickness of lectin in 1 μ M DON-treated oocytes was significantly decreased at 44 h of IVM, indicating that rearrangement of cortical granules in MII oocytes was increased. Furthermore, distributions of mitochondria and actin filaments in MII oocytes, two key indexes of cytoplasmic maturation of oocytes (Sun *et al.* 2001, Sha *et al.* 2010), were investigated. In the present study, DON treatment significantly increased diffuse mitochondrial distribution and decreased semi-peripheral mitochondrial distribution, indicating that cytoplasmic maturation of oocytes was improved by DON supplementation during the first half period of IVM. However, there was no significant difference in distribution of actin filaments between control and DON treatment groups. It might be because control group already had high normal actin distribution (83.3%). Based on these results, it was demonstrated that DON-assisted IVM improved cytoplasmic maturation of oocytes through fine-tuning of ERK phosphorylation kinetics, cortical granule rearrangement and mitochondrial distribution. Furthermore, cytoplasmic maturation of oocytes can be assessed by indirect parameters, including monospermic fertilization, blastocyst formation rate, total cell numbers, and cellular survival in blastocysts. The present study showed that 1 μ M DON treatment during the first half of IVM period led to the improvements in these indirect parameters for cytoplasmic maturation after PA, IVF and SCNT. Therefore, supplementation of DON at optimal

concentration (1 μ M) significantly improved direct and indirect parameters of cytoplasmic maturation of oocytes.

In conclusion, DON is demonstrated to be a useful supplement during the first half period of porcine IVM by balancing between nuclear and cytoplasmic maturation. Furthermore, the present study provides the first line of evidence that DON supplementation improves the direct and indirect parameters for cytoplasmic maturation, such as ERK phosphorylation, cortical granule rearrangement, monospermic fertilization, blastocyst formation rate, total cell numbers, and cellular survival in blastocysts. These findings may contribute to our understanding of the mechanisms of oocyte maturation and be useful for improving porcine IVM by providing oocytes with more appropriate culture environments.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-19-0235>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

H-J Y, S L and B-W S conceived the study, carried out the experiments, and then performed the statistical analysis and drafted the manuscript. P-S J, S-A C, Y-H P, B-S S, S-B Y, P K, K-J J, Y-H K, J-W H, S-R L, D-B K and Y-K C assisted the project and revised the manuscript. J-S K and S-U K supervised the research and supplied the funding. All authors read, revised, and approved the final manuscript.

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