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—Original Article—

Vitrification of porcine cumulus-oocyte complexes at the germinal vesicle stage does not trigger apoptosis in oocytes and early embryos, but activates anti-apoptotic *Bcl-XL* gene expression beyond the 4-cell stage

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Abstract. The aim of the present study was to clarify whether or not our vitrification procedure at the germinal vesicle (GV)-stage triggers the apoptotic cascade in oocytes and subsequent embryos. Immature porcine cumulus-oocyte complexes were either vitrified and warmed (vitrified group) or subjected to cryoprotectant agents (CPA group) or cultured without any treatment (control). Oocytes of all treatment groups were subjected to *in vitro* maturation (IVM), fertilization, and embryo culture. Apoptosis was assayed in live oocytes at the end of IVM culture and in cleavage-stage embryos after *in vitro* fertilization (IVF). We detected similar frequencies of DNA fragmentation, levels of caspase activity, phosphatidylserine externalization, and mRNA levels for pro-apoptotic *Bax* and *CASP3* genes in oocytes at the end of IVM and in early embryos among all groups. However, in the vitrified group, the anti-apoptotic *Bcl-XL* gene was upregulated in 4–8 cell embryos, which caused an 8-fold significant increase in the *Bcl-XL*/*Bax* mRNA ratio compared with the control and CPA groups ($P < 0.05$). In conclusion, vitrification of porcine oocytes at the GV stage by our method did not trigger the apoptotic cascade in oocytes and subsequent embryos but triggered the upregulation of the anti-apoptotic *Bcl-XL* gene in embryos.

Key words: Apoptosis, Immature oocyte, Porcine, Vitrification

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In mammals, cryopreservation of oocytes is an important approach for *ex-situ* gene banking of female germplasm [1]. Additionally, cryopreservation enables flexible use of oocytes in time and space for assisted reproductive techniques such as *in vitro* embryo production (IVEP) or cloning. Although porcine oocytes are extremely sensitive to low temperatures and cryopreservation procedures [2], they can be preserved by vitrification; however, the production of offspring by this approach was reported only recently [3, 4]. Despite relatively high survival rates, the competence of porcine oocytes to develop to blastocyst stage embryos is greatly compromised by the vitrification process applied either at the mature metaphase-II (MII) stage [5] or at the immature germinal vesicle (GV) stage [3].

In pigs, perhaps uniquely in farm animals, vitrification at the GV stage seems to be more advantageous than that at the MII stage [6]. Therefore, in a series of studies, we developed a vitrification protocol for immature porcine oocytes [3, 7–10]. Porcine oocytes survive this procedure at high rates without major reduction in their ability to resume and complete the meiotic process during *in vitro* maturation (IVM) [11]. However, although live offspring could be obtained by *in vitro* fertilization (IVF) of such oocytes, embryonic developmental competence of vitrified oocytes remained lower than that of non-vitrified ones [3, 10]. The most notorious manifestation of detrimental effects of oocyte vitrification/warming at the GV stage were reduced cleavage rates and compromised ability of cleaved embryos to reach the blastocyst stage [3, 10]. The exact reason for this phenomenon has not been clarified thus far.

In previous studies, vitrification at the MII stage reportedly triggered the apoptotic cascade in porcine oocytes, which is believed to contribute to their low developmental performance [12–14]. Accordingly, application of reagents with anti-apoptotic activities such as resveratrol [15] or caspase inhibitor Z-VAD-FMK [16] during or after vitrification, reduced the incidences of apoptosis and improved developmental ability of MII stage oocytes. Additionally, when applied during the post-warming IVM, resveratrol improved embryo developmental competence of porcine oocytes vitrified at the GV stage; however, the effects of neither the vitrification process nor resveratrol on the apoptotic status of oocytes were confirmed in that report [17].

The aim of the present study was to clarify whether or not our vitrification procedure at the GV stage triggers the apoptotic cascade in oocytes and subsequent embryos. Immature porcine cumulus-oocyte complexes (COCs) were either vitrified and warmed or subjected

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to cryoprotectant agents (CPA) or cultured without any treatment (control). We assayed apoptosis in surviving oocytes at the end of IVM culture and also in cleavage-stage embryos after IVF and subsequent embryo culture by the measurement of 1) frequency of DNA fragmentation, 2) cytoplasmic caspase activity, 3) phosphatidylserine externalization and 4) real-time PCR of pro-and anti-apoptotic genes.

Materials and Methods

Oocyte collection and vitrification

Collection and vitrification of COCs were performed according to our previous report [10]. Briefly, ovaries of prepubertal cross-bred gilts (Landrace × Large White) were collected at a local abattoir and transported within 1 h at 35−37°C to the laboratory in Dulbecco's phosphate-buffered saline (PBS) (Nissui Pharmaceutical, Tokyo, Japan). COCs were collected from 3 to 6 mm follicles into a collection medium of Medium 199 (with Hanks' salts; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5% fetal bovine serum (Gibco; Invitrogen, Carlsbad, CA, USA), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Dojindo Laboratories, Kumamoto, Japan) and antibiotics [100 IU/ml penicillin G potassium (Sigma-Aldrich) and 0.1 mg/ml streptomycin sulfate (Sigma-Aldrich)].

Basic medium (BM) for vitrification and warming was modified NCSU-37 [18] without glucose, but supplemented with 20 mM HEPES, 50 μM β-mercaptoethanol, 0.17 mM sodium pyruvate, 2.73 mM sodium lactate and 4 mg/ml polyvinylpyrrolidone (PVP) (Sigma, P0930). The COCs were briefly washed in BM, pre-warmed to 38°C. Thereafter, groups of 50−60 COCs were equilibrated at once in an equilibration medium, composed of BM supplemented with 2% (v/v) ethylene glycol (EG, E-9129), and 2% (v/v) propylene glycol (PG, 29218-35, Nacalai Tesque, Kyoto, Japan). The COCs were incubated in equilibration medium for 13−15 min at room temperature (25°C). After equilibration, COCs were washed three times in 50-μl of vitrification solution, pipetted into a glass capillary tube in groups of 30, and finally, in about 2−3 μl of vitrification solution, dropped onto aluminum foil covered with and floating on the surface of liquid nitrogen (LN). The vitrification solution was composed of BM supplemented with 50 mg/ml of additional PVP (Sigma, P-0930), 0.3 M sucrose (196-00015, Wako Pure Chemical Industries, Osaka, Japan), 17.5% (v/v) EG and 17.5% (v/v) PG. COCs were washed in vitrification medium and microdrops of these were placed onto aluminum foil at room temperature (25°C) within 30 sec in total. The vitrified droplets were collected in 2-ml cryotubes (Iwaki 2732-002; AGC Techno Glass, Tokyo, Japan) partly immersed in LN, and subsequently stored in LN until use. The warming medium was pre-warmed for 3 h to 38.5°C, and, thereafter, maintained at 42°C in a dry block tube heater on a warm plate (SP-45D, Hirasawa, Tokyo, Japan) for an additional 20 min. Vitrified droplets were warmed by transfer into 2.5 ml of warming solution (0.4 M sucrose in BM) in a 35 mm plastic dish (Falcon 351008, Becton Dickinson Labware, NJ, USA) on a warm plate that was maintained at 42°C. After 2−3 min, COCs were transferred for periods of 1 min (each) to 500-μl droplets of BM supplemented with 0.2, 0.1 or 0.05 M sucrose at 38.0°C. Thereafter, they were washed in BM without sucrose at 38.0°C and subjected to IVM.

IVM and the assessment of the live/dead status of oocytes

The maturation medium was porcine oocyte medium (POM; Research Institute for the Functional Peptides, Yamagata, Japan) supplemented with 1 mM dibutyryl cAMP (dbcAMP; Sigma-Aldrich), 10 ng/ml epidermal growth factor (EGF, Sigma-Aldrich), 10 IU/ml eCG (Serotropin; ASKA Pharmaceutical, Tokyo, Japan) and 10 IU/ml hCG (500 units; Puberogen, Novartis Animal Health, Tokyo, Japan). Forty to fifty COCs were cultured in each well of 4-well dishes (Nunc MultiDishes, Thermo Fisher Scientific, Waltham, MA, USA) in 500 -μl droplets of IVM medium without oil coverage for 22 h in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5°C. Thereafter, COCs were cultured in maturation medium without dbcAMP for an additional 22−24 h under the same atmosphere. At the end of IVM, COCs were denuded by treatment with 0.1% (w/v) hyaluronidase in collection medium for 2 min, followed by gentle pipetting through a narrow-bore glass capillary tube in collection medium without hyaluronidase. Thereafter, oocytes were investigated under a stereo microscope. Those with an intact oolemma, a normal spherical shape, a smooth surface, and a dark and evenly granular cytoplasm were considered to be alive. Oocytes with membrane damage and a brownish faded cytoplasm were considered dead. Survival rates are expressed as the living oocytes on the total number of oocytes.

IVF and embryo culture

Oocytes were fertilized *in vitro* by the 2-step method of Grupen and Nottle [19] with some modifications. The medium used for IVF was a modified Pig-FM medium [20] containing 10 mM HEPES, 2 mM caffeine, and 5 mg/ml BSA. Oocytes were washed three times in IVF medium, and transferred into 90-μl IVF droplets (approximately 20 oocytes in each droplet) covered by paraffin oil (Paraffin Liquid; Nacalai Tesque). Frozen-thawed epididymal spermatozoa from a Meishan boar were preincubated at 37°C in Medium 199 (with Earle's salts, Gibco, pH adjusted to 7.8) for 15 min [21]. To obtain the final sperm concentration (1×10^5 cells/ml), 10 μl of the sperm suspension was introduced into the IVF medium containing oocytes and co-incubated for 30 min at 39°C under 5% CO_2, 5% O_2 and 90% N_2. Thereafter, oocytes with zona-bound sperm were transferred to a second droplet of the IVF medium containing no sperm and subsequently incubated for 3 h. At the end of IVF, spermatozoa were removed from the surface of zona pellucida by gentle pipetting with a fine glass pipette. Embryo culture was performed in 50 μl drops of porcine zygote medium (PZM)-5 medium (Research Institute for the Functional Peptides) in 6-well culture dishes (IFP9670, Research Institute for the Functional Peptides) covered with paraffin oil in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 39°C.

Evaluation of DNA fragmentation in oocytes and embryos

DNA fragmentation was assessed in oocytes and cleavage stage embryos by terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) assay based on the method reported by Karja *et al*. [22] with modifications. Briefly, embryos and oocytes were washed four times in PBS containing 3 mg/ml PVP (PBS-PVP), and then fixed at 4°C overnight in 3.7% (w/v) paraformaldehyde diluted in PBS. After fixation, embryos were washed three times in PBS-PVP, permeabilized in 0.1% Triton X-100 (diluted in PBS) for 60 min, and incubated at 4°C overnight in a blocking solution, which was

PBS containing 10 mg/ml BSA. Embryos were then washed four times in PBS-PVP and incubated in fluorescein-conjugated dUTP and TdT (TUNEL reagent; Roche diagnostics, Tokyo, Japan) for 1 h at 38.5°C and 5% CO_2 in air. After TUNEL staining, embryos were incubated with 50 µg/ml propidium iodide (PI) for 20 min to label total DNA. Finally, embryos were washed three times in PBS-PVP, and flat-mounted in anti-fade solution (S2828; Thermo Fisher Scientific) on glass slides with a cover slip. Labeled nuclei were examined under an epifluorescent microscope (BX-51; Olympus, Tokyo, Japan) at excitation wavelengths of 460−495 nm and 520−550 nm for detection of TUNEL reaction and PI, respectively. Cells with DNA labeled by TUNEL were considered be apoptotic. To validate the reliability of the assay, in each experimental replication, 20 cleaved embryos and 10 non-cleaved oocytes from the control group (see *Experimental design* below) were incubated in 1000 IU/ml deoxyribonuclease I (DNase) (2270A, Takara Bio, Kusatsu, Japan) for 20 min before TUNEL staining, which served as a positive control.

Measurement of caspase activity in oocytes and early embryos

The activation of caspases was detected using fluorescein isothiocyanate-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (FITC-VAD-FMK) according to Vallorani *et al*. [12] with a slight modification. VAD-FMK is a cell permeable caspase inhibitor conjugated to FITC that covalently binds activated caspases. Oocytes were washed twice with PBS-PVP and then incubated with 500 µl of PBS-PVP containing 1 µM of FITC-VAD-FMK (CaspACE™ FITC-VAD-FMK In Situ Marker, Promega, Tokyo, Japan) and 0.2 µl of 5 mg/ml Hoechst 33342 (Calbiochem, San Diego, CA) for 30 min at 39°C. During the final 5 min of incubation, 3 µl of 1 mg/ml PI solution were added to detect dead oocytes. Oocytes were then washed twice for 5 min with PBS-PVP and mounted on glass slides. The fluorescence intensity of FITC-VAD-FMK was measured under an epifluorescence microscope at an excitation wavelength of 460−495 nm and emission at 510 nm, only in live oocytes and embryos characterized by PI-negative nuclei. A digital photograph of each oocyte was taken at the equatorial plane at the same magnification, immediately after UV light exposure. Thereafter, the nuclear status and cell number of each oocyte and embryo, respectively were recorded based on Hoechst 33342 staining evaluated at an excitation wavelength of 330−385 nm and emission at 420 nm. The procedure was performed in a dark room. The mean fluorescence intensity for FITC-VAD-FMK and the surface area of each oocyte/embryo were measured from fluorescent images using the NIH ImageJ (v. 1.49) software (https://imagej.nih.gov/ij/). Fluorescent color images were converted to 8-bit grayscale. After inverting each image, fluorescent intensity of the oocyte area was expressed by gray pixel density. The total fluorescence of each oocyte/embryo was calculated as mean fluorescence ("mean gray value") multiplied with oocyte surface ("area"). Relative fluorescence was calculated from total fluorescence where the control group was 100%. For positive controls, representative oocytes and embryos from the control group (see *Experimental design*) were treated with 10 µM staurosporine (Sigma) for 30 min before the assay.

Measurement of phosphatidylserine exteriorization

Phosphatidylserine exteriorization was investigated in oocytes/embryos based on the annexin V binding according to Vallorani *et al*. [12] with a slight modification. In brief, denuded live oocytes and cleavage stage-embryos were washed three times with PBS-PVP and then transferred to 100 µl of a binding buffer (Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit, Molecular Probes, Eugene, USA) with 5 µl of Alexa Fluor Annexin V, 1 µl of PI (100 µg/ml), and 0.2 µl of 5 mg/ml Hoechst 33342 for 20 min at 38.5°C in the dark. After three consecutive washes in binding buffer, oocytes were mounted on glass slides. The fluorescence intensity of annexin V was measured under an epifluorescence microscope at an excitation wavelength of 460−495 nm and emission at 510 nm, only in live oocytes and embryos characterized by PI negative nuclei and the presence of the first polar body (1PB). A photograph of each oocyte was taken at the equatorial plane at the same magnification, immediately after exposure to UV light, using the same camera settings described above. Thereafter, the nuclear status and cell number of each oocyte and embryo, respectively, was recorded based on Hoechst 33342 staining, evaluated at an excitation wavelength of 330−385 nm and emission at 420 nm. The procedure was performed in a dark room. Relative fluorescence of annexin V was assayed from digital images as described above. For positive controls, representative oocytes and embryos from the control group (see *Experimental design*) were treated with 10 µM staurosporine (Sigma) for 30 min prior to the assay.

Quantitative real-time PCR (qRT-PCR) of apoptosis-related genes

The expression of genes related to apoptosis (Table 1) were analyzed in oocytes, cumulus cells, and embryos using Real-Time PCR. Briefly, pooled samples of 30 oocytes or embryos or the total of amount of cumulus cells derived from each treatment group was lysed in RLT buffer (RNeasy Micro Kit, QIAGEN, Hilden, Germany) and stored at −80°C until analysis. Total RNA was extracted and purified from pooled samples using the RNeasy Micro Kit (QIAGEN) according to the manufacturer's instructions. cDNA synthesis was performed using the PrimeScript™ II 1st strand cDNA Synthesis Kit (Takara), following the manufacturer's protocol. The DNA primers of genes investigated in this study are listed in Table 1. Quantitative mRNA analysis of samples was performed using Real-Time PCR at the 1/20 dilution using the LightCycler® 480 SYBR Green I Master (Roche Applied Science, Penzberg, Germany) according to standard protocols. The quantification of transcripts in samples was analyzed using the LightCycler® 480 Instrument (Roche Applied Science). In each sample, cDNA levels of each gene were calculated directly using the LightCycler® 480 software, comparing measured values to standard curves prepared by the measurement of pooled standards diluted 1 ×, 10 ×, and 100 ×. Relative quantifications of transcripts in each gene were normalized to an internal reference transcript that was *PGK1*, based on the report by Kuijk *et al*. [23].

Experimental design

Experiment 1. The effect of oocyte vitrification at the GV stage on DNA fragmentation in cleavage stage embryos and non-cleaved oocytes: Freshly collected COCs were either vitrified and warmed (vitrified group) or treated with CPA and warming solutions without vitrification (CPA group), then subjected to IVM, IVF, and IVC as described above. On Day 2 (Day 0 = IVF), the developmental status

Table 1. List of primers used in the present study

Gene	Code	Function	Primer	Access No.	Product (BP)
Phosphoglycerate kinase 1	*PGK1*	Glycolysis *	*F* (5-3): AGATAACGAACAACCAGAGG *R* (5-3): TGTCAGGCATAGGGATACC	AY677198	126
Bcl-2-associated X protein	*Bax*	Pro-apoptotic	*F* (5-3): ACACCTCATAGCCATGAAAC *R* (5-3): ATGGCTGACATCAAGATACC	NM_004324	232
Caspase 3	*CASP3*	Pro-apoptotic	*F* (5-3): GGGATTGAGACGGACAGTGG *R* (5-3): TGAACCAGGATCCGTCCTTTG	NM_214131	136
B-cell lymphoma-extra large	*Bcl-XL*	Anti-apoptotic	*F* (5-3): GTTGACTTTCTCTCCTACAAGC *R* (5-3):GGTACCTCAGTTCAAACTCATC	AF216205	277

* Reference gene. F, forward, R, reverse.

and frequency (%) of DNA fragmentation in cleavage-stage embryos and non-cleaved oocytes were compared to those generated from the oocytes without treatment (control) and to those treated with DNase I (positive control). Only live oocytes/embryos were selected for analysis. Three biological replications were performed.

Experiment 2. The effect of oocyte vitrification at the GV stage on caspase activity in oocytes and early embryos: Freshly collected COCs were either vitrified and warmed (vitrified group) or treated with CPA and warming solutions without vitrification (CPA group), then subjected to IVM, IVF and IVC as described above. Caspase activity in oocytes at 44 h IVM and a group of 10 cleavage stage embryos and five non-cleaved oocytes on Day 2 were compared to those generated from oocytes without treatment (control) and to those treated with staurosporine (positive control) as described above. Only live oocytes/embryos were selected for analysis by morphological evaluation. Three biological replications were performed.

Experiment 3. The effect of oocyte vitrification at the GV stage on phosphatidylserine exteriorization in oocytes and early embryos: Freshly collected COCs were either vitrified and warmed (vitrified group) or treated with CPA and warming solutions without vitrification (CPA group), and then subjected to IVM, IVF, and IVC as described above. Phosphatidylserine exteriorization in oocytes at 44 h IVM and a group of 10 cleavage stage embryos and five non-cleaved oocytes on Day 2 were compared to those generated from oocytes without treatment (control) and to those treated with staurosporine (positive control) by annexin V binding assay as described above. Five biological replications were performed.

Experiment 4. The effect of oocyte vitrification at the GV stage on RNA expression of pro-and anti-apoptotic genes in oocytes, cumulus cells and embryos: Freshly collected COCs were either vitrified and warmed (vitrified group) or treated with CPA and warming solutions without vitrification (CPA group), then subjected to IVM, IVF, and IVC as described above. Relative mRNA levels of pre-apoptotic *Bax* and *CASP3* and anti-apoptotic *Bcl-XL* genes in MII-stage oocytes (selected by the presence of 1PB), cumulus cells at 44 h IVM, and embryos with at least four cells on Day 3 were compared to those generated from oocytes without treatment (control) by qRT-PCR as described above. Three biological replications were performed.

Overall survival rates and early developmental progress

In each experiment, the survival rates were recorded after IVF. In each treatment group, cleavage rates, and numbers of blastomeres in cleaved embryos were recorded on Day 2 in representative groups of embryos. Overall, survival and cleavage rates were compared among treatment groups in 14 replications. Status of cleaved embryos on Day 2 was compared among treatment groups in 12 replications.

Statistical analysis

Data are expressed as the mean ± SEM. Results of experiments 1−4 were analyzed by the Kruskal-Wallis test, followed by Dunn's multiple comparisons test, using GraphPad Prism software (Version 7.02 for Windows, GraphPad Software, La Jolla, California, USA). Overall, cleavage and early development results were analyzed by one-way ANOVA followed by Dunn's multiple comparisons test. $P < 0.05$ was defined as the significance level.

Results

Experiment 1. The effect of oocyte vitrification at the GV stage on DNA fragmentation in cleavage stage embryos and non-cleaved oocytes

There was no significant difference in the frequency of Day 2 embryos and non-cleaved oocytes with DNA fragmentation among the control, CPA and vitrified groups ranging between 0−4.1% (Fig. 1). However, the percentage of cleaved embryos and non-cleaved oocytes with DNA fragmentation in the positive control (DNase) group was 100 and 96.6%, respectively, which was significantly higher ($P < 0.05$) than those in the other groups. There was no difference in the frequency of DNA fragmentation between cleaved embryos and non-cleaved oocytes in each group.

Experiment 2. The effect of oocyte vitrification at the GV stage on caspase activity in oocytes and early embryos

In both oocytes, at the end of IVM, and embryos, on Day 2, the intracellular levels of caspase were similar in the control, CPA, and vitrified groups (Fig. 2A). However, caspase activity in oocytes and embryos of the positive control (staurosporine) group were significantly higher ($P < 0.05$) than those in the other groups (Fig. 2A). There was no significant difference in caspase activity levels between cleaved embryos and non-cleaved oocytes within each group (Supplementary Fig. 1: online only).

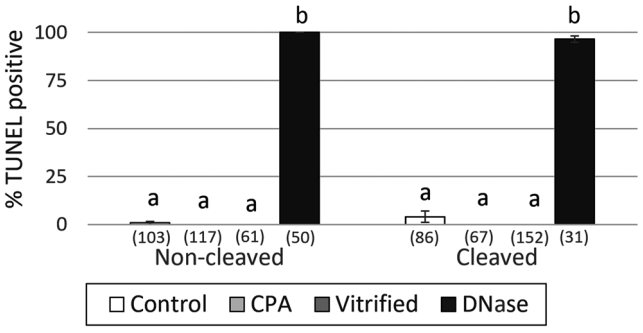

Fig. 1. The frequency (%) of cleaved embryos and non-cleaved oocytes with a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive signal on Day 2 (Day 0 = *in vitro* fertilization (IVF)) obtained from oocytes, either vitrified at the germinal vesicle (GV) stage (vitrified), treated with cryoprotectants (CPA) or without any treatment (control). Treatment of representative embryos and oocytes from the control group with DNase for 30 min before assay served as a positive control. Data are shown as mean ± SEM. Values with different superscripts (a and b) differ significantly (P < 0.05). The experiment was replicated three times. Total number of oocytes/embryos in each group is given in parentheses.

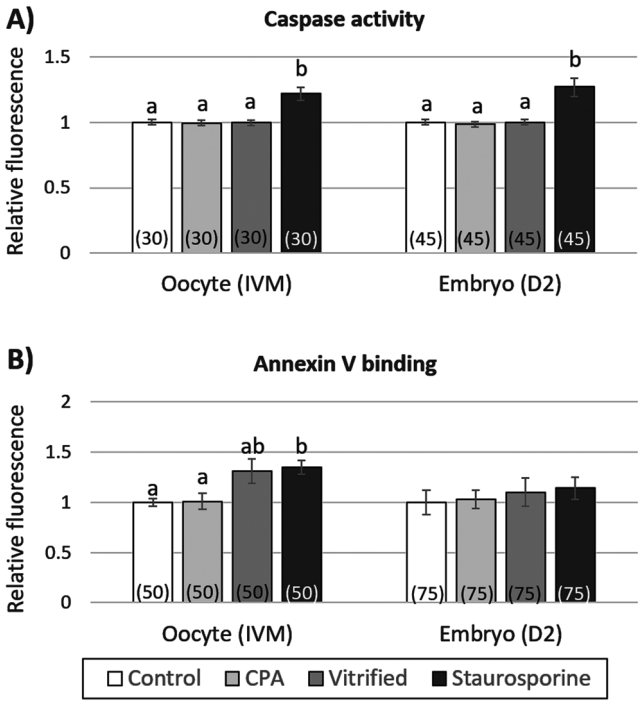

Fig. 2. Relative levels of caspase activity (A) and annexin V binding (B) in matured oocytes after *in vitro* maturation (IVM) and embryos on Day 2 (D2) obtained from oocytes, either vitrified at the germinal vesicle (GV) stage (vitrified), treated with cryoprotectants (CPA) or without any treatment (control). Treatment of representative oocytes and embryos from the control group with staurosporine for 30 min before the assay served as a positive control. Data are shown as the mean ± SEM. Values with different superscripts (a and b) differ significantly (P < 0.05). The experiment was replicated three times for caspase activity and six times for the Annexin V binding test. Total number of oocytes/embryos in each group is given in parentheses.

Experiment 3. The effect of oocyte vitrification at the GV stage on phosphatidylserine exteriorization in oocytes and early embryos

In both oocytes, at the end of IVM, and embryos, on Day 2, there was no significant difference (P > 0.05) in the level of phosphatidylserine exteriorization measured by annexin V binding among the control, CPA and vitrified groups (Fig. 2B). In oocytes, at the end of IVM, annexin V binding in the positive control was significantly higher (P < 0.05) than that of the control and CPA groups, but was not statistically different from that in the vitrified group. However, in embryos on Day 2, the annexin V binding in the positive control (staurosporine) group was not significantly higher (P > 0.05) than in other groups (Fig. 2B).

Experiment 4. The effect of oocyte vitrification at the GV stage on RNA expression of pro- and anti-apoptotic genes in oocytes, cumulus cells and embryos

No significant difference (P > 0.05) was detected in the relative expression of *Bax* and *CASP3* genes among groups in matured oocytes, cumulus cells, and 4−8 cell embryos (Fig. 3). Moreover, the mRNA level of the *Bcl-XL* gene in oocytes and cumulus cells was similar among the control, CPA, and vitrified groups (Fig. 3). However, in 4−8 cell stage embryos, expression of *Bcl-XL* in the vitrified group was significantly higher (P < 0.05) than that in the control group and tendentiously higher than that in the CPA group (P = 0.06). Additionally, in 4−8 cell stage embryos, the ratio of *Bcl-XL* and *Bax* expression in the vitrified group was significantly higher (P < 0.05) than that in the control and the CPA groups, which were in turn, similar to one another.

The effect of oocyte vitrification at the GV stage on cleavage rates and early developmental progress

Survival and cleavage rates in the control and CPA groups were similar to one another but were significantly higher (P < 0.05) than that in the vitrified group (Fig. 4A). Furthermore, on Day 2, distribution of cleaved embryos with different blastomere numbers were similar in the control and CPA groups. However, in the vitrified group, a significantly lower (P < 0.05) proportion of the cleaved embryos were beyond the 4-cell stage and a significantly higher (P < 0.05) proportion of them were at the 2-cell stage, compared with the control and CPA groups (Fig. 4B).

Discussion

Our previous studies have shown that porcine oocytes vitrified at the GV stage by our method survive the procedure at high rates and maintain their ability to reach the MII stage during subsequent IVM; however, their further developmental competence is compromised [3, 9, 10]. Similarly, in the present study, approximately 90% of immature oocytes survived vitrification at the GV stage. However, they showed reduced cleavage rates after IVF and a developmental delay of cleaved embryos, which manifested as lower cell numbers on Day 2.

The aim of the present study was to clarify whether or not vitrification of immature COCs by our method activated the apoptotic cascade in porcine oocytes and subsequent cleavage-stage embryos

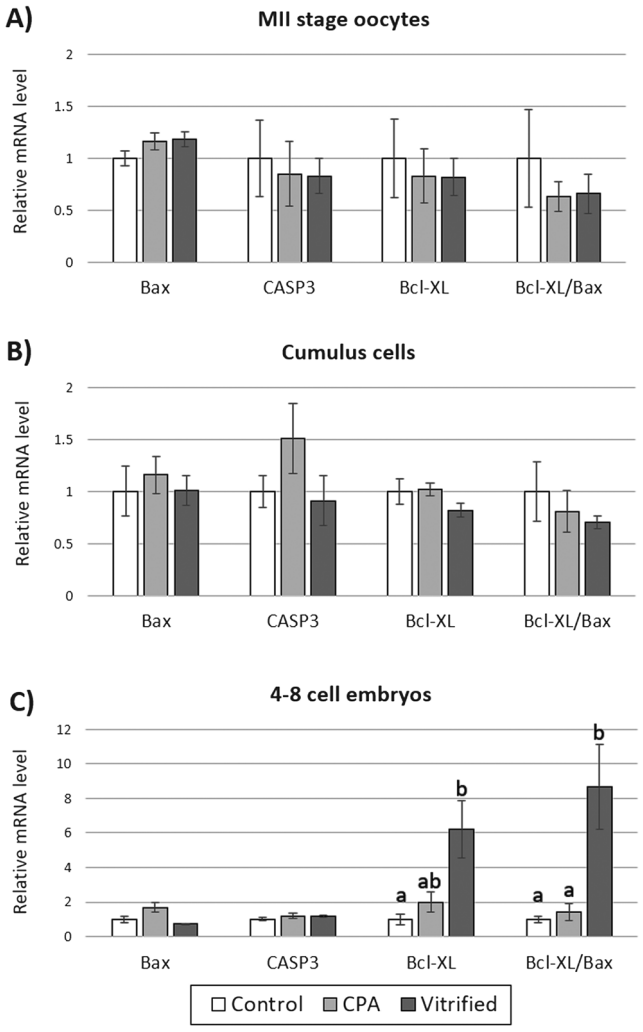

Fig. 3. Relative mRNA levels of pro-apoptotic (Bax, CASP3) and anti-apoptotic (Bcl-XL) genes in matured oocytes (A) and cumulus cells (B) after *in vitro* maturation (IVM) and in 4–8-cell embryos on Day 3 (Day 0 = *in vitro* fertilization (IVF)) obtained from cumulus-oocyte complexes (COCs), either vitrified at the germinal vesicle (GV) stage (vitrified), treated with cryoprotectants (CPA) or without any treatment (control). Data are shown as the mean ± SEM. Values with different superscripts (a and b) differ significantly ($P < 0.05$). The experiment was replicated three times.

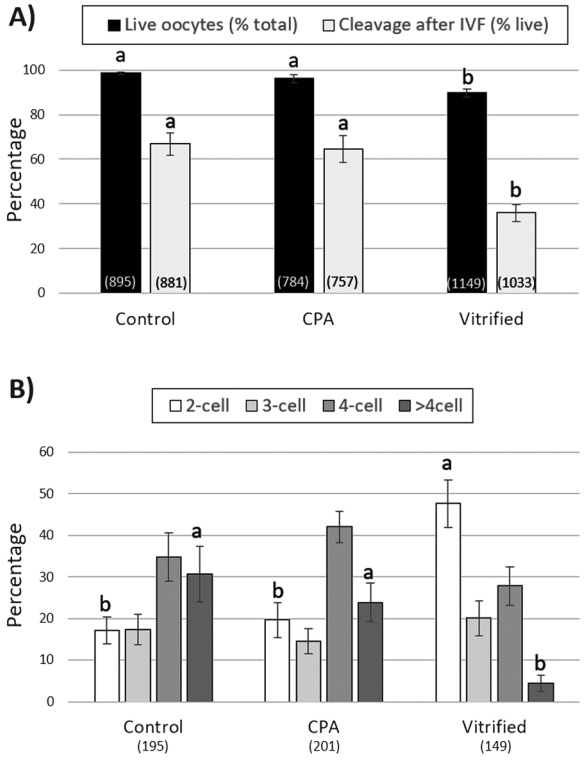

Fig. 4. Survival and cleavage rates (A) and the distribution of cleaved embryos with different cell numbers on Day 2 (B) after *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) of oocytes, either vitrified at the germinal vesicle (GV) stage (vitrified), treated with cryoprotectants (CPA) or without any treatment (control). Data are shown as the mean ± SEM. Values with different superscripts (a and b) among treatment groups differ significantly ($P < 0.05$). Total number of oocytes/embryos in each group is given in parentheses.

after IVF, which could theoretically cause their developmental arrest. In animal cells, apoptosis (programmed cell death) is featured by landmark events including DNA fragmentation, activation of pro-apoptotic genes such as *Bax* and *CASP3* leading to the production of caspases, which execute the disassembly of cellular proteins [24], and the externalization of phosphatidylserine from the inner surface of the phospholipid cell membrane, which serves as a recruitment signal for phagocytes responsible for removal of apoptotic cells from the organism [25]. Our results revealed similar frequencies of DNA fragmentation, levels of caspase activity and mRNA levels for pro-apoptotic *Bax* and *CASP3* genes in oocytes and cumulus cells at the end of IVM and in cleavage stage embryos after IVF among the control, vitrified and CPA-treated groups. Also, phosphatidylserine externalization levels in oocytes and embryos was not significantly different between vitrified and non-vitrified groups. This indicates that our method of vitrification of porcine COCs at the GV stage did not trigger the apoptotic cascade in oocytes and resultant embryos, at least up to the 8-cell stage. However, in several previous reports, the apoptotic cascade was activated by vitrification of porcine oocytes at the MII stage [12–16]. It is possible that vitrification might act differently upon immature (GV stage) and matured (MII stage) oocytes in terms of the activation of apoptotic events. Similarly, we previously demonstrated that vitrification of porcine MII-stage oocytes resulted in an increase in intracellular levels of reactive oxygen species (ROS), a common activator of apoptosis in cells [26], whereas vitrification at the GV stage did not affect ROS levels of cumulus-enclosed porcine oocytes [11, 17]. Alternatively, it is also possible, that differences in the vitrification protocol applied by different groups could cause differences in results. In many previous reports, oocytes were equilibrated in 7.5% EG + 7.5% dimethylsulfoxide (DMSO) for 5−15 min [12–16], in some cases at 39°C [12,

15]. DMSO is known to have the potential to trigger apoptosis in animal cells in a concentration-dependent manner, sometimes even at relatively low concentrations [27]. Furthermore, the toxic effects of permeating CPA on porcine oocytes seem more pronounced at temperatures higher than 25°C [10]. In the report by Vallorani *et al*. [12], treatment of oocyte with CPA without cooling, did in fact cause a slight but significant increase in the frequency of apoptotic oocytes. Therefore, it is plausible that in previous reports, application of permeating CPA during equilibration at higher concentrations and temperatures could contribute to high rates of apoptosis in oocytes after vitrification. In our previous studies, DMSO was omitted and equilibration with CPA before vitrification was performed at much lower percentages of permeating cryoprotectants (i.e. 2% EG + 2% PG for 13–15 min), which itself does not seem to affect the developmental competence of oocytes to the blastocyst stage [8, 10]. Additionally, we applied CPAs at room temperature, which was beneficial for the preservation of oocyte developmental competence, than that at higher temperatures [10].

In the present study, results of TUNEL staining and the caspase activity assay on both oocytes and cleavage-stage embryos were clear and validated by positive control groups. To assay phosphatidylserine exteriorization, similar to the caspase activity assay, we followed the report by Vallorani *et al*. [12] and used the fluorescent Alexa 488-conjugated annexin V binding test. In the original report, this staining was combined with PI, which marks live apoptotic cells with a positive green fluorescent signal without labeling the DNA red, allowing the classification of oocytes into three groups: "live non-apoptotic", live apoptotic" and "dead (membrane damaged)" [12]. However, we found that regardless of the treatment, all oocytes and embryos bear a certain level of green fluorescence. Using our equipment, the classification of Alexa 488-positive and negative oocytes could not be performed objectively. Therefore, we decided to measure the actual fluorescence intensity of Alexa 488 in membrane intact (PI-negative) cells. Treatment of oocytes with staurosporine (i.e., the positive control) resulted in a significant increase in fluorescent intensity of Alexa 488 compared with the control, which suggested that the method was adequate to measure phosphatidylserine exteriorization in oocytes. There was no significant difference in fluorescent intensity levels between the control and vitrified oocytes and their subsequent embryos, which confirms our previous report [17]. However, in vitrified oocytes, the phosphatidylserine exteriorization appeared to be tendentiously increased compared with the control ($P = 0.12$), and it did not differ significantly from that measured in the positive control. In animal cells, phosphatidylserine exteriorization can occur via apoptotic and non-apoptotic pathways as well; the latter by the increase of free cytoplasmic Ca^{2+} in cytoplasm [28, 29]. In mammalian oocytes, cooling and cryoprotectants were reported to trigger the release of Ca^{2+} from intracellular stores [30–32]. These reports and the premature resumption of meiosis, triggered by the vitrification process [11], suggests that vitrification might trigger a release of intracellular Ca^{2+} in immature oocytes, which could explain the slight increase in phosphatidylserine exteriorization in vitrified oocytes in the present study. However, this point requires further investigations. Interestingly, in live Day 2 embryos, fluorescence of Alexa 488-conjugated annexin V was not statistically different amongst all groups, including the staurosporine-treated positive control. We found that, regardless of treatment, most cleaved embryos had a region with a relatively strong Alexa 488 signal, most prominently in cleavage burrows (Supplementary Fig. 2: online only). Therefore, measurement of phosphatidylserine exteriorization based on fluorescent intensity of Alexa 488-conjugated annexin V might not be an adequate method for cleavage stage embryos. Moreover, this emphasizes that the investigation of apoptotic events should not be based on a single type of assay.

In the present study, we investigated apoptosis on the level of mRNA expression in oocytes, cumulus cells and resultant embryos as well. After vitrification, warming and subsequent IVM, mRNA levels of all three apoptosis-related genes examined in this study were similar in MII-stage oocytes. This complies with common knowledge that fully-grown porcine oocytes are transcriptionally inactive, and in porcine embryos, *de novo* mRNA synthesis starts only at the 4-cell stage [33]. On the other hand, an interesting and unexpected finding of the present study, was that vitrification of the oocyte at the GV stage caused a 6-fold increase in *Bcl-XL* gene expression in 4–8 cell stage embryos, whereas *Bax* gene expression remained similar amongst groups. These genes are members of the *Bcl-2* gene family, which controls the initiation of apoptosis in mammalian cells [34]. Their encoded proteins are located in mitochondria. It is generally accepted that upon stresses, the balance of the pro- and anti-apoptotic members of the *Bcl-2* family determines if the cell undergoes apoptosis and self-terminates or remains alive [34]. If the proportion of pro-apoptotic homodimers of the *Bcl-2* family such as *Bax* increase (which was not the case in our study), mitochondrial pores are formed, which lead to the release of caspase activators such as cytochrome c, triggering apoptosis [34]. On the other hand, the role of the *Bcl-XL* gene and its encoded protein, is the prevention of mitochondrial content leakage [35], hence, generally considered an anti-apoptotic gene. Our present results revealed that in the vitrified group, the upregulation of the *Bcl-XL* gene in Day 3 embryos caused an 8-fold significant increase in *Bcl-XL*/*Bax* expression ratio compared with the control. This suggests, that rather than undergoing apoptosis, embryos obtained from vitrified oocytes were trying to survive the consequences of the stresses of vitrification, which were exerted at the immature oocyte stage, 5 days before the assay. Furthermore, since such an alteration was not observed in the CPA group, the upregulation of the *Bcl-XL* gene was triggered by the cooling procedure or the combined effects of cooling and CPAs. The role and importance of the *Bcl-XL* upregulation in the development of embryos obtained from vitrified oocytes is not clear thus far. It is possible that the embryo is producing Bcl-XL protein to restore mitochondrial integrity, which is compromised by vitrification, and thus upregulation of the *Bcl-XL* gene is related to a recovery process to normalize the developmental competence of embryos generated from stressed oocytes. Previously, we reported that blastocyst stage embryos obtained from porcine oocytes vitrified at the GV stage were similar to those obtained from non-vitrified oocytes in terms of total cell numbers [3, 7, 8] and that they had the ability to develop to term [3]. Vitrification causes several different sub-lethal damages in porcine oocytes, including mitochondrial alterations [36]. Surviving GV stage oocytes can recover from damage such as partial disruption of the cytoskeleton and nucleolus fragmentation, during subsequent culture [11]. Recovery processes from sub-lethal damages might have

key importance for oocytes to regain developmental competence after cryopreservation. The understanding of damage-repair processes in vitrified oocytes and subsequent embryos will require further research.

Previous studies have shown that besides their role in apoptosis regulation, members of the Bcl-2 family including *Bcl-XL* also cause cell cycle arrest at the G_0 stage and delayed cell cycle entry in animal cells [37, 38]. Our previous results have demonstrated that oocyte vitrification at the GV stage reduces both cleavage and competence of cleaved embryos to reach the blastocyst stage [10], and delays the timing of blastulation [3]. Therefore, it is also possible that activation of *Bcl-XL* plays a role in the molecular mechanism of the developmental arrest and/or delay of embryos in the vitrified group. Our present results also revealed reduced cell numbers in cleaved embryos obtained from vitrified oocytes on Day 2. Further research is needed to clarify if this phenomenon is caused by a delay of the first embryonic cleavage or the extension of subsequent cell cycle durations, and if embryos with low blastomere numbers on Day 2 are permanently arrested or have the ability to develop to the blastocyst stage. Furthermore, it is yet to be elucidated if this developmental delay during the cleavage stage contributes to delayed timing of blastulation.

In conclusion, vitrification of porcine COCs at the GV stage by our method did not trigger the apoptotic cascade in oocytes and resultant embryos, at least up to the 8-cell stage. In contrast, it triggered anti-apoptotic *Bcl-XL* gene expression in resultant embryos at the 4−8 cell stage.

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