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Changes in mitochondrial function in porcine vitrified MII-stage oocytes and their impacts on apoptosis and developmental ability

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ABSTRACT

The purpose of this study was to investigate the changes in mitochondria in porcine MII-stage oocytes after open pulled straw (OPS) vitrification and to determine their roles in apoptosis and in vitro developmental ability. The mitochondrial membrane potential ($\Delta\Psi$m), reactive oxygen species (ROS) level, adenosine-5′-triphosphate (ATP) concentration, mitochondrial distribution, mitochondrial ultrastructure, early-stage apoptosis with Annexin V-FITC staining, survival rate, parthenogenetic developmental ability and related gene expression were measured in the present experiments. The results showed that: (1) the mitochondrial $\Delta\Psi$m of vitrified-thawed oocytes (1.05) was lower than that of fresh oocytes 1.24 ($P < 0.05$). (2) ROS level in the OPS vitrification group was much higher than that of the fresh group, while the ATP concentration was much lower than that of fresh group ($P < 0.05$). (3) Early-stage apoptosis rate from the OPS vitrification group (57.6%) was much higher than that of fresh group (8.53%) ($P < 0.05$), and the survival rate and parthenogenetic cleavage rate of OPS vitrified oocytes were much lower than those from fresh ones ($P < 0.05$). (4) Vitrification not only disrupted the mitochondrial distribution of porcine MII-stage oocytes, but also damaged the mitochondrial ultrastructure. (5) After vitrification, the gene expression level of *Dnm*1 was up-regulated, and other four genes (*SOD*1, *Mfn*2, *BAX* and *Bcl*2) were down-regulated. The present study suggested that not only the morphology and function of mitochondria were damaged greatly during the vitrification process, but also early-stage apoptosis was observed after vitrification. Intrinsic mitochondrial pathway could be in involved in the occurrence of apoptosis in vitrified-thawed porcine oocytes.

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1. Introduction

As one of the main means to conserve animal genetic resources, porcine oocyte cryopreservation is not yet successful. Porcine oocytes are particularly sensitive to low temperature. It was confirmed that their survival was essentially lost after exposure to a temperature of 15 °C or below for a short period [2,30,35]. High lipid content in porcine oocytes was considered to be one of the main reasons for their sensitivity to low-temperature [23]. Other reasons, such as mitochondrial damage, may affect the survival or apoptosis of vitrified porcine oocytes and therefore are in need of further studies.

Many studies have focused on the damage to microtubules, microfilaments, chromosomes and lipid droplets in the cryopreservation of porcine oocytes [20,33,48]. The data on changes to mitochondria and their relationship to apoptosis after vitrification are few. Mitochondria are the main cellular components related to energy supply and organelle migration; therefore, damage to mitochondria or changes in mitochondrial distribution could have strong impacts on oocyte development [13,36]. Mitochondria are also involved in the so-called intrinsic apoptotic pathway where they release soluble proteins, including cytochrome c, from the intermembrane space to initiate caspase activation in the cytosol [46]. The release of some proteins is a consequence of the integrity of the mitochondrial outer membrane (OMM) being compromised, a process called mitochondrial outer membrane permeabilization (MOMP) [24]. In mammals, MOMP is under the control of the

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proapoptotic Bcl-2 family members and has a direct relationship to the apoptotic process [28].

Due to the importance of mitochondrial function in the porcine oocytes and lack of systematic studies on its changes after vitrification, this paper reports the use of the OPS vitrification method to vitrify porcine MII-stage oocytes. The distribution, ultrastructure and function of mitochondria were observed after thawing; the apoptotic status and related gene expression level of vitrified porcine oocytes were also detected.

2. Materials and methods

All chemicals for this study were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless stated otherwise. Plastic dishes, four-well plates and tubes were obtained from Nunc (Roskilde, Denmark). The OPS was made in the laboratory with 0.25 ml plastic straws according to the method of Vajta et al. [42].

2.1. Source of porcine oocytes

Porcine ovaries recovered from a local slaughterhouse were placed in 37 °C, 0.9% NaCl solution containing antibiotics and transported to the laboratory within 1 h of collection. GV-stage oocytes with cumulus cells were aspirated from 3 to 8 mm diameter follicles. About 70 oocytes were cultured in a 500 μl maturation medium (TCM199 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 69 μg/ml L-cysteine, 10% porcine follicular fluid (lab-made), 10% FBS (fetal bovine serum), 10 IU/ml PMSG (pregnant mare serum gonadotropin), and 10 IU/ml hCG (human chorionic gonadotropin) in a humidified atmosphere with 5% CO_2 at 38.5 °C for 44 h. Matured oocytes were treated with 0.1% (w/v) hyaluronidase for 5 min, pipetted to strip away cumulus cells and washed 3–4 times with TCM199. Those oocytes with first polar body were considered to have reached MII-stage and selected for further studies [19].

2.2. Vitrification and warming of porcine oocytes

The basic medium for vitrification and warming solution was TCM199 with 20% FBS.

Briefly, oocytes were initially equilibrated for 5 min in cryoprotectant I (7.5% (v/v) EG (ethylene glycol) +7.5% (v/v) Me_2SO (dimethyl sulphoxide), and then transferred into the vitrification medium (17% EG + 17% Me_2SO + 0.4 M sucrose). Oocytes were loaded into OPS within 30 s and immediately immersed in liquid nitrogen.

After storage for at least 2 wks, oocytes were thawed with 0.5 M sucrose at 37 °C for 5 min, then were transferred to a 0.25 M sucrose for an additional 5 min. Oocytes were washed 2–3 times in TCM199 and were recovered in TCM199 (with 10% FBS) for 1 h in a CO_2 incubator.

2.3. Measurement of mitochondrial $\Delta\Psi m$ by JC-1 staining

To monitor $\Delta\Psi m$ changes, porcine MII oocytes were incubated with 2 μM JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide) in TCM199. All oocytes were stained in a CO_2 incubator at 37 °C for 30 min. Samples were analyzed using a laser scanning confocal microscope (LSCM, Leica, TCS SP2) with fluorescein isothiocyanate (FITC, green) and rhodamine isothiocyanate (RITC, red) channels. Two fluorescent images were recorded in the largest diameter of each oocyte and were analyzed by confocal software, which allows for quantitative values of the signal intensity of green and red fluorescence. The ratio of RITC to FITC for each oocyte is the point for the $\Delta\Psi m$. The experiment was replicated 3 times with about 25 oocytes each time [47].

2.4. Measurement of intracellular ROS level

Intracellular ROS level was detected by 2′,7′-dichlorofluorescein (DCF) fluorescence assay described by Gupta et al. [12]. Briefly, oocytes were incubated with 10 mM DCF for 20 min at 39 °C, washed three times in TCM199, and immediately observed and photographed using an epifluorescence microscope. The mean gray values of fluorescent oocytes were calculated using Image pro-Plus 6.0 software. Background fluorescent values were subtracted from the final values before analyzing for the statistical difference between the groups. The experiment was replicated 3 times with 20–25 oocytes each time.

2.5. Measurement of cytoplasmic ATP concentration

ATP concentration in the cytoplasm was measured by an ATP assay kit (ATP Bioluminescence Assay Kit, Roche, Germany). Briefly, 20 μl cell lysis reagent was added to 0.5 ml centrifuge tube containing 10 oocytes, which were homogenized by repeated pipetting until lysed. 100 μl ATP detection solution were added to 96-well dishes, then the 20 μl sample were added to each well. Luminescence was immediately measured using a luminometer (Synergy 2, Biotek, USA). Sample ATP concentration was calculated using a standard curve generated from 11 ATP gradient concentrations ranging from 10 fmol to 10 pmol. The experiment was replicated 3 times.

2.6. Annexin V-FITC staining for early stage apoptosis detection

Early-stage apoptosis of vitrified oocytes was detected by Annexin V-FITC apoptosis detection Kit (Beyotime, China). Briefly, oocytes were washed three times with DPBS and transferred into 195 μl Annexin V-FITC binding buffer with 5 μl Annexin V-FITC for 20 min at room temperature in the dark. After incubation, oocytes were washed two times with binding buffer and were observed and photographed using an epifluorescence microscope. The experiment was replicated for 3 times with about 30 oocytes each time.

2.7. Evaluation of oocyte survival by FDA and DAPI staining

This study modified traditional FDA survival staining by using simultaneous staining of FDA and DAPI. Vitrified-warmed oocytes were transferred in TCM199 containing 5 μg/ml FDA and 500 ng/ml DAPI for 20 min, and then washed two to three times in TCM199. Live oocytes accumulate intracellular fluorescein and appear green under an epifluorescent microscope. Oocytes chromosome with intact membrane cannot be stained with DAPI. The experiment was replicated 3 times.

2.8. Oocyte parthenogenesis

Oocytes were placed in the 0.5 mm gap chamber of an Electro Cell Manipulator (BTX 2001, A Division of Genetronics, Inc., San Diego, CA, USA). An electrical pulse of 60 V for 60 μs was used. The component of electric activation medium and the method were referred from the article by Li et al. [19]. After activation, embryos were cultured in NCSU-23 medium containing 0.4% BSA (bovine serum albumin). Each group of 10–15 embryos was cultured in 50 μl drops of culture medium at 39 °C in a humidified atmosphere of 5% CO2.

2.9. Observation of mitochondrial distribution

For mitochondrial distribution, MII-stage oocytes after warming were incubated in PBS supplemented with 10 μg/ml R-123

Table 1
Primers used for RT-PCR analysis.

Gene	Primers	Sequence (5′–3′)	Product size (bp)
GAPDH	Forward primer	CGATGGTGAAGGTCGGAGTG	159
	Reverse primer	TGCCGTGGGTGGAATCATAC	
SOD1	Forward primer	GTGCAGGGCACCATCTACTT	191
	Reverse primer	TCTTGATCCTTTGGCCCACC	
Mfn2	Forward primer	TGTCCAAAGTGAGGGGCATC	115
	Reverse primer	TTGTCCCAGAGCATGGCATT	
Dnm1	Forward primer	ATCTTCCATGAGCGCTTCCC	136
	Reverse primer	CAAAGGCCATGTCTGGGGTA	
Bax	Forward primer	AGCTGAGCGAGTGTCTCAAG	95
	Reverse primer	AGAAGAGACCACTCCTGGGT	
Bcl2	Forward primer	GAACTGGGGGAGGATTGTGG	164
	Reverse primer	CATCCCAGCCTCCGTTATCC	

(Rhodamine-123) at 39 °C for 15 min. After three times wash in TL-HEPES-PVA, five oocytes with about 3 μl volume were mounted on a glass slide, and the stained oocytes were examined under an epifluorescent microscope. The experiment was replicated for 3 times with 20–30 oocytes each time.

2.10. Preparation of transmission electron microscopy (TEM) samples

Briefly, oocytes were fixed in 2.5% glutaraldehyde in 0.1 M PBS at 4 °C overnight and washed in PBS. Subsequently, samples were embedded in 4% agar, post-fixed for 1 h with 1% OsO_4 (osmium tetroxide) in PB at 4 °C, and dehydrated in ascending concentrations of ethanol solutions (50–100%), substituted in propylene oxide, embedded in Epon 812, and serially semithin-sectioned. Selected semithin sections were subsequently re-embedded, and ultrathin sections were stained with uranyl acetate and lead citrate and examined under TEM (HITACHI H-600, Japan).

2.11. RNA extraction, reverse transcription and Real time PCR

MII-stage oocytes were collected with or without vitrification treatment. Three pools of 100 oocytes each were used to carry out the RNA extraction and qPCR analysis. RNA was extracted from the samples using RNAprep pure MicroKit (Tiangen). Reverse transcription was carried on with FastQuant RT Kit (With gDNase) (Tiangen), and cDNA samples from RT reaction were diluted with nuclease-free water to 60 μl. The quantification of all gene transcripts (SOD1, Mfn2, Dnm2, Bax, Bcl2 and GAPDH) was done by real-time quantitative RT-PCR using SYBR Green I chimeric fluorescence method (SYBR® Premix Ex Taq™ II, TaKaRa). Gene annotations were obtained from Genbank. Accession number, primer sequence, and product length of target genes are presented in Table 1. Each reaction mixture (20 μl) consisted of 10 μl SYBR® Premix Ex Taq II, 0.8 μl each of forward (10 μM) and reverse (10 μM) primers, 0.4 μl Rox Reference Dye II, 2 μl cDNA and 6 μl dH_2O. cDNA was conducted using the 7500 Real-time PCR system (Applied Biosystem) under the following condition: 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The data were subsequently analyzed using porcine GAPDH as housekeeping genes and the SDS software for Relative Quantification (Applied Biosystems).

2.12. Statistical analysis

Experiments were replicated three times or more, and data were pooled for statistical analysis. The percentages were subjected to an arc-sine transformation, and the transformed values were analyzed by ANOVA. The level of significance was set at $P < 0.05$.

3. Results

3.1. OPS vitrification decreased mitochondrial $\Delta\Psi$m level of porcine MII-stage oocytes

Representative image of mitochondria $\Delta\Psi$m are shown in Fig. 1. Oocytes with high mitochondrial $\Delta\Psi$m always show orange color. As shown in Table 2, the average value of mitochondria $\Delta\Psi$m from the fresh oocytes group was 1.24, which was significantly higher than that of OPS vitrification group.

3.2. Effect of OPS vitrification on ROS level and ATP concentration of porcine MII-stage oocytes

In this experiment, ROS level and ATP concentration of porcine MII-stage oocytes from fresh and OPS vitrification groups were compared. As shown in Table 3, ROS level from OPS vitrified oocytes was 69.5, which was much higher than that of fresh oocytes group (46.8, $P < 0.05$). The average ATP concentration from

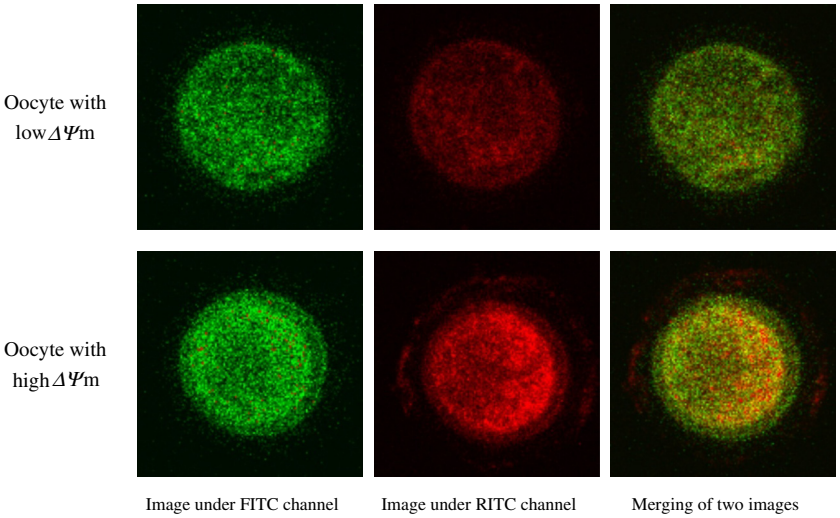


Fig. 1. Confocal micrographs of porcine oocytes labeled with JC-1 (200×).

Table 2
Effect of OPS vitrification on mitochondrial $\Delta\Psi$m changes in porcine oocytes.

Group	No. of oocytes	$\Delta\Psi$m
Fresh	64	1.24 ± 0.425[b]
OPS vitrification	72	1.05 ± 0.366[a]

[a,b] Values with different letter superscripts within columns are significantly different, $P < 0.05$.

Table 3
Effect of vitrification of porcine oocytes on ROS level and ATP concentration.

Group	ROS level	ATP concentration (pmol per oocyte)
Fresh	46.8 ± 3.58 ($n = 66$)[a]	0.47 ± 0.05 ($n = 30$)[b]
OPS vitrification	69.5 ± 4.23 ($n = 74$)[b]	0.34 ± 0.06 ($n = 30$)[a]

[a,b] Values with different letter superscripts within columns are significantly different, $P < 0.05$.

each vitrified oocyte was 0.34 pmol, and fresh oocyte was 0.47 pmol per oocyte ($P < 0.05$).

3.3. Effect of OPS vitrification on mitochondrial distribution and ultrastructure of porcine MII-stage oocytes

In this experiment, mitochondrial distribution was classified into two categories: (1) normal distribution: intact mitochondria evenly distributed in the ooplasm of MII oocytes; (2) abnormal distribution: mitochondrial reduced uneven distribution or mitochondrial broken distribution. (Fig. 2). The results were showed in Table 4. There were significant differences in the mitochondrial distribution between the two groups ($P < 0.01$). Fresh oocytes had the higher mitochondrial normal distribution rate (88.24%) with the OPS vitrification group having 48.65%. In the OPS vitrification group, 39.19% of the oocytes showed mitochondrial reduced uneven distribution and 12.16% oocytes were considered as broken distribution.

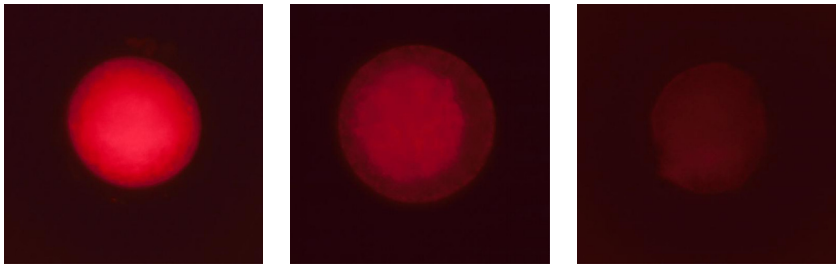
With the observation of TEM (Fig. 3), most mitochondria from fresh oocytes were round, and some of them showed vacuoles (A, B, C), the mitochondrial membrane was smooth and flat, and had a clear mitochondrial ridge (B, C, arrowed) in some mitochondria. In vitrified MII-stage oocytes (D, E, F), some mitochondrial membranes maintained their appearance, but most were destroyed and became coarse and indistinct (arrow). None of normal mitochondrial ridge could be seen in the vitrified porcine oocytes.

3.4. Effect of OPS vitrification on apoptosis, survival rate and parthenogenetic development ability in porcine MII-stage oocytes

The results of the apoptotic rate, survival rate and parthenogenetic cleavage rate from fresh and OPS vitrification groups are shown in Table 5. In Fig. 4, oocyte with arrowhead pointed is the apoptotic positive oocyte after Annexin V-FITC staining. Apoptotic rate from the OPS vitrification group was 57.6%, which was much higher than that of fresh group (8.53, $P < 0.01$). The survival rates of FDA staining and FDA-DAPI simultaneous staining from the OPS vitrification group were 72.50% and 60.00%, which were much lower than those of fresh group (98.00% and 92.00%, $P < 0.05$). Oocytes from the OPS vitrification group achieved only 14.56% parthenogenetic cleavage rate, while the fresh group achieved 80.91% cleavage rate.

3.5. Effect of OPS vitrification on gene expression level related with mitochondrial function and apoptosis

The gene expression related to mitochondrial function (*SOD*1, *Mfn*2 and *Dnm*1) and apoptosis (*BAX* and *Bcl*2) after vitrification were detected by RT-PCR in this study, and the results are showed in Fig. 5. In mitochondrial genes, *SOD*1, *Mfn*2 were weakly expressed in the OPS vitrification group, while *Dnm*1 was strongly expressed. In apoptosis related genes, both *BAX* and *Bcl*2 with vitrification were lower than those of oocytes without vitrification.



Intact and even mitochondrial distribution | Reduced mitochondrial distribution | Broken mitochondrial distribution

Fig. 2. Graphic examples for normal and abnormal distribution of mitochondria (200×).

Table 4
Effect of vitrification on mitochondrial distribution of porcine oocytes.

Group	No. of recycled oocytes	Mitochondrial normal distribution rate (%)	Mitochondrial abnormal distribution rate	
			Mitochondrial reduced uneven distribution rate (%)	Mitochondrial broken distribution rate (%)
Fresh	85	88.24[b] (75/85)	9.41[a] (8/85)	2.35[a] (2/85)
OPS vitrification	74	48.65[a] (36/74)	39.19[b] (29/74)	12.16[b] (9/74)

[a,b] Values with different letter superscripts within columns are significantly different, $P < 0.05$.

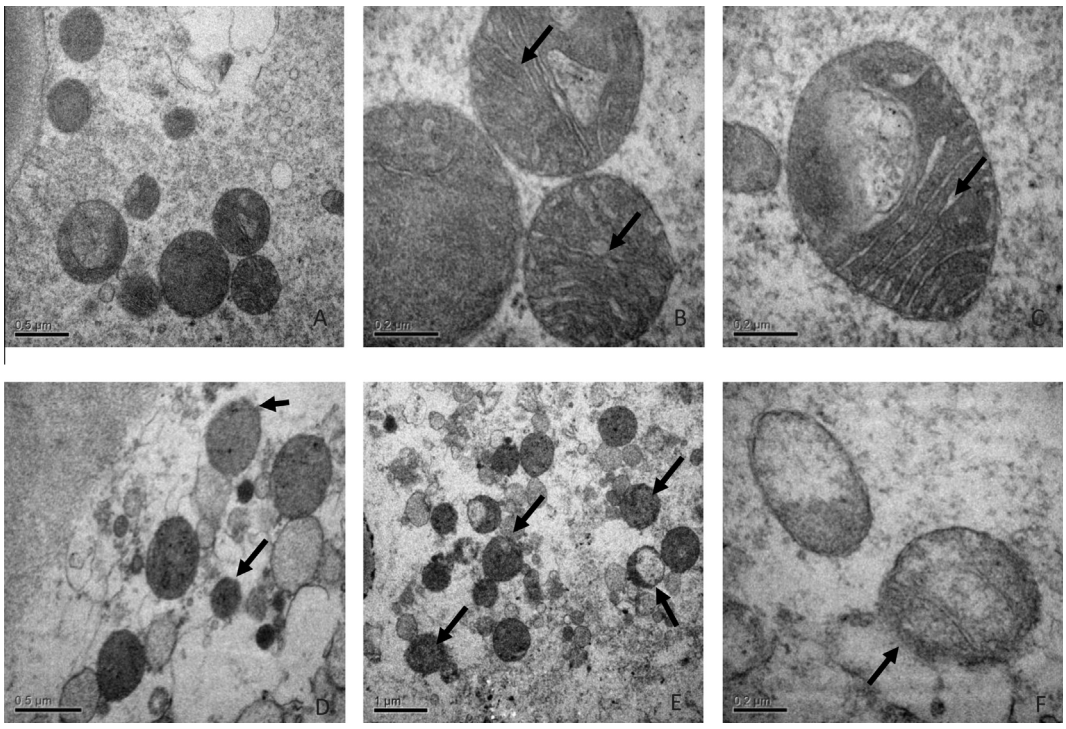


Fig. 3. Mitochondrial changes of vitrified MII-stage oocytes with ultrastructure observation.

Table 5
Effect of vitrification on apoptosis, survival rate and developmental ability of porcine oocytes.

Group	Apoptotic rate (%)	FDA staining survival rate (%)	Parthenogenetic cleavage rate (%)
Fresh	8.53 (7/82)[a]	98.00 (49/50)[b]	80.91 (89/110)[b]
OPS vitrification	57.6 (53/92)[b]	72.50 (29/40)[a]	14.56 (15/103)[a]

[a,b] Values with different letter superscripts within columns are significantly different, $P < 0.05$.

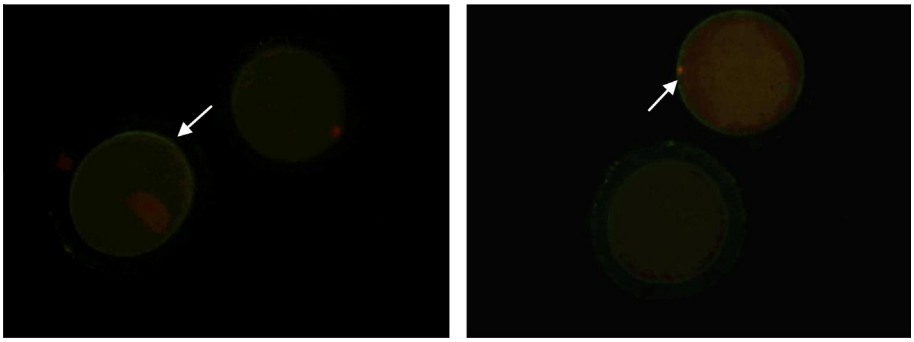


Fig. 4. Fluorescent micrographs for oocyte apoptosis detection with Annexin V-FITC staining (100×).

4. Discussion

Porcine oocyte cryopreservation is still not successful with resulting in vivo or in vitro developmental ability still very low. Studies on improving freezing efficiency have been mainly focused on modification of freezing methods. Research on mitochondrial injury and apoptosis is still rare. The main results in this study revealed that vitrification of porcine MII-stage oocytes altered mitochondrial distribution, destroyed mitochondrial ultrastructure and mitochondrial function, and induced the occurrence of oocyte apoptosis.

JC-1 is widely used in the detection of mitochondrial $\Delta\Psi$m. JC-1 can accumulate in mitochondrial matrices and form aggregates

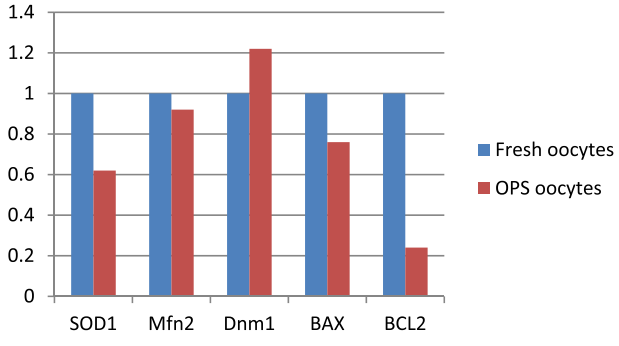


Fig. 5. Effect of vitrification on gene expression level of porcine oocytes.

from monomers and can stain red under epifluorescent microscope. The ratio of red to green fluorescence intensity is usually used to show the functional status of mitochondria [29]. Mitochondrial membrane potential $\Delta\Psi$m is easily damaged during cryopreservation and thawing. This study showed that vitrification could affect the mitochondrial function, and the average value of $\Delta\Psi$m from vitrified-thawed oocytes (1.24) was greatly decreased compared with that of fresh oocytes (1.05). Lei et al. [17,18] suggested that vitrification significantly affects mitochondrial function and caused the failure of embryo development after fertilization in both mouse oocytes and human IVM oocytes. Jones et al. [15] and Chen et al. [5] used programmed freezing method and vitrification method, respectively, to cryopreserve human MII-stage oocytes; both groups reported that the irreversible loss of high $\Delta\Psi$m in thawed oocytes may be associated with their poor developmental ability after in vitro fertilization. Zander-Fox et al. [50] showed that mouse oocytes vitrification altered mitochondrial distribution and membrane potential. Zhao et al. [52] found that vitrification had a significantly negative impact on the mitochondrial function of bovine oocytes, and cyclosporine (a specific inhibitor of mitochondrial permeability transition (MPT) pretreatment before vitrification could increase the mitochondrial $\Delta\Psi$m and improve the developmental ability of vitrified oocytes. Results from these reports were similar to those reported here and indicated that vitrification of porcine MII-stage oocytes resulted in serious damages to mitochondrial membrane potential.

As is well known, mitochondria are the major source of energy in eukaryotic cells, producing ATP via oxidative phosphorylation and the citric acid cycle. ATP concentration in the oocyte is directly related with its fertilization and embryo development [37,54]. When the mitochondrial membrane potential is low, the efficiency of energy supply by oxidative phosphorylation will drop [45,51]. Cryopreservation compromises mitochondrial activity and decreases ATP concentration in mouse, bovine and human oocytes [22,49,52,53]. This present study showed the ATP concentration of vitrified porcine oocytes after 1 h thawing decreased greatly compared with that of fresh oocytes. However, some authors described similar ATP concentrations within cryopreserved and fresh human [15] and bovine [4] oocytes, which questioned cryopreservation-related ATP loss as the main factor contributing to reduced oocyte competence. Jones et al. [15] suggested that irreversible loss of high $\Delta\Psi$m in thawed human oocytes may be associated with defects in Ca^{2+} signaling, and had nothing to do with the decreasing ATP concentration. In their study, they incubated post-thawed oocytes for 6 h (compared with 60 min in our study) before analyzing for the intra-oocyte ATP concentration; this prolonged incubation time might allow the oocytes to recover [15]. Manipalviratn et al. [22] demonstrated that vitrification/thawing procedure has a negative effect on intra-oocyte ATP level; however, with a 180 min post-thaw incubation period, the intra-oocyte ATP level returned to a level significantly higher than that of immediately-thawed oocytes. The present study showed that the ATP concentration of porcine vitrified-thawed oocytes decreased greatly with 1 h incubation; whether ATP concentration can recover after longer incubation periods after thawing needs to be further studied.

ROS is formed during the intermediate steps of oxygen reduction: the superoxide anion radical, hydrogen peroxide, and the hydroxyl radical [11]. Mitochondria damage can induce ROS. ROS can alter cell conformation and activity by directly affecting kinases and transcription [1]. Moreover, ROS can decrease embryo developmental ability and promote the occurrence of apoptosis in oocytes and early embryo [8]. Several past studies have emphasized the damage caused by the formation of ROS during cryopreservation processes, leading to oxidative stress [12,21,52]. ROS formed during the cryopreservation process can degrade essential molecules to cells, including the polyunsaturated lipids present in the cell membrane (lipid peroxidation), leading to oocyte death [40]. The present study in porcine oocytes also demonstrated that ROS level increased greatly after thawing with the damage to the mitochondria.

Intracellular distribution of mitochondria is closely related to the level of cell metabolism, proliferation and differentiation [25]. Disruption of the plasma membrane and mitochondria has been observed in vitrified-thawed human [32], mouse [7] and porcine oocytes [33]. The present study also showed that mitochondria abnormal distribution ratio after vitrification was much higher than that of fresh oocytes ($P < 0.01$). It has been reported that irreversible damage to the cytoskeleton could be seen in porcine GV and MII oocytes after vitrification [48]. The movement of mitochondria within different areas of oocytes is mediated by a cytoskeletal network of microtubules [39,44], so damage to the cytoskeleton during vitrification may affect the movement of mitochondria within oocytes [34]. The study of Fu et al. [9] showed a similar result; they vitrified porcine MII-stage oocytes with treatment of taxol (a cytoskeleton stabilizer) and achieved higher mitochondria normal distribution ratio than that of the vitrification group without treatment.

In the present study, the ultrastructure of mitochondria was observed. Vitrification had a negative effect on the ultrastructure of mitochondria. In fresh oocytes, the mitochondrial membrane was smooth and flat with a clear mitochondrial ridge. In vitrified oocytes, mitochondria were elongated, and their surface became coarse, indistinct and cracked. These ultrastructural changes were similar to those observed in vitrified bovine [10,31] and horse [14] oocytes.

The degeneration of oocytes and embryos after cryopreservation is thought to be caused by apoptosis. Meng et al. [26] first found that cell degradation in cattle oocyte after freezing was due to apoptosis. Vallorani et al. [43] vitrified porcine oocytes with the cryotop method and found that vitrification could cause apoptosis by activating caspase activity and loss of mitochondrial membrane potential. Men et al. [27] improved the survival rate and decreased the apoptotic level of vitrified porcine embryos by partial delipation treatment, and also proved that apoptosis in vitrified-thawed embryos was closely related to cell degradation. Sudano et al. [38] compared lipid content and apoptosis of in vitro-produced bovine embryos as determinants of susceptibility to vitrification and found that apoptosis level could reflect the embryos anti-freezing ability. This study showed that porcine vitrified MII-stage oocytes had an obvious increased apoptotic rate and decreased survival rate and parthenogenetic cleavage rate. Mitochondria play an important role in the development of oocytes and embryos, and their damage is closely related to apoptosis [16]. In the present study, not only the ultrastructure and distribution of mitochondria, $\Delta\Psi$m, ATP concentration, ROS level after vitrification decreased greatly, but also the gene expression (*SOD*1, *Mfn*2 and *Dnm*1) of vitrified oocytes could reflect the injury of mitochondrial function. The damage to mitochondria after vitrification could stimulate the intrinsic apoptotic pathway and induce the occurrence of apoptosis. It is well known that *Bcl*2 is anti-apoptosis and promotes cell survival, and *BAX* is pro-apoptosis and leads to cell death. We found that both *Bcl*2 and *BAX* were weakly expressed in vitrified oocytes. The result of down-regulation of *Bcl*2 gene in this study was similar to the report in mouse embryos [6] and human embryos [3]. *Bcl*2 may be used to evaluate the apoptotic status and developmental ability of vitrified porcine MII-stage oocytes. However, there are some opposing reports; Turathum et al. [41] showed that *Bcl*2 had a greater increase in the vitrified canine oocytes compared with fresh oocytes. Different species, different developmental stage and different cryopreservation method may influence the *Bcl*2 expression model

after thawing. In the present study, there was slightly lower *BAX* expression in verified porcine oocytes compared with fresh oocytes, which is similar to results in mouse embryos [6]. In vitrified canine germinal vesicles oocytes, *BAX* gene expression was not detected in either fresh or vitrified groups [41]. *BAX* may be not a suitable reference to predict the quality of mammalian oocytes and embryos after cryopreservation.

In conclusion, the present study showed that vitrification induced great damage to mitochondrial function (decreased $\Delta\Psi$m, ATP concentration, ROS level), mitochondrial distribution, mitochondrial ultrastructure and gene expression related mitochondrial function in porcine MII-stage oocytes. Intrinsic mitochondrial pathway could be involved in the occurrence of apoptosis for porcine vitrified oocytes. As an important apoptotic gene, *Bcl2* could be used to evaluate the apoptotic statue of vitrified porcine MII-stage oocytes after thawing.

Acknowledgments

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References

[1] V. Adler, Z. Yin, K.D. Tew, Z. Ronai, Role of redox potential and reactive oxygen species in stress signaling, Oncogene 18 (1999) 6104–6111.
[2] G. Bou, L.Q. Liu, Z. Zheng, J.T. Tian, Q.R. Kong, J. Song, X.D. Wang, Z.H. Liu, Effect of chilling on porcine germinal vesicle stage oocytes at subcellular level, Cryobiology 59 (2009) 54–58.
[3] C.A. Brenner, G.E. Exley, M. Alikani, J. Cohen, A.S. McElhinny, J.J. Stachecki, R.T. Scott, C.M. Warner, Apoptosis and human embryo survival, Fertil. Steril. Supp. 1 (1997) 86.
[4] V. Chankitisakul, T. Somfa, Y. Inaba, M. Techakumphu, T. Nagai, Supplementation of maturation medium with L-carnitine improves cryotolerance of bovine in vitro matured oocytes, Theriogenology 79 (2013) 590–598.
[5] C. Chen, S. Han, W. Liu, Y.P. Wang, G.N. Huang, Effect of vitrification on mitochondrial membrane potential in human metaphase II oocytes, J. Assist. Reprod. Genet. 29 (2012) 1045–1050.
[6] A. Dhali, V.M. Anchumparuthy, S.P. Butler, R.E. Pearson, I.K. Mullarky, F.C. Gwazdauskas, Gene expression and development of mouse zygotes following droplet vitrification, in: Theriogenology 68 (2007) 1292–1298.
[7] A. Eroglu, T.L. Toth, M. Toner, Alterations of the cytoskeleton and polyploidy induced by cryopreservation of metaphase II mouse oocytes, Fertil. Steril. 69 (1998) 944–957.
[8] M.D. Evans, M. Dizdaroglu, M.S. Cooke, Oxidative DNA damage and disease: induction, repair and significance, Mutat. Res. 567 (2004) 1–61.
[9] X.W. Fu, W.Q. Shi, Q.J. Zhang, X.M. Zhao, C.L. Yan, Y.P. Hou, G.B. Zhou, Z.Q. Fan, L. Suo, A. Wusiman, Y.P. Wang, S.E. Zhu, Positive effects of taxol pretreatment on morphology, distribution and ultrastructure of mitochondria and lipid droplets in vitrification of in vitro matured porcine oocytes, Anim. Reprod. Sci. 115 (2009) 158–169.
[10] E. Fuku, L. Xia, B.R. Downey, Ultrastructural changes in bovine oocytes cryopreserved by vitrification, Cryobiology 32 (1995) 139–156.
[11] P. Guérin, S.E.I. Mouatassim, Y. Ménézo, Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings, Hum. Reprod. Update 7 (2001) 175–189.
[12] M.K. Gupta, S.J. Uhm, H.T. Lee, Effect of vitrification and beta-mercaptoethanol on reactive oxygen species activity and in vitro development of oocytes vitrified before or after in vitro fertilization, Fertil. Steril. 93 (2010) 2062–2067.
[13] A.N. Ha, S.R. Lee, J.S. Jeon, H.S. Park, S.H. Lee, J.I. Jin, B.R. Sessions, Z. Wang, K.L. White, I.K. Kong, Development of a modified straw method for vitrification of in vitro-produced bovine blastocysts and various genes expression in between the methods, Cryobiology 68 (2014) 57–64.
[14] S. Hochi, M. Kozawa, T. Fujimoto, E. Hondo, J. Yamada, N. Oguri, In vitro maturation and transmission electron microscopic observation of horse oocytes after vitrification, Cryobiology 33 (1996) 300–310.
[15] A. Jones, J. Van Blerkom, P. Davis, A.A. Toledo, Cryopreservation of metaphase II human oocytes effects mitochondrial membrane potential: implications for developmental competence, Hum. Reprod. 19 (2004) 1861–1866.
[16] S.K. Lee, M.H. Zhao, J.W. Kwon, Y.H. Li, Z.L. Lin, Y.X. Jin, N.H. Kim, X.S. Cui, The association of mitochondrial potential and copy number with pig oocyte maturation and developmental potential, J. Reprod. Dev. 60 (2014) 128–135.
[17] T. Lei, N. Guo, M.H. Tan, Y.F. Li, Effect of mouse oocyte vitrification on mitochondrial membrane potential and distribution, J. Huazhong Univ. Sci. Technol. Med. Sci. 34 (2014) 99–102.
[18] T. Lei, N. Guo, J.Q. Liu, M.H. Tan, Y.F. Li, Vitrification of in vitro matured oocytes: effects on meiotic spindle configuration and mitochondrial function, Int. J. Clin. Pathol. 7 (2014) 1159–1165.
[19] Y. Li, J. Liu, J. Dai, F. Xing, Z. Fang, T. Zhang, Z. Shi, D. Zhang, X. Chen, Production of cloned miniature pigs by enucleation using the spindle view system, Reprod. Dom. Anim. 45 (2010) 608–613.
[20] R.H. Liu, Q.Y. Sun, Y.H. Li, L.H. Jiao, W.H. Wang, Effects of cooling on meiotic spindle structure and chromosome alignment within in vitro matured porcine oocytes, Mol. Reprod. Dev. 65 (2003) 212–218.
[21] H.K.M. Luz, L.S. Wanderley, L.R. Faustino, C.M.G. da Silva, J.R. de Figueiredo, A.P. R. Rodrogies, Role of antioxidants agents in germ cells and embryos cryopreservation, in: Acta Sci. Vet. 39 (2011) 956.
[22] S. Manipalviratn, Z.B. Tong, B. Stegmann, E. Widra, J. Carter, A. Decherney, Effect of vitrification and thawing on human oocyte ATP concentration, Fertil. Steril. 95 (2011) 1839–1841.
[23] F. Marco-Jiménez, L. Casares-Crespo, J.S. Vicente, Porcine oocyte vitrification in optimized low toxicity solution with open pulled straws, Zyogote 22 (2014) 204–212.
[24] I. Masgras, A. Rasola, P. Bernardi, Induction of the permeability transition pore in cells depleted of mitochondrial DNA, Biochim. Biophys. Acta 2012 (1817) 1860–1866.
[25] H. Matsumoto, N. Shoji, N.S. Sugawara, M. Umezu, E. Sato, Microscopic analysis of enzyme activity, mitochondrial distribution and hydrogen peroxide in two-cell rat embryos, in: J. Reprod. Fertil. 113 (1998) 231–238.
[26] H. Men, R.L. Monson, J.J. Parrish, J.J. Rutledge, Degeneration of cryopreserved bovine oocytes via apoptosis during subsequent culture, Cryobiology 47 (2003) 73–81.
[27] H. Men, Y. Agca, L.K. Riley, J.K. Critser, Improved survival of vitrified porcine embryos after partial delipation through chemically stimulated lipolysis and inhibition of apoptosis, Theriogenology 66 (2006) 2008–2016.
[28] M.S.O.M. Nawaz, H. Ahsan, Role of Bcl-2 family protein and caspases in the regulation of apoptosis, Mol. Cell. Biochem. 351 (2011) 41–58.
[29] X.H. Ou, S. Li, Z.B. Wang, M. Li, S. Quan, F. Xing, L. Guo, S.B. Chao, Z. Chen, X.W. Liang, Y. Hou, H. Schatten, Q.Y. Sun, Maternal insulin resistance causes oxidative stress and mitochondrial dysfunction in mouse oocytes, Hum. Reprod. 27 (2012) 2130–2145.
[30] J.E. Parks, N.A. Ruffing, Factors affecting low temperature survival of mammalian oocytes, Theriogenology 37 (1992) 59–73.
[31] G.J. Rho, S. Kim, J.G. Yoo, S. Balasubramanian, H.J. Lee, S.Y. Choe, Microtubulin configuration and mitochondrial distribution after ultra-rapid cooling of bovine oocytes, Mol. Reprod. Dev. 63 (2002) 464–470.
[32] A.H. SathananthanA, L. Trounson, T. .Brady. Freemann, The effects of cooling human oocytes, Hum. Reprod. 3 (1988) 968–977.
[33] L.Y. Shi, H.F. Jin, J.G. Kim, B.M. Kumar, S. Balasubramanian, S.Y. Choe, G.J. Rho, Ultra-structural changes and developmental potential of porcine oocytes following vitrification, Anim. Reprod. Sci. 10 (2007) 128–140.
[34] W.Q. Shi, S.E. Zhu, D. Zhang, W.H. Wang, G.L. Tang, Y.P. Hou, S.J. Tian, Improved development by Taxol pretreatment after vitrification of in vitro matured porcine oocytes, Reproduction 131 (2006) 795–804.
[35] T. Somfai, M. Ozawa, J. Noguchi, H. Kaneko, M. Nakai, N. Maedomari, J. Ito, N. Kashiwazaki, T. Nagai, K. Kikuchi, Live piglets derived from in vitro-produced zygotes vitrified at the pronuclear stage, Biol. Reprod. 80 (2009) 42–49.
[36] J.C. St John, The transmission of mitochondrial DNA following assisted reproductive techniques, in: Theriogenology 57 (2002) 109–123.
[37] M. Stojkovic, S.A. Machado, P. Stojkovic, V. Zakhartchenko, P. Hutzler, P.B. Gonçalves, E. Wolf, Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture, Biol. Reprod. 64 (2001) 904–909.
[38] M.J. Sudano, D.M. Paschoal, T.S. Rascado, L.C. Magalhães, L.F. de Lima-Neto, F.C. Landim-Alvarenga, Lipid content and apoptosis of in vitro-produced bovine embryos as determinants of susceptibility to vitrification, Theriogenology 75 (2011) 1211–1220.
[39] Q.Y. Sun, G.M. Wu, L.X. Lai, K.W. Park, B.N. Day, R.S. Prather, H. Schatten, Translocation of active mitochondria during pig oocyte maturation, fertilization and early embryo development in vitro, Reproduction 122 (2001) 155–163.
[40] C. Tatone, G. Di Emidio, M. Vento, R. Ciriminna, P.G. Artini, Cryopreservation and oxidative stress in reproductive cells, Gynecol. Endocrinol. 26 (2010) 563–567.
[41] B. Turathum, K. Saikhun, P. Sangsuwan, Y. Kitiyanant, Effects of vitrification on nuclear maturation, ultrastructural changes and gene expression of canine oocytes, Reprod. Biol. Endocrinol. 70 (2010) 153–164.
[42] G. Vajta, P. Holm, T. Greve, H. Callesen, Vitrification of porcine embryos using the open pulled straw (OPS) method, Acta Vet. Scand. 38 (1997) 349–352.

[43] C. Vallorania, M. Spinacia, D. Buccia, E. Porcu, C. Tamanini, G. Galeati, Pig oocyte vitrification by Cryotop method and the activation of the apoptotic cascade, Anim. Reprod. Sci. 135 (2012) 68–74.
[44] J. Van Blerkom, Microtubule mediation of cytoplasmic and nuclear maturation during the early stages of resumed meiosis in cultured mouse oocytes, PNAS 88 (1991) 5031–5035.
[45] J. Van Blerkon, Mitochondrial function in the human oocyte and embryo and their role in developmental competence, Mitochondrion 11 (2011) 797–813.
[46] D.L. Vaux, Apoptogenic factors released from mitochondria, Biochim. Biophys. Acta 2011 (1813) 546–550.
[47] M. Wilding, B. Dale, M. Marino, L. di Matteo, C. Alviggi, M.L. Pisaturo, L. Lombardi, G. De Placido, Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos, Hum. Reprod. 16 (2001) 909–917.
[48] C.H. Wu, R. Rui, J.J. Dai, C.Y. Zhang, S.Q. Ju, B. Xie, X.F. Zheng, Effects of cryopreservation on the developmental competence, ultrastructure and cytoskeletal structure of porcine oocytes, Mol. Reprod. Dev. 73 (2006) 1454–1462.
[49] C.L. Yan, X.W. Fu, G.B. Zhou, X.M. Zhao, L. Suo, W.E. Zhu, Mitochondrial behaviors in the vitrified mouse oocyte and its parthenogenetic embryo: effect of taxol pretreatment and relationship to competence, Fertil. Steril. 93 (2010) 959–966.
[50] D. Zander-Fox, K.S. Cashman, M. Lane, The presence of 1 mM glycine in vitrification solutions protects oocyte mitochondrial homeostasis and improves blastocyst development, J. Assist. Reprod. Genet. 30 (2013) 107–116.
[51] X. Zhang, X.Q. Wu, S. Lu, Y.L. Guo, X. Ma, Deficit of mitochondria-derived ATP during oxidative stress impairs mouse MII oocyte spindles, Cell Res. 16 (2006) 841–850.
[52] X.M. Zhao, W.H. Du, D. Wang, H.S. Hao, Y. Liu, T. Qin, H.B. Zhu, Effect of cyclosporine pretreatment on mitochondrial function in vitrified bovine mature oocytes, Fertil. Steril. 95 (2011) 2786–2788.
[53] X.M. Zhao, W.H. Du, D. Wang, H.S. Hao, Y. Liu, T. Qin, H.B. Zhu, Recovery of mitochondrial function and endogenous antioxidant systems in vitrified bovine oocytes during extended in vitro culture, Mol. Reprod. Dev. 78 (2011) 942–950.
[54] J. Zhao, Y. Li, Adenosine triphosphate content in human unfertilized oocytes, undivided zygotes and embryos unsuitable for transfer or cryopreservation, J. Int. Med. Res. 40 (2012) 734–739.