REPRODUCTION
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Improved development by Taxol pretreatment after vitrification of *in vitro* matured porcine oocytes

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Abstract

This study was designed to examine the effect of Taxol pretreatment on vitrification of porcine oocytes matured *in vitro* by an open pulled straw (OPS) method. In the first experiment, the effect of Taxol pretreatment and fluorescein diacetate (FDA) staining on parthenogenetic development of oocytes was evaluated. In the second experiment, viability, microtubule organization and embryo development of oocytes were assessed after oocytes were exposed to vitrification/warming solutions or after vitrification with or without Taxol pretreatment. The results showed that Taxol pretreatment and/or FDA staining did not negatively influence the oocyte's developmental competence after parthenogenetic activation. After being exposed to vitrification/warming solutions, the survival rate (83.3%) of the oocytes was significantly ($P < 0.05$) reduced as compared with that in the control (100%). Vitrification/warming procedures further reduced the survival rates of oocytes regardless of oocytes being treated with (62.1%) or without (53.8%) Taxol. The proportions of oocytes with normal spindle configuration were significantly reduced after the oocytes were exposed to vitrification/warming solutions (38.5%) or after vitrification with (10.3%) or without (4.1%) Taxol pretreatment as compared with that in control (76.8%). The rates of two-cell-stage (5.6–53.2%) embryos at 48 h and blastocysts (0–3.8%) at 144 h after activation were significantly reduced after exposure to vitrification/warming solutions or after vitrification as compared with control (90.9% and 26.6% respectively). However, the proportion of vitrified oocytes developed to two-cell stage was significantly higher when oocytes were pretreated with (24.3%) than without (5.6%) Taxol. These results indicate that pretreatment of oocytes with Taxol before vitrification helps to reduce the damage induced by vitrification and is a potential way to improve the development of vitrified porcine oocytes.
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Introduction

The development of cryopreservation technology in non-human mammalian oocytes can be applied to human-assisted reproductive technology, in which oocyte cryopreservation shows many advantages for infertility treatment (Arav *et al.* 2002). Recently, vitrification has been widely used to cryopreserve oocytes in many mammals (Kono *et al.* 1991, Arav & Zeron 1997, Isachenko *et al.* 1998), including man (Kuleshova *et al.* 1999, Yoon *et al.* 2000). As vitrification does not require a programmed freezer and the technique itself is easy, safe and highly efficient, it will become an indispensable method in cryobiology in the future (Kuleshova & Lopata 2002, Liebermann *et al.* 2002). Normal offspring have been produced from the vitrified mature (Kono *et al.* 1991, Hamano *et al.* 1992, Kuleshova *et al.* 1999, Yoon *et al.* 2000) and immature oocytes (Vieira *et al.* 2002). However, it has been found that vitrification of porcine oocytes (Rojas *et al.* 2004) is much more difficult than in other mammals, such as mice (Wood *et al.* 1993) and cattle (Vieira *et al.* 2002). Various vitrification methods have been used to cryopreserve porcine oocytes (Ahn *et al.* 2004, Dinnyes *et al.* 2004, Rojas *et al.* 2004), but live births from vitrified oocytes have not been reported. Rojas *et al.* (2004) compared the viability of immature and mature porcine oocytes vitrified in ethylene glycol (EG)-based solution by open pulled straw (OPS) vitrification, and found that porcine metaphase II (MII) oocytes (2–4 layers of cumulus cells remain) had better resistance to

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vitrification than germinal vesicle (GV) stage oocytes. The authors also found that 10.4% of vitrified oocytes could develop to the two-cell stage after *in vitro* fertilization (IVF). Recently, the vitrification solution (VS) with both EG and dimethyl sulfoxide (DMSO) has been successfully used to vitrify human and other mammalian oocytes (Hyttel *et al.* 2000, Vieira *et al.* 2002). Le Gal & Massip (1999) reviewed the literature and found that only the OPS method (Vajta *et al.* 1998) yielded approximately 3% blastocysts in mouse oocyte vitrification. Chen *et al.* (2000) reported that OPS can preserve higher rates of normal meiotic spindle morphology (78%) and chromosome distribution (87%) than the conventional straw method. It is well known that the porcine oocyte is more sensitive to low temperature than other mammalian oocytes; thus, successful methods for vitrification of porcine oocytes have not been developed.

Taxol has been used in cryopreservation of embryos (Dobrinsky *et al.* 2000), and it has been found that Taxol pretreatment can improve developmental competence of vitrified mouse (Park *et al.* 2001) and human (Fuchinoue *et al.* 2004) oocytes. However, to our knowledge, no study has reported the effect of Taxol treatment of porcine oocytes during vitrification. Therefore, in the present study, we investigated whether porcine oocytes matured *in vitro* can be vitrified by OPS and whether Taxol pretreatment can improve the vitrification efficiency in porcine oocytes.

Materials and Methods

All chemicals for this study were purchased from Sigma-Aldrich (St Louis, MO, USA) unless stated otherwise.

In vitro maturation of oocytes

The collection and culture of porcine oocytes were based on the protocol reported by Wang *et al.* (2000). Briefly, ovaries were collected from prepubertal pigs at a local slaughterhouse and transported to the laboratory within 2 h in 0.9% (w/v) NaCl solution containing 75 μg penicillin G/ml and 50 μg streptomycin sulfate/ml maintained at 33–37 °C. The room was always air-conditioned at a temperature of 25 ± 1 °C. The oocytes were aspirated from medium-sized follicles (3–6 mm in diameter) with a 20 gauge needle fixed to a 10 ml disposable syringe. Cumulus–oocyte complexes (COCs) surrounded by a compact cumulus mass with evenly granulated cytoplasm were selected. The COCs were washed four times in Tyrode's lactate (TL)-Hepes (Ca^{2+}-free) medium with 0.01% (w/v) polyvinyl alcohol (TL-Hepes-PVA), and washed three times with maturation medium: TCM-199 (Gibco, Invitrogen Corporation, Grand Island, NY, USA) supplemented with 0.57 mmol cysteine/l, 10 ng epidermal growth factor (EGF)/ml, 10 IU equine chorionic gonadotropin (CG)/ml, 10 IU human (h)CG/ml and 10% (v/v) porcine follicle fluid. A group of 20–25 oocytes was cultured at 39 °C in an atmosphere of 5% CO_2 in air and saturated humidity. The oocytes were freed of cumulus cells at 44 h after culture by pipetting in the TL-Hepes-PVA medium containing 0.02% (w/v) hyaluronidase. Cumulus-free oocytes with the first polar body and even-granulated cytoplasm were used in the following experiments.

Vitrification and warming of oocytes

OPS was made by the method described by Vajta *et al.* (1998) with some modifications. Briefly, the straws (250 μl; IMV, L'Aigle, France) were heat-softened and pulled manually. The pulled straws (2–3 cm in length) were cut at the tapered end with a blade to ensure an inner diameter of about 0.2 mm and a wall thickness of about 0.02 mm, which were measured with a microforge. The VS was modified from EFS40 (Zhu *et al.* 1993), which contained 20% (v/v) EG, 20% (v/v) DMSO, 18% (w/v) Ficoll (mol. wt 70 000) and 0.3 mol sucrose solution/l in a modified PBS solution without Ca^{2+} and Mg^{2+} (m-PBS^-). The oocytes were equilibrated for 90 s in equilibration solution (ES), that is, 10% (v/v) EG +10% (v/v) DMSO in m-PBS^-. The oocytes were loaded into the pulled end of straws by negative pressure and then exposed to VS for 30 s before the straws were plunged into liquid nitrogen (LN). The straws were stored in the LN for at least 24 h before warming. The warming solution (WS) was m-PBS^- containing various concentrations of sucrose. Stepwise warming was performed by placing the pulled end of the straws directly into 0.5 mol sucrose/l solution for 2 min. The oocytes were then rinsed with other solutions of 0.5, 0.25 and 0.125 mol sucrose/l, each for 2 min. All warming procedures were performed at 38.5 °C on a warming stage fixed on the stereomicroscope, and the ambient atmosphere was always air-conditioned at a temperature of 25 ± 1 °C.

Assessment of viability and survival of oocytes

For assessment of viability, oocytes exposed to different chemicals or vitrified were incubated for 30 min in a CO_2 incubator, and then quickly washed three times in m-PBS^- solution. Finally, the oocytes were cultured in m-PBS^- containing 2.5 μg fluorescein diacetate (FDA)/ml for 3 min at 38.5 °C and then washed three times in m-PBS^- solution before examination of membrane integrity under an inverted fluorescence microscope by the method of Zeron *et al.* (1999). High intensity of flourescence level was regarded as high viability, low fluorescence intensity was regarded as low viability, and nonfluorescence indicated dead oocytes. All images were acquired with a CCD video camera (DP70; Olympus, Tokyo, Japan) connected to the computer. Oocytes with high level of fluorescence and regular,

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spherical shape; without lysis; and not shrunken, swollen, or blackened were regarded as surviving.

Labeling of oocytes for examination of microtubules (MT) and chromosomes

The methods for examination of spindle morphology were based on a previous study (Sun *et al.* 2001) with some modifications. Briefly, cumulus-free oocytes were treated with 0.5% (v/v) TritonX-100 in PHEM buffer (60 mmol Pipes/l, 25 mmol Hepes/l, 10 mmol EGTA/l, 4 mmol $MgSO_4$/l (pH 7)) for 5 min before being fixed in 4% formaldehyde in PHEM for 20 min. After being washed in PBS, the oocytes were blocked with 1% BSA in PHEM for 1 h at room temperature and incubated in PHEM with 1% BSA containing monoclonal mouse antitubulin antibody (Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:16 000 overnight at 4 °C. The oocytes were washed four times in PBS with 0.05% Tween-20 (PBST) and then stained with fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (Jackson ImmunoResearch) diluted 1:200 in PHEM with 1% BSA for 45 min. After four washes in PBST, the oocytes were stained with propidium iodide (PI) for chromosomes for 2 min.

Confocal microscopy of stained oocytes

The oocytes were washed in PBST after staining and mounted with antifade solution (0.5% *n*-propyl gallate in 20 mmol Tris/l, with 90% glycerol (pH 8)) on the slides and examined by confocal laser scanning microscope (Zeiss LSM-510). Normal and abnormal morphologies were assessed by the former criterion (Liu *et al.* 2003). In brief, normal spindles show barrel-shaped morphology with the chromosomes aligned regularly at the metaphase plate and microtubules (MT) traversing the spindle from pole to pole or extending from the two poles to chromosomes; otherwise, those with unorganized, disassembled or absent MT were all classified as abnormal spindles. The chromosomes which aligned in a discrete bundle on the equator of the spindles were classified as normal chromosomes; otherwise, those with scattered or dispersed chromatids in the cytoplasm were all classified as abnormal chromosomes. Each experiment was repeated four times, and at least 30 oocytes were examined each time.

Oocyte activation and embryo culture

The oocytes were washed three times in TL-Hepes-PVA medium (Ca^{2+}-free) and then rinsed twice in the activation medium (0.3 mol mannitol/l, 0.05 mmol $CaCl_2$/l, 0.1 mmol $MgCl_2$/l and 0.4% (m/v) BSA). The oocytes were transferred between two electrodes covered by activation medium in a chamber connected to an electrical pulsing machine (Fujihira Industry, Tokyo, Japan). The oocytes were stimulated with a direct current of 100 V/mm for three consecutive 80-μs pulses at 100-ms intervals. All oocytes activated were washed and cultured in North Carolina State University (NCSU)-23 medium (Petters & Wells 1993) containing 7.5 μg cytochalasin B/ml (pre-equilibrated for 4–6 h in 5% CO_2 incubator). After treatment, the oocytes were completely washed, and each group of 20–25 oocytes cultured in NCSU-23 medium containing 0.4% (m/v) BSA (A-8022) in 100 μl drops under mineral oil (pre-equilibrated for 4–6 h in 5% CO_2 incubator). Oocyte cleavage and blastocyst formation were examined and recorded at 48 and 144 h after activation respectively.

Total nuclear counts

Blastocysts were stained with 10 μg bisbenzimide (Hoechst 33342)/ml in NCSU-23 at 38.5 °C for 10 min and then mounted and viewed under ultraviolet light on the same day. The total nuclear number of every blastocyst was counted by the number of nuclei under fluorescence microscopy.

Experimental design

In experiment 1, the effect of Taxol pretreatment and FDA staining on developmental competence of oocytes was tested. *In vitro* matured oocytes were randomly assigned to one of the following four experimental groups:

1. *Control*. No treatment was performed.
2. *Taxol treatment*. Oocytes were treated by 1 μmol Taxol (T-7402)/l/m-PBS^- for 30 min in the incubator.
3. *FDA treatment*. Oocytes were treated by 2.5 μg FDA/ml for 3 min on a warm stage (38.5 °C) without light.
4. *Taxol plus FDA*. Oocytes were stained by FDA after Taxol pretreatment. Cleavage rate, blastocyst rate and mean number of nuclei in blastocysts were examined after treatment and culture.

Table 1 Effect of taxol pretreatment and FDA staining on development of parthenogenetically activated porcine oocytes.

No. oocytes	Taxol pretreatment	FDA staining	No. cleaved (%)	No. blastocysts (%)	No. of nuclei/Blastocysts Mean±S.E.M.
192	−	−	155 (80.7 ± 1.3)[a]	48 (25.0 ± 2.8)[a]	26.2 ± 1.7
201	+	−	171 (85.2 ± 1.7)[a]	56 (27.9 ± 1.1)[a]	25.1 ± 1.0
200	−	+	157 (78.8 ± 3.7)[a]	46 (23.1 ± 2.1)[a]	24.4 ± 0.7
196	+	+	160 (81.6 ± 1.3)[a]	43 (21.9 ± 3.6)[a]	24.0 ± 0.7

[a]Values with same superscript within same column are not significantly different at 0.05 level.

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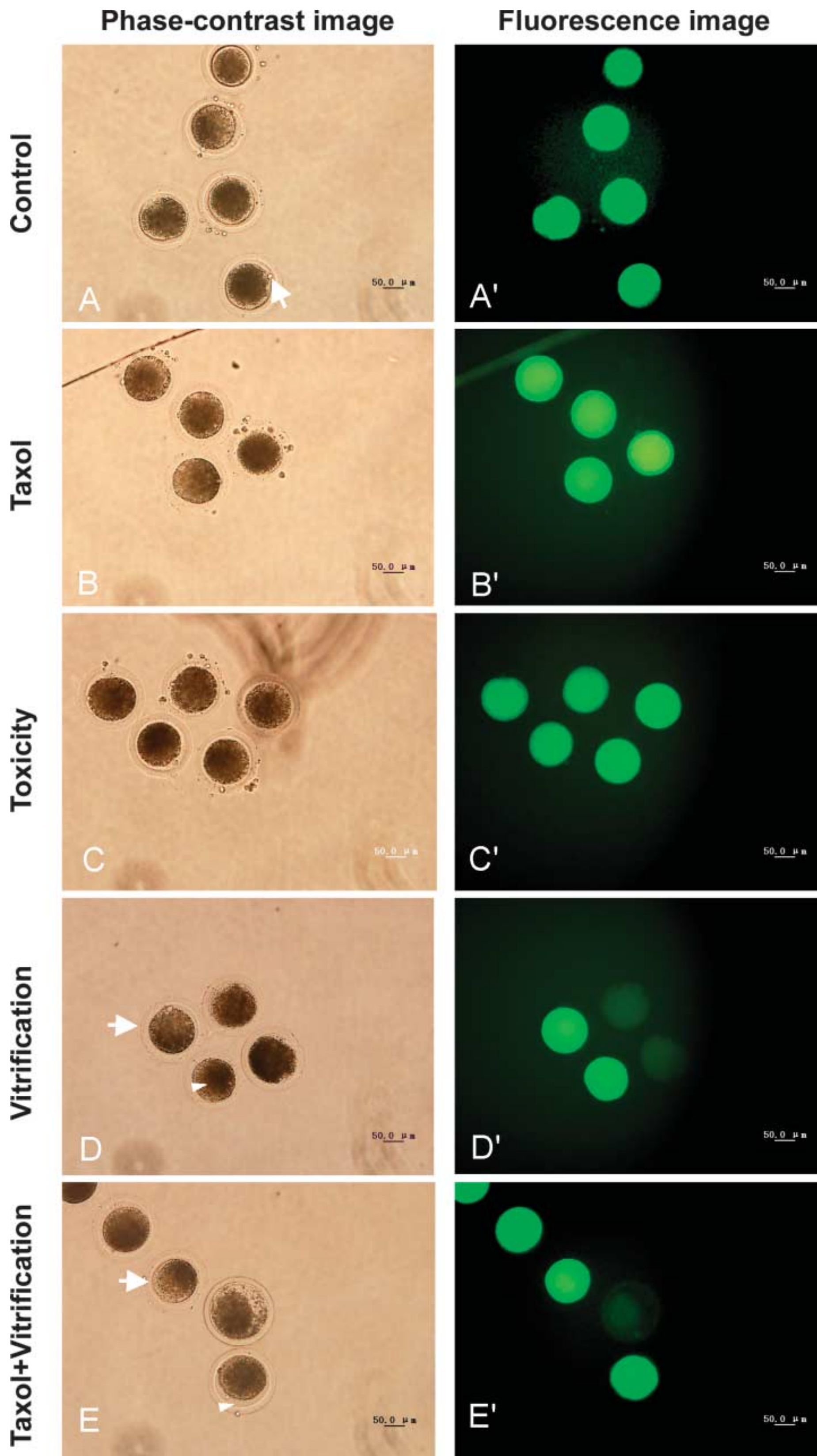

Figure 1 Phase-contrast and fluorescence microscope images of *in vitro* matured porcine oocytes exposed to vitrification solutions or Taxol, or after vitrification. Left column: phase-contrast microscope images; right column: fluorescence microscope images. (A, A′) Control group: oocytes were cultured in the medium without extra treatment. (B, B′) Toxicity-test group: oocytes were exposed to ES for 90 s, VS for 30 s and WS (0.5, 0.25 and 0.125 mol sucrose/l/m-PBS$^-$ solutions), each for 2 min. (C, C′) Taxol group: oocytes were cultured in 1 μmol Taxol/l/m-PBS$^-$ for 30 min at 39 °C. (D, D′) Vitrification group: oocytes were vitrified- warmed. (E, E′) Taxol plus vitrification: oocytes were pretreated with 1 μmol Taxol/l for 30 min at 39 °C and then vitrified-warmed. Oocyte viability was indicated by intense fluorescence; nonfluorescence indicated nonviability. Bar = 50 μm. Arrow in panel A indicates first polar body; Arrow in panel D indicates unsmooth Zona Pellucida; Arrow head in panel D indicates very dark cytoplasm; Arrow in panel E indicates uneven cytoplasm; Arrow head in panel E indicates bigger perivitelline space.

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Table 2 Effect of different treatment on spindle morphology and chromosomes alignment in porcine oocytes.

Groups	No. examined	No. (%) of oocytes with	
		Normal spindle configuration	Normal chromosome alignment
Control	124	95 (76.8 ± 1.27)[a]	119 (81.6 ± 1.70)[a]
Toxicity	115	45 (38.5 ± 5.02)[b]	84 (73.5 ± 6.80)[a]
Vitrification	122	5 (4.1 ± 0.80)[c]	68 (55.6 ± 5.75)[b]
Taxol + vitrification	116	12 (10.3 ± 1.27)[d]	81 (68.1 ± 1.58)[ab]

[abcd] Values with different superscripts within same column are significantly different at 0.05 level.

More than 40 oocytes in very group were examined each time, and experiments were repeated four times.

In experiment 2, the effect of different treatments and vitrification on oocyte survival, MT and chromosomes, and developmental competence was examined. *In vitro* matured oocytes were randomly classed into the following four groups:

1. *Control*. No treatment was performed.
2. *Toxicity test of VS/WS*. Oocytes were exposed to ES for 90 s and VS for 30 s, and then rinsed in WS (0.5, 0.25 and 0.125 mol sucrose/l/m-PBS^{-} solution), each for 2 min.
3. *Vitrification*. Oocytes were vitrified and warmed as in the OPS vitrification procedure mentioned above.
4. *Taxol plus vitrification*. Oocytes were pretreated with 1 μmol Taxol/l for 30 min at 39 °C and vitrified-warmed as in the OPS vitrification procedure.

After treatment and/or vitrification/warming, half of the oocytes from each group were fixed and labeled for examination of MT and chromosomes by confocal microscopy. Another half of the oocytes were examined for viability and survival. Only survival oocytes were further activated, and their parthenogenetic developmental competence was assessed by cleavage and blastocyst development.

Statistical analyses

All data are presented as the mean ± S.E.M. All percentage data were subjected to arcsine transformation before statistical analysis. Data were analyzed by one-way ANOVA by Duncan's test. A value of $P < 0.05$ was considered statistically significant.

Results

Experiment 1

Effect of Taxol and FDA pretreatment

As shown in Table 1, the developmental competence of MII oocytes after Taxol pretreatment or FDA staining was the same as that in the control, showing no statistical differences ($P > 0.05$) in the cleavage rates (78.8–85.2%), blastocyst formation rates (21.9–27.9%) and mean numbers (24.0–26.2) of nuclei in the blastocysts.

Experiment 2

FDA staining and survival assessment of oocytes

Figure 1 shows normal phase-contrast images (panels A–E) and fluorescence images (panels A′–E′) of oocytes. Only cytoplasm was stained green by FDA. After treatment and short-time culture, the oocytes exposed to VS/WS could recover normal shape (Fig. 1B and C), and their relative viability was the same as control oocytes. However, after vitrification and warming procedures, some oocytes did not recover normal morphology (Fig. 1D and E). These oocytes were considered as nonsurviving: the cytoplasm was very dark (Fig. 1D, arrowhead) or uneven (Fig. 1E, arrow), or the zona pellucida was not smooth (Fig. 1D, arrow) or swollen (Fig. 1D, arrowhead).

Chromosomes and spindle configuration of oocytes after treatment

As shown in Table 2, most oocytes had normal spindle configuration (76.8%) and chromosome alignment (81.6%) in the control. Toxicity treatment with VS/WS reduced the rate of normal spindle configuration to 38.5%, but had little influence on the rate (73.5%) of normal chromosome alignment in the oocytes. After vitrification and warming, however, 4.1–10.3% of oocytes had normal spindle configuration, and 55.6–68.1% of oocytes had normal chromosome alignment (Fig. 2). When Taxol was used before vitrification, the rate of oocytes with normal spindles was significantly ($P < 0.05$) higher (10.3%) than with vitrification without Taxol pretreatment (4.1%).

Survival and development of the treated oocytes

Survival rates of oocytes after treatment are shown in Table 3. After exposure to VS/WS, most of the oocytes survived, although the survival rate (83.3%) was lower than that in the control (100%). However, 62.1% (Taxol plus vitrification) and 53.8% (vitrification) of the oocytes survived after vitrification and warming for 30 min, rates that were significantly ($P < 0.05$) lower than those in control and toxicity groups.

As shown in Table 3, 90.9% of the control oocytes developed to two-cell stage after 48-h culture and 26.6% reached blastocyst stage after 144-h culture. However, in the toxicity-tested group, both cleavage rate and blastocyst formation rate were reduced significantly (53.2% and

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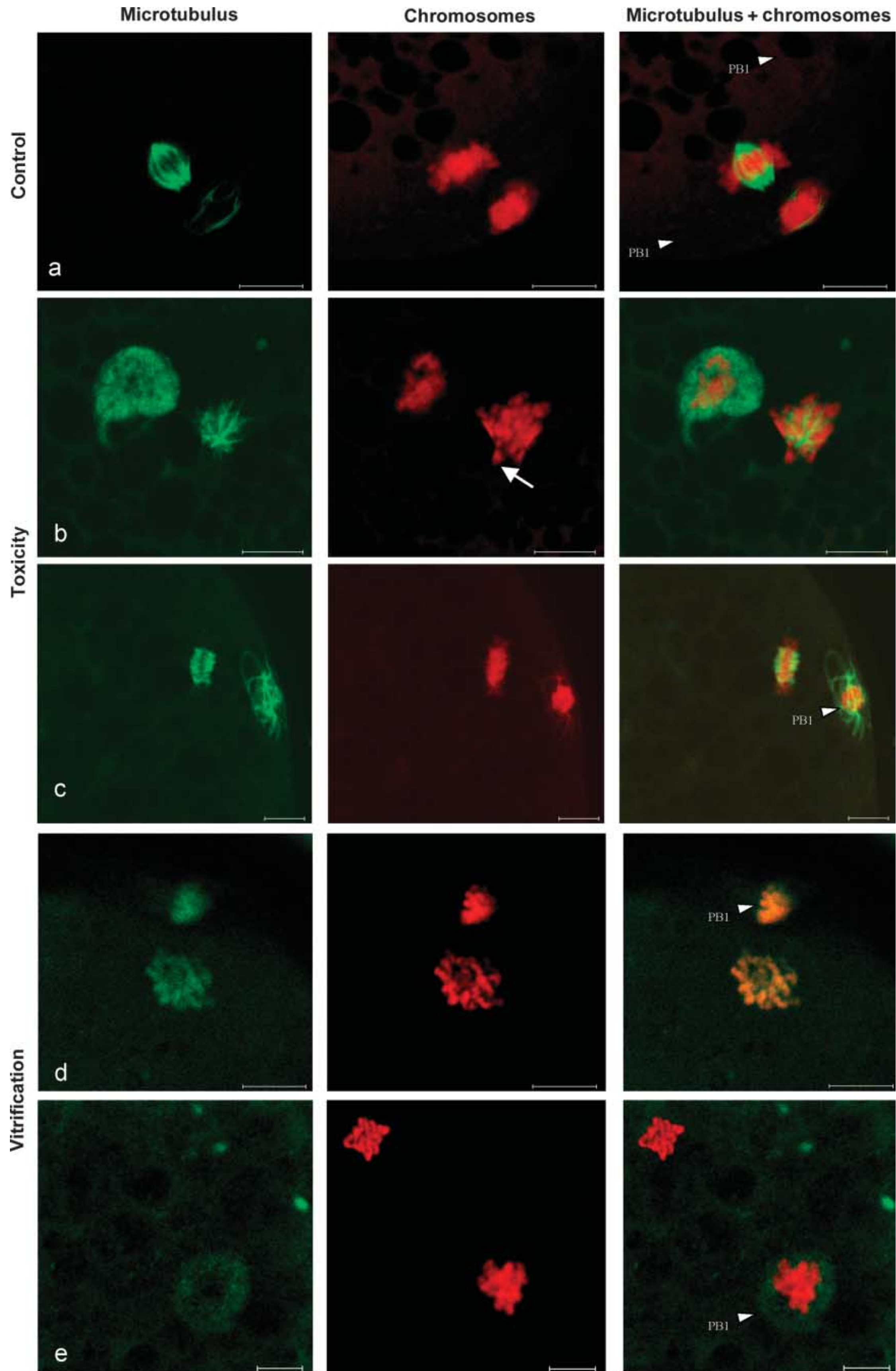

Figure 2 (Continued)

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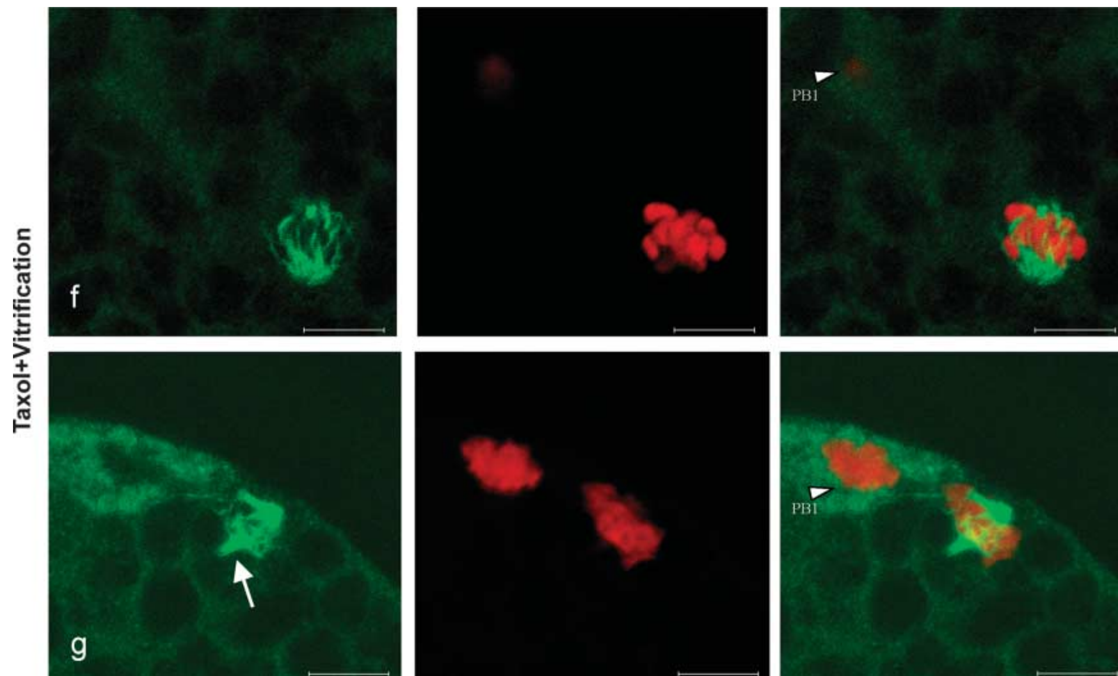

Figure 2 Confocal micrographs of spindles and chromosomes in the porcine oocytes exposed to vitrification solution or after vitrification. Panel (a) shows an oocyte (without treatment) with normal spindle configuration, indicating a barrel-shaped structure and the chromosomes arranged on the equator of the metaphase plate. Panels (b) and (c) show oocytes exposed to ES-VS and WS with abnormal spindle, with some chromosomes displaced from the metaphase plate (b, arrow), and an oocyte with a normal spindle and chromosomal organization. Panels (d) and (e) show oocytes vitrified without taxol pretreatment. The spindles are obviously disorganized (d) or almost completely disassembled (e). The chromosomes are dispersed and less condensed. Panels (f) and (g) show oocytes vitrified after taxol pretreatment. The spindle is obvious with rounded poles and the chromosomes arranged on the plate of the equator but condensed (f), or microtubules from one pole to another are assembled into big bundles (arrow) and the spindle shows pointed poles but normal chromosomes (g). Green images represent microtubules, red images represent chromosomes, and yellow images represent the merging of green and red images. Arrowheads indicate the first polar body (PB1). Bar = 10 μm.

3.8% respectively) as compared with control ($P < 0.05$). Vitrification further reduced both cleavage and blastocyst formation (5.6–24.3% and 0% respectively). The cleavage rates of oocytes vitrified by Taxol pretreatment (24.3%) was higher ($P < 0.05$) than that without Taxol pretreatment (5.6%), although all two-cell embryos did not develop to blastocyst stage.

Discussion

The present study indicates that porcine oocytes matured *in vitro* can be vitrified by the OPS method, and pretreatment of oocytes with Taxol significantly improved the developmental competence of oocyte after vitrification.

Our present study also indicates that Taxol treatment itself does not affect oocyte quality, which has been assessed by the blastocyst development of parthenogenetically activated oocytes. The viability or survival of oocytes was affected by both VS/WS and vitrification/warming procedures. FDA has been previously used to assess the viability of mouse embryos (Mohr & Trounson 1980), porcine GV oocytes (Didion *et al.* 1990) and bovine oocytes (Zeron *et al.* 1999). Oocyte viability was evidenced by intense fluorescence; nonfluorescence indicated nonviability. Therefore, FDA staining is useful and more objective in survival assessment. In the present experiment, the validity of MII porcine oocytes was proved by FDA staining.

Cryopreservation always induces oocyte damage. Cryodamage to oocytes is caused by mechanical, thermal or chemical factors. Vitrification can prevent the formation of intracellular ice crystals that may cause mechanical damage (Rall & Fahy 1985). In the present study, the inner diameter of the pulled straws allows just a porcine oocyte (without cumulus cell) to go through by negative pressure, and the volume of VS can be limited (~1 μl VS in the pulled straw) so that the cooling rate can be increased. By this method, intracellular cryodamage to oocytes can be avoided or minimized. However, our results showed that porcine oocytes suffer irreversible damage after vitrification, especially on the meiotic spindle configuration. Furthermore, we found that spindles were also damaged when the oocytes were treated by EG and DMSO only, indicating that the cooling rate is not the only key factor to affect oocyte viability during vitrification. Further studies must determine how to optimize the vitrification procedure and modify the VS in order to achieve higher

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survival rates after vitrification of porcine oocytes. These results also suggest that injury to the oocyte cytoskeleton is an important cryoinjury during vitrification.

Chemicals in the VS are the major factors that affect oocyte survival rate during vitrification. Oocytes exposed to cryoprotective agents (CPAs) could depolymerize MT (Johnson & Pickering 1987). This depends on the concentration of CPAs. Chen *et al.* (2000) found that the spindles in mouse oocytes became disorganized or disappeared when they were exposed to VS containing 5.5 mol EG/l and 1.0 mol sucrose/l. In the present experiment, when oocytes were exposed to ES-VS and WS, their developmental competence was significantly reduced, suggesting that these chemicals, and their concentrations and exposing time are not suitable for porcine oocyte vitrification under the present experimental conditions, and that further studies are necessary.

The survival rate of vitrified oocytes is not sufficient to evaluate potential quality. Testing the developmental competence of oocytes is indispensable to examine their viability. Because of the high rate of polyspermy in *in vitro* fertilized pig oocytes (Nagai 1994), artificial activation, especially electrical activation of oocytes, becomes a useful approach to test porcine oocyte developmental competence. The electrical activation method in porcine oocytes has proved to be effective and has been used in somatic nuclear transfer in pig (Onishi *et al.* 2000, Polejaeva *et al.* 2000); 25–26.6% of oocytes developed into blastocysts in our control experiments. However, vitrification and exposing the oocytes to VS/WS dramatically reduced oocyte development, indicating that vitrified porcine oocytes have very low viability even if they survive.

In the present study, our results indicate that low concentrations of Taxol did not reduce the development of parthenogenetically activated porcine oocytes. Taxol can lower the critical tubulin concentration *in vitro* for assembly and promote tubulin to reassembly *in vitro* (Schiff *et al.* 1979, De Brabander *et al.* 1981), and it can be used to investigate the system of microtubule assembly/disassembly in pig oocytes at different developmental stages (Sun *et al.* 2001). MT in porcine oocytes are vulnerable to cryoinjury *in vitro* (Didion *et al.* 1990, Wang *et al.* 2001, Liu *et al.* 2003). Morphology of MT and microfilaments in porcine oocytes can resume after 1-h culture, after oocytes are thawed or warmed, and the distribution of MT and microfilaments recovers, but low temperature leads to distinct impairment of the fertilization of oocytes and the growth of embryos (Wu *et al.* 1999). Moreover, cryopreservation can affect configuration of MT, block the formation of the first meiotic spindle (Rojas *et al.* 2004), and lead to cell death or abnormal development (Shaw *et al.* 2000). In the present experiment, the cleavage rate of oocytes vitrified by OPS increased from 5.6% to 24.3% by pretreatment of Taxol, the survival rates of porcine MII oocytes vitrified with Taxol pretreatment increased from 53.9% to 62.1%, and normal spindle configuration rates increased from 4.1% to 10.3%. From these results, it can be concluded that Taxol pretreatment of oocytes before vitrification significantly improves the parthenogenetic development of oocytes after subsequent vitrification and warming. These results also indicate that stabilization of the MT in the oocytes may reduce the damage induced by vitrification.

Chen *et al.* (2000) examined the intracellular cryodamage of mouse oocytes by immunofluorescence staining of spindles and found that 1–3-h incubation after thawing can significantly increase rates of oocytes with normal spindles. In the present study, rates of oocytes with normal spindles remained low after 30-min incubation. It is still unclear whether normal cytoskeleton configuration in porcine oocytes can be increased by longer incubation.

Partial removal of lipid from porcine embryos (Nagashima *et al.* 1995) and oocytes (Nagashima *et al.* 1999) also reduces sensitivity to chilling and improves development after vitrification. There are abundant cytoplasmic lipids in porcine oocytes, and the amount of triglyceride, the main component of the lipids, is threefold higher than that in cattle and sheep oocytes (McEvoy *et al.* 2000). These lipids may play a significant role in cell structure and function, especially in biological membranes, but it is unclear why pig oocytes contain so much lipid (Sturmey & Leese 2003). The maturation of vitrified immature porcine oocytes can be improved (from 3.7% to 15.0%) by partial removal of cytoplasmic lipids (Park *et al.* 2005). The effect of delipidization on oocyte vitrification has been reported (Nagashima *et al.* 1999), and it was found that about 56% of the oocytes vitrified after delipidization survived, and 6% of oocytes could develop to morula stage after fertilization by subzonal sperm injection.

In conclusion, we found that both exposure to VS/WS and vitrification/warming procedures can cause spindle

Table 3 Effect of different treatments on development of parthenogenetically activated oocytes.

Groups	No. of oocytes examined	No. (%) of surviving oocytes	No. (%) of oocytes developed to	
			Two-cell stage	Blastocyst stage
Control	122	122 (100 ± 0)[a]	112 (90.9 ± 3.59)[a]	32 (26.6 ± 2.65)[a]
Toxicity	138	115 (83.3 ± 3.48)[b]	72 (53.2 ± 10.36)[b]	5 (3.8 ± 2.01)[b]
Vitrification	158	85 (53.8 ± 5.62)[c]	9 (5.6 ± 0.79)[c]	0[b]
Taxol + vitrification	132	82 (62.1 ± 1.14)[c]	32 (24.3 ± 2.78)[d]	0[b]

[abc] Values with different superscripts within same column are significantly different at 0.05 level.

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disassembly and chromosome misalignment, reducing the survival and developmental competence in porcine oocytes. Taxol pretreatment can reduce the damage induced by vitrification, thus improving the development of porcine oocytes after vitrification. As no blastocyst development was obtained after the oocytes were vitrified under the present conditions, further studies are necessary to optimize the VS/WS or vitrification/warming procedures.

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