Production of Plasminogen Activators during *In Vitro* Maturation of Fresh or Frozen-Thawed Oocytes in the Pig

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ABSTRACT

This study were examined whether plasminogen activators (PAs) are produced by porcine fresh or frozen-thawed cumulus-oocytes complexes (COCs) and cumulus cell free-oocytes. In fresh or frozen-thawed COCs and oocytes for 0 hour cultured, no activity of PAs was detected. However, at 24 hours of culture urokinase-type plasminogen activator (uPA) was detected in COCs and denuded oocytes. In the frozen-thawed COCs and cumulus cell free-oocytes cultured for 24 hours, no PAs were observed. After COCs were cultured for 48 hours, tissue-type plasminogen activator (tPA) and tPA-PAI were observed in COCs only. In the frozen-thawed COCs and cumulus cell free-oocytes cultured for 48 hours, no PAs were observed. These results suggest that uPA, tPA and tPA-PAI are produced by porcine COCs, but only uPA by oocytes during maturation for 24 hours. Only tPA, and tPA-PAI are produced by COCs cultured for 48 hours, and no PAs are produced by denuded-oocytes cultured for 48 hours. In all of the frozen-thawed groups, no PAs are observed by COCs and denuded-oocytes.

(Key words : *In vitro* maturation, Pig, Frozen-thawed oocytes, Plasminogen activators)

INTRODUCTION

Ovaries obtained from the slaughterhouse have become a widely used source of oocytes for procedures such as IVF, cloning or other reproductive technologies. The cryopreservation of embryos of most domestic species has become a routine procedure in embryo transfer, and recently, advances have been made in the cold storage of mammalian oocytes. The ability to sustain viable oocytes and embryos from mammalian species at low temperature for prolonged periods of time has important implications to basic and applied biotechnology. Thus far the oocytes of some mammalian species have been cryopreserved successfully through slow freezing procedures or vitrification, but rates of subsequent fertilization and development are much lower than those obtained using fresh oocytes. The first successful blastocysts and offspring achieved from cryopreserved oocytes in cattle were obtained by slow-rate freezing (Lim et al., 1991a). This was followed by reports of similar development rates and offspring after vitrification (Hamano et al., 1992). Since these initial attempts, new vitrification techniques for the cryopreservation of bovine oocytes have emerged (Martino et al., 1996a; Arav and Zeron, 1997; Vajta et al., 1998a). The open pulled straw (OPS) method developed by Vajta et al. (1998b) uses a minimum amount of vitrification solution and is reported to achieve blastocyst rates of 25% for *in vitro* matured/fertilized bovine oocytes.

The loss of developmental potential after cryopreservation makes mammalian oocytes probably one of the most difficult cell types to cryopreserve. Indeed, the survival and developmental capacity of the cryopreserved oocyte are greatly impaired, probably as a consequence of morphological and cytological damage induced by the cryopreservation process. Ultrastructural studies on vitrified bovine oocytes have revealed that intercellular communication between the cumulus cells and oocytes might be interrupted and that the zona pellucida may be modified by premature cortical granule release (Fukui et al., 1995). Changes in the structure of the cytoskeleton, mitochondria, cortical granules and nucleoli have also been observed in bovine oocytes (Saunders and Parks, 1999; Wu et al., 1999; Hyttel et al., 2000). However, the factors most likely to affect

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the success of bovine oocytes cryopreservation are the particular structural and functional characteristics of the oocytes, such as its size cumulus-oocytes complex, maturation status (Men et al., 2002; Le and Massip, 1999; Hochi et al., 1998) and the dynamics of subcellular organelles during meiosis (Shamsuddin et al., 1996).

Plasminogen activators (PAs) are serine proteases, known to be secreted by a large number of cell types that convert plasminogen to plasmin. PAs are classified into two groups on the basis of molecular mass: urokinase-type PA (uPA), which is secreted as an inactive single-chain molecule of 31~54 kDa, and tissue-type PA (tPA), which is secreted by an active form with a molecular mass of around 70 kDa. PAs play roles not only in fibrinolysis but also in various reproductive processes including ovulation and implantation.

Although PA activity in rat (Liedholm and Astedt, 1975), mouse (Strickland et al., 1976; Sherman, 1980), porcine (Fazleabas et al., 1983), ovine (Menino et al., 1989) and bovine (Menino and Williams, 1987; Kaaekuahiwi and Menino, 1990; Dyk and Menino, 1991; Berg and Menino, 1992) embryos, and trypsin-like activity in hamster (Gwatkin et al., 1973) and mouse (Wolf and Hamada, 1977) oocytes have been reported, reports describing proteases in the mammalian oocytes itself are limited. The first identification of an oocyte-produced protease was reported by Huarte et al. (1985) who found that resumption of meiosis in mouse and rat oocytes triggers the production of tPA. The production of tPA and uPA in cultured cumulus-oocyte complexes (COCs) has also been reported in rats (Liu et al., 1986; Liu et al., 1987). The activity of tPA, but not of uPA, in rat oocytes and cumulus cells is stimulated by FSH and forskolin (Liu et al., 1986). It has been suggested that the stimulation of PA activity in rat COCs by FSH and GnRH is through protein kinases and C, respectively (Ny et al., 1987; Salustri et al., 1985). It was also reported that PA production by porcine COCs is influenced by protein kinases A and C and kinase inhibitors (Kim and Menino, 1995).

Furthermore, to our knowledge no information on the production of PAs by porcine oocyte is available. Therefore, the present study was undertaken 1) to identify PAs in porcine COCs maturation, 2) to identify PAs in the frozen-thawed porcine COCs maturation, and 3) to clarify the effects of cumulus cells on PA production by oocytes during *in vitro* maturation.

MATERIALS AND METHODS

In Vitro Maturation

Porcine ovaries were collected from a local slaughterhouse and transported to the laboratory at 35~37℃ in 0.85% saline. Cumulus-oocytes complexes (COCs) with uniform ooplasm and a compact cumulus cell mass were prepared in Hepes-buffered TALP medium containing 0.1% (v/v) polyvinylalcohol (H-TL-PVA). Group of 15 COCs were matured in 100 μL of BSA-free NCSU23 (NCSU23; Petters and Wells, 1993) supplement with 10% (v/v) pig follicular fluid (pFF), 0.6 mM cysteine, 10 IU/mL human chorionic gonadotropin (hCG; Sigma, St Louis, MO) and 10 IU/mL pregnant mare's serum gonodotropin (PMSG; Sigma) under mineral oil at 38.5℃, 5% CO_2, for 24 and 48 hours.

OPS Vitrification

The vitrification procedure was essentially as described by Vajta et al. (1998a). All manipulations were performed on a 41℃ hot plate in a room at 25~27℃. Oocytes were first equilibrated in a holding medium (TCM199-HEPES supplemented with 20% FBS) for 3 min and initially dehydrated by 3 min exposure to 1 mL 10% EG + 10% DMSO in TCM199-HEPES + 20% FBS + 0.6 M sucrose. Straws were loaded with three to five oocytes each by touching the surface of a 1~1.5 μL droplet of vitrification solution containing the oocytes with the narrow tip of the OPS. The loaded straw was then directly plunged into liquid nitrogen within 25 sec. Warming was performed by exposing the OPS straw to air for 3 sec prior to directly immersing the narrow tip of the straw into 1.2 mL of TCM199-HEPES medium containing 20% FBS + 0.3 M sucrose. The oocytes were directly expelled into the medium after the vitrified medium became liquid. After about 5 min, the oocytes were transferred into 0.15 M sucrose for further rehydration. They were then washed in the 0.8 mL of holding medium for 5 min and *in vitro* maturation continued in the original dish for 24 and 48 hours culturing.

Electrophoresis and Zymography

SDS-PAGE and zymography were carried by the procedures described by Dyk and Menino (1991) with a slight modification. Frozen samples were thawed and homogenized with a sonicator. As a standard of tPA, stock solution of 0.5 ng/mL tPA from human melanoma cell culture was prepared in sample buffer. Each homogenized sample (15 μL) being compared in each experiment, a stock solution of human tPA (5 μL) and molecular mass markers (10 μL) were placed in a castellated well in a 4.5 % stacking gel with a 10% separating gel. Electrophoresis was conducted at 20 mA for 2 hours.

After electrophoresis, the polyacrylamide gels were gently shaken in 2.5% Triton X-100 for 30 min, rinsed with distilled water three times, and incubated for 30 min at 3 9℃ in PBS. Each gel was carefully laid on a casein-agar gel (zymogram) containing purified human plasminogen supported in a plastic chamber, and the other zymogram without shuman plasminogen was overlaid as a control to detect nonspecific proteases. For preparing zymogram, 0.8 g of nonfat dry milk was dissolved in 19 mL of buffer containing 0.0013 M $CaCl_2$, 0.1 M glycine, 0.038 M Tris, and

0.005 M sodium azide. The mixture was heated to 55°C and combined with 19 mL of 2% (v:v) melted agarose maintained at 55℃. One milliliter of purified human plasminogen stock solution was added to 19 mL of the warmed mixture to yield a final plasminogen concentration of 50 μg/mL. For zymograms without human plasminogen, 1 mL water was added to 19 mL of the warmed mixture. Each 10 mL of these mixtures was cast onto a warmed dish (100 × 15 mm; Falcon 1012; Becton and Dickinson) and allowed to cool. Each polyacrylamide gel exposed simultaneously to the two zymograms, i.e., casein-agar gel with and without plasminogen, was incubated at 39℃ for 12~18 h to allow the development of lytic bands. After the distance from the edge of the separating gel to the center of the clear lytic bands in each lane was measured, incubation of zymograms was terminated by separating the gels. The the zymograms were fixed with 3% (v:v) acetic acid for 10 min, rinsed under tap water, dried, and stained with 0.1% (w:v) Amido Black 10B dye (Bio-Rad) in acetic isopropanol (25% [v:v] isopropanol and 10% [v:v] acetic acid in water) for permanent storage. Polyacrylamide gels were stained with 0.05% Coomassie Brilliant Blue R-250 dye (Bio-Rad) in acetic isopropanol overnight without fixing and rinsing. The molecular mass of PAs was determined from the regression calculations of log molecular mass and relative mobility.

Orcein Staining

At the end of examination, cumulus cells removed by repeated passage through a fine pipette. The cumulus-free oocytes were transferred on to the center of a glass slide with four vaselin spots, gently compressed with a cover slip, immersed in 25% acetic alcohol for 2~3 days for complete fixation and stain with 1% orcein in 45% acetic acid. Excess stain was removed by infiltrating aceto-glycerol medium under the phase-contrast microscope at a magnification of ×200 or ×400.

Experimental Designs

Experiment 1

In experiment a): To identify the presence of the plasminogen activators in the GV stage of cumulus- oocytes complexes (COCs) or cumulus cell free-oocytes. Parts of porcine COCs with uniform ooplasm and a compact cumulus cell mass were prepared in Dubelcco's Phosphate Buffered Saline (D-PBS ; Gibco, USA) added in 0.1% polyvinyl-alcohol (PVA; Sigma, USA). COCs were aspirated from 2 to 6 mm follicles with a 10 mL syringe with 18-G needle. Parts of the collected COCs were washed three times in D-PBS-PVA and parts of COCs were freed from the cumulus cells by repeated passage through fine pipette in D-PBS-PVA with 0.1% hyaluronidase (sigma). The COCs and denuded oocytes were putted into microtubes containing 15 μL of sample buffer (5% [w:v] SDS, 20% [v:v] glycerol, and 0.0025% [w:v] bromophenol blue in 0.125 M Tris-HCl buffer) and frozen at -20℃ until used for zymographic analysis.

In experiment b): To identify the presence of plasminogen activators in the GV stage of frozen-thawed COCs or cumulus cell free-oocytes. After the COCs were washed three times in D-PBS-PVA, the COCs were frozen-thawed by OPS method. Parts of COCs were freed from cumulus cells. The frozen-thawed COCs and cumulus cell free-oocytes were sampled for determination of PAs activity.

Experiment 2

In experiment a): To determine the changes of PAs activity in COCs or cumulus cell free-oocytes after maturation for 24 hours. The COCs were washed three times in D-PBS-PVA and then cultured in NCSU-23 medium for 24 hours. After 24 hours cultured, parts of COCs were freed from cumulus cells. The denuded oocytes and COCs were sampled for determination of PAs activity.

In experiment b): The GV stage COCs were frozen-thawed and then cultured for 24 hours. After cultured, the COCs and denuded oocytes were used for zymographic analysis.

Experiment 3

In experiment a): To determine the changes of PAs activity in COCs or denuded oocytes after maturation for 48 hours. The methods is the same as the experiment 2.

In experiment b): The GV stage COCs were frozen-thawed by OPS method and then cultured for 48 hours. The frozen-thawed COCs or denuded oocytes cultured for 48 hours were sampled for determination of PAs activity.

Statistical Analysis

Statistical analyses of data were carried out by ANOVA and fishers protected least significant difference test using the SAS program. A probability of $P<0.05$ was considered to be statistically significant.

RESULTS

The morphological changes of nuclear during *in vitro* maturation in porcine oocytes were shown as the Table 1. Most of fresh oocytes cultured for 24 hours were reached the prophase-I stage (70.7%) and a little of them reached Metaphase-I stage (5.7%). The percentage of oocytes matured with metaphase-II stage was significantly lower in frozen-thawed oocytes (5%) compared to the fresh oocytes (72.8%) cultured for 48 hours. However, after cultured for 24 hours the fresh and frozen oocytes reached to meta

Table 1. The morphological changes of nuclear during *in vitro* maturation in porcine oocytes

Conditioins	No. of oocytes cultured	No.(%) of oocytes at stage of					
		GV	P-I	A-I	M-I	T-I	M-II
24h fresh	123	21(17.0)	87(70.7)	0(0.0)	7(5.7)	1(0.8)	4(3.3)[b]
24h frozen	106	26(24.5)	66(62.3)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
48h fresh	136	10(7.4)	9(6.6)	2(1.5)	7(5.1)	3(2.2)	99(72.8)[a]
48h frozen	118	46(38.9)	50(42.3)	0(0.0)	4(3.4)	0(0.0)	6(5.0)[b]

phase-II stage percentage was 3.3% and 0%.

No Detection of PA Activity in COCs Cultured for 0 hour

No PA activity was detected either in denuded oocytes or in oocytes just after aspiration of COCs from follicles (Fig. 1). Also, no PA activities were observed in porcine frozen-thawed COCs or oocytes cultured during 0 hours.

Detection of PA Activity in COCs Cultured for 24 h

One plasminogen-dependent lytic zone (58.5 kDa) was detected in COCs and denuded oocytes cultured for 24 h (Fig. 2). Addition of amiloride to the zymogram eliminated activity in the zone of 58.5 kDa, suggesting that this band was a uPA. However, the PA activity was not detected in the frozen-thawed COCs and denuded oocytes cultured for 24 hours.

Detection of PA Activity in COCs Cultured for 48 h

Two plasminogen-dependent lytic zones (70 kDa, 114 kDa) were detected in COCs cultured for 48 hours (Fig. 3). Addition of amiloride to the zymogram, the activity in these two zone of 70 kDa and 114 kDa, was not eliminated, suggesting that these represented different forms of PA.

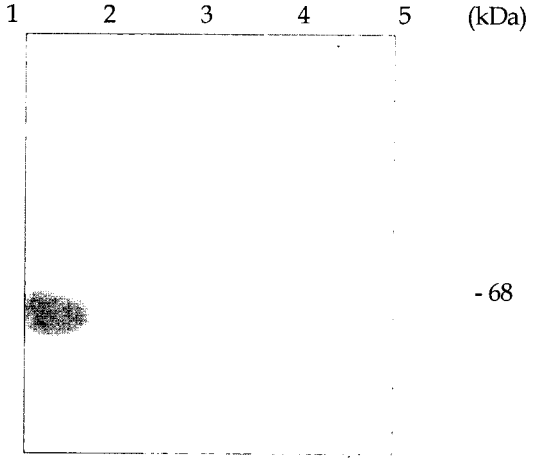

Fig. 1. Zymographic analysis of fresh and frozen-thawed porcine COCs and cumulus cell free-oocytes cultured for 0 hours. 1: human tPA, 2: COCs, 3: oocytes, 4: frozen-thawed COCs, 5: frozen-thawed oocytes.

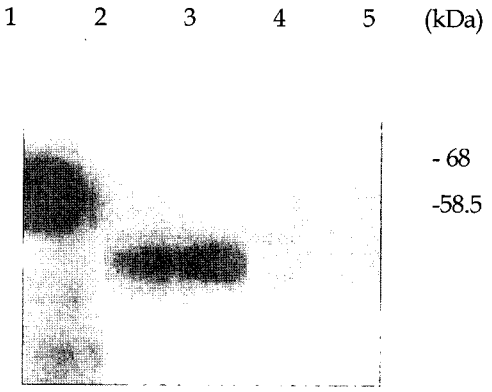

Fig. 2. Zymographic analysis of fresh and frozen-thawed porcine COCs and cumulus cell free-oocytes cultured for 24 hours. 1: human tPA, 2: 24 hours COCs (uPA: 58.5 KDa), 3: 24 hours oocytes (uPA: 58.5 KDa), 4: 24 hours frozen-thawed COCs, 5: 24 hours frozen-thawed oocytes.

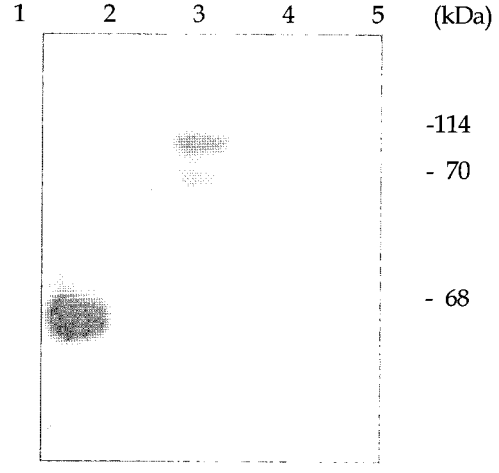

Fig. 3. Zymographic analysis of fresh and frozen-thawed porcine COCs and cumulus cell free-oocytes cultured for 48 hours. 1: human tPA, 2: 48 hours oocytes , 3: 48 hours COCs, (tPA-PAI: 114 kDa, tPA: 70 kDa), 4: 48 hours frozen-thawed COCs, 5: 48 hours frozen-thawed oocytes

Since PA activity and form an SDS-stable complex with a high molecular mass retained in the zymogram, it is suggested that the 114 kDa band was a tPA-PAI complex

and the 70 kDa band was a tPA.

DISCUSSION

The present study demonstrates that in the fresh or frozen-thawed COCs and denuded oocytes for cultured 0 hour, no activity of PAs was detected. However, after cultured for 24 hours, uPA was observed in the COCs and denuded oocytes. Also, in the frozen-thawed COCs and denuded oocytes cultured for 24 hours, no PAs were observed. After COCs were cultured for 48 hours, tPA and tPA-PAI were observed. On the other hand, in the denuded oocytes cultured for 48 hours, no PAs were observed. In the frozen-thawed COCs and oocytes cultured for 48 hours, no PAs were also observed.

In present study, it has been found that three plasminogen-dependent protease are produced by porcine COCs during maturation *in vitro*. The resistance to amiloride treatment in zymographic analysis and molecular mass suggested that the 58.5 ± 3.5 kDa, 79.0 ± 3.0 kDa, and 113.5 ± 6.5 kDa species that were detected in COCs and oocytes are uPA, tPA, and tPA-PAI, respectively. Although the activity of these PAs was not detected in COCs and denuded oocytes freshly collected from follicles, it was detected during maturation *in vitro* for 24, and 48 hours. In frozen-thawed group, however, no PAs was detected. The increase of PA activity in COCs during maturation *in vitro* or *in vivo* is reported in rats (Liu et al., 1986). However, the type of PAs detected in COCs seems to be different according to the different species: in rat, low amounts of tPA are detected in freshly obtained COCs, but both tPA and uPA activity increases during maturation *in vivo* (Liu et al., 1987) and *in vitro* (Liu et al., 1986). Although the precise role of PAs in COCs has not yet been elucidated, their possible involvement in last period of maturation of oocytes and ovulation (Liu et al., 1987; Beers et al., 1975; Huseh, et al., 1988), or cumulus expansion or dispersion (Liu et al., 1986; Liu et al., 1987) is suggested.

It is reported that rat denuded oocytes freed from cumulus cells just after collection from follicles do not contain tPA activity or contain (Liu et al., 1986; Liu et al., 1987; Bicsak et al., 1989) low amounts, but the activity was time-dependently increased during maturation *in vivo* (Liu et al., 1987; Bicsak et al., 1989) or *in vitro* without cumulus cells (Huarte et al., 1985; Liu et al., 1986). An increase of tPA activity during maturation *in vitro* is also reported in cumulus-free mouse oocytes (Huarte et al., 1985). In these species, however, uPA and tPA-PAI activity are not detected in maturing oocytes. In rats and mice, resumption of meiosis triggers the production of tPA by oocytes, probably by translation of stored mRNA (Huarte et al., 1985; Huarte et al., 1987a,b). It has been also demonstrated that rat oocytes contain mRNA for tPA, suggesting that oocytes synthesize tPA themselves and not simply take up tPA from the extracellular space (Bicsak et al., 1989).

However, these findings in rats and mice are not consistent with those of the present study using porcine oocytes. In the present study, no PA activity was detected in oocytes just after collection from follicles; but after 24 hours of maturation *in vitro*, only uPA, but not tPA and PAI, activity was detected at COCs and denuded oocytes. After 48 hours of cultured, tPA and PAI activities was detected in COCs, but in the denuded oocytes cultured for 48 hours, no activities was detected. It is suggested that tPA, and tPA-PAI activities may not be of oocytes origin but is acquired from cumulus cells. In all groups of the frozen-thawed oocytes, no activities was detected, suggesting that frozen-thawed COCs and oocytes, no activities was detected. It is not clear how oocytes acquire the ability to produce uPA from cumulus cells, but it is possible that tPA, and tPA-PAI from cumulus cells, but it is possible that tPA and tPA-PAI produced by cumulus cells is transported into oocytes through gap junctions between both cells, since cumulus expansion is not extensive, and thereby gap junctions may still be kept firmly, during the maturation. However, there is no direct evidence for production of tPA, tPA-PAI by cumulus cells without the influence of the oocytes because cumulus cells from which oocytes had been removed were not cultured in the present study. As another possibility, some unknown signals from cumulus cells may stimulate oocytes to produce tPA, tPA-PAI.

Huarte et al.(1985) have suggested that spontaneous increases in oocyte tPA activity correlated with germinal vesicle breakdown in rats and mice. These results are consistent with further reports showing the presence of tPA activity in rat oocytes but again are not consistent with results of the present study using porcine oocytes. In the present study, nuclear maturation of porcine oocytes was not directly examined, but 60~75% of COCs cultured for 48 hours had a polar body, indication that possibly these oocytes completed meiotic maturation reaching metaphase II. Nevertheless, absolutely no production of uPA was observed in these oocytes, suggesting that there is no correlation between meiotic maturation and production of uPA in porcine oocytes. However, at present, the reason for the discrepancy between our results using porcine oocytes and others using rat and mouse oocytes is not known.

The OPS method developed by Vajta et al. (1998a), besides being simple and inexpensive, achieves a vastly increased cooling speed by reducing the volume to be vitrified and narrowing the insulating layer between the cooling agent and the vitrification solution. Using this method, these authors have reported rates of up to 50% cleavage and up to 25% blastocyst development after vitrification. Moreover, in accordance with the results of Martino et al. (1996a) and Rho et al. (2002), control oocytes exposed to cryoprotectant additive (CPA) without further cooling showed reduced development over controls, suggesting that osmotic shock plays an important role in

the success of cryopreservation procedures. This deficient developmental capacity of oocytes is most likely due to the abnormal cytoplasmic maturation of these porcine oocytes. Exposure to the cryoprotectants EG and DMSO had a drastic effect on the arrangement of microtubles and chromosomes in porcine oocytes, while the vitrification procedure seemed to more severely affect the spindle configuration of porcine oocytes.

Several authors have described that the main hurdle in developing successful protocols for the cryopreservation of mammalian oocytes is being able to preserve the integrity of the meiotic spindle when the oocytes are cooled. Temperature fluctuations directly affect the cytoskeletal and chromosome organization of mature bovine (Saunders and Parks et al., 1999; Aman and Parks, 1994) and human oocytes (Almeida and Bolton, 1995). The main consequence of cooling is pronounced depolymerization and disappearance of microtubule organizing centers. Chilling leads to the disassemble of spindle fibers within minutes, followed by an equally rapid reassembly of the spindle after the return to normal temperatures. Magistrini and Szollosi (1980) reported that the meiotic spindles of mouse oocytes were sensitive to cooling, with complete disassembly occurring after 45~60 min at 0℃. The effects of cooling on the spindle appeared to be reversible in the mouse oocytes, with normal spindle formation occurring after step-wise re-warming. Pickering et al. (1990) found that the meiotic spindle of human oocytes completely disassembled, and this was accompanied by chromosomal dispersion in 60 % of the oocytes after 30 min at room temperature. This meiotic spindle becomes completely disassembled when *in vitro* matured bovine oocytes are maintained for 10~20 min at 4℃ (Richardson and Parks, 1992). When pig oocyes were kept for 5 min at 4℃, microtubles in the spindles of most oocytes partially or completely disassembled (Liu et al., 2003).

Cryoprotectants are known to induce changes in microtuble organization in several species, including the mouse (Cooper et al., 1996; Vincent and Jonhson, 1992), rabbit (Vincent et al., 1989), human (Sathananthan et al., 1988) and cow (Saunders and Parks et al., 1999). The exposure of bovine oocytes to EG did not affect spindle arrangement after 20 min re-warming, but a lower percentage of oocytes with abnormal spindle configuration was seen after 1 and 3 hours (Saunders and Parks et al., 1999). At room temperature, the addition of a cryoprotectant (propanediol or DMSO) to rabbit oocytes led to disorganization of spindle mictotubules (Vincent, et al., 1989). In mouse oocytes, a similar effect of DMSO (Johnson and Pickering, 1987) and propanediol has been noted, and a consequent dispersal of chromosomes is often seen in both species.

Vitrification was, however, founded to modify the organization of actin filaments. Freezing has also been described to causes changes in the organization of cytoskeleton actin in rabbit and mouse oocytes (Vincent and Johnson 1992; George and Johnson,1993), although these changes were often reversible upon thawing. Dramatic effects on crytoskeletal actin were observed after freezing bovine oocytes (Saunders and Parks, 1999). Because of the association of microfilaments with other structures, it is possible that their disruption is the result of damage to another cell component such as the plasma membrane or mitochondria. Disruption of the cytoskeleton may be intrinsic to the changes in shape and shrinkage related to cryopreservation procedures, which in turn may lead to irreversibly changes in the structure of the plasma membrane or cytoskeleton so that it can no longer adjust to changing conditions, including those associated with fertilization. Thus, even when the normal microfilament distribution of oocytes is restored after CPA exposure and vitrification, irreversible alterations to other cell components may already have occurred such as the early release of cortical granule enzymes and zona hardening. These changes may eiher prevent fertilization completely or incompletely block polyspermy, both leading to decreased cleavage rates after insemination.

Generally, the ultrastructure of oocytes is particularly sensitive to the changes of temperature and extracellular osmotic pressure during freezing and thawing. Various cellelar damage, such as cytoskeleton disorganization, chromosome and DNA abnormality, spindle distintegration, premature cortical granule exocytosis and its related hardening of zona pellucida and plasma membrane disintegrity, are frequently found when oocytes are placed at a low temperature, below 0℃. All of these impairments negatively affect the developmental competence of frozen oocytes. Al-Hasani et al. (1987) reported that only 11% of cryopreserved oocytes from 48 patients were fertilized *in vitro*. Lower maturation, fertilization and cleavage rates were also found in slowly frozen oocytes than in fresh oocytes (Son et al., 1996). Park et al.(1997) reported that the high incidence of chromosome and spindle abnormalities was detected in frozen oocytes.

On the other hand, the maturation stage of oocytes at the time of freezing greatly affects the crypreservation capacity and survival after thawing. In another study using an animal model (Lim et al., 1991a), one-cell embryos has higher post-thawed survival and developmental capacities than oocytes frozen slowly at the metaphase-II (M-II) stage. Furthermore, more oocytes survived, fertilized and developed after slowly freezing at the M-II stage than after slowly freezing at the germinal vesicle (GV) stage (Lim et al., 1991b). To enhance the viability of oocytes after thawing, we should consider the type of cryopreservation programs and the stage of oocyte maturation at the time of freezing.

We used ethlene glycol (EG), which has higher membrane permeability and lower cytotoxicity than other cryoprotectants, as a permeable cryoprotectant for this method. Also, appropriate concentrations of sucrose were used as a nonpermeable cryoprotectant. The use of sucrose as a cryoprotectant contributes to regulating intracellular concentration of EG during equilibration and vitrification and

to effectively remove EG from oocyte cytoplasm during thawing and dilution procedures.

These results show that the frozen-thawed oocytes and COCs cultured for 0, 24, and 48 hours, no PAs activities were observed. Maybe various cellelar damage, such as cytoskeleton disorganization, chromosome and DNA abnormality, spindle disintegration, premature cortical granule exocytosis and its related hardening of zona pellucida and plasma membrane disintegrate, effected on oocytes to produce PAs.

These results strongly suggest that porcine oocytes and COCs have no ability to produce PAs at 0 hours. However, after porcine oocytes cultured for 24 hours can produce uPA. The tPA and tPA-PAI activity were founded at 48 hours of culure is not clear. The tPA and tPA-PAI may play a role in cytoplasmic maturation of oocytes, which results in normal fertilization and development of oocytes. In all groups of the frozen-thawed oocytes, there were no PAs observed.

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