Effects of Cooling on Meiotic Spindle Structure and Chromosome Alignment Within In Vitro Matured Porcine Oocytes

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ABSTRACT   Meiotic spindle structure and chromosome alignment were examined after porcine oocytes were cooled at metaphase II (M II) stage. Cumulus–oocyte complexes (COCs) collected from medium size follicles were cultured in an oocyte maturation medium at $39°C$, 5% CO_2 in air for 44 hr. At the end of culture, oocytes were removed from cumulus cells and cooled to 24 or $4°C$ for 5, 30, or 120 min in a solution with or without 1.5 M dimethyl sulfoxide (DMSO). After being cooled, oocytes were either fixed immediately for examination of the meiotic spindle and chromosome alignment or returned to maturation medium at $39°C$ for 2 hr for examination of spindle recovery. Most oocytes (65–71%) cooled to $24°C$ showed partially depolymerized spindles but 81–92% of oocytes cooled at $4°C$ did not have a spindle after cooling for 120 min. Quicker disassembly of spindles in the oocytes was observed at $4°C$ than at $24°C$. Cooling also induced chromosome abnormality, which was indicated by dispersed chromosomes in the cytoplasm. Limited spindle recovery was observed in the oocytes cooled to both 4 and $24°C$ regardless of cooling time. The effect of cooling on the spindle organization and chromosome alignment was not influenced by the presence of DMSO. These results indicate that the meiotic spindles in porcine M II oocytes are very sensitive to a drop in the temperature. Both spindle and chromosomes were damaged during cooling, and such damage was not reversible by incubating the oocytes after they had been cooled. *Mol. Reprod. Dev. 65: 212–218, 2003.* © 2003 Wiley-Liss, Inc.

Key Words: pig; oocyte; cooling; spindle; chromosomes

INTRODUCTION

Meiotic spindle is composed of microtubules and is the crucial structure in the oocyte for normal chromosome alignment and separation of maternal chromosomes during meiosis I and meiosis II. It has been found that meiotic spindles are very sensitive to low temperature in most mammalian oocytes (Magistrini and Szöllözi, 1980; Moor and Crosby, 1985; Pickering and Johnson, 1987; Pickering et al., 1990; Aman and Parks, 1994; Almeida and Bolton, 1995). Partial or complete disassembly of microtubules in the spindle was observed when oocytes were cooled to room or lower temperature (Moor and Crosby, 1985; Pickering and Johnson, 1987; Pickering et al., 1990; Parks and Ruffing, 1992; Aman and Parks, 1994; Almeida and Bolton, 1995). The recovery of spindle after oocytes were rewarmed was also examined but species-specific differences were found. For example, spindles of mouse oocytes underwent complete disassembly after 45–60 min at $0°C$, but most oocytes reformed spindles normally after rewarming (Magistrini and Szöllözi, 1980). However, spindles of human (Almeida and Bolton, 1995; Wang et al., 2001), bovine (Aman and Parks, 1994), and sheep oocytes (Moor and Crosby, 1985) exhibited only a limited recovery after cooling–rewarming treatment. The disrupted spindles also contributed to abnormal chromosome distribution (Eroglu et al., 1998) and insemination of such oocytes may cause failed fertilization or abnormal fertilization, such as aneuploidy.

To our knowledge, there is still no report to examine the effects of cooling on spindle morphology in in vitro matured porcine oocytes and their relationship with chromosome alignment. Therefore, in this study, experiments were designed to examine spindle structure and chromosome alignment of in vitro matured porcine oocytes after cooling and rewarming. It has been found that dimethyl sulfoxide (DMSO) is one of the most popular cryoprotectants used for cryopreservation of germ cells in mammals (Hunter et al., 1991; Candy et al., 1994; Bouquet et al., 1995; Hovatta et al., 1996) and it has been successfully used to freeze mammalian oocytes (Siebzehnruebl et al., 1989; Todorow et al., 1989a,b; Hunter et al., 1991; Pickering et al., 1991; Agca et al., 1998; Newton et al., 1999; Wu et al., 1999), early stage embryos (Siebzehnruebl et al., 1989; Van der Elst et al., 1995), blastocysts (Li et al., 1990; Li and Trounson, 1991; Oberstein et al., 2001), and ovarian tissues (Hovatta

et al., 1996). Therefore, in the present study, the effects of DMSO, as a cryoprotectant, during cooling were also examined to test if addition of DMSO in cooling solution can protect porcine oocytes from damages induced by cooling. Furthermore, as the morphology of meiotic spindle is one of the most important markers to predict the efficacy of cooling and cryopreservation (Johnson and Pickering, 1987; Vincent et al., 1989, 1990; Aigner et al., 1992; Rho et al., 2002), in the present study, we also examined the spindle morphology so as to address the relationship between spindle morphology and cooling.

MATERIALS AND METHODS
Medium

Unless otherwise stated, all chemicals used in this study were purchased from Sigma (Sigma Chemical Co., St. Louis, MO). The basic medium used for oocyte maturation was tissue culture medium (TCM)199 (with Earle's salts; Gibco, Grand Island) supplemented with 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 75 µg/ml potassium penicillin G, 50 µg/ml streptomycin sulphate, 0.1% polyvinyl alcohol (PVA), 10 IU hCG/ml, 10 IU eCG/ml, and 10 ng/ml epidermal growth factor.

Collection and Culture of Oocytes

Ovaries were collected from a slaughterhouse and transported to the laboratory ($\sim$3 hr) in warm (30°C) 0.9% (w/v) NaCl solution containing 75 µg/ml potassium penicillin G and 50 µg/ml streptomycin sulphate. Cumulus−oocyte complexes (COCs) were aspirated from medium size antral follicles (2–6 mm in diameter) and washed three times with HEPES−TL-PVA medium and two times with maturation medium. Each group of 50 cumulus-enclosed oocytes were then transferred to 500 µl maturation medium, which had been covered previously with paraffin oil in a polystyrene culture dish (35 mm × 10 mm, Nunc) and equilibrated in an atmosphere of 5% CO_2 in air for about 3 hr. COCs were cultured at 39°C for 44 hr in the same atmospheric conditions. In this IVM system, $\sim$85% of oocytes reach M II after 44 hr of culture. Because it is difficult to observe the first polar body in in vitro matured porcine oocytes, we use morphologically normal oocytes for experiments after culture.

Cooling of Oocytes

Matured oocytes were removed from the cumulus cells in maturation medium containing 0.1% hyaluronidase and then cooled by placing oocytes directly into 100 µl drops of HEPES−TL-PVA with or without 1.5 M DMSO to 24 or 4°C for 5, 30, or 120 min. The dishes were pre-cooled to 24 or 4°C for 2 hr. so the oocytes were rapidly cooled to 24 or 4°C in the study. After being cooled, some oocytes were fixed immediately and others that had been first cooled were returned to the maturation medium at 39°C for 2 hr and then fixed for immunofluorescence staining. For warming, the warming solution was kept at 39°C before oocytes were warmed so that rapid warming was performed in the study. Control oocytes were kept at 39°C in the maturation medium.

Immunofluorescence Staining

Oocytes were fixed in 3.7% paraformaldehyde in PBS for 30–40 min at room temperature. After fixation, oocytes were washed in PBS containing 0.5% Triton X-100 and 3% bovine serum albumin (BSA) for 40 min and then blocked in PBS containing 115 mM glycine and 3% BSA for 40 min at 37°C or overnight at 4°C. For microtubule detection, oocytes were incubated in PBS-Triton (0.01%) with mouse anti-α-tubulin (1:250) for 40–60 min, washed three times for a total of 15 min and then stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:80) for 40–60 min. After three additional washes, oocytes were stained with 10 µg/ml propidium iodide for 5 min for examination of nuclear status. All treatments were processed at 37°C. After being stained, oocytes were mounted on slides and examined with a Leica DM-IRB scanning confocal microscope with an argon laser (488 nm wavelength).

Spindles morphology in the oocytes was classified as three categories: (1) Normal spindles: barrel-shaped spindle with the chromosomes clustered in a discrete bundle at the metaphase plate and microtubules traversing the length of the spindle from pole to pole or extending from the spindle poles to chromosomes; (2) Abnormal spindles: microtubules were not organized to form typical spindles or some microtubules disassembled; (3) No spindle: no microtubules were found around chromosomes.

Chromosomes were classified as two categories: (1) Normal chromosomes: chromosomes aligned on the equator of the spindles or remained in one place in the cytoplasm if there were no typical spindles due to cooling; and (2) Chromosomes were scatted in the cytoplasm or dispersed in a few places in the cytoplasm.

Statistical Analysis

Experiments were replicated three times and the data were pooled for statistical analysis. The percentages were subjected to an arc-sine transformation, and the transformed values were analyzed by ANOVA.

RESULTS
Spindle Disassembly and Chromosome Alignment in In Vitro Matured Oocytes During Cooling

As shown in Table 1, 79% of oocytes in control had normal spindles (Fig. 1A), 19% had abnormal (Fig. 1B), and 2% did not have a spindle. Chromosomes aligned in the spindle plate in all oocytes with normal spindles and 7 of 8 oocytes with abnormal spindle. One oocyte with abnormal spindle and one oocyte (out of 45) without spindle had chromosomes dispersed in the cytoplasm. When oocytes were cooled to 24°C in medium with or without DMSO for 5–120 min, the proportions of oocytes with normal spindles decreased in a time-dependent manner and reached to 19–23% after 120 min.

TABLE 1. Meiotic Spindle Organization and Chromosome Distribution of In Vitro Matured Porcine Oocytes Cooled to 24 and 4°C

Temp. (°C)	Time (min)	DMSO during cooling	Total no. of oocytes examined	Spindle morphology			Chromosomes	
				Normal (%)	Abnormal (%)	None (%)	Normal (%)	Dispersed (%)
Control (39°C)			45	36 (79)[a]	8 (19)[a]	1 (2)[a]	43 (97)[a]	2 (3)[a]
24	5	+	63	41 (65)[a,b]	21 (33)[a,b]	1 (2)[a]	61 (97)[a]	2 (3)[a]
		−	59	37 (62)[b]	20 (34)[a,b]	2 (4)[a]	56 (95)[a,b]	3 (5)[a,b]
	30	+	67	27 (41)[c]	34 (51)[b]	6 (8)[a]	60 (90)[a,b]	7 (10)[a,b]
		−	61	32 (52)[c,d]	22 (36)[ab]	7 (12)[a]	55 (92)[a,b]	6 (8)[a,b]
	120	+	70	13 (19)[d]	50 (71)[c]	7 (10)[a]	58 (83)[b]	12 (17)[b]
		−	63	14 (23)[d]	41 (65)[b,c]	8 (12)[a]	57 (89)[a,b]	6 (11)[a,b]
4	5	+	65	10 (15)[e,d]	14 (21)[a,b]	41 (54)[b]	54 (83)[b]	11 (17)[b]
		−	63	8 (12)[e,d]	23 (37)[b]	32 (51)[b]	49 (78)[b,c]	14 (22)[b,c]
	30	+	70	7 (10)[e,d]	12 (17)[a]	49 (83)[c]	52 (74)[b,c]	18 (26)[b,c]
		−	67	2 (3)[e]	7 (17)[a]	58 (87)[c]	51 (76)[b,c]	16 (24)[b,c]
	120	+	72	0 (0)[e]	14 (19)[a]	58 (81)[c]	50 (69)[c]	22 (31)[c]
		−	72	0 (0)[e]	6 (8)[a]	66 (92)[c]	56 (78)[b,c]	16 (22)[b,c]

[a,b,c,d,e]Values with different superscripts within each column are significantly different, $P < 0.05$.

Accordingly, the proportions of oocytes with abnormal spindles (partially depolymerized spindle; Fig. 1C,E) increased and reached to 65–71% after 120 min. The proportions of oocytes without spindle (Fig. 1D) increased during cooling but there was no significant difference being observed as compared with control. The proportion of oocytes with dispersed chromosomes increased as cooling time was increased. However, most oocytes (83–97%) still had normal chromosome alignment even without a normal spindle (Fig. 1D).

On the other hand, when oocytes were cooled to 4°C, microtubule in the spindles of most oocytes partially (Fig. 1F) or completely (Fig. 1G) disassembled (Table 1), so that the proportions of oocytes without spindle increased in a time-dependent manner. When oocytes were cooled for 120 min at 4°C, microtubules in most oocytes depolymerized and 81–92% of oocytes did not have a spindle. As compared with control, the proportions of oocytes with normal chromosomes decreased and 17–31% of oocytes had dispersed chromosomes (Fig. 1G) in the cytoplasm

DMSO did not have any beneficial effects on the cooling induced spindle disassembly and chromosome abnormalities regardless of oocytes being cooled to 24 or 4°C (Table 1). It would appear that longer exposure of oocytes to DMSO was detrimental to oocytes as more oocytes had dispersed chromosomes.

Spindle Recovery After Rewarming Oocytes

When oocytes were rewarmed at 39°C for 2 hr, it was found that limited spindle recovery was observed. When the results in Tables 1 and 2 were compared, it was found that complete spindle recovery was observed only in a limited number of oocytes, depending on the cooling time. Most oocytes had abnormal or partially recovered spindles even after 2 hr of rewarming (Fig. 1H,I). The proportion of oocyte with an abnormal chromosome distribution also increased as the cooling time was increased, but when the results in Table 1 were compared

with those in Table 2, it was shown that most of the oocytes with abnormal chromosomes, such as dispersed chromosomes (Fig. 1I), was induced during cooling.

Again, addition of DMSO during cooling did not have any beneficial effects on subsequent spindle recovery and chromosomes distribution after rewarming.

DISCUSSION

The present study indicates that the microtubules of meiotic spindles in in vitro matured porcine oocytes are sensitive to reduced temperature and the spindles in most oocytes undergo partial disassembly at 24°C and complete disassembly at 4°C. Spindle disassembly during cooling is accompanied by chromosome disperse in the cytoplasm. Limited spindle recovery was observed in the oocytes cooled at 24 or 4°C. DMSO did not have any beneficial effects during cooling on maintaining spindle morphology and chromosome distribution. Our results also indicate that chromosome abnormalities during cooling–rewarming are mainly resulted from cooling, not from rewarming, probably due to cold shock.

Cooling pig oocytes has been tried earlier but the results were not satisfied (Miyamoto et al., 1988). This may be due to two reasons. One is that porcine oocytes, even other germ cells, such as sperm and embryos, are very sensitive to temperature changes. Many lipid drops are present in the cytoplasm and it is believed that a higher ratio of lipid to protein in the cytoplasm is a main factor affecting sensitivity to chilling and cryopreservation for porcine oocytes and embryos (Nagashima et al., 1994). Nagashima et al. (1994, 1995) succeeded in cryopreservation of porcine bastocysts after centrifuging and removing the lipid from blastocysts. Another is that the methods for cooling and rewarming of porcine oocytes have not been established. Because there are many differences in the oocyte structure between pig and other mammals, it would appear that the information obtained from other animals is not completely useful for porcine oocytes. Further researches by using

TABLE 2. Meiotic Spindle Organization and Chromosome Distribution of In Vitro Matured Porcine Oocytes After Cooling and Rewarming

Temp. (°C)	Time (min)	DMSO during cooling	Total no. of oocytes examined	Spindle morphology			Chromosomes	
				Normal (%)	Abnormal (%)	None (%)	Normal (%)	Dispersed (%)
Control (39°C)			36	27 (74)[a]	8 (23)[a]	1 (3)[a]	35 (98)[a]	1 (2)[a]
24	5	+	67	45 (67)[a,b]	21 (31)[a,b]	1 (2)[a]	65 (97)[a,b]	2 (3)[a,b]
		−	62	39 (63)[a,b]	21 (34)[a,b]	2 (3)[a]	60 (96)[a,b]	2 (4)[a,b]
	30	+	62	34 (55)[b]	25 (40)[b]	3 (5)[a,b]	55 (89)[a,b]	7 (11)[a,b]
		−	70	43 (61)[a,b]	25 (35)[a,b]	2 (4)[a,b]	66 (95)[a,b]	4 (5)[a,b]
	120	+	70	20 (29)[ce]	45 (64)[c]	5 (7)[a,b]	59 (85)[b]	11 (15)[b]
		−	73	27 (37)[c]	43 (59)[c]	3 (4)[a,b]	64 (87)[a,b]	9 (13)[a,b]
4	5	+	54	11 (21)[e]	33 (61)[c]	10 (19)[b]	47 (88)[a,b]	7 (12)[a,b]
		−	71	13 (19)[e,f]	43 (61)[c]	15 (20)[b]	65 (92)[a,b]	6 (8)[a,b]
	30	+	60	10 (16)[e,f]	41 (68)[c]	9 (26)[b,d]	50 (81)[b,c]	12 (19)[b,c]
		−	59	6 (11)[e,f]	37 (62)[c]	16 (27)[b,d]	48 (82)[b,c]	13 (18)[b,c]
	120	+	65	6 (9)[e,f]	35 (54)[b,c]	24 (36)[d,e]	41 (63)[c]	14 (37)[c]
		−	62	2 (4)[f]	32 (52)[b,c]	28 (44)[e]	42 (68)[c]	20 (32)[c]

[a,b,c,d,e]Values with different superscripts within each column are significantly different, $P < 0.05$.

porcine oocytes are necessary. As more effective IVM methods for porcine oocytes collected from ovaries obtained at slaughterhouse have been established (Wang et al., 1997, Abeydeera et al., 2000), it is necessary to use these oocytes to study the cooling protocols in the pigs.

We found that spindles in porcine oocytes started to depolymerize when the oocytes were exposed to room temperature (24°C) for 5 min. However, during in vitro fertilization, fluctuations in temperature inevitably occur, even in the most careful hands when oocytes are manipulated in vitro. Thus low fertilization rate (Saunders and Parks, 1999; Wu et al., 1999) or increased aneuploid formation (Eroglu et al., 1998) may be due to disruption of spindle integrity by low temperature. As compared to 24°C, cooling to 4°C induced a quicker disassembly of spindle. However, limited recovery was observed in both cooling treatments. These results are consistent with those observed in bovine oocytes (Aman and Parks, 1994). Moor and Crosby (1985) found that sheep oocytes at metaphase I to anaphase I stages cooled below 29°C for 3 hr had the least degree of disruption and the oocytes undergoing germinal vesicle breakdown was particularly sensitive to cooling during maturation. During these periods, oocytes underwent dramatic changes in protein synthesis, protein phosphorylation and membrane transport (Crosby et al., 1984). Therefore, the susceptibility of the oocytes to temperature changes at this time may not be entirely due to nuclear events. Cooling bovine oocytes at germinal vesicle stage for 10 min at 31 or 24°C did not significantly alter the formation of microtubules and the meiotic spindles (Wu et al., 1999). However, the microtubules of the meiotic spindles in bovine oocytes started to depolymerize after oocytes at M II stage were cooled at 25°C for 5 min (Aman and Parks, 1994). In the mouse oocytes, it was found that cooling to ~25°C for 60 min induced complete disassembly of the spindle, but incubation at 37°C for 60 min resulted in recovery of normal spindles (Pickering and Johnson, 1987). Also mouse oocytes subjected to cooling

at 4°C for 60 min followed by 60 min at 37°C were able to recover their normal spindle morphology in 89% of the oocytes (Pickering and Johnson, 1987). However, spindles in human oocytes are more sensitive to temperature decline (Pickering et al., 1990; Wang et al., 2001). It was found that 5–10 min cooling of human oocytes to 33°C induced complete spindle disassembly and limited spindle recovery was observed when human oocytes were rewarmed after being cooled to room temperature for 10 min (Wang et al., 2001).

The main structural elements of spindle fibers are microtubules (Inoue, 1981). Low temperatures induce disassembling of microtubules and this disassembly is the result of the depolymerization of the major structural protein of microtubules, the tubulin (Moor and Crosby, 1985). In M II oocytes, microtubules are assembled into the meiotic spindles and no microtubules are shown outside the spindles. Depolymerization of the microtubules induces the disruption of the meiotic spindle (Magistrini and Szöllözi, 1980; Trounson, 1986; Pickering et al., 1990). Studies have shown that chromosomes enhance spindle microtubule assembly and play an essential role in the initiation of spindle formation (Zhang and Nicklas, 1995). Meanwhile, kinetochores in chromosomes capture microtubules in the spindles and make chromosome accurate segregation (Nicklas, 1997). The organization of the meiotic spindle requires both chromosomes, which causes a local reduction in the threshold for microtubule polymerization, and the pericentriolar material to nucleate microtubule polymerization. Only in the vicinity of the chromosomes is the pericentriolar material able to sustain nucleation of microtubules, whereas conditions in the remainder of the cells favor rapid polymerization (Pickering et al., 1990). Thus, if the chromosomes and the pericentriolar material are functional, microtubules could assemble into a normal spindle structure. By contrast, if the chromosomes were damaged, microtubules could assemble around the chromosomes, but normal spindles could
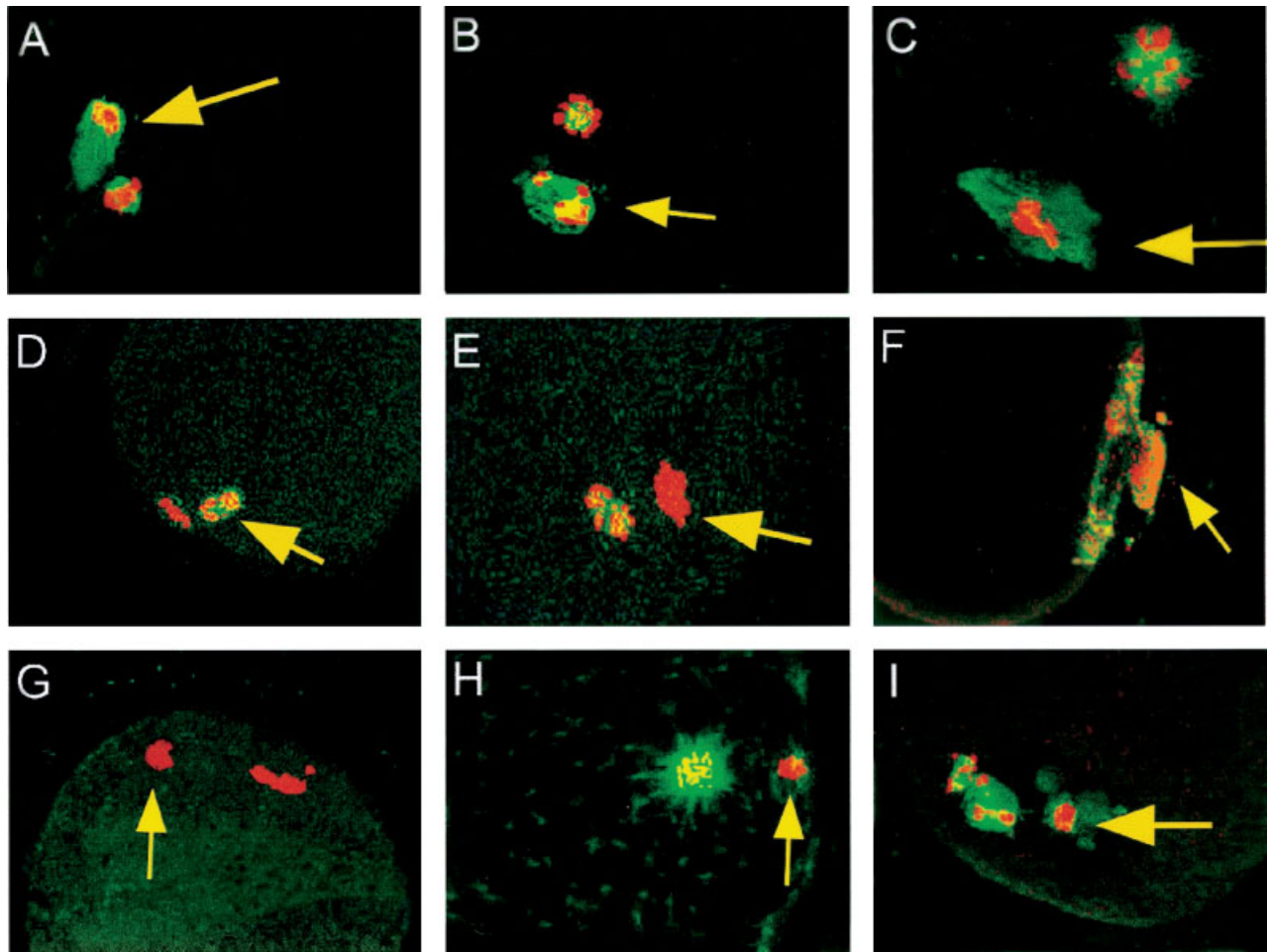
Fig. 1. Confocal micrographs of spindle organization and chromosome alignment of porcine oocytes during cooling and rewarming. **A**: An oocyte in control shows a normal meiotic spindle organization and chromosome alignment. **B**: An oocyte in control has a partially depolymerized spindle, but the chromosomes are normal. **C–E**: Oocytes cooled at 24°C for 5 min (C) and 30 min (D, E) show partially (C, E) and completely (D) disassembled spindles, with normal chromosome distribution. **F**: Oocyte cooled at 4°C for 5 min shows partially disassembled spindle with dispersed chromosomes. **G**: Oocyte cooled at 4°C for 30 min shows no microtubules and dispersed chromosomes. **H**, **I**: Oocytes after cooling-rewarming show abnormal spindles formation and chromosomes distribution. Chromosomes are normal in (H) and scatted in (I) and microtubules are repolymerized around the normal and dispersed chromosomes, but no normal spindles have been formed. Arrows indicate the first polar body (400×). Red images: chromosomes; green images: microtubules; yellow images: overlay of green and red.

not form. In the present study, we found that cooling can induce chromosome dispersion in the cytoplasm and no normal spindle was observed in these oocytes. Furthermore, a limited number of oocytes recovered the spindles after cooling–rewarming. It is possible that other elements related to spindle formation were damaged during cooling. Thus, the oocytes can't reorganize the normal spindles after rewarming although some microtubules were repolymerized around the chromosomes. It seems that dispersed chromosomes observed in the present study were mainly resulted from cooling, as the proportion of oocytes with dispersed chromosomes was not increased after rewarming.

DMSO, as a cryoprotectant, has been successfully used for the cryopreservation of the somatic cells (Saeed et al., 2000) and embryos (Siebzehnruebl et al., 1989; Todorow et al., 1989a,b; Li et al., 1990; Li and Trounson, 1991; Van der Elst et al., 1995; Oberstein et al., 2001).

For cryopreservation of oocytes, it has been found that DMSO exhibits the same effects as other cryoprotectants (Siebzehnruebl et al., 1989; Todorow et al., 1989a,b; Hunter et al., 1991; Newton et al., 1999). However, the positive effects of DMSO were not observed when mouse oocytes at the GV stage were cryoproserved (Van der Elst et al., 1992). It seems that cooling speed, oocyte stage or concentration of cryoprotectants affect the results (Hunter et al., 1991; Aigner et al., 1992; Bouquet et al., 1995; Agca et al., 1998). In the present study, we directly exposed oocytes to a solution containing DMSO to 24 or 4°C, it may limit the penetration of cryoprectant into the oocytes, thus the concentration of DMSO within the cytoplasm may be low. It is also possible that a temperature shock during cooling affects the subsequent oocyte recovery and viability.

Furthermore, previous studies have shown that cryoprotectants disrupt the cortical microfilament network

(Vincent et al., 1989, 1990; Aigner et al., 1992) and caused depolymerization and disorganization of the spindle microtubules, which in turn results in chromosomal scattering (Johnson and Pickering, 1987; Vincent et al., 1989). Eroglu et al. (1998) also found that when mouse M II oocytes were cryopreserved in medium with DMSO, some oocytes were lysed because of excessive swelling during the thawing procedure. However, when both sucrose and DMSO were added to the freezing medium to reduce the difference in osmolarity between the inside and outside of the cells, an increased survival rate (up to 95%) was observed. Such a possibility in porcine oocyte cooling or cryopreservation needs further investigation.

In summary, our study indicates that spindles in porcine oocytes at M II stages are very sensitive to temperature decline. Temperature decline induces most microtubules in spindles to undergo disassembly and only a limited number of oocytes can recover their normal spindle structure after rewarming. Chromosome abnormalities induced by temperature dropping may be due to dispolymerization of microtubules, which in turn induce abnormal chromosome alignment and separation. It is suggested that temperature fluctuation may also affect other oocyte functions associated with subsequent fertilization, such as polyspermic penetration, which is one of the unresolved problems during in vitro fertilization of porcine oocytes.

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