Effect of Temperature Decline on the Cytoskeletal Organization of the Porcine Oocyte

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Abstract: The purpose of this study was to evaluate effect of cooling on the cytoskeletal organization affecting the distribution and structure of the meiotic spindle in the porcine metaphase II (MII) oocyte. Fluorescence staining was done for visualization of microtubules, microfilaments and chromosomes. In vitro-matured oocytes were either maintained at 37°C (for controls) or cooled abruptly to 5 or 18°C. Microtubules were preferentially labeled at the meiotic spindle and microfilaments were mainly detected at the cortex of the MII oocyte. The majority of controls (87%, n=97) had a barrel-shaped spindle, whereas the remaining 13% showed a broad, box-shaped spindle. After exposure to 18°C for 30 min, 94% of oocytes (n=48) possessed either a box-shaped spindle (63%) with a shortened interpolar distance (7.4 ± 0.4 μm vs. 9.8 ± 0.2 μm for controls $P<0.05$), or no spindle (31%). Exposure of oocytes to 5°C for 30 min caused complete disassembly of the spindle in 81% of the oocytes (n=103). Such spindle-disassembled oocytes had increased intensity of microtubule signaling in the cytoplasm and irregular staining of cortical microfilaments. Cortical microfilament staining decreased in intensity in 64% (n=33) and 89% (n=74) of the oocytes cooled to 18 and 5°C, respectively. When oocytes were rewarmed at 37°C after exposure to 5°C for 30 min, the spindles were not restored to normal in any oocyte (n=138) regardless of the rewarming duration (0, 1, 10, 20 or 60 min), although microtubules and microfilaments were reassembled. However, more oocytes contained dispersed chromosomes with no spindle and collapsed microfilament architecture, as rewarming time increased beyond 20 min. These results suggest that in porcine MII oocytes both microtubular spindle and cortical microfilaments are irreversibly affected by a 5°C cold shock, and that the polar microtubule-organizing centers malfunction even after rewarming.

Key words: Microfilament, Microtubule, Pig oocyte, Spindle, Temperature changes

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Introduction

Meiotic spindles are composed of microtubules and are important for chromosome alignment and separation of maternal chromosomes during maturation and fertilization. The oocytes also contain distinct elements of actin microfilaments, which control cytoplasmic events including the spindle orientation and migration to the periphery, cortical granules migration, and polar body formation [1–6]. Microtubules and microfilaments may mutually control the function of the meiotic spindle apparatus to complete chromosome conformation and segregation. Meiotic spindles of oocytes are known to be particularly sensitive to cooling; namely, exposure to low temperature causes microtubules to undergo depolymerization. Partial and complete depolymerization of microtubules and/or altered morphology of the spindle have been reported in murine [7, 8], bovine [9], porcine [10], ovine [11] and human oocytes [12–16]. In porcine MII oocytes, Liu *et al.* [10] reported that both spindle and chromosomes were damaged during cooling and the resultant disruption of spindles was not reversible by rewarming the oocytes after exposure to low temperature. However, little attention has been paid to the effect of temperature fluctuations on microfilaments in the oocyte. We examined distributional changes in the microtubules, microfilaments and chromosomes of porcine MII oocytes subjected to simple cooling in order to assess the effects on configuration of the meiotic spindle. This is a fundamental work, exploring the

possibility of controlling assembly and disassembly of microtubules under an experimental condition in order to study the organization of the cytoskeleton in the oocyte.

Materials and Methods

Oocyte collection and maturation

Cumulus-oocyte complexes (COCs) were aspirated from antral follicles (3–6 mm in diameter) of ovaries collected from slaughtered prepubertal gilts. Only oocytes with multiple layers of compact cumulus cells were selected for maturation [17,18]. After being washed with Dulbecco's phosphate buffered saline (DPBS) containing 0.1% polyvinyl alcohol, groups of 10-15 oocytes were transferred to NCSU23 medium supplemented with 10% (v/v) porcine follicular fluid, 10 i.u./ml eCG (Teikoku Hormone Mfg. Co. Ltd., Tokyo, Japan) and 10 i.u./ml hCG (Mochida Pharmaceutical Co. Ltd., Tokyo, Japan). The oocytes were cultured for 24 h, then incubated in NCSU23 without hormonal supplements for a total period of 44 h in an atmosphere of 5% CO_2 at 39°C, as reported previously [19]. Cumulus cells were removed from mature oocytes by vigorous vortexing in a 1.5-ml microtube for 1–2 min in a solution of 0.1% hyaluronidase in calcium-free DPBS.

Cooling of oocytes

Cumulus-denuded oocytes were cooled by placing them directly into 100-μl drops of DPBS containing 0.3% BSA (DPBS-BSA) under oil at 5 or 18°C for 30 min according to the method of Pickering and Johnson [8]. The media were equilibrated to either 5 or 18°C by placing dishes of droplets in a 5°C refrigerator or in an 18°C incubator (Cell Incubator, Carson Electronic Co, Ltd, Korea) before use. Control oocytes were transferred to DPBS-BSA and kept at 37°C for 30 min. Some oocytes cooled at 5°C for 30 min were directly placed in 37°C DPBS-BSA for 0, 1, 10, 20 or 60 min. After cooling or rewarming, oocytes were fixed (see below). The oocytes, which were cooled at 5°C and then directly transferred to the prewarmed fixative at 37°C, were referred to as the group of "0 min".

Fluorescence observations

To assess the nuclear configuration and the distribution of microtubules and microfilaments, the oocytes were processed as reported previously [18]. Briefly, denuded oocytes were fixed in a microtubule stabilization fixative at 37°C for 1 h, washed extensively and blocked overnight at 5°C in the washing medium (calcium-free DPBS containing 2% BSA, 2% goat serum, 0.2% milk powder, 0.2% sodium azide and 0.1% Triton-X). The fixed samples were then exposed overnight to anti-β tubulin primary antibody (1:200; Sigma Chemical Co., MO, USA) at 5°C, and incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:200; Sigma) at 37°C for 2 h. After rinsing, the samples were stained with rhodamine-phalloidin (1:1000; Molecular Probes, Eugene, OR, USA) for microfilaments for 1 h, and then stained for DNA with Hoechst 33342 (10 μg/ml) in mounting medium containing PBS and glycerol (1:1).

The samples were viewed on a fluorescence microscope (BX-FLA, Olympus, Tokyo, Japan). A U-MNIBA filter set (Olympus) was used for FITC, a U-MWIB set (Olympus) was used for rhodamine, and a U-MWU set (Olympus) for Hoechst. A CCD digital camera system (DP70, Olympus) was used to obtain images on a computer, and color adjustment and image analysis were performed by DP Manager (Olympus) and ImageJ 1.36b (Wayne Rasband, NIH, USA).

Statistical analysis

The distance between spindle poles was analyzed by Student's *t*-test. Proportional data were analyzed by the chi-squared test or Fisher's exact probability test.

Results

The incidences of different types of microtubular spindles and microfilament architecture in the oocytes exposed to 18 and 5°C are shown in Tables 1 and 2, respectively. The majority of control MII oocytes (87%, n=97) incubated at 37°C for 30 min after maturation had anastral, barrel-shaped spindles with a single cluster of chromosomes at the equatorial plate (Fig. 1a,a'). The remaining 13% of controls showed box-shaped spindles. In 8 of 13 oocytes, each spindle pole was broadened in a direction parallel to the equatorial plane (Fig. 1b,b'), whereas in the others (5/13) the broad spindle poles were shortened in distance. Such box-shaped spindles were more commonly observed in oocytes exposed to 18°C (see below). Therefore, it is possible that the oocytes incubated at 37°C had been affected by temperature changes at room temperature during manipulation of the oocytes, including removal of the cumulus cells. In the control oocytes, microfilaments were strongly stained on the cortical cytoplasm (Fig. 1a"; Table 2) and on the contact surfaces of the oocyte and the polar body.

Exposure to 18°C for 30 min produced changes of

Table 1. Meiotic spindle organization of porcine MII oocytes cooled to 18 and 5°C

Incubation conditions*	No. of oocytes	Number (%) of oocytes showing:					Dispersed chromosome (%)
		Barrel-shaped spindle	Box-shaped spindle**	Reduced spindle***	No spindle****	Aster spindle*****	
37°C	97	84 (87)	13 (13)	0	0	0	0
18°C	48	3 (6)	14 (29)	16 (33)	15 (31)	0	0
5°C	103	0	9 (9)	11 (11)	83 (81)	0	0
Rewarmed at 37°C after 5°C for:							
0 min	19	0	4 (21)	8 (42)	7 (37)	0	0
1 min	40	0	14 (35)	5 (13)	9 (23)	12 (30)	0
10 min	21	0	7 (33)	1 (5)	12 (57)	1 (5)	0
20 min	39	0	5 (13)	2 (5)	30 (77)	2 (5)	2 (5)
60 min	35	0	3 (9)	0	30 (86)	2 (6)	5 (14)

*, Porcine oocytes were matured in NCSU23 at 39°C and then maintained at 37°C or cooled abruptly to 5 or 18°C for 30 min. Some were rewarmed at 37°C after cooling to 5°C for 0–60 min. **, Spindles with broadened poles. ***, Partially depolymerized spindles frequently showing a short interpolar distance. ****, No spindle was found around the chromosome. *****, Spindles with microtubules radiating outward.

Table 2. Microfilament organization of porcine MII oocytes cooled to 18 and 5°C

Incubation conditions*	No. of oocytes	Number (%) of oocytes with cortical microfilaments showing:		
		Overall strong	Irregular staining	Overall weak
37°C	70	70 (100)	0	0
18°C	33	12 (36)	12 (36)	9 (27)
5°C	74	8 (11)	48 (65)	18 (24)

*, Porcine oocytes were matured in NCSU23 at 39°C and then maintained at 37°C or cooled abruptly to 5 or 18°C for 30 min.

microtubular organization in 93% of the oocytes, including box-shaped spindles, and partially or completely depolymerized spindles (Table 1). The majority of oocytes (12/14), showing a box-shaped spindle (Fig. 1c,c'), possessed a shortened interpolar distance. The mean interpolar distance of shortened spindles was 7.4 ± 0.4 μm, which was significantly different from that of control barrel-shaped spindles (9.8 ± 0.2 μm, $P<0.05$). In some of the shortened spindles, microtubules decreased in number and in intensity, due to the partial depolymerization (Fig. 1c'). This type of the spindle was referred to as a "reduced spindle" in Table 1.

Cooling of oocytes to 5°C produced more oocytes showing complete depolymerization of the spindle microtubules than those showing partial disassembly (11% and 81% for reduced and no spindles, respectively; Fig. 1d,d'; Table 1). Such spindle-disassembled oocytes had increased intensity of microtubule signaling in the cytoplasm (Fig. 1d') and irregular staining of cortical microfilaments (Fig. 1d"; Table 2). Interestingly, a one-pole spindle was noted in 19% (n=16) and 36% (n=11) of oocytes cooled to 18 and 5°C, respectively (Fig. 1e,e'). In this case, the one-pole spindle was always observed on the side of the outer pole only. Cortical microfilaments were also depolymerized by cooling (Fig. 1e"). The proportions of the oocytes having decreased cortical microfilaments were 64% and 89% for 18 and 5°C, respectively (Table 2). The layer of microfilaments at the cortex appeared to be fragmented or to have disappeared at 5°C in particular. Since the cortical layer of microfilaments decreased in density, the cortex overlying the chromosomes on the MII spindle presented a thicker actin rich domain compared to the rest of the cortex (Fig. 1e"). This feature is very similar to that observed in mouse oocytes [1]. In this study, the incidences of spindle abnormality were significantly greater in the oocytes exposed to 18 or 5°C than in controls ($P<0.001$, respectively). In this case, movements of chromosomes toward the cell surface seemed to be frequently induced by temperature reduction (Fig. 1e,e'). The chromosome dispersion was not obvious after cooling even in the oocytes without spindle.

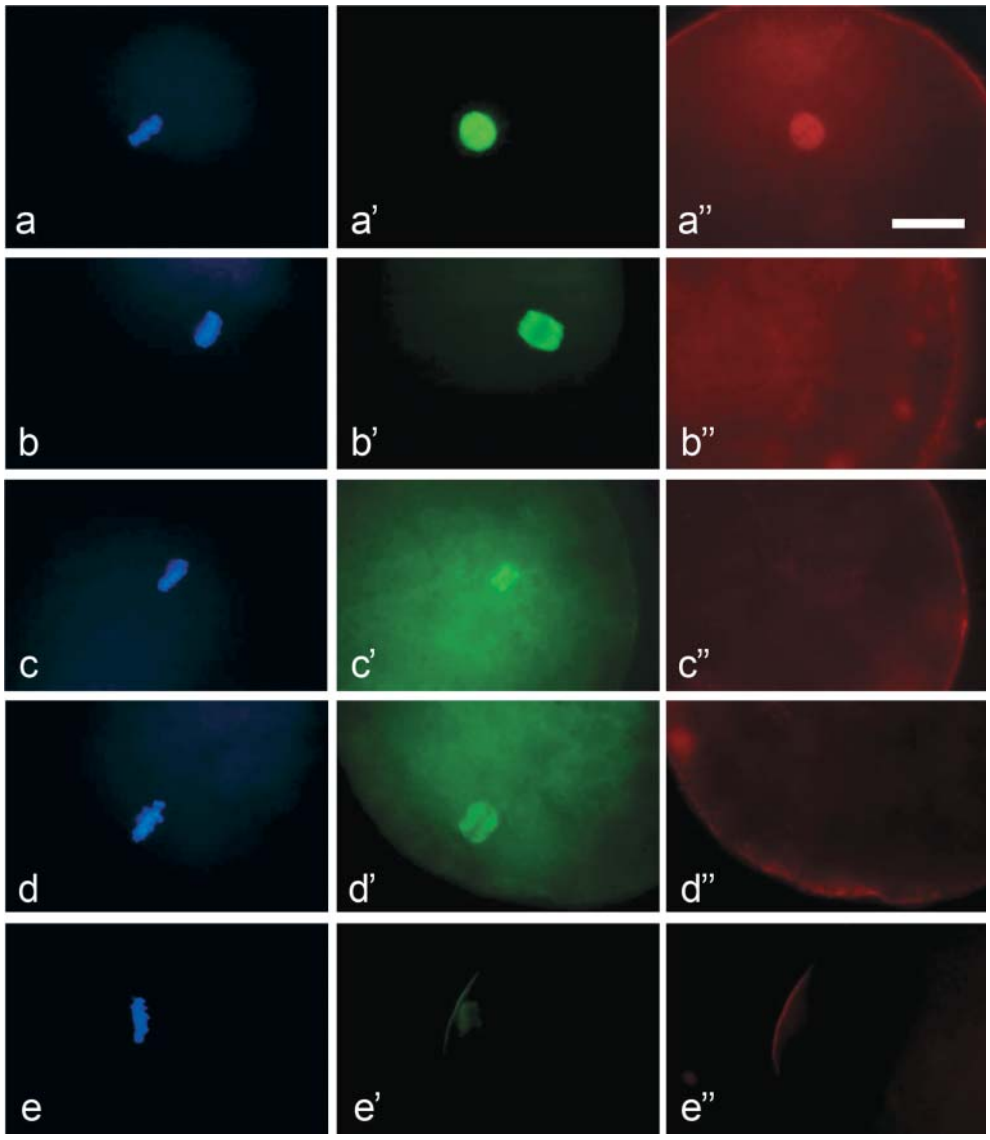

Fig. 1. Fluorescence micrographs of porcine oocytes cooled to low temperature. The left panel (a–e) shows DNA labeling (blue), the middle panel (a'–e') shows microtubule labeling (green), and the right panel (a"–e") shows microfilament labeling (red) of the same oocyte. The bar in (a") represents 20 μm for all micrographs. A control oocyte matured *in vitro* (a): microtubules are concentrated in the spindle located on both sides of the metaphase plate, showing a barrel shape with a radial axis (a,a'). Cortical microfilaments are uniformly and strongly stained (a"). Another control oocyte matured *in vitro* (b): note a box-shaped spindle (b,b') and intense staining of microfilaments in the cortex and cytoplasm (b"). An oocyte cooled to 18°C (c): note a shortened, box-shaped spindle (c,c') and irregular staining of the cortical microfilaments (c"). An oocyte cooled to 5°C (d): note a reduced spindle with it's pole-to-pole axis perpendicular to the cell surface and increased staining of microtubule in the cytoplasm (d,d'). The staining of microfilaments is irregular (d"). Another oocyte cooled to 5°C (e): note a one-pole spindle where the microtubules are seen only from the outer pole (e,e'). Cytoplasmic microfilaments are absent and the cortical microfilament-rich area is identified (e").

We examined the effect of rewarming at 37°C for 0, 1, 10, 20 or 60 min after exposure to 5°C for 30 min. Repolymerization of microtubules and microfilaments became obvious after 0–10 min of rewarming (Fig. 2a',a"). However, a barrel-shaped spindle was never observed in all the oocytes examined (n=138). Another

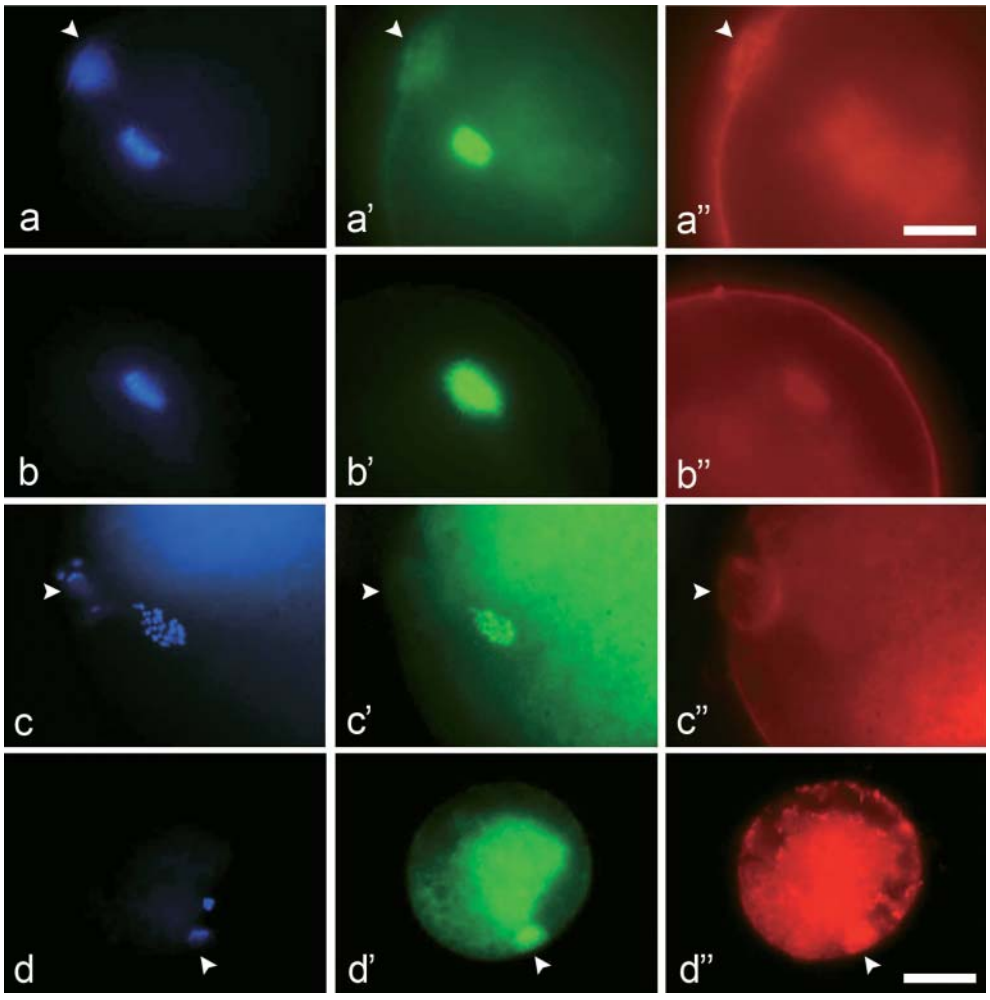

Fig. 2. Fluorescence micrographs of porcine oocytes cooled and rewarmed. The left panel (a–d) shows DNA labeling (blue), the middle panel (a'–d') shows microtubule labeling (green), and the right panel (a"–d") shows microfilament labeling (red) of the same oocyte. The bar in (a") represents 20 μm for (a–c, a'–c', a"–c"). The bar in (d") represents 50 μm for (d–d"). An oocyte 1 min after rewarming (a): note a box-shaped spindle with it's axis perpendicular to the cell surface (a,a') and a thin layer of cortical microfilaments (a"). The arrow indicates the position of the polar body. Another oocyte 1 min after rewarming (b): note an astral spindle (b,b') and recovery of microfilaments (b"). An oocyte 20 min after rewarming (c): the chromosomes are dispersed with no spindle (c,c') and the staining of microfilament decreases in the cortex (c"). An oocyte 60 min after rewarming (d): the microtubular spindle disappears (d,d') and the microfilament architecture is disrupted (d").

type of spindle, an astral spindle (Fig. 2b,b'; Table 1), was found in all rewarming groups, except for the 0 min group, with normal thick cortical microfilaments. These observations suggest that reassembly of microtubules may not proceed normally with bipolar organization. Rewarming of oocytes resulted occasionally in the appearance of multiple cytoplasmic asters as well as polar asters (not shown). At 20 min or later after rewarming, spindle microtubules and cortical microfilaments were depolymerized again in most oocytes (Fig. 2c',c",2d',d"). In these oocytes (5% and 14% for 20 and 60 min, respectively; Table 1), the chromosomes were dispersed (Fig. 2c).

Discussion

The present study revealed that exposure of porcine MII oocytes to 5°C produced more marked effects on

the spindle organization and the cortical microfilaments than exposure to 18°C, and that the cooling seemed to have only mild effects on the assembly of microfilaments compared to that of microtubules. This is the first report showing changes in the microfilament organization of the porcine oocyte induced by temperature fluctuations. Our results on microtubular organization correspond with the observations of Liu *et al.* [10], who reported that disassembly of microtubular spindles in the porcine MII oocytes was quicker at 4°C than at 24°C.

Partial or complete disassembly of microtubules in the spindle was observed when oocytes were cooled to room or lower temperatures [9–16]. In mouse oocytes, the recovery of the spindle after rewarming was reported by Magistrini and Szollozi [7], who observed that most oocyte spindles were restored to normal by rewarming after exposure to 0°C for 45–60 min. Pickering and Johnson [8] also found that cooling to 25°C for 60 min induced complete disassembly of the spindle in mouse oocytes, but subsequent incubation at 37°C for 60 min resulted in recovery of normal spindles. However, spindles of bovine [9], porcine [10], ovine [11] and human [12–16] oocytes exhibited only limited recovery after temperature fluctuations of cooling and rewarming. The discrepancies among the reports noted above may be explained by cooling speed and/or species differences. In the present study, we did not find any oocytes with a normally recovered spindle after cooling-rewarming in contradiction to the observation of Liu *et al.* [10], who found that spindles occasionally recovered in rewarmed porcine oocytes which had been cooled to 4°C. The reason for this discrepancy is not clear. Recently, it has been reported that irreversible damage occurs to the cytoskeleton of porcine GV- and MII-oocytes after vitrification [20].

In the present study, cooling affected the MII spindle, changing it from a barrel shape to a box shape. We observed some oocytes having a box-shaped spindle with the normal interpolar distance at 37 and 18°C. The box-shaped spindle poles had broadened in a direction parallel to the equatorial plane. More oocytes showed a box-shaped spindle with shortened interpolar distance at 18°C. It is suggested, therefore, that the polar areas become wider first, and then the distance between the poles shortens. Kinetochore microtubules being attached to the kinetochores of the chromosomes may be more stable and cold-resistant than the pole-to-pole microtubules [21]. The kinetochore microtubules are believed to be involved in generating and/or transmitting the force for chromosome movement. The pole-to-pole microtubules make antiparallel interactions within the spindle and are required for keeping the two-spindle poles apart [5, 22]. Therefore, the reduced spindles observed in this study may have been derived from disassembly of the pole-to-pole microtubules. In addition, we frequently observed movements of chromosomes toward the cell surface in the oocytes exposed to the low temperatures. Such movement of chromosomes has been reported in mitosis, too [22]. Interestingly, one-pole spindles were seen in the oocytes cooled to 5°C. We always observed one microtubular spindle on the side of the outer pole only, suggesting that the bundles of microtubules on the side of the inner pole disassemble first and then those on the side of the outer pole disappear. Finally, complete disassembly of spindle microtubules may follow.

The spindle poles of the oocyte are known as microtubule-organizing centers (MTOC) [5, 23–25]. In a box-shaped spindle, the polar areas became wider and flattened, probably due to elongation of the polar MTOC. The broadened spindle was unable to recover to the normal spindle after cooling-rewarming, suggesting that this alteration is irreversible. It is possible, therefore, that other elements including MTOC-related proteins, such as γ-tubulin and pericentrin [24, 25], are damaged during cooling. Thus, the oocyte spindles can't recover to normal after rewarming, even though some microtubules were repolymerized around the chromosomes and the majority of cortical microfilaments were reassembled. The effect of cooling on MTOC-related proteins and the mechanism controlling the organization of microtubules and microfilaments in the oocyte remain to be elucidated at the cellular and molecular levels.

In conclusion, cooling of porcine MII oocytes to 5°C induced most of the spindle microtubules and the cortical microfilaments to undergo disassembly from which no oocyte could recover their normal spindle structure after rewarming. Therefore, in association with reorganization of microtubules and microfilaments, temperature fluctuations may influence crucial events of the cortical ooplasm, such as normal alignment and segregation of chromosomes, the eccentric anchorage of the spindle, the polar body formation [1–6], and the migration of organelles, including mitochondria [26], and subsequent fertilization and development.

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