Influence of Incubation Temperature on Meiotic Progression of Porcine Oocytes Matured In Vitro

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Abstract: *Oocyte maturation is a key issue in current animal biotechnology. This study was designed to examine effects of incubation temperature and cumulus investment on in vitro maturation (IVM) of porcine oocytes. Cumulus-oocyte complexes were recovered from slaughterhouse ovaries. The oocytes, surrounded completely or partially with cumulus cells (completely- and partially-enclosed groups, respectively), were incubated for 44 h at either 37°C or 39°C, and progression of meiosis and changes in the cytoskeleton distribution were evaluated by fluorescence staining. Prior to maturation culture (0 h), 94–100% of the oocytes were at the germinal vesicle (GV) stage. At 24 h IVM, a significantly greater number of the oocytes showed signs of GV breakdown (GVBD) when incubated at 39°C than at 37°C (96% vs. 61% and 92% vs. 40% for completely- and partially-enclosed groups, p<0.01, respectively). Percentages of the oocytes which reached metaphase-II (M-II) at 36 h IVM was higher at 39°C than at 37°C (p<0.01), whereas no significant difference was found in the maturation rate at 44 h IVM. The completely-enclosed oocytes had a significantly higher maturation rate than did the partially-enclosed oocytes (90% vs. 68% for 37°C and 91% vs. 53% for 39°C, p<0.01 respectively). Fluorescence staining showed that transzonal microfilaments were abundant at the GV and M-I stages (0 to 24 h), but decreased in number at the M-II stage (36 to 44 h) and that there was no remarkable difference in the distribution of microtubules and microfilaments within the ooplasm, irrespective of the incubation temperature or the condition of cumulus attachment. These observations suggest that GVBD and meiotic progression may be drastically delayed by a 2°C decrease in incubation temperature and that the cumulus condition may affect porcine oocyte maturation.*

Key words: *Porcine oocyte, Incubation temperature, Cumulus cell projection, In vitro maturation*

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The events occurring during maturation and fertilization are temperature sensitive [1, 2]. In the bovine, the maximal cumulus expansion was obtained between 35 and 39°C, but not at 41°C, and the oocyte maturation was also inhibited at 41°C [1]. In pigs, a higher percentage of polar body formation was noted when cultured at 39°C than at 37°C, although meiotic progression during *in vitro* maturation (IVM) of the oocytes was not mentioned [3]. Another factor affecting the IVM of oocytes is the contribution of the cumulus investment. Cumulus cells play a very important role during oocyte growth and maturation by supplying nutrients [4–6] and/or messenger molecules [7–9] and mediating the effects of hormones on the cumulus-oocyte complex (COC) [10]. The proportion of oocytes capable of IVM is lower when cumulus cells are previously removed [11, 12]. The maturation status of the oocyte may be directly influenced via modifications in the mode of interaction between the oocyte and the surrounding follicular cells [13]. Our previous study [14] showed that the cumulus cell projections consist mainly of microfilaments, which are abundant at the germinal vesicle (GV) and metaphase-I (M-I) stages, but which are fewer in number at the M-II stage. In the bovine, it is well known that the cumulus investment character and the ooplasm appearance of the oocyte are good indicators of the oocyte potential to mature *in vitro* [15, 16]. But no data concerning this have been available on pigs. Thus the objective of this study was to investigate the influence of incubation temperature and the cumulus investment character on IVM of porcine oocytes. With this aim porcine oocytes, surrounded completely or partially with cumulus cells, were matured *in vitro* at either 37°C or 39°C. The progression of meiosis and changes in the cytoskeleton distribution were evaluated by fluorescence staining.

Materials and Methods

Preparation and culture of oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory within 1–1.5 h in Dulbecco's phosphate buffered saline (Gibco, Grand Island, NY) containing 0.1% polyvinyl alcohol (Sigma, St. Louis, MO)(DPBS-PVA) at 37°C. Porcine COCs were aspirated from antral follicles (2–5 mm in diameter) with an 18-gauge needle fixed to a 10 ml disposable syringe. Groups of 10–15 COCs were washed and transferred to NCSU23 medium supplemented with 10% (v/v) porcine follicular fluid, 10 i.u./ml eCG (Teikoku Hormone Mfg. Co., Ltd., Tokyo) and 10 i.u./ml hCG (Mochida Pharmaceuticals, Tokyo, Japan) [14]. The COCs were cultured for 24 h, then incubated in NCSU23 without hormonal supplements until 44-h time point in an atmosphere of 5% CO_2 at 37°C or 39°C. At 0, 24, 36 and 44 h IVM, oocytes were processed for fluorescence staining.

Classification of the oocytes

COCs were classified into two groups according to the condition of cumulus investment: completely- or partially-enclosed groups. The former were oocytes with compact cumulus oophorus which tightly adheres to the zona pellucida in more than four-layers thick. The latter were oocytes containing less than three layers of cumulus cells not completely surrounding the oocyte. Denuded oocytes were not used in the present study.

Fluorescent observations

Methods for preparing samples for fluorescent observations have been reported previously [14]. Oocytes with or without cumulus cells were fixed in a microtubule stabilization buffer [17] at 37°C for 1 h, washed extensively and blocked overnight at 4°C in the wash 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 (at 4°C) to anti-β tubulin primary antibodies (1:200; Sigma), washed, and then 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, washed again, then stained for DNA with Hoechst 33342 (10 μg/ml) in mounting medium containing PBS and glycerol (1:1). The oocytes were finally mounted on slides and examined to assess the nuclear configuration and the distribution of microtubules and microfilaments.

The samples were viewed under an Olympus 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 cooled CCD video system (ImagePoint, Photometrics Ltd., Tucson, AZ, USA) was used to obtain images on a computer and color adjustment was done with IPLab-Spectrum P software (Signal Analytics Corporation, Vienna, VA, USA).

Data analysis

Proportional data were analyzed by chi-square test.

Results

Meiotic progression at different temperatures and cumulus conditions are summarized in Table 1. Percentages of GV breakdown (GVBD) and maturation during incubation in the experimental groups are compared in Fig. 1. Prior to maturation culture (0 h), almost all oocytes (94–100%) were at the GV stage. In the completely-enclosed oocytes, the percentage of oocytes showing GV breakdown was 96% after 24 h of incubation at 39°C, but decreased to 61% when incubated at 37°C ($p<0.01$). This is also true of the oocytes partially surrounded with cumulus cells (92% vs. 40%, $p<0.01$). Likewise, at 36 h IVM, the percentage of oocytes that reached M-II was significantly higher at 39°C than at 37°C (69% vs. 12% for the completely-enclosed oocytes, $p<0.01$ and 46% vs. 19% for the partially-enclosed oocytes, $p<0.01$). After 44 h of incubation, however, the frequency of oocytes that finally reached M-II did not differ significantly with temperature in each cumulus condition (Table 1). On the other hand, it was evident that the completely-enclosed oocytes matured in significantly higher proportion than did the partially-enclosed oocytes (90% vs. 68% for 37°C, $p<0.01$ and 91% vs. 53% for 39°C, $p<0.01$).

Fluorescence staining showed that the cumulus cell projections, which consisted mainly of microfilaments, were abundant at the GV and M-I stages (0 to 24 h, Fig. 2a), but decreased in number at the M-II stage (36 to 44 h, Fig. 2b). The degree of cumulus expansion and the distribution of microtubules and microfilaments in the M-II oocytes in the completely- and partially-enclosed groups and at different incubation temperatures did not differ remarkably. Microtubules were mainly located in the meiotic spindle (photo not shown) and microfilaments were strongly stained on the peripheral ooplasm

Table 1. Meiotic progression of the porcine oocytes matured in various conditions

Temp. (°C)	Condition of cumulus[a]	Hours of maturation	Number of oocytes	Number (%) of oocytes at each maturational stage[b]				
				GV	ProM	MI	AI-TI	MII
37	Completely	0 h	52	51 (98)	1 (2)			
		24 h	133	52 (39)	44 (33)	36 (27)	1 (1)	
		36 h	65	10 (15)	5 (8)	25 (39)	17 (25)	8 (12)
		44 h	162	13 (8)	1 (1)	2 (1)	0 (0)	146 (90)
	Partially	0 h	19	19 (100)				
		24 h	37	22 (60)	9 (24)	6 (16)		
		36 h	37	4 (11)	5 (14)	18 (49)	3 (8)	7 (19)
		44 h	59	6 (10)	7 (12)	3 (5)	3 (5)	40 (68)**
39	Completely	0 h	102	96 (94)	6 (6)			
		24 h	105	4 (4)	25 (24)	63 (60)	12 (11)	1 (1)
		36 h	126	1 (1)	6 (5)	11 (9)	21 (17)	87 (69)
		44 h	124	2 (2)	1 (1)	2 (2)	6 (5)	113 (91)
	Partially	0 h	92	89 (97)	2 (2)	1 (1)		
		24 h	80	6 (8)	18 (23)	32 (40)	14 (18)	10 (13)
		36 h	79	7 (9)	5 (6)	8 (10)	23 (29)	36 (46)
		44 h	68	6 (9)	5 (7)	3 (4)	18 (27)	36 (53)**

[a] See details in text.; [b] GV, germinal vesicle; ProM, prometaphase, MI, metaphse-I, AI-TI, anaphase-I to telophase-I; MII, metaphse-II. **, Final maturation rates in the partially-enclosed groups at 37 and 39°C were significantly different from that in the completely-enclosed group at 39°C ($p<0.01$).

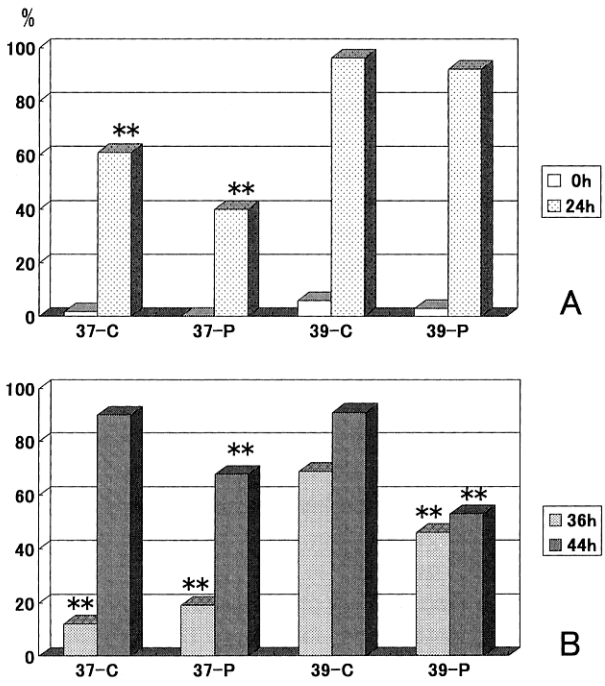

Fig. 1. Percentages of germinal vesicle breakdown at 0 and 24 h of incubation (A) and maturation rates at 36 and 44 h of incubation (B) in each experimental group: namely, completely- and partially-enclosed groups at 37°C (37-C and 37-P, respectively) and corresponding groups at 39°C (39-C and 39-P, respectively). Double asterisks (**) indicate highly significant difference from the rate in the 39-C group at each time point ($p<0.01$).

and on the contact surfaces of the oocyte and the polar body (Fig. 2b).

Discussion

The inability of oocytes lacking cumulus investments to mature *in vitro* has been previously shown for bovine [11, 12, 15, 16] and porcine follicular oocytes [18, 19]. In the present study, maturation rates decreased in the oocytes surrounded partially with cumulus cells compared to those completely surrounded with cumulus cells at both 37°C (90% vs. 68%) and 39°C (91% vs. 53%), supporting the previous observations mentioned above. The present fluorescence observations demonstrated that a great reduction in the number of microfilament-filled, transzonal cumulus cell projections occurred between the M-I and M-II stages, suggesting a disruption of cumulus cell projections during final maturation. A close association between cumulus cells and the oocyte has been shown to be important in the production of developmentally-competent oocytes during IVM [10, 20–22]. It has therefore been speculated that through dynamic contacts via transzonal cumulus cell projections and via inter-cumulus projections, some substance(s) may actively modulate the maturation of COCs [14], but no remarkable differences in the distribution of microtubules and microfilaments within M-II oocytes were noticed due to the different cumulus con-

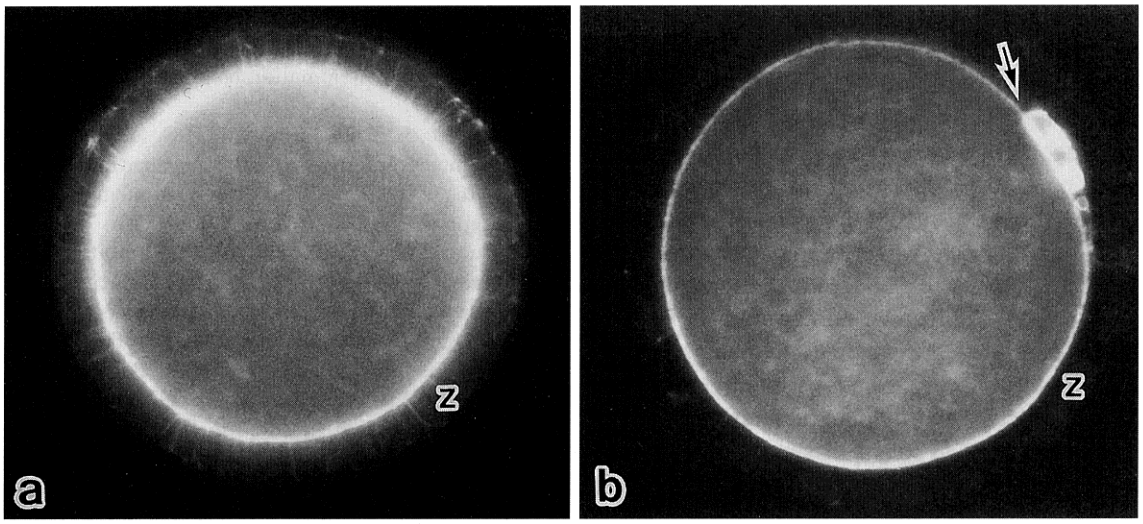

Fig. 2. Fluorescent micrographs of immature (a) and mature (b) porcine oocytes showing the distribution of microfilaments. × 460. Z indicates zona pellucida. a) An oocyte at the germinal vesicle stage before incubation, showing microfilament-rich peripheral region of the ooplasm. Note abundant transzonal cell projections. b) A metaphase-II oocyte at 44 h of incubation. The contact surfaces of the oocyte and the polar body (arrow) show strong response to actin staining. The number of transzonal cell projections has diminished dramatically.

ditions examined here. Certain factors produced by cumulus cells during follicular growth or IVM are essential for the oocyte to acquire the ability to be fertilized and to sustain normal embryo development [7–9]. Because it has also been reported that the presence of the follicle wall during IVM of pig oocytes induces some modifications in the process of sperm penetration into the oocyte and the formation of the male pronucleus [23], further studies are needed to clarify the biological significance of those factors.

The present study also demonstrated that high maturation rates were obtained after 44 h IVM at both 37°C and 39°C. But this is not in accordance with the results obtained by Eng *et al.* [3], who reported a higher percentage of polar body formation when the porcine oocytes were cultured at 39°C than at 37°C (71% vs. 37%). The difference between the results of the current and the previous studies [3] may be due to differences in the culture media used and in the evaluation methods. In the present study, the final maturation rates at 44 h IVM were similar at 37°C and 39°C, whereas the frequency of GVBD at 24 h IVM and that of the oocytes at the M-II stage at 36 h IVM were higher at 39°C than at 37°C. These observations suggest that GVBD and the meiotic progression were drastically affected by a 2°C decrease in incubation temperature.

Lenz *et al.* [1] reported that temperatures ranging between 35 and 39°C had no deleterious effects on resumption and completion of meiosis of bovine oocytes, but the oocyte maturation was inhibited at 41°C. They also showed that oocytes matured at 39°C (the core body temperature of the species) had significantly higher rates of *in vitro* fertilization than at 35°C, 37°C, or 41°C. In mice, pronuclear embryos suffered deleterious effects on 39°C incubation, 2°C higher than the core body temperature of the species (37°C), whereas two-cell embryos seemed to be less susceptible to high incubator temperature [24, 25]. These results suggest that the oocytes may be more sensitive to mild increases (as higher as a 2°C increase) in incubation temperature than the embryos. A drastic delay in meiotic progression responding to a 2°C decrease in incubation temperature might be associated with a decline in the cellular metabolic rate, depending upon the temperature change, and subsequently progression in the cell cycle may be decelerated. It has been reported that culturing at 39°C is more advantageous than at 37°C for the development of porcine ova after sperm penetration [26].

In the routine work of the *in vitro* fertilization/embryo culture programs, recovery, transfer and culture of oocytes (and also embryos) have been performed to avoid extreme temperature shock, but with reduced temperatures. In addition, temperature fluctuation might occur

in incubators, when the incubator door is frequently opened. Such a decrease in temperature might ultimately slow the rate of cellular division and affect meiotic progression and also further development. In human *in vitro* fertilization programs, it has been suggested that the number of incubator door openings, resulting in rapid temperature changes, affects the rates of fertilization and pregnancy [27]. The IVM sheep oocytes developed chromosomal anomalies if allowed to cool approximately 12°C in 3 h [28]. More attention should be paid to minimizing temperature fluctuation when the oocytes are manipulated *in vitro*. In conclusion, it is suggested that meiotic maturation of the porcine oocytes may be affected by cumulus-oocyte communication and the meiotic progression may be affected by incubation temperature.

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