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Maturation of porcine oocytes after cooling at the germinal vesicle stage

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Summary

Maturation of porcine oocytes was examined after oocytes were cooled at the germinal vesicle stage. Cumulus–oocyte complexes (COCs) collected from medium-sized follicles were cooled at 24 °C or 4 °C for 5, 30 or 120 min in a solution with or without 1.5 M dimethylsulfoxide (DMSO). After rewarming, COCs were cultured in maturation medium at 39 °C, 5% CO_2 in air for 44 h. Meiotic spindle organisation (by immunostaining and confocal microscopy), nuclear maturation (by orcein staining) and cytoplasmic maturation (by intracellular glutathione assay) of oocytes were examined after maturation. When COCs were cooled at 24 °C for various times in the medium without DMSO, a tendency to decreased spindle formation, nuclear maturation and cytoplasmic maturation was observed, but there was no statistical difference compared with controls. Addition of DMSO during cooling inhibited subsequent nuclear maturation and spindle formation. When COCs were cooled at 4 °C, both nuclear and cytoplasmic maturation as well as spindle formation were inhibited in most oocytes in a time-dependent manner. DMSO during cooling did not have any beneficial effect on subsequent oocyte maturation and spindle formation. These results suggest that porcine oocytes are very sensitive to a drop in the temperature before exposure to culture. Cooling oocytes before maturation inhibits their subsequent spindle organisation, nuclear and cytoplasmic maturation. Addition of DMSO to the cooling solution did not protect porcine oocytes from cooling-induced damage.

Keywords: Cooling, Maturation, Oocyte, Pig, Spindle

Introduction

Most porcine oocytes exposed to *in vitro* maturation (IVM) can reach metaphase II (M-II) after 36–44 h of culture (Wang *et al*., 1994; Kim *et al*., 1996). However, incomplete cytoplasmic maturation after IVM and a high rate of polyspermic penetration after *in vitro* fertilisation (IVF) are two unsolved problems for porcine embryo production *in vitro*. For IVM, ovaries are usually collected from a slaughterhouse and transported to laboratory, where cumulus—oocyte complexes (COCs) are aspirated from medium-sized follicles at room temperature (Wang *et al*., 1997; Abeydeera *et al*., 2000) and then exposed to maturation medium. Almost all oocytes are at germinal vesicle (GV) stage at this point. After 36–44 h of culture, most oocytes undergo GV breakdown (GVBD) and reach M-II stage. During IVM, the cell cycle transition is accompanied by extensive assembly/disassembly of the microtubule network. The proper assembly, functioning or disassembly of microtubules is an absolute prerequisite for the proper completion of oocyte maturation. As oocytes are more sensitive to temperature changes than other germ cells and somatic cells, *in vitro* manipulation may be important for normal oocyte maturation. Although the effects of temperature changes on oocyte maturation in some mammals have been reported (Moor & Crosby, 1985; Vincent *et al*., 1989; Wu *et al*., 1999), to our knowledge, there is still no report of a systematic examination of the effects of cooling on porcine oocyte maturation. In addition, only spindle formation was examined in most reports; nuclear maturation and cytoplasmic maturation have

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not been examined after cooling treatment. Therefore, in this study experiments were designed to examine whether cooling of porcine oocytes before IVM affects subsequent spindle formation, nuclear maturation and cytoplasmic maturation. The effects of dimethylsulfoxide (DMSO) as a cryoprotectant during cooling were also examined. Our results indicate that porcine oocytes are very sensitive to low temperature; detrimental effects of cooling on porcine oocytes were observed after IVM, including reduced normal spindle formation, and decreased nuclear and cytoplasmic maturation. Furthermore, we found that DMSO does not protect oocytes from cooling-induced damage.

Materials and methods

Medium

Unless otherwise stated, all chemicals used in this study were purchased from Sigma (Sigma Chemical, 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 sulfate, 0.1% polyvinyl alcohol (PVA), 10 IU hCG/ml, 10 IU eCG/ml and10 ng/ml epidermal growth factor (EGF).

Collection and culture of oocytes

Ovaries were collected from a slaughterhouse and transported to the laboratory (~3) in warm (30 °C) 0.9% (w/v) NaCl solution containing 75 μg/ml potassium penicillin G and 50 × μg/ml streptomycin sulfate. COCs were aspirated from medium-sized antral follicles (2- 6 mm in diameter) and washed three times with HEPES-TL-PVA medium before cooling. After cooling, COCs were washed and cultured in TCM 199 supplemented with chemicals mentioned above, 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 h. COCs were cultured at 39 °C for 44 h in the same atmospheric conditions.

Immunofluoresence staining

COCs from each experimental condition were removed from the cumulus cells in maturation medium containing 0.1% hyaluronidase and fixed in 3.7% paraformaldehyde in phosphate-buffered saline (PBS). After fixation for 30–40 min at room temperature, oocytes were cultured in PBS containing 0.5%Triton X-100 and 3% bovine serum albumin (BSA) for 40 min at 37 °C or overnight at 4 °C and then blocked in PBS containing 115 mM glycine and 3% BSA for 40 min. 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 (FITC)-labelled goat anti-mouse serum (1:80) for 40–60 min. After three additional washes, oocytes were incubated in PBS containing 10 ng/ml propidium iodide (PI) for 5 min for nuclear staining. All treatments were carried out at 37 °C. After staining, oocytes were mounted on slides and examined using a Nikon epifluorescence microscope or Leica scanning confocal microscope.

Spindles morphologies in the oocytes were classified into three categories (Fig. 1): (1) Normal spindles: barrel-shaped spindle with the chromosomes clustered

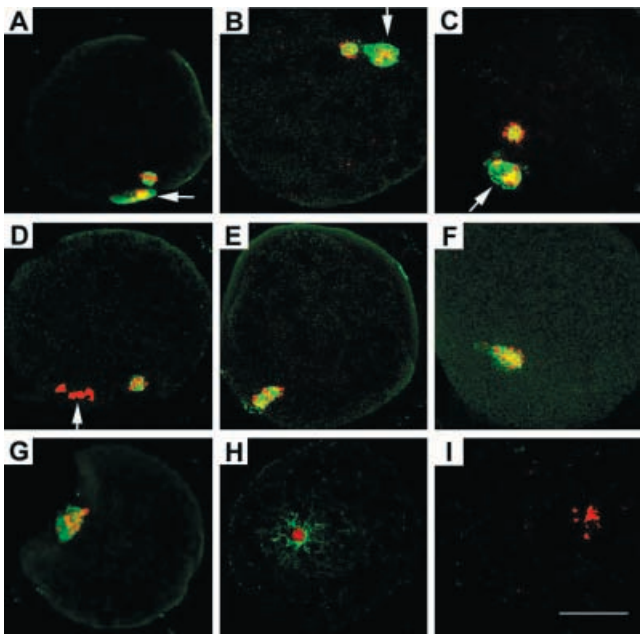

Figure 1 Spindle formation and chromosome alignment in oocytes after cooling at the GV stage for various times and then culture for 44 h. Oocytes were stained with FITC-labelled anti-α-tubulin and propidium iodide and examined by microscopy. (*A*) A control oocyte has a normal spindle and chromosome alignment. (*B*)–(*D*) Normal nuclear maturation and spindle formation in oocytes after cooling–rewarming and IVM. The oocytes were cooled at 24 °C for 30 min (*B*), 24 °C for 120 min (*C*) and 4 °C for 5 min (*D*). (*E*)–(*I*) Abnormal nuclear maturation and spindle formation in oocytes after cooling–rewarming and IVM. (*E*) A-I oocytes cooled at 4 °C for 5 min before IVM; (*F*) M-I oocytes cooled at 4 °C for 30 min and (*G*) M-I oocytes cooled for 24 °C for 120 min before IVM; and (*H*) pre-M-I and (*I*) GV stages cooled at 4 °C for 30 min before IVM. Arrows indicate the first polar body. Red represents chromatin, green represents microtubules and yellow represents the overlay of the green and red images. Scale bar represents 25 μm.

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 organised to form typical spindles or some microtubules were missing between poles; (3) No spindle.

Orcein staining

Orcein staining was conducted as described by Wang *et al*. (1994). Briefly, cumulus-free oocytes were mounted on slides, fixed for 48–72 h in 25% (v/v) acetic acid in alcohol at room temperature, stained with 1% (w/v) orcein in 45% (v/v) acetic acid and examined by phase-contrast microscopy at a magnification of ×400 for assessment of nuclear stages.

Glutathione assay

The glutathione assay was based on the procedures reported by Wang *et al*. (1997). Briefly, after culture, oocytes were completely denuded of cumulus cells and washed three times with a stock buffer (0.2 M sodium phosphate-containing 10 mM sodium EDTA, pH 7.2). Five microlitres of stock buffer containing 20–30 oocytes was transferred to a 1.5 ml microfuge tube. The oocytes were ruptured by agitation with a narrow-bore glass pipette and 5 µl of 1.25 mM phosphoric acid was added to the tube. The tubes containing samples were kept at –30 °C until assay. The content of glutathione in the oocytes was determined by the 5,5′-dithiobis-(2-nitobenzoic acid) and glutathione disulfide reductase recycling assay (Anderson, 1985).

Experimental designs

COCs were either maintained at 39 °C as control or cooled to 24 °C or 4 °C for 5, 30 or 120 min before IVM in Petri dishes containing 100 µl of HEPES-TL-PVA with or without 1.5 M DMSO that had been equilibrated at 4 °C or 24 °C (room temperature) for about 3 h before putting oocytes. After cooling treatment, oocytes were washed twice with maturation medium and cultured for 44 h at 39 °C, in 5% CO_2 in air. After culture, nuclear maturation, glutathione content and spindle morphology were examined.

Statistical analysis

Experiments were replicated five times. The data from maturation and spindle morphology were pooled for statistical analysis (50–90 oocytes per treatment). The percentages were subjected to an arcsine transformation, and the transformed values were analysed by ANOVA. The data for glutathione content were expressed as mean ± SD and analysed by Student's *t*-test.

Results

In a preliminary experiment, we found that all oocytes were at GV stage after aspiration from medium-sized follicles. Therefore, in this study, we considered that all oocytes were cooled at the GV stage. As compared with the control, exposing oocytes to 24 °C in the medium without DMSO for up to 120 min did not sig-

Table 1 Maturation of porcine oocytes cooled to 24 or 4 °C at the germinal vesicle stage and then cultured *in vitro* for 44 h

Temp (°C)	Time (min)	DMSO during cooling	Total no. of oocytes examined	No. (%) of oocytes at the stage of				
				GV	GVBD~PreM-I	M-I	M-II	Deg
Control			68	0(0)	2 (3)	8 (12)	58 (85)^{a}	0
24	5	+	84	7 (8)	13 (15)	11 (13)	52 (63)^{b}	1 (1)
		–	80	1 (1)	6 (7)	10 (13)	61 (76)^{ab}	2 (3)
	30	+	65	2 (3)	8 (13)	8 (12)	43 (66)^{b}	4 (6)
		–	71	3 (4)	5 (7)	7 (10)	54 (76)^{ab}	2 (3)
	120	+	77	4 (5)	11 (14)	10 (13)	44 (57)^{b}	8 (11)
		–	70	4 (6)	4 (5)	9 (13)	50 (71)^{ab}	3 (5)
4	5	+	73	12 (16)	25 (35)	13 (18)	22 (30)^{c}	1 (1)
		–	79	16 (21)	22 (29)	9 (11)	28 (35)^{c}	3 (4)
	30	+	83	6 (7)	36 (43)	6 (7)	31 (38)^{c}	4 (5)
		–	98	17 (17)	46 (47)	2 (2)	32 (33)^{c}	1 (1)
	120	+	65	14 (23)	36 (57)	9 (10)	9 (14)^{d}	4 (6)
		–	70	29 (42)	30 (43)	2 (3)	6 (8)^{d}	3 (4)

GV, germinal vesicle; GVBD, germinal vesicle breakdown; M-I, metaphase I; M-II, metaphase II; Deg, degenerated.
^{a,b,c,d}Values with different superscripts within each column are significantly different, $p < 0.05$.

Table 2 Spindle morphology of porcine oocytes cooled to 24 or 4 °C at germinal vesicle stage and then cultured for 44 h

Temp (°C)	Time (min)	DMSO during cooling	Total no. of oocytes examined	Spindle morphology		
				Normal %	Abnormal %	None %
Control (39°C)			37	28 (76)[a]	8 (22)[a]	1 (2)[a]
24	5	+	72	52 (73)[a]	19 (26)[ab]	1 (1)[a]
		–	65	48 (74)[a]	16 (25)[ab]	1 (1)[a]
	30	+	65	45 (69)[ab]	18 (28)[ab]	2 (3)[ab]
		–	60	44 (73)[a]	13 (21)[a]	3 (6)[ab]
	120	+	67	40 (59)[b]	21 (31)[ab]	6 (10)[ab]
		–	71	48 (67)[ab]	18 (25)[a]	5 (8)[ab]
24	5	+	60	31 (52)[b]	24 (40)[b]	5 (8)[ab]
		–	63	35 (55)[b]	23 (36)[ab]	5 (9)[ab]
	30	+	52	26 (37)[c]	34 (49)[bc]	10 (14)[ab]
		–	70	15 (29)[c]	28 (53)[bc]	9 (18)[b]
	120	+	63	12 (20)[d]	30 (56)[c]	14 (24)[b]
		–	59	8 (12)[d]	36 (57)[c]	19 (31)[b]

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

nificantly ($p > 0.05$) affect subsequent oocyte maturation and spindle formation but there was a tendency to reduced maturation and spindle formation with prolonged cooling time (Table 1). When oocytes were cooled at the same temperature (24 °C) in the medium with DMSO, nuclear maturation and normal spindle formation were significantly reduced. Accordingly, the proportions of oocytes with condensed and dispersed chromosomes increased.

When oocytes were exposed to 4 °C for 5, 30 or 120 min and then cultured, the proportions of oocytes reaching M-II stage were significantly reduced from 85% in the control to 30–35%, 33–38% and 8–14% ($p < 0.01$) in medium with and without DMSO, respectively. High proportions of oocytes were at GVBD/prometaphase I stages after being cooled for 30 min (43–47%), but were at GV stage (23–42%) or at GVBD/premetaphase I stages (57–43%) after being cooled for 120 min. The proportions of oocytes with normal spindle morphology also decreased significantly ($p < 0.05$) in a time-dependent manner after oocytes were cooled in the medium with (52–20%) and without (55–12%) DMSO (Table 2). The proportions of oocytes with abnormal (36–57%) and missing (8–31%) spindles also increased in a time-dependent manner when oocytes were cooled at 4 °C. There was no difference between the groups with and without DMSO at each time point. The spindle disassembled more quickly at 4 °C than at 24 °C ($p < 0.05$) as shown by more oocytes having abnormal and missing spindles in the 4 °C group than in the 24 °C group.

As shown in Fig. 2, glutathione contents in oocytes exposed to 24 °C for 5 min (9.25 ± 0.36 pmol/oocyte), 30 min (7.95 ± 1.49 pmol/oocyte) or 120 min (9.89 ± 0.92 pmol/oocyte) were not statistically different from that in the control (10.59 ± 1.25 pmol/oocyte). However, when oocytes were exposed to 4 C for 5, 30 or 120 min, it significantly reduced glutathione synthesis (5.04 ± 0.17 to 5.93 ± 0.5 pmol/oocyte) ($p < 0.05$).

Discussion

This study examined the effects of cooling on spindle formation and meiotic progression of porcine oocytes cooled at 24 °C or 4 °C for various periods at GV stage. The effect of DMSO, as a cryoprotectant, was also examined. The results indicate that reducing the temperature before IVM induces subsequent alterations in spindle organisation, and cooling oocytes before IVM inhibits both nuclear and cytoplasmic maturation. The results also indicate that DMSO does not protect oocytes from the damage induced by cooling.

The response of the microtubules of meiotic spindles to cooling was different in different species. Sheep oocytes at M-I to A-I stages cooled below 29 °C for 3 h had the least degree of disruption and the oocytes undergoing GVBD were particularly sensitive to cooling during maturation (Moor & Crosby, 1985). During these periods, oocytes underwent a dramatic reprogramming involving 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 due entirely to nuclear events. Cooling bovine oocytes at GV stage for 10 min at 31 °C or 24 °C did not signif-

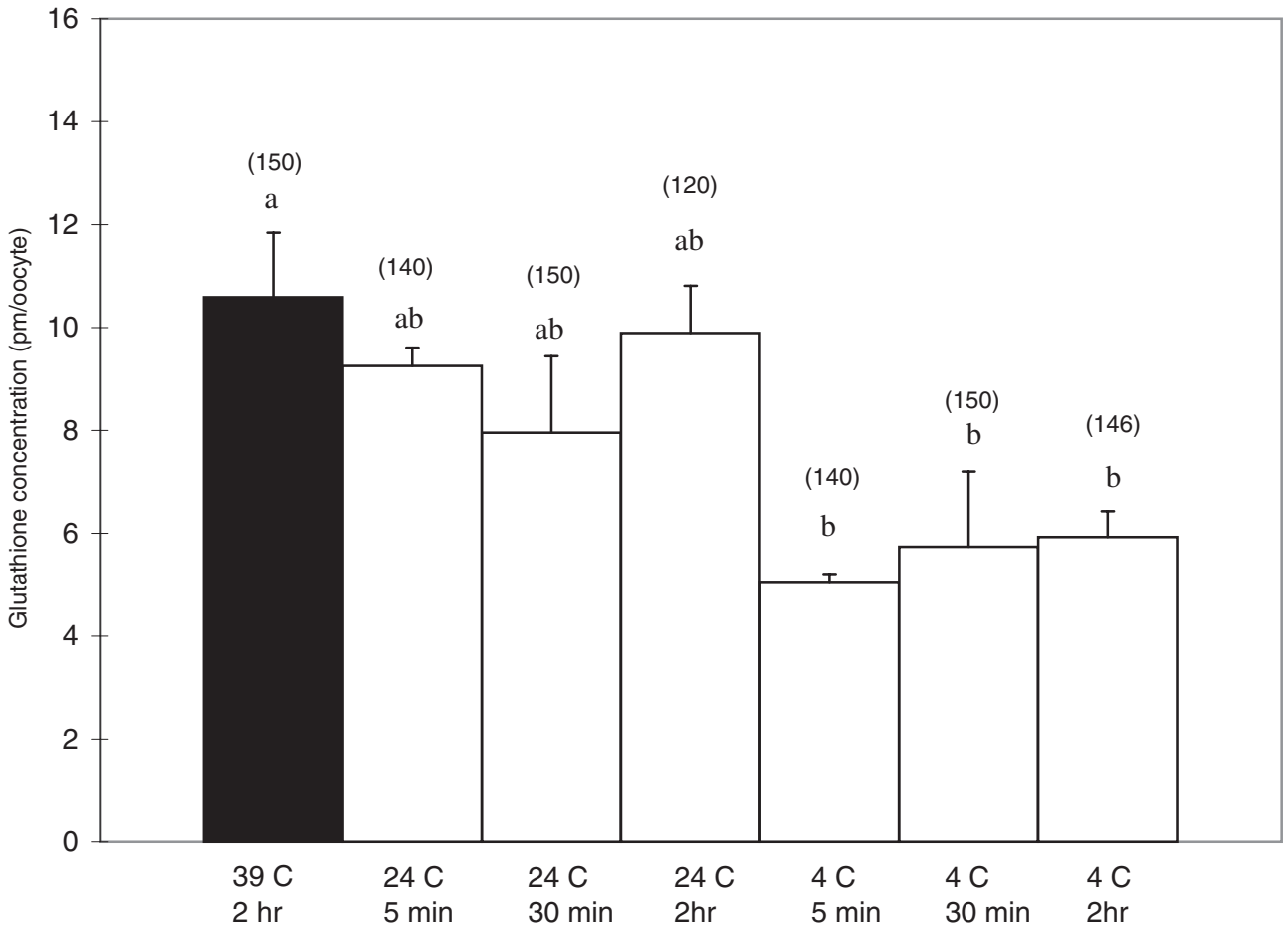

Figure 2 Glutathione concentration in oocytes exposed to 24 °C or 4 °C for 5, 30 or 120 min and then cultured in a maturation medium for 44 h. The numbers of oocytes examined are shown in parentheses. a,b: $p < 0.05$.

icantly alter the formation of microtubules and meiotic spindles (Wu *et al*., 1999). However, when cooling bovine oocytes at M-II stage for 5 min at 25 °C, the microtubules of the meiotic spindle started to depolymerise (Aman & Parks, 1994). On the other hand, mouse oocytes subjected to cooling at approximately 25 °C for 60 min followed by 60 min at 37 °C showed a normal spindle (Pickering & Johnson, 1987). Also mouse oocytes subjected to cooling at 4 °C for 60 min followed by 60 min at 37 °C recovered normal spindles in 89% of cases. However, limited spindle recovery was observed in human oocytes after cooling–rewarming, which was examined in greater detail in individual oocytes with polarised light microscopy (Wang *et al*., 2001).

The main structural elements of spindle fibres are microtubules (Inoue, 1981). Low temperature induces disassembly of the microtubules and this disassembly is the result of the depolymerisation of tubulin, the major structural protein of microtubules (Moor & Crosby, 1985). In contrast, the microtubules in GV oocytes have not been assembled into the spindle structure and there is enough time for microtubules in cooled oocytes to polymerise to form a spindle during *in vitro* culture. Studies have shown that chromosomes enhance spindle microtubule assembly and play an essential role in the initiation of spindle formation (Zhang & Nicklas, 1995). Meanwhile, two kinetochores of chromosomes capture microtubules in the spindles from opposite poles and ensure the chromosomes segregate accurately (Nicklas, 1997). The organisation of the meiotic spindle requires both the chromosomes, which cause a local reduction in the threshold for microtubule polymerisation, and the pericentriolar material, which nucleates microtubule polymerisation. Only in the vicinity of the chromosomes is the pericentriolar material able to sustain nucleation of microtubules, whereas conditions in the remainder of the cell favour rapid polymerisation (Pickering *et al*., 1990). Thus, if the chromosomes and the pericentriolar material are functional, the microtubules can assemble a normal spindle structure. By contrast, if the chromosomes are damaged, microtubules could assemble around the chromosomes but a normal spindle could

not form, as seen in the oocytes cooled at 4 °C. Therefore, abnormal spindle formation and low nuclear and cytoplasmic maturation of oocytes cooled at 4 °C may be due to damage to the cytoplasm and/or nucleus during cooling and rewarming.

The process of oocytes maturation is complicated, as it includes chromosomes finishing the first meiosis and being arrested at the middle stage of the second meiosis; microtubules being assembled into a barrel-shaped spindle preparatory to the correct division of chromosomes; and sufficient RNA and proteins being synthesised and stored for the following early embryo development. However, the mechanisms of oocyte maturation are not completely understood. At present, nuclear maturation of oocytes, which is identified by the first polar body being released from oocytes and the oocyte acquiring a metaphase plate (i.e. M-II oocytes), can easily be induced during IVM (Wang *et al*., 1997). However, cytoplasmic maturation of oocytes that results in oocytes being fertilised normally and early embryos being completely developed is not fully achievable. It has been found that an increased intracellular concentration of glutathione in maturing pig oocytes could induce full decondensation of sperm nuclei, removing sperm protamine and replacing it with somatic histones which are essential for male pronuclear formation and subsequent embryo development: therefore the concentration of glutathione in the oocyte now is an important marker for predicting cytoplasmic maturation, especially in pig oocytes (Sawai *et al*., 1997).

We examined the nuclear status and glutathione content in cooled oocytes at GV stage followed by 44 h of *in vitro* culture. The results showed that cooling GV oocytes at room temperature even for 2 h did not significantly affect either nuclear or cytoplasmic maturation, whilst cooling GV oocytes at 4 °C significantly reduced both nuclear and cytoplasmic maturation.

Cooling bovine oocytes at GV stage to 24 °C did not affect cleavage rates after the oocytes were rewarmed, then matured and inseminated (Wu *et al*., 1999). But cleavage rates were significantly decreased when oocytes were chilled to 4 °C or 0 °C. Vincent *et al*. (1989) also found reduced fertilisation and development rates of frozen M-II oocytes in cattle. Taken together, the inhibition of maturation in oocytes subjected to various chilling treatments may be due to the detrimental effect of cooling on microtubules, chromosomes and the synthesis of some important cytokinesis factor, such as glutathione.

DMSO, as a cryoprotectant, has been successfully used for the cryopreservation of somatic cells (Saeed *et al*., 2000) and embryos (Nowshari *et al*., 1995). However, the positive effects of DMSO were not observed when oocytes were cryopreserved (Magistrini & Szöllösi, 1980; Van der Elst *et al*., 1992). Eroglu *et al*. (1998) 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 control the osmotic forces, an increased survival rate (up to 95%) was observed. Such a potential in porcine oocyte cooling or cryopreservation needs further investigation.

Acknowledgements

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