Attempts to Cool Pig Oocytes

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Abstract The present study was carried out to clarify the tolerance of pig oocytes toward cooling to temperatures above freezing. Oocytes at the germinal vesicle stage were recovered from ovaries. Oocytes at 30°C were cooled at 0.5 or 3°C/min from 30°C to 0, 10 and 20°C, and held for 0.1–60 min at these temperatures. They were then cultured by the microdrop method at 37°C for 40–43 hr in 5% CO₂ in air. Both before cooling, and after the oocytes were cooled to 0, 10 and 20°C at 0.5°C/min, the proportion of oocytes maturing to metaphase II during culture was 54, 0, 0, and 18%, respectively. The cooling rate of 3°C/min was more damaging to oocytes cooled to 20°C than that of 0.5°C/min. Holding for 60 min at 20°C decreased the proportion of oocytes maturing to metaphase II, whereas holding for 60 min at 30°C had no effect. The addition of cryoprotectants to the phosphate-buffered solution showed no adverse effects on the tolerance of cooled oocytes. These results indicate that pig oocytes at the germinal vesicle stage appear to be extremely sensitive toward cooling.

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Since the first successful preservation of mouse embryos at low temperatures¹⁾, the deep freezing of embryos has now been achieved in a number of farm and laboratory animals²⁾. However, pig embryos have not been frozen successfully to date^{2,3)} and less attention has been devoted to the low temperature preservation of pig embryos compared to cattle embryos. Pig embryos survived when cooled to 15–20°C, whereas embryos cooled to 0–10°C died^{4,5)}. In contrast, recently it has been shown that pig embryos have survived after cooling to –5°C and being cultured *in vitro*⁶⁾.

Instead of the greater difficulty of obtaining a large number of embryos from farm animals, many oocytes can be obtained easily from ovarian follicles and are available for experimentation. Recently it has been reported that a high proportion of bovine oocytes at the germinal vesicle stage can mature *in vitro* to metaphase II after cooling to 4°C⁷⁾.

In a preliminary paper, we examined the susceptibility of pig oocytes to cooling to temperatures above freezing⁸⁾. The present study reports in detail all the experiments to clarify the tolerance of pig oocytes to cooling to temperatures around 0°C.

Materials and Methods

Oocyte collection

Pig oocytes were obtained from prepubertal gilts of Landrace, 6–7 months old, at

a slaughterhouse. The ovaries were transported to our laboratory in a physiological saline solution at 30–35°C. The oocytes at the germinal vesicle stage were liberated from follicles (2–5 mm in diameter) with a needle at 30–35°C.

Suspension solution and cryoprotectants

The suspension solution used for the collection of oocytes was a modified DULBECCO's phosphate-buffered saline⁹⁾ supplemented with a 20% heat-inactivated fetal calf serum (PBS). In each experiment, the oocytes with corona cells from 2 to 4 females were used. The oocytes were washed twice with fresh PBS, and 15–30 oocytes were pipetted into each test-tube (10 × 100 mm) containing 0.2 ml PBS at 30°C. In some experiments the oocytes were placed into test-tubes containing 0.2 ml 0.5 M sucrose, 1.5 M dimethyl sulphoxide (DMSO) or 1.5 M glycerol in PBS.

Cooling and recovery of cooled oocytes

Except for the samples held at 30°C, the remaining samples were initiated into the cooling process immediately after the oocytes recovered from ovaries were transferred into a test-tube at 30°C. The samples were cooled at 0.5 or 3°C/min from 30°C to 0, 10 and 20°C. Immediately upon cooling to these temperatures, the oocytes were recovered in a watch glass and washed several times with the culture medium at room temperature.

In some experiments the oocytes were held for 0.1, 20 and 60 min at 0–30°C before recovery in a watch glass at room temperature. The holding time at 30°C means the duration incubated at 30°C after the oocytes, recovered from the ovaries, were transferred into a test-tube at 30°C.

Culture

After the oocytes were washed, they were cultured by the microdrop method in a modified Krebs-Ringer bicarbonate medium¹⁰⁾ supplemented with a 20% heat-inactivated fetal calf serum at 37°C for 40–43 hr in 5% CO₂ in air.

Survival assay

After culture, the oocytes were mounted on slides and fixed in acetic acid-ethanol (1 : 3) for 4–7 days. Then they were stained with aceto-orcein and examined for maturation. The viability of the cooled oocytes was assessed by their ability to mature to metaphase II from the germinal vesicle stage during culture. Experiments were replicated 2–5 times and the data were analyzed for statistical significance using an χ^2 test.

Results

Before cooling or after oocytes at the germinal vesicle stage without cryoprotectant were cooled to 0, 10 and 20°C at 0.5°C/min, the proportion of oocytes matured to metaphase II during culture was 54, 0, 0 and 18%, respectively (Table 1). In the next experiments the oocytes were cooled at 3°C/min. Before cooling or after having been cooled to 0, 10 and 20°C at 3°C/min, the rate of maturation was 49, 0, 0 and 3%, respectively (Table 1). The rate (3%) of maturation after having been cooled at 3°C/min to 20°C was lower than that (18%) of oocytes cooled at 0.5°C/min ($P < 0.01$).

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Table 1. The proportion of pig oocytes at the germinal vesicle stage matured to metaphase II during culture after cooled at 0.5 or 3 °C/min to temperatures above freezing

Cooling rate (°C/min)	Minimum temperature (°C)	No. of oocytes recovered after cooling	% of oocytes matured to metaphase II during culture
0.5*	Before cooling	81	54
	20	85	18
	10	74	0
	0	77	0
3*	Before cooling	57	49
	20	90	3
	10	82	0
	0	66	0

* Experiments were carried out independently.

Immediately upon cooling to 0, 10 and 20°C, the oocytes were recovered and washed with culture medium at room temperature before culture.

Table 2. The proportion of pig oocytes at the germinal vesicle stage matured to metaphase II during culture after cooling in the presence of cryoprotectants

Cryoprotectant	Minimum temperature (°C)	No. of oocytes recovered after cooling	% of oocytes matured to metaphase II during culture
Without cryoprotectant	Before cooling	87	51
	20	78	15
	10	71	0
1.5 M DMSO	Before cooling	73	48
	20	68	7
	10	79	0
1.5 M glycerol	Before cooling	82	55
	20	85	18
	10	79	0
0.5 M sucrose	Before cooling	92	56
	20	80	20
	10	63	0

The oocytes were cooled at 0.5°C/min from 30°C to various minimum temperatures. Immediately upon cooling to 10 and 20°C, the oocytes were recovered and washed with culture medium at room temperature before culture.

As shown in Table 2, all the cryoprotectants (sucrose, DMSO and glycerol) examined had no adverse effects on the sensitivity of oocytes to cooling damage.

As shown in Table 3, the proportion of oocytes matured to metaphase II after being held for 0.1, 20 and 60 min at 30°C was 46, 52 and 41%, respectively. By

Table 3. The proportion of pig oocytes at the germinal vesicle stage matured to metaphase II during culture after cooling and holding for 0.1-60 min at various minimum temperatures

Minimum temperature (°C)	Holding time (min)	No. of oocytes recovered after cooling	% of oocytes matured to metaphase II during culture
30	0.1	65	46
	20	75	52
	60	61	41
20	0.1	72	21
	20	66	12
	60	81	1
10	0.1	58	0
	20	63	0
0	0.1	52	0

The oocytes were cooled at 0.5°C/min from 30°C to various minimum temperatures and held for 0.1-60 min.

The holding time at 30°C means the duration incubated at 30°C after the oocytes recovered from ovaries were transferred into test-tube at 30°C.

contrast, the rate of maturation was 21, 12 and 1% when held for 0.1, 20 and 60 min at 20°C. Hence, holding time of 60 min at 20°C decreased the survival of oocytes, whereas holding time at 30°C had no effects.

Discussion

In the present study, pig oocytes at the germinal vesicle stage, cooled to 0 and 10°C, completely lost their ability to mature to metaphase II during culture. Attempts of slow cooling were unsuccessful. By contrast, when cattle oocytes at the germinal vesicle stage were cooled to 4°C, 62% of the oocytes matured to metaphase II during culture and 34% of the oocytes after being held for 6 hr at 4°C⁷⁾. The sensitivity of pig oocytes to cooling appears to be even more marked than that of cattle oocytes.

Deep freezing of cattle embryos is now well established and commonly employed in cattle breeding, whereas no pig embryos at 8-cell and blastocyst stages survived even after cooling to 0-10°C^{4,5)}. These results show that there are differences between cattle and pig in the responses of embryos to cooling as observed in oocytes. Recently it has been shown that Days-5-6 pig embryos survived after cooling to -5°C and culturing *in vitro*⁶⁾. Furthermore, a few of the fully expanded pig blastocysts could hatch from the zona pellucida after deep freezing and culturing, but none survived to term after transplantation¹¹⁾. Considerable progress in the preservation of pig embryos and oocytes will be made through experiments designed to study an understanding of the nature of differences between species.

The reasons for the extreme sensitivity of pig oocytes and embryos to cooling are still obscure. It has been shown that triacylglycerol is the major lipid component of

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pig oocytes followed by cholesterol and diacylglycerol and that while the lipid composition of pig oocytes is similar to that of other cell types, there are some striking differences¹²⁾. Since during cooling and warming there was an abundant loss of intracellular lipids in the pig embryos, the damage caused to pig embryos during cooling may be associated with lipid phase changes within the cell membranes²⁾. Further studies are necessary for the cryopreservation of pig embryos and oocytes.

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ブタ卵胞卵の冷却の試み

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ブタ卵胞卵を 0-20℃ の温度に冷却したときの生存性を明らかにするために, 本実験を行なった. 直径 2-5 mm の卵胞から卵核胞期の卵胞卵を採取し, 20% の牛胎児血清を含む修正リン酸緩衝液 (30℃) に浮遊させた. 卵胞卵を 0.5 または 3℃/分の速度で 0, 10 および 20℃ に冷却し, これらの温度に到達直後, または 0.1, 20 および 60 分間保った後に室温下の培養液で洗浄後, 培養した. 卵胞卵を, 冷却前または 0.5℃/分の速度で 0, 10, 20℃ に冷却し, ただちに室温下の培養液で洗浄後培養した場合, 卵核胞期から第 2 成熟分裂中期に移行した割合はそれぞれ 54, 0, 0 および 18% であった. また卵胞卵を 3℃/分の速度で 0, 10, 20℃ に冷却した場合, 第 2 成熟分裂中期に移行した割合はそれぞれ 0, 0 および

3% であり, 20℃ に冷却した卵の移行率は 3℃/分より 0.5℃/分の方が高かった. 30℃ で卵巣から採取した卵胞卵を洗浄し小試験管に移した後, 30℃ で 60 分間保持しても, 培養後第 2 成熟分裂中期へ移行する割合は低下しなかったが, 20℃ で 60 分間保持すると培養後の移行率は低下した. ジメチルスルフォキシド, グリセロールおよびショ糖を添加しても, 冷却された卵胞卵の生存性は影響を受けなかった. 以上の成績から, 本研究における実験条件下では卵核胞期のブタ卵胞卵を 0-10℃ に冷却すると死滅し, ブタ卵胞卵は冷却処理にきわめて弱いと思われる.

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