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Fertilization *in vitro* and development to term of unfertilized mouse oocytes previously stored at −196°C

D. G. Whittingham

M.R.C. Mammalian Development Unit, Wolfson House, 4 Stephenson Way, London NW1 2HE, U.K.

Summary. Unfertilized mouse oocytes were frozen and stored at −196°C. Immediately after thawing 331 out of 463 MF1 oocytes (71·5%) and 271 out of 410 F_1 (C57BL × CBA) hybrid oocytes (66·1%) were morphologically normal. No significant difference was found between the survival of oocytes frozen and thawed with (70%) or without (66%) the surrounding cumulus cells. Fertilization *in vitro* of frozen–thawed oocytes was significantly lower than that of freshly collected control oocytes. The overall fertilization rate *in vitro* for MF1 oocytes was lower than with F_1 (C57BL × CBA) hybrid oocytes. The presence or absence of cumulus cells during fertilization *in vitro* did not affect the fertilization rate. Live 14-day fetuses were obtained after transfer of frozen–thawed unfertilized oocytes directly to the oviducts of females mated with fertile males. However, much higher rates of survival (up to 45%) to 14-day fetuses and live-born were found after the fertilization of frozen–thawed oocytes *in vitro* and subsequent transfer at the 2-cell or blastocyst stage to pseudopregnant recipients.

Introduction

During the past few years, preimplantation embryos of several mammalian species have been successfully stored at sub-zero temperatures (see review by Whittingham, 1975a). The earliest preimplantation stage that has survived freezing and thawing is the fertilized 1-cell mouse ovum (Whittingham, Leibo & Mazur, 1972), but no successful attempts of the storage of this stage have so far been reported for other species. The advantages of storing the early embryo rather than the separate gametes have been discussed by Whittingham (1975a), but the storage of the unfertilized oocyte could be of practical importance in the establishment of a bank from which particular genetic combinations could be originated. This paper reports on the storage of unfertilized mouse oocytes at −196°C and their subsequent fertilization *in vitro* and development to term after transfer.

Materials and Methods

Source of oocytes

Freshly ovulated oocytes were obtained from random bred MF1 (Olac Ltd) and F_1 (C57BL × CBA) females (approx. 6–12 weeks old) between 13 and 16 h after injection of HCG. The females were induced to superovulate with i.v. injections of 5 i.u. PMSG and 5 i.u. HCG 46–48 h apart. The oocytes were released from the excised oviducts into PB1 medium (Whittingham, 1974). At each collection the oocytes from the females of each strain were divided into 2 groups. In one group the oocytes were left with the cumulus cells intact and in the other group the cumulus cells were removed with hyaluronidase (150 units/ml) and the oocytes washed twice in PB1 medium.

Freezing and thawing

The general procedures for freezing and thawing mouse embryos are fully described elsewhere (Whittingham *et al.*, 1972; Whittingham, 1974). Between 1 and 3 cumulus masses or 20 and 30

cumulus-free oocytes were transferred to 0·15 ml PB1 medium contained in glass test-tubes or glass ampoules (2 ml borosilicate glass: see Whittingham, 1976). The samples were immediately transferred to an ice bath at 0°C and equilibrated with 1·5 M-dimethylsulphoxide (DMSO) 15 min later. At −80°C the samples were transferred directly to liquid nitrogen and stored for times varying from 24 h to 3 months. Samples were cooled from −10 to −65°C at rates between 0·25 and 0·58°C/min (mean 0·39 ± 0·04°C/min) and warmed from −65 to −10°C at rates between 3·4 and 14·4°C/min (mean 7·8 ± 0·6°C/min). Samples were held at −196°C for 1–2 days in the experiments in which the oocytes were subsequently fertilized *in vitro* and for 2–9 weeks in the experiments in which the oocytes were directly transferred to the oviducts after thawing. The DMSO was diluted out at 0°C and the oocytes recovered. After assessment of normality of appearance of the oocytes from which the cumulus cells had been removed, all oocytes were transferred directly without further washing to previously prepared sperm suspensions for fertilization *in vitro* or to the oviducts of recently mated females (see below). The zona was intact in all the eggs, whether normal or degenerating.

Fertilization and development in vitro

The procedures for fertilization *in vitro* were essentially those described previously (Kaufman & Whittingham, 1972; Hoppe & Pitts, 1973). The contents of a cauda epididymidis and vas deferens from each of 2 fertile MF1 males were expressed into 1 ml modified Tyrode's medium (Whittingham, 1971) containing 0·5 mM-pyruvate and 32 mg bovine serum albumin (Armour)/ml. For each experiment several suspensions were prepared and only that judged to have dispersed uniformly after 30 min incubation, i.e. with minimal agglutination, was diluted 1:10 for fertilization. The final sperm concentration ranged between 1450 and 2970/μl.

The frozen–thawed oocytes and control freshly collected oocytes (13–16 h after HCG) were added directly to the sperm suspensions. After incubation for 5–6 h at 37°C in 5% CO_2 in air, the oocytes were recovered and washed in several changes of a mouse embryo culture medium (Whittingham, 1971). Fragmented, misshapen and degenerate oocytes were discarded and the remainder were cultured in drops of medium overlaid with paraffin oil (Brinster, 1963). After culture for 24 h the number of 2-cell ova was recorded and these ova were either transferred or left in culture to develop to the early blastocyst stage before transfer. The MF1 ova rarely progressed beyond the 2-cell stage in culture but the F_1 ova developed to the blastocyst stage *in vitro* (Whittingham, 1975b).

Development of frozen-thawed oocytes in vivo

Three types of transfers were made to examine the survival of frozen–thawed oocytes. (1) MF1 oocytes, cumulus-free and cumulus intact, were transferred, directly after thawing, to the oviducts of F_1 (C57BL × CBA) females (kept on reversed daylight; dark period 10.00 to 18.00 h) which had mated with fertile MF1 males 2–4 h before. (2) Frozen–thawed oocytes (MF1 and F_1 (C57BL × CBA) fertilized *in vitro* were transferred at the 2-cell stage to the oviducts of pseudopregnant females on the day of finding the vaginal plug (Day 1 of pseudopregnancy). (3) Frozen–thawed F_1 oocytes fertilized *in vitro* were tranferred at the morula and early blastocyst stages, i.e. approximately 72–96 h after insemination, to the uterine horns of females on Day 3 pseudopregnancy.

Females were either examined on Day 14 of pregnancy and the number of live fetuses and resorbing implantation sites noted or they were allowed to litter.

Statistics

Data on the survival of frozen oocytes immediately after thawing and the postimplantation development of frozen–thawed oocytes fertilized *in vitro* were compared by χ^2 analysis using Yates' correction for continuity (Maxwell, 1964). The results of the experiments on fertilization *in vitro* of frozen–thawed and control oocytes were assessed by analysis of variance following angular transformation of the data (Snedecor & Cochran, 1967).

Initial survival of frozen–thawed unfertilized oocytes

In 4 experiments oocytes from MF1 and F_1 (C57BL × CBA) females were frozen either in the presence or absence of the cumulus cells. The number of oocytes frozen with cumulus cells could not be counted but the total number of oocytes recovered was recorded approximately 2 h after incubation with spermatozoa. In all 4 treatments the χ^2 tests for heterogeneity between experiments were not significant and therefore the combined data are presented in Table 1. There was no significant difference between the presence and absence of cumulus cells during freezing and thawing or between the source of oocytes: 331 out of 463 frozen–thawed MF1 oocytes (71·5%) and 271 out of 410 frozen–thawed F_1 oocytes (66·1%) were morphologically normal at recovery.

Table 1. The effect of cumulus cells on the survival of unfertilized mouse oocytes frozen and thawed in the presence of 1·5 M-DMSO

		Oocytes	
Source of oocytes	Cumulus cells	No. recovered/total no. frozen (%)	No. morphologically normal (%)
MF1	Present	279*	208 (74·6)
	Absent	184/223 (82·5)	123 (66·8)
F_1 (C57BL × CBA)	Present	186*	125 (67·2)
	Absent	224/251 (89·2)	146 (65·2)

* Only the total numbers of oocytes recovered are given because the number of oocytes frozen with cumulus cells could not be counted.

Fertilization in vitro *of frozen–thawed oocytes*

Initially, oocytes (MF1 and F_1) were examined to determine whether the cooling process *per se* or exposure to DMSO stimulated parthenogenetic activation. Oocytes with and without cumulus cells were recovered at various stages of the freezing procedure: (a) after 30 min exposure to 0°C without equilibration with DMSO; (b) 15 min after equilibration with DMSO at 0°C; (c) 10 min after induction of ice formation at −6°C, and (d) after storage at −196°C for 1 h. After recovery, they were cultured and subsequently examined for pronuclei and/or cleavage. No evidence was found that any of these treatments increased the incidence of activation above the level observed in the controls (2/124 (1·6%) for MF1 and 4/131 (3·1%) for F_1 oocytes). The removal of the cumulus cells with hyaluronidase did not affect activation.

The results of the 4 experiments comparing the fertilization *in vitro* of frozen–thawed and control oocytes with and without cumulus cells are given in Table 2. The data for each experiment are tabulated separately since there is a significant variation between the fertilization rate in the 4 experiments ($F_{3,21} = 29{\cdot}23$; $P < 0{\cdot}001$). This is mainly due to the lower fertilization rate observed in all treatments in the third experiment. A significantly greater proportion of control than frozen–thawed oocytes was fertilized *in vitro* ($F_{1,21} = 17{\cdot}82$; $P < 0{\cdot}001$), and a higher proportion of F_1 oocytes than MF1 oocytes was fertilized *in vitro* ($F_{1,21} = 8{\cdot}63$; $P < 0{\cdot}01$). The presence or absence of the cumulus cells did not significantly affect the fertilization rates of control or frozen–thawed oocytes. No significant interactions were found between any of the variables studied.

Transfer of frozen–thawed oocytes

Immediately after thawing. Ten groups of frozen–thawed oocytes (MF1) surrounded by cumulus cells and 48 frozen–thawed oocytes (MF1) devoid of cumulus were transferred to the oviducts of 5 and 4 F_1 (C57BL × CBA) females, respectively, mated 2–4 h previously with fertile MF1 males (1 cumulus mass or 6 cumulus-free oocytes per oviduct). All females were pregnant when examined on Day 14 of pregnancy. Two out of 5 females receiving oocytes plus cumulus cells had 'foreign' pink-

Table 2. The percentage of frozen–thawed and control unfertilized mouse oocytes at the 2-cell stage 24 h after the start of fertilization *in vitro*

	Source of oocytes							
	MF1				F_1 (C57BL × CBA)			
	Frozen–thawed		Control		Frozen–thawed		Control	
Exp.	+ Cumulus	− Cumulus	+ Cumulus	− Cumulus	+ Cumulus	− Cumulus	+ Cumulus	− Cumulus
1	53·4	60·0	53·8	90·0	79·3	68·9	95·2	87·0
	(88)	(25)	(13)	(10)	(29)	(45)	(21)	(23)
2	37·3	52·1	100·0	71·4	72·0	82·4	84·6	93·1
	(51)	(46)	(8)	(7)	(25)	(34)	(26)	(29)
3	19·4	15·0	22·7	30·0	19·2	17·6	36·4	25·9
	(36)	(20)	(22)	(20)	(52)	(34)	(33)	(27)
4	60·6	53·1	70·8	68·4	63·1	75·8	93·6	80·0
	(33)	(32)	(24)	(38)	(19)	(33)	(16)	(25)
Total	51·4	48·0	55·2	61·3	50·4	61·6	71·9	71·2
	(208)	(123)	(67)	(75)	(125)	(146)	(96)	(104)

Numbers in parentheses represent total number of 1-cell oocytes recovered after incubation with spermatozoa for 4–5 h.

eyed fetuses (2 and 3 respectively) and 2 out of 4 females receiving cumulus-free oocytes had pink-eyed fetuses (4 and 3 respectively). The number of oocytes plus cumulus cells transferred could not be counted but it was estimated that 10 oocytes were transferred per oviduct. Survival to Day-14 fetuses was therefore about 5% for oocytes frozen with cumulus cells and 15% (7/48) for frozen–thawed cumulus-free oocytes.

After fertilization in vitro. Two-cell embryos from frozen–thawed and control oocytes (MF1 and F_1) fertilized *in vitro* were transferred to the oviducts of recipients on Day 1 of pseudopregnancy. The results are summarized in Table 3. The numbers of control embryos transferred for both strains were small, and the validity of statistical comparisons is therefore questionable. Nevertheless, a significantly higher proportion of F_1 than MF1 frozen–thawed oocytes developed to 14-day fetuses and live young ($\chi^2_{[1]} = 9{\cdot}12$, $P < 0{\cdot}01$).

Table 3. Development of frozen–thawed and control mouse oocytes fertilized *in vitro* and transferred to pseudopregnant recipients

		Embryos transferred					
Source and treatment of oocytes	Embryonic stage at transfer	Total no. (No. of recipients)	To females becoming pregnant (no.)	No. of implantation sites	No. of fetuses at 14 days of gestation	No. of live young at birth	% fetuses and live young
MF1							
Frozen	2-cell	104 (10)	63 (5)	7	6	—	15·7
			20 (2)	—	—	7	
Control	2-cell	35 (3)	23 (2)	8	8	—	34·3
			12 (1)	—	—	4	
F_1 (C57BL × CBA)							
Frozen	2-cell	87 (7)	49 (4)	29	17	—	38·4
			24 (2)	—	—	11	
	Early blastocyst	33 (3)	21 (2)	16	5	—	36·4
			12 (1)	—	—	7	
Control	2-cell	20 (2)	20 (2)	9	9	—	45·0
	Early blastocyst	12 (1)	12 (1)	7	3	—	25·0

All 2-cell embryos which were not transferred were left in culture. Only a few 2-cell embryos from control and frozen–thawed MF1 oocytes developed to the 4-cell stage during the next 72 h in culture. However, 86% (57/66) and 80% (98/123) of the 2-cell embryos obtained from the frozen–thawed and control F_1 oocytes, respectively, developed to the early blastocyst stage after a further 72 h in culture. Some of these were transferred to the uterine horns of recipients on Day 3 of pseudopregnancy (Table 3). Again the numbers were small but there was no apparent difference in the viability of frozen–thawed oocytes transferred at the 2-cell or early blastocyst stage.

Discussion

For the first time the freshly ovulated egg of a female mammal has been successfully preserved at −196°C. Live 14-day fetuses were obtained after the transfer of frozen–thawed unfertilized mouse oocytes directly to the oviduct after thawing. The overall survival rate was low but this may have been due to the competition between transferred (foreign) and native oocytes. However, after fertilization *in vitro*, significantly higher survival rates to 14-day fetuses and liveborn were obtained when the frozen–thawed oocytes were transferred at either the 2-cell or early blastocyst stage. The proportion of unfertilized mouse oocytes appearing morphologically normal at recovery was higher than that reported for the recovery of the fertilized 1-cell mouse ovum (Whittingham *et al.*, 1972). The present study was not set up to determine the optimal ranges for cooling and thawing the unfertilized oocyte, although the rates used were within the ranges previously found to be optimal for the preservation of all the preimplantation stages of development in the mouse (Whittingham *et al.*, 1972; Leibo, Mazur & Jackowski, 1974).

The presence of cumulus cells during freezing and thawing did not affect the survival rate of the unfertilized oocyte or fertilization *in vitro* of frozen–thawed or control oocytes, although earlier reports have indicated that the absence of cumulus cells during fertilization *in vitro* significantly lowers the fertilization rate of unfrozen oocytes (Pavlok & McLaren, 1972; Hoppe & Pitts, 1973). The fertilization rate of the frozen–thawed oocytes was significantly lower than that of the freshly collected controls indicating that some membrane damage may have occurred during freezing and thawing and be interfering with the subsequent fertilization of the oocyte. There was no indication that any of the procedures involved in freezing and thawing, e.g. cold shock or exposure to DMSO, stimulated parthenogenetic activation (Tarkowski, 1975). The survival of oocytes from MF1 and F_1 (C57BL × CBA) strains was similar but the fertilization rate and development following transfer was higher with oocytes of F_1 origin.

Although the freshly ovulated mouse oocyte survives freezing and thawing, very limited numbers (<10%) of ovarian oocytes frozen at the germinal vesicle stage develop to the Metaphase II stage of meiosis *in vitro* following thawing (unpublished observations). Permeability and other membrane changes probably take place in the oocyte before ovulation and change the sensitivity of the oocyte to freezing and thawing. To date, no-one has successfully preserved mouse spermatozoa at low temperatures and therefore the preservation of the unfertilized oocyte provides the first opportunity of storing the haploid mouse genome.

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