

Developmental capacity of mouse oocytes cryopreserved before and after maturation *in vitro**

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Summary. The survival and developmental capacity of cumulus cell-enclosed oocytes frozen (1) at the germinal vesicle (GV) stage, after maturation *in vitro* with (2) and without (3) FSH, and (4) after gonadotrophin-stimulated ovulation were assessed. Survival, defined as the number of morphologically normal oocytes, after freeze–thaw at the GV stage (69%), was lower than for oocytes frozen after ovulation (84%), and after maturation *in vitro* with FSH (88%) and without FSH (81%). Treatment with DMSO without freezing had no effect on survival when compared with untreated controls except in immature GV-stage oocytes for which there was a slight reduction. After insemination *in vitro*, 9% of frozen–thawed GV-stage oocytes cleaved to two equal blastomeres, but none developed to blastocysts. Of oocytes matured *in vitro* before freezing, 17% cleaved to the 2-cell stage and 18% of these developed to blastocysts. When oocytes were matured *in vitro* in the presence of FSH, however, the percentage cleaving to the 2-cell stage after freeze–thaw was improved to 55%, and 77% of 2-cell stage embryos developed to blastocysts. When ovulated cumulus cell-enclosed oocytes were frozen, 88% cleaved and 67% of the cleaved embryos developed to blastocysts. When 158 two-cell embryos resulting from oocytes matured *in vitro* with FSH were transferred to the oviducts of pseudopregnant foster mothers, 41 genetically marked live young were produced (26%). These results demonstrate (1) that ova frozen after ovulation undergo normal preimplantation development with frequencies at or near those for unfrozen control oocytes, (2) FSH treatment of oocytes maturing *in vitro* greatly improves the capacity to cleave and develop to blastocysts after freeze–thaw and (3) oocytes frozen at the GV stage survive cryopreservation and undergo maturation *in vitro* but their developmental ability is severely impaired.

Keywords: freezing; oocyte maturation; oocyte preservation; fertilization; mouse

Introduction

The developmental capacity of mouse oocytes isolated at the germinal vesicle stage (GV) and matured *in vitro* is similar to that of superovulated eggs (Schroeder & Eppig, 1984, 1989). Oocyte maturation *in vitro* with subsequent embryonic development has also been achieved in other species (Van Blerkom & McGaughey, 1978; Shalgi *et al.*, 1979; Staigmiller & Moore, 1984; Goto *et al.*, 1988; Sirard *et al.*, 1988; Vanderhyden & Armstrong, 1989). Improved species-specific maturation protocols may ultimately enable investigators to take advantage of the large number of immature oocytes contained in the mammalian ovary for increasing populations of valuable animals. To maximize the practical effectiveness of such protocols, however, techniques for long-term storage of oocytes similar to those currently employed for spermatozoa would be very useful.

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Since the first demonstration that ovulated mouse ova could be cryopreserved (Tsunoda *et al.*, 1976; Parkening & Chang, 1977; Whittingham, 1977), several investigators have attempted to freeze metaphase II oocytes with varied success (summarized in Friedler *et al.*, 1988). There have been fewer reported attempts to freeze GV-stage oocytes (Whittingham, 1977; Heyman *et al.*, 1986; Pellicer *et al.*, 1988; Sathananthan *et al.*, 1988) perhaps because, from the first description of oocyte maturation *in vitro* (Pincus & Enzmann, 1935) until recently, attempts to achieve embryonic development of *in-vitro* matured oocytes have met with limited success. Attempts to freeze oocytes after maturation *in vitro* have not been reported. The study reported here evaluated the cryopreservation of cumulus cell-enclosed oocytes, frozen before and after maturation *in vitro* and after ovulation. Survival was compared with that of corresponding control groups treated with cryoprotectant and cooling but not freezing while the developmental capacity of each group was compared with corresponding untreated and unfrozen oocytes.

Materials and Methods

Animals. (C57BL/6J × SJL/J)_F₁ mice were used for all experiments. Immature GV-stage, cumulus cell-enclosed oocytes were obtained from juvenile 22–24-day-old mice 44–52 h after administration of 5 i.u. PMSG (Diosynth, Chicago, IL 60618, USA). Ovulated oocytes were retrieved from the oviducts of PMSG-primed mice 14–15 h after administration of 5 i.u. hCG. Spermatozoa for fertilization *in vitro* were obtained from 4-month-old males housed individually from the time of weaning.

Oocyte maturation, fertilization, embryonic development in vitro, and embryo transfer. The oocyte maturation medium was Eagle's Minimum Essential Medium (MEM), supplemented with 5% fetal bovine serum (FBS; Hyclone, Logan, UT 84321, USA), 0.23 mM-pyruvic acid, 0.01% phenol red (Difco, PO Box 1058, Detroit, MI 48232, USA), 50 mg streptomycin sulphate/175 mg penicillin G potassium salt/l and Earle's salts (components purchased from Sigma Chemical Co., PO Box 14508, St Louis, MO 63178, USA). Media were made up with water collected from a spring in Franklin, Maine, USA, and distilled once. FSH (ovine FSH-17), kindly provided by the pituitary hormone distribution program (NIADDK), was used at a concentration of 1 µg/ml. Medium for fertilization and embryo culture was Whitten's medium (Whitten, 1971) containing 3 mg bovine serum albumin/ml (BSA crystallized; ICN, PO Box 1200, Lisle, IL 60532, USA). Oocyte maturation, fertilization, and embryo culture were carried out as described previously (Schroeder & Eppig, 1984). Genetically marked 2-cell stage embryos were transferred to the oviduct ampullae of Day-1 pseudopregnant foster mothers with the aid of a No. 8 acupuncture needle (Pease *et al.*, 1989).

Oocyte freezing and thawing. Four different groups of cumulus cell-enclosed oocytes were subjected to a slow-freeze/slow-thaw technique originally devised for mouse embryos by Whittingham *et al.* (1972) and currently used in the frozen embryo repository at the Jackson Laboratory (Mbraatten, 1986). The groups were (1) GV-stage oocytes isolated in 50 µM-1-isobutyl-3-methylxanthine (IBMX; Aldrich, PO Box 355, Milwaukee, WI 53201, USA) which reversibly maintains meiotic arrest, (2) oocytes matured *in vitro* for 15–16 h, (3) oocytes matured *in vitro* for 15–16 h in medium containing 1 µg FSH/ml, and (4) ovulated oocytes. Before freezing, the cumulus cell-enclosed oocytes were washed three times in Dulbecco's phosphate-buffered saline (PBS) containing 0.01% phenol red and 5% FBS to prevent zona hardening which inhibits fertilization (Downs *et al.*, 1986a). Groups of 25–50 oocytes in 0.1 ml PBS + FBS were placed into a round-bottomed freezing vial (Nunclon; Wescor, Billerica, MA 01221, USA) on ice. After 15 min, freshly prepared PBS (0.1 ml) containing 2 M-dimethyl sulphoxide (DMSO; Aldrich) was added and the tubes were equilibrated for 30 min on ice. Ice nucleation (seeding) was induced in a salted ice bath at –6°C. The tubes were then cooled to –80°C in a freezing apparatus (Cryomed, Mt Clemens, MI 48045, USA) at a rate of 0.5°C/min then immersed in liquid nitrogen at –196°C.

Oocytes were stored for periods ranging from 2 days to 2 months. For thawing, tubes were removed from liquid nitrogen and held in nitrogen vapour at –101°C for 10 min then transferred to the thawing apparatus (Planer, Sunbury-on-Thames, Middlesex TW16 7HD, UK) and warmed at 8°C/min to +12°C. The contents of the tubes were diluted by 10 sequential additions of 0.1 ml PBS + FBS at 1-min intervals. The contents of the tube were then quickly transferred to 1.5 ml PBS + FBS in a 35 × 10 mm Corning tissue-culture dish, washed twice in PBS + FBS and twice in MEM or Whitten's medium. Percentage survival was assessed after 1 h in culture by determining the numbers of oocytes that appeared normal and those that were pycnotic. The effects of DMSO and cooling without freezing were determined for some of the oocytes in each group. The developmental capacity of frozen-thawed oocytes was determined in a second experiment. Surviving oocytes were prepared for fertilization or maturation *in vitro*, immediately upon thawing. Unfrozen, non-cooled and non-DMSO treated oocytes from each of the four groups served as controls.

Statistics. Data from 4 separate experiments were pooled and the results subjected to χ^2 analysis with Yates' correction.

Results

Table 1 compares the survival frequencies of 4 groups of oocytes 1 h after thawing, and control groups treated with DMSO and cooled but not frozen. Nearly 100% of the control DMSO-treated unfrozen oocytes in each group survived except for immature germinal vesicle-stage oocytes (88%). Immature GV-stage oocytes frozen immediately after their isolation from antral follicles had a lower survival frequency (69%) than oocytes frozen after maturation *in vitro* with or without FSH or frozen ovulated oocytes, all of which had approximately the same survival frequency. After maturation *in vitro* without FSH and freeze–thaw, 81% of ova appeared morphologically normal which was significantly lower than for unfrozen DMSO treated controls. The remainder of the ova showed a darkened central area and were assumed to be degenerate. The cumulus cells of ova matured *in vitro* remain compacted around the ova. After freeze–thaw, however, in contrast to DMSO-treated controls, many of the cumulus cells appeared pycnotic and only a few remained attached to the oocyte. Cumulus cell-enclosed ova frozen after maturation in medium containing FSH, which stimulates the synthesis of hyaluronic acid and cumulus expansion (Eppig, 1979), had a survival frequency of 88%. The cumulus cells in this group appeared identical to those of DMSO-treated controls, and the gel-like hyaluronic acid matrix of the expanded complexes remained intact (Fig. 1). For the ovulated cumulus cell-enclosed ova, which were dissected from the clutch into individual cumulus cell-enclosed eggs before freezing, 84% survived and they appeared identical to DMSO-treated controls and to the group matured *in vitro* with FSH and then frozen–thawed.

Table 1. Survival* of cumulus cell-enclosed oocytes frozen before and after maturation *in vitro* and after gonadotrophin-stimulated ovulation

Group	Treatment	No. of oocytes	No. recovered (%)	No. morphologically normal (%)
Germinal vesicle stage†	Frozen	300	282 (94)	194 (69) ^a
	DMSO-cooled‡	342	339 (99)	300 (88)
Matured <i>in vitro</i>	Frozen	296	284 (96)	230 (81) ^b
	DMSO-cooled	201	201 (100)	199 (99)
Matured <i>in vitro</i> + FSH (1 µg/ml)	Frozen	270	270 (100)	237 (88) ^c
	DMSO-cooled	139	139 (100)	137 (99)
Ovulated	Frozen	237	233 (98)	196 (84) ^{bc}
	DMSO-cooled	110	110 (100)	107 (97)

Values represent data pooled from 4 experiments. Values with different letters are significantly different, $P < 0.05$ at least. DMSO treatment groups are significantly different from their corresponding frozen–thawed group, $P < 0.01$.

*Morphologically normal 1 h after thawing.

†Cumulus cell-enclosed oocytes were collected in medium containing 50 µM-IBMX to maintain meiotic arrest during processing for freezing.

‡Cumulus cell-enclosed oocytes were subjected to DMSO treatment and cooling as described in 'Materials and Methods' then warmed and examined after 1 h for survival.

Table 2 compares the percentage of successful development to the 2-cell and blastocyst stages of surviving frozen–thawed ova from the four groups and also compares each group with corresponding unfrozen non-DMSO-treated control ova. Although the oocytes that survived freezing at the GV stage underwent maturation *in vitro* as indicated by germinal vesicle breakdown (GVB) (>95%) and polar body formation (>90%), only a few developed to the 2-cell stage and none of the embryos developed to blastocysts. Of surviving ova frozen after maturation *in vitro* without FSH, a greater percentage developed to the 2-cell stage after insemination and 18% of these

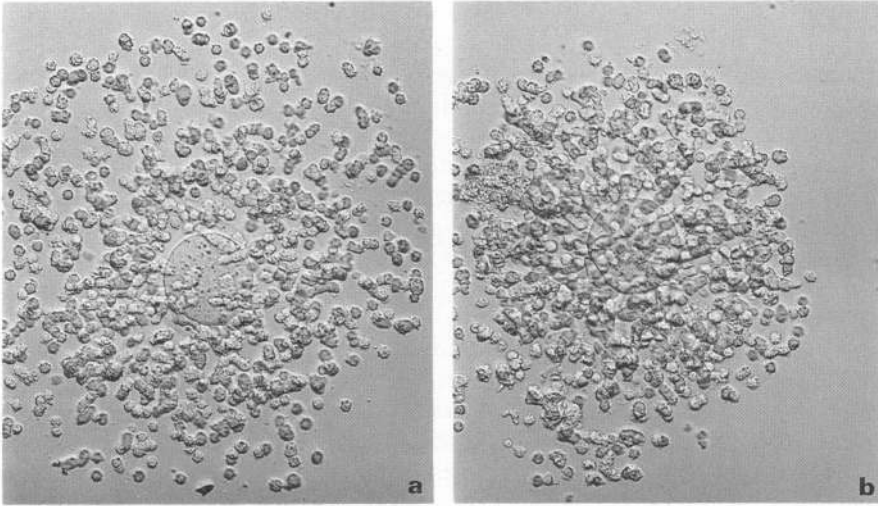


Fig. 1. Cumulus cell-enclosed ova (a) after maturation *in vitro* in medium containing FSH, (b) after maturation *in vitro* in medium containing FSH followed by freeze-thaw. $\times 185$.

developed to blastocysts, but the values were significantly below those for corresponding unfrozen *in-vitro* matured controls.

Ova frozen after maturation in medium containing FSH developed to the 2-cell stage and blastocysts at significantly higher frequencies than those which were matured without FSH then frozen. Although a significantly greater percentage of the corresponding unfrozen control ova developed to the 2-cell stage, there was no difference in blastocyst development. In an experiment not shown in Table 2, when oocytes matured *in vitro* with FSH were subjected to cooling and DMSO treatment but not freezing, 72% developed to the 2-cell stage ($n = 212$) which was significantly lower than in the corresponding untreated group (89%; $n = 158$), but significantly above that for frozen-thawed oocytes (53%; $n = 144$).

Table 2. Developmental capacity of cumulus cell-enclosed oocytes frozen before and after maturation *in vitro* and after gonadotrophin-stimulated ovulation

Group	No. of ova inseminated	No. of 2-cell stage embryos (%)	No. of embryos continued in culture	No. of blastocysts (%) from 2-cell stage embryos
Germinal vesicle stage (frozen)*	263	24 (9) ^a	24	0 (0) ^a
In-vitro matured (frozen)	170	29 (17) ^b	23	4 (17) ^a
In-vitro matured (not frozen)	192	123 (64) ^c	120	77 (64) ^b
In-vitro matured + FSH (frozen)†	487	267 (55) ^d	109	84 (77) ^c
In-vitro matured + FSH (not frozen)	211	175 (83) ^e	175	123 (70) ^{bc}
Ovulated (frozen)	147	129 (88) ^e	111	74 (67) ^c
Ovulated (not frozen)	240	192 (80) ^e	158	122 (77) ^c

Values represent data pooled from 4 experiments. Values in a column with different letters are significantly different, $P < 0.05$, at least.

*GV-stage, cumulus cell-enclosed oocytes were isolated in medium containing IBMX (50 μM) to maintain meiotic arrest during processing for freezing. Oocytes were matured *in vitro* after thawing. Control is the same as *in-vitro* matured (not frozen).

†Transfer of 158 2-cell stage embryos from this group produced 41 live young (26%).

There was no difference between the frequencies of ovulated frozen–thawed ova and control ova which developed to the 2-cell stage, but the development of ovulated ova to the 2-cell stage was significantly better than for oocytes frozen after maturation *in vitro* with FSH. There was, however, no difference in the percentage that reached the blastocyst stage for these two groups when compared with their respective control group or with each other.

When 158 two-cell stage embryos, derived from oocytes frozen after maturation *in vitro* with FSH, were transferred to the oviducts of pseudopregnant foster mothers, 41 genetically marked live young were born (26%).

Discussion

This study reports the successful cryopreservation of mouse ova after maturation *in vitro*. The survival and developmental capacity of four groups were compared: (1) immature GV stage oocytes which were frozen–thawed then matured *in vitro*, oocytes matured *in vitro* in the presence (2) and absence (3) of FSH and then frozen–thawed, and (4) ovulated eggs which were frozen–thawed. When cumulus cell-enclosed oocytes were matured *in vitro* then frozen–thawed, percentages of surviving oocytes were significantly higher than for oocytes frozen–thawed at the GV stage then matured *in vitro* and equivalent to survival of frozen–thawed ovulated oocytes. Forty-one genetically marked live young (26%) were produced from transferred embryos that resulted from *in-vitro* fertilization of ova frozen–thawed after maturation *in vitro* with FSH.

Survival of immature oocytes frozen at the GV stage was improved over that reported earlier for the mouse (Whittingham, 1977; Sathanathan *et al.*, 1988). This may be the result of inclusion of IBMX in the isolation medium to inhibit GVB reversibly during the processing steps for freezing. GVB is initiated immediately upon liberation from the follicular environment and is completed in virtually all cumulus cell-enclosed oocytes after 75 min. This may be a particularly sensitive period since there is movement and rearrangement of various organelles and microtubules within the ooplasm (Calarco *et al.*, 1972; Wassarman & Fujiwara, 1978; Van Blerkom & Runner, 1984; Albertini, 1987; Alexandre & Mulnard, 1988). Spindle microtubule reorganization is particularly sensitive to cold (Magistrini & Szollosi, 1980; Johnson & Pickering, 1987; Pickering & Johnson, 1987; Sathanathan *et al.*, 1988). Maintenance of meiotic arrest with IBMX or other meiosis-arresting agents followed by maturation *in vitro* resulted in normal frequencies of development (Downs *et al.*, 1986b). In the present study, more than 90% of immature GV-stage frozen–thawed oocytes underwent GVB and extrusion of a polar body. This suggests that the microtubular spindle apparatus was functional. The kinetics of GVB were not assessed in this study, but Pellicer *et al.* (1988) have shown that freezing slows subsequent GVB in rat oocytes. In agreement with previous reports (Whittingham, 1977; Sathanathan *et al.*, 1988), embryonic development of immature frozen oocytes after thawing, maturation *in vitro*, and insemination *in vitro*, was deficient compared with that in unfrozen controls. This could be the result of damaged cumulus cells being unable to support maturation fully and/or possible damage to microtubule organization which is reflected at the first mitotic division. When oocytes matured in the presence of FSH were subjected to DMSO treatment and cooling but not freezing, their developmental capacity was better than for corresponding frozen oocytes but below that of untreated controls. This suggests a possible additive effect of cooling–DMSO treatment with freezing on the developmental ability of this group.

When ovulated metaphase II cumulus cell-enclosed oocytes were frozen–thawed, subsequent embryonic development was equivalent to that of unfrozen controls. The improved frequencies reported here for ovulated oocytes when compared with previous studies for the mouse and other species (summarized by Friedler *et al.*, 1988) may be strain and species dependent. Alternatively, the ovulated clutches containing 20–40 oocytes were carefully dissected into individual cumulus cell-enclosed oocytes with the aid of sterile needles, and groups of 25–50 oocytes were then frozen with the cumulus intact. When the entire clutch of eggs from superovulated mice was

frozen–thawed, survival was markedly lower (data not shown). Improved success in freezing ovulated oocytes may also be due to inclusion of serum in the solutions for the freeze–thaw processing steps up to the time of insemination when the ova were quickly washed and placed into Whitten's medium containing BSA. Serum has been shown to prevent hardening of the zona pellucida of oocytes maturing *in vitro* and metaphase II oocytes (Downs *et al.*, 1986a). Carroll *et al.* (1989) observed only a 46% cleavage rate for ovulated oocytes processed in serum containing PB1 medium (Whittingham, 1974), but the oocytes were treated with hyaluronidase before processing to remove the cumulus cells and there was a prolonged period after thawing before insemination during which the ova were in PB1 that did not contain serum.

Attempts to freeze–thaw ovulated eggs, *in-vitro* matured oocytes, or GV-stage oocytes after they were first denuded of their cumulus cells resulted in lower survival when compared with cumulus cell-enclosed oocytes (data not shown). These results for immature oocytes agree with those of Pellicer *et al.* (1988), who found that the more layers of cumulus cells, the higher the survival in frozen–thawed rat oocytes. However, Whittingham (1977) found no difference in the survival or ability to cleave to the 2-cell stage for ovulated ova frozen–thawed with or without cumulus.

Survival of oocytes frozen–thawed after maturation *in vitro* in the presence or absence of FSH was about equal but the developmental capacity of those matured with FSH was significantly better. These findings confirm previous results (Downs *et al.*, 1986b), showing that inclusion of FSH in the maturation medium significantly improves the developmental potential of oocytes matured *in vitro*. The percentage of 2-cell stage embryos, derived from ova frozen–thawed after maturation *in vitro* with FSH, that developed to blastocysts was the same as for unfrozen controls as well as frozen and unfrozen ovulated oocytes. The improved survival and developmental capacity of maturing oocytes treated with FSH compared to those which were matured untreated may be due to FSH-stimulated cumulus expansion. Frozen–thawed FSH-treated oocyte–cumulus cell complexes as well as ovulated frozen–thawed complexes appeared morphologically identical to unfrozen FSH-treated controls and ovulated control oocytes. The gel-like hyaluronic acid matrix appeared unperturbed by freezing and may provide a cryoprotective effect. Moreover, the cumulus cells within the expanded hyaluronic acid matrix did not appear pycnotic, in contrast to the cumulus cells from the groups in which the cumulus cell-enclosed oocytes were frozen without expansion. In these groups, many of the cumulus cells were pycnotic after freeze–thaw, and the oocyte was often denuded of remaining cells as a result of the manipulations for maturation or fertilization. Oocytes denuded of cumulus cells before maturation *in vitro* exhibit a lower developmental capacity than do cumulus cell-enclosed oocytes (Schroeder & Eppig, 1984).

The experiments reported here were performed under controlled laboratory conditions with cumulus cell-enclosed oocytes isolated from PMSG-primed juvenile mice. Nevertheless, administration of exogenous gonadotrophins is not necessary to recover developmentally competent GV-stage oocytes (Schroeder & Eppig, 1989) and live young were produced from *in-vitro* matured oocytes isolated from unprimed adult cyclic females at all stages of the oestrous cycle. In the present study, embryonic development was obtained after *in-vitro* matured oocytes isolated from such unprimed mice were frozen–thawed (data not shown).

The study reported here suggests that oocyte banking of ovulated and/or *in-vitro* matured oocytes is now possible for the mouse. The potential advantages of gamete banking in mammals are many, including the easy establishment of genetic combinations when male and female gametes in the desired combination are not simultaneously available. The practical advantages of oocyte development *in vitro* include the recovery of a greater number of developmentally competent oocytes from an ovary than will ever be ovulated. Also, maturation *in vitro* could serve to rescue mice that are otherwise reproductively impaired (Schroeder & Eppig, 1989). Thus refined species-specific techniques of oocyte maturation *in vitro* coupled with ova freezing could ultimately increase the repertoire of current reproductive technology in other mammals, particularly valuable zoological specimens, rare species, and agricultural animals.

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