Journal of Assisted Reproduction and Genetics, Vol. 14, No. 10, 1997

Cryopreserved Immature Mouse Oocytes: A Chromosomal and Spindle Study

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Submitted: July 30, 1997
Accepted: September 10, 1997

Purpose: *Cryopreservation of human oocytes might provide an alternative approach to freezing supernumerary embryos obtained during IVF. This process, performed on immature denuded prophase I mouse oocytes, was investigated.*
Methods: *We first investigated the capacity of frozen, immature, murine oocytes to continue in vitro maturation after thawing. We then evaluated the risk to offspring from chromosomal damage by cytogenetical and cytological (spindle) analysis. Finally, we attempted to determine the reasons for and the stage of maturation failure.*
Results: *A total of 700 immature oocytes was frozen, 629 (90%) were recovered intact after thawing, and 53% extruded the first polar body, versus 74% for the control group. Freezing was not accompanied by an increase in aneuploidy in maturing oocytes (18 and 15% for thawed and control oocytes, respectively). Consequently, the first meiotic division occurred normally, without an increase in nondisjunction. Spindle analysis demonstrated only a few abnormalities (15 and 2% for thawed and control oocytes, respectively) incompatible with further development. Oocytes arrested during in vitro maturation were mainly at the metaphase I stage (64 and 76% for thawed and control oocytes, respectively). Whereas 17% of thawed oocytes were blocked before the formation of the first meiotic spindle, this never occurred in the control group.*
Conclusions: *Immature murine oocytes can withstand cryopreservation, which is encouraging for future human application of this technique.*

KEY WORDS: chromosomes; cryopreservation; immature oocyte; in vitro maturation; spindle.

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INTRODUCTION

The use of ovarian hyperstimulation for in vitro fertilization (IVF) procedures results in the recovery of excess oocytes and the development of supernumerary embryos, which can be frozen. This option is associated with legal, ethical, and religious problems. Cryopreservation of human oocytes might provide an alternative approach. Furthermore, this technique is of interest in other medical areas (autopreservation before chemotherapy or radiotherapy and constitution of an oocyte library for donation). The cryopreservation of mature oocytes has a poor success rate. An explanation could be that these oocytes present a metaphase II spindle with microtubules bound to maternal chromosomes. Indeed the microtubules have been observed to be highly sensitive to thermal changes (1–3), and the last stage of meiosis seems to be altered by this treatment (4). Therefore, immature oocytes [germinal vesicle (GV) stage] with DNA enclosed in the nucleus and protected by the nuclear membrane might be less sensitive to the cryopreservation process (as are embryos or other cells) than mature oocytes. Indeed, results concerning the cryopreservation of human immature oocytes appear encouraging: with a slow freezing and rapid thawing procedure, a survival rate of 59% (n = 123) and a maturation rate of 83% have been reported (5).

The aims of our study were to assess the feasibility of immature oocyte cryopreservation followed by in vitro maturation using a mouse model, to determine the reasons for and the stage of the possible failures of maturation, and to evaluate the chromosomal innocuity of the procedure for future progeny.

MATERIALS AND METHODS

All manipulations of the oocytes were carried out using M2 medium at 38°C.

1058-0468/97/1100-0617$12.50/0

Collection of Oocytes

GV-stage, denuded oocytes were collected from unstimulated (C57-CBA) F_1 hybrid mice at 21–28 days of age. The ovaries were dissected in M2 medium (6) containing 0.25 m*M* dibutyryl cyclic AMP (dbcAMP; Sigma), added to the culture medium to maintain the oocytes at the GV stage (7). The oocytes were denuded mechanically and checked for the presence of a GV.

Freezing and Thawing

The method used was as described by Candy *et al.* (8), except that those authors froze enclosed mouse oocytes. Briefly, denuded oocytes were submitted to slow freezing (Minicool cell freezer AS25), using dimethylsulfoxide (DMSO; Sigma) as cryoprotectant. The straws were stored in liquid nitrogen at −196°C for 1–4 weeks. For thawing, the DMSO was rapidly diluted in M2 using a procedure known to result in a good survival rate after thawing (8).

In Vitro Maturation

Frozen–thawed and freshly (control) collected GV-stage oocytes were washed three times in M2 and then cultured (5% CO_2, 38°C, under oil BDH) in medium (MEM; GIBCO) supplemented with 50 mg/ml sodium pyruvate (Sigma); 50 mg/L streptomycin sulfate, 100 IU/ml benzylpenicillin, 100 m*M* L-glutamine (BioMerieux), and 4 g/L bovine serum albumin (Sigma).

After 12–14 hr the oocytes were assessed for stage of meiotic maturation and classified as follows: oocytes that had extruded the first polar body (PB1), oocytes that had undergone GV breakdown (GVBD), and oocytes containing an intact GV.

Oocyte Fixation and Immunocytochemical Staining at 12–14 hr

Only intact oocytes, regardless of their stage (PB1 or the GVBD), were selected for immunostaining: zona pellucida-free oocytes (the zona pellucida was removed from oocytes by brief exposure to Tyrode acid) were placed in specially designed chambers coated with 0.1 mg/ml concanavalin A (Sigma). The samples (fresh and thawed) were centrifuged at 450*g* for 10 min at 37°C and then fixed with 0.1% glutaraldehyde (Sigma) diluted in phosphate-buffered saline (PBS) containing 1% Triton X-100 (Boehringer).

After rinsing for 5 min in PBS, the samples were extracted with 2% Triton X-100 for 30 min at room temperature, incubated for three periods of 10 min in 10 mg/ml $NaBH_4$ in PBS, and processed for immunofluorescence as described by Maro *et al.* (9). The tubulin was visualized using the rat monoclonal antibody YL1/2 to tyrosinated α-tubulin (10) followed by a fluorescein-labeled anti-rat antibody (Miles). The chromatin was stained with propidium iodide (Molecular Probe; 5 mg/ml in PBS). The coverslips were removed from the chambers and mounted on glass slides in Citifluor (City University, London) to prevent bleaching. Samples were examined under a Leitz Diaplan microscope, and photographs were taken on Ektachrome 160 film using a Bio-Rad MRC-600 confocal microscope mounted on an Optiphot II Nikon microscope equipped with a ×60 objective (Plan Apo; NA 1,4) and a dual argon and helium–neon laser system adjusted to excitation wave lengths of 488 and 534 nm, respectively.

Three types of abnormal spindle morphology were defined: elongated, totally absent, and absent but with remnant asters of microtubules.

Cytogenetic Analysis

After 12–14 hr of in vitro maturation, the oocytes were fixed according to the method of Tarkowski (11). Slides were stained with Giemsa and examined with a Univar photomicroscope (Reichert).

Statistics

The data were analyzed using the chi-square test. Differences were considered significant at P less than 0.05.

RESULTS

Efficiency of In Vitro Maturation

A total of 700 immature oocytes was frozen, of which 629 (90%) were recovered intact after the thawing procedure; the remaining 71 oocytes were degenerated. Significantly more frozen than control oocytes (Table I) remained blocked at the GV stage (26 vs 18%) and significantly fewer frozen than control oocytes extruded a first polar body (53 vs 73%).

Chromosomal Damage

Freezing Was Not Accompanied by an Increase in the Aneuploidy Rate. No significant difference

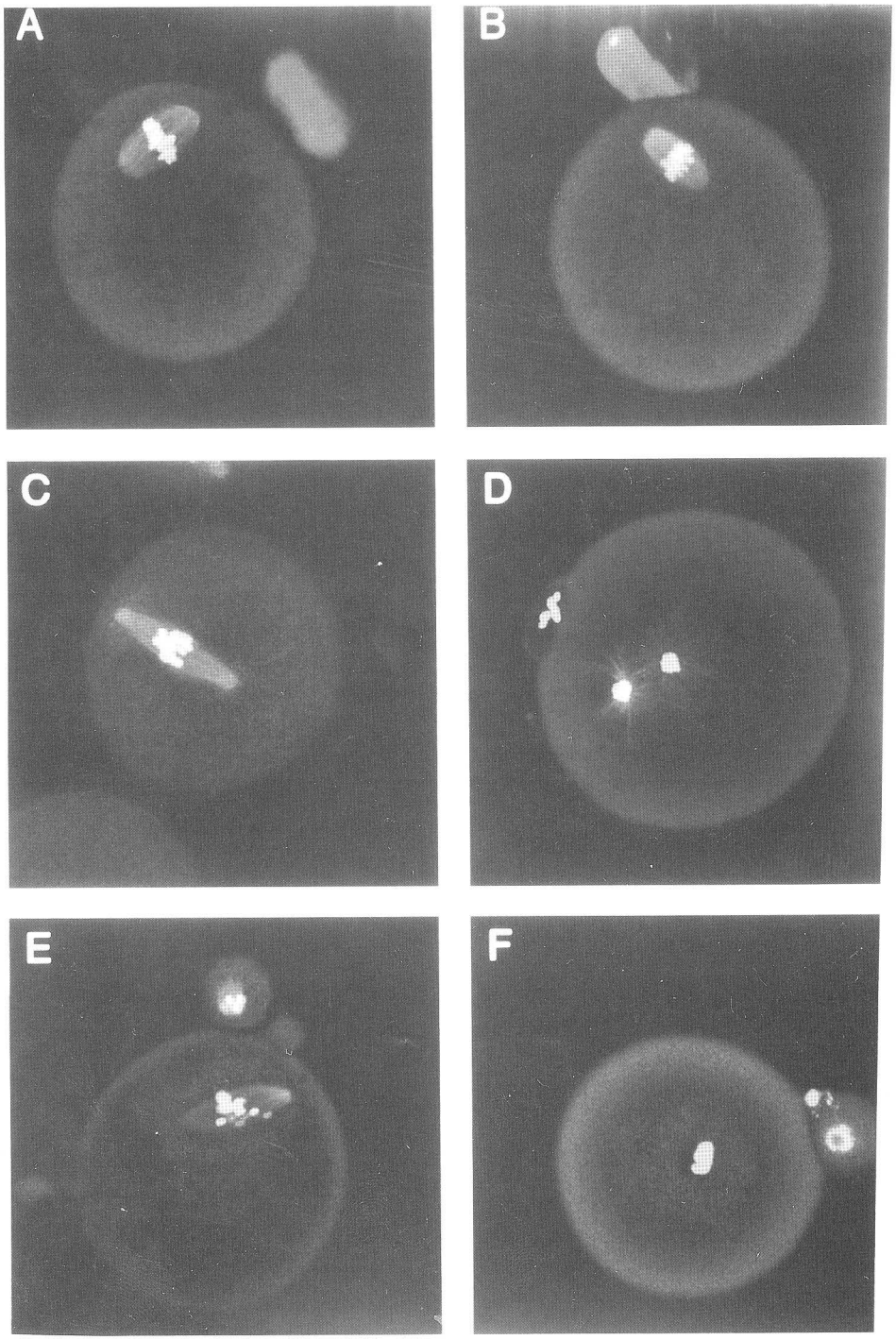

Fig. 1. Confocal microscopy of the second meiotic spindle. (A, B) Normal barrel-shaped spindle (A, fresh oocytes and B, thawed one), (C) Elongated spindle with condensed chromosomes, (D) Asters of microtubules with dispersed chromosomes, (E) Activated oocyte, (F) Oocyte with no spindle but with remaining chromosomes.

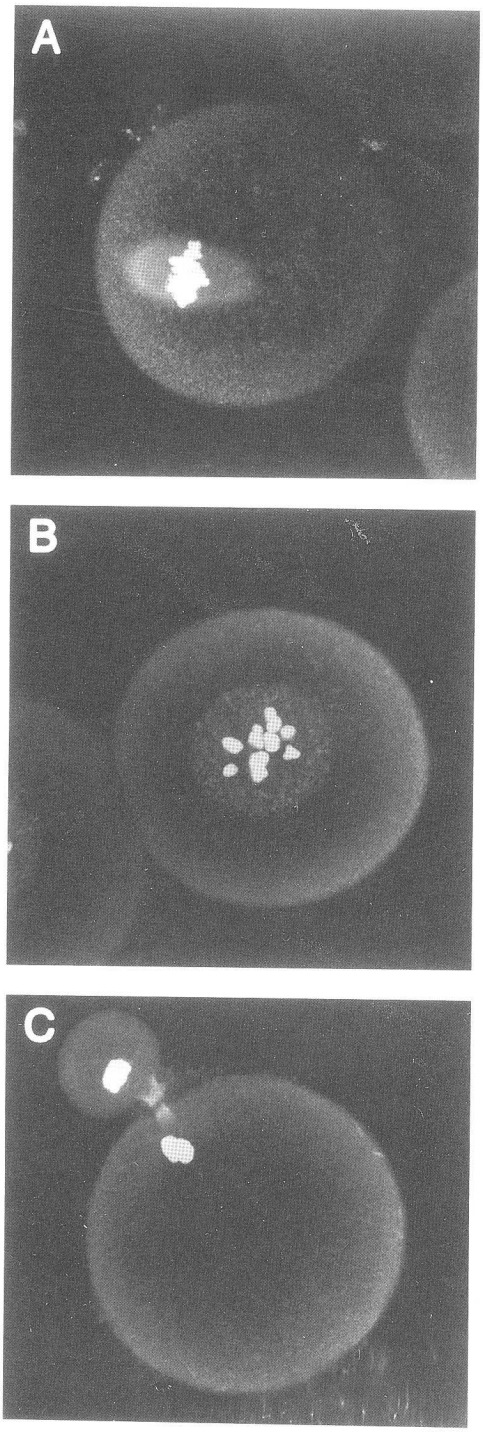

Fig. 2. Confocal Microscopy of the First Meiotic Spindle. (A) Normal barrel-shaped spindle (chromosomes are bivalent), (B) Oocyte with no spindle and with dispersed chromosomes, (C) Midbody with two haploid complements.

Table I. Oocyte Morphology Observed After 12–14 hr of Culture*

	GV	Maturing oocytes	GVBD	PB1
Control (n = 1674)	304 (18%) a[a]	1370 (82%) c[a]	369 (27%) e[b]	1001 (73%) g[b]
Thawed (n = 629)	166 (26%) b[a]	463 (74%) d[a]	220 (47%) f[b]	243 (53%) h[b]

[a] Percentages calculated in relation to the number of GV cultured
[b] Percentages calculated in relation to the number of maturing oocytes
* (ab), (cd), (ef), and (gh): significant difference.

was found between the thawed and the control oocyte groups (Table II) with respect to the rate of euploidy (82 vs 85%, respectively), hypohaploidy (10 vs 8.4%), or disomy (8 vs 6.6%, respectively).

Freezing Increased Spindle Abnormalities. There was a significant difference (Table III) in the total number of spindle anomalies (15 vs 2%; $P < 0.001$) found for the thawed (n = 81) and for the control group (n = 203).

In the control group, chromosomes were located on the spindle equatorial plane (98% of cases), apart from activated oocytes, in which chromosomes were located at the two spindle poles. In nonactivated, thawed oocytes, chromosomes were also located on the spindle equatorial plane, apart from cases with remnant asters of microtubules, where the chromosomes were dispersed in the cytoplasm.

Stage of Failure of Maturation

Chromosomes of Oocytes Stopped at the Metaphase I Stage Were Usually Associated in 20 Divalents. In the majority of cases (95% of thawed oocytes and 94% of fresh controls), paired homologous chromosomes were found associated in 20 divalents (typi-

Table II. Karyotypic Analysis of Fresh and Thawed Metaphase II-Stage Oocytes*

	Oocytes		
Treatment	Euploidy	Hypohaploidy	Hyperhaploidy
Control (n = 155) (12–14 hr)	132 (85%) a	13 (8.4%) c	10 (6.6%) e
Thawed (n = 88) (12–14 hr)	72 (82%) b	9 (10%) d	7 (8%) f

* (ab), (cd), and (ef): no significant difference.

Table III. Spindle Analysis in Metaphase II-Stage Oocytes*

	Spindle				Chromosomes		
PB1 (12–14 hr)	Barrel-shaped	None	None (remnant asters)	Elongated	Condensed	Dispersed	Activated
Control (n = 203)	199 (98%) a	1 (0.4%) c	0 e	3 (1.6%) g	198 (98%) i	0 k	4 (2%) m
Thawed (n = 81)	69 (85%) b	2 (2.8%) d	6 (7.3%) f	4 (4.9%) h	74 (91.4%) j	6 (7.3%) l	1 (1.3%) n

* (ab), (ef), and (kl): significant difference ($P < 0.01$). (cd), (gh), (ij), and (mn): no significant difference.

cal configuration of metaphase I). The remaining oocytes contained 40 separated chromosomes.

A Metaphase I Spindle Was Found in the Majority of Cases. A normal metaphase I spindle was found (a barrel-shaped spindle with an increased size compared to metaphase II) in 64% (Table IV) of the thawed oocytes and 76% of the control oocytes (significantly different $P < 0.01$). In 17% of the thawed oocytes, no spindle was found (absent or had not yet appeared), and this was never observed in the control group. In the two groups there was a similar frequency (19 vs 24%) of anaphase I being followed by the extrusion of the polar body and the formation of a broad microtubular structure joining the polar body and the oocyte through the midbody, with two haploid complements. Chromosomes were always located in the equatorial plane of the spindle, with the exception of cases in which no spindle was viewed (thawed group), where the chromosomes were dispersed in the cytoplasm.

DISCUSSION

Efficiency of Maturation In Vitro

The survival rate of oocytes after thawing was 90%, which is in the same range as the results reported from earlier studies carried out in denuded immature mouse oocytes: 96 and 80% (12,13). Thus, we confirm the capacity of denuded immature oocytes to resist a freezing/thawing cycle. A severe deleterious effect was observed after freezing/thawing of cumulus intact mouse oocytes (14–16), suggesting that the presence of cumulus cells renders the oocyte more fragile to cryopreservation, possibly due to augmentation of dehydration. The cumulus and corona radiata may also form a more rigid structure, limiting the distortion of oocyte shape which occurs during ice formation in the cryoprotectant (17). Finally, the cumulus is not always a good indicator of nuclear maturity and cytoplasmic integrity, and furthermore, it prevents morphological assessment before freezing, thus, morphological damage observed after cryopreservation is impossible to attribute to cryopreservation alone. Therefore, to evaluate directly the effects of cryopreservation, it was better to freeze denuded rather than enclosed oocytes.

The maturation rate obtained in this work with fresh controls, 74%, confirms previous results reported by several authors: 80% (13), 74% (8), and 62% (18). Significantly more frozen than control oocytes remain arrested at the GV stage (26 vs 18%). Significantly fewer frozen than control oocytes extruded a first polar body (53 vs 73%).

Chromosomal Damage

Our results indicate that the freezing procedure of immature oocytes (GV stage) was not accompanied by a significant increase in aneuploidy in the metaphase II stage after in vitro maturation. These results confirm the observations of Van Blerkom and Davis (13), using vitrification. Therefore, immature oocytes seem to be

Table IV. Morphology of the First Meiotic Spindle in Stopped Oocytes*

	Spindle			Chromosomes		
Treatment	Barrel-shaped	None	Midbody	Equatorial plane	Dispersed	Two haploid complements
Fresh control (n = 46)	33 (76%)	0 a	11 (24%)	35 (76%)	0 c	11 (24%)
Thawed (n = 77)	49 (64%)	13 (17%) b	15 (19%)	49 (64%)	13 (17%) d	15 (19%)

* (ab) and (cd): significant difference ($P < 0.01$).

less prone to microtubular disruption, because the chromatin is found in an uncondensed form enclosed in the nucleus, protected by the nuclear membrane, with most of the microtubular system not organized in microtubules (14,19).

In our study, after cryopreservation of prophase I oocytes, the spindle of mature metaphase II oocytes was found to be normal in 85% of cases, confirming the results observed with human oocytes, where 81% (n = 42) of spindles were found to be normal after cryopreservation in a small series (20). Furthermore, the most common abnormality observed is the absence of a spindle (in totality or with remnant asters of microtubules) and therefore no future oocyte activation and egg development are possible (12).

Stage of Failure of Maturation

Oocytes failing to extrude the PB1 during in vitro maturation are usually arrested in metaphase I. Nevertheless, during meiotic maturation, the appearance of the first meiotic spindle is a very long process (6 to 8 hr) and the cytogenetic findings (chromosomes in 20 divalents) are not proof of the presence of a spindle. Indeed, looking at the first meiotic spindle, we confirmed that oocytes that had not progressed to extrusion of the polar body were mainly arrested during the metaphase I stage, as suggested by the cytogenetic results. However, surprisingly, 17% of frozen oocytes at the GVBD stage showed an absent spindle with dispersed chromosomes. These oocytes seem to be arrested early during the in vitro maturation process and before spindle formation. Nevertheless, it should be noted that our observations were made after 12 hr of culture and not continuously from the start, thus microtubule depolymerization cannot be excluded, even if it seems improbable.

Equivalent numbers of oocytes (19 vs 24% in the thawed and control groups, respectively) observed during the GVBD stage were extruding a polar body during the fixation process and had a microtubular structure joining the polar body and the oocyte (midbody). Thus, these oocytes were "activated" by our micromanipulations before fixation. This has been observed in other situations: metaphase I oocytes penetrated by spermatozoa (22) or after contact with a calcium ionophore (23).

In conclusion, despite the good survival rate after cryopreservation, the ability of oocytes to resume meiosis is reduced by this treatment. Oocytes arrested during in vitro maturation are usually at the metaphase I stage, but a few thawed oocytes seem to be blocked before the formation of the first meiotic spindle. Cytogenetic analysis of mature oocytes showed that the first meiotic division happened normally, without an increase in aneuploidy. Cytological analysis showed a few abnormalities, which were not compatible with fertilization and development in vitro. However, a cytogenetic study needs to be performed after in vitro fertilization as done previously for mature oocyte cryopreservation: a mutagenesis test demonstrated an increase in sister chromatid exchanges (24) and an increased triploidy rate in these embryos (4,25). We intend to perform the same type of studies after in vitro fertilization of freeze/thawed murine oocytes to evaluate their developmental capacity and, only, then to apply this approach to human oocytes.

ACKNOWLEDGMENTS

This work was supported by the Association Claude Bernard, Paris, France (Unit No. 1376 and Grant CRC).

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