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Effects of slow and ultrarapid freezing on morphology and resumption of meiosis in immature cat oocytes

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This study examined the ability of immature cat oocytes to survive after cryopreservation by evaluating their subsequent development following maturation *in vitro*. The effect of slow and ultrarapid freezing using one of two cryoprotectants dimethylsulphoxide (DMSO) or 1,2-propanediol (PROH) at different concentrations (1.5 or 3.0 mol l^{-1}) and the slow freezing with the cryoprotectant ethylene glycol (EG 1.5 mol l^{-1} and 3.0 mol l^{-1}) were tested. Morphology, resumption of meiosis and metaphase II rates of oocytes were recorded after thawing. Freshly collected oocytes were used as controls. Results indicate that immature cat oocytes can survive, resume meiosis and achieve metaphase II *in vitro* after freezing. The highest rates of resumption of meiosis and metaphase II were achieved with slow freezing and 1.5 mol DMSO or EG l^{-1} (DMSO: 47.4%, 18/38 and 23.7%, 9/38 and EG: 52%, 13/25 and 20%, 5/25, respectively). The ultrarapid procedure did not result in resumption of meiosis *in vitro*, despite intact morphology of the oocytes after thawing. These results suggest that morphology of oocytes after freezing and thawing has no predictive value for their ability to resume meiosis.

Introduction

An objective of the development of assisted reproduction techniques in domestic cats is their application to non-domestic feline species, most of which are considered threatened or endangered. In the last decade, progress has been made in assisted reproduction techniques including *in vitro* maturation and fertilization (IVM/IVF) and embryo transfer. The effectiveness of such protocols in the preservation of genetic material of feline species can be improved by developing better techniques for long-term storage of gametes and embryos.

Sperm cells and cat embryos derived *in vivo* or *in vitro* have been frozen (Platz *et al.*, 1978; Dresser *et al.*, 1988; Pope *et al.*, 1994), but no information is available regarding oocyte cryopreservation in the feline species. The cryopreservation of oocytes would greatly increase their availability for a range of reproductive techniques and would allow an easy establishment of genetic combinations when male and female gametes in the desired combination are not simultaneously available. Therefore, the captive propagation of felids and other rare carnivores would be facilitated.

Attention has focused on the cryopreservation of immature oocytes as it has been reported that mature oocytes are damaged by freezing because of the fragility of their meiotic spindle (Pickering and Johnson, 1987; Van der Elst *et al.*, 1988). Since immature oocytes (germinal vesicle stage) have the genetic material contained in early prophase within the contours of a nucleus and no spindle is present, freezing them appeared to be an alternative approach to the cryopreservation of the female gamete.

Cryopreservation of immature oocytes has been performed in several mammals, including rats (Pellicer *et al.*, 1988), mice (Schroeder *et al.*, 1990; Van der Elst *et al.*, 1992, 1993); cattle (Lim *et al.*, 1992; Suzuki and Nishikata, 1992; Schellander *et al.*, 1994) horses (Hochi *et al.*, 1994) and humans (Mandelbaum *et al.*, 1988; Toth *et al.*, 1994a, b). Different freezing procedures and cryoprotectants have been used for immature oocytes. Both dimethylsulphoxide (DMSO) and 1,2-propanediol (PROH) were effective for immature mouse oocytes frozen with a slow or an ultrarapid procedure (Schroeder *et al.*, 1990; Van der Elst *et al.*, 1993). Toth *et al.* (1994a) demonstrated that immature human oocytes cryopreserved with PROH can mature to metaphase II (M II) and retain the same capacity for fertilization as unfrozen oocytes.

Recently attention has focused on ethylene glycol (EG). The molecular weight of this cryoprotectant is lower than that of cryoprotectants commonly used, and its beneficial effect in cryopreservation may be partly due to its high permeability. Since it has been shown that oocytes are less permeable than embryos to the cryoprotectant (Fuller and Bernard, 1984) highly permeant cryoprotectants may be more suitable for freezing oocytes. Thus, Hochi *et al.* (1994) showed that immature equine oocytes mature to M II stage *in vitro* following slow freezing in EG.

In the present study some freezing procedures mentioned in the literature were applied to immature cat oocytes. The ability of immature cat oocytes to survive after cryopreservation was examined by evaluating their subsequent development following *in vitro* maturation.

Experiments and Oocyte Collection

In Expt I a comparison between a slow and an ultrarapid freezing procedure with two different cryoprotectants (DMSO and PROH) was carried out. For both cryoprotectants a concentration of 1.5 mol l^{-1} and 3.0 mol l^{-1} was tested. Expt II used slow freezing with the cryoprotectant EG (1.5 mol l^{-1} and 3.0 mol l^{-1}) or with DMSO (1.5 mol l^{-1}).

Ovary collection and oocyte recovery. Domestic cat ovaries were recovered from queens subjected to ovariectomy. Ovaries were stored at 38°C in PBS supplemented with 100 iu penicillin-G potassium salt ml^{-1} and 100 μg streptomycin sulphate ml^{-1} (Sigma Chemical Co., St Louis, MO) for 1–4 h before processing.

Oocytes were released by repeatedly puncturing the ovaries with a 22-gauge needle. Immature oocytes with an intact corona radiata, attached cumulus cells, and medium to dark pigmented cytoplasm were selected for the experiments. Oocytes were rinsed in PBS and antibiotics with 0.1% (w/v) polyvinyl alcohol (Sigma Chemical Co.) and held at room temperature in PBS and antibiotics with 20% heat-inactivated FBS (fetal bovine serum, Sigma Chemical Co) before exposure to the cryoprotectant solutions.

Oocyte Freezing and Assessment

Oocyte freezing and thawing

In Expt I, oocytes were exposed for 5 min at 5°C at each increasing concentration (0.5, 1.0, 1.5 mol l^{-1} or 0.5, 1.5, 3.0 mol l^{-1}) of DMSO (BDH, Chemicals Ltd, Poole, Dorset, UK) or PROH (Sigma Chemical Co.) in PBS + 20% FBS and then exposed to the final cryoprotectant solution consisting of 0.2 mol sucrose l^{-1} (BDH Chemicals Ltd) and 1.5 or 3.0 mol l^{-1} DMSO or PROH. Five to ten oocytes were loaded into 0.5 ml straws. After a 10 min equilibration period at 5°C, the straws were plunged directly into liquid nitrogen (ultrarapid freezing) or placed in a programmable freezer (Planer Kryo 10 System) and cooled at a rate of -2°C min^{-1} from 5°C to -7°C. After 5 min, each straw was seeded manually with chilled forceps, held at -7°C for an additional 5 min, and cooled at a rate of -0.3°C min^{-1} to -30°C, before being plunged into liquid nitrogen (slow freezing).

Oocytes were thawed by exposure to air at 22°C for 5 s followed by a 10 s hold in a water bath at 30°C. After the recovery of oocytes from the straw, the cryoprotectant was removed by sequential

5 min rinses of the oocytes in PBS + 20% FBS with decreasing concentration of DMSO or PROH (1.5, 1.0, and 0.5 mol l^{-1} or 3.0, 1.5, and 0.5 mol l^{-1}). Finally, the oocytes were rinsed twice in the maturation medium.

On the basis of the results of Expt I, in Expt II the slow freezing procedure was chosen. Two groups of oocytes were exposed for 20 min at room temperature in the cryoprotectant solution consisting of 0.2 mol sucrose l^{-1} and 1.5 or 3.0 mol EG l^{-1} (Sigma Chemical Co.) in PBS + 20% FBS and were frozen slowly (as in Expt I). After thawing performed as in Expt I, the oocytes were equilibrated for 5 min in the freezing solution and then transferred to PBS + 20% FBS, before washing in the maturation medium. A third group of oocytes was frozen with 1.5 mol DMSO l^{-1} as in Expt I. This served to verify the corresponding results of Expt I.

Assessment of morphology

After thawing the straws and diluting the cryoprotectant, the morphology of the oocytes was examined. Oocytes with an evenly granulated ooplasm and with an intact oolemma and zona pellucida were considered to be morphologically intact.

Oocyte maturation in vitro

Intact oocytes were subjected to *in vitro* maturation and cultured in 100 µl drops (five to ten oocytes per drop) under oil for 48 h at 38.5°C in 5% CO_2 in air, in modified Kreb's Ringer bicarbonate (Toyoda and Chang, 1974) medium supplemented with 100 iu penicillin-G potassium salt ml^{-1}, 100 µg streptomycin sulphate ml^{-1}, 3 mg BSA ml^{-1} (bovine albumin fatty acid free; Sigma Chemical Co.) and 0.5 iu FSH ml^{-1} + 0.5 iu LH ml^{-1} (Pergovet 500, Serono, Rome) to evaluate their developmental potential. Freshly collected oocytes were used as unfrozen controls.

Assessment of resumption of meiosis and metaphase II

After culture for 48 h, the oocytes in each experimental group were fixed, stained and examined for the resumption of meiosis after thawing. This process involved removal of the cumulus cells by mechanical displacement, fixation of the oocytes in acetic acid:ethanol (1:3) for 24 h, staining with 1% (w/v) aceto-orcein (Sigma Chemical Co.). Oocytes that showed nuclear stages ranging between germinal vesicle breakdown and metaphase II were considered to have resumed meiosis.

Statistical analysis

The proportions of morphologically intact oocytes after freezing and thawing and the percentages of oocytes resuming meiosis and reaching M II after IVM were evaluated by Chi-square analysis. Values were considered to be significant when $P < 0.05$.

Effects of Slow versus Rapid Cooling – Expt I

Frozen–thawed oocytes were recovered at a rate of 91.2% (404/443). Morphological evaluation of the oocytes indicated that, following the ultrarapid freezing procedure, 81.7% (174/213) of the oocytes were intact versus 46.6% (89/191) for slow freezing ($P < 0.001$) (Table 1). These results were independent of the choice of the cryoprotectant or its concentration, except for the case of DMSO 3.0 mol l^{-1} with ultrarapid freezing which gave a lower result (62.5%, 35/56, $P < 0.05$). None of the oocytes frozen with the ultrarapid procedure resumed meiosis, while oocytes were able to resume meiosis following the slow freezing procedure (Table 1). The highest resumption rate (44.4%, 12/27 of morphologically intact oocytes, $P < 0.025$) was achieved in the presence of 1.5 mol DMSO l^{-1}, although this result was lower than that of unfrozen oocytes (82.1%, 23/28, $P < 0.005$). Full nuclear maturation post-thawing was

Table 1. Morphology of and resumption of meiosis in immature cat oocytes after slow or ultrarapid freezing with dimethylsulphoxide (DMSO) and 1,2-propanediol (PROH)

	Slow freezing		Ultrarapid freezing	
Treatment	Oocytes after thawing Morphologically intact No. (%)	Oocytes after IVM 48 h Resumption of meiosis* No. (%)	Oocytes after thawing Morphologically intact No. (%)	Oocytes after IVM 48 h Resumption of meiosis* No. (%)
Unfrozen control	—	23/28 (82.1)^{A}	—	23/28 (82.1)^{A}
DMSO 1.5 mol l^{-1}	27/52 (51.9)^{a}	12/27 (44.4)^{B}	46/57 (80.7)^{b}	0/46 (0)^{D}
DMSO 3.0 mol l^{-1}	19/46 (41.3)^{a}	1/19 (5.3)^{C}	35/56 (62.5)^{c}	0/35 (0)^{C,D}
PROH 1.5 mol l^{-1}	23/48 (47.9)^{a}	3/23 (13.0)^{C}	44/47 (93.6)^{b}	0/44 (0)^{D}
PROH 3.0 mol l^{-1}	20/45 (44.4)^{a}	1/20 (5.0)^{C}	49/53 (92.4)^{b}	0/40 (0)^{C,D}
Total	89/191 (46.6)^{a}	17/89 (19.1)^{C}	174/213 (81.7)^{b}	0/174 (0)^{D}

Data are pooled from six replicates.
Different superscripts within columns and within rows denote significant differences ($P < 0.05$).
*Oocytes showing nuclear stages between germinal vesicle breakdown and metaphase II as a percentage of the morphologically intact oocytes.

achieved only in the group frozen slowly with 1.5 mol DMSO l^{-1} where 18.5% (5/27) of intact oocytes were in M II stage after culture for 48 h. However, there was a significant difference in M II rate with the unfrozen oocytes (50%, 14/28, $P < 0.025$).

Effects of EG Versus DMSO as Cryoprotectant – Expt II

Frozen–thawed oocytes were recovered at a rate of 83% (174/209). No significant difference in the number of morphologically intact oocytes after thawing was observed among treatments (Table 2). Resumption of meiosis occurred in all the treatments, with significantly higher percentages in the presence of 1.5 mol l^{-1} EG and DMSO (52%, 13/25 and 47.4%, 18/38, respectively) compared with 3.0 mol EG l^{-1} (17.6%, 6/34; $P < 0.01$) (Table 2). Moreover, meiosis resumption rates of oocytes frozen with 1.5 mol EG l^{-1} and unfrozen oocytes were not significantly different (72.7%, 40/55, $P > 0.05$). Full nuclear maturation (M II) was obtained in 23.7% (9/38) and 20% (5/25) of intact thawed oocytes frozen with 1.5 mol DMSO or EG l^{-1}, although with lower rates than those of unfrozen oocytes (47.3%, 26/55; $P < 0.025$). The results for 1.5 mol DMSO l^{-1} are the same as in Expt I.

Discussion

These results show that immature cat oocytes can survive, resume meiosis and achieve metaphase II *in vitro* after freezing. The best results were obtained with the slow freezing method and 1.5 mol DMSO or EG l^{-1}. The ultrarapid procedure never resulted in resumption of meiosis *in vitro*, despite the intact morphology of the oocytes after freezing.

Table 2. Morphology, resumption of meiosis, and metaphase II rates of immature cat oocytes after slow freezing with ethylene glycol (EG) and dimethylsulphoxide (DMSO)

	Slow freezing		
	Oocytes after thawing	Oocytes after IVM 48 h	
Treatment	Morphologically intact No. (%)	Resumption of meiosis* No. (%)	Metaphase II** No. (%)
Unfrozen control	—	40/55 (72.7)^{A}	26/55 (47.3)^{x}
EG 1.5 mol l^{-1}	25/50 (50.0)^{a}	13/25 (52.0)^{A,B}	5/25 (20.0)^{y,z}
EG 3.0 mol l^{-1}	34/51 (66.7)^{a}	6/34 (17.6)^{C}	2/34 (5.9)^{y}
DMSO 1.5 mol l^{-1}	38/73 (52.0)^{a}	18/38 (47.4)^{B}	9/38 (23.7)^{z}

Data are pooled from three replicates.
Different superscripts within columns denote significant differences ($P < 0.05$).
*Oocytes showing nuclear stages between germinal vesicle breakdown and metaphase II as a percentage of the morphologically intact oocytes.
**Oocytes showing nuclear stage of metaphase II as a percentage of the morphologically intact oocytes.

This finding demonstrates that morphology of oocytes after freezing and thawing is not predictive of the ability to resume meiosis. It underlines the importance of damage to the cytoplasmic system by freezing, which is not revealed by a morphological evaluation, on maturation. In addition, Hochi *et al.* (1994) showed that M II rates for equine oocytes are not related to the morphological assessment after thawing. Therefore, survival rates based on the capability of thawed oocytes to develop after culture *in vitro* are more relevant than survival rates based on the number of oocytes that are morphologically intact after thawing. The latter criterion has been used by some authors (Schroeder *et al.*, 1990; Van der Elst *et al.*, 1993).

The results for maturation suggest that DMSO and EG may provide better intracellular cryoprotection than PROH in cat oocytes, even though PROH has been successfully used for cryopreservation of oocytes of different species (Suzuki and Nishikata, 1992; Toth *et al.*, 1994a, b) and of cat embryos (Pope *et al.*, 1994). PROH is a well-known permeating cryoprotectant, but its permeability through the oocyte membrane may differ considerably for different species.

We cannot state that the procedures we used are the most suitable for cat oocytes. Further studies about intrinsic characteristics of cat oocytes are needed, since the permeability of the membrane or the presence of the intracellular lipid droplets can be responsible for uneven intracellular ice formation (Toner *et al.*, 1986), which could severely affect the freezing and thawing process. Furthermore, since successful cryopreservation usually requires optimization of each procedural step, further studies are necessary to evaluate different cryoprotectant exposures and dilutions, cooling rates, and thawing procedures, as well as different cryoprotectants or freezing procedures (vitrification).

In conclusion, the results of this study indicate that immature cat oocytes can resume meiosis and mature to the metaphase II stage *in vitro* following slow freezing in either 1.5 mol l^{-1} DMSO or EG. Although the fertizability and developmental capacity of these oocytes was not determined, it is hoped that these results and ongoing experiments will demonstrate that cryopreservation of immature cat oocytes coupled with *in vitro* maturation and fertilization could ultimately help the conservation of endangered felids species.

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