Viability of small preantral ovarian follicles from domestic cats after cryoprotectant exposure and cryopreservation

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About 1500 preantral follicles can be recovered from a single cat ovary by mechanical dissection. This is a potentially rich source of genetic material if ova could be preserved and grown *in vitro*, especially from rare or endangered species that die abruptly or are ovariectomized for medical reasons. The aims of this study were to examine cryoprotectant toxicity and then the potential of successfully cryopreserving preantral cat follicles. In the initial toxicity trial, isolated cat follicles (40–90 µm) were exposed to dimethylsulfoxide, glycerol, 1,2-propandiol or ethylene glycol at 0°C for 15 min. Follicle viability was assessed by supravital staining using a combination of Trypan blue and Hoechst 33258 at 0 h, and after 18 h and 1 week of culture. Percentages of follicles with intact oocytes and granulosa cells were similar ($P > 0.05$) among control (no cryoprotectant), dimethylsulfoxide, 1,2-propandiol and ethylene glycol treatments at all time points, but were reduced ($P < 0.05$) after glycerol exposure. On the basis of this finding, dimethylsulfoxide and 1,2-propandiol were used to cryopreserve intact follicles, and post-thaw viability was assessed by supravital staining and 5-bromo-2'-deoxyuridine uptake into oocytes and granulosa cells during culture. Of control (noncryopreserved) follicles, $31.4\% \pm 2.9\%$, $18.8\% \pm 1.9\%$ and $16.2\% \pm 1.6\%$ were intact after 0 h, 18 h and 1 week of culture, respectively. Uptake of 5-bromo-2'-deoxyuridine occurred in approximately 20% of follicles at all time points. On the basis of the presence of both a healthy oocyte and granulosa cells, cryopreservation in dimethylsulfoxide or 1,2-propandiol allowed approximately 19% of follicles to survive. Approximately 10% demonstrated clear evidence of cell activity that was sustainable for 1 week. In conclusion, the cat ovary contains a population of preantral follicles that are not adversely affected by short-term exposure to most conventional cryoprotectants. Furthermore, there is a subpopulation of these follicles capable of surviving cryopreservation, remaining structurally intact and physiologically active after thawing.

Introduction

Cryopreservation and assisted reproduction techniques, developed to exploit the availability of germ cells in domesticated mammals, have profound potential for maintaining genetic diversity in wildlife populations living in zoos and in the wild (Wildt, 1990). Combinations of techniques such as artificial insemination, *in vitro* oocyte maturation (IVM), *in vitro* fertilization (IVF) and embryo transfer, together with gamete and embryo cryopreservation, offer unique opportunities for assisting in the management and propagation of small populations. In particular, cryopreservation of germ cells would extend the generation interval and protect the genetic diversity of valuable founder animals, making these genes available for future generations (Wildt *et al.*, 1993).

Rescue of female germ cells from valuable animals that die abruptly or undergo ovariectomy for medical reasons

requires the successful recovery, preservation, growth and fertilization of ovarian oocytes. The development of IVM/IVF systems for cat oocytes has been in progress for almost a decade (Goodrowe *et al.*, 1988; Johnston *et al.*, 1989; Lengwinat and Blottner, 1994; Wood *et al.*, 1995; Wolfe and Wildt, 1996), and includes work with several wild felid species (Johnston *et al.*, 1991). All of these studies have focused on antral oocytes, but the number of oocytes could be vastly increased if it was possible to recruit earlier oocyte stages. The preantral pool of primordial, primary and secondary follicles comprise > 99% of ovarian oocytes (Gougeon, 1993). Because the ovary of a fertile, adult cat contains approximately 50 000 oocytes (Göritz and Jewgenow, 1993), this is a rich source of genetic material if these gametes could be collected and grown *in vitro* to maturity. We have developed a mechanical dissection tool for harvesting many preantral follicles from the ovaries of the domestic cat (Jewgenow and Göritz, 1995) and its wild relatives (Jewgenow and Stolte, 1996).

The required growth period for preantral rodent oocytes is < 3 weeks (Eppig and O'Brien, 1996), but is several months for

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similar stage cattle (Lussier *et al.*, 1987) and human (Gougeon, 1993) oocytes. Mouse preantral follicle–oocyte complexes can be grown *in vitro* and the oocytes recovered, successfully matured, fertilized *in vitro* and then used to produce young after embryo transfer (Eppig and Schroeder, 1989; Eppig and O'Brien, 1996). Mouse preantral follicles also have been cryopreserved successfully as demonstrated by the production of live young after IVM, IVF and embryo transfer (Carroll *et al.*, 1990).

We anticipate that a practical and comprehensive gamete rescue programme for felids will involve long-term culture for growth, as well as an ability to cryopreserve freshly collected oocytes. Therefore, as a first step to achieving this long-term goal, the objectives of this study were: (1) to determine the ability to recover and sustain preantral cat follicles *in vitro* for as long as 1 week; and (2) to examine the influence of four common cryoprotectants (dimethylsulfoxide (DMSO), glycerol (GLYC), 1,2-propanediol (PROH) and ethylene glycol (ETOH)) on follicle viability at a chilling temperature, and the effects of DMSO and PROH on the viability of follicles after cryopreservation. Because the follicle complex comprises an oocyte and granulosa cells, a secondary aim was to determine the need to use a multiple evaluative approach to ensure accurate estimation of viability in terms of plasma membrane integrity and cell proliferation.

Materials and Methods

Follicle recovery

Ovaries were obtained from domestic cats ovariohysterectomized at local veterinary clinics. The exact age and reproductive history of each donor almost always was unknown; however, most donors were 12–24 months old and believed nulliparous. After excision, ovaries were placed into 50 ml centrifuge tubes (Corning Glass Works, Corning, NY) containing 15 ml PBS, stored at 4°C and then processed within 24 h. Number of follicles (recovery) and viability (see below) were evaluated on the basis of cool ovarian storage for one of two time periods: < 12 h or 12–24 h.

After being washed, ovaries were dissected to recover oocytes from antral follicles and these were used in another unrelated study. The dissection procedure for antral follicles is described by Wolfe and Wildt (1996). Ovaries then were carefully pressed through a cell dissociation sieve (60 mesh, Sigma Chemie GmbH, Deisenhofen) together with 20 ml Hepes buffered Minimum Essential Medium (MEM; Gibco Laboratories, Grand Island, NY) containing 3 mg BSA ml⁻¹ (Sigma Chemical Company, St Louis, MO; A-9418, lot 53H00685), hereafter referred to as HMEM. The resulting cell suspension was passed through a series of nylon sieves (100 µm and 40 µm cell strainers; Falcon, Becton Dickinson Labware, Franklin Lakes, NJ). The 40 µm cell strainer was rinsed in 10 ml HMEM allowing the recovery of all follicles with a diameter of 40–90 µm. This follicle suspension (a composite of 2–4 ovaries processed together) consisted of a combination of primordial and primary (80–90%) and small secondary follicles (Jewgenow and Göritz, 1995). The suspension was centrifuged at 300 g for 5 min and resuspended in 1 ml MEM (Sigma)

containing Earle's salts, 30 iu streptomycin ml⁻¹, 120 iu penicillin ml⁻¹ (Gibco), 0.026 g pyruvate l⁻¹, 0.292 g L-glutamine l⁻¹ and 3 mg BSA ml⁻¹ (Sigma). A 20 µl aliquot was taken for counting the number of follicles.

In vitro culture of small preantral follicles

By adding MEM, follicle concentration was adjusted to 1000 ml⁻¹, and approximately 200 follicles (approximately 200 µl) were transferred into each well of a series of four-well culture dishes (Nunc, Roskilde) containing 300 µl MEM covered with mineral oil (Sigma). Follicles were cultured at 38°C in 5% CO₂ in air in a standard laboratory incubator. Random aliquots of follicles were removed immediately before culture (0 h) or after an 18 h or 1 week incubation, and were then assessed for viability.

Viability assessment

Viability assessment of follicle cells was performed using two supravital stains, Trypan blue (TB, Sigma) and Hoechst 33258 (H58, Sigma). Both stains have proved useful for characterization of cell membrane integrity by dye exclusion from vital cells (Trypan blue: Phillips, 1973; Hoechst 33258: Singh and Stephens, 1986). In a preliminary experiment, aliquots of preantral follicle populations with presumed high (immediately after isolation) and low (killing follicles by heating and cooling) viability were stained using Trypan blue and Hoechst 33258 separately. The percentage of dead (fully stained) follicles was confirmed with Trypan blue and Hoechst 33258 ($n = 25$; $P < 0.05$; viability range, 0–60%). Owing to their complex structure, many follicles were partially stained (intermediate stages). For enhanced differentiation of these intermediate stages, the combination of Trypan blue and Hoechst staining was used to take advantage of the different staining pattern (Trypan blue is enriched in the cytoplasm and Hoechst 33258 binds to the DNA of dead cells; Phillips, 1973; Singh and Stephens, 1986).

In the present study, the combined viability staining was used as follows. A 50 µl aliquot containing preantral follicles was collected from each culture well and was supplemented with 0.5 µl Hoechst 33258 stock solution in PBS (1 mg ml⁻¹) for 15 min at 38°C. This step was followed by adding 2.5 µl 0.4% Trypan blue solution for 1 min. The aliquot then was mounted on a slide, and each follicle was evaluated under phase contrast and fluorescence excitation using a Leitz Diaplan microscope (Leica, Wetzlar). Follicles were categorized into one of five viability classes (Fig. 1): I, follicles with 0–3 granulosa cells (< 10%) stained with Trypan blue and with no visible germinal vesicle within the oocyte; II, follicles with 4–15 granulosa cells (10–50%) stained and with no visible germinal vesicle within the oocyte; III, follicles with 0–3 granulosa cells (< 10%) stained and with a visible germinal vesicle (detectable by Hoechst treatment); IV, follicles with 4–15 granulosa cells (10–50%) stained and with a visible germinal vesicle; V, follicles in which > 50% of the granulosa cells stained. Follicles

categorized as class I or II were considered to contain viable oocytes, whereas class I and III follicles were considered to contain viable granulosa cells.

Incorporation of 5-bromo-2'-deoxyuridine as an index of DNA synthesis

At 0 h, 18 h and 1 week of culture, follicles were supplemented for an additional 12 h with 10 μ l 5-bromo-2'-deoxyuridine (BrdU) labelling reagent (0.5 mmol l⁻¹; BrdU-Kit II; Boehringer Mannheim Biochemica, Mannheim). After incubation, follicles were transferred into siliconized glass tubes containing 10 ml washing buffer (BrdU-Kit II), centrifuged at 300 g for 5 min, and resuspended in 50 μ l buffer. All follicles were mounted on a glass slide and air-dried. Cells were fixed in 70% ethanol (in glycine buffer, 50 mmol l⁻¹; pH 2.0) for at least 20 min at -20°C. Further steps were performed according to the assay procedure described in the Boehringer Kit. In brief, slides were washed and subsequently covered with anti-BrdU- and anti-mouse-IgG-alkaline phosphatase working solutions (30 min, 37.5°C). The substrate reaction was visible 30 min after adding the colour substrate solution. Slides were covered with glycerol-gelatin and evaluated under a light microscope at a magnification of $\times 100$. Preantral follicles that incorporated BrdU into nuclei were considered to have proliferating cells. Control follicles were processed without adding BrdU to the culture medium ($n = 400$) and by heat-treating follicles (75°C for 20 min; $n = 400$).

Cryoprotectant toxicity

The sources and concentration of the various cryoprotectant solutions were as follows: 1.5 mol DMSO l⁻¹ (DOMOSO[®], Syntex Animal Health, West Des Moines, IA); 10% (v/v) GLYC (Fisher Scientific, Fair Lawn, NJ); 1.5 mol PROH l⁻¹ (J. T. Baker Inc., Phillipsburg, NJ); and 1.5 mol ETOH l⁻¹ (J. T. Baker). All cryoprotectants were prepared at a twofold concentration in HMEM with 20% (v/v) fetal calf serum (Sigma). Aliquots (200 μ l) of follicle suspension were transferred to 1.8 ml Eppendorf tubes and cooled in ice water (0–1°C) for 10 min before adding an equal volume of ice-cold cryoprotectant. Follicles were left to equilibrate in each cryoprotectant at 0°C for an additional 15 min. Tubes then were warmed by shaking in a 37°C water bath for 1 min. Cryoprotectant was removed by adding 400 μ l HMEM twice at room temperature followed each time by a 5 min incubation. Diluted follicle suspensions were centrifuged at 300 g for 2 min, washed with 1 ml HMEM and finally resuspended in 200 μ l culture medium. Follicles were counted, and viability was estimated using Trypan blue–Hoechst-staining after 0 h, 18 h and 1 week of culture. Controls consisted of follicles to which HMEM was added, without cooling to 0°C (hereafter called FRESH) and with cooling to 0°C and incubation for 15 min at 0°C (hereafter called COOL). Control follicles were washed as described above before assessing viability.

Follicle cryopreservation and thawing

On the basis of findings from the toxicity experiment above and earlier results from freezing cat embryos and preantral

follicles (Pope *et al.*, 1994; Jewgenow and Göritz, 1995), DMSO and PROH were selected for the cryopreservation trial. Follicle samples (200 μ l) were transferred into 1.8 ml Eppendorf tubes and placed in ice water for 10 min. An equal volume (200 μ l) of ice-cold (0°C) twofold cryoprotectant was added slowly, and the vial was equilibrated at 0°C for 15 min. Samples were transferred into 0.25 ml plastic straws, sealed and cooled slowly at 0.5°C min⁻¹ (Biocool, FTS Systems Inc., Stone Ridge, NY). After seeding at -7°C, straws were cooled further to -70°C at 0.5°C min⁻¹ before being plunged into liquid nitrogen (-196°C) for a minimum of 24 h.

Straws were thawed rapidly in a water bath (37°C, approximately 2500°C min⁻¹) for 1 min. Each follicle suspension (two straws) was flushed into an Eppendorf tube with 200 μ l HMEM per straw, diluted by adding 400 μ l HMEM at room temperature followed each time by a 5 min incubation. After centrifugation at 300 g for 5 min, follicles were washed with 1 ml HMEM and resuspended in 100 μ l of culture medium, counted and aliquots were stained for viability or cultured for testing *in vitro* DNA synthesis.

Statistical analyses

Mean $\pm$ SEM values are presented. Data were analysed using a multi-way analysis of variance (MANOVA). For calculating significant differences among test groups in the toxicity (6 $\times$ 3 matrix) and cryopreservation (3 $\times$ 3 matrix) trials, the LCD test for planned comparison (*t* test for dependent samples) was applied. All statistical procedures were performed with the software program Statistika for Windows (Release 4.5, copyright StatSoft Inc., 1993).

Results

Recovery and culture of preantral follicles

Ovaries from 52 queens were subjected to mechanical isolation, resulting in an average of 1472 ± 288 small (40–90 μ m diameter) preantral follicles recovered per ovary. On the basis of the separate viability assessments of oocytes and granulosa cells, different classes of harvestable preantral follicles were found in the cat ovary. Follicles were considered to be healthy if they contained both a viable oocyte and granulosa cells (class I). All other classes were compromised either by a poor oocyte or by poor granulosa cells, or both (Fig. 1). The number of healthy follicles depended on the length of cold ovarian storage before dissection (Table 1). When ovaries were processed on the day of ovariectomy (< 12 h storage), the percentage of intact follicles with viable oocytes and granulosa cells (class I) was > 10% greater ($P < 0.05$) than for ovaries stored for 12–24 h. However, by 1 week of culture, the percentage viability was similar between the groups.

Cryoprotectant toxicity assessment

Trypan blue combined with Hoechst staining allowed the effective independent assessment of cryoprotectant effects on

Table 1. Recovery and viability of preantral follicles 40–90 µm in diameter from domestic cat ovaries after storage for < 12 h and 12–24 h at 4°C

Ovarian storage interval (h)	Number of ovaries	Number of follicles per ovary	Percentage viable (class I) preantral follicles after	
			Immediate isolation	1 week culture
< 12	32	1622 ± 289	42.3 ± 3.5 ^a	22.5 ± 1.8
12–24	72	1269 ± 808	31.7 ± 1.8 ^b	18.5 ± 2.7

^{a,b}Different superscripts within columns indicate significantly different values ($P < 0.05$).

oocyte and granulosa cell integrity (Table 2, Fig. 1). Follicle culture always resulted in a decrease ($P < 0.05$) in overall viability of oocytes and granulosa cells with time (0 h versus 18 h versus 1 week; Table 2). Decline in viability affected only the oocyte, since there was no obvious effect on class III follicles in which granulosa cells remained healthy. Approximately 5% of all preantral follicles contained a healthy oocyte but poor granulosa cells (class II), whereas approximately 12% contained a poor oocyte but viable granulosa cells (class III). During the 1 week culture, class II follicles (with unhealthy granulosa cells) were not sustained. In contrast, class III follicles (dead oocyte inside a viable granulosa cell layer) tended to survive (Table 2).

Cooling follicles to 0°C for 15 min did not reduce ($P > 0.05$) the number of healthy (class I) follicles. However, follicles with partly affected granulosa cells (class II and IV) could not withstand low temperature and died ($P < 0.05$). Adding DMSO, PROH or ETOH did not result in further loss of viability compared with FRESH controls. However, supplementing with GLYC decreased viability by approximately 15% ($P < 0.05$) at 0 h, although this difference was not evident at 18 h or 1 week.

Cryopreservation of preantral follicles

Approximately 16 000 small, preantral follicles isolated from 25 cat ovaries stored > 12 h were cryopreserved. Upon thawing, 66.3 ± 5.6% of these ($n = 10\,786$) were recovered intact, suitable for assessment and further culture. Overall, cryopreservation and thawing decreased ($P < 0.05$) the percentage of viable oocytes (class I and II follicles) regardless of cryoprotectant used (Fig. 2). Before cryopreservation, 33.9 ± 2.7% of follicles contained viable oocytes, and > 21% experienced BrdU incorporation (Table 3). After thawing, 19.3 ± 2.8% of DMSO- and 18.5 ± 2.7% of PROH-exposed oocytes were viable when stained with Trypan blue–Hoechst (Fig. 2). *In vitro* DNA synthesis occurred in 15.5 ± 5.4% and 13.9 ± 4.5% of these oocytes, respectively (Table 3). Follicular

granulosa cells also were affected by cryopreservation. The percentage of follicles with viable granulosa cells (class I and III) at 0 h (FRESH) was 45.1 ± 3.7% compared with 32.6 ± 5.6% and 28.6 ± 5.6% for cryopreservation with DMSO and PROH, respectively ($P < 0.05$) (Fig. 2). BrdU incorporation into granulosa cells was similar ($P > 0.05$) for FRESH, DMSO and PROH treatments (mean range 18.5–24.8%).

For FRESH, class I follicles, there was a gradual decline in oocyte/granulosa cell viability over time (Fig. 2). At 18 h and 1 week of culture, cryopreserved–thawed follicles were less viable ($P < 0.05$) than their FRESH counterparts. Nonetheless, approximately 10% of follicles cryopreserved with DMSO or PROH retained viable oocytes, and 20% contained healthy granulosa cells. *In vitro* DNA synthesis in oocytes or granulosa cells did not change during culture, regardless of treatment (Table 3).

Labelling of control follicles resulted in staining of fewer than 1% of oocytes and 3% of granulosa cells in the negative control (without BrdU) versus 6.8% of oocytes and 1.7% of granulosa cells in the heat-treated control (dead follicles).

Discussion

This study determined that it was feasible to cryopreserve successfully a subpopulation of small preantral follicles (40–90 µm in diameter) from the domestic cat ovary. These follicles could be sustained either fresh or after freezing and thawing for as long as 1 week *in vitro*, and could contain both viable oocytes and granulosa cells. Because growth to a more mature (larger) stage was not expected within 1 week culture, we assessed follicle survival with cryoprotectant and cryopreservation on the basis of plasma membrane integrity and cell proliferation status. Carroll *et al.* (1990) reported the survival of mouse preantral follicles assessed morphologically after freezing. Because mouse preantral follicles can grow to maturity *in vitro*, it was possible in that study to test survival on the basis of oocyte fertilization and embryo development to term.

Fig. 1. Preantral cat follicles stained with Trypan blue (left column) and Hoechst 33258 (right column). (a) Class I follicle, no staining of germinal vesicle and < 4 granulosa cells (< 10%) stained. (b) Class II follicle, unstained germinal vesicle and 4–15 granulosa cells (10–50%) stained. (c) Class III, stained germinal vesicle (Hoechst) with < 4 granulosa cells (< 10%) stained. (d) Class IV, germinal vesicle and 4–15 granulosa cells (10–50%) stained. (e) Class V, staining of entire follicle. Arrowheads represent stained germinal vesicle; asterisks show stained granulosa cells; scale bar represents 10 µm.

Table 2. Viability of preantral follicles 40–90 µm in diameter from domestic cat ovaries during 1 week culture before (FRESH) and after cooling (COOL) and then exposure to different cryoprotectants at 0°C for 15 min

Duration of culture	Treatment	n	Viability class				
			I + oocyte + granulosa	II + oocyte ± granulosa	III – oocyte + granulosa	IV – oocyte ± granulosa	V – oocyte – granulosa
0 h	FRESH	665	39.9 ± 4.3% ^a	4.9 ± 1.8% ^a	11.7 ± 1.3%	4.8 ± 1.5% ^a	38.7 ± 5.1% ^a
	COOL	468	33.0 ± 5.7% ^a	0.8 ± 0.6% ^b	12.2 ± 0.6%	0.4 ± 0.2% ^b	53.6 ± 5.7% ^b
	DMSO	666	32.3 ± 3.6% ^a	6.9 ± 3.3% ^a	11.5 ± 1.4%	3.4 ± 1.5%	45.9 ± 5.9%
	GLYC	701	25.0 ± 3.3% ^b	4.4 ± 2.1% ^a	14.1 ± 2.2%	4.2 ± 2.0% ^a	52.3 ± 3.7% ^b
	PROH	705	36.5 ± 3.7% ^a	5.1 ± 2.1% ^a	13.1 ± 1.9%	2.7 ± 1.7%	42.7 ± 4.9%
	ETOH	672	33.5 ± 3.3% ^a	4.0 ± 1.5%	11.6 ± 1.5%	2.9 ± 1.7%	47.9 ± 4.6%
18 h	FRESH	713	20.9 ± 2.6%	2.3 ± 0.6%	10.6 ± 1.7%	2.5 ± 0.6%	63.7 ± 2.6%
	COOL	559	22.8 ± 1.4%	0.8 ± 0.4%	12.3 ± 1.8%	1.8 ± 1.1%	62.2 ± 2.8%
	DMSO	650	22.3 ± 1.0%	2.4 ± 1.0%	13.1 ± 2.7%	1.7 ± 0.7%	60.5 ± 2.0%
	GLYC	587	17.9 ± 3.7%	3.1 ± 1.4%	11.2 ± 1.7%	2.5 ± 1.0%	65.4 ± 4.0%
	PROH	656	22.0 ± 2.0%	2.0 ± 1.0%	13.8 ± 2.2%	2.2 ± 1.0%	59.9 ± 2.0%
	ETOH	640	25.0 ± 3.4%	2.0 ± 1.0%	8.2 ± 1.1%	1.1 ± 0.7%	63.7 ± 4.4%
1 week	FRESH	477	13.5 ± 1.6%	0.4 ± 0.3%	14.3 ± 2.4%	0.9 ± 0.5%	69.2 ± 3.0%
	COOL	474	14.9 ± 2.0%	0.5 ± 0.2%	8.9 ± 2.1%	0.2 ± 0.2%	75.5 ± 1.9%
	DMSO	456	13.5 ± 2.5%	1.2 ± 0.5%	10.7 ± 2.5%	1.4 ± 1.0%	73.0 ± 1.4%
	GLYC	479	11.4 ± 1.5%	0.0 ± 0.0%	9.5 ± 2.3%	0.8 ± 0.5%	78.1 ± 1.8%
	PROH	500	13.4 ± 1.5%	0.6 ± 0.2%	11.4 ± 3.0%	0.4 ± 0.3%	74.2 ± 3.1%
	ETOH	501	11.8 ± 1.5%	0.4 ± 0.2%	9.8 ± 2.3%	1.4 ± 0.6%	76.7 ± 2.6%

+, healthy cells; ±, for 10 to 50% viable granulosa cells; –, nonviability.
DMSO, dimethylsulfoxide; GLYC, glycerol; PROH, 1,2-propanediol; ETOH, ethylene glycol.
^{a,b}Different superscripts within columns and time intervals indicate significantly different values (*P* < 0.05).

Fig. 2. Viability of preantral cat follicles during 1 week of culture *in vitro* assessed by combined Trypan blue–Hoechst 33258 staining. (■) viable follicles (class I); (□) viable granulosa cells (class I and III). FRESH, untreated control follicles. DMSO and PROH include follicles previously cryopreserved in 1.5 mol dimethylsulfoxide l^{-1} (DMSO) or 1.5 mol 1,2 propandiol l^{-1} (PROH). Within different main treatment groups, bars with different letters differ significantly ($P < 0.05$). Numbers within each open bar indicate total numbers of preantral follicles tested within that treatment during that time period.

Table 3. *In vitro* DNA synthesis in preantral follicles 40–90 μm in diameter from domestic cat ovaries before (FRESH) and after cryopreservation using dimethylsulfoxide (DMSO) and 1,2-propandiol (PROH)

Treatment	Time of adding BrdU	Number of replicates	Number of follicles	Percentage of labelled	
				Oocytes	Granulosa cells
FRESH	0 h	5	241	21.9 $\pm$ 1.9%	24.8 $\pm$ 5.1%
	18 h	5	237	17.8 $\pm$ 2.1%	23.2 $\pm$ 5.0%
	1 week	5	289	23.6 $\pm$ 4.7%	22.3 $\pm$ 3.8%
DMSO	0 h	6	486	15.5 $\pm$ 5.4%	23.6 $\pm$ 3.7%
	18 h	6	454	12.9 $\pm$ 2.6%	19.0 $\pm$ 4.8%
	1 week	5	383	16.8 $\pm$ 3.5%	22.8 $\pm$ 3.7%
PROH	0 h	6	451	13.9 $\pm$ 4.5%	18.5 $\pm$ 4.3%
	18 h	6	368	11.9 $\pm$ 3.0%	19.8 $\pm$ 6.7%
	1 week	5	287	15.1 $\pm$ 4.0%	18.5 $\pm$ 3.7%

BrdU, 5-bromo-2'-deoxyuridine.

Oocytes from 6-day-old mice require 16 days of culture to reach the diameter and developmental competence of oocytes fully grown *in vivo* (Eppig and O'Brien, 1996). However, the growth period of preantral follicles from other mammals appears to be considerably longer (Gougeon, 1993), and *in vitro* culture of primary stage follicles to the antral stage has not yet been achieved. Long-term culture of human preantral follicles with measurable growth in diameter has been shown after 40 days culture (Zhang *et al.*, 1995). Sirard and Coenen (1995) cultured bovine follicles for more than 3 months but, although viability was maintained, did not report follicle growth.

Immediately at collection, fewer than 50% of domestic cat preantral follicles were viable. The low post-isolation viability agreed with previous data (Jewgenow and Göritz, 1995) and is

probably a result of the isolation procedure and natural amounts of atresia. Cats normally experience a high incidence of continuous follicular atresia (Wood *et al.*, 1997). Approximately 65% of preantral follicle–oocyte complexes in cats examined histologically show clear evidence of slight or severe degeneration. In the present study, we determined that storage of ovaries for > 12 h resulted in an additional loss in follicle viability. In contrast, cat oocytes collected from antral follicles can be stored at 4°C for 24 h without losing the ability to mature and develop *in vitro* after fertilization (Wolfe and Wildt, 1996). Small preantral follicles seem to be more sensitive to prolonged cold storage than larger preantral follicles.

Combined use of the supravital stains Trypan blue and Hoechst 33258 was effective for determining overall follicle

viability. Trypan blue staining is a means of assessing granulosa cell viability (Jewgenow and Göritz, 1995), but Hoechst-staining is required to determine oocyte viability in the presence of dense granulosa cells. The unique structure of germinal vesicles meant that what could be considered the DNA of surrounding granulosa cells and the DNA of the oocytes could be distinguished. The present results confirmed that oocytes can be healthy in the presence of poor quality granulosa cells, and that granulosa cells can be viable in the presence of a dead or dying oocyte. However, a healthy follicle must contain both a viable oocyte and viable granulosa cells (Bachvarova *et al.*, 1980; Canipari *et al.*, 1984; Salustri *et al.*, 1993), because intact granulosa cells are important for nutrient and metabolic precursor transport (Buccione *et al.*, 1990) and for reducing cryoinjury (Imoedemhe and Sigue, 1992). Therefore, it was intriguing that small, preantral cat follicles containing degenerate oocytes could survive 1 week of culture.

Successful oocyte cryopreservation was demonstrated originally by Whittingham (1977) in mice, with similar procedures applied to a few other species (for review, see Aigner *et al.*, 1992). However, cryopreserving female germ cells has proven difficult, because mature (metaphase II) oocytes are more sensitive than embryos to requisite cryopreservation steps, including cooling and cryoprotectant addition and removal. Whereas cooling to 4°C appears to impair the meiotic spindle in most species, cryoprotectant exposure can increase zona pellucida resistance, disrupt cytoskeleton and metaphase plate organization, parthenogenetically activate thawed oocytes, and inhibit first cell division after fertilization (Trounson and Kirby, 1989; Vincent *et al.*, 1990; Hunter *et al.*, 1991; Garrisi and Navot, 1992; Gook *et al.*, 1995).

To avoid such problems with mature oocytes, it may be preferable to cryopreserve immature, prophase I oocytes (Candy *et al.*, 1994; Baka *et al.*, 1995). These young oocytes may be less susceptible to microtubular disruptions, because most of the microtubular system remains unorganized, and the chromatin is in a decondensed form, protected by the nuclear membrane (Mattson and Albertini, 1990). However, until now, no study has examined the sensitivity of immature oocytes in isolated, preantral follicles to different cryoprotectants.

The impact of prefreezing steps (cooling, adding and diluting out cryoprotectant) on both oocyte and granulosa cell viability was also assessed. Cooling of cat preantral follicles to 0°C was not accompanied by a decrease in the proportion of class I (healthy) follicles. In contrast, follicles with already poor quality oocytes (class II and IV) were affected adversely by simple cooling. Additional exposure to cryoprotectant (at the concentrations tested) appeared unrelated to the ability of follicles to be sustained *in vitro*. The only exception was an acute negative influence of 10% glycerol, an effect that was not evident by 18 h of culture. Although the 0 h negative influence of glycerol may have had no impact on freezability, follicles were cryopreserved only in DMSO and PROH, in part because DMSO has been used successfully for cryopreserving mouse (Carroll *et al.*, 1990) and cat (Jewgenow and Göritz, 1995) preantral follicles and PROH has been used successfully for cat embryos (Pope *et al.*, 1994). Approximately 15% of the cryopreserved class I oocyte-granulosa cell complexes (50% of the non-cryopreserved controls) survived the freeze-thaw

process on the basis of the multiple viability assessments used. Granulosa cells appeared to be less sensitive to the cryopreservation process than the enclosed oocyte. Nonetheless, regardless of cryoprotectant used, those follicles surviving cryopreservation were relatively consistent in their ability to maintain both the oocyte and granulosa cells through the 1 week culture interval.

Cryopreservation of feline preantral follicles would be one of several components to a gamete rescue programme for rare species. At the very least, it would provide the means to preserve genetic material from valuable individuals until a culture system, or appropriate alternative, is developed to transform preantral follicles into antral follicles. A substitute to media culture could involve microsurgical nucleus transfer from preantral follicle oocytes into oocytes of fully grown follicles. Liu *et al.* (1996) have demonstrated that cryopreserved primordial germ cells are competent to undergo nuclear remodelling and cleavage after nuclear transfer to enucleated pig oocytes. Another option is follicle transfer to the kidney capsule (Telfer *et al.*, 1989), an approach that already has resulted in the recovery of viable oocytes that were fertilized and used to produce live young in mice (Carroll *et al.*, 1990). The cryopreservation of isolated, preantral follicles followed by transplantation could be an alternative to the orthotopic transplantation of ovarian pieces (Candy *et al.*, 1995). Gosden (1992) reported the development of cat ovaries xenografted under the kidney capsule of ovariectomized, immunodeficient mice. In this case, the feline follicles grew to maturity in 1 month. A transplantation of isolated follicles is attractive because many germinal cells can be included in a single transplant with a high likelihood of graft revascularization (Scott *et al.*, 1981).

In conclusion, approximately 1500 preantral follicles can be recovered from a single cat ovary, and about 20% of these can be considered healthy after 1 week of *in vitro* culture. High quality preantral oocytes also appear resilient to exposure to conventional cryoprotectants. Furthermore, approximately 10% of cat preantral follicles remain structurally intact and metabolically active after freeze-thawing. Thus, it is estimated that at least 150 healthy cat preantral follicles with a viable germ cell can be rescued from a cat ovary stored < 12 h at 4°C after excision. If similar extrapolations can be made to other felids, then serious consideration should be given to the development of oocyte banks as another tool for helping retain genetic diversity in rare species.

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