Development to blastocysts of domestic cat oocytes matured and fertilized *in vitro* after prolonged cold storage

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Four experiments determined the kinetics of *in vitro* maturation and fertilization of cat oocytes and the effects of prolonged cold storage of ovaries before oocyte recovery on *in vitro* maturation/*in vitro* fertilization (IVM/IVF) success. Domestic cat ovaries were collected at ovariohysterectomy and stored at 4°C in PBS until oocyte recovery and culture in Eagle's minimal essential medium (EMEM) containing FSH, LH, oestradiol and BSA for maturation. In Expt 1, meiotic maturation was assessed at 0, 12, 24, 38 and 48 h of culture. After 24 h, > 61% of oocytes were in telophase I or metaphase II. In Expt 2, oocytes were recovered from ovaries stored for 24, 48 or 72 h and cultured in EMEM for 24 h. There was no difference among groups ($P > 0.05$) in the ability to achieve nuclear maturation (mean ± SEM, 57.1 ± 5.3%, 60.4 ± 5.4%, 55.4 ± 15.1% for 24, 48 and 72 h, respectively). Fertilization and embryo development after insemination at 16, 24, 32, 40 and 48 h of culture were examined in Expt 3. Of 98 oocytes inseminated at 32 h, 69% cleaved, 59% developed into morulae and 13% into blastocysts, more ($P < 0.05$) than those oocytes inseminated at earlier and later times. Development to blastocysts occurred after insemination at 16 (1.2%), 24 (9.1%) and 32 (13.3%) h of culture, but not after insemination at 40 or 48 h. Expt 4 involved cold storage of ovaries for 24, 48 or 72 h before oocyte recovery and insemination at 32 h of culture (the optimal time measured in Expt 3). Compared with storage for 24 h, fertilization success was lower ($P < 0.05$) in the 48 and 72 h groups, and, although 9.1% of inseminated oocytes from the 24 h storage group developed to blastocysts, none ($P < 0.05$) achieved this stage after 48 or 72 h of storage. These results indicate that domestic cat oocytes reach nuclear maturity by 24 h in culture and can be fertilized and develop to blastocyts optimally after insemination at 32 h. Oocytes recovered from ovaries stored at 4°C for up to 72 h are capable of reaching telophase I or metaphase II *in vitro*. However, only oocytes stored within the ovary for 24 h produce blastocysts, indicating that the ability to achieve nuclear maturation is an inadequate indicator of fertilization and developmental competence.

Introduction

In vitro maturation (IVM) and fertilization (IVF) of oocytes collected from excised ovarian tissue have reached a level of consistency, in certain species, to allow replacement of the costly and labour-intensive processes of *in vivo* embryo production and recovery (for review see Trounson *et al.*, 1994). The ability to grow and fertilize immature oocytes is useful for producing large numbers of embryos for developmental biology, cryopreservation and genetic studies, as well as for live animal production. The domestic cat is a valuable model for studying human genetic diseases (for review see Goodrowe *et al.*, 1989) and for developing assisted reproduction in taxonomically related endangered felids (Wildt, 1991). The success of IVM/IVF in cats, however, has been partially confounded by the unique reproductive physiology of the species.

The cat is a reflex ovulator, usually requiring multiple copulations to induce oocyte maturation and ovulation (Concannon *et al.*, 1980; Wildt *et al.*, 1980). Ovulation has been reported to occur from as early as 24 h (Shille *et al.*, 1983) to more than 46 h (Paape *et al.*, 1975; Wildt *et al.*, 1981) after the initial mating stimulus. The number and frequency of copulations influence the timing, magnitude and duration of the pituitary LH surge (Concannon *et al.*, 1980; Wildt *et al.*, 1981; Shille *et al.*, 1983) and probably the onset of nuclear maturation and ovulation of the oocyte. Consequently, it is difficult to measure the timing of oocyte maturation *in vivo*. Significant meiotic maturation *in vitro* has been reported to begin as early as 24 h after the onset of oocyte culture (Johnston *et al.*, 1989; Luvoni and Oliva, 1993; Pope *et al.*, 1994) and to peak as late as 48 h (Johnston *et al.*, 1989; Goodrowe *et al.*, 1991). Successful fertilization and *in vitro* development of IVM cat oocytes have been reported when oocytes are inseminated from 24 h to 56 h after initiation of culture (Johnston *et al.*, 1989; Pope *et al.*,

1994). However, no definitive study has been conducted that evaluates the timing of insemination of IVM cat oocytes in the context of embryo development. Therefore, one of our objectives was to combine elucidation of the timing of cat oocyte meiotic maturation in our culture system with a comprehensive analysis of the impact of insemination timing on fertilization and developmental competence, including blastocyst production.

Felid oocytes also demonstrate a unique ability to mature *in vitro* after prolonged cold storage (Johnston *et al.*, 1989). This is in contrast to the finding in cattle (Yang *et al.*, 1990) that oocytes recovered from ovaries stored at 4°C for 2–24 h before recovery are developmentally incompetent after insemination. The ability to store feline ovaries before IVM/IVF has profound implications for applying this technology to conservation (Johnston *et al.*, 1991a). For example, if intraovarian oocytes can remain sufficiently viable under prolonged cold conditions to allow successful IVM/IVF, then it should be possible to 'rescue' genes from valuable individuals post-mortem or after ovariectomy by shipping the ovarian tissues to a central facility for processing and embryo production. There has been no systematic and comprehensive analysis of the time oocytes may be successfully stored *ex situ* before *in vitro* maturation, fertilization and development. Therefore, our second objective was to examine the meiotic, fertilization and developmental competence of oocytes stored at 4°C for increasing time intervals.

Materials and Methods

Oocyte recovery and culture

Ovaries from healthy, adult domestic queens were recovered as surgical waste from ovariohysterectomies performed at local veterinary clinics. Most of the donors (about 85%) were in the luteal phase of the reproductive cycle or were inactive, based on the presence of corpora lutea (60%) or no visible follicles (about 25%) on the ovaries. No attempt was made to separate ovarian tissue on the basis of the reproductive status of the donor. Each ovary was placed intraoperatively into 15 ml PBS containing 100 iu penicillin ml^{-1} and 0.1 mg streptomycin ml^{-1} (Sigma Chemical Co., St Louis, MO) at 24°C and stored at approximately 4°C in a refrigerator or on ice packs in a styrofoam container until oocyte recovery. At that time, ovaries were minced in minimal essential medium (Gibco Laboratories, Grand Island, NY) containing Hank's salts, 25 mmol Hepes l^{-1} (Sigma), 2.0 mmol glutamine l^{-1} (Sigma), 0.4% (w/v) BSA (Sigma), 100 iu penicillin ml^{-1} and 0.1 mg streptomycin ml^{-1} (HMEM). Liberated oocytes exhibiting uniform, darkly pigmented ooplasm and an intact cumulus cell investment were chosen for culture (Johnston *et al.*, 1989) and washed in HMEM, and then in culture medium, consisting of minimal essential medium containing Earle's salts and bicarbonate buffer (Gibco) supplemented with 0.4% (w/v) BSA, 2.0 mmol glutamine l^{-1}, 1.0 mmol pyruvate l^{-1} (Sigma), 1 µg FSH ml^{-1} (NIADDK-oFSH-17; National Hormone and Pituitary Program, Rockville, MD), 1 µg LH ml^{-1} (NIADDK-oLH-25; National Hormone and Pituitary Program) and 1 µg oestradiol ml^{-1} (Sigma) (EMEM). Oocytes were cultured in groups of 10–20 per 50 µl drop of EMEM under light mineral oil (Sigma) at 38°C in a humidified environment of 5% CO$_2$ in air.

Semen collection and processing

Two normospermic (Wildt, 1991) domestic cats served as sperm donors, and each was used for every treatment group to avoid a male-specific effect (Fukui *et al.*, 1988; Goodrowe *et al.*, 1988). Semen was collected by electroejaculation (Wildt *et al.*, 1983) and subjected to swim-up processing (Goodrowe *et al.*, 1988; Johnston *et al.*, 1989). Briefly, freshly collected semen was diluted 1:1 in Ham's F10 medium (HF10; Irvine Scientific, Irvine, CA) supplemented with 1.0 mmol pyruvate l^{-1}, 2.0 mmol glutamine l^{-1} and 5% (v/v) fetal calf serum (Sigma) and centrifuged at 300 *g* for 8 min. Supernatant was discarded, the remaining pellet overlaid with 100 µl HF10 and the sample maintained at room temperature undisturbed for 1 h. Next, 50 µl was removed from the top layer and evaluated for sperm motility, forward progression and concentration as described by Wildt *et al.* (1983). Samples with 80% or more progressively motile cells and a 3.5 or greater forward progression rating were used to inseminate oocytes.

In vitro *fertilization*

Cultured oocytes were washed twice in HF10 and maintained in 90 µl fertilization drops of HF10 under oil in 5% CO$_2$ in air at 38°C. Processed sperm samples were diluted in HF10 to 2 × 10^6 cells ml^{-1}, and 10 µl was added to the 90 µl drops (total number of spermatozoa, 2 × 10^4 cells per drop). After coincubation for 18 h, oocytes were washed to remove cumulus cells and loosely attached spermatozoa and returned to fresh 50 µl drops of HF10 under oil. At 30 h after insemination, oocytes were visually assessed for cleavage to the two-cell stage as an index of fertilization (Goodrowe *et al.*, 1988; Johnston *et al.*, 1991b, c). Subsequent embryonic development was assessed at intervals of 24 h. For each group of oocytes collected on a given day, 5–10% were removed before insemination and cultured separately (parthenogenetic controls).

Assessment of nuclear maturation

After culture in EMEM, oocytes were incubated for 15 min in HMEM containing 0.2% (w/v) hyaluronidase (Sigma) at 38°C, then physically denuded of cumulus cells. Denuded oocytes were fixed in buffer containing 25 mmol Hepes l^{-1}, 60 mmol Pipes l^{-1} buffer (Sigma), 10 mmol EGTA l^{-1} (Sigma), 1 mmol MgCl$_2$ l^{-1}, 2% (v/v) formaldehyde and 0.04% (v/v) Triton X-100 (Sigma) for 30 min at room temperature, and then stained with 5 µg Hoechst 33342 ml^{-1} (Sigma) DNA-specific stain for 30 min (Goodrowe *et al.*, 1988; Johnston *et al.*, 1989). Fixed oocytes were compressed on glass slides and observed using epifluorescence to assess meiotic status. Oocytes exhibiting germinal vesicles, germinal vesicle breakdown or metaphase I plates were categorized as immature. Oocytes in telophase I or metaphase II were termed mature, indicating that they had progressed beyond metaphase I and were in the process of completing meiotic maturation. Those

with diffusely staining cytoplasm characteristic of nonviable cells were considered degenerate, and those in which chromatin was unidentifiable or not visible were designated as 'unknown'.

Experimental design

Four experiments were conducted to address the specific objectives stated above. Expt 1 focused on defining the kinetics of oocyte maturation in the IVM system described. Pairs of ovaries from 43 cats were processed within 12 h of ovario-hysterectomy, and recovered oocytes were denuded, fixed and stained immediately ($n = 65$), or cultured in EMEM for maturation *in vitro* for 12 h ($n = 61$), 24 h ($n = 104$), 38 h ($n = 101$) or 48 h ($n = 108$) before fixation. All oocytes were assessed for meiotic status as described.

Expt 2 examined the ability of cat oocytes to mature *in vitro* after various intervals of ovarian tissue storage at 4°C. Collected ovaries ($n = 23$ pairs) were stored as described for 24 h, 48 h or 72 h before 70, 67 and 67 oocytes were recovered, respectively, and processed. On the basis of results of Expt 1, meiotic status was assessed after 24 h of maturation culture.

Expt 3 was conducted to evaluate the influence of timing of insemination on the fertilization and subsequent development of IVM oocytes *in vitro*. Forty pairs of cat ovaries were collected and stored at 4°C for < 12 h. Recovered oocytes were cultured in maturation medium for 16 h ($n = 85$), 24 h ($n = 99$), 32 h ($n = 98$), 40 h ($n = 70$) or 48 h ($n = 73$) before insemination, and then evaluated for fertilization and developmental competence.

Expt 4 evaluated the effects of the duration of cold storage on fertilization and development of IVM oocytes. Twenty-nine pairs of ovaries were stored for 24 h, 48 h or 72 h before oocyte recovery and *in vitro* culture. On the basis of results from Expt 3, oocytes were inseminated at 32 h of culture and assessed daily for fertilization and development.

Statistical analyses

In each experiment, the incidence of oocyte maturation, fertilization and embryo development was expressed as a percentage of oocytes subjected to treatment. In Expts 1 and 2, arcsin transformations of maturation frequencies were subjected to analysis of variance, with multiple comparisons performed using the Scheffé test (Zar, 1984). Results are presented as mean ± SEM. In Expts 3 and 4, fertilization and embryo development were compared between groups by contingency table analysis with Yates' continuity correction (Zar, 1984).

Results

Experiment 1

Immediately after collection, 78.4 ± 4.6% of oocytes not subjected to culture contained a germinal vesicle or were in the process of germinal vesicle breakdown (Fig. 1). After culture for 12 h, 34.7 ± 8.3% were in metaphase I, whereas 57.7 ± 8.2% remained in the germinal vesicle stage. Fewer than 3.8 ± 2.7% of all oocytes had achieved telophase I or metaphase II by this
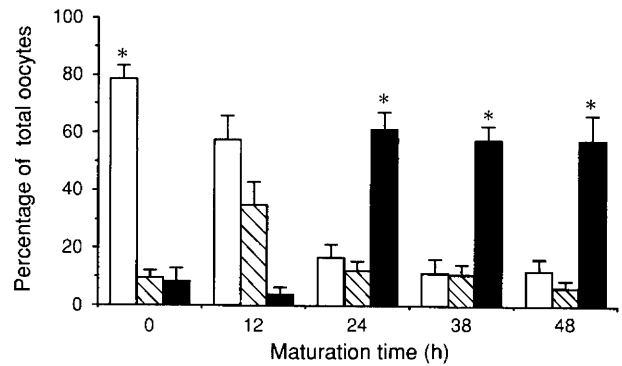
Fig. 1. Nuclear maturation of domestic cat oocytes *in vitro* as a function of time. (□), germinal vesicle or germinal vesicle breakdown; (▨), metaphase I; (■), telophase I or metaphase II. *Column values are greater ($P < 0.05$) than all unmarked counterpart columns within treatment groups.

time. After 24 h of culture, most (61.3 ± 6.0%) of the oocytes were in telophase I or metaphase II of meiosis, and the incidence of oocyte maturation did not increase or change further ($P > 0.05$) over time. The proportion of degenerate oocytes was 1.6 ± 1.6% at 0 h, and no oocytes were degenerate at 12 h of culture. Although not significant ($P > 0.05$), the proportion of degenerate oocytes tended to increase over time (5.7 ± 2.0% at 24 h; 9.4 ± 3.0% at 38 h; 12.5 ± 3.7% at 48 h). Likewise, the proportion of oocytes with unidentifiable chromatin was 1.6 ± 1.6% at 0 h, 3.7 ± 3.7% at 12 h, 3.7 ± 2.1% at 24 h, 10.4 ± 7.2% at 38 h and 11.2 ± 3.2% at 48 h ($P > 0.05$).

Experiment 2

Maturation competence of oocytes stored at 4°C within ovaries for 24 h, 48 h or 72 h before recovery and processing did not differ ($P > 0.10$) among groups (Fig. 2). The proportion of oocytes achieving telophase I/metaphase II within 24 h of culture was 57.1 ± 5.3%, 60.4 ± 5.4% and 55.4 ± 15.1% after 24 h, 48 h and 72 h storage, respectively. There was a non-significant ($P > 0.05$) increase in the proportion of degenerate oocytes with storage time (from 6.7 ± 3.8% after 24 h to 14.1 ± 8.4% after 72 h of storage). Fewer than 5.0% of oocytes in any group contained unidentifiable chromatin.

Experiment 3

As judged by embryo cleavage, fertilization of oocytes matured *in vitro* occurred at all insemination times (Table 1). However, cleavage and development to morulae were highest ($P < 0.01$) in the group inseminated at 32 h. Blastocyst development occurred in groups inseminated at 16 h, 24 h and 32 h, but not 40 h or 48 h, and the incidence was not different ($P > 0.05$) between the 24 h and 32 h treatments (9.1% and 13.3%, respectively). Typical blastocysts resulting from insemination of IVM oocytes at 32 h are presented (Fig. 3a). Parthenogenetic cleavage occurred in 2 of 54 (3.7%) oocytes that were not inseminated.
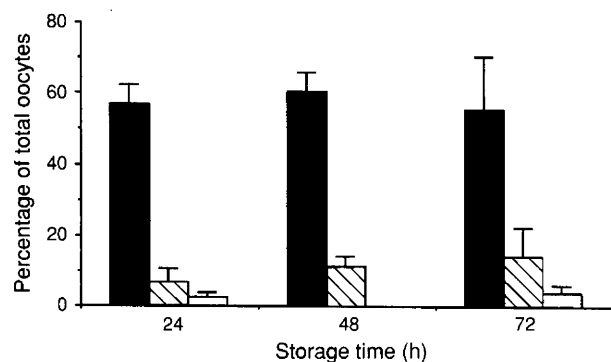
Fig. 2. Nuclear maturation *in vitro* of domestic cat oocytes collected from ovaries stored at 4°C. Maturation culture period was 24 h. (■), mature: telophase I or metaphase II; (▨), degenerate: nonviable egg; (□), unknown: unidentifiable chromatin. No differences (*P* > 0.05) were noted among storage groups.

Table 1. Cleavage and development of *in vitro*-matured domestic cat oocytes after insemination at different intervals of maturation culture

Insemination time (h)	*n*	Cleaved (%)	Morulae (%)	Blastocysts (%)
16	85	37 (43.5)[a]	32 (37.6)[a]	1 (1.2)[a]
24	99	48 (48.5)[a]	36 (36.4)[a]	9 (9.1)[b]
32	98	68 (69.4)[b]	58 (59.2)[b]	13 (13.3)[b]
40	70	27 (38.6)[a]	18 (25.7)[ac]	0[a]
48	73	31 (42.5)[a]	13 (17.8)[c]	0[a]

[abc]Different superscripts denote column differences (*P* < 0.05) by contingency table analysis.

The kinetics of embryonic development *in vitro* in this experiment were similar to those reported for domestic cat oocytes matured *in vivo* and fertilized *in vivo* or *in vitro* (Roth *et al.*, 1994; Swanson *et al.*, 1994). By 30 h after insemination, most embryos had cleaved to the two- or four-cell stage and appeared to undergo approximately one cell division every 24 h, reaching the morula stage (> 16 cells) by approximately 100 h after insemination. Compaction occurred on days 5 and 6 after insemination (day 0), and blastocyst formation was observed from late day 6 to day 8. Blastocyst hatching (Fig. 3b), which occurred by rupture of a small area of the zona pellucida (described by Roth *et al.*, 1994), began on days 8 and 9 and was initiated in 26.1% (6 of 23) of blastocysts, but was completed in only 8.7% (2 of 23).

Experiment 4

After cold storage for 24 h, more than half of the inseminated oocytes cleaved, and 45.5% and 9.1% developed to morulae and blastocysts, respectively (Table 2). The frequency of development to blastocysts and the developmental kinetics were comparable to those of unstored oocytes in Expt 3. There
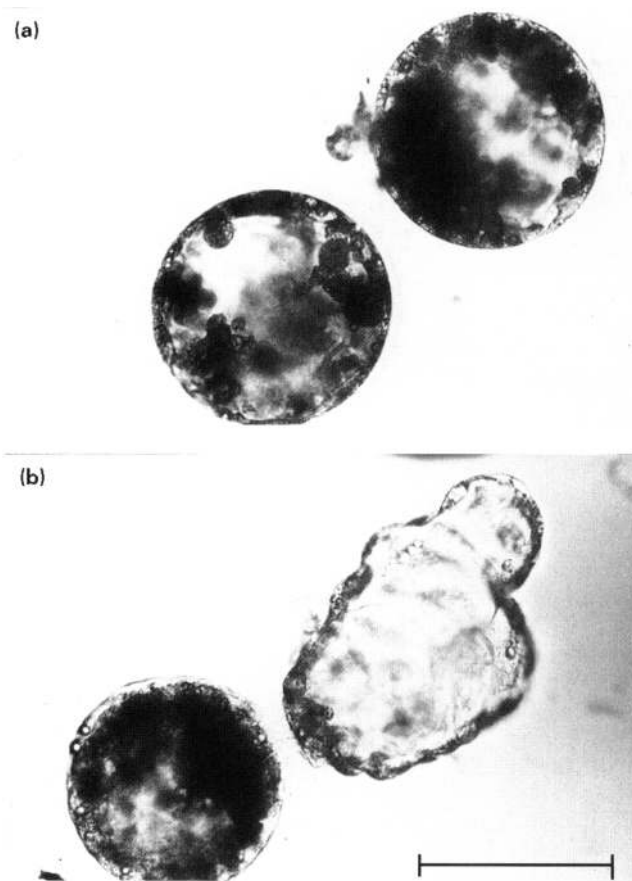
Fig. 3. Typical blastocysts produced from *in vitro* fertilization of domestic cat oocytes matured *in vitro*. (a) day 7 after insemination, expanded blastocysts; (b) day 8 after insemination, hatching blastocyst. Scale bar represents 100 µm.

Table 2. Cleavage and development of domestic cat oocytes collected from ovaries stored at 4°C and inseminated after maturation for 24 h *in vitro*

Storage time	*n*	Cleaved (%)	Morulae (%)	Blastocysts (%)
24 h	99	51 (51.5)[a]	45 (45.5)[a]	9 (9.1)[a]
48 h	87	22 (25.3)[b]	14 (16.1)[b]	0[b]
72 h	48	2 (4.2)[c]	0[c]	0[c]

[abc]Different superscripts denote column differences (*P* < 0.05) by contingency table analysis.

was a precipitous decline (*P* < 0.05) in the ability to cleave when oocytes were stored within ovaries for 48 h, and normal cleavage did not occur after 72 h of storage. Likewise, there was a decrease (*P* < 0.05) in the ability to develop into morulae after 48 h of storage, and no blastocysts were produced from any oocytes maintained at 4°C for 48 h or longer. No parthenogenetic cleavage was observed (0 of 22; 0%) in controls.

Discussion

These studies indicated that, under the laboratory conditions described, maturation of domestic cat oocytes *in vitro* effectively was completed by 24 h of culture, and fertilization and development of these oocytes were highest when insemination was performed 8 h later, at 32 h after recovery. Fertilization and development to blastocysts of oocytes stored at 4°C within ovaries for 24 h before *in vitro* processing were observed for the first time in any mammal. Furthermore, although oocyte maturation was possible after cold storage for up to 3 days, successful nuclear maturation was found to be uncoupled from fertilization and developmental competence. Therefore, after 24 h of storage at 4°C, cat oocytes began to lose the ability to become fertilized and cleave *in vitro*.

Earlier studies of oocyte maturation in domestic cats have reported significant proportions of meiotically mature oocytes at 24 h, with increasing (Goodrowe *et al.*, 1991) or equivalent (Luvoni and Oliva, 1993) proportions reaching maturity at 48 h. In our initial study of cat oocyte maturation *in vitro* (Johnston *et al.*, 1989), we determined that most oocytes are not mature until 30 h of culture, with the proportion of mature oocytes increasing through 48 h. The observation that oocyte maturation *in vitro* can occur over a broad interval of time is not unique to cats (King *et al.*, 1986; Süss *et al.*, 1988) and is likely to be related to variation among laboratories in the culture system used and to changes in laboratory procedures over time.

One factor in the variation in results is likely to be the inclusion of fetal calf serum (FCS) (Johnston *et al.*, 1989, 1993; Goodrowe *et al.*, 1991; Luvoni and Oliva, 1993). We have documented the inhibitory effects of FCS on domestic cat oocyte maturation, which appear attributable to dialysable factors of low molecular mass (Wood *et al.*, 1995). For this reason, the current study used BSA as the sole protein source for promoting maturation. Furthermore, the buffer system of the current working medium was altered to provide a more stable pH during manipulations. Preliminary findings indicated that the bicarbonate-buffered medium reached a pH value of 8.5–9.0 during oocyte recovery; therefore, Hepes buffer and Hank's salts were added to the oocyte recovery medium. Another source of variation in cat IVM is the method of assessing meiotic status. Certain preparatory techniques requiring disruption of the oolemma appear to yield a high proportion of oocytes (37–73%) with unidentifiable chromatin (Johnston *et al.*, 1989; Goodrowe *et al.*, 1991). As the proportion of oocytes reported as degenerate or unidentifiable commonly increases with time in culture, this may erroneously raise the percentage of oocytes categorized as mature in later culture groups. This is particularly important when the degenerate or unidentifiable oocytes are excluded from analysis. For example, if those oocytes in which the chromatin is not visible are arrested in prophase I (when the chromatin is decondensed and more difficult to see), then excluding this group from the data set falsely increases the incidence of maturation in the remaining population. In the current system, maintaining the chromatin within the oolemma during assessment of meiotic status decreased chromosomal loss and improved the likelihood of identifying the chromatin with a given oocyte.

The optimal time for inseminating IVM cat oocytes to obtain embryos competent to develop into blastocysts *in vitro* was 32 h after first exposure to IVM conditions. The observation that fertilization and development to early pre-implantation stages, but not to blastocysts, occurred after insemination at 40 h and 48 h of culture, was consistent with our observation (Johnston *et al.*, 1989) that IVM cat oocytes inseminated at 48–55 h of culture can fertilize and cleave, but fail to develop into blastocysts. This suggests that any cat oocyte matured *in vitro* for 40 h or more is aged beyond developmental competence. The time of insemination has been demonstrated to affect fertilization and subsequent development of cattle IVM oocytes to blastocysts, and aged oocytes are more likely to be polyspermic (Chian *et al.*, 1992; Long *et al.*, 1994). The effect of oocyte ageing on the incidence of polyspermy in cats has not been studied and requires attention, since this species appears to have a different mechanism for inducing the block to polyspermy (Byers *et al.*, 1992).

Development to the blastocyst stage was observed in 18.8% and 19.1% of cleaved embryos in the groups inseminated at 24 h and 32 h, respectively, in this study. These values were similar to (Johnston *et al.*, 1991c; Pope *et al.*, 1993) or higher than (Roth *et al.*, 1993, 1994) the percentage of blastocyst development in IVF embryos produced from oocytes matured *in vivo* (after gonadotrophin therapy) and collected by laparoscopic aspiration. The developmental block *in vitro* at the morula stage, commonly seen in IVF cat embryos (Roth *et al.*, 1994; Swanson *et al.*, 1994), has been investigated in detail with regard to specific culture conditions *in vitro*. Temperature, gas phase, culture medium, protein supplement and oviductal cell co-culture have been eliminated as causative or ameliorative factors (Johnston *et al.*, 1991b, c; Pope *et al.*, 1993; Roth *et al.*, 1993). We suspect that the block is a result of deficiencies in the culture conditions of the oocyte or early embryo, because embryos fertilized *in vivo* and flushed from the uterus of the donor can develop into blastocysts *in vitro* (Roth *et al.*, 1994). In this context, and in light of the common finding that IVF embryos block *in vitro* at the morula stage, the successful formation of blastocysts from oocytes matured *in vitro* was surprising. However, the marked effect of oocyte ageing on developmental competence suggests that the timing and conditions of maturation and insemination are the keys to developmental competence. We observed in Expt 1 that, although most IVM oocytes achieved nuclear maturity by 24 h of culture, insemination 8 h later (at 32 h) yielded a higher proportion of developmentally competent oocyes than did earlier insemination. Similarly, Dominko and First (1992) found that when bovine oocytes are matured *in vitro* and inseminated at 24 h, those oocytes demonstrating nuclear maturity (polar bodies) at 16 h were more likely to become blastocysts than were oocytes that matured later. In contrast, Byers *et al.* (1994) found that only 38.2% of cat oocytes aspirated transabdominally from gonadotrophin-stimulated females were meiotically mature at aspiration. These oocytes appear to progress through meiosis during co-culture with spermatozoa. Therefore, if developmental competence is optimal 8 h after nuclear maturation, oocytes matured *in vivo* are not optimally capable of being fertilized until 10–12 h after insemination. Furthermore, oocytes matured *in vitro* are exposed to gonadotrophins up to

the time of insemination, whereas oocytes matured *in vivo* are removed from the ovarian environment and presumably complete maturation in the absence of FSH and LH. This may help explain the discrepancy in developmental competence between oocytes matured *in vivo* and *in vitro* subjected to IVF and culture.

In this study, cat oocytes stored for up to 72 h at 4°C could undergo nuclear maturation *in vitro*. However, the ability of these oocytes to be fertilized and develop further declined markedly after 24 h of cold storage. This indicated that nuclear and cytoplasmic maturation were uncoupled in this system, but that both were necessary to achieve complete developmental competence. Although nuclear maturation (indicated by progression of the chromatin through metaphase II) can occur spontaneously in oocytes removed from the follicular environment (Pincus and Enzmann, 1935), coincident cytoplasmic maturational events are required to initiate sperm head decondensation, DNA synthesis and pronuclear formation (Longo, 1981; Yoshimura and Wallach, 1987). Proposed regulators of cytoplasmic maturation include the follicular steroids oestrogen and progesterone (Wramsby *et al.*, 1981; Osborn and Moor, 1983; Laufer *et al.*, 1984) and serum factors such as meiosis-inducing substance (Westergaard *et al.*, 1985) and oocyte maturation inhibitor (Channing *et al.*, 1983), presumably produced by follicular somatic cells. The loss of cytoplasmic maturational competence may, therefore, be due to protein degradation or loss of certain functions in the follicular cells during storage.

On the basis of data currently available, storage of oocytes followed by successful recovery, maturation, fertilization and embryo development in cats appears unique, both in terms of time interval and manner of storage. Bovine IVM/IVF programmes typically limit storage of ovaries to transport times of 8 h or less at 25–35°C (Eckert and Niemann, 1995) before oocyte recovery. Yang *et al.* (1990) found that storage of bovine oocytes at 25°C for up to 11 h resulted in fertilization and development to blastocysts, but longer storage, or storage for any time at 4°C, yielded few fertilizable oocytes. Rather than presume that the cat oocyte is uniquely tolerant to cold, we suspect that further study will reveal similar storage capabilities in the oocytes of other species. Species variation is expected, however, on the basis of previous observations of differential meiotic responses to cold exposure by mouse and human oocytes (Pickering and Johnson, 1987; Pickering *et al.*, 1990).

The results of this study are encouraging because we confirm that about 60% of cat ovarian oocytes can be induced to mature *in vitro*, and, of these, about 70% can be fertilized after insemination. The developmental kinetics of these embryos are not different from those measured in culture after IVF or *in vivo* fertilization of oocytes matured *in vivo*. Particularly exciting is the ability to produce blastocysts, especially from oocytes stored at 4°C within the ovaries for 24 h. We have used our earlier IVM/IVF approaches (developed in the domestic cat) in comparative studies of 13 species of wild felids (Johnston *et al.*, 1991a). With these new domestic cat IVM/IVF data, including the storage of oocytes before processing, we can begin applying this improved knowledge to further studies of the wild relatives of cats and to more systematic efforts to rescue genetic diversity.

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