# *In vitro* maturation and degeneration of domestic cat oocytes collected from ovaries stored at various temperatures

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**ABSTRACT**: Cat oocytes have the unique ability to mature *in vitro* after temporary storage at 4°C which can provide opportunities to rescue oocytes from the ovaries of endangered felids after sudden death or medical ovariohysterectomy. It has been demonstrated that factors such as season, culture conditions and morphological quality of oocytes influence the meiotic competence of domestic cat oocytes. In the present study we determined the meiotic maturation rate and incidence of apoptosis or necrosis in domestic cat oocytes collected from ovaries stored at different temperatures. Nuclear status and the presence of the first polar body were evaluated by fluorescence DAPI staining. Cell death was detected using Annexin-V, a phospholipid-binding protein that detects translocation of phosphatidyl-serine to the outer cytoplasmic membrane. Most oocytes (77.5%) collected from ovaries immediately after ovariohysterectomy (control group) resumed meiosis and reached metaphase II. A similarly high percentage of oocytes underwent nuclear maturation after recovery from ovaries stored for 6 h at 4°C (68.6%) or at room temperature (55.5%), but the rate of maturation after recovery from ovaries stored for 24 h at 4°C was greatly reduced (15.3%) Not surprisingly, the highest percentage of apoptotic oocytes were seen in Group 3, and the lowest frequency of apoptotic oocytes were observed in Group 1. Correspondingly, Group 1 had the highest percentage of necrotic oocytes. Thus, our results indicate that storage of domestic cat ovaries at room temperature, even for a short time, can negatively influence the competence of oocytes to undergo nuclear maturation *in vitro*.

**Keywords**: IVM; feline COC; apoptosis; necrosis

The domestic cat is an important experimental model for developing assisted reproductive technologies potentially useful for the conservation of non-domestic felidae. Development of reliable methods for IVM and IVF of oocytes collected from domestic cat ovaries can be subsequently used to preserve endangered species (Nagano et al., 2008). In domestic cats, *in vitro* maturation (IVM) studies of intraovarian oocytes have been conducted since 1989 (Johnston et al., 1989). It is well established that the use of strict selection criteria for freshly collected oocytes assists in ensuring higher rates of subsequent IVM and IVF success (Wolfe and Wildt, 1996; Wood and Wildt, 1997). Under optimal culture conditions and using grade I oocytes, 59–80% of immature oocytes achieve nuclear maturation (Wolfe and Wildt, 1996; Spindler and Wildt, 1999; Gomez et al., 2000; Bogliolo et al., 2001; Otoi et al., 2001). Incidence of vitro maturation of cat oocytes depends on such factors as the stage of the estrous cycle (Donoghue et al., 1993; Spindler and Wildt, 1999), quality of the cumulus-oocyte complex (Wood et al., 1997; Pope et al., 1997), duration of culture (Goodrowe et al., 1989; Luvoni and Oliva, 1993; Wolfe and Wildt, 1996) and hormonal supplementation (Goodrowe et al., 1991; Schramm and Bavister, 1995; Wood et al., 1995; Pope et al., 1997). Unlike some other species, cat oocytes have a unique ability to mature *in vitro* after temporary storage at 4°C (Wolfe and Wildt, 1996; Pope et al.,

2003). Temporal storage of ovaries can provide opportunities to rescue oocytes from ovaries of endangered felids after sudden death in the field or to rescue ovaries after medical ovariohysterectomy (Naoi et al., 2007). The aim of the present study was to examine the meiotic competence and incidence of apoptosis and necrosis of domestic cat oocytes collected from ovaries stored for 6 or 24 h at various temperatures.

## MATERIAL AND METHODS

### Oocyte recovery

Ovaries from domestic cats were obtained following ovariohysterectomy at the veterinary clinic near the University of Life Sciences in Poznan and kept in physiological saline. Cumulus oocyte complexes (COC) were collected immediately after ovariohysterectomy (control group) or storage of the ovaries at room temperature for 6 h (Group 1), at 4°C for 6 h (Group 2) or 24 h (Group 3).

To collect COC, ovaries were sliced with a scalpel blade in a 35 mm Petri dish containing Hepes buffered medium. COCs were evaluated according to criteria described by Wood and Wildt (1997) and only grade I and II oocytes were used.

### *In vitro* maturation

Maturation medium consisted of TCM-199 (Earle's salt; Gibco Laboratories) supplemented with 25mM HEPES, 4 mg/ml BSA (Sigma-Aldrich Chemical Co., St. Louis, MO, USA), 0.5 μg/ml of FSH (Sigma Chemical Co.), 1 μg/ml of estradiol-17β (Sigma Chemical Co.), 0.2mM sodium pyruvate, and 50 μg/ml of gentamicin sulfate. COC were cultured in 50 μl droplets of maturation medium (10–15 COCs/droplet) covered with paraffin oil at 39.5°C for 30 h in a humidified atmosphere of 5% $CO_2$ in the air.

### Evaluation of nuclear maturation

Nuclear status and the presence of the first polar body were evaluated by fluorescence DAPI staining. After maturation, oocytes were incubated with 300 μg/ml bovine testicular hyaluronidase (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) for 5 min in 38°C to remove cumulus cells. Then, oocytes were fixed with 4% paraformaldehyde in PBS at 4°C, washed three times in PBS/PVP (0.2%) and mounted on glass slides in a drop of Vectashield (Vector Laboratories, LTD UK) with 1.5 μg/ml DAPI (4',6-diaminoino-2-fenylindol) drop and observed using a confocal system (LSN 510) on a Carl ZEISS microscope (Axiovert 200M). DAPI produces a blue fluorescence with excitation at 360 nm and emission at 460 nm.

### Annexin V binding

Cells were stained with Annexin-V, a phospholipid-binding protein that detects translocation of phosphatidyl-serine to the outer cytoplasmic membrane, an event which takes place during the early stages of apoptosis. Annexin-V-FLUOS binds in a $Ca^{2+}$ dependent manner to negatively charged phospholipid surfaces and shows high specificity to phosphatidylserine; therefore, it stains apoptotic as well as necrotic cells. Simultaneously, cells were stained with propidium iodide (PI), a membrane impermeable stain, to distinguish between live cells and dead cells. PI can only enter the cell when the cytoplasmic membrane has lost its integrity. Briefly, samples were stained using the Annexin-V-FLUOS Staining Kit (Roche Diagnostics, Mannheim, Germany).

Oocytes were placed in 35 μl droplets containing Annexin-V buffer, Annexin-V/FITC and PI and incubated for 15 min at 37°C in the dark. After incubation, cells were mounted on microslides and observed using a fluorescence microscope (CKX41, Olympus) equipped with a FITC filter at 40×. After Annexin-V staining, oocytes were classified into three groups: (1) Annexin-V negative: no signal in the ooplasmic membrane; (2) Annexin-V positive (apoptotic): a clear green signal observed in the oocyte membrane; (3) Necrotic: propidium iodide (PI) positive – red nucleus indicative of membrane damage and green signal inside of oocyte.

### Experimental design

COC were collected from ovaries either immediately after ovariohysterectomy (control group), after 6 h storage at room temperature (Group 1) or 4°C (Group 2) or after 24 h storage at 4°C (Group 3).

In experiment 1, we evaluated the influence of ovarian storage on incidence of nuclear maturation *in vitro*. The rate of nuclear maturation was defined as the number of oocytes that were at the telophase I or metaphase II stage of meiosis relative to the total number of oocytes cultured *in vitro*. Oocytes that arrested at the germinal vesicle breakdown (GVBD) stage or that only progressed to the metaphase I stage were considered immature (Wolfe and Wildt, 1996; Spindler and Wildt, 1999).

In experiment 2, we evaluated the incidence of apoptosis in oocytes from the control and the three experimental groups according to classification described in the Material and Methods.

### Statistical analysis

Statistical analysis was performed with PROC FREQ in SAS 9.1 (SAS Institute Inc., Cary, NC, 1998) using the χ-square test of goodness of fit.

## RESULTS

### Experiment 1

The highest rate of oocyte nuclear maturation was seen in the control group (77.5%; $P < 0.01$). A similarly high percentage was seen after 6 h storage at room temperature; however, after storage at 4°C the incidence of meiotic maturation was slightly (Group 2) or greatly reduced (Group 1), depending on the duration of storage. The highest frequency of degeneration (39.8%; $P < 0.01$) occurred in oocytes recovered from ovaries stored at room temperature for 6 h (Group 1). Oocytes recovered from ovaries stored for 6 h at room temperature had the highest rate of blockage at the GV stage (28.6%) in comparison with the other groups ($P < 0.01$) (Table1).

### Experiment 2

The aim of the second experiment was to study if the degree of oocyte apoptosis was related to ovarian storage temperature. After Annexin-V staining, we detected a difference in the percentage of live oocytes between the control group and both Group 1 and Group 2 (77.1, 55.0 and 55.2%, respectively) (Table 2). The highest percentage of apoptotic oocytes was seen in Group 3, and the lowest percentage of apoptotic oocytes was in Group 1. Conversely, the highest percentage of necrotic oocytes was found in Group 1.

## DISCUSSION

In the present study we examined the ability of domestic cat oocytes to undergo *in vitro* meiotic maturation after recovery from ovaries stored for 6 h at room temperature or after 6 h or 24 h at 4°C. Also, we evaluated the effect of storage time and temperature on the incidence of apoptosis after *in vitro* maturation. The effect of ovary storage interval and temperature on the subsequent ability of oocytes to complete in vitro maturation and

Table 1. Effect of storage interval and temperature on domestic cat oocyte nuclear maturation *in vitro*

| Experimental groups[#] | Oocytes (*n*) | Meiotic stage of oocytes | | | | | | Degenerated | |
|---|---|---|---|---|---|---|---|---|---|
| | | GV | | GVBD | | MII | | | |
| | | *n* | % | *n* | % | *n* | % | *n* | % |
| Control | 129 | 4 | 3.1[a] | 12 | 9.3[a] | 100 | 77.5[a] | 13 | 10.1[a] |
| 1 | 98 | 28 | 28.6 | 16 | 16.3[a] | 15 | 15.3 | 39 | 39.8 |
| 2 | 118 | 4 | 3.4[a] | 12 | 10.2[a] | 81 | 68.6[ab*] | 21 | 17.8[ab] |
| 3 | 191 | 13 | 6.8[a] | 25 | 13.1[a] | 106 | 55.5[b*] | 47 | 24.6[b] |

[#]experimental groups are as follows: control group = oocytes recovered immediately after ovariohysterectomy; Group 1 = oocytes recovered after 6 h storage of ovaries at room temperature; Group 2 = oocytes recovered after 6 h storage of ovaries at 4°C; Group 3 = oocytes recovered after 24 h storage of ovaries at 4°C

Within a column, groups with the same letter are not significant different ($P < 0.05$)

*indicates that groups are not highly significantly different ($P < 0.01$)

Table 2. Incidence of apoptosis and necrosis of oocytes after recovery from ovaries stored at room temperature or 4°C for 6 or 24 h

| Experimental groups | Oocytes (*n*) | Annexin-V negative (viable) | | Annexin-V positive (apoptotic) | | PI positive (necrotic) | |
|---|---|---|---|---|---|---|---|
| | | *n* | % | *n* | % | *n* | % |
| Control | 140 | 108 | 77.1[a] | 18 | 12.9[a] | 14 | 10[a*] |
| 1 (6 h, RT) | 120 | 66 | 55[bc*] | 8 | 6.7[a] | 46 | 38.3 |
| 2 (6 h, 4°C) | 128 | 87 | 68[ac*d*] | 17 | 13.3[a] | 24 | 18.8[a*b] |
| 3 (24 h, 4°C) | 134 | 74 | 55.2[bd*] | 29 | 21.6[a] | 31 | 23.1[b] |

Within a column, groups with the same letter are not significant different ($P < 0.05$)
*indicates that groups are not highly significantly different ($P < 0.01$)

undergo further development varies among species. The present results provide further evidence that cat oocytes exhibit more tolerance to ovarian storage than do some other species.

Adequate temperatures for ovary storage have a subsequent influence on oocyte maturation. Bovine oocytes from ovaries held at 25°C had a higher developmental competence than oocytes from ovaries held at 37°C (Yang et al., 1990). Blondin et al. (1997) found that storage of cow ovaries at 30–37°C for 7–8 h reduced the rate of blastocyst formation after IVM/IVF/IVC. In contrast, others have reported that storage of cow ovaries at room temperature for > 12 h did not decrease their capacity for fertilization and blastocyst development (Yang et al., 1990; Schernthaner et al., 1997). The plasma membrane of bovine oocytes has been found to be very sensitive to cooling below 20°C (Arav et al., 1996). At room temperature, the membrane lipids go through phase transition from a liquid phase to a gel phase, which results in a hardening of the *zona pellucida* (Arav et al., 1996). In mammalian oocytes, the transition of the membrane lipids is connected with a disruption of microtubules in the meiotic spindle and causes a decrease in fertilization frequency of oocytes (Pickering et al., 1990; Parks and Ruffing, 1992). Cooling oocytes at various stages of meiosis to 20°C has been demonstrated to lead to induction of chromosomal abnormalities (Moor and Crosby, 1985). Storage of horse ovaries at 4°C, meanwhile, has had damaging effects on oocytes (Love et al., 2003). Guignot et al. (1999) observed that mare ovaries may be held for up to 6 to 8 h at 27 to 37°C without affecting the rate of oocyte harvest, cytoplasmic membrane integrity and nuclear maturation. Storage at a warmer temperature conditions increased the percentage of porcine oocytes with DNA fragmented nuclei by altering the follicular pH and resulted in a lower rate of oocyte in vitro maturation. Wongsrikeao et al. (2005) observed that storage of porcine ovaries at 4°C to 35°C for 6 h decreased the pH of follicular fluid and induced DNA frag-

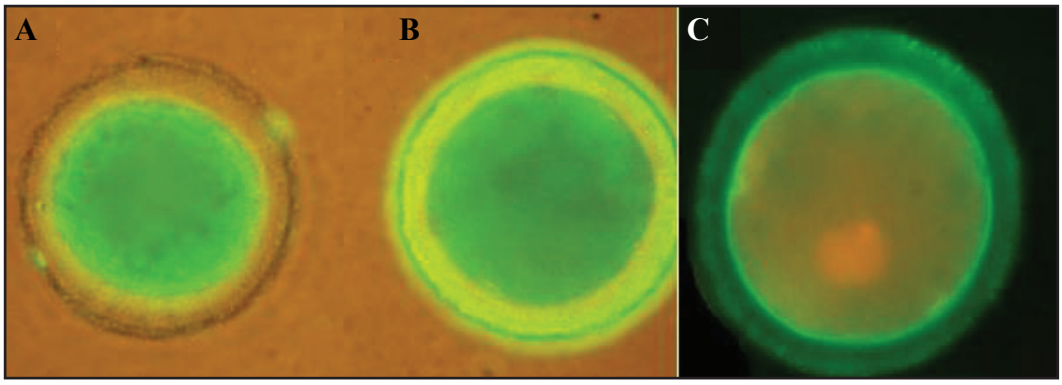


Figure 1. Oocyte classification by Annexin-V staining: (A) Oocyte Annexin-V negative: no signal in the ooplasmic membrane; (B) Annexin-V positive (apoptotic): a clear green signal is observed in the oocyte membrane; (C) Necrotic oocyte. Scale bar = 50 μm

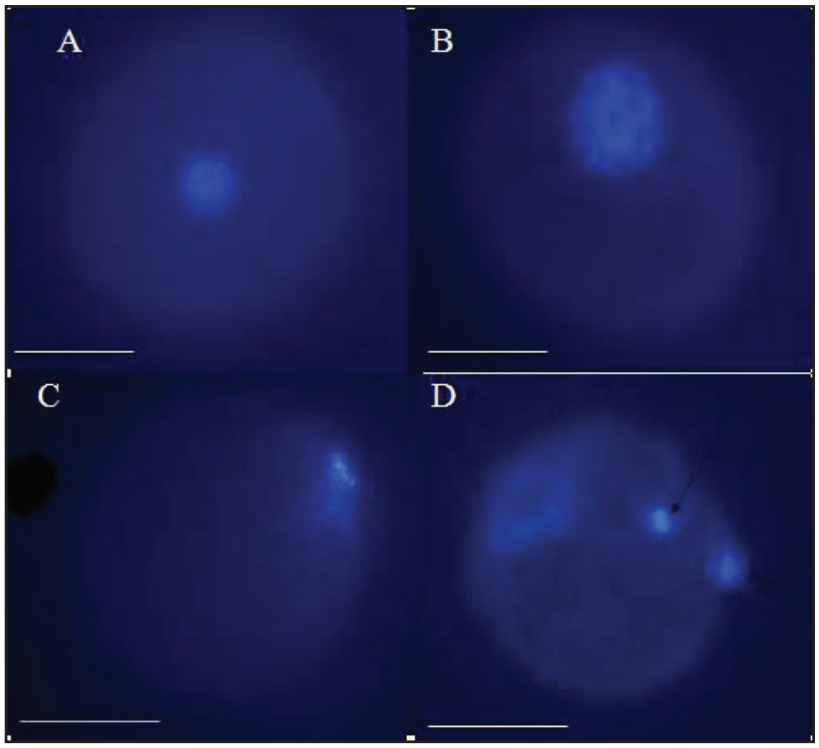


Figure 2. Feline oocytes stained with DAPI showing: (A) fluorescent germinal vesicle (GV); (B) fluorescent nuclear labeling with chromatin condensation and dissolution of the nuclear membrane, germinal vesicle break down (GVBD); (C) fluorescent nuclear labeling with chromosomes in first metaphase (MI); (D) fluorescent nuclear labeling metaphase II with extrusion of first polar body. Scale bar = 50 µm

mentation in oocytes stored at higher temperatures (≥ 15°C). Porcine oocytes have been described to be very sensitive to chilling, leading to a reduction in membrane integrity in oocytes stored < 15°C (Didion et al., 1990; Yuge et al., 2003). Lower temperatures adversely affected the viability of oocytes, and none of the oocytes collected from ovaries stored at 4°C reached the MII stage after IVM. Wongsrikeao et al. (2005) also reported that storage of porcine ovaries at 25°C to 35°C was more successful at maintaining the development rates of oocytes than was storage of ovaries for 3 h at ambient temperature.

Naoi et al. (2007) reported that feline oocytes recovered from ovaries stored at 4°C for 24 h had a significantly higher rate of meiotic competence than did oocytes stored at room temperature or at 38°C. It has been demonstrated that storage of cat ovaries in saline at 4°C protected oocytes from taphonomic changes, such as more vacuolization and increased loss of membrane integrity in granulosa cells and oocytes (Wood and Wildt, 1997). Following recovery from ovaries stored at 4°C for 12 h, feline oocytes show an increase in the rate of apoptosis in granulosa cells (Jewgenow et al., 1997). Possibly, metabolism and enzyme activity was less efficient at the cooler temperature than at body temperature, which may reduce accumulation of acid byproducts and apoptotic processes (Wongsrikeao et al., 2005).

We found that the rate of apoptotic positive cells increased during in vitro maturation. Using the TUNEL assay, Lobascio et al. (2007a) were able to identify most of the mouse fetal oocytes undergoing degeneration in culture. They reported that the frequency of TUNEL positive oocytes increased from early stages of MPI to the pachytene/diplotene stage. During four days of culture the percentage of apoptotic cells rose to 50% (Lobascio et al., 2007a). Annexin-V-positive oocytes showed a markedly lower ratio of Bcl-xL/Bax transcripts in comparison to AnnexinV-negative oocytes after three days of culture (Lobascio et al. 2007b).

In conclusion, long-time storage at 4°C could reduce the quality of ovaries and developmental competence of oocytes. Also, ovaries stored at room temperatures provided the highest number of degenerated oocytes. We suggest that the best way to protect feline oocytes from degeneration, apoptotic and necrotic changes is to store ovaries at 4°C for less than 6 h after ovariohysterectomy.

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