

Overcoming poor *in vitro* nuclear maturation and developmental competence of domestic cat oocytes during the non-breeding season

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The domestic cat experiences circannual variations in ovarian activity and intrafollicular oocyte quality. One result is poor nuclear and cytoplasmic maturation during *in vitro* maturation (IVM) conducted during the annual non-breeding season (July through November). In an attempt to overcome this seasonal phenomenon immature oocytes were collected from July through November and cultured in a conventional IVM medium (IVM1) or in IVM1 supplemented with different FSH concentrations and antioxidant (ascorbic acid or cysteine). Nuclear status of oocytes was assessed after IVM or IVF. Embryo stage and blastocyst quality were evaluated after 7 days of *in vitro* culture. Although the addition of antioxidant alone had no effect, the presence of 10 μg FSH ml^{-1} improved nuclear maturation ($75.4 \pm 4.1\%$ versus $48.7 \pm 8.8\%$ in IVM1; $P < 0.05$) and fertilization success ($47.9 \pm 3.2\%$ versus $35.0 \pm 5.1\%$ in IVM1; $P < 0.05$). Furthermore,

developmental competence of fertilized oocytes was enhanced ($P < 0.05$) only in the presence of ascorbic acid ($30.6 \pm 6.7\%$) or cysteine ($33.6 \pm 5.1\%$) compared with IVM1 ($8.1 \pm 8.8\%$). Consequently, blastocyst yield (17% of total oocytes cultured) was highest when oocytes were matured in medium containing higher FSH concentration and antioxidants. The results of this study demonstrate that meiotic and developmental competences are inherent to the immature cat oocyte collected during the non-breeding season. However, appropriate mechanisms (perhaps seasonal variation in FSH receptors or lack of antioxidant capacity of the cumulus–oocyte complex) are inadequate during this period of gonadal quiescence. Regardless, this compromised oocyte function during the non-breeding season can be overridden by altering *in vitro* culture conditions to include supplemental FSH and antioxidant.

Introduction

In domestic cats, *in vitro* maturation (IVM) studies of intraovarian oocytes have been conducted since 1989 (Johnston *et al.*, 1989). The success of various IVM conditions has been based on nuclear maturation and successful embryo development after IVF (Johnston *et al.*, 1989; Schramm and Bavister, 1995; Wolfe and Wildt, 1996; Spindler and Wildt, 1999; Karja *et al.*, 2002a), intracytoplasmic sperm injection (Gomez *et al.*, 2000; Bogliolo *et al.*, 2001) and nuclear transfer (Skrzyszowska *et al.*, 2002; Gomez *et al.*, 2003; Kitiyanant *et al.*, 2003), including the production of live offspring after embryo transfer (Pope *et al.*, 1997, 2003). In addition, 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, 1997a). It is clear and unsurprising that there are vast differences in the quality

of oocytes recovered from ovarian tissue (Wood and Wildt, 1997a), which no doubt explains some of the variation in success of IVM across laboratories. Under optimal culture conditions and using grade I oocytes, 59–80% of immature oocytes, on average, achieve nuclear maturation (Wolfe and Wildt, 1996; Spindler and Wildt, 1999; Gomez *et al.*, 2000; Bogliolo *et al.*, 2001; Otoi *et al.*, 2001). When inseminated with conspecific spermatozoa, 45–50% of cultured oocytes are fertilized and develop *in vitro* to the blastocyst stage (Wolfe and Wildt, 1996; Wood and Wildt, 1997a; Spindler and Wildt, 1999; Freistedt *et al.*, 2001; Karja *et al.*, 2002a).

Spindler and Wildt (1999) first reported the marked effect of season on IVM success in cat oocytes. When oocytes were recovered from cat ovaries during the traditional non-breeding season in North America (from July through November), the incidence of nuclear maturation was approximately 20% and no embryos developed to the blastocyst stage after fertilization. Other studies have confirmed this effect associated with quiescent ovarian

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activity and reduced follicular growth, including studies in other geographic regions and at times of the year that differ from the North American breeding season (Freistedt *et al.*, 2001). FSH is known to be an important supplement for IVM success when using oocytes collected during the breeding season (Johnston *et al.*, 1989; Schramm and Bavister, 1995; Wood *et al.*, 1995). For this reason, Spindler and Wildt (1999) speculated that either lower circulating FSH concentrations or fewer FSH receptors may be contributing to reduced IVM efficiency during the non-breeding season, possibly by adversely affecting oocyte quality. It remains unclear whether the poor nuclear maturation observed during the seasonal quiescent period of the cat is linked to a poor meiotic competence, to a failure to meet a threshold concentration of FSH to induce nuclear maturation, or to both factors.

As FSH receptors are located on cumulus cells, the induction of nuclear maturation must be mediated by these cells, which coincidentally also play a key role in cytoplasmic maturation (Shimada *et al.*, 2002; Tanghe *et al.*, 2002; Webb *et al.*, 2002). Cytoplasmic maturation is generally considered to be the ability of an oocyte to support monospermic fertilization, pronuclear formation and early embryo development (Mermillod *et al.*, 1999; Tanghe *et al.*, 2002). A major cytoplasmic factor regulating sperm decondensation and chromatin remodelling is the reduced form of glutathione (GSH) (de Matos *et al.*, 1997). As a result of an increase in oocyte GSH content, the cumulus cells maintain the redox state while simultaneously protecting the oocyte against oxidative stress (de Matos *et al.*, 1997; Sutovsky and Schatten, 1997). Furthermore, high GSH content in the oocyte is also required for supporting successful embryo development in cattle (de Matos *et al.*, 1997; de Matos and Furnus, 2000). It is also known that supplementing pig oocytes during IVM with cysteine, a precursor of GSH synthesis, enhances cytoplasmic maturation (Yoshida *et al.*, 1993; Abeydeera *et al.*, 1999). Likewise, in rats and mice another extracellular antioxidant, ascorbic acid, protects cumulus cells against apoptosis and improves maturation (Behrman *et al.*, 1996; Takami *et al.*, 1999; Eppig *et al.*, 2000). Acquisition of oocyte competence may be lost because of oxidative stress during IVM. However, the impact of antioxidants on IVM during the non-breeding season in carnivore species, including the domestic cat, is unknown.

The aim of the present study was to determine whether the poor nuclear and cytoplasmic maturation observed during the non-breeding season in the cat is related to decreased sensitivity to FSH and increased oxidative stress. This possibility was examined by characterizing the influence of supplemental FSH (at different concentrations) and antioxidants (ascorbic acid or cysteine) on *in vitro* nuclear and cytoplasmic maturation of intraovarian cat oocytes recovered during the non-breeding season.

Materials and Methods

Oocyte collection and IVM

Ovaries from the adult domestic cat (*Felis catus*) were collected during the non-breeding season (July to November) from local veterinary clinics and transported in PBS at 4°C to the laboratory within 6 h of ovariectomy. Immature oocytes were then recovered by slicing the ovaries with a scalpel blade in Hepes-buffered minimum essential medium (H-MEM; Gibco Laboratories, Grand Island, NY) supplemented with 1.0 mmol pyruvate l⁻¹, 2.0 mmol L-glutamine l⁻¹, 100.0 iu penicillin ml⁻¹, 100.0 mg streptomycin ml⁻¹ and 4.0 mg BSA ml⁻¹ (Sigma Chemical Co, St Louis, MO). Only grade I immature oocytes (with homogeneous dark cytoplasm, surrounded by several layers of compacted cumulus) (Wood and Wildt, 1997a) were used. These oocytes were selected and pooled before they were cultured in different IVM media for 32 h in 50 µl microdrops (10 oocytes per microdrop) under mineral oil (38.5°C in air with 5% CO₂).

IVM control medium (designated IVM1) was used in our laboratory (Wolfe and Wildt, 1996; Spindler and Wildt, 1999) and comprised MEM (Gibco) supplemented with 1.0 mmol L-glutamine l⁻¹, 1.0 mmol pyruvate l⁻¹, 100.0 iu penicillin ml⁻¹, 100.0 mg streptomycin ml⁻¹, 4.0 mg BSA ml⁻¹, 1.0 µg FSH ml⁻¹ (1.64 iu ml⁻¹; NIDDK-ovine FSH-18; National Hormone and Pituitary Program, Rockville, MD), 1.0 µg LH ml⁻¹ (1.06 iu ml⁻¹; NIDDK-oLH-25; National Hormone and Pituitary Program) and 1.0 µg oestradiol ml⁻¹ (Sigma).

In Expt 1, oocytes were cultured in IVM1 supplemented with different FSH concentrations: 1 (control), 5, 10 or 15 µg ml⁻¹. After 32 h of culture, oocytes were fixed in ethanol overnight at room temperature and stained with 10 µg Hoechst 33342 ml⁻¹ (Sigma) (Comizzoli *et al.*, 2001). The meiotic stage of the oocytes was assessed by epifluorescence microscopy (Olympus BX 41, Olympus Corporation, Melville, NY) (Johnston *et al.*, 1989; Wood *et al.*, 1995; Wolfe and Wildt, 1996; Spindler and Wildt, 1999).

The results of Expt 1 were used to design the composition of the IVM media used in Expts 2 and 3. For these studies, oocytes were cultured in IVM1 supplemented with either 10.0 µg FSH ml⁻¹ (FSH+), 0.5 mmol ascorbic acid l⁻¹ (AS; Sigma), 0.13 mmol cysteine l⁻¹ (CYS; Sigma), 10.0 µg FSH ml⁻¹ + 0.5 mmol ascorbic acid l⁻¹ (FSH+/AS) or 10.0 µg FSH ml⁻¹ + 0.13 mmol cysteine l⁻¹ (FSH+/CYS). The dosage of the two antioxidants was based on previous studies in pig (Abeydeera *et al.*, 1999) and mouse oocytes (Eppig *et al.*, 2000).

In vitro fertilization and development

IVF was performed in Expts 2 and 3 using a standard protocol originally developed in our laboratory for oocytes matured *in vivo* (Johnston *et al.*, 1991a) and later

for oocytes matured *in vitro* (Wood *et al.*, 1995). The most recent description of the protocol has been provided by Spindler and Wildt (1999). Briefly, frozen-thawed motile spermatozoa from a single sperm donor were selected by swim-up processing (Goodrowe *et al.*, 1988) in Ham's F-10 medium (Irvine Scientific, Santa Ana, CA) supplemented with 25 mmol Hepes l^{-1} , 1 mmol pyruvate l^{-1} , 2 mmol glutamine l^{-1} , 100 iu penicillin ml^{-1} , 100 mg streptomycin ml^{-1} and 5% (v/v) fetal calf serum (complete Ham's medium with Hepes). Oocytes were inseminated with 5×10^5 motile spermatozoa ml^{-1} in 50 μl microdrops of complete Ham's medium without Hepes under equilibrated mineral oil at 38.5°C in air with 5% CO₂. Some of the oocytes ($n=12$ per IVM condition) were incubated without spermatozoa to assess the incidence of parthenogenetic activation. At 16 h after insemination, cumulus cells were removed by vortexing at maximum setting (Vortex-Genie, Scientific Industries, Bohemia, NY) for 2 min. For Expt 2, presumptive zygotes were fixed and stained as described by Comizzoli *et al.* (2001) to assess pronuclear formation. Pronucleus morphology was recorded according to its size and localization within the cytoplasm (Hewitson *et al.*, 2000). For Expt 3, presumptive zygotes were cultured *in vitro* for 7 days in complete Ham's F-10 (38.5°C in air with 5% CO₂). *In vitro* development was observed on day 6 and again on day 7 (day 0 corresponding to the day after the *in vitro* insemination). Embryos were then fixed and stained as described by Comizzoli *et al.* (2001) to determine the developmental stage and number of blastomeres.

Experimental design and statistical analysis

Grade I immature oocytes from different ovaries were pooled before random and equal distribution across different IVM conditions. In Expt 1, incidence of nuclear maturation was defined as the number of oocytes at the telophase I or the metaphase II stage 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). Oocytes with fragmented cytoplasm or without chromatin were considered degenerated. In Expt 2, oocytes were considered fertilized when two pronuclei were present in the cytoplasm. The number of unfertilized oocytes in metaphase II was also assessed in this experiment to determine the incidence of nuclear maturation at the time of *in vitro* insemination. In Expt 3, the percentage of cleaved embryos and blastocyst yield were calculated relative to the total number of oocytes cultured *in vitro*. Developmental competence was defined as the number of blastocysts produced relative to the total number of cleaved embryos. All experiments were replicated three or four times on different days with different batches of oocytes. Average values were ex-

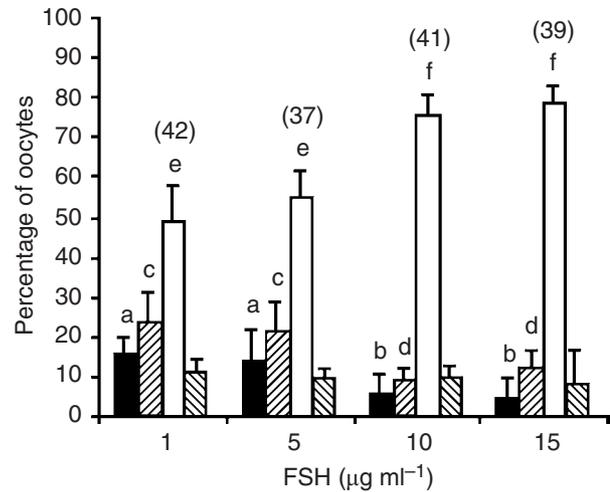


Fig. 1. Percentage of grade I cat oocytes at different nuclear status (germinal vesicle breakdown (■); metaphase I (▨); telophase I and metaphase II (□); degenerated (▩)) after *in vitro* maturation in conventional IVM medium (IVM1) plus different FSH concentrations (three replicates per condition; values are mean \pm SD; values above bars represent total number of oocytes per treatment). ^{a-f}Among FSH concentrations, bars with different superscripts are significantly different ($P < 0.05$).

pressed as mean \pm SD. Percentage data were transformed using arcsin transformation before analysis. Comparison between IVM condition and among replicates were analysed by ANOVA and Tukey's multiple comparison testing (SigmaStat, SPSS, Chicago, IL).

Results

Ovarian activity and oocyte recovery during the non-breeding season

Of the 414 ovaries collected from July through November for this study, none contained corpora lutea or corpora haemorrhagica. Furthermore, all antral follicles observed were < 1 mm in diameter. The mean number of grade I oocytes recovered per ovary was 2.7 ± 0.7 .

Effects of FSH on oocyte nuclear maturation

In this experiment designed to assess the impact of FSH concentration, an examination of oocytes before fixation revealed no apparent gross influence of gonadotrophin concentration on expansion of the surrounding cumulus mass. However, in the presence of 10 or 15 μg FSH ml^{-1} , the percentage of oocytes in telophase I or metaphase II was significantly higher ($P < 0.05$), and the percentage of oocytes undergoing GVBD and reaching metaphase I was significantly lower ($P < 0.05$) compared with oocytes in the presence of 1 or 5 μg FSH ml^{-1} (Fig. 1). The incidence of oocyte degeneration was not significantly different among treatments. As nuclear maturation was significantly enhanced in the presence of

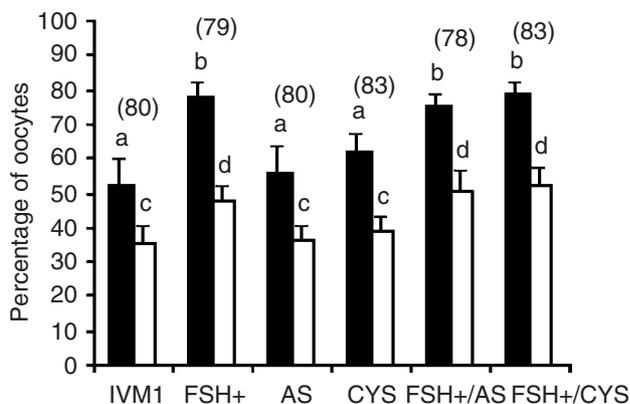


Fig. 2. Percentage of grade I cat oocytes reaching metaphase II (■) and undergoing fertilization (□) (16 h after insemination) after *in vitro* maturation (IVM) in different conditions (IVM1: conventional *in vitro* maturation medium; FSH+: IVM1 with FSH; AS: IVM1 with ascorbic acid; CYS: IVM1 with cysteine; FSH+/AS: IVM1 with FSH and ascorbic acid; FSH+/CYS: IVM1 with FSH and cysteine) (four replicates per condition; values are mean \pm SD; numbers above bars represent total number of oocytes per treatment). ^{a-d}Among IVM conditions, bars with different superscripts are significantly different ($P < 0.05$).

10 $\mu\text{g FSH ml}^{-1}$ and was not further increased by 15 $\mu\text{g FSH ml}^{-1}$, subsequent experiments were carried out in the presence of 10 $\mu\text{g FSH ml}^{-1}$.

Effect of higher FSH concentration in the presence or absence of antioxidant on oocyte nuclear maturation and fertilization

Expt 2 replicated the findings of Expt 1 in that nuclear maturation was positively influenced ($P < 0.05$) by the higher FSH concentration (FSH+ versus IVM1; Fig. 2). This improved maturation was also noted in the presence of 10 $\mu\text{g FSH ml}^{-1}$ combined with ascorbic acid (FSH+/AS) or cysteine (FSH+/CYS) (Fig. 2). As might be expected, the percentages of oocytes in GVBD and at metaphase I were significantly lower ($P < 0.05$) in all groups with the 10 $\mu\text{g FSH ml}^{-1}$ (data not shown). The addition of antioxidant to the IVM medium alone (AS or CYS) or in combination with the higher FSH concentration (FSH+/AS, FSH+/CYS) had no significant accentuating effect on nuclear maturation, and the incidence of degeneration (approximately 10%) was not significantly different among treatments. The percentage of fertilized oocytes was significantly higher ($P < 0.05$) after IVM in the presence of all the higher FSH concentration treatments (FSH+, FSH+/AS, FSH+/CYS) compared with the other IVM conditions (IVM1, AS, CYS).

The number of fertilized oocytes relative to the number of matured oocytes did not differ significantly ($P > 0.05$) among the five treatments (data not shown) and averaged approximately 66%. No parthenogenetic activation was detected and fertilized oocytes with more

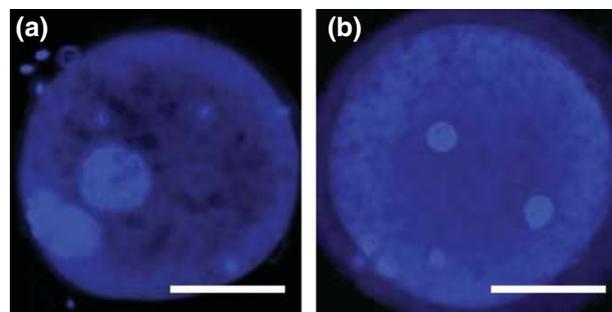


Fig. 3. Pronuclear morphology at 16 h after insemination after *in vitro* maturation of grade I cat oocytes in the (a) presence or (b) absence of ascorbic acid or cysteine. Scale bars represent 50 μm .

than two pronuclei were not observed. At 16 h after insemination, the proportion of zygotes with two fully developed pronuclei (Fig. 3a) was higher ($P < 0.05$) after IVM in the presence of antioxidant (AS, $66.7 \pm 5.4\%$; CYS, $66.0 \pm 5.2\%$; FSH+/AS, $63.5 \pm 3.9\%$; FSH+/CYS, $62.8 \pm 5.9\%$) compared with IVM1 only ($32.5 \pm 2.1\%$) or the higher concentration of FSH in the absence of antioxidant (FSH+, $32.5 \pm 3.8\%$). For the last two treatments, pronuclei were generally smaller and more distantly spaced at 16 h after insemination (Fig. 3b).

Effect of higher FSH concentration in the presence or absence of antioxidant on embryo cleavage, developmental competence and blastocyst yield

In Expt 3, the same proportion of fertilized oocytes progressed to the first cleavage stage regardless of the IVM treatment. Compared with the IVM1 control, the percentage of cleaved embryos was higher ($P < 0.05$) when IVM was performed in the presence of 10 $\mu\text{g FSH ml}^{-1}$ with or without the antioxidants (FSH+, FSH+/AS, FSH+/CYS; Fig. 4). There was no enhancement effect by the presence of either ascorbic acid or cysteine, and neither antioxidant alone promoted cleavage. The percentage of blastocysts relative to the number of cleaved embryos (developmental competence) was always significantly higher ($P < 0.05$) after IVM in the presence of the antioxidants and with or without the higher FSH concentration (AS, CYS, FSH+/AS, FSH+/CYS; Fig. 4). The presence of higher FSH concentration and antioxidant during oocyte IVM (FSH+/AS, FSH+/CYS) increased ($P < 0.05$) blastocyst yield (number of blastocysts relative to the total number of oocytes) compared with all other IVM treatments (that is, IVM1, FSH+, AS, CYS; Fig. 4).

The developmental stages of embryos across treatments are shown (Fig. 5). More embryos ($P < 0.05$) developed beyond the 2- to 8-cell stage after IVM in all supplemented media compared with IVM1. Embryo development was mainly arrested at the 8–16-cell stage ($P < 0.05$; Figs 5, 6b) when the original

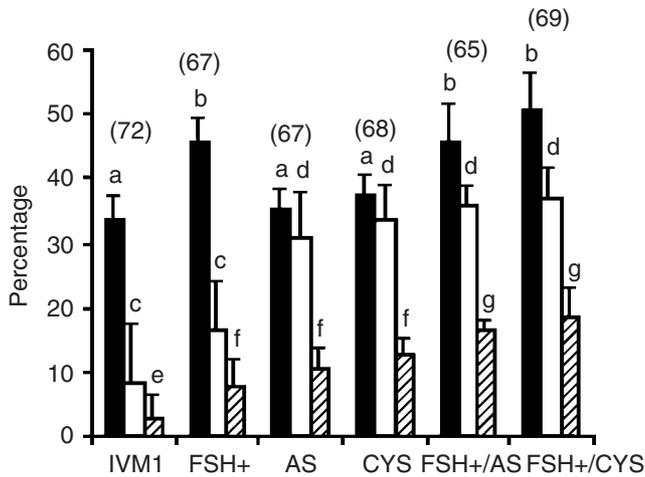


Fig. 4. Percentages of cleaved embryos (■), percentages of blastocysts relative to number of cleaved embryos (□, developmental competence) and percentages of blastocysts relative to total number of oocytes (▨, blastocyst yield) on day 7 after *in vitro* maturation (IVM) in different conditions (IVM1: conventional *in vitro* maturation medium; FSH+: IVM1 with FSH; AS: IVM1 with ascorbic acid; CYS: IVM1 with cysteine; FSH+/AS: IVM1 with FSH and ascorbic acid; FSH+/CYS: IVM1 with FSH and cysteine) (three replicates per condition, values are mean ± SD; values above bars represent the total number of oocytes per treatment). ^{a–g}Among IVM conditions, bars with different superscripts are significantly different ($P < 0.05$).

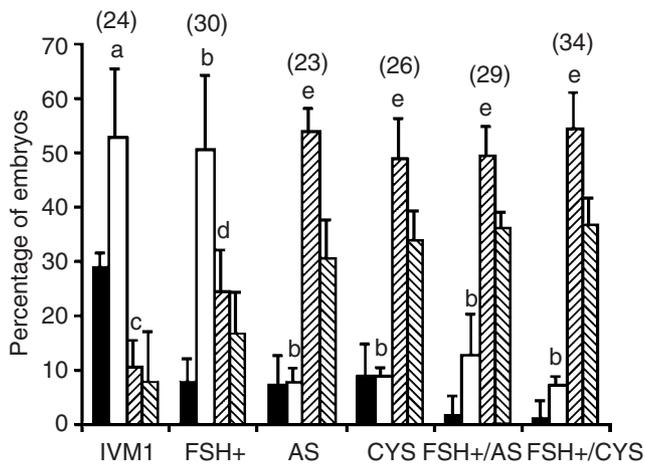


Fig. 5. Proportion of embryos at different stages of development (■, 2–8 cells; □, 8–16 cells; ▨, morulae; ▩, blastocysts) on day 7 (three replicates per condition; values are mean ± SD; values above bars represent the total number of embryos per treatment). ^{a–g}Among IVM conditions, bars with different superscripts are significantly different ($P < 0.05$). IVM1: conventional *in vitro* maturation medium; FSH+: IVM1 with FSH; AS: IVM1 with ascorbic acid; CYS: IVM1 with cysteine; FSH+/AS: IVM1 with FSH and ascorbic acid; FSH+/CYS: IVM1 with FSH and cysteine.

oocytes were cultured in IVM1 or supplemented with higher FSH alone. In contrast, the presence of the antioxidant with or without the higher FSH concentration enhanced ($P < 0.05$; Fig. 5) the proportion of resulting

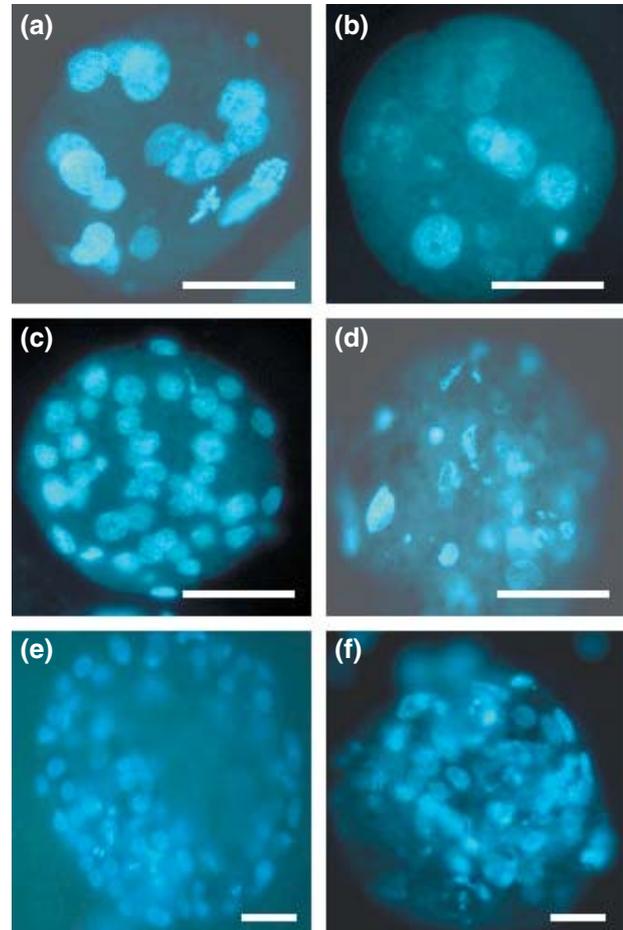


Fig. 6. Morphology of cat embryos after *in vitro* maturation of grade I oocytes in the (a,c,e) presence or (b,d,f) absence of ascorbic acid or cysteine. (a,b) 8–16-cell stage, (c,d) morula stage and (e,f) blastocyst stage (after 7 days of *in vitro* development). Scale bars represent 50 μm .

morulae and blastocysts (Fig. 6c,e). On day 6 of culture *in vitro*, blastocyst formation was observed only in the four treatments containing antioxidant. The morphological benefits of supplemental ascorbic acid or cysteine are shown (Fig. 6). In addition to more uniform blastomeres and the absence of pycnotic nuclei at different developmental stages, the mean number of cells for blastocysts was higher ($P < 0.05$) after IVM in the presence of antioxidant (88.4 ± 10.3 cells, combined average for AS, CYS, FSH+/AS, FSH+/CYS) compared with the absence of antioxidant (59.6 ± 8.8 cells, combined average for IVM1 and FSH+).

Discussion

The present study provides important insight into two potential causes of compromised IVM/IVF capacity in intraovarian cat oocytes collected during the traditional non-breeding season. First, it was discovered that the

conventional IVM medium for this species benefited from a higher FSH concentration that, in turn, increased both nuclear maturation and fertilization success. As there was no difference in the results from using 10 or 15 $\mu\text{g FSH ml}^{-1}$, there appeared to be a threshold effect after which there was no further advantage of more gonadotrophin in the culture medium. Second, supplementing the IVM medium with the antioxidants ascorbic acid or cysteine had a particularly beneficial impact on advanced embryo development *in vitro*, especially from the 8–16 cell to the blastocyst stage. Both of these findings were significant in helping to speculate about arrested or dysfunctional mechanisms in oocytes during intervals of reproductive acyclicity in this species. Finally, the results of the present study demonstrate that simple supplements to conventional IVM conditions circumvented the maturational and developmental compromise that, in turn, resulted in healthy embryo numbers near those reported in studies conducted during the breeding season (Wolfe and Wildt, 1996; Spindler and Wildt, 1999; Freistedt *et al.*, 2001; Karja *et al.*, 2002a).

More specifically, the percentages of cat oocytes reaching metaphase II in the control group of the present study were comparable using the same IVM medium in our laboratory and 1 $\mu\text{g FSH ml}^{-1}$ (previous study, breeding season) (Spindler and Wildt, 1999) versus 10 $\mu\text{g FSH ml}^{-1}$ (the present study, non-breeding season). The findings of the present study were similar to percentages of oocytes reaching metaphase II or undergoing degeneration after IVM using oocytes collected during the breeding season by other laboratories (Pope *et al.*, 1998; Gomez *et al.*, 2000; Bogliolo *et al.*, 2001; Otoi *et al.*, 2001; Karja *et al.*, 2002a). Culturing in the presence of higher concentrations of FSH also promoted meiotic resumption from both the GVBD stage and the metaphase I stage. Although increased FSH concentration in the culture medium has been shown to enhance nuclear maturation in bovine oocytes (Izadyar *et al.*, 1998; Choi *et al.*, 2001), the present study was the first to report that supplementation with high concentrations of gonadotrophins enhanced oocyte function *in vitro* during a seasonally related interval of compromised gamete function. It was apparent that immature cat oocytes recovered during the non-breeding season had full meiotic competence, but required supplemental FSH to advance the maturation process. Although circannual FSH patterns have not been studied in the cat, it is well known that GnRH and FSH secretion patterns decrease in other seasonally breeding mammals (Lehman *et al.*, 1997). In addition, treating the squirrel monkey (Yeoman *et al.*, 1994) or ewe (Stenbak *et al.*, 2001) (the whole animal) with FSH during seasonal periods of ovarian inactivity is known to improve oocyte nuclear maturation and developmental competence. Particularly interesting from the present study is that FSH priming in cats can be delayed until the oocytes are placed in culture. The mechanism by which higher FSH

exerts an effect in culture remains unknown, although a plausible explanation could be a circumvention of a naturally depressed number of functional FSH receptors. Supplementing FSH may largely allow targeting all available receptors to facilitate communication between the somatic cells and the oocyte itself (Eppig *et al.*, 2000; Tanghe *et al.*, 2002; Webb *et al.*, 2002). Concentrations of FSH are difficult to compare among IVM studies in the domestic cat from 0.02 iu bovine FSH ml^{-1} (Freistedt *et al.*, 2001) to 1.0 $\mu\text{g FSH ml}^{-1}$ (Schramm and Bavister, 1995) because of different purity and origin of the hormones. However, the highly purified FSH used in the present study did not contain any contaminants that might have influenced the nuclear maturation and the cumulus expansion.

Although the higher FSH concentration promoted fertilization success similar to that measured during the breeding season (Spindler and Wildt, 1999; Freistedt *et al.*, 2001; Karja *et al.*, 2002a), the addition of only ascorbic acid or cysteine to the IVM medium failed to enhance either nuclear maturation or fertilization. The latter observation is consistent with previous reports for pig (Yoshida *et al.*, 1993; Tatemoto *et al.*, 2001), rat (Takami *et al.*, 1999) and goat oocytes (Mayor *et al.*, 2001). However, these antioxidants profoundly influenced the morphology of cat pronuclei at 16 h after insemination, indicative of a more rapid and improved chromatin remodelling which was probably due to enhanced cytoplasmic maturation (Yoshida *et al.*, 1993; Collas, 1998; Tatemoto *et al.*, 2001).

As more oocytes exposed to higher FSH concentration underwent successful maturation in culture in the present study, more fertilized and cleaved. As others have already asserted (Schramm and Bavister, 1995; Wood *et al.*, 1995), the benefits of this gonadotrophin are exerted via its impact on nuclear maturation rather than improved fertilization and developmental competence. After 7 days in culture, the proportion of embryos developing beyond the 2- to 8-cell stage was increased markedly in all supplemented media. This finding was not influenced by parthenogenetic activation. Although IVM culture supplementation influenced nuclear and cytoplasmic maturation, the percentage of blastocysts eventually developing was 10% less than that reported in comparable studies conducted during the breeding season (Wolfe and Wildt, 1996; Spindler and Wildt, 1999; Freistedt *et al.*, 2001; Karja *et al.*, 2002a). Nonetheless, blastocyst quality in terms of developmental pace *in vitro*, total number and uniformity of blastomeres produced was better in the presence, rather than the absence, of either antioxidant. Furthermore, these traits were similar to those reported for IVM/IVF oocytes collected from cats during the breeding season (Spindler and Wildt, 2002; Karja *et al.*, 2002b).

The benefits of adding ascorbic acid to IVM culture conditions have been reported for pigs (Tatemoto *et al.*, 2001). Extracellular cysteine supplementation too has

been shown to enhance embryo development in bovine (de Matos *et al.*, 1997; de Matos and Furnus, 2000) and pig (Yoshida *et al.*, 1993) oocytes. It is possible that the maternal–zygotic transition and the onset of embryonic transcription (which occurs at the 5- to 8-cell stage in cats; Hoffert *et al.*, 1997) was improved in the presence of antioxidant because we observed more embryos developed beyond the 8–16-cell stage when IVM medium contained ascorbic acid or cysteine. Clearly, the presence of these antioxidants promoted developmental competence. As ascorbic acid and cysteine provided similar beneficial effects on early chromatin remodelling (either by protecting cumulus cells via the former or improving GSH synthesis via the latter), it appeared that these early stage, immature oocytes were particularly sensitive to oxidative stress during the non-breeding season. The antioxidant capacity of the oocyte could be decreased during this period because of a depletion of GSH synthesis or an altered metabolism (Sutovsky and Schatten, 1997; Spindler *et al.*, 2000). It is highly likely that the beneficial effect observed in this study was induced by a positive signal, which then was transferred from cumulus cells to the oocyte (Behrman *et al.*, 1996) without any interaction with the FSH concentration. Regardless, although supplemental antioxidant seemed to ensure improved chromatin remodelling (as demonstrated by excellent pronuclear morphology after insemination) and maternal–zygotic transition, embryos arrested at the morula–blastocyst transition. This block is not directly related to IVM conditions or the onset of embryonic transcription, but rather to inadequate culture conditions during the early stages of embryo development (Schramm and Bavister, 1995; Hoffert *et al.*, 1997).

The present study has demonstrated that cat follicles during the non-breeding season (most of which are <1 mm in diameter) contain oocytes that already have meiotic and developmental competence. Furthermore, despite an impressive amount of follicular atresia (approximately 65%) occurring at any given time in this species (Wood *et al.*, 1997b), a significant number of viable oocytes are recoverable and can undergo embryogenesis by altering culture conditions. Nuclear and cytoplasmic maturation benefit from supplemental FSH, and developmental competence is improved by the antioxidants ascorbic acid or cysteine. The exact mechanism responsible for this phenomenon is currently under investigation in our laboratory. Specifically, the seasonal changes in FSH receptor concentration and functionality as well as modulation of GSH contents in the cumulus–oocyte complex are being investigated.

Finally, these studies have application to our parallel efforts to manage small populations of endangered felid species. Some basic studies in these species have been carried out in our laboratory (Johnston *et al.*, 1991b) as well as the laboratories of others (Jewgenow *et al.*, 1997). Wild felid species are especially prone to seasonal

breeding patterns to ensure the production of young at times of the year most conducive to offspring survival (Wildt *et al.*, 1998). Thus, the findings of the present study will also be used to continue comparative assessments of reproductive mechanisms among species of the Felidae as well as hopefully to improve IVM–IVF results in zoo-housed, valuable specimens that die unexpectedly during seasonal anoestrus.

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During the transgenerational breeding of embryos, the reference population can be updated as frequently as needed. In general, individual production performance and genome-wide single-nucleotide polymorphisms (SNPs) are required to construct original reference population under the breeding plan guidelines as discussed in detail previously [19]. The proposed novel embryo-stem cell breeding system is mainly based on the recently developed technologies for in vitro germ cell induction and the established routines including IVF, genome sequencing, and genomic selection. Recently, functional haploid male and female gametes have been successfully induced in vitro in mice. Since the domestic cat is used as a model for the development of cryopreservation strategies transferable to its wild relatives (1), survival and development of feline oocytes after vitrification and warming are critical steps to make gamete biobanks worth the effort. Immature vitrified oocytes (VOs) experience several cryoinjuries, which affect their ability to resume meiosis and develop properly into embryos after warming. Its influence on the same apoptosis markers and on the in vitro development of cat VOs was assessed. Nuclear evaluation was performed under a fluorescence microscope (Axiovert 200M, Zeiss) at 200 \times magnification, considering that the PI maximum excitation and emission wavelengths are 535 and 617 nm, respectively.

Domestic Cat, Oocytes, In Vitro Maturation, Culture Time, Estrus Cycle Stages. Share and Cite

Experiment 1. Maturation determination of domestic cat oocytes in different reproduction stages at 24 h of culture. A total of 789 oocytes were evaluated with DAPI (8 replicates): 319, 170, 95 and 205 oocytes from prepubertal, follicular, pregnant and anestrus, respectively. The IVM of the oocyte is essential for achieving nuclear maturation [26], an essential process for fertilization and embryonic development [7]. Currently, there is not enough information on the efficiency of IVM using different culture times [13], while considering the reproductive stages of the domestic cat [12]. Overcoming poor in vitro nuclear maturation and developmental competence of domestic cat oocytes during the non-breeding season. It is demonstrated that meiotic and developmental competences are inherent to the immature cat oocyte collected during the non-breeding season and can be overridden by altering in vitro culture conditions to include supplemental FSH and antioxidant.

Expand. 57. KEYWORDS: Domestic cats; Immature oocytes; Mature oocytes; Proteomics. 1. Introduction. Most wild members of the felidae family are vulnerable, threatened, or in danger of extinction in nature. The domestic cat (*Felis catus*) serves as an animal model in the reproductive studies of endangered or nondomestic species. Figure 1. Morphological characterization of feline oocytes during in vitro maturation. (A) An intact germinal vesicle; (B) Metaphase α stage with extrusion of the first polar body (arrow). Bongkoch Turathum et al. To improve the rate of maturation and subsequent fertilization, it is essential to understand the molecular mechanisms and proteins associated with meiotic maturation.