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Effect of Medium-199 and fetal calf serum on *in vitro* maturation of domestic cat oocytes

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Summary. *In vitro* maturation (IVM) and fertilization are potentially useful for propagating threatened or endangered species. The domestic cat is currently used in this field as an experimental model for studies aimed at non-domestic Felidae. At present optimal conditions for obtaining IVM of cat oocytes have not yet been completely defined. The aim of this study was to evaluate two parameters derived from the procedures that currently ensure high maturation rates in domestic ruminants: (1) the suitability of a complex medium (M-199) for IVM of cat oocytes; (2) the effect of different concentrations of fetal calf serum in the culture medium with or without the addition of gonadotrophins. The maturation rate at two different intervals from the onset of culture (24 and 48 h) was also evaluated. The use of M-199 allowed resumption of meiosis in 4·3–18·7% of cat oocytes, according to the supplements and culture periods used. No significant differences were recorded among the treatment groups ($P > 0{\cdot}05$). Meiosis was completed in 90·9% of cases within 24 h with no significant differences between the three treatment groups ($P > 0{\cdot}05$). A 3·2% rate of parthenogenesis was observed at the end of the maturation period with no significant differences between the culture systems ($P > 0{\cdot}05$). However, the percentage cleavage of oocytes was much higher (29%) when correlated with the percentage that had matured. In this case significant differences among treatments were also observed ($P < 0{\cdot}05$). It is concluded that the use of a complex medium supplemented with serum supports the ability of cat oocytes to resume meiosis *in vitro*, but overall results are still lower than those obtained in farm animal species and in the cat using simple media. These results suggest that fetal calf serum at high rates is detrimental to resumption of meiosis but this negative effect is at least partially counteracted by the presence of gonadotrophins. In the system described here, full maturation was usually achieved within the first 24 h of culture. A high incidence of parthenogenesis was noted.

Keywords: In vitro maturation; cat; oocytes; parthenogenesis

Introduction

The development of reliable methods for the *in vitro* maturation (IVM) and fertilization (IVF) of follicular oocytes collected from ovaries obtained *post mortem* or at ovariectomy, is important for preventing the extinction of endangered species. The domestic cat is currently used as an experimental model for studies aimed at non-domestic Felidae.

IVF in the domestic cat has been attained with oocytes matured both *in vivo* (Goodrowe *et al.*, 1988) and *in vitro* (Johnston *et al.*, 1989). On the basis of these results the IVM of oocytes has also been obtained in several non-domestic feline species, although with highly variable rates of success (Johnston *et al.*, 1991a). However, optimal conditions for IVM in the cat have not yet been fully

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defined. At present, results indicate that IVM of cat oocytes can be achieved in relatively simple media supplemented with bovine serum albumin (BSA), and that the presence of gonadotrophins markedly improves the rate of maturation (Johnston *et al.*, 1989; Goodrowe *et al.*, 1991) whereas the use of natural oestrous cat serum seems to be detrimental for the resumption of meiosis (Goodrowe *et al.*, 1991). However, even under optimal conditions the results obtained are still poorer than those currently obtained in domestic ungulates.

The aim of this study was, therefore, to evaluate two different culture conditions derived from the procedures that currently ensure high maturation rates in farm animal species: (1) the suitability of a complex medium (Medium-199) for IVM of cat oocytes; (2) the effect of different concentrations of fetal calf serum (FCS) in the culture medium with or without gonadotrophins.

As previous reports did not clearly describe the optimal culture period to obtain full maturation *in vitro*, the maturation rate was evaluated at two different intervals from the onset of culture.

Materials and Methods

Ovary collection and oocyte recovery. Domestic cat ovaries were recovered from queens subjected to ovariectomy or ovariohysterectomy. Ovaries were stored at room temperature in phosphate-buffered saline (PBS) supplemented with 100 iu penicillin-G potassium salt ml^{-1} and 100 μg streptomycin sulfate ml^{-1} (Sigma Chemical Co., St Louis, MO, USA) for 30–120 min before processing.

Oocytes were released by repeatedly puncturing the ovaries with a 22-gauge needle. Oocytes with an intact corona radiata, attached cumulus cells, and medium to dark pigmented cytoplasm were selected for this experiment and washed twice in PBS and antibiotics with 0·1% (w/v) polyvinyl alcohol (Sigma Chemical Co., St Louis, MO, USA).

Oocyte culture. The medium used for IVM of oocytes was Medium-199 with Earle's salts, 25 mmol Hepes l^{-1} and sodium bicarbonate (M-199; Sigma Chemical Co., St Louis, MO, USA) supplemented with 0·058 mg L-glutamine ml^{-1}, 100 iu penicillin-G potassium salt ml^{-1} and 100 μg streptomycin sulfate ml^{-1} (Sigma Chemical Co., St Louis, MO, USA), and heat-inactivated FCS (Gibco Ltd, Grand Island, NY, USA) at different concentrations according to the treatments.

Hormonal supplementation was with human urinary follicle-stimulating and luteinizing hormone (FSH/LH; Pergovet 500, Serono, Rome, Italy).

Oocytes were randomly divided into three treatment groups (A, B, C) and cultured in 50 μl drops of M-199 (10–25 oocytes per drop) under oil for 48 h at 38·5°C in 5% CO_2 in air with (treatment A) 5% FCS, (treatment B) 10% FCS or (treatment C) 10% FCS + 0·5 iu FSH ml^{-1} + 0·5 iu LH ml^{-1}.

Assessment of maturation and parthenogenesis. Assessment of maturation rate was carried out at 24 h and 48 h on approximately 50% of all cultured oocytes. In brief, this process involved removal of the cumulus cells with mechanical displacement, fixation of the oocytes in acetic acid:ethanol (1:3) for 24 h, staining with 1% (w/v) aceto-orcein (Sigma Chemical Co., St Louis, MO, USA), and morphological evaluation for meiotic status: those achieving telophase I or metaphase II were classified as mature. The appearance of two equally spread groups of chromosomes was judged as a telophase I, and as a metaphase II when one of these groups formed a cluster within the polar body.

At the same intervals, oocytes were evaluated under a stereomicroscope for morphological evidence of parthenogenetic cleavage and the rate and stage of cleavage were recorded.

At 48 h of incubation all cleaved oocytes were fixed and stained as described above.

Statistical analysis. A Fisher's exact test was used to evaluate differences in rates of oocyte maturation and parthenogenesis among the treatment groups and the different culture periods. Values were considered to be significant when $P < 0·05$.

Results

From 56 ovaries 1202 oocytes were recovered (mean of 21·46 oocytes per ovary), of which 622 (51·74%) were selected for this experiment (mean of 11·1 oocytes per ovary). Treatment A was applied to 228, treatment B to 210 and treatment C to 184 oocytes. The remaining 48·25% of oocytes recovered were judged unsuitable for maturation on the basis of morphological criteria: some presented an irregular shape or fragmented cytoplasm and were considered to be degenerate; in others, the cumulus cells were enlarged or absent, and others were already mature, as judged by the presence of a polar body.

As shown in Table 1, the use of M-199 allows meiosis to resume in 4·3–18·7% of cat oocytes according to the supplements and to the culture periods used (Figs 1a, b). No significant differences were recorded among the treatment groups ($P > 0·05$).

Table 1. Maturation of domestic cat oocytes cultured *in vitro*

	In vitro maturation					
	24 h			48 h		
Treatment[a]	Oocytes	Mature[b] (*n*)	(%)	Oocytes	Mature[c] (*n*)	(%)
A	36	4	11·1	38	4	10·5
B	46	2	4·3	78	4	5·1
C	56	8	14·3	64	12	18·7
Total	138	14	10·1	180	20	11·1

[a]Oocytes were cultured in 50 µl drops of M-199 (10–25 oocytes per drop) under oil at 38·5°C in 5% CO_2 in air, with (treatment A) 5% fetal calf serum (FCS), (treatment B) 10% FCS or (treatment C) 10% FCS and 0·5 iu follicle-stimulating hormone ml^{-1} + 0·5 iu luteinizing hormone μl^{-1}; [b,c] values within columns are not significantly different.

Comparing the percentage maturation rates at 24 h and 48 h, it appears that meiosis is completed in 90·9% of cases within 24 h, with no significant differences between the three treatment groups ($P > 0·05$).

A 3·2% (20/622) incidence of parthenogenesis was observed at the end of the maturation period with no significant differences between the culture systems ($P > 0·05$). However, the incidence of cleavage is much higher (29·0%) when correlated with the percentage of mature oocytes (Table 2). In this case significant differences among treatments are also observed ($P < 0·05$).

The demonstration of multiple nuclei corresponding to the number of blastomeres in all stained preparations excluded any fragmentation process and 20% (4/20) of parthenogenetic eggs reached the six-cell stage after 48 h (Figs 1c, d).

Discussion

These preliminary data suggest that a complex medium (M-199), supplemented with serum, supports the ability of cat oocytes to resume meiosis *in vitro* but overall results are still lower than those obtained in farm animal species and in the cat using simple media. In fact, even the highest percentage of mature oocytes in this study is significantly lower than that obtained by Johnston *et al.* (1989), who observed that 54% of oocytes had matured with, and 37% had matured without, hormonal supplementation; Goodrowe *et al.* (1991) observed that 38% of oocytes had matured with FSH in the culture medium and 55% had matured without, after 32 h of culture.

In the study described here, however, full maturation was usually achieved within the first 24 h of culture, in contrast to the findings of other workers (Johnston *et al.*, 1989; Goodrowe *et al.*, 1991). Although no significant differences were observed between the treatments, the results of this study suggest that high concentrations of FCS are detrimental to resumption of meiosis but this negative effect is at least partially counteracted by the presence of gonadotrophins. Perhaps BSA, as used by Johnston *et al.* (1989) and Goodrowe *et al.* (1991), would be more suitable than serum for maturation of cat oocytes.

Finally, the high incidence of parthenogenesis of the matured oocytes must be noted – an incidence much higher than that (0–6%) reported by other workers (Goodrowe *et al.*, 1988; Johnston *et al.*, 1989, 1991b; Donoghue *et al.*, 1992).

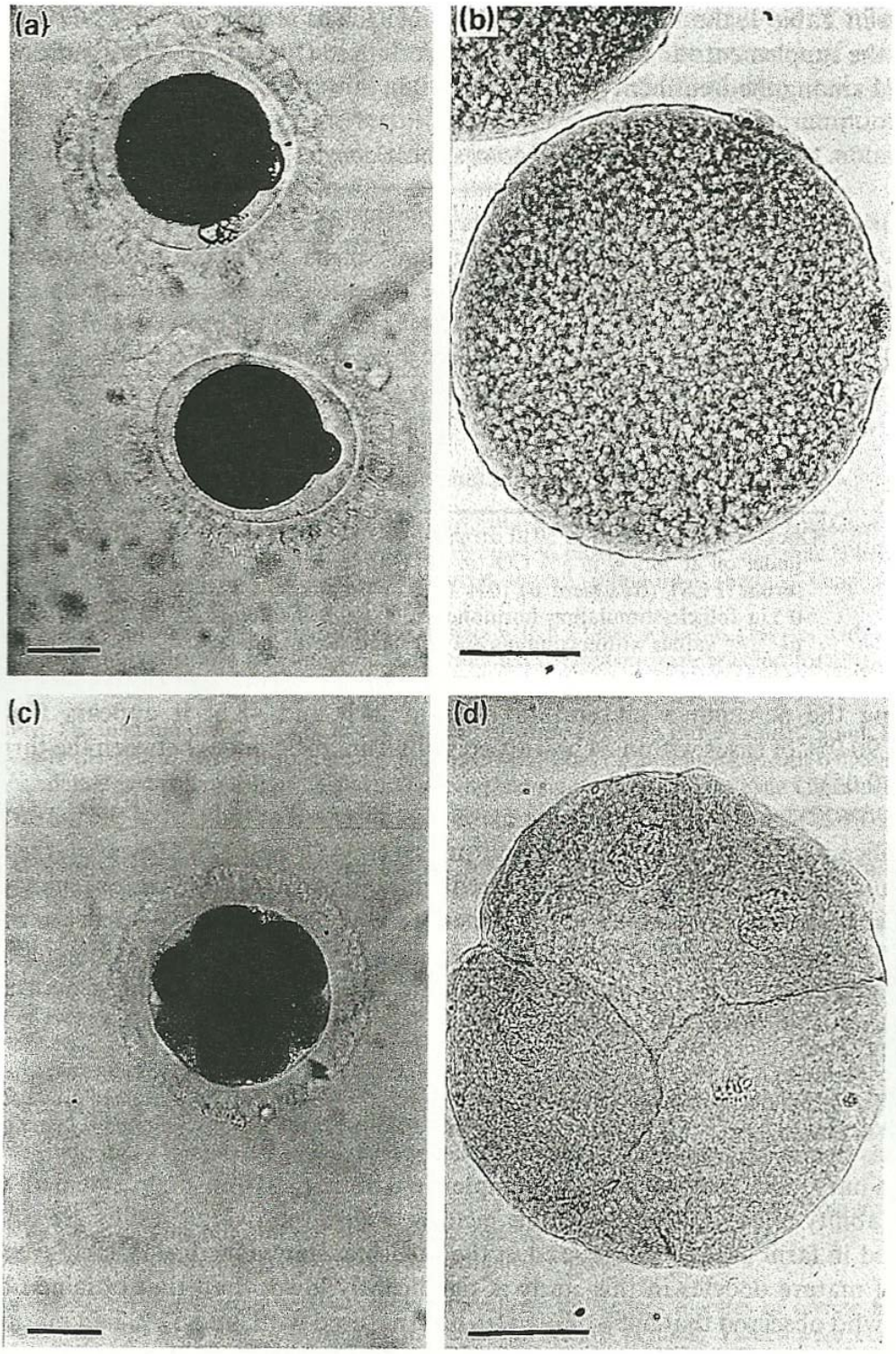

Fig. 1. Cat oocytes (a) at metaphase II, (b) fixed and stained with aceto-orcein; (c) cat oocyte at six-cell stage of parthenogenesis; (d) fixed and stained with aceto-orcein. Bar: 50 μm.

Parthenogenesis can occur spontaneously in a wide array of mammals, in the absence of any detectable stimuli, because the female germ cells have an inherent tendency to divide and differentiate; however, a variety of physical and chemical stimuli applied to oocytes are known to induce parthenogenetic activation (Whittingham, 1980) and the oocytes in this study might have been exposed inadvertently to one or more of these stimuli. Nevertheless, such activation generally appears to occur in oocytes that have undergone nuclear maturation when cultured beyond the time required for maturation to metaphase II, as reported previously in bovine oocytes (Ware *et al.*, 1989). This suggests that changes occur in the mature oocyte that make it more sensitive

Table 2. Parthenogenesis of domestic cat oocytes cultured *in vitro* for 48 h

Treatment	Oocytes	Mature (*n*)	Mature (%)	Cleaved (*n*)	Cleaved (%)	Percentage cleaved/matured
A	228	24	10·5	8	3·5	33·3[a]
B	210	11	5·1	8	3·8	72·7[b]
C	184	34	18·7	4	2·2	11·8[c]
Total	622	69	11·1	20	3·2	29·0

[a,b,c]Values with different superscripts are significantly different ($P < 0·05$).

to environmental factors that are able to destabilize the meiotic block. Although the reason for the high incidence of parthenogenesis in this study, in comparison with the lower percentage of maturation, is not entirely clear, it may be attributable to the more rapid maturation (within 24 h) that causes oocytes to be aged following 48 h of culture.

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