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Original article

Influence of co-culture with oviductal epithelial cells on in vitro maturation of canine oocytes

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Abstract — The process of oocyte maturation in the canine species is unique among mammals: oocytes are immature at ovulation and the resumption and progression of meiotic maturation occur in the oviduct. This study was performed to investigate (i) the effect of co-culture with infundibulum and ampullar oviductal epithelial cells on the in vitro maturation of canine oocytes and (ii) the culture time necessary to reach full meiotic maturation. For this purpose the oocytes, collected from the ovaries of bitches undergoing ovariectomies, were divided into three groups and cultured for 48 and 72 h with the following systems: (A) TCM 199 + 10% oestrus bitch serum + FSH (0.1 IU·mL^{-1}), LH (0.1 IU·mL^{-1})+ progesterone (1 μg·mL^{-1}) + oestradiol (1 μg.mL^{-1}) + cysteamine (100 μM); (B) medium A plus infundibulum cells; (C) medium A plus ampullar cells. Infundibulum and ampullar cells were recovered from the oviducts of bitches at the oestrus stage of their cycle. The results showed that after 48 h of incubation, a significantly higher meiotic resumption ($P < 0.01$) was observed in the oocytes cultured with infundibulum (59%) and ampullar cells (60.0%), than in the control group (40.0%). There was also a significantly ($P < 0.01$) higher meiotic progression to the MII in systems B and C (15.6% and 16.7%) than in system A (4.0%). After 72 h of culture, the percentages of meiotic resumption and progression were unchanged. These results showed that both the infundibulum and the ampullar oviductal epithelial cells positively influence the meiotic resumption and progression of canine oocytes and that 48 h are sufficient for the completion of nuclear maturation.

bitch / oocyte / in vitro maturation / oviductal cells / oestrous cycle

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1. INTRODUCTION

The development of the assisted reproductive techniques including in vitro maturation (IVM), in vitro fertilization (IVF) and embryo culture (IVC) in Canids has been relatively slow compared to that of other species. Current methods for inducing bitch oocyte maturation in vitro are suboptimal and the results obtained are poor compared to those achieved routinely in most other domestic animals [7]. The perfection of in vitro maturation in the bitch is a prerequisite for in vitro embryo production for future salvage programmes in endangered canine species.

Several studies have attempted to improve in vitro maturation of canine oocytes using different culture systems and media [10–14]. Reports in the literature describe the effect of the addition to the culture medium of different types and concentrations of protein sources including fetal calf serum [18, 28], oestrus bitch serum [19, 20], bovine serum albumin [10, 12, 14] and the addition of gonadotrophins or steroids [10, 12]. Some authors [14] have shown that supplementation with 0.3% BSA has a positive effect on the in vitro maturation of canine oocytes, others [19, 20] have obtained a high meiotic maturation of oocytes after the addition of 10% oestrus bitch serum to the culture medium. The stage of the oestrous cycle of the donor animal [10, 19], the age of the bitches, oocyte size [11, 21] and nuclear and cumulus morphology are also important factors related to the in vitro meiotic competence of canine oocytes [19].

The time necessary for oocyte maturation in vitro in this species is still an open question. Some authors [19, 23] report that full meiotic maturation occurs after 24–48 h of culture while others indicate the need to culture oocytes for 72–96 h to achieve the highest maturation rates [8, 14, 22, 28].

The difficulty in obtaining high rates of in vitro matured bitch oocytes is probably due to the peculiar reproductive process of this species including the hormonal environment and meiotic resumption and progression. In fact, preovulatory follicular luteinization occurs in bitches, resulting in the exposure to increasing concentrations of progesterone before ovulation. In most mammals the oocyte is primarily exposed to high concentrations of oestrogen before ovulation. In addition, canine oocytes are ovulated at the germinal vesicle stage; the completion of meiotic maturation takes place in the middle part of the oviduct and the oocytes reach metaphase II by 3–4 days after the LH peak [15], whereas other mammalian oocyte maturation occurs within the ovarian follicle.

In the present study we investigated the benefits of co-culturing canine oocytes with infundibulum or ampullar oviductal epithelial cells collected during the oestrus stage. We studied its effect on the in vitro maturation of bitch oocytes and the time necessary to induce full meiotic maturation.

2. MATERIALS AND METHODS

2.1. Chemicals

All chemicals in this study were purchased from the Sigma Chemical Company (St. Louis, MO) unless otherwise stated.

2.2. Oocyte recovery

Ovaries were harvested from 23 bitches at various stages of the oestrus cycle during routine ovariohysterectomy at local veterinary clinics. No attempt was made to divide the oocytes according to the stage of the oestrus cycle of the animals. The bitches, of various breeds and aged 1–7 years, were all healthy at the time of surgery. The ovaries were immediately placed in phosphate-buffered saline (PBS) containing penicillin-G potassium (100 $\mu g.mL^{-1}$) and streptomycin sulphate (100 $\mu g.mL^{-1}$) at 37 °C. Within 2 h of collection the ovaries were

washed several times in fresh PBS and sliced repeatedly to release oocytes. Only cumulus-oocyte complexes with two or more dense layers of cumulus cells and darkly granulated cytoplasm were selected for in vitro maturation. Moreover, according to Hewitt and England [11], oocytes > 100 μm in diameter were cultured to evaluate the meiotic competence.

2.3. Oocyte culture

A total of 1329 oocytes were recovered from 23 bitches; amongst these, 667 (50.1%) were selected for in vitro maturation. The mean number of oocytes recovered from individual bitches was 57.8 ± 20.9 (SD).

For in vitro maturation, the selected oocytes were randomly divided into three treatment groups for in vitro maturation: (A) TCM 199 + 10% oestrous bitch serum + FSH (0.1 IU·mL^{-1}, Pluset, Serono, Rome, Italy), LH (0.1 IU·mL^{-1}, Pluset, Serono, Rome, Italy) + progesterone (1 μg·mL^{-1}) + oestradiol (1 μg·mL^{-1}) + 100 μM cysteamine (n = 201; control group); (B) medium A plus canine infundibulum oviductal epithelial cells (n = 239); (C) medium A plus canine ampullar oviductal epithelial cells (n = 227). The oocytes were cultured in 1 mL of medium in groups of 30 per well, in four well dishes at 39 °C in a humidified environment of 5% CO_2 in air.

2.4. Assessment of nuclear maturation

Assessment of nuclear maturation was carried out after 48 h and 72 h of incubation. This process involved the removal of the cumulus cells of the oocytes by mechanical displacement with a small-bore glass pipette. The denuded oocytes were then stained with a solution of glycerol-bisbenzimide (10 μg·mL^{-1}; Hoechst 33342) between a slide and a coverslip. After 1 h of incubation at 4 °C in the dark, the slides were observed under a fluorescent microscope (Diaphot, Nikon, Tokyo, Japan) and the chromatin configuration was evaluated.

2.5. Infundibulum and ampullar oviductal epithelial cell collections

The infundibulum and ampullar oviductal epithelial cells used in co-culture with the oocytes during in vitro maturation were obtained from bitches in the oestrous cycle immediately after ovariohysterectomy. Oestrous was revealed by vaginal cytology and measurement of plasma progesterone [6].

The oviducts were dissected from the ovarian tissue and washed in PBS and antibiotics. The epithelial cells from the infundibulum portion of the oviduct were collected by scraping the surface of the infundibulum using a microblade. To recover the ampullar cells, the oviducts were slit lengthways and the luminal surface of the ampulla was lightly scraped with a microblade. After several washes in TCM 199 + Hepes, oviductal epithelial cells were suspended in the maturation medium immediately before culture with oocytes at a concentration of approximately 10^4 cells·mL^{-1}. The cell concentration was measured using a Bürker haemocytometer.

2.6. Statistical analysis

Data were evaluated by the chi-square test. Differences were considered to be significant at $P < 0.05$.

3. RESULTS

The results of the in vitro maturation after 48 h of culture (Tab. I) show that a significantly ($P < 0.01$) higher rate of meiotic resumption was recorded in the systems containing infundibulum (59%) and ampullar cells (60%, Fig. 1) than in the culture system without cells (40%). Similar differences

Table I. Meiotic progression of bitch oocytes after 48 h of culture with infundibulum and ampullar oviductal epithelial cells.

Culture system	N° oocytes examined	N° oocytes degenerated	N°(%) oocytes that resumed meiosis				
			TOTAL	GVBD	MI	AT	MII
A	115	15 (13.0)	40 (40.0)[a]	36 (36.0)	0	0	4 (4.0)[a]
B	135	13 (9.6)	72 (59.0)[b]	31 (25.4)	19 (15.6)	3 (2.4)	19 (15.6)[b]
C	131	11 (8.4)	72 (60.0)[b]	33 (27.5)	15 (12.5)	4 (3.3)	20 (16.7)[b]

(GVBD: germinal vesicle breakdown; MI: metaphase I; AT: anaphase-telophase; MII: metaphase II). Different superscripts are statistically different[a] vs.[b] $P < 0.01$.

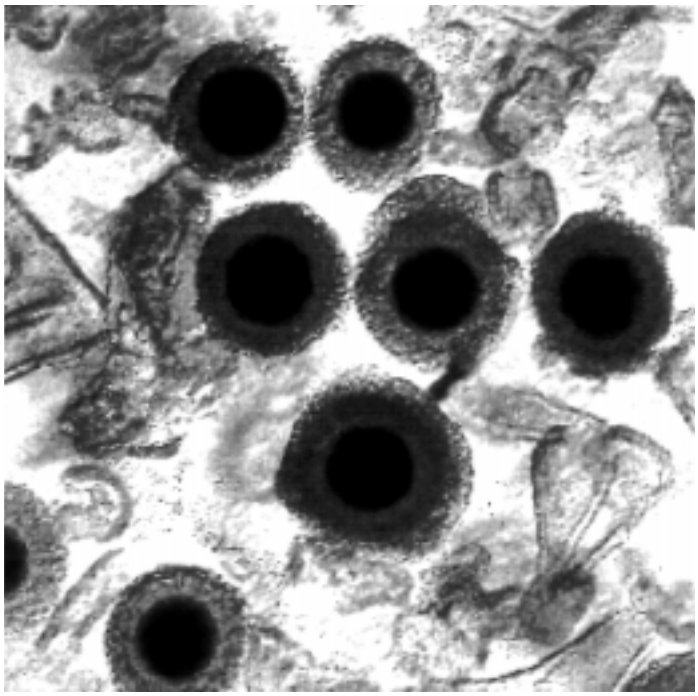

Figure 1. Canine oocytes matured in vitro with oviductal epithelial cells.

($P < 0.01$) in the percentages of oocytes that reached metaphase II (MII, Figs. 2 and 3D) were observed after co-culture with infundibulum (15.6%) and ampullar cells (16.7%) compared to the control group (4.0%). In the culture system without cells, all the oocytes that resumed meiosis but did not reach MII were arrested at the germinal vesicle stage (GV), while in the systems with infundibulum and ampullar cells

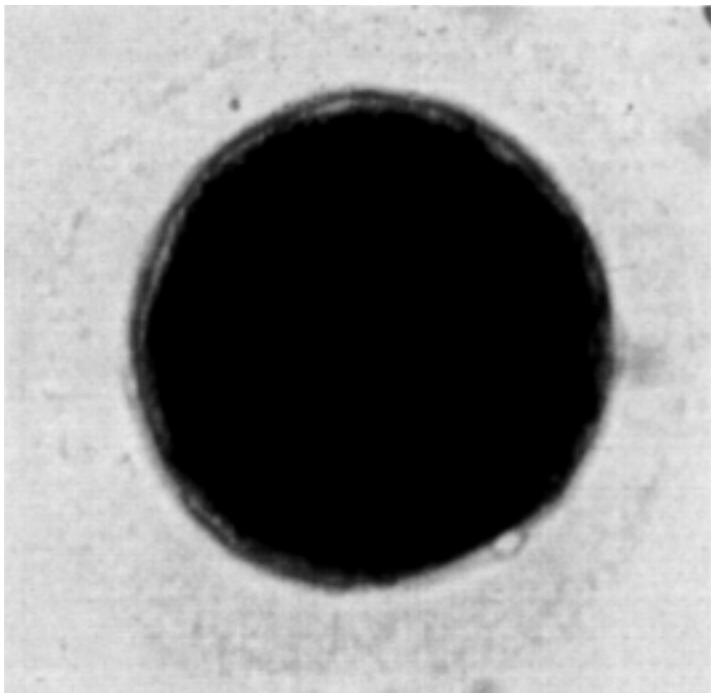

Figure 2. In vitro matured canine oocytes after the extrusion of the first polar body.

respectively 25.4% and 27.5% were at the GVBD stage (Fig. 3B), 15.6% and 12.5% progressed to MI and 2.4% and 3.3% were at the A/T stage (Fig. 3C). No significant differences were recorded between the systems with infundibulum and ampullar cells in the percentage of oocytes resuming and completing meiosis. When oocytes were cultured for 72 h the proportion of oocytes that resumed meiosis and reached MII did not change significantly (Tab. II) compared to those with 48 h of culture.

4. DISCUSSION

In the present study, we evaluated (i) the effect of co-culture with infundibulum or ampullar oviductal epithelial cells recovered from bitches at the oestrus stage on the in vitro maturation of canine oocytes and (ii) the culture time required to obtain a complete nuclear maturation.

Our results showed that the presence of oviductal cells collected from the infundibulum and the ampulla region in the culture medium increased the number of canine oocytes resuming and completing meiosis.

Several authors have reported the beneficial effects of the use of cell co-culture or medium conditioned by cell culture for in vitro embryo development in other species [5, 9, 26]. Moreover, recent findings in pigs [5, 25] and horses [16] have shown that the co-culture with oviductal epithelial cells during in vitro maturation does not affect nuclear maturation rates but positively influences in vitro embryo development. Thus it is possible that co-culture with oviductal epithelial cells during maturation enhances cytoplasmic maturation in oocytes and thereby contributes to its potential for embryo development. In the canine species, Hewitt et al. [13] previously investigated the effect of oviductal epithelial cells on the in vitro maturation of canine oocytes,

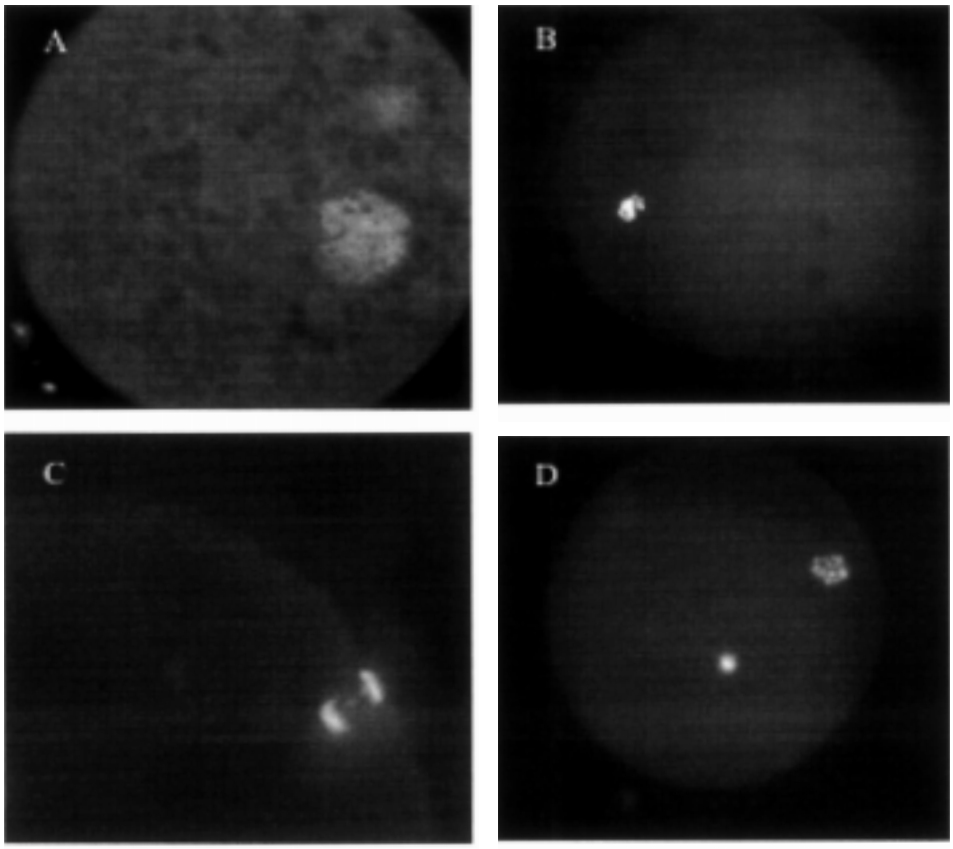

Figure 3. In vitro meiotic progression of canine oocytes stained with bisbenzimide: (A) germinal vesicle stage; (B) germinal vesicle breakdown; (C) telophase I; (D) metaphase II.

Table II. Meiotic progression of bitch oocytes after 72 h of culture with infundibulum and ampullar oviductal epithelial cells.

Culture system	N° oocytes examined	N° oocytes degenerated	N°(%) oocytes that resumed meiosis				
			TOTAL	GVBD	MI	AT	MII
A	86	20 (23.3)	28 (42.4)[a]	24 (36.4)	0	0	4 (6.0)[a]
B	104	12 (11.5)	54 (58.7)[b]	26 (28.3)	11 (11.9)	0	7 (18.5)[b]
C	96	10 (10.4)	50 (58.1)[b]	25 (29.1)	5 (5.8)	0	20 (23.2)[b]

(GVBD: germinal vesicle breakdown; MI: metaphase I; AT: anaphase-telophase; MII: metaphase II). Different superscripts are statistically different [a] vs.[b] $P < 0.01$.

reporting that the oviductal cells do not exert any beneficial effect on meiotic resumption and maturation of bitch oocytes. In fact, after 96 h of co-culture they recorded a low percentage (9%) of oocytes maturing to MI/AI/MII and this rate of maturation was not different from that observed in the system without the cells. Our results were higher than those reported by Hewitt et al. [13]. The reason for this could be related to several factors, including the stage of the oestrus cycle when the cells were collected and the various regions of the oviduct where the cells were recovered. In fact, these authors used co-culture with epithelial cells obtained from the whole oviduct of bitches not at the oestrus stage of the cycle, whereas in our study we supplemented the maturation medium with cells collected from the infundibular and ampullar regions of the oviduct of bitches at the oestrous stage.

In other species, many histological studies have demonstrated regional variations in both the morphological and ultrastructural features of the secretory cells in the oviduct epithelium during the different stages of the oestrus cycle [1, 24]. Histochemical and immunocytochemical analysis have also revealed regional differences in the localization of various materials in the oviduct epithelium, suggesting the possibility of regional specificity in the production of various secretory materials by the oviductal epithelial cells [2]. In addition, recent biochemical and immunoelectron microscopical research have shown that the biosynthesis of specific proteins or glycoproteins, is associated with region-specific variations in epithelial cells in different oviductal segments and with the different stages of the oestrus cycle [3].

Moreover, at the oestrus stage the oviductal epithelial cells were primed with oestrogen, so their secretory activity could be different and could consequently positively influence the process of meiotic maturation of bitch oocytes. In other species, recent studies have demonstrated that oestrogen is responsible for the induction of de novo synthesis and secretion of certain oviductal secretory proteins and the inhibition of others [4] and that the oestradiol treatment of oviductal epithelial cells may increase the rate of zygote cleavage during early development in vitro [27]. We can therefore suppose that the positive effect of oviductal epithelial cells collected at oestrus on nuclear maturation of canine oocytes may be related to the secretion of specific oviduct-related factors that are present in the upper regions of the oviduct after oocyte ovulation and are involved in the mechanism of the resumption and progression of meiosis.

In addition to the cycle stage at the moment of cell collection and the regions where they were recovered, in our work, the culture of cells in the presence of oestrus bitch serum could have sustained their viability and their metabolic and secretory activity better than the system used by Hewitt et al. [14] in which bovine serum albumin was used.

To date, the highest rate of maturation to MII (39%) of oocytes has been reported by Nickson et al. [19] who used a medium containing 10% oestrus bitch serum and 20 $\mu g \cdot mL^{-1}$ oestradiol. Yamada et al. [28] demonstrated that 32% of preovulatory oocytes collected from superovulated bitches reached MII. In the present study, oocytes were cultured in a medium with oestrus bitch serum but with a lower concentration of oestradiol than that used by Nickson et al. [19] and were collected from animals at different stages of the oestrus cycle. These differences may, therefore, account for the lower rate of maturation of canine oocytes observed in our research. In fact, as Yamada et al. [28] observed, oocytes from anoestrous bitches showed no tendency to resume meiosis. A similar effect of the phase of the oestrous cycle on meiotic competence has been described by Luvoni et al. [17] whose results indicate that dog cumulus-oocyte complexes isolated from

the ovaries during anoestrus are unable to complete meiosis and that the communications between the germinal and somatic compartment through gap junctions are absent, thus suggesting a relation between the presence of communications and meiotic competence.

In the present study, the analysis of the influence of different incubation times (48 and 72 h) showed that full maturation is generally achieved within 48 h of culture in both systems used. Similar results have been reported in other studies [20, 28], while in previous research the highest rate of nuclear maturation was observed after 24 h [19, 24] or 72–96 h [10, 11, 14] of incubation.

The difference in maturation time may be related to the different culture conditions used, the quality of the oocytes and the age and stage of the oestrus cycle of the donor animals.

In conclusion, the results of our experiment demonstrated that the introduction in the culture medium of epithelial cells collected from the infundibulum and ampullar region of oviducts of bitches in oestrus positively influences the in vitro maturation of canine oocytes. In this system of culture the completion of meiotic maturation was obtained after 48 h of culture.

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