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The effect of oviductal cells on in vitro maturation of canine oocytes in different culture media

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Abstract: The aims of this study were to investigate the effect of oviductal cells on in vitro maturation (IVM) of canine oocyte in Tissue Culture Medium 199 (TCM-199) or synthetic oviductal fluid (SOF) supplemented with bovine serum albumin (BSA) or fetal calf serum (FCS) and to compare the maturation rates of oocytes from the diestrus and anestrus stages. Following ovariohysterectomy, 13 pairs of ovaries were collected from bitches in anestrus (n = 10) or diestrus (n = 3) and oocytes were harvested by slicing. The oviducts were flushed with TCM-199 containing 10% FCS and were scraped and squeezed into a tube in order to obtain oviductal cells. Selected oocytes were divided into groups for IVM over 48 h for each of the diestrus and anestrus stages as follows: Group Ia, SOF+BSA; Group Ib, SOF+BSA+oviductal cells; Group IIa, SOF+FCS; Group IIb, SOF+FCS+oviductal cells; Group IIIa, TCM-199+BSA; Group IIIb, TCM-199+BSA+oviductal cells; Group IVa, TCM-199+FCS; and Group IVb, TCM-199+FCS+oviductal cells. Afterwards, oocytes were fixed with acetic acid-ethyl alcohol and stained with aceto-orcein to determine nuclear maturation. When compared between anestrus and diestrus stages for all parameters (undetermined nuclear material, germinal vesicles, germinal vesicle break down, metaphase I, metaphase II, and degenerated) in different media, the differences were found to be significant statistically in Group IIa (22.9%) and Group IIIb (35.7%) for the germinal vesicle stage ($P < 0.05$) as compared to the other groups. In conclusion, in the oocytes obtained from bitches in diestrus and anestrus supplemented with FCS or BSA in SOF medium without oviductal cells, more positive effects were seen on canine oocyte maturation than with TCM-199 medium supplemented with same protein sources and oviductal cells.

Key words: Canine, oocyte, in vitro maturation

1. Introduction

The reproductive physiology of the dog is different when compared to other species, as the dog is a nonseasonal and monoestric species, ovulating only once or twice a year at an interval of 5–12 months (1). The bitch ovulates a primary oocyte that needs 48–72 h to complete postovulatory maturation to the metaphase II stage in the isthmus of the oviduct (2,3). The maturation rate of the canine oocyte beyond the germinal vesicle stage is 0% to 58% and the proportion of those reaching the metaphase II stage was generally found to be 20% (4). Lopes et al. (5) reported that most mammals ovulate in metaphase II due to estradiol exposure from the follicular fluid, unlike the bitch, which has a follicular environment around ovulation dominated by progesterone due to the preovulatory luteinization of the follicle (6). Willingham-Rocky et al. (7) reported that the reason for this low in vitro maturation (IVM) rate could be because of the estrous cycle stage of the dog and also an insufficient IVM medium. The oocytes recovered from the proestrus stage were found to have lower rates of maturation to the metaphase II stage when compared to oocytes recovered from the estrus or diestrus stage. The same components required in other mammalian species for mature oocytes may elicit different responses in canine species and need to be investigated (7).

Canine female gamete cells are different when compared to other mammalian oocytes. The main differences are the follicular environment, dark cytoplasm due to high lipid content, highly compact and unexpanded corona radiate cells, and the meiotic stage at ovulation (8). Before ovulation, the canine oocyte is arrested in the first prophase of the first meiotic (MI) division in foxes and bitches. The germinal vesicle (GV) or oocyte nucleus breaks down (GVBD) after ovulation (4). Improvement in in vitro canine oocyte maturation techniques is very important in canine embryo production, because assisted and natural breeding are elusive in canids (9).

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As is well known in cattle IVF procedures, Özdaş et al. (10) reported that the addition of oviductal cells to the in vitro culture medium improves embryo development to expanded blastocyst and hatching blastocyst stages in cattle embryos. Oviductal fluid composition and oviductal structure may influence the oocyte maturation, fertilization, and early embryonic development (2). Standard or complex culture media [i.e. Tissue Culture Medium 199 (TCM-199) and synthetic oviductal fluid (SOF)] have been used for IVM of canine oocytes (4). SOF medium containing bovine serum albumin (BSA) and oviduct cells may act positively on the maturation of canine oocytes (11).

The development of in vitro techniques for canine oocytes is a priority among reproductive techniques. The objectives of this study were to evaluate the effect of oviductal cells on IVM of canine oocytes in different culture media (TCM 199 and SOF) with 2 different protein sources [BSA or fetal calf serum (FCS)] and to compare the maturation of oocytes obtained from the ovaries of bitches in the diestrus and anestrus stages.

2. Materials and methods

2.1. Collection of ovaries and oocyte retrieval

A total of 13 healthy cross-breed bitches (from 8 months to 2 years of age) were used in this study. Thirteen pairs of ovaries were obtained by ovariohysterectomy in the Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, İstanbul University, İstanbul, Turkey. Ovaries were transferred to the laboratory in phosphate-buffered saline (PBS) solution at 30–35 °C within 2 h following ovariohysterectomy. The ovaries were categorized according to the stage of external morphology of ovarian tissue as being in stages of anestrus (n = 10 dogs; ovaries without follicles or pronounced luteal tissue) or diestrus (n = 3 dogs; ovaries with 1 or more corpora lutea) and also according to the vaginal cytology of the bitches prior to ovariohysterectomy. The oocytes were harvested by ovarian slicing in TCM-199 with HEPES (washing medium; 30–35 °C) supplemented with 10% FCS, washed 3 times, and then selected.

Selection of oocytes was conducted according to the following criteria: smooth vitellin membrane, a homogeneous ooplasm, a smooth and undamaged zona pellucida, and surrounded with at least 3 or 4 layers of compact cumulus oophorus. From the bitches in anestrus 624 oocytes and from the bitches in diestrus 192 oocytes were collected; thus, a total of 816 Grade 1 oocytes were obtained and incubated for maturation. Oocytes were not classified according to their size and follicle diameter.

2.2. Recovery of oviductal tissue

After ovariohysterectomy, ovaries were maintained at 30–35 °C in PBS supplemented with 10% FCS. The oviducts were dissected from the ovarian bursa immediately and were put into another petri dish containing TCM-199. The lumen of the oviduct was flushed with the same medium and lightly scraped and squeezed into an Eppendorf tube. The oviductal cells were washed via centrifugation of the Eppendorf tubes at 3500 rpm for 1.5 min and cells were resuspended in TCM-199 twice. The supernatant was discarded, and the remaining pellets were added with up to 100 µL of maturation medium.

2.3. Maturation of oocytes and experimental design

The selected oocytes were washed once with washing medium TCM-199 (45 mL) containing: TCM-199 medium, 660 mg; sodium bicarbonate, 99 mg; gentamicin sulfate, 50 µg/mL; L-glutamine, 1 mM; and heparin, 20 U/mL (1000 U/mL, 50×). The oocytes were then washed twice with the same medium without heparin.

TCM-199 maturation medium (20 mL) contained 190 mg of TCM-199 without HEPES, 44 mg of sodium bicarbonate, 30 µL of 200 mM L-glutamine, 200 µL of 20 mM sodium pyruvate (100×), 50 µL of 10 µg/mL epidermal growth factor (EGF; 200×), 100 µL of 10 µg/mL luteinizing hormone (LH), 5 µL of 10 µg/mL follicle stimulating hormone (FSH), and 100 µL of 10 mg/mL gentamicin sulfate (200×).

SOF medium contained 39 mL of H_2O, 0.0050 g of Tri-Na-citrate dihydrate, 0.0250 g of myo-inositol, 1.5 mL of BME (50×), 500 µL of MEM (100×), 50 µL of 200 mM L-glutamine, and 250 µL of 10 mg/mL gentamicin sulfate. Twenty milliliters of this solution was taken and hormones [5 µL of 10 µg/mL FSH, 100 µL of 10 µg/mL LH, 50 µL of 10 µg/mL EGF (200×)] were added. The two media were supplemented with 10% FCS or 3 mg/mL BSA.

Five microliters of the diluent containing oviductal cells was added to 4 wells containing selected oocytes [1–10 µL per cumulus-oocyte complex (COC)] and maturation medium (TCM-199 or SOF) with or without BSA and FCS incubated at 39 °C under 5% CO_2 for TCM-199 medium and under 5% CO_2, 5% O_2, and 90% N_2 (gas mixture) for SOF medium at high humidity for 48 h. When added, the oviductal cells of diestrus were put into the 4 wells containing the diestrus oocytes, and the oviductal cells of anestrus were put into the 4 wells containing the anestrus oocytes.

Osmotic pressure of the TCM-199 washing and maturation medium was 280 ± 10 mOsm/kg and pH was 7.2–7.4; osmotic pressure of SOF medium 275–285 mOsm/kg and pH was 7.4.

The experimental design included 4 different IVM media × 2 (addition of oviductal cells or not into IVM media) × 2 (anestrus and diestrus stages).

Therefore, there were 16 groups in this study, as follows: Group Ia (anestrus), SOF+BSA; Group Ib (anestrus), SOF+BSA+oviductal cells; Group IIa (anestrus), SOF+FCS; Group IIb (anestrus), SOF+FCS+oviductal cells; Group IIIa (anestrus), TCM-199+BSA; Group IIIb (anestrus), TCM-199+BSA+oviductal cells; Group IVa (anestrus), TCM-199+FCS; Group IVb (anestrus), TCM-199+FCS+oviductal cells (Table 1); Group Ia (diestrus), SOF+BSA; Group Ib (diestrus), SOF+BSA+oviductal cells; Group IIa (diestrus), SOF+FCS; Group IIb (diestrus), SOF+FCS+oviductal cells; Group IIIa (diestrus), TCM-199+BSA; Group IIIb (diestrus), TCM-199+BSA+oviductal cells; Group IVa (diestrus), TCM-199+FCS; Group IVb (diestrus), TCM-199+FCS+oviductal cells (Table 2). After maturation, oocytes were fixed with an acetic acid-ethyl alcohol combination (1:3) and were stained with aceto-orcein. Undefined (undetermined) nuclear material (UDNM) was defined as when there were still a few COCs around the oocyte and in those oocytes the chromosome structure was unidentifiable or not visible.

2.4. Statistical analyses

Data of oocytes arrested at GVBD, GV, MI, and MII stages along with UDNM and degenerated oocytes of bitches in anestrus and diestrus in different media (SOF or TCM-199) supplemented with either FCS or BSA and addition of oviductal cells or not were analyzed with the chi-square test using SPSS 13.0. The level of significance was $P < 0.05$.

3. Results

When compared between anestrus and diestrus stages for all parameters (UDNM, GV, GVBD, MI, MII, and degenerated) in different media, the differences were found to be significant statistically in Group IIa (22.9%) and Group IIIb (35.7%) for the GV stage ($P < 0.05$) as compared to the other groups (Tables 1 and 2).

No statistical difference was found for the other parameters in all stages. Some of the oocytes were degenerated because of zona damages during pipetting, staining, and maturation; therefore, 416/624 oocytes from bitches in anestrus and 103/192 oocytes from bitches in diestrus were used in the study.

Oocytes were arrested at almost the same ratios of GV stage in the anestrus group (Figure 1). GVBD stage rate was found to be numerically higher in Group IIb (SOF+FCS+oviductal cells; 31.1%) compared to Group IIa (SOF+FCS; 27.1%) in anestrus oocytes (Figure 2). The highest MI stage of oocytes from bitches in anestrus was detected numerically in Group IIIb (TCM-199+BSA+oviductal cells; 22.2%) and no significance was detected statistically (Table 1).

Table 1. Distribution of in vitro maturation rates of oocytes in different IVM media from dog in anestrus stage. No statistical difference was found among the groups. UDNM: Undetermined nuclear material, GV: germinal vesicle, GVBD: germinal vesicle break down, MI: metaphase I, MII: metaphase II, a: without oviductal cells, b: with oviductal cells.

Stages of maturation		Group I SOF+BSA		Group II SOF+FCS		Group III TCM-199+BSA		Group IV TCM-199+FCS	
		a	b	a	b	a	b	a	b
UDNM	n	10	11	7	9	7	12	11	11
	%	19.6	18.0	14.6	14.8	16.3	22.2	24.4	20.8
GV	n	9	13	11	15	10	7	10	11
	%	17.6	21.3	22.9	24.6	23.3	13	22.2	20.8
GVBD	n	11	12	13	19	9	10	7	7
	%	21.6	19.7	27.1	31.1	20.9	18.5	15.6	13.2
MI	n	8	8	9	9	6	12	9	9
	%	15.7	13.1	18.8	14.8	14.0	22.2	20.0	17.0
MII	n	0	0	0	0	0	0	0	0
	%	0	0	0	0	0	0	0	0
Degenerated	n	13	17	8	9	11	13	8	15
	%	25.5	27.9	16.7	14.8	25.6	24.1	17.8	28.3

Table 2. Distribution of in vitro maturation rates of oocytes in different IVM media from dog in diestrus stage. No statistical difference was found among the groups. UDNM: Undetermined nuclear material, GV: germinal vesicle, GVBD: germinal vesicle break down, MI: metaphase I, MII: metaphase II, a: without oviductal cells, b: with oviductal cells.

Stages of maturation		Group I SOF+BSA		Group II SOF+FCS		Group III TCM-199+BSA		Group IV TCM-199+FCS	
		a	b	a	b	a	b	a	b
UDNM	n	4	3	3	4	3	5	4	4
	%	26.7	17.6	37.5	40.0	18.8	35.7	36.4	33.3
GV	n	3	3	5	1	5	5	5	3
	%	20	17.6	62.5	10.0	31.3	35.7	45.5	25.0
GVBD	n	3	3	0	1	1	2	1	1
	%	20	17.6	0	10.0	6.3	14.3	9.1	8.3
MI	n	0	1	0	0	3	1	0	0
	%	0	5.9	0	0	18.8	7.1	0	0
MII	n	0	0	0	0	0	0	0	0
	%	0	0	0	0	0	0	0	0
Degenerate	n	5	7	0	4	4	1	1	4
	%	33.3	41.2	0	40	25	7.1	9.1	33.3

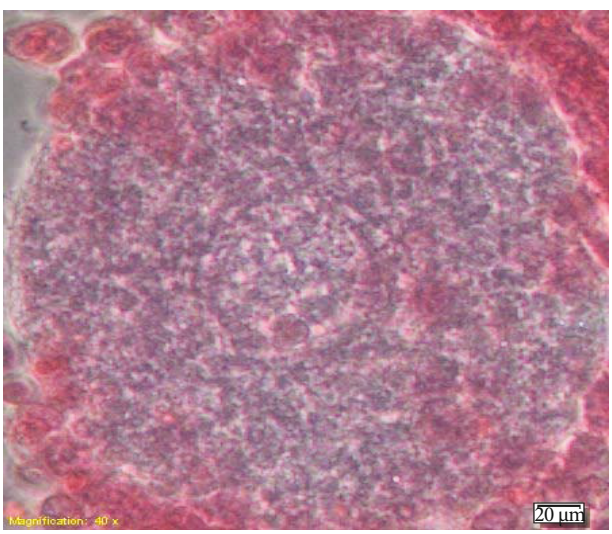

Figure 1. Maturation of canine oocytes at GV stage (arrow).

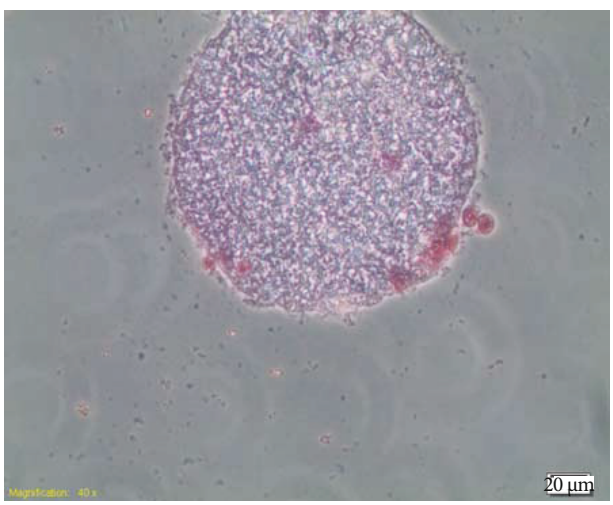

Figure 2. Maturation of canine oocytes at GVBD stage.

The ratio of oocytes reaching the GVBD stage in the bitches in diestrus was found to be the highest numerically in Group Ia (SOF+BSA; 20%), but the ratio of oocytes arrested at the GV stage in bitches in diestrus was found to be the highest numerically in Group IIa without oviductal cells (SOF+FCS 62.5%) (Table 2). Numerically, the highest ratio of MI stage in bitches in diestrus was detected in Group IIIa (TCM-199+BSA; 18.8%) (Table 2).

4. Discussion

Currently, IVM of canine oocytes is not yet clarified. Many researchers are still striving to establish optimal culture conditions and limited success rates in IVM are obtained for canine oocytes and embryos (5,12).

In this study, effects of estrus cycle stages (diestrus or anestrus), different culture media (TCM-199 or SOF), and

presence of proteins and oviductal cells on maturation rates of canine oocytes were investigated.

In the dog, meiotic resumption is completed in 2–3 days after ovulation and 5 days after the LH peak (4). Some researchers reported the completion of the meiotic maturation of canine oocytes at 24–48 h (13,14), whereas others reported this period as 72–96 h (15,16). Saint-Dizier et al. (14) reported the rate of MII oocyte maturation in 24–48 h as 9.4%, whereas in this study, at the end of 48 h none of the oocytes had reached the MII stage. However, Saint-Dizier et al. used 20% FCS for TCM-199 medium for canine oocyte maturation, while in this study we used a lower amount of FCS (10%); this may be why the oocytes in this study could not reach the MII stage.

It is well known that the progesterone level increases before ovulation and maturation occurs after 3–4 days in dogs, whereas in other species, ovulation occurs in the preovulatory follicular fluid (12). In this study, it is thought that lack of MII stage oocytes could be attributed to the anestrus stage and lack of progesterone effects. However, Rodrigues and Rodrigues (3) reported that the stages of the estrus cycle in the dog have no effect on reaching the MII stage. In the anestrus stage, the percentage of oocytes reaching the MI stage in TCM-199 was 20% in current study, whereas Rodrigues and Rodrigues (3) reported that 5.2% of anestrus oocytes reached metaphase I in TCM-199 with serum. Hewitt and England (2) reported that the oviductal cells did not positively influence meiotic resumption and maturation of canine oocytes.

In canine oocytes, the degeneration rate was found to be 20%–68% (4). Oocytes were classified as degenerate due to light cytoplasm, intracytoplasmic vacuoles or a ruptured zona pellucida, or incomplete cumulus mass (under 2 layers), and these types of degenerations were found in a high prevalence in canine oocytes (17). Furthermore, the oocytes were obtained from the follicles of 70–130 µm in diameter. The best result was achieved from oocytes of >100 µm in diameter that reached the metaphase II stage, at 19% (18). In the current study, the oocytes were not classified according to their sizes.

Farstad (4) reported that canine oocytes can be brought to meiosis in vitro spontaneously (GVBD) using adaptations of cattle in vitro techniques. As a result, canine oocytes reaching beyond the GV stage can be affected by season, content of medium, protein supplementation, and adaptation to cattle IVM techniques and culture systems. In the anestrus period, supplementation of oviductal cells with BSA or FCS for the maturation of canine oocytes has no apparent positive effects, at least not in the early stages.

In the bitches in diestrus, the percentage of oocytes having reached the GVBD stage was 20% in Group Ia. In Group IIa, none of the oocytes reached the GVBD stage. However, this rate was 10% when oviductal cells were added to Group IIb. In Group IIIb, the highest GVBD rate was found to be 14.3% (Table 2) after the addition of oviductal cells.

None of the oocytes in the 2 different estrus cycle groups reached the MII stage. This indicates the arrest of the canine oocytes' maturation in the MI stage prior to ovulation. The reason for this is not known, but oocyte proteins, mitogen-activated protein kinase, and maturation-promoting factor cdc2-kinase provide the oocyte maturation and their amounts and sequels are different among mammals (19,20). This situation allows us to suggest less adaptability of the canine oocyte to the in vitro environment when compared with other mammals, owing to hormonal variations in the anestrus stage, and maturation of the oocytes after ovulation, and the fact that these maturation factors in oocytes are not fully activated.

Although Hewitt and England (2) did not report the reproductive stage of the estrus cycle of the dogs in their similar study, when they added 4% and 0.3% BSA to SOF medium, the maturation rate of oocytes were found low in MI/AI/MII at 7% and 5%, respectively. Özdaş et al. (10) recommended that the addition of oviductal cells to SOF medium in 5% CO_2 can improve bovine embryo development. In the present study, TCM-199+BSA with oviductal cells had more influence on reaching the GV stage than SOF medium without oviductal cells, whereas the effects of the 2 media were found to be statistically significant on the GV stage.

In conclusion, the current results showed that supplementation with oviductal cells or manipulation of microenvironmental conditions with addition of sera and proteins during the early maturation of canine oocytes is not sufficient to overcome the block from the MI to the MII stage. It was concluded that in the oocytes obtained from bitches in diestrus and anestrus supplemented with either FCS or BSA in SOF medium without oviductal cells, more positive effects on canine oocyte maturation were seen than with TCM-199 medium supplemented with the same protein sources and oviductal cells.

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