Effects of oocyte culture density on meiotic competence of canine oocytes

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This study was conducted to determine a suitable ratio of oocytes to medium for *in vitro* maturation (IVM) of cumulus–oocyte complexes (COCs) collected from bitches at anoestrus and dioestrus and to examine the meiotic competence of COCs cultured singly or in different group sizes. In the first experiment, different numbers of COCs (5, 10, 15 and 20 per drop) were cultured for 72 h in 100 µl drops of maturation medium. The meiotic competence of oocytes from ovaries at anoestrus was affected by the number of COCs incubated, whereas at dioestrus, the incubation number of COCs had no effect. In the second experiment, COCs were cultured singly or in different group sizes for 72 h by suitable oocyte density according to the reproductive cycle of the donor. In the

anoestrous group, 1, 5 and 10 COCs were cultured in 10, 50 and 100 µl drops of the medium (10 µl per COC), respectively. In the dioestrous group, 1, 5 and 15 COCs were cultured in 7, 35 and 105 µl drops of the medium (7 µl per COC), respectively. There were no differences in the proportions of oocytes reaching metaphase II among the different group sizes in each stage of the reproductive cycle of the donor. The results indicate that the influence of oocyte density on the meiotic competence of oocytes differs according to the stage of the reproductive cycle of the donor. Moreover, the group sizes have no effect on the meiotic competence of oocytes cultured at suitable oocyte density according to the reproductive cycle of the donor.

Introduction

Successful maturation, fertilization and embryo development *in vitro* is a prerequisite for the techniques required for assisted reproduction of endangered canine species. Although studies have examined the feasibility of *in vitro* maturation (IVM) of canine oocytes (Yamada *et al.*, 1992; Nickson *et al.*, 1993; Hewitt and England, 1997; Otoi *et al.*, 2000a), the rates of maturation of oocytes to metaphase II still remain low (<40%). Therefore, the low meiotic competence of canine oocytes cultured *in vitro* is a major obstacle to the *in vitro* production of embryos. Oocytes used for IVM in the dog are usually obtained from deeper cortical follicles by slicing ovaries, to increase the number of oocytes recovered from canine ovaries. The rates of meiotic completion of canine oocytes have varied, because the oocytes used for IVM experiments have been collected from different sources, and cultured by a variety of different culture systems and media (Farstad, 2000). The age of the donor bitch (Hewitt and England, 1998), oestrous cycle stage of the donor (Yamada *et al.*, 1993; Otoi *et al.*, 2001) and oocyte diameter (Hewitt and England, 1998; Otoi *et al.*, 2000a) influence the IVM rates of the oocytes collected from ovaries obtained by ovariectomy. In most

studies, canine oocytes have been cultured using adaptations of bovine and pig IVM techniques. Studies in other species (mice, hamsters and cows) showed that the volume of medium and oocyte or embryo density (number per unit volume) are important factors that influence IVM, *in vitro* fertilization and embryo development (Kato and Tsunoda, 1994; Carolan *et al.*, 1996; Kito *et al.*, 1997).

However, in studies of IVM on canine oocytes, oocytes have been cultured in various volumes of medium and at various oocyte densities that may influence IVM rates of the oocytes (Mahi and Yanagimachi, 1976; Yamada *et al.*, 1993; Hewitt *et al.*, 1998; Fujii *et al.*, 2000).

Nickson *et al.* (1993) reported that the morphological quality of oocytes affects the IVM rates of canine oocytes. They suggested that only oocytes with at least two layers of cumulus cells reached metaphase II. Although a large number of oocytes can be collected from the ovarian tissue of bitches, the proportions of good quality oocytes with at least two layers of cumulus cells from individual ovaries vary greatly from 0% to 50% (Nickson *et al.*, 1993; Fujii *et al.*, 2000). Thus, canine oocytes need to be cultured singly or in small groups for IVM and subsequent culture.

The reproductive biology of bitches is unique. The bitch is monoestrous, and has a 2 month luteal phase and a prolonged, but variable, non-seasonal anoestrus of 3–10 months at the end of both pregnant and non-pregnant

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cycles. The ovaries obtained by ovariectomy in veterinary clinics are mainly at anoestrus or dioestrus, in which follicles on the surface of the ovary are not visible. It has been demonstrated that the meiotic competence of oocytes collected from ovaries of bitches at oestrus is significantly higher than that of oocytes at anoestrus and dioestrus (Yamada *et al.*, 1993; Hewitt and England, 1997). Therefore, improvement of meiotic competence of oocytes from anoestrus and dioestrus has been required for *in vitro* embryo production.

The objectives of the present study were to examine a suitable ratio of oocyte to medium for IVM of canine oocytes collected from ovaries at two stages of the oestrous cycle (anoestrus and dioestrus), and to investigate the effect of culturing oocytes singly or in small groups on the meiotic competence of oocytes.

Materials and Methods

Oocyte preparation

Ovaries were collected from bitches by ovariectomy following anaesthesia at local veterinary practices. The animals were of various breeds and ranged in age from 6 months to 3 years. Both ovaries from each bitch were brought to the laboratory in physiological saline (0.85% (w/v) NaCl) at 30°C within 6 h of removal. Bitches were categorized according to the stage of oestrous cycle by the examination of morphology of reproductive tissue: specific morphological criteria were used to identify these stages according to the study of Hewitt *et al.* (1998). The reproductive status of the donors was categorized as: (1) anoestrus, ovaries without follicles or pronounced luteal tissues; (2) oestrous (follicular phase), one or more visible follicles (2–10 mm in diameter) were present; and (3) dioestrus, one or more pronounced corpora lutea were present. Only ovaries at anoestrus (33 pairs) and dioestrus (46 pairs) were used for this study. The ovaries were placed in TCM-199 medium (Hank's salts) buffered with 25 mmol Hepes buffer l⁻¹ (Gibco, Grand Island, NY) supplemented with 50 µg gentamicin ml⁻¹ (Sigma, St Louis, MO) at 37°C, and then sliced repeatedly to release oocytes. Only non-degenerate cumulus–oocyte complexes (two or more dense layers of cumulus cells and darkly granulated cytoplasm) were collected and suspended in culture medium (TCM-199 medium (Earle's salts) buffered with 25 mmol Hepes buffer l⁻¹ supplemented with 10% (v/v) bitch serum, and 50 µg gentamicin ml⁻¹). The bitch serum used for culture was collected from healthy bitches at the time of ovariectomy and was heat-inactivated for 30 min at 56°C and stored at –30°C until it was used. The concentrations of progesterone and oestradiol of the heat-inactivated serum were 1.6 ng ml⁻¹ and 31.5 pg ml⁻¹, respectively. The concentrations were measured using a luminescence-enhanced immunoassay system (Immulate; Diagnostic Products Co., Los Angeles, CA).

The vitelline diameter of the cumulus–oocyte complexes

(COCs) from ovaries at anoestrus and dioestrus was measured using a calibrated ocular micrometer (OSM-4; Olympus, Tokyo), and the oocytes were divided into two groups according to their diameter: < 110 and > 110 µm. Only oocytes with diameter > 110 µm were selected and used for this study because oocytes of this size have greater potential for maturation than those with diameter < 110 µm (Theiss, 1997).

Oocyte culture

In the first experiment, the effect on IVM rates of the number of COCs incubated and collected from ovaries at each stage of the oestrous cycle was tested. Different numbers of COCs (5, 10, 15 and 20 per drop) were transferred into 100 µl drops (four drops per dish) covered with warm paraffin oil (3.5 ml, Sigma) in a polystyrene culture dish (35 mm × 10 mm; Falcon; Becton Dickinson Labware, NJ), which had been equilibrated in a CO₂ incubator at 38.5°C for 2 h. Subsequently, the COCs were cultured for 72 h at 38.5°C in a humidified atmosphere of 5% CO₂ in air.

In the second experiment, the effect of culturing COCs singly or in different group sizes on IVM rates was tested. As the suitable number of COCs to a 100 µl drop was found to differ at different stages of the oestrous cycle of ovary in the first experiment, different volumes of culture medium to a COC were used in this second experiment. In the anoestrous group, 1, 5 and 10 COCs were incubated in 10 µl drops (ten drops per dish), 50 µl drops (two drops per dish) and 100 µl drops (one drop per dish) respectively, and covered with paraffin oil (3.5 ml) in a polystyrene dish. The volume of culture medium to a COC was 10 µl. In the dioestrous group, 1, 5 and 15 COCs were placed in 7 µl drops (15 drops per dish), 35 µl drops (three drops per dish) and 105 µl drops (one drop per dish) covered with paraffin oil (3.5 ml) in a polystyrene culture dish. The volume of culture medium to a COC was 7 µl. In each group, the ratio of volume of mineral oil to total volume of culture medium in a dish was the same between experimental treatments because the mineral oil may absorb oestradiol in culture medium (Funahashi *et al.*, 1994). These COCs were cultured for 72 h at 38.5°C in a humidified atmosphere of 5% CO₂ in air.

Oocyte fixing and staining

After incubation for 72 h, the COCs were transferred into TCM-199 medium (Hank's salts) buffered with 25 mmol Hepes buffer l⁻¹ supplemented with 0.3% (w/v) hyaluronidase (Sigma) and then vortexed for 5 min to remove cumulus cells. After vortexing, the remaining cumulus cells surrounding oocytes were removed using small glass pipettes. Denuded oocytes were fixed and permeabilized for 15 min at room temperature in Dulbecco's phosphate-buffered saline (PBS; Gibco) supplemented with 3.7% (w/v) paraformaldehyde and 1% (v/v) Triton-X100 (Sigma), and then placed in PBS containing 0.3% (w/v) polyvinylpyrrolidone for 15 min at

Fig. 1. Chromatin configurations in canine oocytes. (a) germinal vesicle; (b) condensed chromatin; (c) metaphase I; (d) metaphase II (m) and polar body (pb). Magnification is $\times 200$.

room temperature. The oocytes were transferred into the small drop comprising PBS supplemented with 90% (v/v) glycerol (Sigma) and $1.9 \mu\text{mol}$ bis-benzimide I⁻¹ (Hoechst 33342, Sigma) on a slide. Subsequently, the oocytes were overlaid with a coverslip supported by four droplets of vaseline/paraffin and incubated overnight at 4°C . The oocytes were examined using a fluorescence microscope with a 355 nm wavelength excitation filter and classified according to chromatin configuration as 'germinal vesicle', 'condensed chromatin', 'metaphase I' or 'metaphase II' (Fig. 1). Those with diffusely stained cytoplasm characteristic of non-viable cells, and those in which chromatin was unidentifiable or not visible, were considered as degenerated.

Statistical analysis

Each experiment was repeated five to seven times. The percentages of oocytes reaching each stage of meiosis after maturation culture were subjected to arc sin transformation before analysis, and then were tested by a post hoc, Fisher's protected least significant difference test (PLSD test).

Differences at $P \leq 0.05$ were considered significant. Data are expressed as means $\pm$ SEM.

Results

Experiment 1

The effect of the incubation number of COCs collected from ovaries at anoestrus and dioestrus on meiotic competence of oocytes cultured in $100 \mu\text{l}$ drop of the maturation medium are shown (Table 1). All oocytes were fixed and stained after culture for 72 h, of which 6.2% (27 of 435) and 4.9% (25 of 510) of oocytes from ovaries at anoestrus (19 pairs) and dioestrus (28 pairs) were lost during fixation and staining, respectively, and the remaining oocytes were analysed for nuclear maturation. In the anoestrous group, the number of COCs incubated affected the meiotic competence of oocytes. When ten COCs were cultured in $100 \mu\text{l}$ drops of the maturation medium, a significantly lower proportion of oocytes remained at the germinal vesicle (GV) stage, compared with groups in which 15 or 20 COCs were cultured ($P < 0.05$). Moreover,

Table 1. Meiotic maturation of canine oocytes cultured for 72 h in 100 µl drop

Stage of oestrous cycle	Number of oocytes cultured (per 100 µl)	Number of oocytes examined*	Mean ± SEM of oocytes at stage				Degenerated
			GV	CC	MI	MII [†]	
Anoestrus	5	96	59.3 ± 5.2 ^{a,b}	6.9 ± 3.1 ^{a,b}	16.2 ± 2.8 ^a	4.6 ± 1.9 ^a	13.1 ± 4.5 ^{a,b}
	10	121	50.1 ± 6.3 ^a	10.9 ± 3.0 ^a	4.7 ± 2.9 ^{b,c}	16.2 ± 4.5 ^b	18.1 ± 4.1 ^{a,b}
	15	92	72.5 ± 3.8 ^{b,c}	6.9 ± 2.6 ^{a,b}	6.0 ± 0.9 ^{a,b}	5.6 ± 1.9 ^{a,b}	9.0 ± 5.3 ^{a,b}
	20	99	84.7 ± 2.0 ^c	2.5 ± 1.5 ^b	1.7 ± 1.1 ^b	7.0 ± 2.5 ^{a,b}	4.0 ± 1.4 ^a
	Total	408	65.3 ± 3.9 ^A	7.0 ± 1.4	7.5 ± 1.7	8.6 ± 1.8	11.5 ± 2.3 ^A
Dioestrus	5	132	46.4 ± 7.9 ^a	10.9 ± 1.7 ^a	7.9 ± 1.5 ^{a,b}	5.6 ± 1.2 ^a	29.2 ± 8.9 ^b
	10	119	49.4 ± 2.6 ^a	8.7 ± 2.0 ^{a,b}	5.8 ± 3.0 ^{b,c}	10.4 ± 5.5 ^{a,b}	25.7 ± 6.5 ^{a,b}
	15	131	39.3 ± 10.1 ^a	6.7 ± 2.7 ^{a,b}	12.3 ± 4.3 ^{a,c}	11.3 ± 4.0 ^{a,b}	30.3 ± 10.7 ^b
	20	103	50.2 ± 10.4 ^a	7.7 ± 1.6 ^{a,b}	13.7 ± 5.6 ^{a,c}	2.2 ± 1.3 ^a	26.3 ± 11.9 ^{a,b}
	Total	485	46.1 ± 3.9 ^B	8.5 ± 1.0	9.8 ± 1.9	7.6 ± 1.9	28.0 ± 4.4 ^B

GV: germinal vesicle; CC: condensed chromatin; MI: metaphase I; MII: metaphase II.

*Of the oocytes examined in each group, 1–17 oocytes were lost during removal of cumulus cells, fixation and staining.

†The MII oocytes had metaphase chromosomes and polar body.

^{a-c}, ^{A-B}Values with different superscripts in the same column differ significantly ($P < 0.05$).**Table 2.** Meiotic maturation of canine oocytes cultured for 72 h by suitable oocyte density according to the reproductive cycle of the donor

Stage of oestrous cycle	Number of oocytes cultured	Volume of medium (µl)	Number of oocytes examined*	Mean ± SEM of oocytes at stage				Degenerated
				GV	CC	MI	MII [†]	
Anoestrus	1	10	85	48.1 ± 3.4	7.9 ± 3.3	21.2 ± 7.4 ^a	7.0 ± 3.3	15.9 ± 4.5
	5	50	109	48.5 ± 5.9	9.2 ± 3.3	12.2 ± 2.0 ^{a,b}	5.3 ± 1.6	24.8 ± 2.5
	10	100	104	45.6 ± 9.7	9.9 ± 4.0	6.1 ± 2.2 ^b	10.4 ± 4.9	28.1 ± 4.8
	Total		298	47.3 ± 3.8	9.0 ± 2.0	13.2 ± 3.1	7.7 ± 2.1	22.8 ± 2.6
Dioestrus	1	7	112	52.6 ± 9.1	6.2 ± 1.3	10.4 ± 1.6 ^{a,b}	11.5 ± 3.7	19.2 ± 10.3
	5	35	92	44.9 ± 8.7	6.0 ± 1.0	11.0 ± 4.0 ^{a,b}	9.9 ± 2.9	28.2 ± 10.9
	15	105	88	68.3 ± 6.5	3.4 ± 0.1	6.8 ± 3.4 ^{a,b}	11.3 ± 5.9	10.2 ± 2.0
	Total		292	53.3 ± 5.4	5.5 ± 0.7	9.8 ± 1.7	10.9 ± 2.0	20.6 ± 5.7

GV: germinal vesicle; CC: condensed chromatin; MI: metaphase I; MII: metaphase II.

*Of the oocytes examined in each group, 2–8 oocytes were lost during removal of cumulus cells, fixation and staining.

†The MII oocytes had metaphase chromosome and polar body.

^{a,b}Values with different superscripts in the same column differ significantly ($P < 0.05$).

significantly more oocytes reached metaphase II in the group cultured with 10 COCs than in the group cultured with 5 COCs ($P < 0.05$). In contrast, there were no effects of the incubation number of COCs on the meiotic competence of oocytes collected from ovaries at dioestrus. However, the proportion of oocytes that reached at least metaphase I tended to increase in the group cultured with 15 COCs, compared with other groups. When the data for the four different incubation numbers of COCs were combined to investigate any effect of the oestrous cycle stage of the ovary, the percentage of oocytes that remained at the GV stage was significantly lower in the dioestrous group than in the anoestrous group (46.1% versus 65.3%, $P < 0.01$).

However, the degeneration rate of oocytes from ovaries at the dioestrous stage significantly increased, compared with that of oocytes at the anoestrous stage (28.0% versus 11.5%, $P < 0.01$). There were no significant differences between the anoestrous and dioestrous groups with respect to the proportion of oocytes reaching metaphase II (8.6% versus 7.6%).

Experiment 2

The effect of culturing COCs singly or in different group sizes on meiotic competence of oocytes cultured in a drop of maturation medium at the fixed ratio of oocyte density

according to the stage of the reproductive cycle of the donor is shown (Table 2). All oocytes were fixed and stained after culture for 72 h, of which 5.4% (17 of 315) and 5.8% (18 of 310) of oocytes from ovaries at anoestrus (14 pairs) and dioestrus (18 pairs) were lost during fixation and staining, respectively. In the anoestrous group, the proportion of oocytes that remained at metaphase I was lower in the group cultured with 10 COCs than in the single-cultured group. However, there were no differences in the proportions of oocytes reaching metaphase II among the different group sizes in each stage of the reproductive cycle of the donor. In comparison with the combined data for the three different incubation numbers of COCs in each oestrous cycle of ovary, there were no significant differences between the anoestrous and dioestrous groups with respect to the proportions of oocytes reaching each meiotic stage.

Discussion

The results of the present study show that oocyte density (number per unit volume) influences the meiotic resumption of oocytes collected from ovaries at anoestrus, but has no apparent effects on the meiotic resumption of oocytes at dioestrus. Even though many studies have been published regarding the meiotic ability of canine oocytes, none has provided data about the oocyte density to medium volume. Petr *et al.* (1989) reported that the incidence of germinal vesicle breakdown (GVBD) decreases with increasing numbers of pig COCs cultured in a drop. Similarly, increasing the number of bovine COCs to a small drop resulted in a significant inhibition of meiotic competence (Sirard *et al.*, 1992). It has been proposed that the factors inhibiting GVBD are produced by the cumulus cells and secreted into a culture medium (Isobe and Terada, 2001). Isobe and Terada (2001) suggested that the GVBD-inhibiting factors inhibit the disruption of gap junctions, thus preventing oocytes from undergoing GVBD by continuous transfer of the inhibitor through the mediation of cumulus cells. It has been shown that a loss of communication between the oocyte and the granulosa cells via gap junctions is responsible for the resumption of oocyte meiotic maturation that occurs after the LH surge (Dekel and Beers, 1978; Gilula *et al.*, 1978; Wert and Larsen, 1990). Wiesen and Midgley (1993) reported that only the granulosa cells of healthy and developing antral follicles express large amounts of connexin 43 gap junction mRNA, but very little connexin 43 gap junction mRNA was detected in the corpora lutea or in follicles undergoing atresia. They suggested that the expression of the gap junction gene is hormonally regulated during follicular growth and development. In the present study, an increase in the proportions of canine oocytes that remained at the GV stage was observed when a large number of COCs from ovaries at anoestrus were cultured in the same volume (100 μ l). At dioestrus, however, the quantitative influence of COCs on the meiotic resumption of oocytes was not

observed under the same conditions. In the combined data, the percentage of oocytes that remained at the GV stage was significantly lower in the dioestrous group than in the anoestrous group, indicating that the number of COCs in which communication was lost between the oocyte and the cumulus cells via gap junctions might increase in the dioestrous group. Therefore, the differences in the quantitative effect of COCs on the meiotic resumption of oocytes between anoestrus and dioestrus may result from the differences of gap junctional communication that occur in the processes of follicular development. However, the degeneration rate of COCs was higher in the dioestrous group than in the anoestrous group. Studies in other species (mice, rats, pigs and cattle) have shown that some cumulus-denuded oocytes can complete meiotic resumption (Binor and Wolf, 1979; Magnusson, 1980; Yamauchi and Nagai, 1999; Geshi *et al.*, 2000), but the removal of cumulus cells before oocyte maturation results in a significant reduction in the nuclear maturation rate (Das *et al.*, 1997). The oocyte and the cells of cumulus and granulosa are interconnected by numerous gap junction channels that pass nucleotides, amino acids and sugars from the cells to the oocyte for growth and development (Zamboni, 1974; Eppig, 1979). Therefore, the increase in the degeneration rate of COCs at dioestrus may be related to the disruption of cumulus–oocyte gap junctions.

The results of the present study show that there were no significant differences between the anoestrous and dioestrous groups with respect to the proportion of oocytes reaching metaphase II. This observation is consistent with a finding by Hewitt and England (1997) who reported that there were no differences in the maturation rates between oocytes collected from ovaries at anoestrus and dioestrus. However, the suitable oocyte density for reaching metaphase II was slightly different between the anoestrous and dioestrous groups in the present study. The oocytes were cultured for 72 h in the same culture medium, indicating that the culture of many COCs in a small amount of medium for a long time raises the possibility that nutrients for oocytes may be depleted from the medium. In contrast, the presence of supplementary granulosa cells during *in vitro* maturation of oocytes has been shown to exert beneficial effects (Fukui *et al.*, 1991; Mochizuki *et al.*, 1991). We previously reported that co-culture with bovine cumulus cells improved the rates of maturation of canine oocytes to metaphase II (Otoi *et al.*, 2000b). It has been demonstrated that a significant amount of protein is accumulated in cumulus cells and secreted during oocyte maturation (Rabahi *et al.*, 1991; Wu *et al.*, 1996). Parrott and Skinner (1999) suggested that the paracrine factors secreted by granulosa cells promote germ cell survival and oocyte growth in cell culture systems. Moreover, the cumulus or granulosa cells act as scavengers, that is, they remove toxic materials from the culture medium (Khurana and Niemann, 2000). Therefore, the difference of suitable oocyte density between the anoestrous and dioestrous groups may indicate that the number of cumulus cells in

COCs that reached the critical level required to ensure full meiotic maturation is slightly different between groups.

In bitches, most studies on IVM have been carried out on group cultures, but the meiotic competence of oocytes cultured singly or in small groups has not been determined. In practice, the culture of canine oocytes singly or in small groups is required, because the numbers of oocytes collected from individual ovaries vary greatly (Nickson *et al.*, 1993; Fujii *et al.*, 2000). In other mammals, it has been demonstrated that the rate of embryo development is density dependent, with single oocyte cultures resulting in inferior rates of development compared with group cultures in drops of the same volume (Lane and Gardner, 1992; Gardner *et al.*, 1994). The developmental competence was improved by culturing embryos in reduced volumes (Lane and Gardner, 1992; Gardner *et al.*, 1994; Kato and Tsunoda, 1994). However, there appears to be little information on the IVM of oocytes cultured singly or in small groups. Fukui *et al.* (2000) reported that the proportions of bovine oocytes that reached metaphase II were not different with regard to the single and group culture in drops of the same volume. In the present study, when canine oocytes were cultured at suitable oocyte density according to the reproductive cycle of the donor, there were no differences in the proportions of oocytes reaching metaphase II among the different group sizes, irrespective of the oestrous cycles of ovaries. Therefore, the present results indicate that the group sizes have no effect on the meiotic competence of oocytes cultured at suitable oocyte density.

Most studies of IVM in bitches have used standard culture techniques using culture media such as TCM199 and Krebs–Ringer bicarbonate solution with or without serum or hormonal supplementation (Yamada *et al.*, 1992; Nickson *et al.*, 1993; Hewitt and England, 1999; Otoi *et al.*, 1999; Saint-Dizier *et al.*, 2001). However, the completion of meiosis obtained in these studies was low, and less than 40% of cultured oocytes reached metaphase II. Songsasen *et al.* (2002) reported that addition of growth hormone or β -mercaptoethanol to maturation medium did not improve nuclear maturation of canine oocytes, and a high concentration of glucose in the medium had a detrimental effect on meiotic maturation of the oocytes. In the present study, the average percentage of oocytes reaching metaphase II was very low (< 11%), irrespective of oocyte source and culture method. Therefore, further study will be required to determine the culture requirements of canine oocytes for completion of meiotic maturation.

In conclusion, the present study indicates that the influence of oocyte density on the meiotic competence of canine oocytes differs according to the oestrous cycle stage of the donor, but the oocyte cultured singly at suitable oocyte density during IVM can be matured to a similar stage to oocytes cultured in groups, irrespective of the oocyte source.

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References

- Binor Z and Wolf DP (1979) *In vitro* maturation and penetration of mouse primary oocytes after removal of the zona pellucida *Journal of Reproduction and Fertility* **56** 309–314
- Carolán C, Lonergan P, Khatir H and Mermillod P (1996) *In vitro* production of bovine embryos using individual oocytes *Molecular Reproduction and Development* **45** 145–150
- Das SK, Chauhan MS, Palta P and Tomer OS (1997) Influence of cumulus cells on *in vitro* maturation of denuded buffalo oocytes *Veterinary Record* **141** 522–523
- Dekel N and Beers WH (1978) Rat oocyte maturation *in vitro*: relief of cyclic AMP inhibition by gonadotropins *Proceedings National Academy of Sciences USA* **75** 4369–4373
- Eppig JJ (1979) A comparison between oocyte growth in coculture with granulosa cells and oocyte with granulosa cell–oocyte junctional contact maintained *in vitro*. *Journal of Experimental Zoology* **209** 345–353
- Farstad W (2000) Assisted reproductive technology in canid species *Theriogenology* **53** 175–186
- Fujii M, Otoi T, Murakami M, Tanaka M, Une S and Suzuki T (2000) The quality and maturation of bitch oocytes recovered from ovaries by the slicing method *Journal of Veterinary Medical Science* **62** 305–307
- Fukui Y, MacGowan LT, James RW, Pugh PA and Tervit HR (1991) Factors affecting the *in vitro* development to blastocysts of bovine oocytes matured and fertilized *in vitro*. *Journal of Reproduction and Fertility* **92** 125–131
- Fukui Y, Kikuchi Y, Kondo H and Mizushima S (2000) Fertilizability and developmental capacity of individually cultured bovine oocytes *Theriogenology* **53** 1553–1565
- Funahashi H, Cantley T and Day BN (1994) Different hormonal requirements of pig oocyte–cumulus complexes during maturation *in vitro*. *Journal of Reproduction and Fertility* **101** 159–165
- Gardner DK, Lane M, Spitzer A and Batt PA (1994) Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage *in vitro* in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development *Biology of Reproduction* **50** 390–400
- Geshi M, Takenouchi N, Yamauchi N and Nagai T (2000) Effects of sodium pyruvate in nonserum maturation medium on maturation, fertilization, and subsequent development of bovine oocytes with or without cumulus cells *Biology of Reproduction* **63** 1730–1734
- Gilula NB, Epstein ML and Beers WH (1978) Cell-to-cell communication and ovulation. A study of the cumulus–oocyte complex *Journal of Cell Biology* **78** 58–75
- Hewitt DA and England GCW (1997) Effect of preovulatory endocrine events upon maturation of oocytes of domestic bitches *Journal of Reproduction and Fertility Supplement* **51** 83–91
- Hewitt DA and England GCW (1998) The effect of oocyte size and bitch age upon oocyte nuclear maturation *in vitro*. *Theriogenology* **49** 957–966
- Hewitt DA and England GCW (1999) Influence of gonadotrophin supplementation on the *in vitro* maturation of bitch oocytes *Veterinary Record* **144** 237–239
- Hewitt DA, Watson PF and England GCW (1998) Nuclear staining and culture requirements for *in vitro* maturation of domestic bitch oocytes *Theriogenology* **49** 1083–1101
- Isobe N and Terada T (2001) Effect of the factor inhibiting germinal vesicle breakdown on the disruption of gap junctions and cumulus expansion of pig cumulus–oocyte complexes cultured *in vitro*. *Reproduction* **121** 249–257
- Kato Y and Tsunoda Y (1994) Effects of the culture density of mouse zygotes on the development *in vitro* and *in vivo*. *Theriogenology* **41** 1315–1322
- Khurana NK and Niemann H (2000) Effects of oocyte quality, oxygen tension, embryo density, cumulus cells and energy substrates on cleavage and morula/blastocyst formation of bovine embryos *Theriogenology* **54** 741–756
- Kito S, Iritani A and Bavister BD (1997) Effects of volume, culture media and type of culture dish on *in vitro* development of hamster 1-cell embryos *Theriogenology* **47** 541–548

- Lane M and Gardner DK (1992) Effect of incubation volume and embryo density on the development and viability of mouse embryos *in vitro*. *Human Reproduction* **7** 558–562
- Magnusson C (1980) Role of cumulus cells for rat oocyte maturation and metabolism *Gamete Research* **3** 133–140
- Mahi CA and Yanagimachi R (1976) Maturation and sperm penetration of canine ovarian oocytes *in vitro*. *Journal of Experimental Zoology* **196** 189–196
- Mochizuki H, Fukui Y and Ono H (1991) Effect of the number of granulosa cells added to culture medium for *in vitro* maturation, fertilization and development of bovine oocytes *Theriogenology* **36** 973–986
- Nickson DA, Boyd JS, Eckersall PD, Ferguson JM, Harvey MJA and Renton JP (1993) Molecular biological methods for monitoring oocyte maturation and *in vitro* fertilization in bitches *Journal of Reproduction and Fertility Supplement* **47** 231–240
- Otoi T, Fujii M, Tanaka M, Ooka A and Suzuki T (1999) Effect of serum on the *in vitro* maturation of canine oocytes *Reproduction, Fertility and Development* **11** 387–390
- Otoi T, Fujii M, Tanaka M, Ooka A and Suzuki T (2000a) Canine oocyte diameter in relation to meiotic competence and sperm penetration *Theriogenology* **54** 535–542
- Otoi T, Murakami M, Fujii M, Tanaka M, Ooka A, Une S and Suzuki T (2000b) Development of canine oocytes matured and fertilised *in vitro*. *Veterinary Record* **146** 52–53
- Otoi T, Ooka A, Murakami M, Karja NW and Suzuki T (2001) Size distribution and meiotic competence of oocytes obtained from bitch ovaries at various stages of the oestrous cycle *Reproduction, Fertility and Development* **13** 151–155
- Parrott JA and Skinner MK (1999) Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis *Endocrinology* **140** 4262–4271
- Petr J, Zetova L, Fulka JJ and Jilek F (1989) Quantitative inhibitory influence of porcine cumulus cells upon the maturation of pig and cattle oocyte *in vitro*. *Reproduction Nutrition Development* **29** 541–550
- Rabahi F, Monniaux D, Pisselet C, Chupin D and Durand P (1991) Qualitative and quantitative changes in protein synthesis of bovine follicular cells during the preovulatory period *Molecular Reproduction and Development* **30** 265–274
- Saint-Dizier M, Renard JP and Chastant-Maillard S (2001) Induction of final maturation by sperm penetration in canine oocytes *Reproduction* **121** 97–105
- Sirard MA, Coenen K and Bilodeau S (1992) Effect of fresh or cultured follicular fractions on meiotic resumption in bovine oocytes *Theriogenology* **37** 39–57
- Songsasen N, Yu I and Leibo SP (2002) Nuclear maturation of canine oocytes cultured in protein-free media *Molecular Reproduction and Development* **62** 407–415
- Theiss T (1997) *Investigations on the Collection, In Vitro Maturation and Fertilization of Dog Oocytes* PhD Thesis, Tierärztliche Fakultät der Ludwig-Maximilian Universität München
- Wert SE and Larsen WJ (1990) Pre-endocytotic alterations in cumulus cell gap junctions precede meiotic resumption in the rat cumulus–oocyte complex *Tissue and Cell* **22** 827–851
- Wiesen JF and Midgley ARJ (1993) Changes in expression of connexin 43 gap junction messenger ribonucleic acid and protein during ovarian follicular growth *Endocrinology* **133** 741–746
- Wu B, Ignatz GG, Currie WB and Yang X (1996) Temporal distinctions in the synthesis and accumulation of proteins by oocytes and cumulus cells during maturation *in vitro* of bovine oocytes *Molecular Reproduction and Development* **45** 560–565
- Yamada S, Shimazu Y, Kawaji H, Nakazawa M, Naito K and Toyoda Y (1992) Maturation, fertilization and development of dog oocytes *in vitro*. *Biology of Reproduction* **46** 853–858
- Yamada S, Shimazu Y, Kawano Y, Nakazawa M, Naito K and Toyoda Y (1993) *In vitro* maturation and fertilization of preovulatory dog oocytes *Journal of Reproduction and Fertility Supplement* **47** 227–229
- Yamauchi N and Nagai T (1999) Male pronuclear formation in denuded porcine oocytes after *in vitro* maturation in the presence of cysteamine *Biology of Reproduction* **61** 828–833
- Zamboni L (1974) Fine morphology of the follicle wall and follicle cell–oocyte association *Biology of Reproduction* **10** 125–149

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