Effects of oocyte culture density on meiotic competence of canine oocytes

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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 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 in different group sizes for 72 h by suitable oocyte density according to the reproductive cycle of the donor. In the 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).

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 anoestru and dioestru 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 anoestru was affected by the number of COCs incubated, whereas at dioestru, 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.

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cycles. The ovaries obtained by ovariohysterectomy in veterinary clinics are mainly at anoestrous or dioestrous, 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 dioestrous 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 ovariohysterectomy 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) anoestrous, 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) dioestrous, one or more pronounced corpora lutea were present. Only ovaries at anoestrous (33 pairs) and dioestrous (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 0.3% (w/v) hyaluronidase and 10% (v/v) bitch serum, and medium (Earle’s salts) buffered with 25 mmol Hepes

buffer L−1 supplemented 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

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
room temperature. The oocytes were transferred into the small drop comprising PBS supplemented with 90% (v/v) glycerol (Sigma) and 1.9 μmol bis-benzimide l⁻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 ± 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 μl 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 μl 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

<table>
<thead>
<tr>
<th>Stage of oestrous cycle</th>
<th>Number of oocytes cultured (per 100 μl)</th>
<th>Number of oocytes examined*</th>
<th>Mean ± SEM of oocytes at stage</th>
<th>Degenerated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GV</td>
<td>CC</td>
</tr>
<tr>
<td>Anoestrus</td>
<td></td>
<td></td>
<td>5</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>121</td>
<td>50.1 ± 6.3a</td>
<td>10.9 ± 3.0a</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>92</td>
<td>72.5 ± 3.8bc</td>
<td>6.9 ± 2.6a,b</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>99</td>
<td>84.7 ± 2.0c</td>
<td>2.5 ± 1.5b</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>408</td>
<td>65.3 ± 3.9A</td>
<td>7.0 ± 1.4</td>
</tr>
<tr>
<td>Dioestrus</td>
<td></td>
<td></td>
<td>5</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>119</td>
<td>49.4 ± 2.6a</td>
<td>8.7 ± 2.0b</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>131</td>
<td>39.3 ± 10.1a</td>
<td>6.7 ± 2.7ab</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>103</td>
<td>50.2 ± 10.4a</td>
<td>7.7 ± 1.6b</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>485</td>
<td>46.1 ± 3.9b</td>
<td>8.5 ± 1.0</td>
</tr>
</tbody>
</table>

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 chromosomes and polar body.

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

<table>
<thead>
<tr>
<th>Stage of oestrous cycle</th>
<th>Number of oocytes cultured (μl)</th>
<th>Number of oocytes examined*</th>
<th>Mean ± SEM of oocytes at stage</th>
<th>Degenerated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GV</td>
<td>CC</td>
</tr>
<tr>
<td>Anoestrus</td>
<td>1</td>
<td>85</td>
<td>48.1 ± 3.4</td>
<td>7.9 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>109</td>
<td>48.5 ± 5.9</td>
<td>9.2 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>104</td>
<td>45.6 ± 9.7</td>
<td>9.9 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>298</td>
<td>47.3 ± 3.8</td>
<td>9.0 ± 2.0</td>
</tr>
<tr>
<td>Dioestrus</td>
<td>1</td>
<td>85</td>
<td>52.6 ± 9.1</td>
<td>6.2 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>92</td>
<td>44.9 ± 8.7</td>
<td>6.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>88</td>
<td>68.3 ± 6.5</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>292</td>
<td>53.3 ± 5.4</td>
<td>5.5 ± 0.7</td>
</tr>
</tbody>
</table>

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 chromosomes 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 anoaestrous 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 anoaestrous stage (28.0% versus 11.5%, P < 0.01). There were no significant differences between the anoaestrous 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 groups sizes on meiotic competence of oocytes cultured in a drop of maturation medium at the fixed ratio of oocyte density...
Oocyte density and meiotic competence of canine oocytes

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 μl). 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 (Rabah 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; Fuji 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. Fuji 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; Otô 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


Magnusson C (1980) Role of cumulus cells for rat oocyte maturation and metabolism Gamete Research 3 133–140


Mochizuki H, Fukui Y and Ono H (1991) Effect of the number of granulosa cells added to the culture medium for in vitro maturation, fertilization and development of bovine oocytes Theriogenology 36 973–986


Parrott JA and Skinner MK (1999) Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis Endocrinology 140 4262–4271


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


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