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Original article

Influence of maturation culture period on the development of canine oocytes after in vitro maturation and fertilization

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Abstract – The objective of this study was to determine an optimum maturation period of canine oocytes for the development in vitro after in vitro fertilization (IVF). Canine oocytes larger than 110 micrometers in diameter, which were collected from ovaries at the follicular phase of the reproductive cycle, were cultured for each time (48, 72 and 96 h) in TCM 199 medium supplemented with 10% canine serum, fertilized, and then cultured in vitro for 8 days. Significantly more oocytes reached metaphase II (MII) in the 72-h culture group than in the 48-h culture group (25.6% vs. 41.0%). The percentages of oocytes that reached MII or beyond after maturation culture did not differ significantly between the 72- and 96-h culture groups, but the percentage of parthenogenetically activated oocytes in the 96-h culture group was significantly higher than that in the 72-h culture group. The percentages of cleaved embryos after IVF were significantly higher in the 48- and 72-h culture groups than in the 96-h culture group. In the 48-h culture group, 3.9% of fertilized oocytes developed to the 16-cell stage or beyond, but none of the cleaved embryos in the 72- and 96-h culture groups developed to the same stage. These results indicate that full nuclear maturation of oocytes collected from ovaries at the follicular phase occurs after 72 h of in vitro culture. However, an optimum maturation period (48 h) for the in vitro development of canine oocytes after IVF may be different from the period necessary to reach the maximal oocyte maturation rate, when based on the developmental stage of the cleaved embryos.

developmental competence / incubation duration / meiotic maturation / oestrous stage

1. INTRODUCTION

The techniques of in vitro maturation (IVM), fertilization (IVF) and culture (IVC) of canine oocytes may provide useful information for gamete salvage programs and may be applied to the conservation of endangered Canidae. However, due to the particular physiology of the canine female, progress in this area has been relatively slow compared with other species. Canine oocytes are ovulated as immature oocytes at

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the germinal vesicle stage and require 2 to 5 days for the completion of meiotic maturation within the oviducts [1, 2]. Moreover, spermatozoa are already present around ovulated oocytes before the completion of meiotic maturation, because ovulation occurs approximately 2 to 3 days after the onset of oestrus, during which bitches are receptive to mating before ovulation [3]. In the IVM of canine oocytes, some studies have demonstrated that full meiotic maturation occurs after 24–48 h of in vitro culture [4, 5], while other studies indicate that the oocytes cultured for 72–96 h achieved the highest maturation rates [6–8]. Yamada et al. [9] suggested that oocytes from superovulated bitches achieve the highest maturation rates at 72 h of maturation culture, while oocytes from non-superovulated bitches show no tendency for increased maturation rates on culturing for up to 144 h. On the contrary, only four reported studies have shown the development of in vitro-matured and fertilized oocytes to the early embryo stage [9–12]. In the four studies, the oocytes were collected from bitches at various oestrous stages, matured for various incubation times (24–72 h) and fertilized with spermatozoa. Therefore, the relationship between the maturation culture period and development of IVF oocytes remains unclear.

The present study was conducted to determine an optimum oocyte maturation time for the in vitro development of canine oocytes after IVF.

2. MATERIALS AND METHODS

2.1. Oocyte preparation and culture

The ovaries were collected from bitches by ovariohysterectomy following anesthesia at local veterinary practices. The animals were of various breeds and ranged in age from 1 to 3 years. Both ovaries from each bitch were brought to the laboratory in physiological saline (0.85% (w/v) NaCl) at approximately 30 °C within 6 h of removal. The bitches were categorized according to the stage of the oestrous cycle by the examination of the morphological appearance of reproductive tissue, in which specific morphological criteria were used to identify these stages according to the study of Hewitt et al. [7]. The reproductive status of the donors was categorized as follows: (1) anoestrous, ovaries without antral follicles (≥ 2 mm in diameter) and pronounced luteal tissues; (2) oestrous (follicular phase), one or more visible follicles (2–10 mm in diameter); and (3) dioestrous, one or more pronounced corpora lutea (CL). It has been suggested that the stage of the reproductive cycle of the donor influences the maturation frequencies of canine oocytes [8, 13, 14]. If the oocytes collected from ovaries at various stages of the reproductive cycle are used for experiments, the data may compromise the optimum maturation culture period for the development of oocytes after IVF. Moreover, the percentage of oocytes reaching metaphase II (MII) has been shown to be higher in oocytes collected from the ovaries of prooestrous and oestrous bitches than anoestrous and dioestrous bitches [8, 14, 15]. In a previous study, we found that when the reproductive status of the donors was categorized by the morphological criteria described above, more oocytes from ovaries at the follicular phase of the reproductive cycle reached MII [14]. Therefore, only ovaries (20 pairs) at the follicular phase were used for this study. The ovaries were placed in a TCM199 medium (Hank's salts) buffered with 25 mM HEPES buffer (Gibco, Grand Island, NY, USA) supplemented with 50 $\mu g \cdot mL^{-1}$ gentamicin (Sigma, St. Louis, MO, USA) at 37 °C, and then sliced repeatedly to release oocytes. Only non-degenerated cumulus-oocyte complexes (two or more dense layers of cumulus cells and darkly granulated cytoplasm) were collected and then suspended in a maturation medium (TCM199 medium (Earle's salts) buffered with 25 mM HEPES buffer (Gibco) supplemented with 10% (v/v) bitch serum, 1.5 mM sodium pyruvate, 2.9 mM calcium lactate pentahydrate and 50 $\mu g \cdot mL^{-1}$ gentamicin). The bitch serum used for culture was collected from bitches at various stages of the

reproductive cycle and pooled. The serum was heat-inactivated for 30 min at 56 °C and stored at –30 °C until use. The concentrations of progesterone and estradiol 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 (Immulite; Diagnostic Products Co., Los Angeles, CA, USA).

After collection, the oocyte cytoplasm diameter of the cumulus-oocyte complexes (COCs) was measured using a calibrated ocular micrometer (OSM-4; Olympus, Tokyo, Japan), and the oocytes were divided into two groups according to their diameter size: < 110 and > 110 µm. After being measured, only oocytes > 110 µm were selected and used for this study, because a difference in maturation rate has been found for canine oocytes with diameters < 110 µm versus > 110 µm [16, 17].

After the measurement of the oocyte cytoplasm diameter, COCs with > 110 µm in diameter were transferred into 100 µL-drops (10 COCs/drop) of maturation medium covered with warm paraffin oil (3.5 mL, Sigma) in a polystyrene culture dish (35 × 10 mm; Falcon; Becton Dickinson Labware, NJ, USA), which had been equilibrated in a CO_2 incubator at 38.5 °C for at least 2 h. Subsequently, the COCs were cultured for various times (48, 72 and 96 h) at 38.5 °C in a humidified atmosphere of 5% CO_2 in air.

2.2. Oocyte fixing and staining

At the end of the maturation culture period (48, 72 and 96 h), some of COCs (2–7 COCs/group) were transferred into TCM199 medium (Hank salts) buffered with 25 mM HEPES buffer supplemented with 0.3% (w/v) hyaluronidase (Sigma), and then the cumulus cells surrounding the oocytes were removed using small glass pipettes. Denuded oocytes were fixed and permeabilized for 15 min at room temperature in Dulbecco phosphate-buffered saline (PBS; Gibco) supplemented with 3.7% (w/v) paraformaldehyde and 1% (v/v) triton X-100 (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 µM bis-benzimide (Hoechst 33342; Sigma) on a slide. Subsequently, the oocytes were overlaid with a coverslip supported by 4 droplets of vaseline/paraffin and incubated for one night at 4 °C. The oocytes were examined using a fluorescence microscope with a 355 nm wavelength excitation filter. They were classified according to chromatin configuration as the "germinal vesicle", "germinal vesicle breakdown", "metaphase I", or "metaphase II". The oocytes containing a pronucleus or two polar bodies were considered to be parthenogenetically activated. Those with a diffusely stained cytoplasm characteristic of nonviable cells, and those in which chromatin was unidentifiable or not visible, were classified as others.

2.3. In vitro fertilization and culture

In the present study, epididymal spermatozoa were used for IVF, because the fertility of epididymal sperm has been shown to be similar to that of ejaculated sperm [18]. Testes were obtained from adult male dogs of various breeds aged from 1.5 to 3 years after routine castration at local veterinary practices. They were brought to the laboratory in physiological saline (0.85% (w/v) NaCl) at 4 °C within 6 h of removal. The cauda epididymis and vas deferens were removed from each testis. Spermatozoa were collected in a 15 mL-tube by flushing the lumen of the cauda epididymis and vas deferens with about 10 mL of Brackett-Oliphant medium [19] supplemented with 137 µg·mL^{-1} sodium pyruvate and 50 µg·mL^{-1} gentamicin (BO medium). The sperm suspension was centrifuged at 500 *g* for 5 min. The supernatant was removed, and the sperm pellet was resuspended in 12 mL of the BO medium. Subsequently, spermatozoa were allowed to swim-up for 30 min at 38.5 °C

Table I. Meiotic maturation of canine oocytes cultured for various times.

Incubation time (h)	No. of oocytes examined	No. (%) of oocytes at the stage[a]				No. (%) of activated oocytes[b]	No. (%) of oocytes classified as others[c]
		GV	GVBD	MI	MII		
48	82	27 (32.9)[d]	8 (9.8)	10 (12.2)	21 (25.6)[d]	1 (1.2)[d]	15 (18.3)[d]
72	122	32 (26.2)[d,e]	8 (6.6)	18 (14.8)	50 (41.0)[e]	5 (4.1)[d]	9 (7.4)[e]
96	79	13 (16.5)[e]	6 (7.6)	16 (20.3)	14 (17.7)[d]	17 (21.5)[e]	13 (16.5)[d]

[a] GV: germinal vesicle, GVBD: germinal vesicle breakdown, MI: metaphase I, MII: metaphase II.
[b] Oocytes containing a pronucleus or two polar bodies were considered to be parthenogenetically activated.
[c] Oocytes with diffusely stained cytoplasm characteristic of nonviable cells or unidentifiable chromatin were classified as others.
[d,e] Values with different superscripts in the same column differ significantly ($P < 0.05$).

in a humidified atmosphere of 5% CO_2 in air. The swim-up layer was carefully recovered and centrifuged at 500*g* for 5 min. The supernatant was removed, and the sperm pellet was resuspended in 500 µL of the BO medium before the assessment of sperm concentration and motility. Semen was assessed for sperm motility using an optical microscope. Only samples with sperm motility > 80% were used for IVF. After the assessment of motility, the spermatozoa were adjusted to 1×10^7 sperm·mL^{-1} in the BO medium, and then diluted with additional BO medium supplemented with 0.6% bovine serum albumin and 20 µg·mL^{-1} heparin (Novo Industry A/S, Osaka, Japan) to a final concentration of 5×10^6 sperm·mL^{-1}.

The COCs which had been previously matured, were incubated with the sperm suspension (100 µL) for 15 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air. After co-incubation with sperm, these oocytes were transferred into fresh 100 µL-drops (10 oocytes/drop) of culture medium (TCM199 medium (Earle's salts) buffered with 25 mM HEPES buffer (Gibco) supplemented with 10% (v/v) bitch serum, 5 µg·mL^{-1} insulin (Sigma) and 50 µg·mL^{-1} gentamicin) and were cultured for further development at 38.5 °C in a humidified atmosphere of 5% CO_2 in the air. At 42 h after insemination, the culture medium was replaced with fresh medium and the cumulus cells surrounding the zygotes were removed by pipetting. The cumulus layer attached to the bottom of the culture dish, however, was not removed. For examination of the development of inseminated oocytes, the embryos were cultured for 8 days after insemination, then fixed and stained by Hoechst 33342 as described above. The cell number per embryo was examined using a Nikon Diaphot microscope fitted with epifluorescent illumination.

2.4. Statistical analysis

Six to nine independent trials for each experiment were conducted and the results from all replicates were pooled for analysis. The percentages of oocytes reaching each stage of meiosis after maturation culture and embryos developed to each stage were analyzed by Chi-square analysis. When some expected values were ≤ 5, Fisher's exact probability test was used. Differences at a probability $P \leq 0.05$ were considered significant.

3. RESULTS

A total of 604 COCs with > 110 µm in diameter were obtained from 20 bitches at the follicular phase of the reproductive cycle (30.2 ± 3.6 COCs per bitch). Table I shows the effect of the culture period of COCs on the meiotic maturation of the oocytes. The proportion of oocytes that

Table II. Developmental competence of canine oocytes matured for various times followed by co-incubation with sperm.

Incubation time (h)	No. of oocytes inseminated	No. (%) of embryos developed to					Total No. % of cleaved embryos
		2-cell	4-cell	8-cell	16-cell	Morula	
48	103	6 (5.8)[a]	15 (14.6)[a]	5 (4.9)	3 (2.9)	1 (1.0)	30 (29.1)[a]
72	121	17 (14.0)[b]	22 (18.2)[a]	6 (5.0)	0 (0)	0 (0)	45 (37.2)[a]
96	45	0 (0)[a]	1 (2.2)[b]	0 (0)	0 (0)	0 (0)	1 (2.2)[b]

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

were arrested at the germinal vesicle stage gradually decreased as the culture period increased. Significantly more oocytes reached MII in the 72-h culture group than in the 48-h culture group (25.6% vs. 41.0%; $P < 0.05$). The total percentages of oocytes that reached MII and were activated parthenogenetically after maturation culture did not differ significantly between the 72- and 96-h culture groups (45.1%, 55/122 at 72 h and 39.2%, 31/79 at 96 h; $P > 0.05$), while the percentage of parthenogenetically activated oocytes in the 96-h culture group was significantly higher ($P < 0.05$) than that in the 72-h culture group.

The developmental rates of oocytes inseminated after maturation culture for each time are shown in Table II. The percentages of cleaved embryos in the 48- and 72-h culture groups were significantly higher ($P < 0.05$) than those in the 96-h culture group. None of the cleaved embryos in the 72- and 96-h culture groups developed to the 16-cell stage or beyond. In the 48-h culture group, 3.9% of fertilized oocytes developed to the 16-cell stage or beyond, and only one embryo developed to the morula stage containing 48 cells.

4. DISCUSSION

There is little literature on the in vitro production of canine embryos because of the low meiotic competence of the oocytes. To date, the highest rate (40%) of maturation to MII of the oocytes from non-stimulated bitches was reported by Nickson et al. [4] in which the oocytes were cultured for 48 h. In other studies, even though the oocytes were cultured for 72–96 h, the rates of maturation to MII of oocytes from non-stimulated bitches at various stages of the reproductive cycle were less than 20% [7, 8, 12, 21]. In the present study, however, when the oocytes collected from ovaries at the follicular phase were cultured for 72 h, 45% of the oocytes achieved MII, including parthenogenetically activated oocytes (4%). This rate was higher than that reported by Yamada et al. [9] in which 32% of the oocytes from superovulated bitches reached MII. In the present study, only oocytes with > 110 μm were used for maturation culture and they were cultured in medium supplemented with bitch serum. It has been suggested that the stage of the reproductive cycle of the donor [8, 13, 14], oocyte diameter [14] and protein supplementation of IVM medium [21] influence the maturation frequencies of canine oocytes. Therefore, the variations of maturation rates between these studies may result from the differences of oocyte source and in vitro culture methods used for IVM.

Yamada et al. [8] reported that oocytes from superovulated bitches achieved the highest maturation rates after 72 h of culture. In the present study, similarly, the highest rate of oocytes reaching MII was observed at 72 h of culture, while the rates significantly increased during 48–72 h of culture and did not change during 72–96 h of culture. This observation was different

from that of previous reports in which the rate of oocytes reaching MII did not increase significantly during 48–72 h of culture [4, 5, 8]. The difference in the relationship between the incubation period and maturation rates of oocytes is unclear, but our results indicate that the maximum rate of meiotic maturation of the oocytes collected from ovaries at the follicular phase occurs after 72 h of in vitro culture.

Songsasen et al. [12] obtained canine embryos at the cleavage stage after IVM/IVF, in which the oocytes were matured for 48 h. England et al. [10] demonstrated a single pregnancy after transferring 1- and 2-cell zygotes/embryos from IVF of canine oocytes matured between 24 and 72 h. Only one study reported the production of a single blastocyst from oocytes matured for 72 h followed by IVF [11]. However, an optimum maturation period for in vitro development of canine oocytes after IVF remains unclear. It has been shown that sperm penetration induces a resumption of meiosis in some oocytes fertilized at the germinal vesicle stage [5]. In cattle, only oocytes that reach metaphase I or beyond are stimulated by sperm penetration to complete meiotic maturation [22]. When we assessed the presence of spermatozoa in the cytoplasm of canine oocytes collected from ovaries at the follicular phase, matured for 72 h and co-incubated with spermatozoa for 15 h, 42% (22/52) of the oocytes were penetrated by spermatozoa but only 19% (10/52) of the oocytes reached MII or the pronuclear stage (unpublished data). The percentage of the oocytes reaching MII after IVF was lower than that of the oocytes matured for 96 h in the maturation medium. Since we used so few oocytes for the assessment of fertilization, it was not possible to draw any conclusions regarding the effects of sperm penetration on the nuclear maturation of oocytes. In the present study, we observed that the cleavage rates of oocytes matured for 48 h and 72 h were higher than those of oocytes matured for 96 h. About 4% of the oocytes matured for 48 h could develop to the 16-cell stage or beyond, but none of the oocytes matured for 72 h developed to the same stage. These results indicate that the developmental competence after cleavage is superior when the oocytes are matured for 48 h and then fertilized in vitro.

In the present study, the rates of parthenogenetic activation significantly increased when the oocytes were matured for 96 h. Moreover, most oocytes cultured for 96 h could not cleave after IVF. A previous study has demonstrated that aged bovine oocytes are more easily activated by chemical treatment [23]. The activation process evokes exocytosis of cortical granules followed by modification of the zona pellucida that may result in a block to fertilization by sperm [20, 24]. Studies employing in vitro fertilization have shown that postovulatory aging of oocytes directly contributes to a reduction in embryonic development in the hamster [25] and the mouse [26]. Chian et al. [27] suggested that aged bovine oocytes have a smaller ability to cleave, even though normal monospermic fertilization may occur. Therefore, the low rates of development of oocytes matured for 96 h may result from the increased proportions of activated and aged oocytes after culture. These results indicate that the maturation culture for beyond 72 h is detrimental to the development of oocytes after IVF.

In conclusion, the results of our experiment demonstrate that maximum meiotic maturation of oocytes occurs after 72 h of in vitro culture, but 48 h of maturation culture is superior to 72 h of maturation culture based on the developmental stage of the oocytes after IVF. Therefore, our results indicate that an optimum maturation period (48 h) for in vitro development of canine oocytes after IVF may be different from the period necessary (72 h) for a maximal proportion of oocytes to complete meiotic maturation.

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