Effects of Estrus Status, Oocyte Diameter and Supplementations on *In Vitro* Maturation of Canine Immature Oocytes

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ABSTRACT

The present study was performed to determine the ability of canine oocytes to achieve nuclear maturation according to oocyte diameter and different culture environments. All of the collected oocytes were classified by grade 1 to 3 and by their diameters such as <100μm, <100μm to <110μm, <110μm to <120μm, >120μm. Oocytes were cultured in culture medium supplemented with 10% FBS, 0.4% BSA, 10% porcine follicular fluid (pFF), 10% canine serum (CS), or 10% canine estrus serum (CES). The mean number of oocytes recovered from estrus status ovaries was significantly higher than that of anestrus status ovaries ($p<0.01$). The maturation rate of grade 1 oocytes (>120μm) was significantly higher than that of the other groups ($p<0.05$). Nuclear maturation to MI to MII in diameter of >110 μm groups was significantly higher than that in <100μm group ($p<0.05$). The oocytes cultured in 10% FBS-supplemented group were significantly higher rate of GVBD compared to the other supplemented groups ($p<0.05$), and oocytes maturation to MI to MII in 10% FBS-, 0.4% BSA-, and 10% pFF-supplemented groups were significantly higher than those in 10% CS-supplemented group ($p<0.05$). Based on these results, the estrus status and the size of oocyte affect positively to improve nuclear maturation of canine immature oocytes *in vitro*. Among several protein sources, porcine follicular fluid was the most effective supplementation to culture medium to achieve higher *in vitro* maturation rate.

(Key words : *In vitro* maturation, Estrus cycle, Oocyte diameter, Protein sources, Canine)

INTRODUCTION

Compared to the efficiency of *in vitro* maturation (IVM) of oocytes from many mammalian species, that of canine oocytes is very low (Hewitt and England, 1998a,b; 1999a,b; Otoi et al., 1999; Farstad, 2000; Saint-Dizier et al., 2001; Songsasen et al., 2002). The lack of an effective procedure for IVM of canine oocytes is a major impediment to the derivation of other assisted reproductive technologies (ART) for this species.

Reproduction in the dog differs significantly from that in other species. Unlike other mammalian species, canine oocytes are ovulated at the germinal vesicle (GV) stage, and undergo maturation in the distal part of the oviduct (Holst and Phemister, 1971; Phemister et al., 1973). Canine oocytes require at least 48hr to 72hr to complete meiotic maturation both *in vivo* and *in vitro* (Mahi and Yanagimachi, 1976; Nickson et al., 1993).

These differences may contribute to the unsuccessful application to the dog of IVM methods developed for other species. To improve the IVM rate to metaphaseII (MII), several categories have been considered such as estrus cycle (Yamada et al., 1992, 1993; Lonergan et al., 1996; Willingham-Rocky et al., 2003; Kim et al., 2004), grade or diameter of oocyte (Hewitt and England, 1997, 1998a; Otoi et al., 2000), and different protein sources into culture medium (Hewitt and England, 1998b; England et al., 2001). However, there are some controversies on the maturation rate among researchers.

The present study was performed to investigate the effect of ovarian estrus stage on the number of oocytes collected and the oocyte diameter and supplementation of medium with different protein sources on IVM of canine immature oocytes.

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MATERIALS AND METHODS

Evaluation of Estrus Cycle Stages

Ovaries were placed into phosphate buffered saline (PBS, Sigma, USA) supplemented with 100IU/ml penicillin (Sigma) and 50μg/ml streptomycin (Sigma) for transport. All tissue was transported to the laboratory in PBS at 37℃ and was processed within 2h of surgery. The stage of the estrus cycle of each bitch was determined using both histological observation and measurement of plasma progesterone concentrations (Hewitt and England, 1998b).

Oocyte Collection, Grading, and Culture

Reproductive tracts from normal bitches 1 to 2 year of age were collected after routine ovariohysterectomy at private clinics, placed immediately into physiological saline at 37℃ and transported to the laboratory within 1h. Ovaries removed from the tract were washed free of blood in fresh PBS and minced with a #10 scalpel blade at room temperature in washing medium (TCM-199, Life Technologies, Rockville, MD) supplemented with 25mM HEPES (Life Technologies), 0.3% BSA (Sigma, USA) and 1% penicillin-streptomycin solution (Life Technologies). Sliced pieces were washed in washing medium and examined under a dissecting microscope (Nikon, Japan); cumulus-oocyte-complexes (COCs) were collected, and washed three times in washing medium prior to transfer to maturation medium. The COCs were selected with criteria of their size, morphological normality in the cytosol and zona, and number of layers of cumulus cells under an inverted microscope (Olympus Co., Japan) at × 400 magnification. The COCs were graded according to the following criteria: Grade 1: COCs were darkly pigmented and completely surrounded by one or more layers of cumulus cells; Grade 2: COCs were lightly pigmented with incomplete layers of cumulus cells; and Grade 3: COCs were pale colored, often misshapen and without any cumulus cells attached (Hewitt and England, 1997).

The diameter of the oocyte was measured using an eyepiece graticule and stage micrometer system under an inverted microscopy. The COCs were assigned to 4 groups according to size. These were 1) less than 100μm, 2) from 100 μm to 110μm, 3) from 110μm to 120μm, and 4) greater than 120μm in diameter.

Culture Medium

The basic culture medium used was TCM-199 with the addition of 50μg/ml gentamycin, and supplemented with 5 μg/ml FSH, 1μg/ml estradiol-17 (Sigma), 20ng/ml epidermal growth factor (Sigma). The supplementations in culture medium were 10% FBS, 0.4% BSA, 10% porcine follicular fluid (pFF), 10% canine serum (CS, ♂), or 10% canine estrus serum (CES, ♀). And to evaluate the efficiency of coculture system, the COCs were cultured with canine oviduct epithelial cells (COEC). Oocytes were cultured for 96h at 39°C in a humidified environment of 5% CO_2 in air.

Oocyte Staining

Oocytes were placed in a 1% sodium citrate solution for 10 min at room temperature to allow for the dispersal of chromatin. They were then vortexed for 3 min to remove cumulus cells and then positioned on a grease-free slide and overlaid with a coverslip supported by 4 droplets of a vaseline / paraffin mixture (40 : 1). Some pressure was placed on each droplet to secure the oocyte and to lightly compress it onto the slide. The compression was necessary to bring the nuclear material into focus when viewed under light microscopy. The slide was placed in an acetic acid : methanol: chloroform fixative (3 : 6 : 2, v/v) for 2 to 3 rain prior to acetic acid: methanol (1 : 3, v/v)) fixing for 48 h. Oocytes were stained for 30 rain with aceto-orcein (1% in 45% acetic acid) followed by aceto-glycerol (glycerol 20%, acetic acid 20%, distilled water 60%) for differential visualization. Oocytes were examined using phase contrast microscopy at a magnification of ×400.

Determination of the Stage of Nuclear Maturation

At the end of maturation (IVM) culture, COCs were transferred to PBS containing 0.25% (w/v) hyaluronidase (Sigma-Aldrich Corp., St. Louis, MO) for 1min and the cumulus cells were subsequently removed by gentle pipetting. The denuded oocytes were fixed in a 4% formaldehyde-Triton X-100 solution (Sigma-Aldrich) for 15min and were mounted on a slide and stained with 1.9mM Hoechst 33342 (Sigma-Aldrich) in glycerol (Sigma-Aldrich). Chromatin state and position as well as spindle formation of oocytes were evaluated under UV light to determine the stage of meiosis as follows (Hewitt et al., 1998a; Rodrigues and Rodrigues, 2003): germinal vesicle breakdown (GVBD), metaphase I (MI) and metaphase II (MII) stage.

Statistical Analysis

Data were analyzed using the statistical analysis system program (Release 9.1. Cary, NC, USA: Inst. Inc.; 2002). Oocytes were randomly distributed within each experimental group and experiments were repeated at least three times. Experiments were first analyzed for interaction among experimental parameters. The data were further analyzed using Duncan's multiple range tests. Differences of $p<0.05$ were considered statistically significant.

RESULTS

The number of oocytes recovered from ovaries on di-

Table 1. The effect of estrus status on the number of canine oocytes recovered

Status	No. of		No. of oocytes per ovary
	Ovaries	Oocytes	
Estrus	24	1,383	57.6 ± 22.1[a]
Anestrus	25	298	11.9 ± 8.3[b]

[a,b]; $p<0.01$.
The data were expressed as mean ± SD.

fferent estrus status was shown in Table 1. The mean number of oocytes per ovary in estrus cycle was significantly higher than that of anestrus cycle ($p<0.01$). Table 2 was shown the rate of oocyte grades according to the diameter of oocytes. Bigger than 110μm of diameter, the rate of grade 1 oocytes was increased. Especially the rate of grade 1 oocytes in >120μm of diameter group was significantly higher than grade 2 and 3 oocytes ($p<0.05$). The effects of oocyte diameter upon oocyte maturation *in vitro* were shown in Table 3. The number of GVBD was not statistically significant among different size of oocyte groups, however, the M I~M II oocytes was significantly higher in >110μm of diameter groups than in <100μm of diameter group ($p<0.05$). The effect of supplementations added into culture medium upon oocyte maturation *in vitro* was shown in Table 4. Only in 10% FBS group showed significantly higher GVBD rate compared to other groups ($p<0.05$), however, the rate of oocytes reached to M I ~M II in 10% CS group were significantly lower that those of oocytes in 10% FBS, 0.4% BSA, and 10% pFF supplementation groups ($p<0.05$).

DISCUSSION

Ström Holst et al. (2001) reported that the mean number of COCs recovered was 37.2±34.1 per ovary. And age sig-

Table 3. The effect of oocyte diameter upon oocyte maturation *in vitro*

Oocyte diameter (μm)	No. (%) of oocytes*	
	GVBD	MI to MII
<100	20/81 (24.6)	15/81 (18.5)[a]
>100 to <110	22/84 (26.1)	18/84 (21.4)
>110 to <120	15/78 (19.2)	25/78 (32.0)[b]
>120	20/78 (25.6)	26/78 (33.3)[b]

GVBD: germinal vesicle breakdown, M: metaphase.
*; Expressed as number / total (%) oocytes after 96 hours of culture period.
The data for oocytes undergoing the process of germinal vesicle breakdown and those which had completed maturation to metaphase I - metaphase II are combined for the 96-hour culture periods.
[a,b]; $p<0.05$.

Table 4. The effect of supplementations into culture medium upon oocyte maturation *in vitro*

Supplementations	No. (%) of oocytes*	
	GVBD	MI to MII
10% FBS	44/126 (34.9)[a]	38/126 (30.2)[a]
0.4% BSA	22/152 (14.5)[b]	42/152 (27.6)[a]
10% pFF	11/110 (10.0)[b]	39/110 (35.5)[a]
10% CS (♂)	7/139 (5.0)[b]	21/139 (15.1)[b]
10% CES (♀)	16/141 (11.3)[b]	29/141 (20.6)

FBS: fetal bovine serum, BSA: bovine serum albumin, pFF: porcine follicular fluid, CS: canine serum, CES: canine estrus serum, GVBD: germinal vesicle breakdown, M: metaphase.
*; Expressed as number / total (%) oocytes after 96 hours of culture period.
The data for oocytes are combined for the 96-hour culture periods.
[a,b]; $p<0.05$.

Table 2. The rate of oocyte grades according to the diameter of oocytes

Grades†	No. (%) of oocytes*			
	<100μm	>100 to <110μm	>110 to <120μm	>120μm
Grade 1	15/80 (18.8)	24/80 (30.0)	27/80 (33.8)	14/80 (17.5)[a]
Grade 2	26/94 (27.6)	32/94 (34.0)	28/94 (29.8)	8/94 (8.5)[b]
Grade 3	24/80 (30.0)	30/80 (37.5)	20/80 (25.0)	6/80 (7.5)[b]

*; Expressed as number / total (%) oocytes after 96 hours of culture period.
The diameter of the oocyte was measured using an eyepiece graticule and stage micrometer system under an inverted microscopy.
†; Grade 1: COCs were darkly pigmented and completely surrounded by one or more layers of cumulus cells, Grade 2: COCs were lightly pigmented with incomplete layers of cumulus cells, Grade 3: COCs were pale colored, often misshapen and without any cumulus cells attached.
[a,b]; $p<0.05$.

nificantly affected COC recovery rates. From bitches 1~6 years old, 54.2±35.1 COCs/ovary were recovered, compared to 26.4±29.0 from bitches 7~13 years old. However, they didn't examine the number of COC on estrus cycles. Several papers commented that an estrus cycle affects the nuclear maturation (Luvoni et al., 2001; Otoi et al., 2001), but not about the relations between the number of COC and estrus status. In the present study, although the range of age did not differ between two groups, the mean number of oocytes collected is increased significantly from ovaries in estrus cycle compared to those in anestrus group. To obtain higher amount of COC per ovary, the estrus status of donor bitches should be considered.

The recovered oocytes are a heterogeneous population made of competent and non-competent oocytes derived from growing or atretic follicles. In order to select the oocytes, which potentially undergo a successful IVM, some morphological criteria are needed. Among these, oocytes morphology and diameter, as well as cumulus conformation are the most commonly considered. The dog oocyte is distinguished by the presence of large amounts of lipid yolk material that gives a dark and homogeneous appearance to the oocyte (Guraya, 1965). Recently, the grade of oocyte was considered the color of the cytoplasm and the layer of cumulus cells (Hewitt and England, 1997). The diameter of the oocytes is related to the follicular stage at the time of collection and the acquisition of meiotic competence of canine oocyte has been found to increase as the diameter of the oocyte increases.

When oocytes were divided into three groups based on different diameters (>100µm; 100µm; <100µm), only the oocytes >100µm diameter reached meiotic stages ranging from MI to MII in a higher proportion than smaller oocytes (Hewitt and England, 1998a). In subsequent studies (Otoi et al., 2000, 2001), it was reported that oocytes with at least 120 µm achieved MII and oocytes <110µm of diameter had no ability to reach this stage. Taken together of these reports, the good quality canine oocyte must show the following morphological patterns: darkly pigmented and homogeneous cytoplasm, a diameter >100µm, and must be completely surrounded by two or more layers of cumulus cells. In the present study, bigger than 110µm of oocyte diameter groups showed higher portion of grade 1 oocytes, especially in larger than 120µm. And the rate matured to MI to MII in bigger than 110µm of oocyte diameter groups was significantly higher than those in smaller than 100µm group. Like previous studies, it can be suggested that the oocytes bigger than 110µm of diameter showed positive effects to obtain good quality of oocytes before IVM and to achieve higher maturation rate developed to MI to MII.

In vitro maturation of canine oocytes has been performed in media containing concentration ranging from 5~20% of bovine or canine serum and 0.3~0.4% of BSA. Rodrigues and Rodrigues (2003) reported that a comparison between estrous canine and estrous bovine serum showed the greater efficiency at promoting maturation to MI-AI stages of the canine serum, however, the highest rate was less than 10%. On the other hand, Otoi et al. (1999) revealed that a higher rate of maturation was obtained with 10% estrous compared to anestrous canine serum. In the present study, we obtained similar maturation results compared to previous papers, however, the supplementation of canine serum regardless of estrus cycle into culture medium showed lower maturation rate than that of FBS, BSA, and pFF. This study used porcine follicular fluid as a supplementation into culture medium for the first time. Interestingly, the addition of pFF into culture medium showed the highest maturation rate. Although it is difficult to explain exactly why porcine follicular fluid obtains the highest maturation rate compared to other protein sources, it is well known that follicular fluid of dominant follicle contains several hormones such as gonadotropins, steroids and growth factors and for this reason it is difficult to attribute to a specific compound the positive effect on oocyte maturation.

In conclusion, the estrus status and the bigger size of oocyte affect positively to improve the nuclear maturation of canine immature oocytes *in vitro*. Among several protein sources, porcine follicular fluid is one of the most effective supplementations into culture medium to obtain higher *in vitro* maturation rate.

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(Received: 15 June 2005 / Accepted: 25 June 2005)