Morphological Assessment of Ovulated and *In Vitro* Immature Canine Oocytes and Biological Availability according to the Size at Different Reproductive Stages

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ABSTRACT

The growing oocytes become progressively capable of resuming meiosis, and full meiotic competence appear when they are about 80% of the size of fully grown oocytes. As hormonal influences vary at different stages of reproductive cycle, the size of oocytes may vary according to the reproductive stages. The present study was designed to compare the diameter between the ovulated and freshly collected immature canine oocytes. The ovulated oocytes were collected 72 hr after ovulation by oviductal tube flushing by laparotomy under general anesthesia. Immature oocytes were collected by ovarian slicing method. Diameter of all oocytes was measured directly using epiflurescence microscope with a calibrated micro-eyepiece micrometer at ×200 magnification. The thickness of zona pellucida and diameter of cytoplasm were measured separately and recorded. A total of 2209 zona intact oocytes were collected, among them 628 from anestrus, 675 from follicular, 838 from luteal and 68 by fallopian tubes flushing methods. The average number of oocytes was 104.7, 168.8, 119.7 and 11.3 for anestrus, follicular, luteal and fallopian tubes flushing methods, respectively. The average diameters of the ooplasm and oocyte were significantly varied in different reproductive stages as well as with ovulated oocytes ($P<0.05$). The average diameter of ooplasm and oocyte was 115.6 and 127.7, 143.0 and 162.0, 134.6 and 150.6, 159.6 and 185.6 for anestrus, follicular, luteal and ovulated oocytes, respectively. Highest number of oocytes with larger diameter could be collected from the follicular and luteal stages. In conclusion, the follicular and luteal ovaries are the best sources of oocytes for canine IVM.

(Key words : Reproductive stage, Maturation, Oocyte, Diameter, Canine)

INTRODUCTION

Immature canine oocytes, retrieved from ovaries at various reproductive stages, can resume meiosis and complete the nuclear maturation *in vitro* (Hewitt and England, 1997; Otoi *et al.*, 2000; Oh *et al.*, 2005). However, the maturation rate up to the metaphase II (M II) stage is very low compared to other mammals. On average, only 15 to 20% of ovarian oocytes complete nuclear maturation after 48 to 72 hrs culture (Kim *et al.*, 2004; Songsasen and Wildt, 2005). An array of canine *in vitro* maturation (IVM) studies has attempted to associate the stages of reproductive cycle and/ or size of the donor follicle with the meiotic and developmental competence of oocytes. Willingham-Rockey *et al.* (2003) reported that oocytes collected from the bitches in oestrus or luteal stage has increased meiotic competence. In contrast, in a recent study, Songsasen and Wildt (2005) reported that the size of donor follicle influences the developmental competence of oocytes rather than the stages of reproductive cycle.

Bitches are non-seasonal monoestrous animals. The estrous cycle is characterized by a follicular phase (proestrus and early estrus) of 21 days, luteal phase of 2 months and anoestrus of 3~10 months at the end of both pregnant and non-pregnant cycle (Johnston *et al.*, 2001). The hormonal influence on ovary varies in different reproductive stages like in proestrus stage is dominated by gonadotrophin hormones (follicle stimulating hormone and Luteinizing hormone) and estrogens where as luteal stage is dominated by progesterone hormones (Johnston *et al.*, 2001). Accordingly, the ovarian structures like presence or absence of dominant follicle and corpus luteum also vary at different reproductive stages. So, it is likely that the number and the size of oocytes and their developmental competence may vary with the reproductive status of donor bitches.

Diameter of immature oocytes selected for IVM is crucial for the outcome of IVM. Oocytes with larger diameter have been showed more developmental competence. Songsasen and Wildt (2005) reported that nearly 80% of oocytes subjectively selected based upon

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size and obtained from follicles >2 mm in diameter are able to complete nuclear maturation *in vitro*. In contrast, those from smaller follicles either failed to mature or reached MII in much lower proportions. It has been suggested that the dog oocyte must be at least 110 μm in diameter to be able to resume meiosis (Otoi *et al.*, 2001). Besides these, cumulus cell layers surrounding the oocytes and its health are an important parameter for selection of oocyte. Niekson *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 can reach up to the MII stage.

The availability of oocytes, based on the quality, at various reproductive stages has already been reported (Durrant *et al.*, 1998; Hewitt and England, 1998; Hishinuma *et al.*, 2004). However, information regarding the diameter of ovulated oocytes and distribution of oocytes according to size at different reproductive stage are lacking. The objectives of this study were to compare the diameter between the ovulated and *in vitro* collected immature oocytes, and to evaluate the number of oocytes according to the oocyte size that can be recovered from canine ovaries at various reproductive stages. The hypothesis was that the diameter and the number of harvested oocytes may vary with stages of reproductive cycle.

MATERIAL AND METHODS

Care and Use of Animals

Ovulated oocytes were collected from mixed breed bitches, aged between 1 to 7 years. The dogs were cared using facilities, and using procedures, which exceeded the standards established by the Seoul National University for Accreditation of Laboratory Animal Care. The study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals in Seoul National University.

Collection and Assessment of Ovulated Oocytes

The time of ovulation was determined based on our previous experiments (Kim *et al.*, 2004; Lee *et al.*, 2005). Briefly, vaginal histology were performed by staining with with Diff-Quik® stain (International Reagents Corp., Kobe, Japan), examined under light microscope, and classified as described by Concannon and DiGreorio (1986). Blood was assayed for serum progesterone concentration by DSL-3900 ACTIVE® Progesterone Coated-Tube Radioimmunoassay Kit (Diagnostic Systems Laboratories, Inc., Webster, TX, United States). The day on which the progesterone concentration initially reached 4.0 ng/ml or more was regarded as the day of ovulation (Hase *et al.*, 2000) while in vaginal cytology, superficial cells were usually greater than or equal to 90% of epithelial cells (Evans and Savage, 1970). The laparotomy was performed at 72 hr after ovulation following the procedure adopted in our laboratory (Lee *et al.*, 2005). Briefly, the oviductal lumen was cannulated using a 24 gauze hypodermic needle (Angiocath TMPlus, Becton Dickison Korea, Inc). Approximately 7 ml of collection medium (TCM 199 supplemented with HEPES (Invitrogen Corporation, Carlsbed, CA) was flushed through individual oviduct and the media was collected in a sterile plastic petridish. Oocyes were collected and preliminary morphological evaluations were done under stereomicroscope. After that, the oocytes were denuded by agitating them up and down in a mouth pipette in 0.5% (w/v) hyaluronidase solution (Sigma, St Louis, MO) and washed three times with TCM 199 with 2.5 mM HEPES. Then they were stained with 5 μg/ml bisbenzimide (Sigma, St Louis, MO) to determine their nuclear status. The oocytes with a MII nucleus and an extruded polar body were considered matured oocytes.

Determination of Reproductive Stage and Collection of Samples

Estrous cycle stage was evaluated for each bitch through a combination of ovarian morphology and vaginal cytology (Feldman and Nelson, 1996). Briefly, in anestrus, ovaries without follicles or pronounced luteal tissues; follicular stage, one or more visible follicles (2~10 mm in diameter) were present; and luteal stage, one or more pronounced corpora luteal were present. The characteristic ovarian and oocyte morphology at various reproductive stage are shown in Fig. 1. Samples were collected following ovariohysterectomy at various reproductive stages and transported to the laboratory within 1 hr in a physiological saline solution (0.9% sodium chloride) at 37℃. Ovaries were minced with a #10 scalpel blade at room temperature in a handling medium (TCM-199; Invitrogen Corporation) supplemented with 25 mM HEPES (Invitrogen Corporation), 1% charcoal-extracted fetal calf serum (Hyclone, Logan, UT) and 1% penicillin- streptomycin solution (Invitrogen Corporation). Minced ovaries were washed in the handling medium and all oocytes with intact zona pellucida were collected under light microscope.

Experimental Designs

The experiment was conducted to determine the numbers of oocytes that could be retrieved at different reproductive stage according to the diameter. Diameter of all oocytes was measured directly using epifluorescence microscope with a calibrated microeyepiece micrometer at ×200 magnification. The thickness of zona pellucida and diameter of cytoplasm were measured

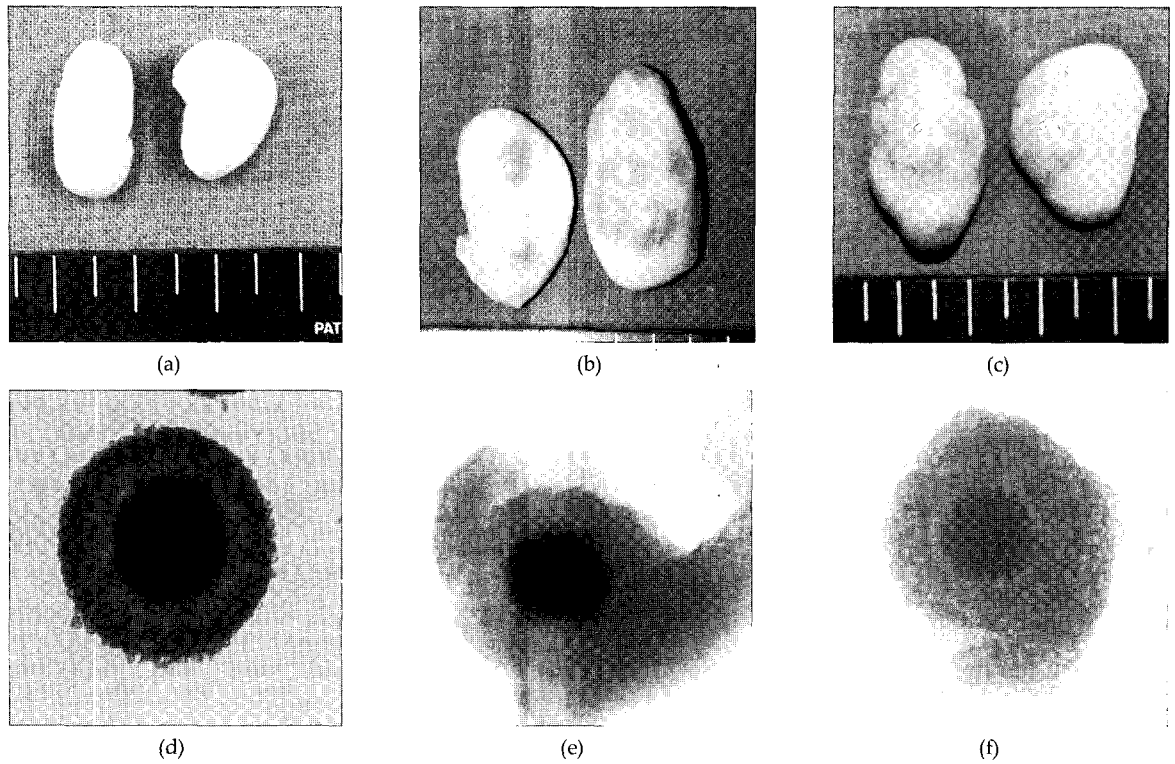

Fig. 1. Canine Ovaries and oocytes collected from various reproductive stages. (a) ovary collected from anestrus stage characterized by smooth ovarian surface, no visible follicle or corpus luteum present; (b) ovary collected from follicular stage characterized by preovulatory follicle; (c) ovaries collected from luteal stage characterized by many corpus lutea; (d) an acceptable quality immature oocytes collected from anestrus stage having more than three layers of cumulus cell and no granulose cell; (e) an acceptable quality immature oocytes collected from follicular stage having more than three layers of cumulus cell and many granulosa cells; (f) an acceptable quality immature oocytes collected from luteal stage having more than three layers of cumulus cell and few granulosa cells. The oocytes were magnified (×200).

separately and recorded.

Statistical Analysis

Data were analyzed using GraphPad Prism version 4.0 (Motulsky, 2003). The descriptive statistics were used to present the data. All the data were compared by one way analysis of variance (ANOVA) followed by least square differences (LSD). In each case, a P value less than 0.05 was considered as significant.

RESULTS

A total of 2209 zona intact oocytes were collected, among them 628 from anestrus, 675 from proestrus, 838 from diestrus and 68 by oviductal tubes flushing methods. The average oocyte number, the average and range of ooplasm and oocyte diameter at various reproductive stages are presented in Table 1. The aver-

Table 1. Average number of oocyte, diameter of ooplasm and oocyte, and ranges of ooplasm and oocyte diameter collected at various stages of reproductive cycle

Parameter	Anestrus	Proestrus	Diestrus	Ovulated
Number of bitches	6	4	7	6
Average no. of oocyte per bitch	104.7±21.4[a]	168.8±47.3[a]	119.7±20.8[a]	11.3±0.4[b]
Average ooplasm diameter (μm)	115.6±0.7[a]	143.0±0.5[b]	134.6±0.6[c]	159.6±2.3[d]
Range of ooplasm diameter (μm)	65 to 150	70 to 175	70 to 160[c]	130 to 225
Average oocyte diameter (μm)	127.7±0.8[a]	162.0±0.4[b]	150.6±0.6[c]	185.6±2.1[d]
Range of oocyte diameter (μm)	70 to 170	105 to 200	80 to 185	165 to 245

The values are mean of percent values ± SEM.
[a-d] Values with different superscripts within the same row are significantly different (P<0.05).

age number of oocytes was 104.7, 168.8, 119.7 and 11.3 for anestrus, follicular, luteal and fallopian tubes flushing methods, respectively. There was no difference in the number of total oocytes in different reproductive stages but a significantly lower number of oocytes were recovered by flushing of fallopian tubes, as it was anticipated.

The average diameter of the ooplasm and oocyte are significantly varied in different reproductive stage. The average diameter of ooplasm and oocyte was 115.6 and 127.7, 143.0 and 162.0, 134.6 and 150.6 for anestrus, follicular and luteal stage, respectively. In case of ovulated oocytes the diameter of ooplasm and oocytes were 159.6 and 185.6 μM, respectively. The diameter of oocyte varies fro 70 to 170, 105 to 200, 80 to 185, and 165 to 245 μM for anestrus, follicular, luteal stage, and ovulated oocytes, respectively. The diameter of oocytes collected from anestrus bitches were 68.8% of the diameter of ovulated oocytes, where as oocytes from follicular and luteal stage were 87.2 and 81.1, respectively.

The distribution of immature canine oocytes according to the ooplasm and oocyte diameter is shown in Fig. 2 and 3, respectively. In case of anestrus stage, 24.1% oocytes were less than 100 μm diameter where as only 1.6% oocytes in luteal stage and no oocytes in follicular stage was less than 100 μm in diameter. The majority of anestrus oocytes (39.4%) were within the diameter of 101 to 130 μm where as luteal oocytes (54.5%) were within 131 to 160 μm and follicular stage oocytes (53.1%) were more than 160 μm in diameter.

DISCUSSION

The success of IVM largely depends on the reproductive stage of donor bitch and size of the selected follicles or oocytes undergoing maturation culture. The present study evaluated the suitability of anestrus, follicular and luteal stage ovaries as a source of oocytes for IVM, and compared the diameter of immature oocytes among different reproductive stage, and with the ovulated oocytes. In the present study, significantly more oocytes with larger diameter were retrieved from the follicular and luteal stage compared to anestrus st-

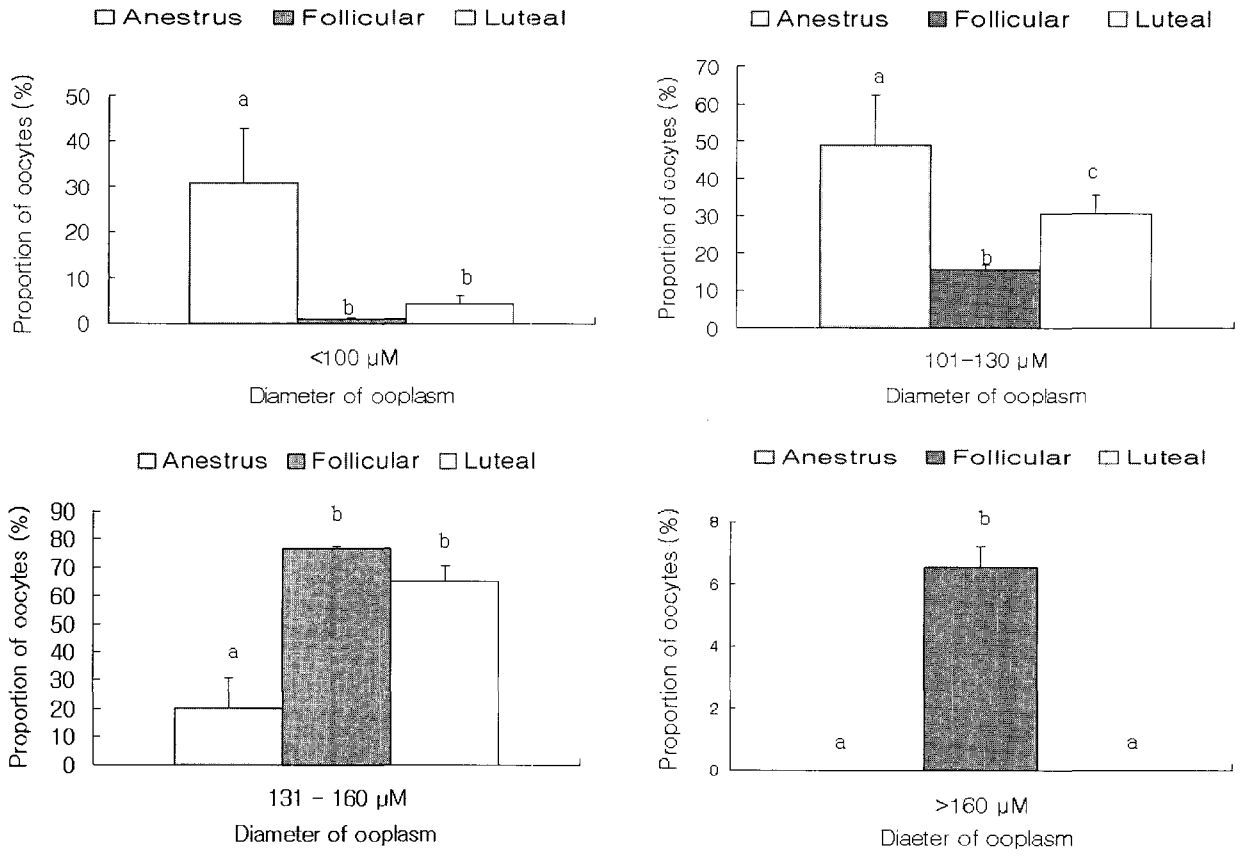

Fig. 2. Frequency distribution of canine oocytes according to the diameter of ooplasm. The ooplasm of all the oocytes with intact zona pellucida were directly measured under an epiflurescence microscope with a calibrated micro eyepiece at ×200. Oocytes obtained from bitches in follicular and luteal stage has larger diameter of ooplasm. [a,b] Different letters indicate significant differences ($P<0.05$).

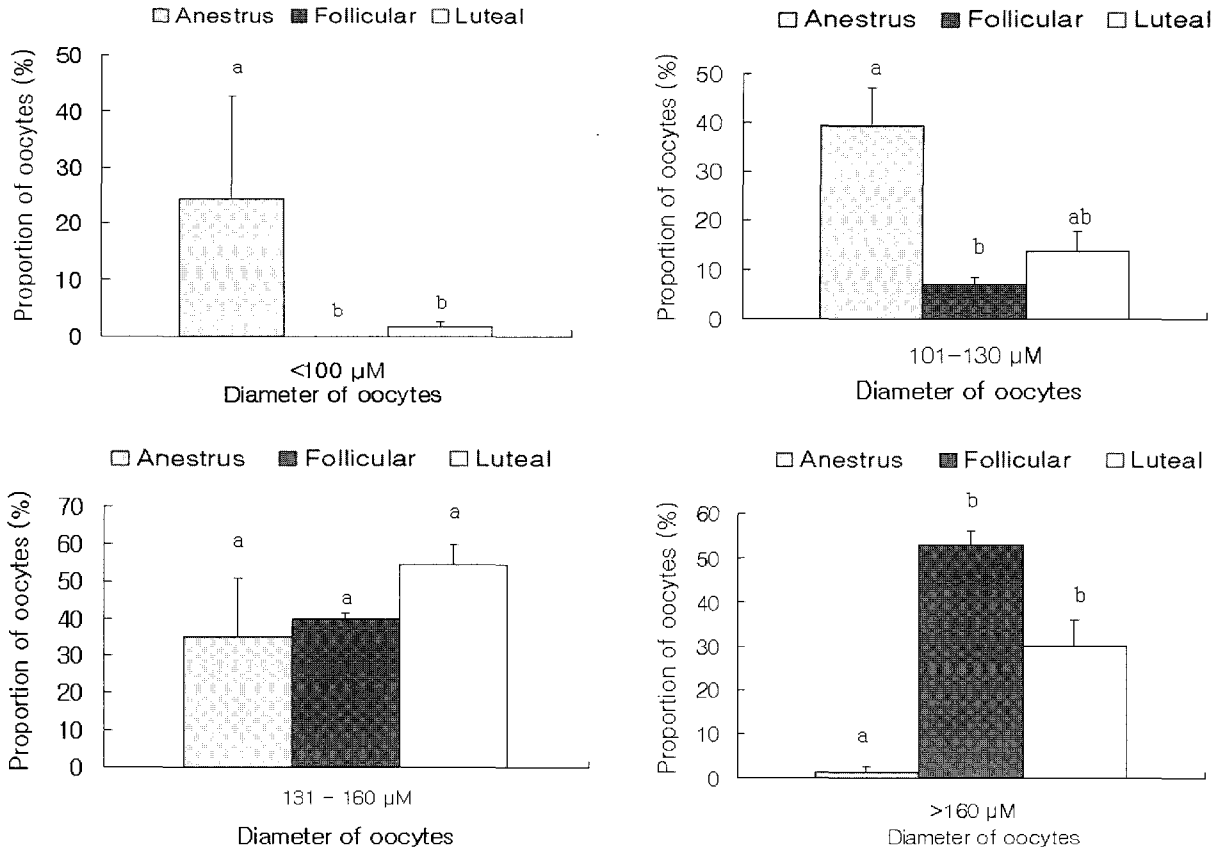

Fig. 3. Frequency distribution of canine oocytes according to the diameter of oocyte. All the oocytes with intact zona pellucida were directly measured under an epiflurescence microscope with a calibrated micro eyepiece at ×200. Oocytes obtained from bitches in follicular and luteal stage has larger diameter. [a,b] Different letters indicate significant differences ($P<0.05$).

age.

The immediate challenge for assisted reproductive technology in canine species is the severe scarcity of mature oocytes. IVM system represents a rich source of mature oocytes. Ovaries, for retrieval of immature oocytes, could be collected from animal hospital following therapeutic or routine ovariohysterectomy, even at post mortem or from convalescent animals. Unfortunately, the IVM procedures used in domestic animals with success have been found to be unsatisfactory for use in canine species (Luvoni *et al.*, 2005). This low rate of maturation may be due to the low intrinsic quality of oocytes selected for IVM. Lonergan *et al.* (2003) reported that the intrinsic quality of the oocyte is the key factor on developmental competence of oocytes. The size of the oocytes may be considered as one of most important parameters of oocytes intrinsic quality. In the present study, the average size of oocytes is found to be varied according to reproductive stages.

There is much debate about the influence of reproductive stage of donor bitch and oocytes size on the success of canine IVM. In general, the developmental competence of an immature oocyte greatly depends on its diameter at collection. According to Thibault *et al.* (1987) the growing oocyte becomes progressively capable of resuming meosis; meotic competence appears when the oocyte is about 80% the size of the fully grown oocyte. In canine species, no information is available at which size an immature oocyte becomes capable of resuming meiosis, at single oocyte level. However, studies on canine IVM revealed that the acquisition of meiotic competence of oocytes increase as the diameter of oocytes increase (Otoi *et al.*, 2000; Hewitt and England, 1998). Otoi *et al.* (2000) reported that canine oocytes acquire the ability to develop to MII at a diameter of 120 μm. In the present study, the average size of an ovulated oocyte was 185.6 μm. Based on these findings and our results, it can be hypothesized that canine oocytes acquire the competence to complete nuclear maturation *in vitro* when they are about 65% of mature oocytes.

The acquisition of meiotic competence in oocytes varies with species. In rodents, oocytes have reached full size and have attained meiotic competence by the time follicle develop an anestrus; in cattle the oocytes are

still growing when follicle reach to the antral size (Gordon, 1994). In canine, growing follicle progressively achieve developmental competence (Songsasen and Wildt, 2005). In the present study, the average size of mature oocytes was significantly higher than immature oocytes, irrespective of the reproductive stage. The oocytes recovered from follicular ovaries were largest and was around 87% of the size of mature oocytes at collection.

The number total of oocytes collected from different reproductive stage did not vary in the present study. Likewise, Durrant *et al.* (2003) reported no significant effect of ovary status on the total number of follicles recovered or the number of follicles per gram of ovary. However, we found more oocytes with larger diameter from follicular and luteal stage compared to anestrus stage. Songsasen and Wildt (2005) reported that higher proportion of oocytes obtained during anestrus and diestrus were from small follicles.

The success of IVM in canine may be associated with the reproductive stage when ovaries were collected (Oh *et al.*, 2005). In a recent study, Songsasen and Wildt (2005) reported that neither stage of reproductive cycle nor season of the year appeared to play major roles in regulating the ability of the intrafollicular oocyte to mature in culture. Rather, size of the 'donor' follicle was the driving force behind the oocyte's ability to progress to MII *in vitro*. The present study revealed that there variation in the availability of larger oocytes at different reproductive stage. In case of anestrus stage, although oocytes have more than three layers of cumulus cells and apparently healthy looking appearance, their average size was smaller compared to luteal and follicular stage.

In conclusion, more oocytes with larger diameter may be collected from follicular and luteal stage ovaries compared to anestrus ovaries. So, follicular and luteal stage ovaries are the best source of immature oocytes for IVM in canine.

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