Size of the Donor Follicle, but not Stage of Reproductive Cycle or Seasonality, Influences Meiotic Competency of Selected Domestic Dog Oocytes

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ABSTRACT Ability of ovarian oocytes from the domestic dog to complete nuclear maturation in vitro (IVM) varies markedly among donors and generally is 20% or less of all oocytes cultured. To identify the cause(s) underlying these significant variations in meiotic maturation (to metaphase II; MII), we retrospectively analyzed data from 1,643 oocytes recovered from 90 bitches for which stage of reproduction and season of year were known. Neither stage of reproduction (proestrus/estrus, diestrus, anestrus, or prepuberty) nor season ($P > 0.05$) influenced the ability of oocytes to achieve nuclear maturation in vitro. A second study was conducted to examine the impact of follicular size on meiotic maturation. Populations of large oocytes were recovered from four categories of follicles (ranging from <0.5 to >2 mm in diameter) and cultured in TCM 199 for 48 hr. Follicular size influenced ($P < 0.05$) meiotic competence. Mean percentages of MII oocytes were 16.9 ± 9.2, 26.1 ± 7.6, 38.4 ± 9.2, and 79.5 ± 10.9 for oocytes recovered from <0.5 mm, ≥0.5–<1 mm, 1–2 mm, and >2 mm diameter follicles, respectively. In summary, stage of reproduction and season have no impact on the ability of dog oocytes to achieve nuclear maturation in vitro. However, we demonstrated for the first time that dog oocytes acquire meiotic competency during follicular development. IVM success of selected oocytes from large size follicles (almost 80%) is about 60% higher than measured in most previous studies involving randomly collected oocytes. *Mol. Reprod. Dev.*

Key Words: dog; oocyte; in vitro maturation; follicle; meiotic competence

INTRODUCTION

The domestic dog has become increasingly important as a model to study human genetic diseases (Patterson, 2000), and its popularity as a companion species is well known (Patronek, 1994). However, it remains difficult to manage valuable genotypes in this species using assisted reproductive technologies, especially through in vitro embryo production. It is generally accepted that lack of an effective procedure to in vitro mature oocytes is the major impediment to using embryo transfer in the dog (see reviews, Farstad, 2000; Luvoni, 2000). Since the first report on in vitro maturation of domestic dog oocytes in 1976 (Mahi and Yanagimachi, 1976) and despite significant efforts (Yamada et al., 1993; Hewitt and England, 1998, 1999; Saint-Dizier et al., 2001; Bogliolo et al., 2002; Bolamba et al., 2002; Otoi et al., 2002; Songsasen et al., 2002, 2003; Luvoni et al., 2003; Rodrigues and Rodrigues, 2003; Willingham-Rocky et al., 2003), little progress has been made in developing a culture system that will consistently achieve nuclear maturation in canine oocytes. On average, only 15%–20% of ovarian oocytes complete nuclear maturation after 48–72 hr of culture.

The domestic bitch is monestrus, ovulating once or twice per year followed by a 2-month luteal phase (irrespective of pregnancy) and a prolonged and variable anestrus (see review, Concannon et al., 1989). Season has no influence on incidence of estrus or anestrus, with the latter characterized by nadir concentrations of circulating progesterone, estrogen and luteinizing hormone (LH, Wildt et al., 1979; Concannon, 1991). The LH surge produces a rapid, final enlargement and luteinization of mature follicles causing ovulation that may occur several days after the LH peak (Wildt et al., 1977; Concannon et al., 1989). Behavioral estrus occurs in the presence of declining circulating estrogen and rising progesterone (Wildt et al., 1979) with the oocytes released from ovarian follicles of 4–13 mm diameter (Wildt et al., 1977; England and Allen, 1989). Unlike other mammalian species studied to date, these oocytes are at the prophase stage of the first meiotic cell cycle (i.e., the germinal vesicle, GV, stage) (Holst and Phemister, 1971; Renton et al., 1991). While in the

Grant sponsor: Smithsonian Institution's Sichel Endowment.

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Received 26 October 2004; Accepted 5 January 2005
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/mrd.20330

oviduct, oocytes undergo germinal vesicle breakdown (GVBD) within 48 hr (Holst and Phemister, 1971; Tsutsui, 1989; Renton et al., 1991) with nuclear maturation completed by 48–72 hr post-ovulation in the presence of elevated, circulating progesterone (Wildt et al., 1979; Concannon et al., 1989).

Our previous IVM studies in the dog have involved collecting ovaries randomly from ovariohysterectomy material provided by local veterinary clinics and then recovering and culturing intra-ovarian oocytes. Particularly interesting has been the wide variation among bitches in the ability of oocytes to complete nuclear maturation in vitro. Proportions of oocytes completing nuclear maturation have ranged from 0% to 70% (Songsasen et al., 2002). Stage of reproductive cycle has been shown to influence meiotic and developmental competence of oocytes in vitro in the human (Trounson et al., 2001) and horse (Goudet et al., 1997). This variable has been studied some in the domestic dog with contradictory results ranging from no association (Hewitt and England, 1997; Rodrigues and Rodrigues, 2003) to a significant relationship between reproductive stage and developmental capacity in vitro (Luvoni et al., 2001; Willingham-Rocky et al., 2003). In terms of seasonal effects, there are no data for the dog, although our laboratory recently reported a strong seasonal influence on the ability of immature cat oocytes to achieve meiotic competence, fertilize and develop to blastocyst in vitro (Spindler and Wildt, 1999).

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., 1997, 2001). However, oocyte size does not appear to be the sole limiting factor influencing in vitro maturation success, largely because only a small proportion of oocytes of this size (~14%) actually develop to MII stage in culture (Nickson et al., 1993; Otoi et al., 1997; Hewitt and England, 1998). Perhaps more significant is follicular size. In other mammals, oocyte meiotic and developmental competence is progressively acquired during follicular growth (Christmann et al., 1994; De Smedt et al., 1994; Lonergan et al., 1994; Crozet et al., 1995; Goudet et al., 1997; Pavlok et al., 1997). For example, in the goat, oocytes from follicles of 0.5–0.8 mm in diameter are only able to begin meiotic resumption, whereas those from follicles of 1–1.8 mm are capable of meiotic progression to metaphase I (MI), while those from follicles >3 mm are able to reach MII (De Smedt et al., 1994). Unlike in the cow, goat, pig, or cat, dog follicles generally remain below the ovarian surface within the cortex and are not visible until late proestrus (Wildt et al., 1977). Although the dog ovary is comprised of many follicular sizes, most are in the early antral stage (i.e., <2 mm in diameter) (Songsasen et al., 2003) and require substantial growth to become preovulatory (i.e., 4–13 mm).

The objectives of this study were to assess the influence of (1) stage of reproductive cycle of the donor, (2) season of year when the oocytes were recovered, and (3) follicular size on the oocyte's ability to complete nuclear maturation. The hypothesis was that stage of reproductive cycle and season were negligible factors, whereas follicle size (especially the predominance of small follicles) is the most significant limiting factor to achieving consistent IVM success in this species. We did not measure the diameter of the oocytes, but rather subjectively selected a population of large oocytes from a pool obtained each day.

MATERIALS AND METHODS

Source of Oocytes

Ovaries were obtained from bitches (6 months–9 years of age) undergoing routine ovariohysterectomy at private animal hospitals. Ovaries were placed in 0.9% NaCl containing 0.06 mg/ml penicillin G sodium and 0.1 mg/ml streptomycin and were transported to the laboratory at room temperature (22–25°C). All oocytes were recovered within 6 hr after ovarian excision. All chemicals were obtained from Sigma Chemical Company (St. Louis, MO), unless otherwise stated.

Study 1: Influence of Stage of Reproductive Cycle and Seasonality on Meiotic Competence

A retrospective analysis was made of data collected over a 3.5 year interval involving 1,643 oocytes obtained from 90 bitches. Numbers of ovarian pairs obtained from January through December (none were obtained in June) were 8, 4, 13, 17, 6, 6, 5, 11, 17, 1, and 2, respectively. In each of these cases, stage of reproduction for the donor was known based on a detailed history provided by the veterinarian as well as our own direct observations of ovarian morphology. Using these metrics, each pair of ovaries was classified as being derived from a bitch in: (1) anestrus, when there were no large follicles or corpora lutea (CL) on the ovarian surfaces; (2) proestrus/estrus, when there were large follicles (≥2 mm) on the ovarian surfaces; (3) diestrus, when there were solely prominent CL on the ovarian surfaces; and (4) prepuberty, when ovaries originated in females 6–8 months of age with neither follicles nor CL evident. Of the 90 ovarian pairs, 17, 23, 25, and 25 were classified as being derived from bitches in anestrus, proestrus/estrus, diestrus, or prepuberty, respectively. Oocytes were collected by repeatedly slicing the ovaries with a scalpel blade followed by repeated rinsing in TCM199 containing HEPES. Only a population of large oocytes with homogeneous dark cytoplasm and ≥2 layers of surrounding cumulus cells were recovered and cultured for 48 hr in TCM 199 plus 0.1% polyvinyl alcohol at 38.5°C in 5% CO_2 in humidified air under various experimental conditions.

Study 2: Influence of Follicular Size on Meiotic Competence

A total of 405 oocytes was obtained from the ovaries of 26 bitches in various stages of the reproductive cycle. Each ovary was horizontally dissected (~5 mm thickness) so that individual follicles could be observed, measured using a stage micrometer and then divided into four classes: (1) <0.5 mm diameter (n = 60); (2) ≥0.5–<1 mm

(n = 176); (3) 1–2 mm (n = 116); and (4) >2 mm (n = 53). The cumulus–oocyte-complex (COC) from each follicle was recovered using a 20-gauge needle to tear the follicular wall. COCs obtained from the same follicular class were grouped together, washed three times in TCM199 containing 25 mM HEPES and cultured (5–10 oocytes/80 μl of IVM medium) for 48 hr in TCM199 plus 0.25 mM pyruvate, 2.0 mM glutamine, 10 ng/ml epidermal growth factor, and 25 μM β-mercaptoethanol at 38.5°C under a humidified atmosphere of 5% CO_2.

Evaluation of Nuclear Status

At the end of a 48 hr maturation period, oocytes were denuded by vigorous shaking in a 3% (w/v) sodium citrate solution followed by fixation in a freshly prepared 1:3 acetic acid:ethanol solution for 48–72 hr. Fixed oocytes were stained with aceto-orcein (1% [w/v] orcein in 45% [v/v] acetic acid), washed in aceto-glycerol (1:1:3 glycerol:acetic acid:distilled water) and then evaluated by light microscopy (400×). Oocytes were categorized as being at one of the following stages: germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), anaphase/telophase I (AI/TI), or metaphase II (MII). In Experiment 1, degenerate oocytes (~20% of cultured oocytes) were included in data analysis. For Experiment 2, data were analysed with and without degenerated oocytes being included.

Statistical Analysis

Average proportions of oocytes developing to various meiotic stages were expressed as mean ± SEM. Proportional data of each meiotic stage were subjected to arcsin transformation. Evaluation of transformed data were performed using analysis of variance (SigmaStat, SPSS, Chicago, IL). Comparisons of means among treatments were conducted using Tukey's multiple comparison test. The distribution of follicular populations among reproductive cycle stages was analyzed using a Chi-square test. The level of significance was set at 95%.

RESULTS

Study 1: Influence of Stage of Reproductive Cycle and Seasonality on Meiotic Competence

Stage of the reproductive cycle had no influence ($P > 0.05$) on the ability of the dog oocyte to complete nuclear maturation in vitro. Average percentages of oocytes developing to MII were 20.8 ± 4.7, 20.5 ± 2.8, 23.8 ± 4.7, and 17.8 ± 5.2 for oocytes recovered from anestrus, proestrus/estrus, diestrus, and prepubertal bitches, respectively. Likewise, months during which oocytes were obtained did not affect overall ability to achieve meiotic competency ($P > 0.05$, Fig. 1). Because oocytes were not available at all reproductive stages during every month, data collected from within the same season (spring [Mar–May], summer [Jun–Aug], fall [Sep–Nov], and winter [Dec–Feb]) were pooled. This allowed examining any potential interaction between reproductive stage and season. Data revealed that oocytes from various reproductive stages completed nuclear maturation at the same incidence regardless of time of year when the oocytes were recovered ($P > 0.05$, Fig. 2).

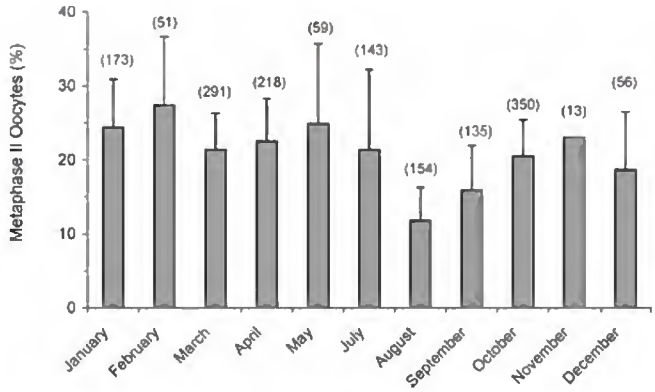

Fig. 1. Proportion of dog oocytes obtained during various months developing to metaphase II stage after 48 hr of culture. Numbers in parentheses above bars indicate number of oocytes. Only one pair of ovaries was obtained in November, and none was obtained in June.

Study 2: Influence of Follicular Size on Meiotic Competence

Of 405 oocytes, 103 were degenerate after 48 hr culture. Although there were no statistical differences among follicular sizes, there was a tendency towards more degeneration in smaller follicles after 48 hr of culture (29.8% ± 13.9%, 22.3% ± 8.2%, 13.1% ± 6.7%, and 12.3% ± 6.3% for <0.5 mm, ≥0.5–<1 mm, 1–2 mm, >2 mm, respectively). Follicular size significantly influenced nuclear maturation of dog oocytes ($P < 0.05$, Table 1) with ~80% of oocytes obtained from >2 mm in diameter follicles having the capacity to reach MII. Although ~12%–21% more oocytes from the 1 to 2 mm follicles developed to MII compared to the smaller follicle groups, the overall differences among these three diminutive size groups were not significant ($P > 0.05$). There were no differences within other meiotic classes from different follicular sizes ($P > 0.05$) with one exception. Overall, more oocytes from the 1 to 2 mm and >2 mm

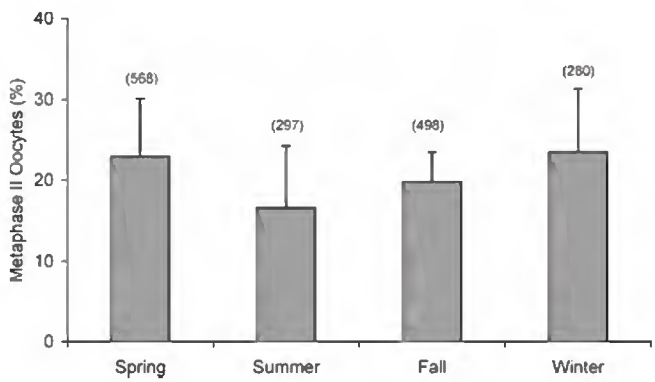

Fig. 2. Proportion of dog oocytes obtained in spring (Mar–May), summer (Jun–Aug), fall (Sep–Nov), and winter (Dec–Feb) developing to metaphase II stage after 48 hr of culture. Numbers in parentheses above bars indicate number of oocytes.

TABLE 1. Meiotic Stages of Dog Oocytes Obtained From <0.5 mm, ≥0.5–<1 mm, 1–2 mm, and >2 mm in Diameter Follicles and Then Cultured for 48 hr In Vitro

Follicle diameter (mm)	Total oocytes	Meiotic stages (mean ± SEM)				
		GV	GVBD	MI	AI/TI	MII
<0.5	38	32.6 ± 17.9	20.8 ± 7.4	19.7 ± 9.4	10.0 ± 10.0	16.9 ± 9.2[b]
≥0.5–<1	122	21.4 ± 7.9	30.5 ± 7.6	21.2 ± 5.3	1.7 ± 1.2	26.1 ± 7.6[b]
1–2	95	5.0 ± 2.8	36.0 ± 3.6	19.0 ± 7.5	1.0 ± 1.2	38.4 ± 9.2[a,b]
>2	46	3.8 ± 2.4	4.1 ± 4.1	9.7 ± 5.0	3.1 ± 3.1	79.5 ± 10.9[a]

GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; AI/TI, anaphase I/telophaseI; MII, metaphase II.
[a,b]Different letters within the same column indicate differences ($P < 0.05$).

diameter follicles resumed meiosis (GVBD/MI/AI/MII) after 48 hr culture compared to counterpart follicles ($P < 0.05$).

There was a relationship between stage of the reproductive cycle and distribution of different size follicles. For example, ovaries of anestrus and diestrus bitches contained more ($P < 0.05$) of the smallest size follicles (<0.5 mm) compared to dogs that were in proestrus or prepuberty (Table 2). Nonetheless, the ovaries of females in proestrus did contain smaller size follicles (>0.5–<1 mm), and, just as interesting, bitches in diestrus maintained larger size follicles, including those that were 1–2 mm and >2 mm in diameter. As expected, most of the >2 mm follicles were recovered from bitches in proestrus. When degenerated oocytes were included in the analysis, a higher proportion of oocytes recovered from females in proestrus developed to MII ($P < 0.05$) compared to those obtained during other reproductive stages (Table 2). However, when degenerated oocytes were excluded, there were no differences ($P > 0.05$) in the numbers of MII oocytes among groups, despite about a 20% higher incidence of nuclear maturation in oocytes from females in proestrus (Table 2).

DISCUSSION

This is the first study to demonstrate that dog oocytes progressively acquire developmental competence during folliculogenesis. Nearly 80% of oocytes subjectively selected based upon size and obtained from follicles >2 mm in diameter had the ready capacity to complete nuclear maturation in vitro. In contrast, those from smaller follicles either failed to mature or reached MII in much lower proportions. Our findings also revealed 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. We suspect that this finding largely explains the wildly variable and overall low IVM success rate for the domestic dog (Songsasen et al., 2002, 2003).

For years, the traditionally low IVM efficiency in the dog has been suspected to be associated with the species' rather peculiar estrual cyclicity. The bitch can show one or two periods of protracted estrus annually that is preceded by a comparatively long proestrus and, in the absence of mating, a prolonged luteal phase followed by widely variant intervals (2–10 months) of anestrus. Although the endocrine events associated with these stages of the cycle have been long characterized and well understood (Wildt et al., 1979; Concannon et al., 1989), knowledge about what triggers the end of anestrus and onset of proestrus remains somewhat mysterious. Circulating FSH increases as anestrus progresses (Concannon et al., 1989), and its increased production seems to be key to the transition from anestrus to proestrus and perhaps even folliculogenesis (Kooistra et al., 1999). Nonetheless, the ability to consistently control ovarian activity by stimulating ovulation with exogenous gonadotropins also remains elusive (Concannon et al., 1997; Jeukenne and Verstegen, 1997). Given these unknowns and the difficulty of provoking follicular growth in vivo, it is natural to assume that perhaps these still-unknown mechanisms also are related to

TABLE 2. Distribution of Oocytes From Each Follicular Class and Meiotic Competence During Various Stages of Reproductive Cycle

Reproductive stage	Total oocytes		Follicular classes (mm) distribution				% oocytes in metaphase II (mean ± SEM)	
	Include degenerated	Exclude degenerated	<0.5	>0.5–<1	1–2	>2	Include degenerated	Exclude degenerated
Anestrus	112	62	39	55	18	0	22.7 ± 7.1[b]	33.1 ± 7.6
Proestrus	103	90	0	19	34	50	46.7 ± 9.6[a]	53.2 ± 10.9
Diestrus	131	101	21	57	50	3	24.5 ± 3.7[b]	34.3 ± 5.9
Prepuberty	59	53	0	45	14	0	11.9 ± 4.2[b]	27.1 ± 9.6

[a,b]Different letters within the same column indicate significant differences ($P < 0.05$).

challenges in achieving IVM success. An array of dog studies has attempted to associate stage of reproductive cycle during which an oocyte is recovered with its meiotic competency. Our finding of no relationship agreed with a report by Hewitt and England (1997) who compared oocytes from proestrus versus estrus and found similar abilities to resume meiosis and develop to the MI/AI/MII stage (19% and 11%, respectively). In our study, ~20% of all oocytes completed nuclear maturation regardless of being recovered from dogs during anestrus, proestrus/estrus, diestrus or prepuberty. Rodrigues and Rodrigues (2003) also reported a similar proportion of oocytes (5%) achieving nuclear maturation from donors in a follicular, luteal or anestrual phase. However, others (Luvoni et al., 2001; Willingham-Rocky et al., 2003) have argued that dog oocytes obtained during anestrus are incapable of completing nuclear maturation in vitro. In our study, we found that higher proportion of oocytes obtained during anestrus and diestrus were from small follicles. High proportions of these oocytes were degenerated after 48 hr culture, and this might affect the overall maturational ability of oocytes obtained from bitches in anestrus and diestrus. However, even given this finding, we concluded that stage of reproductive cycle was not the definitive cause of overall low IVM success in this species. This is because our (1) Study 1 demonstrated no difference among reproductive stages, with equal maturational success (~20%) between nonproliferative (i.e., anestrus, diestrus) and proliferative phases (proestrus/estrus) and (2) Study 2 revealed that larger follicles can exist in the dog cycle during diestrus and smaller follicles during proestrus. It would be worthwhile to examine follicular (and oocyte) status and IVM ability during different times (early, mid, and late) of the anestrual interval. This period of the dog's reproductive cycle is vastly understudied, and more careful characterization and examination may reveal interesting differences in follicle and oocyte populations and status.

The present study also determined that time of year has no significant impact on meiotic competency, as dog oocytes completed nuclear maturation at a similar rate regardless of month. This finding was in contrast to another domesticated carnivore, the cat, where reproductive activity can vary with season (Robinson and Cox, 1970; Johnston, 1987). Nuclear maturation of cultured cat oocytes and ability to fertilize in vitro are greatly diminished during the nonbreeding season of August through October (Spindler and Wildt, 1999). However, the domestic dog is not considered to be a seasonal breeder (Concannon, 1991), so, therefore, it makes sense that our data revealed that season or time of the year had no influence on meiotic competency of immature oocytes.

The incidence of 80% nuclear maturation (from the >2 mm diameter follicles) was easily >50% higher than rates reported when oocytes were not categorized on the basis of donor follicle size (Bogliolo et al., 2002; Bolamba et al., 2002; Otoi et al., 2002; Songsasen et al., 2002, 2003; Luvoni et al., 2003; Rodrigues and Rodrigues, 2003; Willingham-Rocky et al., 2003). The incidence of MII (15%–30%) in smaller size follicles was quite comparable to a host of other reports for this species (Nickson et al., 1993; Yamada et al., 1993; Otoi et al., 2001; Willingham-Rocky et al., 2003). At the same time, the positive impact of larger follicle size in the dog was analogous to findings in other mammals, including the goat (De Smedt et al., 1994; Crozet et al., 1995), pig (Christmann et al., 1994), cow (Lonergan et al., 1994; Pavlok et al., 1997), and horse (Goudet et al., 1997). In fact, the common denominator for all of these species is that oocytes have more capacity to complete nuclear maturation when the host follicle is ≥2 mm in diameter. In the case of the dog, it now is apparent that the wide variability in IVM success observed among studies and bitches most likely is due to the status of the follicular population at the time of ovarian harvest. When larger size follicles are present, the overall incidence of nuclear maturation will be vastly improved compared to times when only small follicles exist.

In terms of mechanisms explaining successful completion of maturation in oocytes from large compared to small size follicles, we suspect the latter fail to provide intrinsic factors essential for driving oocytes to complete the meiotic cell cycle in vitro. In all species studied to date, the initiation and completion of nuclear maturation are driven by maturation promoting factor (MPF), which consists of a protein kinase subunit, $p34^{cdc2}$ and a regulatory subunit, cyclin B1 (Norbury and Nurse, 1992). The activation of MPF triggers a cascade of events associated with nuclear maturation including GVBD and chromosome condensation (Kotani and Yamashita, 2002). MPF activation is regulated by its subunit concentration, intracellular localization and the phosphorylation-dephosphorylation of specific residues (Norbury and Nurse, 1992; Motlik et al., 1998; Abrieu et al., 2001). It has been shown that meiotically incompetent oocytes (i.e., growing or fully grown oocytes obtained from small follicles) are incapable of activating MPF due to the lack of (1) one of the MPF subunits (Chesnel and Eppig, 1995; de Vantéry et al., 1996; Mitra and Schultz, 1996), or (2) upstream regulators driving the cell cycle (Christmann et al., 1994; Dedieu et al., 1998). Mitogen activated protein kinase (MAPK) also has been shown to be associated with events leading to nuclear maturation completion, including microtubule dynamics, spindle assembly, chromosomal condensation and maintaining oocytes at the MII stage (Kotani and Yamashita, 2002; Sun et al., 2002). MPF and MAPK activities recently have been observed in all stages of meiotic maturation in the dog, with increased activity occurring coincidentally with the MI and MII stages (Saint-Dizier et al., 2004). Thus, the mechanisms driving meiotic maturation that were first identified in other species seem to be conserved in the dog, and these are likely immature or incompetent in oocytes recovered from smaller-size follicles. However, this hypothesis requires testing by studying the expression of genes controlling the cell cycle in oocytes recovered from various follicular sizes.

CONCLUSIONS

We conclude that overall low IVM success historically measured in the dog, as well as variations in meiotic capability among bitches, are caused by widely variant populations of follicles of diverse size. Since the sources of most dog oocytes are follicles <2 mm in diameter, IVM success will continue to hover around 20% because oocytes from diminutive follicles likely do not have the appropriate meiotic promoting mechanisms inherent in counterparts from larger follicles.

ACKNOWLEDGMENTS

The authors thank the following veterinary hospitals for providing ovaries for study: Royal Oak Animal Hospital, Stephens City Animal Hospital, Animal Medical Center, New Town Veterinary Clinic, and Cedarville Veterinary Clinics.

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