The Effect of Ovarian Status and Follicular Diameter on Maturational Ability of Domestic Cat Oocytes

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ABSTRACT. The objective of this study was to clarify the effect of ovarian status and follicular size on morphological normality and maturational ability of cat oocytes. Ovarian status was classified into inactive, follicular, luteal and prepubertal, and follicles were classified into three groups according to their diameter (400–800, 800–1200 and 1200–2000 μm). In each ovarian status, the number of follicles decreased but the percentage of morphologically normal oocytes increased with the growth of follicles ($p<0.05$). Only a single follicle that was 1200–2000 μm in diameter was observed in two of the five prepubertal cats. In follicles that were 800–1200 μm in diameter, the percentage of normal oocytes and maturation rate were higher in prepubertal cats than in mature cats ($p<0.05$). Oocyte diameter tended to increase with the growth of follicles. After oocytes were cultured individually in droplets of maturation medium, the oocyte maturation rate increased with the growth of follicles in each ovarian status ($p<0.05$). In conclusion, oocytes collected from larger follicles possess higher maturational ability *in vitro* in sexually mature cats. In prepubertal cats, a higher maturation rate can be obtained from oocytes derived from small follicles compared with in mature cats.
KEY WORDS: feline, follicle, oocyte maturation, ovary, prepuberty.

According to the 2007 IUCN (International Union for Conservation of Nature) Red List [6], most feline species are classified as threatened, vulnerable or endangered. The important role of artificial reproductive technology (ART) as part of a multifaceted captive breeding program for selected feline species is gradually gaining acceptance [19]. Previously, we have reported that the follicular oocytes derived from ovaries of prepubertal cats could develop to blastocysts *in vitro* [28]. However, antral follicles in prepubertal cats were smaller than those in sexually mature cats, and the developmental competence of follicular oocytes in prepubertal cats was lower than that in mature cats. In bovine subjected to advanced ART procedure, the size of the antral follicles has an influence on the *in vitro* development of oocytes [11, 18]. In cats, it has been reported that the *in vitro* maturational and developmental competence of the oocytes were influenced by season [2, 25], ovarian status [2, 8, 9, 16] and morphological quality of the cumulus and oocyte [10, 16, 20, 29]. However, the follicular population in the cat ovaries and influence of follicular size on *in vitro* maturation (IVM) of cat oocytes are not clear. For efficient *in vitro* production of feline embryos, knowledge concerning the maturational competence of cat oocytes should be accumulated. Therefore, in this study, we collected follicular oocytes from ovaries of mature and prepubertal cats and cultured them to examine the effect of ovarian status and follicular size on morphological normality and maturational

ability of cat oocytes.

MATERIALS AND METHODS

Animals for ovarian collection: The ovaries of 22 sexually mature cats were obtained following ovariohysterectomy at the veterinary teaching hospital of Tottori University and a local veterinary clinic in Tottori City. The ovaries of five prepubertal cats (body weights of 740–1270 g and estimated ages of 60–120 days) were donated by a cat shelter with permission from the Eastern Tottori General Office of Tottori Prefecture. The ovaries were collected from January to October 2008. The ovarian status of mature cats was classified into three groups: inactive stage without follicles $\geq$2 mm in diameter nor corpora lutea (CL) (n=8), follicular stage with one or more follicles 2 mm in diameter in one or both ovaries (n=7) and luteal stage with one or more CL in one or both ovaries (n=7).

Antral follicle isolation and oocyte recovery: The collected ovaries were kept in normal saline at room temperature and delivered to the laboratory within 1 hr after collection. They were then transferred to TCM–199 (Cat. No. 31100, Invitrogen, Grand Island, NY, U.S.A.) containing 0.1% polyvinyl alcohol (Sigma-Aldrich, St. Louis, MO, U.S.A.), 25 mM HEPES (Sigma-Aldrich), 0.85 mg/ml of NaHCO$_3$ (Kanto Chemical Co., Inc., Tokyo, Japan) and 50 μg/ml of gentamicin sulfate (Sigma-Aldrich; isolation medium, pH 7.4) [5]. The ovaries were cut with scissors along their long axis. The ovarian medulla was removed, and the cortex was cut to form small pieces in the isolation medium. It was difficult to identify the antrum formation in follicles < 400 μm in diameter. The antral follicles $\geq$400

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μm in diameter were isolated with an 18-gauge needle under a stereomicroscope and transferred to fresh isolation medium. Using an ocular micrometer, follicular diameter was measured as the mean length of two perpendicular axes. The follicles were classified into three groups according to diameter: 400–800, 800–1200 and 1200–2000 μm. They were then dissected with a 25-gauge needle under a stereomicroscope. Cumulus oocyte complexes (COCs) were collected, and their morphological characteristics were determined under a stereomicroscope (× 15 to 50). Oocytes that were tightly surrounded by more than two layers of cumulus cells and had evenly granulated black ooplasm were defined as morphologically normal and culturable and were used for IVM. Photos of the COCs were taken by a digital camera connected to an inverted microscope, and the diameters of the oocytes (excluding the zona pellucida) were measured as the mean length of two perpendicular axes using a personal computer.

IVM culture of oocytes: The COCs were washed once in the maturation medium, composed of 25 mM HEPES-buffered TCM 199 (Cat. No. 12340, Earle's salt, Invitrogen) supplemented with 3 mg/m*l* fatty-acid free BSA (Sigma-Aldrich), 0.02 IU/m*l* of FSH (from the porcine pituitary, Sigma-Aldrich), 1 μg/m*l* of estradiol-17β (Sigma-Aldrich), 0.2 mM sodium pyruvate (Sigma-Aldrich) and 50 μg/m*l* of gentamicin sulfate. They were then cultured individually in 10-μ*l* droplets of maturation medium covered with paraffin oil at 39°C in a humidified atmosphere of 5% CO_2 in air for 30 hr.

Evaluation of nuclear maturation by a whole mount procedure: After IVM culture, the cumulus investments were removed from the oocytes by gentle pipetting. They were then fixed with a mixture of ethanol:acetic acid (3:1) and stained with 1% aceto-orcein solution. The nuclear status was evaluated using a phase-contrast microscope. When the nuclear envelope was clearly visible, the nuclear stage was classified as germinal vesicle (GV). Oocytes with germinal vesicle breakdown to telophase I were classified as GVBD to TI stage. When chromosomes were condensed and present in equatorial view with extrusion of the first polar body, the nuclear stage was classified as metaphase II (MII). Oocytes at the MII stage were considered meiotically mature. Oocytes with more than two blastomeres and those with shrinkage and/or vacuolization of the ooplasm were considered to be parthenogenetically activated (parthenotes) and degenerated, respectively.

Experimental design: Firstly, for evaluating the relationship between ovarian status and follicular growth, the number of antral follicles in each ovarian status was counted, and the diameters of isolated follicles were measured. Secondly, for evaluating the relationship between follicular growth and oocyte normality, the morphologies of COCs derived from different follicular diameter groups were investigated in each ovarian status. The data from individual cats were pooled and calculated as the percentage of normal oocytes for the total number of follicles examined. Finally, for evaluating the relationships between follicular and oocyte growth, and between follicular growth and maturational ability of oocytes, the diameters of oocytes derived from each follicular diameter group were measured in each ovarian status. These oocytes were cultured individually for IVM, and nuclear status after culture was investigated. The data were pooled and calculated as the percentage of oocytes with each nuclear status for the total number of oocytes cultured.

Statistical analysis: Statistical analysis was performed using the JMP Version 7.0.1 software (SAS Institute Inc., Cary, NC, U.S.A.). Values for number of follicles and oocyte diameter were presented as means ± SD. Total number of follicles per cat was analyzed by Turkey-Kramer's honestly significant difference (HSD) test. Influence of ovarian statuses (inactive, follicular, luteal and prepubertal) and follicular size (400–800, 800–1200 and 1200–2000 μm in diameter) on number of follicles and oocyte diameter was also analyzed by the HSD test. The data for morphological normality and maturation rate of oocytes were analyzed by the chi-square test. Values were considered statistically significant at p<0.05.

RESULTS

The relationship between ovarian status and follicular population per cat is shown in Table 1. No significant difference was observed in the total number of follicles among the ovarian statuses. In each ovarian status, the number of follicles decreased with the growth of follicles (p<0.05). In follicles that were 400–800 and 800–1200 μm in diameter, the number of follicles was not different among the ovarian statuses. Only a single follicle that was 1200–2000 μm in diameter was observed in two of the five prepubertal cats.

The relationship between follicular diameter and morphological normality of oocytes in cats with various ovarian statuses is shown in Table 2. In each ovarian status, the percentage of normal and culturable oocytes increased with the growth of follicles (p<0.05). In follicles that were 800–1200 μm in diameter, the percentage of normal oocytes was higher in prepubertal cats than that in mature cats (p<0.05).

The influence of ovarian status and follicular size on oocyte diameter is shown in Table 3. In each ovarian status, the diameters of the cat oocytes collected from follicles that were 800–1200 μm in diameter were larger than those from follicles that were 400–800 μm in diameter (p<0.05) and similar to those from follicles that were 1200–2000 μm in diameter. The diameters of luteal stage oocytes were larger than those of follicular and inactive stage oocytes collected from follicles that were 400–800 and 800–1200 μm in diameter, respectively (p<0.05). The difference in oocyte diameter among the ovarian statuses in the mature cats was not detected in the follicles that were 1200–2000 μm in diameter.

As shown in Table 4, after IVM culture, the maturation rate of the cat oocytes increased with the growth of follicles in each ovarian status (p<0.05). In each follicular diameter group, there was no significant difference in the maturation

Table 1. Follicular population in cats with various ovarian statuses

Follicular diameter (μm)	No. of follicles per cat in each ovarian status (No. of cats)			
	Inactive	Follicular	Luteal	Prepubertal
400–800	48.3 ± 34.7[a)]	43.6 ± 23.7[a)]	37.4 ± 18.1[a)]	66.2 ± 18.3[a)]
800–1200	33.4 ± 14.2[a,b)]	20.0 ± 14.4[b)]	22.3 ± 11.8[a,b)]	36.0 ± 11.6[b)]
1200–2000	10.6 ± 4.8[b,A)]	8.0 ± 5.0[b,A)]	11.7 ± 5.3[b,A)]	0.4 ± 0.5[c,B)]
Total	92.3 ± 41.1 (8)	71.6 ± 30.0 (7)	71.4 ± 26.9 (7)	102.6 ± 25.3 (5)

Values are means ± SD.
a, b) Values with different superscripts in the same column differ significantly ($p<0.05$).
A, B) Values with different superscripts in the same row differ significantly ($p<0.05$).

Table 2. Relationship between follicular size and morphological normality of oocytes in cats with various ovarian statuses

Follicular diameter (μm)	% of normal oocytes in each ovarian status (No. of follicles examined)*			
	Inactive	Follicular	Luteal	Prepubertal
400–800	19.4[a)] (386)	19.3[a)] (305)	22.9[a)] (262)	18.7[a)] (331)
800–1200	36.0[b,A)] (267)	39.3[b,A)] (140)	36.5[b,A)] (156)	53.3[b,B)] (180)
1200–2000	62.4[c)] (85)	60.7[c)] (56)	64.6[c)] (82)	100[b)] (2)

* Values in each ovarian status were pooled data from 8 cats in the inactive group, 7 cats in the follicular group, 7 cats in the luteal group and 5 cats in the prepubertal group.
a–c) Values with different superscripts in the same column differ significantly ($p<0.05$).
A, B) Values with different superscripts in the same row differ significantly ($p<0.05$).

Table 3. Relationship between follicular size and oocyte diameter in cats with various ovarian statuses

Follicular diameter (μm)	Mean diameter (μm) of oocytes in each ovarian status (No. of oocytes examined)			
	Inactive	Follicular	Luteal	Prepubertal
400–800	104.0 ± 5.1[a,A,B)] (72)	103.5 ± 5.0[a,A)] (57)	106.5 ± 5.7[a,B)] (57)	105.1 ± 6.4[a,A,B)] (63)
800–1200	108.1 ± 5[b,A)] (96)	109.6 ± 4.3[b,A,B)] (56)	110.9 ± 5.2[b,B)] (58)	109.2 ± 4.8[b,A,B)] (99)
1200–2000	109.9 ± 4.6[b)] (50)	110.6 ± 4.1[b)] (33)	112.1 ± 4.5[b)] (51)	109.0 ± 3.5* (2)

Values are means ± SD.
a, b) Values with different superscripts in the same column differ significantly ($p<0.05$).
A, B) Values with different superscripts in the same row differ significantly ($p<0.05$).
* Data were excluded from statistical analysis because of the few number of oocytes.

rate of oocytes among ovarian statuses in the mature cats. In follicles that were 400–800 and 800–1200 μm in diameter, the maturation rate of oocytes was higher in prepubertal cats than in mature cats ($p<0.05$). The percentage of degenerated oocytes after IVM culture was higher in the oocytes collected from small follicles in each ovarian status ($p<0.05$). The degeneration rate of oocytes from follicles that were 400–800 and 800–1200 μm in diameter was lower in prepubertal cats than that in mature cats ($p<0.05$).

DISCUSSION

This is the first report to describe the number of antral follicles ≥400 μm in diameter in ovaries of sexually mature and prepubertal cats. Total number of follicles that were 400–2000 μm in diameter per cat was not influenced by ovarian status. The number of follicles that were 400–800, 800–1200 and 1200–2000 μm in diameter did not differ among ovarian statuses in mature and prepubertal cats, except for the presence of few follicles that were 1200–2000 μm in diameter in the prepubertal cats. These results suggest that antral follicles grow similarly until they are 2000 μm in diameter in all ovarian statuses in mature cats but only until 1200 μm in diameter in prepubertal cats. Luteinizing hormone (LH) is an essential factor for terminating follicular development [13], and LH binding sites were intensively detected in the granulosa cell layer of cat follicles with ≥800 μm in diameter [22]. Therefore, it is speculated that

Table 4. Relationship between follicular size and *in vitro* maturation (IVM) of follicular oocytes in cats with various ovarian statuses

Ovarian status (No. of cats)	Follicular diameter (μm)	No. of oocytes cultured	% of oocytes in each nuclear morphology after IVM culture*				
			GV	GVBD to TI	MII	Parthenote	Deg
Inactive	400–800	72	40.3[a,A]	1.4[A]	19.4[a,A]	1.4	37.5[a,A]
(8)	800–1200	96	9.4[b]	4.2[A]	69.8[b,A]	2.1	14.6[b,A]
	1200–2000	50	0[c]	2.0	86.0[c]	4.0	8.0[b]
Follicular	400–800	57	36.8[a,A]	0[a,C]	22.8[a,A]	0	40.3[a,A]
(7)	800–1200	56	7.1[b]	17.9[b,B]	55.4[b,A]	5.4	14.3[b,A]
	1200–2000	33	0[b]	0[a]	93.9[c]	6.1	0[c]
Luteal	400–800	57	54.4[a,A]	5.3[A,B]	10.5[a,A]	1.8	28.1[a,A,B]
(7)	800–1200	58	8.6[b]	6.9[A,B]	55.2[b,A]	3.4	25.9[a,A]
	1200–2000	51	2.0[b]	3.9	82.4[c]	5.9	5.9[b]
Prepubertal	400–800	63	17.5[a,B]	11.1[a,B]	55.6[a,B]	1.6	14.3[a,B]
(5)	800–1200	99	5.1[b]	4.0[b,B]	83.8[b,B]	4.0	3.0[b,B]
	1200–2000	2	0[a,b]	0[a,b]	100[a,b]	0	0[a,b]

* GV: germinal vesicle; GVBD: germinal vesicle breakdown; TI: telophase I; MII: metaphase II; Parthenote: more than two blastomeres; Deg: degeneration.
a–c) Values with different superscripts in the same column within the same ovarian status differ significantly (p<0.05).
A–C) Values with different superscripts in the same column among the same follicular diameter differ significantly (p<0.05).

the development of follicles may be suppressed at a size less than 1200 μm in diameter due to insufficient LH secretion or weak expression of LH receptor in the granulosa cells of prepubertal cats.

In the present study, it was demonstrated that the number of follicles decreased but that the percentage of morphologically normal oocytes in the follicles increased with follicular growth in cats. It was also clarified that oocyte diameter and maturational ability increased with follicular growth. Oocytes derived from small follicles showed a lower percentage of morphological normality after collection and a higher degeneration rate after IVM culture in each ovarian status. These results indicate that healthy oocytes having high maturational competence are selected during follicular growth. The relationship between follicular growth and acquirement of maturational competence of oocytes differed among species [26]. Meiotic competence of oocytes was shown concomitantly with antrum formation in adult mice [24], hamsters [7] and marmoset monkeys [24], while it was not strictly correlated with antrum formation but increased with follicular growth in cows [1], sheep [13], pigs [14, 15], horses [4], humans [27] and rhesus monkeys [23]. Otoi *et al.* [17] reported no significant relationship between oocyte diameter (range 83.5 to 126.4 μm) and maturation rate in cat ovaries stored at 38°C for 2 hr. In the present study, cat oocytes derived from follicles that were 1200–2000 μm had larger ooplasm (approximately 110 μm in diameter) and showed higher maturational ability than those from small follicles that were 400–800 μm in diameter (approximately 105 μm in diameter) in each ovarian status. On the other hand, oocytes from follicles that were 800–1200 μm in diameter had similar sizes but showed lower maturation

rates when compared with those from follicles that were 1200–2000 μm in diameter. This discrepancy may be due to the status of degeneration of follicles and oocytes. The percentage of morphologically normal oocytes in follicles that were 800–1200 μm in diameter was lower than that in follicles with 1200–2000 μm in diameter. Follicles that were 800–1200 μm in diameter may contain large oocytes with swelling of the ooplasm by membrane dysfunction even if the COC morphology looks normal.

In mature cats, the maturation rates of oocytes derived from each follicular group were similar among ovarian statuses. However, Johnston *et al.* [8] reported that maturational competence of cat oocytes in the inactive and follicular stages was higher than that in the luteal stage. The relationship between ovarian status and developmental competence of cat oocytes was also different among the reports. The cleavage and developmental rates of cat oocytes in the inactive and freshly ovulated stages were lower than those in follicular and luteal stages [2], while the cleavage and developmental rates of oocytes in the follicular stage were lower than those in the inactive and luteal stages [9]. Recently, Naoi *et al.* [16] reported no difference in cleavage and developmental rates of cat oocytes among the ovarian statuses. Although we reported previously the maturation rate of oocytes collected from follicles with various sizes and pooled for culture in mature cats [28], our present results suggest that the size of the follicles/oocytes and their qualities may affect *in vitro* maturation and subsequent development of cat oocytes. To evaluate maturational and developmental competence of cat oocytes, methods to select healthy and high quality oocytes should be developed in further study.

In prepubertal cats, the number of small follicles (400–800 μm in diameter) and the percentage of normal oocytes in the small follicles were not different from those in mature cats. However, these prepubertal cat oocytes showed higher maturation rates and lower degeneration rates when compared with oocytes from mature cats. The reason for the high maturational competence of the prepubertal cat oocytes derived from the small follicles is not clear. We previously reported that the success rate of IVM, in vitro fertilization, cleavage and development to blastocysts in prepubertal cats were lower than that in mature cats [28]. The low maturation rate (48.3%) of the prepubertal cat oocytes in the previous study [28] may have been due to culture of oocytes derived from small follicles (620 μm in diameter by histological investigation). In the present study, the maturation rate of oocytes derived from follicles that were 400–800 μm in diameter was also around 50% in the prepubertal cats. In bovine and ovine, the number of antral follicles increased just prior to puberty, and the size of the antral follicles did not change during the prepubertal period (reviewed by Rawlings et al. [21]). The prepubertal cats in the present study might be in a period of temporary increase of healthy antral follicles in the ovaries.

In conclusion, the number of antral follicles (400 μm in diameter) in the ovaries of sexually mature cats and percentage of normal oocytes in the follicles were not different among ovarian statuses. Oocytes collected from larger follicles possess higher maturational ability in vitro in mature cats. In prepubertal cats, a higher maturation rate can be obtained from oocytes derived even from small follicles compared with in mature cats.

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