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Morphological characterization and *in vitro* maturation of follicular oocytes from the owl monkey (*Aotus lemurinus*)

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Abstract: The morphological characterization of ovaries and ovarian follicular oocytes obtained from the owl monkey is detailed in the present paper. *In vitro* maturation of oocytes, using a method which has proved successful for other mammalian oocytes, was also evaluated and the maturation rate was compared with that obtained with squirrel monkey oocytes. The ovaries of the owl monkey are oval in shape (long axis of about 10 mm, short axis about 7 mm) and their oocytes are spherical. The mean diameter of owl monkey oocytes is significantly larger than that of squirrel monkey oocytes (145.7 ± 16.3 *μ*m vs 112.0 ± 12.4 *μ*m). The owl monkey oocytes were incubated in HTF medium containing 10% FBS at 37 °C (5% CO_2 in air) for 22–23 hours. The *in vitro* maturation rate of the owl monkey oocytes was higher than that of the squirrel monkey oocytes (83.3% vs 40.0%); therefore, our maturation conditions were as suitable for them. This study is the first to detail differences in the follicular oocytes of the owl monkey and squirrel monkey. Further studies using owl monkey gametes might yield results leading to a greater understanding of primate reproduction.

Key words: Owl monkey, Oocytes, *in vitro* maturation, Ovary, Squirrel monkey

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Introduction

Primates are the closest animals to the human. Obtaining information about their reproduction can contribute to saving them as genetic resources and as potential models for human reproduction in assisted reproductive technologies (ART). *In vitro* maturation (IVM) of oocytes is an important technique in ART that can be used in conjunction with intra-cytoplasmic sperm injection (ICSI), and the cryopreservation of gametes, etc.

Owl monkeys (*Aotus lemurius*) are new world monkeys living in South America; they are monogamous non-seasonal breeders with estrus cycles of approximately 16 days [1]. They are used for research in microbiology and physiology [2, 3], but little is known about the potential uses of owl monkey gametes in the investigation and developments of reproductive technologies that could be used for many mammalian species. Squirrel monkeys (*Saimiri boliviensis*) are also new world monkeys from South America. They show estrus cycles of approximately 10 days in the breeding season, from September to March, and form a harem, socially [4, 5]. A few studies have reported successful *in vitro* fertilization using squirrel monkey gametes [6–8].

To obtain more information about the reproductive aspects of the owl monkey, we recently undertook a morphological study of owl monkey spermatozoa [9]. In the present study, we examined the morphology of owl monkey ovaries and immature oocytes, and estimated the maturational competence of the oocytes by employ-

ing an *in vitro* maturation method used successfully for follicular oocytes obtained from the ovaries of mice and cattle. The results were compared with those obtained for squirrel monkey oocytes.

Materials and Methods

Animals

The present study was conducted in the period from 2012 to 2014, only one experiment per year using limited numbers of animals. All animal experiments were performed according to the Regulations for Animal Care and Use of The University of Tokyo and were approved by the Animal Experiment Committee of the Institute of Medical Science of The University of Tokyo (approval number: PA14-40).

The owl monkeys and the squirrel monkey used in the experiments were kept at the Amami Laboratory of the Institute of Medical Science, The University of Tokyo, which is located on Amami Island, Kagoshima, Japan.

A total of three female owl monkeys (4, 5 and 13–15 years old) and three female squirrel monkeys (5, 6 and 13 years old) were used. These monkeys were individually housed in cages (L: 630 mm, H: 900 mm, W: 630 mm) in the breeding rooms of each species and exposed to a natural light/dark cycle that tracked local sunrise and sunset. Food and water were available to the monkeys *ad libitum*, and a commercial pellet diet (CMS-1M, CLEA Japan, Inc., Tokyo) was supplemented daily with fresh fruits and sweet potato. Individual cages had bars that the monkeys could use for climbing up and down. The monkeys could see and hear each other. All monkeys were euthanized by exsanguination after anesthesia with a combination of ketamine (30 mg/kg) and medetomidine (0.05 mg/kg). None of the animals used in the study could be checked for their estrus cycles, and none were administered any hormone; however, it was considered that the squirrel monkey was at the end of the breeding season.

Observation of ovaries

After the monkeys had been euthanized, their ovaries were removed. The morphology of 5 ovaries from three owl monkeys (one ovary was used in another study by our group) and 2 ovaries from the 5 year old squirrel monkey was evaluated.

Obtaining oocytes and measuring the sizes

Oocytes observed below the surface of ovaries were scooped out of follicles teased with the tip of a 25G needle, in HEPES-buffered HTF medium (Irvine Scientific, Irvine, CA) containing 10% FBS. Oocytes were additionally obtained by chopping up the 5 ovaries from 3 owl monkeys and from 6 ovaries of 3 squirrel monkeys.

Cumulus-oocyte complexes were classified into 3 categories according to the layers of surrounding cumulus cells: oocytes with more than 3 layers - excellent; those with 1 or 2 layers - good; those with less than 1 layer - poor (Fig. 1). The diameters of oocytes plus the zona pellucida and the oocytes themselves, plus the thickness of each zona pellucida, were also measured.

In vitro maturation of oocytes

Preliminary experiments to ascertain which medium (HTF or medium-199) and incubation time (22 h or 41 h) would be suitable for *in vitro* maturation of owl monkey oocytes suggested the use of HTF medium and an incubation time of 22 h. For *in vitro* maturation, oocytes were incubated in 100 *μ*l droplets of HTF medium containing 10% FBS in a 35 mm diameter culture dish (Nunc, Roskilde, Denmark) in the incubator at 37 °C (5% CO_2 in air) for 22–23 h. After *in vitro* maturation, oocytes with an extruded first polar body were judged to be mature.

Fluorescent antibody staining of oocytes

Some of the matured oocytes were fixed and evaluated using fluorescent antibody staining to confirm their maturation. This was performed as described previously with slight modifications [10, 11]. Oocytes were fixed in 3.7% formaldehyde in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (D-PBS) at room temperature for 30 min, and permeabilized in 2.5% Tween-20 in D-PBS for 5 min. They were then incubated overnight at 4 °C with mouse monoclonal anti-α-tubulin antibody (Sigma, St. Louis, MO, USA) as the primary antibody. The oocytes were washed with D-PBS containing 0.5% Triton X-100 and 0.5% BSA, and incubated with FITC conjugated goat anti-mouse antibody (MP Biomedicals, Morgan Irvine, CA, USA) as the secondary antibody for 1 h at room temperature. After the oocytes had been washed with D-PBS containing 0.5% Triton X-100 and 0.5% BSA, their nuclei were labeled with 10 *μ*g/ml of propidium iodide (PI) (Sigma) for 30 min at room temperature. The oocytes were mounted on glass-bottomed dishes (Nippon Genetics Co. Ltd., Tokyo, Japan) and viewed with an FV1000-D confocal scanning laser microscope (Olympus, Tokyo, Japan).

Statistical analysis

The percentages of matured oocytes of the total numbers of oocytes examined were analyzed using Fisher's exact test. The results of measurements of oocytes and zonae were analyzed using Student's t-test.

Results

The ovaries of the owl monkeys were oval in shape and yellow-brown in color (Fig. 2). The average of the long axis of 5 ovaries from three owl monkeys was 10.3 ± 1.2 mm, and the average of the short axis was 7.4 ± 1.2 mm (Table 1). Some follicles could be seen in the surface layer of the ovaries. The ovaries of the squirrel monkey evaluated were rounder in shape (long axis of 12.0 mm, and short axes of 11.5 and 10.0 mm), but they had the same yellow-brown color. The cortex was thick, and few follicles could be seen.

Table 1. Morphological characteristics of owl monkey and squirrel monkey ovaries

Animal species	diameter (mm)		shape
	long axis	short axis	
Owl monkey (3*, 5**)	10.3 ± 1.2	7.4 ± 1.2	oval
Squirrel monkey (1*, 2**)	12.0, 12.0	11.5, 10.0	round

Nos. of individuals* and ovaries**. Mean ± SD mm.

The morphology of excellent or good oocytes collected from follicles was investigated in three owl monkeys and two squirrel monkeys (Table 2). Oocytes from the owl monkeys were spherical or ovoid (Fig. 1) and had a semi-transparent ooplasm with a few cytoplasmic lipid droplets. Oocytes from the squirrel monkeys were also spherical or ovoid but had a transparent ooplasm and very few cytoplasmic lipid droplets. The median diameters of owl monkey oocytes + zona pellucida, the oocytes themselves and the thickness of the zona pellucida (145.7 ± 16.3 μm, 98.2 ± 11.4 μm and 19.6 ± 4.4 μm, respectively) were significantly larger than those of the squirrel monkey (112.0 ± 12.4 μm, 80.7 ± 11.9 μm and 13.0 ± 2.1 μm, respectively).

Oocytes graded as described in Materials and Methods were obtained from 2 ovaries of the 5 years old owl monkey and 3 squirrel monkeys, while oocytes of ovaries from the 4 years old and an ovary of the 13–15 years old owl monkeys were selected as excellent or good without grading (Table 3). The numbers of oocytes collected from

Table 2. Comparison of the morphological characteristics of owl monkey and squirrel monkey oocytes

Animal species	diameter of oocyte + zona (μm)	diameter of oocyte (μm)	thickness of zona pellucida (μm)
Owl monkey (3*, 41**)	145.7 ± 16.3[a]	98.2 ± 11.4[a]	19.6 ± 4.4[a]
Squirrel monkey (3*, 17**)	112.0 ± 12.4[b]	80.7 ± 11.9[b]	13.0 ± 2.1[b]

Nos. of individuals* and oocytes**. Median ± SD μm. Values with different superscript letters within the same column are significantly different ($P < 0.05$).

Table 3. Results of *in vitro* maturation of oocytes graded according to cumulus cell layers or not graded

Animal species	age	oocytes graded	oocytes collected	oocytes cultured	oocytes with 1st polar body (%)
Owl monkey	5	excellent	8	6	5 (83.3)
		good	24	20	10 (50.0)
		poor	3	2	0
	4	not graded	21	19	3 (15.8)
	13–15	not graded	8	8	3 (37.5)
Squirrel monkey	5	excellent	3	3	2 (66.7)
		good	9	9	0
		poor	27	0	0
	6	excellent	3	3	1 (33.3)
		good	4	4	0
		poor	25	9	1 (11.1)
	13	excellent	4	4	1 (25.0)
		good	9	9	1 (11.1)
		poor	8	0	0

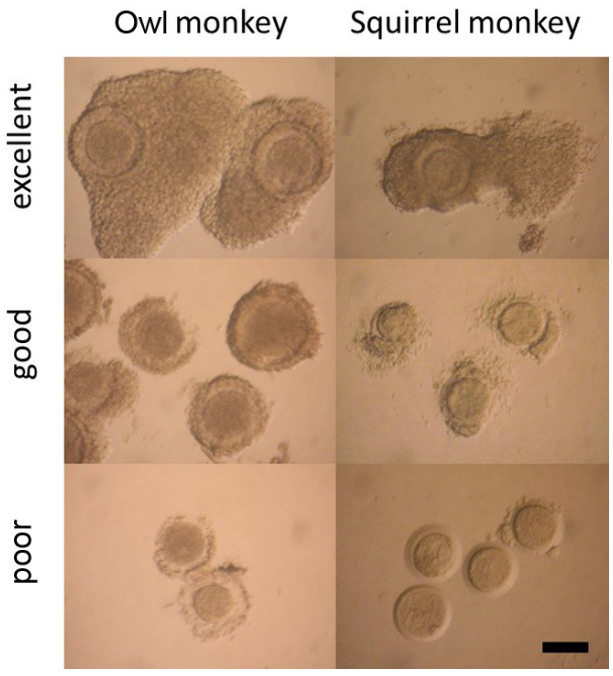

Fig. 1. Follicular oocytes of the owl monkey and the squirrel monkey classified according to surrounding-cumulus cell layers (bar=100 μm).

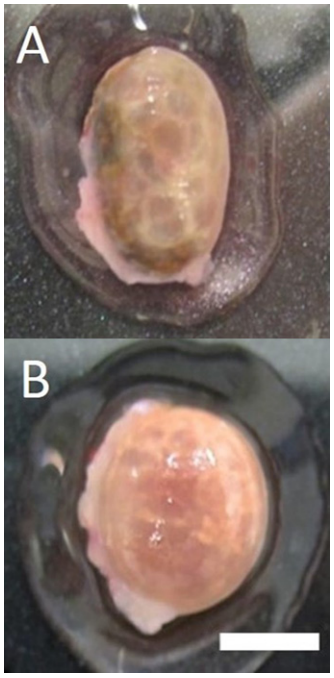

Fig. 2. Ovaries of the owl monkey and the squirrel monkey. The owl monkey ovary is oval in shape (A), while the squirrel monkey ovary is round (B) (bar=5 mm).

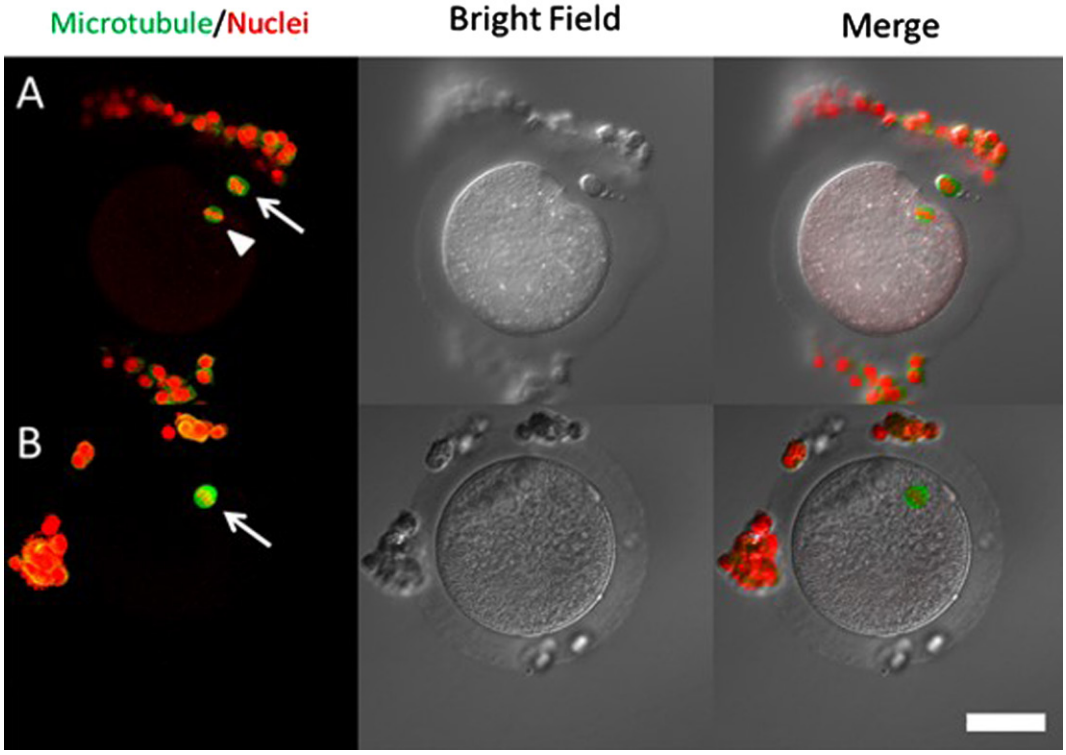

Fig. 3. Results of fluorescent antibody staining of owl monkey oocytes. Meiotic spindles and chromosomes can be seen in the oocytes (bar=50 μm). A mature oocyte has the metaphase form of the second meiosis (arrowhead in A) and 1st polar body (arrow in A); an immature oocyte has only the metaphase form of the first meiosis (arrow in B).

owl monkeys were larger in the young monkeys than in the oldest monkey. A similar result was also found for the oldest squirrel monkey.

After incubating the oocytes to allow maturation *in vitro*, oocytes were considered to be mature if they had a 1st polar body. The fixed and stained oocytes were evaluated for their maturation state by the presence of a meiotic second metaphase and the 1st polar body (mature oocytes), or only the first meiotic metaphase (immature oocytes), by fluorescent antibody staining (Fig. 3). The maturation rate of owl monkey oocytes graded as excellent (5/6) was higher than that of the squirrel monkey oocytes (4/10). Similar results were found for oocytes graded as good: owl monkey oocytes (10/20) compared to squirrel monkey oocytes (1/22). A significant difference ($P < 0.01$) was found between the maturation rates of pooled oocytes graded as excellent and good: owl monkey (15/26) and squirrel monkey (5/32).

Discussion

Morphologically, the ovaries of owl monkeys and squirrel monkeys are similar in color and size but differ in shape: owl monkey ovaries are oval while squirrel monkey ovaries are round. In addition, owl monkey oocytes have a thicker zona pellucida and are larger in diameter than squirrel monkey oocytes. Owl monkey oocytes are also larger than those of rhesus monkeys [12] and equal in size or slightly larger than human oocytes [13]. Their ooplasm is semitransparent and contains a few cytoplasmic lipid droplets. The owl monkey is non-seasonal breeder and a monogamous species with males and females forming pair bonds [14]. Since the biological behavior of owl monkeys is somewhat similar to humans, the owl monkey could perhaps be a good animal model for at least some aspects of human reproduction. Because the total number of oocytes collected from young monkeys was larger than that from the oldest monkey, the number of collected oocytes may be related to the age of owl monkeys.

The maturation rates of owl monkey oocytes *in vitro* (83.3% in the excellent grade and 50.0% in the good grade) were comparable to results reported for the macaque monkey, which is also a primate and a non-seasonal breeder [15]. In contrast, the squirrel monkey oocytes showed poor maturation rates (40.0% in the excellent grade and 4.5% in the good grade oocytes), compared with those of owl monkey oocytes. However, there is a possibility that, since the squirrel monkey ovaries were obtained at the end of the breeding season, it might have made the oocytes less responsive to the culture conditions used for *in vitro* maturation [8].

In conclusion, the present study is the first to report successful *in vitro* maturation of owl monkey oocytes, and we were able to demonstrate differences between immature oocytes from owl monkeys and squirrel monkeys. Further studies using owl monkey gametes might yield results leading to a greater understanding of primate reproduction.

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