

Cumulus-Oocyte Complexes from Small Antral Follicles During the Early Follicular Phase of Menstrual Cycles in Rhesus Monkeys Yield Oocytes That Reinitiate Meiosis and Fertilize In Vitro¹

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ABSTRACT

The stage at which follicle-enclosed cumulus-oocyte complexes achieve developmental competence in primates is unknown. Therefore, studies were designed to characterize the ability of oocytes in small antral follicles present during the menstrual cycle to spontaneously resume meiosis, fertilize, and support early embryo development. Ovaries were removed from adult rhesus monkeys (n = 12) during the early follicular phase (Days 3–4) of spontaneous cycles. Small antral follicles were divided into five groups according to their diameter; group I: <0.5 mm; group II: 0.5–0.99 mm; group III: 1.0–1.49 mm; group IV: 1.5–1.99 mm; and group V: 2.0–2.5 mm. The cumulus-oocyte complex from healthy small antral follicles (devoid of dark oocytes or granulosa cells) were extracted (n = 199) and cultured for 48 h under different conditions: in TALP (tyrode, albumin, lactate, pyruvate) medium alone, SAGE medium alone, or plus gonadotropins. At 48 h, oocyte meiotic status and diameter were measured after treatment of cumulus-oocyte complexes with hyaluronidase. Cumulus-oocyte complexes derived from follicles of 0.5- to 2-mm diameter contain oocytes that typically reinitiate meiosis in the absence or presence of gonadotropins and fertilize via in vitro fertilization or intracytoplasmic sperm injection. Moreover, the inseminated oocytes can reach the morula stage but arrest. Thus, the ability of these oocytes to complete maturation, as monitored from subsequent embryonic development after fertilization, is suboptimal. Further studies on primate IVM of oocytes from SAFs are warranted in order for them to be considered as an additional, novel source of gametes for fertility preservation in cancer patients.

cumulus-oocyte complex, follicular phase, oocyte maturation, rhesus monkeys

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INTRODUCTION

There has been a remarkable increase in the number of young cancer survivors as a consequence of improvements in therapies [1]. As of 2007, nearly 12 million people were estimated to be living with a history of cancer in the United States [2]. Unfortunately, cancer therapies usually lead to impaired fertility and premature ovarian failure [3]. Approaches for fertility preservation in women have been proposed and are under investigation [4]. The most successful approach involves the traditional reproductive technologies of in vitro fertilization (IVF) and cryopreservation of embryos prior to cancer therapy. However, for girls and many young women, this is not an option. Successful human oocyte cryopreservation for fertility preservation has also been reported [5, 6]. Moreover, cryopreservation of the ovarian cortex is one experimental option for restoring fertility in cancer survivors [7]. So far, two approaches have been considered to support the maturation of cryopreserved immature follicles from the cortex: ovarian tissue transplantation or in vitro follicle maturation (IFM [8]). Transplantation of cryopreserved ovarian cortical strips has been successfully performed in several patients [9, 10]. However, this approach has the risk of introducing cancer cells back into the patient [11, 12]. In contrast, in vitro ovarian follicle culture to achieve mature oocytes for IVF is an alternative without this risk. To date, live offspring in mice have been produced using IFM [13], but limited studies have been performed in primates.

Xu et al. [14] recently reported that an encapsulated three-dimensional (3D) culture system utilizing biomaterials to maintain cell-cell communication [15–17] permitted follicle growth to the small antral stage and supported steroidogenesis of individual secondary macaque follicles. Furthermore, the encapsulated 3D culture system supported the in vitro development of human follicles [18]. However, since mature preovulatory follicles achieve diameters in vivo up to 6 mm [19] in macaques and 20 mm in women [20], the technical challenges of follicle culture in primates relative to mice are obvious. It is estimated that it takes approximately 90 days for a preantral follicle that has entered the growing pool to become a preovulatory follicle in women [21]. But whether this time interval or a preovulatory size follicle is required in vitro to produce an antral follicle that contains a competent oocyte remains unknown. In our laboratory, encapsulated 3D culture of secondary follicles produced antral follicles of ≤ 1 mm in diameter [14], but whether the oocytes are developmentally competent is under investigation. Therefore, by determining the smallest diameter of an antral follicle that yields a developmentally competent oocyte, this option for fertility preservation can be advanced.

The key process required for growing follicles to yield healthy mature oocytes is the acquisition of developmental competence that includes the ability of the oocyte to spontaneously resume and complete meiosis (on removal from follicles) as well as to support embryonic development after fertilization. In rodents, development competence of oocytes is associated with antrum formation, maximum oocyte size, and nuclear encapsulation or rimming [22–24]. However, in Old World monkeys such as the rhesus macaque, meiotic competence increases with follicle size but has not correlated with antrum formation or maximum oocyte diameter [25, 26]. Moreover, nuclear rimming in primates does not coincide with the maximum oocyte size [25, 26]. In primates, the stage at which the antral follicle-enclosed oocyte achieves developmental competence, such that removal from the follicle permits the spontaneous resumption of meiosis, is unknown. Therefore, studies were designed to characterize the pool of small antral follicles (SAFs) present during the natural menstrual cycle in the monkey ovary for development competence of the oocyte.

The clinical use of in vitro maturation (IVM) of the oocyte as an assisted reproductive technology is increasing, and there are successful clinical IVM protocols even though its efficiency remains low [27–33]. We used similar culture conditions in a nonhuman primate model to assess the maturation of oocytes from SAFs during the natural menstrual cycle. In rhesus monkeys, the highest yield of healthy oocytes and highest ratio of healthy to degenerating oocytes were obtained from SAFs during the early follicular phase compared to those from late follicular or luteal phase of the menstrual cycle [34]. Moreover, the cohort of antral follicles in ovaries from the early follicular phase (Days 1–5 of menstrual cycle) represents those from which the dominant follicle will eventually be selected [35]. Therefore, this study determined the minimal diameter of an antral follicle from the early follicular phase of spontaneous menstrual cycles in macaques that contained a cumulus-oocyte complex (COC) capable of oocyte maturation in vitro, plus the ability of meiotically mature oocytes to fertilize and support preimplantation embryonic development.

MATERIALS AND METHODS

Animals and COC Recovery

The general care and housing of monkeys (*Macaca mulatta*) at the Oregon National Primate Research Center (ONPRC) was previously described [36]. The studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the ONPRC Animal Care and Use Committee.

Adult female rhesus monkeys (n = 12; 5–13 yr of age) exhibiting normal menstrual cycles of approximately 28 days based on serum levels of estradiol and progesterone [14] were used in this study. The first day of menses was considered as Day 1 of the cycle. Ovaries were removed from anesthetized monkeys during the early follicular phase (Days 2–4) of spontaneous cycles by laparoscopy, as previously described [37]. The excised ovaries were transported immediately to the laboratory in holding media (SAGE; CooperSurgical, Inc., Trumbull, CT) supplemented with 0.1% serum protein substitute plus gentamycin. After a gentle treatment with collagenase (0.1 mg/ml collagenase-DNAase-PBS) at 37°C for 20 min, the SAFs were dissected from the ovarian medulla under a stereoscopic microscope using 30-gauge needles. The diameter of healthy SAFs (devoid of dark oocytes or granulosa cells) were measured, and their COCs were extracted (n = 199).

In Vitro Culture of COCs

Individual COCs from each group were cultured for 48 h under different conditions: in tyrode, albumin, lactate, and pyruvate (TALP) alone (n = 45 COCs); TALP + follicle-stimulating hormone (rhFSH; NV Organon, Oss, Netherlands; 9.2 ng/ml) + luteinizing hormone (rhLH; Ares Serono,

Randolph, MA; 0.9 ng/ml) (n = 56 COCs); SAGE alone (n = 39 COCs); or SAGE + rhFSH + rhLH (0.75 IU each/ml, as suggested by the manufacturer) (n = 59 COCs). Images of the individual COCs were acquired 0, 24, and 48 h posttreatment using a digital camera attached to an Olympus CK40 microscope. At 48 h, the COCs were treated with hyaluronidase (2 mg/ml) in TALP HEPES-BSA (0.3%) for 30 sec to dissociate the cumulus and obtain denuded oocytes for analysis of the stage of nuclear maturation (germinal vesicle [GV]-intact; metaphase I [MI], and metaphase II [MII]) and diameter. Image Tool Software V3.0 was employed to measure oocyte diameter.

Sample Preparation for Microscopy

A cohort of denuded MII-stage oocytes from each of the four treatment groups was randomly selected and fixed in 4% paraformaldehyde for indirect immunofluorescence to ascertain nuclear maturation, spindle and polar body (PB) organization, and transzonal projections (tzps) after 48 h of oocyte maturation [38]. Oocytes were incubated in primary antibody overnight at 4°C with gentle agitation, followed by three 10-min washes in wash buffer and then a 1-h incubation of secondary antibody at room temperature with agitation. Spindle microtubules were labeled with a-tubulin clone DM1A (1:100; Sigma, St. Louis, MO) followed by Alexa 633 goat anti-mouse IgG (1:500; Invitrogen, Carlsbad, CA); F-actin was probed with Alexa 488-phalloidin (1:50; Invitrogen); chromosomes were labeled by 5 µM ethidium homodimer (Invitrogen). Oocytes were mounted on slides with 2–5 µl of glycerol/PBS solution (1:1) containing 25 mg/ml sodium azide. Samples were analyzed on a Leica SP5 confocal-equipped DM6000 CFS microscope with resonance scanner and argon, HeNe 543, and HeNe 633 lasers. Full Z-stack data sets were collected with a HCX PL Apo CS 40× oil objective (1.25 na) for each oocyte, with images taken every 0.3 µm.

Semen Preparation and IVF/ICSI

Semen was collected by the assisted reproductive technology (ART) core from fertile male rhesus monkeys as previously described [39, 40]. Samples were allowed to liquefy for 15 min and then washed twice in HEPES-buffered TALP medium containing 0.3% bovine serum albumin (BSA; Sigma, St. Louis, MO) by centrifugation (7 min, 1400 rpm). The washed sperm were resuspended in TALP containing 0.3% BSA. Sperm motility and concentration were examined in each sample.

Sperm samples were either used for in IVF of oocytes or intracytoplasmic sperm injection (ICSI), as previously described [40, 41]. Sperm used for IVF was previously activated by a sperm activator containing caffeine and db-cAMP. A cohort of MII oocytes (n = 26) was transferred individually to 100-µl droplets of TALP (serum free) under oil for insemination IVF at 37°C in 5% CO₂. Oocytes were examined 16 h postinsemination for the presence of two PB and two pronuclei to confirm fertilization. Fertilized oocytes were transferred to four-well dishes (Nalge Nunc International Co., Naperville, IL) containing 500 µl HECM-9 (serum free) equilibrated at 37°C in 6% CO₂, 5% O₂, and 89% N₂ and covered with tissue culture oil (SAGE) [42, 43]. Embryos were cultured under these conditions for 48 h and thereafter in HECM-9 with 5% FBS. Media was changed every other day, and pictures were taken daily to document embryonic development. Reagents and protocols for embryo culture are routinely used by the ART core and quality control tested every week [44].

An additional cohort of IVM mature oocytes (n = 16) was fertilized within the ART core by ICSI [45]. The micromanipulation chamber (Falcon 1009; Becton-Dickinson, Franklin Lakes, NJ) contained oil and two drops: a sperm drop consisting of 4 µl of 10% polyvinylpyrrolidone (Irvine Scientific, Santa Ana, CA) in TALP-hepes and 1 µl of spermatozoa (3 million/ml) and an oocyte drop, 20 µl of TALP-Hepes, into which mature oocytes were placed. Only progressively motile spermatozoa with normal morphology were selected for ICSI. Individual spermatozoa were immobilized by striking the tail and then injected into oocytes away from the PB using a 7-µm-outer-diameter micropipette (Humagen, Charlottesville, VA). Injected oocytes were transferred and cultured as described after IVF above.

Statistical Analysis

Statistical calculations were performed using Sigma Stat software package (Systat Software, Inc., Richmond, CA). Chi-square or Fisher exact tests were used to analyze differences in proportions among different treatments. Differences in oocyte diameters (MI–MII) among groups were analyzed using a one-factor analysis of variance followed by comparison among means using Kruskal-Wallis tests. Differences were considered significant at $P < 0.05$.

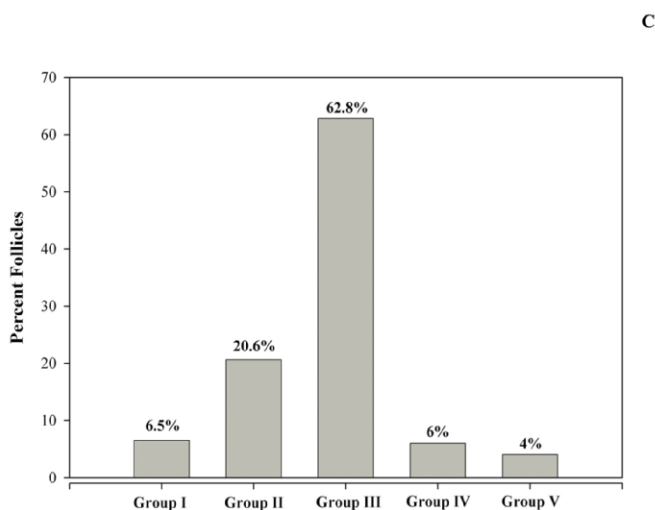
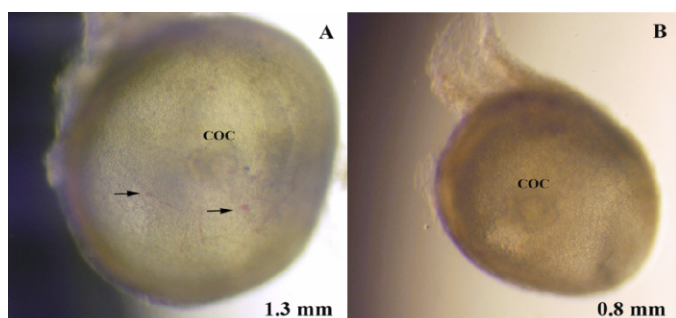


FIG. 1. **A** and **B**) Representative pictures of healthy small antral follicles (SAFs) dissected from the ovaries of adult monkeys during the early follicular phase of the menstrual cycle. COCs are easily observed through SAFs of different diameters. Arrows denote the presence of blood vessels containing red blood cells. Size distribution of healthy SAFs dissected from all animals is summarized in **C**. SAFs were divided into five groups according to their diameter; group I: <0.5 mm; group II: 0.5–0.99 mm; group III: 1.0–1.49 mm; group IV: 1.5–1.99 mm; and group V: 2.0–2.5 mm. Not every animal yielded SAFs in each size group.

RESULTS

Size Distribution of SAF

The isolated healthy SAFs (Fig. 1, **A** and **B**) were measured and divided into five groups according to their diameter (Fig. 1 **C**); group I: <0.5 mm; group II: 0.5–0.99 mm; group III: 1.0–1.49 mm; group IV: 1.5–1.99 mm; and group V: 2.0–2.5 mm. Of the total SAFs collected, the majority distributed into group III (1.0–1.49 mm; 62.8%), with fewer ($P < 0.05$) in groups II (0.5–0.99 mm; 20.6%), I (<0.5 mm; 6.5%), IV (1.5–1.99 mm; 6%), and V (2–2.5 mm; 4%). The number of SAFs per animal varied from 3 to 31, with an average of 17 ± 3 . Not every animal yielded SAFs in each size category.

Oocyte Maturation After 48 h of Culture

Although we carefully dissected what appeared to be healthy SAFs avoiding those with dark oocytes or granulosa cells, 46% of oocytes within the total number of COCs collected contained vacuoles (Fig. 2A) at 48 h postculture. COCs from group III provided the fewest vacuolated oocytes among the groups. Vacuolated oocytes were considered degenerate and discarded from the statistical analysis. Figure

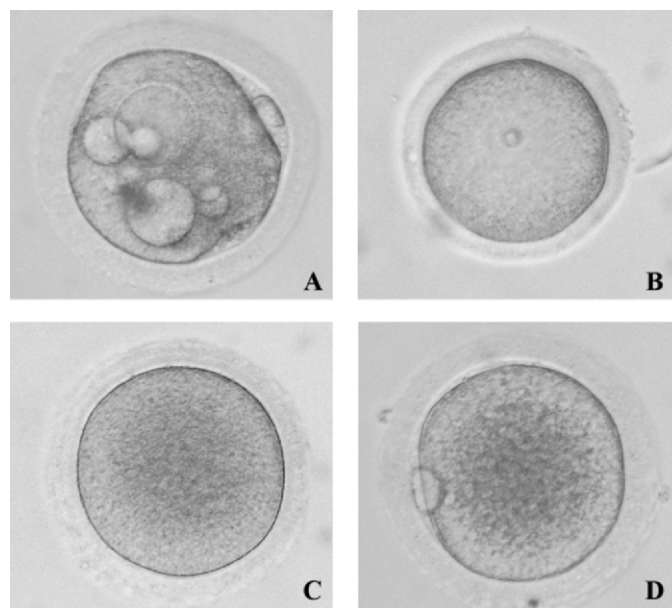


FIG. 2. Representative pictures of monkey oocytes at different stages of nuclear maturation after isolation from SAFs during the early follicular phase of the menstrual cycle and 48 h of culture (GV: **B**; MI: **C**; MII: **D**) as well as degenerating (**A**). The surrounding cumulus cells were removed by hyaluronidase treatment. Original magnification $\times 20$.

2 also shows representative pictures of healthy (54%), nonvacuolated oocytes at different stages of nuclear maturation after culture (GV-intact, Fig. 2B; MI, Fig. 2C; and MII, Fig. 2D). The percentage of healthy oocytes resuming maturation to MI and continuing meiosis to MII did not significantly differ between media, nor with or without gonadotropins (Table 1).

Oocyte nuclear maturation as a function of SAF diameter was also examined (Table 2). Since there were no differences in oocyte maturation between treatments, the data are pooled. Also, not every animal ($n = 12$) yielded SAFs in each size category. The few oocytes collected in group I did not resume meiosis. In contrast, oocytes from groups II, III, and IV resumed meiosis to the MI stage (Table 2). Moreover, half the oocytes from groups II, III, and IV matured to MII relative to group I. The very few oocytes collected from group V resumed meiosis but precluded statistical analysis.

Representative MII oocytes derived from SAFs after 48 h under different culture conditions (TALP + FSH + LH, SAGE + FSH + LH, TALP alone, SAGE alone) were analyzed using immunofluorescence to visualize chromatin, spindles, and actin (Fig. 3). The majority of the MII oocytes showed normal spindle and PB positions regardless of the culture conditions (Fig. 3, **A–E**). However, some of the spindles and/or PBs were smaller than the expected size (Fig. 3, **C–E**), and a larger gap

TABLE 1. Percentage of oocytes from healthy COCs at given stages of nuclear maturation after 48 h in the different culture media.*

Treatment [†]	GV	MI	MII
TALP alone (n = 24)	27 \pm 12	19 \pm 11	54 \pm 14
TALP + FSH + LH (n = 31)	28 \pm 11	19 \pm 9	54 \pm 11
SAGE alone (n = 19)	27 \pm 11	24 \pm 12	31 \pm 12
SAGE + FSH + LH (n = 34)	21 \pm 8	14 \pm 7	57 \pm 12
All media (n = 108)	29 \pm 7	19 \pm 7	53 \pm 9

* Values represent mean \pm SEM from 12 animals per treatment group.

[†] n = Number of COCs per treatment group.

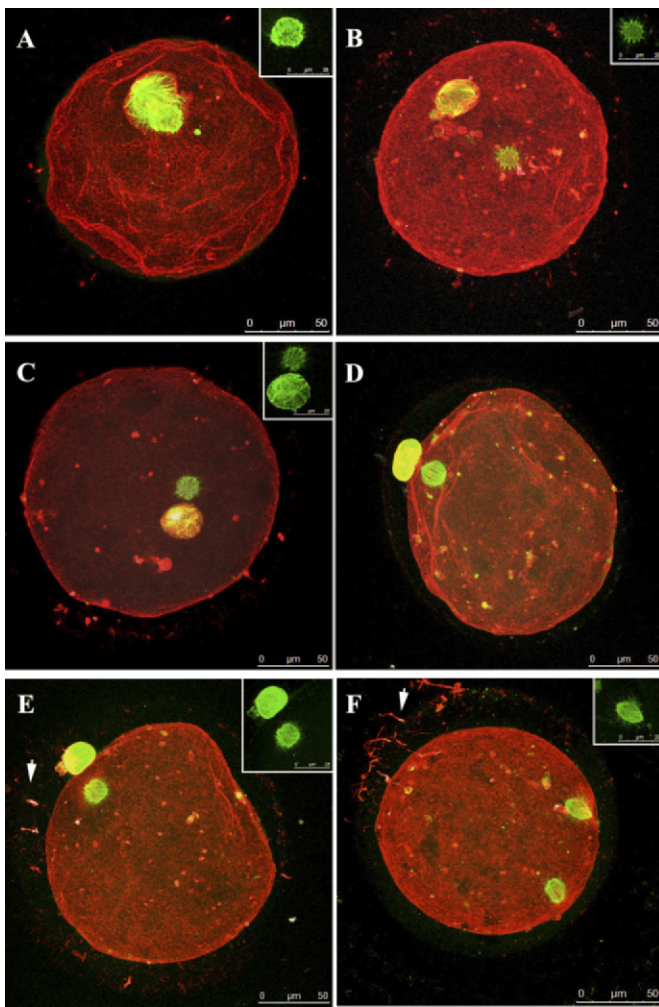


FIG. 3. Confocal microscopy images of representative oocytes from SAFs after 48 h of culture in media with or without gonadotropins. **A**) Oocyte matured in SAGE media without supplementation. Oocyte shows first PB adjacent to MII spindles. Spindle shown separately in inset stained with TUBA (green). **B**) Oocyte matured in TALP without supplementation. The oocyte shows a MII spindle that is somewhat adjacent to the first extruded PB; tzp fragments remain along the oolemma (red). TUBA staining alone (green) is shown in the inset. **C** and **D**) Oocytes matured in SAGE media with LH and FSH. MII oocytes (**C** and **D**) showing adjacent spindle to PB placement. The oocytes have small MII spindles and small, extruded first PBs. Bright red staining (F-actin) shows remnants of tzp after IVM. Tubulin staining alone (green) is shown in the inset. **E** and **F**) oocytes cultured in TALP with LH and FSH. MII oocyte (**E**) with spindle placement adjacent to extruded polar body. An oocyte (**F**) with two spindles (green) from incomplete cytokinesis. Numerous transzonal projections (tzps, arrows) seen with F-actin staining (red). Numerous tzps are also seen around the oocyte (red). TUBA staining alone (green) is shown in the inset. Bars = 50 μ m and 25 μ m (insets).

between spindle and PB was detected in one of the MII oocytes (Fig. 3B). Abnormal maturation was observed in only one oocyte (Fig. 3F), in which the second spindle was not extruded into a PB showing an incomplete cytokinesis. In several oocytes, tzps were seen (arrows), with F-actin staining along the oolemma (Fig. 3, E and F).

Oocyte Diameter After 48 h of Culture

Oocyte diameters were analyzed as a function of SAF diameter and meiotic status (Table 3). The minimum and maximum diameters for a GV oocyte were 74 and 119 μ m, for

TABLE 2. Percentage of oocytes from healthy COCs at given stages of nuclear maturation after 48 h in culture, as a function of SAF size.*

Group	SAF diameter [†]	GV	MI	MII
I	<0.5 mm (n = 7)	100 \pm 0	0	0
II	0.5–0.99 mm (n = 16)	38 \pm 17	11 \pm 7	51 \pm 14
III	1–1.49 mm (n = 77)	26 \pm 7	22 \pm 8	52 \pm 9
IV	1.5–1.99 mm (n = 5)	13 \pm 13	25 \pm 25	63 \pm 24
V	2–2.5 mm (n = 3)	0	100	0

* Values represent mean \pm SEM from 3 animals in group I, 7 animals in group II, 12 animals in group III, 4 animals in group IV, and 2 animals in group V.

[†] Not every animal yielded SAFs in each size category; n = number of healthy (nonvacuolated) follicles-COCs per group.

an MI oocyte 78.5 and 118 μ m, and for an MII oocyte 94.1 and 121.9 μ m, respectively. The diameter of MII oocytes was similar regardless of SAF diameter and did not differ between media. When data for all SAFs are pooled, the average diameter of GV oocytes (102 \pm 2 μ m) was less ($P < 0.05$) than that of MII oocytes (110 \pm 1 μ m). Only SAF of <0.5-mm diameter tended to enclose smaller oocytes, none which were capable of resuming meiosis.

IVF or ICSI of MII Oocytes

Table 4 depicts fertilization and embryonic development following IVF and ICSI of MII oocytes derived from five and four animals, respectively. Five of 26 oocytes from SAFs fertilized by IVF, and four zygotes cleaved but arrested at the 8- to 16-cell stage. Ten of 16 oocytes that underwent ICSI also fertilized, and zygotes underwent cleavage. In addition, three oocytes from one animal continued cleavage to the morula stage before arrest. In contrast, the current blastocyst rate for rhesus macaque embryos at ONPRC cultured in HCEM-9 media is 53% (Data not shown). Figure 4 shows embryonic development from representative MII oocytes inseminated by ICSI.

DISCUSSION

This study characterizing the pool of SAFs present in the primate ovaries during the early follicular phase of the natural menstrual cycle, established the ability of the cumulus-enclosed oocytes complexes derived from healthy SAFs to reinitiate meiosis in vitro in culture media with or without gonadotropins. It also demonstrated that the SAFs had to reach at least 0.5 mm in diameter in order for the oocyte to display meiotic competence. Moreover, some inseminated oocytes did fertilize and even reached the morula stage but arrested.

While the number and sizes of presumably healthy SAFs obtained from each animal varied, the majority (63%) measured between 1 and 1.49 mm, followed by 0.5–0.99-mm diameter (21%). The diameters of these macaque SAFs would be equivalent to 4–5 and 2–3 mm, respectively, human follicles [46], which are much smaller than the large, preovulatory follicles from which COCs for human IVF protocols are derived. A recent study from our laboratory using ultrasound imaging identified SAFs (<2 mm) in monkeys throughout the menstrual cycle on the ovary bearing the dominant follicle/corpus luteum and the contralateral ovary [47]. On the first day of the menstrual cycle (first day of menses), these ovaries had between 10 \pm 3 (mean \pm SEM, larger ovary) and 8 \pm 2 (mean \pm SEM, smaller ovary) SAFs, respectively. In accordance with these in vivo results, in the present study we obtained an average of 17 \pm 3 SAFs (mean \pm SEM) per animal. Scheffer et al. [48] reported that during the early

TABLE 3. Diameter of oocytes obtained from SAF of different sizes, as a function of nuclear maturation.*

Group	SAF diameter [†]	GV	MI	MII
I	<0.5 mm (n = 7)	99 ± 2 μm	NA	NA
II	0.5–0.99 mm (n = 16)	104 ± 4 μm	108 μm	111 ± 4 μm
III	1–1.49 mm (n = 77)	101 ± 4 μm	106 ± 3 μm	111 ± 1 μm
IV	1.5–1.99 mm (n = 5)	116 μm	113 μm	103 ± 5 μm
V	2–2.5 mm (n = 3)	99 μm	111 μm	NA
	Average (n = 108)	102 ± 2 μm ^a	107 ± 2 μm ^{ab}	110 ± 1 μm ^b

* Values represent mean ± SEM from 3 animals in group I, 7 animals in group II, 12 animals in group III, 4 animals in group IV and, 2 animals in group V; NA, not available.

[†] Not every animal yielded SAFs in each size category; n = number of healthy (nonvacuolated) follicles-COCs per group.

^{a,b} Different superscript letters represent a significant difference ($P < 0.05$).

follicular phase, the number of SAFs (2–5 mm) in women with regular menstrual cycle was around five to six. The wide range of age (25–46 yr) used to calculate the median for SAFs count in this study may explain the lower number of SAFs reported in women in comparison to rhesus monkey data. It has also been described that the number of SAFs declined with age, whereas the number of larger follicles (7–10 mm) remained nearly constant [46, 48]. However, the number of SAFs obtained per animal was not necessarily related to the ability of oocytes to mature during 48 h in vitro. For example, one animal yielded three SAFs, and all the oocytes resumed meiosis reaching the MII stage. In contrast, several animals yielded many SAFs, but only a few oocytes attained MII in vitro. Thus, this may indicate an inverse correlation between the number and quality of the SAFs. Moreover, perhaps only a small cohort of the SAF population selectively possesses the capacity to continue their development in the early follicular phase of the cycle. Bishop et al. [47] proposed that the dominant follicle in macaques appeared to be selected on the basis of its size differential (>2 mm) from other antral follicles before Day 3 of the follicular phase.

Although we carefully dissected healthy SAFs avoiding dark oocytes or granulosa cells, unexpectedly almost half (46%) of the total COCs contained one or more vacuoles after 48 h of culture. Since the oocytes were not evaluated until removal of the cumulus, it is not clear whether the vacuoles were present at collection or developed during culture. In previous studies on macaque oocytes from COCs, vacuolated oocytes were not subtracted from the MI or MII oocyte

categories; thus, higher percentages of maturation were described compared to our results [26, 49]. However, a vacuolated oocyte cannot be considered a healthy oocyte; hence, results from our study may more accurately depict developmental competence of only healthy oocytes.

Notably, we found that oocytes within COCs derived from healthy SAFs of at least 0.5-mm diameter obtained during the early follicular phase can reinitiate meiosis in vitro. However, following fertilization in vitro, embryonic development arrests; therefore, oocyte competence is not fully achieved in these SAFs of ≤2-mm diameter. In our laboratory, the encapsulated 3D culture model permitted isolated secondary follicles from macaques to grow to the small antral stage (≤1 mm) [14]. Whether oocytes derived from the encapsulated 3D culture model are developmentally competent is under investigation.

The current results suggest that the macaque oocyte must achieve a diameter of ≥103–110 μm to reinitiate meiosis. In contrast, it has been reported that human oocytes from early antral follicles may reach a diameter of approximately 120 μm [50], while in rodents the maximum diameter of 70 μm has already been reached at the end of the preantral stage when the oocyte acquires the competence to resume meiosis [50]. The growth phase of the oocyte allows development of the zona pellucida and production of mRNA and proteins required for subsequent fertilization and early embryonic development [50]. However, size cannot be the only requirement for developmental competence in primates since GV oocytes of up to 116 μm were obtained from larger (1.5–1.99 mm) SAFs.

Nuclear events endow oocytes with the competence to resume and complete meiosis whereas, cytoplasmic programs enable the oocyte to be fertilized successfully and support embryo development to term. Failure to complete cytoplasmic maturation can block the development at fertilization, embryonic genome activation, blastocyst formation, or even post-implantation events [51]. To date, the ability of the primate oocyte to complete nuclear and cytoplasmic maturation in vitro is markedly inferior to that of oocytes from other species [52–58]. In addition, the quality of primate oocytes matured in vitro is much less compared with their counterparts matured in vivo [26, 49, 59–62]. Acquisition of oocyte meiotic maturation potential, especially developmental ability, requires mutual interactions between the oocyte and its surrounding somatic cells (cumulus cells plus mural granulosa cells) throughout oogenesis. However, oocyte maturation encompasses both nuclear and cytoplasmic programs, and the full developmental competence is conferred only when these two processes are closely integrated. The intimate associations between these two cell types are established through gap junctions as well as paracrine interactions and persist until ovulation [51, 63, 64].

TABLE 4. Fertilization and embryonic development in a cohort of MII oocytes after 48 h of culture and insemination using IVF or ICSI.

No. of oocytes inseminated from an individual animal	Fertilized	2-Cell	4-Cell	8-Cell	16-Cell	Morula
IVF						
4	3	3	3	3	2	–
4	0	–	–	–	–	–
3	0	–	–	–	–	–
9	1	–	–	–	–	–
6	1	1	1	1	–	–
Total 26	5	4	4	4	2	0
ICSI						
2	1	1	1	–	–	–
5	5	5	5	4	4	3
2	2	1	–	–	–	–
7	2	–	–	–	–	–
Total 16	10	7	6	4	4	3

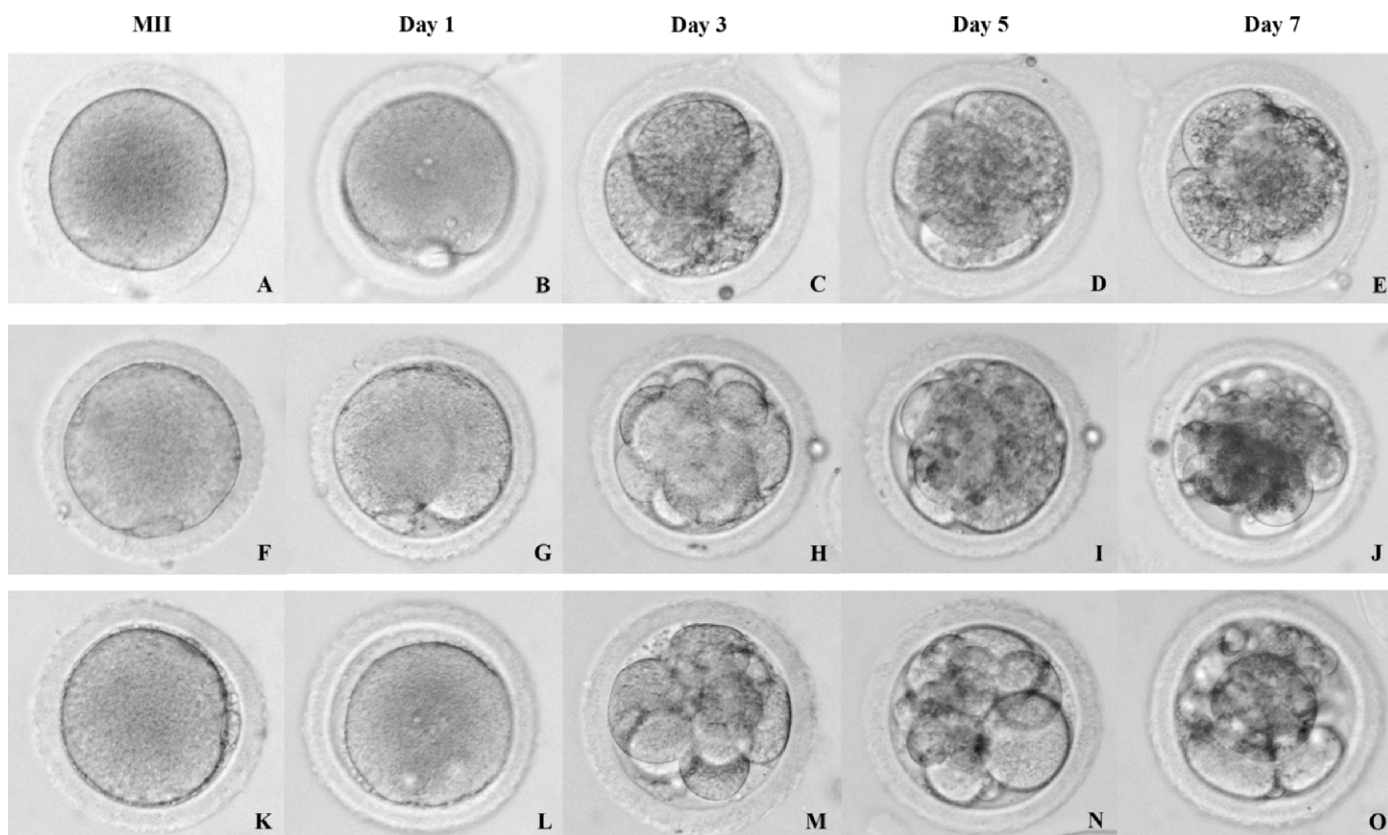


FIG. 4. Representative pictures of MII oocytes prior to (A, F, and K) and after insemination using ICSI (Day 1: B, G, and L; Day 3: C, H, and M; Day 5: D, I, and N; Day 7: E, J, and O). After insemination, the presence of two pronuclei (PN) and two polar bodies (PB) was checked to confirm fertilization (B, G, and L). Some embryos arrested at very early stages (e.g., four cell; D and E), whereas others continued cleaving until the morula stage (J and O), when they arrested. Original magnification $\times 20$.

Therefore, the early interruption that occurs when the COCs are dissected from the follicle for in vitro culture may compromise the meiotic and developmental competence of the oocytes. However, it remains to be determined whether the failure in embryo development in oocytes derived from SAF is due to suboptimal culture conditions or a requirement for the COC to reside for a longer time within the growing antral follicle in primates.

Gonadotropins (FSH and LH) are not required for the cumulus-enclosed oocyte from macaque SAFs to resume meiosis since COCs cultured in either TALP or SAGE media without gonadotropins yielded MI and MII oocytes. These results agree with a previous report in primates wherein no differences were observed between the proportion of MI or MII oocytes obtained from COCs cultured in the absence or presence of gonadotropins [49, 65]. In primates, although follicles that reach the antral stage become dependent on these pituitary hormones for further growth and maturation [66], their involvement in oocyte nuclear and/or cytoplasmic development remains unknown. In mice, low doses of FSH in vitro improve the rates of oocyte fertilization and blastocyst development, whereas high doses reduce oocyte competence to undergo fertilization and preimplantation development but increase granulosa cell differentiation [67, 68]. The use of FSH experimentally, in vitro, avoids the fact that mouse cumulus cells reportedly lack LH receptors and thus cannot respond to LH in vitro [67]. Whether primate cumulus cells are LH insensitive is unknown.

In the present study, all the COCs from SAFs of young adult rhesus monkeys were obtained during the early follicular phase

(Days 2–4) of natural menstrual cycles, and the oocyte's ability to resume meiosis in vitro was carefully analyzed. This source of the COCs is distinct from those in previous reports using both nonhuman primates and women. Most prior studies evaluating IVM in nonhuman primate models used oocytes from large antral follicles of monkeys primed with FSH for 6–7 days [42, 60, 69, 70]. Both human and macaque oocytes isolated from large antral follicle resume spontaneous meiosis [52, 71–74]. In nonhuman primates [42, 60, 69, 70], as in women [75, 76], gonadotropin priming in vivo has been shown to increase the number of oocytes collected and improve their maturation as well as to enhance the embryonic competence. In contrast, oocytes from small follicles unresponsive to an ovulatory gonadotropin surge or from dissected ovaries collected at unknown stages of the menstrual cycle, presumably from small follicles not exposed to gonadotropins, are also used in human IVM, but their competence is lower [29, 31, 32, 77–81]. Furthermore, the specific gonadotropin regimen to use in vitro (FSH alone, hCG alone, or the combination of both) to obtain mature oocytes remains controversial [28, 29, 31, 32, 78, 82]. Although the efficiency of IVM remains low, over 900 babies have currently been born following this technique (with or without gonadotropin priming) around the world [83]. Further studies on primate IVM of oocytes from SAFs are warranted in order to provide an additional, novel source of gametes for fertility preservation in cancer patients.

In summary, primate COCs derived from SAFs of 0.5- to 2-mm diameter during the early follicular phase contain oocytes that can reinitiate meiosis and be fertilized in vitro. Thus, the cohort of SAF present in the medulla of ovaries from

spontaneous menstrual cycles prior to selection of the dominant follicle can provide COCs as an additional source of oocytes for IVM and fertilization. However, the competence of these oocytes to achieve cytoplasmic maturation prior to fertilization with subsequent embryonic development is still under investigation.

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