Short-Term Culture of Ovarian Cortical Strips From Capuchin Monkeys (*Sapajus apella*): A Morphological, Viability, and Molecular Study of Preantral Follicular Development In Vitro

A. B. Brito, MS^{1,2}, R. R. Santos, PhD^{1,2,3}, R. van den Hurk, PhD³, J. S. Lima, MS^{1,2}, M. S. Miranda, PhD⁴, O. M. Ohashi, PhD⁴, and S. F. S. Domingues, PhD^{1,2}

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Abstract

The aim of this study was to evaluate whether an in vitro culture (IVC) medium containing either or not β -mercaptoethanol (BME), bone morphogenetic protein 4 (BMP4), or pregnant mare serum gonadotrophin (PMSG) could be able to promote the development of capuchin monkeys' preantral follicles enclosed in ovarian cortical strips. Follicular viability after IVC was similar to control (89.32%). Primordial follicle recruitment to primary stage was not reached with IVC, but the rate of secondary follicle formation was increased in the medium supplemented with BME, BMP4, and PMSG (44.86%) when compared to IVC control (9.20%). In the medium supplemented with BME, BMP4, and PMSG, contrary to other media, anti-müllerian hormone-messenger RNA (mRNA) expression in ovarian tissue was upregulated (3.4-fold), while that of growth differentiation factor-9 was maintained. The BMP4-mRNA expression, however, appeared downregulated in all cultured tissues. Our findings show a favorable effect of BME, BMP4, and PMSG on the in vitro development of secondary follicles from capuchin monkeys.

Keywords

viability, growth factors, qRT-PCR, nonhuman primates

Introduction

Primatology has been receiving great attention, certainly because of the anatomical and physiological similarity with the human species. As studies develop, the fragility of most primates species has been found to be caused mainly by destruction of the natural primate habitat.¹ In this context, captive breeding programs for endangered primates need to be developed. At present, many reproductive biotechnology programs have been developed in domestic animals, which includes the manipulation of oocytes enclosed in preantral follicles; this process seeks to isolate, culture, and/or preserve preantral follicles.² However, in New World Primates such as capuchin monkeys (*Sapajus apella*), knowledge is restricted to the late folliculogenesis³ and no advances have been obtained in the in vitro development of preantral follicles.

Preantral follicles (ie, primordial, primary, and secondary follicles) can be cultured enclosed in the ovarian tissue^{4,5} or in 3-dimensional (3D) alginate tissue scaffolds,⁶ and both methods guarantee a 3D structure in in vitro culture (IVC). Although IVC in 3D alginate scaffolds allows a long-term culture period instead of ovarian cortical tissue that is restricted for short periods, IVC of preantral follicles enclosed in the ovarian tissue is very practical and avoids the risks of follicular isolation.

As key markers for preantral follicle development or functionality, growth factors involved in the activation and development of preantral follicles could be used such as those previously described in rodents,⁷⁻⁹ humans,¹⁰⁻¹³ and nonhuman primates:¹⁴ connective tissue growth factor (CTGF),^{7,8} growth differentiation factor 9 (GDF9),^{10,11} bone morphogenetic proteins (BMPs),^{11,12} anti-müllerian hormone (AMH),^{13,14} and stem cell factor or c-Kit ligand (KL).⁹ Hence, evaluation of genes encoding the mentioned growth factors seems to be a usable method to assess follicular development in vitro.

¹ Laboratory of Wild Animal Biology and Medicine, Federal University of Pará, Pará, Brazil

² Animal Science Post-graduation Program, Federal University of Pará, Pará, Brazil

³ Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

⁴ Institute of Biological Sciences, Federal University of Pará, Brazil

Corresponding Author:

R. R. Santos, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 104, 3584 CM, Utrecht, the Netherlands.

Email: R.Rodriguesdossantos@pq.cnpq.br

To improve preantral follicular survival and development during IVC, medium has been supplemented with reducing agents such as β -mercaptoethanol (BME),¹⁵ growth factors such as BMP4,¹⁶ and a glycoprotein hormone such as pregnant mare serum gonadotrophin (PMSG), which has both follicle-stimulating hormone (FSH-) and lutenizing hormone (LH)-like activities.¹⁷ Except for the studies such as those from Nilsson and Skinner,¹⁶ Abir et al,¹² and Silva et al,¹⁸ assessment of follicular development is mainly or exclusively based on morphological and viability parameters.^{15,17} Viable and morphologically normal follicles are, however, not unconditionally able to further develop into more advanced stages, since they might be functionally unable to do so. Therefore, the aim of the present study was to assess the viability, the development, and the molecular profile of in vitro cultured capuchin preantral follicles.

Materials and Methods

Chemicals

Unless mentioned otherwise, the chemicals used in the present study were purchased from Sigma Chemical Co (St. Louis, Missouri).

Animals

Our study was approved by the local Ethical Committee in Animal Research (no.029/2009/CEPAN/IEC/SVS/MS) and by the Brazilian Institute for Wildlife and Environment (IBAMA). Four healthy and sexually mature capuchin female monkeys (8-12 years old, weight range 1.9-2.8 kg; all in the follicular phase of the reproductive cycle) were selected for our study. The females were kept indoors under a natural photoperiod and housed individually in cages (80 × 90 × 80 cm) at the National Primate Center, Ananindeua, Pará, Brazil.

Ovarian Tissue Collection

All procedures were performed under general anesthesia. Each animal was anesthetized with ketamine hydrochloride (10 mg/kg; intramuscular [IM]; Vetanarcol, Köning S.A., Avellaneda, Argentina) and xylazine hydrochloride (1 mg/kg; IM; Kensol, Köning S.A.). The females were placed in dorsal recumbence and ovarian biopsies were collected by exploratory laparoscopy. For this, a ventral midline skin incision was made to reach the ovaries. To obtain the ovarian tissue samples, we used a modified trap door technique.¹⁹ Briefly, we performed a 1-mm thick circular incision in the surface of the ovarian tissue and an ovarian cortical envelope flap was raised by means of a N° 15 blade (Paramount Surgimed LTDA -New Delhi, Delhi, India). Suture was unnecessary and bleeding was repressed by gently pressing the sampled area with cold (15°C) 0.9% saline-soaked gauze.

Experimental Design

Biopsies from each ovary were divided into 9 ovarian cortical pieces of 1 mm³. On completion of the procedure, 1 ovarian

Figure 1. Experimental protocol for the IVC of capuchin monkeys' preantral follicles enclosed in ovarian tissue fragments. Viability analysis was performed by labeling preantral follicles with a mixture of propidium iodide (dead cell fluorescent marker) and Hoescht (total cells fluorescent marker). The qRT-PCR was performed to quantify the relative expression (fold-change) of the genes encoding AMH, CTGF, BMP4, BMP15, KL, and GDF9. AMH indicates anti-müllerian hormone; BMP4, bone morphogenetic protein 4; BMP15, bone morphogenetic protein 15; CTGF, connective tissue growth factor; GDF9, growth differentiation factor-9; IVC, in vitro culture; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

fragment (control) was immediately divided into 2 pieces, which were subjected to follicular isolation and viability analysis, or snap frozen in liquid nitrogen and stored at -80°C until RNA extraction. The remaining 8 fragments were individually cultured in vitro without (IVC control) or with supplementation. Each treatment was repeated 4 times, and each repetition was performed in duplicate (Figure 1).

In Vitro Culture

Ovarian fragments were placed in 500 μL of culture medium in 24-well plates for 24 hours. The basic culture medium consisted of TCM-199 (pH 7.2-7.4) supplemented with 10% fetal calf serum (FCS), 100 ng/mL epidermal growth factor (control for cultured follicles), 1.0 mg/mL insulin, 5.5 mg/mL transferin, and 5.0 ng/mL selenium (ITS), either or not added with 10 $\mu\text{mol/L}$ BME,¹⁵ 100 ng/mL BMP4,¹⁶ or 25 IU PMSG¹⁷ alone or in combination. After IVC, all fragments were divided into 2 equal parts and submitted to follicular isolation or quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

Follicular Isolation

After 3 washes in sterile medium, the ovarian fragments were placed in 2 mL tubes containing 500 μ L of TCM with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 200 IU/mL penicillin, and 200 μ g/mL streptomycin, and submitted to a combination of mechanical and enzymatic isolation modifying a method previously described.²⁰ To this end, fragments were cut with a pair of 12-cm straight-bladed (f/f) scissors, homogenized 40 times in 3 mL of medium, and incubated with collagenase type IV (200 IU/mL) at 37°C for 20 minutes, being mixed every 10 minutes. After that, 10% FCS was added to the medium to stop collagenase activity. This homogenate was filtered through a 500- μ m nylon mesh. Spherical follicles smaller than 500 μ m in diameter were collected under a dissecting stereomicroscope and transferred to a fresh Hepes-buffered TCM (holding medium).

Follicular Viability

Isolated follicles were incubated in the holding medium for 10 minutes at 37°C in a mixture of propidium iodide and Hoechst 33342 to detect follicular viability and to enable the counting of the nuclei, respectively. After being labeled, stained follicles were washed 3 times in holding medium, mounted on a glass microscope slide, and examined by epifluorescent microscopy equipped with a digital camera. Oocytes and granulosa cells were classified as degenerating if chromatin was stained positively with propidium iodide, and as viable if chromatin was unlabeled with propidium iodide.²¹ The percentages of viable granulosa cells were calculated in relation to the total number of Hoechst-positive nuclei. Follicles with a viable oocyte surrounded by $\geq 90\%$ viable granulosa cells were considered viable.²²

Follicular Activation and Development to the Secondary Stage

The numbers of primordial, primary, and secondary follicles were, respectively, calculated in cortical fragments that were uncultured (hour 0) and in fragments that were 24 hours in vitro cultured in the various media under study. Likewise, the ratios of secondary follicles were calculated in these fragments. Developmental stages of viable follicles were classified based on the granulosa cell morphology surrounding the oocyte (1) primordial, oocyte surrounded by a layer of flattened granulosa cells; (2) primary, oocyte surrounded by 1 layer of cuboidal granulosa cells; (3) secondary, oocyte surrounded by 2 or more layers of cuboidal granulosa cells.

RNA Extraction and Complementary DNA Synthesis

Total RNA was isolated from control and cultured ovarian tissue homogenized in Trizol reagent (Invitrogen, Carlsbad, California) as described by Nilsson and Skinner.¹⁶ The RNA concentration was estimated by reading the absorbance at

260 nm and was checked for purity at 280 nm in a spectrophotometer (NanoDrop ND-1000 and ND-8000 8-Sample, NanoDrop, Wilmington, Delaware). For each sample, the RNA concentrations were adjusted to 45 ng/mL and used to synthesize complimentary DNA. The reverse transcription was performed in a total volume of 20 μ L composed of 10 μ L of sample RNA, 3.2 μ L of ultra-pure water, 2 μ L reverse transcriptase buffer (High Capacity Reverse Transcription kit, Applied Biosystems, Foster City, California), 0.8 μ L dNTP Mix (10 mmol/L), 2 μ L random primers, 1 μ L MultiScribe reverse transcriptase, and 1 μ L RNase inhibitor (High Capacity Reverse Transcription kit, Applied Biosystems). The mixture was incubated at 42°C for 1 hour, subsequently at 80°C for 5 minutes, and finally stored at -20°C. The negative control was prepared under the same conditions, but without addition of the nucleic acid.

qRT-PCR

Quantitative reverse transcriptase polymerase chain reaction was performed using a thermocycler (ABI PRISM 7500 Real Time PCR system, Applied Biosystems). The primers, chosen to carry out amplification of different reference gene candidates, are shown in Table 1. Amplification efficiency was determined per plate using LinRegPCR.²⁴ Data were analyzed using the efficiency corrected Delta-Delta-Ct method.²⁵ The fold-change values of the genes encoding AMH, CTGF, BMP4, BMP15, KL, and GDF9 were normalized using the geometric average of the fold-change values of 2 reference genes, hypoxanthine phosphoribosyltransferase 1 and TATA box binding protein.

Statistical Analysis

The viability of oocytes and granulosa cells and the numbers of total nuclei per follicle as well as molecular data were calculated using 1-way analysis of variance (ANOVA) and unpaired t test. Rates of viable primordial, primary, and secondary follicles were calculated using 2-way ANOVA. The differences were considered significant when $P < .05$. All data are presented as mean values $\pm$ standard error of the mean, the standard errors being calculated from the variance between the samples.

Results

In total, the viability of 588 preantral follicles (at least 50 per treatment) was examined in the control uncultured group and in the various experimental groups of IVC follicles (IVC control and IVC supplemented groups). Although it is expected that the ovarian cortex is very heterogeneous, the rates of primordial follicles (viable and nonviable) in the uncultured controls was similar (approximately 40%), being possible to use these samples as the standard for follicular class counting. Figure 2 (panels A-C) shows representative images of viable and nonviable preantral follicles. The percentage of viable

Table 1. Pairs of Primers Used in qRT-PCR for Quantification of Reference and Target Genes in the Ovarian Tissue From Capuchin Monkeys.

Gene	Accession no.	Sequence	Fragment size (bp)	Primer position
HPRT1 ²³	S43335.1	5'-TTATGGACAGGACTGAACGTCTTGCT 3'-TCCAGCAGGTCAGCAAAGAATTTATAGC	107	59
TBP ²³	AB178959.1	5'-TGCACAGGAGCCAAGAGTGAA 3'-CACATCACAGCTCCCCACCA	132	889
AMH	XM_002761553.1	5'-CTGCTGCGTGCCCACCGCCTA 3'-CAGCCACACTCGGTGGCCAC	106	1575
BMP4	XM_002805069.1	5'-CGAGAGAGACGCAGACGCAGA 3'-AATGGCTCCATGTTCCCGAG	82	56
BMP15	XM_001083980.2	5'-GCCACTGTGGTTTACCGCCA 3'-GGGAAGGTTTTGAGGAATCTCCTTC	130	446
CTGF	NM_001901.2	5'-TGCATCTTCGGTGGCACG GT 3'-ATGCTGCACAGGGGCATGCA	110	513
GDF9	NM_001168763.1	5'-GCCTTCCCAGGGTCCTGACCA 3'-TGACCTCTGCGGTGACGGTGA	105	861
KL	NM_003994.5	5'-GCTCGGGCTACCCAATGCGTG 3'-CGCTGCGATCCAGCACAAACAG	112	72

Abbreviations: AMH, anti-müllerian hormone; BMP4, bone morphogenetic protein 4; BMP15, bone morphogenetic protein 15; bp, base pair; CTGF, connective tissue growth factor; GDF9, growth differentiation factor-9; HPRT1, hypoxanthinephosphoribosyltransferase 1; KL, kit ligand; TBP, TATA box binding protein.

Figure 2. Panel A-C, Representative images of 2 preantral follicles from capuchin monkeys before (A) and after (B, C) staining with Hoechst (blue; general nuclear staining) and propidium iodide (red; stains dead cells). Absence of propidium iodide staining in the left follicle (C) indicates its viability, whereas the presence of propidium iodide in the right follicle (C) is indicative of its death. $\times 400$. For reference to color, please see online version.

preantral follicles in control uncultured ovarian fragments (89.83%) was not different ($P > .05$) from those after IVC. Furthermore, there was no difference between the IVC control and the rest of the IVC groups ($P > .05$; Table 2).

The rate of activation of primordial follicles that is a decrease in the percentages of primordial follicles accompanied by an increase in the percentages of primary follicles was unaffected ($P > .05$; Figure 3). However, there was an increase ($P < .05$) in the percentages of secondary follicles at the cost of primary follicles, when PMSG, BME, and BMP4 were added to the culture medium (Figure 3).

Figure 4 depicts the relative expression of genes encoding AMH, BMP4, BMP15, CTGF, GDF9, and KL. Expression of genes encoding BMP15, CTGF, and KL was unaltered by the different IVC treatments. The BMP4-messenger RNA (mRNA) expression was decreased ($P < .05$) in all cultured fragments, thus independent of the culture medium. With exception of ovarian tissue cultured in medium supplemented with BME, BMP4, and PMSG, IVC led to decreased GDF9 mRNA expression. In contrast, AMH-mRNA expression was increased when ovarian tissue was cultured in a medium

containing BME, BMP4, and PMSG, whereas its expression was unaffected by other IVC treatments.

Discussion

In the present study, 24 hours IVC of ovarian tissue from capuchin monkey in medium supplemented with PMSG, BME, and BMP4 combined, and not alone, resulted in maintenance or follicular viability combined with the follicular transition from primary to secondary stages. Among these 3 used supplements, PMSG is known to act like FSH and LH and to enhance follicular growth.²⁶ According to Cannon et al,²⁷ PMSG induces follicular growth by stimulating the proliferation and differentiation of granulosa cells that reach a peak in the S phase 24 to 30 hours post-PMSG administration. The BMP4 is known as a promoter of follicular development acting not entirely only on the promotion of the primordial-primary follicle transition,¹⁶ but also promoting granulosa cell proliferation that may increase the secretion of AMH by growing follicles and subsequently inhibit primordial follicle recruitment.²⁸ However, IVC medium supplementation with a combination of PMSG and

Table 2. Mean ($\pm$ SEM) Percentages of Viable Preantral Follicles (Primordial, Primary, and Secondary) in the Control Group and After 24 Hours IVC.^a

	% Viable Follicles $\pm$ SEM (n Viable/n Total)			
	Primordial	Primary	Secondary	Preantral follicles ^b
Control	85.00 $\pm$ 3.54 (17/20)	88.00 $\pm$ 4.64 (22/25)	100.00 $\pm$ 0.00 (07/07)	88.46 $\pm$ 4.64 (46/52)
Control IVC	82.76 $\pm$ 10.9 (24/29)	74.36 $\pm$ 12.1 (29/39)	75.00 $\pm$ 1.84 (06/08)	77.63 $\pm$ 11.9 (59/76)
+ BME	80.00 $\pm$ 1.26 (16/20)	83.33 $\pm$ 1.36 (30/36)	76.92 $\pm$ 1.28 (10/13)	81.16 $\pm$ 3.52 (56/69)
+ BMP4	87.50 $\pm$ 6.97 (14/16)	92.59 $\pm$ 4.57 (25/27)	75.00 $\pm$ 4.52 (06/08)	88.24 $\pm$ 3.31 (45/51)
+ PMSG	75.00 $\pm$ 5.25 (09/12)	86.96 $\pm$ 7.40 (20/23)	80.00 $\pm$ 9.09 (16/20)	81.81 $\pm$ 8.38 (45/55)
+ BME + BMB4	92.86 $\pm$ 4.57 (13/14)	83.33 $\pm$ 4.10 (25/30)	84.21 $\pm$ 7.26 (16/19)	85.71 $\pm$ 5.89 (54/63)
+ BME + PMSG	100.0 $\pm$ 0.00 (15/15)	85.71 $\pm$ 7.05 (24/28)	81.48 $\pm$ 8.22 (22/27)	87.14 $\pm$ 6.33 (61/70)
+ BMP4 + PMSG	94.74 $\pm$ 3.79 (18/19)	93.33 $\pm$ 3.44 (28/30)	95.00 $\pm$ 5.56 (19/20)	94.20 $\pm$ 3.71 (65/69)
+ BME + BMB4 + PMSG	90.48 $\pm$ 5.38 (19/21)	88.46 $\pm$ 4.19 (23/26)	94.44 $\pm$ 3.82 (34/36)	91.57 $\pm$ 4.28 (76/83)
Total evaluated follicles				(507/588)

Abbreviations: BME, β -mercaptoethanol; BMP4, bone morphogenetic protein 4; IVC, in vitro culture; PMSG, pregnant mare serum gonadotrophin; SEM, standard error of the mean.

^a No significant differences were observed among groups and follicular classes regarding the percentages of viable follicles.

^b Total preantral follicles is the sum of primordial, primary, and secondary follicles.

Figure 3. Rates of viable primordial, primary, and secondary follicles before and after in vitro culture. Follicles were considered viable when a viable oocyte was surrounded by $\geq 90\%$ viable granulosa cells. Follicles were classified as primordial when the oocyte was surrounded by a layer of flattened granulosa cells, primary when the oocyte was surrounded by 1 layer of cuboidal granulosa cells, or as secondary when the oocyte was surrounded by 2 or more layers of cuboidal granulosa cells. *Indicates significant differences between control group and treatments in each follicular class ($P < .05$).

BMP4 only was not sufficient to improve follicular transition to secondary stages, but medium supplementation with BME was necessary. As a reducing agent, one can state that this compound might help the follicular development by counteracting the production of reactive oxygen species during IVC. However, no differences were found in the follicular viability when groups cultured with or without BME were compared. Another explanation for our findings, therefore, is the cell proliferative effect of BME. By culturing different cell lines, it has been shown that BME acts not only as an antioxidant but also by stimulating cell proliferation,^{29,30} which supports our findings regarding the increase in secondary follicles.

Independent of the culture medium tested, follicular viability was similar to control values after 24 hours of IVC,

probably because such short-term culture did not require extra-nutritional supplements for follicle survival. Furthermore, the presence of serum in the culture medium might provide extra nutrients to the follicles.³¹ A 24-hour IVC may appear as a too short period to achieve follicular assembly of even the transition to secondary stages. However, one must bear in mind that under in vitro conditions stimulatory and inhibitory factors act more robustly. As already shown by Abir et al,^{32,33} human primordial follicles cultured in vitro for 24 hours were able to develop into secondary follicles. Such results were reached when these former authors had performed IVC of isolated follicles, that is free from the factors secreted by stromal cells, which may lead to a release of factors that control follicular development. In the present study, medium supplementation

Figure 4. Comparison of mean ($\pm$ SEM) relative expression of AMH, CTGF, BMP4, BMP15, KL, and GDF9 in ovarian tissue from the control, IVC control group, and ovarian tissue that was cultured in vitro in the presence of BME, BMP4, or PMSG alone or in mixtures of these compounds. Fold-changes in relative mRNA expression were quantified relative to the expression in the control group by qRT-PCR analyses ($P < .01$). Data were normalized using 2 housekeeping genes (HPRT1 and TBP). AMH indicates anti-müllerian hormone; BME, β -mercaptoethanol; BMP4, bone morphogenetic protein 4; BMP15, bone morphogenetic protein 15; CTGF, connective tissue growth factor; GDF9, growth differentiation factor 9; HPRT1, hypoxanthine phosphoribosyltransferase 1; KL, kit ligand; IVC, in vitro culture; PMSG, pregnant mare serum gonadotrophin; mRNA, messenger RNA; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; SEM, standard error of the mean; TBP, TATA box binding protein.

with BMP4, BME, and PMSG may lead to the similar effects observed by Abir et al³² as shown by qRT-PCR analysis. Merely in this medium, GDF9 expression is maintained and AMH expression is increased. Expression of BMP15, KL, and CTGF was not influenced by all tested culture media. In contrast, BMP4 expression was decreased in this medium as it was in all other tested media.

Anti-müllerian hormone was previously found to be expressed in human¹³ and monkey¹⁴ granulosa cells from secondary and early antral follicles. Upregulation of AMH was found to inhibit activation of primordial follicles³⁴ which might be related to the stimulation of proliferation and differentiation of granulosa cells in the present study. The AMH expression has been demonstrated in the granulosa cells from growing follicles, mainly secondary ones from human¹³ and monkeys.¹⁴ These studies have shown that AMH inhibits primordial to primary follicle transition, and so controls primordial follicle recruitment. The GDF9 is expressed in oocytes^{7,11} and is essential for early folliculogenesis.⁶ Its downregulation might have caused the absence of stimulated secondary follicle formation in other media than that containing PMSG, BME, and BMP4.

Connective tissue growth factor is required for normal follicle development and is expressed in preantral follicles during all developmental phases,³⁵ while its expression rises only in antral follicles.³⁶ Therefore, change in expression could be unexpected in the present IVC conditions. Like CTGF, KL and BMP15 are important in the maintenance of follicular survival and normal follicular development.^{6,9,37} The KL is found in pregranulosa cells and promotes primordial follicles activation.⁹ In our cultured ovarian tissues not only KL expression, but also primordial follicle recruitment was unaltered. Within the ovary, BMP15 is exclusively expressed in the oocyte^{38,39} with expression increasing in relation to the follicle growth and development until a peak is reached in maturing antral follicles.^{39,40} The BMP-15 furthermore stimulates KL expression in granulosa cells,⁴¹ while KL inhibits BMP-15 expression in the oocytes. In this way, BMP-15 and KL form a negative feedback loop between the oocyte and the surrounding granulosa cells,⁴² which made it difficult to observe change in their expression at mRNA level in our IVC study.

In all ovarian tissues that have been in vitro cultured for 24 hours, BMP4 appeared to be downregulated. This, however, did not negatively affect follicular viability, primordial follicle activation, and secondary follicle development. Previously, in vitro BMP4 suppression for 14 days resulted in apoptosis of rat ovarian stromal cells and a progressive loss of follicles.¹⁶ This indicates that BMP4 suppression may have a detrimental effect on follicle development after a longer culture period than 24 hours.

In conclusion, 24 hours IVC of ovarian tissue from capuchin monkeys in a medium supplemented with PMSG, BME, and BMP4 favors follicular transition from primary to secondary stages. Therefore, it can be used as a second step to be added after activation of primordial follicles in vitro. However, studies on synchronic follicular development are still needed.

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Declaration of Conflicting Interests

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