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Optimized study of an *in vitro* 3D culture of preantral follicles in mice

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ABSTRACT

Background: *In vitro* culture of preantral follicles is a promising technology for fertility preservation.
Objectives: This study aims to investigate an optimized three-dimensional (3D) fetal bovine serum (FBS)-free preantral follicle culture system having a simple and easy operation.
Methods: The isolated follicles from mouse ovaries were randomly divided in an ultra-low attachment 96-well plates supplement with FBS or bovine serum albumin (BSA) culture or encapsulated with an alginate supplement with FBS or BSA culture. Meanwhile, estradiol (E_2) concentration was assessed through enzyme-linked immunosorbent assay of culture supernatants. The diameter of follicular growth was measured, and the lumen of the follicle was photographed. Spindle microtubules of oocytes were detected via immunofluorescence. The ability of oocytes to fertilize was assessed using *in vitro* fertilization.
Results: The diameters were larger for the growing secondary follicles cultured in ultra-low attachment 96-well plates than in the alginate gel on days 6, 8, and 10 ($p < 0.05$). Meanwhile, the E_2 concentration in the BSA-supplemented medium was significantly higher in the alginate gel than in the other three groups on days 6 and 8 ($p < 0.05$), and the oocytes in the FBS-free system could complete meiosis and fertilization *in vitro*.
Conclusions: The present study furnishes insights into the mature oocytes obtained from the 3D culture of the preantral follicle by using ultra-low attachment 96-well plate with an FBS-free system *in vitro* and supports the clinical practices to achieve competent, mature oocytes for *in vitro* fertilization.

Keywords: Preantral follicles; 3D culture; ultra-low attachment plate; sodium alginate

INTRODUCTION

The rejuvenation of tumors has recently made numerous adolescent women with cancer lose fertility even at a young, childbearing age. Studies show that the use of radiotherapy and chemotherapy in cancer treatment can damage 40%–80% of fertility so that patients face the risk of ovarian function loss or infertility [1]. According to the fertility preservation recommendations of female cancer patients, for women with a reproductive will, ovarian tissue cryopreservation, oocyte freezing, embryo freezing, artificial ovary transplantation, and

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Conflict of Interest

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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other approaches of fertility preservation can be used [2]. Currently, with the help of ovarian tissue freezing technology, more than 200 babies have been born worldwide, and the first in China was successfully born in 2021 [3]. However, the tumor cells will accompany the blood circulation into the ovarian tissue, and transplantation of ovarian tissue containing tumor cells may make the cancer relapse [4]. Currently, *in vitro* culture of preantral follicles and obtaining mature oocytes can effectively avoid the risk of tumor recurrence after remigration.

To date, mouse [5], bovine [6], pig [7], sheep [8], and human [9] follicles have successfully acquired mature oocytes. However, the oocytes derived from preantral follicles have unstable *in vitro* maturation rates (varying from 20% to 70%) [10]. Furthermore, the culture methods contained fetal bovine serum (FBS), which limited this technology in fertility preservation and application in humans. FBS contains nutrients, growth factors, cytokines, extracellular matrix proteins and hormones that stimulate cell division [11]. Nowadays, the desire to minimize the introduction of xenogeneic proteins or pathogens and to standardize cellular products intended for clinical application has driven the search for alternatives to FBS. Bovine serum albumin (BSA), as the most abundant protein in plasma, is probably better than FBS owing to lower xenogeneic proteins or pathogens [12].

To establish a stereoscopic three-dimensional (3D) follicle culture system, the preantral follicles were mainly cultured by microdroplet method in the early stage, but the ovarian somatic cells (theca and granulosa cells) were adherent, leading to a low rate of follicle development [13]. Subsequently, while the alginate hydrogel culture system could support individual follicle growth with 3D, the early follicular development stage requires a longer time period, and *in vitro* matured oocyte was collected after degrading alginate sodium by alginate lyases [14]. Recently, ultra-low attachment 96-well plates were used to form 3D spheroids, including organoids, since hydrogel was attached to the surface of the plate, mimicking the extracellular matrix [15], and soluble factors such as cytokines and growth factors can pass freely in hydrogel [16,17].

Therefore, this study aims to develop an FBS-free *in vitro* ovarian follicle culture system that can enable a convenient operation and provide a new avenue for the advancement of fertility preservation technology for cancer patients.

MATERIALS AND METHODS

Animals and ethical approval

Pregnant Institute of Cancer Research (ICR) mice, purchased from the Laboratory Animal Center of Ningxia Medical University, were maintained in a specific pathogen-free laboratory. The experiments performed here were approved by the Animal Care Committee of the Ningxia Hui Autonomous Region (IACUC No. SCXK (Ning) 2020-0001). To abide by the 3R Principle of Animal Experiments, the number of mice was minimized so that the smallest number was experimentally used while still retaining statistical significance.

Preantral follicle isolation

Female ICR mice were sacrificed by cervical dislocation 12.5 d after birth. The skin and muscle were cut layer by layer from the rib ridge angle to expose the ovaries. After the ovaries were removed, they were immediately placed in the operating culture medium, which consisted of L-15 (Invitrogen Life Science, USA) supplemented with 3% BSA (Sigma, USA) or

10% FBS (Gibco, USA), 1% penicillin (Gibco). Isolation of mice follicles was achieved using 30-G needles. The separation time of each ovary *in vitro* was guaranteed to be no more than 10 min. Under an anatomic microscope, the preantral follicles of diameter between 100 and 130 μm were selected. It has an intact basement membrane and clear oocyte structure and is surrounded by two to three layers of granulosa cells with a few adhering theca cells. Preantral follicles were washed thrice in the growth medium (GM), which consisted of MEM-alpha (Invitrogen Life Science) supplemented with 0.33 mM pyruvate (Sigma), 3% BSA or 10% FBS, 1% penicillin, 1% ITS (Gibco), and 100 mIU/mL FSH (ShuSheng, China) to remove the operating medium.

Cultures of preantral follicles *in vitro*

As illustrated in **Fig. 1**, the materials for 3D follicle culture, including ultra-low attachment 96-well plate and alginate hydrogel, were used to culture the early secondary preantral follicles *in vitro*. The specific experiments are described as follows.

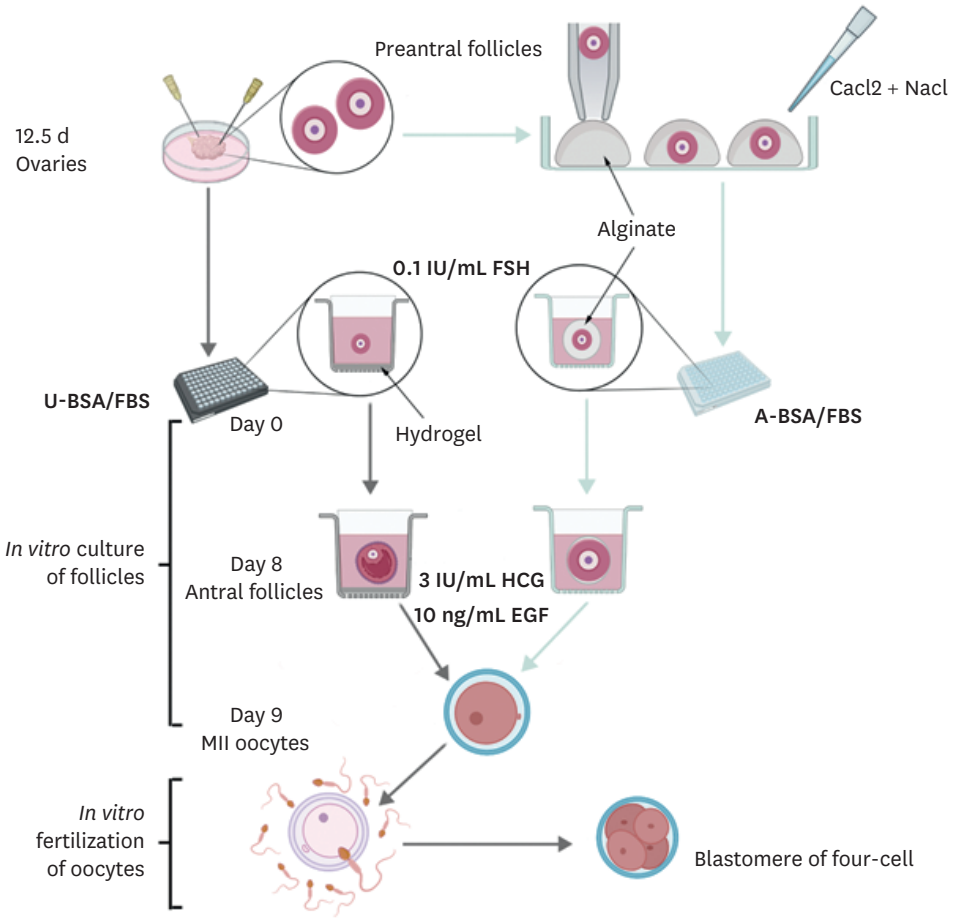

Fig. 1. A schematic illustration of the present research. The preantral follicles were first cultured in an ultra-low attachment 96-well plate or alginate to obtain antral follicles using a BSA or FBS medium, followed by the IVF of oocytes from the COCs.
BSA, bovine serum albumin; FBS, fetal bovine serum; A-FBS, alginate supplemented with fetal bovine serum; A-BSA, alginate supplemented with bovine serum albumin; U-FBS, ultra-low attachment 96-well plate supplemented with fetal bovine serum; U-BSA, ultra-low attachment 96-well plate supplemented with bovine serum albumin; IVF, *in vitro* fertilization; COC, cumulus-oocyte complex; FSH, follicle stimulating hormone; MII, meiosis II; hCG, human chorionic gonadotropin; EGF, epidermal growth factor.

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Ultra-low attachment 96-well plate method

The volume of each well in the GM was 200 μL, which was incubated for about 4 h with 5% CO_2 in air and 100% humidity at 37°C. Each follicle was mechanically isolated from the ovaries in the operating medium and placed individually in the ultra-low attachment 96-well plate. After that, follicles were cultured for 8 d in an incubator with 5% CO_2 in air and 100% humidity at 37°C. Day 0 represents the day on which the culture was started. On day 1 of culture, 100 μL of the culture medium was added to each well. On day 8, follicles were further cultured in the mature medium (MM), which contained of GM supplemented with 1.5 IU/mL human chorionic gonadotropin (hCG; ShuSheng) and 10 ng/mL of mouse epidermal growth factor (PeproTech, USA) for another 16 h for ovulation. Next, the cumulus-oocyte complexes (COCs) were expelled from follicles. The COCs were digested in 0.5 mg/mL of hyaluronidase (Sigma) within 2 min. The oocytes were measured for their ability to fertilize. Half of the medium was refreshed, and the medium was collected every day after day 2 of culture and pooled at −20°C. The concentration of estradiol (E_2) was measured using the enzyme immunoassay (Elabscience Biotechnology Co., Ltd, USA). Diameters and morphological structures of cultured follicles were observed using an inverted microscope every day from day 2 of culture.

Encapsulation of isolated follicles in alginate beads

After washing in the GM, preantral follicles were sucked into the pipette tip with 0.7 μL of 0.5% alginate solution (Sigma) solution. Alginate beads are typically made by dropping the alginate solution and follicles from a pipette tip directly into a crosslinking bath consisting of 140 mM calcium solution (Sigma). After washing in the GM with BSA or FBS thrice, beads were placed individually in a 96-well plate well incubated about 4 h with 5% CO_2 in air and 100% humidity at 37°C. On day 9, 16 h post-hCG on day 8, follicles embedded by alginate were mechanically teared using 30-G needles to release COCs. The subsequent steps are the same as above.

In vitro fertilization

After 16 h, COCs ovulated from follicles were retrieved. COCs were digested by hyaluronidase (Sigma) in about 2 min at 37°C. Oocytes with first polar bodies and homogeneous cytoplasm were considered normal mature oocytes. Mature oocytes that are at the meiosis II (MII) stage are known as MII oocytes. As illustrated in **Fig. 1**, mature oocytes were counted and transferred to a drop of G-IVF medium covered (Vitrolife, Sweden) with paraffin liquid (Sigma) in a 35-mm dish, which was incubated for about 4 h with 5% CO_2 in the air and 100% humidity at 37°C. Mature spermatozoa were collected from the cauda epididymis of ICR mice over 8 wk of age [18]. Spermatozoa were added at a final concentration of 2×10^6/mL to a drop of G-IVF medium containing the MII oocyte and incubated for 20 h at 37°C. Spermatozoa were removed by pipetting, and the eggs were cultured for 72 h in the G-1 medium (Vitrolife).

In the positive control group, female mice were injected intraperitoneally with 10 IU of pregnant mare serum gonadotropin (PMSG; ShuSheng), followed by 10 IU hCG (ShuSheng) at 48 h post-PMSG. Ovulated COCs were retrieved from the oviduct at 16 h post-hCG [19].

Determination of exocrine hormone E_2

The supernatant from each cultured follicle was used for E_2 analysis. E_2 was detected using an enzyme-linked immunosorbent assay (ELISA) kit (Elabscience Biotechnology Co., Ltd) in accordance with the manufacturer's instructions. This is summarized as follows: (i) add the standard working solution and samples to wells (50 μL for each well); (ii) immediately add

50 μL of biotinylated detection Ab working solution to each well; (iii) incubate for 45 min at 37°C; (iv) wash thrice after step (iii); (v) add 100 μL of HRP conjugate working solution to each well; (vi) incubate for 30 min at 37°C; (vii) add 90 μL of substrate reagent to each well; (viii) incubate for about 15 min at 37°C; (ix) protect the plate from light; (x) add 50 μL of stop solution to each well; and finally (xi) determine the optical density value of each well at once with a micro-plate reader set to 450 nm.

Measurement of follicle diameter

The follicles were photographed on days 2, 4, 6, 8, and 10 by an inverted fluorescence microscope (Nikon, Japan). The follicle diameter was measured using NIS-Element D software. This software was applied to determine the three outermost points of the follicle, and the circle formed by these three points enclosed the follicle as much as possible. The diameter of the circle counts as the diameter of the follicles.

Tissue fixation and histological analysis

On days 5 and 8, follicles were mixed with 3% agarose (low-melting gel; Solarbio, China) and fixed in 4% paraformaldehyde (ZSGB-BIO, China) overnight. All fixed tissues were processed using an automated tissue processor (Leica, Germany) and embedded in paraffin. Next, 5 μm sections were performed and stained with hematoxylin and eosin (H&E).

Immunofluorescence staining

Follicles on day 9 embedded by alginate beads were mechanically teared using 30-G needles to release COCs. Control groups were retrieved from the oviduct of 21-d mice. COCs from each group were digested in hyaluronidase (Sigma) for 2 min and washed thrice (5 min each) with phosphate-buffered saline (PBS). Oocytes with first polar bodies and homogeneous cytoplasm were considered at the MII stage. Oocytes only with homogeneous cytoplasm were considered at the meiosis I (MI). Oocytes with germinal vesicles and homogeneous cytoplasm were considered at the germinal vesicle stage (GV) [20]. Corresponding oocytes at GV, MI, MII stages were counted. Oocytes at the MI stage were collected and fixed with 2% paraformaldehyde and 0.02% Triton X-100 for 30 min. After that, oocytes were washed with PBS thrice for 5 min. The oocytes were blocked with 3% BSA (Sigma) for 1 h at room temperature. Thereafter, the oocytes were incubated with rabbit α-tubulin antibodies (1:800; Abcam, USA) overnight at 4°C. After washing thrice, FITC-labeled goat anti-rabbit secondary antibodies (Gibco) were added, and the samples were further incubated at 37°C for 1 h. Next, the slides were incubated with 10 ng/mL DAPI at room temperature for 15 min for nuclei staining. Observation and photographing were carried out using a confocal laser scanning microscope (Nikon) using the appropriate excitation wavelength filters.

Statistical analyses

All data are expressed herein in terms of mean ± SD. The statistical analyses were performed using a one-way analysis of variance and Student's *t*-test using Prism v.8 software (GraphPad Software, Inc., USA). Furthermore, the graphs were plotted using Prism v.8 software. $p < 0.05$ was regarded as the statistical significance, while $p < 0.01$ was considered to indicate a highly significant difference among the different treatment groups.

RESULTS

In vitro culture of early preantral follicles with a different 3D culture system

To obtain a large number of preantral follicles (100–130 μm) with whole intact outer membrane of theca cells and layers of granulosa cells in the middle together with primary oocyte, ovaries were isolated using a mechanical method from 12.5 d of postcoitum fetuses. Typical images showing the morphology of early preantral follicles appeared to be surrounded by two to four layers of granulosa cells and a small number of theca cells with no follicular cavity (**Fig. 2**).

Morphologically, in the process of culture in alginate, the follicles showed a 3D spherical growth state (**Fig. 2**). With the increase in culture days, the number of layers of granulosa cells increased. However, it was difficult to form follicular antrum. After 8 d of culture, the follicles were deformed, and some of them displayed abnormal growth like long strips, and no COCs with complete structure were excreted after maturation after 12 d (**Fig. 2**). Simultaneously, most follicles undergo apoptosis, atresia, and degeneration during culture. Compared with FBS, the follicles showed a higher number of granulosa cells by adding BSA, but without any significant difference (**Table 1**).

With hydrogels on their surfaces, ultra-low attachment 96-well plates minimize cell attachment and are often used to study *in vitro* cell self-assembly into organoids or tissues [21,22]. Therefore, placing follicles into 96-well can simulate a 3D culture environment, making it difficult for the proliferating granulosa cells to contact the bottom of the hole. The follicles became round and increased in size suspended in the ultra-low attachment 96-well plates. As mentioned above, the follicles were isolated and washed in the GM. Follicular growth was observed on days 2, 4, 6, 8, and 10 (**Fig. 2**). In the process of culture, the number of layers of granulosa cells increased with the increase in culture days. Generally, the structure of the follicles appeared loose and lost their integrity when compared with the alginate group. Follicular volume increased and follicular antrum began to form after 4 d. On day 8, the follicular antrum continued to increase, and the follicles were close to maturity. COCs were released after 16 h by adding the MM (**Fig. 2**). These results indicated that follicular growth rate was faster than alginate to mimic the growth pattern *in vivo* with ultra-low attachment 96-well plates. However, some follicles that did not ovulate COCs were cultured until day 10, when they were considered to be in the stage of developmental arrest.

We then performed the statistical analysis for the diameter of follicles. After 8 d in culture, the follicles were observed and photographed, and the follicles had increased by 230% in diameter (**Table 1**). We found no significant difference in follicular diameter between different culture methods on days 0 4 ($p > 0.3$ and $p > 0.23$ respectively). However, the follicular diameter from ultra-low attachment 96-well plate supplemented with the BSA (U-BSA) was significantly different from that of alginate supplemented with FBS on day 2 ($p < 0.05$). In addition, on days 6 and 8 of culture, the diameter of follicles cultured by the alginate supplemented with BSA (A-BSA) group was significantly higher than that of the other three groups. No statistically significant differences were observed among these groups ($p < 0.05$). On day 10 of culture, the diameter of follicles cultured by ultra-low attachment 96-well plate with the FBS medium (U-FBS) was significantly higher than U-BSA.

Next, we performed statistical analyses for the number of antrum formation, survival follicles on day 8, and ovulating COCs, the number of GV, MI, and MII on day 9 from follicles (**Table 2**). We found no significant difference in the number of survival follicles between different culture

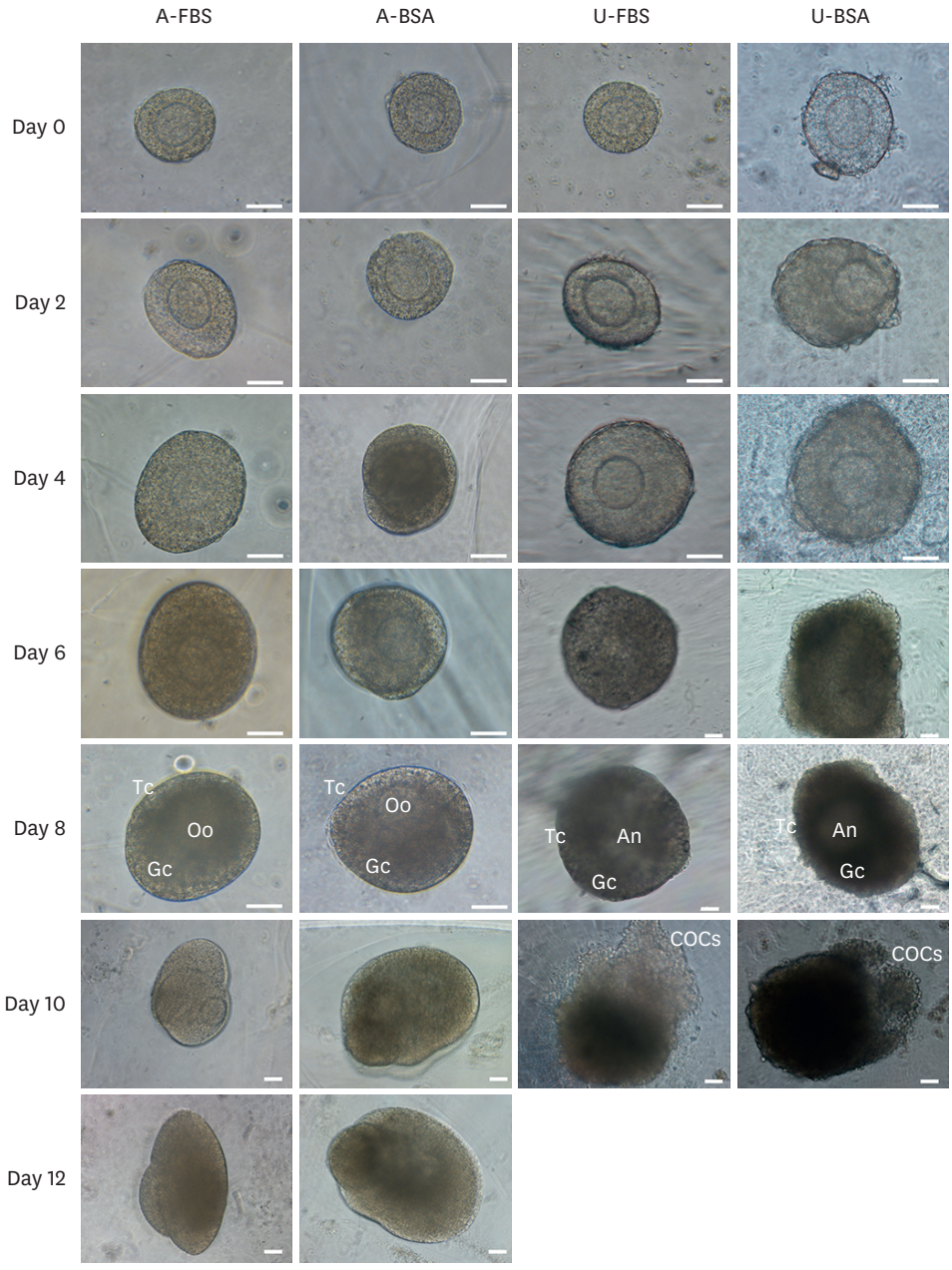

Fig. 2. Growth and development of preantral follicles *in vitro*. Day 4, follicles that increased the number of layers of granulose cells. Day 6, follicles become spherical. Day 8, antral follicles. Day 10, follicles close to maturity and expelled COCs after hCG at day 8 in an ultra-low attachment 96-well plate. Day 12, follicles embedded in alginate cannot form antrum. Scale bar: 50 μm.
Tc, theca cell; Gc, granulosa cell; Oo, oocyte; An, antrum; A-FBS, alginate supplemented with fetal bovine serum; A-BSA, alginate supplemented with bovine serum albumin; U-FBS, ultra-low attachment 96-well plate supplemented with fetal bovine serum; U-BSA, ultra-low attachment 96-well plate supplemented with bovine serum albumin; COC, cumulus-oocyte complex; hCG, human chorionic gonadotropin.

methods on day 8 ($p > 0.39$). However, the number of antrum formation from U-BSA were significantly different from U-FBS and A-BSA on day 8 (44.44% and 24.65%, respectively; $p < 0.05$). In addition, on day 9 of culture, the number of ovulating COCs cultured by U-BSA was significantly different from U-FBS (6.94% and 12.15%, respectively; $p < 0.05$). Furthermore, compared with ultra-low attachment 96-well plate group, the number of GV oocyte was

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Table 1. Effects of different groups on the diameter of preantral follicles *in vitro*

Group	No. of preantral follicles	Diameter of follicles					
		Day 0	Day 2	Day 4	Day 6	Day 8	Day 10
A-FBS	150	125.8 ± 16.0	137.5 ± 20.0	161.9 ± 29.0	185.9 ± 28.0	207.5 ± 32.0	252.3 ± 56.0
A-BSA	150	130.5 ± 14.0	141.7 ± 17.0	154.6 ± 25.0	217.0 ± 55.0	278.1 ± 59.0	286.1 ± 64.0
U-FBS	150	127.6 ± 16.0	130.0 ± 9.0	161.9 ± 25.0	271.1 ± 46.0[c]	358.5 ± 31.0[c]	365.4 ± 46.0[c]
U-BSA	150	129.8 ± 17.0	139.3 ± 13.0	155.1 ± 19.0	164.5 ± 25.0[a,b]	232.7 ± 65.0[a,b]	328.6 ± 70.0
p					< 0.05	< 0.05	< 0.05

Data are presented herein as mean ± SD.
A-FBS, alginate supplemented with fetal bovine serum; A-BSA, alginate supplemented with bovine serum albumin; U-FBS, ultra-low attachment 96-well plate supplemented with fetal bovine serum; U-BSA, ultra-low attachment 96-well plate supplemented with bovine serum albumin.
[a]$p < 0.05$, U-BSA compared with U-FBS; [b]$p < 0.05$, U-BSA compared with A-BSA; [c]$p < 0.05$, U-FBS compared with A-FBS.

Table 2. Effects of different groups on the development of preantral follicles *in vitro*

Group	Total	No. of survival follicles	No. of antral follicles	No. of ovulate COCs	No. of GV oocytes	No. of MI oocytes	No. of MII oocytes	No. of fertilization
A-FBS	96	84 (87.9 ± 4.0)	30 (31.6 ± 7.5)	0	72 (75.4 ± 4.0)	5 (5.2 ± 1.0)		0
A-BSA	96	73 (76.4 ± 6.1)	24 (24.7 ± 6.0)	0	65 (68.1 ± 5.5)	2 (2.1 ± 1.0)		0
U-FBS	96	74 (77.1 ± 13.5)	43 (44.4 ± 4.5)	12 (12.2 ± 1.5)	2 (2.1 ± 1.0)[c]	5 (4.9 ± 0.6)	3 (3.1 ± 1.0)	1 (1.3 ± 0.6)
U-BSA	96	80 (83.7 ± 6.4)	31 (32.6 ± 6.4)	7 (6.9 ± 1.2)[a]	1 (1.0 ± 1.0)[b]	2 (2.4 ± 0.6)[a]	4 (3.8 ± 0.6)	2 (1.7 ± 0.6)
p				< 0.05	< 0.05	< 0.05		

Data were shown as mean ± SD.
COC, cumulus-oocytes complex; GV, germinal vesicle; MI, meiosis I; MII, meiosis II; A-FBS, alginate supplemented with fetal bovine serum; A-BSA, alginate supplemented with bovine serum albumin; U-FBS, ultra-low attachment 96-well plate supplemented with fetal bovine serum; U-BSA, ultra-low attachment 96-well plate supplemented with bovine serum albumin.
[a]$p < 0.05$, U-BSA compared with U-FBS; [b]$p < 0.05$, U-BSA compared with A-BSA; [c]$p < 0.05$, A-BSA compared with U-FBS.

significantly different from alginate groups (1.56% and 71.71%, respectively; $p < 0.05$) and was thus considered to be in the stage of developmental arrest. These data suggested that ultra-low attachment 96-well plates had greater development potential than alginate in the 3D culture environment *in vitro*.

Detection of E_2 levels in different culture methods

E_2 can maintain secondary sexual characteristics in female animals and affect follicular development [23]. In this essay, E_2 levels on days 2, 4, 6, and 8 of culture was detected via ELISA (**Fig. 3**). The mean E_2 secretion on days 2 and 4 remained stable, while E_2 levels in each group increased on days 6 and 8. However, the E_2 levels from the BSA medium on days 6 and 8 were significantly higher than those from the FBS medium with ultra-low attachment 96-well plate or sodium alginate embedding ($p < 0.05$). The E_2 levels from the sodium alginate embedding on days 6 and 8 were significantly higher than those from the ultra-low attachment 96-well plate with the BSA medium ($p < 0.05$). These data suggested that sodium alginate embedding with the BSA medium stimulated the secretion of E_2 by the follicles more effectively on days 6 and 8.

Detection of the internal morphology of follicles cultured *in vitro*

Next, we used H&E staining to detect the internal morphology of follicles. After that, follicles were grown on days 5 and 8 in the ultra-low attachment 96-well plate group shown 3D spherical growth state. However, the follicles in the alginate group were irregular in shape and heterogenous in density, with cell membrane breakage (**Fig. 4**). U-BSA follicular theca was thicker than in the U-FBS group on day 5, although no significant difference was found in the diameter (**Table 1**). On day 8, the follicle cavity continued to increase, and the follicles were close to maturity. However, as shown in the **Fig. 2**, although the follicle cavity and diameter in the U-BSA group were smaller than those in the U-FBS group (**Table 1**), the number of granular layers in the U-BSA group was more than that in the FBS group.

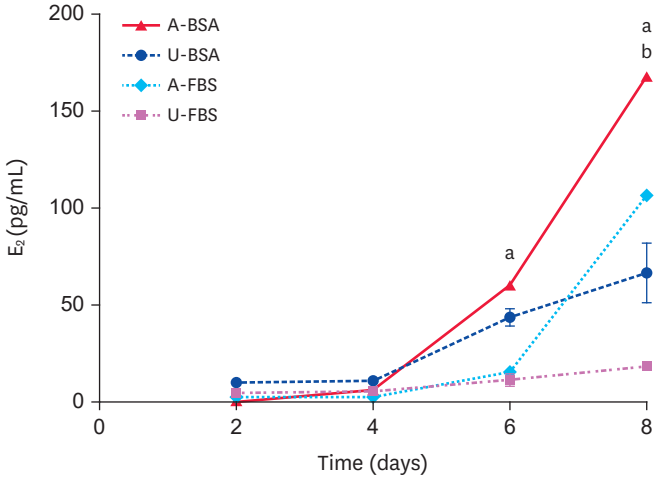

Fig. 3. Endocrine function of preantral follicles growing *in vitro*. Changes in the E_2 concentrations in medium cultured in the presence of U-BSA, U-FBS, A-BSA, and A-FBS. The E_2 concentration in culture medium was significantly higher in the BSA group than in the FBS group whether in sodium alginate or ultra-low attachment 96-well plates on days 6 and 8.
E_2, estradiol; A-FBS, alginate supplemented with fetal bovine serum; A-BSA, alginate supplemented with bovine serum albumin; U-FBS, ultra-low attachment 96-well plate supplemented with fetal bovine serum; U-BSA, ultra-low attachment 96-well plate supplemented with bovine serum albumin; BSA, bovine serum albumin; FBS, fetal bovine serum.
$^{a}p < 0.05$; $^{b}p < 0.05$, U-FBS compared with A-FBS.

Moreover, the granular cytoplasm around the oocyte was deeply stained. In contrast, the inner of the follicle lacked the cumulus granulosa cell, and mural granulosa cells exhibited deep staining in the U-FBS group, suggesting that the cells were necrotic. These results indicated that the internal structure of follicles was normal, and the number of apoptotic cells induced by spatial location was relatively lower in the U-BSA group.

Effects of different culture methods on microtubule distribution of oocyte spindles *in vitro*

To further ascertain whether oocytes were activated after *in vitro* maturation, we stained them with the combination of an anti-α-tubulin antibody and DAPI. On day 9 of follicular culture *in vitro*, oocytes at the germinal vesicle stage were allowed to undergo germinal vesicle breakdown in U-FBS and U-BSA groups, and then microtubules organized into the MI spindle, with chromosomes aligning at the equatorial plate (**Fig. 5A**) [24]. Oocytes from the ultra-low attachment 96-well plate showed a representative organization of spindles (α-tubulin as green) with chromosomes (blue). Spindle microtubules were organized in a characteristic barrel shape at metaphase in control and U-FBS groups. However, attenuated astral microtubule formations were found in the U-BSA group. Furthermore, the number of oocytes in the U-FBS group with organized spindle were higher compared with that in the U-BSA group, while significant difference was observed (**Fig. 5B**, $p < 0.05$). However, the assembly of spindles was not observed in the alginate group (**Fig. 5**) at the same time point. Furthermore, oocytes germinal vesicle did not breakdown while nucleus showing some fragments in alginate group. These results suggested that oocytes could form an assembly spindle in an ultra-low attachment 96-well plate compared with the alginate group.

In vitro fertilization of oocytes from preantral follicles

As spindles were difficult to organize in the sodium alginate group, we used ultra-low attachment 96-well plates for subsequent follow-up experiments. To further assess the

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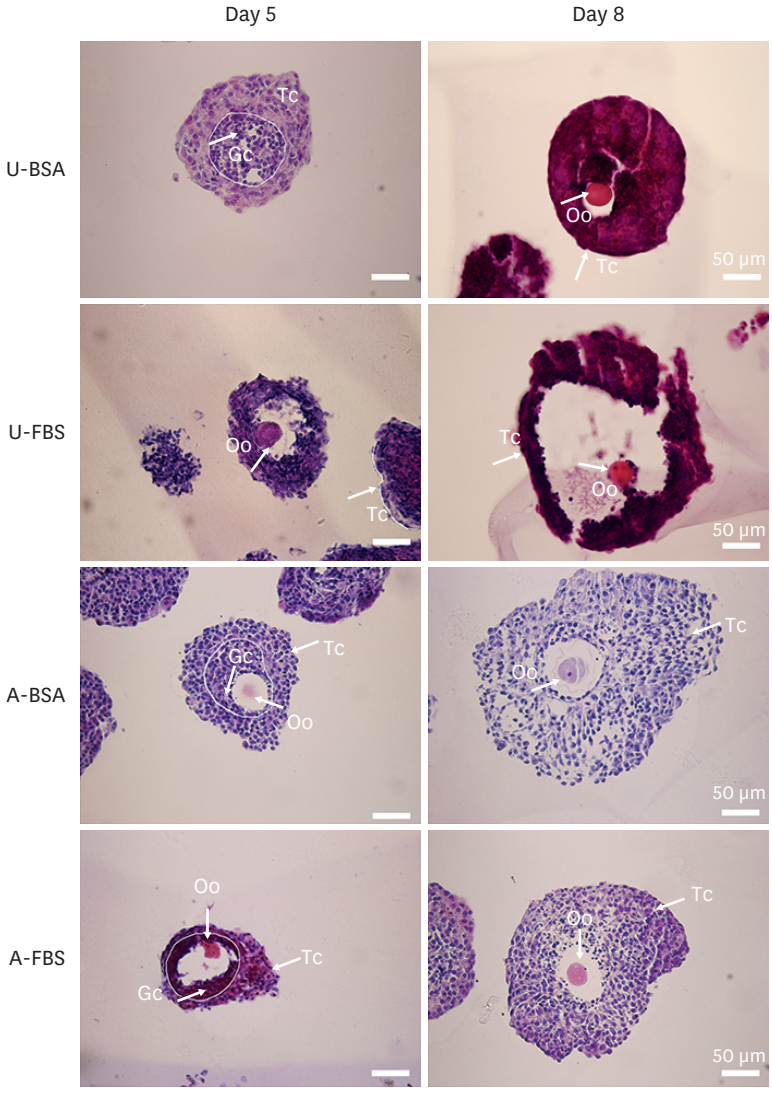

Fig. 4. The internal morphology of follicles cultured *in vitro*. Histological appearance of follicles after culture in alginate or ultra-low attachment 96-well plate. On day 5, the U-FBS group had more Tc layers than the U-BSA group, although no significant difference was found in the diameter. At day 8, a mature follicular lumen was observed in the U-FBS group. Scale bar: 50 μm. The white arrow indicates Gc, Oo, and Tc.
A-FBS, alginate supplemented with fetal bovine serum; A-BSA, alginate supplemented with bovine serum albumin; U-FBS, ultra-low attachment 96-well plate supplemented with fetal bovine serum; U-BSA, ultra-low attachment 96-well plate supplemented with bovine serum albumin; Tc, theca cell; Gc, granulosa cell; Oo, oocyte.

fertilization capacity of oocytes, we derived MII oocytes by culturing preantral follicles coming from ultra-low attachment 96-well plates and performed *in vitro* fertilization experiments. As shown in **Fig. 6**, 16 h after *in vitro* maturation, few oocytes were able to properly extrude the polar body. With 96 preantral follicles in each group, no significant difference was found in oocyte maturation rates in U-FBS and U-BSA groups (3.13% and 3.82%, respectively). Also, no significant difference was noted in the fertilization rates between FBS and BSA groups (1.3% and 1.7%, respectively). However, the rate of ultra-low attachment 96-well plate group MI were significantly higher than the alginate group's MI ($p < 0.05$). The fertilized eggs then developed into two-cell stage embryos, even four-cell embryos (**Fig. 6**). However, the number of blastomeres was abnormal in these embryos, and they could not develop to blastocysts compared with *in vivo* maturation zygotes (**Fig. 6**). These

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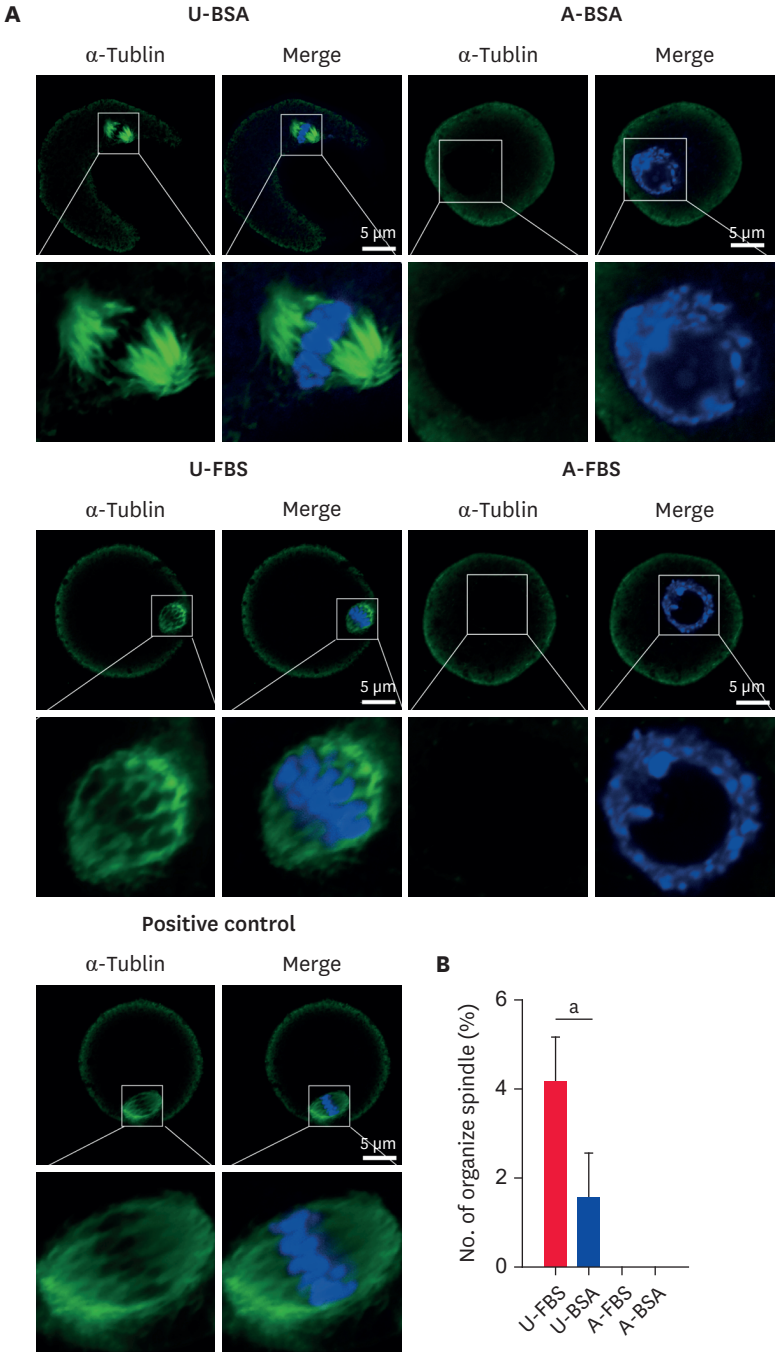

Fig. 5. Meiosis of oocytes after culture for 9 d in alginate and ultra-low attachment 96-well plate. The distribution of α-tubulin in spindle after oocyte maturation was detected via immunofluorescence. The α-tubulin was stained green, and the nuclei were stained blue. The positive control group included normal 21-day-old female mice. (B) Quantitative analyses of oocytes with organized spindle and chromosomal morphologies. Oocytes were obtained from COCs in U-FBS or U-BSA. Oocytes were obtained from mechanically torn follicles in A-FBS or A-BSA.
A-FBS, alginate supplemented with fetal bovine serum; A-BSA, alginate supplemented with bovine serum albumin; U-FBS, ultra-low attachment 96-well plate supplemented with fetal bovine serum; U-BSA, ultra-low attachment 96-well plate supplemented with bovine serum albumin.
[a]$p < 0.05$, U-FBS compared with U-BSA.

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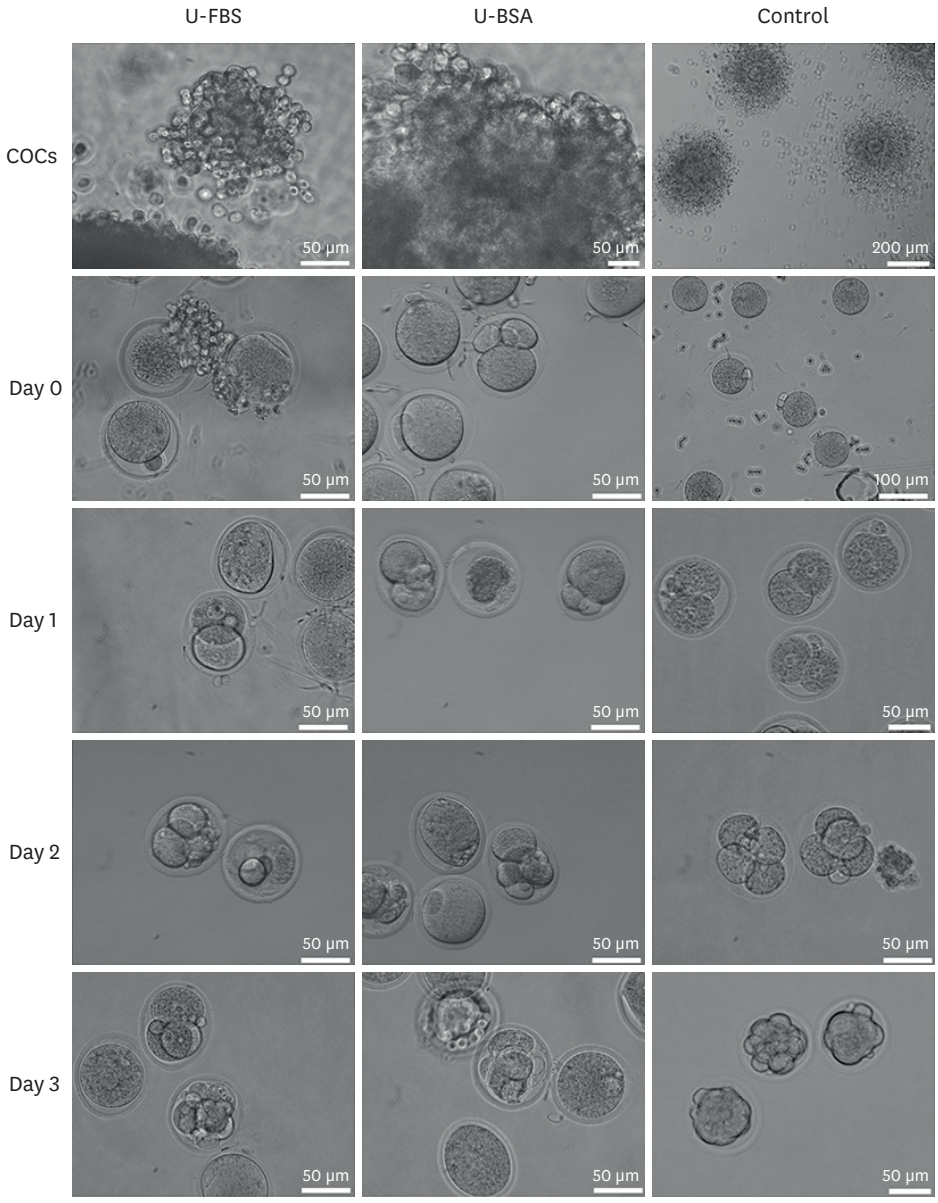

Fig. 6. *In vitro* fertilization of functional oocyte from follicles. Cumulus-oocyte complexes separated from preantral follicles grown *in vitro* at day 10. Day 0: Overview of follicle-derived mature oocytes after *in vitro* maturation. Day 1: Overview of follicle-derived two-cell embryos after *in vitro* fertilization. Day 3: Representative follicle-derived embryos.
U-FBS, ultra-low attachment 96-well plate supplemented with fetal bovine serum; U-BSA, ultra-low attachment 96-well plate supplemented with bovine serum albumin.

results showed that although the preantral follicles cultivating in BSA medium with ultra-low attachment 96-well plate were capable of fertilization *in vitro*, the embryo development was highly defective with only a few embryos reaching the blastocyst stage.

DISCUSSION

The follicle is the structural and functional unit of the female reproductive system. It is of great significance for the preservation of female fertility to establish a culture system of

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preantral follicles *in vitro* to collect more oocytes with simple operation and no exogenous pollution in the culture medium [25]. In this study, we generated an ultra-low attachment 96-well plate with BSA medium (U-BSA), which simplified the operation and prevented the heterogeneous contamination, which could be used for fertilization-competent oocyte generation and toxicological drug detection in clinics.

To ensure the uniformity of follicular development and reduce biases, we selected follicles from 12.5 d after the birth of mice for culture. Brown et al. [26] presumed that preantral follicles began to accept the regulation of hormone levels through vascular construction. In addition, we adopted the mechanical separation method in our experiment [10]. This method reduces the secondary separation of mechanical follicle stimulation and ensures the structure and function of the follicle. The results showed that follicles separated by mechanical method had a uniform diameter in size, with three to four layers of granulosa cells and intact follicular theca structure, consistent with the reported article [10].

Currently, the most commonly used 3D droplet method for *in vitro* culture of mouse preantral follicles mainly involves sodium alginate embedding culture method [14]. In this study, isolated preantral follicles survived and developed when composed with the alginate scaffold. However, the follicle growth was related to the shape of alginate beads. It was also difficult to form antral follicles and release COCs even on day 12 of culture. However, the morphology of the follicle was round, smooth, and neat edges, as well as had a compact structure. These results likely indicate that the operation time affected the development of follicles or oocytes, and this process was usually affected by lower room temperature, carbon dioxide concentration, and external pressure coming from the surrounding gel [27]. In addition, sodium alginate can also affect the exchange between follicles and culture medium and reduce the development ability of oocytes [28].

The surface of the ultra-low attachment 96-well plate was attached with hydrogel, which has been reported to minimize cell attachment [29], an important feature in the study of self-assembly of cells *in vitro* into organoids or tissues [17]. The hydrogel can replace the extracellular matrix, and the V-shaped plate bottom of the pore plate also promotes the formation of spherical objects [30]. Therefore, placing follicles into a plate can simulate a 3D culture environment, making it difficult for the proliferating granulosa cells to contact the bottom of the hole and always maintain a suspended state, facilitating the antrum cavity formation and COC release [31]. In our study, for preantral follicles cultured in ultra-low attachment 96-well plate, a rate of 12.5% of COCs ovulating was obtained. In addition, our data suggest that preantral follicles cultured in ultra-low attachment 96-well plate grew faster after antrum formation. It suggests that all type of cells in follicles can directly contact the culture medium for nutrient exchange, reducing the risk of cell apoptosis, thus increasing the efficiency of follicular growth. Furthermore, COCs from small antral follicles were released into culture medium with expansion state. Therefore, under the same culture medium, the preantral follicles developed more rapidly when cultured in the ultra-low attachment 96-well plate.

To date, FBS remains a nutrient additive for follicle *in vitro* culture, including activation, growth, and maturation. It contains nutrients, growth factors, cytokines, extracellular matrix proteins, and hormones that stimulate cells division [11]. However, the desire to minimize or remove the introduction of xenogeneic proteins or pathogens and to standardize cellular products intended for clinical applications has driven the search of alternatives to FBS. BSA as one of the most important nutrients and is probably better than FBS owing to higher antral

follicle formation (42.67%). Furthermore, the E_2 level in the BSA group was significantly higher than that in the FBS group from day 4 to day 8. These results are in agreement with Mohammadzadeh et al. [32], who documented a lower number of abnormal follicles in the BSA group during sheep ovarian follicle culturing within 2 wk. However, another research group reported greater efficiency of FBS [33]. The difference between these studies and our work is that they used other supplements, including insulin [34], stem cell factor [35], and other growth factors. For clinical purpose, a previous study demonstrated that human serum albumin instead of human serum and ITS could support human follicle growth and showed less atresia in ovarian tissue-slice culture [36].

We also noticed that spindle assembly and chromosome congression of most oocytes from cultured follicles were abnormal, although chromosome congression at the spindle plates were observed in oocytes in both FBS and BSA groups after *in vitro* mutation. These oocytes also showed significantly lower embryonic development with unevenly cleaved embryos, and they were difficult to develop into a four-cell stage. These results may be correlated with impaired mitochondrial function because of mitochondrial distribution [37]. While this study has a limitation that the preantral follicle growth rate was high in both U-BSA and U-FBS groups, the developmental potential of the oocytes has been reported as being positively correlated with granulosa cell numbers, apoptosis, follicle size, and cumulus expansion. Consequently, the improved developmental potential of oocytes from FBS-free follicular culture *in vitro* may be related to adequate central mitochondrial by adding growth factors and improving the microenvironment for follicular development.

In summary, we have established a fully defined serum-free culture system for the purposes of standardizing culture methods and protocols for *in vitro* growth and maturation of ovarian follicles. Our system will provide unique opportunities for clinical applications.

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