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Comparison of Bovine Small Antral Follicle Development in Two- and Three-Dimensional Culture Systems

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Abstract: To compare the effects of two-(2D, microplate) and three-dimensional (3D, alginate) culture systems on the in vitro growth of small antral follicles in cattle, individual follicles were separately cultured in the two culture systems for 8 days. Half of the culture medium was replaced by fresh medium every 2 days; the former medium was used to assess the amount of follicular hormone secretion using ELISA. Individual follicle morphology, diameter, and survival rate were recorded every alternate day. The results showed that in 4 days, there was no significant difference between the two systems, except that the growth rate of follicles in 2D system was relatively faster. After 4 days, estradiol concentration in 3D system was higher than that in 2D system. However, progesterone concentration was lower than that in the 2D system. The survival rate and oocyte quality of follicles in 2D system were significantly lower than those in 3D system on day 8. The follicle diameter slightly increased (30-60 μm) in the entire process. Taken together, for in vitro culture of follicles within 4 days, the 2D culture system is more suitable. However, when the culture duration is >4 days, the 3D culture system is more suitable.

Key words: Cattle, follicle in vitro culture, small antral follicles, two culture systems.

INTRODUCTION

Understanding the regulatory mechanism of ovarian follicle growth and atresia is clinically significant in the selection of high-quality germ cells for assisted reproduction. In mammals, many follicles are activated into the growth phase from fetal life through reproductive age. However, most follicles become atretic during the growing period, which results in considerable wastage of follicles in single fetus mammals who have a low follicle density, long reproductive cycle, and low reproductive capacities, such as humans (Xu et al. 2006), horses, and cattle (Petro et al. 2010). In vitro culture is a great way to increase the utilization rate of follicles (Gomes et al. 2015, Haag et al. 2013). Over the past decade, there was a great deal of research on follicle culture. For example, various murine in vitro culture systems have been successfully established for the growth of mature mouse oocytes from primordial follicles, in vitro fertilization, and development of fertile progeny after embryo transfer (Itami et al. 2014). Also, studies have reported breakthrough progress in the in vitro development in large nonrodent species. Culture systems have been established for culturing preantral follicles, such as those found in sheep (Praveen Chakravarthi et al. 2015), goat (Silva et al. 2015), buffalo (Gupta et al. 2008), pig (Wu et al. 2001), and cattle (Petro et al. 2010). Although there are many studies on

bovine follicles, most are based on culturing preantral follicles (Araujo et al. 2015). There are few studies on in vitro culture of bovine small antral follicles.

Culturing bovine small antral follicles in vitro will be of great value for experimental purposes. Compared with human ovaries, bovine ovaries have highly similar anatomical, physiological, and oocyte characteristics; hence, the bovine model has been increasingly recommended and accepted as a female ovarian disease model in research. Furthermore, the ability to culture follicles in vitro is significant for maintaining fertility for delayed childbearing or in females whose follicles are destroyed due to cancer (Salem et al. 2017). Small antral follicles have a developed follicular structure and morphology, which is very helpful for follicle culture. However, a stable in vitro culture system supporting the long-term survival of bovine small antral follicles and completing oocyte meiosis has not been developed.

The current in vitro follicle culture systems show wide variation. Several in vitro follicle culture methods have been successfully developed, such as in mice (Higuchi et al. 2015), sheep (Cecconi et al. 2004), goat (Huanmin & Yong 2000), monkey (Xu et al. 2013), and humans (Xu et al. 2009), including two-dimensional (2D) systems and three-dimensional (3D) systems. The 2D follicle culture systems show great diversity, such as the droplet method (Jorssen et al. 2015), microplate culture (Telfer 1996), and culture plate inversion culture (Wycherley et al. 2004). Among these, the microplate method is the most commonly used, which helps in the optimal use of various hormones secreted by the follicle itself. Moreover, it is convenient and easy to evaluate. However, in long-term in vitro follicle culture, the theca cells can easily attach to the petri dish bottom, which can seriously damage the 3D follicular structure (Wan et al. 2009). On

the other hand, 3D follicle culture methods also show great diversity, such as hanging drop (Tang et al. 2011), alginate hydrogel system, and alginate and fibrin mixture (Shikanov et al. 2011, Xu et al. 2011). Among these, alginate is the most commonly used substrate. Alginate is a biologically inert molecule (Rinaudo 2008), and has the characteristics of easy encapsulation, gelation and digestion, mildness, maintenance of follicle activity and integrity of the 3D structure, and scope for follicular expansion (West et al. 2007). However, follicular growth can be affected by the surrounding alginate.

In this report, we cultured bovine small antral follicles using 2D microplate and 3D alginate systems and compared the growth and development of bovine follicles between the two culture systems. The growth, morphology, hormone production, and the number of competent oocytes were evaluated. By comparing the advantages and disadvantages of both methods, we aimed to find a more suitable culture method for the growth and development of follicles in cattle and other animals.

MATERIALS AND METHODS

Chemicals and media

All chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless mentioned otherwise.

The basal medium (BM) was McCoy's 5A supplemented with 10% FBS (GIBCO, Grand Island, NY, USA), 50 mg/mL streptomycin, 2 mmol/L glutamine, and 100 IU/mL penicillin. The follicle culture medium (FCM) was McCoy's 5A supplemented with 10% FBS, 50 mg/mL streptomycin, 2 mmol/L glutamine, 100 IU/mL penicillin, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL sodium selenite, 4 ng/mL FSH, 3 mg/mL BSA(GIBCO, Grand Island, NY, USA), 50 μg/mL ascorbic acid, and 2 mmol/L hypoxanthine.

The pH of the medium was adjusted between 7.2 and 7.4 before filtration, following which the medium was sealed at a temperature of 4°C for preservation.

Collection of ovarian tissue

We used Qinchuan cattle aged 5-10 years old. Bovine ovaries were collected from a slaughterhouse 90 km away from Xi'an, Shanxi Province, and transported to the laboratory in a thermos flask filled with sterile saline at 35°C-37.5°C within 6 h. The ovaries were rinsed 3-4 times in PBS, and the residual ovarian mesangial and adipose tissue were cut using sterile scissors. Next, the treated ovaries were placed in a 50 mL centrifuge tube containing BM.

Isolation and selection of small antral follicles

The processes of follicle isolation and selection have been previously reported (Xu et al. 2016b). Briefly, ovarian cortical slices (5-mm thick) were vertically cut from the ovarian surface using surgical scissors under sterile conditions (Telfer 1996). Small antral follicles (<2 mm in diameter, the average diameter of follicles: 778.65 ± 199.56 µm) were mechanically isolated using tweezers and ophthalmic scissors, selected under a stereomicroscope (Nikon, Tokyo, Japan), manually dissected from the ovarian cortical strips using a 1-mL syringe and transferred to Petri dishes with FCM for further evaluation of the follicle quality.

The follicles were divided into three groups according to the morphological characteristics of the follicles observed by an Olympus CK40 inverted microscope. The survival rate of granulosa and theca cells in each group was determined using trypan blue. The survival state of the oocyte was analyzed based on the morphology of the oocyte and the results of the live/dead staining test. According to the test results, follicles suitable for culture are selected.

Two-dimensional culture system

The selected small antral follicles were individually cultured in each well of a 48-well plastic plate (one follicle/well) containing 800 µL FCM and cultured for 8 days in the incubator at 37.5°C and 5% CO_2. Every two days, half the medium was replaced by FCM medium and the 400 µL of old FCM was collected and stored at –80°C for the analysis of steroid hormones (Xu et al. 2013).

Three-dimensional culture system

Sodium alginate solution (0.25% (w/v) was prepared (Xu et al. 2009) and filter sterilized. Then, 50 µL of this solution was seeded onto 96-well plates (Corning Incorporated) and incubated at 37.5°C and 5% CO_2 for 15 min before the addition of small antral follicles (Green & Shikanov 2016). To form the beads, a solution containing 50 mM $CaCl_2$, 140 mM NaCl, 10 mM HEPES (pH 7.2) was slowly transfused into the 96-well plate for cross-linking for 2 min (Tingen et al. 2011). Every bead was cultured in a 48-well plastic plate containing 800 µL FCM and incubated at 37.5°C and 5% CO_2 for 10 days. Every other day, half the amount of the medium was replaced by fresh FCM medium and the old FCM was collected and stored at –80°C for the analysis of steroid hormones (Xu et al. 2013).

Follicle survival and growth

Follicle survival, diameter, and growth rate were assessed once every 2 days using an Olympus CK40 inverted microscope and Image J software, as previously described (Xu et al. 2013). The diameter of the follicles was determined by evaluating the distance from the outer layer of follicles at the widest diameter and the next diameter perpendicular to the first measurement (Cadoret et al. 2017) and calculating the mean of the two values (Tingen et al. 2011). The survival rate was determined

according to follicular morphology. Follicles were considered atretic if the integrity of the follicle was destroyed, the oocyte was dark or not surrounded by the granulosa cell layer, the granulosa cells appeared dark or fragmented, or the follicle diameter was decreased (Rossetto et al. 2013). Follicle images were imported into the Image J software, and each follicle's diameter was evaluated.

Follicle viability was checked using live/dead staining (2 µM calcein AM, 4 µM EthD-1) and inverted fluorescence microscopy. The control group comprised of newly isolated follicles, whereas the test group comprised cultured follicles (Amorim et al. 2009). Calcein AM and EthD-1 were used as staining solutions, which are taken up by live cells, emitting a strong green fluorescence; the dead cells emit bright red fluorescence (Araujo et al. 2015, McNatty et al. 2006). Follicles were incubated in 80 µL PBS containing 2 µM calcein AM and 4 µM EthD-1 for 10 min at 37.5°C in a CO_2 incubator (Araujo et al. 2015), followed by washing twice in FCM and observing under an inverted fluorescence microscope.

Assessment of oocytes

Some cultured follicles were randomly selected, and their oocytes were isolated to evaluate the quality every other day. The alginate from 3D system was degraded using a gel with 10 U/mL alginate lyase for 45 min in a CO_2 incubator to release the follicles. The oocytes from both the culture systems were then denudated by treating with 0.3% hyaluronidase and mildly aspirating through a smoothed drawn glass pipette. Oocyte conditions were assessed using the same camera, software, and staining as described above. If a clear gap existed between the zona pellucida and oocyte cytoplasm, or if the oocyte cytoplasm was dark and condensed, the oocytes were considered unhealthy.

Hormone assay

The hormone level of follicles was an important basis for evaluating follicle quality. To assess the quality of follicles in group I, II and III, follicular fluid was collected and refrigerated at –80°C. To examine and appraise follicular steroidogenesis in vitro, half the amount of the culture medium was pipetted at 2, 4, 6, and 8 days and refrigerated at –80°C. Progesterone (P4) and estradiol (E2) levels in the culture medium and follicular fluid were determined using the progesterone ELISA kit (the minimum detectable dose of bovine Pg is typically <10 pmol/L; Donglin Sci & Tech Development Co., Ltd., Wuxi, China) and estradiol ELISA kit (sensitivity <0.422 ng/mL; Ke Lei Biological Technology, Shanghai, China), respectively, according to the manufacturer's protocols.

Statistical analysis

Statistical analysis was performed with SPSS 20.0 (IBM Corporation, Armonk, NY, USA) software. A probability (P) of < 0.05 was considered statistically significant. All data are presented as the mean ± SD unless mentioned otherwise.

RESULTS

Selection of follicles for culture

According to morphological characteristics, 225 small antral follicles were divided into three categories: group I, in which 71 follicles have an oocyte surrounded by cumulus cells, with intact mural granulosa cells and limited by an intact basement membrane and outer thecal-stromal layer rich in blood vessels (Fig. 1 aI); group II, in which 76 follicles have a disrupted architecture in the mural granulosa cell layer inside the basal membrane (Fig. 1 aII); and group III, in which 78 follicles, the mural granulosa cell layers inside
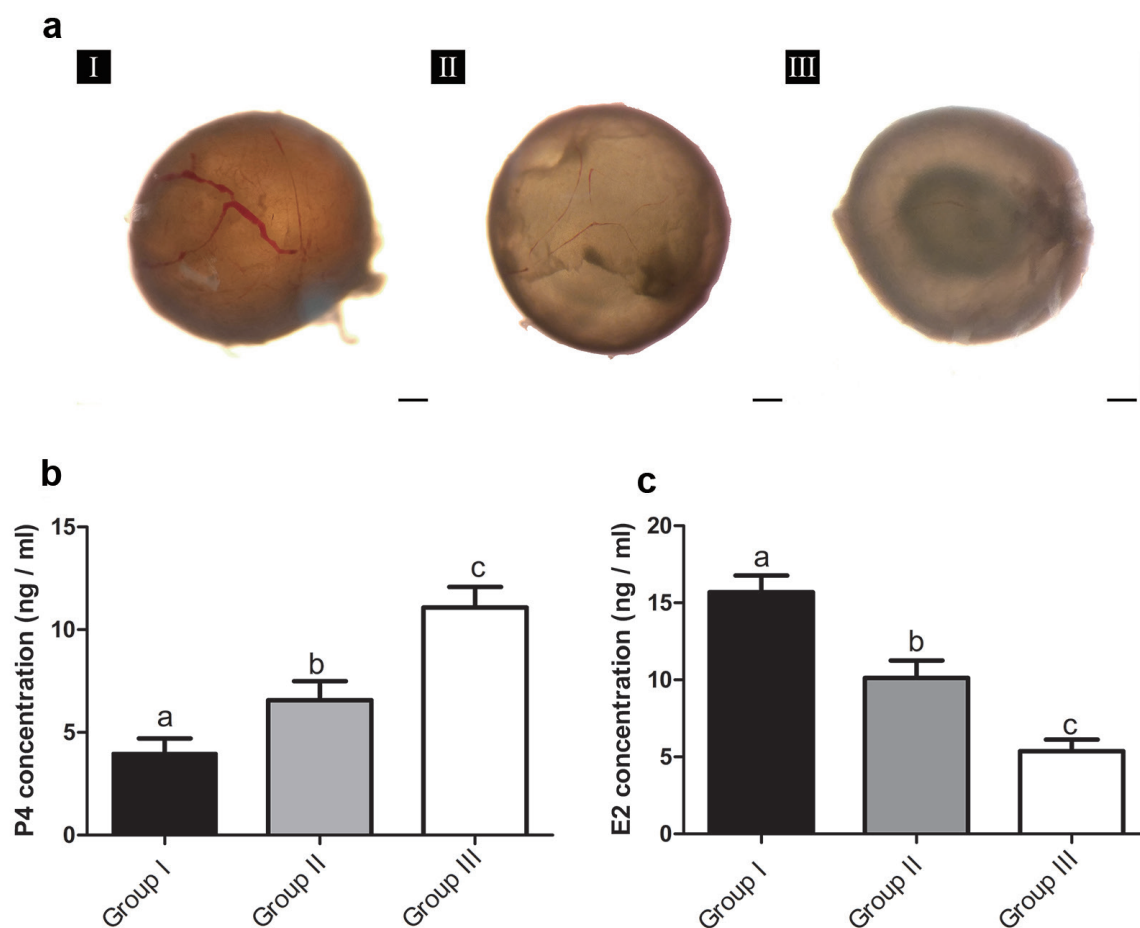
Figure 1. Morphological features and Steroidsteroid hormone secretion of different follicle groups follicles. Group I, bovine follicle with complete cell membrane structure, granulosa cells, and rich in blood vessels on the surface (I). Group II, bovine follicle with complete cell membrane structure, incomplete granulosa cell layer, and rich in blood vessels on the surface (II). Group III, bovine follicle with the mural granulosa cell layers inside the basal membrane are completely separated from the basement membrane (III). P4 levels in the follicular fluid of Group I follicles was significantly lower than those in other group follicles (b). E2 levels in the follicular fluid of group I follicles were significantly higher than those in other group follicles (c). Values with different superscripts are significantly different (P < 0.05). Data are presented as the mean ± SD (Bars = 100 μm).

the basal membrane are completely separated from the basement membrane (Fig. 1 aIII).

P4 level in the follicular fluid of group I follicles was significantly lower than that in other groups; the E2 level in the follicular fluid of group I was significantly higher than in other groups. As shown in Figure 1, the P4 level (3.98 ± 0.62 ng/mL) in group I was significantly lower than those in the other two groups (6.57 ± 0.72 and 11.08 ± 0.90 ng/mL; Fig. 1b). E2 level (15.69 ± 1.0 ng/mL) in group I was significantly higher than those in the other two groups (10.13 ± 1.1 ng/mL and 5.37 ± 0.75 ng/mL; Fig. 1c).

There was no significant difference in the survival rate of theca cells in the three groups (Table I). In contrast, there were significant differences in the survival rates of granulosa cells between the three groups. As shown in table I.

Table I. The survival rate of granulosa and theca cells in different groups of follicles.

Items	Group I	Group II	Group III
Survival rate of granulosa cells (%)	92.50% ± 6.50%[a]	79.75 ± 5.70%[b]	45.70% ± 6.45%[c]
Survival rate of theca cells (%)	96.75% ± 3.25%	97.85% ±2.75%	96.40% ± 4.16%

Note: Values are presented as the mean ± SD of different follicle groups from at least three independent cultures. Different letters within each row indicate statistically significant differences (P < 0.05).

The health status of oocytes in follicles of the three groups cultured in vitro was labeled with calcein-AM and ethidium homodimer-1 (Fig. 2). According to experimental results, the rate of healthy oocytes (%) was 81.78 ± 6.2%, 37.61 ± 4.6%, and 0.00 % in group I, II, and III, respectively (Fig. 3).

In summary, the group I follicles were of the best quality among the three groups, thus they were selected and used on the further steps of the study.

Evaluation of small antral follicle survival

516 follicles with the same morphological characteristics of group I follicles were selected for in vitro culture. Among them, 256 follicles were cultured in a 2D system, and 260 follicles were cultured in a 3D system.

Follicle morphology

Morphological changes in small antral follicles within 8 days of culture in the two culture systems were evaluated. In the first 4 days, the granulosa cell layer and follicular fluid were homogeneous, and follicles were slightly transparent, which strongly indicated 3D morphology (Fig. 4) in both the systems. After 4 days, in the 2D system, theca cells adhered to the culture plate and began proliferating, and some morphological integrity of the follicles was destroyed (Fig. 4d). In the 3D system, the majority of follicles maintained the 3D morphology (Fig. 4f-j) and did not bind

the bottom of the plate, but there was cell mass emigration, and cells existed in the sodium alginate gel layer; besides, with the increase in the culture duration, the number of migrating cells also increased (Fig. 4j).

Diameter change

The diameters of follicles cultured in the 2D system increased slightly faster than those of follicles in the 3D system (P < 0.05) compared with the follicles cultured in the 3D system in the first 4 days (Fig. 5). Then, the diameters of follicles in the 3D system increased relatively faster, whereas those of follicles in the 2D culture system increased slowly or even ceased to increase (Fig. 5).

### Viability assessment of follicles cultured in vitro by live/dead fluorescence labeling

There were differences in the morphology of granulosa cells between the two systems on day 4. Follicular granulosa cells cultured under 2D conditions were a structure resembling a mycelial morphological characteristic that seems to connect the granulosa cells. However, follicles cultured in 3D conditions had no significant changes in morphology. On day 8, the green fluorescence intensity of the follicles in the 2D system was significantly lower than that of the follicles in the 3D system (Fig. 6).
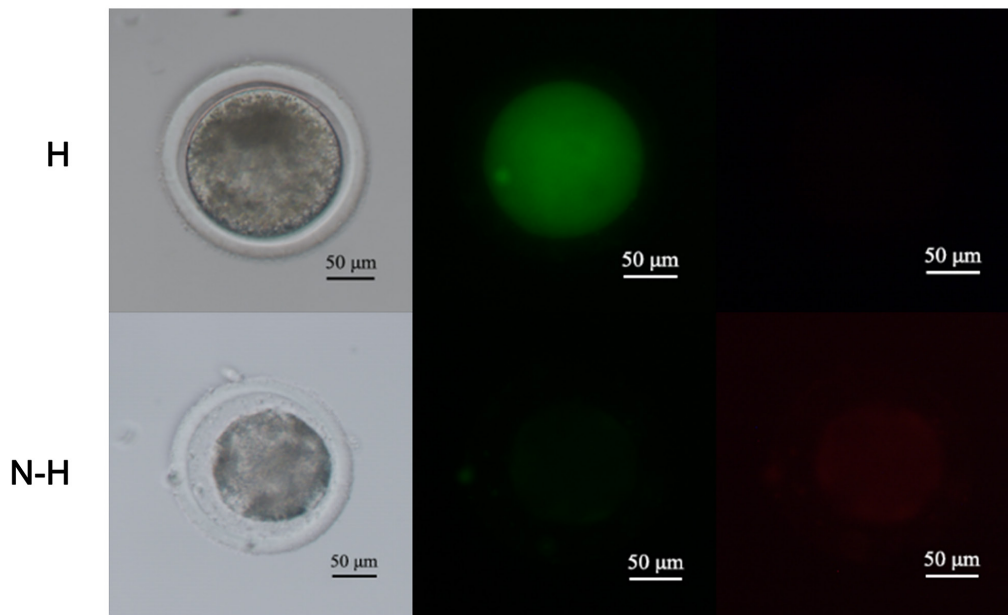
Figure 2. Healthy and unhealthy oocyte morphology. Oocytes stained for viability appear healthy (H) and unhealthy (N-H). Oocytes are labeled with calcein-AM (live cells, green) and ethidium homodimer-1 (dead cells, red). H, healthy oocyte; N-H, unhealthy oocyte; Bars = 50 μm.
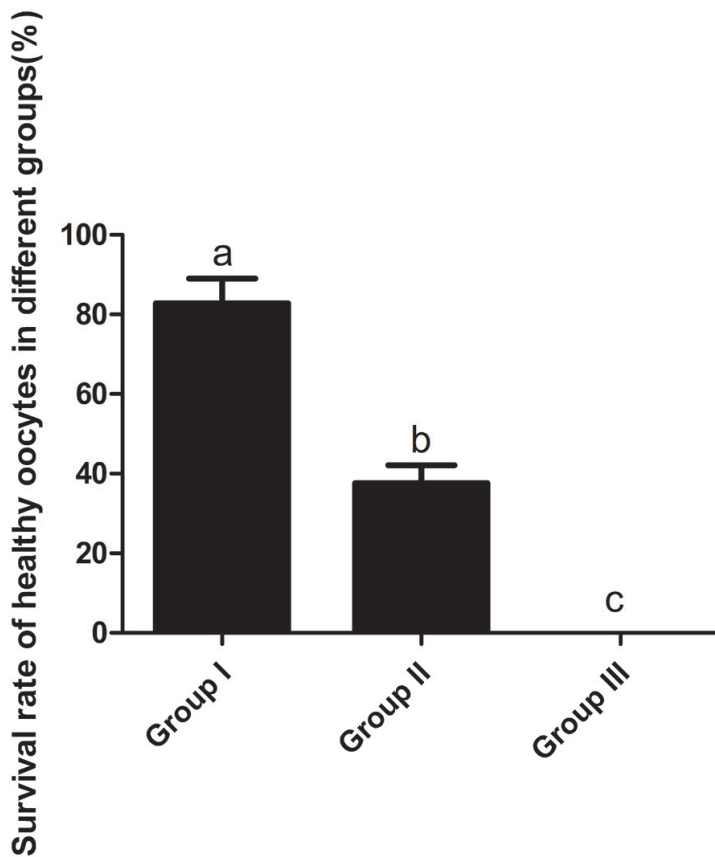
Figure 3. The rate of healthy oocytes in different groups of follicles. In group I, the percentage of healthy oocytes in the follicles was significantly higher than that in the other two groups. There were no healthy oocytes in group III follicles. Values with different superscripts are significantly different (P < 0.05). Data are presented as the mean ± SD.
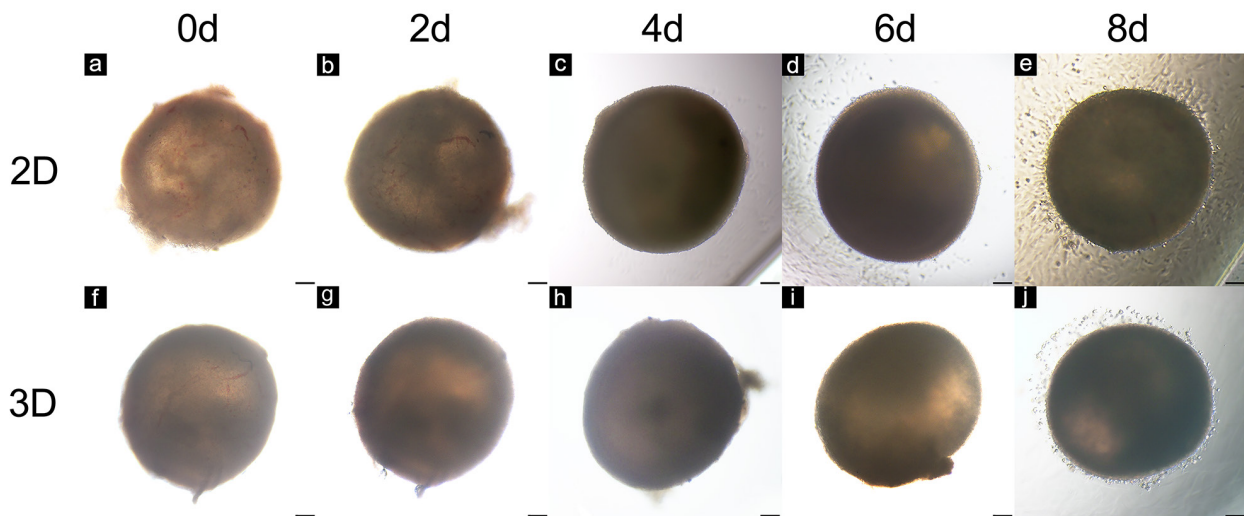
Figure 4. Micrographs showing morphological changes of follicles within 8 days of culture in the two culture systems. In the 2D system, a-c, days 0, 2, and 4, intact and healthy small antral follicles; d, day 6, parts of follicles bind to the basal plate and begin to proliferate cells; e, day 8, the follicles are completely combined with the plates and a large number of cells are produced. In the 3D system, f-i, days 0, 2, 4, and 6, intact and healthy small antral follicles maintain their 3D structure; j, day 8, follicles still maintain their 3D structures, but some cells proliferate out [×40; Bars = 100μm (a-j)].
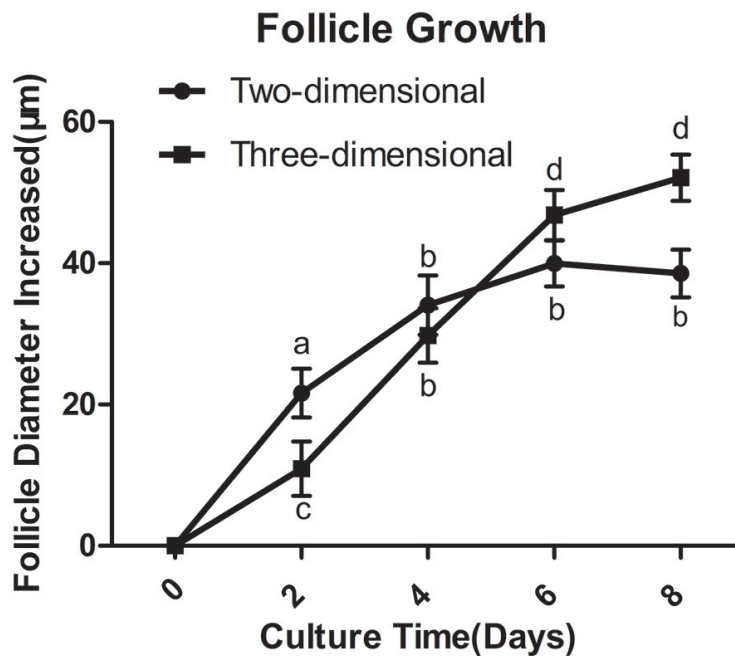
Figure 5. The effects of different culture systems on follicle growth rate. The diameters of follicles at 0, 2, 4, 6, and 8 days are relative diameters, i.e., the values obtained by subtracting the follicle diameter on day 0 of the same follicle. The actual mean diameter of the follicles placed in culture is 778.65 ± 199.56μm. In the first 4 days, the diameters of follicles cultured in the two-dimensional (2D) system increased slightly faster. Four days later, the follicle diameters in the three-dimensional system grew faster. The follicular diameter increased slowly or even ceased to grow in the 2D culture system. Different letters indicate statistical significance in the culture systems (a, b, c, d) ($P < 0.05$). Data are presented as the mean ± SD.
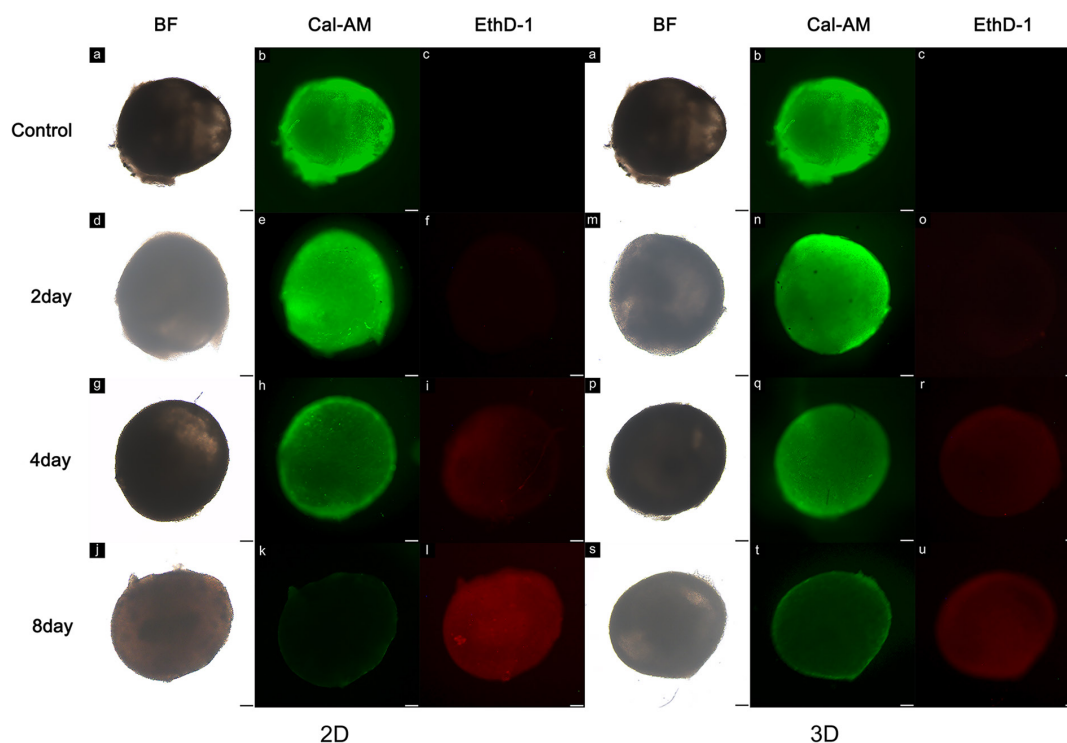
Figure 6. Micrographs showing the viability of follicles within 8 days of culture in the two culture systems. The experiment was divided into two groups: two-dimensional (2D) and three-dimensional (3D). Each group was cultured with 16 follicles at a time. All the experiments were repeated many times. Follicles are labeled with Calcein-AM (live cells, green) and ethidium homodimer-1 (dead cells, red). a-c, the newly isolated follicle is a negative control. d-l, days 2, 4, and 8, follicles cultured in 2D conditions. Follicular granulosa cells of follicles cultured in 2D system were the structure resembling a mycelial morphological characteristic that seems to connect the granulosa cells to each other on day 4, and the fluorescence intensity of the follicles was significantly low on day 8. m-u, days 2, 4, and 8, follicles cultured in 3D conditions. Follicular granulosa cells of follicles cultured in 3D system were the structure resembling a mycelial morphological characteristic that seems to connect the granulosa cells to each other on day 8 [×40; Bars = 100μm (a-u)].
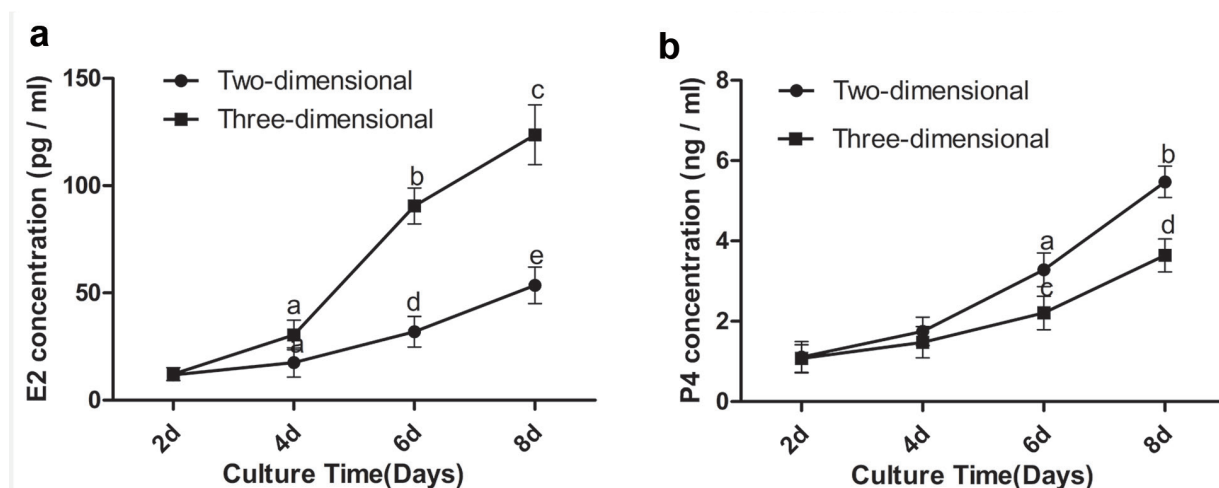
Steroid production

In the first four days, there was no significant difference in E2 and P4 production by follicles grown in the two culture systems. Four days later, the P4 level significantly increased over time in the 2D system, whereas the E2 level rapidly increased in the 3D system. At day 8, P4 secretion was remarkably higher in follicles cultured in the 2D system than in those cultured in the 3D system; E2 levels showed the opposite trend (Fig. 7).

Oocyte quality

There was no significant difference in the proportion of healthy oocytes in both the culture system in the first four days. On day 6, the ratio of healthy oocytes in follicles cultured in the 2D system is less than half of that cultured in the 3D system. On day 8, the ratio of healthy oocytes of the 2D system was significantly lower than that in the 3D system. (Table II).

Abbreviations

2D: Two-dimensional; 3D: Three-dimensional; E2: Estradiol; P4: Progesterone; FBS: Fetal bovine serum; ITS: insulin, transferrin, sodium-selenite;

Figure 7. The effects of different culture systems on E2 and progesterone production by bovine follicles in vitro. No differences were observed between the two groups for P4 and E2 in the first 4 days. At day 8 of culture, the P4 concentration was significantly higher in follicles cultured in the 2D system than in the follicles cultured in the 3D system. E2 concentration was significantly lower in follicles cultured in the 2D system than those cultured in the 3D system. Data are presented as the mean ±SD. Different letters indicate statistical significance in the culture systems (a, b, c, d, e) (P < 0.05).

Table II. The rate of healthy oocytes of follicles cultured in two culture systems.

Cultural method	Nª (%)	Rate of healthy oocytes (%)			
		2 d	4 d	6 d	8 d
Two-dimensional culture	86.16±4.95 (n = 58)	79.37±5.20[a,A] (n = 36)	54.45±4.40[b,B] (n = 34)	16.67±3.55[c,A] (n = 35)	0[d,A] (n = 32)
Three-dimensional culture	87.07 ± 5.20 (n = 67)	78.95±4.50[a,A] (n = 35)	53.84±4.79[b,B] (n = 37)	38.29±3.87[c,B] (n = 36)	19.30±3.50[d,B] (n = 35)

Note: Values are the average SD of multiple follicles or oocytes from at least three independent cultures;
Nª, rate of healthy oocytes from fresh ovarian antral follicles; n, total number of follicles for oocytes analysed;
Different letters indicate statistical significance in the same system on different days (a, b, c, d) or on the same day in the different culture systems (A, B) (P < 0.05).

FSH: Follicle-stimulating hormone; BSA: Albumin from bovine serum; BM: The basal medium; FCM: The follicle culture medium.

DISCUSSION

Assessment of the quality of follicles is an important component in the study of follicle selection and culture. There are many studies on the quality of bovine small antral follicles, which are histologically classified as healthy or atretic (Irving-Rodgers et al. 2009). A healthy follicle has a complete cell membrane structure (Rodgers & Irving-Rodgers 2010), uniform cytoplasm, increased blood vessels on the surface (Rinaudo 2008), and an intact granulosa cell layer (Araujo et al. 2014). However, atretic follicles with turbid follicular fluid look dim, and the granulosa cell layer shows serious litter with obvious signs of atresia (Jin et al. 2010). In this study, according

to the morphology of follicles, follicles were divided into three groups. The quality of each group follicles was analyzed by comparing the survival rate of granulosa cells and theca cells, the expression levels of P4 and E2, and the quality of oocytes. Compared with group II and group III, we found that group I follicles have an intact Slavjanski (basal) membrane structure, a typical and intact granulosa cell layer, uniform cytoplasm, rich in blood vessels on the surface, a spherical central oocyte (Fig. 1 aI), the highest rate of survival granulosa cells (Table I) and healthy oocytes (Fig. 3), including low P4 secretion and high E2 secretion (Fig. 1c). The results are consistent with the results of some classification studies on follicle quality (Araujo et al. 2014, Higuchi et al. 2015).

Culturing bovine small antral follicles in vitro may be a valuable alternative to ovarian disease research in women, and research on follicle in vitro culture systems has been a hot spot. However, the mechanisms of action by which the specific advantages and disadvantages of different cultural systems occur remain unclear. The current study used bovine small antral follicles as models, and 2D microplate method and 3D sodium alginate system as culture methods to study follicular growth in vitro. We demonstrated that follicles cultured in the 2D system grew faster than those in the 3D culture system in the first 4 days. After 4 days, follicles cultured in the 3D system grew faster. This may be due to the following reasons: First, in the first 4 days, small antral follicles cultured in 2D systems became flattened for the lack of 3D support. At the same time, the rigidity of alginate affected follicle growth, which is consistent with a previous study result stating that mouse follicles embedded in alginate gel showed growth inhibition and reduced steroidogenesis (Rossetto et al. 2013). Second, after 4 days, the 3D culture system can

well support the three-dimensional structure of bovine follicles in vitro (Qiu et al. 2013) compared with the 2D system (Araújo et al. 2011), which was very beneficial to the survival of follicles. However, the follicles cultured in the 2D culture system are often combined with the bottom of the culture dish, which will destroy the three-dimensional structure of the follicle.

According to the literature, the diameter of rhesus monkey follicles increases from 150 to ≥700 μm (Xu et al. 2016a). The diameter of mice follicles increases by 560 μm (Sun et al. 2004), that of pig follicles increases by approximately 100 μm (Araujo et al. 2015), and that of sheep follicles increases by only 32 μm (Pangas et al. 2003). In our study, although the follicles grew gradually, the increase in follicle diameter was not large (approximately 30-60 μm) in both the systems. There was a difference in the survival rate of follicles on day 6, and bovine small antral follicles (<2 mm) cultured in 3D system could successfully remain active until day 8 without losing their normal 3D structure. The above indicators are in agreement with the results of Qiu et al who stated that goat follicle diameter is similar to that of cattle (Qiu et al. 2013). This may be due to the following reasons: First, the status of the follicles are affected by follicular wave. Follicle growth is promoted at the beginning of the follicle wave and inhibited at the end of the wave (Nagai et al. 2016). During different periods of follicular waves, E2 and P4 concentrations in the follicular fluid also change (Xu et al. 2016a), which may affect the growth of follicles. Second, as mono-ovular animal species, the living environment of bovine follicles is more complex, with less vitality. Most mouse follicles can grow, probably because of the strong vitality of the species. Third, compared with other species, bovine in vitro experiments with small antral follicles are rare, and the concentration of various components in the follicle culture

medium is variable, which may affect follicle growth. Besides, many different factors may affect the growth of follicles, such as the method of medium replacement (Shikanov et al. 2009), BM used (Pangas et al. 2003), and addition of supplements (Hasegawa et al. 2006).

Interestingly, after living/dead staining, follicular granulosa cells of follicles cultured in 2D system were the structure resembling a mycelial morphological characteristic that seems to connect the granulosa cells. The longer the culture duration, the more obvious the phenomenon. We speculated that during culture, (1) The granulosa cells originally attached to the inner membrane of the follicle were detached from the follicular lining and dissolved. 2) the adhesive secreted by follicles results in the adhesion of the falling granulosa cells. The specific reason for this phenomenon needs further study.

For the past several years, in follicular atresia studies, P4 and E2 concentration ratio in culture have been widely used to determine the degree of follicular atresia (Silva et al. 2015). Increased E2 levels and decreased P4 levels in the follicular fluids suggest that the quality of follicles is improving (Rodrigues et al. 2015, Jin et al. 2010). Comparing with the changes in E2 levels and the degree of DNA shrinkage of granulosa cells, a decrease in E2 synthesis and secretion occurs before a large number of granulosa cells undergo nuclear pyknosis. Our results indicated that, during the first 4 days, the concentration of the same hormone secreted by follicles was not significantly different in the follicle culture medium between the two culture systems. Four days later, the trend of follicular secretion of E2 and P4 showed significant differences. P4 levels in the 2D culture system increased faster than those in the 3D culture system, which is consistent with the literature (Kreeger et al. 2005). The results showed that the efficacy of the short-term culture of follicles in both the culture systems was similar. However, for long-term culture, the 3D culture system was more conducive to the growth of follicles.

The quality of oocytes is an important reference for evaluating follicular status. In the study, after 4 days, the rate of healthy oocytes under 2D conditions was significantly lower than that under 3D conditions. We speculated that the reason for this low ratio may be as follows: a) Due to the loss of the three-dimensional structure, the follicles under 2D conditions have a higher mortality rate after four days of culture. b) During different periods of follicular waves, E2 and P4 concentrations in the follicular fluid also change (Xu et al. 2016a). Hormones have a significant effect on the quality of oocytes in the follicles (He 2017).

CONCLUSION

In conclusion, our studies revealed that there was no difference between the two in vitro culture systems in the first 4 days of culture. For >4 days, the 3D culture system is better. However, if the aim is to cultivate follicles for a longer duration, the culture system should be improved.

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