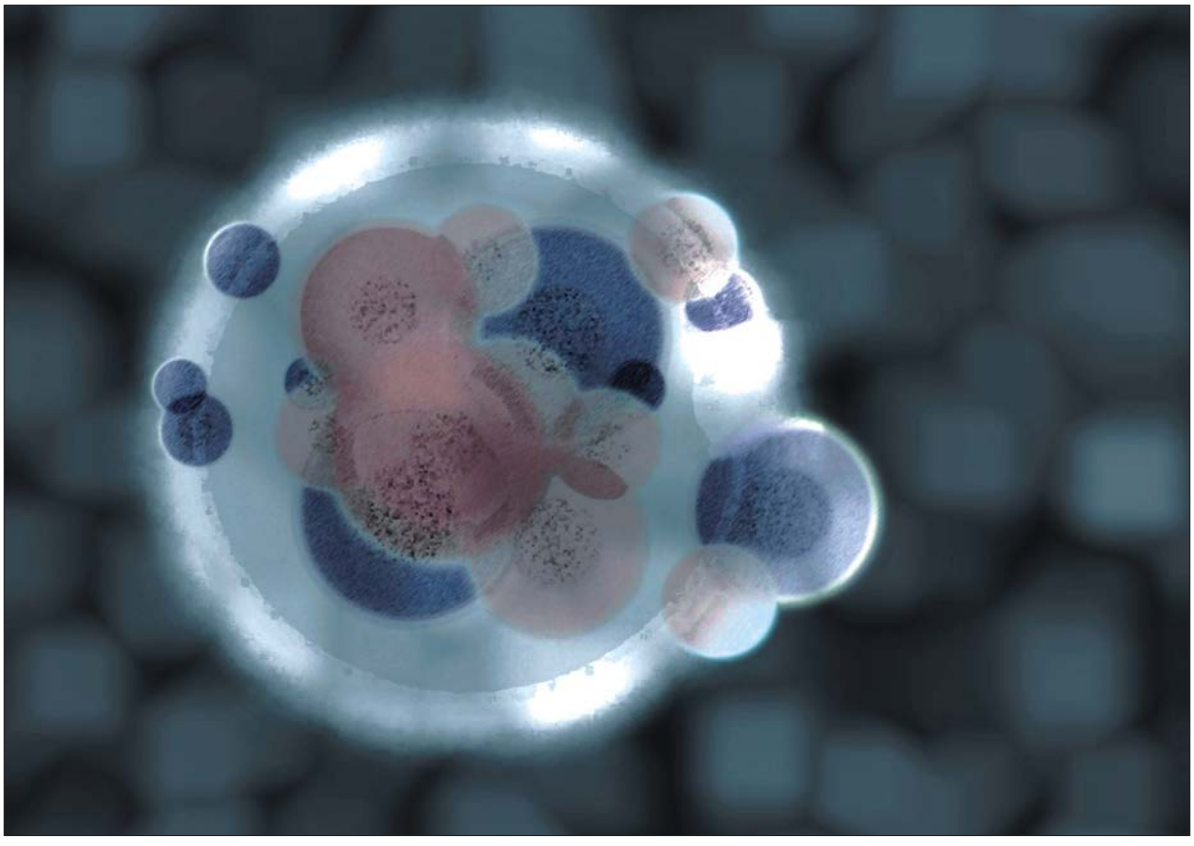

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Long-Term *in vitro* Culture of Bovine Preantral Follicles by Using Different Culture Media

Khairy M.A. Zoheir, Nermin El-Halawany, Xiang Li, Moaeen-ud-Din and Li-Guo Yang
China Education Ministry's Key Laboratory in Agricultural Animal Genetics,
Breeding and Reproduction, Huazhong Agricultural University, Wuhan 430070, China

Abstract: The aim of the present study is to develop a new culture system, capable of producing healthy preantral follicles, which can reach full maturity especially in a long-term culture. To achieve this task we used CR1aa medium in combination with endocrine hormone (FSH), growth factor (EGF) and/or Insulin-Transferrin-Selenium (ITS). More over, we compared its efficacy with TCM-199 medium plus the same supplements. Culturing of the preantral follicles with diameters of 60, 80 and 100 μm were commenced at follicle recovery (day 1) and continued for 26 days. Follicle diameters and morphological deformity or degeneration were monitored on the day of collection and during the culture (days 5, 10, 15, 20 and 26). Compared with TCM-199 medium, culture system using CR1aa medium showed a significant increase in the size and viability of the follicles of all size classes regardless of the used supplements. The follicular survival rate at 26th day of the culture in CR1aa culture system reached 28%. Regardless of culture treatments, the greatest follicular growth rate was between days 5, 10 and 15 of culture. The survived follicles maintained their morphology throughout the culture, with presence of a thecal layer and basement membrane surrounding the granulosa cells. In conclusion, CR1aa medium plus FSH, EGF and/or ITS can be a promising culture system for long-term culture of bovine preantral follicles.

Key words: Preantral follicles, CR1aa, long term culture, bovine

INTRODUCTION

Thousands of small oocytes are contained in mammalian ovaries, but about 99.9% of them undergo atresia (Ireland, 1987). It would be of great practical benefit if these follicles, destined to become atretic, could be rescued before they degenerate.

The long-term culture requirements have hindered the advance of preantral follicle cultures. In cattle, a follicle that has formed an antrum needs 40 days to reach ovulatory size (Lussier *et al*., 1987) and it was estimated that the oocyte could take a number of month to reach ovulatory size. Attempts have been made to develop a culture system for cattle preantral follicles using different culture media in combination with various endocrine and paracrine factors (Figueiredo *et al*., 1994; Itoh and Hoshi, 2000; Saha *et al*., 2000). Cattle preantral follicles were cultured individually or in groups for 7-12 days. Large preantal follicles were shown to have more growth rate than the small preantral follicles in culture (Katska and Rynska, 1998). In other study, the authors succeeded to culture bovine preantral follicle (168 μm) up to 28 days (Gutierrez *et al*., 2000). More recently, a 40 days culture of buffalo preantral follicles with growth rate of 17% were reported (Gupta *et al*., 2002).

The ability of gonadotropins to stimulate the proliferation of granulosa cells in primary follicles has been demonstrated *in vivo* and *in vitro* (Chiras and Greenwald, 1978). FSH has been demonstrated to be essential in proliferation and differentiation of preantral granulosa cells *in vitro* and thus in growth and normal *in vitro* development of preantral follicle of cows and buffalos (Ralph *et al*., 1995; Santos *et al*., 2006). In addition, FSH is an anti-apoptotic factor and helps to maintain the viability of granulosa cells (Chun *et al*., 1996). A minimal concentration of 10 UI mL^{-1} of FSH has been shown to be essential during *in vitro* culture of intact preantral follicles (Cortvrindt *et al*., 1997). In this culture system, full differentiation of the preantral follicles *in vitro* was proven to achieve only in the presence of FSH at least from the late preantral stage (Adriaens *et al*., 2004). Using the same culture model, a follicular survival rate of only 10% in the absence of FSH during *in vitro* growth was reported (Mitchell *et al*., 2002).

Other factors were shown to promote preantral follicular development *in vitro*. Epidermal Growth Factor (EGF) has been shown to have a role in proliferation of granulosa cells and decreasing apoptosis in pigs (Morbeck *et al*., 1993; Mao *et al*., 2004), hamsters (Roy and Treacy, 1993) and cows (Wandji *et al*., 1996).

Corresponding Author: Li-Guo Yang, China Education Ministry's Key Laboratory in Agricultural Animal Genetics, Breeding and Reproduction, Huazhong Agricultural University, Wuhan 430070, China

On other hand, association of gonadotropins and insulin was considered optimal during intact preantral follicular culture for follicular proliferation, differentiation and to generate mature oocytes (Liu *et al*., 2002). Other study reported that using Insulin-Transferrin-Selenium (ITS) is effective to maintain granulosa cells within the follicular microenvironment and greatly improve development of the follicles (Katska and Rynska, 1998).

Several authors have studied the effect of CR1aa medium for oocyte maturation and embryo culture. Oocytes matured in CR1aa or CR2aa media have been reported to express higher cleavage and developmental rates than oocytes matured in TCM-199, Minimal Essential Medium (MEM) or RPMI-1640 (Abdoon *et al*., 2001). Similarly, Rosenkrans and First (1991) concluded that a simple medium, *Charles rosenkranl* (CR1) that contains essential and non-essential amino acids were beneficial to bovine embryo development *in vitro* in the absence of feeder cells. A comparison between CR1 medium supplemented with amino acids and BRL cell monolayer gave a similar outcome in terms of embryo yield (Moreno and Westhusin, 1993).

To our knowledge no one has used CR1aa medium to culture preantral follicles. Hence, in the present study we tried to develop a new culture system using CR1aa medium plus some supplements such as FSH, EGF and ITS to culture the preantral follicles over a period of 26 days. We also compared the effect of the modified CR1aa with TCM-199 medium to evaluate its effect on the follicular growth and development.

MATERIALS AND METHODS

Isolation of preantral follicles: Ovaries were obtained from a local slaughterhouse and placed in isotonic sodium chloride solution (0.9% NaCl) supplemented with penicillin (200 IU mL^{-1}) and streptomycin (200 μg mL^{-1}) at 30-35°C. Sections of the ovarian cortex were excised by scalpel and placed into a tissue chopper adjusted to produce 500 μm sections. These small to minute segments were placed in Dulbecco's phosphate-buffered solution supplemented with 36 μg mL^{-1} sodium pyruvate, 1 μg mL^{-1} glucose and 3 mg mL^{-1} BSA. After several washings and repeated pipetting, the samples were filtered first through a 500 μm filter to remove all large fragments and debris, then through a 38 μm filter to exclude blood cells and other minute particles. The tissue remaining on the 38 μm filter was washed and collected in an embryo searching dish (100×20 mm, Falcon).

Culture of isolated preantral follicles: The freshly isolated follicles were examined under the inverted microscope; the morphologically normal follicles were selected (healthy appearing, spherical with one or more compact layers of granulosa cells around the oocyte with an intact basal membrane, with no apparent sign of necrosis and no antrum). Preantral follicles with diameters of 60, 80 and 100 μm were collected and cultured *in vitro* using one of the following systems: CR1aa medium + Follicle Stimulating Hormone (FSH) (100 ng mL^{-1}) + EGF Epidermal growth factor (100 ng mL^{-1}), basal medium (TCM-199) + FSH (100 ng mL^{-1}) + (EGF) (100 ng mL^{-1}), CR1aa medium + FSH + insulin-transferring selenium ITS (1%), TCM-199 medium + FSH + ITS (1%) and TCM-199 medium without any addition as control. The patent for CR1aa medium is held by Infigen. TCM-199 supplemented with 10% Fetal Calf Serum (FCS), 0.1 mg mL^{-1} sodium pyruvate and 100 IU mL^{-1} of penicillin and 100 mg mL^{-1} streptomycin. Preantral follicles were cultured in groups of three in 500 μL culture medium in 24-well dishes (Falcon) at 38.5°C in 5% CO_2 for 26 days (harvested on day 26). Every third day, half the medium was removed and replaced by freshly prepared medium. Follicular diameters were measured with an ocular micrometer on the day of collection and during the culture (days 5, 10, 15, 20 and 26). To monitor the morphological deformity or degeneration, at the end of culture, half of the cultured preantral follicles were stained with trypan blue to categorize their viability on the basis of the degree of dye exclusion. Unstained follicles were classified as viable and fully stained follicles as dead. Follicles with medium staining were regarded as damaged. And the rest of the cultured preantral follicles were double stained by bisbenzimide (H 33342) plus propidium iodine (10 μg mL^{-1} each in PBS) and compared with fresh controls to examine the morphological aspects such as the presence of an intact follicle membrane, a flattened or cuboidal granulosa cell layer and presence of a nucleus. Double staining helped us to analyze the proportions of live and dead granulosa cells and also the nuclear components.

Statistical analysis: Data were subjected to analysis of variance using a computer package of the Statistical Analysis System (SAS) and results were compared by Duncan's Multiple Range Test. Comparison was made between the follicles of different size classes included in the same treatment group as well as between treatment groups for each follicle size class. The analysis for maintenance of viability and morphology was done by Chi-square test.

RESULTS

Generally the follicles size has increased during the *in vitro* culture regardless of the initial size classes or the used treatments. The greatest increases in the

Table 1: Effect of the different culture systems on 60 μm preantral follicles

Treatments	No. of cultured follicles (Mean±SE)	Follicular size and No. of growing follicles at different days of treatments					
		Day 1 (60-65 μm)	Day 5 (80-85 μm)	Day 10 (100-105 μm)	Day 15 (120-125 μm)	Day 20 (130-135 μm)	Day 25 (140-145 μm)
FSH + EGF + TCM-199	30±1.2	30±1.2^{a} (100%)	20.8±0.8^{b} (69.3%)	15.4±1.4^{b} (51.3%)	10.2±0.58^{b} (34.0%)	8.8±1.50^{b} (29.3%)	6.00±1.05^{b} (20.0%)
FSH + EGF + CR1aa	30±0.8	30±0.8^{a} (100%)	26.4±0.54^{c} (88.0%)	18.6±0.85^{c} (62.0%)	14.4±1.05^{c} (48.0%)	10.05±1.25^{bc} (33.5%)	8.06±0.95^{b} (26.9%)
FSH + ITS + TCM-199	30±0.5	30±0.5^{a} (100%)	21.2±1.05^{b} (70.7%)	14.04±0.8^{b} (46.8%)	10.08±0.95^{b} (33.6%)	11.04±1.04^{bc} (36.8%)	8.5±1.06^{b} (28.3%)
FSH + ITS + CR1aa	30±0.5	30±0.5^{a} (100%)	25.52±1.04^{c} (85.1%)	19.08±1.06^{c} (63.6%)	15.02±1.04^{c} (50.7%)	12.08±0.86^{c} (40.3%)	10.08±1.02^{b} (33.6%)
Control (TCM 199)	30±0.4	30±0.4^{a} (100%)	15.08±1.02^{a} (50.3%)	6.08±1.05^{a} (20.3%)	5.25±1.5^{a} (17.5%)	3.40±1.06^{a} (11.3%)	2.05±0.66^{a} (6.8%)

SE: Standard Error, Symbol a, b and c refer to the differences between the groups, Significant differences $p<0.05$ and $p>0.01$

Table 2: Effect of the different culture systems on 80 μm preantral follicles

Treatments	No. of cultured follicles (Mean±SE)	Follicular size and No. of growing follicles at different days of treatments					
		Day 1 (80-85 μm)	Day 5 (100-105 μm)	Day 10 (120-125 μm)	Day 15 (130-135 μm)	Day 20 (140-145 μm)	Day 25 (150-155 μm)
FSH + EGF + TCM-199	30±0.8	30±0.5^{a} (100%)	21.8±0.54^{b} (72.7%)	15.04±1.2^{b} (50.1%)	11.6±1.3^{b} (38.7%)	8.80±1.0^{b} (29.3%)	6.45±1.08^{b} (21.5%)
FSH + EGF + CR1aa	30±1.05	30±1.6^{a} (100%)	25.45±1.8^{c} (84.8%)	20.6±1.2^{c} (68.7%)	17.05±1.3^{c} (56.8%)	10.55±1.5^{b} (35.2%)	7.56±1.04^{b} (25.2%)
FSH + ITS + TCM-199	30±1.2	30±1.2^{a} (100%)	20.20±0.80^{b} (67.3%)	17.04±1.04^{b} (56.8%)	14.06±1.06^{b} (46.9%)	9.05±1.04^{b} (30.2%)	6.00±1.06^{b} (20.0%)
FSH + ITS + CR1aa	30±0.8	30±0.8^{a} (100%)	26.00±1.00^{c} (86.7%)	21.00±1.00^{c} (70.0%)	16.54±1.60^{c} (55.1%)	11.06±0.80^{b} (36.9%)	9.04±1.02^{c} (30.1%)
Control (TCM 199)	30±1.4	30±1.4^{a} (100%)	18.06±0.8^{a} (60.2%)	7.08±0.92^{a} (23.6%)	5.25±1.0^{a} (17.5%)	3.2±1.20^{a} (10.7%)	2.0±0.86^{a} (6.7%)

SE: Standard Error, Symbol a, b and c refer to the differences between the groups, Significant differences $p<0.05$ and $p>0.01$

Table 3: Effect of the different culture systems on 100 μm preantral follicles

Treatments	No. of cultured follicles (Mean±SE)	Follicular size and No. of growing follicles at different days of treatments					
		Day 1 (100-05 μm)	Day 5 (120-125 μm)	Day 10 (140-145 μm)	Day 15 (160-165 μm)	Day 20 (180-185 μm)	Day 25 (195-200 μm)
FSH + EGF + TCM-199	30±0.8	30±0.85^{a} (100%)	23.8±0.60^{b} (79.3%)	15.4±1.45^{b} (51.3%)	10.2±1.50^{b} (34.0%)	9.80±1.50^{b} (32.7%)	7.45±1.00^{b} (24.8%)
FSH + EGF + CR1aa	30±1.0	30±1.4^{a} (100%)	22.4±0.54^{b} (74.7%)	16.6±0.80^{b} (55.3%)	11.4±1.00^{b} (38.0%)	9.05±0.80^{b} (30.2%)	8.06±0.95^{b} (26.9%)
FSH + ITS + TCM-199	30±0.6	30±0.6^{a} (100%)	19.25±1.00^{b} (64.2%)	15.04±0.85^{b} (50.13%)	10.08±0.90^{b} (33.6%)	8.05±1.00^{b} (26.8%)	7.07±1..8^{b} (23.6%)
FSH + ITS + CR1aa	30±1.2	30±1.0^{a} (100%)	20.02±1.04^{b} (66.7%)	17.08±1.06^{b} (56.9%)	11.02±1.04^{b} (36.7%)	8.08±0.80^{b} (26.9%)	6.88±1.02^{b} (22.9%)
Control (TCM 199)	30±0.6	30±0.6^{a} (100%)	12.08±1.02^{a} (40.3%)	5.08±1.05^{b} (16.9%)	3.52±1.58^{a} (11.7%)	2.4±1.06^{a} (8.0%)	1.5±0.54^{a} (5.0%)

SE: Standard Error, Symbol a, b and c refer to the differences between the groups, Significant differences $p<0.05$ and $p>0.01$

proportional size were observed between days 5, 10 and 15 of culture. The culture media supplements caused a significant increase ($p<0.01$) in the follicle size comparing to control for all follicle size classes. However, no significant differences between different supplement systems (FSH+EGF and FSH+ITS) in combination with the same medium were detected. Both types of the used media with different supplements did not affect the 100 μm size follicles where no significant differences between them were detected (Table 3, 4). However, the overall follicular viability and morphology were better maintained with the tested culture systems than the controls.

Effect of culture media supplemented with FSH and EGF: CR1aa medium supplemented with the FSH and EGF showed a significant increase ($p<0.01$) in the size of the follicles with an initial diameter of 60 μm, at days 5, 10 and 15 of culture than TCM-199 medium with the same supplements. Similarly the same supplements in combination with CR1aa medium were more effective than with TCM199 regarding to follicles viability on the 26th day (22.7 vs 16.2%, respectively) (Table 1, 4). Follicles with initial diameter of 80 μm showed response to the used culture systems similar to that of 60 μm follicles. There were significant increases ($p<0.01$) in both follicles

Table 4: Percentage of viable follicles of different size classes at the end of culture (day 26th)

	Follicular size		
Treatments	60 μm	80 μm	100 μm
FSH + EGF + TCM-199	4.85±1.4^{b} (16.2%)	5.0±1.05^{b} (16.7%)	6.50±1.8^{b} (21.7%)
FSH + EGF + CR1aa	6.80±0.55^{c} (22.7%)	7.0±0.80^{c} (23.3%)	7.65±1.55^{b} (25.5%)
FSH + ITS + TCM-199	6.9±1.06^{b} (23.0%)	4.80±1.06^{b} (16.0%)	6.00±1.06^{b} (20.0%)
FSH + ITS + CR1aa	8.5±1.05^{c} (28.3%)	7.50±0.8^{c} (25.0%)	6.25±1.05^{b} (20.8%)
Control (TCM 199)	1.55±0.50^{a} (5.2%)	1.00±0.54^{a} (3.3%)	1.0±0.54^{a} (3.3%)

Symbol a, b and c refer to the differences between the groups, Significant differences $p<0.05$ and $p>0.01$

size and follicles viability (23.3% vs. 16.7%) in CR1aa supplemented with FSH+EGF than TCM-199 with the same supplements (Table 2, 4).

Effects of culture media supplemented with FSH and ITS: Regarding to 60 μm follicles, CR1aa medium supplemented with FSH and ITS was more effective than TCM-199 medium with the same supplements for both follicles size ($p<0.01$ at days 5, 10 and 15) and follicles viability (23.0% vs. 28.3% at 26th day of culture) (Table 1, 4). Similarly, follicles of 80 μm diameter cultured in CR1aa with FSH and ITS showed significant increase in the size ($p<0.01$) and in the viability (25.0% vs, 16%) than those cultured in TCM-199 with the same supplements (Table 2, 4).

DISCUSSION

Culture of preantral follicles has important biotechnological implications through its potential to produce large quantities of oocytes for embryo production and transfer. Moreover, the ability to culture isolated preantral follicles constitutes a new tool for investigation of paracrine and autocrine factors involved in early folliculogenesis.

In this study, we used CR1aa media plus some supplements such as FSH, EGF and IT in a trial to improve the growth and development of bovine preantral follicles. We succeeded to extend the culture period to 26 day.

The greatest increases in the proportional size were observed at the beginning of culture (days 5, 10 and 15) with gradual reduction during the later part in all size classes of the follicles. This finding is in agreement with previous studies on bovine and buffalo preantral follicles (Gutierrez *et al*., 2000; Gupta *et al*., 2002). The 100 μm size follicles respond similarly to both CR1aa and TCM-199 media with different supplements where no significant differences between them were detected (Table 3, 4). However, the overall follicular viability and morphology were better maintained with the tested culture systems than the controls. This similar efficacy of both culture systems on the larger sized follicles may be due to the intrinsic follicular factors, which make them less vulnerable to external influences. This opinion is strengthened by previous study which suggested that culture conditions of large preantral follicles necessarily different from the smaller follicles (Van den Hurk *et al*., 1998).

Regardless of the initial size classes or the used treatments, follicles size has been increased during the *in vitro* culture compared to control. Moreover, all of the used culture systems produced more viable and more morphologically good follicles than control that prove the beneficial effects of the used supplements. This findings are in agreement with the previous studies which reported that the FSH, EGF and ITS promote follicle development and granulosa cell proliferation in cultured preantral follicles *in vitro* (Morbeck *et al*., 1993; Ralph *et al*., 1995; Roy and Treacy, 1993; Chun *et al*., 1996; Wandji *et al*., 1996; Katska and Rynska, 1998; Mao *et al*., 2004). No significant differences in the follicle size or viability between the different supplements combinations (FSH+EGF and FSH+ITS) with CR1aa medium were detected suggesting similar efficacy for both combinations.

Follicular growth was significantly higher ($p<0.01$) in CR1aa medium with different supplements than in the TCM-199 media with the same supplements in the case of smaller follicles (60 and 80 μm). This finding is in agreement with previous study, which reported that, oocytes matured and cultured in CR1aa medium expressed higher cleavage and developmental rates than oocytes mature in TCM-199 (Abdoon *et al*., 2001). This better growth rate of the follicles referred to the amino acids that included in the CR1aa media as discussed by Rosenkrans and First (1991).

To successfully culture preantral bovine follicles, it is necessary to define appropriate culture conditions, which would allow follicular (granulosa) cells as well as oocytes to survive and grow *in vitro* for a prolonged period. Several studies, using mainly small secondary follicles, have shown that follicles cannot grow for more than 6 or 7 days in culture (Spears *et al*., 1994; Magarelli *et al*., 1996; Gutierrez *et al*., 1997; Armstrong *et al*., 2000). In other study by Gutierrez *et al*. (2000), he succeeded to extend the culture period to 28 day but he used a relatively large bovine preantral follicles (168 μm). In this study we found that the morphologically normal structure of smaller preantral follicles (60, 80 and 100 μm) was preserved in approximately 25-28% of the follicles that survived *in vitro* culture for 26 days. This relatively higher

percentage of normal follicles in a long-term culture indicates the efficacy of our culture system using CR1aa media plus FSH, EGF/and or ITS for preantral follicles. However, further research is needed to evaluate whether this culture system are able to survive an extended culture period. Also further histological characterization and ultrastructure are required to investigates whether the increase in diameter of the follicles were due to increased oocyte diameter, granulosa cell proliferation or accumulation of fluids.

In conclusion, we succeed to culture bovine preantral follicles (60, 80 and 100 μm) for a relatively long period (26 day) in a new culture system using CR1aa in combination with FSH, EGF and/or ITS. The follicles increased in size and maintained their viability in a relatively high percentage. Hence, this culture system can provide a new rout for *in vitro* culture of bovine preantral follicles.

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