Effects of Serum Addition and Different Culture Media on Growth of Porcine Preantral Follicles *In Vitro*

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ABSTRACT

Current developments in IVF and animal cloning have resulted in increasing demand for large quantities of oocytes and ovarian follicles at specific stages of development. These medical and scientific needs may be met by developing an optimal culture system for preantral follicles. In this study, we investigated the growth of porcine preantral follicle cultures in different media and in the presence and absence of serum. Follicles were manually dissected from ovaries obtained from prepubertal gilts at a local slaughterhouse, and cultured for 3 days in M199 or NCSU23 medium supplemented with porcine FSH, transferrin, L-ascorbic acid and insulin. Follicle diameters were measured on day 1 and 3 of culture. In Experiment 1, the effect of supplementing culture medium with fetal calf serum (FCS) on porcine preantral follicle growth was examined. In the group of cultures supplemented with FCS, follicle diameter after 3 days of culture, survival rate and antrum formation rate in the FCS group were significantly higher than those of the control group. In Experiment 2, the effects of culture medium (M199 and NCSU23) on follicle growth were compared. Follicle diameters were increased in the M199 group, compared with those in NCSU23 ($p<0.05$), but we observed no significant differences in survival and antrum formation rates between cultures grown in the two media. In conclusion, supplementation of the culture medium with serum enhances preantral follicle growth and antrum formation, and M199 is superior to NUSU23 for porcine preantral follicle culture *in vitro*.

(Key words : Porcine preantral follicle, Serum, Culture medium, *In vitro*)

INTRODUCTION

Harvesting oocytes is crucial for *in vitro* embryo production procedures, including *in vitro* fertilization, nuclear transfer, intracytoplasmic sperm injection, and transgenic technology, among other processes. Currently, the recovery rate of oocytes from antral follicles is poor, owing to the small number of large antral follicles in the ovary. In mammalian ovaries, the majority of follicles are present in the cortex as preantral follicles. Ovaries of human, sheep and rat at birth contain 1×10^6, 1×10^5 and 2×10^4 follicles, respectively, which predominantly exist as primordial and primary follicles. The average preantral follicle number in goat ovaries is 37,646. In cattle, the ovary contains more than 100,000 preantral follicles. However, during oocyte growth and development, less than 1% of follicles continue normal growth, with most of the rest undergoing atresia (Fortune, 1994; Evans, 2003). Hence, the development of an effective *in vitro* culture system for preantral follicles is important to ensure the provision of large quantities of oocytes for embryo production. Furthermore, *in vitro* follicle culture is an essential tool to elucidate the underlying mechanisms of oocyte growth and differentiation, and may also be used for preservation and long-term storage of female germ cells.

Several recent studies have focused on improving the preantral follicular culture system. Various culture systems for follicles have been developed in several species, including mouse (Eppig and Schroeder, 1989; Gong *et al.*, 2009), rat (Cain *et al.*, 1995), hamster (Roy and Greenwald, 1985), pig (Hirao *et al.*, 1994), bovine (Wandji *et al.*, 1996; Lim *et al.*, 2009), and human (Abir *et al.*, 1997). However, limited information is currently available on the *in vitro* mechanisms of folliculogenesis and oocyte growth and differentiation. Moreover, the efficiency of preantral follicle culture *in vitro* is still low, and optimal culture conditions for preantral follicle growth are yet to be established in domestic animals.

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The objective of this study was to investigate the effects of different culture media (M199 and NCSU23) and fetal calf serum (FCS) on the growth of porcine preantral follicles *in vitro*, with a view to establishing an optimal culture system to support follicle development *in vitro*.

MATERIALS AND METHODS

Reagents

All chemicals were obtained from Sigma, unless otherwise indicated.

Animals

Ovaries from prepubertal gilts were collected at a local abattoir, and rinsed in PBS supplemented with 100 IU/ml penicillin and 50 μg/ml streptomycin. Ovaries were maintained at 37℃ during 1 to 2 h of transportation from the slaughterhouse to the laboratory.

Preantral Follicle Dissection and Collection

Ovaries were washed with PBS, and transferred into PBS containing 3 mg/ml BSA (A 8022, Sigma). Ovarian cortical tissue was sliced (<1 mm thickness) from the ovarian surface with a blade. Preantral follicles were visualized under a dissecting microscope, and manually isolated with two 28-gauge needles in PBS with 3 mg/ml BSA. Follicles with diameters of 270 to 320 μm, in which oocyte and granulosa cells were completely surrounded by the basement membrane, thecal cells, and some stromal tissue, were collected into 4-well multidishes that contained collecting medium consisting of Medium199 (M4530, Sigma) supplemented with 3 mg/ml BSA.

Preantral Follicle Culture *In Vitro*

Follicles were collected and transferred into culture medium (M199 or NCSU23) supplemented with 3.5 μg/ml insulin (I5523, Sigma), 10 μg/ml transferrin (T5391, Sigma), 100 μg/ml L-ascorbic acid (A4544, Sigma), and 2 mIU FSH (F2293, Sigma). Depending on the experiment, two culture media, M199 and NCSU23, were supplemented with 7.5% FCS (26010-074, Gibco) or left untreated. Follicles were randomly distributed to different experimental groups, and cultured for 3 days in 24-well cell culture cluster plates, with four follicles per well in 280 μl medium. The culture was performed at 38.5℃ in 5% CO_2 and air. Half of the culture medium was replaced every day with freshly prepared medium. Follicle diameters were measured under a stereomicroscope with an ocular scale at a magnification of ×50 at days 0 and 3 of culture. Follicle survival rates were determined using trypan blue staining (15250-061, Gibco).

Experimental Design

Experiment 1 was conducted to assess the effects of FCS on porcine preantral follicular survival, growth and antrum formation following *in vitro* culture. Based on the culture system described by Wu *et al.* (2001), 7.5% FCS was added to the medium. Preantral follicles of the control group were cultured without FCS, while the treatment group was cultured in M199 medium supplemented with 7.5% FCS.

Experiment 2 was performed to evaluate the effects of two different culture media, M199 and NCSU23, on survival, growth and antrum formation of porcine preantral follicles.

Statistical Analysis

Experiments were repeated at least four times. Statistical analysis was performed using the SPSS statistical software with Student *t*-test. Data were presented as mean values±SEM.

RESULTS

Effects of FCS on Porcine Preantral Follicular Survival, Growth and Antrum Formation

All follicles used for the experiment were manually isolated. Follicle growth and antrum formation are presented in Fig. 1. The survival rates of preantral follicles in cultures *in vitro* were determined (Fig. 2). Notably, the survival of preantral follicles in medium supplemented with 7.5% FCS was significantly higher than that of the control group (75.1% vs 54.6%). The initial follicular diameter (Day 0 of culture), shown in Table 1, was similar between groups (299.9 μm in the FCS group vs 298.4 μm in the control group). After 3 days of culture, the mean diameter of follicles cultured in medium containing 7.5% FCS was significantly larger than that of the control group (382.4 μm vs 335.8 μm). Moreover, the antral formation rate of the FCS-supplemented group was higher, compared to the control group (74.9% vs 41.3%). These results clearly indicate that 7.5% FCS promotes the growth of porcine preantral follicles in culture medium *in vitro*.

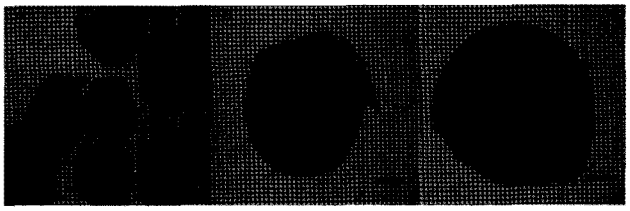

Fig. 1. Growth of porcine preantral follicle cultures *in vitro*. (A) Preantral follicle on Day 0 of culture, (B) Preantral follicle on Day 1 of culture, (C) Antral follicle on Day 3 of culture. (Bar=100 μm).

Table 1. Effect of FCS on growth of porcine preantral follicle cultures *in vitro*

FCS	No. of follicles	Repeats	Follicle diameter (μm)		Antrum formation (%)
			Day 0 (mean±SEM)[1]	Day 3 (mean±SEM)	
0	29	4	299.9±1.37	335.8±11.49[a]	12 (41.3±3.69)[a]
7.5%	29	4	298.4±1.20	382.4±8.78[b]	22 (74.9±5.31)[b]

[a,b] Within a column, different superscripts imply significant differences ($p<0.05$).
[1] Follicle diameters at Day 0 display no significant differences ($p>0.05$).

Table 2. Effects of M199 and NCSU23 on growth of porcine preantral follicle cultures *in vitro*

Medium	No. of follicles	Repeats	Follicle diameter (μm)		Antrum formation (%)
			Day 0 (mean±SEM)[1]	Day 3 (mean±SEM)	
M199	31	4	295.2±4.13	399.6±4.26[a]	21 (69.1±4.83)
NCSU23	31	4	297.3±1.48	376.4±6.07[b]	21 (68.5±6.88)

[a,b] Within a column, different superscripts imply significant differences ($p<0.05$).
[1] Follicle diameters at Day 0 have no significant differences ($p>0.05$).

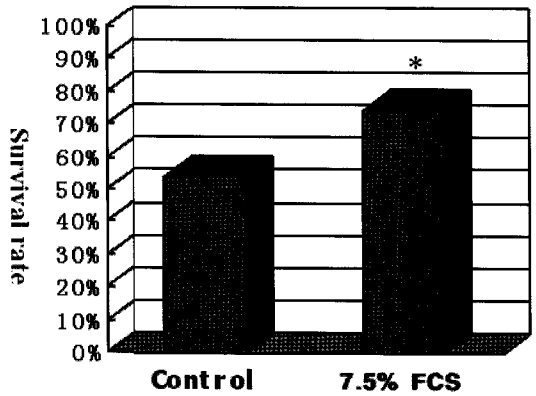

Fig. 2. Effect of FCS on the survival rates of porcine preantral follicle cultures *in vitro*. Control: Preantral follicle culture in the absence of FCS, FCS: Preantral follicle culture in medium supplemented with 7.5% FCS. *Significant difference between the two groups ($p<0.05$).

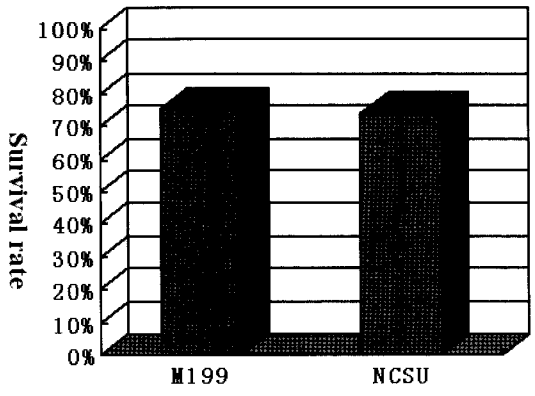

Fig. 3. Effects of different culture media (M199 and NCSU23) on the survival rates of porcine preantral follicle cultures *in vitro*.

Effects of Different Culture Media (M199 and NCSU23) on Survival, Growth and Antrum Formation of Porcine Preantral Follicles

The survival rates of preantral follicles cultured *in vitro* in different media were analyzed (Fig. 3). We observed no marked differences in the survival rates of preantral follicle cultures between the two medium groups (75.1% with M199 vs 74.2% with NCSU23). Follicular diameters on day 0 of culture were similar between the two groups (295.2 μm in M199 vs 297.3 μm in NCSU), as shown in Table 2. However, after 3 days of culture, the diameters of follicles cultured in M199 were significantly larger than those in the NCSU23 group (399.6 μm vs 376.4 μm). We observed no differences in antrum formation rates between the two culture medium groups (69.1% with M199 vs 68.5% with NCSU23). Our results suggest that M199 is superior to NCSU23 for culture of porcine preantral follicles *in vitro*.

DISCUSSION

Porcine folliculogenesis from the primordial to preovulatory follicle is a lengthy process regulated by interactions among endocrine, paracrine and autocrine factors in the ovary (Morbeck *et al.*, 1992; Mao *et al.*, 2002). Many researchers have used serum as a component of culture media, as it provides growth factors and improves preantral follicular growth (Wu *et al.*, 2001; Hovatta *et al.*, 1999). Fetal calf serum is often used in bovine preantral follicle culture (Saha *et al.*, 2000; Katska and

Rynska, 1998). Serum contains proteins, amino acids, carbohydrates, trace elements, hormones and growth factors, as well as extracellular matrix components that promote cell adhesion and proliferation (Picton *et al.*, 2008). Extracellular matrix moderates follicle survival in organ culture. Serum provides a source of albumin, and can balance osmolarity and scavenge harmful molecules and metal ions. Serum additionally acts as a source of precursors for steroid biosynthesis.

In our experiments, supplementation of the culture medium with FCS improved preantral follicle growth. The diameters of preantral follicles after 3 days of culture were significantly enhanced following supplementation with FCS. Serum contains growth factors and other unknown components that may promote the proliferation of granulosa cells in follicles, leading to increased follicular diameters. The antrum formation rate in the FCS supplementation group was also significantly higher than that in control cultures, indicating that FCS promotes antrum formation of porcine preantral follicle culture *in vitro*.

Various base media have been employed for *in vitro* culture of follicles in different species, including minimum essential medium (Jewgenow, 1998; Newton *et al.*, 1999), Waymouth medium (Eppig and O'Brien, 1996; Muruvi *et al.*, 2005), and McCoy's 5a medium (Telfer *et al.*, 2008). M199 and NCSU23 media are frequently used for porcine follicle culture. In this study, we observed no differences between the survival rates and antrum formation of preantral follicles cultured in the two media (M199 and NCSU23). However, the diameters of follicles after 3 days of culture in M199 were significantly increased, compared to those cultured in NCSU-23, consistent with the finding of Mao *et al.* (2002). Our results indicate that M199 medium promotes faster growth of porcine preantral follicles than NCSU23. In the murine species, the oocyte is almost fully developed in the preantral follicle, but the porcine oocyte needs to continue growing after antrum formation. The oocyte is characterized by higher proliferation of granulosa cells and enhanced RNA and protein synthesis. In relation to NCSU23, M199 contains more amino acids, vitamins, ribonucleosides, and deoxyribonucleosides. Therefore, porcine preantral follicles appear to require more nutrient components to support their development. NCSU23 is a simple medium, and may not satisfy the demands for porcine preantral follicle development *in vitro*, which would account for the slower growth of follicles. Our findings clearly suggest that M199 is the superior medium for porcine preantral follicle culture *in vitro*.

In conclusion, supplementation of culture medium with serum enhances preantral follicle growth and antrum formation, and M199 is more suitable than NU-SU23 for *in vitro* culture of porcine preantral follicles.

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