Asian-Aust. J. Anim. Sci.
Vol. 21, No. 4 : 532 - 537
April 2008

www.ajas.info

In vitro Culture Conditions for the Mouse Preantral Follicles Isolated by Enzyme Treatment

Dong-Hoon Kim*, Hwan-Hoo Seong and Ho-Joon Lee[1]
Animal Biotechnology division, National Institute of Animal Science
564 Omockcheon-dong, Kwonsun-gu, Suwon 441-706, Korea

ABSTRACT : In order to investigate the factors affecting the culture of mouse preantral follicles *in vitro*, we examined the effect of culture media, protein supplements, and culture period on their growth. The oocyte diameter (initial size: 55.6±2.5 μm) was progressively increased during culture, and the maximum size (72.0±2.4 μm) was reached on day 10 of the *in vitro* culture. The chromatin configuration in the germinal vesicle (GV) oocyte progressively shifted from a non-surrounded nucleolus (NSN) to a surrounded nucleolus (SN). On day 10 of the culture, most of the oocytes progressed to the SN pattern. The survival and metaphase II rates of the oocytes in alpha-minimal essential medium (alpha-MEM) were significantly higher ($p<0.05$) than those in Waymouth and tissue culture medium (TCM)-199. As a protein source, fetal bovine serum (FBS) was more suitable for the culture of mouse preantral follicles as compared to human follicular fluid (hFF) and bovine serum albumin (BSA); the optimal concentration of FBS was 5%. These results suggest that in a culture of mouse preantral follicles, alpha-MEM and 5% FBS are an optimal medium and a protein source, respectively; further, the 10 days of culture is required for the complete growth of oocytes in this culture system. (**Key Words :** Chromatin Pattern, Media, Mouse Preantral Follicle, Protein)

INTRODUCTION

Recent advances in *in vitro* reproductive technologies have opened up new opportunities in assisted reproduction technology (ART) and biotechnology. However, these techniques depend on the predictable production of fully developed oocytes, and currently, their availability is limited by the number of antral follicles present in the ovaries. The development of a preantral follicle culture system that can potentially produce large quantities of oocytes with uniform developmental status will significantly advance the use of these techniques. Additionally, it might enable the preservation and long-term storage of the female germ cells (Gutierrez et al., 2000).

Various methods have been developed to isolate and culture preantral follicles from mouse ovaries (Eppig and Schroeder, 1989; Cortvrindt et al., 1996). In general, complex media have been used for the culture of preantral follicles. A large variety of additives, such as serum or serum supplements and growth factors, have been employed for medium supplementation. Under appropriate conditions, the meiotically incompetent oocytes from the preantral follicles can grow to their final size and complete nuclear maturation *in vitro*. Furthermore, studies regarding the culture of mouse preantral follicles have demonstrated successful growth and maturation as well as the fertilization and development of oocytes from follicles cultured *in vitro* (Eppig and Downs, 1989; Nayudu and Osborn, 1992; Cortvrindt et al., 1996; Kim et al., 2004). However, synthetic studies concerning the culture conditions of mouse preantral follicles *in vitro* have not been accomplished.

In order to establish a suitable culture condition for the mouse preantral follicles that were enzymatically isolated from the ovary, we conducted a series of experiments with the following aims: (1) to determine the optimal culture period of preantral follicles according to the change in oocyte diameter and chromatin configuration; (2) to compare the effects of the culture medium and protein sources on the survival and maturation rates of preantral follicles *in vitro*; and (3) to examine the optimal concentration of fetal bovine serum (FBS) for the growth

* Corresponding Author: D-H. Kim. Tel: +82-31-290-1633, Fax: +82-31-290-1622, E-mail: kdh1010@rda.go.kr
[1] Vincent Center for Reproductive Biology, Vincent Obstetrics and Gynecology Service, Harvard Medical School, Boston, Massachusetts 02114, USA..
Received September 1, 2007; Accepted November 29, 2007

Table 1. Change in the oocyte diameter according to the culture period

	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
Oocyte diameter (SEM, μm)	55.6±2.8 (57)*	59.3±3.5 (54)*	62.4±3.1 (61)*	66.0±3.0 (53)*	70.2±2.1 (56)*	72.0±2.4 (55)*	71.0±2.2 (57)*	72.3±4.0 (67)*

* The number of oocytes examined.

and development of the preantral follicles *in vitro*.

MATERIALS AND METHODS

Isolation of preantral follicles

The ovaries were aseptically removed from the 12-day-old ICR female mice. The ovaries were immersed into Leibovitz L-15 medium (Gibco-BRL, Carlsbad, CA, USA) containing 1 mg/ml collagenase (Type 1A; Sigma, St. Louis, MO, USA) and 0.2 mg/ml DNase I (Sigma) for 20 min at 37°C and repeatedly drawn in and out of the pipette until the ovaries were dissociated into individual follicles. The preantral follicles (100-120 μm in diameter) to be cultured were selected based on the following criteria: i) intact round follicular structure with 2-3 layers of granulosa cells and ii) the oocyte had to be visible, round, and centrally located within the follicle. All the selected follicles were pooled and randomly divided over the culture conditions under study.

In vitro growth and maturation of the preantral follicles

The culture medium was supplemented with a protein supplement and 100 mIU/ml follicle stimulating hormone (FSH, Metrodin-HP; Sereno, Switzerland) and 10 mIU/ml luteinizing hormone (LH; Sigma). The follicles were cultured on Transwell-COL membrane inserts (3.0 μm pore size, 24.5 mm diameter; Costar, NY, USA) in 6-well cluster dishes to prevent the loss of structural integrity between the oocyte and the granulosa cells. The follicles were cultured for 10 or 14 days at 37°C in 5% CO_2 in air. Half of the medium was changed every 2 days. After 10 days of growth *in vitro*, the follicles were allowed to mature for 16-18 h in a medium supplemented with 1.5 IU/ml human chorionic gonadotrophin (hCG, Profasi; Sereno). The diameter of the morphologically normal metaphase II oocytes, excluding the zona pellucida, was examined with an inverted microscope and an ocular micrometer.

Preparation of human follicular fluids

During oocytes aspiration in the human IVF programs, human follicular fluids (hFF) were collected from the preovulatory follicles of the patients. The blood cells and cell debris were removed from the follicular fluids by centrifugation at 3,000 rpm for 30 min. The supernatant was heat-inactivated at 59°C for 35 min and filtered with a 0.22-μm microfilter (Millex-GV; Millipore, Bedford, MA, USA). All the hFF samples were stored at -70°C.

Nuclear chromatin staining

The chromatin configuration of the germinal vesicle (GV) oocytes during culture was evaluated by fluorescence staining. The oocytes were fixed in 2% formaldehyde in phosphate buffered saline (PBS) for 10 min at room temperature. The fixed oocytes were then placed on slides with a drop of the mounting medium consisting of glycerol and PBS containing 2.5 mg/ml sodium azide and 2.5 μg/ml Hoechst 33342 (Sigma); glycerol and PBS were in the ratio of 3:1. A coverslip was placed on top of the oocytes, and the edge was sealed with fingernail polish. The stained oocytes were examined under a fluorescence microscope.

Experimental designs

Experiment 1 : To determine optimal culture period for mouse preantral follicles, the mouse preantral follicles were cultured in α-minimal essential medium (αMEM) supplemented with 5% FBS, 100 mIU/ml FSH, and 10 mIU/ml LH for 14 days. The assessment of the oocyte diameter and nuclear configuration was carried out by the mechanical removal of the oocytes from their follicular complex on days 2, 4, 6, 8, 10, 12, and 14.

Experiment 2 : To compare the effects of different culture media (αMEM, Waymouth, and tissue culture medium (TCM)-199) and protein sources (FBS, hFF, and bovine serum albumin (BSA)) on the survival and maturation rates of the preantral follicles *in vitro*, the mouse preantral follicles were cultured for 10 days and further cultured in a medium supplemented with 1.5 IU/ml hCG for 16-18 h to induce meiotic maturation.

Experiment 3 : To examine the effect of the various concentrations of FBS (5, 10, 15, and 20%) on the preantral follicle growth and development, the mouse preantral follicles were cultured in αMEM as described in experiment 2.

Statistical analysis

Data were subjected to a Generalized Linear Model procedure (PROC-GLM) of the Statistical Analysis System (SAS Institute, Cary, NC, USA). Differences among treatment means were determined by using the Duncan's multiple range tests. Statistical significance was established at $p<0.05$.

RESULTS

Change of oocyte diameter during the culture period

The change in oocyte diameter within the preantral

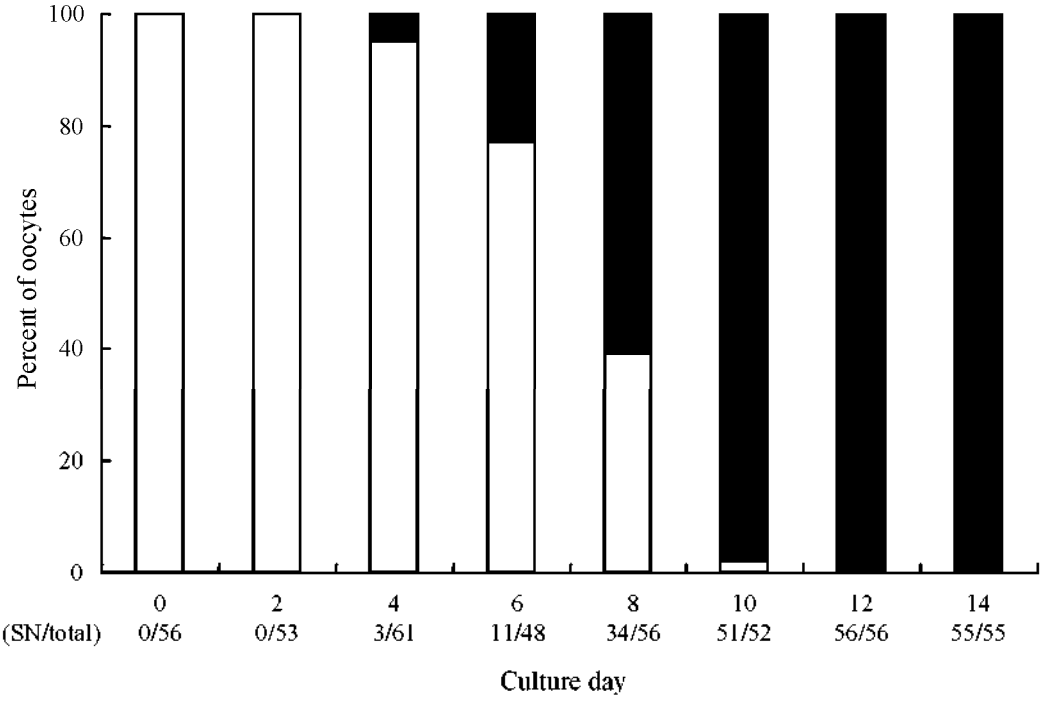

Figure 1. Change in the nuclear chromatin pattern of oocytes within mouse preantral follicles according to the culture period. Bars represent the proportion of total oocytes observed in each category. Solid, SN (surrounded nucleolus); open, NSN (non-surrounded nucleolus).

Table 2. Effect of media on the *in vitro* growth and maturation of mouse preantral follicles

Medium	No. of follicles cultured	No.(%) of oocytes survived*	GV (%)	GVBD (%)	Meta II (%)	Oocyte diameter (SEM, μm)
αMEM	164	112 (68.3)^{a}	30 (18.3)	9 (5.5)	73 (44.5)^{a}	67.7±0.9
Waymouth	164	90 (54.9)^{c}	51 (31.1)	13 (7.9)	26 (15.9)^{b}	69.3±1.1
TCM199	164	64 (39.0)^{b}	37 (22.6)	2 (1.2)	25 (15.2)^{b}	70.0±1.1

GV = Germinal vesicle, GVBD = Germinal vesicle breakdown, Meta II = Metaphase II.

* Survival was defined as those oocytes (GV, GVBD and metaphase II) which showed normal morphology after *in vitro* growth (10 days) and maturation (16-18 h), and was expressed as a percentage of preantral follicles put into culture.

$^{ab, bc}$ Significant differences within the same column ($p<0.05$).

follicle during the period of the *in vitro* growth is summarized in Table 1. The mean oocyte diameter at the beginning of the culture was 55.6±2.5 μm. During the culture period, the oocyte diameter increased progressively. The maximum size (72.0±2.4 μm) of the oocytes was reached at 10 days after culture.

Change in nuclear chromatin pattern in the oocyte during the culture period

Mouse GV oocytes show 2 different patterns of chromatin organization; one with chromatin surrounding the nucleolus, i.e., surrounded nucleolus (SN), and one without chromatin surrounding the nucleolus, i.e., non-surrounded nucleolus (NSN). The change in the nuclear chromatin pattern of the oocyte during the *in vitro* culture period is summarized in Figure 1. Initially, all the oocytes had an NSN pattern of chromatin organization but with the growth of the culture, they progressively shifted into the SN pattern. On day 10 of the *in vitro* culture, most of the oocytes progressed from the NSN to the SN pattern, and this pattern change was correlated with the increase in oocyte diameter as shown in Table 1.

Effect of culture medium

In order to determine the optimal culture medium, the mouse preantral follicles were cultured in 3 different culture media. As shown in Table 2, the survival and metaphase II rates of the preantral follicles following *in vitro* growth and maturation was significantly higher in αMEM (68.3% and 44.5%, respectively) than in Waymouth (54.9% and 15.9%, respectively) and TCM-199 (39.0% and 15.2%, respectively). With respect to the mean diameter of the matured oocytes, there was no difference among those cultured with αMEM (67.7±0.9 μm), Waymouth (69.3±1.1 μm) and with TCM 199 (70.0±1.1 μm).

Effect of protein sources

In order to examine the optimal protein sources, the

Table 3. Effect of protein sources on the *in vitro* growth and maturation of mouse preantral follicles

Protein	No. of follicles cultured	No.(%) of oocytes survived*	GV (%)	GVBD (%)	Meta II (%)	Oocyte diameter (SEM, μm)
5% FBS	197	141 (71.6)	49 (24.9)[a]	20 (10.2)	72 (36.5)[a]	67.3±2.1
5% hFF	197	130 (66.0)	59 (29.9)[a]	41 (20.8)	30 (15.2)[b]	69.5±1.6
0.3% BSA	197	145 (73.6)	112 (56.9)[b]	32 (16.2)	10 (5.1)[c]	70.4±2.0

GV = Germinal vesicle, GVBD = Germinal vesicle breakdown, Meta II = Metaphase II.

* Survival was defined as those oocytes (GV, GVBD and metaphase II) which showed normal morphology after *in vitro* growth (10 days) and maturation (16-18 h), and was expressed as a percentage of preantral follicles put into culture.

[ab, ac] Significant differences within the same column ($p<0.05$).

Table 4. Effect of FBS concentrations on the *in vitro* growth and maturation of mouse preantral follicles*

Conc. (%)	No. of follicles cultured	No.(%) of oocytes survived**	GV (%)	GVBD (%)	Meta II (%)	Oocyte diameter (SEM, μm)
5	148	90 (60.8)	19 (10.7)	15 (8.4)	56 (37.8)	69.2±2.9
10	147	95 (64.6)	29 (19.7)	15 (10.2)	51 (34.7)	68.8±2.7
15	148	71 (48.0)	20 (13.5)	4 (2.7)	47 (31.8)	68.4±2.6
20	147	71 (48.3)	12 (8.2)	13 (8.8)	46 (31.3)	69.0±1.8

GV = Germinal vesicle, GVBD = Germinal vesicle breakdown, Meta II = Metaphase II.

* There were no significant differences among the four concentration treatments in oocyte survival and metaphase II rates and oocyte diameter.

** Survival was defined as those oocytes (GV, GVBD and metaphase II) which showed normal morphology after *in vitro* growth (10 days) and maturation (16-18 h), and was expressed as a percentage of preantral follicles put into culture.

mouse preantral follicles were cultured with 3 different proteins. As shown in Table 3, the survival of the preantral follicles after *in vitro* growth and maturation demonstrated no difference among the 3 protein sources. However, the metaphase II rates of the oocytes were significantly higher in FBS (36.5%) than that in FF (15.2%) and BSA (5.1%). With respect to the mean diameter of the matured oocytes, there was no difference between those cultured with FBS (70.4±2.0 μm) and with FF (69.5±1.6 μm); however, the mean diameter of the oocytes derived from these protein sources was slightly larger than that of the oocytes cultured with BSA (67.3±2.1 μm).

Effect of FBS concentration

To examine the optimal concentration of FBS on the culture of mouse preantral follicles, the preantral follicles were cultured with various concentrations of FBS. As shown in Table 4, the survival rates of the preantral follicles following *in vitro* growth and maturation were higher at 5% and 10% concentrations (60.8% and 64.6%, respectively) than at 15% and 20% concentrations (48.0% and 48.3%, respectively), but there was no statistical significance. The metaphase II rates of the oocytes were slightly higher at 5% concentration (37.8%) than at 10%, 15% and 20% (34.7%, 31.8% and 31.3%, respectively), although there was no statistical significance among the concentration of FBS. The mean diameter of the matured oocytes did not differ among the treatment groups.

DISCUSSION

The preantral follicles were isolated for culture from the ovaries of 12-day-old mice. In these ovaries, most of the follicles consist of approximately 1-3 layers of granulosa cells around the oocyte. Such oocytes are approximately in a mid-growth phase and are incompetent to undergo a germinal vesicle breakdown (GVBD) at the time of isolation (Eppig and Downs, 1989); however, the follicular cells possess all the morphological and functional abilities to sustain follicular growth. In this experiment, we also confirmed that the oocytes were incapable of resuming meiosis at the time of isolation from preantral follicles.

Based on the purpose of the experiment, various culture methods for the growth of preantral follicles to maturity *in vitro* have been developed, such as agar or collagen-gel embedding (Torrance et al., 1989; Carroll et al., 1991; Roy and Treacy, 1993), collagen-impregnated membranes (Eppig and Schroeder, 1989), agar-coated plastic petri dishes (Hirao et al., 1990), 96-V-well microtiter plates (Hartshorne et al., 1994; Spears et al., 1994), and microdroplets covered with mineral oil (Cortvrindt et al., 1996; Choi et al., 2007). In the present study, we used collagen-coated Transwell-COL membrane inserts (Eppig and Schroeder, 1989) to culture mouse preantral follicles. These membranes were treated with an equimolar mixture of Type I and III collagen produced from bovine placentae, and they allowed the attachment and maintenance of the complexes with only minimal migration of the granulosa cells from the oocytes as compared to the general petri dishes. Generally, it is more difficult to maintain a three-dimensional structure between the oocyte and granulosa cells in the enzymatically isolated preantral follicles because theca cells and the basal lamina are partially damaged by collagenase. However, the culture of preantral follicles on the Transwell-COL membrane makes it possible for the oocyte and granulosa cells to maintain the complex

as a three-dimensional structure that helps in the survival and growth of oocytes and follicles.

The diameter of the oocyte is an important index for the cytoplasmic maturation and developmental competence of oocytes. In the present study, the mean oocyte diameter at the start of culture was 55.6±2.5 μm and progressively increased during the culture period. The maximum diameter (72.0±2.4 μm) of the oocytes was reached at 10 days after culture. The diameter of these oocytes was comparable to that of the oocytes (73.3±3.0 μm) from 22-day-old mice (after ovarian stimulation and *in vitro* maturation) (Kim et al., 2004). Thus, the results from the present study confirmed that in this culture condition, the optimal culture period for the growth of mouse preantral follicles is 10 days.

Earlier studies have shown that 2 different classes of oocytes are present within the antral compartment of the mouse ovary (Mattson and Albertini, 1990; Debey et al., 1993; Zuccotti et al., 1995). One class known as SN is characterized by the presence of a ring of Hoechst-positive chromatin around the nucleolus and a thread-like nuclear chromatin organization; the other class NSN has more homogeneously widespread nuclear chromatin and less well-defined chromatin surrounding the nucleolus. Several observations suggest that the SN configuration is a prerequisite for GVBD and is a stage of the GV oocyte that is more advanced toward ovulation (Wickramasinghe et al., 1991; Zuccotti et al., 1998; Bouniol-Baly et al., 1999). The normal sequence of GV progression from dispersed chromatin toward the formation of a nucleolar rim has been demonstrated in mouse preantral follicles cultured *in vitro* (Hartshorne et al., 1994; Johnson et al., 1995; Kim et al., 2004). In the present study, the chromatin configuration in the GV oocytes progressively shifted from the NSN to the SN pattern. On day 10 of the *in vitro* culture, most oocytes progressed into the SN pattern, and this change in the nuclear chromatin configuration was closely correlated with an increase in the oocyte diameters. Therefore, this result reconfirmed that the optimal culture period for the growth of mouse preantral follicles is 10 days.

The mouse preantral follicles were grown in various media, such as Waymouth (Eppig et al., 1992), αMEM (Nayudu and Osborn, 1992; Cortvrindt et al., 1996), Dulbecco's modified Eagle's media (DMEM) (Liu et al., 1998), F12-DMEM (Li et al., 1995) and TCM-199 (Christmann et al., 1994; Harada et al., 1997). In the present study, we found that αMEM is an optimal culture medium for the growth of mouse preantral follicles. Microscopic observations showed strikingly different effects of culture medium on the development of the granulosa cells. In αMEM, the structure of the follicle showed a rapid proliferation of the granulosa cells surrounding oocyte when compared to other culture media. This result may be due to beneficial effect of higher concentrations of ribonucleosides and deoxynucleosides in αMEM medium. It is known that precursors of DNA, ribonucleosides and deoxynucleosides, are suitable for rapidly dividing cell types (Hartshorne, 1997).

The medium is generally supplemented with a protein source, and this may affect the growth of the preantral follicles (Hulshof et al., 1995). Boland et al. (1993) reported that homologous mouse serum (5%) and sera from hypogonadal mice are suitable for this purpose. Post-menopausal human serum, which contains substantial endogenous gonadotrophins, has also been used for the culture of mouse preantral follicles (Qvist et al., 1990). Fetal calf serum (FCS), hFF and bovine or human serum albumin resulted in the poor growth of mouse preantral follicles in spherical culture systems (Nayudu and Osborn, 1992); however, FCS was successful in supporting the development of follicular morphology and the oocyte maturation of attached cultures (Cortvrindt et al., 1996). In the present study, we found that FBS is the optimal protein source for the culture of the mouse preantral follicles and its optimal concentration is 5%.

In conclusion, we have described a suitable culture system for the *in vitro* growth of mouse preantral follicles. We demonstrated that αMEM and 5% FBS are an optimal medium and a protein source, respectively; further, 10 days of culture is required for the complete growth of oocytes in this culture system. Therefore, the culture system described may be useful not only investigate the critical questions of follicular development, but also give a assistance on the future management of conservation in endangered and rare animals.

REFERENCES

Boland, N. I., P. G. Humpherson, H. J. Leese and R. G. Gosden. 1993. Pattern of lactate production and steroidogenesis during growth and maturation of mouse ovarian follicles *in vitro*. 48:798-806.

Bouniol-Baly, C., L. Hamraoui, J. Guibert, N. Beaujean, M. S. Szollosi and P. Debey. 1999. Differential transcriptional activity associated with chromatin configuration in fully grown mouse germinal vesicle oocytes. Biol. Reprod. 60:580-587.

Carroll, J., D. G. Whittingham and M. J. Wood. 1991. Effect of dibutyryl cyclic adenosin monophosphate on granulosa cell proliferation, oocyte growth and meiotic maturation in isolated mouse primary ovarian follicles cultured in collagen gels. J. Reprod. Fertil. 92:197-207.

Choi, J. K., J. H. Lee, S. T. Lee, M. H. Choi, S. P. Gong, E. J. Lee and J. M. Lim. 2007. Developmental competence of intrafollicular oocytes derived from preantral follicle culture with different protocols after parthenogenetic activation. Asian-Aust. J. Anim. Sci. 20:1190-1195.

Christmann, L., T. Jung and R. M. Moor. 1994. MPF components and meiotic competence in growing pig oocytes. Mol. Reprod. Dev. 38:85-90.

Cortvrindt, R., J. Smitz and A. C. Van Steirteghem. 1996. *In-vitro* maturation, fertilization and embryo development of immature oocytes from early preantral follicles from prepuberal mice in a simplified culture system. Hum. Reprod. 11:2656-2666.

Debey, P., M. S. Szollosi, D. Szollosi, D. Vautier, A. Girousse and D. Besombes. 1993. Competent mouse oocytes isolated from antral follicles exhibit different chromatin organization and follow different maturation dynamics. Mol. Reprod. Dev. 36:59-74.

Eppig, J. J. and S. M. Downs. 1987. The effect of hypoxanthine on mouse oocyte growth and development *in vitro*: maintenance of meiotic arrest and gonadotrophin-induced oocyte maturation. Dev. Biol. 119:313-321.

Eppig, J. J. and A. C. Schroeder. 1989. Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation and fertilization *in vitro*. Biol. Reprod. 41:268-276.

Eppig, J. J., A. C. Schroeder and M. J. O'Brien. 1992. Developmental capacity of mouse oocytes matured *in vitro*: effects of gonadotrophic stimulation, follicular origin and oocyte size. J. Reprod. Fert. 95:119-127.

Gutierrez, C. G., J. H. Ralph, E. E. Telfer, I. Wilmut and R. Webb. 2000. Growth and antrum formation of bovine preantral follicles in long-term culture *in vitro*. Biol. Repord. 62:1322-1328.

Harada, M., T. Miyano, K. Matsumura, S. Osaki, M. Miyake and S. Kato. 1997. Bovine oocytes from early antral follicles grow to meiotic competence *in vitro*: effect of FSH and hypoxanthine. Theriogenol. 46:743-755.

Hartshorne, G. M. 1997. *In vitro* culture of ovarian follicles. Rev. Reprod. 2:94-104.

Hartshorne, G. M., I. L. Sargent and D. H. Barlow. 1994. Meiotic progression of mouse oocytes throughout follicle growth and ovulation *in vitro*. Hum. Reprod. 9:352-359.

Hirao, Y., T. Miyano and S. Kato. 1990. Fertilization of *in vitro* grown mouse oocytes. Theriogenol. 34:1071-1077.

Hulshof, S. C., J. R. Figueiredo, J. F. Beckers, M. M. Bevers, J. A. van der Donk and R. van den Hurk. 1995. Effects of fetal bovine serum, FSH and 17beta-estradiol on the culture of bovine preantral follicles. Theriogenol. 44:217-226.

Johnson, L. D., D. F. Albertini, L. K. McGinnis and J. D. Biggers. 1995. Chromatin organization, meiotic status and meiotic competence acquisition in mouse oocytes from cultured ovarian follicles. J. Reprod. Fertil. 104:277-284.

Kim, D-H., D-S. Ko, H-C. Lee, H-J. Lee, W-I. Park, S. S. Kim, J-K. Park, B-C. Yang, S-B. Park, W-K .Chang and H-T. Lee. 2004. Comparison of maturation, fertilization, development, and gene expression of mouse oocytes grown *in vitro* and *in vivo*. J. Assist. Reprod. Genet. 21:233-240.

Li, R., D. M. Phillips and J. P. Mather. 1995. Activin promotes ovarian follicle development *in vitro*. Endocrinol. 136:849-856.

Liu, X., K. Andoh, H. Yokota, J. Kobayashi, Y. Abe, K. Yamada, H. Mizunuma and Y. Ibuki. 1998. Effects of growth hormone, activin and follistatin on the development of preantral follicle from immature female mice. Endocrinol. 139:2342-2347.

Mattson, B. A. and D. F. Albertini. 1990. Oogenesis: chromatin and microtubule dynamics during meiotic prophase. Mol. Reprod. Dev. 25:374-383.

Nayudu, P. L. and S. M. Osborn. 1992. Factors influencing the rate of preantral and antral growth of mouse ovarian follicles *in vitro*. J. Reprod. Fertil. 95:349-362.

Qvist, R., L. F. Blackwell, H. Bourne and J. B. Brown. 1990. Development of mouse ovarian follicles from primary to preovulatory stages *in vitro*. J. Reprod. Fertil. 89:169-180.

Roy, S. K. and B. J. Treacy. 1993. Isolation and long-term culture of human preantral follicles. Fertil. Steril. 59:783-790.

Spears, N., N. I. Boland, A. A. Murray and R. G. Gosden. 1994. Mouse oocytes derived from *in vitro* grown primary ovarian follicles are fertile. Hum. Reprod. 9:527-532.

Torrance, C., E. Telfer and R. G. Gosden. 1989. Quantitative study of the development of isolated mouse pre-antral follicles in collagen gel culture. J. Reprod. Fertil. 87:367-374.

Wickramasinghe, D., K. M. Ebert and D. F. Albertini. 1991. Meiotic competence acquisition is associated with the appearance of M-phase characteristics in growing mouse oocytes. Dev. Biol. 143:162-172.

Zuccotti, M., P. Giorgi Rossi, A. Martinez, S. Garagna, A. Forabosco and C. A. Redi. 1998. Meiotic and developmental competence of mouse antral oocytes. Biol. Reprod. 58:700-704.

Zuccotti, M., A. Piccinelli, P. Giorgi Rossi, S. Garagna and C. A. Redi. 1995. Chromatin organization during mouse oocyte growth. Mol. Reprod. Dev. 41:479-485.