MOLECULAR REPRODUCTION AND DEVELOPMENT 75:89–96 (2008)

Effects of Gonadotrophins, Growth Hormone, and Activin A on Enzymatically Isolated Follicle Growth, Oocyte Chromatin Organization, and Steroid Secretion

SAFIRIYU IDOWU OLA,[1,2] JUN-SHU AI,[1] JING-HE LIU,[1] QIANG WANG,[1] ZHEN-BO WANG,[1] DA-YUAN CHEN,[1] AND QING-YUAN SUN[1]*

[1]*State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China*
[2]*Department of Animal Science, Obafemi Awolowo University, Ile-Ife, Nigeria*

ABSTRACT So far, standard follicle culture systems can produce blastocyst from less than 40% of the in vitro matured oocytes compared to over 70% in the in vivo counterpart. Because the capacity for embryonic development is strictly associated with the terminal stage of oocyte growth, the nuclear maturity status of the in vitro grown oocyte was the subject of this study. Mouse early preantral follicles (100–130 μm) and early antral follicles (170–200 μm) isolated enzymatically were cultured for 12 and 4 days, respectively, in a collagen-free dish. The serum-based media were supplemented with either 100 mIU/ml FSH (FSH only); 100 mIU/ml FSH + 10 mIU/ml LH (FSH-LH); 100 mIU/ml FSH + 1 mIU/ml GH (FSH-GH) or 100 mIU/ml FSH + 100 ng/ml activin A (FSH-AA). Follicle survival was highest in follicle stimulating hormone (FSH)-AA group in both cultured preantral (91.8%) and antral follicles (82.7%). Survival rates in the other groups ranged between 48% (FSH only, preantral follicle culture) and 78.7% (FSH only, antral follicle culture). Estradiol and progesterone were undetectable in medium lacking gonadotrophins while AA supplementation in synergy with FSH caused increased estradiol secretion and a simultaneously lowered progesterone secretion. Chromatin configuration of oocytes from surviving follicles at the end of culture revealed that there were twice more developmentally incompetent non-surrounded nucleolus (NSN) oocytes (>65%) than the competent surrounded nucleolus (SN) oocytes (<34%). We conclude that the present standard follicle culture system does not produce optimum proportion of developmentally competent oocytes. *Mol. Reprod. Dev. 75: 89–96, 2008.* © 2007 Wiley-Liss, Inc.

Key Words: follicle culture; oocyte chromatin; gonadotrophins; growth hormone; activin A

INTRODUCTION

The capability of mouse oocyte to undergo embryonic development has been linked to its acquisition of the condensed form of chromatin organization (termed surrounded nucleolus, SN) during follicle growth (Zuccotti et al., 1998, 2002; De La Fuente, 2006). But exactly how the reorganization of the chromatin from the previously diffused form (termed non-surrounded nucleolus, NSN) to the SN form is directed awaits clarifications.

Over the last two decades culture systems for in vitro production of eggs are being improved. In standard follicle cultures, it is presumed that the necessity of follicle stimulating hormone (FSH) in the culture medium depends on other conditions particularly the presence of serum. Liu et al. (1998) showed that FSH alone had no effect on the growth of the preantral follicle in serum-free culture except in combination with growth hormone (GH), insulin-like growth factor I (IGF-1) or activin A (AA). In the presence of serum FSH played a complementary role with luteinizing hormone (LH) at optimally balanced concentrations of the three to induce rapid growth of the preantral follicle (Wu et al., 2000). LH also stimulates antral cavity formation and luteinization of the follicle (Cortvrindt et al., 1998a). Thyroid hormone T_3 at supraphysiological levels in culture medium was shown to cause decreased follicle growth and oocyte meiotic competence (Cecconi et al., 2004). Apart from the follicle isolation method and the size or category of follicle chosen for culture, other factors that have also been shown to be of significant importance in the development of follicle culture systems include oxygen supply to the medium, vitamin C, and the presence or absence of oil covering (Nayudu et al.,

Grant sponsor: National Basic Research Program of China; Grant numbers: 2006CB944001, 2006CB504004; Grant sponsor: National Natural Science Foundation of China; Grant numbers: No. 30430530, 30570944; Grant sponsor: Knowledge Innovation Program of the CAS; Grant number: KSCX2-YW-R-52.

*Correspondence to: Qing-Yuan Sun, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, #5 Da Tun Road, Chaoyang, Beijing 100101, China.
E-mail: sunqy@ioz.ac.cn

Received 6 November 2006; Accepted 20 March 2007
Published online 7 May 2007 in Wiley InterScience (www.interscience.wiley.com).
DOI 10.1002/mrd.20762

2001; Smitz and Cortvrindt, 2002; Wycherley et al., 2004).

An important controversial issue in the in vitro culture of follicle is what should be the valid endpoint for assessing the follicle after culture if production of live offspring seems too distant. Moreover, many other factors come into play after ovulation. Many of the available reports used meiosis completion rate as the end point while some went ahead to the blastocyst stage to judge the follicular culture system. In view of the convincing reports (Zuccotti et al., 1998, 2002) that both NSN and SN antral oocytes have the capability to resume meiotic maturation and form MII oocytes but, only the SN oocytes are able to develop up to blastocyst stage after fertilization, we hypothesize that the acquisition of the condensed chromatin structure (SN) at the appropriate size of the oocyte before meiotic maturation could give the shortest and reliable indication of the oocyte developmental potential after culture. Thus in our study we examined the final chromatin structure and size of oocyte grown from enzymatically isolated early preantral and antral follicle stages in system cultures supplemented with FSH in combination with LH or GH or AA. We chose to use the enzymatic method of follicle isolation because of its relative simplicity and also with reference to the report of Demeestere et al. (2002) that showed follicles are able to regenerate theca cells after enzymatic treatment and thus, preserve the steroidogenic and developmental capabilities of the follicle and oocyte, respectively.

MATERIALS AND METHODS

Mice

Animal care and handling were conducted in accordance with policies on the care and use of animals promulgated by the ethical committee of Institute of Zoology, Chinese Academy of Sciences. The mice with color gene type of aabbcc used for the study were from an inbred strain of Kunming white mice, a native breed widely used in biological research in China.

Chemicals

Porcine pituitary somatotropin/GH and recombinant human AA were procured from Sigma Chemical Co. (St. Louis, MO). Porcine pituitary FSH was obtained from Sansheng Co. (Ninbo, China) while the porcine pituitary LH was locally produced in our institute. All other chemicals used in this study were purchased from Sigma Chemical Co. except for those specifically mentioned.

Isolation and Culture of Follicles

Early preantral (100–130 μm in diameter) and early antral (170–200 μm in diameter) follicles were obtained from 11 and 18 day old mice, respectively. The mice were killed by cervical dislocation and their ovaries were aseptically removed into D-MEM (Invitrogen corp, Grand Island, NY) medium supplemented with 5% fetal bovine serum, (FBS, Invitrogen corp), 45 μg/ml penicillin G and 350 μg/ml streptomycin. This mixture is hereafter referred to as basal culture medium (BCM). Under the dissecting microscope the ovarian bursa was carefully removed and the naked ovary was placed in fresh BCM containing 0.5 mg/ml crude collagenase type I (~160 collagen digestion unit per mg) and 0.01 mg/ml (18 U) DNase I, type II. The mixture was incubated at 37°C for 1 hr and triturated by gentle pipetting every 15 min to facilitate follicle dissociation. After digestion the solution was centrifuged and the supernatant was removed. The follicles were resuspended in fresh BCM without enzyme and follicles of the appropriate size and morphology were picked for culture as described below in the experimental design.

Experimental Design

Experiment 1: Culture of early preantral follicle to early antral stage. Early preantral follicles measuring 100–130 μm with 1–2 layers of granulosa cells and basal membrane surrounding a centrally located oocyte from 11-day-old mice were cultured for 8 days in either: (i) BCM (serum only); (ii) BCM supplemented with 100 mIU/ml FSH (FSH only) or (iii) BCM supplemented with 100 mIU/ml FSH and 10 mIU/ml LH (FSH-LH).

Experiment 2: Culture of early preantral follicles to late antral stage. Early preantral follicles measuring 100–130 μm with 1–2 layers of granulosa cells and basal membrane surrounding a centrally located oocyte from 11-day-old mice were cultured for 12 days in either: (i) BCM supplemented with 100 mIU/ml FSH (FSH only); (ii) BCM supplemented with 100 mIU/ml FSH and 10 mIU/ml LH (FSH-LH); (iii) BCM supplemented with 100 mIU/ml FSH and 1 mIU/ml GH (FSH-GH) or (iv) BCM supplemented with 100 mIU/ml FSH and 100 ng/ml AA (FSH-AA).

Experiment 3: Culture of early antral follicles to late antral stage. Early antral follicles measuring 170–200 μm with three or more layers of granulosa cells and basal membrane around a centrally located oocyte from 18-day-old mice were cultured for 4 days. The culture media treatments were the same as experiment 2.

Generally, 20–25 follicles were cultured in each replicate of each experiment. Each experiment was repeated three times. For each replicate the follicles were cultured individually in 20 μl microdrop of medium under mineral oil in a 60 mm culture dishes in 5% CO_2 in air at 37°C.

Measurement of Follicle Survival, Oocyte Growth and Chromatin Organization in Cultured Follicles

The culture system in this study allowed the follicles to attach to the collagen-free culture dish (Demeestere et al., 2002). Initially on the starting day (Day 0) of each experiment the follicles were individually placed in 10 μl of medium. The diameter of each follicle was measured 24 hr later (Day 1) with an ocular micrometer under 200× of a Nikon T300 inverted microscope, 10 μl of fresh medium was then added to each drop and incubation

was continued. On Day 2 half (10 μl) of the culture medium was siphoned and replaced with equal amount of fresh medium. This was repeated in surviving follicles every 48 hr in experiment 1 and until Day 8 in experiment 2, and every 24 hr after Day 8 in experiment 2 as well as throughout experiment 3. Our medium renewal method followed the report of Liu et al. (2002) that showed that daily renewal of culture media, especially during the last phase of maturation, ensured optimal nutrition of oocyte and E_2 secretion in cultured follicles. Follicles were regarded as surviving when the oocyte maintained contact with and remained surrounded by granulosa cells and the complex remained attached to the culture dish (Fig. 1G,H) (Demeestere et al., 2002; Lenie et al., 2004). The conditioned media of surviving follicles was pooled within each experimental treatment for each day of medium renewal and stored frozen for estradiol (E_2) and progesterone (P_4) assay. Analysis was performed in duplicate and 50 μl of media was used for each replicate. The final concentration reported is the average hormonal level in the culture media from surviving follicles irrespective of their number.

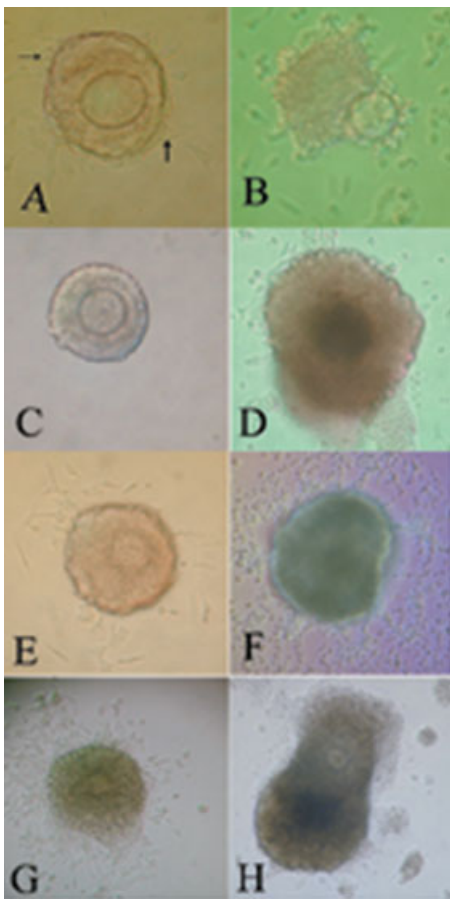

Fig. 1. Different morphological phases of mouse follicles cultured in vitro. **A**: Early preantral follicle on Day 1 in serum-based medium with no hormonal or growth factors supplementation. Even though the follicles initially formed fibroblast-like outgrowth (arrow) and attach to the culture plate, majority of the follicles regressed, that is, dead follicle (**B**) by Day 6 of culture. **C**: On Day 1 of culture of the early antral follicles in medium supplemented with FSH or plus LH or GH, the follicular nature of the follicles is still evident and antral-like cavity is poorly evident at the end of culture (**D**) whereas in the FSH plus activin A supplemented medium follicles rapidly formed fibroblast attachment, granulosa proliferate, and become diffuse (**E**) and antral-like cavities are clearly evident at the end of culture (**F**). Throughout culture period surviving follicles were regarded as those attached to the culture dish with oocytes surrounded by granulosa cells (**G**), but not necessarily centrally located within the follicle (**H**). [See color version online at www.interscience.wiley.com.]

On the last day of culture (Days 8, 12, and 4 in experiments 1, 2, and 3, respectively) surviving follicles were detached from the dish and placed in BCM containing 1.5 mg/ml crude collagenase and 0.1 mg/ml DNase digestion medium and the mixture was vigorously pipetted to release the oocyte. The oocytes were immediately fixed in 4% paraformaldehyde for 30 min and then incubated at room temperature in PBS containing 5 μg/ml Hoechst 33258 for 15 min to stain the DNA. The oocytes were thereafter individually observed under 400× of the Nikon inverted microscope. Their diameters were measured and the chromatin appearance depicting SN (brightly stained chromatin rim around the nucleolus) or NSN (no chromatin rim around nucleolus) was checked under UV illumination.

Hormone Assay

Both P_4 and E_2 levels in the conditioned media were assayed by a competitive enzyme immunoassay using the commercial EIA kits (Cat. No 582601 for P_4 and 582251 for E_2) obtained from Cayman Chemical Company (Ann Arbor, MI). The sensitivity of the assay, defined as the amount of steroid giving a 10% drop in the binding of the enzyme conjugated steroid, was 9 and 12 pg/ml for E_2 and P_4, respectively. Intra-assay and inter-assay coefficients of variation for replicate determination of sample was 3.8% and 5.5% for E_2 and 4.5% and 6.0% for P_4.

Statistical Analysis and Data Representation

Replicate experiments were combined for one-way ANOVA of the treatment effect separately between and within culture days. Where significant difference at $P < 0.05$ was indicated, Duncan multiple range test was used to separate the means. Analyses were performed with the SPSS 13 software. Results were presented as mean ± SEM. The concentrations of E_2 and P_4 in the media were reported in ng/ml with error bar charts.

RESULTS

Follicle Survival and Development In Vitro

Follicles were allowed to settle down and attach to the bottom of the culture dish in the first 24 hr of culture for proper evaluation of their initial morphology. In experiment 1 and 2 involving early preantral follicles (100–130 μm) cultures, there were no differences in the follicle size between the different hormonal supplements after 24 hr of culture. In experiment 3 in which larger early antral follicles (170–200 μm) were cultured, follicles in all the four treatment groups increased in diameter

and particularly, FSH-AA supplement group was significantly larger than the rest at 24 hr of culture (Table 1). Because of the rapid disruption of the basal membrane and the subsequent disproportionate spreading of the growing granulosa cells in almost all the follicles, it became difficult to measure the diameter of the growing follicles. In some cases the rupture of the basal membrane was followed by the extrusion of the oocyte but the granulosa cells soon multiplied to recapture the oocyte (Fig. 1H). Thus we regarded surviving follicles as those in which the oocyte maintained contact with but not necessarily positioned centrally within the granulosa cell layers (Lenie et al., 2004). In experiment 1, 90.5% of follicles in culture with only FSH supplement survived the 8-day culture, a percentage significantly higher than the 61.7% and 4.8% in cultures with FSH plus LH and no hormonal supplementation (serum only), respectively (Table 1).

Having established that the preantral follicles would not survive the serum-based culture lacking in gonadotrophins, we combined FSH with LH, GH or AA in a 12-day culture of preantral follicles in our second experiment. FSH-AA supplementation resulted in a significantly higher percentage of follicles (91.8%) surviving the culture conditions compared to 76.0% in the FSH-GH group which was also significantly higher than the 56.0% or 48.0% in the FSH-LH and FSH only group, respectively. The survival rates when early antral follicles were cultured for 4 days (experiment 3) using the same treatment groups were slightly different. Follicle survival in FSH only and FSH-GH groups were statistically similar but higher than the FSH-LH group.

In the culture of preantral follicles, antral-like cavity were first noticed on Day 6 in about 15% each of follicles in FSH-AA and FSH-GH groups and in 10% of FSH-LH group. Antral cavities were noticed on Day 8 in FSH only group. In the antral follicles cultured for 4 days, antral-like cavities were seen from Day 2 of culture. In all the three experiments the percentage of surviving follicles that showed antral-like cavities was always higher in the FSH-LH than the FSH only group. In the 12-day culture of preantral follicles, FSH-AA and FSH-GH supplementation resulted in follicles with similar rate of antral-like differentiation whereas in the 4-day culture of early antral follicles, FSH-AA supplementation proved superior to other supplementations in terms of antral-like cavity formation.

Steroid Hormone Secretion by Cultured Follicles

Figure 2 A–C depicts the estradiol (E_2) secretion of surviving follicles in experiments 1, 2, and 3, respectively. The corresponding progesterone (P_4) secretions are depicted in Figures 3 A–C. Generally, E_2 levels in the media were below 1.5 ng/ml and P_4 lower than 1 ng/ml throughout the culture periods. E_2 was undetectable in the media of follicles cultured with no hormonal supplementation whereas it was above 1 ng/ml in the media of both FSH only and FSH-LH groups and marginally increased between Days 4 and 8 of culture (Fig. 2A). However, the E_2 levels in these two groups were statistically similar. Figure 2B appeared to give a better view of the difference in E_2 secretion between the FSH only and FSH-LH groups. The later showed consistent significant higher concentration than the former although both groups showed increasing concentration over time in culture. In the same experiment, FSH-GH and FSH-AA cultures showed higher E_2 secretion than FSH only and FSH-LH groups throughout the culture period and significantly so from Day 8 to 12 of culture.

P_4 concentrations during the 8-day preantral follicle culture (Fig. 3A) followed the same pattern as E_2 (Fig. 2A) but were significantly higher in FSH only than FSH-LH on Days 6 and 8 of culture.

P_4 secretion in preantral follicles cultured for 12 days (Fig. 3B) appeared to take the reverse order of E_2 secretion (Fig. 2B). P_4 was consistently and significantly lower in the FSH-AA group and was actually undetectable at the start of culture in this group. There were no significant differences in P_4 secretions between FSH only, FSH-LH and FSH-GH groups until the 10th day of culture when secretions in the FSH only rose above the

TABLE 1. Development of Early Preantral and Antral Mouse Follicles Cultured With Different Hormone Supplementation

Experiment	Supplement in culture	Follicle size (μm)—after 24 hr of culture	% Surviving follicle—last day of culture	% Antral-like formation—last day of culture[d]
Experiment 1: culture of early preantral follicle for 8 days	FSH	120.0 ± 3.7 (63)[e,f]	90.5 ± 5.5^{a} (57)	47.4 ± 3.5^{b} (27)
	FSH-LH	122.1 ± 2.3 (60)	61.7 ± 4.2^{b} (37)	59.5 ± 6.2^{a} (22)
	Serum only	118.8 ± 2.4 (63)	4.8 ± 0^{c} (3)	0^{c}
Experiment 2: culture of early preantral follicles for 12 days	FSH	124.8 ± 1.9 (75)	48.0 ± 4.4^{c} (36)	38.9 ± 3.1^{c} (14)
	FSH-LH	126.0 ± 2.4 (75)	56.0 ± 3.7^{c} (42)	47.6 ± 4.2^{b} (20)
	FSH-GH	130.4 ± 1.6 (75)	76.0 ± 5.3^{b} (57)	71.9 ± 8.1^{a} (41)
	FSH-AA	128.3 ± 1.9 (73)	91.8 ± 6.9^{a} (67)	68.7 ± 7.7^{a} (46)
Experiment 3: culture of early antral follicles for 4 days	FSH	194.2 ± 3.7^{b} (75)	78.7 ± 3.8^{b} (59)	30.5 ± 5.3^{c} (18)
	FSH-LH	193.7 ± 3.2^{b} (75)	66.7 ± 6.6^{c} (50)	54.0 ± 5.0^{b} (27)
	FSH-GH	188.8 ± 3.5^{b} (75)	74.6 ± 6.4^{b} (56)	48.2 ± 3.6^{b} (27)
	FSH-AA	217.6 ± 4.7^{a} (75)	82.7 ± 4.5^{a} (62)	71.0 ± 5.8^{a} (44)

[abc] Values in the same column with different letters within each experiment are significantly different, $P < 0.05$.
[d]Expressed as percentage of surviving follicle.
Values are given as means ± SEM.
Values in bracket represent the total number of follicles.

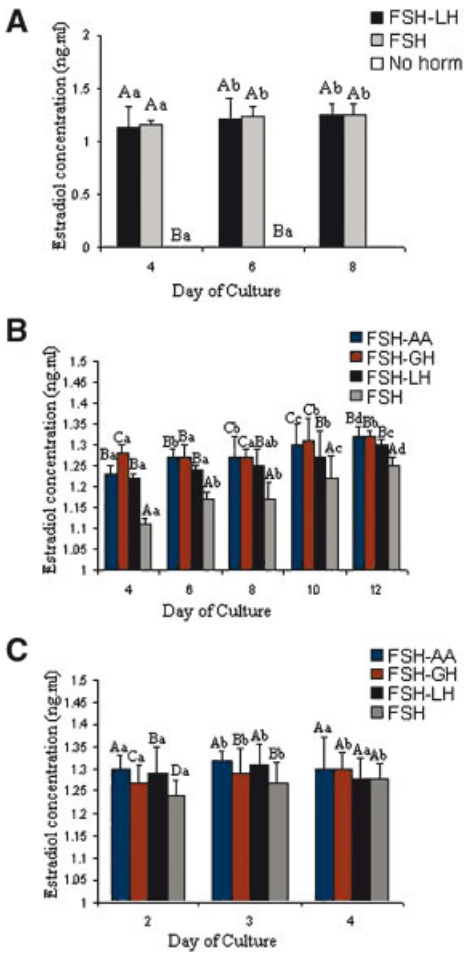

Fig. 2. Estradiol levels in culture media of enzymatically isolated mouse follicles. A–C depicts the estradiol (E_2) secretion of surviving follicles in experiments 1, 2, and 3, respectively. ABCD above error bars indicate significant difference between treatment groups on that day of culture while abcd indicates significant difference between culture days in a particular treatment group. [See color version online at www.interscience.wiley.com.]

other two groups. E_2 and P_4 secretions by the cultured early antral follicles (Figs 2C and 3C, respectively) were a close replica of the secretions in the last 4 days of the cultured early preantral follicles of experiment 2 (Figs 2B and 3B).

Oocyte Growth and Chromatin Configuration After Follicle Culture

Oocyte size at the beginning of culture could not be accurately determined because of the surrounding granulosa cells and it was not possible to determine the chromatin pattern of the oocytes at this time either. We relied on the reports Zuccotti et al. (1995) and Bouniol-Baly et al. (1999) that showed that oocytes from mouse preantral follicles are of 40–50 μm big with about 95% showing NSN configuration while oocytes from antral follicles measure between 50 and 60 μm of which about 85% show the NSN chromatin configuration. In our study oocyte size and percentage of oocyte with the SN chromatin conformation did not follow the pattern

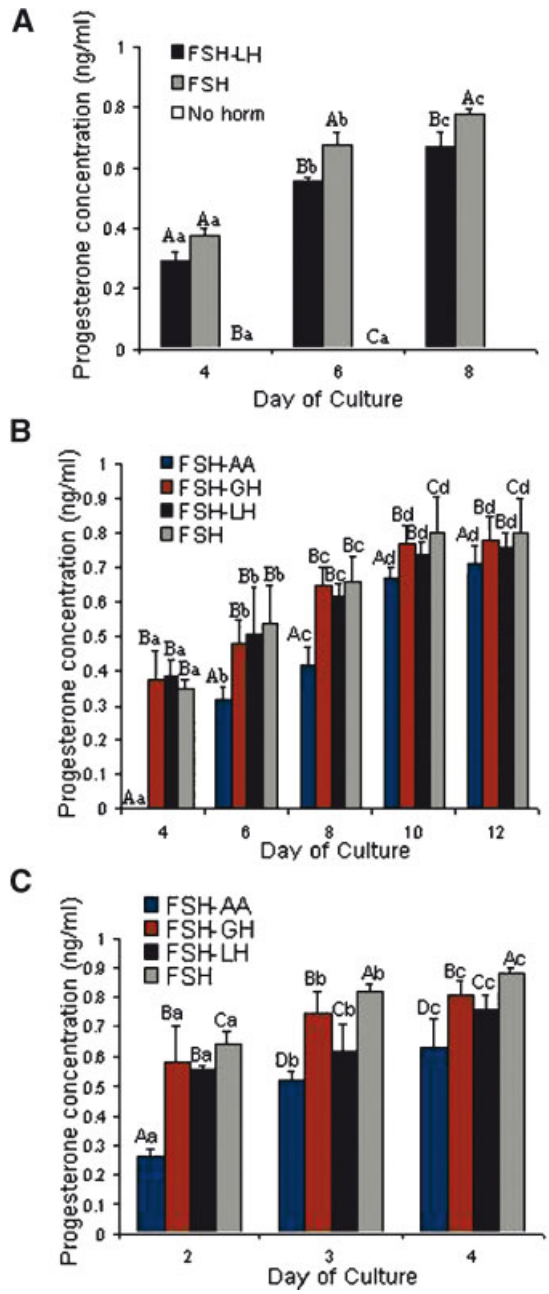

Fig. 3. Progesterone levels in culture media of enzymatically isolated mouse follicles. A–C depicts progesterone (P4) secretions of surviving follicles in experiments 1, 2, and 3, respectively. ABCD above error bars indicate significant difference between treatment groups on that day of culture while abcd indicates significant difference between culture days in a particular treatment group. [See color version online at www.interscience.wiley.com.]

observed for follicle survival or antral-like cavity formation. After culture of preantral follicles for 8 days, oocyte size was significantly larger in gonadotrophins supplemented groups than in the serum only group, and oocyte size was not different between the FSH only and FSH-LH groups (Table 2). However, oocyte size was significantly higher in the FSH-LH group after 12-day culture as well as after 4-day culture of antral follicles.

TABLE 2. Oocyte Growth and Chromatin Status After Culture of Early Preantral and Antral Mouse Follicles With Different Hormone Supplementation

Experiment	Supplement in culture	Oocyte size (μm)–Last day of culture[d]	% SN oocyte
Experiment 1: culture of early preantral follicle for 8 days	FSH	63.3 ± 1.5^{a} (50)[e,f]	10.0 ± 4.5a (5)
	FSH-LH	64.1 ± 2.5^{a} (35)	21.4 ± 5.4b (11)
	Serum only	60.2 ± 2.2^{b} (3)	0c
Experiment 2: culture of early preantral follicles for 12 days	FSH	66.4 ± 1.5^{c} (30)	26.7 ± 3.3 (8)
	FSH-LH	68.0 ± 3.3^{b} (35)	34.3 ± 6.1 (12)
	FSH-GH	68.2 ± 2.4^{b} (50)	32.0 ± 2.8 (16)
	FSH-AA	70.5 ± 3.3^{a} (64)	34.4 ± 3.4 (22)
Experiment 3: culture of early antral follicles for 4 days	FSH	68.3 ± 2.7^{a} (50)	30.0 ± 5.3 (15)
	FSH-LH	69.5 ± 2.3^{b} (48)	33.3 ± 4.6 (16)
	FSH-GH	69.8 ± 4.3^{b} (50)	34.0 ± 6.2 (17)
	FSH-AA	71.6 ± 3.6^{b} (60)	33.3 ± 2.1 (20)

[abc] Values in the same column with different letters within each experiment are significantly different, $P < 0.05$.
[d]Some oocytes retrieved from the follicles were either degenerated, lost or had resumed meiotic maturation.
Values are given as means ± SEM.
Values in bracket represent the total number of oocytes.

Oocytes from FSH-AA group were larger after 12-day culture of preantral follicles but were similar to FSH-GH and FSH-LH groups after 4-day culture of early antral follicles. In the three experiments, the proportion of the NSN oocytes (>65%) was generally double or more that of the SN oocytes at the end of culture. Only in the 8-day culture of preantral follicles (experiment 1) was there significantly higher percentage (21.4%) of SN oocytes in the FSH-LH group than the FSH only (10%) and serum only (0%) groups. In the remaining two experiments there was no significant difference ($P > 0.05$) between the four hormonal supplementation groups and the proportion of SN oocytes ranged between 26.7% and 34.4%.

DISCUSSION

Earlier during the development of follicle culture systems, enzymatic separation of the individual follicle from the ovarian connective tissues was the favored technique employed by many researchers in this field (Roy and Greenwald, 1985; Torrance et al., 1989; Hirao et al., 1994; Eppig et al., 1996). Follicles isolated by enzymatic digestion are now regarded as partial follicle because of the absence of theca cells and the degradation of the basal membrane (Nayudu et al., 2001). The perforation of the basal membrane in these follicles allows the spreading of the granulosa cells out of the follicular wall boundary and the consequent separation of the granulose–oocyte contact and extrusion of oocyte during culture. To correct these deficiencies, oocyte–granulosa cell complexes were cultured embedded within a collagen matrix to preserve the spherical morphology of the follicle (Roy and Greenwald, 1985; Torrance et al., 1989; Hirao et al., 1994). However, recovery of the oocyte in this culture system appears to be more difficult. Alternatively, non-intact follicles could be cultured attached to a collagen or serum coated dish, in which case the spherical structure is lost but oocyte recovery is easier (Eppig et al., 1996). This culture system has been reported with over 90% follicle survival rate after 10 days of culture (Eppig et al., 1998) and currently yields the highest blastocyst rate of all culture systems (Nayudu et al., 2001). More interesting is the recent report by Demeestere et al. (2002), which showed that follicles isolated enzymatically were able to grow in culture dish without collagen membrane support with a 12-day survival rate of 76% compared to 94% in the mechanically isolated follicles under the same culture conditions. We eventually employed this culture system for our study and recorded 12-day survival rates ranging from 48% to ~92% of enzymatically isolated follicles. The higher 12-day survival rate of 92% in our study was obtained when the culture medium was supplemented with FSH and AA whereas Demeestere et al. (2002) recorded 76% with FSH and LH supplementation.

In our study, variations in the survival rates due to different hormonal supplementation largely agreed with many previous reports which had employed mechanically isolated follicles. With no hormonal or growth factor supplementation in the serum-based culture, we observed that preantral follicles failed to grow and survive beyond 6 days in culture and when FSH was the only supplement many follicles remained follicular (i.e., poor growth) and antral-like cavity formation was significantly low. Previous reports have shown serum alone does not support the in vitro growth of preantral follicles (Cortvrindt et al., 1998a; Wu et al., 2000) and in the absence of serum FSH requires the potentiation of hormones like GH and/or growth factors like IGF-I and AA to cause rapid growth of preantral follicles (Liu et al., 1998). FSH plus AA supplementation caused the highest follicle survival in our study of both preantral and early antral follicles culture. This could be adduced to the somatogenic action of AA on the granulosa cells (Li et al., 1995; McGee et al., 2001). It is suggested that the synergistic effect of AA and FSH on

the follicle may be mediated by the effect of AA to increase the number of FSH receptors in the preantral follicle (Xiao et al., 1992) as well as to stimulate the differentiation of the follicle cells (Yokota et al., 1997). But FSH alone does have stimulatory effect on the growth of early antral follicles because this stage is undoubtedly FSH dependent (Greenwald and Roy, 1994). In our study, this position was reflected in the higher survival rate of FSH only group in the culture of antral follicle (78.7%) than that of preantral follicles (48.0%). Contrary to the report of Liu et al. (1998), our result indicates that GH may have synergistic somatogenic effect with FSH in preantral follicles because the rate of antral-like cavity formation after 12-day culture was similar to that obtained in FSH-AA group and survival rate was higher than in the FSH only or FSH-LH group. These effects were however not reproduced in the culture of antral follicles, suggesting that the effect of GH could be stage specific. LH on the other hand has been shown to be required primarily for the initiation of growth in vitro of preantral follicles less than 150 μm (Wu et al., 2000). These authors provided evidence that a careful balance of the concentration of FSH (400 mIU/ml), LH (10 mIU/ml), and serum (7.5%) is a prerequisite for the initiation of growth of small preantral follicles to develop through the antral stage. Intact preantral mouse follicles >150 μm with two to three granulosa cell layers can be routinely cultured to the preovulatory stage in a virtually LH-free mixture containing FSH and 5% serum in 3–5 days (Vitt et al., 1998). This could further explain the low survivability of preantral follicles cultured for 12 days in FSH only group (Table 1) in our study compared to similar follicle size cultured for 8 days and antral follicles cultured for 4 days. However, it appears that the potentiation effect of LH to cause rapid growth in preantral follicle also results in more extruded oocyte in enzymatically isolated follicle leading to reduced follicle survivability (Table 1, experiment 1). FSH-LH supplementation stimulated antral formation over FSH alone in our study in agreement with the findings of Cortvrindt et al. (1998a). However, antral cavities formation in less than 70% of the cultured follicles after 12 days of culture or up to the age of late antral stage can be considered low in comparison to what is obtainable in same sized follicles in vivo. This is an attestation that the present culture system is lacking in an important factor necessary for optimum growth of the follicle in vitro. In whole our results showed that GH and AA augment the stimulatory effect of FSH on follicle growth in serum-based culture of enzymatically isolated follicles.

In vivo, increasing amount of E_2 is produced by the growing follicle from its androgen precursor secreted by the theca cells. Expectedly therefore follicles isolated by enzymatic method which removes the theca cells produced lower concentration of E_2 during in vitro culture (Kobayashi et al., 2000). Without theca cells, estrogen concentrations in rFSH-supplemented cultures were 100 times lower than their theca-containing counterparts (Cortvrindt et al., 1998a). The concentrations of E_2 in our study (below 1.5 ng/ml) agreed with this position in contrary to the report of Demeestere et al. (2002) which used a similar culture system like ours. During a 12-day culture Demeestere et al. (2002) reported a significantly higher E_2 levels, ranging between ~3 and ~18 ng/ml, from the enzymatically isolated follicles, than from the mechanically isolated counterpart which ranged between ~1 and ~12 ng/ml. They justified their report by showing that a positive alkaline phosphatase staining on Day 4 of culture in 90% of the enzymatically isolated follicles indicated the presence of theca cells. They suggested that theca cells may have rapidly differentiated from certain precursors during the first days of culture. Since we didn't perform the alkaline phosphatase test in our study we are unable to corroborate their finding. Moreover the higher estrogen production by the enzymatically isolated follicle did not result in better developmental capability of their oocytes.

Although the pathways of action of AA and GH on the follicle are not clear, both are known to cause increased E_2 secretion (Liu et al., 1998; Smitz et al., 1998; Kobayashi et al., 2000) in cultured follicles. Higher E_2 production from LH supplemented medium can be expected during in vitro culture with theca cells because these cells expresses LH receptors early in their development, whereas granulosa cells express FSH receptors first and acquire LH receptors only at a later differentiation stage (O'Shaughnessy et al., 1997). That E_2 levels were higher in the FSH-LH than the FSH only culture in our study of enzymatically isolated follicle thought to be free of theca cells may thus corroborate the opinion of Demeestere et al. (2002) that theca cells may have differentiated from certain unknown precursors.

During folliculogenesis the amount of P_4 remains low until ovulation when the peripheral levels rise suddenly as an indication of a functional luteinized follicle (Pedersen, 1970; Cortvrindt et al., 1998b). In vivo a rapid surge in P_4 concentration signifies ovulation and the luteinization of the granulosa layers. High P_4 during in vitro culture therefore indicates expulsion of the oocyte and non-survival of the follicles (Cortvrindt et al., 1998b). P_4 concentrations in our study were generally low indicating that the follicles sampled were actually the surviving ones. However, there were significant variations between the different supplementation groups that appeared to be related to the rate of development of the follicles. P_4 levels were lowest in FSH-AA group which showed rapid follicular development and differentiation.

The novel finding of the present report is the fact that none of the hormonal supplementation (LH, GH, AA) in this culture system was able to produce more oocytes with developmental capability, that is, SN chromatin configuration, at the end of a 12-day culture, indicating that the culture system is far from being perfect. Cortvrindt et al. (1998b) showed that 12-day culture appears to be the optimum period to initiate mucification and ovulation in in vitro grown preantral mouse follicle, so we chose to analyze the chromatin

configuration after 12 days of culture of the preantral follicles and 4 days for the bigger early antral follicles. As shown on Table 2, NSN configured oocytes generally made up of two-third or more of the oocytes derived from in vitro grown follicles and there was no difference in the SN proportions among the different hormonal supplementations. This finding showed that most of the in vitro grown oocytes are not developmentally competent (Zuccotti et al., 2002) at the time of harvest and this corroborated many previous reports that obtained low blastocyst rate (usually <40%) from in vitro grown oocyte compared to the in vivo ovulated egg (usually >70%) (Spears et al., 1994; Nayudu et al., 2001; Smitz and Cortvrindt, 2002). The significance of our finding is that the current follicle culture systems are yet to support the complete developmental potential of the oocyte in vitro. It still remained unknown the factors responsible for the transformation of the growing oocyte from NSN to SN status which eventually confers developmental competence on the oocyte.

In conclusion, our study added to the body of evidence that enzymatically isolated mouse follicles can be successfully cultured after attaching to collagen-free dish and that FSH is required in serum-based media for follicle development. FSH stimulatory effect is augmented by the addition of GH or AA which also caused increased secretion of estradiol. In addition, we showed that over 60% of oocytes produced from this standard culture have NSN chromatin configuration. Thus, the NSN to SN chromatin transformation does not occur in most oocytes from cultured follicles and this may be responsible for their low developmental competence.

REFERENCES

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

Cecconi S, Rossi G, Coticchio G, Macchiarelli G, Borini A, Canipari R. 2004. Influence of thyroid hormone on mouse preantral follicle development in vitro. Fertil Steril 81:919–924.

Cortvrindt R, Hu Y, Smitz J. 1998a. Recombinant luteinizing hormone as a survival and differentiation factor increases oocyte maturation in recombinant follicle stimulating hormone-supplemented mouse preantral follicle culture. Human Reprod 13:1292–1302.

Cortvrindt RG, Hu Y, Liu J, Smitz JEJ. 1998b. Timed analysis of the nuclear maturation of oocytes in early preantral mouse follicle culture supplemented with recombinant gonadotropin. Fertil Steril 70:1114–1125.

De La Fuente R. 2006. Chromatin modifications in the germinal vesicle of mammalian oocytes. Dev Biol 292:1–12.

Demeestere I, Delbaere A, Gervy C, Van den Bergh M, Devreker F, Englert Y. 2002. Effect of preantral follicle isolation technique on in vitro follicular growth, oocyte maturation and embryo development in mice. Human Reprod 17:2152–2159.

Eppig JJ, O'Brien MJ, Pendola FL, Watanabe S. 1998. Factors affecting the developmental competence of mouse oocytes grown in vitro: Follicle stimulating hormone and insulin. Biol Reprod 59:1445–1450.

Eppig JJ, O'Brien MJ, Wigglesworth K. 1996. Mammalian oocyte growth and development in vitro. Mol Reprod Dev 44:260–273.

Greenwald GS, Roy SK. 1994. Follicular development and its control. In: Knobil E, Neil JD, editors. The Physiology of Reproduction. New York: Raven Press. pp 629–724.

Hirao Y, Nagai T, Kubo M, Miyano T, Miyake M, Kato S. 1994. In vitro growth and maturation of pig oocytes. J Reprod Fertil 100:333–339.

Kobayashi J, Mizunuma H, Kikuchi N, Liu X, Andoh K, Abe Y, Yokota H, Yamada K, Ibuki Y, Hagiwara H. 2000. Morphological assessment of the effect of growth hormone on preantral follicles from 11 day-old mice in an in vitro culture system. Biochem Biophy Res Comm 268:36–41.

Lenie S, Cortvrindt R, Adriaenssens T, Smitz J. 2004. A reproducible two-step culture system for isolated primary mouse ovarian follicles as single functional units. Biol Reprod 71:1730–1738.

Li B, Phillips DM, Mather JP. 1995. Activin promotes ovarian follicle development in vitro. Endocrinology 136:849–856.

Liu HC, He Z, Rosenwaks Z. 2002. In vitro culture and in vitro maturation of mouse preantral follicles with recombinant gonadotrophins. Fertil Steril 77:373–383.

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

McGee EA, Smith R, Spears N, Nachtigal MW, Ingraham H, Hsueh AJW. 2001. Müllerian inhibitory substance induces growth of rat preantral ovarian follicles. Biol Reprod 64:293–298.

Nayudu PL, Fehrenbach A, Kiesel P, Vitt UA, Pancharatna K, Osborn S. 2001. Progress toward understanding follicle development in vitro: Appearances are not deceiving. Arch Med Res 32:587–594.

O'Shaughnessy PJ, McLelland D, McBride MW. 1997. Regulation of luteinizing hormone-receptor and follicle-stimulating hormone-receptor messenger ribonucleic acid levels during development in the neonatal mouse ovary. Biol Reprod 57:602–608.

Pedersen T. 1970. Follicle kinetics in the ovary of the cyclic mouse. Acta Endocrinol 64:304–323.

Roy SK, Greenwald GS. 1985. An enzymatic method for the dissociation of intact follicles from the hamster ovary: Histological and quantitative aspect. Biol Reprod 32:203–215.

Smitz J, Cortvrindt R, Hu Y, Vanderstichele H. 1998. Effects of recombinant activin A on in vitro culture of mouse preantral follicles. Mol Reprod Dev 50:294–304.

Smitz JEJ, Cortvrindt RG. 2002. The earliest stages of follicullogenesis in vitro. Reproduction 123:185–202.

Spears N, Boland NI, Murray AA, Gosden RG. 1994. Mouse oocytes derived from in vitro grown primary ovarian follicles are fertile. Human Reprod 9:527–532.

Torrance C, Telfer E, Gosden RG. 1989. Quantitative study of the development of isolated mouse preantral follicles in collagen gel culture. J Reprod Fertil 87:367–374.

Vitt VA, Kloosterboer HJ, Rose UM, Mulders JWM, Kiesel PS, Bete S, Nayudu PL. 1998. Isoforms of human recombinant follicle-stimulating hormone: comparison of effects on murine follicle development in vitro. Biol Reprod 59:854–861.

Wu J, Nayudu PL, Kiesel PS, Michelmann HW. 2000. Luteinizing hormone has a stage-limited effect on preantral follicle development in vitro. Biol Reprod 63:320–327.

Wycherley G, Downey D, Kane MT, Hynes AC. 2004. A novel follicle culture system markedly increases follicle volume, cell number and oestradiol secretion. Reproduction 127:669–677.

Xiao S, Robertson DM, Findlay JK. 1992. Effects of activin and follicle-stimulating hormone (FSH)-suppressing protein/follistatin on FSH receptors and differentiation of cultured rat granulosa cells. Endocrinology 131:1009–1016.

Yokota H, Yamada K, Liu X, Kobayashi J, Yumiko A, Mizunuma H, Ibuki Y. 1997. Paradoxical action of activin A on folliculogenesis in immature and adult mice. Endocrinology 138:4572–4576.

Zuccotti M, Piccinelli A, Rossi PG, Garagna S, Redi CA. 1995. Chromatin organization during mouse oocyte growth. Mol Reprod Dev 41:479–485.

Zuccotti M, Ponce RH, Boiani M, Guizzardi S, Govoni P, Scandroglio R, Garagna S, Redi CA. 2002. The analysis of chromatin organisation allows selection of mouse antral oocytes competent for development to blastocysts. Zygote 10:73–78.

Zuccotti M, Rossi PG, Martinez A, Garagna S, Forabosco A, Redi CA. 1998. Meiotic and developmental competence of mouse antral oocytes. Biol Reprod 58:700–704.