Human Reproduction vol 11 no 12 pp.2656–2666, 1996

In-vitro maturation, fertilization and embryo development of immature oocytes from early preantral follicles from prepuberal mice in a simplified culture system

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A simplified culture system was developed for the in-vitro maturation of early preantral mouse ovarian follicles. The follicles were cultured singly in 20 μl droplets under oil in medium supplemented with recombinant follicle stimulating hormone (r-FSH) at 37°C and 5% CO_2 in air. The follicles grew and became attached to the bottom of the dish, progressively lost their spherical structure by outgrowth of the granulosa cells through the basal membrane and developed follicles with antral-like cavities. The normal three-dimensional follicular structure was lost but all components, i.e. theca, granulosa and oocyte, remained functional, as was proven by the oestradiol, inhibin and progesterone secretion patterns. Follicle survival exceeded 80% and histological analysis proved the absence of atresia and cell death in granulosa cells up to day 16. Oocytes of 55 (± 4) μm diameter on the day of isolation reached 74 (± 3) μm by day 16 of culture. The optimal moment for inducing the final meiotic maturation with human chorionic gonadotrophin was investigated: the highest absolute numbers of metaphase II oocytes were obtained on days 12 and 14 (39 and 41%). The fertilizing potential of the in-vitro matured oocytes was comparable to in-vivo matured controls. A 50% hatched-blastocyst development rate was observed.

Key words: follicle/in-vitro/maturation/mouse/oocyte

Introduction

Early stages of follicles present in the ovary are a potential source for supplies of oocytes since freezing of ovarian tissue and of early developmental stages of follicles is possible (Carroll and Gosden, 1993; Candy *et al.*, 1995; Zhang *et al.*, 1995). However, the ability to bring primary or early preantral follicular stages with immature oocytes to maturity *in vitro* is a prerequisite.

To accomplish this, isolation of ovarian follicles and insights into the requirements, metabolism and differentiation processes of in-vitro maturation of ovarian follicles have already been studied by many researchers, using rodents (Eppig and Schroeder, 1989; Torrance *et al.*, 1989; Qvist *et al.*, 1990; Gore-Langton and Daniel, 1990; Carroll *et al.*, 1991; Nayudu and Osborn, 1992; Boland *et al.*, 1993; Boland and Gosden, 1994; Hartshorne *et al.*, 1994a,b; Spears *et al.*, 1994; Almahbobi *et al.*, 1995; Johnson *et al.*, 1995) and also using other mammals, e.g. cows (Figueiredo *et al.*, 1993), pigs (Hirao *et al.*, 1994) and even humans (Roy and Treacy, 1993).

Basically, two types of system have been developed to grow immature oocytes from mouse ovaries: cultures of enzymatically isolated oocyte–granulosa cell complexes, or of mechanically dissected intact preantral follicles. The first system was developed by Eppig's group (Eppig and Schroeder, 1989): ovaries of prepuberal mice were enzymatically digested and granulosa–oocyte complexes were cultured in large groups on collagen membranes for 10 days before in-vitro maturation. The cultured material probably involved a mixture of early stages of preantral follicles. Eppig and Schroeder (1989) proved the capacity of this system by obtaining live young, although at a low rate. Torrance *et al.* (1989) and Carroll *et al.* (1991) cultured a similar population of complexes but these were embedded in collagen gels. The second system, described by many others (Nayudu and Osborn, 1992; Boland *et al.*, 1993; Boland and Gosden, 1994; Hartshorne *et al.*, 1994a,b; Spears *et al.*, 1994; Almahbobi *et al.*, 1995; Johnson *et al.*, 1995), studied the growth and development of mechanically isolated intact follicular structures at somewhat later stages (late preantral) from older mice. Most authors used a short period of culture (4–6 days), cultured either singly or in groups on membranes (Nayudu and Osborn, 1992), or singly and transposed the follicles in order to preserve follicular integrity (Boland and Gosden, 1994; Hartshorne *et al.*, 1994a,b; Spears *et al.*, 1994; Almahbobi *et al.*, 1995; Johnson *et al.*, 1995). The culture media used were simple media supplemented with a large variety of additives, such as serum or protein supplements, growth factors, more or less purified hormone preparations and inhibitors of meiotic progression such as hypoxanthine, dibutyryl-cyclic AMP (dbcAMP) or 3-isobutyl-1-methylxanthine (IBMX) (Eppig and Schroeder, 1989; Carroll *et al.*, 1991; Hartshorne *et al.*, 1994a,b).

The primary aim of our work being the assessment of cryopreserved immature oocytes from early follicle stages for potential oocyte supplies, we wished to develop an in-vitro culture system that allowed easy manipulation with a view to morphological and metabolic study of individual follicles. The culture design had to permit daily microscopical evaluation under an inverted microscope and provide easy access to the conditioned medium surrounding single follicles during their entire growth phase up to final maturation. This paper describes the characteristics of a 12 day in-vitro culture system for early preantral mouse follicles which permits the production of meiotically competent and fertilizable oocytes.

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Materials and methods

Female F1 hybrid (C57bl/8j×CBA/cα) mice housed and bred according to national standards were used throughout the study Permission was obtained from the Ethical Committee of the University Hospital Medical School, Brussels, to perform this research project on mice

Ovaries from mice aged 7, 14 and 21 days were examined using standard histological techniques. It was determined (see below) that the optimal age for isolation of early preantral follicles was 14 days.

Mechanical isolation and selection of early preantral mouse follicles for in-vitro culture

Mice aged 14 days old were killed by cervical dislocation and the ovaries were aseptically removed and collected in L15 Leibovitz–glutamax with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 mg/ml streptomycin (L15* medium) (all obtained from Life Technologies, Merelbeke, Belgium) Ovaries were freed from all connective tissues and each ovary was placed in 500 µl of L15* Follicles were released from the ovary using $25\frac{1}{2}$-gauge needles (Becton Dickinson, Erembodegem, Belgium) for blunt dissection Follicles to be cultured were selected in three washing steps by the following criteria: (i) intact round follicular structure with one or two layers of granulosa cells and at least some adhering thecal cells; (ii) the oocyte had to be visible, round and centrally located within the follicle All selected follicles were pooled and randomly divided over the culture conditions under study.

In-vitro maturation of early preantral mouse follicles

The culture medium consisted of α-minimal essential medium (MEM) enriched with 5% heat-inactivated FCS (Life Technologies), 10 µg/ml transferrin, 5 µg/ml insulin (Boehringer Mannheim, Mannheim, Germany) and 100 mIU/ml of recombinant follicle stimulating hormone (r-FSH) (kindly donated by Ares Serono, Geneva, Switzerland and Organon, Oss, The Netherlands) Both r-FSH preparations were completely luteinizing hormone (LH)-free The dose of 100 mIU/ml of r-FSH was chosen as higher concentrations did not increase growth further (Nayudu and Osborn, 1992). Culture dishes (60 mm Petri dishes, Falcon®, Becton Dickinson) contained 20×10 µl culture droplets and 2×10 µl washing droplets, covered with 5 ml of washed mineral oil (Sigma, Bornem, Belgium) Selected early preantral mouse follicles (n = 20) were washed in the washing droplets and placed one by one in the culture droplets. On day 2 of culture, 10 µl of medium was added to each droplet and thereafter half of the medium was refreshed every other day. Follicles were cultured in an incubator at 37°C, 100% humidity and 5% CO_2 in air

Exact characterization of the cultured follicles was done on day 1 by means of an inverted microscope with a Hoffman contrast-modulation system.

The diameter of the follicle within the basal membrane, the oocyte–granulosa cell apposition and the presence of theca cells were scored. Follicle survival in culture was considered positive as long as an oocyte remained surrounded by granulosa cells attached to the culture dish

Indication of meiotic maturation of in-vitro grown oocytes from early preantral mouse follicles

Ovulation induction was carried out by adding 1.5 IU/ml recombinant human chorionic gonadotrophin (r-HCG; kindly donated by Ares Serono) with 5 ng/ml human epidermal growth factor (EGF; Boehringer Mannheim) to the in-vitro matured early preantral follicle cultures. EGF was added to improve oocyte maturation (Dekel and Sherizly, 1985, Downs, 1989; Das *et al.*, 1992) In preliminary controlled experiments using 80 follicles, we found an increased rate of metaphase II-stage (MII) oocytes when EGF was added to HCG (unpublished personal observations). Mucification of the cumulus–oocyte complexes (COC) was observed 14–16 h later under the inverted microscope The nuclear maturation of the oocytes was assessed after denudation. Oocytes were scored as GV when the germinal vesicle (GV) was recognizable, as GVBD when the GV was absent and as MII when the first polar body was extruded

In-vitro fertilization of the in-vitro grown and matured oocytes

The in-vitro released mucified COC were put one by one into 20 µl fertilization droplets of Whittingham's medium [containing NaCl (5.803 g/l), $NaHCO_3$ (2.106 g/l), α-D-glucose (1.0 g/l), KCl (0.201 g/l), Na_2HPO_4 (0.02 g/l), sodium pyruvate (0 055 g/l), penicillin-G (0.063 g/l), streptomycin sulphate (0 05 g/l), $CaCl_2{\cdot}2H_2O$ (0.264 g/l), $MgCl_2$ $6H_2O$ (0.102 g/l), sodium lactate syrup (3.5 ml of 60% syrup), phenol red (0 01 g/l)] with 3% (w/v) bovine serum albumin (BSA) fraction V (Sigma), and 10^6/ml spermatozoa. After 4 h, oocytes were washed and cultured singly in 10 ml droplets of Earle's medium with 0 5% crystalline BSA under oil for 5 days until the blastocyst stage.

Histological evaluation of the cultured follicles

Follicles were cultured on 13 mm Thermanox glasses (ICN Biomedicals, Asse, Belgium) in 4-well culture dishes, two culture droplets of 20 µl/glass with one follicle each, covered with 0.5 ml of washed mineral oil On the relevant day, the follicles were washed in phosphate-buffered saline (PBS) and fixed in 2.5% glutaraldehyde in 0 1 M cacodylate buffer, pH 7.3 After postfixation with 1% osmium tetroxide in H_2O, block staining with uranyl acetate and dehydration, follicles were embedded in Epon. After polymerization, the Thermanox glass was pulled from the block after local gentle heating of the glass, leaving the follicle in the Epon block without altering its orientation or disrupting the three-dimensional structure developed *in vitro*. Serial semi-thin sections of 1 µm were cut throughout the follicle and stained with Toluidine blue.

Assessment of hormone production in vitro

In the 10 µl medium droplets, which were sampled and subsequently replaced by fresh medium, the following secretory products were measured: oestradiol, inhibin and, at the end of the culture period, progesterone. By measuring oestradiol and progesterone, we wished to obtain information on the steroidogenic pathways functioning throughout the culture period and on the differentiation of the granulosa cells in culture. We were interested in evaluating the expression of LH receptors indirectly by measuring the progesterone output after the HCG stimulus Inhibin, being a specific parameter for granulosa cell growth, was also included. Every other day, all 10 µl samples from surviving follicles were pooled together. All samples were diluted appropriately to correct for matrix effects and to measure concentrations within the most precise part of the calibration curve.

Steroids were measured using commercially available radioimmunoassays. For oestradiol, the radioimmunoassay from Clinical Assays (Sorin, Belgium), with a sensitivity of 4 ng/l, and a total precision of <10% (% coefficient of variation, CV) was used. Samples were measured after an appropriate dilution in steroid-free serum. The dilutions were perfectly linear, and medium from days 4 and 6 was measured after diluting 1/6; medium from later days was measured after diluting 1/16

Progesterone was measured with a radioimmunoassay from bio-Mérieux (Marcy-l'Etoile, France) The sensitivity of this assay is 0.04 µg/l and its total precision <8% (CV).

Inhibin was measured after dilution in inhibin-free serum with a commercial immunoenzymatic assay using antibodies directed against

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the α-subunit (Medgenix, Fleurus, Belgium) Culture droplets from days 4 and 6 were diluted 1/121, those from days 8–16 1/451. Linearity on dilution was ascertained. The calibration was expressed in Medgenix Units (MU); 1 MU is ~400 pg of recombinant α,β-human inhibin A from Genentech (CA, USA). The sensitivity of this assay reached 0.1 MU/ml and its precision was <12% (CV).

Statistics

Significances as regards response data were calculated using contingency table analysis and χ^2 for trend analysis. For comparison of small groups, Fisher's exact test was used.

Results

Early preantral follicles to mature *in vitro* were isolated from 14 day old mice. At this age, only a few follicles had reached the antral stage and no corpora lutea had yet been formed. The population of early preantral follicles (one and two layers of cuboidal granulosa cells) predominated over the other follicular classes.

Isolation, selection and characterization of the early preantral follicles for in-vitro culture

Isolation by mechanical dissection of a 14 day old mouse ovary yielded an average of 30–40 early preantral follicles suitable for culture. The oocyte diameter of the selected follicles was determined after removal of the granulosa cells. The mean diameter (±SD) of the denuded oocytes without zona pellucida was 55 (±4) μm.

On day 1 of culture, follicles were characterized under the inverted microscope equipped with a Hoffman contrast-modulation system. The diameter of the cultured follicles ranged from 85 to 145 μm. Thecal cells were recognized as flattened cells covering the outer part of the basal membrane in 82% of the follicles. The oocyte–granulosa cell apposition was judged intact in 85% of the follicles; in the remaining 15%, a localized gap was discerned between granulosa cells and the oocyte. Very rarely, a collapsed oocyte was found within a follicle; these follicles were excluded from the study.

Morphological observations on growth, differentiation and survival of cultured early preantral follicles

Evaluation of follicles in culture

On day 2 of culture, spindle-shaped cells originating from the surface of the follicle attached themselves to the dish and proliferated, with the formation of a monolayer surrounding and attaching the follicle to the dish (Figure 1 and Table I). By day 4, all follicles, including those without clearly visible thecal cells on day 1 of culture, had become attached to the dish. Granulosa cells proliferated and broke through the basal membrane, spreading over the basal membrane and the monolayer already formed The initial follicular structure was lost and the follicles developed a 'diffuse appearance'. On day 4 of culture, 53% of the follicles had already lost their follicular structure. All follicles reached a 'diffuse' pattern by day 10. Antrum-like cavity formation (indicated by the appearance of lucid patches within the granulosa cell mass) was recognized from day 8 onwards. By day 16, clearly visible and well-developed antral-like cavities were recognized in 84% of the surviving follicles.

Table I. Growth characteristics of early preantral follicles observed every 2 days under the stereomicroscope

	No of days of culture						
	4	6	8	10	12	14	16
Survival rate[a] (%)	99	87	84	84	83	83	82
Morphology (% of surviving follicles)							
Follicular structure[b]	47	14	6	1	1	–	–
Granulosa cell outgrowth[c]	53	86	93	93	82	43	16
Antrum formation[d]	–	–	1	6	17	57	84

[a]Survival was defined as those follicles which retained their oocyte within the granulosa cell mass and was expressed as a percentage of follicles put into culture

[b]Follicles containing granulosa cells and oocyte enclosed by the basal membrane.

[c]Loss of the follicular structure by the outgrowth of granulosa cells through the basal membrane

[d]Follicles having a translucent zone within the granulosa cell mass

During the long-term culture period of 16 days, 80% of the follicles retained their oocytes within the follicle. Follicles showing a spontaneous release of the oocyte were discarded from further evaluation. In this respect, days 6 and 8 of culture were critical for premature oocyte release and follicle degeneration

Histological evaluation of the growth and differentiation of the cultured early preantral follicles

Histological evaluation of the cultured follicles was done on serial semi-thin 1 μm plastic sections after Toluidine blue staining.

On day 4 of in-vitro culture, the follicular structure was composed of an eccentrically located oocyte, surrounded by 3–4 layers of granulosa cells, enclosed by the basal membrane and covered on the outside by a few layers of elongated fibroblast-like thecal cells. The basal membrane was occasionally interrupted locally at one or more locations, permitting granulosa cell outgrowth through the breaks. Follicles cultured for 6–8 days showed granulosa cells in and outside the basal membrane. No signs of cell death were detectable at this time. Each oocyte was enclosed by a rim of tightly packed granulosa cells. Sections through follicles cultured for 10, 12 and 14 days revealed similar histological and morphological features (Figure 2). In the first sections, at the attachment pole of the follicle to the glass support, a mass of highly vacuolized (granulosa) cells was recognized. Within the granulosa cell mass a curled membrane-like structure was frequently encountered, probably representing the remnant of the basal membrane. In some sections deeper in the follicle, the antrum appeared as a cavity in the granulosa cell mass. The mural border of the antrum was lined by 12–15 granulosa cell layers. The cytoplasm of these granulosa cells was highly vacuolized (i.e. secretory vesicles, possibly indicative of steroid production). Necrotic cells were encountered on the inner border of the granulosa cell mass and within the antrum itself. The oocyte, surrounded by some shells of tightly packed granulosa cells,

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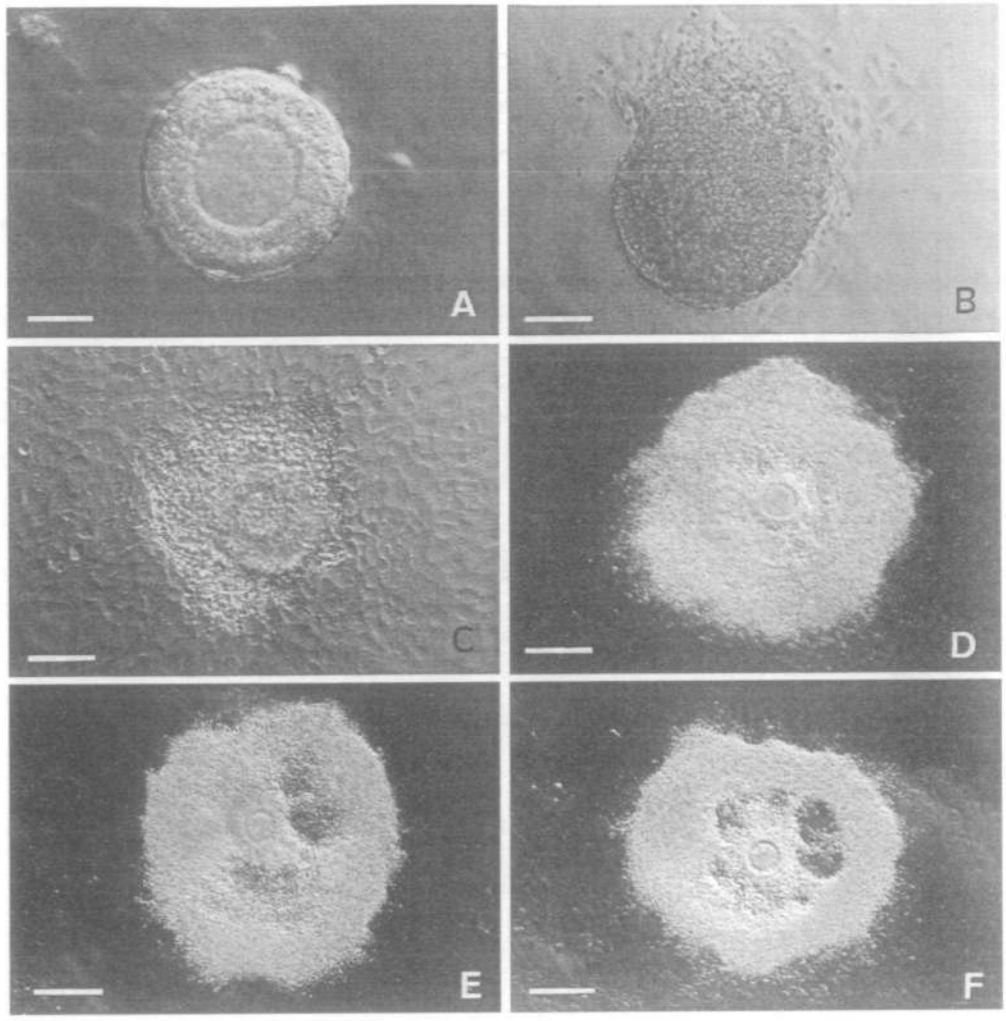

Figure 1. In-vitro growth and differentiation of early preantral mouse follicles from 14 day old mice (**A**) Early preantral follicle cultured for 1 day· central germinal vesicle (GV)-stage oocyte with a thin zona pellucida, surrounded by two layers of granulosa cells, a basal membrane and a single layer of thecal cells. Some cells have started to colonize the culture dish, attaching the follicle to the plate (scale bar = 40 μm). (**B**) Early preantral follicle after 4 days of culture: formation of a monolayer around the follicle and outgrowth of granulosa cells through the basal membrane, spreading over the monolayer (scale bar = 40 μm). (**C**) Early preantral follicle cultured for 6 days well-developed monolayer around the follicular structure, granulosa cell outgrowth around the follicle and over the monolayer The oocyte is centrally located (scale bar = 80 μm) (**D**) Early preantral follicle cultured for 10 days onset of antrum formation around oocyte covered by tightly packed corona cells. The mural granulosa cells are more loosely interconnected (scale bar = 240 μm) (**E** and **F**) Follicles cultured for 12 and 14 days: development of antral cavities (scale bar = 240 μm)

was most often located on top of the antral cavity Microvilli from the granulosa cells adjacent to the oocyte traversed the zona pellucida and revealed intricate contacts with the oocyte. The cytoplasm of the oocyte was homogeneous and the perivitelline space between the zona pellucida and the oocyte was minimal.

Variability of the in-vitro culture system

The survival rate of follicles (with an initial mean diameter of 100–130 mm) within the culture system was considered over a period of 1 year. For this, 32 culture plates (Petri dishes containing 20 follicles) with similar culture conditions were selected at random and the survival rate was compared on day 12 of culture The survival rate varied between 75 and 100%. In only five out of 32 cultures was the survival rate <70% (53–69%), but here too at least half of the follicles had grown from the early preantral to a functional ovulatory stage

Culture of follicles <100 and >130 μm at day 1 of culture

Follicles (n = 68) <100 μm diameter on day 1 of culture were analysed separately. One-third of these follicles did not start in-vitro growth and degenerated. Of those follicles showing progressive enlargement of the granulosa cell mass, only 37% reached day 12 of culture and 8% had started the development of an antral cavity by this day. Survival of this group of follicles was thus impaired relative to the selected preantral (100–130 μm) group (P < 0 001). However, 85% of the growing follicles (those surviving the 12 day culture period) responded to the HCG stimulus on day 12 with mucification of the COC. Premature oocyte release during culture was observed in 45% of the growing small follicles

All the follicles (n = 56) >130 μm grew *in vitro* and reached the final maturational status earlier than the class of follicles described above These follicles often

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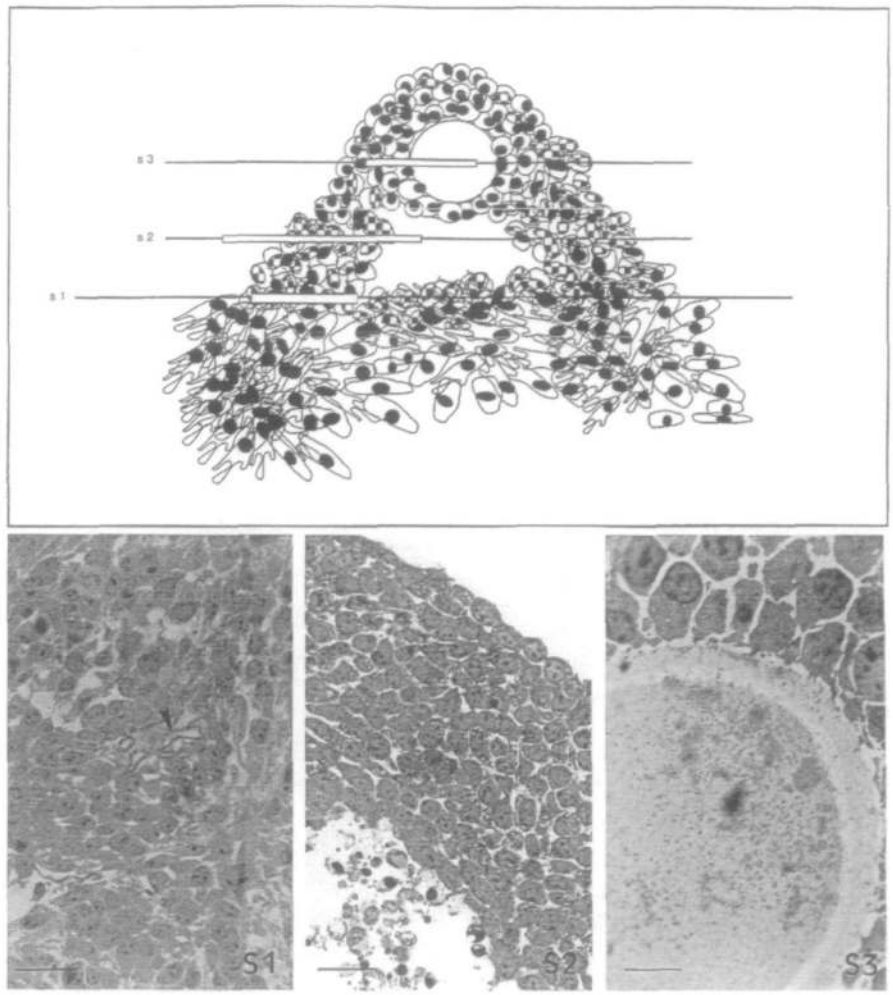

Figure 2. Histological sections through a cultured follicle with an antral-like cavity. (**S1**) A curled fibrous-like structure (arrowhead), the remnant of the basal membrane, is found back within the follicle wall close to the bottom of the plate (scale bar = 3 μm). (**S2**) The follicular wall: the mural granulosa cell layer consists of 12–15 layers of well-individualized cells. These cells are filled with secretory vesicles. Within the wall no pyknotic cells are visible Some detached dead cells are seen within the antral-like cavity (scale bar = 4 μm). (**S3**) Section through the oocyte–granulosa junction. The oocyte's cytoplasm is homogeneous. The well-differentiated corona cell layer sends microvilli through the zona pellucida, making close contact with the oocyte (scale bar = 10 μm).

demonstrated extensive antrum formation by day 12 of culture, but did not often respond to an HCG stimulus, and more often harboured a degenerated oocyte within their follicular structure by day 12 of culture.

Selection of a restricted early preantral class for culture

The determination of the optimal culture conditions for the in-vitro maturation of early preantral follicles was based on the observation of a precise class of early preantral follicles measuring between 100 and 130 μm. We chose to study a rather narrow class of follicles as preliminary experiments had taught us that there existed an optimal culture duration which was strictly dependent on the stage of follicle development at isolation. Further data in this paper concern this selected class of follicles.

Oocyte growth and maturation during culture

Oocyte growth assessment was carried out by mechanical removal of the oocytes from their follicular environment on days 4, 6, 8, 10, 12, 14 and 16. Diameter (mean ± SD) and nuclear status were recorded immediately and after 24 h. During the first 4 days of culture the oocytes had grown up to 63 (±5) μm, by days 6 and 10 the oocytes had reached a mean diameter of 66 (±2) and 65 (±3) μm respectively. On day 12 a diameter of 71 (±3) μm was recorded. On day 14 the oocytes had grown up to 72 μm (±3) and finally reached 74 (±2) μm on day 16 of culture Oocytes ovulated from 26 day old mice (after ovarian stimulation and HCG injection) were measured in a supplementary experiment (data not shown): their diameter was 72 (±3) μm. Immediately after the removal of the granulosa cells, >97% of the oocytes were found arrested in the GV stage (Table II). Owing to the interruption of the connections between granulosa cells and oocyte, meiosis was reinitiated. Depending on the day of culture (days 8–16) on which the mechanical denudation procedure took place, the observations of 24 h later revealed that 6–28% of the oocytes remained blocked in the GV stage, 67–94% had undergone GVBD, but only a minority (2–23%) had extruded the first polar body spontaneously and were MII. Only very few follicle-enclosed oocytes had degenerated

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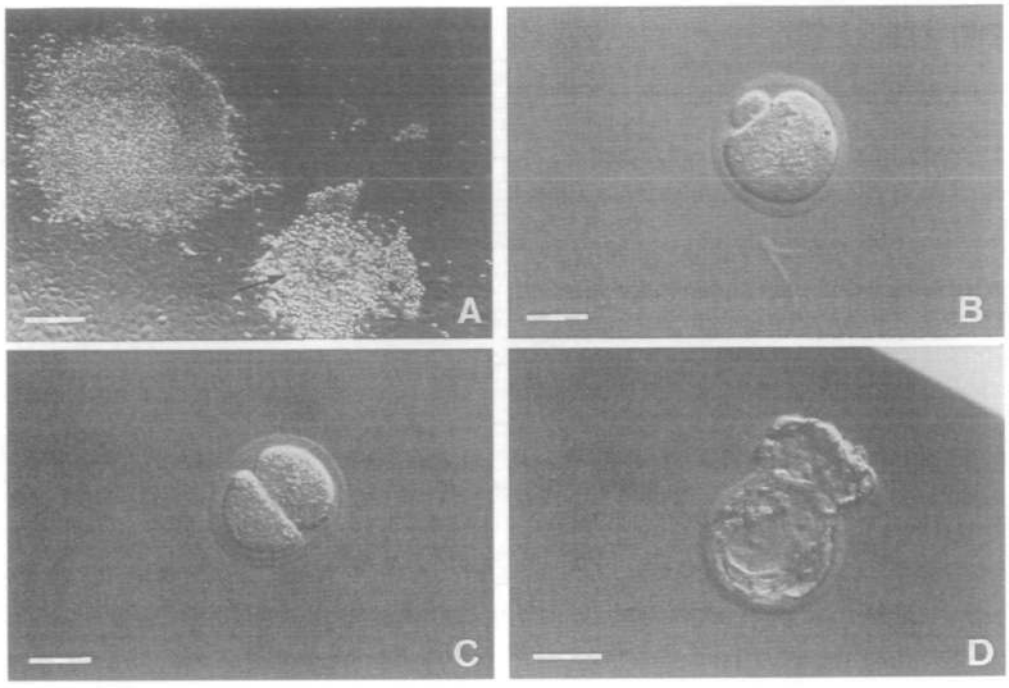

Figure 3. In-vitro ovulation and oocyte fertilization of a 12 day old cultured early preantral follicle. **(A)** In-vitro ovulated 12 day old cultured early preantral follicle· mucified cumulus–oocyte complex floating free in the culture droplet (arrow) (scale bar = 240 μm), **(B)** fertilized oocyte; **(C)** 2-cell embryo; **(D)** hatched blastocyst (scale bar = 40 μm)

Table II. Progression of nuclear maturation during culture in response to mechanical denudation and human chorionic gonadotrophin (HCG) addition

Culture day	Mechanical denudation												
	% at denudation				% 24 h after denudation				% 14–16 h after HCG stimulus				
	No	GV	GVBD	Deg	GV	GVBD	MII	Deg	No	GV	GVBD	MII	Deg
8	20	100	0	0	11	83	2	6	30	40	60	23	0
10	32	97	3	0	28	67	6	6	39	15	77	36	8
12	18	99	1	0	20	78	23	2	41	2	93	39	5
14	35	100	0	0	6	94	10	0	34	0	97	41	3
16	17	100	0	0	16	80	12	4	39	8	57	18	36

GV = germinal-vesicle-stage oocyte, GVBD = germinal vesicle breakdown, Deg = degenerated oocyte, MII = metaphase II oocyte

spontaneously. The data showed that the culture system preserves meiotic arrest up to day 16. The oocytes acquired the potential for GVBD at least from day 8. After granulosa cell removal, the potential to proceed with meiosis up to MII happened at a low frequency from day 10 onwards.

The meiotic progression of oocytes after HCG administration

The optimal time for retrieval of the in-vitro matured oocytes was determined by stimulating the follicles with 1.5 IU/ml r-HCG and 5 ng/ml human EGF on days 8, 10, 12, 14 and 16 of culture. At 14–16 h after the stimulus, mucification of the COC was scored under the stereomicroscope as an expanded mass of granulosa cells, which was eventually released from the follicle to float free in culture medium (Figure 3A). Mucification was influenced to a large extent by the length of time of culture. From day 8 up to day 12, HCG stimulation induced mucification of the COC in 87–100% of the surviving follicles, independently of whether they had developed a clearly recognizable antrum. A significant ($P < 0.01$) decline in the rate of mucification was noted from day 14 onwards (70%) and, on day 16 of culture, only 19% of the follicles, which had almost all become antral, responded to the HCG stimulus by mucification. The HCG/EGF stimulus had a clear effect on GVBD· significantly more oocytes underwent nuclear maturation (total χ^2 20.1; $P < 0.001$) after this hormonal stimulus than after mechanical removal of the granulosa cells from the oocyte.

Oocytes, freed from the mucified complex by gentle pipetting, were observed under an inverted microscope for classification of nuclear maturation, degeneration and diameter measurement. The number of degenerated oocytes was significantly increased to up to 36% in day 16 cultures (total χ^2: 29.2, $P < 0.001$). Days 12 and 14 of culture provided the highest rates of GVBD ($P < 0.05$). The highest percentage of MII oocytes was obtained when the final HCG/EGF stimulus was given on days 10, 12 or 14 of culture (36, 39 and 41%), after which it declined to 18% on day 16 of culture ($P < 0.005$). It was concluded

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that the best absolute yield of MII oocytes was obtained on days 12 and 14, as in day 10 cultures 15% of the oocytes had still not yet reached the potential for GVBD.

Steroid and inhibin production by the cultured follicles

In Figure 4 the production of oestradiol, inhibin and progesterone shown is a representative example.

The production of oestradiol increased progressively up to day 16 (Figure 4A). On this day the production of oestradiol per 48 h reached 7000 ng/l. Progesterone production remained below the sensitivity of the radioimmunoassay up to day 12 (Figure 4C). Basal progesterone production increased moderately after this day up to 0.6 μg/l/48 h.

Inhibin production increased progressively up to day 12, after which the concentration tended to level out (Figure 4B). After the HCG stimulus on day 12, luteinization was clearly illustrated by a dramatic increase in progesterone production (values >3 μg/l/16 h) and an arrest in the further increase of the oestradiol production (post-HCG data not shown).

In-vitro fertilization of in-vitro matured oocytes

One experiment was performed to evaluate the fertilizing potential of in-vitro matured oocytes. In all, 60 mucified COC released after in-vitro ovulation induction with 1.5 IU/ml r-HCG were individually fertilized *in vitro*. Fertilization was scored as the percentage of 2-cell embryos observed 24 h after fertilization. Since the oocytes were not denuded, the exact fertilization potential could not be scored. In the four plates containing cultured oocytes that were inseminated, 50% of the COC were fertilized. Since the yield of MII oocytes after in-vitro maturation in this culture system was 36–41%, the fertilizing potential of in-vitro matured primary oocytes is of the same order of magnitude as in-vivo matured oocytes obtained from adult mice superovulated with pregnant mare's serum gonadotrophin and HCG (unpublished personal observation). Of the in-vitro fertilized oocytes, 50% developed *in vitro* to the hatched blastocyst stage in a culture system where single embryos were placed in culture droplets (Figure 3A–D).

Discussion

Early preantral follicles for culture were isolated from ovaries of 14 day old mice. In these ovaries, early preantral follicles predominate and antral follicles are rarely encountered. Morphological identification of the mechanically isolated early preantral follicles selected for culture revealed that these follicles (100–130 μm in diameter) were composed of an oocyte surrounded by a thin zona pellucida and 1–2 layers of granulosa cells enclosed by an intact basal membrane to which at least some thecal or interstitial cells were attached. This characterization means that the follicles selected for in-vitro maturation were type 3b and type 4 follicles, according to the classification of Peters (1969), or primary follicle stages II and III, according to the classification of Qvist *et al.* (1990). They possessed all the morphological and functional compartments of an ovarian follicle for sustaining oocyte growth and maturation. These follicles differed from the follicles used by other investigators in comparable culture systems in which follicles

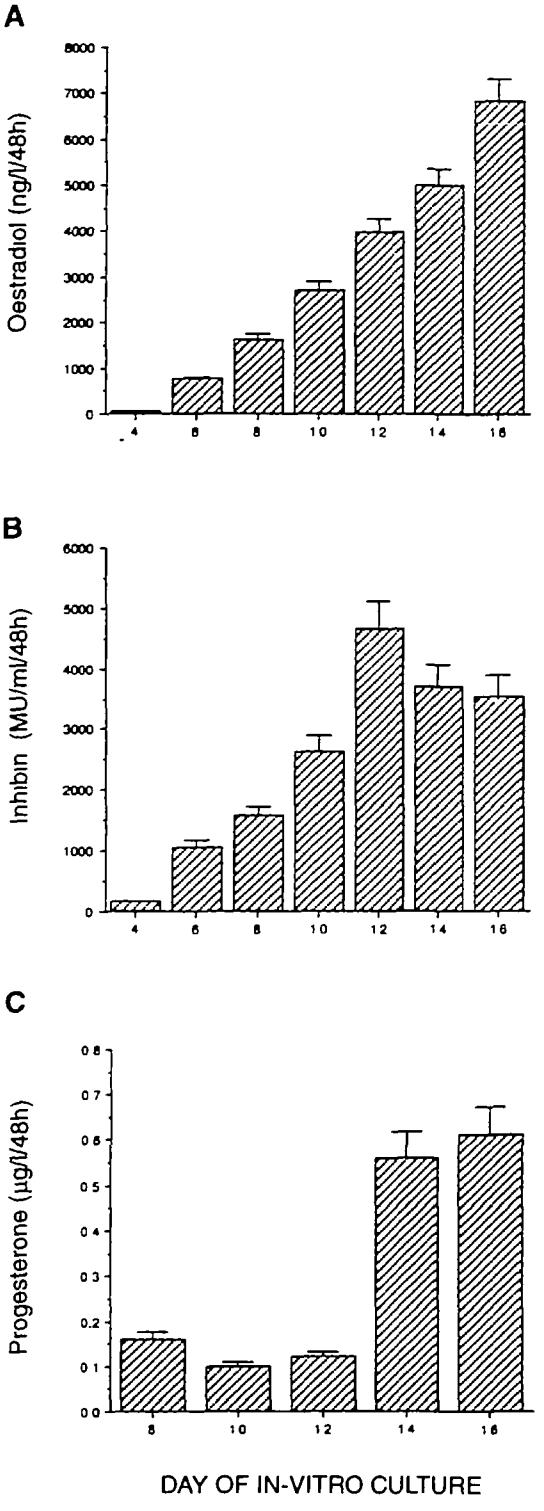

Figure 4. Analysis of conditioned medium collected from growing (diffuse) follicles. Results are concentrations of pools per 48 h with error bars. (**A**) Oestradiol produced per 48 h: oestradiol secretion increased exponentially during growth up to day 16 of in-vitro culture (conversion of mass to SI units: ng/l×272.4 = nM/l). (**B**) Inhibin production per 48 h increased exponentially until day 12 of culture, and flattened out thereafter (**C**) Basal progesterone secretion in conditioned medium was not detectable until day 14 of culture (conversion of mass to SI units: μg/l×3 = nM/l).

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from 25–28 day old mice were isolated, without restriction as to the follicle class used (130–300 μm) for in-vitro maturation (Nayudu and Osborn, 1992; Hartshorne *et al.* 1994a,b). The authors who used ovaries from mice of a similar age (12 days) treated the ovaries enzymatically and cultured the pool of retained oocyte–granulosa cell complexes in groups (Eppig and Schroeder, 1989; Torrance *et al.*, 1989; Carroll *et al.*, 1991) and were therefore working in a completely different environment.

As we wished to have the possibility of tracing each follicle during its entire growth, the option was taken to culture them singly in 20 μl droplets of medium under a protective oil layer in a Petri dish. In this system, daily observation at high magnification can be carried out more conveniently than in V-shaped culture wells, and individual droplets can easily be reached for retrieval of samples from conditioned medium and for medium replenishment. The culture medium was composed of standard reagents as described above. It was not, however, very clear in the current literature whether hormones or growth factors were essential for complete in-vitro maturation and whether the culture of follicle-enclosed oocytes needed a normal or a reduced oxygen atmosphere. In contrast to the culture of granulosa–oocyte complexes, where development is impaired in a 20% atmosphere (Eppig and Wigglesworth, 1995), we have demonstrated the essential requirement of a normal oxygen tension for normal oocyte and follicle development (Smitz *et al.*, 1996) in a follicle-enclosed oocyte culture system

Evidence as to whether to use gonadotrophins as supplements in the in-vitro maturation medium was gained from previous studies. In prepuberal mice the serum FSH concentration reaches its maximum at the age of 14 days (Dullaart *et al.*, 1975; Michael *et al.*, 1980). Since FSH up-regulates the expression of its own receptor, high numbers of FSH-receptors will be present on the granulosa cells of the preantral follicles, rendering them highly responsive to an FSH stimulus (Ryle, 1972; Fortune and Eppig, 1979; Smith and Ojeda, 1986; Roy and Greenwald, 1989). The thecal cell layer begins to differentiate from the interstitial–stromal ovarian cell compartment around types 3b and 4 follicles according to Peters' (1969) classification. The genes for LH receptors and steroidogenic enzymes are expressed prior to morphological differentiation and before the differentiating theca becomes responsive to LH (Magoffin *et al.*, 1994). FSH was an essential factor in our culture system. Without the addition of r-FSH, in-vitro growth and differentiation of the early preantral follicles were compromised. Besides showing an arrest of mitosis and an absence of antrum formation, significantly more denuded oocytes were released from the cultured follicles in FSH-free medium (R.Cortvrindt *et al.*, unpublished observations).

The observations made with regard to the absence of FSH in our culture system were different from those for other culture environments. Gonadotrophins did not seem to be essential in the long-term cultures of COC from early follicle stages described by Eppig and Schroeder (1989) and Daniel *et al.* (1989). These authors cultured COC in the presence of synthetic exogenous factors inhibiting spontaneous progression of meiosis. Boland and Gosden (1994) were also unable to demonstrate an effect of FSH on follicle cell numbers; an explanation here could be that the larger follicle class originating from older mice had already been primed endogenously and that these follicles had been exposed to enough FSH to cover the 6-day culture period. More recently, Eppig and O'Brien (1996), culturing primordial follicle stages in a two-step system, first by organ culture on nucleopore polycarbonate membranes and then by culture of oocyte–granulosa cell complexes on Transwell-Col membranes, showed an increase of 15–30% in potential to undergo GVBD in oocytes cultured in FSH-containing medium. Our medium was supplemented with r-FSH as sole gonadotrophin instead of the purified FSH used in other follicle culture systems, which is always contaminated with some small amounts of LH (Qvist *et al.*, 1990; Nayudu and Osborn, 1992; Boland *et al.*, 1993). Nevertheless, the measurement of oestradiol in the conditioned medium revealed normal oestrogen production in our cultures in comparison to literature data, and this even in the total absence of any LH activity in the medium. It seemed that the substrate for aromatization was synthesized *de novo*, possibly stimulated by insulin present in the culture medium or through paracrine signalling from the inhibin produced by the proliferating granulosa cells. Insulin as well as inhibin might induce steroid production in the thecal compartment (Hillier *et al.*, 1991; Magoffin and Magarelli, 1995).

Daily oestrogen production continued to increase in a linear fashion up to day 16, while inhibin concentrations levelled off from day 14 onwards. From day 14, basal luteinization took place, as illustrated by a discrete increase of progesterone production. The luteinization and the arrest of mitotic activity within the granulosa cell mass (also suggested by the drop in inhibin production) might reflect that the follicle had reached maturity.

There is evidence from our experiments that optimal maturity in our culture system is obtained after 12–14 days; the 16 day cultures were mucified at a significantly lower rate and a lower final yield of GVBD was observed. Probably, the oocytes had exceeded their normal life span on day 16, degenerative processes had commenced and the signalling processes upon HCG were unable to function normally. The process of cumulus expansion or mucification is a reflection of final oocyte maturation and gonadotrophins play critical roles in its regulation (Dekel *et al*, 1979; Salustri *et al.*, 1989; Chen *et al.*, 1994). In the culture system described here, a high rate of mucification followed by in-vitro release of COC was obtained when LH activity was added in time Our in-vitro results confirm the role of LH activity in stimulating mucification of follicle-enclosed oocytes (Dekel *et al*, 1979). The basal increase in progesterone in our cultures might equally well be a reflection of the normal differentiation of some of the granulosa cells in response to the meiotic status of the oocyte, or an indication of oocyte degeneration. It was demonstrated that oocytes produce luteinization-inhibiting factors and, after oocyte death, inhibition of luteinization is abolished (El-Fouly *et al.*, 1970). From the progesterone measurements in the conditioned media after induction of final maturation with HCG, we ascertained the presence of LH receptors on the

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granulosa cells: 16 h after the stimulus, progesterone production had increased.

For the full maturation of oocytes, a precise balance and sequence of steroid secretion during follicular growth might be necessary (Moor *et al.*, 1980; Zhang and Armstrong, 1989). In the present follicle culture system we observed a normal sequence of steroid secretion as compared to the in-vivo situation. Although the observations from oestradiol and progesterone measurements were concordant with what is known of ovarian follicle physiology under in-vivo and in-vitro conditions, we must interpret our data with caution. One of the drawbacks of a culture system under a paraffin-oil layer is that lipid-soluble steroids could easily diffuse into the oil, making accurate quantification difficult (Miller and Pursel, 1987). This is especially the case as regards testosterone, progesterone and, to a lesser extent, oestradiol. We therefore decided also to measure inhibin, a non-lipid-soluble protein that is also secreted specifically by granulosa cells in large amounts in the culture fluid.

In most intact follicle maturation systems described in the literature, follicular attachment was actively prevented by transposing the follicles every day. In this way, the three-dimensional follicular structure *in vitro* was kept intact and release of the oocyte was prohibited (Hartshorne *et al.*, 1994a; Spears *et al.*, 1994; Johnson *et al.*, 1995). In the present culture system, the follicular cells were allowed to attach to the culture dish. The follicular cells on the outer site of the basal membrane (from the thecal–interstitial compartment) migrated away from the basal membrane within 2 days and colonized the bottom of the dish. These cells formed a monolayer and attached the follicle to the culture plate Granulosa cells proliferated under the FSH stimulus, migrated through the basal membrane and spread over the monolayer so formed. The in-vivo tissue organization of the follicle was lost by day 4–6 of culture. The original follicular structure, limited by a basal membrane boundary, grew into a dome-like structure. The oocyte, located on top of this structure, was always enclosed by a rim of tightly packed granulosa cells. The basal membrane did not grow in this in-vitro model but was colonized by granulosa cells on its outer side. Finally, the basal membrane lost its functional significance and ended as an amorphous curled thread somewhere in the wall of the follicle. It was not clear why the basal membrane did not grow parallel to the granulosa cell proliferation. We postulated that, in the absence of theca cell–granulosa cell interaction on the basal membrane, the synthesis of membranous proteins was arrested. Although the in-vivo follicular architecture was not maintained in this culture system, all cellular compartments (thecal cells, granulosa cells and oocyte) remained present and maintained an organized structure. The granulosa cell mass did not spread out into a monolayer but kept a clearly delineated boundary. As cultures progressed, a cellular differentiation gradient was established within the granulosa cell mass, which was comparable to that of natural follicles. Despite the absence of a functional basal membrane growing in relation to the proliferation of the granulosa cell mass, the granulosa cells did differentiate into two distinct cell types. The cytoplasm of the outermost layers of granulosa cells was larger and was loaded with secretory vesicles, whereas the granulosa cells around the oocyte were smaller and no secretory vesicles could be recognized. Antral-like cavity development started on about day 8 of culture, proving that the localization of differentiated theca cells was not essential for cavitation of the follicle (Nayudu and Osborn, 1992). The antral-like cavities became confluent during subsequent days, and 84% of all cultured follicles presented an antrum on day 16.

As our primary aim was to obtain meiotically competent oocytes, we were interested in the intrafollicular growth profile of the oocyte. During the whole culture period up to 16 days, the oocytes grew from 55 (±4) to 74 (±2) μm diameter, and almost all were kept under meiotic arrest until administration of the HCG stimulus. Maintenance of meiotic arrest proved that the gap junctions and the signal transduction mechanisms between the granulosa cells and oocyte were fully functional under our culture conditions. The oocyte diameter that could finally be obtained after in-vitro culture fell within the range of size of ovulated oocytes retrieved out of the ampulla of 26 day old mice. Apparently, it is possible to obtain fully grown oocytes with this culture system.

We could keep carefully selected follicles of 100–130 μm diameter in culture up to 16 days therefore we wondered when would be the optimal moment to induce the final maturation. We determined this critical period by examining the responses of follicle cells and oocytes to an HCG stimulus. The percentage of mucified COC was also maximal up to this period and it decreased thereafter. The percentage of oocytes progressing from GV to MII stage was maximal on days 12 and 14. It is as yet unclear whether the drop in meiotic competence after day 14 is due to a loss of LH receptors or whether intrinsic oocyte changes were taking place after a critical period once oocyte growth was completed.

The observation that complete nuclear maturation up to the MII stage was significantly increased after the HCG/EGF stimulus compared to mechanical denudation of the cumulus cells from the oocyte supports the fact that the hormones provide a positive stimulatory effect for completion of nuclear maturation.

The optimal period of 12–14 days for growing from early preantral to Graafian follicle stage was no different from the in-vivo situation (Pedersen, 1970). It became clear throughout our experiments that culture duration was specific for each class of early preantral follicles. Follicles of <100 μm diameter were not successfully grown in our standard system and probably needed other factors that are essential at earlier stages, such as EGF (Eppig and O'Brien, 1996). Stimulatory effects of EGF on the smallest classes of preantral follicles have been described in the hamster and pig ovary (Roy and Greenwald, 1991). It might be difficult to culture individual primordial follicles, as the specific needs for this follicle class are largely unknown and they might also be dependent on extra-ovarian factors. The larger-sized (130–150 μm) group of preantral follicles pursued a normal follicular growth pattern and had to be ovulated ~2 days earlier (after 10 days of culture) to avoid overmaturity, shown by the absence of mucification upon HCG stimulation at day 12. This illustrates

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the need to adapt the culture conditions to each follicle class at isolation.

The final outcome parameter of an oocyte culture system is definitely the procurement of normal young. When mucified COC were used for in-vitro fertilization, approximately half of them developed to the 2-cell stage. Denudation of the mucified COC revealed that 40% contained a potentially fertilizable MII oocyte. This might suggest that most oocytes which matured *in vitro* to MII had fertilized and that the receptor systems for sperm binding had developed *in vitro*. Presumably, the granulosa cell coat preserved the functional integrity of the zona.

Of the 2-cell embryos, 50% developed *in vitro* up to the hatched blastocyst stage, indicating that the in-vitro matured early oocyte stages were able to reach full cytoplasmic maturity. The final validity of this culture system has still to be proven by the transfer of blastocysts to pseudopregnant foster mothers.

The number of immature oocytes from early preantral follicles that successfully matured in this in-vitro culture is encouraging. Hypotheses to explain the high follicle survival rate in our system include (i) the use of follicles which are very early in the recruitment process, originating from an ovary containing exclusively very early-stage follicle classes; (ii) the stringent selection of one class of healthy follicles to which the culture conditions were adapted; (iii) the endocrine milieu created through culture under oil, where androgens (which induce atresia) are partially extracted by the lipid layer

This in-vitro maturation system showed that conservation of the three-dimensional structure of the follicle was not absolutely necessary for the maturation of oocytes from primary up to Graafian follicles. This in-vitro maturation system offers practical advantages: since the follicles are cultured individually in droplets in a flat Petri dish, their growth and differentiation can easily be followed and evaluated at all times during culture by means of a conventional inverted light microscope, without disturbing follicular development. The cultures are very easy to manipulate under the thin oil layer that protects the follicle from environmental factors. Refreshment of the culture medium can be carried out under clear microscopic vision so that handling can be carried out without disturbing the growing follicular structure. Follicle secretions are easily accessible and thus conditioned medium collected every 2 days can be analysed to follow factors reflecting follicle differentiation and oocyte ripening. Finally, the system can be used as an in-vitro bioassay to study ovarian cell interactions and to evaluate effects of hormones and novel drugs on gametogenesis in mammals.

Acknowledgements

Katy Billooye is thanked for her technical help making the semi-thin sections through the follicles and performing the hormone measurements. Bernadette Mannaerts, PhD from Organon BV (Oss, The Netherlands) is thanked for providing the recombinant FSH preparation Drs A.Ythier and E.Loumaye from Ares Serono (Geneva, Switzerland) are acknowledged for donation of recombinant FSH and recombinant HCG. Frank Winter (VUB-Language Education Centre) is acknowledged for correcting this manuscript and Fritz Vanhoef is sincerely thanked for the artwork. J.Smitz holds a fellowship from the Belgian Fund for Medical Research (grant no. G.3C01.93N). This study was financially supported by the Belgian Fund for Medical Research

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Received on July 1, 1996, accepted on September 17, 1996

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