

Effects of follicle-stimulating hormone and serum substitution on the in-vitro growth of human ovarian follicles

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In-vitro maturation (IVM) of human ovarian follicles and oocytes could benefit infertile women, and allow the development of in-vitro systems for the study of human follicular development. Little is known about the initiation of growth of primordial follicles and the regulation of early folliculogenesis. An ovarian tissue-slice culture system was used to examine the effects of media composition, follicle stimulating hormone (FSH) and serum substitution on the development of small human follicles *in vitro*. Human ovarian cortex biopsies were cut into small pieces and cultured for 5, 10 or 15 days. Control (non-cultured) and cultured tissue was fixed, serially sectioned, and stained. The follicles contained within the tissue pieces were counted, measured, and assessed for stage of development and viability. Comparison of the ability of α -minimum essential medium (α -MEM), Waymouth's, or Earle's balanced salt solution (EBSS) culture media (all with 10% human serum) to support follicle growth demonstrated significantly increased initiation and growth of follicles in α -MEM during the first 10 days of culture. The supplementation of α -MEM with 300 mIU/ml FSH significantly reduced levels of atresia and increased the mean diameter of healthy follicles. Follicles in tissue cultured for 10 days with human serum albumin and ITS (insulin/transferrin/selenium mix) were significantly larger, more developed and showed significantly less atresia than those cultured with serum alone. Primordial to small preantral follicles can be grown under serum-substituted conditions in tissue-slice culture, and are responsive to FSH, which is thought to be acting mainly as a survival factor at these early stages.

Key words: atresia/culture/follicle/insulin/ovary

Introduction

Little is known about the mechanisms which regulate early folliculogenesis in the human. The development of a culture system for the maintenance of ovarian tissue, preserving the

integrity of the oocyte–granulosa–theca interactions and the relationship between the intact follicle and the surrounding stroma, would provide a unique opportunity to examine these mechanisms *in vitro*. Furthermore, the ability to mature oocytes *in vitro* would benefit both infertile women and pre-menopausal women with cancer who are likely to become sterile as a result of radio- or chemotherapy. This approach would avoid possible side-effects associated with gonadotrophin treatment, and allow maturation of oocytes for in-vitro fertilization (IVF) from tissue cryopreserved before cancer treatment.

Various culture systems have been used for the in-vitro maturation (IVM) of human follicles which have generally been adapted from those used successfully in other species. Since human stromal tissue is more dense than in rodents, enzymatic isolation of follicles has generally been used. Human preantral follicles have been enzymatically isolated and grown in agar for 5 days (Roy and Treacy, 1993). Primordial follicles from fetal ovaries have been cultured in a similar way for a longer period (Zhang *et al.*, 1995), and primordial follicles from adult ovaries have also been isolated and cultured for 5 days, although no growth was detected (Oktay *et al.*, 1996), and 25 days when some hormone production was exhibited (Osborn *et al.*, 1997). Preantral follicles have also been mechanically isolated and cultured for several weeks, developing antra, but becoming atretic at the end of the culture period (Abir *et al.*, 1997). Recently it has also been reported that preantral follicles obtained during IVF follicular aspiration can be cultured from a diameter of 100 μ m to one of 300 μ m (Wu *et al.*, 1998).

In this study we used a culture system designed for the culture of human primordial, primary and secondary follicles to early antral stages (Hovatta *et al.*, 1997). Follicles are cultured within small pieces of ovary, which has the advantage of maintaining all the normal intercellular contacts and support. We found that a complex medium such as α -minimum essential medium (MEM) supported significant growth of follicles, that supplementation of the medium with follicle-stimulating hormone (FSH) significantly reduced atresia, and that follicle growth and survival was increased when serum was replaced by a combination of human serum albumin and a commercial insulin/transferrin/selenium mix. We have therefore developed a serum-substituted culture system which supports the growth and differentiation of human primordial follicles to secondary and occasionally preantral stages over a 15-day period. This will allow the study of growth factor, steroid, and hormone interactions *in vitro* during human early folliculogenesis.

Materials and methods

Source of ovarian tissue

Ovarian cortex tissue was obtained with informed consent by ovarian biopsy from 38 women (mean age \pm SEM: 34.2 \pm 0.7 years)

undergoing gynaecological investigations either laparoscopically or by laparotomy. Ethical approval of the study was granted by the local ethics committee of Imperial College School of Medicine, Hammersmith Hospital.

Ovarian tissue was collected into HEPES-buffered minimum essential medium (MEM, Gibco, Life Technologies, Paisley, UK), immediately transported to the laboratory and placed in an incubator within 10 min. Two pieces of tissue (3–5 mm diameter) were removed from the biopsy and immediately placed in Bouin's fixative for 1–3 h before being transferred to 70% alcohol for storage. One of these pieces was sent for clinical analysis to assess follicle density for the prediction of ovarian reserve (Lass *et al.*, 1997). The other acted as the control for pieces put into culture.

Culture of ovarian cortex tissue

The remaining tissue was cut into small pieces (1–3 mm thick) with a scalpel and mounted needles, using a stereo microscope. Tissue pieces were randomly distributed to parallel cultures. One to four tissue pieces were transferred to Millicell culture plate well inserts (Millipore, Bedford, MA, USA) which had been pre-coated with artificial extracellular matrix (ECM) (Matrigel; Becton Dickinson, Bedford, MA, USA). This was achieved by diluting ECM 1:3 with serum-free Earle's balanced salt solution (EBSS, Gibco), and placing 100 μ l into each well insert. The inserts rested in 1 ml of pre-equilibrated culture medium in the wells of 24-well plates (Nunclon, Roskilde, Denmark) (Hovatta *et al.*, 1997). This volume was reduced to 0.4 ml for Experiment 3 so that the pieces of tissue were covered only by a thin layer of culture medium. Every second day 150 μ l (or 400 μ l for the 1 ml cultures) of the culture medium was replaced by fresh medium. The plates were incubated at 37°C under 5% CO₂ for 5, 10 or 15 days.

In the first experiment, three basic culture media were compared. EBSS supplemented with 0.47 mmol/l pyruvate (Sigma, Poole, UK), Minimum Essential Medium Alpha (α -MEM, Gibco) and Waymouth's Medium (Gibco) were each supplemented with 10% heat-inactivated human serum (obtained from women undergoing pituitary desensitization for IVF on days 3–5) and antibiotics (50 U/ml penicillin G, 50 μ g/ml streptomycin sulphate, 0.125 μ g/ml amphotericin B; antibiotic antimycotic solution, Gibco). In the second experiment α -MEM was supplemented with either 10% serum and antibiotics, or 10% serum, antibiotics and 300 mIU/ml FSH (Orgafol: Organon, Cambridge, UK). In the third experiment α -MEM was supplemented with either 10% serum and antibiotics, or 2.5% human serum albumin (HSA, Zenalb 20, Bio Products Laboratory, Elstree, UK; effective concentration 10%), 1% ITS (insulin/transferrin/selenium; Gibco; effective concentration: 10 μ g/ml insulin, 5.5 μ g/ml transferrin, 6.7 ng/ml sodium selenite) and antibiotics.

Hormone level measurement

The FSH, luteinizing hormone (LH), and insulin content of 10% serum in α -MEM and HSA in α -MEM at media concentrations was measured by micro-particle enzyme immunoassay (Abbott AxSYM, Maidenhead, UK). For serum in α -MEM, average values were FSH, 0.44 \pm 0.1 mIU/ml; LH, 0.18 \pm 0.04 mIU/ml; insulin, 0.33 \pm 0.03 μ U/ml. For HSA in α -MEM average values were FSH, 0.12 \pm 0.03 mIU/ml; LH, 0.11 \pm 0.02 mIU/ml; insulin, 0.17 \pm 0.03 μ U/ml.

Assessment of follicular development and survival

After 5, 10 and 15 days of culture, pieces of tissue were fixed in Bouin's fixative, dehydrated through an alcohol series, and embedded in paraffin wax. The wax blocks were serially sectioned (5 μ m) on a Leica RM2135 microtome, stained with haematoxylin and eosin, and analysed microscopically using a \times 63 objective. The number of

follicles in the tissue pieces were counted, and growth and development of the follicles was assessed by measuring follicle diameters, stages of development and viability.

Follicular diameter (from edge to edge of basal lamina or edge to edge of theca cell layer(s)) was measured by a microscope graticule at what was considered the centre of the follicle, indicated either by the presence of the nucleolus, or if one was not present, the widest point. Measurements taken were converted from graticule units to micrometres by multiplication by 1.85 (conversion factor for \times 63 objective). The stage of follicle development (primordial, primary, secondary, or preantral) was assessed as follows: follicles with one layer of a majority of flattened pre-granulosa cells were considered to be primordial (Figure 1A,B); those with one layer of a majority of expanded, cuboidal granulosa cells were considered to be primary (Figure 1A,B); those with more than one layer of granulosa cells were considered to be secondary (Figure 1C); and those with many layers of granulosa cells were considered to be preantral follicles (Figure 1D). Follicles were assessed to be healthy or atretic on the basis of morphology. A degenerated oocyte nucleus, uneven or folded nuclear membrane (Figure 1E,G), vacuoles in the oocyte (Figure 1F), or pyknotic nuclei of several granulosa cells (Figure 1E,G) were all regarded as signs of atresia (Gougeon, 1996).

For each experiment, the results obtained for tissue pieces in parallel cultures from all the patients were pooled for each culture condition.

Statistical analysis

χ^2 and Mann–Whitney *U* tests were used for statistical analyses as appropriate.

Results

Follicles within the tissue pieces all grew to a greater or lesser extent during the period of culture regardless of the culture used. As well as showing increases in follicle diameter, which indicated granulosa cell and oocyte growth, the developmental stage of the follicles assessed histologically during culture showed an initiation of development of primordial follicles to the primary stage. Some follicles developed further to secondary and preantral stages (Figure 1C,D,H) and mitoses were seen in several granulosa cells, confirming that cell division was occurring (Figure 1H). Occasionally oocytes were observed with abnormalities such as double nuclei. Atresia of follicles was assessed in the last two experiments and this occurred to a greater or lesser extent depending on the composition of the culture media (Figure 1E,F,G).

Effect of media composition

The first experiment was designed to determine which culture medium to use for subsequent studies and investigated the effect of different media on the growth and development of follicles in the culture system (patient number = 9; mean age \pm SEM: 35.1 \pm 1.3; total number of follicles = 524). Two complex media (α -MEM and Waymouth's) were compared with a simple salt solution (EBSS), all of which contained 10% human serum.

The majority of follicles cultured in all the media tested initiated growth during the first 5 days in culture (Figure 2). On day 0, at the time of ovarian biopsy, 53% of healthy non-atretic follicles in the ovarian cortex were primordial, with

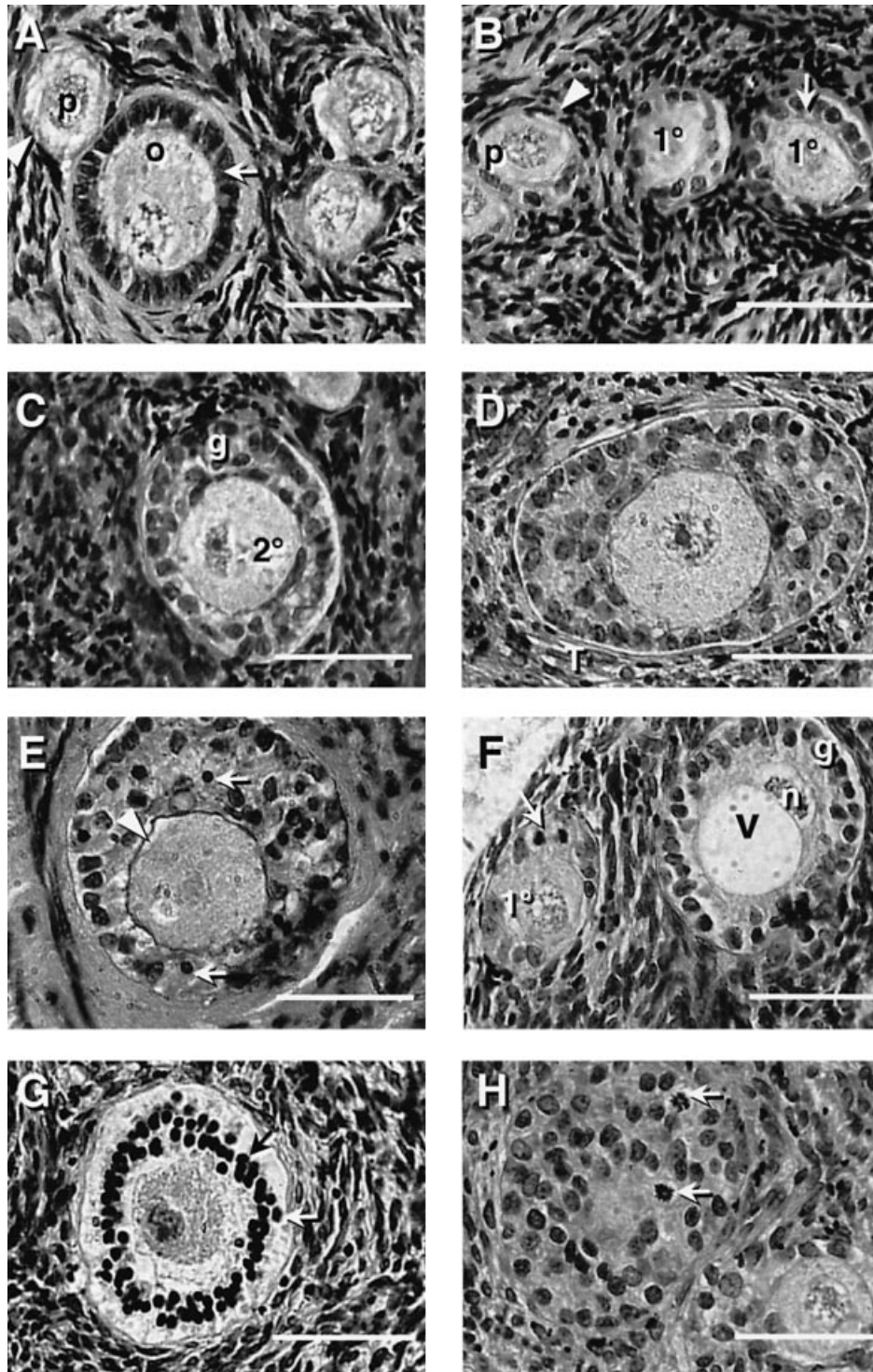


Figure 1. Early human follicles cultured under different conditions. (A) Cluster of primordial (p) and primary (1°) follicles in non-cultured ovarian cortex tissue (control) at the time of biopsy. Note flattened granulosa cells of primordial follicles (\blacktriangleright), cuboidal granulosa cells of primary follicles (\rightarrow), and oocyte (o) with germinal vesicle; (B) Follicles on the borders of growth initiation, showing an uninitiated primordial follicle (p) with more than 50% flattened granulosa cells (\blacktriangleright) and two initiated primary follicles (1°) with more than 50% cuboidal granulosa cells (\rightarrow). (C) Secondary follicle (2°) with 2–3 layers of granulosa cells (g) cultured with α -MEM and 10% serum without FSH for 5 days showing initiation of growth in unsupplemented media; (D) Preantral follicle with several layers of granulosa cells and a theca cell layer (T) cultured with HSA + ITS for 5 days; (E) Atretic secondary follicle cultured with α -MEM and 10% serum for 10 days, showing degenerated oocyte with wrinkled membrane (\blacktriangleright) and pyknotic nuclei of granulosa cells (\rightarrow); (F) Secondary follicle cultured with serum for 5 days with apparently healthy granulosa (g), but vacuolated oocyte (\rightarrow) and displaced nucleus (n), and primary follicle (1°) with newly divided cells (\rightarrow); (G) Atretic secondary follicle cultured with HSA + ITS for 5 days with pyknotic nuclei of granulosa cells (\rightarrow) and degenerated oocyte; (H) Preantral follicle cultured for 10 days with HSA + ITS with mitoses in granulosa cells (\rightarrow). Sections stained with H & E. Scale bars are 50 μ m.

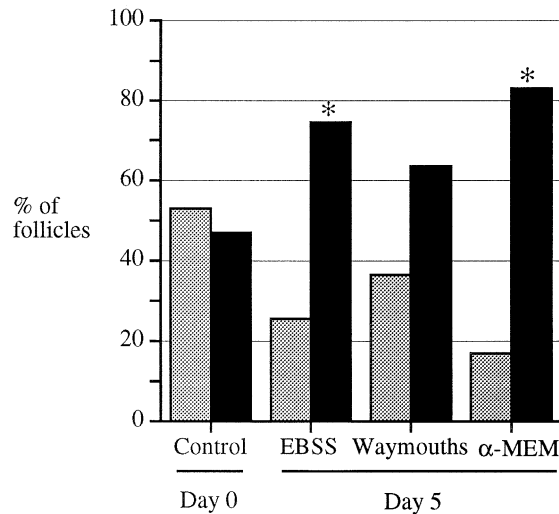


Figure 2. Proportion of non-initiated (■) (primordial) and initiated (■) (primary, secondary and preantral) follicles in ovarian cortical tissue at the time of biopsy (control) and after 5 days of culture in EBSS, Waymouth's, and α -MEM media with 10% human serum. Bars with asterisks mark a significant increase in initiated follicles with α -MEM and EBSS (* $P \leq 0.0002$).

47% at the primary and secondary stages. After 5 days in culture, the proportion of healthy follicles in the primordial pool had fallen to 17% (α -MEM), 26% (EBSS), and 37% (Waymouth's), while the proportion of follicles at primary and secondary stages had increased significantly both in α -MEM (to 83%; $P = 0.0002$) and EBSS (to 74%; $P = 0.0001$). There was also an increase in the proportion of growing follicles in Waymouth's to 63%, although this was not significant (Figure 2).

During the first 5 days of culture, follicular diameter increased in tissue pieces cultured in all three media, although the increase in diameter was only significant ($P < 0.05$) with α -MEM. Follicles cultured with α -MEM continued to increase in size to day 10 ($P < 0.05$), then declined towards the end of culture. The diameter of follicles cultured with Waymouth's also increased over the first 10 days of culture, but this was not significant. Follicles cultured with EBSS did not increase in diameter after day 5 (Figure 3). The decline in follicle growth seen at the end of culture was probably due to larger follicles becoming atretic. However, in this pilot study the extent of atresia was not measured.

A greater proportion of follicles cultured with both α -MEM and Waymouth's media (12 and 16% respectively) reached secondary stages on day 10 of culture than those cultured with EBSS (7%). However, only follicles cultured with α -MEM reached preantral stages on days 10 (2%) and 15 (5%).

On the basis of the significant levels of initiation of growth, significant increases in diameter of follicles and greater numbers reaching secondary and preantral stages, α -MEM was selected as the basal medium for further experiments.

Effect of FSH

The second experiment investigated the effect of addition of 300 mIU/ml FSH to α -MEM medium (with 10% serum) on

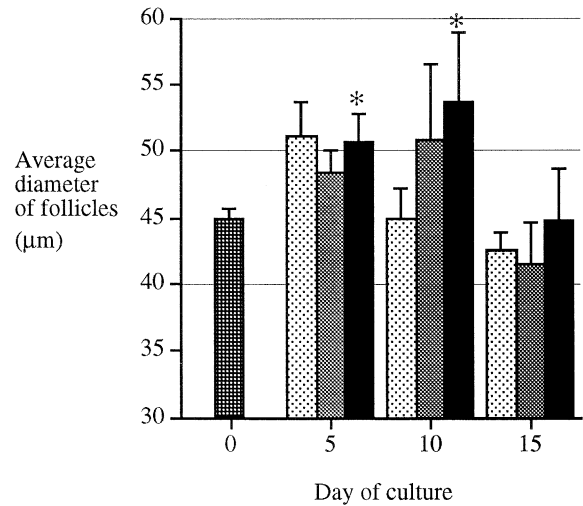


Figure 3. Average diameter of follicles in ovarian cortical tissue at the time of biopsy (control) and after culture in EBSS (■), Waymouth's (■), and α -MEM (■) media with 10% human serum. At 5 and 10 days, the bars with asterisks mark a significant increase in diameter with α -MEM (* $P < 0.05$).

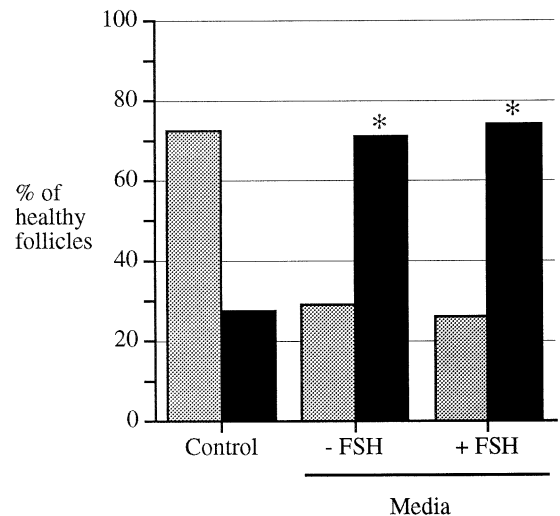


Figure 4. Proportion of non-initiated (■) (primordial) and initiated (■) (primary and secondary) healthy follicles in ovarian cortical tissue at the time of biopsy (control) and after 5 days of culture in α -MEM media with 10% human serum in the presence and absence of 300 mIU/ml FSH. Bars with asterisks mark a significant increase in initiated follicles (* $P = 0.0001$).

both the growth and viability of follicles (patient number = 13; mean age \pm SEM: 33.8 ± 1.2 ; total number of follicles = 626).

The majority of follicles cultured with and without FSH initiated growth during the first 5 days of culture (Figures 1C, 4). On day 0, at the time of biopsy, 72% of healthy non-atretic follicles in the ovarian cortex were primordial, with only 28% at the primary and secondary stages. After 5 days in culture, the proportion of healthy follicles in the primordial pool had dropped by over half to between 29% (-FSH) and 26% (+FSH), while the proportion of follicles at primary and secondary stages had increased in the absence and presence of FSH to 71% ($P = 0.0001$) and 74% ($P = 0.0001$) respectively (Figure 4).

During the 15-day culture period, the proportion of atretic

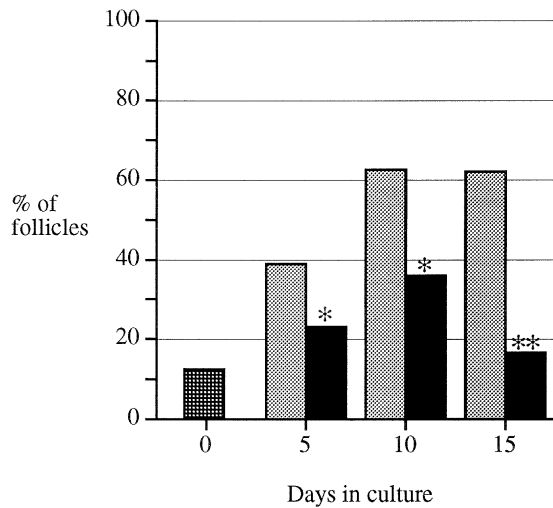


Figure 5. Proportion of atretic follicles in ovarian cortical tissue at the time of biopsy (control) and after culture in the presence ■ and absence □ of 300 mIU/ml FSH. At each time point, bars with asterisks mark a significant reduction in the proportion of atretic follicles with FSH treatment (* $P < 0.01$, ** $P < 0.001$).

follicles increased significantly ($P = 0.01$) whether cultured with or without FSH. After 10 days of culture over 60% of follicles in tissue cultured in the absence of FSH were atretic. However, supplementation of culture medium with FSH dramatically and significantly ($P \leq 0.001$) reduced the proportion of atretic follicles after culture of ovarian tissue for 5, 10 and 15 days (Figure 5).

There was a significant increase in follicular diameter during the first 5 days of culture, both in the presence ($P < 0.05$) and absence ($P < 0.001$) of FSH. During this initial period, FSH had no significant effect on follicle growth, with follicles of similar diameter in both the presence and absence of FSH after 5 days in culture. After day 5, however, follicles cultured without FSH did not significantly increase in size. However, in tissue cultured with FSH, follicles continued to grow steadily, with follicles on day 15 being significantly larger than those on day 5 ($P < 0.005$) and day 10 ($P < 0.01$) (Figure 6). On day 15, follicles cultured with FSH were significantly larger ($P < 0.05$) than those cultured without.

On day 15, 30% of the healthy follicles were at the secondary stage after culture with FSH compared to only 6% in the absence of FSH, and only follicles in media containing FSH reached preantral stages (3% on day 10).

Effect of serum replacement

The third experiment investigated the effect of replacement of 10% human serum with HSA + ITS on the growth and viability of follicles (patient number = 16; mean age \pm SEM: 34.1 ± 1.3 ; total number of follicles = 786).

Most of the follicles cultured with serum and with HSA + ITS initiated growth during the first 7 days of culture (Figure 7). On day 0, at the time of biopsy, 83% of healthy non-atretic follicles in the ovarian cortex were primordial, with only 17% at the primary and secondary stages. After 5 days in culture, the proportion of healthy follicles in the primordial pool had dropped to between 26% (serum) and 37% (HSA + ITS),

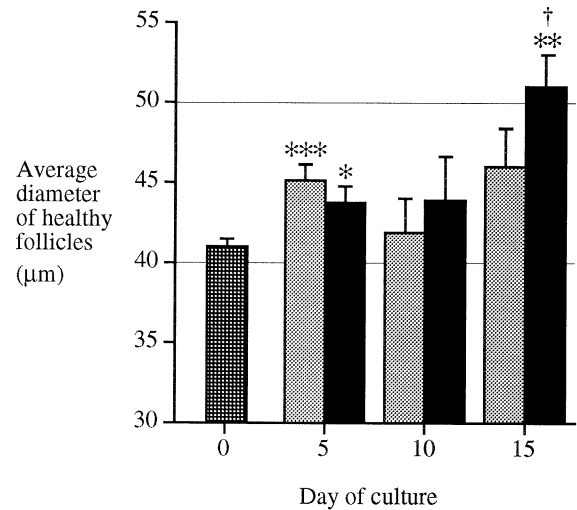


Figure 6. Average diameter of healthy, non-atretic follicles in ovarian cortical tissue at the time of biopsy (control) and after culture in α -MEM media with 10% human serum in the presence ■ and absence □ of 300 mIU/ml FSH. At day 5, the bars with asterisks mark a significant increases in diameter from day 0 with FSH (* $P < 0.05$) and without FSH (***) ($P = 0.001$). Bar with asterisks on day 15 marks significant increases in diameter from day 5 and day 10 (** $P < 0.01$) for follicles cultured with FSH. Follicles cultured with FSH on day 15 are also significantly larger ($\dagger P < 0.05$) than those cultured without.

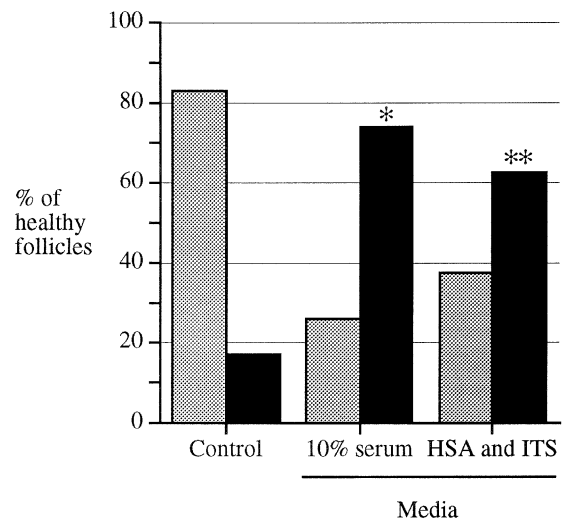


Figure 7. Proportion of non-initiated □ (primordial) and initiated ■ (primary, secondary and preantral) healthy follicles in ovarian cortical tissue at the time of biopsy (control) and after 5 days of culture in α -MEM media with either 10% human serum or HSA + ITS. Bars with asterisks mark a significant increase in initiated follicles cultured with serum (* $P = 0.001$) and HSA + ITS (* $P = 0.0001$).

whilst the proportion of follicles at primary, secondary, and preantral stages had increased to 74% ($P = 0.001$) in the presence of serum and to 63% ($P = 0.0001$) with HSA + ITS (Figure 7).

During the 10-day culture period, the proportion of atretic follicles increased significantly ($P = 0.0001$). After 10 days of culture 49% of follicles in tissue cultured with serum were atretic (Figure 1E,F). However, replacement of serum with HSA + ITS significantly reduced the proportion of atretic

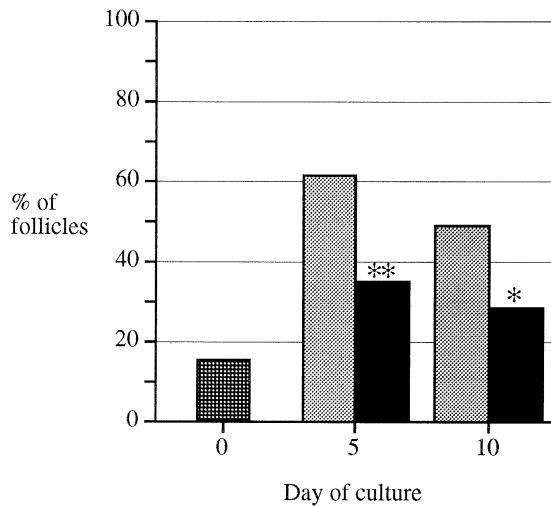


Figure 8. Proportion of atretic follicles in ovarian cortical tissue at the time of biopsy (control) and after culture in α -MEM with either 10% human serum \square or HSA + ITS \blacksquare . At each time point, bars with asterisks mark a significant reduction in the proportion of atretic follicles with HSA and ITS treatment (* $P < 0.002$, ** $P = 0.0001$).

follicles after culture of ovarian tissue for 5 and 10 days ($P = 0.0001$ and $P = 0.002$ respectively) (Figure 8).

There was a significant increase in follicular diameter during the first 5 days of culture, both for follicles cultured with 10% serum ($P = 0.001$) and with HSA + ITS ($P = 0.0001$) (Figure 1D). During this initial period, there was no significant difference in growth between the two groups. After day 5, follicles cultured with 10% serum did not significantly increase in size. However, in tissue cultured with HSA + ITS, follicles continued to grow, with follicles on day 10 being significantly larger than those on day 5 ($P = 0.0001$) (Figure 9). On day 10, follicles cultured with HSA + ITS were significantly larger ($P = 0.0001$) than those cultured with serum (Figure 1H).

On day 10, 38% of the healthy follicles were at secondary and preantral stages after culture with HSA + ITS compared to only 11% in the tissue cultured with serum ($P = 0.0001$).

Discussion

We have demonstrated that initiation of follicle growth in slices of ovarian cortex occurs in a variety of culture media, ranging from a simple salt solution (EBSS) supplemented with 10% human serum, to more complex media, such as α -MEM with serum and FSH. However, a simple medium such as EBSS supplemented only with serum was unable to sustain further follicular growth in the tissue slices, while α -MEM plus serum was able to support significant growth during the first 10 days of culture. The high levels of atresia which are seen after the first 5 days can be reduced significantly by supplementing α -MEM with 300 mIU/ml FSH; however, on average the number of identifiable follicles is reduced by about one-third over the whole culture period, irrespective of culture conditions. Finally, serum can be effectively replaced with a mixture of human serum albumin (HSA) and ITS (insulin/transferrin/selenium mix). This will allow us in future experi-

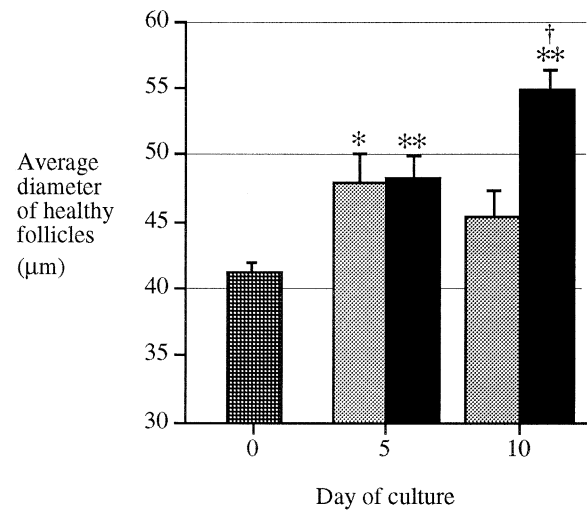


Figure 9. Average diameter of healthy, non-atretic follicles in ovarian cortical tissue at the time of biopsy (control) and after culture in α -MEM media with either 10% human serum \square or HSA + ITS \blacksquare . At day 5, the bars with asterisks mark a significant increase in diameter from day 0 with serum (* $P = 0.001$) and HSA + ITS (** $P = 0.0001$). Bar with asterisks on day 10 marks significant increases in diameter from day 0 and day 5 for HSA + ITS (** $P = 0.0001$), and also that follicles cultured with HSA + ITS are also significantly larger ($\dagger P = 0.0001$) than those cultured with serum.

ments to test growth factor effects without the influence of serum in the media.

A variety of media have been used for the culture of follicles from a number of species. α -MEM, a complex medium, has been successfully used to culture mouse and rat (Eppig and Schroeder, 1989; Carroll *et al.*, 1991; Nayudu and Osborn, 1992; Boland *et al.*, 1993; Spears *et al.*, 1994) bovine (Ralph *et al.*, 1995; Braw Tal and Yossefi, 1997) and human (Abir *et al.*, 1997) follicles. A variation on α -MEM, D-MEM, has also been used to culture human follicles (Roy and Treacy, 1993), and recently MEM has been used in the culture of preantral cat follicles (Jewgenow *et al.*, 1998). Waymouth's, another complex medium, has been used in mouse (Eppig and O'Brien, 1996), cow (Wandji *et al.*, 1996), baboon (Wandji *et al.*, 1997), and fetal human follicle culture (Zhang *et al.*, 1995). Human follicles have also been cultured in EBSS (Hovatta *et al.*, 1997) and Ham's F10 (Wu *et al.*, 1998).

Our experiments using this culture system showed that in tissue cultured in α -MEM, follicle growth was significantly greater and development was improved compared to that seen in follicles cultured with Waymouth's medium and EBSS (Figure 3). α -MEM is a complex medium containing amino acids, vitamins, ribonucleosides, and deoxyribonucleosides, in addition to the usual inorganic salts and energy sources (pyruvate, glucose) of a simple salt solution. It appears that human ovarian tissue requires the metabolic support that these additional components give in culture.

There was an initiation of growth of primordial follicles to primary and secondary stages after 5 days in culture in all the media tested, and indeed in all the experiments performed. This initiation of growth is interesting, since *in vivo* only a small proportion of the primordial follicles would be activated

to begin to grow each day in response to signals which are, as yet, unknown. This growth initiation also occurs in xenografts of human ovary in severe combined immunodeficiency (SCID) mice (Oktay *et al.*, 1998), and in bovine (Wandji *et al.*, 1996; Braw Tal and Yossefi, 1997) and baboon (Wandji *et al.*, 1997) follicles *in vitro*. It may be that on removal of cortical tissue from the ovary, there is a withdrawal of an inhibitory signal (Peters *et al.*, 1973), which allows resting primordial follicles to begin to grow once they are no longer in the normal ovarian environment.

FSH has been used as a media supplement for the culture of follicles from several species. In the mouse addition of FSH to culture media promotes follicle survival and antrum formation in early preantral follicles (Cortvriendt *et al.*, 1997), and follicles cultured from preantral to preovulatory stages (Nayudu and Osborn, 1992; Spears *et al.*, 1994). In the rat preantral follicle *in vitro*, FSH increases growth and viable cell number in combination with cyclic guanosine 3',5'-monophosphate (cGMP) or serum (McGee *et al.*, 1997). In isolated large preantral human follicles, FSH promotes antrum formation and oestrogen production *in vitro* (Abir *et al.*, 1997), and also increases progesterone and androstenedione production (Roy and Treacy, 1993). Furthermore FSH appears to play an important role in follicular atresia. It is known that FSH prevents apoptosis in preantral and antral follicles of mouse (Baker and Spears, 1997). Indeed in rat, FSH appears to be the most important survival factor in early antral follicles (Chun *et al.*, 1996), and will suppress apoptosis in serum-free culture in preantral (McGee *et al.*, 1997), antral (Tilly and Tilly, 1995) and preovulatory (Chun *et al.*, 1994) follicles. In isolated human preantral follicles, FSH was also seen to reduce atresia (Roy and Treacy, 1993).

In our culture system, addition of FSH to the media greatly reduces atresia. It is possible that the main action of FSH is in preventing apoptotic atresia in our cultured follicles, allowing them to survive in culture. However, our results also show that addition of FSH to the culture media significantly increases follicle diameter, so that a mitogenic role for FSH cannot be ruled out. Therefore FSH does have an effect on the growth of primordial, primary and secondary human follicles *in vitro*. Previous observations have implied that FSH is not required *in vivo* until antrum formation. Preovulatory follicles can be induced in the ovaries of women with hypogonadism (who have negligible gonadotrophin levels) 2 weeks after gonadotrophin injection (Santen and Paulsen, 1973), suggesting that preantral follicles may be present. However, ovaries from women with FSH receptor mutation rarely contain follicles past primary stages (Aittomaki *et al.*, 1996). Furthermore, recent studies (Oktay *et al.*, 1998) have shown that human follicles in xenografts require FSH stimulation to progress past early secondary stages. FSH receptor mRNA is present in some primary stage follicles and all follicles from the late secondary stage (Oktay *et al.*, 1997). Therefore human follicles are capable of responding to FSH at early stages both *in vivo* and *in vitro*, and FSH may play a role in follicular development in the ovarian cortex.

Human follicles can also be cultured in this system without addition of serum. HSA and ITS proved to be an effective

substitute for serum and indeed follicle growth later in culture was significantly improved with HSA and ITS. This effect cannot be explained by the possible gonadotrophin content of either the serum or the HSA, as both were negligible. However, ITS provides 10 µg/ml insulin in the media, whereas average levels in media containing just serum were 0.33 ± 0.03 µU/ml, equivalent to 14 ng/ml, about 700-fold lower. Therefore, insulin may be stimulating follicle growth. It has been shown that human granulosa cells respond to insulin by augmenting oestrogen and progesterone production (Erickson *et al.*, 1990; Willis *et al.*, 1996). Insulin also causes cell proliferation in rat granulosa (Peluso *et al.*, 1991) and theca cells (Duleba *et al.*, 1997), and in Chinese hamster ovary cells (Li *et al.*, 1997). The transferrin and selenium present in ITS may also be acting as free-radical scavengers in the media (Roth, 1997), which may affect growth, and are likely to influence atresia. Atresia was reduced in media containing ITS as opposed to that containing serum.

The media which we intend to use in future experiments on the basis of these investigations will consist of α -MEM with FSH, HSA, and ITS supplementation. The culture system can now be used to examine the effects of other growth factors under defined serum-free conditions. However, further optimization of the medium is necessary to obtain maximum growth and minimum atresia of follicles and allow development to antral stages. Once they have reached such stages it may be possible to semi-isolate follicles and grow them further in different culture systems. Immature oocytes could then be removed and matured further in preparation for fertilization. Human oocytes removed from antral follicles (2–15 mm diameter) have been shown to be capable of fertilization, and live births have been reported (Veeck *et al.*, 1983; Cha *et al.*, 1991; Trounson *et al.*, 1994; Barnes *et al.*, 1995; Jaroudi *et al.*, 1997).

The first stage of an IVM system for human follicles has therefore been achieved. Extensive further research is required to achieve full maturation of primordial follicles however. Ovarian biopsy is a relatively simple method of obtaining ovarian tissue and growth of follicles *in vitro* to stages where they can be used for IVF would have major benefits for infertile women, clinicians and scientists. It may be especially important to women who are undergoing procedures likely to result in sterilization such as chemotherapy or radiotherapy (Wood *et al.*, 1997). Ovarian tissue can be removed before treatment begins and cryopreserved until an appropriate time. With IVM there is no known risk of transmission of cancerous cells which could possibly occur if the tissue were to be re-grafted (Gosden *et al.*, 1997; Shaw and Trounson, 1997). Finally, the culture of intact follicles within ovarian stromal tissue provides a unique opportunity to examine the regulation of cell differentiation and follicle growth, particularly at preantral stages.

Acknowledgements

Micro-particle enzyme immunoassay was performed by Steve Verdy and Rob Swan, SAS Endocrinology, Hammersmith Hospital.

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Received on November 16, 1998; accepted on March 8, 1999