A two-step serum-free culture system supports development of human oocytes from primordial follicles in the presence of activin

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BACKGROUND: The objective of this study was to determine whether follicles grown within human ovarian cortical strip culture for 6 days in serum-free medium could be isolated at the secondary stage of pre-antral development and grown in vitro to the late pre-antral/early antral stage during a 4 day culture period. METHODS: Ovarian cortical biopsies were obtained from six women aged 26–40 years, with informed consent, during elective Caesarean section. Small tissue slices of ovarian cortex, with underlying stromal tissue removed, were cultured in serum-free medium for 6 days and at the end of this period pre-antral (secondary) follicles were dissected from the strips. Seventy-four intact pre-antral follicles ranging in size (66–132 μm) (mean size 100 μm ± 3.4) were selected for further culture. Follicles were placed individually within V-shaped microwell culture plates in serum-free medium in the presence (n = 38) or absence (n = 36) of 100 ng/ml of human recombinant activin A. RESULTS: Pre-antral follicles grown for 4 days in the presence of activin A grew to a larger size (mean diameter 143 μm ± 7.4) than those grown in control medium (mean diameter 111 μm ± 8) (P < 0.005). Ninety percent of follicles cultured in the presence of activin A increased in size during the first 2 days of culture compared with only 36% of follicles in control medium (P > 0.005). Of the follicles surviving the entire culture period, 30% of those cultured in the presence of activin A showed normal morphology with intact oocytes and antral formation. None of the follicles grown in control medium developed antral cavities and >90% of those follicles collected at the end of the culture period showed signs of oocyte degeneration. CONCLUSIONS: The results reported here demonstrate that under certain conditions, it is possible to achieve accelerated oocyte/follicle development from human primordial/primary follicles. This provides the first encouraging step towards achieving full in vitro growth of human oocytes.

Keywords: ovarian cortical strips; primordial follicle; preantral follicle; activin; in vitro oocyte culture

Introduction
The limitation in supply of mature fertilizable oocytes represents a major impediment to increasing the success of assisted reproduction techniques (ART), to developing strategies for fertility preservation after chemotherapy and to deriving stem cells in humans. It has long been recognized that the efficiency and efficacy of assisted reproduction programmes could be enhanced by utilizing oocytes from immature follicles for in vitro growth (IVG) and subsequent in vitro maturation (IVM) and these techniques, in combination with cryopreservation, would offer new practical applications for fertility preservation (Thomas et al., 2003a). Primordial follicles represent the most abundant population of oocytes in the ovary at any age (Gosden and Telfer, 1987) and complete growth in culture from this stage with subsequent IVF of oocytes followed by embryo transfer and production of live offspring has been achieved in the mouse (Eppig and O’Brien, 1996; O’Brien et al., 2003).

In species where follicular development in vivo is known to occur over a period of several months (humans and domestic species), in vitro systems that allow the complete development of oocytes contained within primordial follicles have not yet been achieved. Culture systems to support initiation of follicle growth and early development within ovarian cortical strips have been developed (Hovatta et al., 1997, 1999; Wright et al., 1999); however, development to antral stages is inhibited within the cortical strips and it is recognized that isolation of pre-antral follicles is necessary to enable further development and monitoring.

We have developed a serum-free culture system for isolated bovine and ovine pre-antral follicles that maintains their organization and supports oocyte development (McCaffery et al.,
Activin is a member of the TGFβ superfamily and exists as a homo or heterodimer comprising two beta subunits (A or B), with activin A being the predominant activin isoform. Activin is expressed by granulosa cells and oocytes and is known to be involved in pre-antral follicle development (Ethier and Findlay, 2001; Findlay et al., 2002). Systemically activin promotes the release of follicle stimulating hormone (FSH) from the anterior pituitary (Katayama et al., 1990), whereas in the ovary, activin promotes follicle growth by increasing granulosa cell proliferation and enhancing antral formation (Mizunuma et al., 1999; Zhao et al., 2001). Activin-stimulated follicle growth in vitro has been demonstrated in pre-antral ovine follicles (Thomas et al., 2003b), caprine follicles (Silva et al., 2006) and rodent follicles (McGee et al., 2001).

The aims of this study were to determine (i) whether in vitro grown pre-antral follicles could be isolated from human cortical strips cultured in serum-free medium; (ii) whether isolated pre-antral follicles cultured individually could develop in vitro and (iii) whether activin has an effect on growth and development of isolated human pre-antral follicles in vitro.

Materials and Methods

Ovarian material
Ovarian cortical tissue (approximate size 5 × 4 mm, with variable thickness) was obtained by biopsy from consenting patients undergoing elective Caesarean section (n = 6). The mean age of the women was 36.4 ± 5.3 with a range of 26–40 years. The local ethical committee granted approval of this study.

Cortical strip culture
Ovarian tissue was collected, and transported to the laboratory in 10 ml of pre-warmed Leibovitz medium (GIBCO BRL, Life Technologies Ltd., Paisley, Renfrewshire, UK) supplemented with sodium pyruvate (2 mM), glutamine (2 mM), BSA (Fraction V, 3 mg/ml), penicillin G (75 μg/ml), streptomycin (50 μg/ml) and ascorbic acid (50 μg/ml) all obtained from Sigma Chemicals Poole, Dorset, UK. On return to the laboratory, the ovarian biopsy was transferred to fresh Leibovitz medium with supplements and most of the underlying stromal tissue and any visible growing follicles were removed. The cortical ovarian tissue was then cut into small thin slices of ~0.5 mm³. The cut pieces were further pulled mechanically with needles to flatten out the tissue and to minimize the underlying stromal tissue, in preparation for culture. A piece of each biopsy was taken and fixed in 4% paraformaldehyde for histological evaluation whereas the remaining pieces were placed individually in 24-well cell culture plates (Corning B.V. Life Sciences Europe, Amsterdam, The Netherlands) containing 300 μl of McCoy’s 5a medium with bicarbonate supplemented with HEPES (20 mM), BSA (0.1%), glutamine (3 mM), penicillin G (0.1 mg/ml), streptomycin (0.1 mg/ml), transferrin (2.5 μg/ml), selenium (4 ng/ml), insulin (10 ng/ml) and ascorbic acid (50 μg/ml), all obtained from Sigma Chemicals Poole; they were cultured for 6 days at 37°C in humidified air with 5% CO₂ with medium changed every 2 days. After 6 days in culture, growing follicles could be clearly observed within the strips; therefore, it was decided to isolate growing follicles at that time.

A total of 96 cortical strips were cultured and at the end of the culture period, 12 cortical strips (two from each biopsy) were removed and fixed in paraformaldehyde for histological analysis, whereas the remaining tissue was taken for mechanical isolation of pre-antral (secondary) follicles.

Isolation and culture of pre-antral follicles
At the end of the 6 day culture period, cortical strips were transferred to dissection medium [Leibovitz medium (GIBCO BRL, Life Technologies Ltd.) Paisley, Renfrewshire, UK] supplemented with sodium pyruvate (2 mM), glutamine (2 mM), BSA (Fraction V, 3 mg/ml), penicillin G (75 μg/ml), streptomycin (50 μg/ml) and ascorbic acid (50 μg/ml), and pre-antral follicles were dissected from the strips using 25 gauge (25 G) needles following a protocol previously described for bovine follicles (McCaffery et al., 2000). Follicles ranging in size from 66 to 132 μm (mean diameter of 100 μm ± 3.4) with a visible oocyte, an intact basement membrane and no antral cavity were selected for culture. All isolated follicles had adherent theca/stromal cells attached; however, follicle diameter measurements excluded theca layers and refers to the basement membrane enclosed granulosa cell layers only.

Selected follicles were individually placed in 96-well V-bottomed culture plates (Corning Costar Europe, Badhoevedorp, The Netherlands) in 150 μl of culture medium. Isolated follicles with adherent stroma were obtained from each biopsy source, with numbers ranging from 5 to 18 follicles per biopsy, and these were evenly distributed between control and activin groups. Isolated follicles were either cultured in McCoy’s 5a medium with bicarbonate supplemented with HEPES (20 mM), BSA (0.1%), glutamine (3 mM), penicillin G (0.1 mg/ml), streptomycin (0.1 mg/ml), transferrin (2.5 μg/ml), selenium (4 ng/ml), insulin (10 ng/ml) and ascorbic acid (50 μg/ml), all obtained from Sigma Chemicals Poole or medium supplemented with100 ng/ml of activin A (rhAct A) (R&D Systems, Abingdon, UK) (n = 38). Isolated pre-antral follicles were incubated for 4 days at 37°C in humidified air with 5% CO₂. On Days 2 and 4 of culture, follicle diameters were measured using a dissecting microscope with a crossed micrometer and on Day 2, half the culture medium was removed and replaced with fresh medium. Spent medium from each follicle at Days 2 and 4 was stored at −20°C for subsequent estradiol analysis.

Detection of estradiol in culture medium
Concentrations of estradiol in culture media from isolated follicles that had been grown for 2 and 4 days in the presence or absence of activin, and had shown an increase in diameter at day 2, were determined using an ELISA kit (DRG Instruments, GmbH, Germany) as previously described (Murray et al., 1998). The sensitivity of the assay was 15 pg/ml, and the intra-assay coefficient of variation was <5%.

Histological analysis
At the end of the culture period, isolated follicles/cortical strips taken before and after culture were fixed overnight in a 4% paraformaldehyde solution and dehydrated in ethanol (70%, 90% and 100%). Absolute ethanol was replaced with cedar wood oil for a minimum of 24 h, then the oil was cleared using toluene for 30 min. Individual
Follicles were embedded in paraffin wax (60°C), with changes every hour for 4 h to remove all traces of toluene. The samples were sectioned (6 μm) and mounted on charged slides then allowed to dry overnight at 37°C before staining with haematoxylin and eosin.

**Histological evaluation**

**Cortical strips**

Every section of every strip collected for histology was examined by light microscopy. Follicles were classified according to their stage of development based on granulosa cell morphology as follows: (1) primordial stage: oocyte surrounded by a few flattened granulosa cells; (2) transitory stage: oocyte surrounded by flattened and at least one cuboidal granulosa cell; (3) primary stage: oocyte surrounded by a complete layer of cuboidal granulosa cells; (4) secondary stage: oocyte surrounded by two or more complete layers of cuboidal granulosa cells and (5) antral stage: presence of antral cavity within multilaminar follicle.

Follicles were counted and classified when the oocyte nucleolus was present to avoid double counting. Data were expressed as total proportion of stages present before and after a 6 day culture period. Comparisons were made by chi-squared analysis.

**Isolated follicles**

Histological measurements and observations were made under the light microscope with a crossed micrometer (Graticules Ltd.). The section containing the oocyte nucleolus, or if this was absent, the largest cross-section of the oocyte was used for observations and measurements. Follicle and oocyte diameters were measured, and granulosa cell death within a follicle was assessed by counting the number of pyknotic nuclei and expressing them as a percentage of the total number of granulosa cells. An index of health/degeneration from type 1 (healthy) to type 4 (completely degenerate) was used to classify the follicles as follows: type 1: intact oocyte (regular shape and even cytoplasm) in contact with a complete layer of granulosa cells and <10% pyknotic granulosa cells present; type 2: misshapen oocyte still surrounded by granulosa cells with >10% pyknotic granulosa cells; type 3: oocyte dissociated from granulosa cells with >10% pyknotic granulosa cells; type 4: fragmented oocyte with >10% pyknotic granulosa cells.

**Statistical analysis**

Mean values of measurements and estradiol levels were compared using a two sample t-test and proportion data were analysed by chi-squared testing. P-values of <0.05 were considered significant.

**Results**

**Cultured cortical strips**

Histological sections of freshly isolated cortical strips showed that 90% of healthy follicles counted (total of 233) were at the primordial or transitory stage, whereas the remainder were at the primary stage of development (Fig. 1). Degenerating/atretic follicles accounted for 8% of the total follicle population observed at Day 0 and 14% at Day 6. After 6 days in culture, follicles had developed from the primordial/transitory stage since these stages accounted for only 60% of the healthy follicles counted in these samples (total of 162) and 15% (24) of healthy follicles had 2–4 layers of granulosa cells (Fig. 1). The appearance of growing follicles became apparent during the culture period and could be observed microscopically as raised swellings on the surface of some of the cultured strips.

**Figure 1:** Histogram showing the proportion of follicles at the primordial (black bars), transitory (grey bars), primary (white bars) and secondary (hatched bars) stages of development in histological samples taken from six ovarian cortical biopsies at time of collection (Day 0) and after 6 days of culture in serum-free medium (Day 6) (i.e. Step 1 of the two-step culture system). A total of 12 samples were examined for each time point with two samples from each biopsy. Counts are expressed as a total proportion from the pooled material, a total of 233 healthy follicles were counted in Day 0 material and 162 at Day 6. A significantly lower proportion of primordial follicles was found after 6 days compared with the fresh material (P < 0.005) and significantly higher proportions of both primary and secondary follicles were found in Day 6 cultured samples compared with the fresh material. *Significant differences at P < 0.005 using a chi-squared analysis

**Figure 2:** Photomicrographs of ovarian cortical tissue during step one of culture. (A) A piece of ovarian cortex after 2 days *in vitro* in serum-free medium (Step 1) as viewed under the dissecting microscope. Several follicular swellings on the surface can be observed at this time; scale bar (in white) represents 100 μm. (B) Histological section of cultured piece of ovarian cortex, after 6 days *in vitro* and stained with haematoxylin and eosin, showing an intact healthy secondary follicle; scale bar represents 25 μm
indicating follicular growth (Fig. 2A). Growing follicles within the cultured strips were shown to have intact oocytes and healthy granulosa cells after a 6 day culture period (Fig. 2B). No antral follicles were observed in freshly isolated or cultured strips.

**Growth of isolated pre-antral follicles**

From a total of 84 cortical strip pieces removed after 6 days of culture in serum-free medium, 74 pre-antral follicles within the size range of 66–132 μm were isolated. Follicles were obtained from each biopsy source but numbers varied (5–18 per biopsy). Isolated follicles were cultured for 4 days in the presence (n = 38) or absence (n = 36) of activin A in serum-free medium, with measurement of follicle diameters being made every second day. A significant increase in mean follicle diameter was observed in isolated follicles cultured in the presence of activin for 4 days (P < 0.05) with most of the growth taking place during the first 2 days (Fig. 3). Individual follicles cultured in control medium did show an increase in diameter over the culture period but no statistically significant difference was shown in mean follicle diameter during the first 2 days of culture (Fig. 3). The difference observed in mean follicle diameter between follicles grown in control medium and medium containing activin is due to a lower proportion of follicles actually starting to grow in control medium compared with those in the presence of activin. During the first 2 days of culture, 36% of the follicles cultured in control medium showed an increase in diameter compared with 90% of follicles grown in the presence of activin (Fig. 4A), with this proportion decreasing to 58% between Day 2 and Day 4 in the activin-treated group (Fig. 4B).

**Estradiol secretion**

Follicles that increased in size during the first 2 days of culture produced significant levels of estradiol at Days 2 and 4 in the presence or absence of activin (Fig. 5). Follicles grown in the presence of activin produced significantly higher levels (P < 0.01) of estradiol at Days 2 and 4 when compared with those in control medium (Fig. 5). However, levels of estradiol appeared to decline in both groups by Day 4 (P < 0.05) (Fig. 5).

**Follicular health and morphology**

Some follicles became fragile during culture and could not be monitored for the full period; however, in this respect, there were no differences found between control and activin-treated follicles (12 in each group). Differences were found in the health of follicles grown in control or activin media with more healthy follicles being found in medium containing activin.
activin (Fig. 6). Eighty percent of the isolated follicles grown in control medium were classified as either type 3 or type 4 degenerate by Day 4 (i.e. a total of 10 days in vitro, 6 in the strip culture and 4 in isolated culture). Those follicles grown in the presence of activin for 4 days showed significantly less degeneration at the end of the culture period (Fig. 6) with some follicles forming antral cavities and containing intact oocytes that had shown significant growth (Fig. 7A and B). In contrast, follicles grown in control medium were significantly smaller and showed a higher level of granulosa cell death and oocyte degeneration (Fig. 7C and D). The mean oocyte size from histological sections was 31.9 μm ± 1.57 for follicles grown in control medium whereas those follicles grown for 6 days in cortical strips and then a further 4 days as isolated follicles in the presence of activin showed a mean oocyte diameter of 51.33 μm ± 4.2, with the largest antral follicle having an oocyte measuring 60.5 μm.

Discussion

This study has demonstrated that human pre-antral follicles grown in vitro from primordial or early primary stages, within human ovarian cortical strips, can be isolated and have the potential to grow to the antral stage of development at an accelerated rate in the presence of activin. This is the first time that individual follicles grown from cultured human cortical strips have been isolated for further development as previous studies have focused on isolating groups of follicles (Hovatta et al., 1999). Work on rodent ovaries has shown that development from the primordial stage to maturity in vitro can be obtained using a two-step culture system (Eppig and O’Brien, 1996; O’Brien et al., 2003) and using this system pups have been born. In this study, a two-step culture system was utilized and the results obtained provide proof of concept that with modification and optimization of culture conditions, it may be possible to fully develop human oocytes in vitro.

In the mouse two-step culture system, primordial follicles are grown within organ culture of whole ovaries from new born mice. This method is prohibitive in species with large ovaries but the technique of growing primordial/primary follicles within ovarian tissue slices has been well established in humans (Hovatta et al., 1997, 1999). In this study, immature follicles were grown within mechanically loosened cortical pieces for 6 days and follicles developed to pre-antral/secondary stages. The conditions under which the strips were grown differed from those described in previous studies (Hovatta et al., 1997, 1999; Wright et al., 1999) as no serum was present and strips were not cultured on a supported matrix. Most of the underlying stromal tissue was removed from biopsy material so that the cultured pieces consisted predominantly of ovarian cortical tissue which contained only primordial and primary follicles. Under these conditions, follicles were activated to grow and growth to the secondary stage occurred within 6 days and the health of oocytes and somatic cells was maintained in the growing follicles. This relatively short culture period may provide the best strategy for obtaining healthy growing follicles for further development since in this system there appears to be a synchronization of follicle development, whereas in prolonged culture a more heterogeneous population of healthy and unhealthy follicles will be present (Hreinsson et al., 2002; Zhang et al., 2002) and a higher proportion will already be atretic (Webber et al., 2007).

Growing follicles could be observed microscopically in several cultured pieces within 2 days; however, it was difficult to isolate all of the follicles present at Day 6 because of the clustering of follicles within tightly packed stromal cells. Despite this, we were able to obtain a good number of intact pre-antral follicles by mechanical isolation for further culture from a relatively small amount of ovarian material (74 intact pre-antral follicles from six biopsies). Previous studies have mechanically isolated pre-antral follicles from freshly obtained human ovarian biopsies and these showed a
high rate of oocyte degeneration (Abir et al., 1997). This may indicate that already degenerating follicles have been selected, whereas by isolating from the cultured strips we are selecting those follicles that have been initiated to grow in vitro and will not have been subjected to the potential inhibitory influences of serum. The isolation of human pre-antral follicles has also been achieved by enzymatic dissociation of fresh or cryopreserved ovarian tissue (Abir et al., 2001) using modified protocols that have enabled large scale isolation of pre-antral follicles from murine material (Eppig and Schroeder, 1989; Eppig and Telfer, 1993; Telfer et al., 1990). We have found that enzymes are deleterious to bovine oocytes (Telfer, 1996; Telfer et al., 2000) and that follicles isolated without them are more capable of further growth and development in vitro (McCaffery et al., 2000). In addition to avoiding potentially damaging effects of enzymatic treatment, the mechanical isolation method has an advantage in that the follicle retains a theca cell component which has previously been associated with the maintenance of health and differentiation of in vitro grown bovine pre-antral follicles through steroid biosynthesis and the production of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) that are important for tissue re-modelling and basement membrane turnover (McCaffery et al., 2000; Thomas et al., 2001).

In this system, we were able to achieve significant oocyte growth and antral formation within a total of 10 days in vitro (6 days within cortical strips and 4 days as isolated follicles) when activin was present in the medium during culture of isolated follicles. We have previously demonstrated that activin A enhances oocyte development in cultured ovine pre-antral follicles at a concentration of 100 ng/ml (Thomas et al., 2003b). Activins are disulphide-linked dimeric glycoproteins, and the subunits inhibin α, inhibin/activin βA and inhibin/activin βB, together with the activin receptors type I, IIA and IIB are expressed in ovarian cells during follicular development in human ovaries (Roberts et al., 1993; Cameron et al., 1994). Activin A is composed of two βA subunits, and there is growing evidence of its role during folliculogenesis (Ethier and Findlay, 2001); however, the exact stages of development that are regulated by activin is unclear, and this appears to be both species and stage dependent with follicle growth being observed in immature but not mature murine pre-antral follicles in the presence of activin (Liu et al., 1998; Mizunuma et al., 1999). Studies using tissue-specific knockout mice have demonstrated that activins regulate granulosa cell growth and differentiation during follicle development in vivo (Pangas et al., 2007) and activin has been shown to increase the size of pre-antral follicles and promote antrum formation in immature mice (Yokota et al., 1997; Zhao et al., 2001). Activin or activin receptors have not been localized in human primordial or primary follicles but activin type II receptor has been localized in human pre-antral/secondary follicles (Pangas et al., 2002). In this study, we found that only isolated follicles grown in the presence of activin maintained a healthy oocyte and formed an antral cavity. The effect of activin in promoting follicle survival and growth was most dramatic during the initial 2 day culture period of isolated follicles, when 90%
of follicles were capable of growth in the presence of activin compared with only 36% in control medium. Between 2 and 4 days, 58% of follicles continued to grow in the presence of activin, and this reduced effect may be an indication of the necessity for the addition of factors such as FSH later in the culture period.

The growing isolated follicles produced estradiol in both control and activin supplemented medium at levels previously shown in cultured human antral follicles (Abir et al., 1997), indicating that significant granulosa cell differentiation had taken place but it was only in the activin-treated group that this was co-ordinated with antral formation and oocyte development. Estradiol levels were higher in the activin-treated group after 2 days and this reflects the larger size of these follicles rather than the health status of the follicle unit. Estradiol production is not predictive of follicle/oocyte quality since similar levels were measured in follicles that were morphologically degenerate after 4 days in both control and activin treatments.

The rate of oocyte and follicle development achieved in this study was at an accelerated rate taking 10 days to reach antral stage of development from a starting point of either primordial or primary stages when activin was present during the second part of the culture procedure. During follicle development in vivo, the human oocyte increases in size from ~20 to 110 μm and as with other species there is a relationship between oocyte size and developmental competence (Cavilla et al., 2001). The maximum diameter achieved from in vitro grown oocytes in this study was 60.5 μm and this represents a massive increase in oocyte cytoplasmic volume during a relatively short period of time. Human follicle development has always been assumed to take months in vivo (Gougeon, 1986), but it is clear that in vitro this process can be accelerated, indeed antral formation and mature oocytes have been obtained from culturing pig pre-antral follicles for as little as 4 days (Wu et al., 2001) and accelerated development to the antral stage has been observed in cultured bovine and ovine follicles (Cecconi et al., 1999; Walters et al., 2006). Indeed, the rate of follicular development when unimpeded by local inhibitory influences is unknown; however, recent studies where new follicle formation and growth has been demonstrated after bone marrow transplantation indicate that the rate of follicle development can be dramatically shortened within a perturbed system (Johnson et al., 2005). The question is whether this is normal development and this study cannot yet answer that, except that morphologically the oocyte and somatic cells appear intact. Whether normal processes such as epigenetic modifications are taking place would have to be investigated further.

The ability to culture oocytes from the relatively abundant primordial stage would provide a research tool to study the complex process of oocyte development in humans and eventually these techniques could be applied in fertility preservation programmes. This study has shown that accelerated development of human primordial follicles to the antral stage is achievable, but the system reported here requires improvement, the challenge now is to develop further culture steps and to optimize timings of exposure to key factors such as activin and FSH to enable further oocyte development. Our results suggest that complete development of human primordial follicles to a stage where the oocyte can be matured in vitro could be achieved within a multi-step culture system that supports the changing requirements of the oocyte. This work raises the possibility of bridging the gap between IVG of follicles and IVM of oocytes to obtain developmentally competent oocytes.

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