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In vitro culture of ovarian follicles

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This review offers a practically oriented introduction to follicle culture *in vitro*, focusing on mouse follicles, but with reference to other species. The main principles of follicle growth are addressed, including the constraints of tissue culture, methods of follicle isolation, and techniques for individual and collective culture of intact follicles. Culture systems that support a spherical or a non-spherical follicular structure *in vitro* are discussed in terms of follicular and oocyte development, and methods for assessing follicular function *in vitro* are presented. Oocyte development in most *in vitro* culture systems is currently suboptimal and the parallel development of oocytes and follicles is discussed, with a view to maintaining the competence of the oocyte. Finally, some potential future applications of follicle growth *in vitro* are suggested.

Small follicles that are isolated from the ovary and cultured *in vitro* are capable of an extraordinary degree of further development, including substantial growth, oocyte maturation and ovulation. Within the basement membrane surrounding the follicle, the cells interact via various mechanisms including gap junctions, which enable the follicle to operate as a functional unit. The oocyte relies upon the granulosa cells for its survival and in turn it promotes granulosa cell proliferation (Vanderhyden *et al*., 1992). As the follicle grows and becomes antral, subpopulations of granulosa cells are distinguishable according to their location, their density and their specialized responses to, for example, gonadotrophins and epidermal growth factor (EGF) (Amsterdam *et al*., 1975; Kasson *et al*., 1985; Hartshorne 1990). Surrounding the basement membrane, other cells specialize to form the theca interna and externa layers.

Most follicles fail before ovulation, and the largest numbers are lost early in life. At birth, more potential oocytes have degenerated than remain in the ovary; moreover, most of the follicles surviving at birth degenerate even before puberty. Very few, highly selected follicles will reach ovulation. The ovarian follicle management system is therefore highly inefficient and has evolved for intense competition between gametes.

The factors that initiate the growth of primordial follicles are currently unknown, although the size of the remaining stockpile influences the rate at which follicles enter the growing pool in rats (Hirshfield, 1994) and, in cattle, the ovarian stroma may have a restraining influence (Wandji *et al*., 1996). The rate of oocyte utilization in humans is biphasic, relating to the age of the woman (Gougeon and Chainy, 1987; Faddy *et al*., 1992), and evidence in mice suggests that the order in which follicles begin to grow may be programmed even as they are formed in the fetal ovary (Henderson and Edwards, 1968; Polani and Crolla, 1991).

The time for a follicle to grow to preovulatory size seems consistent for a particular species. In mice, follicle growth takes approximately 16 days (Pedersen, 1970) and in humans, about 85 days from primary follicle onwards (Gougeon, 1990). The follicle grows as a result of granulosa cell mitosis which accelerates as the follicle grows larger and is accentuated by the accumulation of fluid in the antrum (see Monniaux *et al*., 1994).

In vivo, successful follicles must have reached an appropriate size and become highly sensitive to gonadotrophins. Stimulation with exogenous gonadotrophins supports the final stages of development and ovulation of more follicles than would occur naturally, yet only those follicles that have reached 2–5 mm in diameter in humans by the early follicular phase are affected (Gougeon, 1990).

Overview of follicle culture

Follicle culture is an experimental technique designed to isolate intact follicles from systemic influences so that their metabolism can be examined scientifically. Various approaches have been used depending upon the types of follicle studied and the endpoint required. A selection is shown diagrammatically (Box 1). The methods adopted have included short-term culture, not anticipating the survival of the follicles at the end of the experiment (for example, Waraksa *et al*., 1995) and organ or fragment culture, in which groups of follicles are cultured together (for example, Baker and Neal, 1973; Qvist *et al*., 1990; Eppig and O'Brien, 1996). Perfusion of ovaries or perifusion *in vitro* may extend the viable lifespan of follicles.

Ovaries of newborn mice, which contain only primordial follicles, can be cultured intact for 8 days, producing preantral follicles with two layers of granulosa cells, the oocytes of which will subsequently develop normally in a collective *in vitro* culture system (Eppig and O'Brien, 1996). Mouse follicles with two granulosa cell layers and an incipient thecal layer can also be cultured individually to preovulatory stages (Nayudu and Osborn, 1992) and some of them will ovulate *in vitro* (Boland *et al*., 1993). It therefore seems feasible that an appropriate combination of methods will permit follicle growth from primordial to postovulatory stages *in vitro*.

Methods for the long-term culture of follicles were developed in mice, but are becoming more widely used in other species including rats, cattle, pigs and humans (Roy and Treacy, 1993; Hirao *et al*., 1994; Smyth *et al*., 1994; Abir *et al*., 1995; Cain *et al*., 1995; Ralph *et al*., 1995). The follicle architecture is broadly similar in these species, but the eventual diameter of mature intact

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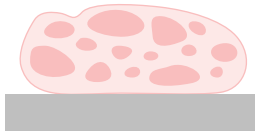
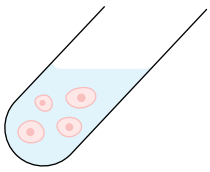
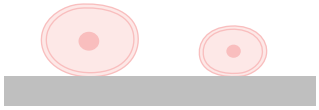
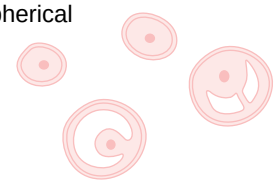
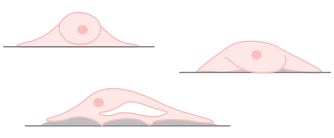
Box 1. Various approaches to follicle culture. The different methods of culture may also be used sequentially if necessary, for example, organ culture followed by individual follicle culture.

Method		Duration	Examples of uses	Species	Example references
Organ culture (+/– perifusion or perfusion)		Usually short because of necrosis unless ovary is very small	Blood flow effects on ovulation, culture of immature whole ovaries	Rabbit, rat, mouse	Bonello *et al.*, 1996; Eppig and O'Brien, 1996
Short-term		<72 h	Steroid production, endocrine responses. (Usually large follicles.)	Hamster, sheep, rabbit	Kitzmann and Hutz, 1992; Tambe and Nandedkar, 1993; Thébault *et al.*, 1983
Group culture	Gel	~7 days	Observation of follicle growth, endocrine studies, preparation for transplantation	Mouse, human, cattle	Carroll *et al.*, 1991a; Roy and Treacy, 1993; Hulshof *et al.*, 1995
	Contact	6–12 days	Interfollicular communication	Mouse	Qvist *et al.*, 1990; Spears *et al.*, 1996
	No contact 		Interfollicular communication	Mouse	Spears *et al.*, 1996
	Non-spherical 	7–9 days	Production of large numbers of oocytes, follicular endocrinology	Mouse, rat	Eppig, 1992; Cain *et al.*, 1995
Individual culture	Spherical 	4–16 days	Follicle metabolism, steroidogenesis and oocyte development	Mouse, rat, pig	Boland *et al.*, 1993; Hartshorne *et al.*, 1994a; Hirao *et al.*, 1994; Smythe *et al.*, 1994
	Non-spherical 	10–16 days	Hormonal influences, oocyte development	Mouse	Cortvindt *et al.*, 1996, in press; Smitz *et al.*, in press.

follicles varies widely. The size that follicles can achieve *in vitro* has physical constraints, including limited diffusion gradients and the absence of the rich blood supply to the theca; however, the interior of follicles is calculated to be quite hypoxic, even *in vivo* (Gosden and Byatt-Smith, 1986; Boland *et al*., 1994a), so follicular oocytes may be quite tolerant of *in vitro* conditions.

The final arbiter of successful follicle growth *in vitro* is the eventual developmental competence of the oocyte. As a follicle initiates growth *in vivo*, its oocyte grows in a tightly controlled manner (Gougeon and Chainy, 1987). During this phase, mitochondria and other organelles are affected, cortical granules develop and the oocyte stores messenger RNA which is essential for its later maturation to a competent gamete. The oocyte reaches full size before the follicle, which continues to grow, its organization and specialization of cells continuing.

In vivo, follicle size is correlated with the developmental potential of the oocyte. The larger the follicle, the more likely it is that the oocyte can undergo germinal vesicle breakdown, fertilization, cleavage, blastocyst formation and implantation. These abilities are acquired sequentially during follicle growth (Tsuji *et al*., 1985; Pavlok *et al*., 1992; Schramm *et al*., 1993).

Since the full size of an intact human follicle exceeds 20 mm in diameter, it is unlikely that such could be grown intact *in vitro*. Alternative approaches will therefore be essential. The method adopted by Cain *et al.* (1995) for the culture of rat primordial and primary ovarian follicles uses a substrate-adherent system, which does not support the spherical structure of the follicles, yet results in a functional entity capable of appropriate endocrine responses and ovulation. Cortvrindt *et al.* (1996, in press) have shown that similar methods used for mouse follicles yield oocytes capable of fertilization and embryo development to hatched blastocysts *in vitro*. This method may offer an opportunity to maintain follicles beyond the limits of intact culture. Perhaps small but mature follicles could be produced by artificially manipulating their hormone environment since granulosa cell proliferation and steroidogenesis can be uncoupled (Monniaux *et al*., 1994). For example, lack of gonadotrophins does not influence the growth of mouse oocytes (Carroll *et al*., 1991a), antigonadotrophins can inhibit mouse follicle growth without reducing oocyte enlargement (Eshkol *et al*., 1970) and egg growth can be accelerated *in vitro* by kit ligand (Packer *et al*., 1994). It may even be possible to reform follicles from monolayers of ovarian cells. Li *et al.* (1995) have used activin A together with FSH to stimulate the formation of follicle-like structures *in vitro* from monolayers derived from rat primary follicles. Whether these methods can be adapted to permit follicle size to be manipulated at will, while retaining normal oocyte development, is currently unknown.

Methods of follicle culture

Follicle isolation

Small follicles are readily dissociated from the ovaries of many small mammals by enzyme action, usually collagenase assisted by DNase. This is also successful for soft tissues, such as fetal or juvenile ovarian samples, which contain large numbers of small follicles; however, enzymatic dissociation of follicles from coarser tissue, such as adult human ovaries, is less easily accomplished. Extended exposure to the enzyme (1 h at 37°C followed by 36 h at 4°C) may produce 140 follicles per ovarian biopsy, but even this is unsuccessful in women approaching the menopause and those with dense stroma, for example having polycystic ovaries (Roy and Treacy, 1993). Others have reported fewer follicles collected with this treatment (Thomas and Shaw, 1995).

Enzyme treatment isolates large numbers of small follicles that lack theca and the basement membrane may be damaged. These follicles often grow poorly or lose their structure *in vitro*. Examples of non-spherical follicles growing in Matrigel after dissociation using collagenase are shown (Fig. 1). Enzyme-dispersed follicles are normally cultured in groups, encased in a gel of, for example collagen or agar (Torrance *et al*., 1989; Roy and Treacy 1993: Hirao *et al*., 1994). Mouse follicles embedded in collagen do not grow beyond preantral stages *in vitro* in the absence of FSH; however, transplantation of follicle-populated plasma clots to sterilized recipient mice restores ovarian function for a time (Gosden, 1990). Basement membrane (Matrigel) rarely supports normal development of mouse follicles (G. M. Hartshorne and C. Clark, unpublished); however, pig follicles cultured in a similar system undergo antrum formation (Hirao *et al*., 1994). Gels of agar support the initial stages of antrum formation when human preantral follicles < 220 μm diameter are cultured for 5 days (Roy and Treacy, 1993).

The effects of dissociating enzymes upon cell surface receptors and other molecules are not known. Physical methods for isolating individual follicles have the disadvantage that smaller numbers can be collected; however, enzyme exposure is avoided, ensuring an intact theca layer. Many manually isolated follicles retain their characteristic structure when cultured individually *in vitro*. The theca is important in sustaining spherical development *in vitro* in the absence of a supporting gel, and also promotes antrum formation (Qvist *et al*., 1990; Gosden *et al*., 1993) as well as having biochemical effects on oocyte development, almost certainly mediated via the granulosa cells (Kotsuji *et al*., 1994; Richard and Sirard, 1996). Small follicles can be isolated readily from quartered mouse ovaries under a microscope. For animals with larger, more fibrous ovaries, it is easier to make slices of the tissue with a scalpel before attempting manual dissection. The slices can be transilluminated so that the location of small intact follicles can be ascertained in advance (Gosden *et al*., 1993). This method is successful in human ovarian biopsies, although very difficult if the woman has polycystic ovaries due to the extremely dense stroma characteristic of this condition. The advantages and disadvantages of enzymic and manual dissociation are presented (Box 2).

Follicle culture techniques

Multiple follicle culture. Culture of ovarian fragments or whole ovaries produces a complex system where viable follicles of different sizes and stages of maturity coexist with atretic follicles and various extrafollicular cell types. This complexity resembles the ovary *in vivo*, and may be used to study hormone responsiveness and ovulation. However, interpretation of the local biochemical and paracrine control pathways is difficult using this approach. Degeneration of tissues owing to inadequate oxygenation is a problem in larger tissue fragments. This approach is successful for newborn mouse ovaries because

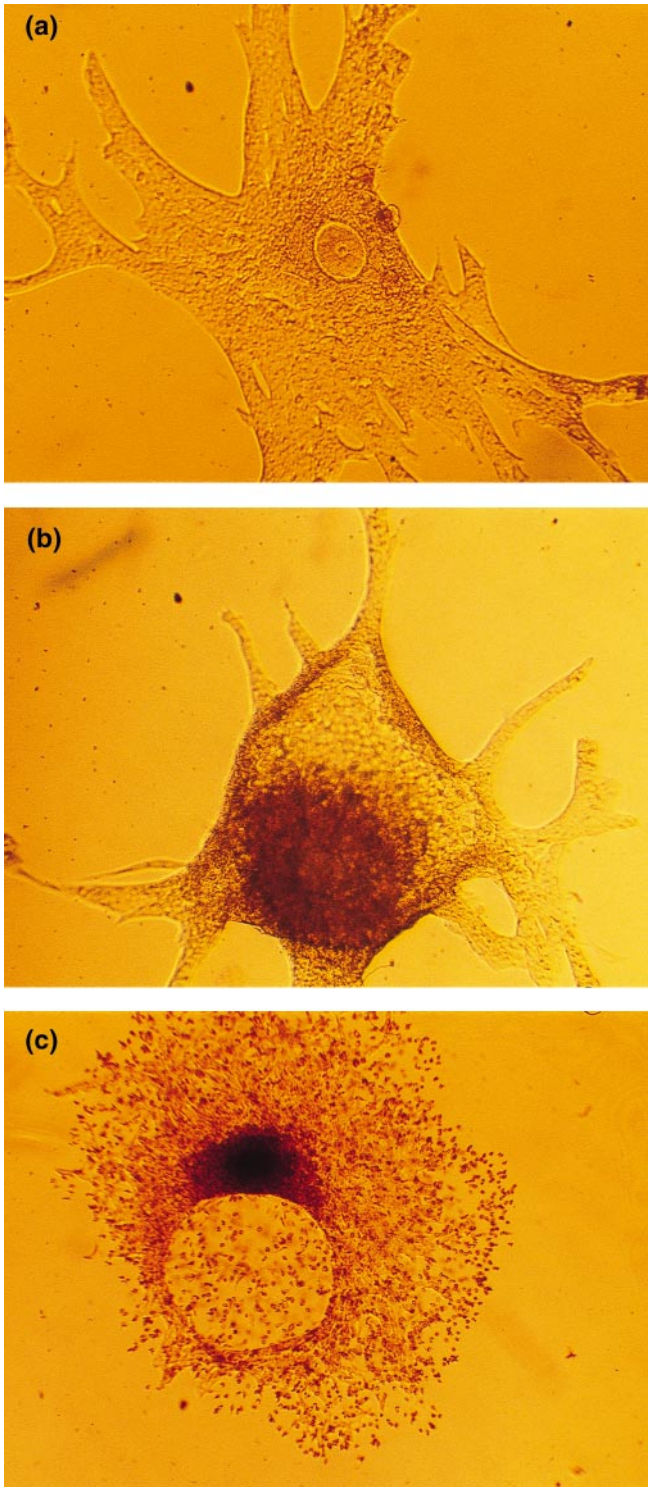


Fig. 1. Three examples of follicles that have lost their normal structure growing in culture. These follicles were isolated enzymatically and cultured for 5–10 days, embedded in 50% Matrigel in Minimal Essential Medium alpha, containing 5% mouse serum. (a) Flattened follicle, oocyte with germinal vesicle is visible. (b) Irregular shaped, possibly antral follicle containing oocyte closely surrounded by granulosa cells. (c) 'Inside-out' follicle appearance. Granulosa cells are multiplying rapidly and migrating into the gel. Magnification × 100.

Box 2. Summary of the advantages and disadvantages of enzymic and manual preparation of follicles.

	Advantages	Disadvantages
Enzyme dissociation	Produces many small follicles Very effective in soft tissues	Unsuitable for large follicles Removes theca and may damage basement membrane May affect surface molecules
Manual dissection	Retains the theca and avoids damage to the basement membrane Suitable for large and small follicles Possible, although difficult, for fibrous tissue	Relatively small numbers can be isolated
Combination	A method combining digestion and dissection may help for fibrous tissues	

of their small size and the uniformity of the small follicles within, which have lower energy requirements and hence suffer less from hypoxia (Eppig and O'Brien, 1996).

Multiple follicle cultures may also be set up by placing several individual follicles into the same vessel, either in large numbers to simulate populations, or in smaller controlled groups, with or without physical contact between them. This offers the opportunity to study interfollicular communication and control. Separated follicles have a tendency to reaggregate *in vitro* and the rates of growth of initially similar follicles may vary (see Fig. 2). Most reports have concluded that follicles cocultured without contact between them grow independently *in vitro*, while differences were observed when follicles were cultured in contact (sheep: Driancourt, 1994; mice: Spears *et al.*, 1996). However, Nayudu and Osborn (1992) noted interactions among follicles cultured in groups larger than two, but without contact. Any factor directly mediating the influence of one follicle upon another is therefore unlikely to travel over distances by diffusion.

Individual follicle culture. Individual ovarian follicles of juvenile mice can be cultured as spherical units from shortly after theca formation (~175 μm) to ovulation *in vitro* (Nayudu and Osborn, 1992; Boland *et al.*, 1993), a period of about 6 days (Fig. 3). Follicles selected for culture have already started their growth phase having progressed from the primordial to the early preantral stages *in vivo* and having some thecal or stromal cells attached (Fig. 3a). Follicles with any irregularities of development, for example, a non-spherical or non-central oocyte, or with evidence of atresia, for example, a dark granulosa layer, are avoided. The largest follicles grown in this system reach

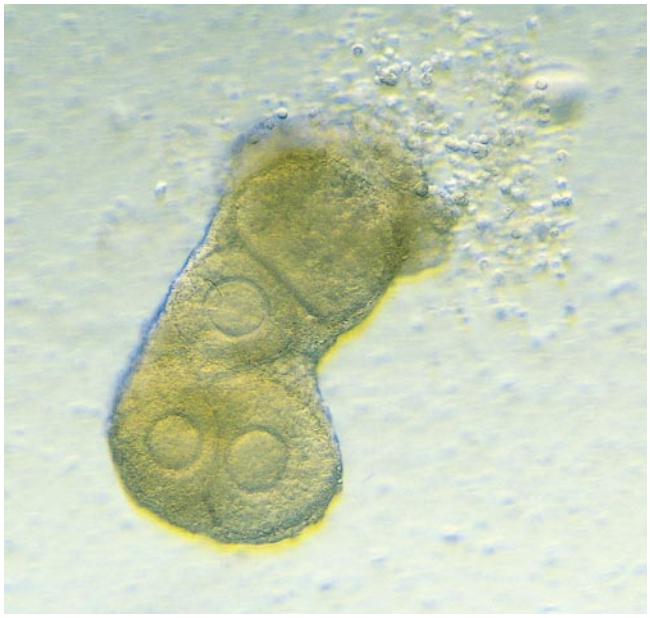


Fig. 2. Cluster of five follicles formed *in vitro* after coincubation of individual theca-free follicles isolated manually. The follicles were approximately 150 μm in diameter at the initiation of culture and were cultured for 5 days on a permeable membrane. On day 5, the follicles were of various sizes: one was atretic (out of focus) and one of the follicles had ovulated (top of figure).

approximately 500 μm in diameter, perhaps slightly smaller than preovulatory follicles *in vivo* (Hartshorne *et al.*, 1994a).

When individual follicles are placed *in vitro*, a proportion grow abnormally because of damage during isolation (about 20%, Hartshorne *et al.*, 1994b; Spears *et al.*, 1994), and some others become atretic during culture. However, the proportion succeeding in antrum formation (Fig. 3c) and subsequent development (about 40–75%) exceeds the anticipated proportion *in vivo* in mice aged 28 days (19 of 94, about 20%; Pedersen, 1970). Gonadotrophins in the culture medium further enhance follicle growth and survival to later stages and show that the follicles are responsive to external stimulation. Dissociation of follicles may therefore be one means of increasing the yield of healthy oocytes and follicles from ovaries under experimental conditions, although it is improbable that follicles already showing signs of atresia can be rescued by culture *in vitro* (Hirshfield, 1989).

Dissociation of follicles from each other may also affect their ability to control the timing of their growth initiation (Gosden, 1990; Spears *et al.*, 1996), yet when the integrity of the ovary of newborn mouse ovaries is maintained *in vitro*, follicles in the medullary region start to grow first, as *in vivo*, showing the local spatial control of this aspect of development (Eppig and O'Brien, 1996).

Follicles *in vivo* are closely surrounded by the complex extrafollicular milieu, including ovarian stroma and thecal cells at various stages of differentiation, branches of the systemic circulation, nervous system and scavenger cells. We are only just beginning to appreciate the influence these cells may exert upon events inside the follicle (for example, bovine thecal cells reversibly inhibit maturation of oocyte–cumulus complexes *in vitro*; Kotsuji *et al.*, 1994; Richard and Sirard, 1996), but the mechanisms for this are not clear. Similarly, isolated ovarian follicles of rabbits will not ovulate *in vitro* without the presence of ovarian tissue in coculture (Thébault *et al.*, 1983). Optimizing culture conditions for the cells surrounding the basement membrane may be rewarding in terms of improved follicular function *in vitro*.

Non-spherical follicle culture. Some methods permit interaction between follicular cells and the oocyte but do not retain the normal follicular architecture, for example, collagenase-dissociated small follicles can be plated onto a porous membrane, retaining some granulosa cells around the oocyte (Eppig, 1992). The oocyte, cumulus and granulosa cells probably communicate via gap junctions and other physical contacts that are essential for a normal rate of oocyte growth *in vitro* (see Wassarman, 1996). The oocyte is raised above the culture surface inside a 'pillar' of cells and remains closely surrounded by granulosa cells. This method can produce abundant fertile oocytes.

In addition, cumulus–oocyte complexes can be cultured on a monolayer of granulosa cells separated from a monolayer of thecal cells by an artificial basement membrane (Kotsuji *et al.*, 1994). Such culture techniques facilitate the study of oocytes, including *in vitro* maturation, since many oocytes can be cultured together and remain easily accessible, while retaining the influence of follicular cells.

Individual follicles can also be cultured in a non-spherical form. Manually dissected preantral mouse follicles placed in a flat tissue culture dish initially adhere to thecal cells migrating onto the surface of the dish. Subsequently, the granulosa layer will breach and grow out over the basement membrane to form a cluster over the flattened thecal layer, retaining the oocyte surrounded by the granulosa cells. Over about 12 days *in vitro*, these follicles will grow and produce an antrum-like structure resembling the normal follicular arrangement (Fig. 4). Such follicles produce oestrogen and can respond to LH by releasing the oocyte with surrounding cumulus cells into the medium. The oocytes can be fertilized and develop at least to the hatched blastocyst stage (Cortvrindt *et al.*, 1996, in press; Smitz *et al.*, in press). This simplified method of culture may have several advantages over the spherical culture systems, perhaps allowing improved oxygenation, nutrition, and access of hormonal support.

Follicle culture media. Mouse ovarian follicles are commonly grown in the alpha modification of Minimal Essential Medium which includes precursors of DNA and is suitable for rapidly dividing cell types. The medium is usually supplemented with a protein source, and this may affect the growth of follicles (Hulshof *et al.*, 1995). Homologous mouse serum (5%) is suitable, as is serum from hypogonadal mice (Boland *et al.*, 1993). Post-menopausal human serum, which contains substantial endogenous gonadotrophins, has also been used successfully for mouse follicle culture (Qvist *et al.*, 1990). Fetal calf serum, human follicular fluid and bovine or human serum albumin usually result in poor growth of mouse follicles in spherical culture systems (Nayudu and Osborn, 1992), but fetal calf serum is successful in supporting the development of follicular morphology and oocyte maturation of 'attached' cultures

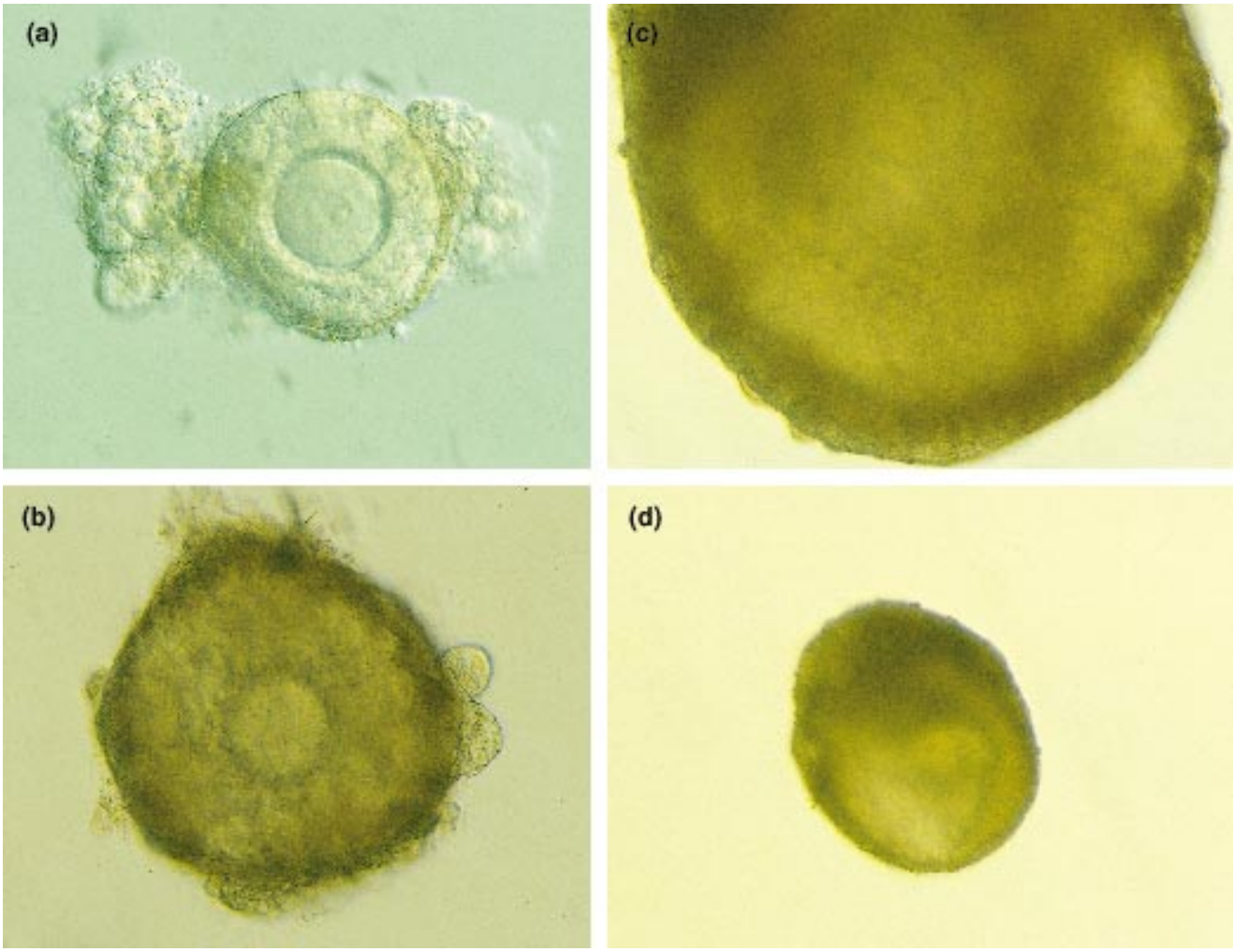


Fig. 3. Manually isolated mouse follicles growing *in vitro* in a system supporting spherical growth and including FSH and 5% mouse serum. (a) Recently dissected follicle showing clear germinal vesicle in oocyte, about three layers of granulosa cells, thin theca and two clumps of stromal cells. This figure was selected for clarity; however, stromal cells often surround the follicle. Magnification × 250. (b). Preantral follicle growing *in vitro*. Theca and stromal layers have surrounded the follicle. The oocyte is still visible, but obscured by surrounding cells. Magnification × 250. (c) Antral follicle growing *in vitro*. Clear areas are evident in the granulosa. The oocyte is visible towards the top of the figure. Magnification × 250. (d) Cultured preovulatory follicle exposed to LH (1 U ml^{-1}) *in vitro* for 20 h. The follicle did not ovulate in response to LH, but a large antral area is visible and the follicle wall has thinned in a localized area at the base of the follicle. The oocyte is visible within the follicle and the cumulus has not expanded. Magnification × 100.

(Cortvrindt *et al.*, 1996). Supplements of insulin, transferrin, selenium, glutamine, pyruvate and insulin-like growth factor I (IGF-I) have proved redundant in mice, but may be recommended for other species or in serum-free conditions (Cain *et al.*, 1995).

Various substances added to the culture medium may influence follicle growth, including gonadotrophins, dibutyryl cyclic AMP, hypoxanthine and relaxin (Carroll *et al.*, 1991b; Nayudu and Osborn, 1992; Boland *et al.*, 1993; Hartshorne *et al.*, 1994b; Cortvrindt *et al.*, in press). The conditions under which the hormones are added may affect their activities, for example, hCG or LH added prematurely results in poor growth, loss of structure of spherical follicles, and reduced post-ovulatory progesterone production, but may induce ovulation if added in a timely manner (Smitz *et al.*, in press). In addition, the use of dynamic cultures, for example rolling bottles or pulsatile administration may enhance hormone action (Goverde and van de Venne, 1994) and the presence or absence of serum may also affect the sensitivity of the cells to exogenous hormonal stimulation.

Follicle growth and differentiation *in vitro*

Enlargement and organization. Follicles appear to be programmed to grow. They may continue to enlarge even if atretic changes have begun and the oocyte has started to degenerate. The rate of growth of the granulosa layers *in vitro* may outstrip the ability of the surrounding thecal layers to keep up, occasionally resulting in a 'bulging' or even a bursting follicle, or follicles that grow slowly or abnormally *in vitro* may become overgrown

with theca (Nayudu and Osborn, 1992). Most data are available on mouse follicles, and various profiles of spherical follicle growth during *in vitro* culture for up to a week have been described, including sigmoid (Nayudu and Osborn, 1992), linear (Boland and Gosden, 1994) and convex curves (Boland *et al.*, 1994b; Spears *et al.*, 1994), possibly depending upon the size of follicles at the start of culture. A selection of mouse follicles at different stages of culture *in vitro* are shown (Fig. 3).

The presence of FSH in the culture medium stimulates glucose utilization and oestradiol production *in vitro* and may also stimulate follicles to enlarge or granulosa cell density to increase (Nayudu and Osborn, 1992; Boland *et al.*, 1994a, Hartshorne *et al.*, 1994b); however, the most obvious effect of FSH is to stimulate antral transformation.

Antrum formation in intact mouse follicles growing *in vitro* without a gel support requires an intact thecal layer (Gosden *et al.*, 1993). FSH is also needed, although inclusion of serum in the medium may provide sufficient gonadotrophic stimulation for some follicles to form antra (Hartshorne *et al.*, 1994b). Human theca-free follicles can form antra *in vitro* during agar gel culture as long as FSH is present in the medium (Roy and Treacy, 1993).

In vivo, the vast majority of follicles eventually undergo atresia, and a variety of evidence suggests an apoptotic mechanism. Established atresia can be recognized under the light microscope as a darkening of the granulosa and the oocyte, and sometimes a slowing of growth. More detail about atretic degeneration usually requires the destruction of the follicle concerned for histological analysis or examination of DNA fragmentation. Oxidative stress and free radical damage have been proposed as triggering factors for atresia in rat follicles (Tilly and Tilly, 1995) and the gonadotrophins appear to alleviate oxidative stress by enhancing superoxide dismutase (SOD) and other scavenger systems (Tilly and Tilly, 1995), promoting the survival of follicles. Insulin and insulin-like growth factor I also offer some protection against atresia induced by gonadotrophin starvation in preovulatory rat follicles *in vitro* (Chun *et al.*, 1994). How the preparation of follicles for culture *in vitro* might affect their oxidative status is uncertain. It is possible that oxidative stress is increased by local tissue damage during the removal of follicles from the intraovarian environment, and also by their culture, frequently in 5% CO_2 in air containing about 20% oxygen, rather than in about 5% oxygen as *in vivo*. However, the proportion of follicles avoiding atresia *in vitro* appears to be greater than that *in vivo*. Other factors may contribute to follicular survival *in vitro*, such as supraphysiological concentrations of gonadotrophins and reduced competition. Much remains to be studied in this area.

Secreted products

Considering their small size, follicles secrete relatively large quantities of steroids. Oestradiol has been detected from the time of antrum formation of mouse follicles *in vitro*, rising subsequently, and falling in response to LH stimulation (Boland *et al.*, 1993). Oestradiol production *in vitro* is FSH-dependent and inhibited by EGF (Boland and Gosden, 1994; Almahbobi *et al.*, 1995). Progesterone was not detected by Boland *et al.* (1993) but was measurable in conditioned media from rat follicle cultures (Smyth *et al.*, 1994).

Enzymes involved in ovulation may also be detected, for example, plasminogen activator, which is also stimulated by FSH *in vitro*. Metabolic products including carbon dioxide and lactate provide a measure of the metabolic activity of the follicle in response to various stimuli (Boland *et al.*, 1994a,b). Inhibin and the activins are also produced by follicles cultured *in vitro* (Smyth *et al.*, 1994).

When individual follicles are cultured, the follicular products are likely to be barely detectable in view of the small numbers of cells present, even when the volume of the culture is small, unless they are produced in copious amounts. Highly sensitive assays may be used but invasive analysis of mRNA concentrations indicating gene expression, or immunohistochemistry using antibodies to specific proteins are more likely to yield measurable results. These experiments are currently unpublished but are ongoing in many laboratories.

Ovulation *in vitro*

Ovulation *in vitro* has been observed both spontaneously occurring and after addition of an ovulatory stimulus. Qvist *et al.* (1990) observed that some cultured follicles extruded their oocytes into the medium in response to hCG, but the oocytes had not resumed meiosis. Naked oocytes are frequently extruded from damaged follicles, and this occurs without hormonal stimulation. It can be easily distinguished from ovulation. A 50% ovulation rate of FSH-stimulated mouse follicles *in vitro* was found by Boland *et al.* (1993), but others have found LH to be ineffective in inducing ovulation in spherical follicles cultured *in vitro* (Johnson *et al.*, 1995). Non-spherical follicles that have attached to the substrate undergo a form of ovulation in response to LH, where the oocyte, surrounded by cumulus cells, is released into the surrounding medium (Smitz *et al.*, in press; Fig. 4f). Although the follicle may not respond with ovulation, stimuli may cause maturation of the intrafollicular oocyte; however, EGF may be a more efficient stimulus than LH in this respect (Spears *et al.*, 1994; Johnson *et al.*,

Fig. 4. Manually isolated mouse follicles growing *in vitro* in a non-spherical follicle growth system including FSH and 5% fetal calf serum. (a) Cultured follicle on day 2 showing thecal cell attachment to the surface of the dish. (b) Cultured follicle on day 4 with increased outgrowth and initial disruption to the basement membrane, shown by the slightly irregular shape of the follicle. (c) Cultured follicle on day 6. The basement membrane has broken down and the granulosa cell layers are beginning to spread over the attached thecal cells. (d) Cultured follicle on day 8. An antrum-like cavity has formed in the granulosa cell layers. (e) Cultured follicle on day 10. The antrum has expanded and a tight cumulus layer is clearly visible around the oocyte. (f) Cultured follicle on day 13, after approximately 16 h exposure to LH. The oocyte cumulus complex has detatched from the rest of the follicle and is visible at the top of the figure. Magnification × 90.

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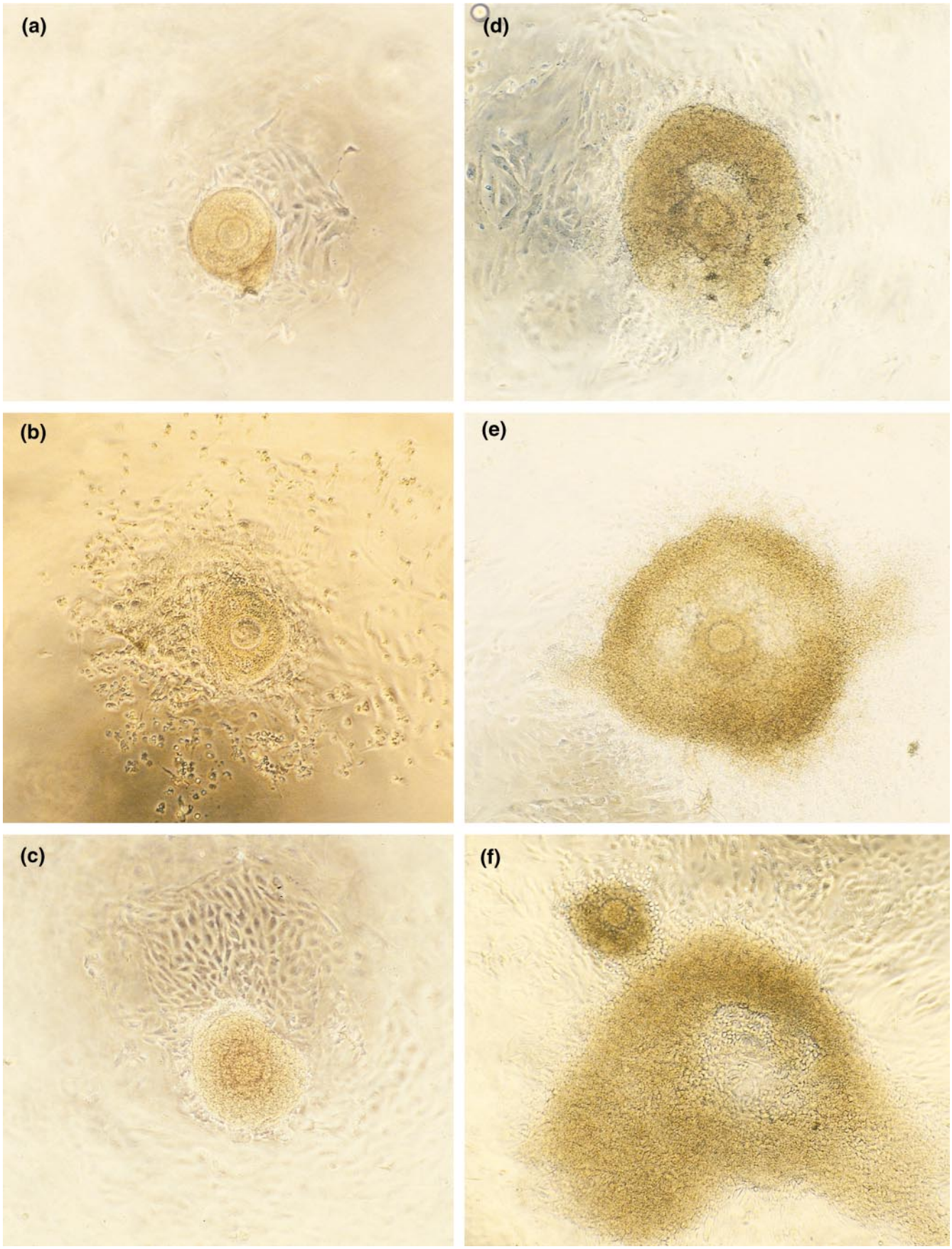

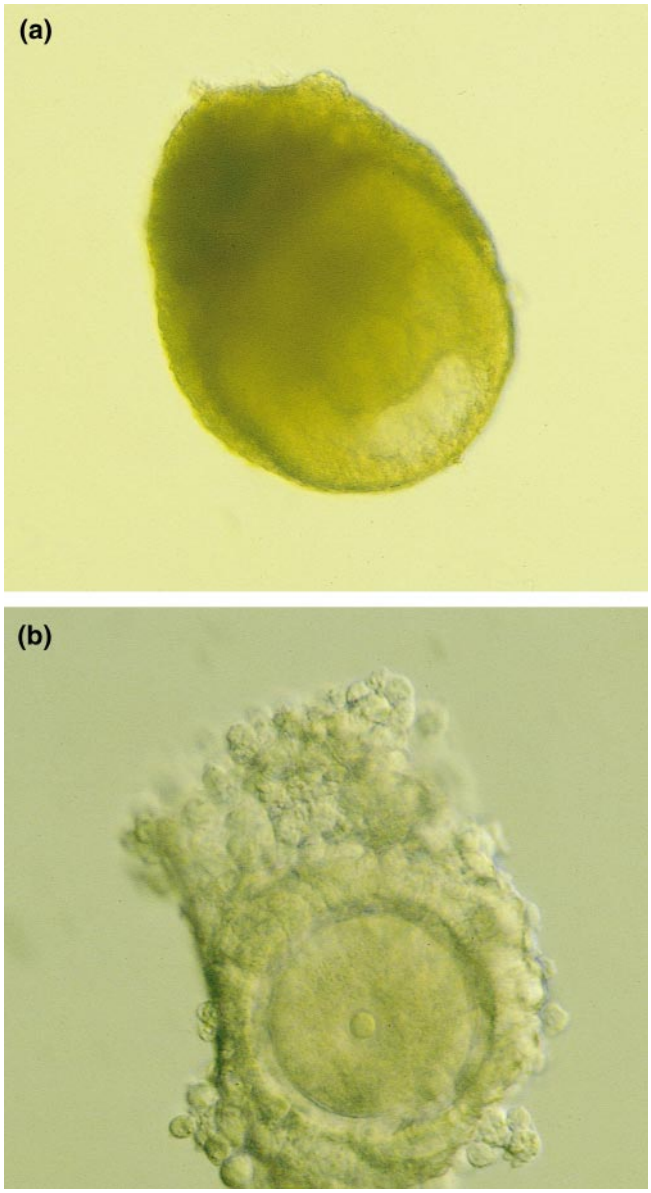

Fig. 5. (a) Follicle cultured for 2 days *in vitro* showing early antrum formation. Magnification × 100. (b) Cumulus–oocyte complex dissected from the follicle in (a). Cumulus cells surround the oocyte tightly. The germinal vesicle disappeared during subsequent culture but the oocyte did not mature to metaphase II. Magnification × 250.

1995). Ovulation itself may be less effective *in vitro* than *in vivo* perhaps because of the involvement of thecal and stromal cells in the ovulation process. These cells are likely to be functioning suboptimally *in vitro*, owing to the loss of their connections with the rest of the ovary, rendering the physical release of the oocyte more difficult to achieve.

Oocyte development within cultured follicles

The normal sequence of germinal vesicle progression from dispersed chromatin to a nucleolar rim has been demonstrated in mouse follicles cultured *in vitro* (Hartshorne *et al.*, 1994a) and the acquisition of meiotic competence during culture has been shown by the ability of oocytes liberated from their cultured follicles to undergo germinal vesicle breakdown in mice (Johnson *et al.*, 1995) and pigs (Hirao *et al.*, 1994). A mouse oocyte dissected out of an antral follicle grown *in vitro* is shown (Fig. 5). Mouse follicles grown from primary to Graafian stages *in vitro* yield oocytes capable of *in vitro* fertilization, implantation and development to term, albeit at a low rate (Spears *et al.*, 1994) and non-spherical cultures of individual mouse follicles also yield fertile oocytes that have been observed to the hatched blastocyst stage *in vitro* (Cortvrindt *et al.*, 1996). Group cultures of small follicles on a membrane substrate also yield fertile oocytes (Eppig, 1992).

The acquisition of meiotic competence by mouse oocytes coincides with the nucleolar chromatin attaining a rimmed appearance at approximately the time of antrum formation. However, in bovine, porcine, primate and probably human oocytes, meiotic and developmental competence are acquired more gradually, subsequent to antrum formation (Adachi *et al.*, 1982; Motlik and Fulka, 1986). In cows, follicles of < 2 mm in diameter, while already antral, were less likely to mature *in vitro* and more likely to have abnormalities of fertilization than oocytes from larger follicles (Pavlok *et al.*, 1992), while development to the blastocyst *in vitro* was compromised in follicles of < 6 mm in diameter (Lonergan *et al.*, 1994). In rhesus monkeys, antrum formation occurred in follicles of about 200 μm in diameter, but the rimmed nucleolar appearance was not evident until about 1 mm in diameter yet the proportion of oocytes completing maturation increased in a graded fashion with antral follicle size beyond about 500 μm in diameter (Schramm *et al.*, 1993).

The developmental capacity of naturally ovulated oocytes is difficult to determine and their fertilization rate *in vivo* is unknown. In humans, only about one quarter of cycles with unprotected and timely intercourse result in a pregnancy. Pregnancy rates per cycle are generally low even after intervention, and very low after *in vitro* maturation (Trounson *et al.*, 1994; Barnes *et al.*, 1995). In contrast, *in vitro* maturation is highly successful in cattle, where material from abattoirs is used routinely to obtain immature oocytes that can be cultured to maturity and fertilized *in vitro* to produce viable embryos.

The critical details of oocyte maturation, including both nuclear and cytoplasmic elements, are poorly understood and may influence subsequent embryonic development (Eppig *et al.*, 1994). The use of follicle culture will provide clues as to particular factors that might be manipulated to improve *in vitro* maturation. This review does not cover *in vitro* maturation specifically; however, such techniques may be an extremely important adjunct to follicle culture for obtaining mature viable oocytes. In humans, the availability of competent mature oocytes from immature follicles would greatly improve the prospects for treatment of infertile couples, both reducing the burden of intensive drug stimulation of the ovaries, and potentially enhancing the pregnancy rate, with the added benefit of providing readily available oocytes for donation and research.

Potential applications of follicle growth technology

Follicle growth *in vitro* has the potential for numerous and varied applications in reproductive biology, medicine and

animal husbandry. Knowledge about ovarian function and follicular control and development (for example, gonadotrophin responsiveness, interfollicular communication, atresia, local biochemistry) obtained by follicular growth *in vitro* may be used in future to manipulate ovarian function.

Gametes are exquisitely sensitive to many toxic compounds, and their damage has severe consequences for fertility and subsequent normal development. It may be possible to determine the toxicity or teratogenic potential of newly developed drugs by assessing follicle growth, oocyte development and embryo formation *in vitro* (Flaws *et al.*, 1994; Nayudu *et al.*, 1994).

Use of follicular growth *in vitro*, or similar methods, in conjunction with cryopreservation of immature follicles (Carroll *et al.*, 1990), would perhaps allow the follicle reserves present in the ovaries of a few females to re-establish populations of species under threat of extinction.

It may be possible to extract immature follicles from valuable livestock for culture in an appropriate system to produce pure-bred embryos to be gestated by genetically unrelated females. The embryos or the follicles could be stored frozen, which would facilitate their management.

The resources of gametes in the ovary are immeasurably valuable. Eggs are the only cells capable of generating embryos and forming the next generation. The requirement for a functional spermatozoon is being continually eroded with advances in intracytoplasmic sperm injection (ICSI) which have provided pregnancies after injection of immature spermatozoa, elongated and round spermatids (Tesarik and Mendoza, 1996). Even freeze-dried or dehydrated fixed spermatozoa may be fertile (Katayose *et al.*, 1992). A male-imprinted haploid complement of chromosomes, and perhaps a centrosome are likely to become the sole requirements for genetic fatherhood in future. Despite this, the requirement for a viable oocyte remains critical and the ovary is still the sole source of these precious cells.

The culture of follicles *in vitro* is one method by which we can learn more about the intimate biology of reproduction. Far-reaching benefits may result from the application of follicle growth and oocyte maturation methods *in vitro* which have the potential to affect profoundly the future management of fertility in animals and man.

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