The production of mature oocytes from adult ovaries following primary follicle culture in a marsupial

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Abstract

A model marsupial culture system has been developed whereby individual primary follicles, obtained from adult ovaries, can be grown *in vitro* to the antral stage and oocytes retrieved from these follicles can achieve nuclear maturation (metaphase II) in the presence of LH. Primary follicles isolated from adult *Sminthopsis macroura* ovaries were cultured individually in one of four systems: microdrops under oil, upright, inverted, or roller culture. After 6 days of culture, cumulus–oocyte complexes (COCs) were excised from early antral follicles and incubated for an additional 24 h to assess meiotic competence and the effects of LH and lithium on oocyte maturation. Histology and transmission electron microscopy established normal *in vivo* standards and verified oocyte and follicular integrity following culture. On day 6 of culture, follicle viability was significantly greater in the inverted system (73%) than in the other three systems (10–46%). The inverted system was the most effective in supporting development with follicles demonstrating progressive growth during culture and showing antral signs by day 4. Meiotic resumption during COC culture was facilitated by LH, but hindered by lithium. The ability to resume meiosis and progress to metaphase II was equivalent in oocytes retrieved following follicle culture and those matured *in vivo*. This study highlights the importance of oxygen and nutrient availability during marsupial follicle culture, and demonstrates for the first time that primary follicles isolated from adult mammalian ovaries can undergo normal growth and development *in vitro*, to produce mature, meiotically competent oocytes.

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Introduction

Assisted reproductive technologies (ART) rely upon the availability of mature, fertilizable oocytes. In the adult ovary, only a small number of oocytes are selected to develop each cycle. Superovulation is routinely used to increase oocyte yield; however, the number of *in vivo* matured oocytes available for ART purposes remains prohibitively low. Limited oocyte number, which compromises the use and success of IVF and ICSI, has, in recent years, prompted researchers to explore the possibility of maturing oocytes outside the ovary.

Follicle culture is an experimental technique that enables the predictable production of mature oocytes by utilizing the rich reserve of growing oocytes within the ovary. The ability to produce fully grown, developmentally competent oocytes *in vitro* from primary or primordial follicles would provide essential material for IVF and ICSI, and used in conjunction with tissue cryopreservation and embryonic transfer could provide new applications for fertility preservation in endangered wildlife species, economically important livestock, or humans undergoing cancer treatment.

To date, the most promising *in vitro* growth systems have been developed in mice, using newborn or prepubertal ovarian tissue. Specifically, oocytes from

primordial and primary follicles can be grown to antral stages using microdrop culture (Eppig & Schroeder 1989, Spears *et al.* 1994, Cortvrindt *et al.* 1996), and using either alginate hydrogel three-dimensional culture (Xu *et al.* 2006) or a two-step system comprising whole ovarian culture and cumulus–oocyte complex (COC) culture, live offspring have been produced (Eppig & O'Brien 1996, O'Brien *et al.* 2003).

Among other eutherian species, culture systems, which enable the initiation of primordial follicle growth and development, have been described in humans (Hovatta *et al.* 1997, 1999, Wright *et al.* 1999), baboons (Wandji *et al.* 1997), sheep (Muruvi *et al.* 2005), and cattle (Braw-Tal & Yossefi 1997). Systems that allow the development of antral follicles from secondary stage follicles have been reported in sheep (Cecconi *et al.* 1999, Newton *et al.* 1999), cattle (Gutierrez *et al.* 2000, McCaffery *et al.* 2000), and pigs (Hirao *et al.* 1994, Wu *et al.* 2001). While these achievements are remarkable, for follicle culture to be of benefit in the conservation of threatened species, or indeed the preservation of human fertility, we cannot rely upon the use of juvenile (neonatal or prepubertal) tissue.

Challenged by a limited source, culture systems that require immature ovaries have little practical application. A more realistic, functional approach is to

develop a system that can support the growth of follicles from adult ovaries, enabling the utilization of tissue from infertile or recently deceased individuals. To date, the use of adult ovaries as a source of immature oocytes has been almost exclusively the domain of human and bovine research. Culture systems that support the initiation of follicle growth within ovarian cortical strips have been established (Hovatta *et al.* 1997, 1999, Wright *et al.* 1999), and isolated secondary follicles can mature *in vitro* to early antral stages (McCaffery *et al.* 2000, Thomas *et al.* 2001, Telfer *et al.* 2008). Nonetheless, progress towards developing a culture system that allows the complete growth of adult-derived primordial or primary follicles through antrum development to oocyte maturity remains a major challenge.

Here, we report the development of a model marsupial culture system, whereby individual, primary follicles from adult ovaries can be grown *in vitro* to antral stages, producing mature, meiotically competent oocytes.

Results

Follicle classification

In vivo matured follicles ($n=2812$) were classified by morphology (Pedersen & Peters 1968) and divided into stages (Table 1). Mean follicle diameter measurements revealed considerable size overlap between follicle categories (Table 1). Cultured follicles were subsequently assigned to stages on the basis of granulosa and theca layer descriptions, rather than size.

Follicle growth

Prior to culture (day 0), the mean diameter of selected primary follicles ranged from 63.6 to 215.5 μm , with an average ($\pm$ S.E.M.) of $163.2 \pm 1.6 \mu\text{m}$. At the start of culture, there was no significant difference in

mean follicle diameter between the four culture systems, $F_{(3, 443)}=5.11$, $P>0.05$ (Table 2). During the first 24 h of culture (day 1), mean follicle diameter increased significantly in the upright, inverted, and roller systems, with follicles demonstrating greatest growth in the inverted system (Fig. 1). This trend continued throughout the 6-day culture period. Follicles cultured in the inverted system showed significant daily growth between days 1–3 and days 4–5, and subsequent stage progression (Table 1) reaching antral stages with a mean follicle diameter of $379.2 \pm 15.9 \mu\text{m}$ by day 6 (Fig. 1 and Table 2). Following the first 24 h, follicles in the upright and roller systems demonstrated progressive but non-significant growth, which appeared to plateau prior during the second half of the culture period (Fig. 1). The final mean diameter of follicles cultured in the upright ($205.8 \pm 13.9 \mu\text{m}$) and roller ($264.8 \pm 14.4 \mu\text{m}$) systems corresponds *in vivo* with the secondary and secondary–tertiary transition stage respectively (Table 1). Both were significantly lower than the final mean diameter achieved in the inverted system (Table 2). The microdrop system was by far the least effective, with no significant change in mean follicular diameter throughout the culture period (Fig. 1 and Table 2).

Follicle morphology

Prior to culture, all primary follicles comprised a single layer of granulosa cells, surrounded by a few fibroblast-like theca cells and varying amounts of stromal tissue. Throughout the culture period, viable follicles maintained their follicular architecture, the oocyte remained relatively central, and the basement membrane appeared intact. The zona pellucida (ZP) initially appeared patchy, gradually developing a uniform appearance. The extent of antrum formation was used to categorize larger follicles into stages. The appearance

Table 1 Classification of *Sminthopsis macroura* ovarian follicles *in vivo*.

Stage	Type ^a	n	Follicle diameter ^b (μm)		Defining characteristics used for classification
			Min	Max	
Primordial	3a	76	27	38	One layer of squamous follicle cells surrounds the oocyte
Primary	3b	1108	36	213	One layer of cuboidal follicle cells (GCs) surrounds the growing oocyte. The ZP appears patchy and irregular
Secondary	4	476	180	256	More than one layer of GCs surrounds the growing oocyte. The ZP appears uniform. TCs begin to proliferate
Transition to tertiary	5	744	221	289	Three or more GC layers surround the growing oocyte. TCs form layers
Tertiary	6	200	277	380	Distinct fluid-filled pockets appear among the GCs, which now comprise many layers. TCs begin to differentiate (interna/externa)
Antral	7	208	301	576	Antral fluid continues to accumulate. Pockets merge into lacunae, eventually coalescing to form a single antrum. The GCs differentiate (cumulus/mural). TC differentiation continues
Preovulatory	8				

GC, granulosa cell; ZP, zona pellucida; TC, theca cell.

^aPedersen & Peters (1968) classification. ^bMean follicle diameter was calculated at the largest follicle cross-section by measuring two perpendicular axes. Minimum and maximum mean diameters are given for each follicle stage.

Table 2 Comparison of *Sminthopsis macroura* primary follicle culture systems.

	Microdrop	Upright	Inverted	Roller
Number of follicles (n)	107	120	152	68
Initial mean diameter (day 0) $\pm$ S.E.M. (μ m)	166.9 $\pm$ 10.1*	164.3 $\pm$ 6.71*	162.3 $\pm$ 5.9*	159.5 $\pm$ 6.2*
Final mean diameter (day 6) $\pm$ S.E.M. (μ m)	156.6 $\pm$ 7.6*	205.8 $\pm$ 13.9 [†]	379.2 $\pm$ 15.9 [‡]	264.8 $\pm$ 14.4 [§]
Most numerous follicle stage after culture (day 6)	Primary	Secondary	Antral	Secondary
Follicle quality				
Viable	10%	32%	73%	46%
Degenerate	90%	68%	27%	54%

Values with different superscripts differ significantly; $P < 0.05$. There was a significant association between follicle quality and the culture system used, $\chi^2(3) = 109.28$, $P < 0.001$.

of small shadowy patches within the granulosa layer of cultured tertiary follicles, type 6 (Pedersen & Peters 1968), indicated the beginning of antrum formation. As antral fluid accumulated, the patches coalesced to form lacunae, type 7 (Pedersen & Peters 1968), and eventually a large single antrum, types 7 and 8 (Pedersen & Peters 1968). Histological (Fig. 2A and B) and ultrastructural (Fig. 2C–H) examination further confirmed that follicles cultured in the inverted system were developing normally and conforming to *in vivo* standards (Kress *et al.* 2001). The oocyte appeared normal, with numerous microvilli extending towards the ZP (Fig. 2C). Outside the ZP, the granulosa cells (Fig. 2E) appeared healthy and were interspersed with antral pockets of varying size. The basement membrane remained intact, and by day 4 onwards the more advanced follicles showed differentiation of thecal cells into the steroidogenic theca interna and the more fibrous theca externa (Fig. 2G).

Oocyte morphology

Following 6 days of inverted follicle culture, 93% (104 out of 111) of intact viable follicles had a diameter $> 300 \mu\text{m}$, and 83% (87 out of 104) of these yielded morphologically normal oocytes. Out of these, 90% (78 out of 87) remained at the germinal vesicle (GV) stage, enclosed by intact cumulus cells (type 2 oocytes). The remaining 10% (9 out of 87) had shed most cumulus cells and undergone GV breakdown (GVBD) with extrusion of the first polar body to form type 3 oocytes. All follicles yielding type 3 oocytes had a diameter $> 350 \mu\text{m}$ (Fig. 3).

COC culture

Three-way log-linear analysis to test the relationship between oocyte origin, COC culture treatment, and oocyte maturation revealed that there was no significant association between the origin of oocytes and their ability to undergo meiotic resumption during COC culture, $\chi^2(3) = 0.314$, $P = 0.957$. χ^2 -tests, performed separately for freshly isolated *in vivo* matured oocytes ($\chi^2(3) = 32.75$, $P < 0.001$) and oocytes retrieved following inverted follicle culture ($\chi^2(3) = 21.37$,

$P = 0 < 0.001$), showed a significant relationship between culture treatment and oocyte maturation. In comparison with control data, the ability of type 2 oocytes to resume meiosis during 24 h COC culture was greatly improved by the addition of LH (10 $\mu\text{g/ml}$). The presence of LiCl (10 mM/ml) had little effect on oocyte maturation frequencies; however, Li_2CO_3 (10 mM/ml) appeared to hinder development (Table 3).

Discussion

This is the first report in mammals of oocyte maturation *in vitro* using primary follicles sourced from adult ovaries, and one of the first descriptions of follicle culture in a marsupial. Using optimized culture conditions, this study has demonstrated that primary follicles harvested from adult ovarian tissue may be grown *in vitro* to antral stages.

The ability to culture follicles from adult ovaries and produce normal oocytes should prove instrumental in enhancing current reproductive technology, providing essential material for IVF and ICSI, and much needed insight into oocyte and follicle development.

Figure 1 *S. macroura* follicle growth *in vitro* showing the mean ($\pm$ S.E.M.) follicle diameter recorded during culture (days 0–6) in each of the four culture systems. The inverted culture system was most conducive to follicle growth. Primary follicles cultured in this system demonstrated progressive growth and stage progression throughout the culture period. *Indicates that the mean follicle diameter was significantly different to the previous day in the given culture system. The horizontal lines approximate transition between follicle stages (labelled on the right) based on measurements collected from *in vivo* samples (Table 1).

Table 3 The influence of culture supplements on the meiotic ability of *Sminthopsis macroura* oocytes during 24 h COC culture. Oocyte frequencies were calculated separately for freshly isolated and cultured follicles.

Treatment	The frequency (%) of oocyte type after COC culture					
	Freshly isolated follicles			Cultured follicles		
	<i>n</i>	Type 2	Type 3	<i>n</i>	Type 2	Type 3
Control	30	21.4	9.2	21	19.7	7.9
LH (10 µg/ml)	30	5.1	25.5	23	7.9	22.4
LiCl (10 mM/ml)	18	13.3	5.1	17	17.1	5.3
Li ₂ CO ₃ (10 mM/ml)	20	18.4	2.0	15	18.4	1.3

The technique would provide greater options for the treatment of premature ovarian failure in humans and enable the propagation of important livestock and threatened wildlife species. The use of adult tissue is of particular interest to endangered species conservation as it would allow the collection of viable oocytes from recently deceased, adult, or infertile animals.

Despite recent eutherian advances (Eppig & O'Brien 1996, Eppig *et al.* 1996, Hardy *et al.* 2000, Liu *et al.* 2002, Rizos *et al.* 2002, Carrell *et al.* 2005, Xu *et al.* 2006), reports of marsupial follicle culture remain scarce and success has been limited by inadequate growth rate and development. The first marsupial study, using preantral follicles from prepubertal ovaries of the grey short-tailed opossum, *Monodelphis domestica*, showed that individual preantral follicles cultured in microdrops grew to antral size without antrum formation, and determined the importance of FSH as a follicle growth stimulant *in vitro* (Butcher & Ullmann 1996). Two additional studies have described marsupial follicle culture, both using follicles collected from adult ovaries and employing a similar microdrop system. In the tammar wallaby, *Macropus eugenii*, secondary follicles demonstrated significant growth over a 4-day culture period, but failed to progress to the tertiary follicle stage (Richings *et al.* 2006). In *Sminthopsis macroura*, early antral (lacunae stage) follicles showed follicle expansion *in vitro* during a 24-h culture period (Maleszewski & Selwood 2004), and this work ascertained the requirement of exogenous LH for oocyte maturation *in vitro*.

The present study comprises the first complete (preantral–antral) description of normal growth and development of marsupial follicles *in vitro*. Having achieved maturation to metaphase II, the next obvious step is to assess the developmental competence of oocytes by examining rates of fertilization and embryonic development. Unfortunately, the techniques required for such analysis are not yet established in marsupials (Mate 1996, Magarey & Mate 2003, Richings *et al.* 2004). To date, successful IVF has only been achieved in *M. domestica* (Moore & Taggart 1993, Taggart *et al.* 1993) and ICSI in *M. eugenii* (Richings *et al.* 2004), neither study, however, attempted surrogate transfer to follow embryonic development to birth.

The use of a microdrop system to culture isolated follicles has proved relatively successful and adaptable across eutherian species; however, this study and previous work (Butcher & Ullmann 1996, Richings *et al.* 2006) suggest that the technique provides inadequate support for the development of marsupial follicles *in vitro*. By comparing the microdrop culture system with three other systems specifically designed to increase nutrient and/or oxygen supply to the follicle, it became apparent that manipulation of these elements is critical to optimizing follicle culture success. Increasing the media volume and thus the availability of nutrients (upright, inverted and roller systems) resulted in a significant improvement in follicle growth and survival in all three systems. Similarly, by increasing oxygen availability (inverted and roller systems) follicle development was further enhanced. The inverted culture system proved by far the most superior, producing antral stage follicles equivalent both in size and morphology to their *in vivo* matured counterparts, suggesting that oxygen access, rather than nutrient supply, may be most crucial to follicle culture success.

The exact mechanism by which LH stimulates meiotic resumption (Channing *et al.* 1978) in arrested oocytes remains unknown, although there is growing evidence to support the involvement of phosphoinositide metabolism and intracellular calcium signaling (Eppig 1991, 1993, Homa *et al.* 1993, Coticchio & Fleming 1998). In mice, the addition of LiCl to COC culture has generated contrasting results (Bagger *et al.* 1993, Pesty *et al.* 1994), leading to the hypothesis that spontaneous and hormone-induced meiotic resumption operate through different mechanisms (Coticchio & Fleming 1998). In the present study, we found that lithium, a known inhibitor of phosphoinositide metabolism, suppressed oocyte maturation during COC culture by delaying GVBD. These results correlate with previous findings in mice (Pesty *et al.* 1994, Coticchio & Fleming 1998), and suggest that activation of the phosphoinositide pathway, following the LH surge, is required for meiotic resumption in both eutherian and marsupial oocytes. This raises the possibility of using lithium as a mammalian fertility control agent. In accordance with previous work

Figure 2 Histological and ultrastructural comparison of *S. macroura* antral stage follicles following maturation *in vivo* and *in vitro* revealed little difference. In addition to follicle growth data, this strongly suggests that primary follicles and their associated oocytes, cultured in the defined inverted system, grew and developed in a manner consistent with *in vivo* maturation. (A and B) Light micrographs of *in vivo* (A) and *in vitro* (B) matured antral stage follicles. Both follicles contain a large, spherical primary oocyte with a central concentration of empty-looking vesicles. The germinal vesicle (arrowhead) is situated in the peripheral, largely vesicle-free region of cytoplasm, which borders the zona pellucida (arrow). Outside the zona, a single layer of cumulus granulosa cells (C) surrounds the oocyte. The fluid-filled antrum (A) is well developed. Multiple layers of mural granulosa cells (M) separate the antrum from the basement membrane, which is surrounded by stromal theca cells (T) (Scale = 100 μ m). (C and D) Electron micrographs showing sections of antral follicle primary oocytes after maturation either *in vivo* (C) or *in vitro* (D). The ooplasm contains an abundance of mitochondria (M) and membrane-bound cytoplasmic vesicles (V). Cortical granules (CG) are predominantly situated beneath the oocyte plasma membrane, and extensive microvilli (MV) processes extend into the zona pellucida (X 6610). (E and F) Electron micrographs of granulosa cells from *in vivo* (E) and *in vitro* (F) matured antral follicles. The granulosa cell nuclei (N) appear large and rounded. The most prominent cytoplasmic organelles are large lipid droplets (L), containing stored cholesterol esters and numerous variably shaped mitochondria (M). Short microvilli (MV) projections of the granulosa cells link one cell to the next (X 5200). (G and H) Electron micrographs of thecal cells from *in vivo* (G) and *in vitro* (H) matured antral follicles. Both theca cells and their nuclei (N) are elongated. Electron density of the nuclei is varied and chromatin appears in clumps along the nuclear membrane (X 6610).

Figure 3 *S. macroura* oocyte maturation *in vitro* following follicle culture. (A) Light micrograph of a type 2 oocyte isolated from an early antral follicle on day 6 of follicle culture. The zona pellucida (arrow) is closely apposed to the oocyte and the germinal vesicle (arrowhead) is eccentric. The oocyte cytoplasm shows polarization with the presence of a yolk mass (YM) in the opposite hemisphere to the germinal vesicle. Cumulus granulosa cells have been removed by aspiration for greater photographic clarity (Scale = 80 μ m). (B) Light micrograph of a type 3 oocyte (which has developed from a type 2 oocyte) after 24 h COC culture in the presence of 10 μ g/ml LH. A distinct perivitelline space has formed between the zona pellucida (arrow) and the oocyte plasma membrane, and within this the first polar body (arrowhead) may be seen. The yolk mass (YM) has concentrated and remains in the opposite hemisphere to the nuclear material (Scale = 80 μ m). (C) Transmission electron micrograph of a type 3 oocyte, following both follicle culture and COC culture. Meiosis has resumed, one pronucleus (PN) has formed and the first polar body (PB) has been extruded into the perivitelline space (PVS). The female pronucleus is surrounded by a region of less dense, relatively organelle-free cytoplasm. Zona pellucida, ZP (X 1200). (D) Transmission electron micrograph of the first polar body (PB) extruded into the perivitelline space (PVS) during COC culture. Containing condensed chromatin, the polar body lies between the zona pellucida (ZP) and the oocyte plasma membrane (not shown), connected to both by microvilli (MV) (X 3900).

(Maleszewski & Selwood 2004), our results also verify that LH influences meiotic resumption in marsupials as it does in eutherian mammals.

In conclusion, this novel study provides proof that adult ovaries may be used as a source of viable oocytes. With species-specific modifications, the inverted culture system reported here may be applied to other marsupial and/or eutherian species, thereby opening new avenues towards assisted reproduction and the conservation of endangered species.

Materials and Methods

Chemicals

Chemicals were purchased from Sigma–Aldrich unless otherwise specified.

Animals

S. macroura were obtained from a colony maintained by L Selwood at The University of Melbourne under Department of Sustainability and Environment permits. Colony maintenance and experiments followed Australian National Health and Medical Research Council Guidelines for the Care and Use of Animals for Scientific Purposes.

Follicle isolation

Mature females were killed by anesthetic overdose (Halothane; Rhone Merieux, West Footscray, VIC, Australia) followed by cervical dislocation. Blood collected from the posterior vena cava using a 21 gauge (G) needle and 3 ml syringe provided the serum required for each experiment. The reproductive tract was removed via a ventral midline incision and washed in calcium- and magnesium-free PBS at 35 $^{\circ}$ C, the basal body

temperature of *S. macroura*. Under a laminar flow hood, the ovaries were removed and transferred immediately into 35 °C HEPES-buffered DMEM containing 3 mg/ml BSA. Individual follicles were microdissected from the ovary at $\times 40$ magnification using 29 G insulin syringes with the plungers removed.

Follicle classification and morphology

In vivo matured ovarian follicles ($n=2812$) were serially sectioned and classified by morphological appearance (Pedersen & Peters 1968), in order to establish standards for the evaluation of cultured follicles. Mean follicle diameter was calculated at the largest follicle cross-section by measuring two perpendicular axes, basement membrane to basement membrane, with a calibrated ocular micrometer. Follicles that presented pyknotic bodies in granulosa cells, low cellular density, a shrunken oocyte or condensed oocyte nucleus were considered atretic and not included in the analysis.

Follicle culture

Following ovarian microdissection, isolated preantral follicles were examined by confocal microscopy. Only follicles that were relatively translucent contained a single layer of cuboidal granulosa cells, Type 3b (Pedersen & Peters 1968), a rounded central oocyte and an intact spherical structure were selected for culture. Follicles were cultured in DMEM with 4.5 g/l glucose, 100 IU/ml penicillin, 50 µg/ml streptomycin, 100 µg/ml glutamine, and 100 µg/ml kanamycin, DMEM⁺ (Merry *et al.* 1995), with the addition of supplements shown to improve follicle development in culture: insulin–transferrin–selenium (Cain *et al.* 1995; ITS-X; insulin, 10 µg/ml; transferrin, 5.5 µg/ml; selenium, 6.7 ng/ml; Gibco-Invitrogen); 100 µg/ml L-ascorbic acid (Murray *et al.* 2001); 1.0 IU/ml recombinant human FSH (Izadyar *et al.* 1998; Gonadotropin; donated by Melbourne IVF); and 5% homologous serum (Nayudu & Osborn 1992), obtained from the experimental animal and heat-inactivated before use. The optimal concentration of each supplement was determined by preliminary trials (data not shown). Follicles were cultured at 35 °C for 6 days in one of four systems, described below.

- (1) Microdrop culture under oil (Butcher & Ullmann 1996): follicles ($n=107$) were cultured individually in 30 µl drops of medium in 35 mm Falcon Petri dishes (Becton Dickinson, Knoxfield, VIC, Australia). The droplets were covered with sterile equilibrated mineral oil to prevent evaporation and fluctuations in pH and temperature. In systems 1–3, incubation occurred in a humidified gas environment of 5% CO₂ in air, and half the volume of media was replaced every 48 h.
- (2) Upright culture (Wycherley *et al.* 2004): to increase nutrient supply, follicles ($n=120$) were cultured in a larger volume of media (100 µl) in 96-well round-bottomed suspension tissue culture plates (Sarstedt Ltd, Leicester, UK).
- (3) Inverted culture (Wycherley *et al.* 2004): follicles ($n=152$) were cultured in 100 µl drops of medium in 96-well

round-bottomed suspension tissue culture plates (as above); however, in this system, the plates were inverted prior to incubation. The medium was held in place by surface tension and the follicle dropped down to lie on the media/gas interface, thereby maximizing oxygen access. This system was employed to increase both nutrient availability and oxygen supply to the follicle. Twelve different 96-well plates were tested in a series of experiments to determine the most conducive to this technique (data not shown).

- (4) Roller culture: individual follicles ($n=68$) were cultured in 300 µl of medium in 2 ml glass bottles. The medium was equilibrated with a gaseous mixture (90% N₂:5% O₂:5% CO₂) previously used in embryo culture (Eppig & Schroeder 1989). Following gas infusion, the bottles were sealed and rotated continuously on rollers within an incubator. The constant movement within the bottle should facilitate gas and nutrient exchange between follicle and medium and also increase oxygenation of the medium by the surrounding gas. Half of the medium was replaced on day 3, and the bottles re-gassed.

In vitro matured follicles ($n=347$) were examined prior to culture (day 0) and then every 24 h using an inverted microscope (Wild Leitz, Melbourne, VIC, Australia) with heated stage. Mean follicle diameter was calculated as previously described. Follicular development and viability were evaluated daily by assessing general morphology and structure, including ZP deposition, antral fluid accumulation, and theca development. Follicles with clear granulosa cells and an intact follicular structure were deemed viable, regardless of growth rate. Follicles assigned to the non-viable (atretic) category demonstrated one or more of the following characteristics: an absence of growth; an irregularly shaped, retracted, or darkening oocyte; dark, granular (apoptotic) granulosa cells; a damaged (thinning/budding) or ruptured thecal layer.

Oocyte morphology

Following 6 days of inverted follicle culture, viable follicles with a diameter >300 µm were carefully ruptured using 29 G needles and oocytes or COCs collected. Oocyte maturity was assessed in terms of follicle type, nuclear content, and cytoplasmic polarity, according to Merry *et al.* (1995). Type 2 oocytes (Merry *et al.* 1995), recovered from early antral follicles, remained in meiotic arrest at the GV stage, contained centrally concentrated cytoplasmic vesicles and adherent cumulus cells. Type 3 oocytes (Merry *et al.* 1995), obtained from late antral follicles, were cumulus free, showed marked cytoplasmic polarity, and had undergone GVBD followed by extrusion of the first polar body.

Oocyte maturation

The ability of type 2 oocytes to resume meiosis and progress to metaphase II *in vitro* was compared between freshly isolated, *in vivo* matured oocytes and those retrieved following follicle culture. Type 2 oocytes and surrounding cumulus cells were transferred to 30 µl drops of the above follicle culture medium supplemented with agents known to affect oocyte maturation:

either 10 µg/ml porcine pituitary LH (Maleszewski & Selwood 2004), a gift from Vetrepfarm (London, Ontario, Canada); 10 mM/ml LiCl (Bagger *et al.* 1993); or 10 mM/ml Li₂CO₃. COCs were incubated at 35 °C in 6% CO₂ in air for 24 h in the previously described microdrop system. After 24 h, oocyte maturity was assessed in terms of chromatin configuration, with oocytes graded as either type 2 (GV stage) or type 3 (Telophase/MII).

Histological and ultrastructural analysis

Isolated, non-cultured, and non-atretic follicles at all stages of folliculogenesis were collected to establish *in vivo* standards. Cultured follicles were randomly selected throughout the culture period and at its conclusion to validate *in vitro* observations and determine culture standards. Selected follicles were immersed in Superfix (2.5% glutaraldehyde (ProSciTech, Thuringowa, QLD, Australia), 3% paraformaldehyde (Merck-BDM), and 0.2 M sodium cacodylate buffer) at room temperature (RT) for 2 h (pH 7.4). After three rinses in 0.1 M sodium cacodylate buffer (pH 7.4), post-fixation took place in 1% osmium tetroxide (ProSciTech) diluted with 0.2 M sodium cacodylate buffer for 2 h at RT. Following rinsing in 0.1 M sodium cacodylate buffer, the tissue was dehydrated in an ethanol series, infiltrated with a propylene oxide – Epon–Araldite mixture, embedded in pure Epon–Araldite and polymerized at 60 °C for 48 h. The resin consisted of a mixture of 25 ml Procure 812, 15 ml Araldite 502, 55 ml dodecyl succinic anhydride, and 1.25 ml benzyldimethylamine (ProSci-Tech). Semi-thin sections (1 µm) were cut with glass knives on an ultramicrotome (Ultracut E; Reichert-Jung, Heidelberg, Germany) and stained with 1% toluidine blue and 1% sodium borate in distilled water at 90 °C for 1–2 min. Thin sections (70 nm), cut with a diamond knife, were stained with 3% uranyl acetate and 0.6% lead citrate, mounted on coated grids, and examined with a transmission electron microscope (CM 10; Philips, Eindhoven, The Netherlands).

Statistical analysis

Mean follicle diameter on day 0 was compared between culture systems by one-way independent ANOVA followed by Bonferroni's *post hoc* correction. Follicle growth during culture was analyzed within each system by one-way repeated-measures ANOVA and Bonferroni's *post hoc* test. χ^2 -tests were used to compare follicle quality between culture systems and oocyte maturation in the presence or absence of supplements. Three-way log-linear analysis was employed to test the relationship between oocyte origin, the effect of culture supplements, and oocyte development. Differences were considered statistically significant when $P < 0.05$. Data analysis was performed using SPSS 14.0 (Chicago, IL, USA).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this research.

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