Zygote **12** (February), pp. 1–18. © 2004 Cambridge University Press
DOI:10.1017/S0967199404002461 Printed in the United Kingdom

Investigations of oocyte *in vitro* maturation within a mouse model

Alexis Heng Boon Chin and Ng Soon Chye
Department of Obstetrics & Gynaecology, National University of Singapore, 5 Lower Kent Ridge Road, Singapore 119074

Date submitted: 24.07.03. Date accepted: 11.09.03

Summary

This study attempted to develop a 'less meiotically competent' murine model for oocyte *in vitro* maturation (IVM), which could more readily be extrapolated to human clinical assisted reproduction. Oocyte meiotic competence was drastically reduced upon shortening the standard duration of *in vivo* gonadotrophin stimulation from 48 h to 24 h, and by selecting only naked or partially naked germinal vesicle oocytes, instead of fully cumulus enclosed oocyte complexes. With such a less meiotically competent model, only porcine granulosa coculture significantly enhanced the oocyte maturation rate *in vitro*, whereas no significant enhancement was observed with macaque and murine granulosa coculture. Increased serum concentrations and the supplementation of gonadotrophins, follicular fluid and extracellular matrix gel within the culture medium did not enhance IVM under either cell-free or coculture conditions. Culture medium conditioned by porcine granulosa also enhanced the maturation rate, and this beneficial effect was not diminished upon freeze–thawing. Enhanced IVM in the presence of porcine granulosa coculture did not, however, translate into improved developmental competence, as assessed by *in vitro* fertilization and embryo culture to the blastocyst stage.

Keywords: Oocyte, *In vitro* maturation, Murine, Follicular fluid, Serum, Granulosa

Introduction

Many studies have established that the *in vitro* maturation (IVM) of germinal vesicle (GV) oocytes to the metaphase II (MII) stage is technically feasible in a range of different mammalian species (Heikinheimo & Gibbons, 1998). In domesticated mammals such as pig and cattle, IVM of GV oocytes derived from slaughterhouse ovaries is routinely used for the commercial production of embryos and hence new livestock (Chanson *et al*., 2001; Kikuchi *et al*., 1999). In laboratory animals such as rats and mice, IVM is also a well-established protocol with high rates of success in the production of embryos and new animals (Zhang *et al*., 1991; Anderiesz & Trounson, 1995). By contrast, the IVM of human GV oocytes in clinical assisted reproduction is much less successful (Trounson *et al*., 1998; Anderiesz *et al*., 2000).

There are various reasons for this discrepancy. Most obviously, IVM studies on the human clinical model comprises a heterogeneous group of subfertile patients, in contrast to the young and healthy subjects used in animal models. IVM studies on the human model can be divided into two categories. The first includes the less maturationally competent GV and metaphase I (MI) oocytes from stimulated human patients that were either slow or had failed to respond to human chorionic gonadotrophin (hCG) stimulation *in vivo*. Such immature oocytes have been reported to have extremely poor maturational and developmental competence, as well as high rates of chromosomal abnormalities (De Vos *et al*., 1999; Nogueira *et al*., 2000; Kim *et al*., 2000). The second category includes the immature oocytes recovered from the small antral follicles of unstimulated or very mildly stimulated polycystic ovarian syndrome (PCOS) patients (Son *et al*., 2002), which are truncated in the early follicular phase of their development, and are therefore maturationally and developmentally compromised. By contrast, IVM studies on animal models selectively retrieve oocytes from larger, more-well-developed follicles, while excluding oocytes from smaller, less-well-developed follicles (Chanson *et al*., 2001; Kikuchi *et al*., 1999). Additionally, most animal models focus solely on GV oocytes within fully enclosed cumulus–oocyte

All correspondence to: B. C. A. Heng, Department of Obstetrics & Gynaecology, National University of Singapore, 5 Lower Kent Ridge Road, Singapore 119074. Tel: +65 6772 4271. Fax: +65 6779 4769. e-mail: bc_heng@hotmail.com

https://doi.org/10.1017/S0967199404002461 Published online by Cambridge University Press

complexes (COCs) while excluding naked or partially naked GV oocytes. There cannot be such selectivity within the human model, because the aim of clinical assisted reproduction is to maximize the number of mature MII oocytes available for fertilization.

One aim of this study would therefore be to develop a murine IVM model with a lower degree of maturational and developmental competence, which might be more readily extrapolated to the human model. This could be achieved by several modifications to the standard laboratory protocol for IVM within the mouse model. First, the immature oocytes will be retrieved much earlier after gonadotrophin (PMSG) stimulation, at 24 h instead of the usual 48 h (Anderiesz & Trounson, 1995; Downs & Mastropolo, 1997; De La Fuente *et al*., 1999). This should increase the proportion of GV oocytes that are truncated during their early follicular growth phase, upon retrieval for IVM. Second, the IVM study could use oocytes retrieved from a mixture of both small and large antral follicles. Last, we would include only the naked or partially naked GV oocytes for the IVM study, while excluding all fully cumulus enclosed GV oocytes. Subsequently, this study looks at various ways to improve IVM within such a putatively less competent murine model. The effects of granulosa coculture and granulosa-conditioned medium on meiotic maturation are investigated, together with various modifications to the culture milieu that might enhance IVM, such as increasing serum concentrations and the supplementation of gonadotrophins and follicular fluid. Additionally, a novel granulosa-coculture system on a bed of extracellular matrix (ECM) gel is investigated.

Materials and methods

Ethics in the use of laboratory animals

All the experiments using animals were in accordance with the International Guiding Principle for Biomedical Research Involving Animals and the experimental protocol was approved by the Ethics Committee for Experimental Animals of the National University of Singapore.

Isolation of immature GV oocytes from the mouse

Immature GV oocytes were collected from gonadotrophin-primed 4–6-week-old F1 (C57BL × CBA) female mice, which were acclimatized under controlled temperature (25 °C), lighting (lights on from 0700–1900) and humidity (50–70%) for at least 1 week before use. Gonadotrophin priming was achieved by intraperitoneal (i.p.) injection of 5 IU pregnant mare's serum gonadotrophin (Folligon; Intervet, Australia) per mouse. Depending on the experiment, the mice were killed by cervical dislocation either 24 h or 48 h after gonadotrophin priming. The ovaries were dissected out and placed in HEPES-buffered M199 medium (Gibco-BRL, USA) supplemented with antibiotics (50 μg ml^{-1} penicillin and 75 μg ml^{-1} streptomycin; Sigma, USA). Under the view of a stereomicroscope, the immature GV oocytes were recovered by manual puncture and dissection of a mixture of large and small antral follicles in HEPES-buffered M199 medium supplemented with antibiotics. Naked and partially naked immature oocytes (in which the GV was clearly visible; Fig. 1a,b) were separated from fully cumulus enclosed oocyte complexes (GV being occluded by surrounding cumulus mass; Fig. 1c), and were then further washed in bicarbonate-buffered M199 medium (Gibco-BRL, USA) before being subjected to IVM. Unlike some earlier studies (Anderiesz & Trounson, 1995; Downs & Mastropolo, 1997; De La Fuente *et al*., 1999), no attempt was made to further denude the partially naked and fully cumulus enclosed oocytes to obtain completely naked GV oocytes.

Collection and cryopreservation of granulosa cells

Granulosa cells were isolated from three different sources in this study: long-tailed macaque, mouse and pig. Granulosa cells from gonadotrophin-primed female macaques were isolated from follicular aspirates (HEPES-buffered TALP medium) obtained through standard laparascopic oocyte retrieval techniques (Ng *et al*., 2002). The macaques (average body weight 2.5 kg) were downregulated with a bolus dose of 1.8 mg GnRH agonist (Decapeptyl; Ferring, Germany) and, 14 days later, were primed with daily intramuscular (i.m.) injections of 37.5 IU of recombinant human follicle-stimulating hormone (FSH) (Gonal-F; Serono, Switzerland) for a total of 12 days (Ng *et al*., 2002), before administration of 1000 IU hCG (Profasi; Serono, Switzerland). Follicular aspiration by laparoscopy was carried out 34 h later. Granulosa cells from the mouse were collected simultaneously with the immature GV oocytes from the ovaries of gonadotrophin-primed 4–6-week-old F1 (C57BL × CBA) female mice (see above). The mural granulosa sheets were released by follicular puncture into HEPES-buffered M199 medium supplemented with antibiotics. Porcine granulosa cells were retrieved in a similar fashion from the ovaries of non-stimulated prepubertal pigs that were obtained from a local abattoir. The granulosa cells from these various sources were then pelleted by centrifugation and resuspended in 1 ml of M199 medium supplemented with 10% (v/v) foetal calf serum (FCS; Gibco-BRL, USA) and 10% (v/v) dimethyl sulfoxide within a cryovial, and left to freeze overnight in a −80 °C refrigerator before being plunged into liquid nitrogen (−196 °C).

https://doi.org/10.1017/S0967199404002461 Published online by Cambridge University Press

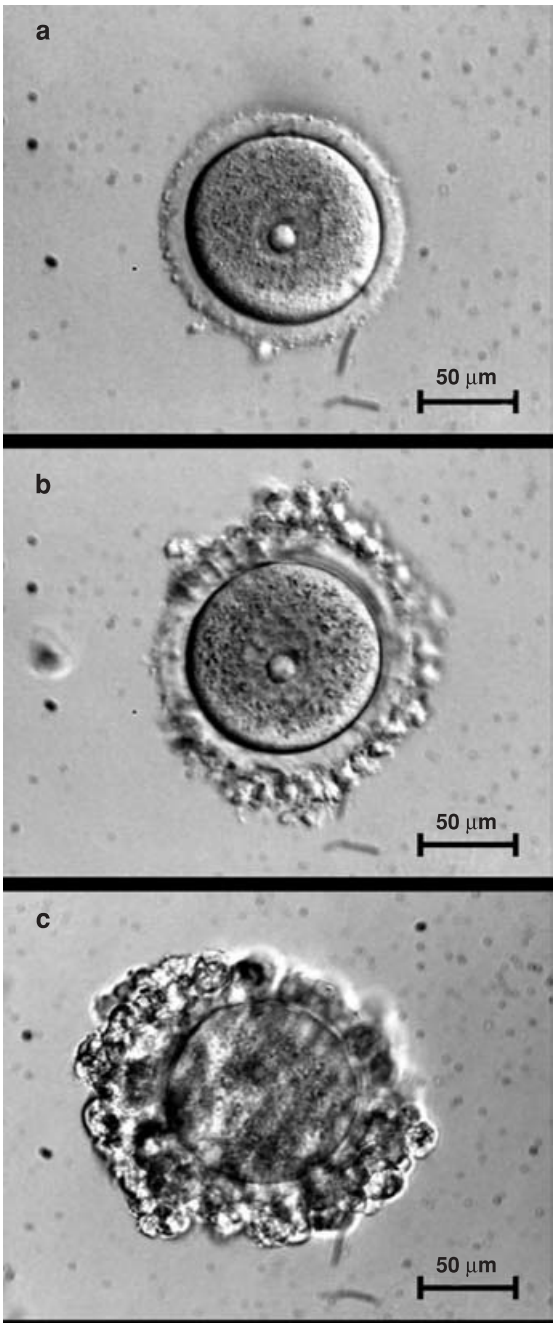


Figure 1 Immature germinal vesicle oocytes recovered from the mouse: (a) naked; (b) partially naked with sparse cumulus mass; (c) fully cumulus enclosed.

Collection of porcine follicular fluid

Porcine follicular fluid (PFF) was obtained by aspiration of selected 3–6 mm follicles of the ovaries of unstimulated prepubertal pigs with a 10 ml syringe and 18G needle. To remove cells and debris, the follicular aspirate was centrifuged at 1000 *g* for 10 min and the supernatant passed through a 0.5 μm filter. The filtered follicular fluid was frozen at −20 °C before being thawed for use.

Thaw and culture of the cryopreserved granulosa cells

The cryopreserved granulosa cells within the cryovials were thawed in a water bath set at 37 °C. The cells were then pelleted by centrifugation at 500 *g* for 10 min and resuspended in M199 medium supplemented with 20% (v/v) FCS, 0.23 mM pyruvate and antibiotics (50 μg ml^{-1} penicillin, 75 μg ml^{-1} streptomycin). This procedure was repeated at least twice to remove all traces of the cryoprotectant (dimethyl sulfoxide). Unlike earlier studies, the freeze–thawed granulosa cells were not subjected to enzymatic treatment (collagenase or trypsin) because the freeze–thaw process dissociated many of the granulosa cells. To remove large clumps of undissociated granulosa cells and other debris, the freeze–thawed cell suspension was passed through a cell strainer (Becton–Dickinson, USA). The granulosa cells were then seeded within a four-well Nunclon dish (Nunclon, Roskilde, Denmark) at a seeding density of 1.0×10^5 vital cells per well (10 mm diameter). The cells were plated either directly on the plastic surface or on a coat of ECM gel (Sigma, USA). After 24 h, unattached and dead cells were removed by gently rinsing the wells with fresh culture medium, which was subsequently allowed to be conditioned by the granulosa cells for a further 24 h before being used for coculture with the immature GV oocytes.

Preparation of conditioned medium

M199 medium supplemented with 10% (v/v) FCS, 0.23 mM pyruvate and antibiotics (50 μg ml^{-1} penicillin and 75 μg ml^{-1} streptomycin) was conditioned by granulosa cells for 24 h. The granulosa cells were either cultured on the plastic surface or on a coat of ECM gel (Sigma, USA) within four-well Nunclon dishes. The conditioned medium was then aspirated from the wells and subjected to centrifugation at 1000 *g* for 10 min to remove cells and other debris, and either used immediately for IVM or frozen at −80 °C before being thawed for use.

In vitro maturation of GV oocytes

In all experiments, the maturation medium used was M199 supplemented with 0.23 mM pyruvate and antibiotics (50 μg ml^{-1} penicillin and 75 μg ml^{-1} streptomycin). Additionally, the concentrations of FCS, PFF and gonadotrophins (FSH, hCG) within the maturation medium were varied according to the experiment. Unless indicated otherwise, all experiments used naked or partially naked oocytes with a clearly visible GV. IVM was carried out under a range of different culture conditions: cell-free or coculture with granulosa cells, in the absence or presence of ECM gel, and with granulosa-conditioned medium.

https://doi.org/10.1017/S0967199404002461 Published online by Cambridge University Press

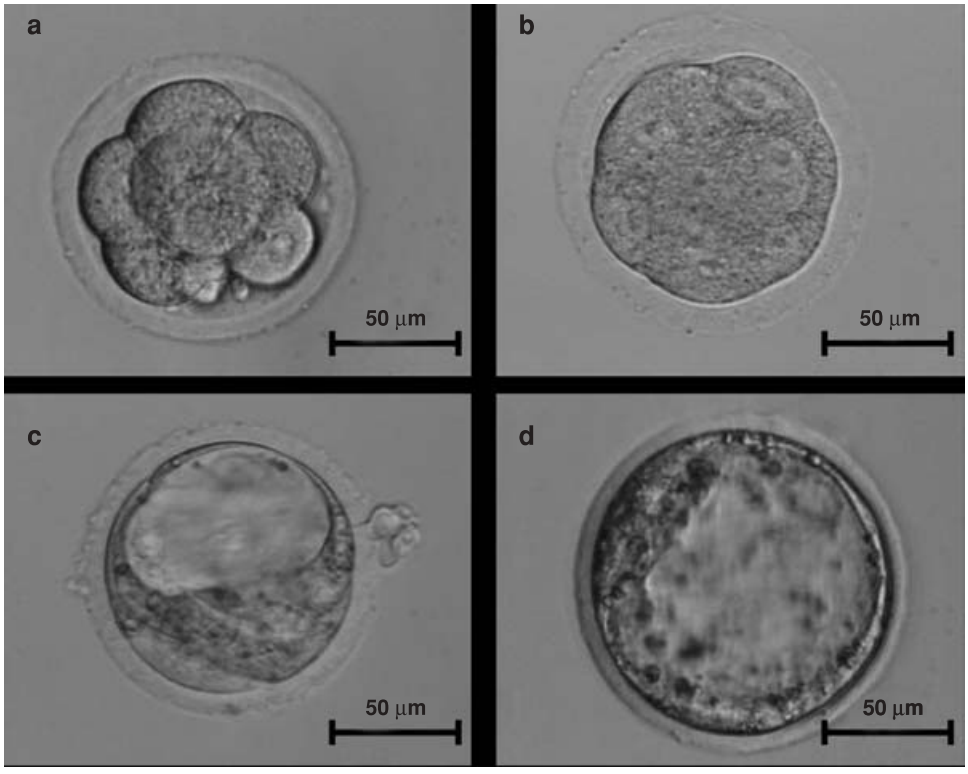


Figure 2 Preimplantation embryos derived from *in vitro* matured oocytes. (a) Compacting morula, day 3. (b) Compacted morula, day 3. (c) Early expanding blastocyst, day 4. (d) Fully expanded blastocyst, day 5.

After 18 h of culture, the maturity of the oocytes was assessed under a Hoffman-modulation contrast microscope. Only oocytes that possessed a distinct first polar body were classified as metaphase II.

In vitro fertilization of IVM oocytes

MII oocytes obtained from IVM were fertilized *in vitro* (IVF) with spermatozoa retrieved from the caudal epididymis of Swiss Albino (SA) male mice. The spermatozoa were teased from the epididymis with a 27-gauge needle, dispersed in MT6 medium (Whittingham, 1971) plus 15 mg ml^{-1} bovine serum albumin and subsequently diluted down to a final concentration of 5.0×10^6 ml^{-1}. 50 μl droplets of this sperm suspension were covered under light mineral oil (Sigma, USA) within a Petri dish, and incubated for at least 90 min within a humidified 5% CO_2 incubator at 37 °C in order to allow capacitation of the spermatazoa to take place. MII oocytes with a distinct first polar body were removed from the maturation medium after 18 h, washed in MT6 + 15 mg ml^{-1} bovine serum albumin and then inseminated by transferring to the sperm droplets under oil. The duration of insemination was 4–5 h. After insemination, the oocytes were washed and subsequently cultured in 50 μl droplets of KSOM medium (Erbach *et al*., 1994) under mineral oil, within the 5% CO_2 incubator at 37 °C.

Embryo culture and assessment of developmental competence

24 h after insemination, fertilization was assessed by cleavage to the two-cell stage. The two-cell embryos were transferred to fresh droplets of KSOM medium under mineral oil and cultured for a further 4 days. On day 3 after insemination, the embryos were assessed for signs of compaction (Fig. 2a,b) and, on day 5 after insemination, the blastulation rate was assessed. Only embryos with a distinct inner cell mass (ICM), trophectoderm (TE) and a clear blastocoelic cavity without any signs of vacuolation (Fig. 2c,d) were considered to be blastocyst stage embryos.

Total cell counts of blastocyst by differential fluorescent staining of ICM and TE

Blastocyst stage embryos on day 5 after insemination were subjected to fluorescent staining according to the methods of Ebert *et al*. (1985) and Papaioannou & Ebert (1988). Blastocysts that have not yet hatched or were in the process of hatching from their zonae pellucidae were enzymatically treated with 0.5% Pronase (Sigma, USA) in KSOM for 3–5 min under constant observation with a stereomicroscope, until their zonae were completely dissolved. The zona-free blastocysts were then washed in droplets of KSOM medium under oil at least three times and

https://doi.org/10.1017/S0967199404002461 Published online by Cambridge University Press

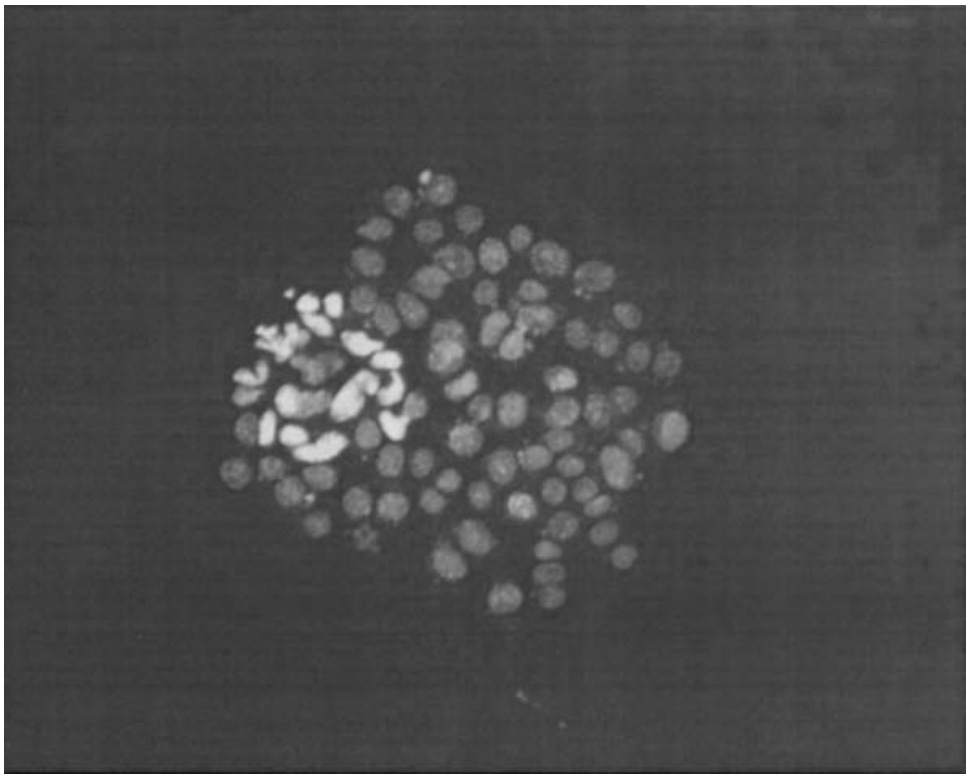


Figure 3 Differential staining of blastocyst for cell counts. The inner cell mass (ICM) is stained blue, whereas the trophectoderm (TE) is stained pink. The combined cell numbers of ICM and TE would give the total cell number (TCN).

then incubated in KSOM + 10% heat-inactivated rabbit anti-mouse serum (RAM) for 30 min at 37 °C. The blastocysts were then washed again and incubated in KSOM medium + 10% non-heat-inactivated guinea pig serum (as a source of complement) + fluorescent stains (10 μg ml^{-1} bisbezimide and 10 μg ml^{-1} propidium iodide; Sigma, USA) for another 30 min at 37 °C. To count the differentially stained nuclei of the ICM and TE, each blastocyst was individually placed within a droplet of medium on alcohol-cleaned microscope slides, squashed under a coverslip and viewed under ultraviolet light with an inverted microscope at 200× magnification. The ICM nuclei which were stained only by bisbenzimide would fluoresce blue, whereas the TE nuclei that were stained by both bisbenzimide and propidium iodide would fluoresce pink (Fig. 3). The combined numbers of differentially-stained ICM and TE nuclei would give the total cell count (TCN) for each blastocyst.

Statistical analysis of data

Immature GV oocytes collected from several females were randomly allocated to the different treatment groups within each set of experiments. At least three replicates of GV oocyte collection and culture were carried out for each treatment group. The proportion of MII oocytes and the proportion of those that remain arrested at the GV stage were expressed as a percentage of the initial number of GV oocytes cultured within each treatment group. Fisher's protected least significant difference (LSD) test and analysis of variance (ANOVA) were used to compare the proportion of immature GV oocytes that matured to MII, as well as the proportion of those that remain arrested at GV, in the different treatment groups. Cells counts of ICM, TE and TCN were presented as mean ± standard deviation, and these were compared with the unpaired Student's *t*-test. A probability of $P < 0.05$ was considered to be statistically significant.

Results

Validation of reduced meiotic competence in the murine model used in this study

The meiotic competence of fully cumulus enclosed oocyte complexes (COCs) recovered after 48 h of *in vivo* PMSG stimulation was compared with that of naked and partially naked GV oocytes retrieved after 24 h and 48 h of *in vivo* PMSG stimulation (Fig. 4). The proportion of GV oocytes from fully cumulus enclosed COCs (collected after 48 h of PMSG stimulation) that matured to MII was 65.3%, which was significantly higher ($P < 0.05$) than the value of 54.4% obtained for naked and partially naked oocytes that were

https://doi.org/10.1017/S0967199404002461 Published online by Cambridge University Press

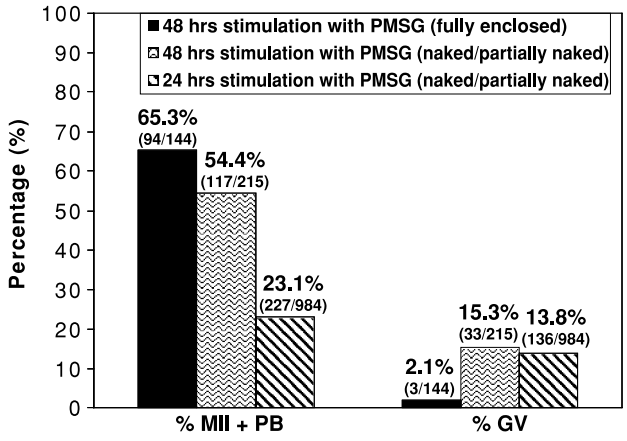


Figure 4 Proportions of fully cumulus enclosed and naked or partially naked GV oocytes that progress to MII and that remain arrested at the GV stage. The percentages are shown above the bars. The numbers in brackets represent the raw data expressed as a fraction, from which the percentages are calculated.

retrieved at the same time. When the duration of exposure to *in vivo* PMSG stimulation was shortened to 24 h, there was a further significant reduction ($P < 0.05$) in the meiotic maturation rate to 23.1%. The proportion of COCs (48 h *in vivo* PMSG stimulation) that were arrested at the GV stage was 2.1%. This was significantly lower ($P < 0.05$) than the values of 15.3% and 13.8% obtained for naked and partially naked GV oocytes that were retrieved after 48 h and 24 h of *in vivo* PMSG stimulation, respectively (Fig. 4). Hence, reducing the duration of *in vivo* PMSG stimulation from 48 h to 24 h and selecting only naked and partially naked GV oocytes led to a significant reduction in meiotic competence in the murine model used in this study.

Morphology of granulosa cells cultured on plastic and on ECM gel

Macaque, murine and porcine granulosa cells that were cultured directly on uncoated plastic surface formed monolayers that displayed characteristic epithelioid morphology, with the cells having a flattened polyhedral appearance (Fig. 5a–c). There were only slight differences in cell morphology between the three different species, with the murine and macaque granulosa cells being less elongated and having a more granular cytoplasm than the porcine granulosa cells. Confluent monolayers were formed by the granulosa cells of all three different species after 48 h of *in vitro* culture (Fig. 5d–f).

Granulosa cells that were cultured on a coat of ECM gel possessed a markedly different morphology to the single monolayer formed on the plastic surface. Instead of having a flattened epithelioid appearance, the granulosa cells of all three species have a spherical morphology when cultured on ECM gel, with their cytoplasm having a much darker and more granular appearance. It was also observed that the cells clustered together to form distinct clumps (Fig. 6a–c), as opposed

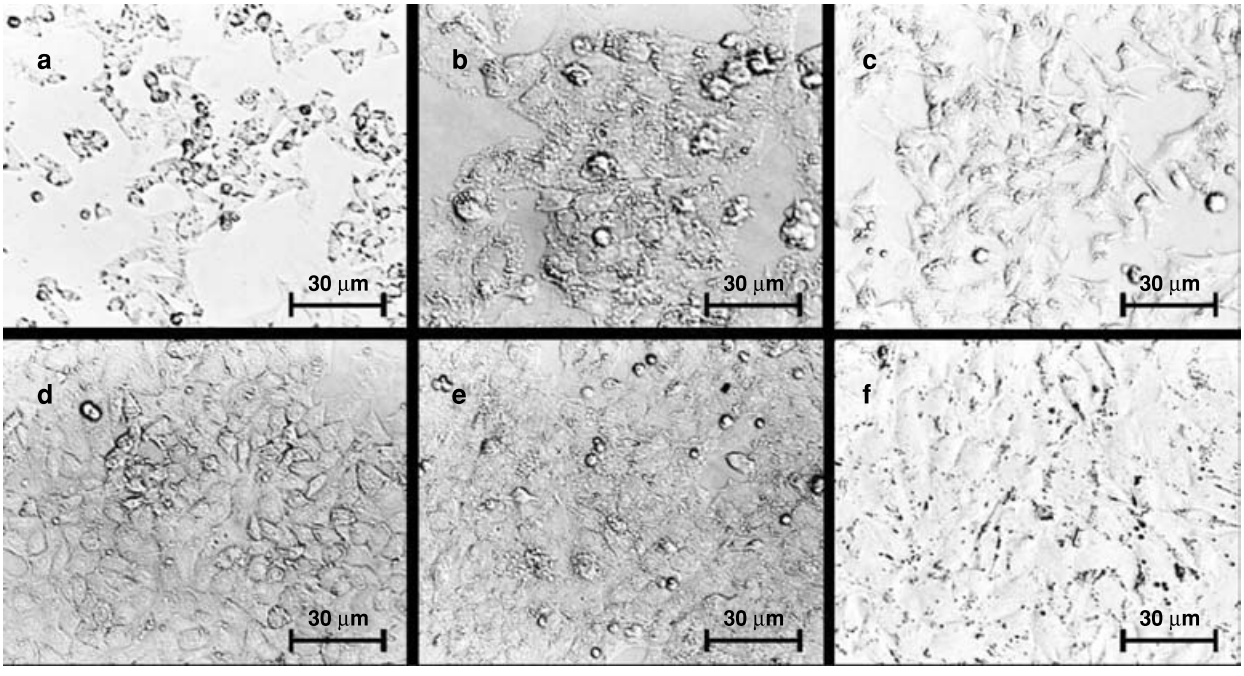


Figure 5 Monolayers of granulosa cells formed on plastic surface. (a) From mouse after 24 h *in vitro* culture. (b) From macaque after 24 h *in vitro* culture. (c) From pig after 24 h *in vitro* culture. (d) From mouse after 48 h *in vitro* culture. (e) From macaque after 48 h *in vitro* culture. (f) From pig after 48 h *in vitro* culture.

https://doi.org/10.1017/S0967199404002461 Published online by Cambridge University Press

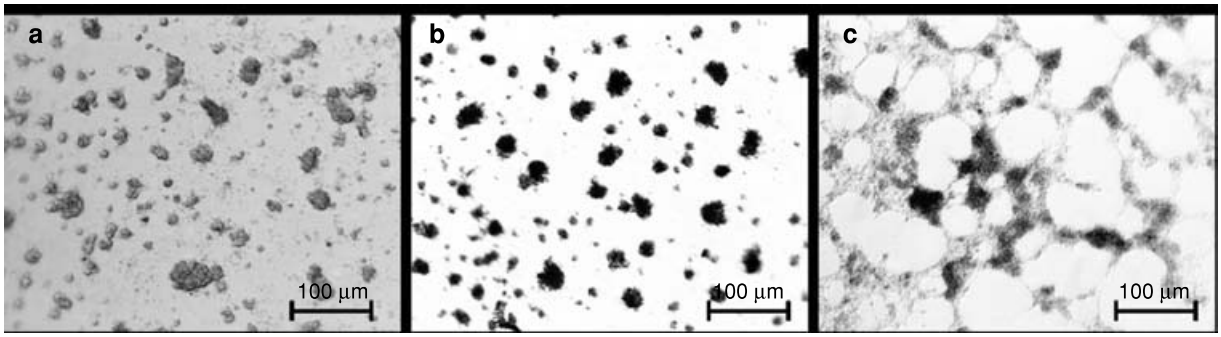


Figure 6 Clusters of granulosa cells formed on ECM gel. (a) From mouse after 48 h *in vitro* culture. (b) From macaque after 48 h *in vitro* culture. (c) From pig after 48 h *in vitro* culture.

to the monolayer formed on plastic. Although the macaque and murine granulosa cells appeared to form discrete isolated cell clumps (Fig. 6a,b), the clumps of porcine granulosa cells appeared to be interconnected through multicellular bridges, giving a web-like appearance on ECM gel (Fig. 6c).

Effects of co-culture and ECM gel on meiotic maturation

On plastic, the proportion of GV oocytes that matured to MII (Fig. 7a) in the presence of porcine granulosa coculture was 37.3%, which was significantly higher ($PF < 0.05$) than the value of 23.1% obtained for the cell-free control. The corresponding values of 19.3% and 28.3% obtained for macaque and mouse granulosa coculture, respectively, were both not significantly different from the cell-free control on plastic ($P > 0.05$). The proportions of immature oocytes that remained arrested at the GV stage (Fig. 7b) in the presence of murine and porcine granulosa coculture were 14.9% and 11.3%, respectively, which were both not significantly different ($P > 0.05$) from the value of 13.8% obtained for the cell-free control on plastic. However, the corresponding value of 22% obtained for macaque granulosa coculture was significantly higher ($P < 0.05$) than the cell-free control on plastic.

On ECM gel, the proportion of GV oocytes that matured to MII (Fig. 7a) in the presence of porcine granulosa coculture was 40.6%, which was significantly higher ($P < 0.05$) than the value of 24.5% obtained for the cell-free ECM gel control. The corresponding value of 29.4% obtained for murine granulosa coculture on ECM gel was not significantly different ($P > 0.05$) from the cell-free ECM gel control, whereas the corresponding value of 7.4% obtained with macaque granulosa coculture on ECM gel was significantly lower ($P < 0.05$). The proportion of immature oocytes that remained arrested at the GV stage (Fig. 7b) in the presence of macaque granulosa coculture on ECM gel was 41.4%, which was significantly higher than

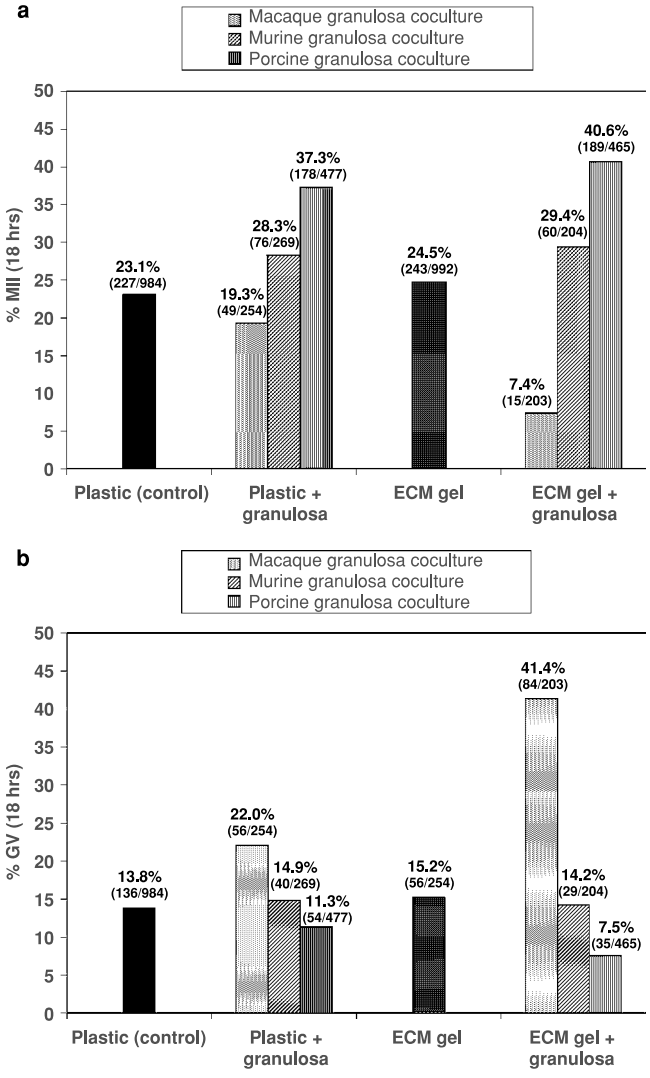


Figure 7 Effects of granulosa coculture (after 18 h culture) on (a) the meiotic maturation rate and (b) the proportion of immature oocytes arrested at the GV stage. The percentages are shown above the bars. The numbers in brackets represent the raw data expressed as a fraction, from which the percentages are calculated.

https://doi.org/10.1017/S0967199404002461 Published online by Cambridge University Press

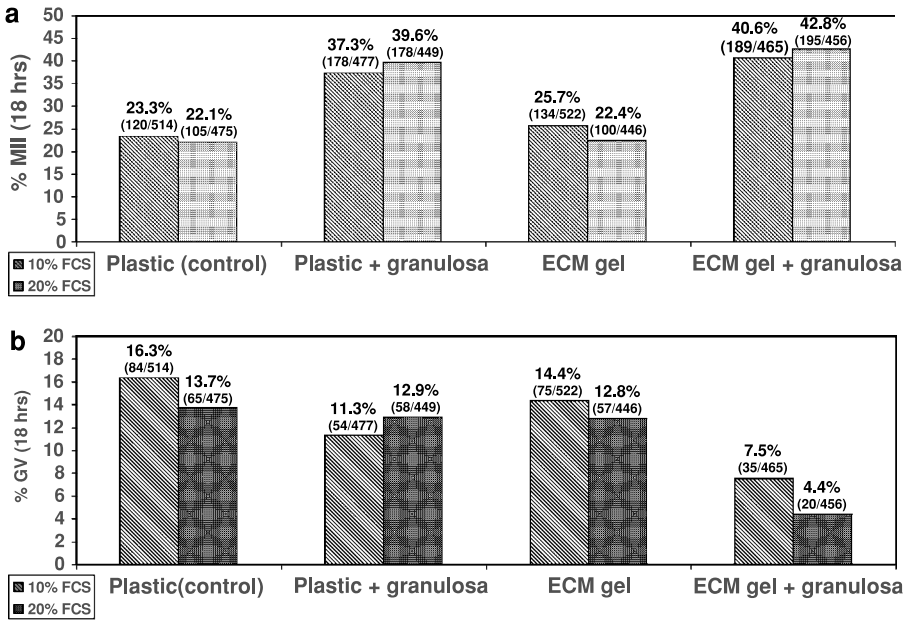


Figure 8 Effects of increasing serum (FCS) concentrations on (a) the meiotic maturation rate and (b) the proportion of immature oocytes arrested at the GV stage. The percentages are shown above the bars. The numbers in brackets represent the raw data expressed as a fraction, from which the percentages are calculated.

the value of 15.2% obtained for the cell-free ECM gel control. The corresponding value of 14.2% obtained for murine granulosa coculture on ECM gel was not significantly different ($P > 0.05$) from the cell-free ECM gel control, whereas the corresponding value of 7.5% obtained for porcine granulosa coculture on ECM gel was significantly lower ($P < 0.05$).

The results therefore show that only porcine granulosa coculture had a beneficial effect on murine meiotic maturation, with the macaque granulosa coculture having a detrimental effect and the murine granulosa coculture having neither a beneficial nor a detrimental effect.

The presence of ECM gel under cell-free conditions did not enhance the meiotic maturation rate (24.5% versus 23.1%, $P > 0.05$) or significantly lower the proportion of immature oocytes arrested at the GV stage (15.2% versus 13.8%, $P > 0.05$). Additionally, the presence of ECM gel did not further enhance the already beneficial effects that the porcine granulosa coculture had on the meiotic maturation rate (40.6% versus 37.3%, $P > 0.05$) or significantly lower the proportion of immature oocytes that remain arrested at the GV stage (7.5% versus 11.3%, $P > 0.05$).

Serum concentration and meiotic maturation

When the serum concentration within the maturation medium was increased from 10% (v/v) to 20% (v/v), there were no significant improvements in the meiotic maturation rates under cell-free conditions in either the presence (22.4% versus 25.7%, $P > 0.05$) or the absence (22.1% versus 23.3%, $P > 0.05$) of ECM gel (Fig. 8a). The proportion of immature oocytes that remained arrested at GV was also unaffected by the increase in serum concentration in either the presence (12.8% versus 14.4%, $P > 0.05$) or the absence (13.7% versus 16.3%, $P > 0.05$) of ECM gel (Fig. 8b).

When the serum concentration within the maturation medium was increased from 10% (v/v) to 20% (v/v) in the presence of porcine granulosa coculture; there were also no significant improvements in the meiotic maturation rates in either the presence (42.8% versus 40.6%, $P > 0.05$) or the absence (39.6% versus 37.3%, $P > 0.05$) of ECM gel (Fig. 8a). Similarly, the proportion of immature oocytes which remained arrested at GV was also unaffected by the increase in serum concentration, both in the presence (4.4% versus 7.5%, $P > 0.05$) and absence (12.9% versus 11.3%, $P > 0.05$) of ECM gel (Fig. 8b). Hence, increasing the serum concentration from 10% (v/v) to 20% (v/v) had no effect on meiotic maturation under either cell-free or coculture conditions.

PFF and meiotic maturation

Under cell-free conditions in the presence of ECM gel (Fig. 9a), when 10% (v/v) FCS within the maturation medium was substituted for 10% (v/v) PFF, there were no significant changes in either the meiotic maturation rate (26.1% versus 24.8%, $P > 0.05$) or the proportion of immature oocytes arrested at the GV stage (14.1% versus 20.3%, $P > 0.05$). When the concentration of PFF was increased from 10% (v/v) to 20% (v/v), there was a

https://doi.org/10.1017/S0967199404002461 Published online by Cambridge University Press

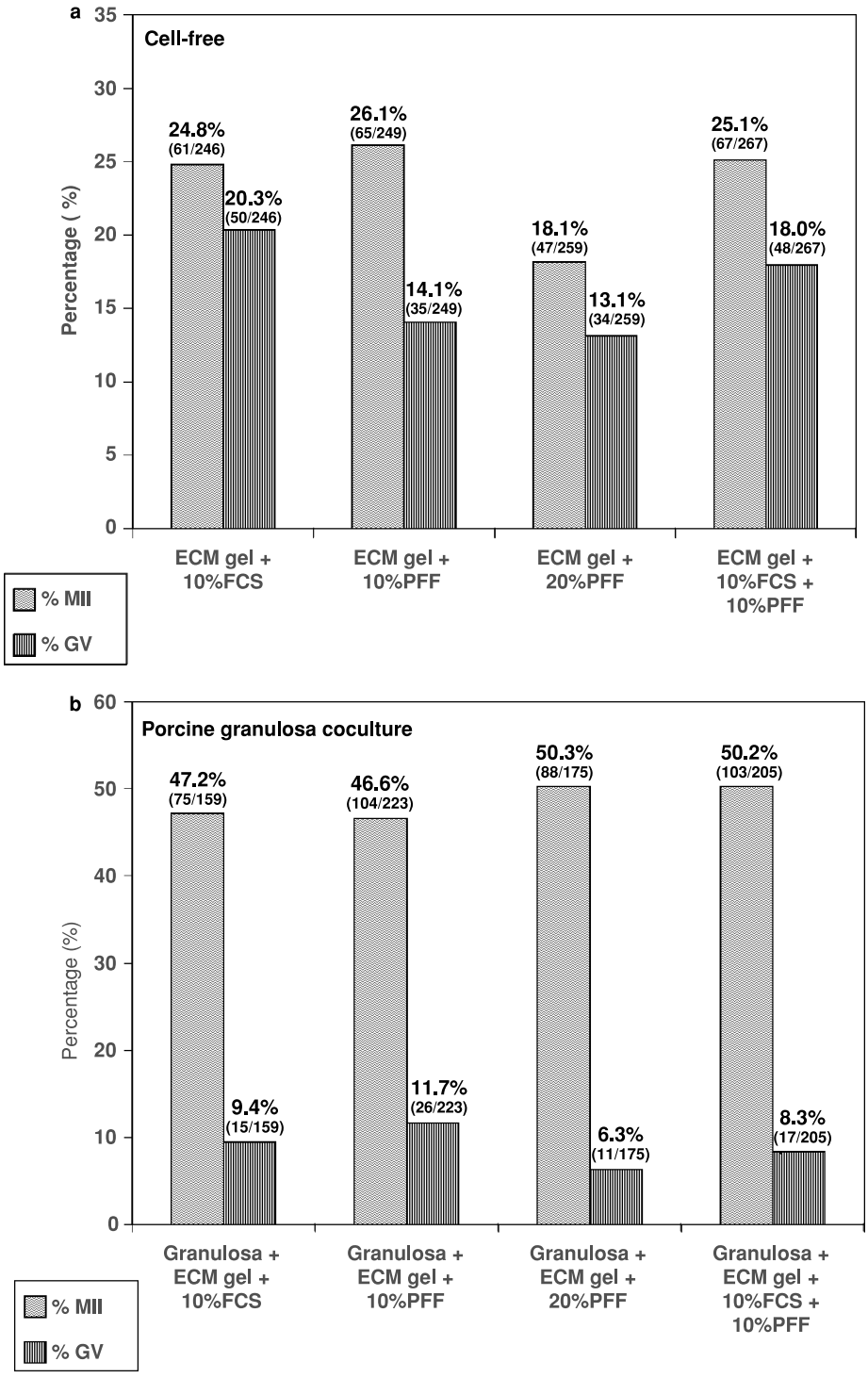


Figure 9 Effects of porcine follicular fluid (PFF) on the proportions of immature oocytes progressing to MII and arrested at the GV stage (a) under cell-free culture conditions and (b) with porcine granulosa coculture. The percentages are shown above the bars. The numbers in brackets represent the raw data expressed as a fraction, from which the percentages are calculated.

significant decrease in the meiotic maturation rate from 26.1% to 18.1% ($P < 0.05$) but no significant change in the proportion of immature oocytes arrested at the GV stage (13.1% versus 14.1%, $P > 0.05$). When 10% (v/v) FCS was combined with 10% (v/v) PFF, there were also no significant changes in either the meiotic maturation rate (25.1% versus 24.8%, $P > 0.05$) or the proportion of immature oocytes arrested at the GV stage (18.0% versus 20.3%, $P > 0.05$) compared with the presence of 10% (v/v) FCS alone.

With porcine granulosa coculture on ECM gel (Fig. 9b), when 10% (v/v) FCS within the maturation

https://doi.org/10.1017/S0967199404002461 Published online by Cambridge University Press

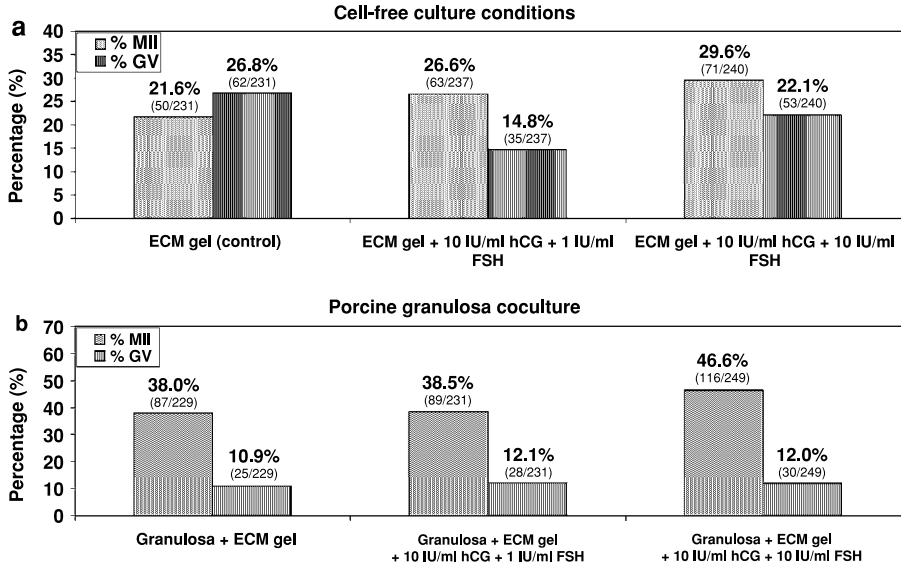


Figure 10 Effects of varying concentrations of gonadotrophins (hCG and FSH) on the proportions of immature oocytes progressing to MII and arrested at the GV stage (a) under cell-free culture conditions and (b) with porcine granulosa coculture. The percentages are shown above the bars. The numbers in brackets represent the raw data expressed as a fraction, from which the percentages are calculated.

medium was substituted for 10% (v/v) PFF, there were no significant changes in either the meiotic maturation rate (46.6% versus 47.2%, $P > 0.05$) or the proportion of immature oocytes arrested at the GV stage (11.7% versus 9.4%, $P > 0.05$). When the concentration of PFF was increased from 10% (v/v) to 20% (v/v), there were no significant changes in either the meiotic maturation rate (50.3% versus 46.6%, $P > 0.05$) or the proportion of immature oocytes arrested at the GV stage (6.3% versus 11.7%, $P > 0.05$). When 10% (v/v) FCS was combined with 10% (v/v) PFF, there were also no significant changes in either the meiotic maturation rate (50.2% versus 47.2%, $P > 0.05$) or the proportion of immature oocytes arrested at the GV stage (8.3% versus 9.4%, $P > 0.05$) compared with the presence of 10% (v/v) FCS alone.

The results (Fig. 9a,b) therefore show that the inclusion of PFF within the maturation medium had no beneficial effects on meiotic maturation under cell-free or coculture conditions. Furthermore, high concentrations of PFF (20% v/v) appeared to be detrimental to meiotic maturation under cell-free conditions (Fig. 9a), but this effect was 'masked' in the presence of porcine granulosa coculture (Fig. 9b).

Gonadotrophins and meiotic maturation

Under cell-free conditions in the presence of ECM gel (Fig. 10a), when 10 IU ml^{-1} hCG and 1 IU ml^{-1} FSH were introduced within the maturation medium, there was no significant change in the meiotic maturation rate (26.6% versus 21.6%, $P > 0.05$), but the proportion of immature oocytes arrested at the GV stage was significantly reduced (14.8% versus 26.8%, $P < 0.05$) compared with the gonadotrophin-free control. When the concentration of FSH was increased from 1 IU ml^{-1} to 10 IU ml^{-1} in the presence of 10 IU ml^{-1} hCG, there was a significant increase in the meiotic maturation rate compared with the gonadotrophin-free control (29.6% versus 21.6%, $P < 0.05$), but no significant change in the proportion of immature oocytes arrested at the GV stage (22.1% versus 26.8%, $P > 0.05$).

With porcine granulosa coculture on ECM gel (Fig. 10b), when 10 IU ml^{-1} hCG and 1 IU ml^{-1} FSH were introduced within the maturation medium, there were no significant changes in either the meiotic maturation rate (38.5% versus 38.0%, $P > 0.05$) or the proportion of immature oocytes arrested at the GV stage (12.1% versus 10.9%, $P > 0.05$) compared with the gonadotrophin-free control. When the concentration of FSH was increased from 1 IU ml^{-1} to 10 IU ml^{-1} in the presence of 10 IU ml^{-1} hCG, there were also no significant changes in either the meiotic maturation rate (46.6% versus 38.0%, $P > 0.05$) or the proportion of immature oocytes arrested at the GV stage (12.0% versus 10.9%, $P > 0.05$) compared with the gonadotrophin-free control.

The results therefore show that the inclusion of gonadotrophins within the maturation medium was beneficial to meiotic maturation only under cell-free conditions, but this beneficial effect was 'masked' within porcine granulosa coculture.

https://doi.org/10.1017/S0967199404002461 Published online by Cambridge University Press

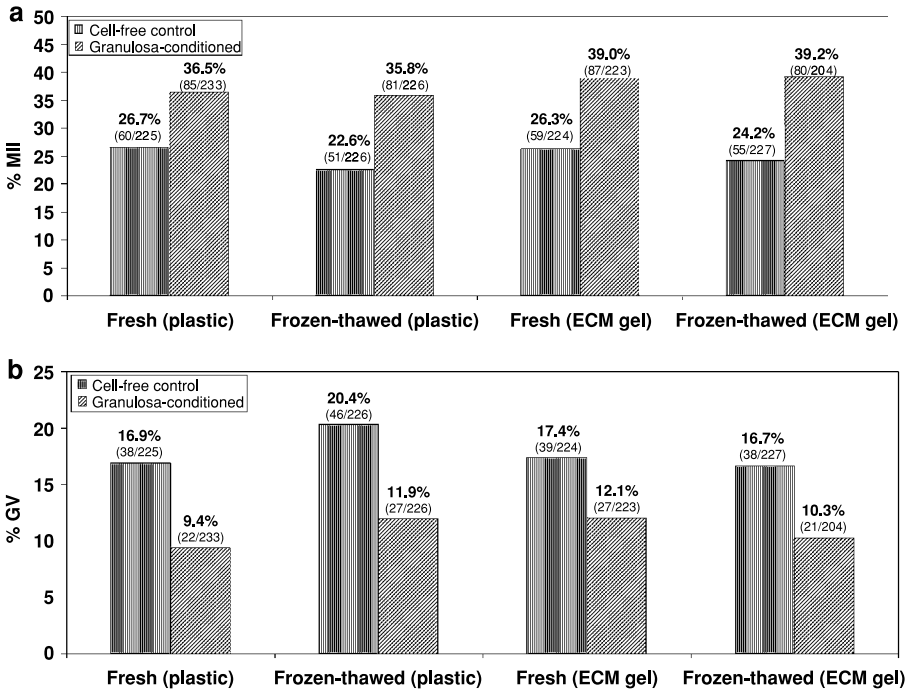


Figure 11 Effects of fresh and freeze–thawed porcine granulosa conditioned medium on (a) the meiotic maturation rate and (b) the percentage of immature oocytes arrested at the GV stage. The percentages are shown above the bars. The numbers in brackets represent the raw data expressed as a fraction, from which the percentages are calculated.

Porcine granulosa coculture conditioned medium and meiotic maturation

Maturation medium that was conditioned by porcine granulosa cells on plastic enhanced the meiotic maturation rate (Fig. 11a) compared with the cell-free control (36.5% versus 26.7%, $P < 0.05$). Additionally, there was also a significant reduction in the proportion of immature oocytes arrested at the GV (Fig. 11b) stage (9.4% versus 16.9%, $P < 0.05$). When the conditioned medium was freeze–thawed, there was also an enhancement of the meiotic maturation rate (35.8% versus 22.6%, $P < 0.05$), as well as a significant reduction in the proportion of immature oocytes arrested at the GV stage (11.9% versus 20.4%, $P < 0.05$).

When maturation medium was conditioned by porcine granulosa cells cultured on ECM gel, there was also an enhancement of the meiotic maturation rate compared with the corresponding cell-free control (39.0% versus 26.3%, $P < 0.05$). However, the reduction in the proportion of immature oocytes arrested at the GV stage was not significant (12.1% versus 17.4%, $P > 0.05$). When the conditioned medium on ECM gel was freeze–thawed, there was also an enhancement of the meiotic maturation rate (39.2% versus 24.2%, $P < 0.05$), as well as a significant reduction in the proportion of immature oocytes arrested at the GV stage (10.3% versus 16.7%, $P < 0.05$).

The results (Fig. 11a,b) therefore show that maturation medium conditioned by porcine granulosa cells had a beneficial effect on oocyte meiotic maturation that was not diminished by freeze–thawing.

Developmental competence of IVM oocytes

The developmental competence of IVM oocytes was assessed by IVF and subsequent embryo culture to the blastocyst stage. On plastic (Fig. 12a), porcine granulosa coculture did not enhance the proportion of IVM oocytes that fertilized and cleaved to the two-cell stage compared with the corresponding cell-free control on plastic (53.0% versus 58.5%, $P > 0.05$). Additionally, there was also no enhancement in the proportions of two-cell embryos able to reach the compacting/compacted stage on day 3 (66.9% versus 76.8%, $P > 0.05$) or the blastocyst stage on day 5 (33.8% versus 41.3%, $P > 0.05$). Hence, the values obtained for the porcine granulosa coculture IVM group on plastic appear to be consistently slightly lower than the corresponding cell-free control, but these were not significantly different ($P > 0.05$).

In the presence of ECM gel (Fig. 12b), the proportion of IVM oocytes that fertilized and cleaved to the two-cell stage was significantly lower for the porcine granulosa coculture group than for the cell-free control on ECM gel (27.0% versus 36.7%, $P < 0.05$). The proportions of two-cell embryos reaching the compacting/compacted stage on day 3 (43.1% versus 47.7%, $P > 0.05$) and the blastocyst stage on day 5 (20.0% versus 27.3%, $P > 0.05$) were slightly lower for

https://doi.org/10.1017/S0967199404002461 Published online by Cambridge University Press

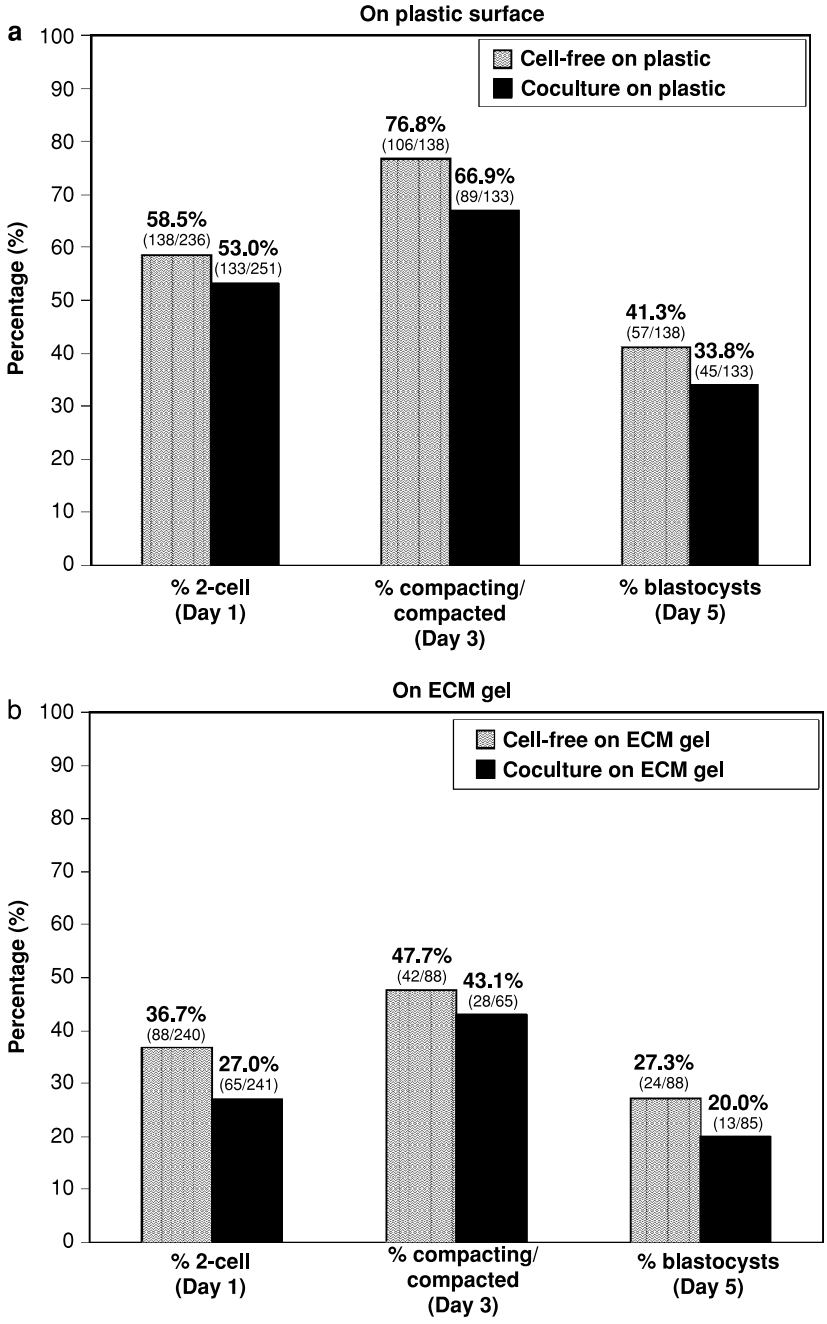


Figure 12 Effects of porcine granulosa coculture on the embryonic development of IVM oocytes that were cultured on (a) plastic surface and (b) ECM gel. The percentages are shown above the bars. The numbers in brackets represent the raw data expressed as a fraction, from which the percentages are calculated.

the porcine granulosa coculture group than for the cell-free control, but these were not significantly different.

The values obtained for IVM oocytes exposed to ECM gel were all consistently and significantly lower ($P < 0.05$) than the corresponding values on plastic under both cell-free and coculture conditions (Fig. 12a,b). Hence, the results show that ECM gel could be detrimental to the developmental competence of IVM oocytes.

Cell counts of blastocysts derived from IVM oocytes

On plastic (Table 1), the use of porcine granulosa coculture for IVM did not significantly increase the total cell numbers of the blastocysts compared to the corresponding cell-free control [61.7 ± 23.0 ($n = 24$) versus 59.7 ± 21.0 ($n = 30$), $P > 0.05$]. There were also no significant differences in the cell counts of the ICM [16.3 ± 6.8 ($n = 24$) versus 16.2 ± 8.0 ($n = 30$), $P > 0.05$]

https://doi.org/10.1017/S0967199404002461 Published online by Cambridge University Press

Table 1 Cell counts of trophectoderm (TE), inner cell mass (ICM) and total cell numbers (TCN) of blastocysts (on day 5) derived from IVM oocytes

Substrate	Conditions	*n*	TE	ICM	TCN
Plastic	Cell free	30	43.4 ± 17.6	16.2 ± 8.0	59.7 ± 21.0
	Porcine granulosa	24	45.4 ± 21.8	16.3 ± 6.8	61.7 ± 23.0
ECM gel	Cell free	18	22.9 ± 8.8	14.3 ± 6.1	37.2 ± 10.5
	Porcine granulosa	7	22.9 ± 11.3	15.7 ± 6.9	38.6 ± 16.6

and TE [45.4 ± 21.8 ($n = 24$) versus 43.4 ± 17.6 ($n = 30$), $P > 0.05$].

In the presence of ECM gel (Table 1), the use of porcine granulosa coculture for IVM also did not significantly change the cell counts of the ICM [15.7 ± 6.9 ($n = 7$) versus 14.3 ± 6.1 ($n = 18$), $P > 0.05$], TE [22.9 ± 8.8 ($n = 7$) versus 22.9 ± 11.3 ($n = 18$), $P > 0.05$] and TCN [38.6 ± 16.6 ($n = 7$) versus 37.2 ± 10.5 ($n = 18$), $P > 0.05$] of the blastocysts compared with the corresponding cell-free control. However, when the IVM oocytes were exposed to ECM gel, the cell counts of the TE were significantly lower than the corresponding values on plastic under both cell-free [43.4 ± 17.6 ($n = 30$) versus 22.9 ± 8.8 ($n = 18$), $P < 0.05$] and coculture [45.4 ± 21.8 ($n = 24$) versus 22.9 ± 11.3 ($n = 7$), $P < 0.05$] conditions. This in turn led to significant differences in the corresponding TCN under both cell-free [59.7 ± 21.0 ($n = 30$) versus 37.2 ± 10.5 ($n = 18$), $P < 0.05$] and coculture [61.7 ± 23.0 ($n = 24$) versus 38.6 ± 16.6 ($n = 7$), $P < 0.05$] conditions when ECM gel was present. Hence, the results show that ECM gel could be detrimental to the developmental competence of IVM oocytes.

Discussion

The murine IVM model that was used in previous studies had a high degree of meiotic competence, with as many as 70–80% of the immature GV oocytes progressing to MII in the presence of standard culture milieu (Table 2). Hence, this study attempted to develop a 'less competent' murine model for oocyte meiotic maturation *in vitro* that could be more relevant to developing IVM protocols for clinical human assisted reproduction. For such a 'less competent' murine IVM model, the omission of *in vivo* gonadotrophin (PMSG) stimulation would be inappropriate because there would be a great deal of variability arising from the endogenous ovulatory cycle within the female mice. This problem could be solved by selectively retrieving COCs from only large antral follicles from ovaries that appear to be well stimulated, as in the case of previous IVM studies on the porcine and bovine model, whereby COCs are retrieved from slaughterhouse ovaries without any prior exogenous gonadotrophin stimulation (Chanson *et al*., 2001; Kikuchi *et al*., 1999). The pitfall of this approach is that such immature oocytes would be near the end of the follicular phase of the endogenous ovulatory cycle within the female mice and would therefore not provide a 'less competent' murine IVM model. What would be required for a 'less competent' model would be to increase the proportion of immature GV oocytes that are truncated mid-way through the early follicular phase of development, when they are retrieved for IVM.

Hence, *in vivo* stimulation with PMSG was provided to increase the number of immature oocytes that are recruited and set on the pathway of follicular development so that, when the immature oocytes are recovered for IVM after a shortened stimulation time of 24 h, a higher proportion of such immature oocytes would be truncated mid-way through the early follicular phase of their development. This would then provide a 'less competent' model for mouse meiotic maturation *in vitro*. Indeed, the results (Fig. 4) do indeed show that a reduction in the duration of *in vivo* PMSG stimulation from 48 h to 24 h led to a significant decrease in meiotic competence.

The putatively 'less competent' murine IVM model developed in this study also used naked and partially naked GV oocytes, as opposed to the fully cumulus-enclosed oocyte complexes used in previous studies. The results (Fig. 4) show that naked or partially naked oocytes that were retrieved after the standard 48 h of *in vivo* PMSG stimulation had reduced meiotic competence compared with fully cumulus-enclosed oocyte complexes that were retrieved at the same time. This is expected from previous studies with different species, which have all shown that the surrounding cumulus cells have an important part to play in the oocyte meiotic maturation process (Gilula *et al*., 1978; Voznesenskaia *et al*., 2001). This is probably mediated through their gap junction cytoplasmic connections to the ooplasm (Carabatsos *et al*., 2000), which traverses the zona pellucida, as well as by the cumulus mass exerting a modulatory effect on the microenvironment surrounding the immature oocytes (Byskov *et al*., 1997; McNatty *et al*., 1980); which could be important if the *in vitro* culture conditions are suboptimal. The use of naked or partially naked immature GV oocytes could also make this study more relevant to the human clinical assisted reproduction model, in which a

https://doi.org/10.1017/S0967199404002461 Published online by Cambridge University Press

https://doi.org/10.1017/S0967199404002461 Published online by Cambridge University Press

Table 2 Comparison of the murine IVM model used in this study with previous studies

Reference	State of cumulus mass	Duration and type of in vivo gonadotrophin stimulation	Duration of in vitro culture	Base medium	Presence of gonadotrophins in culture medium	Maturation rate (% MII)
Andriesz & Trounson, 1995	Fully enclosed COCs	48 h, 5 IU PMSG	18 h	TCM 199 + 10% FCS	4 ng ml^{-1} LH + 4 ng ml^{-1} FSH	80%
Downs & Mastropolo, 1997	Fully enclosed COCs	48 h, 5 IU PMSG	17–18 h	MEM	Nil	75%
Downs & Mastropolo, 1997	Fully enclosed COCs	48 h, 5 IU PMSG	17–18 h	Ham's F10	Nil	77%
Downs & Mastropolo, 1997	Fully enclosed COCs	48 h, 5 IU PMSG	17–18 h	TCM 199 + 0.23 mM pyruvate	Nil	78%
Byskov et al., 1997	Fully enclosed COCs	48 h, Humegon (20 IU FSH + 20 IU LH)	24 h	Mc Coy's 5A Culture medium	36 IU l^{-1} FSH	65%
De La Fuente et al., 1999	Fully enclosed COCs	48 h, 5 IU PMSG	17–18 h	Waymouth medium	100 ng ml^{-1} FSH	54%
Nakano & Kubo, 2000	Denuded or naked oocyte	42–48 h, 5 IU PMSG	24 h	Human tubal fluid	Nil	74.6%
This study	Fully enclosed COCs	48 h, 5 IU PMSG	18 h	TCM 199 + 10% FCS + 0.23 mM pyruvate	Nil	65.3%
This study	Naked or partially naked oocyte	48 h, 5 IU PMSG	18 h	TCM 199 + 10% FCS + 0.23 mM pyruvate	Nil	54.4%
This study	Naked or partially naked oocyte	24 h, 5 IU PMSG	18 h	TCM 199 + 10% FCS + 0.23 mM pyruvate	Nil	23.1%

substantial number of naked or partially naked oocytes are routinely obtained by follicular aspiration or upon denudation of the cumulus mass for intracytoplasmic sperm injection (ICSI).

The next step of the study would be to investigate the use of coculture to improve the meiotic competence of such putatively 'less competent' GV oocytes from the mouse. Ovarian granulosa cells would be the physiological choice for coculture, because these are found *in vivo* within the developing follicle, and undoubtedly play an important role in the oocyte maturation process. Additionally, a novel granulosa coculture system on a bed of ECM gel would also be investigated. Sites *et al*. (1996) reported that the presence of ECM adhesion proteins enhanced FSH receptor expression and intercellular interaction among porcine granulosa cells cultured *in vitro*. Hwang *et al*. (2000) and Richardson *et al*. (2000) reported that the *in vitro* culture of human granulosa cells on ECM probably mimicked the physiological situation *in vivo*, in which sheets of granulosa cells are attached to the basement membrane within the follicle. Indeed, both Hwang *et al*. (2000) and Richardson *et al*. (2000) observed in their studies that human granulosa cells appear to cluster together and to have a more differentiated morphology in the presence of ECM. Hence, the presence of ECM might enhance any beneficial effects that granulosa coculture could have on the IVM of mouse GV oocytes.

The beneficial effect of porcine granulosa coculture on murine meiotic maturation (Fig. 7) is consistent with previous studies that showed that granulosa monolayers (Anderiesz & Trounson, 1995; Dandekar *et al*., 1991) and autologous follicular shells (Abeydeera *et al*., 1998; Hunter, 2000) derived from the pig had a beneficial effect on oocyte meiotic maturation. Granulosa cells have been reported to secrete various meiosis-promoting factors (Byskov *et al*., 1997; Driancourt & Thuel, 1998). It is likely that such factors are not species specific, so that a beneficial effect of porcine granulosa coculture on murine meiotic maturation was observed in this study. Although previous studies (Sites *et al*., 1996; Hwang *et al*., 2000; Richardson *et al*., 2000; Ben Ze'ev & Amsterdam, 1989) have reported that the *in vitro* culture of granulosa cells on ECM enhanced cellular differentiation and impeded senescence by providing a more physiological environment (akin to the cells being attached to basement membrane *in vivo*); the results showed that the use of ECM gel did not further enhance the beneficial effects that the porcine granulosa coculture already had on meiotic maturation (Fig. 7a,b).

The inhibitory effect of macaque granulosa coculture on murine meiotic maturation (Fig. 7) could be because of increased inhibin production by the luteinized granulosa cells (Seifer *et al*., 1996). Unlike the granulosa cells from the mouse and pig, which were non-luteinized, the macaque granulosa cells that were used in this study were luteinized owing to *in vivo* exposure to hCG (34–36 h) before being retrieved by follicular aspiration. The formation of corpus-luteum-like clusters on ECM gel (Fig. 6b) could further increase inhibin production, which could in turn further reduce the meiotic maturation rate, as observed in this study (Fig. 7).

Unlike porcine and macaque granulosa, coculture with murine granulosa either on plastic or ECM gel did not appear to have any significant effect on the meiotic maturation rate or on the proportion of immature oocytes arrested at the GV stage (Fig. 7a,b). This was consistent with the results of Eppig & Downs (1987), who also found no significant effect of mouse granulosa coculture on murine meiotic maturation, both in the presence and absence of FSH.

Previous studies have reported that oocyte meiotic maturation *in vitro* and subsequent developmental competence are enhanced in the presence of serum (Leibfried-Rutledge *et al*., 1986; Kito & Bavister, 1997; Mermillod *et al*., 1999; Yamazaki *et al*., 2001). It has been suggested that serum not only inhibits zona hardening within *in vitro* culture (Eppig *et al*., 1992) but also enhances oocyte cytoplasmic maturity (Mermillod *et al*., 1999; Yamazaki *et al*., 2001). The results (Fig. 8a,b) show that increasing the serum concentration from 10% (v/v) to 20% (v/v) had no significant effect on murine meiotic maturation under either cell-free or coculture conditions, in the absence and presence of ECM gel. It is possible that increased serum concentrations could be more beneficial to fully cumulus enclosed oocyte complexes (Leibfried-Rutledge *et al*., 1986; Kito & Bavister, 1997), as opposed to the naked and partially naked oocytes used in this study. Another possibility is that 10% (v/v) serum concentration within the culture medium is already optimal, so that increasing the serum concentration would have no further beneficial effect on the meiotic maturation rate.

The supplementation of follicular fluid within the culture medium for IVM have led to ambiguous results in previous studies, with some studies reporting a beneficial effect on oocyte meiotic maturation (Reed *et al*., 1993; Khatir *et al*., 1997; Vatzias & Hagen, 1999; Yoon *et al*., 2000; Bøgh *et al*., 2002), whereas other studies reported a detrimental effect (Jagiello *et al*., 1977; Hillensjö *et al*., 1979; Tsafriri & Pomerantz, 1984; Slüss *et al*., 1989; Bhat & Moudgal, 1989; Kokawa *et al*., 1993; Ayoub & Hunter, 1993). Additionally, it is possible that follicular fluid could enhance or stimulate granulosa cell function, because it is the physiological fluid found within the follicle. This could in turn further enhance the beneficial effects of porcine granulosa coculture on murine meiotic maturation (Fig. 7a,b). The results (Fig. 9a,b), however, showed that PFF by

https://doi.org/10.1017/S0967199404002461 Published online by Cambridge University Press

itself or in combination with serum had no significant beneficial effect on murine meiotic maturation, under either cell-free or coculture conditions. In fact, a high concentration of PFF (20%) was detrimental to murine meiotic maturation (Fig. 9a,b). This is consistent with several previous studies that reported the presence of meiosis inhibitory factors within follicular fluid (Jagiello *et al*., 1977; Hillensjö *et al*., 1979; Tsafriri & Pomerantz, 1984; Slüss *et al*., 1989).

The inclusion of gonadotrophins within the culture medium appeared to have a slightly beneficial effect on murine meiotic maturation only under cell-free conditions. This could be mediated by gonadotrophin receptors on the sparse cumulus cells surrounding some of the partially naked GV oocytes. The effects of gonadotrophins appeared to be somewhat 'masked' within porcine granulosa coculture, leading to no further enhancement of the beneficial effects of porcine granulosa coculture on murine meiotic maturation (Fig. 10b).

Because it was shown in this study and in previous studies that granulosa coculture had a beneficial effect on oocyte meiotic maturation (Anderiesz & Trounson, 1995; Dandekar *et al*., 1991), it is likely that the granulosa cells could be secreting some meiosis promoting factors within the culture medium. It is hypothesized that such secreted factors could remain active within the granulosa-conditioned medium under cell-free conditions, which could in turn lead to an enhancement in oocyte meiotic maturation. This hypothesis is supported by previous studies on granulosa-conditioned medium, which have shown the presence of embryotrophic factors secreted into the medium by the granulosa cells (Kobayashi *et al*., 1992; Maeda *et al*., 1996).

Indeed, the results of this study (Fig. 11a,b) showed that the porcine granulosa-conditioned medium enhanced murine meiotic maturation to a similar extent to that observed for granulosa coculture itself. Additionally, the results also showed that the putative meiosis-promoting factors secreted by the granulosa cells into the conditioned medium remained potent after freeze–thawing. This is somewhat consistent with the study of Kobayashi *et al*. (1992), which showed that the embryotrophic activity of granulosa-conditioned medium was stable to freeze–thawing, lysophilization and heating at 56 °C.

Although the earlier part of this study showed that porcine granulosa coculture improved the meiotic maturation rate (Fig. 7), this did not translate into any significant improvement in developmental competence, as assessed by IVF, embryo culture to the blastocyst stage (Fig. 12) and subsequent total cell counts (Table 1). In fact, the proportions of oocytes cleaving to the two-cell stage and developing further to the compacting/compacted and blastocyst stage were consistently lower for the porcine granulosa coculture group than the cell-free control (Fig. 12). One possible hypothesis is that some of the less maturationally and developmentally competent immature oocytes that could not have progressed to MII under cell-free conditions could do so when stimulated by porcine granulosa coculture. Nevertheless, because such MII oocytes are not developmentally competent, a large proportion of them could not undergo cleavage to the two-cell stage after IVF or develop further to the compacting/compacted and blastocyst stage. This could then possibly explain why the corresponding proportions of two-cell, compacting/compacted and blastocyst stage embryos were consistently lower for the coculture-IVM group than the cell-free control (Fig. 12a,b). However, it is difficult to validate this hypothesis experimentally.

The results (Fig. 12b) showed that ECM gel could be detrimental to the fertilization and developmental competence of the IVM oocytes. One possible hypothesis is that ECM gel hardened the zona pellucida of the IVM oocytes. Dolci *et al*. (1991) reported that several different lectins had the ability to harden the zona pellucida of murine oocytes. ECM gel, which is a basement membrane extract of Engelbeth–Swarm mouse sarcoma (Kleinman, 1993), is a complex heterogeneous mixture of various ECM components including lectins, and hence could have a possible effect on zona hardening. Zona hardening would in turn reduce the fertilization rate and hence the proportion of IVM oocytes cleaving to the two-cell stage, which was what was observed in this study (Fig. 12b). Additionally, it is also possible that, with a hardened zona, it might take longer for the sperm to penetrate the zona, which could in turn lead to the oocytes being post-mature when actual fertilization takes place, leading to a detrimental effect on embryonic development.

In conclusion, this study showed that porcine granulosa coculture had a beneficial effect on oocyte meiotic maturation, within a putatively 'less competent' murine IVM model, but this did not necessarily translate to improved embryonic developmental competence. Increased serum concentrations and supplementation with PFF and gonadotrophins (hCG and FSH) have little or no effect on murine meiotic maturation under either cell-free or coculture conditions. ECM gel was not detrimental to meiotic maturation but appeared to have a detrimental effect on fertilization and developmental competence.

Acknowledgements

We are grateful for grant support from the National Medical Research Council, Singapore.

https://doi.org/10.1017/S0967199404002461 Published online by Cambridge University Press

References

Abeydeera, L.R., Wang, W.H., Cantley, T.C., Rieke, A. & Day, B.N. (1998). Coculture with follicular shell pieces can enhance the developmental competence of pig oocytes after *in vitro* fertilization: relevance to intracellular glutathione. *Biol. Reprod*. **58**, 213–18.

Anderiesz, C. & Trounson, A.O. (1995). The effect of testosterone on the maturation and developmental capacity of murine oocytes *in vitro*. *Hum. Reprod*. **10**, 2377–81.

Anderiesz, C., Ferraretti, A., Magli, C., Fiorentino, A., Fortini, D., Gianaroli, L., Jones, G.M. & Trounson, A.O. (2000). Effect of recombinant human gonadotrophins on human, bovine and murine oocyte meiosis, fertilization and embryonic development *in vitro*. *Hum. Reprod*. **15**, 1140–8.

Ayoub, M.A. & Hunter, A.G. (1993). Inhibitory effect of bovine follicular fluid on *in vitro* maturation of bovine oocytes. *J. Dairy Sci*. **76**, 95–100.

Ben Ze'ev, A. & Amsterdam, A. (1989). Regulation of cytoskeletal protein organization and expression in human granulosa cells in response to gonadotropin treatment. *Endocrinology* **124**, 1033–41.

Bhat, P.J. & Moudgal, N.R. (1989). Isolation and characterization of a gonadotropin receptor binding inhibitor from porcine follicular fluid. *Int. J. Pept. Protein Res*. **33**, 59–66.

Bøgh, I.B., Bezard, J., Duchamp, G., Baltsen, M., Gerard, N., Daels, P. & Greve, T. (2002). Pure preovulatory follicular fluid promotes *in vitro* maturation of *in vivo* aspirated equine oocytes. *Theriogenology* **57**, 1765–79.

Byskov, A.G., Yding-Andersen, C., Hossaini, A. & Guoliang, X. (1997). Cumulus cells of oocyte-cumulus complexes secrete a meiosis-activating substance when stimulated with FSH. *Mol. Reprod. Dev*. **46**, 296–305.

Carabatsos, M.J., Sellitto, C., Goodenough, D.A. & Albertini, D.F. (2000). Oocyte–granulosa cell heterologous gap junctions are required for the coordination of nuclear and cytoplasmic meiotic competence. *Dev. Biol*. **226**, 167–79.

Chanson, A., Nocera, D., Senn, A., De Grandi, P. & Germond, M. (2001). Development of a well-defined medium for the *in vitro* maturation of immature bovine cumulus–oocyte complexes. *J. Assist. Reprod. Genet*. **18**, 97–105.

Dandekar, P.V., Martin, M.C. & Glass, R.H. (1991). Maturation of immature oocytes by coculture with granulosa cells. *Fertil. Steril*. **55**, 95–9.

De La Fuente, R., O'Brien, M.J. & Eppig, J.J. (1999). Epidermal growth factor enhances preimplantation developmental competence of maturing mouse oocytes. *Hum. Reprod*. **14**, 3060–8.

De Vos, A., Van de Velde, H., Joris, H. & Van Steirteghem, A. (1999). In-vitro matured metaphase-I oocytes have a lower fertilization rate but similar embryo quality as mature metaphase-II oocytes after intracytoplasmic sperm injection. *Hum. Reprod*. **14**, 1859–63.

Dolci, S., Bertolani, M.V., Canipari, R. & De Felici, M. (1991). Involvement of carbohydrates in the hardening of the zona pellucida of mouse oocytes. *Cell Biol. Int. Rep*. **15**, 571–9.

Downs, S.M. & Mastropolo, A.M. (1997). Culture conditions affect meiotic regulation in cumulus cell-enclosed mouse oocytes. *Mol. Reprod. Dev*. **46**, 551–66.

Driancourt, M.A. & Thuel, B. (1998). Control of oocyte growth and maturation by follicular cells and molecules present in follicular fluid. A review. *Reprod. Nutr. Dev*. **38**, 345–62.

Ebert, K.M., Hammer, R.E. & Papaioannou, V.E. (1985). A simple method for counting nuclei in the preimplantation mouse embryo. *Experientia* **41**, 1207–9.

Eppig, J.J. & Downs, S.M. (1987). The effect of hypoxanthine on mouse oocyte growth and development *in vitro*: maintenance of meiotic arrest and gonadotropin-induced oocyte maturation. *Dev. Biol*. **119**, 313–21.

Eppig, J.J., Wigglesowrth, K. & O'Brien, M.J. (1992). Comparison of embryonic developmental competence of mouse oocytes grown with and without serum. *Mol. Reprod. Dev*. **32**, 33–40.

Erbach, G.T., Lawitts, J.A., Papaioannou, V.E. & Biggers, J.D. (1994). Differential growth of the mouse preimplantation embryo in chemically defined media. *Biol. Reprod*. **50**, 1027–33.

Gilula, N.B., Epstein, M.L. & Beers, W.H. (1978). Cell-to-cell communication and ovulation. A study of the cumulus–oocyte complex. *J. Cell Biol*. **78**, 58–75.

Heikinheimo, O. & Gibbons, W.E. (1998). The molecular mechanisms of oocyte maturation and early embryonic development are unveiling new insights into reproductive medicine. *Mol. Hum. Reprod*. **4**, 745–56.

Hillensjö, T., Kripner, A.S., Pomerantz, S.H. & Channing, C.P. (1979). Action of porcine follicular fluid oocyte maturation inhibitor *in vitro*: possible role of the cumulus cells. *Adv. Exp. Med. Biol*. **112**, 283–91.

Hunter, M.G. (2000). Oocyte maturation and ovum quality in pigs. *Rev. Reprod*. **5**, 122–30.

Hwang, D.H., Kee, S.H., Kim, K., Cheong, K.S., Yoo, Y.B. & Lee, B.L. (2000). Role of reconstituted basement membrane in human granulosa cell culture. *Endocr. J*. **47**, 177–83.

Jagiello, G., Graffo, J., Ducayen, M. & Prosser, R. (1977). Further studies of inhibitors of *in vitro* mammalian oocyte maturation. *Fertil. Steril*. **28**, 476–81.

Khatir, H., Carolan, C., Lonergan, P. & Mermillod, P. (1997). Characterization of calf follicular fluid and its ability to support cytoplasmic maturation of cow and calf oocytes. *J. Reprod. Fertil*. **111**, 267–75.

Kikuchi, K., Kashiwazaki, N., Noguchi, J., Shimada, A., Takahashi, R., Hirabayashi, M., Shino, M., Ueda, M. & Kaneko, H. (1999). Developmental competence, after transfer to recipients, of porcine oocytes matured, fertilized, and cultured *in vitro*. *Biol. Reprod*. **60**, 336–40.

Kim, B.K., Lee, S.C., Kim, K.J., Han, C.H. & Kim, J.H. (2000). *In vitro* maturation, fertilization, and development of human germinal vesicle oocytes collected from stimulated cycles. *Fertil. Steril*. **74**, 1153–8.

Kito, S. & Bavister, B.D. (1997). Gonadotropins, serum, and amino acids alter nuclear maturation, cumulus expansion, and oocyte morphology in hamster cumulus–oocyte complexes *in vitro*. *Biol. Reprod*. **56**, 1281–9.

Kleinman, H.K. (1993). In *Molecular and Cellular aspects of Basement Membranes* (Rohrbach, DH. and Timpl R., eds), pp. 309–26. Academic Press.

Kobayashi, K., Takagi, Y., Satoh, T., Hoshi, H. & Oikawa, T. (1992). Development of early bovine embryos to the blastocyst stage in serum-free conditioned medium from bovine granulosa cells. *In vitro Cell. Dev. Biol*. **28A**, 255–9.

https://doi.org/10.1017/S0967199404002461 Published online by Cambridge University Press

Kokawa, N., Yamoto, M., Furukawa, K. & Nakano, R. (1993). Biological activity of partially purified low molecular weight luteinizing hormone binding inhibitor in porcine follicular fluids. *Acta Endocrinol*. **128**, 88–94.

Leibfried-Rutledge, M.L., Critser, E.S. & First, N.L. (1986). Effects of fetal calf serum and bovine serum albumin on *in vitro* maturation and fertilization of bovine and hamster cumulus-oocyte complexes. *Biol. Reprod*. **35**, 850–7.

Maeda, J., Kotsuji, F., Negami, A., Kamitani, N. & Tominaga, T. (1996). *In vitro* development of bovine embryos in conditioned media from bovine granulosa cells and vero cells cultured in exogenous protein- and amino acid-free chemically defined human tubal fluid medium. *Biol. Reprod*. **54**, 930–6.

McNatty, K.P., Smith, D.M., Makris, A., Osathanondh, R. & Ryan, K.J. (1980). Steroidogenesis by the human oocyte–cumulus cell complex *in vitro*. *Steroids* **35**, 643–51.

Mermillod, P., Oussaid, B. & Cognie, Y. (1999). Aspects of follicular and oocyte maturation that affect the developmental potential of embryos. *J. Reprod. Fertil*. **54** (Suppl.), 449–60.

Nakano, H. & Kubo, H. (2000). Study of the *in vitro* maturation of mouse oocytes induced by microinjection of maturation promoting factor (MPF). *J. Assist. Reprod. Genet*. **17**, 67–73.

Ng, S.C., Martelli, P., Liow, S.L., Herbert, S. & Oh, S.H. (2002). Intracytoplasmic injection of frozen–thawed epididymal spermatozoa in a nonhuman primate model, the cynomolgus monkey (*Macaca fascicularis*). *Theriogenology* **58**, 1385–97.

Nogueira, D., Staessen, C., Van de Velde, H. & Van Steirteghem, A. (2000). Nuclear status and cytogenetics of embryos derived from *in vitro*-matured oocytes. *Fertil. Steril*. **74**, 295–8.

Papaioannou, V.E. & Ebert, K.M. (1988). The preimplantation pig embryo: cell number and allocation to trophectoderm and inner cell mass of the blastocyst *in vivo* and *in vitro*. *Development* **102**, 793–803.

Reed, M.L., Estrada, J.L., Illera, M.J. & Petters, R.M. (1993). Effects of epidermal growth factor, insulin-like growth factor-I, and dialyzed porcine follicular fluid on porcine oocyte maturation *in vitro*. *J. Exp. Zool*. **266**, 74–8.

Richardson, M.C., Slack, C. & Stewart, I.J. (2000). Rearrangement of extracellular matrix during cluster formation by human luteinising granulosa cells in culture. *J. Anat* **196**, 243–8.

Seifer, D.B., Gardiner, A.C., Lambert-Messerlian, G. & Schneyer, A.L. (1996). Differential secretion of dimeric inhibin in cultured luteinized granulosa cells as a function of ovarian reserve. *J. Clin. Endocrinol. Metab*. **81**, 736–9.

Sites, C.K., Kessel, B. & LaBarbera, A.R. (1996). Adhesion proteins increase cellular attachment, follicle-stimulating hormone receptors, and progesterone production in cultured porcine granulosa cells. *Proc. Soc. Exp. Biol. Med*. **212**, 78–83.

Slüss, P.M., Schneyer, A.L., Andersen, T.T. & Reichert, L.E. (1989). Purification and chemical composition of a low molecular weight follicle-stimulating hormone binding inhibitor from porcine follicular fluid. *Biol. Reprod*. **41**, 863–70.

Son, W.Y., Yoon, S.H., Lee, S.W., Ko, Y., Yoon, H.G. & Lim, J.H. (2002). Blastocyst development and pregnancies after IVF of mature oocytes retrieved from unstimulated patients with PCOS after *in-vivo* HCG priming. *Hum. Reprod*. **17**, 134–6.

Trounson, A., Anderiesz, C., Jones, G.M., Kausche, A., Lolatgis, N. & Wood, C. (1998). Oocyte maturation. *Hum. Reprod*. **3** (Suppl.), 52–62; discussion 71–5.

Tsafriri, A. & Pomerantz, S.H. (1984). Regulation of the development of meiotic competence and of the resumption of oocyte maturation in the rat. *Symp. Soc. Exp. Biol*. **38**, 25–43.

Vatzias, G. & Hagen, D.R. (1999). Effects of porcine follicular fluid and oviduct-conditioned media on maturation and fertilization of porcine oocytes *in vitro*. *Biol. Reprod*. **60**, 42–8.

Voznesenskaia, T., Blashkiv, T.V. & Portnichenko, A.G. (2001). Effect of cumulus and granulosa cells on meiosis resumption in murine oocytes *in vitro*. *Tsitologiia* **43**, 250–3.

Whittingham, D.G. (1971). Culture of mouse ova. *J. Reprod. Fertil*. **14** (Suppl.), 7–21.

Yamazaki, Y., Wakayama, T. & Yanagimachi, R. (2001). Contribution of cumulus cells and serum to the maturation of oocyte cytoplasm as revealed by intracytoplasmic sperm injection (ICSI). *Zygote* **9**, 277–82.

Yoon, K.W., Shin, T.Y., Park, J.I., Roh, S., Lim, J.M., Lee, B.C., Hwang, W.S. & Lee, E.S. (2000). Development of porcine oocytes from preovulatory follicles of different sizes after maturation in media supplemented with follicular fluids. *Reprod. Fertil. Dev*. **12**, 133–9.

Zhang, X., Rutledge, J. & Armstrong, D.T. (1991). Studies on zona hardening in rat oocytes that are matured *in vitro* in a serum-free medium. *Mol. Reprod. Dev*. 28:3, 292–6.

https://doi.org/10.1017/S0967199404002461 Published online by Cambridge University Press