Assessment of a new in vitro maturation system for mouse and human cumulus-enclosed oocytes: three-dimensional prematuration culture in the presence of a phosphodiesterase 3-inhibitor

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BACKGROUND: Controlling nuclear maturation during oocyte culture might improve nuclear-cytoplasmic maturation synchrony. In the present study, the quality of mouse and human cumulus-enclosed oocytes (CEOs) was examined after a two-step culture consisting of a three-dimensional prematuration culture (3D-PMC), followed by in vitro maturation (IVM).

METHODS: Mouse and human CEOs were embedded in an extracellular matrix (collagen-gel Type I). The gels containing the CEOs were cultured in medium with a phosphodiesterase 3-inhibitor (PDE3-I; cilostamide 1 μM) for 24 h. Afterwards, CEOs were removed from the gel and washed away from inhibitor then underwent IVM. The optimal concentration of collagen (diluted 1:2 versus not-diluted) was first determined in the mouse model. Cytoplasmic maturation after IVM of human and mouse oocytes was assessed in relation to fertilization and embryonic developmental capacity.

RESULTS: The diluted form of collagen was better for supporting the structure of the expanding CEOs and meiotic competence of the oocytes. Electron microscopy in combination with Lucifer Yellow dye coupling assay revealed that oocyte–cumulus cell connections could be preserved during 3D-PMC. Percentages of mouse 2-cell embryos after IVF were higher in the 3D-PMC group compared with in vitro controls and 2D-PMC oocytes, but lower compared with in vivo controls. In the human model, percentages of polar body-extruded oocytes were significantly higher in the 3D-PMC group compared with conventionally matured oocytes. The 3D-PMC also had a beneficial effect on embryonic development on Day 3 post-ICSI.

CONCLUSIONS: Applying a 3D-PMC in the presence of a PDE3-I preserves oocyte–cumulus cell connections and influences oocyte developmental capacity.

Key words: cumulus-enclosed oocytes / in vitro maturation / oocyte development / phosphodiesterases / three-dimensional culture

Introduction

The availability of viable oocytes is the limiting factor in the development of new assisted reproductive techniques. Oocyte in vitro maturation (IVM) is an important technology, as it bears the prospect of reducing/avoiding ovarian stimulation. However, IVM outcomes remain significantly below the success rates obtained using in vivo-matured oocytes (Chian et al., 2004; Jurema and Nogueira, 2006). Part of this deficiency is due to the fact that this technology must deal with a mixed population of oocytes collected from follicles at varying stages of development, both dominant and atretic (Stouffer and Zelinski-Wooten, 2004). In addition, IVM oocytes undergo nuclear maturation prematurely. Removing oocytes from mid-sized antral follicles interrupts the process of oocyte capacitation, a phase during oogenesis in which the oocyte acquires the cytoplasmic machinery to support preimplantation development (Hyttel et al., 1997; Dieleman et al., 2002; Gilchrist and Thompson, 2007).
These observations have encouraged researchers to seek alternative strategies for the culture of immature oocytes. One approach to optimize developmental potential post-IVM is by temporarily inhibiting spontaneous meiotic maturation in vitro (Loneragan et al., 1998; Anderiesz et al., 2000). In this way, a two-step in vitro culture system is created. In the first step, called the ‘prematuration culture (PMC)’ oocytes are exposed to a meiosis-inhibiting compound to allow time for promoting cytoplasmic maturation. After the PMC period, oocytes are transferred to the classical maturation medium for IVM. The philosophy behind this strategy is to permit (i) synchronization within the mixed population of immature oocytes and (ii) synchronization between cytoplasmic and nuclear maturity within each individual oocyte.

Oocyte-specific phosphodiesterase type 3-inhibitors (PDE3-Is) are potent meiotic arresters. These agents keep intra-oocyte cAMP levels above a threshold that maintains the oocyte arrested at the germinal vesicle (GV)-stage in vitro (Tsafirri et al., 1996; Conti et al., 2002; Thomas et al., 2002). In an attempt to improve oocyte developmental competence, temporary exposure to a PDE3-I during a period of PMC has been tested in several mammals with limited success. Exposure of mouse cumulus-enclosed oocytes (CEOs) to the PDE3-I Org9935 (Nogueira et al., 2003b) or cilostamide (Vanhoutte et al., 2008) improves their quality and developmental potential. A similar observation is found when bovine CEOs are exposed to the PDE3-I milrinone (Thomas et al., 2004b). In addition, enhancement of nuclear maturation rates occurs when human CEOs are prematurely in the presence of the PDE3-I Org9935 (Nogueira et al., 2006a). Furthermore, combined exposure of human CEOs to a PDE3-I (i.e. cilostamide) and an adenylate cyclase activator (i.e. forskolin) has a positive effect on fertilization rates following IVM (Shu et al., 2008). Yet, the results are still suboptimal compared with the in vivo situation. This indicates that IVM techniques require a more robust scientific validation or refining for improved efficiency and acceptability.

The difference between the in vitro situation and the in vivo environment may originate from the extent of the communication between somatic cells and the oocyte. It was observed that during the process of PMC, loss of association between the oocyte and cumulus cells occurred (Nogueira et al., 2003a, b, 2006a; Vanhoutte et al., 2008). The CEOs attach to the two-dimensional (2D) culture surface of the Petri dish and the somatic cells spread out and migrate away from the oocyte. In other cases, the cumulus cells loosen completely from the oocyte in the form of clumps. This spreading or loosening alters the three-dimensional (3D) structure of the CEOs, thereby disrupting the cell–cell interactions. The interruption of normal oocyte–somatic cell interactions during the final hours of oocyte maturation may preclude the establishment of full developmental competence (Albertini et al., 2001; Epig, 2001; Tanghe et al., 2002; Gilchrist et al., 2004).

In accordance with the above observations, the aim of the present study was to establish an optimized two-step culture system that could maintain the bi-directional gap-junctional and/or paracrine communication between the oocyte and the cumulus cells during the period of PMC. One way to preserve 3D structures in vitro and, consequently, to mimic more faithfully the in vivo environment is to embed tissues within an extracellular matrix (ECM). This approach has been tested already on ovarian follicles of different mammalian species, mainly of the mouse (Torrance et al., 1989; Carroll et al., 1991; Gomes et al., 1999; Pangas et al., 2003; Kreeger et al., 2006; Xu et al., 2006). The ECM-embedding method has also been shown to preserve the integrity of bovine (Yamamoto et al., 1999; Alm et al., 2006) and canine (Otoi et al., 2006) CEOs. Many of these studies used collagen-gel as the ECM (Torrance et al., 1989; Carroll et al., 1991; Gomes et al., 1999; Yamamoto et al., 1999; Abir et al., 2001; Itoh et al., 2002; Alm et al., 2006; Otoi et al., 2006).

In the present study, we have employed collagen-gel (Type I) as a 3D matrix for the encapsulation of CEOs during the first step (i.e. the PMC) of our two-step culture. A similar approach has recently been tested by our group on denuded GV-stage oocytes obtained after ovulation induction for ICSI treatment (= ‘rescue IVM’) (Vanhoutte et al., 2009). Denuded oocytes were encapsulated in collagen containing dissociated cumulus cells and prematurated. This co-culture system had a positive effect on the oocytes’ developmental capacity following IVM and IVF. However, if we want to extrapolate a similar two-step culture system to a clinical IVM treatment, it is imperative to use CEOs. Therefore, in the present study, intact CEOs were embedded in collagen matrix. After PMC, CEOs were released from the gel, washed away from PDE3-I and subjected to IVM. This so-called ‘3D-PMC’ system was initially tested and optimized in a mouse model. In addition, we evaluated this culture system on human CEOs derived from small antral follicles after controlled ovarian stimulation (COH) of patients undergoing IVF/ICSI treatment. Outcome parameters were CEO morphology, oocyte–cumulus cell connections, maturation rates, fertilization and embryonic development.

Materials and Methods

The study was undertaken after complete Institutional Review Board approval from the Ghent University Ethical Committee (Project No. EC UZG 2007/142), the Animal Ethical Committee (Project No. ECD 06/05) and the Federal Ethical Committee on Embryos (Belgian Registration No. B67020072076).

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (Bornem, Belgium).

Collection of mouse CEOs

Mice used in this study were 7- to 8-week-old F1 hybrids (B6D2; Iffa Credo, Brussels, Belgium), housed and bred according to national legislation for animal care. Immature CEOs were collected from small antral follicles, 24 h following intraperitoneal injection of 5 IU/ml eCG (Folligon; Intervet, Mechelen, Belgium). Ovaries were dissected and collected in Leibovitz-glutamax medium (Invitrogen, Merelbeke, Belgium), supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 100 µg/ml streptomycin and 100 U/ml penicillin. To prevent spontaneous resumption of meiosis, cilostamide (1 µM) was added to this medium. CEOs were freed mechanically by puncturing antral follicles with fine insulin needles (26 1/2-G; Becton–Dickinson, Erembodegem, Belgium). Only CEOs that consisted of an oocyte surrounded by a compact cumulus cell mass were selected for the experiments.

As in vivo controls, oocytes were obtained by priming mice with 5 IU/ml eCG followed by 5 IU/ml human chorionic gonadotrophin (hCG; Chorulon; Intervet) 48 h later. Mature CEOs were recovered from the ampullae 16–18 h post-hCG.
Collection of human CEOs

Only consenting patients who had undergone routine COH for IVF/ICSI treatment were included in the study. All patients underwent COH after cycle synchronization with a standard contraceptive pill for 2–6 weeks. A short gonadotrophin-releasing hormone agonist protocol was used, consisting of 0.1 mg of triptorelin (Decapeptyl, Ipsen, France) from Day 5 onwards after discontinuation of the oral contraceptive. This was followed by human menopausal gonadotrophin (hMG; Menopur, Ferring, Germany) or follicle-stimulating hormone (FSH; either Gonal-F, Serono, Switzerland or Puregon, Organon, The Netherlands) from Day 7 after discontinuation of the pill onwards. The starting dose of hMG or FSH was 150 IU, which was adjusted on Day 8 of stimulation according to the individual response of the patient. An injection of 5000 IU hCG (Pregnyl, Organon, The Netherlands) was administered when at least two follicles reached a diameter of 20 mm.

To minimize confounding factors, only patients with a good prognosis in terms of ovarian response (e.g., at least seven follicles of ≥12 mm on ultrasound before oocyte retrieval) were included in the study. Oocyte retrieval was scheduled 34–36 h post-hCG, using a 17-G aspiration needle. First, follicles >10 mm were aspirated and the retrieved oocytes were used for the IVF/ICSI treatment of the patient. The needle attached to the test tube was washed with HEPES-containing media, and a new needle was attached to the needle for aspiration of smaller follicles measuring 5–10 mm. Only aspirates from these follicles were used for our study. The pressure to aspirate the smaller follicles was set to 140–150 mm Hg.

After collection, oocyte maturity was evaluated under the microscope with high magnification using the ‘spreading’ method (Veeck, 1988). This procedure consists of placing the CEO in a small droplet of culture medium on the flat surface of a sterile Petri dish and, by jarring the dish, spreading the medium and flattening the cumulus mass. The oocyte is then incubated with Type I collagenase (0.1 mg/ml) for 10 min at 37°C. After this treatment, the CEOs were subjected to gentle and repetitive pipetting until they were free from cumulus cells. The PDE3-I mechanical treatment caused the release of CEOs from the gel, yielding a single CEO per droplet. The gels containing the CEOs were then washed several times in PBS to remove any residual collagen before use.

Culture medium for mouse oocyte maturation

The basal culture medium consisted of alpha-minimal essential medium with glutamax (α-MEM; Invitrogen) supplemented with 5% FCS, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 100 µM cysteamine, 100 IU/ml penicillin G, 100 µg/ml streptomycin sulphate and 10 mlU/ml FSH (Puregon, Organon). The PMC medium was composed of the basal medium plus 1 µM cistolamide, a specific PDE3-I (Calbiochem, Bierges, Belgium; stock 10 mM in DMSO stored at −20°C). This concentration was used because previous studies have shown that 1 µM was the lowest effective dose for a maximum level of inhibition and reversibility of meiosis inhibition in mouse oocytes (Vanhoutte et al., 2008). For the human oocytes, a concentration of 1 µM cistolamide was more likely to preserve meiotic spindle morphology and chromosome alignment after IVM compared with 10 µM cistolamide (Vanhoutte et al., 2007).

The IVM medium was constituted by the basal medium supplemented with 5 ng/ml epidermal growth factor (EGF) and 1.5 IU/ml hCG (Pregnyl, Organon).

Culture medium for human oocyte maturation

The basal medium was Tissue Culture Medium 199 (TCM-199; Invitrogen) supplemented with 0.8% human serum albumin (Red Cross, Brussels, Belgium), 10 ng/ml human recombinant insulin, 5 µg/ml human transferrin, 5 ng/ml sodium selenite, 100 ng/ml long R3 insulin-like growth factor-I, 100 µM cysteamine, 1 mM L-glutamine, 0.3 mM sodium pyruvate, 100 IU/ml penicillin G, 100 µg/ml streptomycin sulphate and 10 mlU/ml FSH.

The PMC medium was composed of the basal medium plus 1 µM cistolamide.

The IVM medium was constituted by the basal medium supplemented with 10 ng/ml EGF, 1 µg/ml estradiol and 0.5 IU/ml hCG.

Three-dimensional prematuration culture

An ECM solution composed of collagen (3.79 mg/ml rat tail collagen Type I; BD Biosciences, Erembodegem, Belgium) was brought to neutral ionic strength and pH (7.4) by quickly mixing eight volumes with one volume of 0.05 N NaOH containing 22 mg/ml NaHCO3 and one volume of 10 x concentrated α-MEM (Invitrogen; for mouse oocytes) or 10 x concentrated TCMM-199 (Invitrogen; for human oocytes). To obtain a 1:2 diluted collagen-gel matrix (see Experiment I), one volume of the neutralized solution was mixed with one volume of PBS. The collagen mixtures were prepared immediately prior to PMC and kept on ice until use.

For mouse 3D-PMC, 200 µl of the collagen mixture was placed in the bottom of a 4-well dish (BD Biosciences) and allowed to polymerize at 37°C. Thirty to 40 CEOs were placed on top of this layer with a small volume of PMC medium. A second layer of 150 µl collagen was put onto the CEOs. The gels containing the CEOs were then placed in an incubator at 37°C for 30 min to polymerize. After gelatinization, 400 µ1 of PMC medium was poured onto the gels containing the CEOs. At this time, the gels were made floating by a gentle rimming of the wells with a 25-G needle.

For human 3D-PMC, microdroplets (4 µl) of the collagen solution were seeded in the wells of a 96-well plate (BD Biosciences). A single CEO was added carefully to each droplet, which was then allowed to polymerize at 37°C for 10 min before adding 80 µl of PMC medium to each well.

The PMC for both human and mouse CEOs took 24 h under standard conditions (37°C in a humidified atmosphere of 5% CO2 in air).

Removal of CEOs from the 3D-PMC and IVM culture

At the end of PMC, mouse and human CEOs embedded in collagen were incubated with Type I collagenase (0.1 mg/ml) for 10 min at 37°C. After this treatment, the CEOs were subjected to gentle and repetitive pipetting with a pulled Pasteur pipette of which the internal diameter was selected to be slightly larger than the complexes. This combined digestion/mechanical treatment caused the release of CEOs from the gel, yielding oocytes still surrounded by several layers of cumulus cells. The PDE3-I was subsequently removed by washing the oocytes several times in fresh IVM medium.

For mouse IVM, CEOs (groups of 15–20) were transferred to microdrops (30 µl) of IVM medium under oil and cultured for 16–18 h under standard conditions. The nuclear maturation stage was classified as GV, GV breakdown (GVBD) or PB extrusion.

For human IVM, CEOs were cultured individually in 25 µl microdrops of IVM medium under oil, after confirming the nuclear maturation stage using the spreading method. Mechanical denudation of the oocytes and evaluation of maturation stages (GV, GVBD or PB) was performed at 24 h of IVM. Immature oocytes remaining at GV- or GVBD stage were further cultured in the same medium and re-examined up to 30 h (from 25 to 30 h) and up to 48 h (from 31 to 48 h).

Evaluation of CEO morphology

Mouse CEOs were embedded in collagen-gel solutions at two different concentrations (not-diluted or diluted 1:2; see description above).
The morphology of the CEOs was evaluated at the end of PMC by light microscopy in relation to the pattern of cumulus cell–oocyte connections: (i) C+ if oocytes were completely surrounded with multiple layers of cumulus cells and (ii) C− if the connections between the two cell types were disturbed (i.e. partially denuded oocytes or presence of gaps between the oocyte and the cumulus cell layers).

The optimal collagen concentration was subsequently tested for the 3D-PMC of human CEOs. The cumulus layers around the oocytes were scored at the beginning of 3D-PMC, the end of 3D-PMC and after 24 h of IVM as follows: Type I, compacted (when one or more compact layers of cumulus cells were present); Type II, moderately expanded (expanded distal layers of cumulus cells but a compact proximal cell layer); Type III, fully expanded; or Type IV, partially denuded (Nogueira et al., 2003a).

Transmission electron microscopy

In order to verify more accurately the presence of cumulus–oocyte connections after 3D-PMC, mouse CEOs were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3). After post-fixation with 1% osmium tetroxide in cacodylate buffer and dehydration in graded series of alcohol, CEOs were embedded in Epon. Serial semithin sections of 1 μm were cut throughout the CEO and stained with Toluidine Blue for light microscopic guidance. Ultrathin sections (0.1 μm) for electron microscopic evaluation were collected at the point where the nucleolus of the oocyte was visible. The sections were transferred on wide single-slot copper grids, coated with carbon film and stained with uranyl acetate and lead citrate. Evaluation was done with a Zeiss EM900 electron microscope (Oberkochen, Germany).

Dye coupling assay

To assess the functionality of oocyte–cumulus connections, a 3% solution of Lucifer Yellow (LY) dye in 5 mM lithium chloride was injected into mouse oocytes and the spread of dye into surrounding cumulus cells was monitored with an inverted fluorescence microscope (Olympus IX70; Aartselaar, Belgium). To reduce the incidence of damaging oocytes during injection, the temperature of the microscope stage was kept at room temperature. The analysis of gap junction functionality was performed within 10 min of the injection by observation of LY dye spreading from the oocyte to the cumulus cells. This was analysed at the start (0 h; positive control) and the end (24 h) of the PMC period. To be certain that the spread of dye observed reflected transfer from the oocyte to the cumulus cells via gap junctions, we also examined a group of CEOs exposed to carbenoxolone (CBX; 500 μM; 20 min), a known gap-junction inhibitor (negative control). Oocyte–cumulus cell communications were classified as open (diffusion in the entire cumulus), partially open (only dye diffusion in a limited number of cells between ooplasm and corona radiata cells) or closed (no dye diffusion) (Luciano et al., 2004).

In vitro fertilization and embryo culture

In the mouse model, the cauda epididymis were removed from adult males. Epididymal contents were carefully squeezed out and the residual caudal tissue was discarded. Sperm suspensions were pre-incubated for 2.5 h to ensure capacitation in KSOM supplemented with 3% BSA (fraction V). CEOs were inseminated into 40 μl droplets and sperm was added at a final dilution of 2 × 10^5/ml and incubated in a humidified atmosphere of 37°C, 5% CO2 in air for 3 h. At the end of this period, inseminated oocytes were washed to remove sperm and cumulus cells by gently pipetting and were then cultured in 40 μl microdroplets of KSOM supplemented with 0.5% crystalline BSA (Calbiochem) for 4 days in a humidified atmosphere of 37°C, 5% CO2, 5% O2 and 90% N2. The percentage of embryos developing to the 2-cell and blastocysts stages was determined.

In the human model, PB-extruded oocytes were fertilized by ICSI between 1 and 4 h after the PB had been visualized. Sperm cells for ICSI were obtained the previous day from several male patients with excellent sperm characteristics (= number of sperm cells, motility and morphology above WHO minima) and who consented to donate supernumerary sperm cells for scientific research. After injection, oocytes were cultured individually in Sydney IVF Cleavage Medium (Cook, Ltd, Limenick, Ireland) for 3 days in a humidified atmosphere of 37°C and 5% CO2 in air.

Fertilization was evaluated 16–18 h post-injection and was considered as normal when two pronuclei were identified. Embryos were scored on Days 2 and 3 post-ICSI and were classified as having a ‘good’ morphology when there were at least two cells on Day 2 and six cells on Day 3, <20% anucleate fragments and no apparent morphological abnormalities.

Experimental groups

A schematic diagram of the different experimental groups in the mouse and human experiments is shown in Fig. 1.

Statistical analysis

The experiments on mouse CEOs were repeated at least three times. Variations between replicates are indicated with the standard deviation (± SD in tables and error bars on graphs). For evaluation of oocyte–cumulus cell morphology and communication (LY dye injection), maturation rates, fertilization and embryonic development, data were analysed with one-way ANOVA and Tukey’s post hoc test. As data were proportions, they were subjected to arcsine square-root transformation before ANOVA to normalize their distribution.

For the experiments on human CEOs, Fisher’s exact test or χ² test was used to evaluate differences in oocyte–cumulus cell morphology, maturation, fertilization and embryo morphological quality. Differences in mean number of blastomeres were calculated using one-way ANOVA, followed by ‘Tukey’s post hoc test’.

The threshold for significant difference was set at P < 0.05.

Results

Experiment 1: effect of different collagen concentrations on mouse CEO morphology and maturation capacity

Mouse CEO morphology was investigated in collagen-gel preparations of two different concentrations (not-diluted versus diluted 1:2) and compared with a group of CEOs prematured in conventional microdroplet cultures (=2D-PMC; Fig. 1A).

CEOs could maintain their 3D structures in the two collagen concentrations tested. In diluted collagen, increase in the cumulus mass could be observed. The cumulus cells stayed in close contact with each other and with the oocyte (Fig. 2A). In the non-diluted gel, although the oocyte was still surrounded by cumulus cells, a gap between the oocyte and the somatic cells was often observed and the oocyte had a shrunken appearance (Fig. 2B). In the 2D-PMC condition, cumulus cells started to spread away from the oocyte and attached to the bottom of the culture dish, leaving the oocyte almost denuded at the end of culture (Fig. 2C). In order to quantify these results, the CEOs in the different groups were classified according to the appearance of their cumulus (C+ or C−). A significant higher proportion of CEOs with a C+ pattern could be recovered after 3D-PMC in diluted collagen...
(80.7 ± 2.8%) compared with 3D-PMC in undiluted collagen (31.9 ± 2.3%) and 2D-PMC (15.2 ± 1.9%; P < 0.05).

After removal from collagen, half of the oocytes were denuded to assess the efficiency of the PDE3-I to maintain mouse oocytes under nuclear arrest in a 3D environment. More than 90% of oocytes exposed to PDE3-I were efficiently arrested at the GV stage, in both the 2D- and the 3D-PMC (Fig. 3A). These results were compared with a group of non-arrested oocytes (in vitro control; Fig. 1A). More than 80% of these oocytes matured spontaneously after isolation.

The remaining CEOs were washed out of PDE3-I and transferred to IVM medium. At the end of IVM, CEOs with a C+ pattern acquired an expanded cumulus layer, whereas CEOs with a C− cumulus were partially or completely denuded (data not shown). Maturation stages were analysed at the end of IVM to evaluate the reversibility of the PDE3-I. The 3D-PMC CEOs were compared with 2D-PMC CEOs and spontaneously matured CEOs (in vitro control) (Fig. 1A).

Oocytes cultured in non-diluted collagen demonstrated the highest rate of GV arrest at the end of IVM compared with the other groups.
(P > 0.05; Fig. 3B). Based on these observations, the diluted form of collagen-gel was applied in all further experiments on mouse and human oocytes.

**Experiment 2: evaluation of mouse cumulus–oocyte connections by transmission electron microscopy and dye coupling assay**

The optimal concentration of collagen solution, as assessed in Experiment 1, was used to test the integrity and functionality of cumulus–oocyte connections at the end of PMC. The presence of transzonal projections (TZPs) in 2D- versus 3D-cultured CEOs was first explored by transmission electron microscopy (TEM) (n = 30 in each group; Fig. 4). In 3D-PMC CEOs, variously shaped TZPs from surrounding cumulus cells were observed within the zona pellucida (Fig. 4A and B). In the 2D-PMC group, on the other hand, big gaps between the oocyte and the cumulus cells were frequently visible (Fig. 4C).

After having confirmed the presence of oocyte–cumulus connections, their functionality was assessed by LY dye microinjection (Fig. 5). The gap junctional statuses in the different treatment groups are shown in Table I. Injection of LY dye in the oocytes immediately (Fig. 5). The gap junctional statuses in the different treatment groups are shown in Table I. Injection of LY dye in the oocytes immediately after collection of CEOs (0 h; positive control) resulted in an immediate spread of the dye into neighbouring corona radiata cells in >80% of CEOs analysed. Compared with the positive control, a dramatic drop in oocyte–cumulus cell coupling occurred in the 2D-PMC group where the majority of CEOs exhibited partially open or closed intercellular junctions (P < 0.05). In the 3D-PMC group, the presence of functional gap-junction-mediated communications between oocytes and surrounding cumulus cells was observed in a significantly higher proportion compared with the 2D-PMC group, although this percentage was lower than for the positive control (P < 0.05). In the CEOs exposed to the gap junction inhibitor CBX (negative control), no dye diffusion was observed between oocyte and cumulus cells.

**Experiment 3: effect of 3D-PMC on human CEO morphology and maturation capacity**

A total of 196 human CEOs out of small antral follicles were retrieved from 57 patients. The mean age of the patients was 31.4 ± 4.52 years (range 23–40). Microscopical evaluation using the spreading method revealed that 60% (n = 118) of the oocytes were at the GV stage at the time of oocyte retrieval. These oocytes were distributed among the two IVM conditions (3D-PMC and in vitro control; Fig. 1B). There were 56 (29%) oocytes at the PB stage at retrieval and these were regarded as the in vivo controls (Fig. 1B).

At the start of 3D-PMC, the pattern of CEO was classified as Type I, II or III (Fig. 6A). A clear modification of the cumulus expansion pattern could be observed during the inhibition period. A significantly higher proportion of CEOs acquired a Type II (moderately expanded) or Type III (fully expanded) configuration at the end of 3D-PMC compared with the start, whereas the percentage of CEOs with a Type I (compact) configuration was significantly reduced (P < 0.05).

A second evaluation of the cumulus layers was performed after 24 h of IVM culture and the results of the 3D-PMC group were compared with conventionally matured oocytes (in vitro control; Fig. 6B). Of interest was the fact that a significantly higher proportion of CEOs in the control group were partially denuded (Type IV) compared with the 3D-PMC group (P < 0.05).

Table II summarizes the nuclear maturation rates in the 3D-PMC versus the in vitro control. Oocytes embedded in collagen and preincubated in PDE3-I-containing medium were efficiently arrested at the GV stage (>90%). After removal from collagen and inhibitor, oocytes were capable of resuming meiosis. At 24 h of IVM, when all CEOs in the two experimental groups were denuded of surrounding cumulus cells, the proportions of GV, GVBD and PB stages did not differ significantly between the in vitro control and the 3D-PMC groups. At 30 h of IVM, a significantly higher proportion of oocytes in the in vitro control group were still arrested at the GV stage compared with the 3D-PMC. Of oocytes evaluated 48 h after IVM, those in the 3D-PMC group acquired significantly higher maturation rates compared with the in vitro controls (P < 0.05).

**Experiment 4: effect of 3D-PMC on fertilization and embryonic development of mouse and human oocytes**

Mouse and human oocytes that matured under different culture conditions were fertilized in vitro at the end of IVM.

In the mouse model, four different experimental groups were evaluated: 3D-PMC, 2D-PMC, in vitro control and in vivo control (Fig. 1A). The results on fertilization and embryonic development are represented in Fig. 7. The percentage of 2-cell embryos on Day 1 was

![Figure 2](image-url) Effect of collagen concentration on mouse CEO pattern. Mouse CEOs were embedded in collagen-gel solutions prepared at two different concentrations (not diluted versus diluted 1:2). The morphology of the CEOs was evaluated at the end of PMC by light microscopy in relation to the pattern of cumulus cell–oocyte connections and compared with 2D-cultured CEOs. (A) C+, the oocyte is completely surrounded by multiple layers of cumulus cells (CEO cultured in diluted 3D-PMC); (B) C−, a gap is present between the oocyte and the cumulus cell layers and the oocyte is shrunken (CEO cultured in non-diluted 3D-PMC); (C) C−, the oocyte is partially denuded (CEO cultured in 2D-PMC). Original magnification: × 400.
higher in the 3D-PMC group compared with the 2D-PMC group and the in vitro control group, but lower compared with the in vivo group ($P < 0.05$; Fig. 7A). The proportion of blastocysts on Day 4 was similar between the 3D- and the 2D-PMC groups ($P > 0.05$), but higher than in the in vitro control group and lower than in the in vivo control group ($P < 0.05$; Fig. 7B). The percentages of developing embryos to the 2-cell and blastocysts stages of the 3D-PMC and 2D-PMC oocytes were higher compared with the in vitro controls ($P < 0.05$).

In the human model, fertilization and embryonic development post-ICSI were assessed in three experimental groups: 3D-PMC, in vitro control and in vivo control groups (Fig. 1B and Table III). Fertilization rates were significantly higher in the in vivo controls compared with the in vitro controls ($P < 0.05$), but similar to the 3D-PMC group ($P > 0.05$; Table III). The majority of fertilized oocytes underwent cleavage on Day 2. Embryo quality on Day 2 was similar among the three groups. On Day 3 post-ICSI, however, a significantly higher proportion of good-morphology embryos were obtained in the in vivo control and 3D-PMC groups compared with the in vitro control group ($P < 0.05$). The mean number of blastomeres on Day 2 post-ICSI did not differ between groups. On Day 3, a significantly higher number of blastomeres were observed in the in vivo control and 3D-PMC groups compared with the in vitro control ($P < 0.05$; Table III).
Oocyte maturation is a complex process involving the interaction of local regulatory factors and endocrine signals. Studies from Nogueira et al. (2003b) and Thomas et al. (2004b) were the first to demonstrate that a two-step culture with cAMP-modulating agents (i.e. PDE3-I alone or in combination with an adenylate cyclase activator) exert a significant positive effect on the IVM outcome of, respectively, mouse and cattle oocytes. Later, similar experiments on human CEOs confirmed that this approach improves meiotic competence (Nogueira et al., 2006a) and fertilization rates (Shu et al., 2008). Although the rationale behind this strategy is to prolong oocyte–cumulus cell gap-junctional communication and to allow for continued mRNA and protein accumulation within the ooplasm (Luciano et al., 2004; Thomas et al., 2004a; Gilchrist and Thompson, 2007), the 2D nature of this culture system alters the 3D architecture of the CEOs.

In the present study, we tried to overcome this deficit by performing the PMC-step in a 3D environment. The feasibility of working with a collagen matrix to create a 3D-PMC was explored for the co-culture of human denuded GV-stage oocytes with dissociated cumulus cells (Vanhoutte et al., 2009). Hence, we applied the same collagen matrix for the first time to encapsulate intact CEOs during PMC, which better approaches the clinical situation of IVM cycles.

The results of the present study demonstrated that embedding mouse and human CEOs in collagen-gel not only maintained their spherical architecture, but also resulted in meiotically competent oocytes. In addition, mature oocytes obtained after 3D-PMC and IVM could be fertilized and sustained preimplantation development in vitro. In the mouse model, an increase in oocyte competence was reflected in higher cleavage rates compared with the 2D-PMC and the in vitro control groups. In the human model, the two-step 3D-PMC had a beneficial effect on oocyte developmental capacity, resulting in an increased yield of matured oocytes and an improved embryonic developmental quality on Day 3 post-ICSI compared with spontaneously matured oocytes.

Providing an appropriate environment to culture CEOs in three dimensions is not easy. The density and mechanical properties of the ECM may influence cellular behaviour in vitro (Cukierman et al., 2002; Berkholtz et al., 2006; West et al., 2007). Therefore, the first experiment in our study was designed to define the optimal concentration of collagen in order to preserve CEO integrity without affecting subsequent meiotic competence. Two collagen-gel preparations varying in matrix stiffness and density were formed by changing the collagen concentration (diluted 1:2 versus not-diluted). Preliminary experiments showed that further dilutions (e.g. 1:3 and beyond) affected the rigidity of the gel and hampered a proper encapsulation of CEOs within the gel (data not shown).

Our results demonstrate that collagen in an undiluted form limits the outgrowth of the cumulus cell layers and influences optimal oocyte maturation. In diluted collagen, on the other hand, CEOs preserved their in vivo-like morphology, with a centrally located oocyte and surrounding layers of cumulus cells. In addition, an increase in the cumulus mass could be observed.

A plausible explanation for this difference is that less rigid gels deform more readily, which creates space as the cumulus mass increases around the oocyte (Xu et al., 2006; West et al., 2007). In addition, oocytes enclosed in undiluted collagen and surrounded by...
multiple layers of cumulus cells may have experienced hypoxic conditions, which may explain the observed shrunken appearance of the ooplasm. This preliminary experiment illustrates that the ECM density is an important aspect to take into account when dealing with a 3D culture in order to obtain satisfactory results.

The next step was to investigate whether the intercellular communication between the two different cell types, cumulus cells and oocytes, was preserved at the end of 3D-PMC. Evaluation of the physical integrity of the connections was initially performed using TEM. Although this type of analysis provides us with a topographically selected kind of information, it showed clearly that big gaps were present between the oocyte and the cumulus cells in 2D-cultured CEOs, whereas numerous TZPs could still be observed at the end of 3D-PMC.

To examine the functionality of these connections, an LY dye coupling assay was performed subsequently. We found that the presence of functional gap-junctions between oocytes and surrounding cumulus cells was significantly higher at the end of 3D-PMC compared with the 2D-PMC group, where an interruption of communication was observed. These results suggest that the presence of the ECM act as a scaffold which preserves the 3D morphology and, as a result, the functional integrity of the CEOs. This is in agreement with findings from Webb et al. (2002), who demonstrate that an intact CEO is required for normal gap-junctional communication between the oocyte and the cumulus cells during IVM.

Our results show also that the culture conditions were not sufficient to maintain functional communication during the entire period of PMC, since the percentage of CEOs with open communications was significantly lower at the end of 3D-PMC compared with the start. This indicates that further fine-tuning of the current culture system may be required. In this respect, the composition of the ECM may be a subject of investigation. Collagen-gel, although being used widely for 3D culture of cells, has some disadvantages as cell scaffold. The composition is very simple, not standardized (it may vary from batch to batch) and removal of the CEO after PMC requires some technical skills and precise adjustment of the enzymatic and mechanical treatment. Since the collagen preparation needs to be chilled to prevent early polymerization, the cells are submitted to an abrupt

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**Figure 5** LY dye diffusion in mouse CEOs cultured for 24 h in 3D-PMC. A solution of LY dye was injected into mouse oocytes and the spread of dye into surrounding cumulus cells was monitored with an inverted fluorescence microscope to assess the functionality of gap junctions. This was analysed at the start and the end of the PMC period. Fluorescence and bright field, respectively, of open (A and B), partially open (C and D) and closed (E and F) gap junction-mediated oocyte–cumulus cell communications. Original magnification: × 200.

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**Table 1** Functional coupling between cumulus cells and oocytes in mouse CEOs at the start of PMC (0 h; positive control), at the end of 2D- and 3D-PMC (24 h) and after exposure to CBX (negative control)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>No. of CEOs analysed</th>
<th>CEOs with open gap junctions</th>
<th>CEOs with partially open gap junctions</th>
<th>CEOs with closed gap junctions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>102</td>
<td>82.9 ± 3.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.2 ± 4.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.9 ± 1.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2D-PMC</td>
<td>101</td>
<td>5.4 ± 5.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>52.8 ± 10.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41.8 ± 4.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3D-PMC</td>
<td>115</td>
<td>64.6 ± 2.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>29.1 ± 2.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.3 ± 3.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Negative control</td>
<td>45</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.4 ± 5.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>85.6 ± 5.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Results are presented as % ± SD. <sup>a,b,c</sup>Different superscripts within columns indicate statistical differences (P < 0.05).

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drop in temperature at the time of loading of the gel. Alternative substrates, such as an alginate hydrogel, may be more appropriate. Alginate exhibits minimal cellular interactions with mammalian cells, which makes it easier to remove the cells from their 3D environment. This substrate has proven to be successful for the culture of mouse pre-antral follicles (Pangas et al., 2003; Kreeger et al., 2003, 2005). More sophisticated and tailor-made matrices could be created by the supplementation of certain growth factors, enzymes and other molecules (Abbott, 2003; Kreeger et al., 2006). In this way, the ECM composition could be adapted more closely to the natural in vivo situation of the CEOs.

Another possible approach to better preserve gap-junctional communication during the course of PMC is to manipulate cAMP levels within the CEO in a different way. We know from previous reports that exposure of CEOs to PDE3-I results in an increase in the intra-oocyte cAMP concentration (Tsafiri et al., 1996, Thomas et al., 2002). Elegant studies from Thomas et al. (2004a, b), Luciano et al. (2004) and Shu et al. (2008) have demonstrated that elevated

Figure 6 Effect of 3D-PMC and IVM on human CEO morphology. CEOs were 3D prematurated for 24 h. Afterwards, CEOs were removed from collagen, washed out of PDE3-I and in vitro matured. Enclosed figures show representative examples of different cumulus configurations: Type I, compact; Type II, moderately expanded; Type III, fully expanded; or Type IV, partially denuded. Original magnification: ×400 (Types I, II and IV) and ×200 (Type III). Evaluation was performed at the beginning of 3D-PMC, the end of 3D-PMC and after 24 h of IVM. (A) Graph illustrating the percentage of CEOs according to the morphological pattern of their cumulus at the start and the end of 3D-PMC; (B) graph illustrating the percentage of CEOs according to the morphological pattern of their cumulus in the 3D-PMC group versus the in vitro control at 24 h of IVM. *P < 0.05.
intra-cellular cAMP levels promotes the gap-junctional crosstalk between the germinal and somatic compartment. Of interest was the fact that this effect was augmented when an adenylate cyclase activator was added in combination with PDE3-I (Thomas et al., 2004a, b; Shu et al., 2008). A similar analysis on mouse CEOs has so far not been performed, but in light of these observations, it might be favourable to enrich our 3D-PMC medium with an adenylate cyclase stimulator, such as forskolin or FSH (at a higher concentration). In this way, cAMP production within the cumulus cells might contribute to the total cAMP content of the oocyte and may enhance gap-junctional communication accordingly. The confirmation of this hypothesis needs further experiments.

After exploring the effect of 3D-PMC on mouse oocytes in detail, we tested our two-step culture in a human model. A valuable source of oocytes for this purpose is the immature oocytes retrieved from small antral follicles (5–10 mm in diameter) after COH for IVF/ICSI treatment. These oocytes are still acquiring cytoplasmic competence and are therefore not routinely punctured for the infertility treatment of the patient (Nogueira et al., 2006b; Son et al., 2008).

This part of the study focused first of all on oocyte–cumulus morphology. During the prematuration step, human CEOs maintained an in vivo-like morphology with a centrally located oocyte and surrounding layers of cumulus cells. A significantly higher proportion of CEOs acquired a moderate or fully expanded cumulus configuration at the end of PMC compared with the start. This may indicate that the presence of collagen did not disturb the ability of the cumulus to expand and develop normally during the prematuration period.

A previous study of Nogueira et al. (2003a) performed on the same type of human CEOs demonstrated that ~65% of oocytes became partially denuded within 24–48 h of culture. This figure corresponds with that observed in our in vitro control group (62% of CEOs partially denuded). In the 3D-PMC group, however, <40% of oocytes became spontaneously denuded after removal from collagen and subsequent IVM culture for 24 h. This illustrates that a 3D environment may preserve oocyte–cumulus cell connections during PMC, leading to a

**Table II** Meiotic progression of human oocytes following 3D-PMC and/or IVM for up to 48 h

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>In vitro control</th>
<th>3D-PMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (%) of GV after inhibition</td>
<td>49/52 (94.2)</td>
<td>49</td>
</tr>
<tr>
<td>No. of GV undergoing IVM</td>
<td>66</td>
<td>49</td>
</tr>
<tr>
<td>Maturation at 24 h of IVM (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GV</td>
<td>37.9</td>
<td>36.7</td>
</tr>
<tr>
<td>GVBD</td>
<td>43.9</td>
<td>46.9</td>
</tr>
<tr>
<td>PB</td>
<td>18.2</td>
<td>16.3</td>
</tr>
<tr>
<td>Degenerated</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Maturation up to 30 h of IVM (between 25 and 30 h) (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GV</td>
<td>30.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GVBD</td>
<td>10.6</td>
<td>22.4</td>
</tr>
<tr>
<td>PB</td>
<td>57.6</td>
<td>65.3</td>
</tr>
<tr>
<td>Degenerated</td>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>Maturation up to 48 h of IVM (between 31 and 48 h) (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GV</td>
<td>27.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GVBD</td>
<td>10.6</td>
<td>6.1</td>
</tr>
<tr>
<td>PB</td>
<td>60.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>81.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Degenerated</td>
<td>1.5</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Values within the same row with different superscripts are statistically different (P < 0.05).

**Figure 7** Effect of PMC conditions on fertilization and embryonic developmental rates in a mouse model. Oocytes that matured under different conditions were fertilized in vitro at the end of IVM. The results were compared between oocytes from 3D-PMC (collagen gels) and 2D-PMC (PMC in microdroplets), in vitro controls (no PMC) and in vivo controls (in vivo matured oocytes). (A) Two-cell stage 1 day after IVF; (B) blastocyst stage 4 days after IVF. Values represent the mean % ± SD (error bars) of five independent replicates. Different letter superscripts denote significant differences (P < 0.05).
slower breakdown of connections during the second step of our culture system. This effect may also explain the improved maturation rates we obtained in the 3D-PMC group compared with the in vitro control, since several studies showed that removal of the cumulus oophorus before IVM is detrimental to oocyte meiotic capacity (Kennedy and Donahue, 1969; Schroeder and Eppig, 1984; Ruppert-Lingham et al., 2003; Nogueira et al., 2006a).

In the last part of our study, we tested the effect of a two-step culture on the cytoplasmic quality of mouse and human oocytes. In order to characterize this parameter, in vitro-matured oocytes were fertilized and the potential for embryonic development was evaluated.

In the mouse model, an increase in oocyte competence after 3D-PMC was reflected in higher cleavage rates compared with the 2D-PMC and the in vitro control groups. The proportion of blastocysts, on the other hand, was similar between 3D- and 2D-PMC oocytes, but higher than in the in vitro control.

It has been demonstrated earlier that mouse oocyte developmental competence can be promoted during meiotic arrest using PDE3-I alone (Nogueira et al., 2003b; Vanhouette et al., 2008), which has now been confirmed in the present report. The additional positive effect we obtained after 3D-PMC, although only observed at the level of cleavage rates, might be explained by a better preservation of cellular connections within the CEO. This hypothesis is supported by previous studies demonstrating that there is a direct correlation between the duration of junctional coupling along IVM and the developmental potential of the oocyte after fertilization (Luciano et al., 1999; Guixue et al., 2001). Elimination of cumulus cells from CEOs prior to IVM decreases sperm penetration rates in mice (Schroeder and Eppig, 1984), rats (Vanderhyden and Armstrong, 1989) and cattle (Zhang et al., 1995). Since most of the oocytes in the 2D-PMC group were partially denuded at the end of PMC, the lower 2-cell rate in this group might be explained by poor sperm penetration and/or a deficient interaction between the spermatozoa and the cumulus oophorus matrix during fertilization (Zhuo and Kimata, 2001). Why this positive effect of 3D-PMC was not visible at the level of blastocyst rates is unclear at the moment.

In the human model, 3D-PMC did not affect fertilization, cleavage rates or Day 2 embryonic quality. On Day 3, however, 3D-PMC resulted in a significantly higher proportion of embryos with less fragmentation and more blastomeres, similar to the in vitro-matured group and superior to the in vitro control. This indicates that improvement of the PMC conditions holds promise, since a previous report on the effect of a PDE3-I alone (in a 2D setting and on the same type of human oocytes) could not show an effect on embryo morphology and blastomere number compared with conventionally matured CEOs (Nogueira et al., 2006a).

An aspect that deserves attention when analysing the present results is the source of in vivo control oocytes used in this study. Several studies indicate that a correlation exists between oocyte developmental competence and follicular size (Triwitayakorn et al., 2003; Nogueira et al., 2006b). Therefore, it must be taken into account that these oocytes come from small follicles and may differ qualitatively from their counterparts derived from larger follicles. However, since their quality is superior compared with in vitro-matured oocytes (Nogueira et al., 2006a), they offer an extra source of mature oocytes for research purposes.

In conclusion, the present study demonstrates that preservation of the 3D morphology of the CEO during a PMC is possible through the use of appropriate collagen-gel culture environments. This approach represents a promising system to optimize PMC. Our data also indicate that the system can be adapted to support IVM from different species. Production of normal offspring might justify the application of this system for clinical settings.

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