Strategies to Improve *In vitro* Maturation of Human and Mouse Oocytes

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Table of contents

List of abbreviations

AC: adenylyl cyclase
ART: assisted reproductive technology
BMP-15: bone-morphogenetic protein 15
cAMP: cyclic adenosine monophosphate
CCPE: cumulus cell process ending
CEC: cumulus-eicel complex
CEO: cumulus-enclosed oocyte
cGMP: cyclic guanosine monophosphate
COH: controlled ovarian hyperstimulation
COC: cumulus-oocyte complex
CSF: cytostatic factor
Cx: connexin
DMAP: dimethylaminopurine
ECM: extracellular matrix
EGF: epidermal growth factor
FF-MAS: follicle fluid meiosis activating sterol
FSH: follicle-stimulating hormone
GC: granulosa cells
GDF-9: growth-differentiation factor 9
GnRH: gonadotrophin-releasing hormone
GPR3: G-protein-linked receptor 3
GSH: glutathione
GV: germinal vesicle
GVBD: germinal vesicle breakdown
hCG: human chorionic gonadotrophin
HX: hypoxanthin
IBMX: 3-isobutyl-1-methylxanthine
ICSI: intracytoplasmic sperm injection
IVF: *in vitro* fertilization
IVM: *in vitro* maturation
LH: luteinizing hormone
MI: metaphase I
MII: metaphase II
MAPK: microtubule-associated or mitogen- activated protein kinase
MPF: maturation, meiosis or mitosis promoting factor
OHSS: ovarian hyperstimulation syndrome
OSF: oocyte-secreted factor
PB: polar body
PCO: polycystic ovaries
PCOS: polycystic ovarian syndrome
PDE: phosphodiesterase
PDE3-I: phosphodiesterase type 3-inhibitor
PGC: primordial germ cell

PKA: protein kinase A or cAMP-dependent protein kinase
PKC: protein kinase C
PMC: prematuration culture
TZP: transzonal projection
ZP: zona pellucida

Summary

Our knowledge of reproductive medicine has expanded rapidly since the birth of Louise Brown, the first baby to be conceived by *in vitro* fertilization (IVF) in 1978. *In vitro* maturation (IVM) of oocytes - a culture technique to support maturation of immature oocytes *in vitro* - could offer an interesting adjunct to the classical assisted reproductive treatments. From a clinical point of view, it could diminish or avoid the use of superovulation drugs, thus reducing treatment length, costs and the risk of severe ovarian hyperstimulation syndrome (OHSS) and other adverse side-effects associated with hormonal stimulation. Other potential benefits include the provision of a source of oocytes for research in the fields of stem cells and cloning, obtaining oocytes for donation and preservation of fertility.
However, several aspects of the technique are still in the process of optimization. A crucial point in this context is the development of new culture strategies for IVM, which is the objective of the present thesis. Immature human and mouse oocytes were used as models to reach this goal.

We first analysed retrospectively the clinical benefit of *in vitro* matured metaphase I (MI) oocytes in a selected population of patients undergoing controlled ovarian hyperstimulation (COH), but with a low number of mature oocytes (≤ 6) available for the infertility treatment (= **'rescue IVM'**). This study was conducted within the framework of a routine IVF-lab, without changing the procedure of ovarian stimulation, ovum pick-up or oocyte culture. Although fertilization rates and developmental capacity of these oocytes was reduced compared to *in vivo* matured controls, a normal pregnancy and live birth could be obtained out of 13 transfers exclusively derived from IVM oocytes. This demonstrated that these oocytes could be worthwhile to increase the number of embryos available for transfer. On the other hand, the oocytes' reduced developmental capacity confirmed that current IVM culture systems are insufficient to support maturation *in vitro* properly.

The next step was trying to modify the existing culture techniques for IVM**.** One of the major problems in IVM is the fact that isolated meiotic-competent oocytes undergo nuclear maturation spontaneously before the cytoplasm achieves full maturity. A possible way to circumvent this deficiency is to apply a **2-step culture** composed of (1) a prematuration culture (PMC) to block temporarily spontaneous nuclear maturation, followed by (2) conventional IVM. By inducing meiotic arrest during PMC, oocytes may have the time to undergo cytoplasmic changes (mRNA storage, protein accumulation, ultrastructural remodelling), needed to enhance the oocytes' capacity to sustain further embryonic development. Oocyte-specific **phosphodiesterase type 3-inhibitors** (PDE3-Is) are potent meiotic inhibitors of the PMC-step.
Since MI oocytes are already on the process of nuclear maturation, the above described hypothesis was applied on germinal vesicle (GV)-stage oocytes. In the second study, mouse cumulus-oocytes complexes (COCs) were utilized to optimize the 2-step culture. We could identify the most effective concentration of the PDE3-I, cilostamide (1 μM), and analyzed the effect of the inhibitor on the kinetics of meiotic progression and aneuploidy rates of the *in vitro* matured oocytes. Improved meiotic spindle morphology and developmental competence demonstrated the potential of this 2-step culture to increase the quality of *in vitro* matured oocytes.

Because of these promising results in mouse oocytes, we applied a similar 2-step culture on human oocytes. In the third study, cumulus-free GV-stage oocytes retrieved from COH cycles were used as source of human experimental material. Temporary nuclear arrest with PDE3-I proved to be beneficial for obtaining normal spindle and chromosome configurations after IVM, similar to *in vivo* matured oocytes. We also observed that the GV oocytes underwent nuclear configurations during PMC, which were characteristic for preovulatory oocytes. However, a clear drawback in the experimental set-up was the fact that the oocytes were denuded from surrounding cumulus cells before starting the 2-step culture. It is known that the bidirectional communication between the oocyte and its surrounding cumulus cells plays an important role in the maturation process. To circumvent this deficiency, we performed the PMC step in the next experiment in a **three-dimensional (3D) co-culture system** of human GV oocytes with dissociated cumulus cells, making use of an extra-cellular matrix (ECM; collagen). The presence of the cumulus cells and/or the ECM during 3D-PMC improved significantly the morphological quality and nuclear constitution of the embryos post-IVM and IVF.

Finally, the type of oocytes that are used clinically for '**true IVM**' are not the denuded 'left over' GV oocytes that failed to mature *in vivo*, but the COCs retrieved from small antral follicles. When a 2-step culture is applied in this type of oocytes (see second study), we observed that the oocytes start to lose their connections with the surrounding cumulus mass, which alters the 3D-structure of the COCs. Therefore, the final step in our research project was to further optimize the PMC conditions in order to **preserve the functional cellular connections** within the COC. This was realized by embedding COCs in an ECM, the same collagen matrix as was tested in the previous study. A mouse model was initially used to test this approach. Lucifer Yellow dye coupling assay revealed that oocyte-cumulus cell connections stayed intact during 3D-PMC. Upon IVF, this strategy resulted in a significant increase in the percentage of mouse 2-cell embryos compared to a 2-step culture in the absence of an ECM (= 2D-PMC). In addition, we evaluated this culture system on human COCs derived from small antral follicles donated by patients undergoing COH for IVF/ICSI treatment. Applying a 3D-PMC improved maturation rates and developmental competence of the oocytes compared to a conventional IVM system.

In conclusion, the results from the above studies might offer significant applications for improving the clinical outcome of IVM technologies. Although the different culture systems still remain to be further explored, this work opens exciting new avenues for future research in this field.

Samenvatting

Onze kennis over reproductieve geneeskunde is snel toegenomen sinds de geboorte van Louise Brown, de eerste baby verwekt via *in vitro* fertilizatie (IVF) in 1978. *In vitro* maturatie (IVM) van eicellen – een cultuurtechniek om maturatie van onrijpe eicellen *in vitro* te ondersteunen – zou een interessante aanvulling kunnen bieden op de klassieke reproductieve behandelingen. Vanuit een klinisch oogpunt zou het het gebruik van superovulatie-medicijnen kunnen verminderen of vermijden en aldus de behandelingstijd, -kosten, het risico op ovarieel hyperstimulatie syndroom (OHSS) en andere ongunstige bijwerkingen geassocieerd met hormonale stimulatie kunnen reduceren. Andere potentiële voordelen zijn de voorziening van een bron van eicellen voor onderzoek in het domein van stamcellen en klonen, het verwerven van eicellen voor donatie en het bewaren van de vruchtbaarheid.
Verschillende aspecten van de techniek bevinden zich echter nog steeds in een proces van optimalisatie. Een cruciaal aspect in deze context is het ontwikkelen van nieuwe cultuurstrategieën voor IVM. Dit is het onderwerp van deze thesis. Onrijpe humane en muizeneicellen werden aangewend als model om dit doel te realiseren.

Allereerst analyseerden we, retrospectief, de klinische bruikbaarheid van *in vitro* gematureerde metafase I (MI) eicellen in een geselecteerde populatie van patiënten die gecontroleerde ovariële hyperstimulatie ondergingen, maar bij wie een laag aantal rijpe eicellen (≤ 6) beschikbaar waren voor de onvruchtbaarheidsbehandeling (= '**rescue IVM'**). Deze studie werd uitgevoerd binnen het kader van een routine IVF-labo, zonder de procedures van ovariële stimulatie, eicelpunctie of eicelcultuur te veranderen.
Hoewel de bevruchtingsgraad en ontwikkelingscapaciteit van deze eicellen gereduceerd was in vergelijking met *in vivo* gematureerde controles, kon uit 13 embryotransfers - exclusief bestaande uit embryo's afkomstig van IVM eicellen - een normale zwangerschap en levendgeborene verkregen worden. Dit toonde enerzijds aan dat deze eicellen waardevol kunnen zijn om het aantal embryo's beschikbaar voor transfer te verhogen. De verminderde ontwikkelingscapaciteit van de eicellen bevestigde, anderzijds, dat de huidige IVM cultuursystemen insufficiënt zijn om maturatie *in vitro* volledig te ondersteunen.

In de volgende stap probeerden we de bestaande cultuurtechnieken voor IVM te wijzigen. Een van de grootste problemen in IVM is het feit dat geïsoleerde meiose-competente eicellen spontane nucleaire maturatie ondergaan alvorens het cytoplasma volledige maturatie heeft bereikt. Een mogelijke oplossing om deze tekortkoming te omzeilen is het toepassen van een **2-staps-cultuur** bestaande uit (1) een pre-maturatie cultuur (PMC) om spontane nucleaire maturatie tijdelijk te blokkeren, gevolgd door (2) conventionele IVM. Door meiotisch arrest te induceren tijdens de PMC krijgen de eicellen de tijd om bepaalde cytoplasmatische veranderingen te ondergaan (mRNA stockage, accumulatie van proteïnes, ultrastructurele modelering). Deze veranderingen kunnen de capaciteit van de eicel verhogen om verdere embryonale ontwikkeling te ondersteunen. Eicel-specifieke **phosphodiesterase type 3-inhibitoren (PDE3-I)** zijn bruikbare meiose-inhibitoren voor de PMC-stap.
Gezien MI eicellen zich reeds in het proces van nucleaire maturatie bevinden, werd de hierboven beschreven hypothese getest op germinale vesikel (GV) eicellen. Cumulus-eicel complexen (CEC-en) van de muis werden gebruikt in de tweede studie om de 2-staps-cultuur te optimaliseren. We konden de meest geschikte concentratie van de PDE3-I, cilostamide

(1μM), identificeren en analyseerden het effect van de inhibitor op de snelheid van meiotische progressie en aneuploidie-graad van de *in vitro* gematureerde eicellen. Verbeterde morfologie van de meiotische spoelfiguur en ontwikkelingscapaciteit toonden het potentieel aan van deze 2-staps-cultuur om de kwaliteit van *in vitro* gematureerde eicellen te verhogen.

Gezien de veelbelovende resultaten op muizeneicellen pasten we een gelijkaardige 2-staps-cultuur toe op humane eicellen. Cumulus-gedenudeerde GV eicellen - verkregen na gecontroleerde ovariële hyperstimulatie - werden gebruikt als bron van humaan experimenteel materiaal in de derde studie. Tijdelijk nucleair arrest met PDE3-I bewees voordelig te zijn om normale spoelfiguren en chromosomale configuraties te verkrijgen na IVM, dit in vergelijking met *in vivo* gematureerde eicellen. We observeerden tevens dat GV-eicellen nucleaire configuraties ondergingen tijdens de PMC die karakteristiek zijn voor pre-ovulatoire eicellen. Niettemin was een duidelijk gebrek in de experimentele set-up het feit dat de eicellen gedenudeerd waren van omringende cumuluscellen alvorens de 2-staps-cultuur te starten. Het is gekend dat de tweerichtings-communicatie tussen de eicel en de omringende cumuluscellen een belangrijke rol speelt tijdens het maturatieproces. Om dit gebrek te omzeilen voerden we in het volgende experiment de PMC-stap uit in een **drie-dimensioneel (3D) co-cultuur systeem** van humane GV eicellen met gedissocieerde cumuluscellen. Hierbij maakten we gebruik van een extra-cellulaire matrix (ECM; collageen). De aanwezigheid van de cumuluscellen en/of de ECM tijdens een 3D-PMC verbeterde significant de morfologie en nucleaire samenstelling van embryo's post-IVM en IVF.

Uiteindelijk is het type eicellen dat klinisch wordt gebruikt voor '**echte IVM**' niet de gedenudeerde 'restant' GV eicellen die faalden om *in vivo* maturatie te ondergaan, maar de CEC-en afkomstig uit kleine antrale follikels. Wanneer we een 2-staps-cultuur toepasten op dit type van eicellen (zie tweede studie) observeerden we dat de eicel de connecties met de omgevende cumulusmassa begint te verliezen. Dit wijzigt de 3D-structuur van het CEC. Daarom bestond de finale stap in ons onderzoeksproject eruit om de PMC condities verder te optimaliseren, zodat **functionele cellulaire connecties binnen het CEC bewaard bleven**. Dit werd gerealiseerd door de CEC-en in te sluiten in een ECM, dezelfde collageen-matrix die was getest in de vorige studie. Een muismodel werd initiëel gebruikt om deze aanpak te testen. Lucifer Yellow koppelingsanalyse toonde aan dat eicel-cumuluscel connecties intact bleven tijdens de 3D-PMC. Na IVF resulteerde deze strategie in een significante stijging van het percentage 2-cellige muizenembryo's in vergelijking met een 2-staps cultuur zonder ECM (= 2D-PMC). Vervolgens evalueerden we dit cultuursysteem op humane CEC-en afkomstig van kleine antrale follikels en gedoneerd door patiënten die gecontroleerde ovariële hyperstimulatie ondergingen voor IVF/ICSI behandeling. Het toepassen van een 3D-PMC verhoogde de maturatiegraad en ontwikkelingscapaciteit van de eicellen in vergelijking met een conventioneel IVM cultuursysteem.

Samenvattend kunnen de resultaten van de bovenvermelde studies significante toepassingen bieden om de klinische resultaten van de IVM technologie te verbeteren. Hoewel de verschillende cultuursystemen verder moeten geëxploreerd worden, opent dit werk boeiende en nieuwe paden voor toekomstig onderzoek in dit domein.

1. Introduction

'Omne vivum ex ovo' (Harvey, 1651) 'all living things come from eggs'

1.1. Oocyte maturation

The mature human oocyte is the key ingredient for fertility, highly specialized and yet totipotent at the same time, invested with the unique ability to be fertilized and form new life. These characteristics make the oocyte perhaps the most precious cell in the body. However, many of the functioning mechanisms of the ooplasm remain ununderstood.

This part of the thesis will provide a concise review of the origin, growth and maturation of the oocyte.

1.1.1. Biological aspects

1.1.1.1. Origin and development of the female gamete

Oogenesis encompasses the differentiation, growth and maturation of the female gamete, in preparation for fertilization and subsequent development of the embryo.
The major milestones in this process are: (a) differentiation of the primordial germ cells (PGCs) from the rest of the cellular population; (b) migration from the site of origin to the developing gonad; (c) further development characterized by initial mitosis, which gradually ceases with the initiation and arrest of meiosis; (d) decline in the number of gametes and (e) re-initiation of meiosis following the preovulatory gonadotrophin surge and re-arrest preceding ovulation and fertilization. In humans, the phenomena (a) and (b) occur during the embryonic period, (c) occurs during the fetal period, (d) starts already before birth and together with (e) encompass almost the entire reproductive period of a woman.

Once oocytes have been formed, they must make connections with somatic cells in order to survive (Gosden *et al.*, 1997). Therefore, follicles are the lifeboats of the ovary because they nourish and control the development of oocytes.
Follicles begin their development as primordial structures that consist of an oocyte arrested at the diplotene stage of the first meiotic division, surrounded by a single layer of flattened or polyhedral granulosa cells (Peters, 1969). Once the pool of primordial follicles has been

established, and in response to either stimulatory or the release from inhibitory factors, follicles are gradually, irreversibly and continuously recruited to grow (= **initial recruitment**; **Fig 1**) (McGee and Hsueh, 2000). Once this process has started, it continues without halting until ovulatory maturity is attained or, in the majority of cases, until the follicle degenerates (atresia). The signals regulating the growth initiation of primordial follicles are still unknown, but are likely to be an interaction of factors such as anti-Müllerian hormone that inhibit initiation (Durlinger *et al.*, 1999) and a range of stimulatory factors such as c-kit and kit-ligand (Skinner, 2005). Follicles at this early stage of development have been shown to express FSH receptors (Bao and Garverick, 1998). However, gonadotrophins do not appear to be essential for early follicle growth, because, in their absence, follicles can still develop to the early antral stage (Awotwi *et al.*, 1984; Gong *et al.*, 1996).

During this early growth period or 'basal growth phase', the oocyte grows and the granulosa cells proliferate to form a multi-laminar structure. The follicle is now called a preantral follicle. At this stage, theca cells (the third follicular cell type) begin to differentiate from the surrounding stroma. Once the follicle reaches a species-specific size, it forms a fluid-filled space called an antrum within the granulosa cell layers (Gosden and Telfer, 1987a,b). When this stage has been reached, follicles become acutely dependent on gonadotrophins for further growth and development (Nayudu and Osborn, 1992) and the oocyte has completed most of its growth by that time.

Increases in circulating FSH during each reproductive cycle, which starts after pubertal onset, rescues a cohort of antral follicles from atresia (= **cyclic recruitment**; **Fig 1**). Among this group of antral follicles, one of the leading follicles grows faster than the rest of the cohort and produces higher levels of estrogens and inhibins (Gougeon, 1996). This exerts a negative feedback upon gonadotrophin release and, as a result, the remaining growing antral follicles are deprived of adequate FSH stimulation required for survival (diZerega and Hodgen, 1981). This allows a positive selection of the dominant follicle, thus ensuring its final growth and eventually ovulation.

At birth, the human ovary is densely packed with primordial follicles, but a number of growing, multi-laminar follicles are usually present (Gosden *et al.*, 1997). During childhood, the ovary contains growing follicles at virtually all developmental stages, except the dominant ones. Thus, in addition to the preantral stages, few antral follicles (> 2 mm) and atretic forms may be found. The follicles in the ovaries become larger as puberty approaches. This stage is somewhat akin to that observed in domesticated animals during their non-breeding seasons.

The pool of primordial follicles is progressively reduced throughout life, even during pregnancy, anovulation periods and lactation, until the store is exhausted shortly after menopause in mid-life (Jones and Krohn, 1961; Gougeon *et al.*, 1994). Less than 1% of primordial follicles present at the time of birth will ever proceed to ovulation (Erickson, 1966), with the majority of follicles degenerating by atresia. For years, it has been assumed that there is a fixed non-renewable population of primordial follicles. However, this assumption has recently been challenged by the publication of two papers that suggest oocyte renewal/regeneration in adult mice and possibly humans (Johnson *et al.*, 2004, 2005), a situation which should be consistent with the ovarian biology of lower vertebrates such as *Xenopus Laevis*. This hypothesis has opened a new debate on the existence of germ-line stem cells in the mammalian ovary (Gosden, 2004).

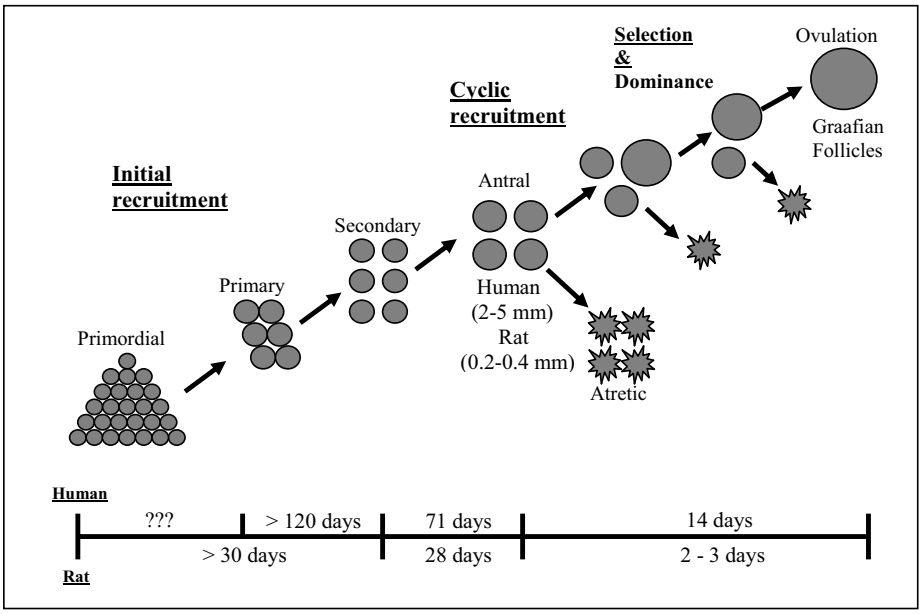

Figure 1: Duration of follicle recruitment and selection in human and rat ovaries. Primordial follicles undergo initial recruitment to enter the growing pool of primary follicles. Due to its protracted nature, the duration required for this step is unknown. In the human ovary, more than 120 days are required for the primary follicles to reach the secondary follicle stage, whereas 71 days are needed to grow from the secondary to the early antral stage. During cyclic recruitment, increases in circulating FSH allow a cohort of antral follicles (2-5 mm in diameter) to escape apoptotic demise. Among this cohort, a leading follicle emerges as dominant, thus ensuring its final growth and eventually ovulation. After cyclic recruitment, it takes only 2 weeks for an antral follicle to become a dominant Graafian follicle. In the rat, the duration of follicle development is much shorter. The time required between the initial recruitment of a primordial follicle and its growth to the secondary stage is more than 30 days, whereas the time for a secondary follicle to reach the early antral stage is about 28 days. Once the early antral stage (0.2 – 0.4 mm in diameter) has been reached, follicles are subjected to cyclic recruitment and only 2-3 days are needed for them to grow into preovulatory follicles. In rodents, multiple follicles become dominant during each estrous cycle (Modified from McGee and Hsueh, 2000).

1.1.1.2. Growth and acquisition of meiotic competence

Non-growing oocytes within primordial follicles are commonly, and misleadingly, described as 'quiescent'. It is only the state of the nucleus that is resting (i.e. dictyate stage of prophase I). The presence of one or more nucleoli, RNA polymerase activity and continuous uptake of amino acids and ribonucleosides demonstrate their active state. Since these cells may survive for 50 years or more in human ovaries, it is not surprising that they continuously repair and replace damaged organelles and macromolecules in order to maintain a *status quo* (Gosden and Bownes, 1995).

During the growth phase, the oocytes remain in the same stage of meiosis, but they undergo a remarkable increase in volume and alteration in cellular behavior. This is indicated by an

intense metabolic activity, which in turn is reflected by marked biosynthetic changes and changes in the oocytes' ultrastructure. It is during this time that many of the macromolecules essential for further development, both before and after ovulation, are produced and stockpiled within the oocyte. Besides accumulating water, ions and lipids, growing oocytes display 2- to 3-fold higher rates of RNA synthesis than non-growing or fully-grown oocytes (Bachvarova, 1981). The rate of protein synthesis and total protein content rises in parallel with expanding cell volume (Schultz and Wassarman, 1977). Transcriptional as well as translational products remain stable over a long period of time, principally due to polyadenylation control (Gosden, 2002). Posttranslational modifications of proteins through phosphorylation are common mechanisms by which proteins are modified for later use (Gould and Nurse, 1989).

Accumulation of RNAs and proteins are not the only major changes taking place during oocyte growth. Ultrastructural studies have demonstrated that complex organelle hypertrophy and reorganization occurs during this accumulative phase (Szollosi, 1972). For example, mitochondria, Golgi complexes and rough endoplasmic reticulum (RER) increase numerically and become dispersed in the oocyte (Gosden and Bownes, 1995). Increased division of mitochondria - from between 50-100 copies to over 200 000 - prepares the oocyte for its intensive metabolic activity before, during and after fertilization. Rearrangement of RER and Golgi complexes is involved in the synthesis and packaging of cortical granules and in the secretion of proteins constituting the zona pellucida (ZP). In addition, the oolemma elaborates a microvillar surface concomitant with the formation of the ZP. Modifications of the cytoskeleton result in the formation of a cytoplasmic framework where membrane-limited organelles translocate and assume defined positions within the growing oocyte (Albertini, 1992).

The coupling between the oocyte and the granulosa cells becomes much more intimate after follicle growth is initiated. Granulosa and theca cells support the oocyte on this journey through the provision of essential nutrients, information molecules, metabolic precursors, growth factors and hormones (Donahue and Stern, 1968; Brower and Schultz, 1982; Gilchrist *et al.*, 2004). However, it is also becoming apparent that the oocyte itself plays an active and dominant role in directing follicle growth by synthesizing factors that regulate the proliferation, function, survival and differentiation of granulosa cells, the recruitment of theca cells and the secretion of extracellular matrix components (Hussein *et al*., 2005, 2006). In this regard, the oocyte manipulates its own microenvironment.

Near the end of the growth phase, the oocyte will acquire the capability to resume meiosis. This important phase during oogenesis is called '**acquisition of meiotic competence'** (Wickramasinghe *et al.*, 1991; Eppig, 1996). It refers to the ability of the oocytes to reinitiate meiosis spontaneously when they are isolated from their follicles (Pincus and Enzmann, 1935; Edwards, 1965). Expression of meiotic competence has been correlated with a species-specific minimal oocyte diameter and it generally coincides with follicular antrum formation in rodents (Eppig and Schroeder, 1989). In humans, the oocyte has a size dependent ability to resume meiosis *in vitro* (Durinzi *et al.*, 1995), but it also depends on the follicle size and the stage of the menstrual cycle. Oocytes retrieved in the follicular phase of the menstrual cycle from follicles of 9 to 15 mm complete meiotic maturation at a higher rate than oocytes from follicles of 3-4 mm. Oocytes retrieved from follicles in the luteal phase will complete meiosis at the same rate irrespective of follicle size (Tsuji *et al*., 1985).

A growing body of evidence indicates that meiotic competence is acquired in individual steps. First, oocytes exhibit the ability to undergo germinal vesicle breakdown (GVBD) (Eppig and Schroeder, 1989; Eppig *et al.*, 1994). Subsequently, they acquire the ability to progress through meiosis I and arrest at metaphase of meiosis II (Wickramasinghe and Albertini, 1991,

1992). A similar process is observed in domestic species as well (Hyttel *et al*., 1997). This shows that acquisition of meiotic competence corresponds to a point at which the oocyte achieves a threshold level of maturation-promoting proteins and/or a correct position of these molecules within the oocyte (Mehlmann, 2005).
In the mouse, the most intensively studied species, it was discovered that critical remodeling in nuclear and cytoplasmic structures occurred during the course of meiotic competence acquisition. These changes include: 1) the appearance of a rim of heterochromatin around the nucleolus; 2) the loss of an interphase array of cytoplasmic microtubules; 3) the appearance of phosphorylated centrosomes; 4) the migration of centrosomes and organelles from a subcortical position to a perinuclear position (Mattson and Albertini, 1990; Wickramasinghe *et al.*, 1991; Wickramasinghe and Albertini, 1992). Since these nuclear and cytoplasmic modifications resembled changes that are normally seen in somatic cells when they move from G2 to the M-phase, it was suggested that a similar transition in the cell cycle of the oocyte occurred during this phase of oogenesis.

Meiotic competence acquisition does not necessarily imply that the oocyte acquires developmental competence. Developmental competence is progressively acquired within the preovulatory follicle, prior to and following the LH surge (Eppig *et al*., 1994). This phase in the maturation process is called 'cytoplasmic maturation'.

1.1.1.3. Cytoplasmic maturation

Cytoplasmic maturation can be defined as the processes in which the female gamete changes from a developmentally incompetent cell to one with the capacity to direct and support the events of fertilization and early embryonic development (Eppig, 1996). This imprecise term covers, for example, the appropriate processing of mRNAs and proteins - previously accumulated during oocyte growth - by modifying the transcription and translation machinery (nucleolus condensation and vacuolization, ribosome depletion, undulation of the nuclear membrane) (Sirard *et al.*, 2006). Other changes are the formation and peripheral arrangement of cortical granules together with relocations of other organelles, such as mitochondria. Another process that is included as part of cytoplasmic maturation is the accumulation of specific molecules - largely unidentified - which prepare the oocyte for post-fertilization events. These include the development of calcium signalling mechanisms for oocyte activation, the ability for embryo cleavage and mitosis and the ability to unpack the fertilizing sperm DNA and enable embryonic genome activation (Eppig, 1996).
These events take place in the pre-ovulatory follicle when the oocyte awaits the ovulation signal and it is only during this phase of development that the oocyte reaches its full developmental capacity. Hence, similarly to the process of sperm capacitation, the term '**oocyte capacitation'** has been introduced (Hyttel *et al.*, 1997; Hendriksen *et al.*, 2000).

A critical challenge in the context of *in vitro* maturation (IVM) is to understand the mechanisms that govern this final peri-ovulatory phase during oocyte development.

1.1.1.4. Regulation of oocyte nuclear maturation

The term 'nuclear maturation' generally encompasses the period between the resumption of meiosis of an oocyte that was arrested in the dictyate stage of prophase I and the transition to the second metaphase stage (MII), when the oocyte undergoes a re-arrest awaiting

fertilization. Morphologically, these events are represented by the breakdown of the GV and asymmetric cytoplasmic division of the oocyte resulting in the extrusion of the first polar body (PB). On fertilization, meiosis is completed by exit from MII and extrusion of the second polar body. Consequently, a truly haploid oocyte does not exist in mammals, because the final reduction in the chromosome number does not occur until fertilization.

Before discussing the external triggers and intracellular events that promote oocyte maturation, the signals that hold oocytes in meiotic arrest must be described.

Meiotic arrest

It is well established that meiotic arrest is regulated by cAMP levels within the oocyte (Conti *et al.*, 1998, 2002; Eppig *et al.*, 2004). The downstream pathway(s) by which high cAMP levels prevent meiotic maturation is incompletely understood and a detailed discussion is beyond the scope of this thesis. Ultimately, the cAMP level within the oocyte affects the activity of the $p34^{cdc2}$ kinase/cyclin B protein complex, also known as maturation, meiosis or mitosis promoting factor (MPF) which is the master orchestrator of the cell cycle. High cAMP levels within the oocyte result in the phosphorylation of $p34^{cdc2}$ kinase on Thr14 and Tyr15, rendering it inactive (= pre-MPF) (Duckworth *et al.*, 2002) (**Fig 2**). A decrease in oocyte cAMP leads to the dephosphorylation of $p34^{cdc2}$ kinase on Thr14 and Tyr 15, and the MPF complex becomes active such that the oocyte can re-enter meiosis. The discrete set of steps through which cAMP activates or inactivates MPF are still under investigation. The major players are protein kinase A (PKA), which, through an undetermined number of steps, regulates the activities of the phosphatase CDC25 and the kinase WEE1/MYT1 (Eppig *et al*., 2004). CDC25 dephosphorylates MPF, while WEE1/MYT1 phosphorylates it. Future studies are needed to clarify the entire pathway by which cAMP levels affect the activity of MPF.

cAMP could be produced either by the oocyte (**internally generated; Fig 2**) or by the follicle cells that surround it (**externally generated**). One long-standing hypothesis is that cAMP is produced by follicle cells and diffuses through gap junctions into the oocyte (Anderson and Albertini, 1976; Dekel *et al*., 1981). As an extension of this concept, it has been proposed that re-entry into the cell cycle is accompanied by the interruption of the communication between the oocyte and the somatic compartment. However, recent findings on mouse oocytes suggest that cAMP diffusion through gap-junctions is not sufficient by itself to maintain meiotic arrest (Vaccari *et al*., 2008). An alternative hypothesis is that the oocyte produces its own cAMP through a G-protein-linked receptor, GPR3 (mouse and human), in the oocyte plasma membrane that stimulates G_s and, subsequently, adenylyl cyclase (AC) (Mehlmann *et al.*, 2004; DiLuigi *et al.*, 2008). It is currently not known whether constitutive activity of GPR3 in the oocyte is sufficient to produce the amount of cAMP required to maintain meiotic arrest, or whether the follicle cells surrounding the oocyte stimulate the intra-oocyte cAMP synthesis by producing a ligand that increases the activity of GPR3.
Alternative possibilities proposed for maintenance of high intracellular cAMP levels are that somatic cells produce hypoxanthine and other purines which, via a paracrine way, block phosphodiesterase 3A (PDE3A), the enzyme involved in the control of cAMP levels in the oocyte (Conti *et al.*, 1998) (**Fig 2**).

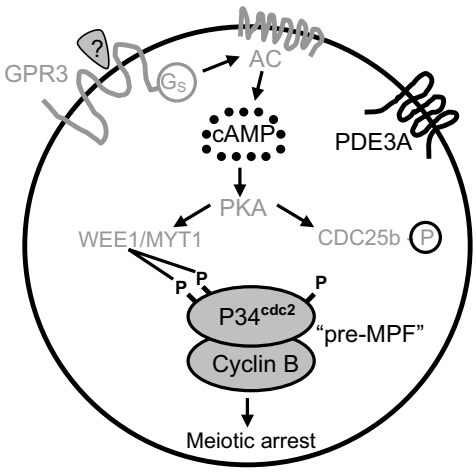

Figure 2: Cell signaling leading to the maintenance of meiotic arrest.
GPR3, activated either constitutively or by an unknown ligand from the follicle cells (?), activates G_S, which stimulates AC to cause an elevation of cAMP. cAMP activates protein kinase A (PKA), which ultimately causes the cell cycle regulatory complex, $P34^{cdc2}$/cyclin B, to be phosphorylated (P) and thereby inactivated. PKA phosphorylates (directly or indirectly) the phosphatase CDC25b (CDC25b-P), which inactivates it. PKA may also stimulate the activity of the WEE1/MYT1 kinase that phosphorylates $P34^{cdc2}$ to keep it inactive and therefore prevent meiotic resumption. The activity of the cAMP phosphodiesterase, PDE3A, is thought to be kept low in the immature oocyte, thus preventing the breakdown of cAMP and maintaining high levels of cAMP within the oocyte (Modified from Mehlmann, 2005).

Resumption of meiosis

The signals that control resumption of meiosis of mammalian oocytes are poorly understood. While it has been difficult to study this phenomenon in situ, two *in vitro* experimental models have provided the framework for much of our insight into this process. In 1935, Pincus and Enzmann observed that mammalian oocytes spontaneously undergo resumption of meiosis when removed from the follicular environment (Pincus and Enzmann, 1935). This observation led to the hypothesis that follicular cells send inhibitory signals to the oocyte to maintain the meiotic arrest. In addition, experiments with cultured follicle-enclosed oocytes have demonstrated that LH promotes oocyte maturation indirectly via activation of the granulosa cells (Tsafriri, *et al.*, 1972; Tsafriri and Dekel, 1994).

In vivo, meiosis resumes in response to a preovulatory surge of luteinising hormone (LH) or the exogeneous administration of human chorionic gonadotrophin (hCG). LH receptors are most concentrated in the theca cells and the outer layers of the granulosa compartment, i.e. the mural granulosa cells. In theca cells, LH elicits the production of aromatizable androgens ('two-cell two-gonadotrophin model'), while in mural granulosa cells, LH triggers ovulation by inducing the rupture of the follicular wall, and thereafter it stimulates luteinization and progesterone production of the corpus luteum. The cumulus cells surrounding the oocytes have relatively few LH receptors (Eppig *et al.*, 1997) and since no functional receptors are present on the oocyte (Patsoula *et al.,* 2001), the mechanism(s) by which LH stimulates oocyte maturation is indirect. Although much is known about LH signalling in the mural granulosa cells (Richards *et al.*, 2002), how the LH signal transmute to the cumulus cells and the oocyte itself is not fully understood. Park *et al.* (2004) has recently characterized a whole network of members of the epidermal growth factor family as paracrine mediators that propagate the LH signal throughout the follicle.

Ultimately, LH action on the mural granulosa cells translates to a change in signalling molecules within the oocyte to initiate meiotic resumption. This action could either remove a **maturation-arresting substance** or it could provide a **maturation-promoting substance** to the oocyte. An attractive hypothesis for a maturation-inducing process is that LH stimulation leads to the activation of oocyte PDE, which hydrolyzes cAMP (Conti *et al.*, 2002). PDE3A activity in cumulus-enclosed mouse oocytes has been shown to increase following the stimulation of LH receptors (Richard *et al.*, 2001).

The role of cAMP

The mechanisms for activation of oocyte maturation vary from one species to another, but what is sure is the fact that cAMP molecules are key regulators of meiosis resumption in amphibian, mammalian and some invertebrate oocytes. Upon release of oocytes from their follicles (i.e. **spontaneous oocyte maturation)**, it is supposed that a drop of intra-oocyte cAMP is enough to cause meiotic arrest release *in vitro*. However, there is evidence that cAMP has both inhibitory and stimulatory actions. Within the follicle (i.e. **induced oocyte maturation**), the gonadotrophin surge leads to opposing changes in cAMP levels in ovarian somatic cells and oocytes: a rise in follicular cAMP mediates LH-induced meiotic maturation, while a rise in intraoocyte cAMP maintains meiotic arrest (Tsafriri *et al.*, 1972). This contradictory observation demonstrates that high levels of cAMP in the cumulus cells may induce the release of a signal or signals that trigger meiotic resumption despite the presence of high levels of cAMP in the oocyte.

It was hypothesized that this paradoxical effect results from the compartmentalization and differential regulation of cAMP levels in the two major compartments of the follicle (i.e. the germ cell and the somatic cells). This differential regulation may result from selective expression and regulation of phosphodiesterases (PDEs) in the somatic and the germ cell compartments of the follicle. These PDEs exist in several isoforms in the ovaries: PDE4 types D and B are present in mural granulosa and theca cells, and PDE3 type A is expressed in oocytes of several species (Tsafriri *et al.*, 1996; Shitsukawa *et al*., 2001; Nogueira *et al*., 2003a). In addition, the effectors of cAMP are the protein kinases (PKA and PKC), among which there is also a compartmentalization of different isoforms. Probably, the magnitude of cAMP levels in the different cell compartments may be the mediator of different signals so that stimulation of an identical nature may result in different responses (Nogueira, 2005).

MPF activation

The common pathways downstream cAMP for both spontaneous and induced (i.e. overriding an inhibitory effect) oocyte maturation involve cAMP-dependent protein kinase A (PKA). Upon deactivation of PKA, progression from the G2- to M-phase in the oocyte requires the activation of MPF. This involves either or both, depending on the species, the synthesis of cyclin B, its relocation from the cytoplasm to the nucleus and the formation of the $p34^{cdc2}$ kinase/cyclin B protein complex together with the dephosphorylation of Thr 14 and Tyr 15 of $p34^{cdc2}$ itself. In meiotic competent mouse oocytes, pre-MPF is already present and de novo protein synthesis is not required to undergo GVBD (Fulka *et al.*, 1986; Wickramasinghe and Albertini, 1993). In humans and other domestic mammalian species, it seems that GVBD requires protein synthesis, because the pattern of proteins is different before and after maturation (Schultz and Wassarman, 1977; Pal *et al.*, 1994).

MPF activity increases until MI and decreases during the anaphase to telophase transition, but remains elevated above a certain baseline (**Fig 3**). This causes the extension of the condensed state of the chromatin and prevention of DNA replication. The activity of MPF increases again, due to neo-synthesis of cyclin B, until the oocyte reaches MII. At this stage, MPF is maintained at a high level by the interaction of cytostatic factor (CSF), which is activated by

the gene product of the proto-oncogene c-mos. Sperm penetration and the resultant increase in oocyte intracellular Ca^{2+} concentrations upon fertilization induce cyclin degradation and the completion of the meiotic cycle.

MAPK activation

Another principal pathway-regulator of the cell cycle that functions in interaction with and parallel to other protein kinases is MAP kinase (MAPK = microtubule-associated or mitogen-activated protein kinase). The activation of the MAPK pathway depends on the conditions in which the oocyte is led to resume meiosis. In mammalian species, activation of MAPK in cumulus cells is necessary for gonadotrophin-induced meiotic resumption, while MAPK activation is not required for spontaneous meiotic resumption (Fan and Sun, 2004). It is proposed that, during induced maturation, an interaction between the cAMP-PKA and the MAPK pathways takes place, while spontaneous maturation acts only via the cAMP-PKA pathway (Leonardsen *et al.*, 2000). The activity of MAPK is associated with a plethora of cytoplasmic events including the regulation of microtubule dynamics, spindle assembly and chromosome condensation (Verlhac *et al.*, 1993). In most oocytes, high concentrations of MAPK are maintained during meiotic progression to MII and concentrations decrease after fertilization or parthenogenetic activation (**Fig 3**).

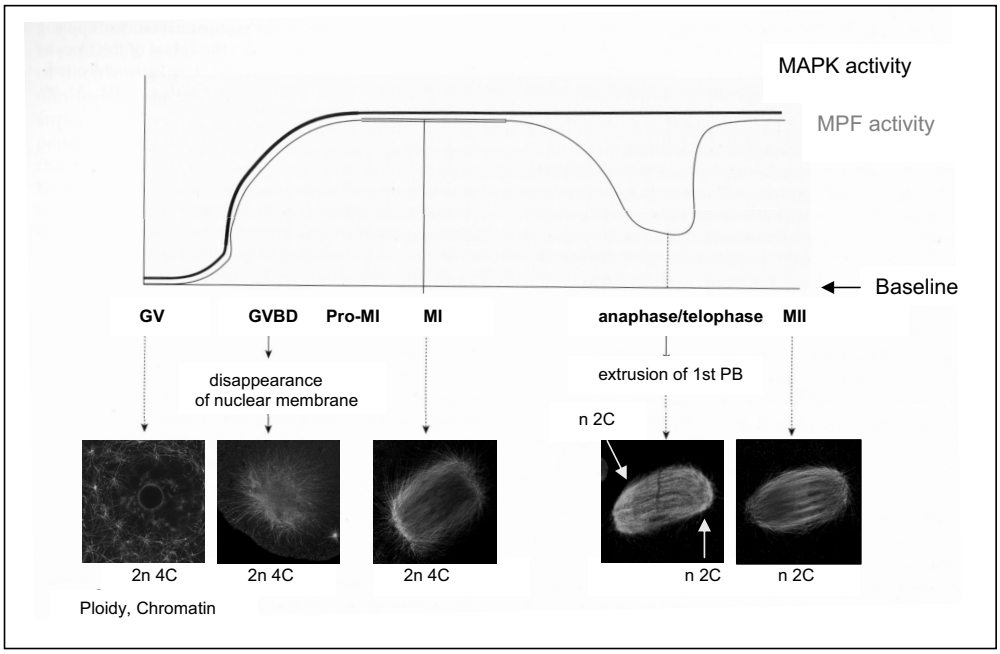

Figure 3: Evolution of MPF and MAPK activity during oocyte meiosis. During anaphase/telophase MPF drops, but remains elevated above baseline (X-axis). This causes the extension of the condensed state of chromatin. MAPK activity increases in oocytes during resumption of meiosis and remains high throughout progression to MII. After GVBD, this component of serine/threonine protein kinase family is involved in microtubule organization and spindle formation (Modified from Smitz *et al.*, 2004).

1.1.2. Clinical aspects

1.1.2.1. Definition of oocyte *in vitro* maturation (IVM)

In vitro maturation (IVM) involves the practice of intentionally retrieving immature oocytes from unstimulated or minimally stimulated ovaries and culturing the obtained cumulus-enclosed oocytes in appropriate medium until they reach metaphase II. In domestic animals, embryo production from unstimulated ovaries using *in vitro* matured oocytes is routine practise. In humans, this technique was employed for the first IVF treatment (Edwards *et al.*, 1969). However, due to the poor success of embryo development, the technique was abandoned to the advantage of *in vivo* matured oocytes until the first pregnancy after IVM was achieved 22 years later (Cha *et al*., 1991). Since then, IVM has become an interesting alternative for the classical assisted reproductive technologies (ARTs).

The main category of patients in whom IVM has been applied initially is constituted by women with polycystic ovaries (PCO) or with polycystic ovarian syndrome (PCOS; Mikkelsen and Lindenberg, 2001; Child *et al.*, 2002; Chian *et al.*, 2004; Le Du *et al.*, 2005). Pregnancy and implantation rates after IVM in these patients are approximately 30-35% and 10-15%, respectively (Chian *et al.*, 2004). Later on, these methods have been applied for patients with regular menstrual cycles and normal appearing ovaries as well. To date, it is estimated that approximately 400 children have been born after IVM. Obstetric and perinatal outcome of these children appears to be normal (Buckett *et al.*, 2005; Söderström-Anttila *et al.*, 2006). The available information is unfortunately limited by the young age of IVM technology.

This new approach in ART is still in an experimental phase and still needs to be improved before routine clinical application, since the outcome measures of IVM (i.e. pregnancy, implantation and spontaneous abortion rates) are often not comparable among studies. This is likely a result of differences in clinical practices such as the population of patients, follicular priming, timing of immature oocyte retrieval, IVM culture conditions and preparation of the endometrium rather than the technique itself (Jurema and Nogueira, 2006). Consequently, the worldwide acceptance of IVM technology is variable. In Canada, Sweden, Norway and Finland, IVM is regarded as a safe technique and is provided as a clinical service to selected patients, while in other countries, like Belgium, IVM is considered experimental and performed only in a research protocol (Jurema and Nogueira, 2006).

1.1.2.2. Rationale to perform IVM

Conventional IVF was originally performed with the single dominant ovarian follicle produced during a spontaneous menstrual cycle. This was very inefficient and pregnancy rates were dismal. Consequently, the ovaries were ‘hyperstimulated’ using exogenous gonadotrophins to induce maturation of multiple follicles. This is obtained by FSH injections after pituitary desensitization with GnRH agonists of antagonists. Oocyte maturation is then induced by hCG administration before transvaginal oocyte retrieval.
Controlled ovarian hyperstimulation (COH) has been shown to be not entirely safe. It can be difficult and inconvenient for the patient. One to three per cent of infertile women develop ovarian hyperstimulation syndrome (OHSS), a severe life-threatening complication which may cause liver dysfunction and thromboembolism (Rizk and Smitz, 1992; Delvigne and

Rozenberg, 2003). Women with PCO or PCOS are more likely to develop OHSS. This complication can be avoided when oocytes are obtained at an immature stage out of small or medium-sized follicles.
There is also growing evidence that the ovary resists to ovarian hyperstimulation by decreasing the quality of the oocytes it produces (Stouffer and Zelinski-Wooten, 2004; Sirard *et al.*, 2006). This brings back in perspective the importance of moderating the hormonal stimulation protocols to optimize the yield of high quality embryos (Baart *et al*., 2007). On the other hand, some studies (both on human and animals) were not able to find a negative effect of ovarian hyperstimulation on oocyte and/or embryo quality (Caligara *et al.,* 2001; Combelles and Albertini, 2003; Xu *et al.*, 2008; Weghofer *et al.*, 2008). Therefore, it is important to determine the underlying defects associated with ovarian hyperstimulation.

Hence, IVM technology offers a simple protocol with decreased or no hormonal stimulation before oocyte retrieval, which will result in a more patient-friendly IVF treatment and, consequently, lower cost. Furthermore, any other potential short- or long-term adverse effects of supraphysiologic concentrations of gonadotrophins on sex steroid hormone-sensitive tissue (i.e. ovaries, endometrium and breasts) are minimized. In this respect, IVM might be suitable for different subgroups of infertile patients, both PCO/PCOS as well as normo-ovulatory women. IVM treatment is also indicated for cancer patients who wish to preserve their fertility, since the high levels of estrogen related to gonadotropin stimulation may be detrimental to patients with estrogen-dependent cancers, such as breast cancer. The reduced costs make IVM to become in clinical demand in developing countries where more than half of the cycle expense is related to gonadotrophins and GnRH analogues (Jurema and Nogueira, 2006). Finally, IVM is fundamentally important to augment our understanding of oocyte maturation processes in the human.

1.1.2.3. Results of IVM in human ART

The application of IVM is very successful in certain mammals and in the livestock industry (Blondin *et al.*, 2002). However, attempts to adopt this technology in human ART have met with mixed success. The most important reason for this is the lower pregnancy rate compared to conventional IVF/ICSI. Although the clinical pregnancy rate per embryo transfer is reasonable, this is still low per oocyte collection (Suikkari and Söderström-Anttila, 2007). However, straight conclusions from clinical studies are sometimes difficult to draw as the data often come from observational evaluations with different patient populations, stimulation regimes and without adequate control groups.

Studies from Child *et al.* (2002) and Cha *et al*. (2000) directly compared the outcome of IVM cycles with conventionally IVF cycles in patients with PCO/PCOS. These studies suggest that IVM may be comparable to IVF in efficacy regarding the number of MII oocytes obtained, fertilization, cleavage and pregnancy rates. In addition, IVM decreased the risk of OHSS compared with IVF. However, implantation rates were found to be lower in the IVM cycles. The reason for this could be related either to poorer embryo quality or suboptimal endometrial preparation (Mikkelsen and Lindeberg, 2001). Lower embryo quality has resulted in the need for more embryos to be transferred. Nevertheless, notwithstanding the fact that multiple embryos are often transferred, the multiple pregnancy rate appears to be lower after IVM than after conventional IVF/ICSI (Cha and Chian, 1998; Chian *et al.*, 2004; Mikkelsen, 2005). Furthermore, cryopreservation of embryos derived from IVM oocytes was found to be less efficient compared to standard IVF (Suikkari and Söderström-Anttila, 2007).

Results from the limited number of experimental studies examining the development of human *in vitro* matured oocytes have shown that several aspects of nuclear and cytoplasmic maturation are abnormal in these oocytes (Combelles *et al.*, 2002, Nogueira *et al.* 2000). An overabundance of transcripts was recently identified in IVM oocytes, which demonstrates a dysregulation in gene transcription or post-transcriptional modification of genes (Jones *et al.*, 2008).
These observations indicate that current maturation systems do not adequately support nuclear and/or cytoplasmic maturation, which subsequently affect embryo development. It should be mentioned that, due to the lack of research material, the immature oocytes in many of these studies came from COH cycles for conventional IVF/ICSI treatment. These oocytes are aspirated out of follicles that did not respond to the hCG stimulus and are considered as a side-product in ART cycles. Hence, maturing this type of oocytes *in vitro* is called '**rescue IVM**'. The results of these studies needs to be interpreted with caution and should not be compared to immature oocytes derived from unstimulated or slightly stimulated cycles.

It is clear that many variables strongly influence the quality of *in vitro* matured oocytes (i.e. etiology of infertility, follicular priming, follicle size, type of oocytes, method of insemination), but a crucial point in the optimization of IVM is the development of new strategies for culturing immature oocytes, which is the topic of the present thesis.

1.2. Concepts and technologies to improve IVM outcome

Progress to improve the efficiency of IVM technology has been slow over the past decade. A critical challenge is to understand what constitutes oocyte developmental competence and the mechanisms governing it. These insights are slowly translated into progressive adaptations of standard culture techniques for IVM. The approaches used in the present thesis serve mainly three goals: one is to prevent spontaneous maturation by establishing a **2-step culture**; the other is to preserve functional cellular interactions within the cumulus-oocyte-complex (COC) by **three-dimensional cultures**; the last one is trying to re-establish the natural *in situ* environment of the COC by **co-culturing** cumulus-denuded oocytes with dissociated cumulus cells. Each concept is outlined hereafter.

1.2.1. Two-step cultures

1.2.1.1. Definition and rationale of 2-step cultures

"The history of the follicle determines the future of its oocyte"
(Hyttel et al., 1997)

IVM differs from *in vivo* oocyte maturation in three fundamentally important ways. Firstly, COCs are generally collected from small or mid-sized antral follicles (i.e. 6-12 mm diameter in humans) that have not completed their final maturation or 'oocyte capacitation' and hence do not possess the cytoplasmic machinery required to support early embryogenesis. Secondly, removing mechanically the COC from the follicle results in loss of the natural meiotic inhibiting environment, leading to spontaneous or 'premature' meiotic maturation *in vitro*. In this way, nuclear maturation occurs before the cytoplasm has reached full maturity. Thirdly, the population of small antral follicles, from which COCs for IVM are collected, is very heterogeneous regarding stages of development and atresia. Such varying degrees of maturity make it difficult to support IVM of all COCs by using one single culture method and/or medium, which may lead to variations in embryo quality.
Hence, it should be expected that embryos and fetuses generated from IVM have impaired developmental potential compared to those generated from *in vivo* matured oocytes, and indeed this is the case.

A possible strategy to circumvent the above evils and, consequently, to improve IVM outcome is to keep oocytes meiotically arrested *in vitro* for a certain period of time. This may promote cytoplasmic maturation by allowing time for the oocyte to undergo structural and biochemical changes which normally occur in the preovulatory follicle (Lonergan *et al.*, 1997). Some examples are the continued transcription of mRNA, post-translational modifications of proteins, relocations and modifications of organelles (Hyttel *et al.*, 1997; Dieleman *et al.*, 2002; Hendriksen *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 temporarily exposed to a meiosis-inhibiting compound. After the PMC period, the inhibitor is removed and the oocytes are transferred to the classical IVM medium to resume nuclear maturation. The philosophy behind this concept is to permit i) synchronization between cytoplasmic and nuclear maturity within each individual oocyte and ii) synchronization within the mixed population of immature oocytes retrieved out of small antral follicles.

1.2.1.2. Approaches to inhibit spontaneous oocyte maturation

The mechanisms governing mammalian oocyte meiotic arrest and resumption have been studied in great detail and there are a wide variety of approaches to artificially induce nuclear arrest *in vitro* (**Fig 4**).

One way is to expose the oocyte to **kinase inhibitors** (e.g. roscovitine, butyrolactone I, 6-DMAP) which prevent MPF and/or MAPK activity. Studies in different species showed that these inhibitors either adversely affects (Adona and Lima Verde Leal, 2004; Lonergan *et al*., 1997; Avery *et al.*, 1998; Anderiesz *et al.*, 2000) or has no positive effect (Adona and Lima Verde Leal, 2004; Lonergan *et al.*, 2000; Grupen *et al.*; 2006) on subsequent oocyte developmental potential, although there is one report of an actual improvement in oocyte quality (Hashimoto *et al.*, 2002).
Another class of inhibitors are the **protein synthesis inhibitors** (e.g. Cycloheximide, puromycin), which act on proteins that needs to be synthesized in order to activate MPF (Motlik *et al.*, 1990; Tatemoto and Terada, 1995).

A negative aspect of the above mentioned inhibitors is that they are not specific to a singular cell type. They can act on the functionality of surrounding cumulus cells, influencing mechanisms related to cumulus cell expansion (Lonergan *et al.*, 2003, Vigneron *et al.*, 2003). Also, they might interfere with different kinase activities that modify the function of proteins related to embryogenesis. Hence, using these inhibitors as PMC additives to promote oocyte developmental potential is probably not realistic.

In contrast, agents that modulate the intra-oocyte concentration of the second messenger cAMP show greater potential. It is widely accepted that the cAMP level within the oocyte plays a crucial role in the regulation of oocyte maturation. Cyclic AMP levels can be increased by exposing the oocyte to: (i) cAMP analogues such as dibutyryl cAMP, (ii) activators of adenylate cyclase, such as FSH, forskolin or invasive adenylate cyclase and (iii) **phosphodiesterase inhibitors (PDE-Is)** (**Fig 4**).
The use of PDE-Is to control cAMP levels has attracted special attention in the field of reproduction in the last decade and is discussed in the next chapter.

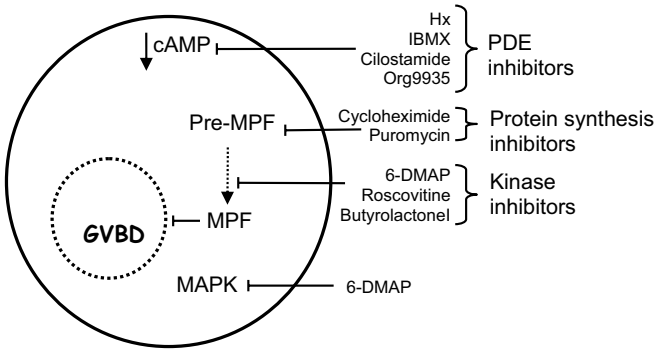

Figure 4: Pharmacological compounds blocking oocyte nuclear maturation *in vitro* (Modified from Nogueira, 2005).

1.2.1.3. Phosphodiesterase inhibitors

Phosphodiesterases (PDEs) are enzymes that are responsible for the hydrolysis of cAMP and/or cGMP. They have a similar structural layout, composed of a conserved catalytic domain and a distinct regulatory domain. They are classified into 11 distinct families on the basis of their kinetic characteristics, substrate specificity and regulation (Manganiello *et al.*, 1995; Conti and Jin, 1999). Two of the PDE families, the PDE3 and PDE4, are strongly associated with intra-follicular cAMP hydrolysis. The **PDE3 family** shows high affinity for cAMP as a substrate and is inhibited by the presence of cGMP and has at least two different gene products: PDE3A and PDE3B. The **PDE4 family** shows high affinity for cAMP as a substrate, but is not inhibited by cGMP. This family has at least four different gene products.
PDE3 and PDE4 are differentially localized within the follicular somatic and germ cells compartments. PDE4D is present in cumulus and mural granulosa cells, PDE4B is localized in theca cells and interstitial tissue and PDE3A is expressed in oocytes of several species (Richard *et al*., 2001; Shitsukawa *et al.*, 2001; Conti *et al.*, 2002; Thomas *et al.*, 2002; Nogueira *et al.*, 2003a; Sasseville *et al.*, 2006) (**Fig 5**). However, a recent report has established the functional presence of a PDE3A in porcine cumulus cells as well (Sasseville *et al.*, 2007).

While **non-specific PDE inhibitors** (PDE-Is; e.g. 3-isobutyl-1-methylxanthine (IBMX), hypoxanthine) inhibit many isoenzymes, **specific PDE-Is or 'second-generation PDE-Is'** will exert their action on only a specific type of PDE. Consequently, specific PDE-Is can be applied to differentially regulate cAMP levels in the oocyte and the cumulus cells. While PDE3 inhibitors (PDE3-Is; e.g. milrinone, cilostamide, Org9935) effectively inhibit oocyte maturation, PDE4 inhibitors (PDE4-Is; e.g. rolipram) cause oocyte maturation in follicle culture in the absence of gonadotrophin stimulation (Tsafriri *et al.*, 1996; Thomas *et al.*, 2002). Because PDE3-Is do not affect PDE4 in the granulosa cells, steroidogenesis and functionality of surrounding cumulus cells appears not to be affected (Nogueira *et al.*, 2005).
In addition, the availability and use of target-specific PDE-Is provide a new opportunity for more extensive examination of oocyte maturation mechanisms and represent new and powerful experimental tools for investigating oocyte-follicular cell interactions during oocyte maturation (Thomas *et al.*, 2002).

The importance of PDE3 in the regulation of oocyte meiosis has been studied in several species, such as Xenopus (Sadler, 1991), rodent (Tsafriri *et al.*, 1996; Richard *et al.*, 2001, Wiersma *et al.*, 1998), bovine (Mayes and Sirard, 2002; Thomas *et al.*; 2002), monkey (Jensen *et al.*, 2002) and human (Nogueira *et al.*, 2003a). Treating oocytes *in vitro* with PDE3-I reversibly prevents meiotic resumption of mouse (Tsafriri *et al.*, 1996; Nogueira *et al.*, 2003b) and human oocytes (Nogueira *et al.*, 2003a, 2006), but exerts only a transitory inhibitory effect on meiotic resumption in bovine oocytes (Thomas *et al.*, 2002, 2004).

Adding PDE3-Is to PMC media often improves or has no detrimental effect on subsequent oocyte developmental potential (summarized in Table I). In addition, meiotic arrest *in vitro* with PDE3-I allows oocytes the time to transit from a dispersed to compacted chromatin configuration and from a non-surrounded to a surrounded nucleoli (Nogueira *et al.*, 2003a, b). These changes indicate transition from a transcriptionally active state to an inactive state, which characteristically occurs during the final phase of acquisition of developmental competence *in vivo*, just prior to the preovulatory LH surge (Hyttel *et al.*, 1997; Combelles *et al.*, 2002; Miyara *et al.*, 2003; De La Fuente, 2006).

As such, these observations suggest that applying a period of PMC in the presence of an oocyte-specific PDE3-I is likely to improve synchronization between nuclear and cytoplasmic maturation. Hence, our laboratory has taken the approach to set-up a 2-step culture with PDE3-I in order to improve the IVM-outcome of human and mouse oocytes.

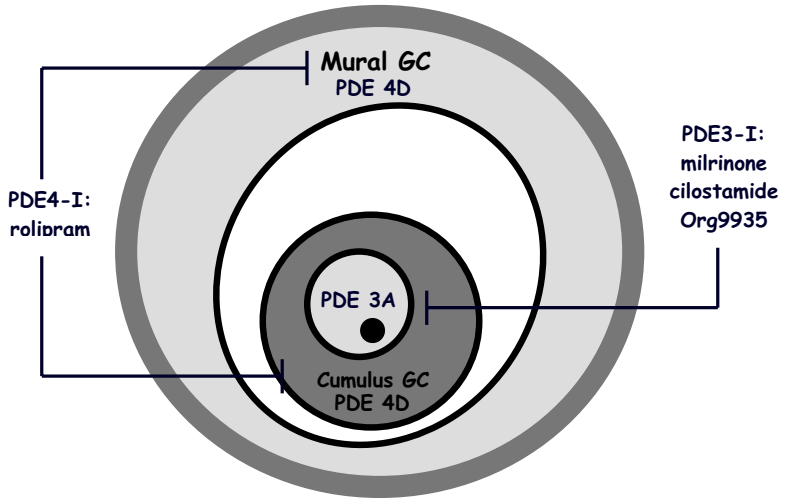

Figure 5: Intrafollicular localization of PDEs and respective selective inhibitors of PDE isoforms schematically represented in an antral follicle (GC: granulosa cells; Modified from Nogueira, 2005).

Table I: Effect of specific PDE3-Is during PMC on subsequent embryo development

Inhibiting agent	Species	Effect on embryo development	Reference
Milrinone	Bovine	Improved	Thomas *et al.*, 2004
Org9935	Murine	Improved	Nogueira *et al.*, 2003b
Milrinone	Porcine	Unchanged	Grupen *et al.*, 2006
Org9935	Human	Improved	Nogueira *et al.*, 2006
Cilostamide	Human	Unchanged	Shu *et al.*, 2008

1.2.2. Three-dimensional cultures

"There's a big difference between a flat layer of cells and a complex, three-dimensional tissue. But until recently, many biologists have glossed over this fact"
(Abbott, 2003)

For biotechnological research *in vitro* in general and tissue engineering specifically, it is essential to mimic the natural condition of the cellular environment as much as possible. Most tissues in the body consist of more than one cell type. The three-dimensional (3D) organization of these cells and there interactions are vital for the tissue's normal development, homeostasis and repair (Hendriks *et al.*, 2007).

When translating the above paragraph to IVM technology, the 'tissue' target for culture is the highly specialized cumulus-oocyte complex (COC) - also called the cumulus-enclosed oocyte (CEO) - present within the antral follicle. The cumulus cells nurture the oocyte throughout its development and maturation, while the oocyte plays an active role in the regulation of ovarian somatic cell function (Eppig, 1991; Gilchrist and Thompson, 2007). Hence, maintenance of the cellular interactions and the 3D morphology of the COC throughout the period of PMC and IVM might be crucial to obtain satisfactory results.

1.2.2.1. Function of cell communication within the cumulus-oocyte complex

The association between the germ cell and somatic granulosa or cumulus cells persists throughout growth, differentiation, maturation and fertilization of the oocyte. While the detailed steps involved in the signalling between these two cell types remain unknown, studies from mice bearing targeted deletions in essential 'communication' genes reveal selective disturbances in oocyte maturation competencies that compromise the oocytes' developmental potential (Carabatsos *et al.,* 1998, 2000).

Cumulus cells have an important function (1) in keeping the oocytes under meiotic arrest, (2) in participating in the induction of meiotic resumption and (3) in supporting cytoplasmic maturation (Tanghe *et al.*, 2002). While the above sections have documented the role of cumulus-oocyte interactions in the regulation of nuclear maturation, the next paragraph will focus on the support of cumulus cells during oocyte cytoplasmic maturation.

From a **metabolic perspective**, cumulus cells fulfill a role as 'nurse cells' through the final phases of oocyte development, by providing the oocyte with essential substances (Moor *et al*., 1998). They reduce cystine to cysteine and promote the uptake of cysteine in oocytes (Takahashi *et al.*, 1993; de Matos *et al*., 1997). This increases the content of intracellular glutathione (GSH), which participates in sperm decondensation and formation of the male pronucleus. GSH also plays an important role in protecting the oocyte against oxidative injury. Another substrate which is not readily metabolized by the oocyte itself is glucose. Cumulus cells metabolize glucose to pyruvate or Krebs cycle intermediates that can pass to the oocyte and are oxidatively metabolized for ATP generation in the oocytes' mitochondria (Downs *et al.*, 2002; Tanghe *et al.*, 2002). The efficiency by which oocytes undergo metabolic activities of these kinds is clearly compromised in the absence of the cumulus (Liu *et al.*, 1997). Maintenance of cumulus contacts throughout the period of maturation is critical for the support of oocyte metabolism, not only prior to and following fertilization, but also

during pre-implantation development. Any procedure that precociously disrupts this metabolic cooperation will compromise oocyte function, even though the consequences of such impairments may not be revealed until later stages of embryonic development (Wang *et al.*, 2008).
Cumulus cells fulfill a **protective role** as well, since they lower oxygen tension in the immediate vicinity of the oocyte. Furthermore, cumulus cells participate in the global **suppression of transcription** in oocytes that occurs before nuclear maturation (De La Fuente and Eppig, 2001). In addition, cumulus cells secrete a wide variety of **growth factors** that may either amplify or attenuate gonadotrophin actions within the COC.

In addition to granulosa cell-to-oocyte communication, it has recently become apparent that the oocyte itself plays a key role in directing its own fate through the synthesis and secretion of **oocyte-secreted factors (OSFs)** which act on granulosa cells to modify their proliferation, function, differentiation, apoptosis and expansion (Eppig, 2001; Gilchrist *et al.*, 2004, 2008; Hussein *et al.*, 2005). The studies of Nalbandov and colleagues were pioneering in this field, observing a precocious luteinization of granulosa cells from antral follicles cultured in the absence of oocytes (Nekola and Nalbandov, 1971). Nowadays, it is becoming clear that this communication axis is mainly regulated by fully grown meiotically competent immature oocytes (i.e. oocytes from antral follicles) (Gilchrist *et al.*, 2004). The exact identities of these OSFs are unknown, but growth-differentiation factor 9 (GDF-9) and bone-morphogenetic protein 15 (BMP-15) are two important examples (Hussein *et al.*, 2005). Recently published studies demonstrated that the addition of these native OSFs during IVM culture can significantly enhance oocyte developmental competence (Hussein *et al.* 2006; Yeo *et al.*, 2008).

The **bi-directional communication axis** within the COC is summarized in Fig 6.

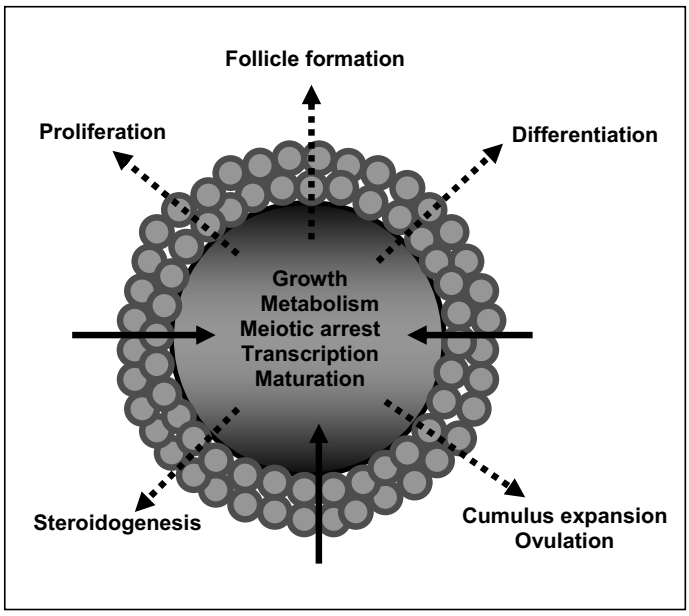

Figure 6: Bi-directional communication between oocytes and companion somatic cells showing the influence of granulosa cells on oocyte development and the processes in granulosa cells controlled by oocytes (Eppig, 2001).

1.2.2.2. Structural basis of oocyte-cumulus cell interactions

In addition to the local production of soluble factors that act in a **paracrine fashion**, oocytes and granulosa cells communicate via **direct physical contacts** (**Fig 7**).

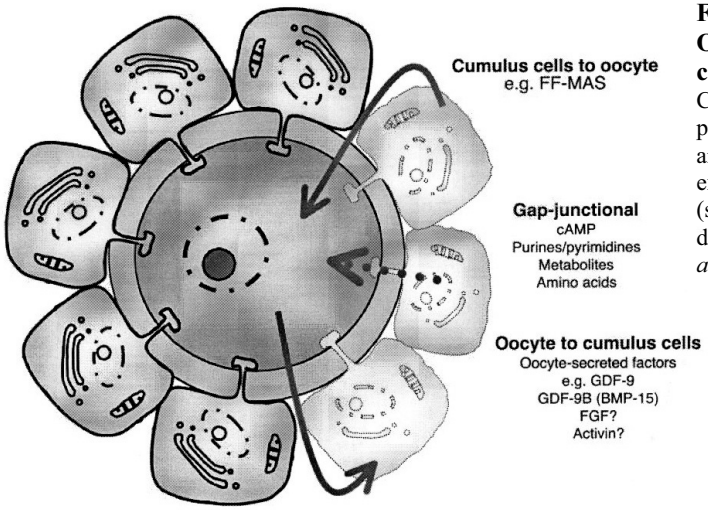

Figure 7: Oocyte-granulosa cell communication. Communication occurs via paracrine signaling (curved arrows) and gap-junctional exchange of small molecules (straight arrow). This is a bi-directional axis (Gilchrist *et al.*, 2004).

Cell junctions of at least two varieties dominate:

- **Gap junctions** composed of connexin subunits (Cx) that assemble to form channels between the cytoplasm of granulosa and oocytes, which facilitate the transport of nutrients and small molecules from cell to cell (Simon and Goodenough, 1998). This includes ions, metabolites and amino acids that are necessary for oocyte growth, as well as small regulatory molecules that control oocyte development (Gilchrist *et al*., 2004).
  While homologous gap junctions are found joining adjacent granulosa cells (containing predominantly Cx43), heterologous gap junctions are found between the oocyte and the granulosa cells. Deletion of the gene for the oocyte-specific gap junctional subunit, Cx37, interferes with antral follicle development and causes female sterility (Simon *et al.*, 1997; Carabatsos *et al.*, 2000), which emphasizes the importance of this interaction.

- **Adhesion junctions** exploit the cytoskeleton between apposed granulosa cells and oocytes using specialized integral membrane proteins to anchor cells and maintain stable and prolonged contact zones between these two cell types. They also connect granulosa cells to the oocytes' 'personal' extra-cellular matrix (ECM), the zona pellucida.
  It has been proposed that these contact sites may serve as active signalling domains for the interaction of certain oocyte- (e.g. GDF-9, BMP-15) and granulosa cell- (Kit Ligand) derived paracrine growth factors with their appropriate receptor kinases (Fagotto and Gumbiner, 1996). However, the exact cellular mechanisms that facilitate bi-directional exchange of paracrine signals are unclear (Albertini *et al*., 2001).

An intriguing feature in the establishment of cell junctions is that the oocyte and cumulus cells are physically separated by a considerable distance because of the zona pellucida surrounding the oocyte. To overcome this distance and to assemble oocyte-granulosa cell (junctional) and/or zona-granulosa cell adhesion, specialized cytoplasmic extensions are derived from granulosa cells that breach the zona pellucida and establish contact with the oolemma. In bovine COCs, these structures are often referred to as cumulus cell process endings (CCPEs) (de Loos *et al.*, 1989; 1991; Hyttel *et al.*, 1997). More generally, they are called **transzonal projections (TZPs)** (Anderson and Albertini, 1976) (Fig 8).
As in the case of neuritic extensions, TZPs have a core of cytoskeletal elements that includes microtubules, microfilaments and intermediate filaments (Combelles *et al.*, 2002; Suzuki *et al.*, 2000). Microtubules within TZPs form tracks for the bi-directional movement of organelles like mitochondria, lysosomes or endosomes (Albertini *et al*., 2001). The abundance of mitochondria indicates that the generation of ATP within these structures may be important for organelle transport, maintenance of cytoskeleton structural integrity and the provision of energy stores required by the oocyte during the metabolically demanding process of oocyte maturation.
An important deficiency in our current knowledge is how TZPs regulate paracrine and gap-junctional signaling within the COC. A model considering different pathways is schematically represented in Fig 8.

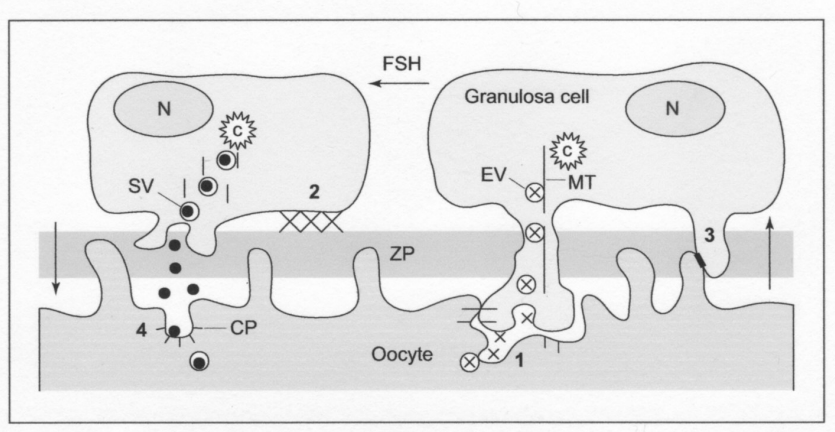

Figure 8: Model proposing different pathways (1-4) of how TZPs regulate paracrine and gap-junctional signaling within the COC. (1) Localized uptake of oocyte factors (x), like GDF-9, by endocytosis at stabilized attachment sites of TZPs at the oolemma; vectorial transport of endocytic vesicles (EV) to granulosa cell body occurs along microtubules (MT) in preparation for intracellular processing and release of factors after transcytosis. (2) Granulosa- ZP anchoring required for TZP orientation. Contact sites may play a signalling role for oocytes and granulosa cells. (3) gap-junctions that allow direct intercellular communication between oocyte microvilli and granulosa cell TZPs. (4) Pathway for delivery of granulosa-derived factors packaged in secretory vesicles (SV) that are subsequently endocytosed by receptor-mediated endocytosis at the oocyte surface through coated pits (CP). FSH modifies oocyte-cumulus interactions by reducing TZP density (Albertini *et al*., 2001).

Studies in humans have demonstrated that there is a dynamic alteration in the number and form of TZPs at specific stages of follicle development (Motta *et al.*, 1994). In preantral follicles TZPs are most numerous forming both adhesive and gap junctional contacts. During

peak periods of oocyte growth, TZPs extend as deep invaginations that impinge upon the GV. Albertini *et al*. (2001) demonstrated that after further antral follicle development, TZPs retract and maintain fewer terminal connections with the oocyte than in preantral follicles. During ovulation, active retraction of TZPs is noted. Typically, all TZPs present in the zona pellucida of human oocytes retrieved for IVF showed **acetylated microtubules** (Albertini *et al*., 2001), indicating that this mechanism for maintaining microtubule stability may persist through the latest stages of the follicle development in order to maintain oocyte-granulosa cell connections during cumulus expansion.

1.2.2.3. Use of an extra-cellular matrix to preserve 3D-structures *in vitro*

The difference between *in vitro* versus *in vivo* matured oocytes may originate from the extent of the communication between somatic cells and the germ cell. There is a growing sense that upon removal of the COC from the follicle, a rapid and irreversible modification in oocyte-cumulus cell interactions occurs, which affects developmental competence of oocytes, even in the presence of the cumulus (Gilchrist *et al.*, 2004). Moreover, during the process of PMC in a 2-step culture, loss of association between the oocyte and cumulus cells was observed (Nogueira *et al.*, 2003a and b, 2006). The COCs attach to the two-dimensional (2D) culture surface of the petri-dish and the somatic cells spread out and migrate away from the oocyte (Fig 9). In other cases, the cumulus cells loosen completely from the oocyte in form of clumps. This spreading or loosening alters the 3D structure of the COC, thereby disrupting the cell-cell interactions. Findings from Webb *et al.* (2002) demonstrate that an intact COC is required for normal gap-junctional communication between the oocyte and the cumulus cells. In addition, disturbed cell-cell orientation may result in the diffusion of paracrine signals away from their target cell-surface receptors.

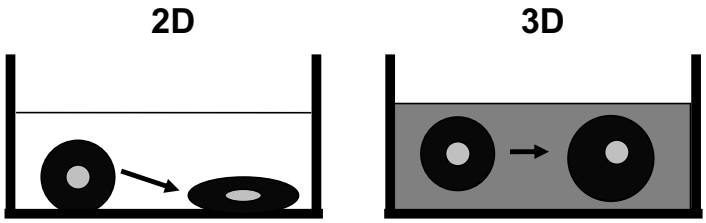

Figure 9: COCs cultured in a 2D-environment (liquid) adhere and flatten readily. Those cultured in a 3D environment maintain their *in vivo*-like architecture.

One way to preserve 3D structures *in vitro* and, consequently, to mimic more faithfully the *in vivo* environment is to embed tissues within an extra-cellular matrix (ECM) (Abbott, 2003). Indeed, in comparison to a 2D environment, a 3D environment results in cell behaviour, signalling and gene expression profiles most resembling those observed in living tissues (Cukierman *et al.*, 2002).
The ECM is the authentic substrate for most cells in living organisms, which is complex and dynamic in molecular composition. In ovarian cells, ECM influences a multitude of cell functions, such as cell morphology, aggregation and communication, proliferation, survival and steroidogenesis (Berkholtz *et al.*, 2006). The structure of the ECM provides mechanical support to the tissue, whereas the biochemical composition can interact directly with cells through specific receptors or can bind growth factors that are released upon matrix

degradation. In addition, the ECM acts as a barrier that restricts cellular access, thereby defining specific cellular compartments and specialization necessary for proper function (Rodgers *et al*., 2003).
The ECM is sensed by cells via integrins, which are membrane-spanning heterodimeric receptors that mediate cell-matrix communication. Upon binding, integrins initiate signalling cascades involved in cell migration, endocytosis and proliferation (Sheppard D, 2000).
The ECM is composed of a variety of molecules, which include collagens, laminin, fibronectin, proteoglycans and polysaccharides. Fibronectin, laminin and certain collagens have been localized in the ovaries of many species and their effects on follicle development have been examined (Huet *et al*., 1997; Rodgers *et al.*, 2003). Ovarian follicles contain distinct ECMs: the ZP, the antrum, basal lamina and the thecal matrix. The composition of these matrix components changes markedly throughout follicle development. During the process of ovulation, a new hyaluronan-rich matrix forms within the COC.

In the field of reproduction, the use of an ECM to preserve 3D morphology *in vitro* has been tested extensively on preantral 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). While flat/adhesive cultures led to distortion of follicular architecture and frequent follicle disruption, 3D-ECM cultures were able to support normal *in vivo*-like follicular morphology, differentiation and growth. The ECM-embedding method has also been shown to preserve the integrity of bovine and canine COCs isolated from antral follicles (Yamamoto *et al.*, 1999; Alm *et al.*, 2006; Otoi *et al.*, 2006). At the level of granulosa cells, many studies showed that cells behave and function in distinct ways when cultured in the presence or absence of ECM and in 3D versus 2D dimensions (Ben-Rafael *et al.*, 1988; Amsterdam *et al.*, 1989; Bussenot *et al.*, 1993).

The composition of the ECM is an essential variable which influences cell behaviour *in vitro*.
For the 3D *in vitro* culture of follicles, most researchers used simple gels consisting of **collagen** Type I (Torrance *et al.*, 1989; Carroll *et al.*, 1991; Gomes *et al*., 1999; Yamamoto *et al.*, 1999; Alm *et al.*, 2006; Otoi *et al.*, 2006). Others used the commercially available **Matrigel**, which consists of structural proteins such as laminin and collagen, plus growth factors and enzymes, all taken from mouse tumours. This approach was tested on human follicles (Hovatta *et al.*, 1997). Another option is the use of **alginate-based matrices** (Pangas *et al.*, 2003; Kreeger *et al.*, 2006). Alginate exhibits minimal cellular interactions with mammalian cells, and thus likely provides only mechanical support. This synthetic matrix can, however, also be modified with intact ECM molecules, as has been tested on mouse preantral follicles (West *et al*., 2007).

Our laboratory has taken the approach to use collagen-gel (Type I) as a 3D matrix for the encapsulation of COCs during the first step (i.e. the PMC) of a 2-step culture. This so-called '3D-PMC' system was tested on mouse and human COCs derived from small antral follicles.

1.2.3. Co-cultures

The somatic cells of the follicle, particularly the cumulus cells, play a key role in the acquisition of oocyte developmental competence. However, the majority of experimental studies on human IVM have utilized immature oocytes denuded of surrounding cumulus cells that were obtained after COH for IVF/ICSI treatment. Enzymatic and physical dissociation of the cumulus at oocyte retrieval is required to assess oocyte maturity and to perform ICSI.

Human oocytes with scanty or no cumulus cells show lower maturation rates after IVM than oocytes surrounded by an intact cumulus oophorus (Kennedy and Donahue, 1969; Schroeder and Eppig, 1984; Ruppert-Lingham *et al*., 2003; Nogueira *et al*., 2006). Moreover, cumulus-free oocytes exhibit accelerated meiotic resumption *in vitro*, a deficiency in the ability of the cytoplasm to maintain M-phase characteristics, a propensity to activate spontaneously after M-phase arrest and a lack of coordination between nuclear and cytoplasmic maturation (Combelles *et al.*, 2002). Therefore, it is essential to consider ways of restoring the structural complexity of COCs in the design and implementation of human **'rescue' IVM**.

Co-cultures of human denuded oocytes with dissociated cumulus cells, either in suspension or monolayer, have been established in an effort to restore support from the surrounding cumulus cells to the oocyte and/or to probe interactions between the two cell compartments (Häberle *et al.*, 1999; Johnson *et al*., 2007; Zhu *et al.*, 2007). This approach offers several theoretical advantages. Cumulus cells produce pyruvate, glutathione, steroid hormones and several growth factors, which may be delivered to the oocyte (Parikh *et al*., 2006). It is also believed that cumulus cells remove toxins from the culture medium. Finally, cumulus cells could participate in the preservation of premature cortical granule exocytosis leading to zona hardening (Ge *et al*., 2008). Most of these studies were, however, unable to show a significant improvement in IVM outcome.

An attempt to better mimic the physiological situation of the COC is to create a system that permits a tight contact between the dissociated cumulus cells and the oocyte in a three-dimensional (3D) structure, making use of an extra-cellular matrix (ECM). Until today, only two studies on human oocytes investigated this approach (Combelles *et al.* 2005; Torre *et al.* 2006).
In the study of Combelles *et al.* (2005), cumulus cells were embedded into a collagen gel matrix with individual immature oocytes added to each gel. The maturation rate of oocytes cultured under these co-culture conditions was comparable with the maturation rate of denuded oocytes in a conventional culture system (microdroplets under oil; no cumulus cells). Nevertheless, co-cultured oocytes exhibited increased activity of MAPK, an important factor in the regulation of oocyte maturation. Whether gap junctional communication was restored between cumulus cells and oocytes was not determined in this study, but microtubule-rich processes resembling transzonal projections were observed at the oocyte-cumulus interface.
In the study of Torre *et al.* (2006) human oocytes were co-cultured with cumulus cells enclosed in the core of a barium alginate capsule, a synthetic ECM product. After 48 h of culture, the oocytes appeared to be surrounded by a pseudo-cumulus structure. This culture system resulted in a significantly higher maturation rate compared to conventional culture techniques in microdroplets.

These innovative culture systems afford several advantages, among which are the ability to reconstitute direct interactions between isolated cell populations and the use of an ECM-3D micro-environment. The positive effect of the ECM on ovarian cell behaviour *in vitro* has been already studied extensively in previous studies. Human mural granulosa cells cultured in ECM, such as collagen, maintain a more highly-differentiated morphology, possess an increased level of steroidogenic activity (Ben-Rafael *et al.*, 1988; Bussenot *et al.*, 1993), produce significantly higher levels of cAMP (Furman *et al.*, 1986) and induce de novo formation of gap junctions (Amsterdam *et al.*, 1989).

A superior **2-step 'rescue IVM' culture system** could be the combination of a 3D co-culture system with a period of PMC. In this way, the unhealthy condition of precocious spontaneous maturation is circumvented, while the surrounding somatic cells may produce a suitable micro-environment to support prematuration events of the cumulus-free oocyte. This hypothesis was tested in our research project on human oocytes.

2. Aims of the thesis

"It is obvious that while one of the goals of oogenesis is to commence embryogenesis, an early objective of this phenomenon is the production of a mature egg"
(Anderson, 1974)

A crucial point in the optimization of IVM technology is the development of new strategies for culturing immature oocytes, which is the aim of the present thesis.

2.1. Clinical outcome of conventional *in vitro* maturation (= 'rescue IVM')

To start-up this research project, we analyzed if there exists a clinical benefit for using *in vitro* matured metaphase I (MI) oocytes in a selected population of patients with a low number of mature oocytes (≤ 6) available for the infertility treatment (= 'rescue IVM') (**Article I**).

2.2. Effect of prematuration culture in the presence of a phosphodiesterase type 3 inhibitor

In the second study, mouse cumulus-oocyte complexes (COCs) were utilized to set-up a 2-step culture composed of a prematuration culture (PMC) to block temporarily spontaneous nuclear maturation followed by IVM. Oocyte quality was evaluated at the end of IVM (**Article II**).

In view of the results obtained, we applied a similar 2-step culture on human oocytes. Spare cumulus-free GV-stage oocytes retrieved from COH cycles were used as source of human experimental material (**Article III**).

2.3. Effect of three-dimensional prematuration culture in the presence of a phosphodiesterase type 3 inhibitor

A clear drawback in the previous experimental set-up (Article III) was the fact that the human GV oocytes were denuded from surrounding cumulus cells before starting the 2-step culture. To circumvent this deficiency, the aim of the next study was trying to perform the PMC step in a three-dimensional (3D) co-culture system of human GV oocytes with dissociated cumulus cells, making use of an extra-cellular matrix (ECM; collagen) (**Article IV**).

The final step in our research project was to further optimize the PMC conditions in order to preserve intracellular connections within an intact COC. For this purpose, COCs were embedded within an ECM, the same collagen matrix as was tested in the previous study (Artikel IV). A mouse model was initially used to test this approach. In addition, we evaluated this culture system on human COCs derived from small antral follicles donated by patients undergoing COH for IVF/ICSI treatment (**Article V**).

3. Scientific papers

3.1. Clinical outcome of conventional *in vitro* maturation (= 'rescue IVM')

Article I: **Clinical benefit of metaphase I oocytes.**

Vanhoutte L, De Sutter P, Van der Elst J, Dhont M

Reproductive Biology and Endocrinology (2005), 3:71

Reproductive Biology and Endocrinology

BioMed Central

Research

Clinical benefit of metaphase I oocytes

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Abstract

Background: We studied the benefit of using in vitro matured metaphase I (MI) oocytes for ICSI in patients with a maximum of 6 mature metaphase II (MII) oocytes at retrieval.

Methods: In 2004, 187 ICSI cycles were selected in which maximum 6 MII oocytes and at least one MI oocyte were retrieved. MI oocytes were put in culture to mature until the moment of ICSI, which was performed between 2 to 11 hours after oocyte retrieval (day 0). In exceptional cases, when the patient did not have any mature oocyte at the scheduled time of ICSI, MI oocytes were left to mature overnight and were injected between 19 to 26 hours after retrieval (day 1). Embryos from MI oocytes were chosen for transfer only when no other good quality embryos from MII oocytes were available. Outcome parameters were time period of in vitro maturation (IVM), IVM and fertilization rates, embryo development, clinical pregnancy rates, implantation rates and total MI oocyte utilization rate.

Results: The overall IVM rate was 43%. IVM oocytes had lower fertilization rates compared to in vivo matured sibling oocytes (52% versus 68%, $P < 0.05$). The proportion of poor quality embryos was significantly higher in IVM derived oocytes. One pregnancy and live birth was obtained out of 13 transfers of embryos exclusively derived from IVM oocytes. This baby originated from an oocyte that was injected after 22 hrs of IVM.

Conclusion: Fertilization of in vitro matured MI oocytes can result in normal embryos and pregnancy, making IVM worthwhile, particularly when few MII oocytes are obtained at retrieval.

Background

A proportion of human oocytes collected during an IVF or ICSI procedure remains meiotically immature at the germinal vesicle (GV) or metaphase I (MI) stage. Several publications have shown that this proportion fluctuates around 15 to 20% [1,2]. It is not exactly known why some of the oocytes are unresponsive to the maturation trigger in vivo. Different explanations are possible. When ovarian hyperstimulation is started, follicles may be at different stages of development, producing oocytes of varying degrees of maturity. Since follicles are aspirated prior to rupture, the collected oocytes come from a heterogeneous pool of follicles, including luteinizing as well as degenerating follicles [3]. It is also possible that smaller antral follicles are aspirated during oocyte retrieval, which can result in the collection of immature oocytes [4,5]. Eventually, the proportion of immature oocytes can be dependent on patients' characteristics (such as cause of infertility, age, ovarian reserve) and the stimulation protocol used.

Immature oocytes from superovulated cycles can undergo the final stages of meiotic maturation spontaneously in vitro. MI oocytes have already undergone the process of germinal vesicle breakdown (GVBD) and may progress to the metaphase II (MII) stage within a few hours of in vitro culture. This allows them to be injected by ICSI at the same time as their sibling mature MII oocytes. The clinical use of MI oocytes from stimulated cycles has been studied by several research groups. It has been reported that these in vitro matured oocytes yield lower fertilization rates [6-8], abnormal embryonic development [7-9] and lower implantation rates [6] than in vivo matured oocytes. Development to term is limited to rare cases [6,8,9]. As a consequence, immature oocytes from stimulated cycles are generally considered to be a side-product and only in vivo matured MII oocytes are used for ICSI. Nevertheless, for patients in whom a low number of MII oocytes are retrieved, the use of in vitro matured MI oocytes may be worthwhile in order to increase the number of injectable oocytes at the time of ICSI.

We therefore designed the present study to determine if, indeed, in vitro matured MI oocytes could have a clinical application in our IVF-program in a selected population of patients with a low number of MII oocytes at retrieval. This was analyzed within the framework of a daily laboratory practice, without changing the routine of ovarian stimulation, oocyte retrieval, ICSI procedure, embryo culture or transfer.

Methods

Patient selection

The study included all ICSI cycles over a one year period (2004) in which two inclusion criteria were fulfilled: 1) a maximum of 6 mature (MII) oocytes and 2) at least one MI oocyte present at retrieval.

Our ICSI program has been approved as infertility treatment by the Ghent University Hospital Ethical Committee.

Ovarian stimulation, IVM and oocyte handling for ICSI

All patients underwent controlled ovarian stimulation after cycle synchronization with a standard contraceptive pill for 2–6 weeks. A short gonadotrophin-releasing hormone (GnRH) 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 was usually 150 IU, but this dose was adjusted after 7 days of hMG or FSH administration, according to the individual response of the patient. Known poor responders were started on 300 IU of hMG or FSH daily. The follicular phase was monitored by means of transvaginal ultrasound scanning of the ovaries and serum estradiol measurement if judged necessary. An injection of 5,000 or 10,000 IU human chorionic gonadotrophin (hCG; Pregnyl, Organon, The Netherlands) was administered when half of all mature follicles had reached a mean diameter of at least 20 mm, measured in two planes. Oocyte retrieval was scheduled 34 to 36 hrs after hCG administration.

Oocytes were denuded enzymatically by a brief exposure of the cumulus-oocyte complexes to 80 IU/ml hyaluronidase (Type VIII; Sigma Chemical Co., Bornem, Belgium), followed by mechanical denudation approximately 1 to 2 hrs after oocyte collection. The nuclear status of denuded oocytes was subsequently recorded. GV oocytes were not considered for ICSI. MI oocytes were defined as those oocytes in which no GV and no first polar body were visible. These oocytes were put in culture to mature. The cul-

Table 1: Comparison of fertilization and embryonic development between in vitro and in vivo matured sibling oocytes

	In vitro matured MI oocytes	In vivo matured sibling oocytes (control)
Total N° of oocytes injected	129	339
N° (%) of normal fertilized oocytes[a]	67 (52)*	229 (68)
Embryo development on day 2 (%)[b]		
Excellent	24*	41
Good	27	32
Poor	49*	27
Embryo development on day 3 (%)[b]		
Excellent	22*	38
Good	28	32
Poor	50*	30

* Statistically different between columns ($p < 0.05$).

[a] Normal fertilized oocytes were defined as oocytes containing 2 pronuclei en 2 polar bodies.

[b] The proportions of excellent, good and poor embryos are expressed per total number of embryos obtained on day 2 or day 3.

ture medium for in vitro maturation (IVM) was either Sydney IVF Fertilization Medium (Cook, Ltd., Limerick, Ireland) or Early Cleavage Medium (Irvine Scientific, Brussels, Belgium).

MI oocytes were left to mature until the time when ICSI for the particular patient was carried out. This was in a time-frame of 2 to 11 hrs after oocyte retrieval (day 0). In exceptional cases, when the patient did not have any mature oocyte at the scheduled time of ICSI, MI oocytes were left to mature overnight and, when matured, were injected between 19 to 26 hrs after oocyte retrieval (day 1).

In vivo and/or in vitro matured oocytes were injected with patient's sperm. Sperm preparation for ICSI and details for the microinjection procedure have been described elsewhere [10]. After injection, oocytes were cultured in either Sydney IVF Cleavage Medium (Cook) or Early Cleavage Medium.

Embryo evaluation and transfer

Assessment of fertilization took place between 16 to 20 hrs after ICSI. Embryos were evaluated based on the number of blastomeres and the degree of fragmentation. Embryos with less than 10% anucleated fragments were classified as 'excellent'. Embryos with either 10–20% or >20% anucleated fragments were classified as 'good' and 'poor' quality embryos, respectively. Embryos with at least one blastomere with more than one nucleus were classified as multinucleated embryos and were considered as 'poor' quality embryos as well, regardless of the degree of fragmentation.

Transfer of embryos was carried out on day 2 or day 3. Embryos from in vitro matured MI oocytes were chosen for transfer only when no other good quality embryos from MII oocytes were available. The number of embryos transferred was limited by Belgian law based on female age, cycle number and embryo quality [11].

Pregnancy was diagnosed by the detection of a positive serum hCG at least 14 days after embryo transfer, followed by a rise in hCG levels. All patients received a transvaginal ultrasound scan between 6 and 7 weeks of pregnancy to differentiate between biochemical and clinical (presence of an intra-uterine gestational sac with fetal heart beat) pregnancies and to diagnose ectopic implantations. All pregnancies were monitored further by transvaginal ultrasound until 12 weeks of amenorrhoea.

Statistical analysis

For comparison among groups, results were analyzed with Chi-square and Fisher's exact test when appropriate. When the P value was <0.05, the difference was considered significant.

Results

Patient and cycle characteristics

In the year 2004, 180 patients underwent 187 ICSI cycles in which maximum 6 MII oocytes and at least one MI oocyte were retrieved. This is 12.7% of the total number of ICSI cycles performed in our infertility center during the same year. The mean age of the patients was 35.9 ± 4.67 years (range 25–48).

A total of 1208 oocytes were collected. Three hundred of these oocytes were at the MI stage at the moment of oocyte denudation (24.8%; mean of 1.6 MI oocytes/cycle; range 1–6). Hundred thirty-two oocytes were at the GV stage (10.9%) and 80 oocytes (6.6%) were degenerated or damaged at the moment of denudation.

Comparison between in vitro matured MI oocytes and in vivo matured oocytes

Overall, 43% (129/300) of MI oocytes matured to the MII stage. Maturation and injection of at least one MI oocyte was achieved in approximately half of the ICSI cycles (55%; n = 102). In vivo matured sibling MII oocytes, retrieved in the same treatment cycles, were injected in parallel and served as the control group. ICSI was performed with fresh ejaculate in 81.4% of the cycles and with frozen ejaculate in 9.8% of the cycles. Frozen epidi-

Table 2: Comparison of maturation rates, fertilization rates and embryo development between different time periods of IVM

Time interval of IVM	N° of MI oocytes	N° (%) of in vitro matured oocytes	N° (%) of fertilized oocytes	N° (%) of excellent + good quality embryos on Day 2 [c]	N° (%) of excellent + good quality embryos on Day 3
2 to 4 hrs	147	60 (41)	26 (43)[a]	15 (58)	14 (61)
>4 to 7 hrs	136	57 (42)	31 (54)[a]	15 (48)	9 (38)
>7 to 11 hrs	9	6 (67)	6 (100)[b]	3 (50)	2 (40)
19 to 26 hrs	8	6 (75)	4 (67)[ab]	1 (25)	2 (100)

[a,b] Different letters indicate significant differences within columns ($p < 0.05$).
[c] Thirteen embryos were transferred on day 2.

Table 3: Clinical pregnancy and implantation rates in cycles with maximum 6 MII oocytes

	cycles with at least one MI oocyte matured (n = 102)			cycles with no MI oocyte matured (n = 85)	Total (n = 187)[a]
	Exclusively embryos derived from MI oocytes	Mixed transfers	Exclusively embryos derived from sibling MII oocytes		
N° of transfers	13	21	63	81	178
N° of embryos transferred	17	54	119	141	331
N° (%) of clinical pregnancies per embryo transfer	1 (7.7)	2 (9.5)	17 (27.0)	15 (18.5)	35 (19.7)
N° (%) of implanted embryos	1 (5.9)	3 (5.6)[b]	18 (15.1)[b]	15 (10.6)	37 (11.2)

No statistical difference between columns.
[a] In 9 ICSI cycles, no embryo transfer was done.
[b] A twin pregnancy was obtained in these groups.

dymal or testicular spermatozoa were used in respectively 1.0% and 7.8% of the cycles.

The results of fertilization and embryo development in the two groups of oocytes (IVM + control) are presented in Table 1. The fertilization rate of matured MI oocytes was significantly lower compared to the fertilization rate of in vivo matured oocytes (52% versus 68%, $P < 0.05$). Also the embryo quality was different between the two groups. In the in vitro matured group, significantly less embryos of excellent quality and more embryos of poor quality were obtained compared to the in vivo matured group. This was observed on day 2 ($p < 0.05$) as well as on day 3 ($p < 0.05$).

Comparison between different time periods of IVM

A second evaluation of the results was done by splitting up the period of IVM, between collection of the oocytes and the time of ICSI, in different time intervals (Table 2).

A number of 147 oocytes, coming from 100 ICSI cycles, were evaluated for maturity within 2–4 hrs of IVM culture, 136 oocytes (77 ICSI cycles) within >4–7 hrs of culture, 9 oocytes (5 ICSI cycles) within >7–11 hrs of culture, and 8 oocytes (5 ICSI cycles) were left to mature overnight and were evaluated the day after retrieval, between 19–26 hrs of culture. A time-dependent increase in the progression to maturation was noted, ranging from 41% mature oocytes after 2–4 hrs of IVM to 75% after 19–26 hrs of IVM, but this trend was not statistically different. The fertilization rate in the group of >7–11 hrs of IVM (100%) was significantly higher compared to 2–4 hrs of IVM (43%) and >4–7 hrs of IVM (54%) ($p < 0.05$), but not to 19–26 hrs of IVM (67%). There was no difference between the number of excellent and good quality embryos in the different IVM time interval groups.

Embryo transfer, clinical pregnancy and implantation rates

The most important parameter to evaluate whether the use of in vitro matured MI oocytes has a clinical benefit is the pregnancy outcome. Table 3 represents the results of embryo transfer, clinical pregnancy and implantation rates. A distinction was made between cycles in which at least one MI oocyte was matured in vitro (n = 102) and ICSI cycles with no matured MI oocytes (n = 85). The group of cycles with matured MI oocytes was further split-up in 3 groups: 1) transfers involving exclusively embryos derived from in vitro matured MI oocytes, 2) mixed transfers and 3) transfers involving exclusively embryos derived from in vivo matured MII oocytes. A double embryo transfer involving embryos exclusively derived from MI oocytes resulted in a singleton pregnancy and the birth of a healthy baby girl. This baby originated from an oocyte that was injected after 22 hrs of IVM. There was no statistical difference between clinical pregnancy rates and embryo implantation rates in the three groups. However, when the numbers of exclusively MI and mixed transfers were pooled and compared to exclusively MII transfers, the clinical pregnancy rates (8.8% versus 27.0%) and embryo implantation rates (5.6% versus 15.1%) were significantly lower in the former group ($p < 0.05$).

Total MI oocyte utilization rate

Forty-two embryos derived from normally fertilized in vitro matured oocytes were used for transfer and 9 embryos were cryopreserved. This means that 51 out of 67 embryos originating from MI oocytes were used. The total

MI oocyte utilization rate (= percentage of embryos transferred and frozen per fertilized oocyte) was 76%.

Discussion

The present study aimed to analyze, for the first time, the clinical benefit of MI oocytes in a selected group of patients with a low number of mature oocytes at retrieval. To achieve this goal, we selected ICSI cycles in which a maximum of 6 mature oocytes and at least one MI oocyte were obtained at oocyte retrieval. The results show that fertilization rate and developmental capacity of the embryos was significantly reduced in IVM oocytes compared with control sibling oocytes. One live birth obtained after transfer of embryos exclusively derived from IVM oocytes illustrates that the use of MI oocytes is not of major issue in IVF programs, but may be an option for patients with low numbers of MII oocytes.

A low number of oocytes at retrieval might be a result of low ovarian response to gonadotrophin stimulation. Low response to stimulation occurs in approximately 10% of the ART population [12]. There is no universally accepted definition for low response. One of the criteria is the number of oocytes retrieved. Faber et al. [13] used an oocyte retrieval rate of ≤ 4 mature oocytes as cut-off limit, while De Sutter et al. [14] and Moreno et al. [15] categorized < 5 and ≤ 6 oocytes retrieved, respectively, as low responding patients, without distinguishing mature and immature oocytes. Based on these different definitions, it can be concluded that the patients in our study can be categorized as 'relatively poor responders'. In each cycle of this study, at least one MI oocyte was present. The proportion of immature oocytes (GV +MI = 35.7%) in this group of relatively poor responding patients is high compared to the percentages described in the literature after ovarian stimulation in a non-selected group of patients (15–20%; [1,2]) and, as a consequence, the total MI oocyte utilization rate of 76% shows that embryos from in vitro matured MI oocytes were used at high frequency for transfer or cryopreservation.

In the majority of the ICSI cycles, the maturation status of the MI oocytes was checked between 2 to 7 hours after oocyte retrieval. Within this time-frame, 41.3% of the collected MI oocytes extruded their polar body. These maturation rates are comparable with those achieved by others working with MI oocytes retrieved from stimulated cycles. Chian et al. [16] obtained a maturation rate of 46.1% and 52.0% after 6 hrs of in vitro culture and Strassburger et al. [8] obtained 45.1% matured oocytes after 4 hrs of culture. Other studies describe lower maturation rates, like the study of Devos et al. [6] (26.7% maturation after 4 hrs of culture) and the study of Chen et al. [9] (16.4% maturation after 9 hrs of culture). Variations in maturation rates between studies might be explained by different starting and ending points for in vitro maturation. Also the culture conditions, the use of more suitable types of media and/or the addition of serum, growth factors and hormones might influence maturation rates, subsequent fertilization and embryo development of in vitro matured oocytes [16-18]. The group of Balakier et al. [7] performed an exact time recording of polar body extrusion during IVM of MI oocytes. They found that the highest fertilization rate and the lowest incidence of multinucleation were obtained when injection of the oocytes was performed between 3 to 6 hrs after extrusion of the first polar body. This indicates that oocyte maturation is not completed upon reaching the MII stage. In the present study, we did not perform an accurate kinetic experiment, but proper timing of polar body extrusion as well as injection may enhance the outcome of in vitro matured oocytes.

Whatever the conditions of in vitro maturation applied, our study and the majority of other studies show consistently lower fertilization rates of in vitro matured MI oocytes compared to sibling in vivo matured oocytes [6-8]. The proportion of poor quality embryos was higher after in vitro maturation. This is in accordance with other publications, which describe more cleavage arrest [7,8], a higher number of multinucleated blastomeres [7] and a reduced development to the blastocyst stage [9] after IVM. On the contrary, the study of Devos et al. [6], performed on a large group of 896 ICSI cycles, found the same proportions of excellent and fair quality embryos after IVM compared to in vivo matured oocytes. This might be explained by the IVM incubation time of maximum 4 hrs applied in this study. Longer IVM incubation times could result in oocyte ageing. However, we were not able to find a statistical difference in fertilization rate and embryo quality between the different IVM time intervals in the in vitro matured group, although a larger sample would be necessary for a more conclusive statement in the IVM time intervals of >7–11 hrs and 19–26 hrs. Nevertheless, of special interest was the fact that the live birth we obtained from exclusively IVM oocytes originated from an oocyte that was matured in vitro for a period of 22 hrs.

Conclusion

We may conclude that the use of in vitro matured MI oocytes can be of benefit to obtain pregnancy in patients with a low number of MII oocytes. IVM culture conditions and time schedule for ICSI must be refined to achieve optimum fertilization and development.

Authors' contributions

LV and JVDE designed the study. LV collected and analyzed the data of the study and wrote the manuscript. All the authors corrected the manuscript and approved the final version. PDS and MD treated the patients.

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3.2. Effect of prematuration culture in the presence of a phosphodiesterase type 3 inhibitor

Article II: **Effect of temporary nuclear arrest by PDE3-I on morphological and functional aspects of *in vitro* matured mouse oocytes.**

Vanhoutte L, Nogueira D, Gerris J, Dhont M, De Sutter P

Molecular Reproduction and Development (2008), 75: 1021-1030

Article III: **Nuclear and cytoplasmic maturation of *in vitro* matured human oocytes after temporary nuclear arrest by PDE3-I.**

Vanhoutte L, De Sutter P, Nogueira D, Gerris J, Dhont M, Van der Elst J

Human Reproduction (2007), 22: 1239-1246

MOLECULAR REPRODUCTION AND DEVELOPMENT 75:1021–1030 (2008)

Effect of Temporary Nuclear Arrest by Phosphodiesterase 3-Inhibitor on Morphological and Functional Aspects of In Vitro Matured Mouse Oocytes

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ABSTRACT The present study aimed to analyze detailed morphological and functional characteristics of mouse in vitro matured oocytes after a prematuration culture (PMC) by temporary nuclear arrest with the specific phosphodiesterase 3-inhibitor (PDE3-I) Cilostamide. In a first experiment the lowest effective dose of Cilostamide was determined. Cumulus–oocyte complexes (COCs), isolated from small antral follicles, were exposed to different concentrations of Cilostamide (ranging from 0 (control) to10 μM) for 24 hr. Afterwards, oocytes were removed from PDE3-I-containing medium and underwent in vitro maturation (IVM) for 16–18 hr. A concentration of 1 μM Cilostamide was the lowest effective dose for maximum level of inhibition and reversibility of meiosis inhibition. This concentration was used in further experiments to evaluate oocyte quality following IVM in relation to different parameters: kinetics of meiotic progression, metaphase II (MII) spindle morphology, aneuploidy rate, fertilization, and embryonic developmental rates. The results were compared to nonarrested (in vitro control) and in vivo matured oocytes (in vivo control). Following withdrawal of the inhibitor, the progression of meiosis was more synchronous and accelerated in arrested when compared to nonarrested oocytes. A PMC resulted in a significant increase in the number of oocytes constituting a MII spindle of normal morphology. None of the oocytes exposed to PDE3-I showed numerical chromosome alterations. In addition, fertilization and embryonic developmental rates were higher in the PMC group compared to in vitro controls, but lower than in vivo controls. These results provide evidence that induced nuclear arrest by PDE3-I is a safe and reliable method to improve oocyte quality after IVM. *Mol. Reprod. Dev. 75: 1021–1030, 2008.* © 2007 Wiley-Liss, Inc.

Key Words: aneuploidy; Cilostamide; fertilization; IVM; meiosis; spindle

INTRODUCTION

In vitro maturation (IVM) of oocytes retrieved from small antral follicles is an alternative approach to avoid or reduce the use of superovulatory drugs. This would be of particular benefit for women who are vulnerable for ovarian hyperstimulation syndrome (OHSS). However, the quality and subsequent developmental competence of in vitro matured oocytes is generally compromised in most mammals, including the human (Trounson et al., 2001; Smitz et al., 2004; Jurema and Nogueira, 2006). This supports the need for improving in vitro culture conditions.

The process of oocyte maturation is a complex orchestration of molecular, cytoplasmic, and nuclear events that must occur in a synchronized manner. When fully-grown germinal vesicle (GV)-stage oocytes are retrieved from antral follicles, they are capable of spontaneously progressing to metaphase II (MII) before the cytoplasm has achieved full maturity (Edwards, 1965; Eppig et al., 1994). In addition, GV-stage oocytes isolated from follicles stimulated by exogenous gonadotrophins are heterogenous in size, cytoplasmic microtubule organization, chromatin, and nucleolar configuration (Mattson and Albertini, 1990; Debey et al., 1993; Zuccotti et al., 1998; Combelles et al., 2002; De La Fuente, 2006). This heterogeneity could also account for the overall low developmental competence after IVM.

A possible strategy to improve the outcome of IVM is to keep oocytes meiotically arrested in vitro for a prolonged period of time rather than allowing the oocytes to undergo germinal vesicle breakdown (GVBD) as soon as oocytes are retrieved. It is hypothesized that a 'prematuration' culture (PMC) gives the oocyte the time to undergo cytoplasmic changes (e.g. storage of mRNA and proteins, morphological changes, ultrastructural remodeling) and might enhance synchronization of the

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starting population of immature oocytes (Anderiesz et al., 2000; Dieleman et al., 2002; Nogueira et al., 2003).

The arrest of meiosis can be induced in vitro by manipulating the intra-oocyte concentrations of the second messenger cAMP. It is widely accepted that the cAMP levels within the oocyte plays a crucial role in the regulation of oocyte maturation (Dekel and Beers, 1978; Downs, 1995; Conti et al., 2002). Inhibition of the oocyte-specific phosphodiesterase type 3 (PDE3) keeps intra-oocyte cAMP levels elevated and, as a consequence, maintains the oocyte arrested at the GV stage without interfering with the functionality of surrounding somatic cells (Tsafriri et al., 1996; Thomas et al., 2002).

The effect of induced nuclear arrest using specific PDE3-inhibitors (PDE3-Is) has been studied in several species. It was demonstrated that PDE3-I promotes developmental competence after reversal from in vitro-induced meiotic arrest in rodent (Nogueira et al., 2003) and bovine (Thomas et al., 2004) oocytes. Furthermore, applying a PDE3-I proved to be beneficial for human IVM by enhancing nuclear maturation rates, without compromising embryonic developmental quality and metaphase-II spindle organization (Nogueira et al., 2006; Vanhoutte et al., 2007).

These observations suggest the potential of specific PDE3-Is to improve synchronization between nuclear and cytoplasmic maturation. Nevertheless, the results are still suboptimal compared to oocytes that have been matured in vivo. Further, detailed insights on the intracellular events during maturation of the oocyte upon release from PDE3-I could clarify the origin for this difference in developmental competence and might be relevant to design new and efficient IVM culture strategies.

The aim of the present study was, therefore, to investigate several morphological and functional aspects of mouse oocytes after temporary exposure to PDE3-I. In order to mimic the human clinical situation, cumulus–oocyte complexes (COCs) were collected at an earlier stage of their development, thus after a short period of ovarian stimulation. This was achieved by shortening the standard duration of eCG stimulation from 48 to 24 hr (see 'Material and Methods' section).

In a first assay, we identified the lowest effective working concentration of the PDE3-I Cilostamide. This concentration was applied in other experiments to assess the effect of a PMC period on the kinetics of meiotic progression, MII spindle morphology and potential risks of induced aneuploidy. Finally, fertilization and embryonic developmental capacity was evaluated as an ultimate assessment for oocyte cytoplasmic quality.

MATERIALS AND METHODS

Chemicals and Drugs

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

The specific PDE3-I Cilostamide (Calbiochem, Bierges, Belgium; Stock 10 mM in dimethyl sulfoxide (DMSO) stored at -20°C) was used at concentrations of 0.001, 0.01, 0.1, 1, and 10 μM. In preliminary experiments, the highest concentration of the DMSO carrier (0.1% v/v) was tested during an 18 hr period of IVM and no effect on maturation, fertilization, and embryonic development was observed (data not shown).

Animals and Ethics

Mice used in this study were 7–8-week-old F1 hybrids (B6D2; Iffa Credo, Brussels, Belgium), housed and bred according to national legislation for animal care. All experimental procedures were done after obtaining written consent from the Animal Ethical Committee of the Ghent University Hospital (Project No. ECD 06/05).

Collection of Cumulus–Oocyte Complexes

For IVM, mice were primed with intraperitoneal injection of 5 IU/ml eCG (Folligon; Intervet, Mechelen, Belgium). The animals were sacrificed 24 hr later by cervical dislocation in order to obtain COCs from small antral follicles. 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, 100 U/ml penicillin. To prevent spontaneous resumption of meiosis, Cilostamide was added to this medium at the same final concentration as used in the subsequent IVM medium (see Experimental Design section). COCs were freed mechanically by puncturing antral follicles with fine insulin needles ($26^{1/2}$-G; Becton Dickinson, Erembodegem, Belgium). Only COCs 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 hCG (Chorulon; Intervet) 48 hr later. Depending on the Experimental Design, COCs were recovered from pre-ovulatory follicles (3–10 hr post-hCG) or from the ampullae (12–18 hr post-hCG).

In Vitro Maturation of Oocytes

The basic culture medium for IVM consisted of α-minimal essential medium with glutamax (α-MEM; Invitrogen) supplemented with 5% FCS, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium and 10 mIU/ml recombinant FSH (recFSH; Puregon, kindly donated by Organon, Oss, The Netherlands).

Nuclear arrest was obtained by adding Cilostamide to the IVM culture medium at final concentrations as specified in the Experimental Design section. The COCs were maintained arrested for 24 hr. Cilostamide withdrawal was carried out by washing the COCs three times in inhibitor-free IVM medium.

Stimulation of nuclear maturation was performed by placing the oocytes in IVM medium supplemented with 1.5 IU/ml recombinant human chorionic gonadotropins (rechCG; Pregnyl, Organon) and 5 ng/ml epidermal growth factor (EGF).

In all cultures, COCs (groups of 15–20) were placed in microdroplets of 30 μl covered with oil and incubated under standard conditions (37°C in a humidified atmosphere of 5% CO_2 in air).

Experimental Design

A schematic diagram of the study is shown in Figure 1.

Experiment 1 was designed to determine the lowest effective dose of Cilostamide that efficiently arrest oocytes at the GV-stage with full reversibility of the inhibitory effect. Six different concentrations were tested: 0 (control), 0.001, 0.01, 0.1, 1 and 10 μM. After 24 hr of inhibitor-culture, half of the oocytes were denuded of cumulus cells by gentle pipetting to facilitate visualization of the nuclear stage. Maturation stages in these oocytes were classified by light microscopy as GV, GV breakdown (GVBD) or polar body extrusion (PB). The remaining COCs were washed out of Cilostamide and cultured in IVM medium supplemented with hCG/EGF for 16–18 hr. Maturation stages were analyzed at the end of IVM to evaluate the reversibility of the inhibitor. The most effective concentration of Cilostamide was used in Experiments 2–5.

Experiment 2 was designed to assess if a PMC alters the kinetics of nuclear progression upon release of meiotic arrest. In the 'Cilostamide' group, COCs were removed from 24 hr PDE3 inhibition and placed in IVM culture. Thereafter, groups of COCs were denuded and evaluated at different time-intervals following IVM: at 3, 4, 6, 8, 10, 12, and 16 hr of culture. In the 'in vitro control' group, COCs were denuded and evaluated at the same IVM time-intervals. For the 'in vivo control', COCs were collected from pre-ovulatory follicles or from the ampulla at these successive time intervals post-hCG priming.

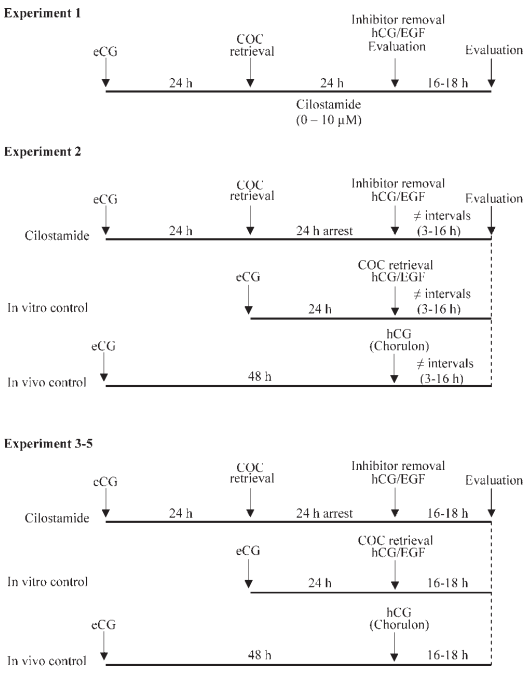


Fig. 1. Schematic representation of the experimental design.

The meiotic stages of individual oocytes were classified on the basis of chromosome configuration and microtubule organization after immunofluorescence staining and the kinetics of meiotic progression were compared between the three groups.

In Experiments 3–5, morphological and functional characteristics of Cilostamide-exposed oocytes were examined. Depending on the experiment, PB oocytes were processed for: (i) immunocytochemistry staining to analyze MII spindle morphology and chromosome alignment (Exp 3), (ii) spreading for chromosome counting (Exp 4), and (iii) fertilization in vitro (Exp 5). The results were compared to nonarrested, spontaneously matured oocytes (in vitro control) and in vivo matured oocytes (in vivo control).

Immunocytochemistry and Confocal Analysis (Experiment 2 and 3)

PB oocytes were simultaneously fixed and extracted in a microtubule-stabilizing buffer, as described elsewhere (Mattson and Albertini, 1990). To visualize microtubules, oocytes were incubated in the presence of a 1:1 mixture of mouse monoclonal anti-α,β-tubulin (1:200) overnight at 4°C, followed by Alexa Fluor conjugated goat-anti-mouse immunoglobulin (Molecular Probes, Eugene, Oregon; dilution 1:200) for 2 hr at 37°C. Chromatin was stained with Ethidium Homodimer-2 (Molecular Probes; dilution 1:500) for 1 hr at 37°C.

Labeled oocytes were mounted on microscope slides in 90% glycerol-PBS solution containing 0.2% 1,4-Diazabicyclo[2.2.2]octane (DABCO) as an antifading reagent. Preparations were observed using a laser scanning confocal microscope (LSCM; Biorad Radiance 2000 mounted on a Nikon inverted microscope; Tokyo, Japan) equipped with a Argon-ion/HeliumNeon (488/543) laser and selective filter sets for Alexa Fluor 488 and Ethidium homodimer-2. Images were obtained using a 100× plan oil immersion objective (numerical aperture 1.3). A three-dimensional image of the microtubular structure and chromosomes was rendered from the collected data by using ImageJ software. This allowed the analysis of spindle size, spindle shape, and chromosome alignment.

Spreading of Oocytes for Chromosome Counts (Experiment 4)

PB extruded oocytes were fixed according to the method described by Tarkowski (1966) with a few modifications. Oocytes were treated with 1.2% hypotonic sodium citrate at room temperature for 2–5 min. The oocytes were then briefly fixed in ice-cold fixative (acetic acid: methanol (1:3)), dropped onto a clean, ice-cold slide, and air dried. The whole process was monitored under a stereomicroscope. Chromosome

preparations were stained with Giemsa and numbers of chromosomes were counted at 1000× final magnification. Only preparations in which chromosomal numbers could be counted unambiguously were included for analysis. Preparations with <17 chromosomes were considered to be technical artifacts.

In Vitro Fertilization and Embryonic Development (Experiment 5)

At the end of the IVM culture period, COCs were fertilized in vitro. The caudae epididymidis were removed from adult males. Epididymal contents were carefully squeezed out and the residual caudal tissue was discarded. Sperm suspensions were preincubated for 2.5 hr to ensure capacitation in KSOM supplemented with 3% BSA (fraction V). COCs were inseminated into 40 μl droplets and sperm was added at a final dilution of 2×10^6/ml and incubated in a humidified atmosphere of 37°C, 5% CO_2 in air for 3 hr. 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% CO_2, 5% O_2, and 90% N_2. The percentages of developing embryos to the 2-cell and blastocyst stages were determined.

Statistical Analyses

All experiments 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 the differences in spindle morphology, chromosome alignment, aneuploidy rate, and kinetics of nuclear progression, data were analyzed with Chi-square test. For evaluation of maturation rates, spindle measurements, fertilization, and embryonic development, data were analyzed with one-way ANOVA and Tukey-post test. Percentages were first subjected to arcsine transformation. Values with $P < 0.05$ were considered as statistically significant.

RESULTS

Experiment 1: Dose-Defining Experiment

The dose-dependent effect of Cilostamide on nuclear arrest is summarized in Figure 2A. In the control group (0 μM Cilostamide) more than 80% of the oocytes matured spontaneously after 24 hr of culture. This was not significantly different from 0.001 and 0.01 μM Cilostamide. It was only at concentrations of 0.1 μM and above that the oocytes were efficiently arrested at the GV stage (>90%).

The reversibility of nuclear arrest following withdrawal of inhibitor and IVM for 16–18 hr is illustrated in Figure 2B. PB and GVBD rates were not significantly different among the groups. The percentage of GV oocytes, however, was significantly higher at 0.1 μM Cilostamide compared to 0 and 1 μM Cilostamide ($P < 0.05$).

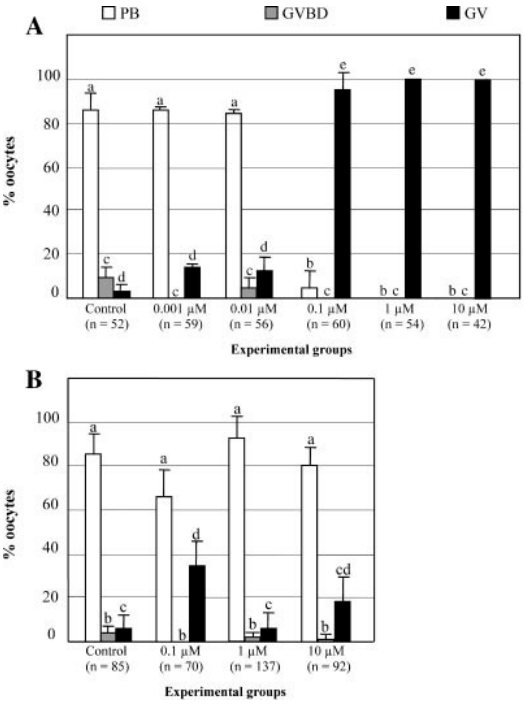


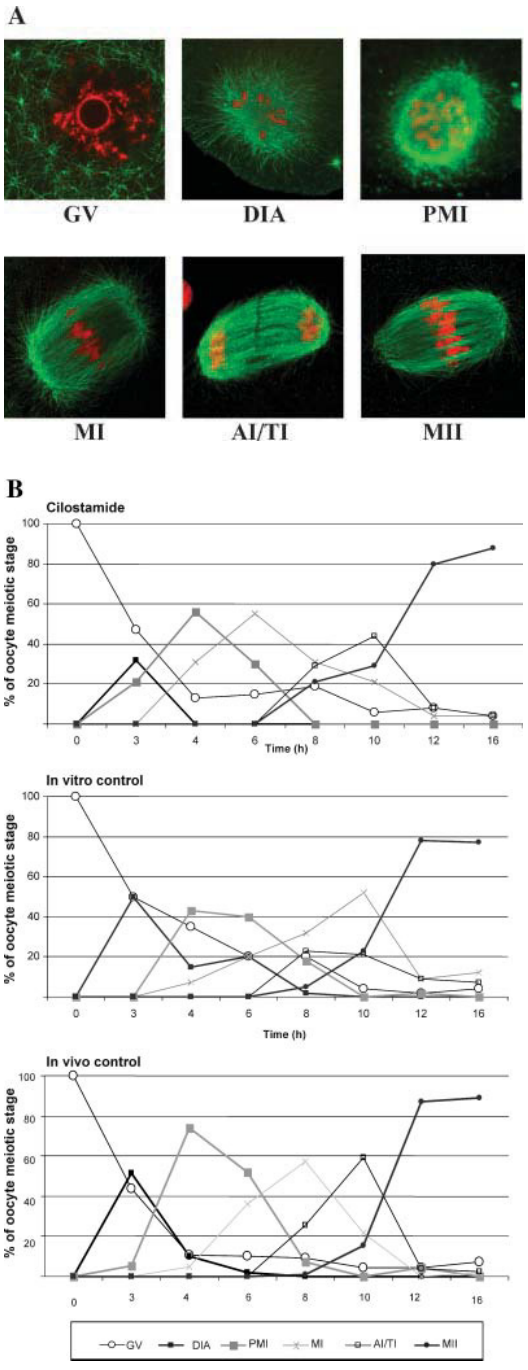
Fig. 2. Effect of different concentrations of Cilostamide (0–10 μM) on nuclear arrest (**A**) and reversibility of meiotic arrest (**B**). Values are expressed as mean % ± SD (error bars). The total number of oocytes analyzed (n) is indicated under each group. Different letters within the same maturation classes represent significant differences ($P < 0.05$). PB, polar body extrusion; GVBD, germinal vesicle breakdown; GV, germinal vesicle.

As a consequence, 1 μM Cilostamide was chosen in the other experiments as the lowest optimal working concentration for efficient inhibition and full reversibility.

Experiment 2: Effect of Cilostamide on Kinetics of Meiotic Progression

Percentages of oocytes at the GV, diakinesis (DIA), prometaphase I (PMI), metaphase I (MI), anaphase I/ telophase I (AI/TI) or MII stage were recorded (Fig. 3A). Different patterns in both the rate and timing of meiotic progression were determined in the temporarily arrested, nonarrested, and in vivo matured oocytes (Fig. 3B).

At 3 hr of IVM, oocytes from the three groups had undergone GVBD in similar proportions. However, the progression of meiosis seemed to be accelerated in the Cilostamide group: 21% of the oocytes had already progressed to PMI, while only 5% in the in vitro control and 0% in the in vivo control did so ($P < 0.05$). One hour later, at 4 hr of IVM, a number of oocytes in the Cilostamide group progressed to MI (31%), which was significantly higher compared to the in vitro (7%) and in



vivo control group (5%; $P < 0.05$). At 6 hr of IVM, the in vitro oocytes had the lowest rate of MI (20%), while these rates were not significantly different between the Cilostamide and the in vivo control group (55% MI and 36% MI, respectively). The first PB extruded oocytes were detected at 8 hr in the three groups and the percentage of MII-stages was higher in Cilostamide-treated oocytes compared to nonarrested and in vivo matured oocytes (21% vs. 5% and 1%, $P < 0.05$). At 10 hr of IVM, 44% of the oocytes from the Cilostamide group were in the process of chromosome segregation, at AI/TI, and 29% had reached the MII stage. This pattern of meiotic progression was similar to the in vivo control. In contrast, the highest proportion of oocytes in the in vitro control group was still at MI by that time (52%). However, the proportion of oocytes that completed meiotic maturation at 12 and at 16 hr was the same in Cilostamide-treated, nonarrested and in vivo matured oocytes (around 80%).

Of notice was the high degree of synchrony during meiotic progression in the in vivo control group. This synchrony was also detectable in the Cilostamide-exposed oocytes: maximal incidence of DIA, PMI, MI, and AI/TI were seen at 3, 4, 6, and 10 hr of IVM, respectively. In contrast, the broader distribution of maturation stages in spontaneously matured oocytes indicated a less synchronous pattern of meiotic progression to MII.

Experiment 3: Effect of Cilostamide on Spindle Morphology and Chromosome Alignment

Spindle morphology in arrested, nonarrested, and in vivo matured MII oocytes was analyzed in a qualitative and a quantitative way (Table 1 and Fig. 4).

For the qualitative analysis (Table 1A), the shape of the spindle was classified as "normal" when it was barrel shaped with slightly pointed poles (Fig. 4A,B) or "abnormal" if it was asymmetrical, elongated, round or disorganized (Fig. 4C). The alignment of the chromosomes on the metaphase plate was categorized as "aligned" when all chromosomes were arranged on a compact metaphase plate at the equator of the spindle (Fig. 4A′), "not aligned" when one or two chromosome were displaced from the metaphase plate (Fig. 4B′) and "chaotic" when several chromosomes were dispersed (Fig. 4C′).

In the Cilostamide group, 89% of the oocytes had a normal spindle. This percentage was similar to the in vivo controls, but significantly higher than the in vitro control group (Table 1A; $P < 0.05$). The percentage of

Fig. 3. Kinetics of meiotic progression in Cilostamide-exposed, in vitro and in vivo matured oocytes. (**A**) Representative confocal images of different meiotic stages. Alfa/beta-tubulin is stained in green, DNA is stained in red. Original magnification = 400×. (**B**) Percentages of oocytes at different meiotic stages in relation to maturation time. The markers on the lines represent the mean ± SD of results obtained from 30 to 120 oocytes/time interval (3 replicates). For significance difference between groups: see text. GV, germinal vesicle; DIA, diakinesis; PMI, prometaphase I; MI, metaphase I; AI/TI, anaphase I/telophase I; MII, metaphase II.

TABLE 1. (A) Effect of Cilostamide on MII Spindle Morphology and Chromosome Organization: Qualitative Analysis; (B) Effect of Cilostamide on MII Spindle Dimensions: Quantitative Analysis

A*

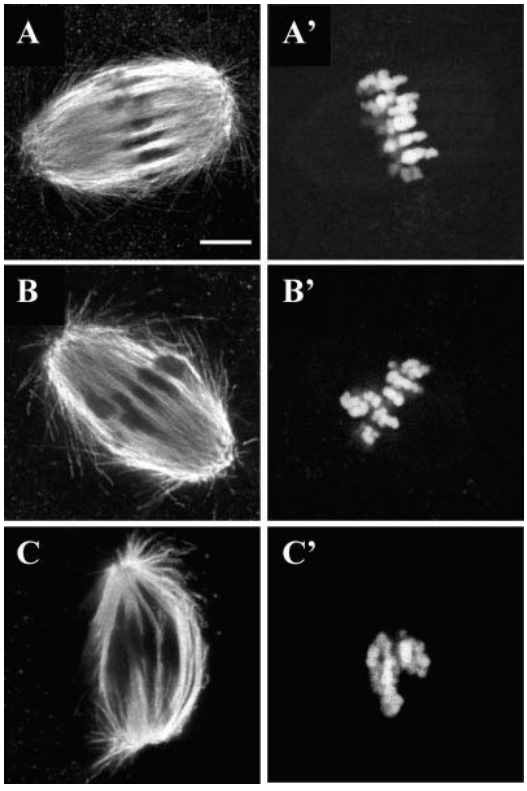
Conditions	No. of oocytes	No. (%) of oocytes with normal spindles	No. (%) of oocytes with chromosomes		
			Aligned	Not aligned	Chaotic
Cilostamide	56	50 (89)[a]	26 (46)[a]	27 (48)[a]	3 (5)[a]
In vitro control	31	21 (68)[b]	15 (48)[a]	14 (45)[a]	2 (6)[a]
In vivo control	50	48 (96)[a]	48 (96)[b]	2 (4)[b]	0[a]

B**

Conditions	No. of spindles measured	Spindle length (μm)	Spindle width at equator (μm)	Average pole width (μm)
Cilostamide	28	23.1 ± 3.0^{a}	13.6 ± 2.8^{a}	6.4 ± 1.5^{a}
In vitro control	17	$21.0 \pm 2.4^{a,b}$	11.1 ± 2.0^{b}	5.6 ± 1.0^{a}
In vivo control	26	20.3 ± 2.5^{b}	11.1 ± 2.5^{b}	5.5 ± 1.7^{a}

*Different superscripts within the same column are significantly different ($P < 0.05$).
**Results are presented as mean ± SD. Different superscripts within the same column are significantly different ($P < 0.05$).

oocytes with normal equatorial "aligned" chromosomes was not different between the Cilostamide group and the in vitro control, but significantly lower than the in vivo control group ($P < 0.05$).



For the quantitative analysis (Table 1B), spindle length, spindle width at the equator and average pole width were measured. Only images with a clear orientation of the spindle were measured. The length and width of the spindles in oocytes cultured in the presence of the Cilostamide group was significantly increased when compared to those of the in vivo control group ($P < 0.05$). The width of spindles in oocytes of the Cilostamide group was also increased when compared to those of oocytes in in vitro controls. There was no difference in the average measurement of spindle poles width between the three groups.

Experiment 4: Effect of Cilostamide on Oocyte Aneuploidy Rate

Chromosome preparations of PB oocytes were analyzed to investigate possible errors that could have occurred during chromosome segregation at MI. Oocytes with 20 chromosomes were classified as haploid, 17–19 chromosomes as hypohaploid, 21–35 chromosomes as hyperhaploid and 36–40 chromosomes as diploid (Carrell et al., 2005). The number of oocytes with aneuploidies in the Cilostamide group was compared to the in vitro and in vivo control group (Table 2).

The overall level of numerical chromosome aberrations was low. Levels of aneuploidy were not significantly different among the treatment groups. Almost all oocytes (>90%) had a normal haploid set of chromosomes (Table 2). Other structural aberrations, such as the presence of single chromatids, were not observed.

Fig. 4. Representative confocal images of mouse MII spindles from Cilostamide-exposed oocytes. Correlative total α/β-tubulin (**A**–**C**) and Ethidium Homodimer-2 (A′–C′) staining patterns. (A, B) Normal barrel-shaped MII spindle with slightly pointed poles, (C) Complete disorganization of the spindle, (A′) Aligned chromosome configuration, (B′) Chromosomes not aligned, (C′) Chaotic chromosome configuration; Scale bar = 5 μm.

TABLE 2. Cytogenetic Analysis of Arrested (Cilostamide), NonArrested (In Vitro Control) and In Vivo Matured (In Vivo Control) Oocytes

		Ploidy (n (%))			
Conditions	No. of oocytes*	Hypohaploid	Haploid	Hyperhaploid	Diploid
Cilostamide	53/72	2 (3.8)	49 (92.4)	1 (1.9)	1 (1.9)
In vitro control	48/65	2 (4.2)	43 (89.6)	1 (2.1)	2 (4.2)
In vivo control	45/69	2 (4.4)	43 (95.6)	0	0

*Number of oocytes successfully analyzed/number processed for spreading. No significant difference was observed among the groups.

Experiment 5: Effect of Cilostamide on Fertilization and Embryonic Developmental Rates

The percentage of 2-cell embryos on Day 1 and blastocysts on Day 4 after insemination was significantly higher in the Cilostamide group compared to the in vitro controls, but lower compared to the in vivo control group ($P < 0.05$; Table 3). The proportion of 2-cell embryos reaching the blastocyst stage was, however, not different between the Cilostamide group and the in vitro control group, but lower than in the in vivo control group (Table 3).

DISCUSSION

The fact that oocyte quality is reduced after IVM remains an intriguing question. Recent results offer a good perspective for the application of isoenzyme-specific or "second-generation" PDE inhibitors to extend the developmental period of the immature oocyte before final IVM (Nogueira et al., 2003, 2006; Thomas et al., 2004). Nevertheless, given the apparent differences with in vivo matured oocytes further studies are mandatory to clarify precisely the effect of PDE3-I on the maturation process itself, which is currently largely unknown. Data on microtubule organization, chromosome alignment, and kinetics of meiotic progression are relevant to study this aspect. In the present study, we were able to collect qualitative and quantitative data on this item, which resulted in some important insights and suggestions for future studies.

Concentration of Cilostamide

The capacity of the specific PDE3-I Cilostamide to induce reversal meiotic arrest in the mouse oocyte has been demonstrated earlier (Tsafriri et al., 1996; Wiersma et al., 1998). Our dose-dependent experiment showed that concentrations of 1 μM Cilostamide and above were efficient for reversal of meiotic arrest, indicating that reconstitution of PDE3A activity had occurred upon inhibitor removal. Surprisingly, at a concentration of 0.1 μM Cilostamide, a significant proportion of oocytes were still blocked at the GV stage after IVM, while higher concentrations (1 and 10 μM Cilostamide) resulted in higher PB rates after IVM. This is an unexpected but very important observation. It demonstrates that the concentration of PDE3-I is a critical factor in the setup of the IVM technique. It is difficult to explain this phenomenon, since the exact regulatory mechanism of PDE3A activation is still unclear. There is enough evidence that this enzyme is involved in the regulation of meiosis (Masciarelli et al., 2004). A drop in the intra-oocyte cAMP concentration is an important trigger to override meiotic arrest (Dekel and Beers 1978; Downs, 1995; Conti et al., 2002; Horner et al., 2003). Cyclic AMP can be produced endogenously by the oocyte (Schultz et al., 1983; Horner et al., 2003) or can diffuse from granulosa cells to the oocyte via gap junctions connecting both cellular compartments (Eppig, 1991; Webb et al., 2002). The magnitude of cAMP levels in the different cell compartments of the COC may result in differential signals. It might be possible that Cilostamide at a concentration of 0.1 μM is able to raise inhibitory cAMP levels above a certain threshold, but has an influence on the activation of other crucial factors, downstream of or separate from the cAMP transduction pathway. A clear demonstration of this relationship requires further systematic studies in which PDE3A and/or cAMP levels are measured in COCs during and after exposure to Cilostamide.

Kinetics of Meiotic Progression

One method to explore possible underlying cell cycle aberrations during IVM is to observe the kinetics of

TABLE 3. Effect of Prematuration Culture in the Presence of Cilostamide on Fertilization and Embryonic Developmental Rates

Conditions	No. of oocytes	% 2-cell/total	% Blasto/total	% Blasto/2-cell
Cilostamide	184	52.3 ± 1.3^{a}	29.8 ± 2.6^{a}	57.3 ± 4.9^{a}
In vitro control	148	20.6 ± 10.3^{b}	11.2 ± 7.9^{b}	50.8 ± 16.6^{a}
In vivo control	177	81.8 ± 4.6^{c}	69.5 ± 8.3^{c}	85.0 ± 9.1^{b}

Results are presented as mean % ± SD.
Different superscripts within the same column are significantly different ($P < 0.05$).

meiotic progression upon removal from the inducer of nuclear arrest. The kinetics of meiosis is a crucial parameter for further developmental competence of the oocyte and has been sparsely documented in previous studies on PDE3-Is.

We found a striking difference in the meiotic progression of Cilostamide-exposed, spontaneously matured and in vivo matured oocytes. Of interest was the higher synchrony in meiotic progression after Cilostamide exposure compared to spontaneously matured oocytes. This might be attributed to a better synchronization of the starting population of GV oocytes after the 24 hr period of PMC. A synchronous pattern of meiotic maturation was found to be a specific characteristic of oocytes that have been matured in vivo compared to in vitro matured oocytes (Sanfins et al., 2004). Our observations indicate that applying a PMC period mirrors the physiological situation of oocyte maturation by creating a more balanced coordination of nuclear and cytoplasmic maturation.

We also observed that the timing of meiotic progression in previously arrested oocytes was initially more rapid compared to the in vitro and in vivo control group. Anderiesz et al. (2000) found a similar accelerated pattern of meiosis in 6-dimethylaminopurine (6-DMAP)-treated mice oocytes. The authors suggested that during the period of PMC there might be extra time for synthesis, translation, and storage of proteins that are necessary to complete meiosis. Although protein synthesis is not initially required to re-enter meiosis in mouse oocytes, it is possible that immediate availability and participation of signaling proteins and/or other key components may account for the accelerated meiotic progression. Alternatively, rapid meiotic progression of mouse IVM oocytes was observed by Sanfins et al. (2004). This phenomena was explained by the authors as a precocious liberation of factors from the oocyte cortex (centrosomes, cytoplasmic microtubules, MPF), leading to uncontrolled G2 to M cell cycle transition. With this in mind, our observation offers some inspiration for future adaptations of the culture technique: adjustment of the PMC to gradually overcome the inhibitory effect of the arrester, rather than removing it abruptly by washing, may recreate a more balanced cell cycle progression during IVM.

Microtubular Organization and Chromosome Alignment

Another potential biomarker of oocyte cytoplasmic quality is the structural normality of the MII spindle and the integrity of their chromosomes constituent. The presence of a well-developed spindle is essential for a correct and balanced distribution of the chromosomes and other molecular factors to the oocyte and the PB (Eichenlaub-Ritter, 2002; Albertini, 2003). Several studies confirmed that IVM conditions can have deleterious effects on the phenotypic properties of the spindle and the organization of the chromosomes. In mice, in vivo ovulated and IVM oocytes show profound differences in spindle shape, size, density, stability and position (Sanfins et al., 2003; Moon et al., 2005; Roberts et al., 2005; Rossi et al., 2006). These differences have been related to the hormonal conditions to which the oocytes are exposed (Sanfins et al., 2003; Roberts et al., 2005; Rossi et al., 2006), unbalanced coordination of nuclear and cytoplasmic maturation (Sanfins et al., 2003), and defects in oocyte-somatic cell communication during meiotic progression (Hodges et al., 2002). As a consequence, microtubular organization and chromosome alignment has been recognized as an important checkpoint for assessing the microenvironment surrounding the oocyte.

It was interesting to observe how the use of a PDE3-I can change the cytoskeleton organization of the oocyte to more closely resemble those of in vivo matured oocytes. Applying a PMC period significantly improved the number of oocytes with normal MII spindle morphology. The vast majority of PB oocytes in the Cilostamide group possessed a normal barrel-shaped spindle with slightly pointed poles as seen in vivo. The improved spindle morphology could be a result of higher nucleation capacity of the centrosomes, tubulin assembly competence or microtubule stability. Whether this is due to cytoplasmic changes induced by a PMC or an indirect result of the increase in cAMP accumulation in the immature oocyte needs to be further researched. Evidence for the latter might be drawn from the study of Duncan et al. (2006). This group demonstrated that cAMP-dependent protein kinase (PKA) appears to be important for appropriate spindle formation. Since an increase in cAMP stimulates PKA activation (Corbin et al., 1978), it is possible that elevated cAMP levels due to PDE3-I exposure are responsible for the improved spindle morphology observed in our study.

Chromosome alignment was, however, not a parameter that improved by applying a PMC period, although gross disturbances in chromosome positioning were not detected. Since the spindle appears normal in most of the arrested oocytes, it seems likely that the defect is not one of spindle formation itself, but rather of chromosome movement and attachment to the spindle, which require kinetochore-fibers (Brunet and Maro, 2005).

The quantitative analysis of the MII spindles in our study revealed that spindle dimensions differ according to the maturation conditions. Spindles of in vivo matured oocytes were clearly shorter and smaller compared to temporarily arrested oocytes. In contrast, spindle length and width was equivalent in the two control groups. Spindle poles tented to be smaller in the control groups compared to the Cilostamide group, although the difference was not significant. Differences in spindle dimensions between in vivo and in vitro matured mouse oocytes have been described by others (Sanfins et al., 2003). It has been proposed that the large, barrel-shaped spindles formed after IVM results from the incorporation of multiple microtubule organizing centers (MTOCs) during spindle assembly, which reduces the stores of available cytoplasmic gamma-tubulin necessary for normal embryonic development

(Combelles and Albertini, 2001; Sanfins et al., 2003, 2004; Barrett and Albertini, 2007).

From these results we may conclude that, although a PMC period improved spindle morphology, the presence of enlarged spindles and disaligned chromosomes reflects a not yet fully optimized spindle assembly mechanism.

Aneuploidy

Before any clinical application of PDE3-Is, further thorough investigations to test the safety of this compound are surely needed. Moreover, it is important to verify if the potential benefits of extending the incubation time is not lost through an increased appearance of chromosomal errors in the meiotic process.

It was previously demonstrated that the majority of aneuploidies arise during meiosis I, probably due to the lack of a checkpoint control mechanism at the MI/AI transition in mammalian oocytes (LeMaire-Adkins et al., 1997; Hassold and Hunt, 2001; Plachot, 2003). For this reasons, we investigated the possible risk of numerical chromosome aberrations during meiosis I by investigating chromosome preparations of PB extruded oocytes.

Temporary inhibition of meiosis by Cilostamide did not increase aneuploidy rates after IVM. The majority of the oocytes were haploid with 20 MII chromosomes. Other structural aberrations, such as the presence of single chromatids, were not observed. This finding demonstrates that the accelerated progression through meiosis I in Cilostamide-exposed oocytes, as observed in our kinetic experiment, did not result in a reduction of the critical period in which chromosome attachment and alignment on the MI spindle equator takes place (Eichenlaub-Ritter and Boll, 1989). Hence, the use of chromosome-specific probes to analyze the chromosome structure more accurately may reveal other abnormalities.

Fertilization and Embryonic Development

Although the beneficial effect of PDE3-I on fertilization rate of mouse in vitro matured oocytes has been described previously (Nogueira et al., 2003), it is important to confirm if the same results can be obtained using another type of molecule (i.e., Cilostamide instead of Org9935). Our results demonstrated that not only the fertilization, but also the blasotcyst rate was increased in arrested oocytes. This might indicate that the use of Cilostamide is preferred above Org9935. It also demonstrated that 1 μM Cilostamide is sufficient to improve oocyte development without causing adverse affects.

All together, this study demonstrated that culturing mouse oocytes for an additional 24 hr in the presence of PDE3-I alters the cytoplasmic quality into a direction more closely related to the in vivo situation. Further improvement of IVM culture techniques might require a combination of a PMC in the presence of an appropriate in vitro environment. We presume that the latter aspect needs to be further optimized in our experiments. It was noticed that cumulus cells started to loose the contact with the oocyte during the PMC period, a process that is associated with loss of gap junctional communication between the two cell types. It is widely recognized that bi-directional communication between the oocyte and follicular somatic cells is fundamentally important for oocyte growth and maturation (Eppig, 2001; Combelles et al., 2004). Modifications of the current culture conditions, aiming for a better oocyte-cumulus contact during PMC and IVM, may result in a better outcome. This can be done by replacing recFSH in the culture medium by forskolin since it was demonstrated that FSH induces a retraction of transzonal projections (Combelles et al., 2004; Thomas et al., 2004). Also the culture of COCs in a synthetic extracellulair matrix might be a possible strategy to improve oocyte-cell connections (Combelles et al., 2005; Torre et al., 2006). Alternatively, co-culturing COCs with oocyte-secreted factors (OSFs) might be another method to enhance oocyte developmental competence, as was recently demonstrated by Hussein et al. (2006) on bovine oocytes.

CONCLUSIONS

This is the first attempt to characterize in detail the phenotype of oocytes after release from PDE3-I. The insights on cytoskeleton organization and meiotic progressing elucidated some unresolved issues of PDE3-I exposed oocytes, which are helpful for future improvement of the IVM culture technique. Although extrapolations from findings obtained in the mouse to the human should be made with caution, these results could have far-reaching implications for improving the efficiency of IVM in human infertility treatment.

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Nuclear and cytoplasmic maturation of *in vitro* matured human oocytes after temporary nuclear arrest by phosphodiesterase 3-inhibitor

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BACKGROUND: The use of hormones for controlled ovarian stimulation results in follicular heterogeneity, with oocytes at diverse stages of nuclear and cytoplasmic development. This study evaluated the impact of temporary nuclear arrest by a specific phosphodiesterase 3-inhibitor (PDE3-I), cilostamide, on nuclear and cytoplasmic maturation of cumulus-free germinal vesicle (GV) human oocytes from controlled ovarian stimulated cycles. METHODS: GV oocytes ($n = 234$) were cultured in: (i) medium without the inhibitor (control); (ii) medium supplemented with 1 μM cilostamide and (iii) medium supplemented with 10 μM cilostamide. Oocytes in groups (ii) and (iii) were exposed to cilostamide for 24 h. The PDE3-I was subsequently removed by transfer of oocytes to fresh *in vitro* maturation (IVM) medium and the reversibility of GV arrest was assessed during IVM culture for maximum 48 h. RESULTS: Cilostamide (1 and 10 μM) could maintain >80% of the oocytes at the GV stage, without affecting subsequent maturation to metaphase II. Oocytes exposed to 1 μM cilostamide were more likely to have normal bipolar spindles with aligned chromosomes than control oocytes ($P < 0.05$). When GV chromatin configurations before and after arrest were compared, a significantly higher proportion of oocytes had acquired a nucleolus completely surrounded by a rim of highly condensed chromatin ($P < 0.05$). CONCLUSIONS: Temporary nuclear arrest of human GV oocytes with PDE3-I proved to be beneficial for obtaining normal spindle and chromosome configurations after IVM. It resulted also in synchronization within the population of GV oocytes.

Key words: *in vitro* maturation/meiosis/oocyte maturation/phosphodiesterases/spindle

Introduction

The application of *in vitro* maturation (IVM) of human oocytes as an assisted reproductive technology treatment could offer several advantages, including lower costs, shorter stimulation protocols and reduced risks of ovarian stimulation syndrome. Oocytes matured *in vitro*, however, do not have the same developmental potential as *in vivo* matured oocytes (Cha and Chian, 1998; Moor *et al.*, 1998; Trounson *et al.*, 2001; Chian *et al.*, 2004). Currently available evidence indicates that the culture conditions adequately support nuclear maturation, but frequently fail to sustain optimal cytoplasmic maturation (Combelles *et al.*, 2002). The nature of this deficiency is still subject to speculation.

Oocyte developmental competence is gradually acquired during the long-lasting period of oogenesis. The final stage for optimal development, prior to ovulation, requires synchronization between nuclear and cytoplasmic maturation (Eppig *et al.*, 1994). Fully grown oocytes from many species can mature spontaneously following release from the follicle (Pincus and Enzmann, 1935; Edwards, 1965). It is believed that extending meiotic arrest *in vitro* by temporary blockage of spontaneous nuclear maturation, a so-called 'prematuration culture' (PMC), might improve the synchronization between nuclear and cytoplasmic maturational status (Anderiesz *et al.*, 2000; Nogueira *et al.*, 2003a). This allows time for the oocyte to undergo structural and biochemical changes that are essential to sustain normal fertilization and further embryonic development, such as the continued transcription of mRNA, post-translational modifications of proteins, relocations and modifications of organelles (Dieleman *et al.*, 2002).

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Furthermore, the use of hormones for controlled ovarian stimulation results in follicular heterogeneity and, consequently, in oocytes with diverse stages of nuclear and cytoplasmic development (Laufer *et al.*, 1984). Hence, different chromatin configurations within the population of fully grown germinal vesicle (GV) oocytes were reported (Combelles *et al.*, 2002; Miyara *et al.*, 2003). A PMC could contribute to a synchronization of the different types of GV oocytes, which may result in an overall improved outcome after IVM (Nogueira *et al.*, 2003b).

The arrest of meiosis can be induced *in vitro* by manipulating the intra-oocyte cAMP levels (Conti *et al.*, 2002). Inhibition of the oocyte-specific phosphodiesterase 3 (PDE3) keeps intra-oocyte cAMP levels elevated and will maintain the oocyte arrested at the GV stage (Tsafriri *et al.*, 1996). This approach has been applied during culture of oocytes from several species, including human. It was demonstrated that human cumulus-enclosed oocytes retrieved from small antral follicles were efficiently arrested by the specific PDE3-inhibitor (PDE3-I) Org 9935 (Nogueira *et al.*, 2003a, 2006). Ultrastructural analysis of the oocytes showed that this period of PMC was not deleterious to oocyte morphology (Nogueira *et al.*, 2003a). Furthermore, applying a PDE3-I proved to be beneficial for human IVM by enhancing nuclear maturation rates, without compromising embryonic development (Nogueira *et al.*, 2006).

On the basis of these results, the present study was designed to determine if the use of a PDE3-I could improve IVM of cumulus-free GV oocytes retrieved from patients stimulated for ICSI treatment. These oocytes are generally considered to be a side product of the treatment and the clinical use is restricted to rare cases (Veeck *et al.*, 1983; Nagy *et al.*, 1996; Edirisinghe *et al.*, 1997; Chen *et al.*, 2000). Nevertheless, cumulus-free GV oocytes are a valuable source of research material to study different effects of PDE3-I on IVM.

The aims of this study were: (i) to evaluate the dose-dependent effect of cilostamide, a specific PDE3-I, on the kinetics and the degree of nuclear maturation; (ii) to assess cytoplasmic quality of arrested oocytes in terms of spindle morphology and associated chromosome alignment after IVM and (iii) ascertain whether a period of PMC could synchronize the population of GV oocytes by evaluating the chromatin organization around the nucleolus.

Materials and methods

Source of oocytes

Spare GV oocytes ($n = 234$) were obtained from 114 consenting women undergoing controlled ovarian stimulation for ICSI treatment. Complete institutional review board approval was obtained for this study (Project N° EC UZG 2006/168). The stimulation protocol used in our centre has been described in detail elsewhere (Vanhoutte *et al.*, 2005). Oocytes were denuded enzymatically by a brief exposure to 80 IU ml^{-1} hyaluronidase (Type VIII; Sigma Chemical Co., Bornem, Belgium), followed by mechanical denudation ~1 to 3 h after oocyte collection. The nuclear status of denuded oocytes was recorded. Oocytes at metaphase II (MII) were used for the ICSI cycle of the patients. GV-stage oocytes were distributed among different culture conditions, according to the experimental design described later.

In vivo matured MII oocytes ($n = 11$) were donated in a case where no sperm was available for ICSI and served as the *in vivo* control for assessment of spindle morphology and chromosome alignment.

Products and IVM culture

All products were purchased from Sigma Chemical Co. (Bornem, Belgium), unless otherwise indicated.

The specific PDE3-I used was cilostamide (Cat # 231085, Calbiochem, Bierges, Belgium; Stock 10 mM in dimethylsulphoxide stored at −20°C) at final concentrations of 1 and 10 μM.

The IVM culture medium consisted of tissue culture medium 199 (TCM 199; M2154) supplemented with 10 ng/ml epidermal growth factor (E9644), 1 μg/ml estradiol (E4389), 10 mIU/ml recombinant FSH (Puregon, Organon, Oss, The Netherlands), 0.5 mIU/ml HCG (Pregnyl, Organon), 1 mM L-glutamine (G7513), 0.3 mM sodium pyruvate (P4562), 0.8% human serum albumin (Red Cross, Brussels, Belgium), 100 IU/ml penicillin G (P4687) and 100 μg/ml streptomycin sulphate (S1277).

All immature oocytes were cultured singly in 25 μl drops of medium overlaid with embryo-tested light mineral oil in a humidified atmosphere at 5% CO_2 in air at 37°C.

Experimental design

GV-stage oocytes from each patient were randomly distributed between three different culture conditions: (i) IVM medium without inhibitor (*in vitro* control); (ii) IVM medium supplemented with 1 μM cilostamide and (iii) IVM medium supplemented with 10 μM cilostamide. This prevented all oocytes from the same patient being assigned to the same treatment group. Oocytes in groups (ii) and (iii) were exposed to cilostamide for 24 h (=PMC). The PDE3-I was subsequently removed by transferring the oocytes to fresh IVM medium and the reversibility of inhibition was assessed during IVM culture for a maximum of 48 h.

The maturational status of the oocytes in the three groups was examined at 24 h or up to 48 h (from 25 to 48 h) of IVM culture and was classified as GV, GV breakdown (GVBD) or polar body (PB) extrusion. When a PB was identified at any of these time points, the oocyte was fixed for immunostaining and confocal imaging of the spindle and the chromosomes.

For analysis of the GV chromatin configuration, immature oocytes were fixed and stained immediately after the 24 h PMC period. Only 1 μM of cilostamide was tested, because the previous experiments in this study proved that this was the optimal concentration (see Results). The organization of chromatin around the nucleolus in arrested oocytes was compared to non-arrested, control GV oocytes that were fixed at time 0 h of culture.

Oocyte fixation, immunostaining and confocal imaging

Oocytes were simultaneously fixed and extracted in a microtubule-stabilizing buffer, as described elsewhere (Mattson and Albertini, 1990). To visualize microtubules, oocytes were incubated in the presence of a mixture of mouse monoclonal anti-α,β-tubulin (1:200; T9026 and T4026) overnight at 4°C, followed by Alexa Fluor 488 conjugated goat-anti-mouse immunoglobulin G (1:200; A11001, Molecular Probes, Eugene, Oregon) for 2 h at 37°C. Chromatin was stained with ethidium homodimer-2 (1:500; E3599, Molecular Probes) for 1 h at 37°C. Labelled oocytes were mounted on microscope slides in 90% glycerol-phosphate-buffered saline solution containing 0.2% 1,4 diazabicyclo[2.2.2]octane (DABCO; D2522) as an antifading reagent. Preparations were observed using a laser scanning

confocal microscope (LSCM; Biorad Radiance 2000 mounted on a Nikon inverted microscope; Tokyo, Japan) equipped with an argon-ion/helium–neon (488/543) laser and selective filter sets for Alexa Fluor 488 and ethidium homodimer-2. Images were obtained using a 100x plan oil immersion objective (numerical aperture 1.3). *Z*-axis stacks and 3D reconstructions were obtained by using 0.25 to 0.5 μm steps.

Statistical analysis

Differences between treatment groups were analysed with chi-square and, when appropriate, two-tailed Fisher's exact test. When the *P*-value was <0.05, the difference was considered significant.

Results

Effect of cilostamide on nuclear maturation

In Table I, the results of the meiotic progression in arrested and non-arrested oocytes are presented. Of oocytes exposed to 1 and 10 μM cilostamide, 88% and 85% were efficiently arrested at the GV stage, respectively. After removal from PDE3-I, oocytes were capable of resuming meiosis. The kinetic progression of PB extrusion was compared with the control group. Twenty-four hours after withdrawal of inhibitor, PB rates in the groups of previously arrested oocytes were lower compared with non-arrested controls ($P < 0.05$; Table I). By the maximum time of IVM (48 h), however, the PB rates of arrested oocytes were not significantly different from that of non-arrested control oocytes. There was no difference in the proportion and the kinetics of PB extrusion between 1 and 10 μM cilostamide.

Table I. Progression of meiosis in non-arrested and temporary arrested germinal vesicle (GV) oocytes, followed by *in vitro* maturation (IVM) for up to 48 h

	Culture conditions		
	Control	1 μM cilostamide	10 μM cilostamide
No. (%) of GV after inhibition		56/64 (88)	51/60 (85)
No. of GV undergoing IVM	43	56	51
Maturation at 24 h of IVM (%)			
GV	26	21	25
GVBD	23[a]	48[b]	51[b]
PB	51[a]	25[b]	22[b]
Maturation up to 48 h of IVM (between 25 and 48 h) (%)			
GV	12	16	24
GVBD	14	13	4
PB	72	63	65

GVBD, germinal vesicle breakdown; PB, polar body.
[a,b]Values within the same row with different superscripts are statistically different ($P < 0.05$). Due to the exclusion of degenerated oocytes, the different proportions may not add up to 100%.

Effect of cilostamide on spindle morphology and chromosome organization

PB oocytes from controls and PMC groups were processed for LSCM and various stages of microtubule organization and chromatin configuration could be distinguished. This allowed us to reclassify 22 IVM oocytes that appeared not to be at the MII stage of the meiotic cycle, as previously assessed by PB extrusion with normal light microscopy (Table II). A few of these oocytes ($n = 13$) were still at the telophase I stage, visible by the presence of two discrete bundles of condensed chromosomes, joined by an attenuated spindle midbody between the oocyte and the PB (Figure 1A and A′). Some oocytes ($n = 9$), present in the two PDE3-I groups, were spontaneously activated. This was characterized by the appearance of a dense and extensive network of interphase microtubules throughout the oocyte cortex and the formation of a single pronucleus. In contrast, all *in vivo* matured oocytes ($n = 11$), which were fixed immediately after retrieval, were at the MII stage at the moment of fixation. This was evidenced by the presence of a MII spindle and one group of condensed chromosomes arranged on a compact plate at the equator of the spindle.

Oocytes that reached the MII stage were classified according to the structure of the spindle (Figure 1B and C) and the organization of the chromosomes on the metaphase plate (Figure 1B′ and C′; Table II). The percentage of oocytes displaying a normal barrel-shaped spindle and well-aligned chromosomes at the equator was significantly higher in the *in vivo* controls compared with the *in vitro* controls and the 10 μM cilostamide

Table II. Microtubule and chromatin organization of *in vivo* matured oocytes (*in vivo* control) and GV oocytes showing a PB after IVM for up to 48 h (*in vitro* control, 1 and 10 μM cilostamide)

	Culture conditions			
	In vivo control	*In vitro* control	1 μM cilostamide	10 μM cilostamide
No. of PB oocytes analysed	11	27	32	30
No. (%) of oocytes at TI stage		6 (22)	5 (16)	2 (7)
No. (%) of oocytes activated			4 (12)	5 (17)
No. (%) of oocytes at MII stage	11 (100)	21 (78)	23 (72)	23 (77)
Normal spindle shape[a]	11 (100)[b]	12 (57)[c]	18 (78)[b,c]	14 (61)[c]
Aligned chromosomes[d]	10 (91)[b]	10 (48)[c]	16 (70)[b,c]	10 (43)[c]

TI, Telophase I.
[a]Defined as a barrel-shaped spindle with slightly pointed, anastral poles. Percentages are calculated over the total number of metaphase II (MII) oocytes.
[b,c]Values within the same row with different superscripts are statistically different ($P < 0.05$).
[d]Defined as condensed chromosomes arranged on a compact plate at the equator of the spindle. Percentages are calculated over the total number of MII oocytes.

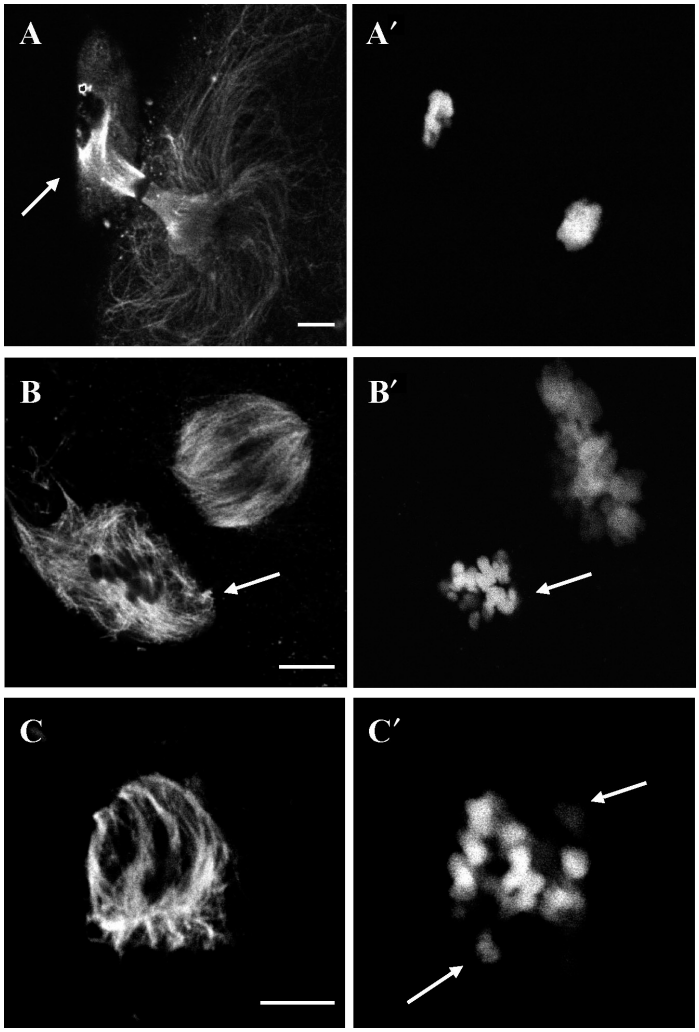

Figure 1. Immunocytochemical staining of different spindle morphologies (**A**–**C**) and correlative chromatin patterns (A′–C′) in human oocytes. **(A and A′)** Telophase I stage: an attenuated spindle midbody is formed between the oocyte and the polar body (PB; arrow in **A**). Two sets of condensed chromosomes are visible; **(B and B′)** Normal metaphase II spindle with aligned chromosomes, orientated perpendicular and in a cortical position in the oocyte. The microtubule and chromatin organization in the PB is visible as well (arrow in B and B′); **(C and C′)** Abnormal spindle with disorganized and partially depolymerized microtubules. In addition, several chromosomes are dislocated from the metaphase plate (arrows in C′). Scale bar = 5 μm.

group ($P < 0.05$; Table II), but similar to the 1 μM cilostamide group.

On the basis of these results, a concentration of 1 μM cilostamide was chosen in the next experiment.

Effect of cilostamide on chromatin configuration within the GV

To evaluate if a PMC for 24 h might exert an influence on nuclear characteristics of the GV oocytes, the pattern of chromatin configurations was evaluated. This was assessed before (control) and after the period of arrest.

Three different patterns could be distinguished: Pattern '1': a nucleolus partially surrounded by chromatin and the presence of fibrillar chromatin distributed throughout the nucleus (Figure 2A); Pattern '2': a nucleolus completely surrounded by chromatin and masses of condensed chromatin or threads of dispersed chromatin occupying a smaller portion of the nuclear area (Figure 2B) and Pattern '3': a nucleolus completely surrounded by a rim of highly condensed chromatin and

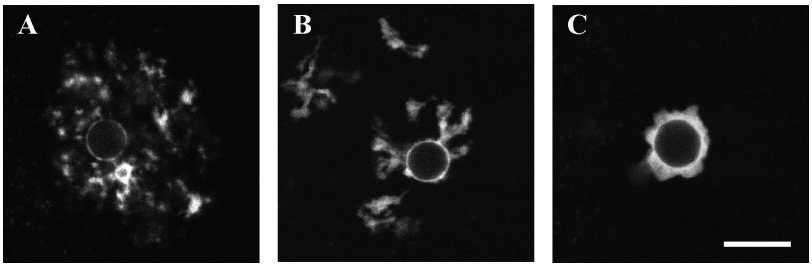

Figure 2. Different chromatin patterns in human germinal vesicle (GV) oocytes after staining with ethidium homodimer-2 and analysis with laser scanning confocal microscopy. (**A**) Pattern '1': partially surrounded nucleolus. Note the presence of fibrillar chromatin distributed throughout the nucleus; (**B**) Pattern '2': identified by a rim of condensed chromatin surrounding the nucleolus and threads of chromatin dispersed in the nucleus; (**C**) Pattern '3': all chromatin surrounds the nucleolus and there is no chromatin staining present throughout the remainder of the nucleoplasm. Scale bar = 10 μm.

no chromatin throughout the remainder of the nucleoplasm (Figure 2C).

The proportions of GV in which the different patterns were observed before and after arrest are represented in Figure 3. In the control group (before meiotic arrest), the three different patterns of chromatin configuration were visualized, but pattern '2' was the most frequent one (53% of the total number of GVs analysed). After 24 h of inhibition, there was a significant increase in the proportion of oocytes that had acquired a pattern '3' configuration, which was significantly different from the control group (81% versus 33%; $P < 0.05$).

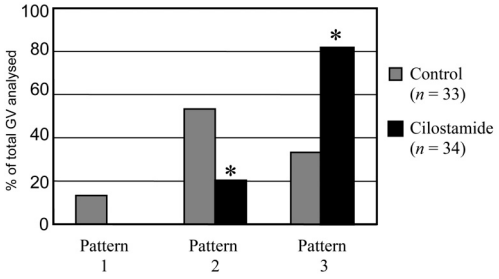

Figure 3. Chromatin configuration of oocyte nuclei before and after arrest by cilostamide (1 μM). Patterns 1, 2 and 3 refer to Figure 2. *Significant different from control ($P < 0.05$).

Discussion

The present study demonstrates that supplementation of the IVM culture medium with the specific PDE3-I cilostamide for 24 h resulted in reversible meiotic arrest of denuded GV oocytes retrieved in ICSI cycles. This temporary induced nuclear arrest was equally effective (>80%) at both concentrations of 1 and 10 μM cilostamide. The high efficiency of specific PDE3-I's to arrest meiosis has been previously demonstrated in human and non-human primate oocytes (Nogueira *et al.*, 2003a, 2006; Jensen *et al.*, 2002).

The period of inhibition proved to be completely reversible. After removal of PDE3-I, GV oocytes were capable of resuming meiosis at the same final rate as non-arrested oocytes. The overall maturation rate obtained in the present study was comparable with those achieved by others working with cumulus-free GV oocytes retrieved from stimulated cycles and matured in TCM-199 (Goud *et al.*, 1998; Cekleniak *et al.*, 2001; Chian and Tan, 2002; Roberts *et al.*, 2002).

Although the final rate of nuclear maturation achieved in the PDE3-I groups was similar to the control group, the time course for PB extrusion after removal of PDE3-I was slowed down. The reason for this event is at the moment unclear. Resumption of meiosis requires PDE3A activity (Tsafriri *et al.*,1996; Richard *et al.*, 2001). It is possible that the delay in meiotic progression observed in the PDE3-I group at 24 h is a result of the time necessary for the reconstitution of PDE3 activity and the breakdown of cAMP prior to GVBD. However, since we did not evaluate the time-lapse between the onset of meiosis and oocytes' PB extrusion, it is still to be evaluated if the lower rate of PB-extruded oocytes at 24 h of IVM is a result of a later onset of GVBD in comparison to the control group or if it is due to a longer gap between GVBD and PB extrusion.

It is still debatable whether this delay in meiotic progression after withdrawal of the inhibitor results in a positive outcome. A study by Son *et al.* (2005) demonstrated that embryos derived from late-maturing oocytes (48 h of IVM and beyond) were of lower quality compared with oocytes maturing between 24 and 30 h of IVM. This suggests that a retarded meiotic progression results in a negative outcome. On the other hand, different studies demonstrated that exposure of oocytes to gonadotrophins (Gomez *et al.*, 1993; Cha and Chian, 1998) and absence of cumulus cells (Goud *et al.*, 1998; Kim *et al.*, 2000) accelerates meiotic progression *in vitro*, which is a non-physiological condition of oocyte maturation. Both of these variables are relevant to the culture conditions applied in the present study. Therefore, it is possible that the initial delay in meiotic progression after removal of inhibitor contributes to a better co-ordination of nuclear and cytoplasmic maturation.

It is difficult to assess the degree of cytoplasmic maturation of oocytes, apart from the ability to fertilize and support embryonic development. A possible marker is the morphology of the meiotic spindle, an essential component of the oocyte. Disruption of microtubule organization might lead to failures in chromosome segregation and organelle allocation during later development (Van Blerkom *et al.*, 1995; Eichenlaub-Ritter *et al.*, 2002). For that reason, spindle morphology and associated chromosome configurations were used in the present study as an index of the cytoplasmic integrity of the oocytes.

LSCM observations revealed that a small proportion of *in vitro* matured PB oocytes were at the telophase I stage at the moment of fixation. Polscope analysis showed that following the extrusion of the first PB, the human oocyte remains at the telophase I stage for ~75–90 min prior to formation of the MII spindle (Montag *et al.*, 2006). This could explain why some of the PB oocytes in our study were still at the telophase I stage. Another portion of oocytes examined showed chromatin and microtubules distinctive of spontaneous activation, which suggests the inability of the *in vitro* matured oocytes to properly maintain M-phase characteristics. This has been previously recognized as one of the deficiencies related to IVM (Combelles *et al.*, 2002). Balakier *et al.* (2004) demonstrated that the highest fertilization rate of human *in vitro* matured oocytes was obtained when ICSI was performed between 3 and 6 h after extrusion of the PB. These observations together led us to conclude that the exact time recording of PB extrusion and fertilization is an essential factor in order to enhance the outcome of IVM.

MII spindle morphology and chromosome organization appeared to be suboptimal in *in vitro* matured control oocytes compared to *in vivo* matured oocytes. This is in agreement with a recent study by Li *et al.* (2006), comparing spindle morphology and chromosome alignment of *in vivo* versus *in vitro* matured oocytes from polycystic ovary syndrome patients. An interesting observation in our study was the tendency to obtain normal spindle/chromosome configurations when oocytes were exposed to 1 μM cilostamide. This could indicate that the period of PMC induced cytoplasmic changes in the oocyte that are necessary for the construction of a normal spindle and the maintenance of a well-aligned MII plate. Microtubule reorganization and stability is influenced by several factors, including protein kinase activity (e.g. mitogen-activated protein kinase), centrosome-based microtubule nucleation and post-translational modifications of tubulin (Albertini, 1992). A PMC could have been attributed to the synthesis, storage and modifications of some of these factors.

The beneficial effect of PDE3-I on spindle/chromosome configuration was not clearly demonstrated when a concentration of 10 μM cilostamide was used. This indicates that the concentration of inhibitor is of importance when applying such IVM systems. The precise reason for this observation is unclear at this stage. Some disorganization of meiotic spindles, abnormalities in microtubule organizing centres and damage to kinetochores and microtubules have been observed in 6-dimethylaminopurine (DMAP)-treated mouse oocytes (Rime *et al.*, 1989; Szöllösi *et al.*, 1991). Whether similar effects were, indirectly, induced by the effect of cilostamide at high concentrations needs further research. However, it is also possible that the findings are influenced by the variability that might exist between individual oocytes. The impact of patient age on spindle morphology has been demonstrated previously (Battaglia *et al.*, 1996; Eichenlaub-Ritter, 1998). Inter-patient variability might be eliminated if a larger number of oocytes per patient could be available.

Another possible marker for oocyte developmental capacity is the pattern of chromatin organization within the GV. The results in this study demonstrate that human denuded GV oocytes, isolated from pre-ovulatory follicles, present heterogeneity in the chromatin configuration. This heterogeneity was previously reported in human oocytes (Parfenov *et al.*, 1989; Combelles *et al.*, 2002; Miyara *et al.*, 2003).

After the 24 h period of meiotic arrest, we could observe an increased proportion of oocytes that had acquired a nuclear configuration in which all of the highly condensed chromatin surrounded the nucleolus (pattern '3'). This finding confirms that a PMC period with a PDE3-I contributes to the synchronization of the population of GV oocytes. Similar effects of the PDE3-I Org9935 were found in mouse oocytes (Nogueira *et al.*, 2003b).

A transition from a decondensed 'non-surrounded nucleolus' to a condensed 'surrounded nucleolus' (SN) chromatin pattern has been related to a transition from an active to an inactive state of nuclear transcription in rodents (Mattson and Albertini, 1990; Zuccotti *et al.*, 1998, De La Fuente, 2006). The presence of a SN configuration has been associated with a higher proportion of oocytes reaching meiotic maturation, better embryonic developmental quality and, hence, represents a more advanced step in oocyte differentiation. In the present study, the increase of the oocyte population in the arrested group (1 μM cilostamide) with a pattern '3' configuration might have contributed to the higher proportion of oocytes with normal spindles and chromosome organization.

At present, it cannot be ruled out that an acceleration of the transition from the active to the inactive state of transcription may have also detrimental effects for some oocytes. Following transcriptional silencing, the oocyte relies on maternal mRNA stores to resume meiosis and sustain the first cleavage divisions after fertilization until activation of the embryonic genome. Experimentally extending the period between transcriptional inactivation in the GV and initiation of meiotic maturation may reduce the competence of the oocytes to complete preimplantation embryonic development (De La Fuente and Eppig, 2001). This suggests that the period of temporary nuclear arrest is crucial and may not be too extensive. Further systematic analysis, e.g. through fluorescent detection of transcriptional activity, could reveal at which time point the transition from an active to an inactive state took place during the PMC period (Fair *et al.*, 1996; Miyara *et al.*, 2003).

An important aspect that deserves attention when analysing the present results is the source of oocytes used in this study. The GV oocytes represent an already compromised group of oocytes retrieved from follicles that failed to conclude maturation *in vivo* in response to the hormonal stimulation. Moreover, the oocytes were denuded of cumulus cells in preparation for ICSI

before culture. Granulosa cells are the production site of steroids, growth factors, proteins and other compounds that contribute to cytoplasmic maturation of oocytes. Beneficial effects of cumulus cells on microtubule dynamics and/or chromatin stability, oocyte maturation and early embryonic development have been reported in many species, including the human (Kennedy and Donahue, 1969; Cha and Chian 1998; Goud *et al.*, 1998; Moor *et al.*, 1998; Ueno *et al.*, 2005). Therefore, these oocytes should not be considered as a first choice for the infertility treatment of the patient. Nevertheless, in some patients with a low number of mature oocytes at the day of retrieval, these GV oocytes could be used in order to increase the number of fertilizable oocytes. One way to approach a more physiological situation for IVM of these spare GV oocytes is to restore the communication between the cumulus cells and the oocyte. A study by Combelles *et al.* (2005) describes a novel co-culture system in which cumulus cells were embedded into a 3D collagen gel matrix and mixed together with individual oocytes. The authors found that microtubule-rich processes, which resemble transzonal projections, were visible at the oocyte–cumulus cell interface. This indicates that the interaction between the isolated cell populations was restored. The next step to improve the outcome of denuded, otherwise clinically useless, human GV oocytes might be the use of a similar co-culture system in combination with temporary nuclear arrest by PDE3-I.

In conclusion, temporary nuclear arrest by the specific PDE3-I cilostamide (1 μM) proved to be beneficial for spindle/chromosome configurations and allowed synchronization of the population of immature oocytes. Modification of the PMC to restore and sustain oocyte-granulosa cell interactions during IVM might be a feasible step to mirror the physiological situation. Finally, before any clinical application, complete testing of possible increased risks of aneuploidy is necessary.

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3.3. Effect of three-dimensional prematuration culture in the presence of a phosphodiesterase type 3 inhibitor

Article IV: **Prematuration of human denuded oocytes in a three-dimensional co-culture system: effects on meiosis progression and developmental competence.**

Vanhoutte L, Nogueira D, De Sutter P

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Article V: **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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ORIGINAL ARTICLE *Reproductive biology*

Prematuration of human denuded oocytes in a three-dimensional co-culture system: effects on meiosis progression and developmental competence

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BACKGROUND: During conventional *in vitro* maturation (IVM), oocytes undergo nuclear maturation but do not attain full cytoplasmic maturity. The present study aimed to evaluate the quality of denuded *in vitro* matured human oocytes (= 'rescue IVM') following a prematuration culture (PMC) in a three-dimensional (3D) co-culture system with cumulus cells.

METHODS: Denuded, germinal vesicle (GV) oocytes retrieved from controlled ovarian hyperstimulation (COH) cycles were embedded in collagen (Type I) gel containing dissociated cumulus cells and a specific phosphodiesterase 3-inhibitor (PDE3-I) for 24 h. Afterwards, oocytes were removed from the gel and transferred to IVM inhibitor-free medium. The reversibility of PDE3 inhibition was assessed following IVM culture for a maximum of 48 h. Cytoplasmic maturation was assessed by the oocytes' capability to fertilize after ICSI and sustain further embryonic development. Conventional microdroplet cultures of denuded oocytes, with and without PMC, served as controls.

RESULTS: Oocytes embedded in collagen gel and exposed to PDE3-I were efficiently arrested at the GV stage. After removal from collagen and inhibitor, oocytes were capable of resuming meiosis: maturation rates (74.5%) were similar compared with non-prematured (68.5%) and prematured oocytes (73.4%) in microdroplets. Fertilization rates after PMC in 3D co-culture were significantly higher compared with non-prematured oocytes (72.2% versus 54.0%; $P < 0.05$), but similar to oocytes prematured in microdroplets (63.8%). Compared with both controls, the PMC in 3D co-culture improved the morphological quality and nuclear constitution of embryos on Day 3 post-ICSI.

CONCLUSIONS: Three-dimensional PMC co-culture using a PDE3-I may enhance cytoplasmic maturation of denuded human GV oocytes retrieved from COH cycles.

Key words: culture conditions / cumulus cells / *in vitro* maturation / oocyte development / phosphodiesterases

Introduction

The major challenge in developing *in vitro* maturation (IVM) systems is to create environmental conditions and to support oocyte development, which approach the *in vivo* situation. Current IVM culture systems are often unable to fulfil these criteria. This is clearly demonstrated by the lower success rates in achieving pregnancy of embryos derived from *in vitro* matured oocytes (for review, see Chian *et al.*, 2004; Mikkelsen, 2005; Jurema and Nogueira, 2006).

In vitro matured oocytes undergo maturation 'precociously' while they are still in the process of acquiring the cytoplasmic machinery needed to fully support preimplantation embryonic development (Dieleman *et al.*, 2002; Sirard *et al.*, 2006; Gilchrist and Thompson, 2007). Therefore, several researchers have tried to improve the oocytes' developmental potential by temporarily inhibiting spontaneous meiotic maturation, in an attempt to arrest oocytes at the germinal vesicle (GV) stage while cytoplasmic maturation takes place. In this way, a two-step culture has been created consisting of a prematuration culture (PMC) followed by IVM (Lonergan *et al.*, 1998; Anderiesz *et al.*, 2000; Nogueira *et al.*, 2003).

Spontaneous maturation can be prevented by exposing the oocytes to compounds that inhibit meiosis. Oocyte-specific phosphodiesterase type-3-inhibitors (PDE3-Is; e.g. cilostamide, milrinone, Org9935) are potent candidates for this purpose. These agents keep intra-oocyte

cAMP levels above a threshold that maintains the oocyte arrested at the GV-stage *in vitro* (Tsafriri *et al*., 1996; Conti *et al*., 2002; Thomas *et al*., 2002). Inclusion of PDE3-I in the culture medium during a period of PMC is likely to improve quality and developmental potential in mouse (Nogueira *et al*., 2003; Vanhoutte *et al*., 2008) and bovine (Thomas *et al*., 2004b) oocytes. In addition, enhancement of nuclear maturation rates occurs when human cumulus-enclosed oocytes (CEOs) are prematured in the presence of PDE3-I (Nogueira *et al*., 2006). Furthermore, combined exposure of human CEOs to PDE3-I and forskolin, an adenylate cyclase activator, has a positive effect on fertilization rates following IVM (Shu *et al*., 2008).

A similar approach has recently been tested by our group on denuded GV-stage oocytes obtained after ovulation induction for ICSI treatment (Vanhoutte *et al*., 2007). Although these oocytes are of poor developmental quality compared with their *in vivo* matured counterparts and, therefore, routinely considered as a side-product of the IVF treatment, they may be valuable for studying new IVM protocols. Furthermore, these dysmature oocytes can be rescued by IVM and could be a surplus material for ICSI patients when a low number of mature oocytes are retrieved.

PMC of those denuded GV-stage oocytes with a PDE3-I contributes to a better coordination between nuclear and cytoplasmic maturation (Vanhoutte *et al*., 2007). However, a clear drawback in this experimental set-up remains the fact that cumulus cells are absent during PMC. Owing to research conducted during the past decade, it is now clear that the bidirectional communication between the oocyte and its surrounding cumulus cells plays an important role in the acquisition of oocyte developmental competence and in subsequent embryogenesis (for review, see Albertini *et al*., 2001; Eppig, 2001; Tanghe *et al*., 2002; Gilchrist *et al*., 2004). Removal of the cumulus oophorus before IVM is detrimental to oocyte quality (Kennedy and Donahue, 1969; Schroeder and Eppig, 1984; Hwang *et al*., 2000; Ruppert-Lingham *et al*., 2003; Nogueira *et al*., 2006). To circumvent this deficiency, some researchers have tried to establish co-culture systems of human denuded GV oocytes with dissociated cumulus cells (Häberle *et al*., 1999; Johnson *et al*., 2008; Zhu *et al*., 2008). These studies, however, have been unable to show a significant improvement in IVM outcome. An attempt to better mimic the physiological situation is to create a system that permits a tight contact between the dissociated cumulus cells and the oocyte in a three-dimensional (3D) structure, by making use of an extra-cellular matrix (ECM) (Combelles *et al*., 2005; Torre *et al*., 2006).

A superior culture condition for human denuded GV oocytes (= 'rescue IVM') could be the combination of a similar 3D co-culture system in a period of PMC. In this way, the unhealthy condition of precocious spontaneous maturation is circumvented by the addition of a PDE3-I, while the surrounding somatic cells may produce a suitable micro-environment to support prematuration events of the oocyte. The current study was undertaken to test this hypothesis. Denuded GV-stage oocytes, retrieved following controlled ovarian hyperstimulation (COH), were prematured in a 3D co-culture with cumulus cells for 24 h. Nuclear maturation capability was assessed upon release from artificial meiotic arrest. Embryonic developmental potential was evaluated subsequently as a marker for oocyte cytoplasmic quality. Possible adverse effects of collagen embedding on cell morphology and viability were analysed under light, confocal and electron microscopy.

Materials and Methods

The study was undertaken after complete institutional review board approval from the Ghent University Ethical Committee (Project no. EC UZG 2006/168 and 2006/128). In addition, the study was filed to the Federal Ethical Committee on Embryos. Written informed consent was obtained from each infertile couple before the use of their donated gametes.

All products were purchased from Sigma-Aldrich (Bornem, Belgium), unless otherwise indicated.

Source of oocytes

Oocytes that failed to mature *in vivo* were donated by women undergoing IVF/ICSI treatment in our Department of Reproductive Medicine. 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, Geneva, Switzerland, or Puregon, Organon, Oss, The Netherlands) from Day 7 after discontinuation of the pill onwards. The starting dose was usually 150 IU, but this dose was adjusted after 7 days of hMG or FSH administration, according to the individual response of the patient. Known poor responders were started on 300 IU of hMG or FSH daily. The follicular phase was monitored by means of transvaginal ultrasound scanning of the ovaries and serum estradiol measurement if judged necessary. An injection of 5000 or 10 000 IU human chorionic gonadotrophin (hCG; Pregnyl, Organon) was administered when half of all mature follicles had reached a mean diameter of at least 20 mm, measured in two planes. Oocyte retrieval was scheduled 34–36 h after hCG administration.

Oocytes were denuded enzymatically by a brief exposure to 80 IU/ml hyaluronidase (Type III), followed by mechanical denudation. In order to perform a more controlled study, all oocytes were completely denuded before starting PMC and IVM. The nuclear status of denuded oocytes was recorded. Only morphologically normal appearing GV-stage oocytes were used in the study.

Isolation and treatment of cumulus cells

Cumulus cells were collected after enzymatic and mechanical removal from the oocytes at the time of oocyte preparation for ICSI. To obtain sufficient numbers, cumulus cells from different patients were pooled and centrifuged for 5 min at 400*g*. The resulting pellet was resuspended and exposed to a lysis buffer to remove red blood cells (8.29 g/L NH_4Cl + 1.0 g/L $KHCO_3$ + 0.58 g/L EDTA in embryo water, pH 7.2; 5 min at 37°C). The cumulus cells were then pelleted down by centrifugation and resuspended in 1 ml of warm Ca/Mg-free EBSS medium (Invitrogen, Merelbeke, Belgium) supplemented with 5% human serum albumin (HSA; Red Cross, Brussels, Belgium). This procedure was repeated at least twice to remove all traces of hyaluronidase and cell debris. Cell clusters were dispersed by repeated gentle pipetting. Finally, cells were resuspended in a small volume of PMC-medium (composition below) and counted in a haemocytometer. Cell viability was estimated by Trypan Blue exclusion and was found to be consistently higher than 80%.

Three-dimensional co-culture

The co-culture was prepared as described by Combelles *et al.* (2005) with some modifications. 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 10× concentrated TCM-199 (Invitrogen) and one volume of 0.05 N NaOH containing 22 mg/ml $NaHCO_3$. This solution was kept on ice until usage. Collected and purified cumulus cells were mixed with this neutralized collagen solution at a final density of $\sim 2 \times 10^6$ cells/ml. Microdroplets (4 μl) of this cell suspension were seeded in the wells of a 96-well plate (BD Biosciences), creating gels that contained around 8000 cumulus cells each. A single immature oocyte was added carefully to each droplet in a minimal volume of medium, which was then allowed to polymerize at 37°C for 10 min before adding 80 μl of PMC-medium (composition below) to each well (final density of 1×10^5 cells/ml/well).

Culture medium for oocyte maturation

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

The PMC-medium was composed of the basal medium plus 1 μM cilostamide, a specific PDE3-I (Calbiochem, Bierges, Belgium; Stock 10 mM in DMSO stored at −20°C) (Vanhoutte *et al.*, 2007).

The IVM-medium was constituted by the basal medium supplemented with 10 ng/ml epidermal growth factor, 1 μg/ml estradiol and 0.5 IU/ml hCG (Pregnyl, Organon).

Experimental design for GV-stage oocyte culture

A schematic presentation of the study design is shown in Fig. 1. Oocytes from each patient were randomly allocated to three different culture conditions:

(i) Conventional IVM: oocytes were cultured individually in 25 μl microdrops of IVM-medium. Evaluation during the IVM period was performed at 24 h, up to 30 h (from 25 to 30 h) and up to 48 h (from 31 to 48 h) of culture. The nuclear maturational stage was classified as GV, GV breakdown (GVBD) or polar body (PB) extrusion.

(ii) PMC in microdroplets + IVM: oocytes were prematured singly in 25 μl microdrops of PMC-culture medium. The oocytes were maintained arrested for 24 h. PDE3-I withdrawal was carried out by washing the oocytes three times in IVM-medium. IVM culture and evaluation of nuclear stage were performed as described in group (i).

(iii) PMC in 3D co-culture + IVM: oocytes were prematured in a 3D co-culture system containing cumulus cells embedded in collagen. The oocytes were prematured for 24 h and were subsequently removed manually with a pulled Pasteur pipette from the gel. Denuded oocytes were washed and placed in IVM-medium and evaluated as described in group (i).

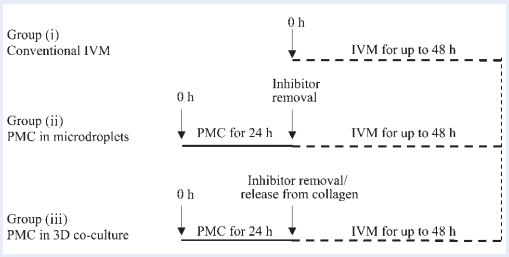

Figure 1 Schematic diagram of the study design.

All cultures were carried out in a humidified atmosphere of 5% CO_2 in air at 37°C.

ICSI procedure and embryo culture

Oocytes matured under different culture conditions 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) and who consented to donate supernumerary sperm cells for scientific research. After injection, oocytes were cultured individually in Sydney IVF Cleavage Medium (Cook, Ltd, Limerick, Ireland) for 3 days.

Fertilization was evaluated 16–18 h post-injection and was considered normal when two pronuclei were identified. Embryo morphology was classified as 'excellent', 'good' or 'poor', based on morphological criteria. Embryos with four or five blastomeres on Day 2 and a minimum of seven blastomeres on Day 3, <20% fragmentation and no visual signs of multinucleation were scored as 'excellent morphology'. Those with two or three blastomeres on Day 2 and a minimum of six blastomeres on Day 3, <20% fragmentation and no visual signs of multinucleation were scored as 'good morphology'.

Immunostaining and confocal imaging of cumulus cells and embryos

Three-dimensional-cultured cumulus cells and embryos of excellent and good morphology on Day 3 post-ICSI were evaluated for cytoskeleton organization and/or nuclear anomalies. The samples were simultaneously fixed and extracted in a microtubule-stabilizing buffer, as described elsewhere (Mattson and Albertini, 1990). To visualize microtubules, cells were incubated in the presence of a mixture of mouse monoclonal anti-α,β-tubulin (1:200) overnight at 4°C, followed by Alexa Fluor 488 conjugated goat-anti-mouse IgG (1:200; Molecular Probes, Eugene, Oregon) for 2 h at 37°C. Chromatin was stained with Ethidium Homodimer-2 (1:500; Molecular Probes) for 1 h at 37°C. Labelled samples were mounted on microscope slides in 90% glycerol–PBS solution containing 0.2% 1,4-diazabicyclo[2.2.2]octane as an antifading reagent. Preparations were observed using a laser scanning confocal microscope (Bio-Rad Radiance 2000 mounted on a Nikon inverted microscope; Tokyo, Japan) equipped with an Argon-ion/HeliumNeon (488/543) laser and selective filter sets for Alexa Fluor 488 and Ethidium Homodimer-2. Images were obtained using a ×60 plan oil immersion objective (numerical aperture 1.2).

For evaluation of embryos, all interphase and metaphase stage nuclei were carefully examined and counted in each blastomere. Interphase nuclei were classified as mono-, bi-, multinucleated or fragmented. Spindle morphology and chromosome alignment in metaphase nuclei were classified as normal (i.e. barrel-shaped spindle and well-aligned chromosomes at the equator) or abnormal.

Transmission electron microscopy

In order to explore morphological characteristics of the 3D-PMC co-culture, a few samples ($n = 6$) of collagen gels containing oocytes and cumulus cells were fixed at the end of PMC 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, cells were embedded in Epon. Serial semithin sections of 1 μm were cut throughout the oocyte 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 E900 transmission electron microscope (Oberkochen, Germany).

Measurement of steroids

At the end of PMC, conditioned media from 3D-cultured cumulus cells with oocytes were collected and stored at −20°C. The media were assayed for progesterone (P) and estradiol-17β (E_2) to validate the functionality of collagen-embedded cumulus cells during the PMC period. PMC-medium placed over collagen with no cells served as the unconditioned medium control. Concentrations of steroid hormones were measured by clinical routine immunoassays after an appropriate dilution in steroid-free culture medium. Kits were obtained from Roche Diagnostics GmbH (Mannheim, Germany; E_2: analytical sensitivity: 5 pg/ml; intra- and inter-assay coefficients of variance: 5.7% and 6.2%; P: analytical sensitivity: 0.03 ng/ml; intra- and inter-assay coefficients of variance: 2.4% and 5.4%). Data are presented as means ± SD from five measurements. For each measurement, the medium of six wells derived from the same cumulus cell preparation was used.

Statistical analysis

The Fisher exact test or contingency χ^2 test was used to evaluate differences in maturation, fertilization, cleavage rate, embryo quality and embryo nuclear status between conventionally *in vitro* matured oocytes and the PMC groups. A Bonferroni correction for multiple testing was performed. Differences in mean number of blastomeres were calculated using one-way ANOVA, followed by Tukey *post hoc* test. A level of $P < 0.05$ was considered statistically significant.

Results

Effect of PMC method on the maturation capacity of oocytes

Table I shows the cumulative time course of meiotic progression under different IVM conditions. There was no difference in the proportion of oocytes maintained at the GV stage (>90%) in the two PMC groups (microdroplet versus 3D co-culture).

Upon 24 h of IVM culture, the percentage of PB-extruded oocytes in the conventional IVM group was significantly higher compared with the PMC groups ($P < 0.05$). However, no difference was observed during the subsequent IVM culture (up to 30 h and up to 48 h). Final maturation rates at the end of IVM were equal among the three experimental groups.

Effect of PMC method on fertilization and embryo development

The results regarding fertilization and embryo development are presented in Table II. The percentage of two pronuclear (PN) embryos was higher in the 3D-PMC compared with the conventional IVM group ($P < 0.05$) and was not different from the microdroplet-PMC group. The majority of fertilized oocytes underwent cleavage on Day 2. Embryo quality on Day 2 was similar among the three groups. On the other hand, a significantly higher number of good

Table I Meiotic progression of immature oocytes cultured under different conditions for up to 48 h

	Culture conditions		
	Conventional IVM	**PMC in microdroplet culture + IVM**	**PMC in 3D co-culture + IVM**
No. (%) of GV after inhibition		94/102 (92.2)	106/117 (90.6)
No. of GV undergoing IVM	92	94	106*
Maturation at 24 h of IVM (%)			
GV	26.1	25.5	32.1
GVBD	38.0[a]	57.4[b]	46.2[a,b]
PB	33.7[a]	17.0[b]	17.9[b]
Degenerated	2.2	0	3.8
Maturation up to 30 h of IVM (between 25 and 30 h) (%)			
GV	22.8	22.3	22.6
GVBD	17.4	16.0	16.0
PB	54.3	61.7	57.5
Degenerated	5.4	0	3.8
Maturation up to 48 h of IVM (between 31 and 48 h) (%)			
GV	13.0	18.1	11.3
GVBD	12.0	8.5	9.4
PB	68.5	73.4	74.5
Degenerated	6.5	0	4.7

[a,b]Values within the same row with different superscripts are statistically different ($P < 0.05$).
*Oocytes were removed from collagen prior to IVM.

Table II Fertilization and embryo development of PB oocytes following IVM under different culture conditions

	Culture conditions		
	Conventional IVM	**PMC in microdroplet culture + IVM**	**PMC in 3D co-culture + IVM**
No. of injected PB oocytes	63	69	79
No. (%) of 2PN	34 (54.0)[a]	44 (63.8)[a,b]	57 (72.2)[b]
Cleaved/2PN (%)	82.4	79.5	82.5
Embryo quality Day 2 (%)			
Excellent	23.5	27.3	29.8
Good	38.2	34.1	42.1
Poor	38.2	38.6	28.1
Embryo quality Day 3 (%)			
Excellent	11.8	11.4	17.5
Good	5.9[a]	9.1[a]	26.3[b]
Poor	82.4[a]	79.5[a]	56.1[b]
Mean blastomere no./embryo ± SD Day 2	2.96 ± 0.96	2.80 ± 1.18	2.87 ± 0.99
Mean blastomere no./embryo ± SD Day 3	4.68 ± 2.00	4.26 ± 2.21	5.17 ± 1.95

[a,b]Values within the same row with different superscripts are statistically different ($P < 0.05$).

quality embryos and a significantly lower number of bad quality embryos were obtained on Day 3 in the 3D-PMC compared with the other groups ($P < 0.05$). The mean number of blastomeres was not statistically different between groups ($P > 0.05$).

Nuclear status of embryos

The double-labelling method allowed the co-observation and localization of the nucleus (chromatin) and cytoskeleton (tubulin) of individual blastomeres in embryos on Day 3 post-ICSI (see Fig. 2 and Table III).

The mean number of blastomeres analysed per embryo was not different among the groups. The 3D-PMC group contained significantly more mononuclear blastomeres compared with the other groups (63.3% versus 47.1% in microdroplet-PMC and 37.5% in conventional IVM; $P < 0.05$). The percentage of multinucleated, fragmented and anuclear blastomeres was lower in the 3D-PMC compared with the conventional IVM ($P < 0.05$), but not different compared with the microdroplet-PMC.

In addition to interphase nuclear abnormalities, we identified some metaphase abnormalities in all groups. This included mostly the disalignment of chromosomes (Fig. 2C). However, the number of blastomeres at metaphase was too low to identify any statistical difference among the groups.

As a result of these observations, 60% ($n = 15$) of the embryos in the 3D-PMC group contained at least 50% blastomeres with normal interphase and/or metaphase configuration, while this was 16.7%

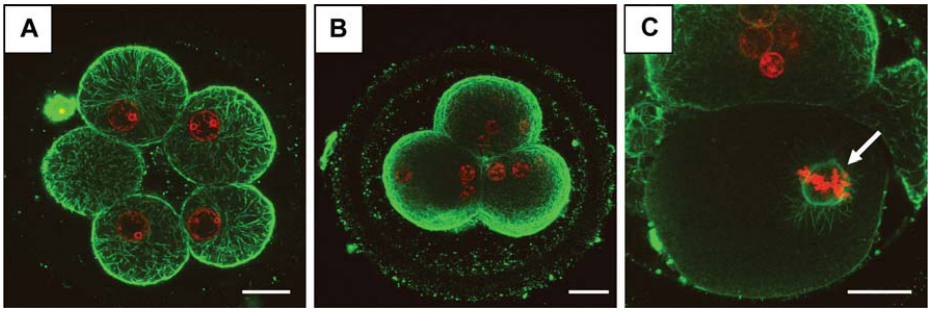

Figure 2 Representative confocal images of good quality embryos on Day 3 post-ICSI from oocytes prematured in 3D co-culture.

Embryos are stained to visualize microtubules (green) and chromatin (red). (**A**) Detailed section through five blastomeres of an 8-cell embryo. A single nucleus is visible in four blastomeres and a dense network of microtubules is present throughout the cytoplasm. (**B**) 3D reconstruction of three multinucleate blastomeres from a 7-cell embryo. (**C**) High-power confocal section of two blastomeres from a 6-cell embryo. One blastomere is at the metaphase stage, with several disaligned chromosomes (arrow). In the other blastomere, multiple nuclei are present. Scale bar = 20 μm.

Table III Nuclear status of embryos and individual blastomeres derived from different IVM groups

	Culture conditions		
	Conventional IVM	PMC in microdroplet culture + IVM	PMC in 3D co-culture + IVM
No. of embryos analysed*	6	9	25
No. of blastomeres analysed	45	65	168
Mean blastomere no./embryo ± SD	7.5 ± 1.5	7.2 ± 1.3	6.7 ± 0.9
No. (%) of blastomeres at 'interphase'	32 (71.1)[a]	51 (78.5)[a]	150 (89.3)[b]
Mononuclear[c]	12 (37.5)[a]	24 (47.1)[a]	95 (63.3)[b]
Binuclear[c]	5 (15.6)	13 (25.5)	23 (15.3)
Multinuclear and/or fragmented nuclei[c]	15 (46.9)[a]	14 (27.5)[a,b]	32 (21.3)[b]
No. (%) of blastomeres at 'metaphase'	8 (17.8)[a]	8 (12.3)[a,b]	12 (7.1)[b]
Normal spindle shape[d]	6 (75.0)	6 (75.0)	10 (83.3)
Aligned chromosomes[e]	3 (37.5)	4 (50.0)	8 (66.7)
No. (%) of 'anuclear' blastomeres	5 (11.1)[a]	6 (9.2)[a,b]	6 (3.6)[b]

[a,b]Values within the same row with different superscripts are statistically different ($P < 0.05$).
[c]Percentages are calculated over the total no. of blastomeres at interphase.
[d]Defined as a barrel-shaped spindle. Percentages are calculated over the total no. of blastomeres at metaphase.
[e]Defined as condensed chromosomes arranged on a compact plate at the equator of the spindle. Percentages are calculated over the total no. of blastomeres at metaphase.
*Only embryos of excellent and good quality on Day 3 were fixed.

($n = 1$) in the conventional IVM group and 44.4% ($n = 4$) in the group of PMC in microdroplets ($P > 0.05$).

Morphological characteristics of the 3D-PMC co-culture

Representative light microscopic and confocal images of the 3D-PMC co-culture are shown in Fig. 3. At the end of PMC, the GV oocytes were surrounded by multiple layers of cumulus cells which covered the whole surface of the zona pellucida (Fig. 3A). Numerous cytoplasmic processes between neighbouring cumulus cells were noticed (Fig. 3B and D). In addition, these processes appeared to have an extension towards the oocyte (Fig. 3C).

Electron microscopy allowed a more accurate observation of the oocyte, cumulus cells and possible interactions at the oocyte–cumulus interface at the end of 3D-PMC (Fig. 4).

Oocytes

Collagen embedding seemed to have no deleterious effect on the ultrastructural morphology of the GV-arrested oocyte. The general nuclear and cytoplasmic organization did not present morphological abnormalities (Fig. 4A). No signs of vacuolization were observed in the cytoplasm. The nuclear membranes of all prematured GV-arrested oocytes had remained intact. Cytoplasmic organelles (e.g. mitochondria, Golgi complexes, elements of smooth endoplasmic reticulum) were dispersed throughout the ooplasm. A few cortical granules were found in the cytoplasm and beneath the oolemma. Microvilli were uniformly distributed over the surface of the oocyte.

Cumulus cells

Three-dimensional-cultured cumulus cells showed well-developed mitochondria, lipid droplets and lysosomes all clustered in the perinuclear region of the cells. The cytoplasm was filled with polyribosomes. Numerous intercellular junctions were seen at the area of contact between neighbouring cells (Fig. 4B). A network of cytoplasmic processes was visible between intercellular spaces, which were often connected with each other (Fig. 4C). Although a few necrotic cells could be observed, their presence was only sporadic.

Connections at the oocyte–cumulus interface were only rarely seen. Flat layers of cumulus cells were visible in close proximity to the zona pellucida, but they did not seem to be tightly connected by transzonal projections (TZPs) (Fig. 4D). In most cases, the zona pellucida had a homogeneous structure, without traces of TZPs. In exceptional cases, some cumulus cell processes extended towards the zona, but did not grow further towards the oocyte (Fig. 4E and F).

Steroid production by cumulus cells during PMC

Steroids were measured in conditioned medium to estimate any adverse effects of collagen embedding and culture of cumulus cells on cell function. The cultured cumulus cells were capable to secrete estradiol (E_2) with a concentration of 340.6 ± 107.6 pg/ml. Progesterone (P) concentration in cumulus-conditioned medium was 472.7 ± 133.1 ng/ml. In the control medium, in the absence of cumulus cells, concentrations were significantly lower (23.1 ± 4.1 pg/ml for E_2 and below the detection limit for P).

Discussion

In the present study, we aimed to design an improved 'rescue IVM' system for human denuded GV-stage oocytes retrieved from COH cycles. Although it is well known that leaving the cumulus oophorus intact during IVM is a prerequisite to obtain satisfactory results, our

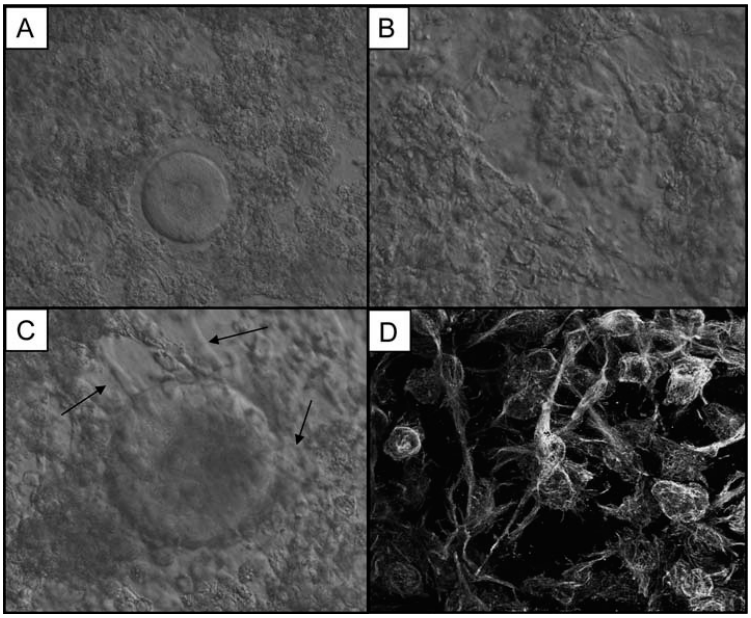

Figure 3 Light microscopic and confocal images of the 3D-PMC co-culture.

(**A**) Representative light microscopic image of a human GV oocyte after 24 h of 3D co-culture PMC with cumulus cells. (**B**) Detail of 3D-cultured cumulus cells: note the cytoplasmic, dendritic processes that interconnect different clumps of cumulus cells. (**C**) The dendritic processes extend from the cumulus cells towards the oocyte (arrow). (**D**) Three-dimensional confocal reconstruction of 3D-cultured cumulus cells stained for anti-a,b-tubulin. Numerous microtubule-rich processes extend from the cumulus cells, giving a web-like appearance. Original magnification: ×400 (A–C) and ×600 (D).

aim here was to apply a methodology which would better rescue the 'left-over' GV-stage oocytes, i.e. those that are punctured in the same follicular pool as the retrieved matured oocytes destined for patients' use.

The oocytes were prematured in a 3D co-culture with cumulus cells embedded in an ECM prior to undergo final nuclear maturation. The rational for this two-step approach was based on the experience gathered from previous publications. Temporary prevention of spontaneous nuclear maturation with an oocyte-specific PDE3-I may allow better synchronization between cytoplasm and nuclear maturity (Nogueira *et al*., 2003, 2006; Thomas *et al*., 2004b). In addition, to overcome the lack of cumulus cells during PMC and to simulate more accurately the naturally 3D-environment of the cumulus–oocyte complex (COC), we opted for the use of an ECM (i.e. collagen) loaded with dissociated cumulus cells (Combelles *et al*., 2005).

The principal findings from our study were a significantly higher fertilization rate of 3D-PMC oocytes compared with oocytes that underwent conventional IVM. Although 3D-PMC did not augment the fertilization capacity when compared with oocytes prematured in microdroplets, embryos on Day 3 post-ICSI from the 3D-PMC groups were of better quality and had fewer nuclear abnormalities compared with the other groups. These results suggest that the combination of a co-culture in the presence of an ECM may serve as a physiological support during the last stages of oocyte development.

The positive effect of the ECM on cellular behaviour *in vitro*, including ovarian cells, has already been explored extensively in previous studies. Human mural granulosa cells cultured on ECM, such as collagen, maintain a more highly differentiated morphology, possess an increased level of steroidogenic activity (Ben-Rafael *et al*., 1988; Bussenot *et al*., 1993), produce significantly higher levels of cAMP (Furman *et al*., 1986) and induce *de novo* formation of gap junctions (Amsterdam *et al*., 1989). It must be noticed, however, that the cells in these experiments were grown on top of an ECM-layer in a 2D way. In the present study, cumulus cells and oocytes were embedded within an ECM to create a 3D nature. Since the collagen preparation needs to be chilled (0°C) to prevent early polymerization, the cells were submitted to an abrupt drop in temperature at the time of loading of the gel. This could be detrimental, especially for oocytes that are highly temperature sensitive (Wu *et al*., 1999; Vandevoort *et al*., 2008). Therefore, one of the aims of the present study was to analyse any adverse effect of collagen-embedding procedure on cell viability and functionality. Although a larger sample would be necessary for a more conclusive statement, the present transmission electron microscopy (TEM) analysis could not reveal a negative effect on cell morphology. Numerous junctional complexes were

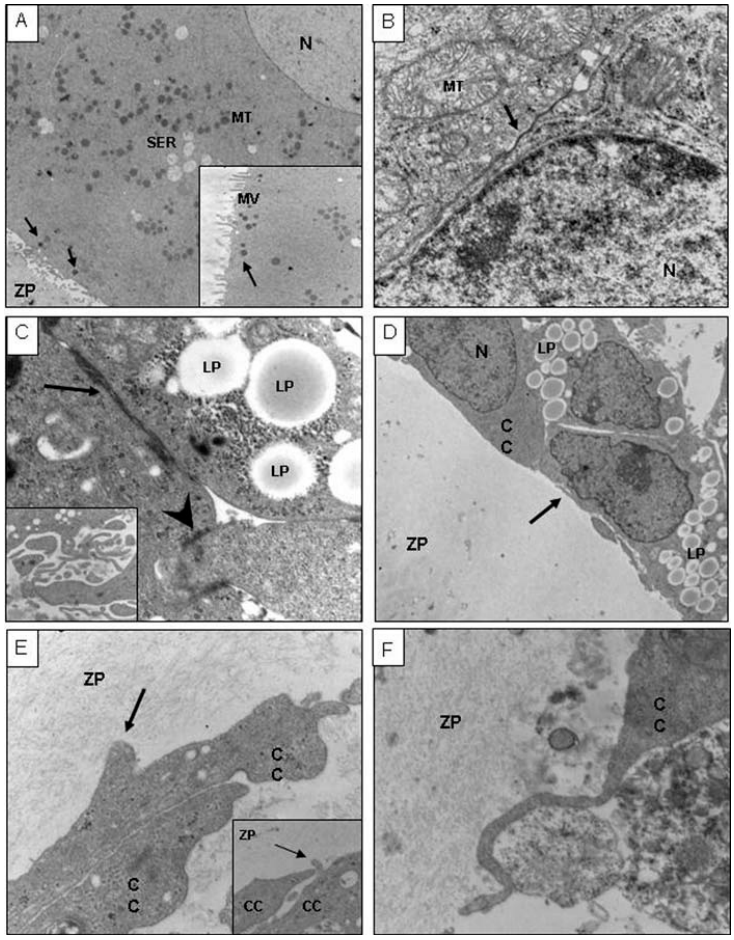

Figure 4 TEM analysis of the 3D-PMC co-culture.

(**A**) Electron micrograph of a prematured 3D co-cultured GV oocyte. The cytoplasm is filled with evenly distributed organelles. The nucleus is spheroid with an intact nuclear membrane. Some cortical granules are visible just below the oocyte plasma membrane (arrows) (×3000). Inset: microvilli are projected from the oolemma to the inner cortex of the zona pellucida (×7000). (**B**) Junctional complex between neighbouring cumulus cells (arrow) (×200 000). (**C**) Detailed image of a cellular process making contact with the cytoplasm of an adjacent cumulus cell (arrowhead) and a junctional complex at the level of the cumulus cell membranes (arrow). Note the accumulation of dense material at the regions of interactions. Many lipid droplets are present in the cytoplasm (×120 000). Inset: dense network of long cellular processes between cumulus cells (×120 000). (**D**) A layer of cumulus cells in close approximation with the zona pellucida. The cell surfaces of the cumulus cells have a smooth appearance. Lipid droplets are widely scattered throughout the cytoplasm. Although a small cell process is formed (arrow), it does not extend towards the zona (×3000). (**E**) Examples of cytoplasmic projections at the oocyte–cumulus interface (see arrows) (×200 000; Inset: ×120 000). (**F**) Detail of a long cytoplasmic process growing around a degenerated cumulus cell and making contact with the zona pellucida. Some cytoplasmic organelles are present within the cell process (×120 000). CC, cumulus cell; LP, lipid droplet; MT, mitochondrion; MV, microvilli; N, nucleus; SER, smooth endoplasmic reticulum; ZP, zona pellucida.

Erratum: Magnification of figures B, C, E, F should be 20 000 and 12 000 instead of 200 000 and 120 000 respectively.

formed between individual cumulus cells. In addition, cumulus cells were capable of secreting estradiol and progesterone, which demonstrates that the cells retained their steroidogenic activity. Furthermore, the presence of the collagen matrix did not reduce the effectiveness of the PDE3-I on keeping the oocytes arrested at the GV stage. Upon release from meiotic arrest and ECM, oocytes were competent to resume meiosis and progress to the metaphase II stage, which is an important functional end-point of the culture system. The kinetics of oocyte nuclear maturation was slowed down compared with the conventionally IVM group. This phenomenon is likely a result of the PDE3-I itself rather than the ECM, since a similar pattern of meiotic progression was seen in the microdroplet-PMC group. Based on these observations, we may conclude that cumulus cells and oocytes were able to withstand the cooling–warming step during

embedding and polymerization of the collagen matrix. Still, it must be noted that there is a lack of information on cooling-induced changes at the biochemical and molecular level. A recent study by Succu *et al.* (2008) observed that vitrifying ovine oocytes negatively affects the content of transcripts related to several cell functions. A similar analysis of differences in gene expression might offer extra information on the effect of cooling during the embedding procedure on oocytes and/or cumulus cells.

In a pilot study, we aimed to re-embed the 3D-cultured oocytes after the 24 h-PMC period in a new collagen gel in order to perform the IVM in a similar 3D-configuration. However, maturation rates never exceeded 50%, which was significantly lower compared with conventional IVM (data not shown). We know from the study of Combelles *et al.* (2005) that nuclear maturation within a collagen gel is feasible, since embedded oocytes matured at similar rates compared with non-embedded, conventionally matured oocytes. Therefore, we may conclude that repeated cooling–warming during the two-step culture should be avoided. Other types of ECMs, which do not have to be chilled before usage, could be tested for the present two-step culture. Such an example is the barium alginate capsule, a synthetic ECM (Torre *et al.*, 2006).

During the final stages of oocyte development, prior to ovulation, the oocyte undergoes a process of 'capacitation'. This process involves structural and biochemical changes within the cytoplasm (e.g. transcription of mRNA, post-translational modifications of proteins, relocations and modifications of organelles etc.) that are essential to sustain normal fertilization and further embryonic development (Hyttel *et al.*, 1997; Dieleman *et al.*, 2002). Recently, Cavilla *et al.* (2008) demonstrated that human GV oocytes from COH cycles increase in size during a 48 h-IVM period, even in the total absence of somatic cellular support. Extending the period of GV-arrest in culture by blocking meiosis progression (i.e. PMC) might allow time to complete this capacitating and growth process. Our results demonstrate that 3D-PMC culture of GV oocytes increases fertilization rates compared with conventional IVM, while the effect of PDE3-I alone (microdroplet-PMC) had only a slight, non-significant influence on this parameter. Of interest is that embryo morphology on Day 3 and nuclear constitution were significantly enhanced in the 3D-PMC compared with the microdroplet-PMC. While the incidence of bi- and multinucleated, fragmented or anuclear blastomeres was not different between the two groups, a significant greater percentage of blastomeres in the 3D-PMC had a mononuclear nucleus compared with their counterparts in the microdroplet-PMC. This suggests that culture protocols for 'rescue IVM' requires a PMC in the presence of an appropriate *in vitro* environment, as we could establish by using the 3D co-culture approach. Future studies on chromosomal analysis should determine whether these embryos are also genetically normal (Nogueira *et al.*, 2000; Emery *et al.*, 2005).

This study focused on oocyte–cumulus cell interactions as well. In the *in vivo* situation, cumulus cells possess highly specialized TZPs which penetrate through the zona pellucida and form gap-junctions at their tips with the oocyte. Via these gap-junctions, cumulus cells deliver numerous substances (e.g. amino acids, energy substrates, purines, cAMP etc.) to the oocyte (Albertini *et al.*, 2001; Tanghe *et al.*, 2002; Gilchrist *et al.*, 2008). There exist several ways to evaluate whether these connections could be reconstituted during the period of PMC (e.g. Lucifer Yellow microinjection; Luciano *et al.*, 2004). In the present study, we used electron microscopy to evaluate this parameter. As expected, newly developed TZPs reaching the oolemma could not be observed. Cytoplasmic processes extending from the cumulus cells might be unable to enter the zona pellucida. It is also possible that the development and outgrowth of TZPs towards the oolemma requires more time, since oocytes and cumulus cells are physically separated a considerable distance by the zona. Extending the PMC, for example, by an additional 24 h, might solve this problem. Another possible solution is the supplementation of the culture medium with compounds that favour the establishment of cellular contacts. An example of such a compound is cAMP. Intracellular cAMP has been shown to be a principal regulator of gap-junctions in various cell types and tissues (Cruciani and Mikalsen, 2002). Treatment of bovine and human COCs with a combination of different cAMP-modulating agents extends the gap-junctional communication between the oocyte and the cumulus cells (Thomas *et al.*, 2004a; Shu *et al.*, 2008). It would be interesting to explore if a similar treatment during prematuration could influence the *de novo* formation of TZPs in our 3D culture system.

On the other hand, details of the role and regulation of TZPs at this final stage of oocyte maturation are scarce. *In vivo*, the structure and number of TZPs changes during the maturation process (Allworth and Albertini, 1993; Motta *et al.*, 1994). While there are numerous junctions present between the oocyte and the cumulus cells at the pre-antral stage, their frequency decreases progressively as ovulation approaches (Gilula *et al.*, 1978; Dekel *et al.*, 1981). Reports on whether junctional association between cumulus cells and oocytes is essential for the beneficial effect of co-culture are controversial (Ge *et al.*, 2008). Our results on embryonic developmental quality on Day 3 indicate that cytoplasmic maturation was improved to some extent without restoration of oocyte–cumulus cell contacts during PMC. Therefore, intracellular communication via paracrine signalling could have been compensated for the absence of oocyte–cumulus cell contacts (Carabatsos *et al.*, 1998; Albertini *et al.*, 2001). The identification of these paracrine factors remains to be explored.

Another aspect that deserves attention is the source of cumulus cells used. These cells were taken from the COCs retrieved after exposure to hCG. This means that the cumulus cells were luteinized, a process which is associated with a decline in proliferative capacity and an increase in the expression of enzymes involved in the synthesis of progesterone (Paton and Collins, 1992). It also leads to an increased production of inhibin (Seifer *et al.*, 1996). One may conclude that this differentiated cell stage is not appropriate to promote prematuration events of the oocytes. A better solution could be the use of granulosa cells which have not yet shifted to the luteinizing phase. Preferable sources to obtain these cells are follicular fluid aspirates retrieved from growing antral follicles during IVM cycles, in which the patient did not receive hCG (Lindeberg *et al.*, 2007). Nevertheless, our results on embryo quality on Day 3 post-ICSI suggest that the presence of cumulus cells during PMC, even if in the luteinized form, exerted a positive effect on oocytes' cytoplasmic maturity. The precise mechanisms causing this beneficial effect are unclear. It could be possible that the oocyte and the cumulus cells reciprocally supported each others physiology during PMC. A study from Nekola and Nalbandov (1971) demonstrated that granulosa cells cultured in close proximity to oocytes appeared to be less luteinized than those cultured without oocytes. A more recent study demonstrated

that denuded bovine oocytes co-cultured with cumulus cells induce a dose-dependent suppression of cumulus cell apoptosis (Hussein *et al*., 2005). This illustrates that the oocyte is not just a passive recipient of developmental signals from its associated somatic cells, but also plays key regulatory roles on neighbouring cumulus cells. The oocyte achieves this through the synthesis and secretion of oocyte-specific factors, such as growth differentiation factor 9 and bone morphogenetic protein 15, which act on cumulus cells to modify their proliferation, function and differentiation (Hutt and Albertini, 2007; Gilchrist *et al*., 2008). Via this pathway, the oocyte is a principal regulator of its own meiotic and developmental competence. In our study, it might be possible that a similar bidirectional feedback loop between the oocyte and the cumulus cells took place during the PMC period, which resulted in the overall improved outcome following IVM.

Another possibility to explain our results is the protective role that cumulus cells may play by reducing cystine to cysteine and promoting the uptake of cysteine in the oocyte (Takahashi *et al*., 1993). Via this pathway, cumulus cells may neutralize the damaging processes that reactive oxygen species may exert on oocytes during the course of PMC (de Matos *et al*., 1997).

Finally, an important aspect that deserves attention when analysing the present results is the source of oocytes used in this study. The GV oocytes represent an already compromised group of oocytes retrieved from follicles that failed to conclude maturation *in vivo* in response to the hormonal stimulation. Therefore, these 'rescue IVM' oocytes should not be considered as a first choice for the infertility treatment of the patient. Nevertheless, in some patients with a low number of mature oocytes at the day of retrieval, these oocytes could be used in order to increase the number of fertilizable oocytes.

Conclusion

In conclusion, the present results provide a proof of concept for a novel and efficient culture approach for human 'rescue IVM'. Before any clinical application in human IVF, complete testing of possible increased risks of aneuploidy is necessary. In addition, further studies involving embryo transfer in large mammals might reveal the level of epigenetic risks that this technique could bring.

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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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Abstract

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 extra-cellular 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, washed away from inhibitor and underwent IVM. The optimal concentration of collagen (diluted 1:2 versus undiluted) was first determined in a 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 supported better 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 upon IVF were higher in the 3D-PMC group compared to in vitro controls and 2D-PMC oocytes, but lower compared to in vivo controls. In the human model, percentages of polar body-extruded oocytes were significantly higher in the 3D-PMC group compared to conventionally matured oocytes. Three-dimensional PMC had also a beneficial effect on embryonic development on day 3 post-ICSI.
Conclusions: Applying a 3D-PMC in the presence of a PDE3-I preserve oocyte-cumulus cell connections and influences oocyte developmental capacity.

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 hyperstimulation. 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 (Dieleman *et al.*, 2002; Hyttel *et al.*, 1997; 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 (Lonergan *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 GV-stage in vitro (Tsafriri *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 prematured 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 to 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 and 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 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 (Eppig, 2001; Albertini *et al.*, 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 2-step culture system which 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 extra-cellular 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 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 2-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 prematured. 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 2-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.

Material and methods

The study was undertaken after complete institutional review board approval from the Ghent University Ethical Committee (Project N° EC UZG 2007/142), the Animal Ethical Committee (Project N° ECD 06/05) and the Federal Ethical Committee on Embryos (Belgian Registration N° B67020072076).

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

Collection of mouse cumulus-enclosed oocyte (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), 100µg/ml streptomycin, 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 hCG (Chorulon; Intervet) 48 h later. Mature CEOs were recovered from the ampullae 16-18 h post-hCG.

Collection of human cumulus-enclosed oocyte (CEOs)

Only consenting patients who had undergone routine controlled ovarian stimulation (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 (GnRH) 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 human chorionic gonadotrophin (hCG; Pregnyl, Organon, The Netherlands) was administered when at least ≥ 2 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 7 follicles of ≥ 12 mm on ultrasound before oocyte retrieval) were included in the study. Oocyte retrieval was scheduled 34 to 36 h post-hCG, using a 17G aspiration needle. First, follicles larger than 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 tube 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 examined under the inverted microscope for the presence of a germinal vesicle (GV). When a well defined nuclear membrane could not been observed, the oocyte was denuded by chemical (80 IU/ml hyaluronidase type VIII) and mechanical means. Polar body (PB)-extruded oocytes were included as in vivo controls, since they were retrieved from small follicles of the same patients.

Culture medium for mouse oocyte maturation

The basal culture medium consisted of alfa-Minimal Essential Medium with glutamax (α-MEM; Invitrogen) supplemented with 5% FCS, 5 µg/ml insulin, 5 µg/ml transferrin, 5ng/ml selenium, 100 µM cysteamine, 100 IU/ml penicillin G, 100 µg/ml streptomycin sulphate and 10 mIU/ml FSH (Puregon; Organon).

The PMC-medium was composed of the basal medium plus 1µM cilostamide, 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 maximum level of inhibition and reversibility of meiosis inhibition in mouse oocytes (Vanhoutte *et al*., 2008). In the human, a concentration of 1 µM cilostamide was more likely to preserve meiotic spindle morphology and chromosome alignment after IVM compared to 10 µM cilostamide (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 (HSA; 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 (IGF-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 mIU/ml FSH.
The PMC-medium was composed of the basal medium plus 1µM cilostamide.
The IVM-medium was constituted by the basal medium supplemented with 10 ng/ml epidermal growth factor (EGF), 1 µg/ml estradiol and 0.5 IU/ml hCG.

Three-dimensional prematuration culture (3D-PMC)
An extra-cellular matrix (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 $NaHCO_3$ and one volume of 10x concentrated α-MEM (Invitrogen; for mouse oocytes) or 10x concentrated TCM-199 (Invitrogen; for human oocytes). To obtain a 1:2 diluted collagen-gel matrix (see Experiment 1), 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 usage.

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 forty 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 µl 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% CO_2 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 microdroplets (30 µl) of IVM-medium under oil and cultured for 16-18 h under standard conditions. The nuclear maturational stage was classified as GV, GV breakdown (GVBD) or polar body (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).

Evalution 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; (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 (TEM)
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 postfixation 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 E900 electron microscope (Oberkochen, Germany).

Dye coupling assay
To assess the functionality of oocyte-cumulus connections, a 3% solution of Lucifer Yellow (LY) dye in 5mM 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 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 carbenoxelone (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 caudae epididymidis were removed from adult males. Epididymal contents were carefully squeezed out and the residual caudal tissue was discarded. Sperm suspensions were preincubated 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 x 10^6/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% CO_2, 5% O_2 and 90% N_2. The percentage of developing embryos 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., Limerick, Ireland) for 3 days in a humidified atmosphere of 37°C and 5% CO_2 in air.
Fertilization was evaluated 16-18 h post-injection and was considered normal when two pronuclei were identified. Embryos were scored on day 2 and day 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, less than 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 Figure 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 injection), maturation rates, fertilization and embryonic development, data were analyzed with one-way ANOVA and Tukey-post 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 chi-square 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 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 CEOs morphology was investigated in collagen-gel preparations of two different concentrations (not-diluted versus diluted 1:2) and compared to a group of CEOs prematured in conventional microdroplet cultures (= 2-dimensional PMC; 2D-PMC; Fig 1A).

CEOs could maintain their 3D structures in the two collagen concentrations tested. In diluted collagen, increase of 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 to 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, either in the 2D- or the 3D-PMC (Fig 3A). These results were compared to 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, while CEOs with a C- cumulus were partially or completely denuded (data not shown). Maturation stages were analyzed at the end of IVM to evaluate the reversibility of the PDE3-I and compared to 2D-PMC cultured oocytes and spontaneously matured oocytes (in vitro control) (Fig 1A).
Oocytes cultured in non-diluted collagen demonstrated the highest rate of GV-arrest at the end of IVM compared to 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 (TEM) 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 TEM (n = 30 in each group; Fig 4). In 3D-PMC cultured CEOs, variously shaped transzonal projections (TZPs) from surrounding cumulus cells were observed within the zona pellucida (Fig 4A, 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 Lucifer Yellow dye (LY) microinjection (Fig 5). The gap junctional statuses in the different treatment groups are shown in Table I. Injection of LY 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 analyzed. A dramatic drop in oocyte-cumulus cell coupling occurred in the 2D-PMC group compared to the positive control, 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-mediate communications between oocytes and surrounding cumulus cells were observed in a significantly higher proportion compared to 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). Fifty six (29 %) oocytes were at the PB-stage at retrieval and 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 to the start, while 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 to 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 to 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 prematured 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 group. At 30 h of IVM, a significantly higher proportion of oocytes in the in vitro control were still arrested at the GV stage compared to the 3D-PMC. Of oocytes evaluated 48 h after IVM, those in the 3D-PMC group acquired significantly higher maturation rates compared to 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 higher in the 3D-PMC group compared to the 2D-PMC and the in vitro control, but lower compared to 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 group ($P>0.05$), but higher than in the in vitro control and lower compared to the in vivo control group ($P<0.05$; Fig 7B). The percentages of developing embryos to the 2-cell and blastocysts stages of 2D-PMC oocytes were higher compared to the in vitro control ($P<0.05$).

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 (Fig 1B and Table

III). Fertilization rates were significantly higher in the in vivo controls compared to the in vitro controls ($P<0.05$), but similar to the 3D-PMC ($P>0.05$; 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 and 3D-PMC groups compared to 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 was observed in the in vivo control and 3D-PMC group compared to the in vitro control ($P<0.05$; Table III).

Discussion

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 2-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 rational behind this strategy is to prolong oocyte-cumulus cell gap-junctional communication and to allow for continued mRNA and protein accumulation within the ooplasm (Thomas *et al.*, 2004a; Luciano *et al*., 2004; 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 to the 2D-PMC and the in vitro control group. In the human model, the 2-step 3D-PMC culture 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 to 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 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, increase of 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 if the intercellular communication between the two different cell types, cumulus cells and oocytes, were preserved at the end of 3D-PMC. Evaluation of the physical integrity of the connections was initially performed using transmission electron microscopy (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, while numerous transzonal projections (TZPs) could still be observed at the end of 3D-PMC.
To examine the functionality of these connections, a 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 to 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 to 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 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 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 preantral 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 the increase of intra-oocyte cAMP concentration (Tsafriri *et al.*, 1996, Thomas *et al.*, 2002). Elegant studies from Thomas *et al.* (2004a + b), Luciano *et al.* (2004) and Shu *et al.* (2008) demonstrated that elevated intra-cellular cAMP levels promotes the gap-junctional cross-talk 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.*, 2004; 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 2-step culture in a human model. A valuable source of oocytes for this purpose are 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 to 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 around 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, less than 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 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 to 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 2-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 to the 2D-PMC and the in vitro control. 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-Is (Nogueira *et al*., 2003b; Vanhoutte *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 oocytes' developmental potential 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 vivo 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 to 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 there quality is superior compared to 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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Figure legends

Figure 1: Schematic diagram of the different experimental groups in mouse (A) and human (B) experiments.
(A) Immature mouse CEOs were collected from small antral follicles and allocated to two different groups: 3D-PMC or 2D-PMC. In the 3D-PMC, CEOs were prematured in collagen. In the 2D-PMC, CEOs were prematured in conventional microdroplet culture. After PMC, CEOs in both groups were in vitro matured. According to the type of experiment, the results

were compared to a group of in vitro controls (non-arrested, spontaneously matured oocytes) or in vivo controls (in vivo matured oocytes retrieved upon priming mice with eCG and hCG). (B) Immature human CEOs were collected from small antral follicles and allocated to two different groups: 3D-PMC or in vitro control. In the 3D-PMC, CEOs were prematured in collagen and subsequently in vitro matured. In the in vitro control, oocytes were immediately in vitro matured. According to the type of experiment, the results were compared to a group of in vivo controls (PB-extruded oocytes retrieved from small follicles).

Figure 2: 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 to 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 x 400.

Figure 3: Effect of PMC on **(A)** nuclear arrest and **(B)** reversibility of nuclear arrest in mouse oocytes.
CEOs were 3D prematured in diluted versus non-diluted collagen for 24 hrs. Afterwards, CEOs were removed from collagen, washed out of PDE3-I and in vitro matured. Maturation rates after PMC (A) and after IVM (B) were compared with a group of CEOs prematured in conventional microdroplet cultures (= 2D-PMC) and non-arrested, spontaneously matured oocytes (= in vitro control). Values are expressed as mean % ± SD (error bars) of four independent replicates. Different letters within the same maturation classes represent significant differences ($P<0.05$). PB = polar body extrusion; GVBD = germinal vesicle breakdown; GV = germinal vesicle.

Figure 4: Representative electron microscopic section of 3D-PMC **(A, B)** and 2D-PMC **(C)** cultured mouse CEOs.
(A) After PMC for 24 h in collagen, cumulus cells were still in close approximation with the oocyte and several transzonal projections were visible in the zona pellucida (arrow). Junctional complexes between neighboring cumulus cells could be observed as well (arrowhead). Note the presence of numerous fibrillar lattices in the oocytes' cytoplasm, which is characteristic for mouse oocytes; **(B)** Detailed image of a long cumulus cell process crossing the zona pellucida (arrow); **(C)** A big gap is visible between the oocyte and the cumulus cells of a 2D-PMC CEO. Although remnants of TZPs could be observed within the zona (arrow), they were disconnected from the cumulus cells.
CC = cumulus cell; FL = fibrillar lattices; MV = microvilli; N = nucleus; O = oocyte; ZP = zona pellucida. Original magnification: x 12 000 (A, B) and x 7000 (C).

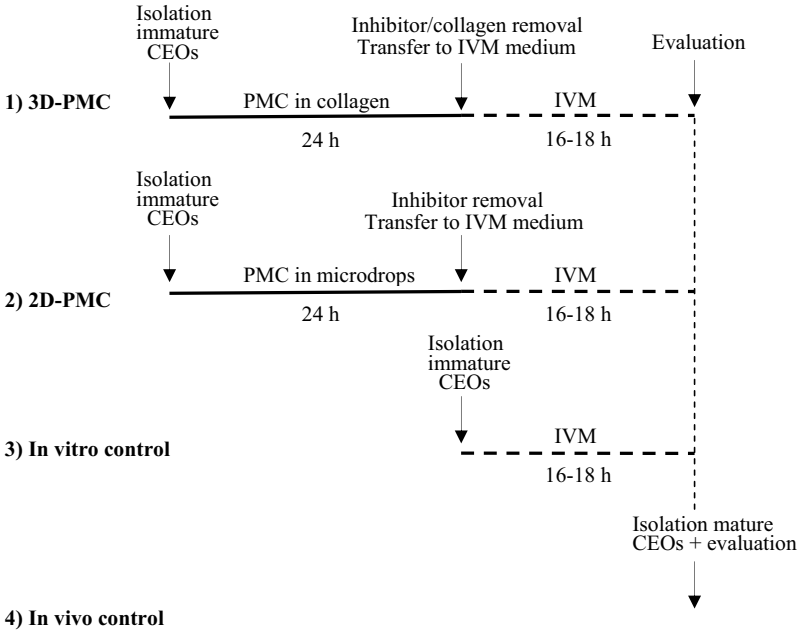
Figure 5: LY diffusion in mouse CEOs cultured for 24 h in 3D-PMC.
A solution of LY dye was injected into mouse oocytes and the spread of dye into surrounding cumulus cells was monitored with an inverted fluorescence microscope to assess the functionality of gap junctions. This was analysed at the start 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 x 200.

Figure 6: Effect of 3D-PMC and IVM on human CEO morphology.
CEO's were 3D prematured for 24 hrs. 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: x 400 (Type I, II and IV) and x 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 % of CEOs according to the morphological pattern of their cumulus at the start and the end of 3D-PMC; **(B)** Graph illustrating the % 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. * = $P<0.05$.

Figure 7: Effect of PMC condition 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 with oocytes from 2D-PMC (PMC in microdroplets), in vitro control (no PMC) and in vivo control (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 ($P<0.05$).

Figure 1:

A) Mouse experiments



B) Human experiments

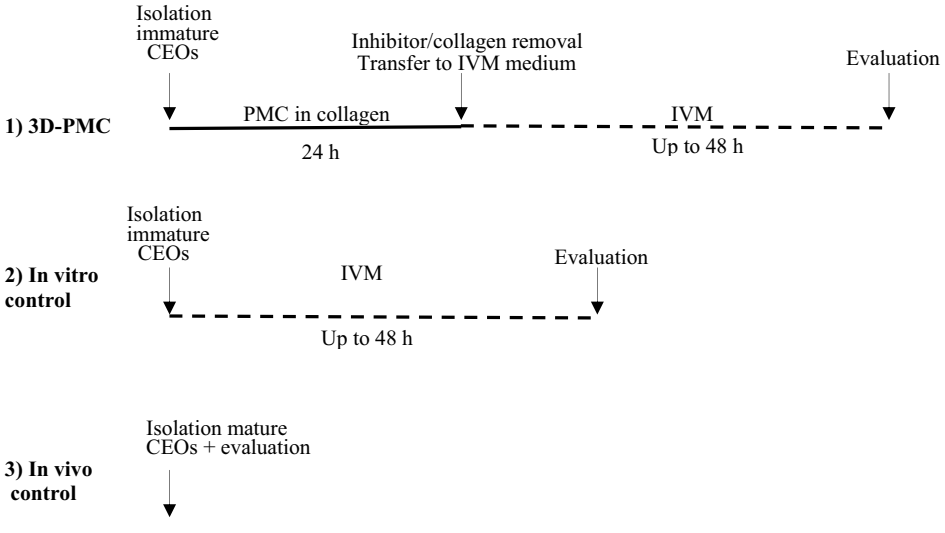
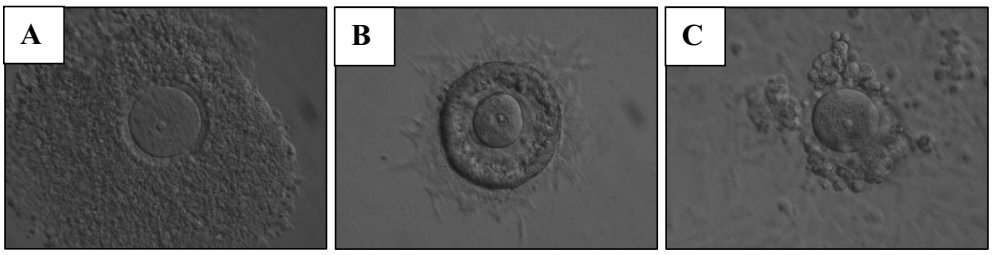


Figure 2:



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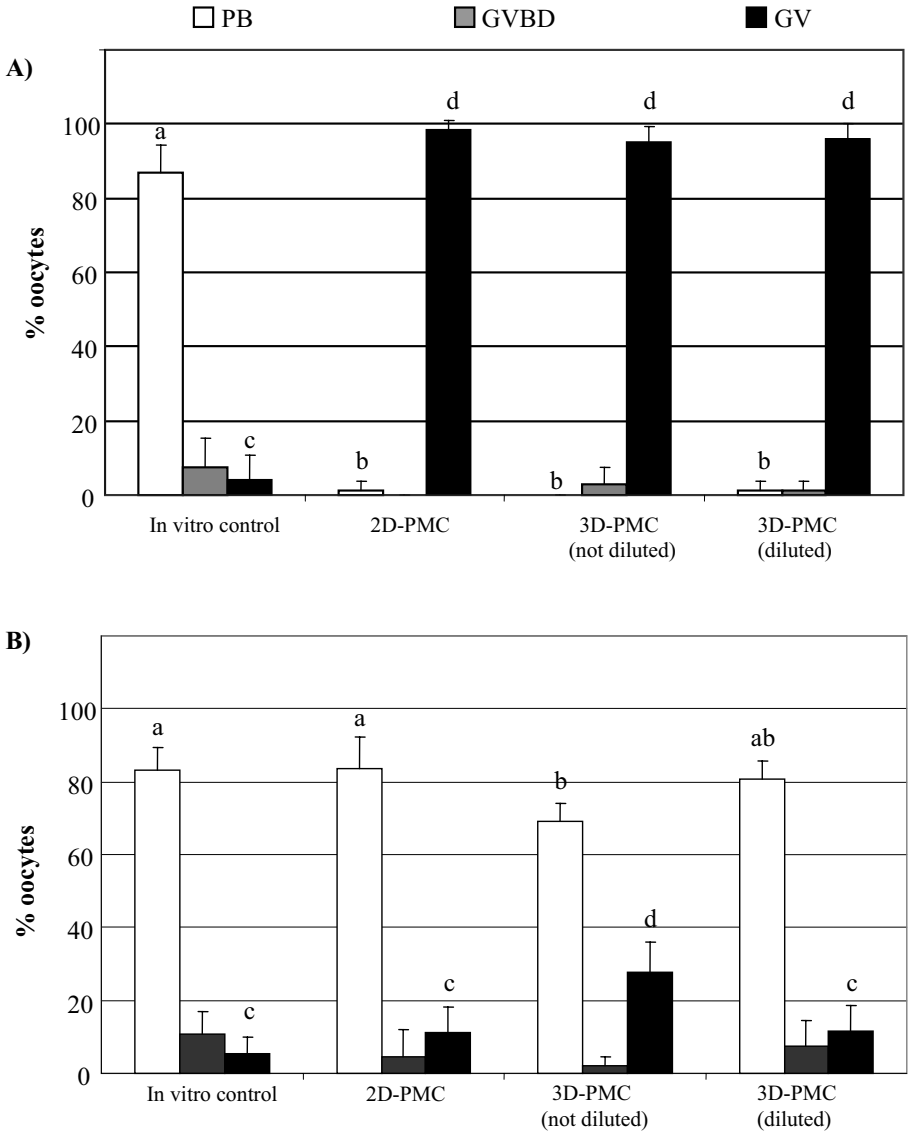

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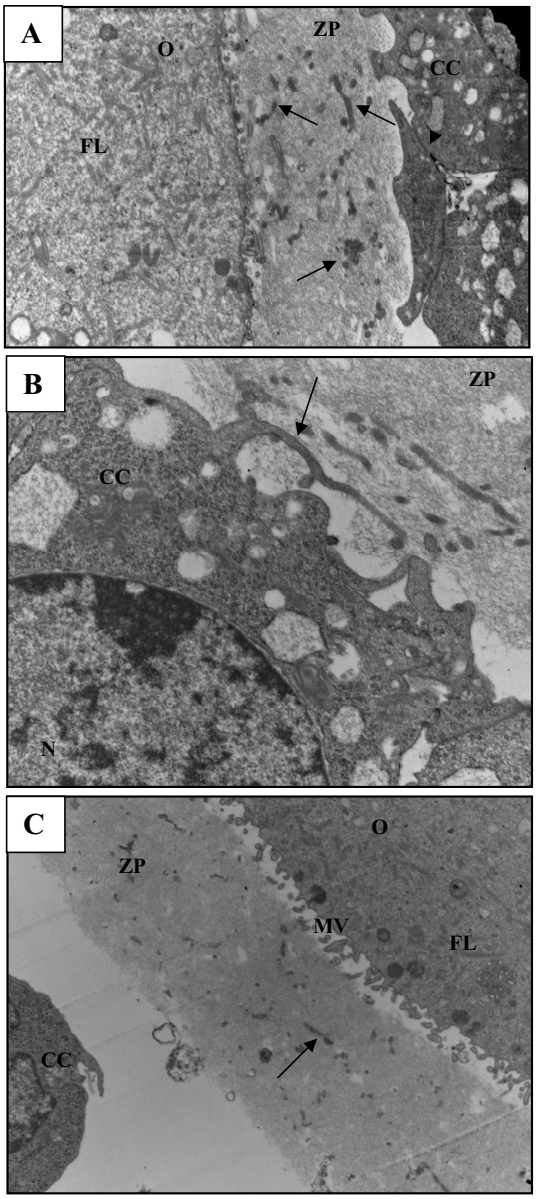

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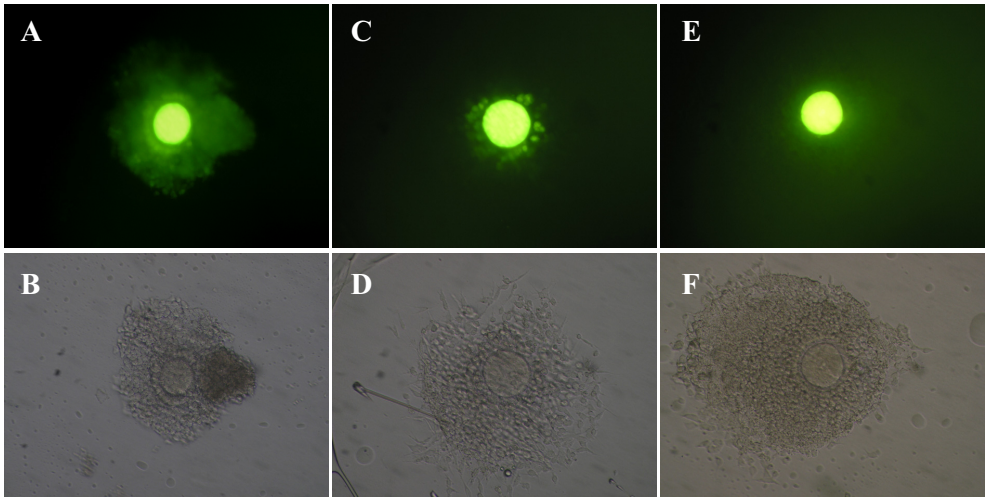
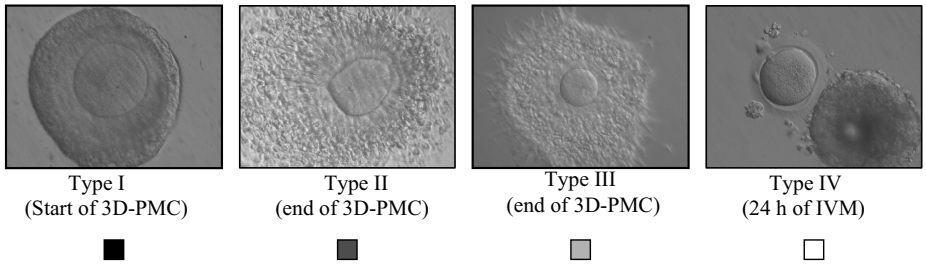
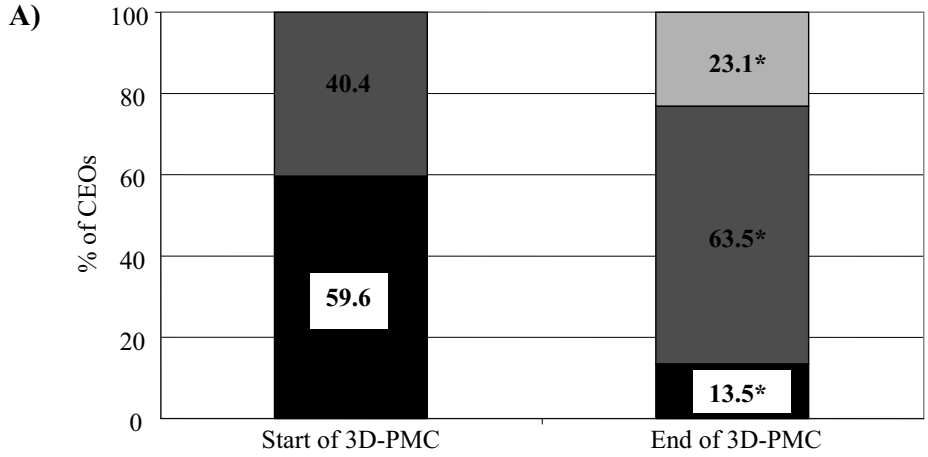


Figure 6:


Type I
(Start of 3D-PMC)
Type II
(end of 3D-PMC)
Type III
(end of 3D-PMC)
Type IV
(24 h of IVM)


A)
100
80
60
40
20
0
% of CEOs
40.4
59.6
23.1*
63.5*
13.5*
Start of 3D-PMC
End of 3D-PMC

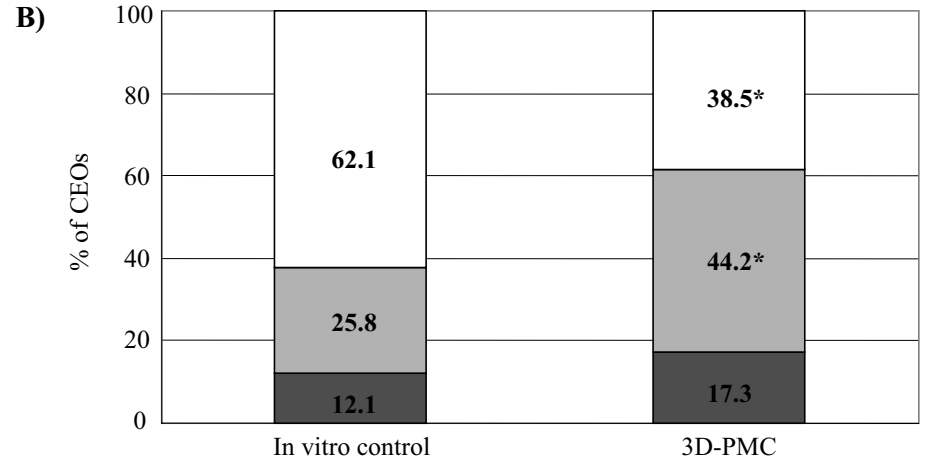

B)
100
80
60
40
20
0
% of CEOs
62.1
25.8
12.1
38.5*
44.2*
17.3
In vitro control
3D-PMC

Figure 7:

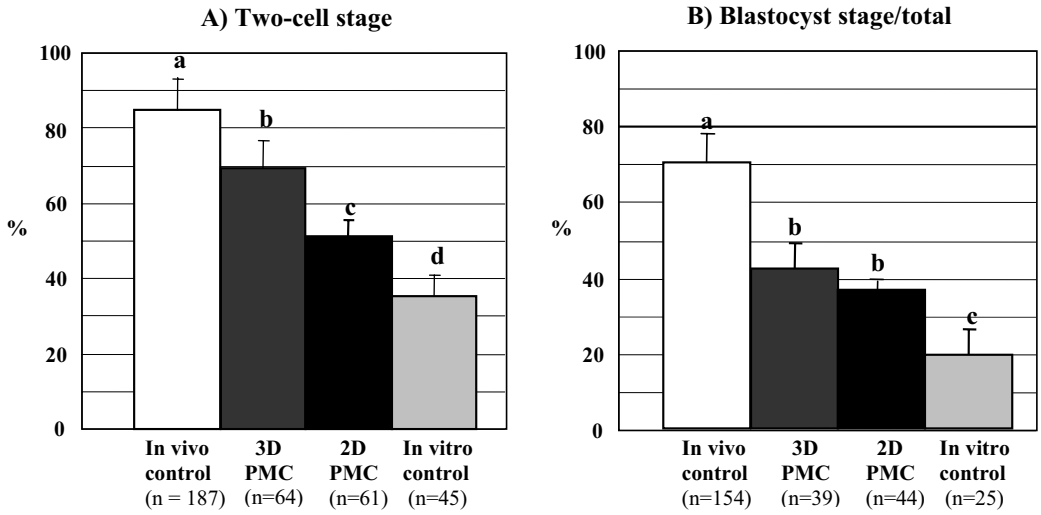


Table I. Functional coupling between cumulus cells and oocytes in mouse CEOs at the start of PMC (0 h; positive control), at the end of 2D- and 3D-PMC (24 h) and after exposure to CBX (negative control)

Treatments	N° of CEOs analyzed	CEOs with **open** gap junctions	CEOs with **partially open** gap junctions	CEOs with **closed** gap junctions
Positive control	102	82.9 ± 3.1^{a}	15.2 ± 4.0^{a}	1.9 ± 1.8^{a}
2D-PMC	101	5.4 ± 5.8^{b}	52.8 ± 10.1^{b}	41.8 ± 4.3^{b}
3D-PMC	115	64.6 ± 2.0^{c}	29.1 ± 2.6^{a}	6.3 ± 3.2^{a}
Negative control	45	0^{b}	14.4 ± 5.1^{a}	85.6 ± 5.1^{c}

Results are presented as % ± SD

[a,b,c] Different superscripts within columns indicate statistical differences ($P<0.05$).

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

	Culture conditions	
	In vitro control	3D- PMC
N° (%) of GV after inhibition		49/52 (94.2)
N° of GV undergoing IVM	66	49
Maturation at 24 h of IVM (%)		
GV	37.9	36.7
GVBD	43.9	46.9
PB	18.2	16.3
Degenerated	0	0
Maturation up to 30 h of IVM (between 25 and 30 h) (%)		
GV	30.3[a]	12.2[b]
GVBD	10.6	22.4
PB	57.6	65.3
Degenerated	1.5	0
Maturation up to 48 h of IVM (between 31 and 48 h) (%)		
GV	27.3[a]	12.2[b]
GVBD	10.6	6.1
PB	60.6[a]	81.6[b]
Degenerated	1.5	0

[a,b] Values within the same row with different superscripts are statistically different (P<0.05).

Table III. ICSI-outcome of human PB oocytes following 3D-PMC and/or IVM.

	Culture conditions		
	In vivo control	In vitro control	3D-PMC
N° of injected PB oocytes	56	40	40
N° (%) of 2PN	43 (76.8)[a]	22 (55.0)[b]	27 (67.5)[ab]
Cleaved/2PN (%)	88.4	81.8	77.8
Good embryo quality on day 2 (%)	69.8	68.2	70.4
Good embryo quality on day 3 (%)	55.8[a]	27.3[b]	55.6[a]
Mean blastomere N°/embryo ± SD day 2	3.3 ± 1.02	2.9 ± 0.88	3.2 ± 1.08
Mean blastomere N°/embryo ± SD day 3	5.8 ± 1.61[a]	4.4 ± 1.66[b]	5.7 ± 2.03[a]

[a,b] Values within the same row with different superscripts are statistically different (P<0.05).

4. General discussion

In vitro maturation (IVM) holds great promise as a potential alternative to classical ART. This technology may even become the procedure of choice, not only for infertile patients but also for obtaining oocytes for donation and preservation of fertility. With the assistance of the cloning technology, *in vitro* matured oocytes could generate embryos for derivation of stem cell lines. However, IVM is still premature and needs to be further developed in order to be as efficient as the routinely used IVF technology.
A major difficulty in developing the technique into a safe and reproducible procedure is the lack of human experimental material. The strategies to be further applied in the human could therefore benefit from experiences obtained in animal studies. In the present research project, results acquired from mouse experiments were translated to humans.

Our main findings include that a 2-step culture composed of (1) a PMC with PDE3-I to induce temporarily nuclear arrest, followed by (2) conventional IVM is a reliable method to improve the quality of mouse cumulus-oocyte complexes (COCs) post-IVM. A similar approach proved to be beneficial for the IVM of human cumulus-free oocytes in relation to stability of spindle morphology and chromosome alignment.
Furthermore, our results indicate that 3D co-culture of human cumulus-free oocytes with dissociated cumulus cells during PMC positively influenced the morphological quality and nuclear constitution of the resulting embryos.
Finally, 3D-PMC followed by IVM of mouse and human COCs, both retrieved from small antral follicles, preserves intra-cellular connections and influences developmental competence. Hence, this innovative 2-step approach with PDE3-I may be an interesting advancement to simulate more precisely the *in vivo* preovulatory maturation conditions.

4.1. Clinical outcome of conventional *in vitro* maturation (= 'rescue IVM')

Around 15 to 20% of human oocytes collected from large (≥ 15 mm) antral follicles following COH for IVF/ICSI treatment are meiotically immature, either at the GV or MI stage (Cha and Chian 1998; Smitz *et al.*, 2004). These oocytes are generally considered to be a side-product of the infertility treatment.
Partially mature MI oocytes have already undergone the process of GVBD and may progress spontaneously to the MII stage within a few hours of *in vitro* culture. This allows them to be submitted to ICSI at the same time as their sibling mature MII oocytes and the same day of sperm collection. As such, IVM might allow these oocytes to be "rescued" and be destined for patients use, which may be particular interesting when a low number of mature oocytes are retrieved. This hypothesis was tested in the first study of our research project. To achieve this goal, we selected ICSI cycles in which a maximum of 6 mature oocytes and at least one MI oocyte were obtained at oocyte retrieval.

In our laboratory, MI oocytes cultured *in vitro* for different periods (2-26 hrs) had the ability to resume meiosis, but the fertilizability and developmental competence was significantly reduced compared to *in vivo* matured sibling oocytes (Vanhoutte *et al*., 2005). These results confirmed the findings of previous studies on this subject (De Vos *et al.*, 1999; Chen *et al.*, 2000; Balakier *et al.*, 2004; Strassburger *et al.*, 2004). Nevertheless, the percentage of embryos transferred or cryopreserved per fertilized oocyte appeared to be relatively high in our study (76% = **total MI utilization rate**). One pregnancy and live birth was obtained out of 13 transfers of embryos exclusively derived from IVM oocytes (Vanhoutte *et al.*, 2005).
In view of the results obtained, we concluded that clinical applications of 'rescue IVM' should be recommended exclusively for selected patient populations, such as women with few mature oocytes (e.g. 'poor responders'). Furthermore, the oocytes' reduced developmental capacity confirmed that IVM culture conditions must be refined to support maturation *in vitro* properly.

4.2. Effect of prematuration culture in the presence of a phosphodiesterase type 3 inhibitor

Isolation of fully-grown GV oocytes out of small or medium-sized follicles truncates the cytoplasmic maturation process by the spontaneous resumption of meiosis. In addition, GV-stage oocytes isolated from follicles stimulated by exogenous gonadotrophins are heterogeneous in size, cytoplasmic microtubule organization, chromatin and nucleolar configuration (Mattson and Albertini, 1990; Zuccotti *et al*., 1998; Combelles *et al.*, 2002; De La Fuente, 2006).
Applying a 2-step culture might synchronise nuclear and cytoplasmic maturation and may reflect more closely the physiological situation. Recent results offer perspectives for the application of 'second-generation' specific PDE-Is to extend the developmental period of the immature oocyte (i.e. PMC) before final IVM (Nogueira *et al.*, 2003b, 2006; Thomas *et al.*, 2004). Hence, our laboratory has taken the approach to set-up a 2-step culture with PDE3-I in order to improve the IVM-outcome of human and mouse oocytes. Since MI oocytes (as used in Article I) are already in the process of nuclear maturation, we continued our experiments with germinal vesicle (GV)-stage oocytes in order to test the above described hypothesis.

4.2.1. Effect of prematuration culture on mouse cumulus-oocyte complexes

In our study on **mouse COCs** retrieved from immature antral follicles, we first identified the lowest effective concentration of the PDE3-I cilostamide (i.e. 1 μM) for maximum level of inhibition and reversibility of meiotic arrest. Thereafter, we aimed to clarify precisely the effect of PDE3-I on the maturation process itself, which is currently largely unknown (Vanhoutte *et al*., 2008).

One method to observe possible underlying cell cycle aberrations during IVM is to explore the **kinetics of meiotic progression** upon removal from the inducer of nuclear arrest. We found a higher synchrony in meiotic progression after PMC compared to spontaneously matured oocytes. This might be attributed to a better synchronisation of the starting population of GV oocytes. A synchronous pattern of meiotic progression was previously found to be a specific characteristic of oocytes that have been matured *in vivo* compared to *in*

vitro matured oocytes (Sanfins *et al.*, 2004). We also observed that the timing of meiotic progression in previously arrested oocytes was initially more rapid compared to *in vitro* non-arrested and *in vivo* control oocytes. The reasons for this acceleration are unclear, but it has been suggested that during the block of meiosis the oocyte might have extra time for the accumulation of factors related to cell cycle progression (Anderiesz *et al*., 2000). Alternatively, rapid meiotic progression may also be explained by a precocious migration of factors from the oocyte cortex (centrosomes, cytoplasmic microtubules, MPF), leading to uncontrolled G2 to M cell cycle transition (Sanfins *et al.*, 2004).

A potential biomarker of oocyte cytoplasmic quality is the **normalcy of the MII spindle structure and the integrity of their chromosome constituents**. The oocyte spindle is crucial for maintaining the fidelity of chromosome segregation at meiotic divisions. Alterations in spindle morphology are therefore indicators of adverse conditions during oocyte development that may result in meiotic aneuploidy (Eichenlaub-Ritter, 2002; Shen *et al*., 2007). Several studies on mouse oocytes confirmed that IVM conditions can have deleterious effects on the microtubular organization of the spindle (Sanfins *et al.*, 2003; Moon *et al.*, 2005; Roberts *et al*., 2005; Rossi *et al.*, 2006; Barrett and Albertini, 2007). It was therefore interesting to observe that a 2-step culture significantly improved the number of oocytes with normal MII spindle morphology. This might be a result of higher nucleation capacity of the centrosomes, tubulin assembly competence or microtubule stability. A quantitative analysis was performed as well by measuring spindle length and width. Spindles of *in vivo* matured oocytes were clearly shorter and smaller compared to 2-step cultured oocytes. It has been shown earlier that large, barrel-shaped spindles are formed during IVM as a result of the recruitment of massive amounts of centrosomal material during spindle assembly. *In vitro* matured oocytes also exhibit a lower number of cytoplasmic microtubule organizing centres (MTOCs) and present increased polar body size (Sanfins et al., 2003). This reduces the stores of available cytoplasmic gamma-tubulin necessary for early cleavage events during embryogenesis (Combelles and Albertini, 2001; Sanfins *et al.*, 2003, 2004; Barrett and Albertini, 2007). Hence, although a 2-step culture improved spindle morphology, the presence of enlarged spindles reflects a not yet fully optimized spindle assembly mechanism.

In addition, we investigated the possible risk of **aneuploidy** during MI by investigating chromosome preparations of 2-step cultured and PB extruded oocytes. Our analysis revealed that a 2-step culture with PDE3-I did not increase aneuploidy rates after IVM. Other structural aberrations, such as the presence of single chromatids, were not observed.

Finally, our results demonstrated that **developmental competence upon IVF** was increased in 2-step cultured oocytes compared to the control group. Not only the fertilization, but also the blastocyst formation rate was significantly increased in arrested oocytes. In a similar study of Nogueira *et al.* (2003b) beneficial effects on fertilization and blastocyst rates were observed, but only the results on fertilization rates were significantly improved using the PDE3-I Org 9935 (10 μM). This might indicate that the type of PDE3-inhibitor and the working concentration can exert different effects on IVM outcome.

All together, this study demonstrates that a PMC in the presence of PDE3-I alters the cytoplasmic quality into a direction more closely related to the *in vivo* situation. Nevertheless, it was noticed that cumulus cells started to lose the contact with the oocyte during PMC, a process that is associated with loss of gap junctional communication. Modifications of the current culture conditions, aiming for a better oocyte-cumulus contact during the 2-step culture, may result in a better outcome (see article V – Vanhoutte *et al*., 2009b).

4.2.2. Effect of prematuration culture on human cumulus-free oocytes

Because of the promising results on mouse oocytes (Vanhoutte *et al.*, 2008), we applied a similar 2-step culture with PDE3-I on human oocytes. Since human oocytes for research purposes is difficult to obtain, we used spare **cumulus-free germinal vesicle (GV)-stage oocytes** retrieved from superovulated women undergoing a regular ICSI attempt (Vanhoutte *et al.*, 2007).

Supplementation of the PMC medium with PDE3-I resulted in efficient meiotic arrest of the GV oocytes. The inhibitory activity was reversible, and oocytes underwent GVBD and extruded the first PB to the same extent as non-arrested oocytes. In contrast to the results obtained in the mouse (Vanhoutte *et al.*, 2008), the time course for PB extrusion was slowed down after PMC. This might be a result of the time necessary for the reconstitution of the PDE3 activity and the breakdown of cAMP prior to GVBD.
Cytoplasmic integrity of the 2-step cultured oocytes was judged by the **morphology of the second meiotic spindle and the associated chromosome configurations**. It has been demonstrated before that human oocytes matured *in vitro* have an increased incidence of spindle abnormalities and chromosomal misalignments compared with human oocytes matured *in vivo*, regardless of whether the immature oocytes are recovered from unstimulated (Racowsky and Kaufman, 1992; Li *et al.,* 2006) or hormonally stimulated ovaries (Cekleniak *et al.*, 2001; Wang and Keefe, 2002). In our study, we observed a tendency to obtain normal spindle/chromosome configurations when oocytes were prematured with PDE3-I at a concentration of 1 μM cilostamide.

The effect of a PMC period on **nuclear configuration within the GV** was analyzed as well. At the end of the PMC, most oocytes had acquired a nucleolus completely surrounded by a rim of highly condensed chromatin, while at the start of PMC heterogeneity in chromatin pattern was present. This illustrates that a synchronization within the mixed population of GV oocytes occurred during the course of prematuration. The presence of a surrounded nucleolus (SN) chromatin pattern has been associated with a higher proportion of oocytes reaching meiotic maturation, better embryonic development and, hence, represents a more advanced step in oocyte differentiation (Mattson and Albertini, 1990; Zuccotti *et al.*, 1998; De La Fuente, 2006).

The results in this section illustrate that applying a 2-step culture with PDE3-I can affect the quality of human oocyte. It should be stressed, however that our first study with PDE3-I in the human was performed using spare GV oocytes, which failed to mature *in vivo*. These oocytes had been disconnected from surrounding granulosa cells to score the oocyte maturation stage. It is questionable whether making use of these GV oocytes for IVM studies is a good choice. One way to approach a more physiological situation for IVM of these ‘left over’ GV oocytes is to restore the communication between the cumulus cells and the oocyte (see article IV – Vanhoutte *et al.*, 2009a).

4.3. Effect of three-dimensional prematuration culture in the presence of a phosphodiesterase type 3 inhibitor

Many of the practices commonly used in human ART lead to alterations in cumulus cell and/or zona integrity and therefore modify the immediate microenvironment of the oocyte. In this context, maintaining and/or re-establishing this microenvironment will be essential in the design and implementation of IVM systems.

4.3.1. Effect of three-dimensional prematuration culture on human cumulus-free oocytes

In the next study, we aimed to further optimize the 2-step 'rescue IVM' for **human cumulus-free oocytes** (see Article III - Vanhoutte *et al.*, 2007). To overcome the lack of cumulus cells during PMC and to simulate more accurately the natural 3D-environment of the COC, the oocytes were prematured in a **3D co-culture with cumulus cells embedded in an extra-cellular matrix** (ECM; collagen) (= 3D-PMC) (Vanhoutte *et al.*, 2009a). Parameters to asses oocyte quality were nuclear maturation, fertilization and pre-implantation embryonic development.

The principal findings from this study were a significantly higher fertilization rate of 3D-PMC oocytes compared to oocytes that underwent spontaneous maturation (= in vitro control). Although 3D-PMC did not augment the fertilization capacity when compared to oocytes prematured without cumulus cells and ECM (= 2D-PMC), embryos on day 3 post-ICSI from the 3D-PMC groups were of better quality and had fewer nuclear abnormalities compared to the in vitro control and 2D-PMC group. These results suggest that the combination of a co-culture in the presence of an ECM may serve as a physiological support during the last stages of oocyte development.

One of the objectives of this study was to analyze **any probable adverse effects of collagen-embedding procedure** on cell viability and functionality. Transmission electron microscopy (TEM) analysis could not reveal a negative effect on cell morphology in cumulus cells and the oocyte. Cumulus cells were capable of secreting estradiol and progesterone, which demonstrates that the cells retained their steroidogenic activity. The presence of the collagen-matrix did not reduce the effectiveness of the PDE3-I on keeping the oocytes arrested at the GV-stage. Upon release from meiotic arrest and ECM, oocytes were competent to resume meiosis and progress to the MII stage, which is an important functional end point of the culture system. Based on these observations we may conclude that cumulus cells and oocytes were able to withstand the cooling-warming step during embedding and polymerization of the collagen matrix.

Furthermore, we evaluated **oocyte-cumulus cell interactions**. A study by Veitch *et al.* (2004) demonstrated that denuded mouse oocyte/granulosa cell co-cultures can re-establish heterocellular coupling, reproducing the intercellular gap-junctional communication that exists *in vivo*. In our study, newly developed TZPs reaching the oolemma could not be observed. The reason for our improved results might, therefore, be explained by the existence of paracrine intracellular communication signalling, which could have compensate the absence of oocyte-cumulus cell contacts (Carabatsos *et al.,* 1998; Albertini *et al.*, 2001; Ge *et*

al., 2008). Another possibility to clarify our improved results on embryo quality post-IVM is the protective role that cumulus cells may play by reducing cystine to cysteine and promoting the uptake of cysteine in the oocyte (Takahashi *et al.*, 1993; de Matos *et al*., 1997). Via this pathway, cumulus cells may neutralize the damaging processes that reactive oxygen species may exert on oocytes.

In summary, this study illustrates that culture protocols for 'rescue IVM' requires a PMC in the presence of an appropriate *in vitro* environment, as we could establish by the 3D co-culture approach (Vanhoutte *et al.*, 2009a).

4.3.2. Effect of three-dimensional prematuration culture on mouse cumulus-oocyte complexes

In our study on mouse COCs, we observed that a proportion of the oocytes had clearly lost cumulus-oocyte contact by the end of the PMC (see Article II - Vanhoutte *et al.*, 2008). We thus tried to overcome this deficit by performing the first step of our 2-step culture (i.e. the PMC) in a 3D-environment. This was realized by embedding COCs in an ECM, the same collagen matrix as was used in the previous study on human cumulus-free oocytes (see Article IV - Vanhoutte *et al.*, 2009a).

This 3D-PMC system was initially tested and optimized in a **mouse model**. The first experiment was designed to define the **optimal concentration of collagen** in order to preserve COC integrity without affecting subsequent meiotic competence. Collagen in undiluted form limited the expansion of the cumulus cell-layers and affected oocyte maturation. In diluted collagen (1:2), on the other hand, COCs preserved their *in vivo*-like morphology and a general growth of the cumulus cell layers due to cell proliferation was observed. This preliminary experiment illustrates that 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 if the **intra-cellular communication** between the two different cell types, cumulus cells and oocytes, were preserved at the end of 3D-PMC. To examine this, a Lucifer Yellow (LY) dye coupling assay was performed. The presence of functional gap-junctions between oocytes and surrounding cumulus cells was significantly higher at the end of 3D-PMC compared to the 2D-PMC group. This suggests that the ECM act as a scaffold to preserve 3D morphology and, as a result, the functional integrity of the COCs. Our results showed also that the culture conditions were not sufficient to maintain functional communication during the entire period of PMC, since the percentage of COCs with open communications was significantly lower at the end of 3D-PMC compared to the start. Hence, further fine-tuning of the current culture system may be required. In this respect, the composition of the ECM may be subject of investigation. Another possibility is to manipulate cAMP levels in a different way, for example by the addition of an adenylate cyclase activator in combination with PDE3-I. This approach has previously shown to promote the gap-junctional cross-talk between the germinal and somatic compartment (Thomas *et al.* 2004, Luciano *et al.*, 2004; Shu *et al.*, 2008).

Mature mouse oocytes obtained after 3D-PMC and IVM could be **fertilized** and sustained **preimplantation development** *in vitro*. An increase in oocyte competence was reflected in higher cleavage rates compared to non-arrested spontaneously matured oocytes and 2D-PMC cultured oocytes. Elimination of cumulus cells from COCs prior to IVM has previously

shown to decrease sperm penetration rates in mice (Schroeder and Eppig, 1984). 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).

4.3.3. Effect of three-dimensional prematuration culture on human cumulus-oocyte complexes

Thereafter, we translated the work we had performed in mice to **human COCs**. Since 'true IVM' is not performed on a routine basis in our Department of Reproductive Medicine, we asked patients to donate immature COCs 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 aspirated for the infertility treatment. Nevertheless, they are a valuable source of research material to study different IVM culture conditions (Nogueira *et al.*, 2006).
Results from 3D-PMC cultured COCs were compared with a group of non-arrested, conventionally matured oocytes (*in vitro* control). Polar body-extruded oocytes, retrieved from small follicles of the same patients, were included as *in vivo* controls.

This part of the study focused first of all on **oocyte-cumulus morphology**. A previous study of Nogueira *et al.* (2003a) performed on the same type of human COCs demonstrated that around 65% of oocytes became partially denuded within 24-48 h of IVM culture. This figure corresponds with what we observed in our *in vitro* control group. In the 3D-PMC group, however, less than 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 slower breakdown of connections during the second step of our culture system.

The 2-step 3D-PMC culture had a beneficial effect on human oocyte developmental capacity. This was visible by an **increased nuclear maturation rate** in 3D-PMC cultured oocytes versus spontaneously matured ones.

Finally, an **improvement in embryonic developmental quality** was obtained as well. The 2-step culture did not affect fertilization, cleavage rates or embryonic quality on day 2 post-ICSI. On day 3, however, 3D-PMC resulted in a significantly higher proportion of embryos with less fragmentation and more blastomeres, similar to the *in vivo* matured group and superior to the *in vitro* control.

This indicates that systems involving PMC with a PDE3-I using culture methods that preserve the 3D architecture of the COC holds promise. This is possible through the use of appropriate collagen gel culture environments. The system can be adapted to support IVM from different species (Vanhoutte *et al.*, 2009b).

5. Perspectives for future research

"Research is what I'm doing when I don't know what I'm doing"
Wernher von Braun

Our understanding of the factors regulating oocyte developmental competence is gradually improving and these insights are slowly translating into progressive enhancement of IVM efficiency. The work presented in this thesis is only one more step on a long road. Many pieces from the puzzle are still missing and substantial adaptations are needed before the '2-step culture' principle can be considered as a valuable strategy in human IVM.

There are several points that remain to be investigated in the near future.

Though our work has contributed towards gaining more insight into the influence of the PDE3-I and 3D culture on oocyte development, mechanisms of PDE3 activation itself and signalling pathways of the oocyte-cumulus cell communication axis remain unknown. Measurement of cAMP concentration could reveal if the positive effects we observed can be explained by a rise in intra-oocyte cAMP concentration. Therefore, a lot of research remains to be done at the molecular level. One of the challenges is the integration of this information into coherent physiological theories on the mechanisms governing oocyte developmental competence.

Also the questions regarding the effect of our newly developed 2-step culture on blastocyst formation and normal fetal development to term remain unanswered. The ability to resume meiosis, to fertilize and to develop into a blastocyst, to induce pregnancy and to generate healthy offspring are all separate events and succeeding in the first event does not ensure the success of subsequent ones (Sirard *et al.*, 2006). It is obvious that, before any clinical application, complete testing of possible increased risks of aneuploidy is necessary. In addition, further studies in large mammals involving embryo transfer might reveal possible epigenetic risks that this technique could induce.

Another consideration that was left unhandled is the choice of culture medium. It is still unknown what constitutes an ideal nurturing medium for oocyte maturation. Although a variety of culture media have been described in the literature, no single system has been shown to be clearly superior (Trounson *et al.*, 2001). Oocyte maturation media are usually formulations designed many years ago for culture of non-ovarian somatic cells. This is the case for the most widely employed oocyte IVM media used today, Tissue Culture Medium-199 and Minimal Essential Medium. There are no studies that directly correlate the metabolic needs of the complex and dynamic requirements of the maturing COC with developmental outcomes (Sutton *et al*., 2003). Therefore, extensive studies on the effects of different types of basal media will be necessary. In this respect, it is possible that the medium composition during the arrest period (i.e. PMC) needs further specific supplements, which may be distinct from supplements needed during the nuclear maturation process (i.e. IVM).
Recent findings on bovine and mouse showed that IVM oocytes benefit from the exposure to exogenous oocyte-secreted factors (OSFs), resulting in improved embryo quality and better fetal survival post-transfer (Hussein *et al.*, 2006; Yeo *et al.*, 2008). These data generate

enthusiasm in the field of IVM, albeit that they are derived from animal models. Supplementing the PMC- and/or IVM-medium with exogenous OSFs (e.g. GDF-9 and BMP-15) may have a major impact on the results of IVM in both animal models and the human.

Further fine-tuning of the ECM composition for culturing the COCs is another 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 COC after PMC is not easy. Alternative substrates, such as the synthetic 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. 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). Finally, the concept of using matrix components normally found in the cell system of interest in the body is gaining interest in other fields. A diversity of ECM molecules is present in ovarian follicles (Rodgers *et al.*, 2003) and proteoglycans, laminin, collagen IV and fibronectin are all present in the hyaluronan-rich matrix of the COC (Zhuo and Kimata, 2001). In this way, the ECM composition could be adapted more closely to the natural intra-follicular *in vivo* situation of the COCs.

Finally, defining objective and non-invasive parameters to predict oocyte quality is of critical importance for future research on IVM. Morphological parameters such as polar body extrusion, spindle morphology, fertilization rate and embryo quality are routinely used, but are often subjective and controversial. Gene expression profiling of cumulus cells may indirectly provide novel and reliable parameters to assess oocyte competence. These cells are easily accessible and their capacity to predict oocyte quality is now emerging (Assidi *et al*., 2008; Bettegowda *et al.*, 2008). With the advent of the functional genomics and metabolomics era, it has become possible to identify the transcriptome of granulosa cells using throughput technology such as the micro-array. Future efforts should focus on integrating these genetic markers as parameters for oocyte/embryo selection, not only in the field of IVM but also for routine IVF/ICSI applications at large. However, consistent molecular markers predictive of oocyte competence are often lacking. Therefore, more comprehensive studies are needed in clinical settings to establish and standardize objective molecular markers.

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Curriculum Vitae

De auteur van dit proefschrift werd geboren op 14 september 1978 te Kortrijk. Na het behalen van het diploma Techniek Wetenschappen aan het St-Vincentiusinstituut te Anzegem in 1996, begon zij aan de studie Industrieel Ingenieur Chemie (optie Biochemie) aan de Hogeschool Gent. Vanaf 2000 was zij werkzaam in het Centrum voor Reproductieve Geneeskunde (eenheid Folliculaire Biologie (FOBI)) van het UZ Brussel. Onder leiding van Prof. Dr. Johan Smitz en Mevr. Rita Cortvrindt was zij betrokken in verschillende onderzoeksprojecten rond folliculaire cultuurtechnieken en *in vitro* maturatie van eicellen. Sinds 2003 werkte ze in de Afdeling Reproductieve Geneeskunde, UZ Gent. Onder leiding van Prof. Josiane Van der Elst en daarna Prof. Petra De Sutter heeft zij onderzoek verricht naar verschillende cultuurmethodes voor *in vitro* maturatie van eicellen, zowel bij de mens als de muis. De resultaten van het doctoraatsonderzoek staan beshreven in dit proefschrift.