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 mM cilostamide and (iii) medium supplemented with 10 mM 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 mM) could maintain >80% of the oocytes at the GV stage, without affecting subsequent maturation to metaphase II. Oocytes exposed to 1 mM 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.
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 (Tsafiri 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 (Vanhouthe 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 \(\sim 1\) 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 \(-20^\circ\)C) at final concentrations of 1 and 10 \(\mu\)M.

The IVM culture medium consisted of tissue culture medium 199 (TCM 199; M2154) supplemented with 10 ng/ml epidermal growth factor (E9644), 1 \(\mu\)g/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 (P4867) and 100 \(\mu\)g/ml streptomycin sulphate (S1277).

All immature oocytes were cultured singly in 25 \(\mu\)l drops of medium overlaid with embryo-tested light mineral oil in a humidified atmosphere at 5% \(\text{CO}_2\) 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 \(\mu\)M cilostamide and (iii) IVM medium supplemented with 10 \(\mu\)M 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 \(\mu\)M 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-\(\alpha\)-\(\beta\)-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 microslide slides in 90% glycerol-phosphate-buffered saline solution containing 0.2% 1,4 diamobicyclo[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.

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 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

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Control</th>
<th>1 μM cilostamide</th>
<th>10 μM cilostamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (%) of GV after inhibition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of GV undergoing IVM</td>
<td>43</td>
<td>56/64 (88)</td>
<td>51/60 (85)</td>
</tr>
<tr>
<td>Maturation at 24 h of IVM (%)</td>
<td>26</td>
<td>21</td>
<td>25</td>
</tr>
<tr>
<td>GV</td>
<td>23a</td>
<td>48b</td>
<td>51b</td>
</tr>
<tr>
<td>GVBBD</td>
<td>51a</td>
<td>25b</td>
<td>22b</td>
</tr>
<tr>
<td>PB</td>
<td>12</td>
<td>16</td>
<td>24</td>
</tr>
<tr>
<td>GVBD</td>
<td>14</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td>PB</td>
<td>72</td>
<td>63</td>
<td>65</td>
</tr>
</tbody>
</table>

GVBD, germinal vesicle breakdown; PB, polar body.

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)

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

TI, Telophase I.
1Defined as a barrel-shaped spindle with slightly pointed, anastal poles. Percentages are calculated over the total number of metaphase II (MII) oocytes.
2Values within the same row with different superscripts are statistically different (P < 0.05).
3Defined as condensed chromosomes arranged on a compact plate at the equator of the spindle. Percentages are calculated over the total number of MII oocytes.
group ($P < 0.05$; Table II), but similar to the 1 $\mu$M cilostamide group.

On the basis of these results, a concentration of 1 $\mu$M 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...
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%; $P < 0.05$).

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 (Tsafri 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.

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.

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 ($P < 0.05$).
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. Polescope 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; Szollösi 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 chromosomes 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; Zucconi 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 ciliostamide (1 μM) proved to be beneficial for spindle/chromosomal configurations and allowed synchronization of the population of immature oocytes. Modification of the PMC to restore and sustain oocyte-cumulus 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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