Developmental Capability of Denuded Bovine Oocyte in a Co-Culture System With Intact Cumulus-Oocyte Complexes: Role of Cumulus Cells, Cyclic Adenosine 3′,5′-Monophosphate, and Glutathione

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ABSTRACT

Cumulus oophorus cells have been implicated in the regulation of female gamete development, meiotic maturation, and oocyte-sperm interaction. Nevertheless, the specific role of cumulus cells (CCs) during the final stages of oocyte maturation and fertilization processes still remains unclear. Several studies have been conducted in order to clarify the role of follicular cells using culture systems where denuded oocytes (DOs) were co-cultured with isolated CCs, or in the presence of conditioned medium. However, those attempts were ineffective and the initial oocyte competence to become a blastocyst after fertilization was only partially restored. Aim of the present study was to analyze the effect of the interactions between somatic cells and the female gamete on denuded oocyte developmental capability using a system of culture where CCs were present as dispersed CCs or as intact cumulus-oocyte complexes (COCs) in co-culture with oocytes freed of CC investment immediately after isolation from the ovary. Moreover, we analyzed the specific role of cyclic adenosine 3′-5′ monophosphate (cAMP) and glutathione (GSH) during FSH-stimulated maturation of denuded oocyte co-cultured with intact COCs. Our data confirm that denuded oocyte has a scarce developmental capability, and the presence of dispersed CCs during in vitro maturation (IVM) does not improve their developmental competence. On the contrary, the co-presence of intact COCs during denuded oocyte IVM partially restores their developmental capability. The absence of CCs investment causes a drop of cAMP content in DOs at the beginning of IVM and the addition of a cAMP analog in the culture medium does not restore the initial oocyte developmental competence. The relative GSH content of denuded oocyte matured in presence of intact COCs is consistent with the partial recovery of their developmental capability. However, the complete restoration of a full embryonic developmental potential is achieved only when DOs are co-cultured with intact COCs during both IVM and in vitro fertilization (IVF). Our results suggest that the direct interaction between oocyte and CCs is not essential during IVM and IVF of denuded oocyte. We hypothesize that putative diffusible factor(s), produced by CCs and/or by the crosstalk between oocyte and CCs in the intact complex, could play a key role in the acquisition of developmental competence of the denuded female gamete. Mol. Reprod. Dev. 71: 389–397, 2005.

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Key Words: denuded oocyte; cumulus-oocyte complex; cAMP; cysteamine; glutathione; secreted factors

INTRODUCTION

In the antral ovarian follicle, the cumulus oophorus is a group of closely associated granulosa cells (namely cumulus cells (CCs)), which plays a crucial role during oocyte growth and development, and ultimately during the maturation and fertilization processes in mammalian oocytes [reviewed in Tanghe et al. (2002)].

CCs exert important biological function: before ovulation they support oocyte cytoplasmic maturation and shortly after ovulation participate in the complex mechanisms controlling sperm–oocyte interaction (Tesarik, 1990). CCs communicate with each other and with the oocyte by means of an extensive network of gap junctions (Gilula et al., 1978), which permits the two-way transfer of small molecules such as nutrients and messengers between somatic and germ cells (Moor...
adenosine 3' monophosphate (cAMP). In particular, cAMP can inhibit or delay the resumption of meiosis, whereas a transient elevation of cAMP has been demonstrated to induce oocyte maturation (Dekel et al., 1988; Downs et al., 1988; Luciano et al., 1999, 2004).

Besides the direct interaction between oocyte and CCs, there is some evidence that factors secreted by CCs are very important in promoting oocyte maturation and acquisition of developmental competence. During natural gonadotropin-induced ovulation at mid-cycle, the meiotic resumption of the oocyte is accompanied with the expansion of cumulus oophorus cells, which is induced in vitro by FSH (Salustri et al., 1990b) via a mechanism that appears to be mediated by cAMP in rat and cow (Dekel and Kraicer, 1978; Ball et al., 1983). Expanded cumulus matrix is composed of glycosaminoglycans such as hyaluronic acid (Ball et al., 1982), which are secreted by CCs and that may contribute to the microenvironment essential to sperm capacitation and fertilization (Tanghe et al., 2003).

In bovine species, removal of CCs at the beginning of in vitro maturation (IVM) or shortly before in vitro fertilization (IVF) (Fukui and Sakuma, 1980; Zhang et al., 1995; Fatehi et al., 2002) has been shown to be detrimental to the correct oocyte maturation and fertilization.

Several studies have been conducted in order to clarify the specific role of follicular cells in the maturation and fertilization process using culture systems where DOs were co-cultured with isolated CCs, either in suspension or monolayer, or in the presence of conditioned medium (Hashimoto et al., 1998; Ikeda et al., 2000; Tanghe et al., 2003). However, those attempts were ineffective and the initial oocyte developmental competence to undergo fertilization and blastocyst formation was only limitedly restored (Geshi et al., 2000).

Aim of the present study was to analyze the interactions between somatic cells and the female gamete using a system of culture where CCs were added as intact COCs in co-culture with oocytes freed of cumulus investment prior to the beginning of IVM.

In particular, we analyzed the specific role of cAMP and GSH intra-oocyte contents during denuded oocyte FSH-stimulated maturation on their subsequent developmental capability.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO), unless otherwise stated.

Oocyte Collection, Selection, and In Vitro Maturation

Bovine ovaries were obtained from a local abattoir and transported to the laboratory, within 2 hr, in sterile saline (9 g NaCl/L) maintained at 32–34°C. As previously described, only ovaries with more than 10 follicles were processed for oocyte aspiration (Gandolfi et al., 1997). All subsequent procedures were performed at 36–38°C.

COCs were retrieved from 2–6 mm follicles with a 16-gauge needle mounted on an aspiration pump (COOK-IVF, Australia) with a vacuum pressure of 28 mmHg. The COCs were examined under a stereomicroscope, and only those medium-brown in color, with five or more complete layers of CCs and a finely granulated homogenous ooplasm were used. Selected COCs were then washed two times in M199 supplemented with HEPES 20 mM and 0.4% of bovine serum albumin (HM199) and two times in the maturation medium, according to the experimental design. The whole procedure was performed in approximately 30 min.

In accord to the experimental design, oocytes were cultured in basic maturation medium (bMM) that was TCM-199 supplemented with 0.68 mM L-glutamine, 25 mM NaHCO₃, 0.4% of bovine serum albumin, fatty acid free, and 0.2 mM of sodium pyruvate or complete maturation media (cMM) that was bMM with 0.1 IU/ml of human recombinant FSH (rhFSH, Gonal-F, Serono, Rome, Italy). Oocytes were matured in 500 µl of medium,
in four-well dishes (NUNC, VWR International, Milan, Italy), and incubated for 24 hr at 38.5 °C under 5% CO2 in humidified air. Groups of about 30 oocytes were cultured either as intact COCs or DOs, obtained by mechanical removal of CCs from COCs by the use of vortex (2 min, 35 Hz) after the selection procedure.

According to the experimental design, groups of DOs were co-cultured either in presence of intact COCs or dispersed CCs. For the former, about 15 COCs and about 15 DOs were co-cultured in a 1:1 ratio during IVM (Fig. 1). For the latter, about 30 DOs were cultured in the presence of dispersed CCs obtained by mechanical removal from 15 complexes, as above described. DOs co-cultured in the presence of intact COCs during IVM are indicated as coDOs while those cultured in presence of CC are indicated as DOs + CC.

**In Vitro Fertilization**

After maturation, oocytes were fertilized as previously described (Luciano et al., 1999). Briefly, the content of a straw of cryopreserved bull spermatozoa (CIZ, S. Miniato Pisa, Italy) was thawed and cells separated on a 45–90% Percoll gradient. Sperms were counted and diluted to a final concentration of 0.5 × 106 spermatozoa/ml in fertilization medium that was TALP supplemented with 0.6% (w/v) BSA fatty acid free, 10 μg/ml heparin, 20 μM penicillamine, 1 μM epinephrine, and 100 μM hypotaurine. Groups of about 30 COCs or DOs or coDOs (Fig. 1A), were incubated in 300 μl of fertilization medium for 18 hr in four-well plates at 38.5 °C under 5% CO2 in humidified air. Before IVF, DOs matured in presence of intact COCs (coDOs) were separated from COCs by using a narrow-bore pipette.

According to the experimental design, groups of about 30 coDOs were maintained in co-culture also during IVF. This group is indicated as coDOF.

**Embryo Culture**

Embryo culture medium was a modified synthetic oviduct fluid (SOF (Tervit et al., 1972)), buffered with 25 mM of NaHCO3, supplemented with MEM essential and nonessential aminoacids, 0.72 mM of sodium pyruvate, 2.74 mM of myo-inositol, 0.34 mM of sodium citrate, and with 5% of calf serum (SOF-C). Embryo manipulation medium was SOF supplemented with 0.5% (w/v) BSA fraction V, fatty acid free, MEM essential and nonessential aminoacids, 0.72 mM of sodium pyruvate, and buffered with 10 mM of HEPES and 5 mM of NaHCO3 (SOF-R).

At the end of fertilization, presumptive zygotes were vortexed for 2 min in 500 μl of SOF-R, rinsed two times, and then transferred in groups of about 30 in 400 μl of SOF-C under 400 μl of mineral oil. After IVF, coDOFs were separated from intact COCs by using a narrow-bore pipette before vortexing.

Incubation was performed in a culture box that was a small desiccators cabinet (Sigma-Aldrich cat #Z11,905–9) with an internal volume of 4 L, with a rubber gasket door for tight seal. The culture box was modified with inlet and outlet gas tubing and placed in a conventional 5% CO2 incubator maintained at 38.5 °C. The box was supplied for 30 min with humidified gas mixture composed by 5% CO2, 5% O2, and 90% N2 with a flux of 15 L/hr. After 48 hr post insemination, the number of uncleaved embryos was rapidly recorded and the 4-well plate was placed back in the culture box. The box was supplied for 30 min with humidified gas mixture as above indicated. At the end of culture period (186 hr post insemination), blastocyst rate was assessed and embryos were fixed in 60% methanol in PBS. Cell nuclei were counted under fluorescence microscopy after staining with 0.5 mg/ml of propidium iodide.

**Determination of Intracellular Concentration of cAMP in Oocyte**

Intra-oocyte cAMP content was determined before maturation, and after 3 hr of culture either in COCs, DOs, or coDOs. COCs were denuded by vortex in SOF-R in which was included 0.5 mM of IBMX in order to preserve the oocyte cAMP concentration (Luciano et al., 2004). The complete removal of CCs from oocytes was monitored using a stereomicroscope. DOs and coDOs were washed two times in the same buffer containing IBMX. Finally, oocytes were washed two times in fresh SOF-R in the absence of IBMX, transferred in a minimum volume of washing buffer ranging between 3 and 5 μl in an eppendorf tube, snap frozen in liquid nitrogen and stored at −20 °C until assayed. Intracellular concentration of cAMP was determined by competitive enzyme immunoassay system (EIA, Biotrak, Amersham Life Science, Milan, Italy) with acetylation protocol for highest test sensitivity, according to the procedure provided with the kit. At the end of the procedures, optical density of samples was determined in a plate reader at 450 nm within 30 min. Each sample was tested in triplicate in each experiment. The assay was validated by adding increasing numbers of oocytes (20, 50, and 100) to a constant volume of extraction medium and recovering a proportional amount of cAMP.

![Fig. 1. Co-culture system of denuded oocytes in presence of intact COCs during IVM.](image)
Determination of Intra-oocyte GSH Concentration

After isolation, oocytes were matured in cMM, in presence or absence of cysteamine, either as COCs, Dos and coDOs. At the end of IVM, COCs were stripped free of CCs and washed three times in PBS containing 1 mg/ml of PVA. Groups of 5–15 matured oocytes were transferred under a stereomicroscope in the bottom of an eppendorf tube with a narrow-bore pipette and the buffer was successively removed by aspiration. Samples were snap frozen and stored at −80°C until assayed. The oocyte GSH content was determined by 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB)-GSH reductase recycling micro-GSH assay according to Baker et al. (1990) and P.J. Hansen, University of Florida, (online protocol: http://www.animal.ufl.edu/hansen) with slight modifications. Briefly, oocytes were thawed on melting ice and 50 μl of deionized water were added to each sample. Each microtube was then frozen in liquid nitrogen and successively thawed. This procedure was repeated three times. Standards containing from 0.19 to 200 pmol in 50 μl were prepared in water, simultaneously with the samples. The samples and standards were kept on ice until being loaded in the microtiter plate. A volume of 50 μl of each sample and standards was added in a 96-well microtiter plate. Reaction mixture was freshly prepared with 0.15 mM of DTNB, 0.2 mM of NADPH, and 1.0 U/ml GSH reductase (final concentrations) in 0.1 M phosphate buffer, pH 7.8, supplemented with 1 mM of EDTA. Immediately, 0.1 ml was pipetted in each well and the plate was analyzed at 405 nm in a microtiter plate reader (SpectraCount, Packard, USA) with one initial mixing and repeated-reads functions at 2 min intervals for 30 min.

Experimental Design

Experiment 1: Effect of co-culture of DOs with intact COCs or dispersed CCs during FSH-stimulated IVM on the subsequent embryonic developmental competence. This experiment was aimed to assess the effect of CCs either as intact COCs or dispersed CCs in co-culture during FSH-stimulated IVM of DOs on their subsequent developmental capability. Part of selected COCs was denuded by vortex. Groups of DOs were matured either as DOs, coDOs, or in presence of dispersed CCs, either in bMM or cMM. Groups of intact COCs were matured in the same conditions, as control.

After maturation, oocytes were fertilized and presumptive zygotes were cultured as above described.

Experiment 2: Role of cAMP during denuded oocyte maturation. In the first part of the experiment, we analyzed the effect of co-culture of denuded oocyte on the cAMP content during 3 hr of IVM. Groups of DOs, coDOs, and intact COCs were cultured in cMM. A group of DOs was cultured also in bMM, in absence of hormonal stimulation, as control. After 3 hr of maturation, oocyte cAMP content in experimental groups was evaluated as above described.

Since FSH stimulation apparently did not induce an appropriate intra-oocyte cAMP concentration, in the second part of the experiment, we evaluated the effect of cAMP analog administration during IVM of co-cultured denuded oocyte. For this purpose, groups of coDOs were cultured in cMM supplemented with non-inhibitory concentration 0.1, 0.05, and 0.01 mM of dybutyril-cAMP (dbcAMP) (Homa, 1988). After maturation, coDOs were separated from intact complexes then fertilized and presumptive zygotes were cultured as above described.

Experiment 3: Role of GSH during denuded oocyte maturation. In the first part of the experiment, we analyzed the effect of the co-culture system and cysteamine administration on the intra-oocyte content of GSH at the end of maturation process. Groups of DOs, coDOs, and intact COCs were cultured in cMM in presence or absence of 100 μM of cysteamine. After 24 hr of maturation, oocyte GSH content was evaluated as above indicated.

In the second part of the experiment, we analyzed the effect of cysteamine administration during IVM of denuded oocyte, incubated either alone or in co-culture with intact COCs, on the subsequent embryonic development. Groups of DOs, coDOs, and intact COCs were matured in cMM in presence or absence of 100 μM of cysteamine. After maturation, oocytes were fertilized and presumptive zygotes were cultured. Cleavage and blastocyst rates were assessed as above described.

Experiment 4: Effect of co-culture during both IVM and IVF on embryonic development of DOs. In order to analyze the effect of co-culture during both maturation and fertilization processes on the developmental capabilities of denuded oocyte, groups of oocytes were matured as coDOs in cMM. After maturation, groups of coDOs were kept as coDOFs. After IVF, coDOFs were separated from intact complexes then presumptive zygotes were cultured and embryonic development was assessed as above described.

Statistical Analysis

All experiments were replicated five times. Results are expressed as mean ± SEM. Data were analyzed using one-way ANOVA followed by Fisher’s protected least significant difference test (SuperANOVA, Abacus Concepts, CA). Probabilities of less than 0.05 were considered statistically significant.

RESULTS

Experiment 1: Effect of Co-Culture of DOs With Intact COCs or Dispersed CCs During FSH-Stimulated IVM, on Embryo Developmental Competence

As reported in Table 1, the removal of CCs before IVM significantly decreased the number of oocytes that cleaved after 48 hr post insemination and the number of those that reached the blastocyst stage after 186 hr post insemination. The administration of FSH during IVM had no effect on the cleavage and blastocyst rates of DOs. The presence of dispersed CCs in the maturation
environment did not improve the developmental capability of DOs + CCs and their cleavage and blastocyst rates were similar to those of DOs. On the contrary, the presence of intact COCs during FSH-stimulated IVM, partially restored the developmental capability of DOs. Cleavage rate of coDOs was similar to that of intact COCs while blastocyst rate was higher than DOs (P < 0.001) but significantly lower than intact COCs.

The presence of the gonadotropin during oocyte maturation of intact COCs induced a cleavage and blastocyst rate of 94.1 and 34.8, respectively, while in its absence, intact COCs exhibited a developmental potential similar to that of DOs.

Experiment 2: Role of cAMP During Denuded Oocyte Maturation

The intra-oocyte content of cAMP significantly increased in all groups after 3 hr of IVM, compared to time 0 hr (Table 2). However, cAMP was significantly higher in oocytes cultured as intact COCs than denuded oocyte, either cultured alone (DOs), or in co-culture (coDOs).

The administration of dbcAMP during IVM of denuded oocyte co-cultured with intact COCs did not increase the percentage of embryos that reached the blastocyst stage of development at the end of culture (Table 3). All the concentrations of dbcAMP added to the culture media in fact resulted in a cleavage and blastocyst rate similar to coDOs matured in cMM alone, significantly lower than intact COCs.

**TABLE 1. Effect of Co-Culture of DOs With Intact COCs During IVM on the Subsequent Developmental Competence of Denuded Oocytes**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IVM medium</th>
<th>Presence of FSH</th>
<th>Total oocytes</th>
<th>% Cleaved</th>
<th>% Blastocyst on cleaved</th>
<th>% Blastocysts on total</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOs</td>
<td>bMM</td>
<td>–</td>
<td>146</td>
<td>86.9 ± 1.3a</td>
<td>11.1 ± 2.2a</td>
<td>9.6 ± 2.1a</td>
</tr>
<tr>
<td>DOs</td>
<td>cMM</td>
<td>+</td>
<td>145</td>
<td>85.6 ± 1.6a</td>
<td>10.1 ± 1.6a</td>
<td>8.6 ± 1.4a</td>
</tr>
<tr>
<td>DOs + CCs</td>
<td>bMM</td>
<td>–</td>
<td>132</td>
<td>82.3 ± 0.9a</td>
<td>7.6 ± 1.9a</td>
<td>6.2 ± 1.6a</td>
</tr>
<tr>
<td>DOs + CCs</td>
<td>cMM</td>
<td>+</td>
<td>128</td>
<td>78.8 ± 1.6a</td>
<td>7.3 ± 1.4a</td>
<td>5.8 ± 1.1a</td>
</tr>
<tr>
<td>coDOs</td>
<td>bMM</td>
<td>–</td>
<td>141</td>
<td>82.7 ± 2.6a</td>
<td>6.9 ± 0.5a</td>
<td>5.7 ± 0.6a</td>
</tr>
<tr>
<td>coDOs</td>
<td>cMM</td>
<td>+</td>
<td>143</td>
<td>93.9 ± 2.1b</td>
<td>23.5 ± 1.3b</td>
<td>22.7 ± 1.4b</td>
</tr>
<tr>
<td>COCs</td>
<td>bMM</td>
<td>–</td>
<td>148</td>
<td>83.5 ± 2.4a</td>
<td>11.5 ± 2.3a</td>
<td>9.8 ± 2.2a</td>
</tr>
<tr>
<td>COCs</td>
<td>cMM</td>
<td>+</td>
<td>159</td>
<td>94.1 ± 0.7b</td>
<td>36.9 ± 3.2c</td>
<td>34.8 ± 3.1c</td>
</tr>
</tbody>
</table>

Different superscripts (a–c) within columns indicate statistical differences (P < 0.05).

*Values are expressed as fmol/oocyte of cAMP (mean ± SEM).

Experiment 3: Role of GSH During Denuded Oocyte Maturation

Before maturation, the average of intra-oocyte GSH content was 5.71 pmol/oocyte (Table 4). The absence of CCs significantly reduced the GSH content in DOs after 24 hr of IVM. However, the presence of intact COCs in co-culture maintained the GSH content of coDOs significantly higher than DOs. The administration of 100 μM of cysteamine in the maturation medium induced a significant increase of intracellular GSH concentration both in DOs and coDOs. At the end of maturation, coDOs showed an oocyte GSH content similar to that retained before the beginning of maturation.

Intact COCs showed a significant increase of GSH content after culture and the addition of cysteamine induced the highest increase of intra-oocyte GSH level after 24 hr of IVM.

As reported in Table 5, the administration of cysteamine during IVM of DOs induced a significant increase of both the cleavage and the blastocyst rates, which resulted similar to those obtained in coDOs, but did not further improve the developmental capability of coDOs. No differences were observed between COCs matured in cMM and those treated with the GSH precursor during IVM. Finally, no differences were observed in the blastocyst cell number among all treatments.

**TABLE 2. Effect of Co-Culture of DOs With Intact COCs and rhFSH Administration During IVM on Intra-Oocyte cAMP Content After 3 hr of Culture**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IVM medium</th>
<th>Presence of FSH</th>
<th>Oocyte cAMP content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before culture</td>
<td></td>
<td></td>
<td>0.29 ± 0.014a</td>
</tr>
<tr>
<td>DOs</td>
<td>bMM</td>
<td>–</td>
<td>0.361 ± 0.042b</td>
</tr>
<tr>
<td>DOs</td>
<td>cMM</td>
<td>+</td>
<td>0.392 ± 0.021b</td>
</tr>
<tr>
<td>coDOs</td>
<td>cMM</td>
<td>+</td>
<td>0.385 ± 0.043b</td>
</tr>
<tr>
<td>COCs</td>
<td>cMM</td>
<td>+</td>
<td>0.497 ± 0.035c</td>
</tr>
</tbody>
</table>

Different superscripts (a–c) indicate statistical differences (P < 0.05).

*Values are expressed as fmol/oocyte of cAMP (mean ± SEM).

DISCUSSION

The results of the present study provide evidence that the addition of intact COCs, during both FSH-stimulated IVM and up to the end of IVF significantly improved the developmental capability of DOs. As shown in Table 6, the percentages of cleaved embryos and blastocysts obtained from coDOFs were significantly higher than those from DOs co-cultured with intact COCs only during IVM (coDOs), and similar to the percentages obtained in the control group (COCs).

No differences were observed in the number of nuclei per blastocyst among all treatments.
Experimental results indicate that the presence of FSH in the maturation media does not influence the cAMP concentration in DOs. On the other hand, oocytes removed from intact COCs, after 3 hr of culture in presence of FSH, showed the highest increase of cAMP concentration as previously observed (Luciano et al., 2004). The localization of FSH receptor in mammalian oocyte is still under investigation. In some species like human, mouse, and pig (Patsoula et al., 2001, 2003; Meduri et al., 2002), the presence of FSH receptor and/or its transcript has been demonstrated while in bovine species contrasting data have been obtained. Our results support previous studies where in bovine antral follicles, FSH receptors were detected on granulosa and CCs but not on the plasma membrane of the oocyte (van Tol et al., 1996). Furthermore, this supports recent findings demonstrating that an increase in cAMP, induced by FSH in ovarian somatic cells, is ultimately communicated from CCs to the oocyte via gap junctions (Webb et al., 2002).

After 3 hr of culture, an increase of cAMP concentration was detected in DOs, significantly higher than that retained by the oocyte at the beginning of culture. The increase of intra-oocyte cAMP was similar either in presence or in absence of FSH but it was significantly lower than in oocytes cultured as intact COCs. This observation could be explained by the fact that bovine as well as mouse oocyte possesses its own adenylate cyclase (Kuyt et al., 1988; Horner et al., 2003) and phosphodiesterase system (Tsafri et al., 1996; Thomas et al., 2002) being able to modulate autonomously the intracellular cAMP content (Thomas et al., 2002). While high levels of cAMP have been proposed as the regulatory mechanism to maintain the oocyte in meiotic arrest (Homa, 1988; Bilodeau et al., 1993), a moderate increase of intra-oocyte cAMP concentration has been suggested as regulatory signal for meiotic resumption (Tsafri et al., 1972, 1996; Luciano et al., 2004). However, in our culture system, the low developmental capability of denuded oocyte was not due to the absence of an adequate cAMP induction. In fact, none of the noninhibitory dbcAMP concentrations added to the culture media showed a restoring effect of the developmental competence in DOs.

In our co-culture system, intracellular GSH concentration seems to play a significant role in the acquisition of developmental competence of DOs. At the end of IVM, DOs showed a decrease of GSH level. However, when
Table 5. Effect of the Administration of Cysteamine 100 μM During IVM on Developmental Competence of Denuded Oocyte Matured in the Presence of Absence of Intact COCs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IVM ± cysteamine</th>
<th>Total oocytes</th>
<th>% Cleaved</th>
<th>% Blastocysts on cleaved</th>
<th>% Blastocysts on total</th>
<th>Blastocyst cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOs</td>
<td>–</td>
<td>145</td>
<td>86.6 ± 0.8a</td>
<td>7.7 ± 1.6a</td>
<td>6.8 ± 1.5a</td>
<td>89.1 ± 4.1</td>
</tr>
<tr>
<td>DOs</td>
<td>+</td>
<td>163</td>
<td>92.6 ± 0.8b</td>
<td>26.2 ± 1.6b</td>
<td>22.6 ± 1.3b</td>
<td>92.8 ± 3.9</td>
</tr>
<tr>
<td>coDOs</td>
<td>–</td>
<td>142</td>
<td>93.8 ± 1.9c</td>
<td>27.4 ± 2.8b</td>
<td>25.9 ± 3.0b</td>
<td>95.1 ± 9.2</td>
</tr>
<tr>
<td>coDOs</td>
<td>+</td>
<td>145</td>
<td>94.3 ± 0.9d</td>
<td>27.3 ± 3.1b</td>
<td>25.8 ± 3.1b</td>
<td>102.1 ± 7.2</td>
</tr>
<tr>
<td>COCs</td>
<td>–</td>
<td>144</td>
<td>97.6 ± 0.7e</td>
<td>36.4 ± 3.3c</td>
<td>35.9 ± 3.4e</td>
<td>99.4 ± 5.7</td>
</tr>
<tr>
<td>COCs</td>
<td>+</td>
<td>171</td>
<td>98.6 ± 0.5f</td>
<td>39.1 ± 1.9f</td>
<td>38.1 ± 2.1f</td>
<td>106.5 ± 6.3</td>
</tr>
</tbody>
</table>

Different superscripts (a–c) within columns indicate statistical differences (P < 0.05).

*Values are expressed as mean ± SEM.

cultured in presence of cysteamine, they were able to retain an intra-oocyte GSH content significantly higher than DOs. The ability of synthesizing GSH from its precursor cysteamine has been previously demonstrated both in cumulus enclosed oocyte and denuded oocyte in bovine (de Matos et al., 1997) and porcine (Yamauchi and Nagai, 1999). A positive effect of GSH content on oocyte developmental competence has been demonstrated in several mammalian species (de Matos et al., 1995, 2002, 2003; Gasparrini et al., 2003). The GSH accumulation during IVM of DOs and coDOs in our experiment could be related to their capability to reach the blastocyst stage of development after fertilization. While DOs cultured alone showed the lowest blastocyst rate, the presence of cysteamine or intact COCs during IVM of DOs induced a significant increase of developmental rate. In fact, oocyte GSH content and blastocyst rate of DOs matured in presence of cysteamine and DOs co-cultured with intact COCs (coDOs) were similar.

The presence of intact COCs during IVM was able to maintain the GSH concentration in coDOs significantly higher than in DOs matured alone and similar to the GSH content of DOs matured in presence of cysteamine. In fact, it could be hypothesized that the achievement of a minimal GSH concentration could promote an improvement of the developmental competence of DOs.

Over the oocyte ability to synthesize GSH from cysteamine precursor even in absence of CCs (de Matos et al., 1997), a direct role of intact COCs in the co-culture environment, in preventing a depletion of GSH storage of the denuded oocyte, cannot be ruled out.

However, the presence of COCs or the addition of cysteamine during IVM of denuded oocytes can recover only partially their initial developmental competence. Only the addition of intact COCs during both IVM and IVF promoted the complete restoration of the initial developmental competence of DOs.

It has been previously demonstrated that rat granulosa cells respond to gonadotropin stimulation by providing a factor(s) that regulate the rate of oocyte maturation and promotes the oocytes capability to undergo fertilization (Vanderhyden and Armstrong, 1990). Moreover, Biskov et al. demonstrated that CCs of mouse COCs secrete a meiosis activating substance when stimulated with FSH (Biskov et al., 1997). This heat-stable substance diffuses into the oocyte and in a paracrine way activates the resumption of meiosis not only in the whole complex but also in DOs. This could suggest that the intimate contact between the CCs and the oocyte is not essential for resumption of meiosis but this contact is needed for the initial production of the meiosis activating substance.

The complex of molecules, which constitute the extracellular matrix of CCs produced through the cumulus expansion or mucification, mediates the achievement of a full oocyte developmental capacity during maturation and fertilization (Zhuo and Kimata, 2001). During gonadotropin-induced ovulation at mid-cycle, the number of gap junction decreases in parallel with the meiotic resumption of the oocyte (Eppig, 1982; Larsen et al., 1987), and this event is accompanied by the progressive CCs expansion and hyaluronic acid synthesis. Both processes are induced in vitro by FSH (Salustri et al., 1990a) via a mechanism that appears to be mediated by cAMP in rat (Dekel and Kraicer, 1978) and cow (Ball et al., 1983). Mucification in vitro is consistent with hyaluronic acid synthesis starting at 3 hr from the

Table 6. Effect of the Presence of Intact COCs During IVM and IVF of Denuded Oocytes on Their Developmental Competence After Fertilization

<table>
<thead>
<tr>
<th>IVM Treatment</th>
<th>Total oocytes</th>
<th>% Cleaved</th>
<th>% Blastocysts on cleaved</th>
<th>% Blastocysts on total</th>
<th>Blastocyst cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>coDOs</td>
<td>149</td>
<td>92.9 ± 1.8</td>
<td>25.6 ± 2.9a</td>
<td>24.1 ± 3.0a</td>
<td>95.3 ± 5.7</td>
</tr>
<tr>
<td>coDOFs</td>
<td>157</td>
<td>97.1 ± 0.7</td>
<td>36.8 ± 2.2b</td>
<td>35.7 ± 2.1b</td>
<td>103.1 ± 7.5</td>
</tr>
<tr>
<td>COCs</td>
<td>159</td>
<td>96.1 ± 1.1</td>
<td>43.2 ± 2.1b</td>
<td>41.5 ± 2.1b</td>
<td>104.7 ± 5.3</td>
</tr>
</tbody>
</table>

Different superscripts (a–c) within columns indicate statistical differences (P < 0.05).

*Values are expressed as mean ± SEM.
beginning of maturation, to 18 hr of incubation with FSH, while in absence of this hormone, only 10% of hyaluronic acid is produced and cumulus expansion does not occur (Salustri et al., 1993). During the maturation process, the secretion and assembly of matrix molecules by CCs are temporally regulated by factors derived from both somatic cells and oocyte (Zhuo and Kimata, 2001). Further studies have identified an oocyte-secreted factor, growth differentiation factor-9 (GDF-9), a member of TGF-β superfamily, which is able to replace the oocyte to induce cumulus expansion (Elvin et al., 2000). It is noteworthy that GDF-9 knockout mice are completely unfertile, and folliculogenesis is arrested to stages preceding cumulus expansion (Dong et al., 1996), strongly suggesting that GDF-9 is the oocyte factor that controls the hyaluronic acid synthesis in CCs.

In conclusion, interactions between the cumulus-oophorus and the oocyte are implicated in the regulation of female gamete development, meiotic maturation, and oocyte-sperm interaction. Data from previous and present study support the central role played by CCs in the acquisition of the developmental competence of the oocyte also by promoting the accumulation of an adequate amount of GSH during IVM. However, the results obtained with our culture system indicate that the direct interaction between oocyte and CCs is not necessary during the maturation and fertilization processes and that presumable diffusible factor(s) produced by CCs and/or by the crosstalk between oocyte and CCs in the intact complex must be deeply investigated in the near future.

Finally, since the removal of CCs from oocyte or zygotes at various stages of development still represents a major limitation in female gamete manipulation techniques in ART, this system could provide a technical approach to IVM and IVF of female gamete after manipulation and CC removal as well as in vitro embryo production procedures of oocyte cryopreserved at the germinal vesicle stage (Modina et al., 2004).

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