Effects of myo-inositol on the in-vitro maturation and subsequent development of mouse oocytes

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BACKGROUND: The study aim was to assess whether the incorporation of myo-inositol (MI) into culture medium could improve oocyte maturation in vitro. METHODS AND RESULTS: We performed a controlled prospective study using female ICR strain mice superovulated with pregnant mare’s serum gonadotrophins. Cumulus-enclosed germinal vesicle (GV) oocytes were randomly cultured in medium with or without MI supplementation. The kinetics of GV breakdown after 4 h of incubation was significantly higher in oocytes incubated with 30 mmol/l of MI than in controls (P < 0.001). Accordingly, this concentration of MI was used for subsequent experiments. The proportion of metaphase II oocytes achieved after 24 h of culture, their fertilization and cleavage rates were significantly higher in the MI-treated group (P < 0.01, P < 0.05, P < 0.05 respectively). This group also demonstrated significant improvement in postimplantation development after transferring the 2-cell embryos to pseudopregnant mice. Confocal microscopy revealed spontaneous intracellular Ca2+ oscillations within competent GV oocytes and treatment with MI caused an earlier onset of these Ca2+ signals. CONCLUSIONS: Our results suggest that MI may affect meiotic progression of mouse GV oocytes possibly by enhancing the intracellular Ca2+ oscillations. Supplementation of MI in culture medium may be useful for human oocyte maturation.

Key words: embryo development/intracellular Ca2+ fluxes/mouse/myo-inositol/oocyte maturation

Introduction

Meiosis in mammalian oocytes is initiated in the fetal ovary and progresses through most of prophase I before becoming arrested at the diplotene stage to form the primordial follicles. After birth, the primordial follicles enter a growth phase that is independent of gonadotrophin concentrations. The oocytes enlarge in size and several surrounding layers of granulosa cells are formed. As the mouse follicles reach ~140 μm in size, fluid starts to fill the antrum and further development of these follicles now becomes gonadotrophin dependent (Hartshorne et al., 1994).

The resumption of meiotic maturation has been proposed to be under dual control involving a loss of inhibitory input and the generation of stimulatory signals (Downs, 1995). It is now established in many species that isolation of oocytes from their follicles can induce resumption of meiosis in vitro, possibly due to the removal of natural inhibitory constraints of the follicles (Eppig et al., 1994; Brzyski et al., 1999; Smith et al., 2000). However, overall pregnancy rates from in-vitro matured oocytes remain very low in most primates, including humans. This decrease in embryo viability may be related to poor oocyte quality after in-vitro maturation (IVM) (Gomez et al., 1993b; Schramm and Bavister, 1995; Smitt and Cortvrindt, 1999). The conditions for in-vitro oocyte maturation remain inferior to the in-vivo environment for reasons that are still not fully understood. Supplementation of different maturation media with growth factors and gonadotrophins has provided minor improvements in the IVM outcome in both animal and human oocytes (Gomez et al., 1993b; Boland and Gosden, 1994; Smitt and Cortvrindt, 1999).

The relevance of phosphatidylinositol (PtdIns) cycle activation to the generation of stimulatory signals for oocyte development has become more apparent in recent years (Downes, 1989; Berridge and Irvine, 1989; Fujiwara et al., 1993; Fissore et al., 1999). Activation can result from either hormonal or other stimuli such as growth factors, and involves a receptor-dependent hydrolysis of inositol phospholipid by phospholipase C to generate two second messengers, inositol 1,4,5-trisphosphate [Ins(1,4,5)P3] and diacylglycerol (DAG). These compounds activate separate but interacting pathways to produce finely tuned signals that help to regulate many types of cellular processes (Downes and Macphee, 1990). Ins(1,4,5)P3 is released into the cytosol and acts to mobilize intracellular Ca2+ (Berridge and Irvine, 1989; Downes, 1989; Downes and Macphee, 1990; Fujiwara et al., 1993). Thus Ca2+ has been suggested to play a role in the regulation of diverse cellular processes.
functions, including cell proliferation, and possibly enhance oocyte maturation (Berridge, 1993; Fujiwara et al., 1993; Mehlmann and Kline, 1994; Pesty et al., 1998).

Myo-inositol is the precursor of the inositol phospholipid that is responsible for the generation of these important intracellular signals (Downes and Macphee, 1990). A number of transport mechanisms for myo-inositol (MI) have been proposed and demonstrated in various cell types (Weigensberg et al., 1990; Fahy and Kane, 1993). Uptake of MI from culture medium is associated with improvement in preimplantation embryo development in a number of mammalian species (Hynes et al., 2000). However, the effect of MI on in-vitro oocyte development has not been fully investigated (Carrasco et al., 1990). There is increasing evidence that both the MI transporter (Matskevitch et al., 1998) and receptor for Ins(1,4,5)P₃ (Fissore et al., 1999) can be isolated and characterized in mammalian oocytes. We hypothesized that direct availability of MI to an oocyte may enhance its meiotic maturation and subsequent developmental competence.

The objective of this study was to investigate whether addition of MI to the maturation medium could enhance in-vitro meiotic maturation and developmental potential of cumulus-enclosed mouse GV oocytes. In addition, we examined the effect of MI on initiation of intracellular Ca²⁺ oscillations within GV oocytes by performing confocal laser scanning microscopy.

Materials and methods

Media and reagents

The handling of the harvested oocytes outside the CO₂ incubator was performed in M2 medium supplemented with 0.4% bovine serum albumin (BSA). The culture medium used for oocyte maturation and IVF was Eagle’s minimum essential medium (MEM) and M16 medium respectively. Both of these media were supplemented with 2 mmol/l glutamine, 100 IU/ml penicillin, 60 µg/ml streptomycin, 5% heat-inactivated fetal calf serum (FCS) and 0.23 mmol/l sodium pyruvate. All these compounds were purchased from Sigma (St Louis, MO, USA).

The fluorescent DNA dye (Hoechst No. 33342; Sigma), for confirmation of oocyte penetration by sperm, was dissolved in phosphate-buffered solution containing 4 mg/ml BSA to give a final concentration of 100 µg/ml and stored as aliquots frozen at −20°C until use. The formalin solution (4%) for initial fixation of the oocytes prior to fluorescent microscopy was purchased from Sigma.

The working solution of the fluorescent probe for Ca²⁺ study consisted of Fluo-3/AM (10 µmol/l) and Pluronic F-127 (0.044%) dissolved in 4.42 ml of M2 medium containing 0.1 mg/ml dibutyryl cAMP (dbcAMP; Sigma). This solution was freshly prepared before each experiment. Fluo-3/AM and Pluronic F-127 were purchased from Molecular Probes (Eugene, OR, USA). The TC chamber slides for confocal microscopy were purchased from Nunc (Nunclon, Nunc A/S, Kamstrupvej, Denmark) and poly-1-lysine was purchased from Sigma.

Animals and oocyte recovery

The animal studies were approved by the Animal Research Ethics Committee at the Chinese University of Hong Kong. Female 5–6 week old albino ICR mice were induced to ovulate using intraperitoneal injection with 10 IU pregnant mare’s serum gonadotrophin (PMSG; Sigma). The animals were killed 48 h later by cervical dislocation. Their ovaries were removed and immediately placed in pre-warmed M2 medium. Cumulus-enclosed oocytes (CEO) at GV stage were harvested by puncturing the large antral follicles with a 26-gauge needle. Denuded oocytes used for the kinetic study of GVBD and confocal microscopy were obtained by repeated pipetting of the CEO through a small-bore pipette to remove the surrounding cumulus cells.

Culture of immature oocytes

Fully grown CEO at GV stage were collected and thoroughly washed three times in fresh prewarmed M2 medium at 37°C. These oocytes were then cultured in a 50 µl drop of preincubated MEM under oil for IVM. This maturation medium (MEM) was either used as control medium (i.e. without the addition of MI) or the required amount of MI had previously been added and equilibrated in an atmosphere of 5% CO₂ in air at 37°C for 24 h before use.

Collection of in-vivo matured oocytes

Oocytes matured in vivo were collected from PMSG-primed mice 14–16 h after injection of 5 IU of hCG (Serono, Aubonne, Switzerland). The surrounding cumulus cells containing the metaphase II oocytes were flushed out from the oviducts/uteri of these superovulated mice with M2 medium supplemented with 0.4% BSA. These oocytes were subsequently subjected to IVF.

IVF and embryo transfer

Sperm were obtained from adult male ICR mice by epididymal extraction, followed by centrifugation for sperm capacitation. The final concentration of capacitated sperm was adjusted to ~1-2×10⁶ sperm/ml and then inseminated into the 50 µl drop of M16 medium (MI-free) containing the in-vivo and in-vitro matured oocytes. After 3–4 h of insemination, the inseminated oocytes were removed from the insemination medium and washed three times in M16 medium. These inseminated oocytes were cultured overnight in groups of 5–10 oocytes per 50 µl drop of M16 medium under oil at 37°C and 5% CO₂ in air. Fertilization assessment was performed in these oocytes over a maximum period of 18–20 h post-insemination. After scoring, these oocytes were cultured until 2-cell embryos were developed. These embryos were subsequently transferred to the oviducts of pseudopregnant ICR mice.

Assessment of postimplantation development

The recipient mice were killed by cervical dislocation on day 10 of gestation. The number of implantation sites and the percentage of normal embryo development were recorded. The assessment of normal postimplantation embryo development was based on criteria that we have reported previously (Tam et al., 1987). In brief, the embryos were dissected from the uterus and decidual tissue and placed in pre-warmed M2 medium set at 37°C. The Reichert’s membrane and most of the eutoplacental cone were then trimmed away to facilitate the observation of yolk sac circulation and heart beat of the embryo. Afterwards, the embryo was removed from the yolk sac and amnion for morphological scoring. Embryos were graded as normal when optimal development of various embryonic structures was observed together with a proper circulatory system.

Detection of intracellular Ca²⁺ oscillations

Immediately after recovery, oocytes were swiftly denuded by gentle suction of the CEO through a finely pulled glass pipette in pre-warmed M2 medium containing dbcAMP. The denuded oocytes were then incubated with a Ca²⁺-sensitive fluorescent dye Fluo-3/AM containing Pluronic F-127 and dbcAMP under oil and put in a dark chamber prewarmed at 37°C for 15 min. Control oocytes (n = 25) and MI-
treated oocytes ($n = 28$) were then placed into microdrops of M2 medium containing 0.5 $\mu$mol/l Fluo-3/AM under oil for subsequent fluorescence measurements. These microdrops were prepared on a TC chamber slide precoated with 1% poly-l-lysine (Sigma). Afterwards, the chamber was placed on a 37°C warm stage mounted on an inverted Zeiss microscope. The detection of $Ca^{2+}$ oscillations as seen in a change in fluorescence intensity was monitored by the use of confocal laser scanning microscopy (Meridian Ultima ACAS confocal laser scanning imaging system) equipped with a 7% transmittance neutral density filter, PMT optics (dichroic mirrors and interference filters) and $\times 10$ objective lens (S PlanApo).

**Experimental design**

**Experiment 1: To study the dose effects of MI on GVBD during meiotic maturation in mouse oocytes**

CEO at GV stage were cultured for 4 h either in MEM medium supplemented with MI at concentrations of 10, 20, 30, 40 and 50 mmol/l in the study group or without MI in the control group. The kinetics of meiotic maturation (GVBD) were assessed by monitoring the disappearance of GV in denuded oocytes at 30 min intervals over the 4 h period with an inverted microscope (Nikon, Diaphot). The assessment of GVBD at 2 and 4 h after in-vitro culture was chosen in accordance to the GVBD assay described by Pesty et al. (1994). The amount of MI corresponding to the maximal percentage of GVBD oocytes after 4 h of in-vitro culture was determined. This concentration was then used in supplementing the maturation medium for subsequent experiments.

**Experiment 2: To study the effect of MI on the fertilization and subsequent development of in-vitro matured mouse oocytes**

Based on the observations obtained in experiment 1, freshly collected CEO at GV stage were cultured for 24 h either in MEM medium supplemented with an optimal dose of MI (study) or without MI (control). The observation of oocytes that had undergone GVBD and the formation of the first polar body were taken as signs of completion of meiotic maturation. The subsequently obtained in-vitro matured oocytes together with the in-vivo matured ones were then placed in M16 medium (MI-free) and inseminated with capacitated sperm for 4 h. These inseminated oocytes were then washed thoroughly before placing in fresh M16 medium (MI-free) for overnight culture. In order to investigate the fertilization outcome of these in-vitro matured oocytes, the inseminated oocytes were checked for the signs of fertilization (2PN) over a maximum period of 18–20 h post-insemination. Those with no signs of fertilization and which failed to reach the 2-cell stage after further culture were subsequently taken as unfertilized oocytes. These unfertilized oocytes were then stained with fluorescent DNA dye (Hoechst No. 33342) for confirmation that sperm penetration had occurred, using the method as described by Spears et al. (1994). The number of 2-cell embryos developed was then noted before transferring them to the oviducts of ICR pseudopregnant mice on day 1 of gestation. Embryos developed from each group were transferred to a single pseudopregnant mouse. About six to eight of the 2-cell embryos were delivered to each oviduct of the recipient mouse.

**Experiment 3: To study the effect of MI on $Ca^{2+}$ oscillations during oocyte maturation**

Denuded GV-intact mouse oocytes from each group were initially arrested at prophase I by incubating in M2 medium containing dbcAMP and loaded with the fluorescence dye Fluo-3/AM at 37°C for 10 min. Prior to confocal microscopy, spontaneous maturation was initiated in synchrony by replacing the oocytes in fresh microdrops of M2 medium without the addition of dbcAMP. The whole of the TC chamber slide was then covered with oil and maintained at 37°C during the entire scanning procedure. Fluorescence measurements were performed every 10 s for a period of up to 1 h with a scan size of $300 \mu m \times 300 \mu m$ in area.

**Statistical analysis**

The calculation of the study sample size was based on the data obtained from an earlier study on the in-vitro development of mouse oocytes (Schroeder and Eppig, 1989). Using these values, the estimated sample size for each group in Experiments 1 and 2 was 100, in order to detect a 15% significant difference in proportion between the two groups with a power of 80% and a type I error of 5%. In accordance with a previous study of $Ca^{2+}$ oscillations in mouse oocytes (Lefevre et al., 1995), 20–30 denuded GV-intact oocytes were used for each group in Experiment 3. Each experiment was repeated four to eight times (Experiments 1 and 2) or five times (Experiment 3) to achieve the desired oocyte number for each experiment. The rates of GVBD, first polar body extrusion, the proportion of unfertilized oocytes that had sperm entry, rates of fertilization and 2-cell embryo formation, the number of implantation sites and of normal fetuses, were compared between study and control group by using the $\chi^2$-test or Fisher’s exact test where appropriate. $P < 0.05$ was considered statistically significant.

**Results**

**Experiment 1: Dose effects of MI on GVBD during meiotic maturation in mouse oocytes**

The kinetic study of GVBD was performed in GV oocytes cultured in MEM containing 0 (control), 10, 20, 30, 40 and 50 mmol/l of MI. The onset of GVBD was detected in both the control and MI-incubated GV oocytes soon after the beginning of the culture period. The dose effects of MI on meiotic resumption, as determined by the percentage of GV oocytes that had undergone GVBD, were determined after 2 and 4 h of in-vitro culture (Figure 1). As shown in Table I, the proportion of GV oocytes maturing to metaphase I oocytes (or undergone GVBD) was significantly higher in MEM supplemented with 20 and 30 mmol/l of MI compared with the controls after 2 h of culture (69.2 versus 52.2%, 74.1 versus 52.2% respectively; $P$
Moreover, by 4 h of in-vitro culture, the group containing 30 mmol/l of MI had the highest proportion of GVBD oocytes (94.1 versus 68.7%; \( P < 0.001 \)). Based on these rates of GVBD, the concentration of MI used in subsequent experiments was 30 mmol/l. The group containing 50 mmol/l of MI showed a significantly lower proportion of oocytes that could undergo in-vitro maturation compared with controls (51.7 versus 68.7%, \( P < 0.05 \)) (Table I).

**Experiment 2: Effects of MI on the outcome of IVF of in-vitro matured mouse oocytes**

When CEO at GV stage were cultured in MI-free MEM medium, the rate of maturation, as judged by the disappearance of GV (GVBD) and first polar body extrusion was 73.6%. A significantly higher rate of maturation was observed when GV oocytes were cultured in MEM medium supplemented with 30 mmol/l of MI (90.0%). As shown in Table II, both the fertilization rate and the proportion of 2-cell embryos developed from fertilized oocytes were also significantly higher in the study group compared with those obtained from the control group (67.5 versus 50.6%, 75.9 versus 53.6% respectively; \( P < 0.05 \)). The proportion of non-activated oocytes that had sperm penetration was significantly higher in the control group (Table II).

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### Table I. Effect of different concentrations of myo-inositol (MI) on the maturing (GVBD) outcome of mouse oocytes after 2 and 4 h of in-vitro culture

<table>
<thead>
<tr>
<th>MI concentrations (mmol/l)</th>
<th>No. of GV oocytes cultured</th>
<th>No. of maturing oocytes after 2 h (%)</th>
<th>No. of maturing oocytes after 4 h (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>115</td>
<td>60 (52.2)</td>
<td>79 (68.7)</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>60 (60.0)</td>
<td>78 (78.0)</td>
</tr>
<tr>
<td>20</td>
<td>130</td>
<td>90 (69.2)(^a)</td>
<td>108 (83.1)(^b)</td>
</tr>
<tr>
<td>30</td>
<td>135</td>
<td>100 (74.1)(^c)</td>
<td>127 (94.1)(^d)</td>
</tr>
<tr>
<td>40</td>
<td>135</td>
<td>85 (63.0)</td>
<td>88 (65.2)</td>
</tr>
<tr>
<td>50</td>
<td>120</td>
<td>50 (41.7)</td>
<td>62 (51.7)(^d)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.
\(^a\)Data were pooled from eight replicate experiments. All data are expressed as the number of positive observations.
\(^b,c,d\)In each column, values with different superscripts are significantly different from control group (\( P < 0.05 \)). \( P \)-Values calculated using \( \chi^2 \)-test.

GVBD = germinal vesicle breakdown.

### Table II. Effect of myo-inositol (MI) on fertilization and developmental potential of in-vitro matured (metaphase II) mouse oocytes after IVF

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of GV oocytes</th>
<th>No. of mature oocytes (%)</th>
<th>No. of 2PN oocytes (%)</th>
<th>No. of 2-cell embryos (%)</th>
<th>No. of penetrated oocytes(^b) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM (control)</td>
<td>110</td>
<td>81 (73.6)(^a)</td>
<td>41 (50.6)(^c)</td>
<td>22 (53.6)(^c)</td>
<td>20 (24.7)(^c)</td>
</tr>
<tr>
<td>MEM + MI (30 mmol/l)</td>
<td>130</td>
<td>117 (90.0)(^b)</td>
<td>79 (67.5)(^d)</td>
<td>60 (75.9)(^d)</td>
<td>12 (10.3)(^d)</td>
</tr>
<tr>
<td>In-vivo matured oocytes</td>
<td>105</td>
<td>96 (91.4)(^e)</td>
<td>88 (91.6)(^e)</td>
<td>2 (2.0)(^e)</td>
<td></td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.
\(^a\)Data were pooled from four replicate experiments. All data are expressed as the number of positive observations.
\(^b\)Unfertilized oocytes analysed for sperm penetration by Hoechst No. 33342.
\(^c,d,e\)In each column, values with different superscripts are significantly different (\( P < 0.05 \)). \( P \)-Values calculated using \( \chi^2 \)-test or Fisher’s exact test.

GV = germinal vesicle; PN = pronuclei; MEM = minimum essential medium.

### Table III. Effect of myo-inositol (MI) on the implantation potential of in-vitro matured (IVM) mouse germinal vesicle oocytes

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of 2-cell embryos</th>
<th>No. of implanted sites (%)</th>
<th>No. of normal embryos (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVM oocytes (control)</td>
<td>120</td>
<td>36 (30.0)(^h)</td>
<td>11 (30.5)(^h)</td>
</tr>
<tr>
<td>IVM oocytes + MI (30 mmol/l)</td>
<td>110</td>
<td>57 (52.8)(^f)</td>
<td>30 (52.6)(^f)</td>
</tr>
<tr>
<td>In-vivo matured oocytes</td>
<td>115</td>
<td>95 (82.6)(^d)</td>
<td></td>
</tr>
</tbody>
</table>

67 (70.5)\(^d\)

Values in parentheses are percentages.
\(^\)Data were pooled from five replicate experiments. All data are expressed as the number of positive observations.
\(^b,d,f,h\)In each column, values with different superscripts are significantly different (\( P < 0.05 \)). \( P \)-Values calculated using \( \chi^2 \)-test.
icated by a significantly higher proportion of implantation sites as well as the rate of development to normal postimplantation embryos observed on day 10 of gestation (Table III). Nevertheless, the overall efficacy in terms of maturation, fertilization and cleavage rates to 2-cell embryos was significantly higher among in-vivo matured oocytes.

Experiment 3: Effects of MI on intracellular Ca\(^{2+}\) oscillations in the mouse GV oocytes

Fully grown denuded GV oocytes (n = 25) had their initial spontaneous intracellular Ca\(^{2+}\) oscillation detected ~40±60 min after release from their antral follicles. These intracellular Ca\(^{2+}\) transients were apparently initiated in the nuclear region and then spread towards the cortical area in both the control and MI-treated oocytes (Figure 2, frame 468). The second and subsequent smaller oscillations were observed soon after the rise of the first Ca\(^{2+}\) signal. The duration of each Ca\(^{2+}\) oscillation lasted ~40±50 s (Figure 2). More than 80% of these maturing oocytes that exhibited spontaneous Ca\(^{2+}\) oscillations were able to undergo GVBD after further culture (data not shown). As observed in Figure 1, the onset of GVBD in both the control and MI-treated oocytes occurred soon when they were placed in culture medium without the addition of dbcAMP. These events took place earlier than the initiation of spontaneous Ca\(^{2+}\) release (Figure 2). Addition of MI to the maturation medium caused additional transient intracellular Ca\(^{2+}\) rises with an interval of 50±100 s (n = 28). The propagation of this intracellular Ca\(^{2+}\) oscillation was detected ~20 min after the release from their antral follicles (Figure 3 and Figure 4). Other than that, the pattern of the spontaneous Ca\(^{2+}\) release observed in the MI-treated oocytes was very much similar to those obtained from the control oocytes (Figure 4).

Discussion

Myo-inositol is essential for the growth of eukaryotic cells and serves as a precursor for biosynthesis of inositol-containing phospholipids (Downes, 1989). Although previous reports suggest that the metabolism of phosphoinositides may be important in the induction of meiotic maturation in mammalian oocytes (Homa, 1991; Downs, 1995), little is known about the association of MI with the resumption of meiotic maturation in...
mammalian oocytes. The results of this study provide the first demonstration that MI has a positive effect on meiotic maturation and developmental competence of maturing mouse oocytes in the absence of exogenous gonadotrophins.

The uptake of MI can occur via a sodium MI transporter (SMIT) found in most living cells and tissues (Hale and Rubin, 1995; Hediger et al., 1995). One previous study has illustrated MI uptake into maturing mouse oocytes (Pesty et al., 1994). Whilst a specific MI transporter remains to be determined, it seems plausible to postulate that the same type of transport mechanism exists. Results from the present study confirm and extend the findings of Pesty et al. (1994) by demonstrating a dose-dependent effect of MI on the normal kinetics of GVBD and a significant increase in the proportion of mouse oocytes that can progress to full meiotic maturation. The enhancing effects of MI on the kinetics of GVBD of mouse oocytes observed in this study are in line with the previous evidence on the role of Ins(1,4,5)P3 and its receptor in maturing mouse oocytes (Mehlmann et al., 1996). The observation on the dose effects of MI on maturing mouse oocytes allows us to postulate that the uptake of extracellular MI may reach a threshold value above which the inositol transporter may become fully saturated. Besides, higher osmolarity can be induced in the culture medium from higher concentrations of MI (i.e. >40 mmol/l) possibly related to the organic osmolyte nature of MI (Matskevitch et al., 1998) and this may have a toxic effect on maturing oocytes.

Ca2+ is a ubiquitous intracellular signal that plays a vital role in the regulation of diverse cellular functions (Berridge, 1993). Previous studies have suggested that Ca2+ signals may trigger meiotic resumption in mammalian oocytes (Lefevre et al., 1995). However, consistent and direct evidence indicating Ca2+ as an essential mediator of meiotic resumption in mammals remains uncertain. In this study, we found that the onset of GVBD in spontaneous maturation after the removal of dbcAMP was not accompanied by a noticeable change in intracellular Ca2+ concentration. This is consistent with the findings from Tombes et al. (1992) in which no detectable Ca2+ change was seen in oocytes during the initial stages of spontaneous meiotic maturation, using the same strain of mouse as used in this study. It is possible that intracellular Ca2+ oscillations seen in mouse oocytes undergoing spontaneous maturation are not causally related with GVBD. Moreover, all oocytes were initially treated with dbcAMP, in an attempt to synchronize the onset of spontaneous maturation of the GV oocytes prior to confocal microscopy. It has been shown that dbcAMP can disrupt the intracellular Ca2+ signals with a much longer inter-spike interval in pre-GVBD mouse oocytes (Lefevre et al., 1995). Such an effect may account both for our failure to detect an intracellular Ca2+ rise in control oocytes prior to GVBD, and for the atypical Ca2+ oscillations observed in the control and MI-treated GV oocytes. In any case, conflicting opinions still exist regarding the association between nuclear and ooplasmic intracellular Ca2+ signals and the induction of meiotic resumption (Lefevre et al., 1995; Mehlmann et al., 1996). These discrepancies between different observations may be related to a number of factors, which include: (i) whether oocytes are matured with or without the presence of surrounding cumulus cells; (ii) whether maturation is spontaneous or hormone driven; (iii) the different animal strains or species used; and (iv) the type of oocyte maturation medium used in the study. Nevertheless, the present results demonstrate that the majority of GV oocytes, that display spontaneous Ca2+ oscillations in both the control and MI-treated groups, are capable of undergoing the full process of GVBD. This is consistent with the current concept that intracellular Ca2+ transients are a phenomenon of meiotically competent mouse oocytes (Homa, 1995). Further studies are required to investigate whether differences in the maturation rates of GV oocytes to metaphase II stage exist between those with intracellular Ca2+ transients accompanying GVBD and those that undergo GVBD alone, amongst both control and MI-
treated GV oocytes. Interestingly, a recent report has demonstrated that calcium current activity and calcium stores are associated with the quality and developmental competence of maturing bovine oocytes (Boni et al., 2002).

In the present study, we observed that spontaneous intracellular Ca\textsuperscript{2+} transients were initiated in the nuclear region and then spread globally towards the cortical area in control GV oocytes undergoing maturation in vitro. The treatment of GV oocytes with an optimal dose of MI can produce an extra intracellular Ca\textsuperscript{2+} rise which is followed by a similar pattern of spontaneous Ca\textsuperscript{2+} oscillations, as seen in the controls. Taken together, our observations suggest that the exogenous MI may not only enhance the basic phospholipid turnover or activate the phosphoinositide metabolism that takes place on the oocyte membrane (Homa, 1991), but may induce a similar phenomenon in the nucleus and ultimately produce Ins(1,4,5)P\textsubscript{3}, which initiates intracellular Ca\textsuperscript{2+} release. Such a mechanism is supported by a previous study which demonstrated the presence of the type-1 Ins(1,4,5)P\textsubscript{3} receptor in the nuclear membrane, and the detection of Ca\textsuperscript{2+} release from nuclear stores (Pesty et al., 1998). Furthermore, the recent detection of cytoplasmic and nuclear phospholipase C-B\textsubscript{1} and their relocation before the onset of GVBD have reinforced the notion that a nuclear phosphatidylinositol cycle may also be involved during resumption of meiosis in mouse oocytes (Avazeri et al., 2000).

Our present observation on the small and scanty intracellular Ca\textsuperscript{2+} oscillations released in GV oocytes undergoing spontaneous maturation may be associated with the sub-optimal calcium release mechanism present in immature mouse oocytes (Carroll et al., 1996). Other studies have indicated that meiotically competent mouse oocytes develop an Ins(1,4,5)P\textsubscript{3}-induced Ca\textsuperscript{2+} release mechanism, possibly related to a structural reorganization of the endoplasmic reticulum, and with a concomitant increase in the number of cortical Ins(1,4,5)P\textsubscript{3} receptors as they undergo the maturation process (Mehlmann et al., 1996; He et al., 1997; Fissore et al., 1999). Indeed, different isoforms of Ins(1,4,5)P\textsubscript{3} receptors have been detected in mouse oocyte, with the type I isoform being mainly associated with oocyte maturation (Parrington et al., 1998). Interestingly, when a monoclonal antibody against the Ins(1,4,5)P\textsubscript{3} type I receptors is injected into the GV of the mouse oocytes, both the nuclear Ca\textsuperscript{2+} oscillations and GVBD are significantly suppressed, suggesting the presence of such receptors within the nuclei of these oocytes (Avazeri et al., 1998). Taken together, these findings provide further support for our postulation that MI may exert its enhancing effect on meiotic maturation via intracellular Ca\textsuperscript{2+} signals.

Complete meiotic maturation of mammalian oocytes requires the proper intracellular changes associated with both the nuclear and cytoplasmic components of maturation. Although most of the morphological and biochemical changes during oocyte maturation are well documented, the identification of specific factors that bring about these changes is incomplete (Fulka et al., 1998). Maturation-promoting factor (MPF) has been well-documented to play a critical role in driving the oocyte meiotic cell cycle progression. Animal studies have shown that MPF is found in growing oocytes and its action seems to precede or coincide with GVBD (Homa, 1995; Wehrend and Meinecke, 1998). In addition to MPF, mitogen-activated protein kinase (MAPK) has also been shown to participate in the regulation of cell cycle progression (Shimada et al., 2001). However, the role of MAPK in meiotic resumption in mammalian oocytes remains to be determined (Shimada et al., 2001).

The fertilizability of the oocytes together with the acquisition of developmental capacity to 2-cell embryos, and preimplantation development are currently taken as an integral part of proper cytoplasmic maturation assessment (Vitt et al., 2001). Fertilization failure seen in incompetent oocytes is often associated with successful sperm penetration but subsequent failure to form pronuclei. We have previously shown that some of the unfertilized human oocytes had cortical granules released after IVF (Tam et al., 1990). Moreover, a recent study has provided the supporting evidence that unfertilized human oocytes have a high incidence of sperm penetration (Neuber and Powers, 2000). The use of the Hoechst dye in this study has provided the evidence that a high proportion of non-activated oocytes are found amongst GV oocytes without exposure to MI during maturation. The inability of the in-vitro matured oocytes to become activated after sperm penetration may be related to incomplete cytoplasmic maturation of these oocytes (Fulka et al., 1998).

In view of the potential occurrence of in-vitro 2-cell block in the majority of mouse strains (Muggleton-Harris et al., 1982), and the fact that subtle imperfections during IVM can accurately be detected after implantation (Moor et al., 1996), the present study used our previously established model of postimplantation embryo development (Tam et al., 1987) as the ultimate assessment of developmental competence and hence the quality of oocytes matured in vitro. Increasing evidence indicates that the type of culture medium and conditions of culture have a profound effect on the in-vitro development of preimplantation embryos (Ho et al., 1995; Niemann and Wrenzycki, 2000). In fact, a recent study has revealed that in-vitro culture conditions can cause deviation from the normal mRNA expression pattern of the H19 gene in mouse embryos (Doherty et al., 2000). Because of the potential effects of culture conditions on embryonic development, the present study minimized the prolonged exposure of embryos to in-vitro culture conditions by transferring the embryos to the oviducts when they were at 2-cell stage. This can provide a more consistent and reliable test for the ultimate assessment of developmental competence of in-vitro matured oocytes.

In this study, we showed that the proportion of fertilized oocytes with 2PN, the number of 2-cell embryos developed, and the percentage of normality of the postimplantation embryos were significantly higher in GV oocytes cultured in maturation medium containing MI compared with control medium. To ensure that the effect of MI can only act on the maturation process of the oocytes, supplementation of MI is performed only in the maturation medium (MEM) but not in subsequent culture medium (M16). Thus the improved outcome of meiotic maturation, fertilization, implantation and postimplantation embryo development may reflect improvements in the quality of in-vitro matured oocytes due to MI exposure. Although the presumed effects of stimulation of
PtdIns pathway on oocyte maturation remains to be elucidated, we have recently shown that higher concentrations of MI in follicular fluids are associated with good quality oocytes (Chiu et al., 2002). On the basis of our previous study and our present findings, we suggest that MI may well be enhancing the rate of meiotic maturation and subsequent development potential of maturing oocytes through triggering premature Ca\textsuperscript{2+} transients that, in turn, may accelerate cell cycle protein synthesis and/or activity. Our present notion is further supported by the recent findings on the role of Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II and Ca\textsuperscript{2+}-dependent protein kinase C in the regulation of meiotic resumption in mammalian oocytes (Downs et al., 2001; Su and Eppig, 2002). Further studies are warranted to investigate the association between the PtdIns pathway and levels of MIF and MAPK in order to provide a better picture of the enhancing effect of MI on mammalian oocyte maturation. It remains to be determined whether the generated Ca\textsuperscript{2+} signals can induce oocytes to reorganize the cytoplasmic environment in the second half of the meiotic maturation process, and this accounts for the oocyte’s subsequent developmental capacity (Moor et al., 1998).

In conclusion, the present study has demonstrated that the exposure of fully grown mouse GV oocytes to MI during IVM can enhance meiotic maturation, and the subsequent developmental potential of these oocytes following fertilization. However, the differences in the proportion of normally implanted embryos found between oocytes matured in vitro (MI-treated) and in vivo suggest that factors other than MI are also required for optimizing the IVM system of mouse oocytes. Further studies are needed to confirm whether the second messengers derived from metabolism of phosphoinositides can also contribute the appropriate signals to regulate the proper nuclear and cytoplasmic maturation in other mammalian oocytes including humans.

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References


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