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Follicular fluid and serum concentrations of myo-inositol in patients undergoing IVF: relationship with oocyte quality

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BACKGROUND: The follicular microenvironment is an important determinant of oocyte development. The aim of this study was to examine whether the myo-inositol (MI) content in human follicular fluid (FF) was associated with better oocyte quality. METHODS: A total of 53 patients treated with IVF was recruited to a prospective observational study. FF and serum samples collected were divided into two groups: group A consisted of FF associated with matured and fertilized oocytes, whilst group B was from follicles with immature and unfertilized oocytes. RESULTS: Patient's age, total ampoules of HMG used, days of stimulation, basal levels of FSH, estradiol (E_2) levels on the day of HCG, and serum MI content were not significantly different between the two groups. FF volume and its MI content were significantly higher in group A compared with group B ($P < 0.05$). The levels of MI in FF were positively correlated with the amount of E_2 in their corresponding FF samples and also correlated with embryo quality. CONCLUSIONS: We propose that higher concentrations of MI and E_2 in human FF appear to play a role in follicular maturity and provide a marker of good quality oocytes.

Key words: estradiol/follicular fluid/IVF/myo-inositol/oocyte

Introduction

Myo-inositol (MI) is an isomer of a C_6 sugar alcohol that belongs to the vitamin B complex group (Kane, 1988). Various studies have suggested that MI plays an important role in cell morphogenesis and cytogenesis, lipid synthesis, structure of cell membranes and cell growth (Berridge, 1987; Downes, 1989). We have previously shown that MI may be a serum trophic factor responsible for promoting in-vitro development of preimplantation embryos (Chiu and Tam, 1992). Other studies have shown that MI is incorporated into phosphoinositides and inositol phosphates in rabbit embryos (Fahy and Kane, 1993) and can enhance bovine blastocyst development from in-vitro culture with medium supplemented with MI (Holm *et al.,* 1999). Taken together, the results from these studies support the notion that MI serves as a precursor for the synthesis of phosphoinositides. This constitutes the phosphatidylinositol (PtdIns) signal transduction system known to be involved in the regulation of diverse cellular functions including cell proliferation (Berridge and Irvine, 1989).

The importance of PtdIns cycle activation in transducing information of various types across the plasma membrane has become more apparent in recent years (Downes, 1989; Berridge, 1993). It is activated in response to hormonal or other types of stimuli, and involves a receptor-dependent hydrolysis of an inositol lipid precursor to generate the inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3]. This is a second messenger that regulates many types of cellular processes by modulating the release of intracellular Ca^{2+} in a variety of cellular systems (Berridge, 1993). Although much of these data derive from studies of somatic cells (Rhodes *et al.,* 1983; Mene *et al.,* 1993), there is increasing evidence that such crucial events are also related to gamete development, including oocyte maturation, fertilization, and early embryonic development (Mehlmann and Kline, 1994; Jones *et al.,* 1995; Stachecki and Armant, 1996).

Since the pioneering study of follicular fluid (FF) started about 20 years ago (Edwards, 1974), increasing knowledge with respect to steroids (Fishel *et al.,* 1983), growth factors (Artini *et al.,* 1994), and protein composition (Spitzer *et al.*, 1996) of FF has greatly contributed to our understanding of the physiological processes related to follicular growth and oocyte development. FF seems to be the ultimate site for these factors to exert their influence on the oocyte. Other substances like hyaluronan, an essential component of extracellular matrices in all tissues, has also been detected in human FF and its concentration may indicate oocyte viability for fertilization (Saito *et al.*, 2000). The presence of MI in our body fluids, its role as a precursor of the inositol phospholipids responsible for the generation of important intracellular signals essential for mammalian oocyte development (Lewin *et al.*, 1973; Indyk and Woollard, 1994; Downes, 1989; Fujiwara *et al.,* 1993) and its effect on improving in-vitro maturation

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in mouse oocytes (Pesty *et al.,* 1994) have prompted us to speculate that a relationship may exist between MI concentrations in FF and the quality of the derived oocytes.

The aims of this study were: (i) to determine the concentrations of MI and E_2 in human FF and serum obtained from patients undergoing IVF treatment, (ii) to correlate the MI content in FF with oocyte quality, fertilizing ability and subsequent IVF outcome of the derived oocyte and (iii) to examine the association between concentrations of MI and E_2.

Materials and methods

Patients and follicle stimulation protocol

A total of 53 infertile couples treated with IVF was recruited into a prospective observational study. The study design and use of patient specimens was approved by the clinical ethics committee of the Chinese University of Hong Kong. The nature of this study was explained in detail and written consent was obtained from all patients.

Patients with polycystic ovarian syndrome were excluded from this study due to the possible association with poor quality oocytes. Other exclusion criteria were couples with infertile male partners according to World Health Organization guidelines (World Health Organization, 1999) who would require ICSI procedures for enhancing fertilization, and diabetic patients who have low levels of detectable MI associated with abnormal transport of myo-inositol (Simmons *et al.,* 1992).

All patients were treated with a similar long protocol of GnRH analogue (Suprecur; Hoechst, Frankfurt, Germany) and HMG (Pergonal; Serono, Aubonne, Switzerland) as described previously (Chiu *et al.,* 2000). In brief, ovarian stimulation with HMG was commenced two weeks after GnRH analogue administration when the patient had evidence of suppression (i.e. LH <5 IU/l, E_2 <200 pmol/l). Follicular development was monitored daily by ultrasound scan and serial hormone measurements. When the leading follicles reached 18 mm in diameter, 10 000 IU of HCG (Profasi; Serono) was administered.

IVF procedures

FF collection and IVF

Oocyte retrieval was performed 36 hours after HCG injection with transvaginal ultrasound guidance. A clearly visible follicle, preferably during the start of the ovum retrieval procedure, was individually aspirated, and flushing was carried out between aspirates with the use of a double lumen aspiration needle. The selection criteria for inclusion of FF in this study were: (i) clear follicular aspirate obtained during oocyte retrieval and; (ii) each specimen of FF contained only one oocyte. After the procedure, the volume of FF was measured and centrifugation was performed at 600 *g* for 10 min to remove debris. The clear FF samples were then stored at –20°C until assayed. Meanwhile, the collected oocyte–cumulus complexes were washed twice in HEPES-buffered human tubal fluid (HTF) medium (Irvine Scientific, Irvine, CA, USA) before culturing in HTF medium (Irvine Scientific) supplemented with 10% serum substitute supplement (SSS; Irvine Scientific) for 4–6 h at 37°C and 5% CO_2 in air prior to insemination. At the appropriate time, standard insemination was performed with motile sperm harvested by the density gradient centrifugation method.

Assessment of oocyte maturity and FF classification

Cumulus masses enclosing the oocytes often made it impossible to determine precisely the nuclear maturity of the oocytes at retrieval time. Therefore, assessment of oocyte quality was based on the fertilization outcome performed at 16–20 h after insemination. Oocytes were classified as good quality if they were mature and successful fertilization occurred as indicated by the presence of two pronuclei and two polar bodies. Oocytes of poor quality were defined as immature and unfertilized. The classification of FF was performed retrospectively by dividing aspirates into two functional groups as described above. The fertilized oocytes were cultured for a further period of 20–24 h in HTF medium supplemented with 10% SSS at 37°C and 5% CO_2 in air. Prior to embryo transfer, the developed embryos were graded in accordance with a previously published embryo grading system (Bolton *et al.,* 1989). According to this system, embryos with regular, spherical blastomeres with no fragments, those with some fragments, those with uneven blastomeres and finally embryos with progressive amount of extracellular fragmentation were referred to as grade 4, grade 3, grade 2 and grade 1 respectively.

Measurement of myo-inositol

The myo-inositol (MI) concentration in FF was determined by an enzymatic assay, based on NAD^+- dependent oxidation of MI by myo-inositol dehydrogenase (MIDH) with the production of NADH, as previously described (Chiu *et al.,* 1992). The generated NADH was then used to react with Fe^{3+}-bathophenanthroline disulphonic acid to produce Fe^{2+}-bathophenanthroline disulphonic acid, which was measured spectrophotometrically by setting the absorbance wavelength at 546 nm. The determination of MI in this study was a fixed-time kinetic assay and required a standard calibration curve of MI. The assay sensitivity was 6.0 µmol/l with intra-assay and inter-assay coefficients of variation (CV) of 7.5 and 9.5% respectively.

Measurement of estradiol

Serum and FF estradiol concentrations were determined by the ACS:180 Estradiol-6 assay kit (Automated Chemiluminescence System, Bayer Corp., NY, USA). This assay is a competitive immunoassay using direct, chemiluminescent technology. The lowest detectable limit of E_2 obtained from this assay is 0.01 ng/ml, and the ranges of intra- and inter-assay CV are 8.1–16.6 and 8.7–17.5% respectively.

Statistical analyses

The calculation of sample size was based on our previous study (Chiu *et al.,* 1992) on the detection of MI in sera collected from women undergoing IVF treatment. We reported a mean difference in serum MI concentration of 13.3 µmol/l and a SD of 7.1 µmol/l between sera with different embryotrophic activity. Using these values, the estimated sample size required when using the Student's *t*-test to compare means of continuous variables was six in each group. This would give a power of 80% and a two-sided significance level of 0.05.

The data presented here are expressed as means $\pm$ SD. The statistical significance of the differences between the means of the two groups were determined and compared by Student's *t*-test. Correlations between parameters were performed by using linear regression analyses and Pearson coefficients of contingence. A *P*-value of $\leqslant 0.05$ was considered statistically significant.

Results

A total of 60 FF specimens containing a single oocyte was collected from 53 patients who had entered into this study. The allocation of the FF was done in a prospective observational manner. A total of 38 FF samples containing mature and fertilized oocytes was allocated to group A and 22 samples to group B consisting of only immature and unfertilized oocytes. The mean ($\pm$ SD) age of the patients in group A ($n = 32$)

Table I. Cycle characteristics in the two groups of patients defined by oocyte maturity and fertilization[a]

	Group A	Group B	*P* value
No. of patients	32	21	–
Age (years)	33.8 ± 3.5 (25–42)	34.4 ± 4.3 (25–42)	NS
FF volume (ml)	4.7 ± 1.4 (2.5–8.0)	3.9 ± 1.0 (2.0–6.0)	< 0.05
No. of oocytes	38	22	–
Days of stimulation[b]	9.0 ± 2.1 (5–17)	8.8 ± 2.8 (6–17)	NS
No. of HMG ampoules	31.8 ± 10.7 (15–62)	34.3 ± 13.8 (16–62)	NS
Basal level of FSH	5.1 ± 2.7 (1.5–10.2)	5.9 ± 3.6 (1.3–12.1)	NS
Estradiol level (Day 0)	1.4 ± 0.8 (0.2–3.0)	1.6 ± 0.8 (0.6–3.0)	NS

Values are means ± SD; NS = not statistically significant. Values in parentheses are range.
[a]Group A = follicles containing mature and fertilized oocytes. Group B = follicles containing immature and unfertilized oocytes.
[b]From the first HMG injection to the day of human chorionic gonadotrophin

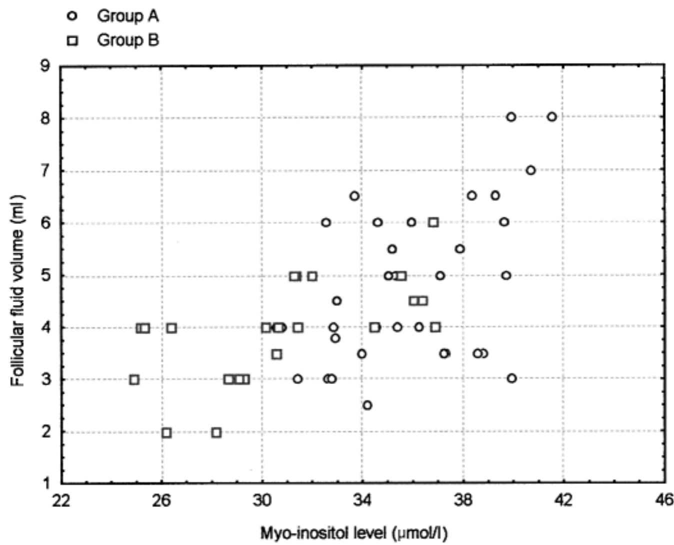

Figure 1. Relationship between the follicular fluid (FF) volume and myo-inositol content in FF samples obtained from IVF patients during oocyte retrieval. ○ = Group A follicles had matured and fertilized oocytes ($r = 0.471$, $P < 0.01$), and □ = Group B follicles had immature and unfertilized oocytes ($r = 0.613$, $P < 0.01$).

and group B ($n = 21$) were 33.7 ± 3.5 years and 33.8 ± 4.1 years respectively. There was no significant difference in the duration of stimulation (days), the number of ampoules of HMG used, basal level of FSH on day 3 of the treatment cycle, and the serum level of E_2 on the day of HCG administration. Significantly larger FF volume was obtained in group A as compared with group B (4.7 ± 1.4 ml versus 3.9 ± 1.0 ml, $P < 0.03$) (Table I). Moreover, positive correlations were found between the concentrations of MI and different volumes in FF for both group A ($r = 0.471$, $P < 0.01$) and group B ($r = 0.631$, $P < 0.01$) as shown in Figure 1.

The mean concentration of MI in FF from group A was significantly higher than that from group B (35.6 ± 3.1 µmol/l versus 30.7 ± 3.9 µmol/l, $P < 0.001$). The concentration of MI in FF in both groups was significantly lower than in the corresponding serum samples ($P < 0.001$). However, the difference in serum MI concentrations between groups A and B was not statistically significant ($P > 0.05$) (Table II).

The concentration of E_2 was significantly higher in FF than

Table II. Concentrations of myo-inositol measured in follicular fluid and serum

	Group A[a]	Group B[a]	*P*-value
Follicular fluid			
n	38	22	
MI (µmol/l)	35.6 ± 3.1 (30.6–41.5)	30.7 ± 3.9 (24.9–36.9)	< 0.005
Serum			
n	32	21	
MI (µmol/l)	38.7 ± 1.5 (36.1–42.9)	39.0 ± 4.0 (27.6–45.8)	NS

Values are means ± SD. Values in parentheses are range.
[a.]See footnote [a] Table I.
MI, myo-inositol; NS = not statistically significant.

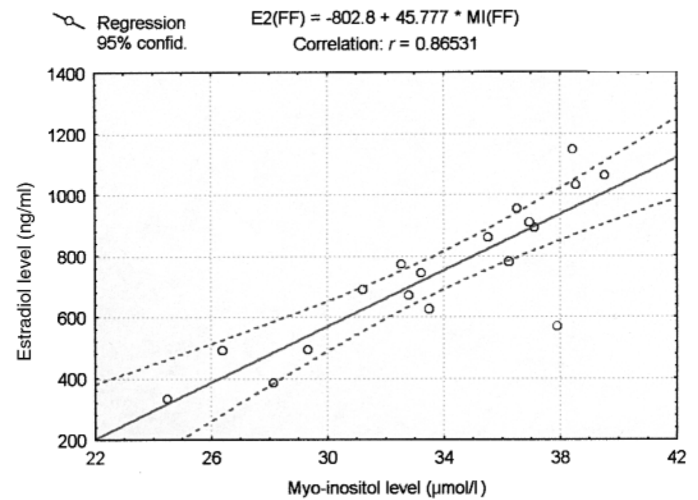

Figure 2. Scatterplot of the concentrations of estradiol and myo-inositol measured in follicular fluid. The straight line shows a positive correlation between variables ($r = 0.865$, $P < 0.001$). The vertical axis represents concentrations of estradiol (ng/ml) and the horizontal axis represents concentrations of myo-inositol (µmol/l).

in the serum (744.4 ± 55.2 ng/ml versus 0.96 ± 0.09 ng/ml, $P < 0.001$). Furthermore, a strong positive correlation was found between the concentrations of MI and E_2 in FF ($r = 0.87$, $P < 0.001$) as shown in Figure 2.

The level of MI in FF specimens and the subsequent

Table III. Content of myo-inositol in follicular fluid and developmental potential of the derived oocytes

	Myo-inositol concentration (μmol/l)
Stage of embryo	
<4-cell	34.2 ± 2.6
⩾4-cell	37.6 ± 2.5[a]
Embryo quality	
< Grade 4	34.5 ± 2.2
= Grade 4	37.1 ± 3.4[b]

Values are mean ± SD.
[a]$P < 0.001$, embryos <4-cell versus embryos ⩾4-cell.
[b]$P < 0.05$, embryos < grade 4 versus embryos = grade 4.

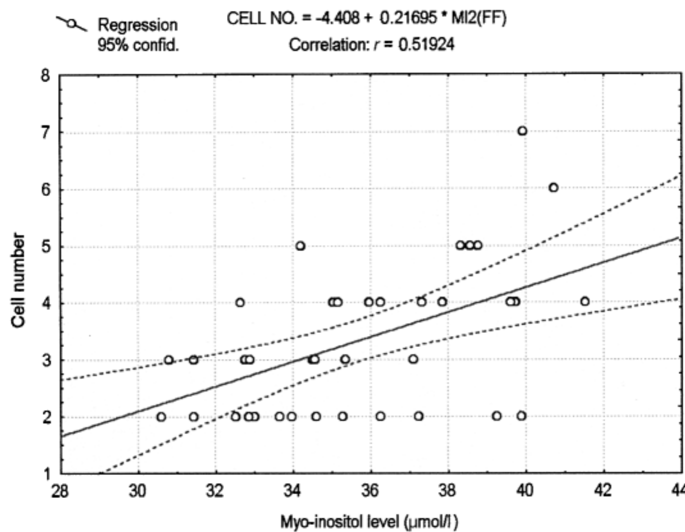

Figure 3. Scatterplot of the developmental stage of embryo and myo-inositol level in follicular fluid (FF). The straight line shows a positive correlation between variables ($r = 0.519$, $P < 0.01$). The vertical axis represents the stages of embryo development in terms of cell number (1 – 8) and the horizontal axis represents concentrations of myo-inositol (μmol/l) in FF.

developmental outcome of the derived oocytes are shown in Table III. The mean concentration of MI was found to be significantly higher in FF containing oocytes that developed into 4-cell embryos with good morphology (grade 4). A positive correlation was found between the concentrations of MI and the cleavage rate of fertilized oocytes harvested from the respective follicles ($r = 0.52$, $P < 0.01$) as illustrated in Figure 3. Furthermore, the morphological grading of the embryos was also related to the amount of MI present in FF ($r = 0.53$, $P < 0.01$) (Figure 4).

Discussion

MI is the main isomer of cyclohexane hexol that occurs in eukaryotic cells. It is a precursor of the inositol phospholipids that undergo a series of complex interconversion pathways in response to stimulation by a wide variety of hormones. The subsequent generation of important intracellular signals can regulate diverse cellular functions (see Introduction). Results of this study have demonstrated, for the first time, the presence

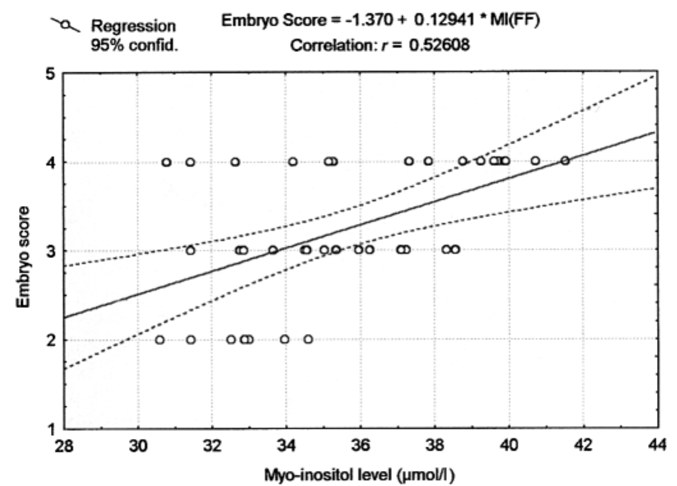

Figure 4. Scatterplot of the embryonic morphological score and myo-inositol level in follicular fluid (FF). The straight line shows a positive correlation between variables ($r = 0.526$, $P < 0.01$). The vertical axis represents morphological score of the embryo (1–4) and the horizontal axis represents concentrations of myo-inositol (μmol/l) in FF.

of MI in human FF. The assay method used here for the determination of the concentration of MI in FF is similar to that used for the detection in serum samples as previously reported (Chiu *et al.,* 1992). Our data have shown a significant difference between the concentration of MI in FF and serum ($P < 0.001$). These findings are consistent with other studies suggesting that the concentrations of MI are not the same among different body fluids (Lewin *et al.,* 1973). It has been proposed that cellular myo-inositol homeostasis can be achieved by contributions from synthesis *de novo*, inositol phosphate re-cycling and accumulation from the extracellular environment (Gani *et al.,* 1993). However, the relative importance of these three mechanisms in maintaining different concentrations of MI within different cell types and body fluids remains to be elucidated.

Investigations into factors affecting oocyte quality in conventional IVF cycles usually assess the oocyte maturity during its retrieval by examining the morphological appearance of the oocyte–cumulus complex (Imthurn *et al.,* 1996). Recent studies reveal that morphology of oocyte–corona–cumulus complex bears little relationship to oocyte maturity (Rattanachaiyanont *et al.,* 1999). Whilst ICSI can provide a more appropriate situation for scoring oocyte maturity, the difficulty of collecting clear FF samples, required by this study, has made it impractical to recruit enough cases solely from ICSI cycles. Moreover, we have observed more unfertilized metaphase I than germinal vesicle (GV)-stage oocytes during the next morning after fertilization check. Therefore, in order to reduce the limitations on our inclusion criteria, we have adopted a similar method of categorizing oocytes as ‘good’ quality when they are matured and fertilized after IVF. Those that are immature and unfertilized are classified as ‘poor’ quality (Lau *et al.,* 1999; Teissier *et al.,* 1999) without further sub-dividing the stage of the immature oocytes.

Calcium has been shown to play a pivotal role in the initiation of mammalian oocyte maturation (Gudermann *et al.,*

1992; Mehlmann and Kline*,* 1994). Increasing evidence has suggested that the phosphoinositide pathway is of prime importance in mobilizing Ca^{2+} within the cells (Downes, 1989; Berridge, 1993). In this study, we have not only demonstrated that MI is found in human FF, but that its concentration is significantly higher in FF containing good quality oocytes than FF containing poor quality oocytes ($P < 0.001$). Since MI is a precursor of the phosphoinositides, we postulate that MI in FF may undergo metabolism to the inositol phospholipids and ultimately to Ins(1,4,5)P_3 during the maturation process of human oocytes. Cells require a constant replenishment with phosphoinositides but only contain a small pool of inositol phospholipids (Downes and Macphee*,* 1990). Inositol transport in different tissues and cultured cell lines can occur either by a diffusion process or by a specific active transport system (Hynes *et al.,* 2000). Pesty *et al.* have demonstrated the uptake of MI in maturing mouse oocytes (Pesty *et al.,* 1994). A myo-inositol transporter (SMIT) has been isolated in Xenopus oocytes (Matskevitch *et al.,* 1998) which is responsible for the uptake of MI into a maturing oocyte. This is further supported by the findings that inositol 1,4,5-trisphosphate receptors (type I) are detected in mammalian GV stage oocytes including humans (Goud *et al.,* 1999). These results, together with those from the present study suggest that MI in FF, Ins(1,4,5) P_3 and its receptors may play an important role during oocyte maturation in humans. The higher concentrations of MI in FF containing good quality oocytes may indicate that an active transport of MI is required to maintain MI homeostasis when phosphoinositide turnover is stimulated during the course of development of a healthy follicle.

Since nuclear and cytoplasmic maturation occur independently during oocyte maturation, these processes need to proceed in a closely integrated manner to ensure developmental competence of the oocyte. Studies from in-vitro maturation (IVM) of human oocytes have shown that most of the matured oocytes derived from IVM are developmentally incompetent, possibly due to incomplete cytoplasmic maturation (Trounson *et al.,* 1998; Mikkelsen and Lindenberg, 2001). Such a poor IVM outcome can be related to the lack of a methodology for in-vitro assessment of cytoplasmic maturation (Rutherford, 1998). Studies from hamster (Fujiwara *et al.,* 1993), mouse (Mehlmann and Kline*,* 1994), and human oocytes (Goud *et al.,* 1999) have shown an increase in the sensitivity of inositol trisphosphate-induced Ca^{2+} release mechanism during the course of oocyte maturation. In our study, there was a positive correlation between the amount of MI in FF and both the cell number and the morphological score of the developed embryos. Since embryos were transferred on day two, whilst surplus ones were cryopreserved during the course of this study, data regarding the further embryo development cannot be obtained. Nevertheless, the present results are in line with other studies suggesting that MI is required to enhance the developmental competence of maturing oocytes.

In this study, we have found that higher level of MI in FF is associated with larger follicles in terms of FF volume as well as the concentration E_2 in FF. Serum concentration of E_2 and follicle size are routinely used as conventional parameters for monitoring follicular development and oocyte maturity during ovulation induction and IVF treatment (Mikkelsen *et al.,* 2000). Furthermore, it has been shown that E_2 can exert a direct nongenomic effect on maturing human oocytes via the induction of intracellular Ca^{2+} oscillations (Tesarik and Mendoza*,* 1995). These findings, together with our results, further support our present hypothesis that MI may represent one of the maturational factors in FF responsible for the in-vitro growth of human oocytes. Perhaps, the content of MI in FF may represent a more appropriate physiological indicator than FF volume for monitoring the status of the developing follicles.

In conclusion, follicles containing good quality oocytes have higher concentrations of MI in FF, probably due to the intricate relationship between MI and inositol phosphates in the PtdIns cycle activation for oocyte maturation. The association between concentrations of MI with FF volume, E_2 and better developmental potential of the oocytes suggests that higher levels of MI in FF may be related to the well being of the follicle and the quality of the oocyte. A larger study is required to examine the association between the pregnancy outcome and the embryos derived from healthy follicles containing higher concentration of MI. Future studies are also needed to elucidate the molecular transport mechanism of MI within the follicles and the molecular effect of MI on maturing oocytes.

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