Immature oocytes grow during in vitro maturation culture

Cavilla JL[4], Kennedy CR[2], Byskov AG[3], Hartshorne GM[1,2]

[1]Clinical Sciences Research Institute, Warwick Medical School, University of Warwick, Clifford Bridge Road, Coventry, CV2 2DX, UK, [2]Centre for Reproductive Medicine, University Hospitals Coventry and Warwickshire NHS Trust, Coventry, CV2 2DX, UK and [3]Laboratory of Reproductive Biology, Juliane Marie Centre, Section 5712, Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen, Denmark.

[4]Current address: Assisted Conception Unit, Kings College Hospital, Denmark Hill, London, SE5 9RS, UK.

[1]To whom correspondence should be addressed at:
Clinical Sciences Research Institute, Warwick Medical School, University of Warwick, Coventry, CV2 2DX,UK
Tel: 02476 968679/528382
Fax: 02476 968880
Email: Geraldine.Hartshorne@warwick.ac.uk

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Abstract

BACKGROUND. Oocyte competence for maturation and embryogenesis is associated with oocyte diameter in many mammals. This study aimed to test whether such a relationship exists in humans and to quantify its impact upon in vitro maturation (IVM). METHODS. We used computer-assisted image analysis daily to measure average diameter, zona thickness and other parameters in oocytes. Immature oocytes originated from unstimulated patients with polycystic ovaries, and from stimulated patients undergoing ICSI. They were cultured with or without meiosis activating sterol (FF-MAS). Oocytes maturing in vitro were inseminated using ICSI and embryo development was monitored. A sample of freshly collected in vivo matured oocytes from ICSI patients were also measured. RESULTS. Immature oocytes were usually smaller at collection than in vivo matured oocytes. Capacity for maturation was related to oocyte diameter and many oocytes grew in culture. FF-MAS stimulated growth in ICSI derived oocytes, but only stimulated growth in PCO derived oocytes if they eventually matured in vitro. Oocytes degenerating showed cytoplasmic shrinkage. Neither zona thickness, perivitelline space, nor the total diameter of the oocyte including the zona were informative regarding oocyte maturation capacity. CONCLUSIONS. Immature oocytes continue growing during maturation culture. FF-MAS promotes oocyte growth in vitro. Oocytes from different sources have different growth profiles in vitro. Measuring diameters of oocytes used in clinical IVM may provide additional non-invasive information that could potentially identify and avoid the use of oocytes that remain in the growth phase.

Key words: diameter/growth/human/IVM/oocyte

Introduction

Studies in several species have highlighted the relationship between oocyte diameter and competence for maturation and embryonic development. However, relatively little information is available in humans despite the accessibility of oocytes during clinical in vitro maturation (IVM). We measured oocytes during maturation culture in order to test the hypothesis that maturation and developmental competence are dependent upon oocyte growth beyond a threshold value. This would provide useful information on the potential of oocyte diameter measurements as a non-invasive predictor of developmental competence.

There is a substantial body of research on oocyte diameter and maturation in animals. Eppig and Schroeder (1989) introduced the concept that competence to develop through successive stages of meiosis and early embryogenesis in mice is dependent upon age and oocyte size. They showed that isolated oocytes from mice ≤13 days of age, having mean diameters >60μm, were able to undergo spontaneous breakdown of the germinal vesicle (GVBD) in culture, but larger oocytes from mice ≥15 days of age were more likely to mature completely to metaphase II (MII) in culture. Hirao *et al.* (1993) confirmed that the threshold diameter of 60μm for GVBD remained the same even when mouse oocytes were grown in vitro. Similar evidence of maturation competence relating to oocyte growth was obtained in rats by Daniel *et al.* (1989) and in pigs by Hirao *et al.* (1994), where the threshold diameters for GVBD were 55μm and 90μm respectively. Continuing transcription in small bovine oocytes indicates that their growth is not complete (Fair *et al.,* 1995) and hence, their complement of maternally derived mRNA, necessary for early embryonic growth, might also be incomplete, providing a possible mechanism for these

observations. However, Canipari *et al.* (1984) observed mouse oocytes that became GVBD competent after being cultured in conditions that did not promote significant growth, suggesting that the events of meiotic resumption and oocyte growth may be separable when non-physiological conditions are applied in vitro.

The capacity to cleave after maturation and insemination in vitro is also acquired with increasing age and oocyte diameter. Bao *et al.* (2000) showed that the developmental competence of mouse oocytes progresses in a stepwise manner as oocyte diameter increases from 65-75μm and that developmental changes occurring during the final stages of oocyte growth are critical for full developmental competence.

In rhesus monkey oocytes, meiotic competence occurs late during oocyte development, however, oocyte diameter appeared relatively constant as competence for GVBD arose, suggesting no close relationship with oocyte diameter (Schramm *et al.,* 1993). Durinzi *et al.* (1995) examined the relationship between oocyte size and maturation in vitro in unstimulated human oocytes from women aged 25-39yrs undergoing gynaecological operations not associated with ovarian pathology. They observed a significant difference in maturation capability of oocytes measuring 86-105μm at collection versus those measuring 106-125μm, leading to the conclusion that, in common with other species, the unstimulated human oocyte has a size-dependent ability to resume meiosis and complete maturation.

During a study of human IVM, fertilization and embryo development (Cavilla *et al.*, 2001), we captured computerised micrographic images over culture periods of up to six days. This afforded the opportunity to quantify human oocyte growth under the in vitro

98 conditions employed, and to explore the possibility of using a non-invasive measure of
99 oocyte development as a predictor for subsequent developmental competence. Our
100 findings confirm the size dependence of human oocyte maturation in vitro, however, they
101 have also highlighted unexpected and interesting growth patterns of maturing oocytes that
102 are novel and of potential importance in the clinical setting.

Methods

The methods of collection and culture of the human oocytes used in this study have been previously described in detail, as have the maturation, fertilization and embryo development results (Cavilla *et al.,* 2001). This manuscript presents additional results obtained on the same source material using image analysis as a non-invasive means of measuring oocyte parameters. The project was approved by Coventry Research Ethics Committee and the Human Fertilisation and Embryology Authority. Briefly, immature oocytes were collected from two sources: (1) 17 women (mean age 28.1 years, range 22-35) with polycystic ovaries undergoing laparoscopic surgery for tubal patency assessment and/or laser drilling of ovaries. These women donated 128 immature oocytes. (2) 28 women (mean age 32.4 years, range 27-40) receiving ovarian stimulation with intracytoplasmic sperm injection (ICSI) treatment for infertility, who donated 72 immature oocytes. Oocytes from these two sources had distinctly different origins. Those from PCO patients had been exposed to a prolonged abnormal endocrine and intrafollicular environment, while those remaining immature in ICSI patients had done so despite an ovulatory stimulus.

Immature oocytes (both GV and GVBD) were randomly allocated to culture with or without meiosis activating sterol derived from human follicular fluid (FF-MAS: 0, 10 or 30μg/ml). Oocytes were checked for maturity at 16, 24, 40 and 48 hours. Those observed to have a polar body were injected promptly with a sperm from a fertile donor. Fertilization and embryo development were monitored.

Oocytes were considered to have reached metaphase II and therefore 'mature' if they extruded a polar body. All oocytes lacking a polar body were considered immature (GV and GVBD oocytes). Oocytes remaining immature after 48 hrs were considered

incompetent for maturation. Atretic oocytes were characterized by a dark appearance and clearly shrunken or irregular ooplasmic outline.

A further group of 20 oocytes, that were mature at the time of their collection from ICSI patients (in vivo matured), had ooplasmic diameter measured once only after cumulus removal and before ICSI on the day of collection, for comparison with the IVM oocytes.

Light microscopic images of individual oocytes and embryos were collected daily using a computerized image analysis system (Image pro-plus, Media Cybernetics) linked via a video camera to an inverted microscope (Nikon) with Hoffman contrast optics. Images were analysed to assess whether any measured parameter related to the culture conditions employed or the subsequent development of the oocyte/embryo. The image analysis package was used to measure the following parameters:

Oocyte diameter: calculated by measuring the mean length of diameters to the oolemma at two-degree intervals passing through the oocyte's centroid. Control experiments, measuring 10 oocytes 10 times each, established the variability of such measurements as <1% (data not shown).

Oocyte+zona diameter: calculated as for oocyte diameter, but measured to the outer circumference of the zona pellucida. It therefore included both the oocyte and its zona pellucida, and incorporated differences in perivitelline space and zona thickness.

Zona pellucida thickness: calculated by averaging measurements of the zona thickness at 2μm intervals around its circumference.

The perivitelline space (PVS) was also measured separately, but tended to vary according to orientation. There were no significant findings in respect of this parameter (data not shown).

Statistics

The measurements for each oocyte over the assessment period were analysed according to the treatment that the oocyte received and the outcome of attempted maturation and fertilization in vitro. Average and threshold values at collection and after IVM culture were identified for various features of oocyte development.

For PCO oocytes , diameters were compared for those with dense cumulus at collection (where measurable), versus those with less or no cumulus cover, using pxq contingency table with χ^2 test. A one-tailed t-test was performed on oocyte diameters on day of collection from the two patient groups.

Within both patient groups the following tests were performed: oocyte diameters on day 0 were compared according to the outcome of in vitro culture (mature, immature, atretic) and tested for statistical significance using the Kruskall-Wallis test (Campbell, 1989). For each patient group, parameters were compared between day of collection and day 0 of oocytes that became atretic, within each culture condition using the Mann-Whitney U-test (Campbell, 1989). Oocyte growth during culture, for those oocytes that matured, was tested for statistical significance using the non-parametric sign test (Campbell, 1989), according to the culture conditions. In Figure 3, data were 'normalised' to day 0 as the day of insemination of mature oocytes. Thus, for the 18 oocytes that matured within 24

177 hours, day 0 was analysed as 1 day after collection, whereas for all other oocytes, day 0 is
178 2 days after collection.
179
180 Only for ICSI oocytes , non-parametric statistical analyses (Mann Whitney U tests) were
181 applied to detect any significant difference in oocyte diameter between oocytes that
182 matured within 24 hr and those that matured within 48 hr. This was performed for oocytes
183 within each culture group, and using pooled data (all culture groups combined) using
184 Kruskall Wallis test.

Results

The 20 in vivo matured oocytes from ICSI patients had a mean ooplasmic diameter of 116μm, ranging from 112-119μm.

A total of 128 oocytes were collected from PCO patients. On the day of collection, 86 (67%) of these oocytes could be measured while 42 could not, due mostly to dense cumulus cells obscuring the oolemma.. In some cases, by enhancing the image contrast and converting to grey scale it was possible to measure the oolemma through the attached cumulus cells.

A total of 72 oocytes were donated by patients undergoing ICSI treatment, 48 oocyte diameters were measured at collection and 24 were not. Eight oocytes were not measured on either the day of collection or day 0 due to camera failure while the others were omitted because of faint oolemmas and/or adherent cumulus cells. The numbers of successful measurements increased between collection and day 0 as a result of improved visibility due to cumulus expansion in vitro and the use of hyaluronidase to remove cumulus cells in preparation for ICSI during the experiment.

Figures 1a and b present the mean diameter at collection and after culture of viable oocytes collected from PCO patients or ICSI patients respectively. For PCO patients, these results approximated a normal distribution with a mean and mode of 106-108μm at the time of collection; whereas the distribution for oocytes from ICSI patients was positively skewed with a mode of 109-111μm. The immature oocytes from ICSI patients were significantly larger at collection than those from PCO patients ($p<0.001$), and they

grew in culture, achieving a mode of 112-114μm in both mature oocytes and those that remained immature (Figure 1b). In contrast, those from PCO patients showed minimal evidence of growth in vitro as a cohort (Figure 1a), however, as shown in Figure 3, individual oocytes either grew or shrank during culture. For oocytes from PCO patients, the chances of atresia during culture reduced with increasing diameter on day 0 (Figure 2a).

At the time of collection, immature oocytes from both PCO and ICSI patients were usually smaller than those that had undergone maturation in vivo, however, there was some overlap with the largest immature oocytes and the smallest of the mature oocytes. After culture, some ICSI derived immature oocytes had grown (see Figure 1B) to more nearly approximate the size range of oocytes that were mature at collection (mean 116μm range 112-119μm).

With the exception of one PCO oocyte (81μm), all oocytes that underwent GVBD in culture had diameters on day 0 of at least 102μm. The threshold diameter for IVM to MII in this study was 100μm at collection and 103μm on day 0. However, most oocytes that matured (82% in PCO group and 100% in ICSI group) had diameters >106μm on day 0. There was no relationship between mean oocyte diameter and the likelihood of maturation in the oocytes from ICSI patients, in contrast to those from PCO patients (Figure 2). The low number of small oocytes from ICSI patients precludes any comment on a threshold size for maturation in oocytes from this source.

Data from the PCO group (Table I) shows that atresia was more likely when cumulus cells were absent, however, maturation of surviving oocytes did not relate to cumulus

levels at collection. There was no relationship between cumulus cover and oocyte diameter at collection or growth in vitro (data not shown). This analysis was not performed for the ICSI group because cumulus cells had already been removed.

Table II shows the diameters of IVM oocytes in relation to fertilisation and cleavage. The same fertile sperm donor was used throughout. The apparent difference in oocyte diameter in the PCO group according to whether or not fertilization occurred was not significant.

Figure 3 shows oocyte diameters during culture with and without FF-MAS. The diameters of individual oocytes were plotted according to the culture conditions (0, 10, 30 μg/ml FF-MAS) and oocyte outcome. In all groups, oocytes that became atretic tended to shrink, while those maturing tended to enlarge, except in the PCO control group. In FF-MAS (10 and 30μg/ml) the mean diameters of mature, immature and atretic oocytes on day 0 were significantly different ($p<0.05$) despite their diameters at collection being similar (Figs 3b and 3c). Interestingly, this difference did not occur in PCO oocytes cultured in control conditions (Fig 3a) and was not significant in those collected from ICSI cycles (Figs 3d-f).

In the ICSI group, 50% of oocytes maturing in vitro had done so by 24 hr, compared to <5% of PCO derived oocytes (Cavilla et al, 2001). There was no significant difference in oocyte diameter on day 0 between those maturing in 24 hr and those in 48 hr, within each culture group (control, 10 μg/ml FF-MAS and 30 μg/ml FF-MAS) or when pooling all the culture groups (24 hr, median 113μm, interquartile range 110-113.75, vs 48 hr, median 112μm, interquartile range 108.5-114.5).

Figure 4 shows the IVM oocytes fertilizing and cleaving according to oocyte diameter for the ICSI group. For oocytes that matured within 24hr of culture, 2/6 (33%) of the fertilized oocytes subsequently cleaved. However, of oocytes that matured within 48hr, 5/7 (71%) fertilized oocytes subsequently cleaved. While this may provide some suggestion that prolonged maturation could be associated with improved cleavage potential, the numbers of embryos were too few for meaningful analysis.

Oocyte + zona diameter

The measurements of 'oocyte+zona' were positively skewed for PCO oocytes, and approximately normal for ICSI derived oocytes (Figure 5), in contrast to the data for oocyte diameter (Figures 1 and 2). The majority (79%) of viable PCO oocytes had mean diameters (including zona) in the 146-163μm range at the time of collection, which showed minimal change after 2 days of culture (Figure 5). As observed for oocyte diameter, oocytes with larger measurements of 'oocyte + zona' in the PCO group appeared more likely to mature in vitro (Figure 6a) however, this was not a significant difference. The diameter of the oocyte/zona complex did not change in culture for oocytes derived from ICSI patients, despite the extensive enlargement of ooplasm that occurred over the same period (Fig 1b vs Fig 5b), and was not associated with maturation in vitro (Fig 6b). There was no significant relationship between oocyte+zona measurements and maturation, fertilisation or cleavage in vitro (data not shown).

Zona pellucida thickness

Frequency distributions were plotted of the mean zona thickness of viable oocytes from both patient groups on the day of collection and for oocytes that did or did not mature in

285 vitro. There were no significant differences in zona thickness between the two groups,
286 and no relationship between zona thickness and FF-MAS (data not shown).
287
288 The zona thicknesses on day 1 and day 2 were compared in matured oocytes that did or
289 did not fertilise after ICSI. PCO oocytes that fertilized had significantly thicker zona
290 pellucidas on day 1 than those that did not (21.8±1.9 vs 16.9±2.7μm, $p<0.05$). No
291 significant differences were observed on day 1 or day 2 for in vitro matured oocytes from
292 ICSI patients (fertilized 20.5±0.8 vs 20.3±0.6μm unfertilized). The results on day 2 were
293 20.1±1.9 (fertilized) vs 17.2±3.4 (unfertilized); and 21.2±0.9 (fertilized) vs 19.3±0.5μm
294 (unfertilized) for the PCO and ICSI groups respectively.

Discussion

Oocyte development in preparation for ovulation includes both increasing size (growth) and maturation of oocyte constituents (ooplasm and genetic material). This report shows that measurable growth of human oocytes may continue during the final hours of oocyte development in vitro and may relate to the eventual outcome of maturation and insemination. This is potentially important because incomplete growth has been linked to reduced developmental capacity (Moor *et al.*, 1998). Moreover, imprinting of certain genes occurs late in the growth phase in mouse oocytes (Lucifero *et al.*, 2004) and imprinting may be disturbed by in vitro conditions in mice (Kerjean *et al.*, 2003). The possibility of incomplete imprinting may therefore be relevant to the safety and clinical outcome of IVM and insemination of oocytes that have not yet achieved their full size.

Oocyte growth

During its growth phase, the human oocyte increases in diameter from ~30µm to >110µm, over a period of at least 8 weeks (Gougeon, 1986). During this time, its nucleus remains arrested in first meiotic prophase. The diameter of the in vivo matured human oocyte, excluding the zona pellucida, is normally approximately 110-120µm (which we confirm here) while the zona pellucida is normally approximately 15-20µm thick (Veeck, 1999). Including the zona pellucida and perivitelline space, the pre-ovulatory oocyte commonly has a diameter around 150µm (Veeck, 1999).

Measurements of oocyte diameter of immature oocytes at collection and after IVM culture confirmed the size dependence of maturation, as has been extensively documented in other species. However, it also resulted in unexpected observations of the relatively

small size of immature oocytes relative to those matured in vivo, as well as evidence of growth of immature oocytes in vitro. An increase of 3μm average diameter from 106 to 109μm (Fig 1b) would result in ~ 54461μm^3 increase in cytoplasmic volume, constituting an astonishing 8% increase in volume over two days. Hence, a relatively small change in diameter that could easily pass unnoticed during routine clinical procedures is associated with a relatively large change in volume. It therefore seems likely to us that growth of human oocytes in vitro has been underestimated and may provide worthwhile information about oocyte potential. Oocyte growth in vitro differed between the patient groups studied, suggesting that endocrine or other patient factors may contribute to its control. Further study is clearly indicated.

The oocytes we observed from patients undergoing ICSI achieved growth in the total absence of somatic cellular support. To our knowledge, this is a novel observation. Others have documented that oocyte growth in fetal ovary cultures does not depend exclusively upon intimate follicular cell communication (McLaren and Buehr, 1990; Zhang *et al*., 1995), however, somatic cells were present in large numbers in these systems. The nature of the oocyte growth observed in our cultures has not been established, however, variables in the medium are not thought to be the cause since oocytes from PCO patients were cultured under identical conditions and did not show the same extent of growth. Control experiments demonstrated that the osmolarity of cultures maintained in a humidified incubator (37°C, 5% CO_2 in air) varied by <1% after 24 hr. Moreover, both increases and decreases in oocyte diameter were observed in the same culture preparations, discounting alterations in media osmolarity as the mechanism by which oocyte size changes occurred.

In this study, oocytes from patients with PCO were retrieved laparoscopically from antral follicles ~10mm diameter or less, whilst oocytes donated by patients undergoing ICSI were retrieved transvaginally from larger follicles >10mm diameter. Other important differences exist between the groups. The endocrine environments in PCO patients and those receiving ovarian stimulation in preparation for ICSI are distinctly different. Moreover, oocytes that remain immature despite an ovulatory stimulus may be defective and harbour cytogenetic abnormalities, even if maturation occurs (Magli et al, 2006). Immature oocytes exposed to an ovulatory stimulus are known to undergo IVM more quickly that those without a stimulus (Chian et al, 2000), as has been documented as a difference between the patient groups in this study (Cavilla et al, 2001). Dubey *et al.* (1995) suggested that competence in human oocytes may normally be conferred relatively late, perhaps only when follicles have reached diameters of >10mm, although occasional pregnancies have resulted from IVM of oocytes from smaller follicles (Trounson *et al.,* 1994). Oocytes retrieved from ICSI patients were significantly larger at collection than those retrieved from PCO patients (mean diameter 111µm vs 106µm), which may have been partially due to the larger size of follicles in patients undergoing ICSI.

Based upon data from unstimulated gynaecology patients, Durinzi *et al.,* (1995) deduced that an oocyte diameter of 105µm at the time of collection was the threshold for GVBD, while oocytes of >115µm would mature to MII. Our data for oocytes retrieved from patients with PCO produced lower thresholds for GVBD (81µm) and MII (103µm), and most of the oocytes reaching MII in our study had a diameter <115µm.

Effect of FF-MAS on oocyte growth

Mature, immature and atretic oocytes cultured with FF-MAS (10 or 30μg/ml), but not those in control conditions, had significantly different diameters on day 0 ($p<0.05$) in the PCO group. For oocytes from ICSI patients, the differences in diameter between mature, immature and atretic oocytes on day 0 were not significant. Interestingly, in the oocytes from ICSI patients, there was significant growth between collection and day 0. Growth was greater in oocytes that became mature than in those that remained immature. Oocytes becoming atretic tended to shrink. The observation of large oocytes from ICSI patients undergoing atresia upon exposure to FF-MAS is intriguing. This could perhaps reflect either an adverse effect of FF-MAS on fully grown oocytes, or that large immature oocytes have a reduced quality and developmental potential. However, the result was non-significant.

The mechanism of action of FF-MAS is not yet known, and its potential as an adjunct to oocyte and embryo cultures is controversial (Downs *et al.,* 2001; Vaknin *et al.,* 2001; Tsafriri *et al.,* 2002, 2005; Bergh *et al.*, 2004; Loft *et al.*, 2004; Marín Bivens *et al.,* 2004). One possibility arising from our data is that FF-MAS may influence oocyte growth. FF-MAS is a steroid related to lanosterol and cholesterol (Byskov *et al.,* 1995, 2002). Cholesterol is known to influence membrane fluidity and the function of membrane proteins (McIntosh and Simon, 2006) and relative levels of cholesterol and MAS change in follicular fluid during maturation (Bokal *et al.,* 2006). While no direct effects of FF-MAS upon membrane fluidity have been reported, oocyte growth from diameters of 106 to 109μm, as exemplified above, would result in an associated increased surface area of 2026μm^2 (5.4%) (assuming the oocyte to be spherical – in fact, if the number of microvilli also increased, the overall surface area could increase more), so membrane elasticity and/or synthetic capacity may be a crucial factor for oocyte growth

and subsequent embryo cleavage. We therefore hypothesise that FF-MAS may be involved in membrane biochemistry, in addition to any role in local communication. There is some evidence in amphibians to support membrane fluidity having a role in meiotic arrest, controlled by progesterone and cAMP, so this idea warrants further study (Morrill *et al.*, 1989; 1993). An alternative perspective, if our hypothesis is correct, is that the ooplasm could become less rigid and oocytes more likely to flatten slightly under their own weight. This could explain the increased diameters of a focal plan observed through the oocyte's centre. Three dimensional imaging will be required to test this idea.

As oocytes from both our patient groups have grown in vitro, it is clear that either the growth phase of these immature oocytes has not been completed in vivo, or that it may be resumed under certain conditions. IVM oocytes are smaller than their in vivo counterparts in mice, however, 87% were capable of emitting a polar body and undergoing normal nuclear maturation (Sun et al, 2005). In 1998, Moor *et al.* suggested that the reduced developmental potential observed in human oocytes matured in vitro might be attributable to incomplete oocyte growth, however, no data were presented on human oocytes to illustrate the point. In the present study, our data provide evidence that in vivo matured oocytes from ICSI patients are larger than immature oocytes, showing that the immature oocytes were not fully grown at collection. Moreover, the prospect that crucial events such as genetic imprinting may be incomplete in such oocytes (Lucifero *et al.*, 2004; Borghol *et al.,* 2006) should promote re-evaluation of IVM protocols to avoid the collection of growing oocytes, or to accommodate their need for further growth.

Zona pellucida

418 The zona pellucida, synthesized by the oocyte, is crucial to fertilization and early
419 development. According to Bertrand *et al.* (1995), human zona thickness varies from 10-
420 31μm, with a mean of 17.5μm. In the present study, on day 0 all mature oocytes had a
421 zona thickness of 15-24μm. This was within the expected range and was unrelated to
422 maturity.
423
424 The oocyte + zona measurements at collection for ICSI patients relative to the PCO group
425 is consistent with their larger oocyte diameter at collection. The oocyte + zona
426 measurement did not offer any additional information over that of oocyte diameter, and
427 may reduce the discriminatory potential of oolemma measurements.
428
429 Various studies of zona pellucida thickness, or thickness variation, as an indicator of
430 oocyte function have resulted in conflicting results (Bertrand *et al.,* 1995, 1996; Garside
431 *et al.,* 1997; Gabrielsen *et al.,* 2001; Pelletier *et al.*, 2004; Shiloh *et al.,* 2004; Shen *et al.*,
432 2005; Sun *et al.,* 2005; Kilani *et al.,* 2006). Both thickening and thinning of the zona have
433 been reported in cultured embryos, however, our study has not identified changes in zona
434 thickness with time, nor was zona pellucida thickness a useful measure related to oocyte
435 maturation.
436
437 The zona thickness measurements obtained for fertilized oocytes matured in vitro in this
438 study were larger than measurements of in vivo matured oocytes obtained by others using
439 differential interference optics (eg day 1, 16.4 ± 3.1μm, Bertrand *et al.,* 1996; 17.7 ±
440 0.14μm, Garside *et al.,* 1997) or computer assisted methods (eg, day 1, 19.9$\pm$1.92 in
441 conception cycles and 18.6$\pm$1.8μm in non-conception cycles, Shen *et al.,* 2005). This

could indicate an effect of culture or differences in the source of oocytes and their developmental potential.

Conclusion

In conclusion, we have extended previous observations on human oocyte maturation in relation to the oocyte's dimensions and origins. Moreover, we have provided the first quantitative non-invasive analysis of oocyte growth during maturation in vitro, highlighting differences from in vivo matured oocytes and demonstrating effects of FF-MAS upon oocyte growth. This work has raised prospects for a non-invasive assessment of oocyte growth in vitro as well as indicating the risks inherent in using oocytes that are not fully grown for clinical application.

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457 **References**
458

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591 **Table I Outcome of oocyte culture according to levels of cumulus on immature**
592 **oocytes (n=128) at collection from patients with PCO.**
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Cumulus grade	Oocyte after culture		
	Mature	**Immature**	**Atretic**
0 (n=65)	13 (20.0%)	23 (35.4%)	29 (44.6%)
1 (n=17)	10 (58.8%)	6 (35.3%)	1 (5.9%)
2 (n=5)	2 (40%)	3 (60%)	0
3 (n=41)	13 (31.7%)	20 (48.8%)	8 (19.5%)

596

597 **Key:** 0 = devoid of cumulus/no more than 10 scattered cells; 1 = partial cover; 2 =
598 complete cover; 3 = substantial multilayered cover.

Table II.

Oocyte diameters on day of maturation according to origin of oocyte and developmental competence in vitro.

		Oocyte diameter (μm) on day 0			
		Surviving but not maturing in vitro	**Maturing in vitro**	**2PN fertilisation by ICSI**	**Cleavage having fertilized with 2PN**
PCO	Median	107	108	112.5	112.5
	Interquartile range	(105-108)	(106-113)	(107-116)	(108-116)
	Range	(81-140)	(103-121)	(105-121)	(105-121)
	n	32	28	10	8
ICSI	Median	115	114	113	113
	Interquartile range	(112-118)	(110-116)	(109-114)	(111-117)
	Range	103-126	(106-131)	(106-125)	(107-125)
	n	25	35	13	7

PCO = polycystic ovaries. These patients underwent laparoscopic retrieval of oocytes without ovarian stimulation.

ICSI = intracytoplasmic sperm injection. These patients underwent transvaginal oocyte collection after ovarian stimulation for a clinical cycle of ICSI as a treatment for infertility.

FIGURE LEGENDS

Figure 1

Frequency histograms of mean oocyte diameter at the time of oocyte collection and after IVM culture.

Only oocytes viable at the time of collection were measured.

A: Oocytes from unstimulated PCO patients (86 measurements at collection, 90 after culture)

B: Oocytes from stimulated ICSI patients (48 measurements at collection, 61 after culture).

Notice that ICSI patient-derived oocytes have grown during the culture while those from PCO patients have not.

Similar results were obtained when only those oocytes having measurements available both at collection and after culture were plotted.

Figure 2

Frequency histograms of mean oocyte diameter after culture for oocytes that either matured in vitro, remained immature or became atretic in culture.

A: Oocytes from unstimulated PCO patients

B: Oocytes from stimulated ICSI patients.

Figure 3

Oocyte diameters during culture in control conditions or with FF-MAS for oocytes from PCO patients or patients undergoing ICSI treatment. Results are presented according to the outcome of in vitro maturation culture.

Oocytes in a-c were collected from unstimulated PCO patients with and those in d-f were collected from patients undergoing ICSI treatment.

The control, 10μg/ml FF-MAS and 30μg/ml FF-MAS results are shown in the top, middle and bottom panels respectively. Mean ± SEM.

Points with similar symbols are significantly different ($p<0.05$).

Figure 4

Numbers of oocytes donated by patients undergoing ICSI treatment, that fertilized and cleaved after maturation in vitro, according to oocyte diameter

a) Oocytes that matured within 24 hr

b) Oocytes that matured within 48 hr

Figure 5

Frequency histograms of mean oocyte+zona diameter at the time of oocyte collection and after IVM culture.

Only oocytes viable at the time of collection were measured.

A: Oocytes from unstimulated PCO patients (48 measurements at collection and after culture)

B: Oocytes from stimulated ICSI patients (46 measurements at collection and 60 after culture).

659 Similar results were obtained when only those oocytes having measurements available
660 both at collection and after culture were plotted.
661
662 **Figure 6**
663 **Frequency histograms of mean oocyte+zona diameter after culture for oocytes that**
664 **either matured in vitro, remained immature or became atretic in culture.**
665 A: Oocytes from unstimulated PCO patients
666 B: Oocytes from stimulated ICSI patients.
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