

## Analysis of meiotic spindle and zona pellucida birefringence of IVM oocytes in PCOS patients

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Received: 19.05.2015 • Accepted/Published Online: 12.06.2016 • Final Version: 27.02.2017

**Background/aim:** Polycystic ovarian syndrome (PCOS) is one of the most common causes of infertility. One of the best therapeutic options for PCOS patients is intracytoplasmic sperm injection (ICSI). In vitro maturation (IVM) can also be a useful technique for these women. The goal of this study was to evaluate both the zona pellucida (ZP) birefringence and meiotic spindle (MS) of in vivo- and in vitro-matured oocytes from PCOS patients using the PolScope system.

**Materials and methods:** Immature oocytes undergoing IVM and MII oocytes were obtained from PCOS patients in an ICSI program. Using PolScope, the presence of MS and ZP birefringence was assessed in both in vivo-matured oocytes (n = 32) and IVM oocytes (n = 24). Oocytes were classified as having highly birefringent (HB) ZP and lowly birefringent (LB) ZP. Furthermore, the rates of fertilization after ICSI were evaluated.

**Results:** The maturation rate was 68.5% after IVM. The percentage of a HB-ZP was significantly higher in the IVM oocytes than in vivo-matured ones (58.3% vs. 31.2%, respectively; P = 0.04). There were similar outcomes for the fertilization rates and MS detection between the two groups (P = 0.80 and P = 0.53, respectively).

**Conclusion:** Clinical IVM is a safe technology for the maturation and maintenance of oocyte integrity in PCOS patients. The use of the noninvasive PolScope is recommended for detection of healthy oocytes in ICSI.

**Key words:** Polycystic ovarian syndrome, in vitro maturation, zona pellucida birefringence, meiotic spindle, fertilization

### 1. Introduction

Polycystic ovarian syndrome (PCOS) is one of the most common causes of infertility in women of reproductive age (1). PCOS is characterized by menstrual cycle irregularity, chronic anovulation, ovarian polycystic morphology on ultrasound, and hyperandrogenemia (2,3). Currently, there are several therapeutic options for infertile women with PCOS, one of which is intracytoplasmic sperm injection (ICSI) following controlled ovarian hyperstimulation (COH). Hence, by using COH multifollicular recruitment and retrieval of immature oocytes with subsequent application of in vitro maturation (IVM) technology, the number of matured oocytes can be increased for ICSI programs (4,5).

IVM can be a useful technique for women with PCOS who are at the risk of ovarian hyperstimulation syndrome (OHSS) (6,7). OHSS is one of the disadvantages of ovarian stimulation with large doses of gonadotropins, which is unnecessary for IVM (8,9). On the other hand, the

oocyte quality is the determining factor for the outcome of assisted reproductive technology (ART) cycles (10). It has been confirmed that assessment of the zona pellucida (ZP) birefringence and meiotic spindle (MS) using PolScope could be helpful to select suitable oocytes for ICSI without negative effects on matured oocytes. The PolScope system is recommended to assess the MS in order to decrease spindle damages during sperm injection (5).

Spindles are subcellular structures that are present in matured oocytes and composed of microtubules. Disturbance of the MS can directly affect the development of embryo, due to abnormal chromosome alignment leading to polyploidy and aneuploidy. By definition, MS microtubules are essential organelles of oocytes. However, they are sensitive to chemical and physical changes, such as temperature and culture conditions (5,11). The ZP is another birefringent structure that could be assessed by PolScope as a reliable marker of oocyte quality and subsequent ART outcomes. The ZP is a unique

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extracellular coat composed of filaments that are organized in a three-dimensional network structure surrounding maturing oocytes during ovulation and fertilization, up to embryonic development. Currently, software programs are available that automatically analyze the inner layer of ZP birefringence scores, relying on intensity and distribution of the birefringence (5,12). Therefore, the objective of the present study was to compare the MS and ZP birefringences of both in vivo- and in vitro-matured oocytes from PCOS patients undergoing an ICSI program.

## 2. Materials and methods

### 2.1. Stimulation protocol and oocyte collection

The oocytes were collected from PCOS patients admitted to the Research and Clinical Center for Infertility, Yazd, Iran. Their ages ranged from 22 to 39 years (mean age  $\pm$  SD: 29.64  $\pm$  5.31 years). Each patient provided written informed consent before the treatment. An independent ethics committee in our institute approved the study. Briefly, pituitary downregulation using an exogenous GnRH agonist or antagonist was started. Ovaries were then stimulated with follicle-stimulating hormone (FSH; Ferring Co., Germany). By monitoring, when two or more follicles measuring  $\geq$ 18 mm in diameter were observed on transvaginal ultrasound, recombinant hCG (rhCG; IBSA Co., Switzerland) was administered, and 36 h later oocyte retrieval was performed by transvaginal ultrasonography-guided aspiration.

### 2.2. Oocyte preparation

All cumulus-oocyte complexes (COCs) were incubated for 2–3 h in culture medium (G-IVF; Vitrolife, Sweden) at 37 °C and 6% CO<sub>2</sub>. The cumulus and coronal cells were removed by 30–60 s of exposure to 80 IU/mL hyaluronidase (Irvine Scientific, USA) and by pipetting the COCs with a pasture pipette. The denuded oocytes were classified according to their stage of maturation. Based on extrusion of the first polar body (PB), oocytes were considered as mature (MII) and used for ICSI procedures, and immature oocytes underwent IVM.

### 2.3. IVM procedures

Initially, immature oocytes were classified as GV- or MI-stage. Then they were washed in 3 drops of washing medium (SAGE IVF) and cultured in maturation medium (SAGE, USA) supplemented with 75 mIU/mL FSH and 75 mIU/mL LH (Ferring, Germany) at 37 °C in an incubator under 5% CO<sub>2</sub> and 95% air with high humidity. Nuclear maturation was evaluated after 24 and 48 h using a stereoscope (Olympus, Japan).

### 2.4. Spindle and ZP birefringence examination

For imaging spindles and ZP birefringence, each mature oocyte was placed individually in an equilibrated 3-mL droplet of buffered medium (G-Mops-V1; Vitrolife)

in a glass-bottomed culture dish (WillCo-Dish; Bellco Glass, USA). Oocytes were immediately imaged under a polarizing optical system (OCTAX PolarAIDE; Octax, Germany), then detection of the MS and homogeneity of the inner layer of the ZP was done. In this manner, oocytes were classified as having high ZP birefringence with a score of  $\geq$ 10 and low ZP birefringence with a score of  $<$ 10.

### 2.5. ICSI procedure

ICSI was performed according to the methodology described by Khalili et al. (13). Before the microinjection, oocytes were incubated for 3 h and then denudation from cumulus cells occurred using 80 IU/mL hyaluronidase (Sigma Chemical Co., USA) along with the mechanical aid of Pasteur pipettes. Each MII oocyte was washed in culture media and then their maturity was evaluated using an inverted microscope (Nikon TE300, Japan). Following semen analysis and sperm preparation, the motile spermatozoa were placed in 10% polyvinylpyrrolidone solution (PVP) droplets (Irvine Scientific) and the best morphologically spermatozoa were selected for the microinjection procedure. The injected oocytes were washed twice and placed individually in fresh droplets of G1 (Vitrolife) covered with mineral oil (ReproLine Co., Germany).

Fertilization was evaluated 16–18 h after ICSI under an inverted microscope (TE300; Nikon, Japan) and injected oocytes checked for the presence of two pronuclei (2PN) and a second PB were considered to be normal. Unfertilized oocytes were incubated longer and checked again.

### 2.6. Statistical analysis

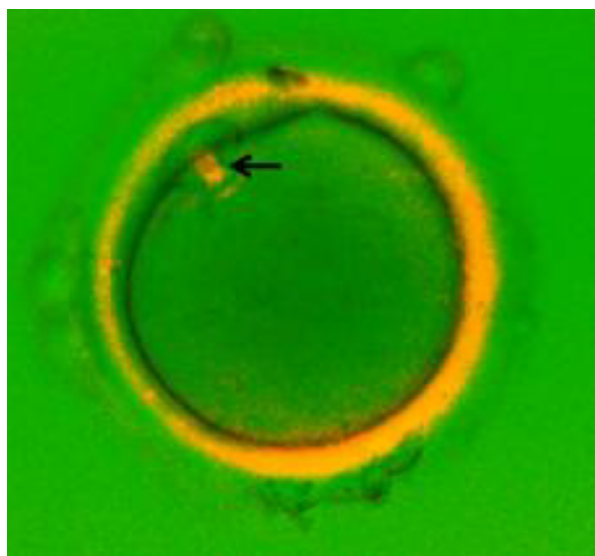
Statistical analysis was carried out using chi-square and Fisher exact tests. Data were presented as mean  $\pm$  SD and as odds ratios (ORs) and 95% confidence intervals (95% CIs). Data were analyzed using SPSS 20 (IBM Corp., USA). Statistical significance was set at  $P < 0.05$ .

## 3. Results

In all, 158 oocytes were retrieved from 11 patients, of which 123 (77.8%) were MII, whereas 30 (18.9%) and 5 (3.1%) were GV and MI oocytes, respectively. After 24–48 h of in vitro culture, 24 (68.5%) oocytes extruded the first PB and reached the MII stage. Thirty-two in vivo-matured oocytes from PCOS patients were compared with IVM oocytes. When the oocytes were examined with PolScope, the percentage highly birefringent ZP was higher in the IVM than the in vivo-matured group (58.3% vs. 31.2%, respectively;  $P = 0.04$ ). A birefringent spindle was detected in 21.9% of in vivo-matured MII oocytes compared with 29.2% of IVM MII oocytes ( $P = 0.53$ ; Table; Figure). After ICSI, the rate of fertilization achieved by in vivo-matured oocytes (65.6%) was slightly higher than the rate for IVM ones (62.5%), but the difference was insignificant ( $P = 0.81$ ).

**Table.** ZP birefringence/spindle visualization and fertilization rates in in vivo- and in vitro-matured oocytes from PCOS patients.

	In vivo		In vitro		Odds ratio (95% CI)	P
	n	%	n	%		
ZP birefringence						
High	10	31.2	14	58.3	0.32 (0.10–0.97)	0.043
Low	22	68.8	10	41.7		
Spindle visualization						
Visible	7	21.9	7	29.2	1.47 (0.43–4.95)	0.533
Not visible	25	78.1	17	70.8		
Fertilization rate						
Fertilized	21	65.6	15	62.5	0.87 (0.29–2.62)	0.809
Unfertilized	11	34.4	9	37.5		

**Figure.** PolScope (OCTAX PolarAID; Octax, Herbon, Germany) image of an IVM human oocyte. A bright meiotic spindle (arrow) is clearly visible (40× magnification).

#### 4. Discussion

To date, PCOS is one of the most prevalent causes of infertility in women of childbearing age. In this study, the majority of patients with PCOS were younger than 35 years (86.6%). Although PCOS patients could benefit from ART, which includes direct manipulation of gametes, several days of treatment are required with gonadotropin to achieve pregnancy. In general, 15%–20% of the oocytes collected in ART cycles are immature (14,15). It has been reported that IVM of these immature oocytes is a potentially useful treatment for increasing the number of embryos, especially in poor responders, such as PCOS patients (16).

Therefore, most notably PCOS patients might benefit from emerging IVM technologies and abbreviated COH protocols (9). The first pregnancy and delivery of a healthy baby after IVM obtained from an anovulatory woman with PCOS was reported by Trounson et al. in 1994 (17). In the present study, the oocyte maturation rate after IVM in PCOS patients undergoing oocyte retrieval for ICSI was 68.5%. However, Child et al. showed that the maturation rate of immature oocytes retrieved during an unstimulated cycle from women with PCOS was as high as 76% (18). In another study by Du et al., the oocyte maturation percentage from nonstimulated cycles in PCOS cases was

only 63%. They concluded that application of the IVM method in the treatment of infertility could be offered as an alternative to conventional IVF (19). It was reported that FSH priming before retrieval of immature oocytes from patients with PCOS could improve the maturational potential of the oocytes but did not increase the number of oocytes harvested from the patient (20). Those results are consistent with a previous study by Cha and Chian (21) that showed that, when oocytes were obtained from stimulated cycles, germinal vesicle breakdown (GVBD) was observed after 30 h of in vitro culture compared with oocytes obtained from unstimulated cycles where GVBD was seen after 48 h. In addition, Barnes et al. (22) compared the maturation and fertilization rates of immature oocytes selected from two different groups including women with normal ovaries and PCOS patients in an unstimulated cycle, and they found these parameters to be significantly higher in the normal group compared to PCOS patients. The present study evaluated the developmental competence of in vivo- and in vitro-matured oocytes of PCOS patients undergoing an ICSI program. In our experience, the rates of fertilization after ICSI did not differ significantly between the two groups.

The MS is a dynamic structure during meiosis (23) and damage to the metaphase spindle may happen in some oocytes during sperm injection if the spindles are away from the first PB (24). It was suggested that the presence of a birefringent spindle in MII oocytes could predict a higher fertilization rate and embryo developmental competence after ICSI (25). It is also well recognized that the spindle and chromosomes have critical importance in fertilization and subsequent embryo development (26). Therefore, spindle imaging could help to identify abnormalities in the oocyte MS (27). Li et al. analyzed the MS via staining by immunocytochemistry. Spindles were observed in 75% of their IVM oocytes and in 81.5% of oocytes matured in vivo. They also found that the rate of spindle abnormality in IVM oocytes was higher compared with those matured in vivo. However, these oocytes retrieved from stimulated and unstimulated ovaries of infertile women with PCOS and their data suggested that IVM could play a deleterious role in MS and chromosome arrangements (26). There are many reports about the sensitivity of spindles to environmental factors (27–29). Some authors reported high rates of abnormal chromosomes in human oocytes following IVM (30–32). Data generated from this study showed that the percentage of oocytes in which a birefringence spindle was detected was insignificantly higher in the IVM group when compared with in vivo-matured oocytes. Moreover, Omidi et al. and Rienzi et al. reported that the rate of spindle presentation in IVM oocytes was lower than in oocytes matured in vivo (5,33). In the last decade, visualization of ZP-BF by using PolScope has been recognized as a noninvasive test of oocyte quality. The ZP is a multilaminar

glycoprotein coat that forms during oogenesis (34,35). At the molecular level, the ZP is composed of three layers that are composed of 4 protein filaments, ZP1, ZP2, ZP3, and ZP4 (12). However, the inner layer exhibits maximum birefringence with application of the PolScope (36). Birefringence is one of the properties of the human ZP that have been suggested as a marker of oocyte quality and can indicate the formation of the ZP during maturation to MII. Different culture conditions may also affect the ZP architecture (37,38). We hypothesized that the zona with high-order structured fibers might reflect the healthiness of an oocyte and its full oocyte maturation (nuclear and cytoplasmic) (12). In addition, in that study we observed that the percentage of a HB-ZP was significantly higher in the IVM oocytes than in vivo-matured ones, similar to findings reported by Omidi et al. According to Braga et al. and Petersen et al. (12,37), the ZP birefringence is unaffected during IVM, and these findings confirm our results. We also observed that the fertilization rate after sperm injection (62.5%) in IVM oocytes was similar to those reported by earlier authors (39,40).

We used ICSI for fertilization of in vitro- or in vivo-matured oocytes and similar fertilization rates were observed between the two groups. It was reported that women with PCOS have low quality oocytes and fertilization rates compared to other infertile women (41,42). Another study by Ebner et al. showed that there was a positive correlation between high/positive ZP birefringence scores and blastocyst formation, but not embryo quality or pregnancy (43). Using a different technique (intensity of the zona inner layer retardance), Raju et al. observed that when the retardance of the ZP inner layer was  $>3$  nm the rate of embryonic development to the blastocyst stage was higher compared to oocytes with retardance of  $<3$  nm (11). Another study reported that there were no differences in the pregnancy rates of in vivo-matured oocytes and IVM oocytes (2), and their findings are similar to our results. In similar study, Child et al. reported insignificant differences between the rates of fertilization and embryo cleavage when oocytes were matured in vitro or in vivo (18).

In conclusion, an absence of a relationship was recognized between in vitro nuclear and cytoplasmic maturation of oocytes from stimulated PCOS patients and deleterious effects on ZP birefringence and MS visualization. To our knowledge, clinical IVM combined with PolScope technology may be safely used for both maturation and visualization of oocyte integrity in ICSI programs for PCOS patients.

#### Acknowledgment

This present study was supported by a grant from the Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

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