Assessment of oocyte quality in polycystic ovarian syndrome and endometriosis by spindle imaging and reactive oxygen species levels in follicular fluid and its relationship with IVF-ET outcome

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ABSTRACT

OBJECTIVES: The aim of this study is to examine meiotic spindle in oocytes along with reactive oxygen species (ROS) levels in follicular fluid of women undergoing IVF and to correlate these findings with embryo quality and pregnancy outcome. **MATERIALS AND METHODS:** 167 women aged 25–35 years with endometriosis (Group A), polycystic ovarian syndrome (PCOS) (Group B) and tubal block (Group C) were included. Long protocol downregulation using recombinant follicular stimulating hormone was used for ovarian stimulation. Aspirated follicular fluid containing mature oocytes were analyzed for ROS levels and the oocytes were assessed for the presence of meiotic spindle using Cri-Oosight™ Polscope. Fertilization, embryo quality, endometrial assessment, and final pregnancy outcome were assessed. **RESULTS:** Meiotic spindles were visualized in a higher proportion of mature oocytes retrieved from women with endometriosis (66%) as compared to those with PCOS (50.5%) and tubal block (62.3%). ROS levels were also observed to be significantly less in the follicular fluid of oocytes in women with endometriosis (Group A) as compared to the other two groups ($P \leq 0.001$). However, pregnancy rates were observed to be lower in Group A (32%) than Groups B (39%) and C (44%), respectively. Within each group, oocytes with spindle visualization yielded a higher number of Grade 1 embryos ($P < 0.05$) as well as lower ROS levels in follicular fluid ($P \leq 0.001$) as compared to those where spindle could not be visualized. **CONCLUSIONS:** There was good correlation between spindle imaging and ROS levels as reliable predictors of oocyte assessment. Women with endometriosis had low ROS levels and good spindle imaging results suggesting a possible role of endometrial receptivity accounting for lower pregnancy rates in these women. Poor oocyte quality, as reflected by higher mean ROS levels and low number of oocytes with spindle visualization, could be the factor impeding pregnancy in women with PCOS as compared to women with tubal block.

KEY WORDS: Endometriosis, meiotic spindle imaging, oocyte quality, PCOS, reactive oxygen species

INTRODUCTION

Advances in reproductive medicine have made clear that one of the most important factors determining the outcome of embryo development is oocyte quality.[1,2] Therefore, numerous prognostic factors based on morphological characteristics of the oocyte have been devised that may allow prediction of oocyte quality, fertilization rates, and embryo development. However, current available techniques are not very reliable in predicting which metaphase II (MII) oocyte will lead to an embryo which will implant and result in a clinical pregnancy.

A promising approach has been proposed that the use of spindle imaging may be a predictor of oocyte quality.[3] The meiotic spindle is responsible for correct chromosome segregation during the process of oogenesis with disturbances in spindle assembly increasing the risk of chromosome mal-segregation and aneuploidy in oocytes.[1,4]

Aneuploidy is one of the most important reasons of abnormal fertilization, early embryo death, poor embryo development, and spontaneous abortion in humans.[5-7] Thus, oocytes possessing a birefringent spindle in Polscope microscopy tend to have a better developmental potential compared to those without a spindle.[3,4,8]

Polarized light microscopy, in combination with imaging process software, allows imaging of non-invasively birefringent spindles in living cells.[2-4] Ovulated oocytes are arrested in MII, at which point a dense array of filaments and bundles forms the meiotic spindle. This molecular order is essential in cytoskeletal components for the optical property known as birefringence.[9]

Several studies in the literature suggest that the presence of a birefringent meiotic spindle predicts a higher embryonic developmental competence. A clear positive correlation between visualization of the meiotic spindle with fertilization rates, embryo development, and progression to blastocyst stage has been described in these studies.[1,3,8] Importantly, no detrimental effects on mouse oocyte and/or embryo development have been found after exposure to the polscope.[10]

There are few studies available at present assessing the association between reactive oxygen species (ROS), oxidative stress, and female infertility[11-13] with most focus currently directed at assessing the impact of oxidative stress on the reproductive potential in men. Oxidative stress is defined as a disequilibrium between the production and neutralization of reactive oxygen species (ROS), which may occur as a result of excess ROS production and/or as a result of deficiency of antioxidant mechanisms.[14] There is no unanimity in the literature regarding the effect of follicular fluid oxidative stress markers on the quality of oocytes and embryos and the subsequent clinical outcome. Whereas Agarwal *et al*.[12] suggest that oxidative stress influences the oocyte and embryo quality and thus the fertilization rate, Jozwik *et al*.[15] did not find the concentration of oxidative stress markers in the follicular fluid to reflect the reproductive potential of oocytes. While Attaran *et al*.[16] reported that women who became pregnant by IVF had higher levels of ROS than those who did not, Pasqualotto *et al*.[11] observed a positive correlation between both lipid peroxidation (LPO) and total antioxidant capacity (TAC) levels (TAC) with the pregnancy rate. Oyawoye *et al*.[13] have also shown that higher total antioxidant capacity level increases fertilization potential in women undergoing IVF. Thus, further studies are required to analyze the relationship between oxidative stress markers in follicular fluid with oocyte quality and subsequent pregnancy outcome as well as to define the ROS, LPO, or TAC levels that should be considered as optimum or abnormal.

According to published data, both endometriosis and polycystic ovarian syndrome (PCOS) may be associated with poor outcomes of assisted reproductive techniques, but what is controversial at present is whether this is due to an impairment of oocyte quality, defect in endometrial receptivity, or other factors. Suboptimal oocyte competence due to aberrant folliculogenesis as a result of both androgen excess and intrinsic molecular defects is the most commonly implicated mechanism leading to lower fertilization and implantation rates in women with PCOS but needs further confirmation.[17,18]

Mechanism of impaired fertility in women with endometriosis is less clear. Some authors have found impaired oocyte/embryo quality[19] or arrested embryo development[20] to be responsible for lower implantation rates in women with endometriosis while others have found alterations in the eutopic endometrium of women with endometriosis to be at least partly responsible for the subfertility in these women. Still others have implicated abnormal cross talk between the developing embryo and the endometrium preceding implantation to play an important role in the implantation failure in these women.[21] This aspect thus remains controversial at present and needs further exploration.

The objective of the current study is to evaluate and compare oocyte quality in women with PCOS, endometriosis and tubal factor (as controls) by means of Polscope imaging as well as by correlation of follicular fluid ROS levels with oocyte quality and to evaluate the effect of follicular fluid ROS levels on oocyte spindle formation in the three groups of women selected for the study and its correlation with embryo quality and pregnancy outcome.

MATERIALS AND METHODS

Approval for this prospective study was obtained from the Research Ethics Committee of the Institute. Written informed consent was obtained from women included in this study.

Subject selection

167 women aged 25–35 years were included in the present study. 56 women with endometriosis were classified as Group A while 48 PCOS women, diagnosed by the Rotterdam 2003 Criteria,[22] were designated Group B. 63 age-matched, regularly menstruating women with normal ovaries and tubal factor infertility served as controls (Group C). Tubal factor infertility included only those women who had salpingectomy for ectopic pregnancy or proximal tubal obstruction because of low-grade infection or fimbrial occlusion with or without mild peritubal adhesions. Tubal infertility associated with gross hydrosalpingeal

changes, dense pelvic adhesions, endometriosis, pelvic inflammatory disease, or tuberculosis was excluded.

Stimulation protocol

Pituitary downregulation was achieved by daily subcutaneous injection of 500 µg of leuprolide acetate (Lupride 4; Sun Pharmaceuticals, Mumbai, India) starting from the mid-luteal phase of the previous cycle and continued for a period of 14 days, or until the onset of next menstruation, whichever was earlier. When optimally downregulated, all patients were stimulated with recombinant follicular stimulating hormone (FSH) (Gonal-F; Serono, Geneva, Switzerland). Ovarian response was monitored regularly by transvaginal ultrasonography and serum estradiol assays and dose adjustment carried out accordingly. When the average diameter of the leading follicle(s) reached at least 18 mm, ovulation was triggered with 10,000 IU of urinary hCG (Pregnyl, Organon, The Netherlands). Oocytes were retrieved around 34–36 hours later under transvaginal ultrasound guidance.

Collection and processing of follicular fluid, ROS estimation and classification of patients based on visualization of meiotic spindle

Follicular fluid (FF) was carefully aspirated in separate tubes during oocyte collection and collected from each follicle of Group A (n = 310), Group B (n = 358), and Group C (n = 332). FF was excluded when the follicle was <15 mm in diameter or when contaminated with blood. FFs containing MII oocytes from each group (Group A, n = 215; Group B, n = 202; Group C, n = 220) were further subdivided based on the presence of MS (Meiotic Spindle) into group A1 (n = 142) and without MS into group A2 (n = 73). Similarly, Group B was classified as Group B1 (n = 102) and Group B2 (n = 100) and Group C as Group C1 (n = 137) and Group C2 (n = 83).

FF samples were centrifuged at 300 *g* for 7 min to remove cellular components and the clear supernatant was used for the measurement of ROS levels. ROS levels in freshly aspirated FF were evaluated by chemiluminescence assay[16] using luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma Chemical Co., St. Louis, MO, USA) as a probe. 400 µl of clear supernatant was placed in the cuvette of the luminometer (Berthold, Sirius Single Tube Luminometer, Model No. 0727) and 10 µl of 5 mM luminol in DMSO was added to it. Each sample was scanned for 10 min. ROS values were expressed as counted photons per second (CPS).

Spindle examination

310 oocytes from Group A, 358 oocytes from Group B, and 332 oocytes from Group C were collected and initially incubated in fertilization medium (Cook IVF, Sydney, Australia) for a minimum of 2 hours. For denudation, these oocytes were exposed to 80 IU/ml of hyaluronidase solution (Cook IVF) for 40 seconds. After this brief exposure, the oocytes were immediately transferred back into the culture medium droplet and reincubated for at least 30 min. The residual cumulus cells were finally removed by repeated aspiration and subsequently replaced into the culture droplets. The oocytes were incubated for at least another hour and then placed in 5 µl fertilization medium covered with mineral oil (Ovoil; Vitrolife, Gothenburg, Sweden) in a glass-bottomed culture dish (Willco Wells, Amsterdam, The Netherlands).

Immediately before the ICSI procedure, oocytes were placed on an Olympus inverted microscope with a heated stage (Tokai Hit, Thermoplate, Japan) at (mean ± SD) 37.0 ± 0.5°C and were observed at 400× magnification for their nuclear maturation stage.[23] MII oocytes were assessed according to the absence of the germinal vesicle and the presence of an extruded polar body. After this, to visualize the meiotic spindle, the oocytes were screened by using a polarization imaging software module, LC PolScope optics, and controller (Oosight TM META Imaging System; CRI, Woburn, MA, USA) having a sensitivity of 0.02 nm combined with a computerized image analysis system (SpindleView software). Oocytes which did not show the MS initially were rotated a maximum of three times to confirm their true absence. On the basis of spindle visualization or not, two groups were established: meiotic spindle present or meiotic spindle absent.

ICSI, Embryo culture, and ET

Immediately after imaging, Intracytoplasmic Sperm Injection (ICSI) was performed in all MII oocytes, with or without MS, within 3–5 hours of oocyte recovery. ICSI was preferred to conventional IVF as all oocytes were denuded for viewing of the MS. Oocytes with or without spindles were cultured separately for 14–16 hours in P1 medium (Irvine Scientific), supplemented with 10% synthesized serum substitute for examination of fertilization.

Pronucleus was noted 16–18 hours after microinjection and embryo quality assessed[23] before ET, approximately 48 hours (4-cell stage) after ICSI. An average of two to three grade I embryos were transferred to all women. Luteal support was given by 600 mg of natural micronized progesterone (Utrogestan; Laboratories Besins International, Paris, France) in divided doses till serum ß-hCG level was estimated on the 13th day of ET. Clinical pregnancy was defined when an ultrasound scan, performed 5 weeks after ET, revealed the presence of an intrauterine gestational sac with an embryo showing normal cardiac activity.

Statistical analysis

Data were analyzed using analysis of variance (one-way ANOVA) wherever appropriate. All analyses were

performed with the statistical software SPSS version 17 (SPSS, Inc., Chicago, IL, USA). Statistical significance was defined as $P < 0.05$.

RESULTS

Baseline characteristics of all groups of patients are summarized in Table 1. Characteristics of oocytes, ROS levels, and clinical outcome are summarized in Table 2.

As depicted in Table 2, although the number of oocytes retrieved was higher in women with PCOS (Group B) than the other two groups, they had a higher proportion of immature oocytes with only 56.42% oocytes graded as MII oocytes (as against 69.35% in endometriosis (Group A) and 66.27% in tubal block (Group C)). Meiotic spindles were visualized in 50.5% of mature (MII) oocytes retrieved from women in Group B, which was significantly lower than that in Group A (66%) ($P < 0.001$) as well as Group C (62.3%) ($P < 0.01$) with spindle visibility comparable in the latter two groups (Groups A and C). Fertilization rate in Group B was also lower as compared to Groups A and C but this was not statistically significant [Table 2]. However, significant difference was observed in Grade I and II embryo formation in Group B (67.9%) which was also low when compared with Group A (80.6%) and Group C (76.5%). The difference, however, was statistically significant ($P < 0.01$) when compared with Group A but not with Group C [Table 2].

Each group was then further analyzed on the basis of presence or absence of MS and its association with fertilization rates, embryo grading, and ROS levels in the follicular fluid. Fertilization rate [Table 3] and grade I and II embryo formation [Figure 1] were significantly higher in oocytes with visualization of MS in each group (A1, B1, C1) as compared to MII oocytes with nonvisualization of meiotic spindle (A2, B2, C2).

ROS in FF was significantly higher in the PCOS and tubal block group as compared to the endometriosis group ($P < 0.001$) [Table 2]. The absence of MS in each group (i.e. A2, B2, C2 versus A1, B1, C1, respectively) was associated with significantly higher ROS values ($P < 0.001$) [Figure 2].

DISCUSSION

The present study indicates the utility of meiotic spindle visualization and assessment of oxidative stress levels in follicular fluid for the assessment of oocyte quality. Even

Table 1: Baseline characteristics of women with endometriosis (Group A), PCOS (Group B), and tubal infertility (Group C)

Baseline characteristics	Group A (mean ± SD) ($n = 56$)	Group B (mean ± SD) ($n = 48$)	Group C (mean ± SD) ($n = 63$)
Mean age (years)	33.42 ± 3.53	32.9 ± 3.3	32.7 ± 3.8
BMI (kg/m^2)	21.42 ± 2.6	23.25 ± 1.23	20.84 ± 1.45
Duration of infertility (years)	7.1 ± 0.5	6.79 ± 2.8	6.84 ± 0.8
Basal FSH (IU/l)	6.84 ± 1.61	6.03 ± 1.39	5.99 ± 1.01
Basal LH (IU/l)	5.41 ± 2.33	7.07 ± 1.79	6.94 ± 0.84
Serum TSH (μIU/ml)	3.13 ± 1.1	3.55 ± 0.6	3.02 ± 0.57
Serum PRL (ng/ml)	14.82 ± 7.8	15.99 ± 6.68	16.00 ± 5.08

SD = Standard deviation

Table 2: Comparison of oocyte characteristics, ROS levels, and outcome parameters between the three groups

Outcome parameters	Group A ($n = 56$) endometriosis (mean ± SD)	Group B ($n = 48$) PCOS (mean ± SD)	Group C ($n = 63$) tubal factor (mean ± SD)	*P* value*
Number of oocytes retrieved (*n*)	5.54 ± 2.8	7.46 ± 3.9	5.27 ± 3.4	AB < 0.001 BC < 0.001 CA NS
Mature MII oocytes (%)	69.35 (215/310)	56.42 (202/358)	66.27 (220/332)	AB < 0.001 BC < 0.01 CA NS
Meiotic spindle present (%)	66 (142/215)	50.5 (102/202)	62.3 (137/220)	AB < 0.001 BC < 0.01 CA NS
ROS (cps)	64.59 ± 18.06	96.18 ± 75.75	98.28 ± 41.4	AB < 0.001 BC NS CA < 0.001
Fertilisation rate (%)	81.4 (175/215)	78.7 (159/202)	81.4 (179/220)	AB NS BC NS CA NS
Grade I + Grade II Embryo formation (%)	80.6 (141/175)	67.9 (108/159)	76.5 (137/179)	AB < 0.01 BC NS CA NS
Endometrial thickness (mm)	9.39 ± 1.76	10.34 ± 1.92	9.47 ± 1.82	AB NS BC NS CA NS
Pregnancy rate (%)	32.1	39.58	43.75	AB NS BC NS CA NS

MII = metaphase II, ROS = reactive oxygen species; cps = counted photons per second, AB = comparison between Group A and Group B, BC = comparison between Group B and Group C, CA = comparison between Group C and Group A, NS = not significant. *Statistically significant = $P < 0.05$ (ANOVA and post-hoc Bonferroni test)

with a higher oocyte yield in women with PCOS (Group B), the proportion of mature oocytes as well as MS visualization in these mature oocytes was lower as compared to the other two groups. The number of good quality embryos were observed to be significantly lower in this group as compared to women with endometriosis (Group A) and comparable to those with tubal factor (Group C). The relatively lower number of zygotes and embryos formed in Group B may be attributed to the higher number of oocytes lacking MS. In their meta-analysis, Heijnen *et al*.[24] similarly demonstrated a significantly higher number of oocytes retrieved with a lower fertilization rate and comparable number of good quality embryos in the PCOS than the control group (tubal factor). This has been confirmed by other authors.[25,26] Furthermore, in agreement with the findings by Heijnen *et al*.,[24] a comparable pregnancy rate was observed between women with PCOS (Group B) and tubal factor (Group C).

Table 3: Comparison of fertilization rate between Groups A1 (oocytes with MS from women with endometriosis), A2 (oocytes without MS from women with endometriosis), B1 (oocytes with MS from women with PCOS), B2 (oocytes without MS from women with PCOS), C1 (oocytes with MS from women with tubal infertility), and C2 (oocytes without MS from women with tubal infertility)

Cause of infertility	Endometriosis (Group A)		PCOS (Group B)		Tubal factor (Group C)		*P* value*
Parameters	Group A1 MS present (n = 142)	Group A2 MS absent (n = 73)	Group B1 MS present (n = 102)	Group B2 MS absent (n = 100)	Group C1 MS present (n = 137)	Group C2 MS absent (n = 83)	
Fertilization rate, %	90.8 (129/142)	63 (46/73)	92.2 (94/102)	65 (65/100)	89.8 (123/137)	67.5 (56/83)	A1A2 < 0.001 B1B2 < 0.001 C1C2 < 0.001

MS = meiotic spindle, A1A2 = comparison between Group A1 and Group A2, B1B2 = comparison between Group B1 and Group B2, C1C2 = comparison between Group C1 and Group C2
*Statistically significant = P < 0.05 (ANOVA and post-hoc Bonferroni test)

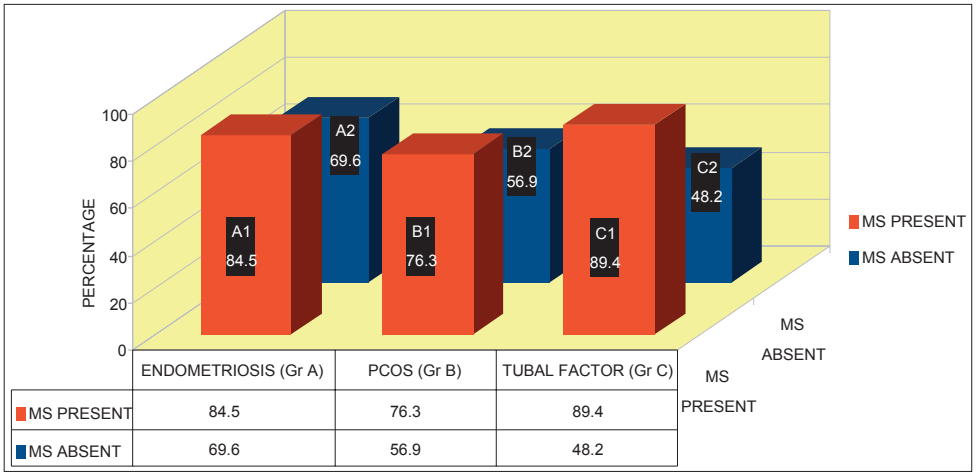

Figure 1: Comparison of percentage of good embryo formation between different groups based on the presence or absence of MS [MS, meiotic spindle A1A2 < 0.05 B1B2 < 0.01 C1C2 < 0.001 A1A2: Comparison between Group A1 and Group A2 B1B2: Comparison between Group B1 and Group B2 C1C2: Comparison between Group C1 and Group C2 *Statistically significant = P < 0.05 (ANOVA and post-hoc Bonferroni test)]

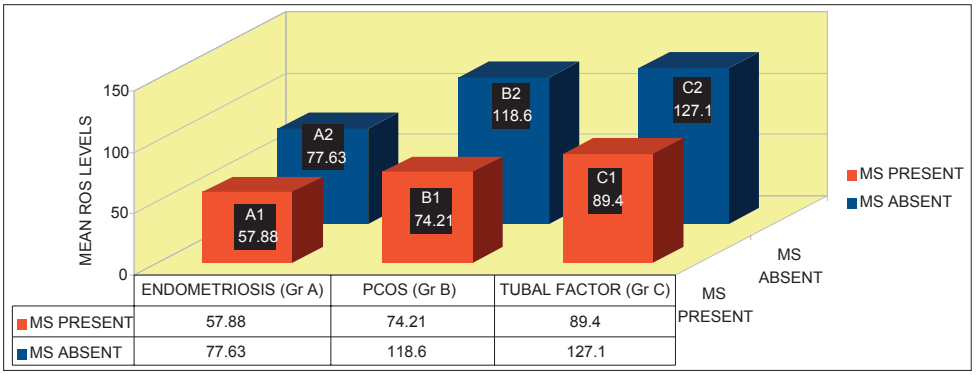

Figure 2: ROS levels in women with endometriosis, PCOS, and tubal factor on the basis of the presence or absence of meiotic spindle [MS, meiotic spindle A1A2 < 0.001 B1B2 < 0.001 C1C2 < 0.001 A1A2: Comparison between Group A1 and Group A2 B1B2: Comparison between Group B1 and Group B2 C1C2: Comparison between Group C1 and Group C2 *Statistically significant = P < 0.05 (ANOVA and post-hoc Bonferroni test)]

In women with endometriosis (Group A), MS could be visualized in two-thirds of mature oocytes with more than 80% good embryo formation rate, which was significantly higher than in the PCOS group and comparable to those with tubal factor. Traditionally, poor oocyte quality has been held responsible for poor ART outcome in women with endometriosis, supported by a meta-analysis by Barnhart *et al*.[27] which observed lower number of oocytes retrieved and lower fertilization rates in oocytes recovered from women with endometriosis as compared to controls of tubal factor infertility but other authors have reported conflicting results. Similar to the findings in the current study, several recent publications[28,29] have found no difference in folliculogenesis or the number of oocytes retrieved in patients with endometriosis as compared to other etiologies such as tubal factor infertility.

In the present study, despite good MS visualization in oocytes and good embryo quality, clinical pregnancy rate was observed to be lower in Group A (endometriosis) compared to Groups B (PCOS) and C (controls) [Table 2]. This is in sync with the reports of Barnhart *et al*.,[27] Kuivasaari *et al*.[30] and Omland *et al*.[31] who have reported lower pregnancy rates following IVF in women with endometriosis. Whether this low pregnancy rate can be attributed to a defect in endometrial receptivity or abnormal embryo cross talk needs further exploration but does not seem to be related to an oocyte defect, based on the findings of the current study.

It is suggested that a certain threshold level of ROS in FF is essential for several reproductive events[32] and may be required for a better reproductive outcome in IVF/ICSI cycles.[33] While some studies suggest optimum level of follicular fluid ROS to be a marker for predicting success in IVF cycles,[12] others have demonstrated a toxic effect of high ROS levels in FF on oocytes and lower pregnancy rates.[13,16,34] A study by Das *et al*. also indicated that high FF ROS levels tend to decrease the fertilization potential of oocytes; furthermore, a higher percentage of good quality embryos was observed corresponding to ROS levels <100 cps.[35] An association between adverse effects of OS on oocyte spindle assembly is suggested in mouse oocytes.[36] These results corroborate our present findings which indicate that in all groups (A, B, C), nonvisualizaton of MS was fairly correlated with higher follicular levels of ROS (r = 0.519, 0.294, 0.543 for Groups A, B and C respectively, $P < 0.01$).

Thus, irrespective of the cause of infertility, be it endometriosis, PCOS, or tubal infertility, the presence of MS and low ROS levels and lower OS is associated with an improved IVF outcome. Higher ROS levels and lower proportion of oocytes with MS in women with PCOS may thus contribute to a lower fertilization rate and lower good quality embryo formation. Though oxidative stress is postulated as one of the possible mechanisms of pathogenesis of endometriosis,[37] we found mean ROS follicular fluid levels to be lower in endometriosis as compared to PCOS and tubal factor controls [Table 2]. Bedaiwy *et al*.[38] and Wang *et al*.[39] have similarly shown no difference between ROS levels in peritoneal fluid between healthy women and those with endometriosis while Van Langendonckt *et al*.[40] observed that reactive oxygen species may be involved in endometriosis-associated infertility.

In summary, there was good analog between visualization of meiotic spindle and levels of oxidative stress markers as reliable predictors of oocyte assessment and embryo development. High oxidative stress appears to inhibit meiotic spindle formation and subsequently affects embryo quality. Women with PCOS had less number of oocytes with meiotic spindle visualization and higher mean ROS levels in follicular fluid indicating a poorer oocyte and thus embryo quality in these women which may be attributed to an excessive generation of ROS and LPO, and to an impaired antioxidant defense mechanism in FF of PCOS women. In contrast, women with endometriosis were observed to have a low FF oxidative stress status and good meiotic spindle imaging results suggesting a possible role of endometrial receptivity rather than oocyte quality accounting for low pregnancy rates in these women.

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