Advanced oxidation protein products from the follicular microenvironment and their role in infertile women with endometriosis

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Abstract. Endometriosis (EM) is associated with oxidative stress. Advanced oxidation protein products (AOPPs) are novel markers of oxidative stress, which serve an important role as an inflammatory mediator in various chronic diseases. In order to examine the role of AOPPs in infertile women with EM, the present study analyzed the levels of AOPPs, estradiol (E_2) and progesterone (P_4) in the follicular fluid (FF) of 89 women with or without EM undergoing *in vitro* fertilization (IVF). The AOPP concentration in the FF of the EM group was significantly higher when compared with that of the control group (51.5±22.4 vs. 41.8±18.3 μmol/l; P<0.05). However, the FF P_4 levels and blastocyst rate were significantly lower in the EM group compared with the control group (P_4:1,249.6±465.4 vs. 1,752.7±565.4 ng/ml, P<0.05; blastocyst rate: 0.511±0.322 vs. 0.662±0.278; P<0.05). The AOPP concentration and P_4 level in the FF presented a significant negative correlation in the EM and control groups, as well as in the total cohort of patients (EM group: r=-0.406, P=0.006; control group: r=-0.315, P=0.035; total: r=-0.421, P<0.001). In addition, there was a significant negative correlation between the FF AOPP concentrations and blastocyst rate in the EM group and in the total cohort (EM group: r=-0.376, P=0.012; total: r=-0.367, P<0.001). In conclusion, these results suggested that AOPPs may be a potentially effective marker for predicting the oocyte quality and outcomes of IVF in infertile women with EM.

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Abbreviations: AOPPs, advanced oxidation protein products; EM, endometriosis; E_2, estradiol; P_4, progesterone; FF, follicular fluid; IVF, *in vitro* fertilization; ROS, reactive oxygen species; LH, luteinizing hormone; hCG, human chorionic gonadotropin; ART, assisted reproductive technology

Key words: advanced oxidation protein products, follicular fluid, hormones, *in vitro* fertilization, endometriosis

Introduction

Oxidative stress results from a disruption of the balance between the pro-oxidation and anti-oxidation systems. The physiological level of reactive oxygen species (ROS) serves an important role in female reproduction, including in ovarian steroidogenesis, oocyte maturation, folliculogenesis, ovulation and luteolysis (1,2). Production of physiological levels of oxygen radicals at ovulation in response to luteinizing hormone (LH) may signal differentiation of the oocyte. However, overproduction may damage the oocytes and mitochondrial membrane potential, reduce the mitochondrial DNA copy number and disrupt the mitochondrial metabolism; all these changes are observed in atresia. Subsequently, the lack of adenosine triphosphate causes severe damage to the ultrastructure of mitochondrial cristae and spindle formation in oocytes (3,4).

Endometriosis (EM) is an estrogen-dependent disease characterized by the presence and growth of endometrial tissue outside the uterine cavity (5). EM is strongly associated with infertility, and has severe effects on ovarian and tubal function, while it may also affect endometrial receptivity (6-11). The development of *in vitro* fertilization (IVF) is promising for infertile patients with EM; however, the success rate of this procedure remains at a low level (12). One of the main causes of failure may be the poor quality of oocytes (13). Thus, research has been focusing on determining the causes of poor oocyte quality in infertile women with EM and improving their quality.

In recent years, the correlation between oxidative stress and EM has received increasing attention. EM is considered to be associated with oxidative stress, and several studies have

demonstrated that the follicular fluid (FF) of patients with EM presented increased levels of ROS and a reduction in the total antioxidant capacity (14-16). In addition, the FF of infertile women with mild EM was demonstrated to greatly impair the meiotic spindle of bovine oocytes matured *in vitro* (17). It has also been observed that decreased percentage of mature oocytes, implantation rate and clinical pregnancy rate were associated with the severity of EM (18). Furthermore, the level of myeloperoxidase as a potential oxidative stress target was revealed to be an indicator of EM-associated infertility (18). The findings of these previous studies suggest that the poor oocytes quality for EM may be associated with oxidative stress.

Advanced oxidation protein products (AOPPs) are a marker of oxidation-mediated protein damage that are usually carried by plasma proteins (19). AOPPs induce an oxidation stress reaction *in vivo* by activating the neutrophil and monocyte oxidative metabolism (20), and circulate for prolonged periods in the patients' blood, since their degradation by cells requires several hours or days since their degradation by cells requires several hours or days (21). Due to the sensitivity, stability, convenience and cost of detection, the role of AOPPs in predicting the severity of oxidative stress and disease prognosis has become increasingly important. AOPPs may accelerate renal fibrosis and atherosclerosis, and may be detrimental to the progression of chronic kidney disease (22-24). In postmenopausal women, AOPPs are negatively associated with a reduced bone mineral density, which increases an individual's risk of developing osteoporosis (25). As a key product in oxidative reactions, AOPPs and their effects on the female reproductive system have received increasing attention. It has been demonstrated that the levels of serum AOPPs are significantly increased in women with polycystic ovarian syndrome (26). In patients with uterine leiomyoma, the serum level of AOPPs increases and the antioxidant capacity decreases (27). In the peritoneal fluid, the level of AOPPs is significantly higher in patients with EM when compared with that in patients without EM (21). However, to the best of our knowledge, there are no previous studies on AOPP levels in the FF of infertile women with EM undergoing IVF.

The FF arises from the secretion of theca and granulosa cells, and forms a direct microenvironment for the development of oocytes, having crucial effects on the oocyte quality (28). Estradiol (E_2) and progesterone (P_4) in the FF are closely associated with the quality of oocytes. In our previous study, a high level of AOPPs in the FF resulted in adverse effects on oocytes and early embryonic development, while it was negatively associated with the outcome of IVF (29).

In the present study, the aim was to examine the role of AOPPs in infertile women with EM undergoing IVF and whether they are involved in altering the follicular environment of the developing oocyte, subsequently affecting the outcome of IVF. The levels of AOPPs, E_2 and P_4 in the FF were detected, and the correlations between gonadal hormones levels, prognosis of IVF and AOPP concentration were analyzed.

Materials and methods

Patients and sample collection. A total of 89 infertile women (age, 20-40 years) were recruited between 01 April 2015 and 31 December 2016. All patients were undergoing IVF at the Center for Reproductive Medicine, Department of Obstetrics and Gynecology, Nanfang Hospital, Southern Medical University (Guangzhou, China). Among the 89 women, 44 were diagnosed with EM (stages II, III and IV) that was confirmed by laparoscopy and biopsy, while 45 non-EM women because of male factors were enrolled into the control group. For the patients with EM, the date of surgical diagnosis was within ~1-4 years, and the minimum period between the last surgery and IVF was at least 6 months. Women in the control group were considered to have no significant infertility factors according to the World Health Organization guidelines (30). All women included in the present study did not receive any medicinal treatment other than the necessary treatment stipulated in the IVF protocol during the past 3 months. IVF stimulation protocols were compliant to the standard treatment dictated by the patients' physicians.

Subsequent to obtaining informed consent from the participants, FF samples were collected on the retrieval day (day of transvaginal oocyte retrieval) from patients undergoing IVF. Biochemical measurement of AOPP concentration was performed in all samples. The outcome of the research did not affect the treatment of participants. In addition, all the information collected in the present study does not identify individual participants. The approval of the study was obtained from the Institutional Research Ethics Board of Nanfang Hospital of Southern Medical University (Guangdong, China).

Oocyte evaluation. Oocyte maturity was examined by embryologists subsequent to transvaginal oocyte retrieval or at the time of intracytoplasmic sperm injection. The evaluation for oocytes was performed according in the following parameters: Expanded cumulus, appropriate cytoplasmic maturation, extruded first polar body and arrest in metaphase II. The embryo quality was scored at day 3 following fertilization and prior to placement in the uterus, as follows: Grade I, very good quality; grade II, good quality; grade III, medium quality; and grade IV, poor embryo quality (13). A good embryo (grades I and II) was defined as having seven or eight blastomeres, which were equally sized, with <20% fragmentation and no multinucleation.

IVF-controlled ovarian hyperstimulation protocol. Stimulation protocols were personalized, and included a leuprolide acetate long protocol and a gonadotropin-releasing hormone antagonist protocol (17). Controlled ovarian stimulation was performed by administering recombinant follicle-stimulating hormone (Gonal-F; EMD Serono, Inc., Rockland, MA, USA; or Puregon; MSD, Kenilworth, NJ, USA). Ovulation was induced with 250 μg recombinant human chorionic gonadotropin (Ovidrel, EMD Serono, Inc.) subcutaneously or 6,000-10,000 U human chorionic gonadotropin (hCG; Chorionic Gonadotropin for injection; Livzon Pharmaceutical Group Inc., Zhuhai, China) intramuscularly at the appropriate time in follicular development. At 34-36 h after hCG administration, transvaginal oocyte retrieval was performed in patients under intravenous sedation.

Collection of the FF. The FF was collected from 89 infertile women, 44 with EM and 45 non-EM with male infertility. At

Table I. Clinical characteristics and *in vitro* fertilization outcome parameters in each group.

Parameters	EM group (n=44)	Control group (n=45)	P-value
Age (years)	31.3±4.0	30.6±3.3	0.542
BMI (kg/m^2)	21.9±2.9	21.2±2.2	0.275
Duration of infertility (years)	3.7±2.3	3.6±2.1	0.980
FF content			
AOPP level (μmol/l)	51.5±22.4	41.8±18.3	0.044[a]
E_2 level (ng/ml)	14.3±5.9	15.4±4.8	0.112
P_4 level (ng/ml)[b]	1,249.6±465.4	1,752.7±565.4	<0.001[b]
Prior to hCG administration			
LH level (mU/ml)	2.2±2.0	2.1±1.5	0.098
E_2 level (pg/ml)	2,471.9±1333.8	2,903.8±1255.5	0.064
P_4 level (ng/ml)	0.90±0.37	0.89±0.32	0.605
No. of follicles (diameter, >14 mm)	9.4±4.9	11.5±5.2	0.032[a]
No. of fertilizations	6.3±3.9	7.9±3.9	0.024[a]
No. of good embryos	2.5±1.8	3.7±2.3	0.006[b]
Good embryo rate (%)	39.0±19.9	47.3±19.0	0.061
Blastocyst rate (%)	51.1±32.2	66.2±27.8	0.022[a]

[a]P<0.05 and [b]P<0.01 indicate a significant difference between the groups. Values are presented as the mean ± standard deviation. EM, endometriosis; BMI, body mass index; FF, follicular fluid; AOPP, advanced oxidation protein product; E_2, estradiol; P_4, progesterone; hCG, human chorionic gonadotropin; LH, luteinizing hormone.

the time of oocyte retrieval, the FF was carefully aspirated from the follicle and stored in sterile containers preheated to 37˚C. The FF sample was collected only from a follicle that was >14 mm in diameter and first punctured. The preparation of FF was conducted as described by Giorgi *et al* (31). Subsequently, the oocytes were microscopically removed from the aspirated FF. Only FF samples with no blood contamination upon visual inspection and presenting a mature oocyte were used. FF samples containing >1 oocyte or from follicles with a diameter of <14 mm were excluded. All FF samples included in the present study were centrifuged at 1,500 x g for 10 min at 4˚C to remove the cellular components, and the clear supernate was stored at -80˚C for later analysis.

Measurements of the levels of AOPPs, E_2 and P_4. The AOPP concentration in the FF samples was determined according to the spectrophotometric method described by Witko-Sarsat *et al* (32), which was expressed in equivalents of chloramine-T. Furthermore, the levels of E_2 and P_4 in the FF were determined using a commercial Iodine (^{125}I) Radioimmunoassay kit (Beijing North Institute of Biological Technology, Beijing, China), according to the manufacturer's protocol. The assays were assessed using competitive radioimmunoassay. The standard curve and assay sensitivity for E_2 was 5-4,000 and 2 pg/ml, respectively, while these values for P_4 were 0.2-100 and 0.2 ng/ml, respectively. For both E_2 and P_4, the intravariabilities were <10% and the interassay variabilities were <15%.

Statistical analysis. The fertilization rate was defined as follows: Number of embryos/number of oocytes. The good embryo rate was defined as follows: Number of grade I or grade II embryos/total number of embryos. The blastocyst rate as defined as follows: Number of embryos which were applied to culture blastocysts/number of mature blastocysts. All values are presented as the mean ± standard deviation. Considering the non-Gaussian distribution of the parameters, statistical analysis between groups was performed with the Mann-Whitney U test. Spearman's rank correlation test was applied to analyze the correlations between the FF AOPP concentration and the levels of hormones, correlations between the FF AOPP concentration and the outcome parameters of IVF. A statistically significant difference was defined as P<0.05. Bar graphs were used to present differences between groups. Data were analyzed by SPSS version 20.0 software (IBM Corp., Armonk, NY, USA).

Results

Clinical characteristics, biochemical and IVF outcome parameters. The clinical characteristics, biochemical and IVF outcome parameters of patients are presented in Table I. There were no significant differences between the two groups in the mean age, BMI, duration of infertility, E_2 concentration in the FF, as well as the serum levels of the LH, E_2 and P_4 at the time of hCG administration (P>0.05; Table I). However, the AOPP concentration in the FF was significantly higher in the EM group when compared with that in the control group (P<0.05; Fig. 1). In addition, the levels of P_4 in the FF were significantly lower in the EM group as compared with the control group (P<0.01; Fig. 2). Regarding the outcome of IVF, the EM group presented reduced numbers of mature follicles (diameter of >14 mm), fertilizations and good embryos as compared with those in the control group (P<0.05; Fig. 3). Furthermore, the blastocyst rate was markedly lower in

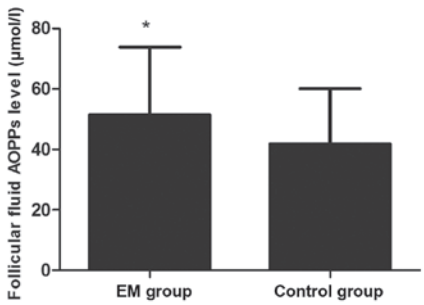

Figure 1. Mean level of follicular fluid AOPPs in the EM (n=44) and control (n=45) groups. Values are presented as the mean ± standard deviation. *P<0.05 vs. the control group. AOPPs, advanced oxidation protein products; EM, endometriosis.

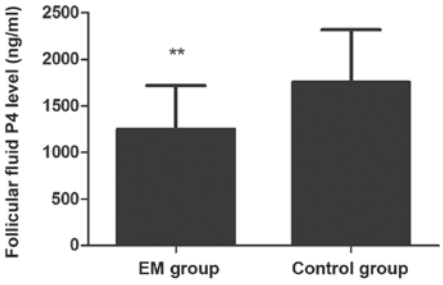

Figure 2. Mean P_4 level in the follicular fluid in the EM (n=44) and control (n=45) groups. Values are presented as the mean ± standard deviation. **P<0.01 vs. the control group. P_4, progesterone; EM, endometriosis.

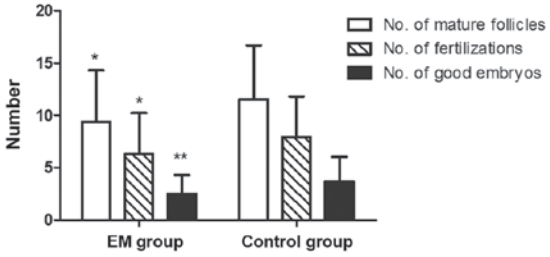

Figure 3. Mean number of mature follicles (with a diameter of >14 mm), fertilizations and good embryos in the EM (n=44) and control (n=45) groups. Values are presented as the mean ± standard deviation. *P<0.05 and ** P<0.01, vs. the control group. EM, endometriosis.

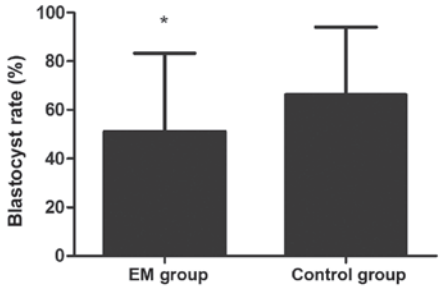

Figure 4. Mean blastocyst rate in the EM (n=44) and control (n=45) groups. Values are presented as the mean ± standard deviation. *P<0.05 vs. the control group. EM, endometriosis.

the EM group in comparison with that in the control group (P<0.05; Fig. 4).

Correlation between FF AOPP concentration and hormone levels. The correlation between the AOPP concentration in the FF and the levels of gonadal hormones is demonstrated in Table II. The FF AOPP concentration exhibited a significant negative correlation with the level of P_4 in the FF (EM group: r=-0.406, P=0.006; control group: r=-0.315, P=0.035; total: r=-0.421, P<0.001). By contrast, no significant correlations were identified between the FF AOPP concentration and the serum levels of LH, E_2, and P_4 at the time of hCG administration.

Correlation between AOPP concentration in the FF and the outcome parameters of IVF. Regarding the outcome of IVF, a negative correlation was observed between the FF AOPP concentration and the blastocyst rate in the EM and the total groups (EM group: r=-0.376, P=0.012; total: r=-0.367, P<0.001; Table III). However, no significant correlation was obsrved between the FF AOPP concentration and the fertilization rate, or between the FF AOPP concentration and the good embryos rate.

Discussion

EM has been demonstrated to be a disease closely associated with oxidative stress and the inflammatory reaction (33). AOPPs are the products of the oxidative stress reaction between chlorinated oxidants and plasma albumin (32,34). In the peritoneal fluid and serum of patients with EM, the AOPP concentration was significantly increased (21,35). To date, the AOPPs in the FF of patients with EM have not been widely investigated, although certain other oxidative stress markers have been reported. For instance, 8-hydroxy-2'-deoxyguanosine, ROS, nitric oxide and lipid peroxidation were all reportedly higher in the FF of EM patients when compared with the healthy individuals (36,37). This suggests that there is more acute oxidative stress reaction in the FF of EM patients. In the present study, the AOPP level in the FF of infertile women with EM was markedly higher in comparison with the controls, which was in agreement with previous studies.

The quality of oocytes is a pivotal factor associated with the success rate of IVF. Oocyte maturation depends on the appropriate acquisition of cytoplasmic and nuclear maturation, with the latter depending on the presence of a normal cell spindle (38,39). The microenvironment in FF is closely associated with the formation of spindles and the distribution of chromosome (40-42). Certain inflammatory factors in the FF, including tumor necrosis factor α and interleukin-17A, have also been reported to adversely affect oocytes (43,44). The FF in EM patients may induce oxidative stress reaction in oocytes and cause DNA damage (45). In addition, AOPPs in the plasma induce endoplasmic reticulum stress in various cells (46-49), and this stress has been observed to cause phenotypic alterations and death in cells (50,51). Under an environment with acute oxidative stress, the oocyte maturation is more likely to be arrested and the developmental potency of embryos is inevitably decreased. As observed in the current study, a reduced number of mature oocytes was retrieved and fertilized in the infertile patients with EM, and consequently, fewer good embryos could be used.

Another key factor for oocyte maturation is the gonadal hormone level, such as the level of P_4. Although no correlation

Table II. Correlations between the AOPP level in the FF and the gonadal hormone levels.

	AOPP level					
	EM group (n=44)		Control group (n=45)		Total (n=89)	
Measurements	r	P-value	r	P-value	r	P-value
FF content						
E_2	-0.052	0.737	-0.203	0.180	-0.162	0.129
P_4	-0.406	0.006[a]	-0.315	0.035[b]	-0.421	<0.001[a]
Prior to hCG administration						
LH	0.273	0.073	0.072	0.639	0.177	0.097
E_2	0.130	0.399	-0.141	0.355	-0.046	0.668
P_4	-0.136	0.380	-0.076	0.620	-0.122	0.254

[a]P<0.01 and [b]P<0.05. All analyses were performed using the Spearman's rank correlation test. EM, endometriosis; AOPP, advanced oxidation protein product; FF, follicular fluid; E_2, estradiol; P_4, progesterone; hCG, human chorionic gonadotropin; LH, luteinizing hormone.

Table III. Correlations between the AOPP levels in the follicular fluid and the outcome parameters of *in vitro* fertilization.

	AOPP level					
	EM group (n=44)		Control group (n=45)		Total (n=89)	
Parameters	r	P-value	r	P-value	r	P-value
No. of follicles (diameter, >14 mm)	0.066	0.670	-0.052	0.736	-0.055	0.609
No. of fertilizations	0.082	0.598	0.027	0.862	-0.019	0.862
Fertilization rate (%)	0.093	0.547	0.107	0.483	0.028	0.797
No. of good embryos	0.086	0.578	-0.100	0.513	-0.067	0.535
Good embryo rate (%)	0.014	0.928	-0.253	0.093	-0.140	0.190
Blastocyst rate (%)	-0.376[a]	0.012	-0.161	0.292	-0.367[b]	<0.001

[a]P<0.05 and [b]P<0.01. All analyses were performed using the Spearman's rank correlation test. EM, endometriosis; AOPP, advanced oxidation protein product.

has been detected between the serum level of P_4 and the prognosis of assisted reproductive technology (ART) (52), the P_4 level in the FF only secreted and synthesized by granulosa cells is more closely correlated with the development of oocytes and the outcome of IVF (53,54). In the present study, the EM group with a higher FF AOPP concentration presented a lower FF P_4 level, thus a negative correlation was observed between AOPPs and P_4 levels. Santulli *et al* (21) and Gomes *et al* (55) reported that the peritoneal fluid of EM patients containing a higher level of AOPPs reduced the P_4 release from granulosa cells. Therefore, the higher level of AOPPs in the FF of EM patients may be the cause of the decreased P_4 level.

The negative influence of oxidative stress on granulosa cells is unimportant; oxdative stress causes mitochondrial and endoplasmic reticulum defects in granular cells and the generation of ROS in granulosa cells is accompanied with caspase3/7 expression, an important indicator for cells apoptosis (56). Extreme oxidative stress is able to induce granulosa cell apoptosis (57); however, the antioxidant factor would exert a positive effect. L-DOPA in FF as an antioxidant factor that exerts positive influences on granular cells, including decreasing H_2O_2 production and promoting cell survival (58). Based on these observations, AOPPs may decrease P_4 production via impairing the function of granulosa cells. In addition, in mammalian preovulatory follicles, P_4 is one of the dominant steroid hormones (59). P_4 regulates meiosis and ovulation by activating the progesterone receptors (60). A high level of P_4 in the FF increases the percentage of oocytes at the germinal vesicle stage, and the maternal P_4 level affects the gene transcripts of cumulus-oocyte complexes (54). By contrast, upon the lack of the necessary hormonal stimulation from P_4, the development and maturation of oocytes would be delayed or may even not reach an acceptable level (61,62).

The present study observed that the EM patients with fewer mature oocytes and fewer good-quality embryos presented a lower blastocyst rate. In the EM group and in the total cohort

of patients, the correlation between AOPP concentration in the FF and blastocyst rate presented a significant negative correlation. The low success rate of ART may be explained by the low oocyte quality and blockage of embryo development (63). In clinical practice, selecting good-quality oocytes and embryos is key for promoting ART. Blastocysts represent a vital stage in the embryo development, and a good-quality blastocyst indicates a greater likelihood of implantation and gives rise to live birth (64-67). Oocytes, one of the origins of embryos, have been demonstrated to be associated with the developmental potential of the embryo; thus, good-quality oocytes are more likely to develop into blastocysts following fertilization (68,69). As described previously, excessive oxidative stress in FF is inversely correlated with the prognosis of pregnancy in ART (70). Furthermore, oocytes with cytoplasmic defects are more likely to occur in an acute oxidative stress environment (71). While the elevated AOPPs may inhibit the appearance of the first polar body and cytoplasmic maturation, thus arresting oocyte maturation and damaging the potential of embryos (45), AOPPs may also damage the function of granulosa cells and decrease P_4 production. Due to the lack of sufficient hormonal support of P_4, oocyte maturation is arrested and the subsequent processes, including fertilization, blastocyst formation and embryo development, are negatively affected. This hypothesis is in accordance with previous experiments on rhesus monkeys, which reported that a higher ratio of P_4 to E_2 in the FF was beneficial for the development of the embryos (72). In addition, this is consistent with the findings of Gustafson *et al* (73), suggesting that a higher E_2/P_4 ratio in the FF was associated with a lower IVF success rate. Therefore, an impairment of oocyte quality, lower implantation and reduced pregnancy rates are observed in patients with EM (74,75).

However, the current study had various limitations. The major limitation is the overall small number of patients included. Furthermore, no significant correlations between the FF AOPP level and other gonadal hormones levels in the FF or serum were observed; however, this does not indicate that there are no correlations between the FF AOPP and other various gonadal hormones A larger sample size may provide more substantive evidence in further studies.

In conclusion, AOPP levels were significantly elevated in the FF of infertile patients with EM in the present study. In addition, the increased FF AOPP level was accompanied with a decreased FF P_4 concentration and blastocyst rate. These findings indicate that AOPP may be a potentially effective marker for predicting the oocyte quality and outcome of IVF, particularly in infertile women with EM. This provides a novel theoretical basis, suggesting that anti-oxidative treatments aimed at reducing the AOPP levels may be a new effective strategy to promote the maturation of oocytes and improve the success rate of IVF.

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