The expression and role of oxidative stress markers in the serum and follicular fluid of patients with endometriosis

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Summary

Objective: To investigate the expression and role of oxidative stress markers in the serum and follicular fluid of patients with endometriosis. *Materials and Methods:* A prospective case-control study was conducted in 42 patients who underwent in vitro fertilization–embryo transfer (IVF-ET). They were divided into Group I: patients with endometriosis (n = 20) and Group II: patients with tubal factor infertility (n = 22). All patients underwent a long gonadotropin-releasing hormone (GnRH) agonist protocol for pituitary downregulation followed by controlled ovarian hyperstimulation. Level of reactive oxygen species (ROS), superoxide dismutase (SOD), and vitamin E (VE) were measured by enzyme-linked immunosorbent assay (ELISA). The results of IVF-ET between the two groups were compared. *Results:* The ROS levels in both serum and follicular fluid of the study group were significantly higher than in the control group. The serum levels of SOD and VE in the study group were significantly lower than those in the control group, but there was no difference in follicular fluid levels of SOD and VE between the two groups. Furthermore, the mature oocyte and fertilization rates in the study group were significantly lower than those of the control group. However, the levels of ROS, SOD, and VE in serum and follicular fluid were not significantly correlated with outcome following IVF-ET. *Conclusion:* Patients with endometriosis have increased oxidative stress, as well as lower mature oocyte rates and fertilization rates. Nevertheless, there is no evidence that the oxidative stress status is directly related to the outcome of IVF treatment.

Key words: Oxidative stress; Endometriosis; In vitro fertilization; Fertilization rate; Pregnancy rate.

Introduction

Endometriosis is a benign disease with malignant tumors and one of common gynaecological diseases causing pelvic cavity pain, menoxenia, infertility, and approximately 30%-50% of these cases evolve into infertility [1]. The underlying mechanism and curative effects of endometriosis-induced infertility remains unclear and unsatisfactory. In vitro fertilization-embryo transfer (IVF-ET) has been regarded as the main treatment of endometriosis-associated infertility, whereas the pregnancy rate was still significantly lower than that of patients with tubal factor infertility [2]. The mechanism of endometriosis-associated infertility and the reason why the women had low pregnant rate following IVF-ET have been the focus for all the clinicians, and oxidative stress is included.

Oxidative stress (OS) is defined as the unbalance between internal oxidative and anti-oxidative reactions, more inclined to oxidation, leading to inflammatory infiltration of neutrophil, increased secretion of protease, and a large amount of intermediate products, which is characterized as the increase in reactive oxygen species (ROS) and the decline or deficiency of anti-oxidants [1]. OS played a certain role during pregnancy maintenance, normal reproduction process, and premature initiation [2-4]. Other previous findings indicated that OS was associated with pathological and physiological mechanism of birth defects and abortion induced by preeclampsia, hydatid mole, and free radicals [5-8]. Although the relationship between endometriosis and OS remains debatable, relevant data indicated that OS was found in the abdominal cavity in endometriosis women. However, whether OS is correlated with the pathogenesis of endometriosis-associated infertility or not remains to be further elucidated. The influence of OS upon reproduction potentiality of human beings has increasingly attracted attention [9-11]. Thereafter, whether OS exists in follicular fluid or granulocytes of women suffering from endometriosis, and whether it is harmful to oocytes quality leading to negative consequences to clinical outcomes have been the main research topic in recent years. Currently, there has been no report investigating the balance status between oxidation and anti-oxidation in serum and follicular fluid of patients with endometriosis-induced infertility, and its effect on the outcome of clinical trials. This clinical trial aimed to investigate the expression and role of OS markers in serum and follicular fluid of women with endometriosis.

Materials and Methods

Subjects

The endometriosis patients and those with tubal factor infertility receiving IVF-ET in Reproduction Center in the Third Hospital, affiliated to Guangzhou Medical College from August to October, 2009 were enrolled in this study.

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The study group included 20 cases diagnosed with endometriosis. Inclusion criteria: 1) the patients with endometriosis were diagnosed by cyst puncture via laparoscopy, laparotomy, or vagina; 2) ages ranged from 25 to 40 years; 3) regular menstrual cycle, 24 to 35 days; 4) received no surgery three months prior to this trial; 5) no gynaecology-related or other diseases. Exclusion criteria: 1) irregular menstrual cycle; 2) diabetes mellitus, angiocardiopathy, dyslipidemia, systemic lupus erythematosus, and other rheumatic diseases, etc; and 3) usual smokers.

The control group included 22 patients with tubal factor infertility but without endometriosis of the same stage. Inclusion criteria: 1) the patients were diagnosed with tubal factor infertility rather than with endometriosis-associated infertility by cyst puncture via laparoscopy, laparotomy; 2) on effusion in bilateral ovarian ducts prior to IVF and during ovarian hyperstimulation by B-mode ultrasound; 3) regular menstrual cycle, 24 to 35 days; 4) received no surgery three months prior to this trial; 5) no gynaecology-related or other diseases. Exclusion criteria: 1) irregular menstrual cycle; 2) diabetes mellitus, angiocardiopathy, dyslipidemia, systemic lupus erythematosus, and other rheumatic diseases, etc., and 3) usual smokers.

Sampling methods

All patients underwent a long gonadotropin-releasing hormone (GnRH) agonist protocol for pituitary downregulation followed by controlled ovarian hyperstimulation.

Serum sampling included periphery blood which was collected on the day of retrieving oocytes, and then centrifuged at 1,000 × g for 20 min. The separated serum was assigned into sterile EP tubes and stored at -70°C.

Follicular fluid sampling included laboratorian's retrieval of the first tube of follicular fluid uncontaminated by blood following oocytes retrieval. The obtained follicular fluid was centrifuged at 1000 × g for ten min. The supernate was collected and assigned into sterile EP tubes and stored at -70°C.

The SOD, ROS, and VE concentrations in both serum and follicular fluid of patients between two groups were detected by using enzyme-linked immunosorbent assay (ELISA).

The main clinical indexes included: retrieved oocytes, mature oocytes rate, fertility rate, good-embryo rate, implantation rate, and clinical pregnancy rate.

Statistical analysis

All the data obtained were statistically analyzed using SPSS 13.0 software package. Categorical data were handled by Chi-Square test. Measurement data were analyzed by independent sample t-test. Correlation analysis between experimental data and clinical evaluation indexes were expressed by Spearman correlation coefficient. A test level of $\alpha = 0.05$ was considered as statistical significance.

Results

Patient characteristics

No significant difference was noted between the study and control groups in terms of age, mean infertility time, and baseline follicle-stimulating hormone (FSH) level ($p > 0.05$), suggesting there was no statistical significance between two groups and all the patients were comparable (Table 1).

ROS, SOD, and VE levels

ROS serum level in the study group was significantly increased compared with that in the control group ($p < 0.05$), and SOD and VE serum concentrations were significantly decreased than those in the control group ($p < 0.05$), which are shown in (Table 2). ROS level in follicular fluid in the study group was significantly increased compared with that in the control group ($p < 0.05$), whereas SOD and VE concentrations slightly decreased than those in the control group with no significant difference ($p > 0.05$) (Table 3) In addition, ROS, SOD, and VE levels in follicular fluid were significantly decreased than their counterparts in serum in both two groups ($p < 0.05$) (Tables 4 and 5).

IVF-ET

No significant difference in retrieved oocytes was noted between two groups ($p > 0.05$). Mature oocyte and fertilization rates in the study group were significantly lower compared with those in the control group ($p < 0.05$). Good oocyte rate, implantation rate, and clinical pregnancy rates in the study group declined compared to those in the control group, whereas no significant difference was noted between the two groups ($p > 0.05$) (Table 6).

ROS, SOD, VE levels, and evaluation factor of IVF-ET

Correlation analysis of ROS, SOD, and VE levels and evaluation factor of IVF-ET outcome revealed that ROS level in serum and follicular fluid negatively correlated with mature oocyte and fertilization rates. SOD level positively correlated with mature oocyte, fertilization, implantation, and clinical pregnancy rates. VE level positively correlated with mature oocyte, fertilization, good-embryo, implantation, and clinical pregnancy rates. However, no significant difference was noted ($p > 0.05$) (Table 7).

Discussion

When internal oxidative level was extraordinarily high, a large amount of oxidants was depleted, and the original oxidation-anti-oxidation balance was destroyed, leading to OS status. As the OS elevated in systemic circulation, the OS level in local environment might be enhanced accordingly, such as, peritoneal fluid, follicular fluid, etc. Until now, the exact relationship between OS and endometriosis has been debatable. Murphy *et al*. [12] indicated that endometriosis originated from OS, or there was relationship between them. For pelvic endometriosis cases, macrophage activation in the peritoneal cavity induced OS, yielding an abundance of peroxides. They also found that lipoprotein level in peritoneal fluid of endometriosis women was increased compared with that of normal counterparts. In addition, VE level in peritoneal fluid was significantly lower than that in serum, indicating that the protective action of anti-oxidants in peritoneal fluid was inferior to that in serum. Szczepan-

Table 1. — *Patient background in the study and control groups.*

	Study group	Control group	*p* value
Mean age (years)	32.0 ± 2.8	32.4 ± 3.1	> 0.05
Mean infertility time (years)	3.3 ± 2.7	4.7 ± 2.7	> 0.05
Mean baseline FSH level (U/l)	5.6 ± 1.4	5.1 ± 1.5	> 0.05

Table 2. — *Comparison of ROS, SOD, and VE levels in serum between the study and control groups.*

	Study group	Control group	*p* value
ROS (ng/ml)	5.49 ± 1.39	3.93 ± 1.22	< 0.05
SOD (U/l)	11.38 ± 4.44	18.99 ± 6.80	< 0.05
VE (μmol/l)	17.66 ± 4.89	23.34 ± 8.14	< 0.05

Table 3. — *Comparison of ROS, SOD, and VE levels in follicular fluid between the study and control groups.*

	Study group	Control group	*p* value
ROS (ng/ml)	1.35 ± 0.38	0.53 ± 0.88	> 0.05
SOD (U/l)	7.65 ± 1.25	9.26 ± 2.70	> 0.05
VE (μmol/l)	6.16 ± 1.95	6.88 ± 2.45	< 0.05

Table 4. — *Comparison of ROS, SOD, and VE levels between serum and follicular fluid in the study group.*

	Serum	Follicular fluid	*p* value
ROS (ng/ml)	5.49 ± 1.39	1.35 ± 0.38	< 0.05
SOD (U/l)	11.38 ± 4.44	7.65 ± 1.25	< 0.05
VE (μmol/l)	17.66 ± 4.89	6.16 ± 1.95	< 0.05

Table 5. — *Comparison of ROS, SOD, and VE levels between serum and follicular fluid in the control group.*

	Serum	Follicular fluid	*p* value
ROS (ng/ml)	3.93 ± 1.22	0.53 ± 0.88	< 0.05
SOD (U/l)	18.99 ± 6.80	9.26 ± 2.70	< 0.05
VE (μmol/l)	23.34 ± 8.14	6.88 ± 2.45	< 0.05

Table 6. — *Evaluation indexes of the outcome of IVF-ET in the study and control groups.*

	Study group	Control group	*p* value
Mean retrieved oocytes	12.6 ± 6.9	12.2 ± 6.4	> 0.05
Mature oocytes rate	86% (211/244)	92% (256/278)	< 0.05
Fertilization rate	70% (171/244)	77% (216/278)	< 0.05
Good-embryo rate	28% (47/169)	31% (66/214)	> 0.05
Implantation rate	20% (8/40)	21% (11/52)	> 0.05
Clinical pregnancy rate	30% (6/20)	36% (8/22)	> 0.05

Table 7. — *Correlation analysis between the outcome of IVF-ET and ROS, SOD, and VE levels in serum and follicular fluid.*

Evaluation indexes	Serum			Follicular fluid		
	ROS	SOD	VE	ROS	SOD	VE
Retrieved oocytes	- 0.01	0.10	0.06	0.004	0.14	0.12
Mature oocytes rate	- 0.27	0.29	0.22	- 0.25	0.36	0.24
Fertilization rate	- 0.13	0.14	0.12	- 0.14	0.18	0.25
Good-embryo rate	- 0.04	0.05	0.17	- 0.04	0.11	0.14
Implantation rate	0.01	0.17	0.22	0.02	0.28	0.26
Clinical pregnancy rate	- 0.04	0.14	0.21	- 0.03	0.27	0.23

ska *et al*. [13] reported superoxide dismutase and glutathione peroxidase levels in peritoneal fluid of endometriosis patients significantly declined compared with those of normal subjects. These two enzymes played a vital role in inhibiting the production of free radicals and ROS, and preventing the occurrence of OS. Moreover, anti-oxidant level in endometriosis women was significantly lower than that in healthy women, whereas lipid peroxide level significantly increased compared with that in normal subjects. Jackson *et al*. [14] also found that anti-oxidant level in peritoneal fluid of endometriosis women was significantly lower than that of healthy counterparts, but lipid peroxide level was significantly higher. However, Polak *et al*. [15] revealed no significant difference in anti-oxidant level of peritoneal fluid between endometriosis and normal patients subjects. Both Arumugam and Dip [16] reported that no statistical significance existed in malondialdehyde (MDA) level in peritoneal fluid among severe endometriosis group, slight-moderate endometriosis group, and control group. One possible explanation may be that OS only occurred in endometriosis lesions, whereas the level of total oxidation products did not increase. In this study, the authors found an increase in serum ROS level in the study group than in the control group and a decrease in SOD and VE levels in the study group, indicating that OS did exist in the patients with endometriosis.

The varying results attribute to the discrepancies among different studies in terms of the inclusion criteria for endometriosis patients and healthy subjects, the selection of OS markers, and the detection methods. In this study, the authors avidly attempted to control the potential interfering factors, take relevant measurements to reduce the influence of interfering factors upon the experimental outcomes, and strictly established the exclusion criteria, excluding those suffering from OS and endometriosis-related diseases, and those having no history of smoking, etc from this clinical trial. Other potential interfering factors were also controlled, such as, patients' ages, infertility time, and baseline FSH level. Comparison results showed that no statistical significance was noted between the study and control groups regarding these indexes. So, the obtained results between the two groups were comparable.

The environments surrounding oocytes played vital roles in regulating ooctyes quality, fertilization, and embryonic development. Under normal circumstances, the redundant ROS in follicular fluid was eliminated by the anti-oxidation system existing in mitochondria of granulocytes, maintaining the biological balance between oxidation-antioxidation reactions, and preventing oocytes from damages induced by ROS. When the host organism was in pathological state, granulocytes presented OS, and released a substantial amount of ROS into follicular fluid. Previous clinical investigations have proved that the fertilization rate of the patients with endometriosis-associated infertility following IVF or ICSI was lower compared with their counterparts with tubal factor infertility

or other patients [17-19]. Alternative findings indicated that the decline in implantation rate of endometriosis patients might be associated with impaired oocytes quality [20, 21]. In addition, other findings revealed the relationship between low oxygenation and the reduction in development potential of oocytes, mainly characterized by the increased defect rate in oocytes cytoplasm, impaired cleavage, and abnormal segregation of oocytes' chromosome [22]. Meantime, ROS increased the production of embryonic segments by accelerating cell apoptosis [23]. Saito *et al*. [24] found that the 8-hydroxy-desoxyguanosine (8-OHdG) and 4-hydroxynonenal (4-HNE) levels in granular cells within endometriosis women were significantly enhanced compared with those within other factor-related infertility cases (tubal factor, male spouse factor, and unknown factors). Moreover, 8-OHdG was regarded as a common evaluation index for oxidation DNA damages and OS, and 4-HNE was a product by lipid peroxides, suggesting that significant OS existed in follicular fluid of endometriosis patients. Simultaneously, they also found that 8-OHdG concentration contained in granular cells negatively correlated with good-embryo rate, and 8-OHdG level in granular cells within endometriosis women were significantly increased compared with those within other factors-related infertility cases (tubal factor, male spouse factor, and unknown factors), collectively indicating granular cells had OS which reduced fertilization rate and embryo quality. In addition, 8-OHdG directly affected the oocytes quality in endometriosis women, which was possibly one of the reasons explaining why endometriosis eventually evolved into infertility. Campos *et al*. [25] found that the serum VE level in endometriosis patients was decreased compared to that in normal controls prior to or after ovulation stimulations. Moreover, the serum MDA levels in endometriosis subjects significantly increased following induced ovulation. However, there was no statistical significance between the endometriosis and control groups in terms of the VE and MDA levels in serum and follicular fluid on the day of oocytes retrieval. To investigate the existence and influence of OS upon oocytes quality in follicular fluid of endometriosis patients, the ROS, SOD, and VE levels in follicular fluid between the study and control groups were detected and compared in this clinical trial. The obtained outcomes showed that the endometriosis patients had a significant rise in follicular fluid ROS level but a slight decline in SOD and VE levels compared with their counterparts in the control group, strongly suggesting the existence of OS in follicular fluid of endometriosis women. Besides, mature oocyte and fertilization rates in the study group were significantly decreased than those in the control group, and ROS level in both serum and follicular fluid negatively correlated with mature oocyte rate and fertilization rate, which indicated that OS indeed affected the oocytes quality, leading to a reduction in oocyte developmental potential and a negative influence on mature oocyte and fertilization rates.

Previously, whether OS existed in endometriosis and endometriosis-associated infertile patients has been widely debated, mainly due to a lacking agreement in the selection and detection methods of OS markers, and the discrepancies in inclusion criteria of the enrolled subjects. Thereafter, establishing a unified standard is the key. The following studies should expand sample size, select, and detect the OS markers especially sensitive to endometriosis patients.

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