



# Evaluation of Oxidative Stress Markers in Serum and Follicular Fluid of Women with Infertility Related to Endometriosis

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## Abstract

**Objective:** To study the levels of four markers of oxidative stress in Follicular Fluid (FF) and serum of patients with infertility related to endometriosis and controls.

**Design:** Experimental study.

**Setting:** University hospital and infertility center.

**Patient(s):** Sixty infertile women were included in the study (30 infertile women with endometriosis and 30 controls including infertile women due to male factor, or unexplained infertility).

**Intervention(s):** Blood was obtained at the time of egg retrieval, and FF from the mature follicles of each ovary was centrifuged and frozen until analysis.

**Main outcome measure(s):** Vitamin C and E, malondialdehyde, and superoxide dismutase concentrations in serum and follicular fluid.

**Result(s):** Women with endometriosis showed a lower vitamin C concentration in serum (1.64 µg/ml to 11.48 µg/ml vs. 6.56 µg/ml to 41.33 µg/ml) and higher superoxide dismutase concentration in FF (30.41 U/mL to 339.53 U/mL vs. 70.95 U/mL to 324.32 U/mL) compared with controls. Vitamin E FF levels were significantly lower in women with endometriosis (0.0 µg/mL to 0.52 µg/mL vs. 0.04 µg/mL to 1.38 µg/mL). A non significant difference in serum and FF concentration of malondialdehyde was found in women in both groups.

**Conclusion(s):** These findings suggest increased oxidative stress in infertile women with endometriosis, which may be manifested by increased activity of SOD in FF and/or consumption of the antioxidant vitamin C in serum, and decrease level of vitamin E in FF of the same affected group.

**Keywords:** Oxidative stress; Endometriosis; Infertility

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## Introduction

Endometriosis is a common benign gynecologic disorder defined as the presence of endometrial glands and stroma outside of the normal location. First identified in the mid-nineteenth century [1].

It is unclear to what degree endometriosis is a detriment to the process of fertilizing oocytes *in vitro*, as several investigations have now reported significantly impaired fertilization rates for these patients. One early study noted fertilization rates per oocyte of 33%, 63%, and 68% for patients with endometriosis, unexplained infertility, and tubal infertility, respectively [2].

Reactive Oxygen Species (ROS) are produced by living organisms as a result of normal cellular metabolism. At low to moderate concentrations, they function in physiological cell processes, but at high concentrations, they produce adverse modifications to cell components, such as lipids, proteins, and DNA [3].

ROS appear to be involved in different facets of endometriosis, peritoneal adhesion formation, and associated infertility, acting at different levels, including fertilization, embryo quality, implantation, and embryonic development [4,5].

Several studies have focused on the microenvironment surrounding the oocyte and ROS and antioxidants found in the Follicular Fluid (FF) [6].

We studied different markers of oxidative stress in serum and FF from infertile patients with endometriosis and patients with sterility not caused by endometriosis (control) in an attempt to investigate a possible role of oxidative stress.

## Materials and Methods

This study included sixty (60) infertile females attending El-Shatby infertility clinic and indicated for ICSI procedure. The patients were divided into 2 main groups namely A and B.

Group (A): Included (30) infertile females suffering from severe endometriosis (according to the revised American society for reproductive medicine classification ASRM: 1996).

Group (B): Included (30) infertile females due to unexplained infertility or mild to moderate male factor (having no endometriosis) as a control group.

All patients signed a well-informed written consent to declare their agreement to be enrolled in this study as agreed upon by the ethical committee.

### All patients were subjected to

A. Thorough history taking about age of the patient, duration of infertility, previous consultations, lab investigations, radiology and previous modalities of therapy received.

B. Physical examination that included: body mass index, pelvic tenderness or masses, uterine size, shape and motility, adnexal or Douglas pouch masses, nodules and tenderness.

C. Transvaginal ultrasonographic examination of the uterine anatomy and tubal pathology, ovarian antral follicular count, signs of ovulation, ovarian pathology like masses and cysts.

D. Diagnostic laparoscopy when there is strong evidence of endometriosis by history, clinical examination or investigations.

Patients who have been infertile for at least one year and indicated for ICSI treatment were classified according to the cause of infertility into diseased and control groups.

Sample size was calculated using NCSS 2004 and PASS 2000 program.

Patients started ovarian stimulation with long agonist protocol and monitored by transvaginal ultrasound and serum levels of Estradiol (E2) in El-Shatby infertility clinic.

### On day of oocyte retrieval

- 5 ml of blood were aspirated from every person included in the study and evacuated in clean vacutainer tubes, left to clot for 30 min, then centrifuged at 3000 rpm for 10 min. Serum was separated and put in aliquots.
- 5 ml of Follicular fluid were aspirated, centrifuged at 3000 rpm for 10 min, and sediment was discarded.
- Serum samples & follicular fluid supernatants from cases & controls were frozen at -20°C until analysis.

### Superoxide dismutase (SOD)

The activity of SOD in serum and follicular fluid was measured using the commercially available test kits supplied by Biodiagnostic Egypt according to the method described by Nishikimi et al. [7] SOD activity levels are expressed in U/ml.

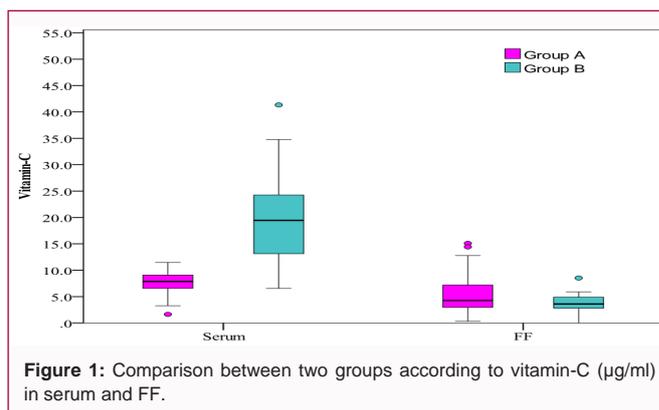


Figure 1: Comparison between two groups according to vitamin-C (µg/ml) in serum and FF.

### Malondialdehyde (MDA)

Serum MDA levels were estimated using commercially available test kits supplied by Biodiagnostic Egypt according to the methods described by Ohkawa et al. [8] (1979) MDA levels are expressed as µmol/L concentrations.

### Vitamin C

Vitamin C was measured in both plasma and FF using spectrophotometry, Ascorbate in sample is expressed in µg/ml [9].

### Vitamin E

Vitamin E was measured in both plasma and FF using High Performance Liquid Chromatography (HPLC), final concentration of the  $\alpha$ -tocopherol in serum and follicular fluid samples express as µ/ml [10].

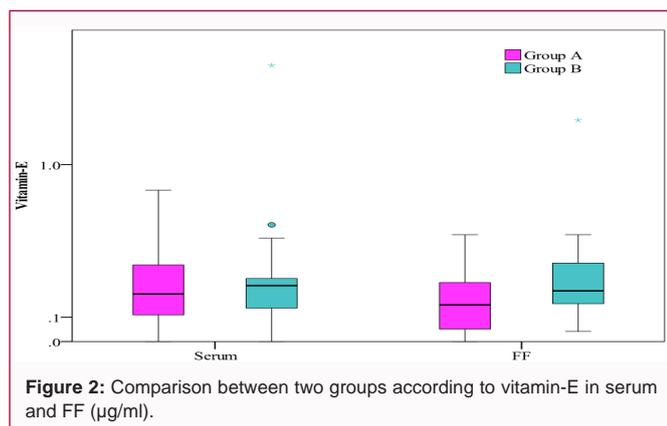
### Statistical analysis of the data

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0 [11,12]. Qualitative data were described using number and percent. Quantitative data were described using Range (minimum and maximum), mean, standard deviation and median. Comparison between different groups regarding categorical variables was tested using Chi-square test. The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, Shapiro-Wilk test and D'Agostino test, also Histogram and QQ plot were used for vision test. If it reveals normal data distribution, parametric tests was applied. If the data were abnormally distributed, non-parametric tests were used. For normally distributed data, comparison between two independent populations were done using independent t-test, also paired t-test is used to analyse two paired data. For abnormally distributed data, comparison between two independent population were done using Mann Whitney test and Wilcoxon signed ranks test used to compare between two techniques Significance of the obtained results was judged at the 5% level.

## Results

### Clinical IVF laboratory data

Group of endometriosis was higher than control group as regards number of oocyte yield and oocyte maturity (presented by number of oocytes with one polar body (M2) cells), but with no statistical significance. About embryo number and quality (presented by number of class an embryo (equal cell sizes and no fragmentation)), again group of endometriosis has higher number of fertilized and class an embryos, but didn't reach statistical significance. The number of embryos transferred was comparable in both groups. The pregnancy rate was equal in both groups.



**Antioxidants and oxidative stress markers**

**Vitamin C:** Vitamin C levels were higher in serum of control group than serum of endometriosis group with high statistical significance (1.64 µg/ml to 11.48 µg/ml vs. 6.56 µg/ml to 41.33 µg/ml), but it was lower in follicular fluid of the same group without statistical significance (0.33 µg/ml to 15.09 µg/ml vs. 0.0 µg/ml to 8.53 µg/ml), as shown in Figure 1.

**Vitamin E:** Vitamin E levels were slightly higher in serum of control group with no statistical significant difference (0.0 µg/ml to 0.81 µg/ml vs. 0.0 µg/ml to 1.95 µg/ml), but it was higher in follicular fluid of the same group than FF of endometriosis group which was of statistical significance (0.0 µg/mL to 0.52 µg/mL vs. 0.04 µg/mL to 1.38 µg/mL), as shown in Figure 2.

**Superoxide Dismutase (SOD):** The activity of the antioxidant enzyme was comparable between the two groups as regard serum levels (166.67 U/ml to 281.25 U/ml vs. 14.58 U/ml to 338.54 U/ml), but was higher in endometriosis group than control group in the FF levels, with statistical significance (30.41 U/mL to 339.53 U/mL vs. 70.95 U/mL to 324.32 U/mL) as shown in Table 1.

**Malondialdehyde (MDA):** As a comparison between the two groups, there were no statistically significant differences in the oxidative stress marker (MDA), either in serum (1.20 nmol/ml to 19.80 nmol/ml vs. 1.0 nmol/ml to 20.80 nmol/ml) or in FF (1.50-7.40 vs. 1.20 to 8.80 nmol/ml), as shown in Table 2.

**Discussion**

Many studies have reported inconsistent results when analyzing ROS concentrations in patients with endometriosis. Therefore, it is virtually impossible to postulate a definitive conclusion regarding the role of OS in endometriosis.

In our study, we demonstrated a slight decrease in ovarian reserve in endometriosis group compared to the control group manifested by a slight elevation in FSH and decrease in AMH; not reaching the level of statistical significance.

Dokras et al. [13] in 2000 and Hock et al. [14] in 2001 evaluated the ovarian reserve using a retrospective design and during Controlled Ovarian Hyperstimulation (COH) cycles. They showed a decrease in inhibin B during COH in infertile patients with endometriosis and, an increased serum FSH level in patients with moderate/severe endometriosis during the early follicular phase.

Nadiane et al. [15] in 2008 found that Serum FSH were not different between endometriosis and control groups. However, infertile

**Table 1:** Comparison between two groups according to SOD activity in serum and FF (U/ml).

SOD (U/ml)	Group A (n=30)	Group B (n=30)	t	p
Serum			0.1	0.921
Min-Max	166.67-281.25	14.58-338.54		
Median	205.73	223.96		
FF			2.405*	0.019*
Min-Max	30.41-339.53	70.95-324.32		
Median	200.17	162.16		
p	0.401	0.014*		

**Table 2:** Comparison between two groups according to MDA in serum and FF (nmol/ml).

MDA(nmol/ml)	Group A (n=30)	Group B (n=30)	Z	p
Serum			1.73	0.084
Min-Max	1.20-19.80	1.0-20.80		
Median	7.25	4.85		
FF			0.821	0.412
Min-Max	1.50-7.40	1.20-8.80		
Median	3.2	3.5		
p	<0.001*	0.008*		

patients with endometriosis had a decreased serum anti-Mullerian hormone (1.26 ng/mL to 0.7 ng/mL) compared to the control group (2.02 ng/mL to 0.72 ng/mL. Minimal/mild endometriosis was associated with a decrease in the follicular ovarian reserve.

Regarding the clinical IVF laboratory data in this study, the endometriosis group has slight increase in the number and maturity of oocytes retrieved, and consequently a slight increase in number and quality of fertilized embryos, both didn't reach statistical significance. The number of pregnant women was higher by only one patient in control group.

Opoien et al. [16] in 2012 described that although patients with endometriosis obtained inferior COH performance and/or inferior embryos when compared to patients with tubal factors, these differences did not transfer to pregnancy results.

However, Coccia et al. [17] in 2011 and Lin et al. [18] in 2012 reported adverse effects of endometriosis on pregnancy results in IVF/ICSI cycles, with respect to decreased implantation rate.

Although many studies demonstrated the ROS and antioxidants in serum of endometriosis patients, little is published on the micro environment around the oocyte-the follicular fluid. In our study, we investigated oxidative marker and antioxidants in both serum and follicular fluid.

In our study, we demonstrated Vitamin C levels lower in serum of endometriosis group with high statistical significance. This finding may be due to increased consumption of the powerful antioxidant in the affected group or due to deficient dietary intake of the vitamin. In the follicular environment, the vitamin levels were slightly higher in endometriosis group, unlike Prieto et al. [19] in 2012 they found lower vitamin C values in FF from patients with endometriosis (12.7 mg/mL to 5.9 mg/mL vs. 9.7 mg/mL to 6.9 mg/mL, P¼.003) compared with infertile patients without the disease.

In that study, Prieto et al. [19] Investigated serum and FF

oxidative marker and antioxidants in 91 patients undergoing ICSI treatment for infertility.

In women with endometriosis, they found reduced SOD enzyme activity in the plasma (0.9 U/mL to 1.4 U/mL vs. 0.55 U/mL to 0.7 U/mL,  $P=0.059$ ) compared with the control group. Vitamin E plasma levels were significantly higher in women with endometriosis (8.1 mg/mL to 3.8 mg/mL vs. 5.2 mg/mL to 3.2 mg/mL,  $P=0.001$ ), although no differences were found in FF. The plasma MDA values in the control group were higher compared with the patients with endometriosis, but the difference was not significant (57.6 mg/mL to 44.2 mg/mL vs. 46 mg/mL to 29.1 mg/mL) [19].

Concerning our results in this study, the activity of the antioxidant enzyme SOD was comparable between the two groups as regard serum levels, but was higher in Endometriosis group than controls in the FF levels, with statistical significance. This may be explained for compensation of increased oxidative stress in follicular micro environment.

Similarly, Pasqualotto et al. [20] in 2004 and Attaran et al. [21] in 2000 found that antioxidant enzymes in FF, such as catalase, xanthine oxidase, and SOD, are over expressed in patients with endometriosis in response to excess free radicals to neutralize the increased oxidative stress in these patients.

Szczepanska et al. [22] reported a statistically significant decrease in peritoneal fluid superoxide dismutase and glutathione peroxidase concentrations in patients with endometriosis compared with those with idiopathic infertility and control patients.

In contrast, Polak et al. [23] in 2000 and Ishikawa et al. [24] in 1993 did not find differences in superoxide dismutase activity in peritoneal fluid from women with idiopathic or structural infertility and infertile women with endometriosis compared to women with other causes of infertility.

Another similar study, done by Aline Zyman de et al. [25] in 2010, conducted on 112 consecutive infertile females divided into three groups (severe, minimal endometriosis and control), vitamin E and glutathione levels were lower in the serum of infertile women with moderate/severe endometriosis ( $21.7 \text{ mMol/L} \pm 6.0 \text{ mMol/L}$  and  $159.6 \text{ nMol/g} \pm 77.2 \text{ nMol/g}$  protein, respectively) compared to women with minimal and mild endometriosis ( $28.3 \text{ mMol/L} \pm 14.4 \text{ mMol/L}$  and  $199.6 \text{ nMol/g} \pm 56.1 \text{ nMol/g}$  protein, respectively). Total hydroxyperoxide levels were significantly higher in the endometriosis group ( $8.9 \text{ } \mu\text{Mol/g} \pm 1.8 \text{ } \mu\text{Mol/g}$  protein) than in the Control Group ( $8.0 \text{ } \mu\text{Mol/g} \pm 2 \text{ } \mu\text{Mol/g}$  protein) and among patients with stage III/IV disease ( $9.7 \pm 2.3 \text{ } \mu\text{Mol/g}$  protein) compared to patients with stage I/II disease ( $8.2 \text{ } \mu\text{Mol/g} \pm 1.0 \text{ } \mu\text{Mol/g}$  proteins). No significant differences in serum malondialdehyde levels were observed between groups.

As regards the oxidative stress marker MDA, our results showed no difference in its levels between the two groups both in serum and FF. this may be due to the control of oxidative stress by the increase SOD activity in FF and decrease level of vitamin C in serum. However the serum levels were higher than the FF levels in both groups, with statistical significance.

A significant increase in lipid peroxide concentrations in peritoneal fluid ( $3.29 \text{ ng/ml} \pm 1.15 \text{ ng/ml}$  vs.  $2.34 \text{ ng/ml} \pm 0.72 \text{ ng/ml}$ ,  $P=0.01$ ) in endometriosis patients was reported by Liu et al. [26] in 2001.

Shanti et al. [5] in 1999 found reversed results in a study comparing women with endometriosis to women having tubal ligation, in which endometriosis was associated with significantly higher levels of malondialdehyde-modified low-density lipoprotein, and oxidized low density lipoprotein as measured in serum compared to tubal ligation; however, no differences were detected in the peritoneal fluid.

Arumugam and Dip [27] in 1995 found no significant differences in malondialdehyde levels measured in peritoneal fluid among women with moderate to severe endometriosis, women with minimal-to-mild endometriosis and women without endometriosis.

In our study, vitamin E values in FF of endometriosis group were significantly lower than controls, again may be due to consumption in removing oxidative stress. But it was slightly lower in serum than controls, which was not significant.

In contrast to Da Broi et al. [28] in 2014, they observed higher serum concentrations of Glutathione (GSH) (220.32 nmol/g to 43.2 nmol/g pt) and SOD (677.9 U/mL to 282.21 U/mL), and higher follicular concentrations of Vitamin E (13.0 mmol/L to 5.33 mmol/L) in infertile women with Endometriosis compared to those without the disease.

Rodrigues et al. [39] in 2009, find that There were no differences between infertile patients with endometriosis and controls with respect to serum levels of MDA (0.0030 [SD 0.0009] mmol/g protein and 0.0033 [SD 0.0006], respectively), vitamin E (21.7 [SD 6.3] mmol/L and 19.2 [SD 6.3] mmol/L, respectively), glutathione (124.8 [SD 32.8] nmol/g protein and 131.6 [SD 24.6] nmol/g protein, respectively), in Ninety-five infertile patients (44 patients with infertility related to endometriosis and 51 with male or tubal factors infertility).

While study results have varied, comparisons across studies have been difficult due to differences in eligibility criteria, selection of control groups, selection of oxidative stress markers, and the biological medium in which oxidative stress was measured.

## Conclusion

We concluded that, there is no increase in oxidative stress marker MDA in serum or follicular fluid of endometriosis patients compared to normal controls, which may be compensated by increased activity of SOD in FF and/or consumption of the antioxidant vitamin C in serum, and decrease level of vitamin E in FF of the same affected group.

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