

# Effects of vitamin C on the outcome of in vitro fertilization–embryo transfer in endometriosis: A randomized controlled study

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## Abstract

**Objective:** This study was performed to investigate the effect of vitamin C (VitC) supplementation on the outcomes of in vitro fertilization–embryo transfer (IVF-ET) in patients with endometriosis (EMs).

**Methods:** A total of 280 patients with EMs underwent IVF-ET (VitC treatment group, n=160; VitC non-treatment group, n=120). An additional 150 patients who did not have EMs but underwent IVF-ET (control group) were also enrolled in this study. Superoxide dismutase (SOD), total antioxidant capacity (TAC), malondialdehyde (MDA) and reactive oxygen species (ROS) were measured to determine the role of VitC on oxidative stress markers in serum and follicular fluid (FF).

**Results:** In total, 245 patients with EMs and 132 patients without EMs underwent successful IVF-ET and follow-up. The serum or FF levels of VitC, SOD, and TAC were lower in the EMs than control group; however, the MDA and ROS levels in serum or FF were higher in the EMs than control group. After 2 months of VitC treatment, the serum VitC levels in serum and FF were significantly increased, while oxidative stress markers were unaffected.

**Conclusion:** Treatment with VitC oral formulation improved the serum and FF levels of VitC but did not affect oxidative stress markers in patients with EMs.

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## Keywords

Endometriosis, in vitro fertilization–embryo transfer, vitamin C, oxidative stress, follicular fluid, biomarker

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## Introduction

Recently, accumulating evidence has strongly implied that female infertility has severely deteriorated during the last few decades, particularly in Western countries.<sup>1</sup> As a leading cause of infertility, endometriosis (EMs) remains one of the most common estrogen-dependent diseases characterized by ectopic proliferation of endometrial tissues in extrauterine sites. EMs leads to severe dysmenorrhea, dyspareunia, chronic pelvic pain, and infertility and affects 6% to 10% of reproductive-aged women worldwide.<sup>2</sup> However, little is known about the pathogenesis of EMs. Despite several explanations of the causes of EMs, the theory of retrograde menstruation proposed by Sampson<sup>3</sup> is still the most widely accepted. Recent studies have highlighted the importance of oxidative stress in the occurrence and development of various diseases, including EMs.<sup>4–6</sup> Indeed, several studies have suggested that oxidative stress is a potential factor largely responsible for local tissue destruction and disease exacerbation in patients with EMs.<sup>7</sup> In fact, EMs has been considered to be a complicated chronic inflammatory process associated with oxidative stress markers such as superoxide dismutase (SOD), total antioxidant capacity (TAC), malondialdehyde (MDA), and reactive oxygen species (ROS).<sup>8–11</sup> Moreover, Ngô et al.<sup>12</sup> found that a significant elevation of endogenous oxidative stress biomarkers resulted in promotion of the proliferative capabilities of endometriotic cells obtained from patients

with EMs via MAP kinase ERK1/2 activation, leading to EMs progression. Furthermore, increasing evidence indicates that patients with EMs have lower levels of several antioxidant components such as vitamin A (VitA), VitC, and VitE and SOD in follicular fluid (FF) surrounding mature oocytes before ovulation and these lower levels reflect the reproductive performance of oocytes.<sup>13</sup> Thus, an imbalance in ROS production in ovarian FF might lead to an adverse effect on oocyte quality, implantation, and early embryo development.

IVF-ET has been shown to be an optimal therapeutic strategy for patients with EMs-related infertility who failed to conceive with insemination.<sup>14</sup> For example, Turocy et al.<sup>15</sup> reported that the frequency of live birth and clinical pregnancy were relatively improved in patients with EMs undergoing frozen embryo transfer (FET). A previous study showed that compared with antioxidant treatment, supplementation with antioxidant vitamins (VitE and VitC) led to a significant decrease in the peritoneal fluid concentrations of inflammatory factors, including the chemokine regulated on activation, normal T cell expressed and secreted (RANTES), interleukin-6, and monocyte chemoattractant protein-1, and a reduction of chronic pelvic pain in patients with EMs.<sup>16</sup> Mier-Cabrera et al.<sup>17</sup> showed that the intake of a high antioxidant diet for 3 months in patients with EMs increased the levels of VitA, VitC, and VitE and SOD activity

but decreased the MDA and lipid hydroperoxidase levels in serum relative to the control diet group. Nonetheless, further studies are needed to evaluate the clinical value of antioxidant application in women with EMs-related infertility.

This study was performed to investigate the effect of VitC on ROS production, antioxidant capacity, and the outcome of women with EMs undergoing IVF-ET.

## Materials and methods

### Participants

Patients who underwent IVF-ET were recruited from the Department of Reproductive Medicine, International Peace Maternity and Child Health Hospital, School of Medicine, Shanghai Jiaotong University from June 2013 to December 2016. Patients who were <40 years of age, had >2 years of infertility and required infertility treatment by IVF-ET for the first time, and had a regular menstrual cycle with a follicle-stimulating hormone (FSH) level of <10 IU/L on cycle day 2 were included in this study. Moreover, patients with EMs that had been diagnosed by conventional laparoscopy or laparotomy and staged according to the revised American Fertility Society classification were included. Patients with tubal factor infertility instead of EMs who received IVF treatment were included in the study as the control group.

The clinical exclusion criteria were as follows: (1) EMs complicated by endocrine diseases such as diabetes mellitus, polycystic ovary syndrome, hypothalamic pituitary dysfunction, or thyroid dysfunction; (2) a history of autoimmune disease, cardiovascular disease, and liver and kidney dysfunction; (3) administration of an intracytoplasmic single sperm injection to the husband because of severe asthenospermia and oligospermia; (4) treatment with

oral contraceptives and gonadotropin-releasing hormone agonists within 3 months; and (5) a history of alcohol and drug abuse and long-term administration of vitamins. All participants provided written informed consent, and this study was approved by the ethics committee of the Medical Faculty of Shanghai Jiaotong University.

### Sample size

We assumed that supplemental nonenzymatic antioxidant VitC could statistically reduce the ROS level ( $P < 0.05$  with a power of 90%) with 10% prospective rate of loss to follow-up. This randomized controlled study should include at least 120 patients per group according to a statistical formula. Eligible patients were randomized in a ratio of 4:3 to receive either 1000 mg/day of oral VitC (Shanghai Sine Pharmaceutical Laboratories Co., Ltd., Shanghai, China) from 2 months before IVF-ET treatment until 2 weeks after ET (EMs treatment group,  $n=160$ ) or no treatment (EMs non-treatment group,  $n=120$ ). In total, 150 patients without EMs were used as the control group.

### IVF-ET procedures

Patients treated with IVF-ET received the long gonadotropin-releasing hormone agonist downregulation protocol during the luteal phase, followed by injection of recombinant FSH (GONAL-f; Merck Serono, Darmstadt, Germany) for controlled ovarian hyperstimulation. The dosage of recombinant FSH was monitored by B-ultrasonography and serum estradiol level measurements. When the three leading follicles reached 18 mm in diameter, 6000 IU of human chorionic gonadotropin (Pregnyl®; N.V. Organon, Oss, the Netherlands) was intramuscularly administered. After 35 to 36 hours, oocytes were

retrieved by transvaginal ultrasonography-guided puncture. The fertilization rate was calculated as the number of cleavage embryos divided by the number of metaphase II oocytes. Embryo quality was assessed using Veeck's classification, and grade I and II were considered high-quality embryos. Two or fewer embryos were transferred to each patient 72 hours after oocyte retrieval. Two weeks after embryo transfer, clinical pregnancy was defined as identification of a gestational sac in serum human chorionic gonadotropin-positive patients through ultrasonographic examination. The implantation rate was defined as the number of gestational sacs per transferred embryo.

### ***Determination of VitC and oxidative stress markers in serum and FF***

The serum and FF levels of VitC and oxidative stress markers (SOD, TAC, MDA, and ROS) were determined in EMs treatment group, EMs non-treatment group, and control group. Fasting peripheral plasma samples were collected from patients in the EMs treatment group on the second day of menstruation before VitC administration and 2 months post-administration. Fasting peripheral plasma samples were collected from patients in the non-treatment and control groups on the second day of menstruation. FF from the first punctured follicle was obtained on the day of retrieval, followed by centrifugation at  $300 \times g$  for 7 minutes to remove cellular remnants. The upper layer was frozen at  $-196^{\circ}\text{C}$  before measurements.

ROS production was measured after the addition of 5  $\mu\text{L}$  of Luminol (0.1 mM) working solution prepared in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) and 2  $\mu\text{L}$  of formyl-methionyl-leucyl-phenylalanine (FMLP) working solution (0.2  $\mu\text{M}$ ) obtained by commixture

of FMLP stock solution and Hank's Balanced Salt Solution. The ROS value was expressed as the relative light units per minute when the chemiluminescence signal was monitored for 15 minutes. The concentrations of TAC and MDA in the serum and FF were assessed by phenanthroline colorimetry using a TAC Assay Kit (Beyotime Biotechnology, Shanghai, China) and thiobarbituric acid chromatometry, respectively, as previously described.<sup>18</sup> The contents of VitC were quantified using spectrophotometry (Model 722 ultraviolet spectrophotometer; Shanghai Jingke Industrial Co. Ltd., Shanghai, China), and the activity of SOD was measured by the xanthine oxidase method using a microplate reader (Shanghai Jingke Industrial Co. Ltd.) with kits supplied by Beyotime Biotechnology. The selected wavelengths were 490 nm for VitC and 550 nm for SOD.

### ***Statistical analysis***

All data analysis was conducted using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). The chi-square test was performed to compare the enumeration data. An independent-sample t test was used for normally distributed data, and a nonparametric rank sum test was used for non-normally distributed data. A P value of  $<0.05$  was considered statistically significant.

## **Results**

### ***Clinical characteristics and pregnancy outcomes***

The 280 patients with EMs were assessed according to the revised American Fertility Society classification. The results showed that among 160 patients in EMs treatment group, 87 had stages I and II EMs and 73 had stages III and IV. Among the 120 patients in the EMs non-treatment group, 66 had stages I and II

EMs and 54 had stages III and IV. The rates of mild and severe EMs were not significantly different between the groups.

As shown in Table 1, there were no significant differences in demographic and clinical data including age, duration of infertility, body mass index, basal FSH level, endometrial thickness, and numbers of transferred embryos among the three groups (Table 1). In the control group, 18 patients failed to receive ET due to absence of transfer embryos (n=9), the impact of endometrium-related factors (n=6), and personal-related factors (n=3). In total, 245 patients in the EMs treatment group (n=137) and non-treatment group (n=108) underwent successful ET and follow-up. The reasons for the lack of success in the 23 and 12 patients, respectively, were a lack of transfer embryos (n=10 in the EMs treatment group and n=9 in the non-treatment group), the impact of endometrium-related factors (n=2 in the EMs treatment group and n=1 in the non-treatment group), hydrosalpinx (n=2 in the EMs treatment group and n=2 in the non-treatment group), and failure to take VitC as required (n=9 in the EMs treatment group).

No significant differences in the fertilization rate, implantation rate, or clinical pregnancy rate were found among these three

groups (Table 2). However, the quantity of retrieved oocytes and frozen embryos in the EMs treatment group and non-treatment group were significantly lower than those in the control group ( $P<0.05$ ) (Table 2), while no prominent differences were observed between the EMs treatment group and non-treatment group (Table 2). The high-grade embryo rate was significantly lower in the non-treatment than control group ( $P<0.05$ ) (Table 2), but there were no significant differences between the EM treatment group and the control group (Table 2).

#### *Levels of VitC and oxidative stress markers in serum and FF*

The FF levels of VitC, SOD, and TAC were significantly lower in patients with EMs than in controls ( $P<0.05$ ) (Figure 1(a)–(c)). The blood levels of VitC, SOD, and TAC were slightly lower in patients with EMs than in controls, without reaching statistical significance (Figure 1(a)–(c)). The FF levels of SOD and TAC in these two groups were significantly higher than the serum levels ( $P<0.05$ ) (Figure 1(b) and (c)).

In contrast, the serum MDA level was significantly higher than the FF level in patients with EMs and controls ( $P<0.05$ ) (Figure 1(d)). However, the FF MDA level

**Table 1.** Demographic and clinical data of patients.

Characteristics	Control group (n=132)	Non-treatment group (n=108)	EMs treatment group (n=137)
Age (years)	32.1±3.1	31.9±3.0	31.5±3.5
Duration of infertility (years)	5.7±3.5	5.6±3.3	6.0±3.2
BMI (kg/m <sup>2</sup> )	22.5±2.3	22.7±2.6	21.9±2.2
Basal FSH level (mIU/L)	7.6±1.4	7.5±1.5	7.8±1.2
Endometrial thickness (mm)	9.3±1.8	9.4±1.6	9.8±2.0
No. of transferred embryos	1.8±0.7	1.8±0.7	1.7±0.6

Data are presented as mean ± standard deviation.

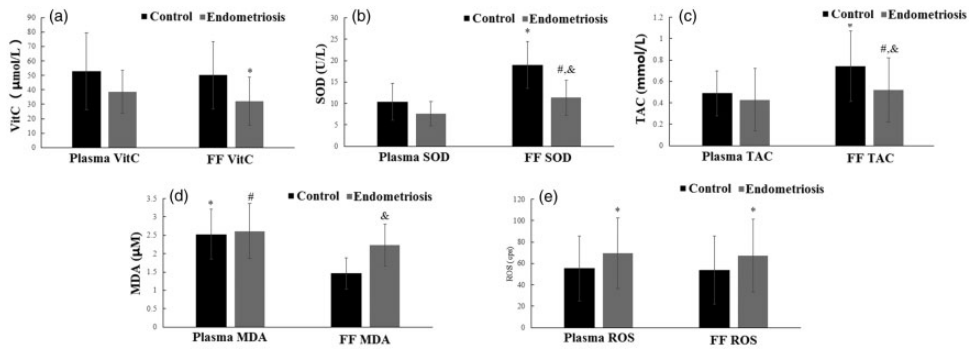
EMs, endometriosis; BMI, body mass index; FSH, follicle-stimulating hormone.

**Table 2.** Laboratory and pregnancy outcomes.

Characteristics	Control group (n=132)	Non-treatment group (n=108)	EMs treatment group (n=137)
Total Gn dosage	2310.0±726.7	2957.5±1009.5*	3015.0±1215.1*
No. of retrieved oocytes	9.1±5.4	7.3±4.0*	7.4±3.7*
Fertilization rate (%)	77.7 (934/1202)	74.8 (590/788)	78.0 (791/1014)
High-grade embryo rate (%)	70.1 (563/803)	58.3 (261/448)*	66.8 (385/576)
Implantation rate (%)	30.4 (72/237)	23.1 (45/195)	28.0 (65/232)
Clinical pregnancy rate (%)	47.7 (63/132)	33.3 (36/108)	39.4 (54/137)
No. of frozen embryos	4.2±3.0	2.5±2.4*	2.9±2.7*

Data are presented as n (%) or mean ± standard deviation.

\*P<0.05 compared with the control group. EMs, endometriosis; Gn, gonadotropin.



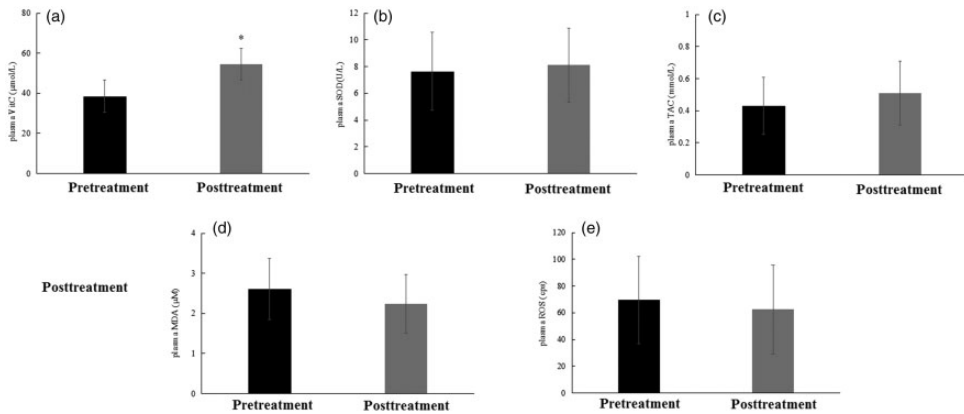
**Figure 1.** Serum and FF levels of vitamin C and oxidative stress markers in patients with and without EMs. (a) Serum and FF levels of vitamin C in patients with and without EMs. \*P<0.05 compared with the control group. (b) Serum and FF levels of SOD in patients with and without EMs. \*P<0.05 compared with the control group; &P<0.05 compared with the EMs group. (c) Serum and FF levels of TAC in patients with and without EMs. \*P<0.05 compared with the control group; &P<0.05 compared with the EMs group. (d) Serum and FF levels of MDA in patients with and without EMs. #P<0.05 compared with the control group; &P<0.05 compared with the EMs group. (e) Serum and FF levels of ROS in patients with and without EMs. \*P<0.05 compared with the control group. EMs, endometriosis; FF, follicular fluid; SOD, superoxide dismutase; TAC, total antioxidant capacity; MDA, malondialdehyde; ROS, reactive oxygen species.

was significantly higher in patients with EMs than in controls (P<0.05) (Figure 1(d)). No difference between the serum and FF levels of MDA was found in the patients with EMs (Figure 1(d)). The ROS levels in serum and FF were significantly higher in patients with EMs than in controls (P<0.05) (Figure 1(e)), but no conspicuous differences between the serum and FF ROS levels were observed (Figure 1(e)).

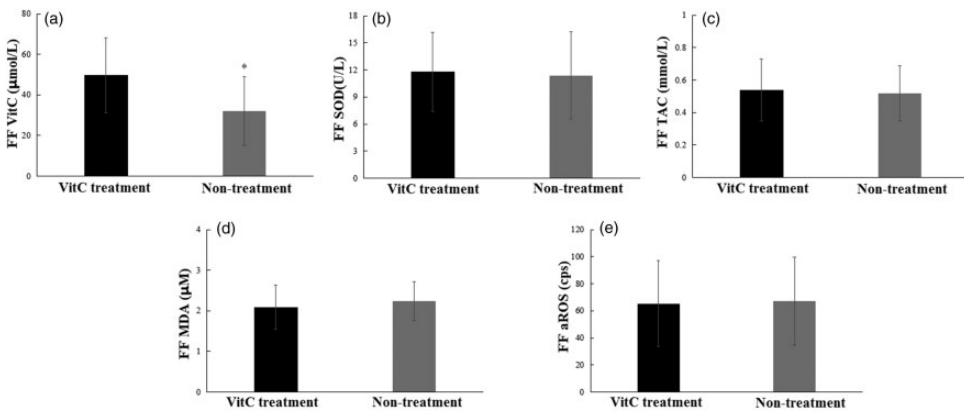
### *Effect of VitC on levels of VitC and oxidative stress markers in patients with EMs*

Treatment with the oral formulation of VitC for 2 months at 1000 mg/day improved the serum and FF levels of VitC in patients with EMs, but it did not affect the oxidative stress markers (ROS, TAC, SOD, and MDA) (Figures 2 and 3).





**Figure 2.** Changes in serum levels of VitC and oxidative stress markers in the VitC treatment group. (a) Serum levels of VitC in patients with endometriosis before and after treatment. (b) Serum levels of SOD in patients with endometriosis before and after treatment. (c) Serum levels of TAC in patients with endometriosis before and after treatment. (d) Serum levels of MDA in patients with endometriosis before and after treatment. (e) Serum levels of ROS in patients with endometriosis before and after treatment. \* $P < 0.05$  compared with pretreatment group. VitC, vitamin C; SOD, superoxide dismutase; TAC, total antioxidant capacity; MDA, malondialdehyde; ROS, reactive oxygen species.



**Figure 3.** Changes in follicular fluid levels of VitC and oxidative stress markers in VitC treatment and non-treatment groups. (a) Serum levels of VitC in patients with endometriosis treated with or without VitC. (b) Serum levels of SOD in patients with endometriosis treated with or without VitC. (c) Serum levels of TAC in patients with endometriosis treated with or without VitC. (d) Serum levels of MDA in patients with endometriosis treated with or without VitC. (e) Serum levels of ROS in patients with endometriosis treated with or without VitC. \* $P < 0.05$  compared with the non-treatment group. VitC, vitamin C; SOD, superoxide dismutase; TAC, total antioxidant capacity; MDA, malondialdehyde.

## Discussion

An estimated 20% to 40% of patients with infertility have EMs-related infertility, which is one of the main indications for

IVF-ET. The FF microenvironment influences IVF outcome parameters such as oocyte quality, fertilization rate, and high-grade embryos.<sup>19</sup> The balance between oxidation and antioxidant action in FF is

associated with the maturation of oocytes as shown by the positive correlation between appropriate ROS levels in FF and the clinical pregnancy rate.<sup>20</sup> Conversely, elevated ROS levels also appear to be responsible for oxidative stress injury, leading to oocyte DNA and cytoskeleton damage, an increase of embryonic debris, and abnormal embryonic development.<sup>21,22</sup> Therefore, an accurate balance of the ROS level and antioxidant capacity in the FF environment is essential for the acquisition of high-quality oocytes and embryos following IVF treatment.

In this study, we found a marked increase in ROS and MDA and a significant decrease in VitC, TAC, and SOD in the FF of patients with EMs, which is consistent with a previous study.<sup>23</sup> We suggest that the abnormal state of oxidative stress and antioxidant capacity in the impaired FF microenvironment, the imbalance between the production and scavenging of ROS, and the high VitC consumption required for neutralization of the excessive ROS production might explain the consequences of EMs including follicular dysplasia and low-quality oocytes, which affect the pregnancy rate and implantation rate. In the present study, the rate of high-grade embryos was lower in patients with EMs than in controls; furthermore, there was a decreasing trend in the implantation rate and clinical pregnancy rate, but this trend did not reach statistical significance. Our data also indicate that the levels of SOD and TAC in FF were higher than those in serum, while the serum level of MDA was higher than that in FF in patients with or without EMs. These findings indicate that there might be a stronger antioxidant capacity in FF to protect oocytes from oxidative stress injury.

Based on the beneficial effect of antioxidant intake and a high antioxidant diet on the oxidative stress status,<sup>16,17</sup> our findings revealed that the peripheral serum and FF levels of VitC in women with EMs

following 2 months of VitC treatment (1000 mg/day) were higher than those in the non-treatment group, demonstrating the effectiveness of the oral administration route. In contrast, the oxidative stress markers never changed, resulting in a consistent retrieved oocyte rate, implantation rate, and clinical pregnancy rate. Additionally, the high-grade embryo rate was better in patients with EMs receiving VitC therapy than in the controls. We speculated that the increased VitC levels in FF might properly neutralize the excessive ROS production, thereby promoting the oxidative stress status in the FF microenvironment and leading to the improvement in the oocyte quality.

One study showed that patients with severe EMs who received VitC (1000 mg/day) and VitE (800 IU/day) for 8 weeks before IVF-ET treatment showed significantly repressed levels of myeloperoxidase in FF.<sup>24</sup> Mier-Cabrera et al.<sup>25</sup> found that VitC and VitE supplementation for 6 months led to a decrease in the plasma and peritoneal fluid concentrations of MDA and lipid hydroperoxides in patients with EMs. In this study, supplementation of VitC was given for 2 months; therefore, further investigation is needed to confirm the effect of the medication time on oxidative stress. Prieto et al.<sup>26</sup> reported that the VitC concentration in FF was significantly associated with the quantity of retrieved oocytes and mature oocytes as well as the fertilization rate in patients with EMs following IVF therapy. Besides the effect of VitC on oxidative stress, other studies have also indicated that VitC supplementation via oral or intravenous administration led to a significant reduction in the volume and weight of the endometriotic cysts and number of natural killer cells and inhibited endometriotic implantation in a dose-dependent manner.<sup>27,28</sup> A randomized controlled trial showed that VitC supplementation at a dosage of 750 mg/day



improved the clinical pregnancy rate of patients with a luteal phase defect.<sup>29</sup> In contrast, Darling et al.<sup>30</sup> showed that intake of foods rich in VitC instead of supplementation of VitC or multivitamins effectively suppressed EMs-related morbidity.

In summary, we analyzed the effect of VitC supplementation in patients with EMs undergoing IVF-ET on oxidative stress markers and the outcome of IVF-ET. Our findings showed that the abnormal state of oxidative stress and impaired antioxidant capacity in FF was discovered in patients with EMs; moreover, 2 months of VitC supplementation accelerated the sample and FF levels of VitC, thereby ameliorating the quality of oocytes and embryos. However, our study had some limitations. First, the sample size was relatively small. Second, a comparison of different time points (e.g., 2, 4, 6 months) was not performed. Finally, assessment of the patients' daily diet was excluded from this study.

### Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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