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N-Acetyl-Cysteine and L-Carnitine Prevent Meiotic Oocyte Damage Induced by Follicular Fluid From Infertile Women With Mild Endometriosis

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Abstract

This study evaluated the potential protective effect of the antioxidants, L-carnitine (LC) and N-acetyl-cysteine (NAC), in preventing meiotic oocyte damage induced by follicular fluid (FF) from infertile women with mild endometriosis (ME). We performed an experimental study. The FF samples were obtained from 22 infertile women undergoing stimulated cycles for intracytoplasmic sperm injection (11 with ME and 11 without endometriosis). Immature bovine oocytes were submitted to in vitro maturation (IVM) divided into 9 groups: no-FF (No-FF); with FF from control (CFF) or ME (EFF) groups; and with LC (C + LC and E + LC), NAC (C + NAC and E + NAC), or both antioxidants (C + 2Ao and E + 2Ao). After IVM, oocytes were immunostained for visualization of microtubules and chromatin by confocal microscopy. The percentage of meiotically normal metaphase II (MII) oocytes was significantly lower in the EFF group (51.35%) compared to No-FF (86.36%) and CFF (83.52%) groups. The E + NAC (62.22%), E + LC (80.61%), and E + 2Ao (61.40%) groups showed higher percentage of normal MII than EFF group. The E + LC group showed higher percentage of normal MII than E + NAC and E + 2Ao groups and a similar percentage to No-FF and CFF groups. Therefore, FF from infertile women with ME causes meiotic abnormalities in bovine oocytes, and, for the first time, we demonstrated that the use of NAC and LC prevents these damages. Our findings elucidate part of the pathogenic mechanisms involved in infertility associated with ME and open perspectives for further studies investigating whether the use of LC could improve the natural fertility and/or the results of in vitro fertilization of women with ME.

Keywords

female infertility, endometriosis, follicular fluid, oocyte quality, oxidative stress

Introduction

Endometriosis is an estrogen-dependent gynecological disease characterized by the presence and growth of endometrial tissue outside the uterine cavity.[1] The disease affects approximately 10% of women of reproductive age[2] and is strongly associated with infertility.[1] It is estimated that more than 30% of infertile women have endometriosis[2] and that 30% to 50% of these women report difficulties in getting pregnant.[3]

However, the pathogenesis of infertility related to endometriosis remains unknown, especially in the early stages of the disease (minimal and mild endometriosis (ME)—stages I and II, respectively)[4] during which no marked changes are detected in pelvic anatomy. However, the presence of lower cumulative pregnancy rates in women in the early stages of endometriosis compared to women with infertility of unknown cause[5-7] supports the association between infertility and endometriosis in the early stages. Although controversial, studies have suggested worsening of oocyte quality as one of the events responsible for the infertility related to endometriosis,[8-11] but the mechanisms leading to this impairment is unknown.[12,13] However, human oocytes are extremely rare for study, and their use in invasive studies usually is unviable because it prevents their use in assisted reproduction techniques. On this basis, studies using animal models may be useful for the elucidation of the mechanisms

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of infertility related to endometriosis. Understanding these etiopathogenic mechanisms may be valuable in designing effective therapeutic approaches to improve the natural fecundity of these patients.

Oocyte quality results from appropriate cytoplasmic and nuclear maturation,[14] which depends on the presence of a normal cell spindle.[15,16] The meiotic spindle of human oocytes in metaphase II (MII) is a structure consisting of microtubules associated with the oocyte cortex and with the subcortical microfilament network.[17-19] The primary function of this microtubular structure is to aid chromatid and chromosome segregation in parallel to the extrusion of the polar bodies, guaranteeing the end of the meiotic process.[20] The meiotic spindle of the oocyte is a structure extremely sensitive to variations in its microenvironment, and oxidative stress (OS) is a factor that changes these conditions[21-24] by provoking meiotic anomalies and chromosome instability in addition to increasing apoptosis and impairing embryonic development before implantation.[25-27]

Oxidative stress occurs when there is disequilibrium between the production of reactive species and antioxidant capacity, and the cell components become vulnerable to attack by free radicals (for a review, see the report by Halliwell[28]). The increase in reactive oxygen species in follicular fluid (FF) may affect embryonic quality and development,[29-31] and recent studies have demonstrated that the FF of patients with endometriosis shows increased levels of reactive species and a reduction in total antioxidant capacity.[31-33] Those studies,[31-33] however, did not analyze the FF from infertile women in the early stages of endometriosis. A study by our group revealed that the FF from infertile women with ME greatly impairs the meiotic spindle of bovine oocytes matured in vitro, even at the lowest concentration tested (1%).[10] However, the mechanisms of meiotic oocyte damage induced by the FF from women with ME remain unknown.

We questioned the potential role of OS, and we asked whether antioxidant supplementation would be able to minimize or reverse the deleterious effects of FF from women with infertility related to ME. L-Carnitine (LC) is a quaternary amine that acts on the clearance of hydrogen peroxides and the products of lipid peroxidation.[34] L-Carnitine is related to cellular energy metabolism, and its primary function is to facilitate the transport of long-chain free fatty acids to the mitochondrial matrix, making them available for β-oxidation, which is one of the main pathways for energy production.[35] Thus, LC is involved in the energy metabolism of fatty acids and contributes to the appropriate functioning of the mitochondria, the organelles responsible for the production of energy in the form of adenosine triphosphate. The metabolic pathways of mitochondria have a high potential for the production of free radicals,[36] which, in excessive concentration, may lead to OS. N-acetyl-cysteine (NAC) is an aminothiol with immunomodulatory, antiapoptotic, and antioxidant functions.[37] N-acetylcysteine acts by reducing the disulfide bridges in proteins, and it is a precursor of intracellular cysteine and reduced glutathione (GSH).[38] In turn, GSH is important for the elimination of a variety of toxic substances, including xenobiotics and peroxide, and performs an essential function in cell protection.[39]

Further evidence of the mechanisms involved in the etiopathognesis of infertility related to endometriosis is necessary for the development of new therapeutic approaches able to contribute for the improvement in the natural fertility of patients with ME. Our objective was to determine whether the addition of the antioxidants LC and NAC, separately or in combination, would prevent the damage to the oocyte meiotic spindle induced by the FF from infertile women with ME during the IVM of bovine oocytes.

Materials and Methods

Patient Selection and FF Collection

Twenty-two FF samples were obtained between February 2009 and February 2011 from infertile women who underwent ovarian stimulation for intracytoplasmic sperm injection in the Sector of Human Reproduction, Department of Gynecology and Obstetrics, Faculty of Medicine of Ribeirão Preto, University of São Paulo (FMRP-USP), Brazil. The study was approved by the Research Ethics Committee of the University Hospital, FMRP-USP (grant number 12201/2008) and by the Ethics Committee for Animal Experimentation of the FMRP-USP (grant number 169/2008), and all patients gave written informed consent to participate.

The endometriosis group comprised patients with infertility associated only with ME, who were diagnosed and classified by videolaparoscopy performed by an experienced surgeon according to the criteria of the American Society for Reproductive Medicine.[4] All women with ME were submitted to destruction or removal of all visible endometriotic implants and the lysis of adhesions. The control group comprised women with tubal or male factor infertility. All control women also underwent videolaparoscopy as part of the protocol of the investigation of infertility, with exclusion of the presence of endometriosis.

Exclusion criteria were as follows: age $\geq$38 years; body mass index $\geq$30 kg/m^2; serum concentration of follicle-stimulating hormone (FSH) on the third day of the menstrual cycle $\geq$10 mIU/mL; chronic anovulation; presence of hydrosalpinx or of chronic diseases such as diabetes mellitus or any other endocrinopathy; cardiovascular disease; dyslipidemia; systemic lupus erythematosus or any other rheumatologic disease; HIV infection or any active infection; smoking habit; and the use of vitamins, hormonal, or nonhormonal medications during a period of 6 months before the inclusion in the study. The exclusion criteria aimed to avoid situations related to OS and/or worsening of oocyte quality as confounding factors.

There was no significant difference between the endometriosis and control groups regarding mean age (32.72 $\pm$ 0.52 and 30.63 $\pm$ 1.36 years, respectively), FSH concentration on the third day of the menstrual cycle (5.02 $\pm$ 0.90 and 5.79 $\pm$ 0.62 mIU/mL, respectively), number of follicles measuring 14 to 17 mm (10.09 $\pm$ 1.43 and 6.11 $\pm$ 1.52 mm, respectively), or number of follicles $\geq$18 mm after ovarian stimulation

(4.89 ± 0.72 to 3.11 ± 0.76 mm, respectively). Data are reported as the mean ± standard error of the mean.

Protocol of Controlled Ovarian Stimulation

Controlled ovarian stimulation (COS) was performed according to the protocol of the sector (long protocol). Pituitary blockade was performed by administering an agonist of gonadotropin-releasing hormone (Lupron, Abbott, São Paulo, Brazil), COS was performed by administering recombinant FSH (Gonal-F, Serono, Geneva, Switzerland; Puregon, Organon, Oss, the Netherlands), and ovulation was induced with human chorionic gonadotropin (Ovidrel, EMD Serono, Rockland, Massachusetts).

Each patient received a daily subcutaneous injection of 0.5 mg leuprolide acetate (Lupron; Abbott) starting 10 days after the first ultrasound exam before ovarian stimulation. Recombinant FSH (Gonal-F and Puregon), 200 to 225 units/day, was administered during ovarian stimulation, and follicular growth was monitored. Ovulation was triggered with Ovidrel, and oocytes were retrieved after 34 to 36 hours.

Collection and Processing of FF Samples

Follicular fluid was collected into individual sterile tubes preheated to 37°C in the absence of culture medium. The FF sample was collected only from the first follicle with a mean diameter ≥15 mm of the first ovary punctured, with aspiration of the full follicular content. Only FF with no blood contamination upon visual inspection and presenting a mature oocyte was used. The samples were centrifuged at 300*g* for 10 minutes to separate the remaining cells, and the supernatant was stored at −80°C in 2 aliquots for future use. The FF was collected from 22 infertile women, 11 with ME and 11 with male and/or tubal infertility.

In the present study, we opted to pool the 11 FF samples of each group for the experiments described subsequently because these samples had already been tested individually in a previous study by our group investigating the role of FF from women with infertility related to ME.[10] In that study, no intragroup difference was detected and there was a homogeneous response in all 11 experiments conducted on both the ME and the control groups. In that same study,[10] 4 different FF concentrations added to the IVM medium were tested (1%, 5%, 10%, and 15%) and no dose-dependent effect was observed. Thus, in the current study, we opted to use an FF pool of each group at the lowest concentration tested (1%) in the experiments described below.

Oocyte Collection

Bovine ovaries were collected immediately after slaughter and transferred in physiological saline at 35°C to 38.5°C. In the laboratory, follicles measuring 2 to 8 mm were aspirated and only cumulus–oocyte complexes (COCs) with homogeneous cytoplasm and at least 3 layers of cumulus oophorus cells were selected.[14,40]

In Vitro Maturation

Cumulus–oocyte complexes were cultured in 400 µL drops of culture medium for IVM (approximately 20 oocytes per drop) at 38.5°C, 95% humidity, and 5% carbon dioxide[14,40,41] in a culture system without mineral oil. The IVM medium used was TCM-199 containing Earle salts and bicarbonate (Invitrogen, Gibco Laboratories Life Technologies, Inc, Grand Island, New York) supplemented with 0.4 mmol/L sodium pyruvate, 0.5 µg/mL gentamicin, 5 µg/mL FSH, 5 mg/mL LH, 1 µg/mL estradiol, and 10% fetal calf serum (Gibco). All culture media and reagents were purchased from Sigma Chemical Company (St Louis, Missouri), except for those followed by a reference in parentheses.

Preparation of Antioxidant (NAC and LC) Solutions

Both stock solutions were prepared at a 100 times higher concentration than the working solution (stock solutions: 150 mmol/L NAC and 60 mg/mL LC) with water of injection and filtration through a filter with 0.22 µm pores. The NAC concentration used to supplement the IVM experiments described subsequently was 1.5 mmol/L,[42] and the LC concentration was 0.6 mg/mL.[43]

Fixation and Immunofluorescence for the Visualization of Microtubules and Chromosomes

After IVM, cumulus cells were separated from the oocytes by pipetting. The oocytes were fixed and left to stand for 30 minutes in a buffer for microtubule stabilization.[14,44] The oocytes were washed and blocked overnight at 4°C in washing medium consisting of phosphate-buffered saline supplemented with 0.02% sodium azide, 0.01% Triton X-100, 0.2% defatted dry milk, 2% goat serum, 2% bovine serum albumin, and 0.1 mol/L glycine. The oocytes were incubated overnight at 4°C with an anti-β-tubulin murine monoclonal antibody (1:1000) and were washed and incubated with a secondary fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G antibody (1:500; Zymed Laboratories, Invitrogen, Carlsbad, California) at 38.5°C for 2 hours. The oocytes were submitted to an additional washing and were labeled with Hoechst 33342 (10 mg/mL) in Vectashield mounting medium (H-1000, Vector, Burlingame, California) on a glass slide covered with a coverslip. The samples were visualized using a high-performance confocal microscope (Confocal Leica TCS SP5, Leica Microsystems, Mannheim, Germany) at 40× magnification with 405 nm diode ultraviolet and 543 nm helium neon lasers.

Oocyte Classification Based on Meiotic Spindle Morphology and Chromosome Alignment

Based on the stage of nuclear maturation, the oocytes were classified as metaphase I (MI), telophase I (TI) or MII, or as parthenogenetically activated (PA). The MII oocytes were

subdivided into analyzable and nonanalyzable groups according to the position of visualization of the metaphase plate. The oocytes were considered to be analyzable when the meiotic spindle was visualized in a lateral or sagittal position (Figure 1A) and nonanalyzable when the spindle was observed in a polar position.[45] In the polar position, although the chromosomes could be aligned, the oocyte spindle was not visualized, and thus the spindle could be abnormal.[10,46,47] So, to avoid underestimating the number of abnormal oocytes, the oocytes in polar position were excluded from the analysis.

The MII oocytes were classified as "normal" when they exhibited a barrel-shaped meiotic spindle with microtubules organized from one pole to the other, chromosomes aligned on the metaphase plate at the equator of the spindle (Figure 1A), and the presence of a polar body (PB), or classified as "abnormal" when they showed an altered meiotic spindle (reduced longitudinal dimension, disorganized or absent microtubules, dispersed or dislocated from the plane of the metaphase plate), and/or altered chromosome configuration (dispersed or displaced from the plane of the metaphase plate; Figure 1B-D). These types of abnormalities (spindle and/or chromosomes) were carefully observed because there are no data about effects of antioxidants on the metaphase plate. Parthenogenetically activated oocytes were characterized by the spontaneous extrusion of a second PB without the occurrence of fertilization.

Experimental Design

Immediately after selection, the COCs were cultured on 4-well NUNC plates for 22 to 24 hours and divided into the following 9 groups:

1. medium with no addition of FF (No-FF);
2. with 1% FF from control patients and no addition of antioxidants (CFF);
3. with 1% FF from control patients and the addition of 1.5 mmol/L NAC (C + NAC);
4. with 1% FF from control patients and the addition of 0.6 mg/mL LC (C + LC);
5. with 1% FF from control patients and the addition of 1.5 mmol/L NAC + 0.6 mg/mL LC (C + 2Ao);
6. with 1% FF from patients with ME and no addition of antioxidants (EFF);
7. with 1% FF from patients with ME and the addition of 1.5 mmol/L NAC (E + NAC);
8. with 1% FF from patients with ME and the addition of 0.6 mg/mL LC (E + LC); and
9. with 1% FF from patients with ME and the addition of 1.5 mmol/L NAC + 0.6 mg/mL LC (E + 2Ao).

After maturation, the oocytes were denuded by pipetting in TCM-199 medium containing Hanks salts, HEPES buffer, and L-glutamine (Invitrogen, Gibco Laboratories Life Technologies, Inc). Nine replicates were performed, each including the 9 groups described earlier (No-FF, CFF, C + NAC, C + LC, C + 2Ao, EFF, E + NAC, E + LC, and E + 2Ao).

Statistical Analysis

Data were analyzed statistically using a generalized linear model (PROC GENMOD) and the SAS 2003 software (2002-2003, SAS Institute, Inc, Cary, North Carolina). Poisson distribution was used for all discrete variables (total number of fixed oocytes, total number of viewed oocytes, number of MI, TI, MII, and PA oocytes, and number of analyzable MII), and gamma distribution for frequencies (frequency of MI, TI, MII, and PA oocytes, and frequency of normal MII oocytes). The groups were compared by the χ^2 test, with the level of significance set at $P < .05$.

Results

A total of 1934 COCs were matured in vitro. Of these, 1682 were fixed and 1572 were analyzed by confocal microscopy (120 oocytes were in MI, 14 in TI, 1400 in MII, and 38 underwent PA). Of the 1400 MII oocytes, 812 were considered analyzable, that is, visualized in a sagittal or lateral position, and 588 were considered nonanalyzable (polar view). There was no difference in the total number of fixed and visualized oocytes or in the number of MI, TI, PA, and MII oocytes in each group (Table 1).

A total of 200 oocytes were evaluated in No-FF group; 182 of them were in MII and 110 of the oocytes in MII were considered analyzable. Ninety-five (86.4%) of the analyzable MII oocytes were considered normal and 15 (13.4%) were considered abnormal (Table 1). Twelve (80%) of the abnormal oocytes exhibited only chromosome misalignment, and 3 (20%) exhibited both chromosome misalignment and an abnormal spindle.

When the groups with FF from control patients were pooled (without and with the addition of antioxidants [CFF, C + NAC, C + LC, and C + 2Ao]), a total of 674 oocytes were evaluated; 593 of them were in MII and 346 of the oocytes in MII were considered analyzable (Table 1). Among the analyzable oocytes in MII, 291 (84.1%) were considered normal and 55 (15.9%) were considered abnormal. Among the abnormal oocytes, 21 (38.2%) showed only chromosome misalignment, 20 (36.4%) showed only spindle abnormalities, and 14 (25.4%) showed both chromosome misalignment and an abnormal spindle.

When the groups with FF from patients with ME (without and with the addition of antioxidants [EFF, E + NAC, E + LC, and E + 2Ao]) were pooled, a total of 698 oocytes were evaluated; 625 of them were in MII and 356 of oocytes in MII were considered analyzable (Table 1). Among the analyzable oocytes in MII, 227 (63.8%) were considered normal and 129 (36.2%) were considered abnormal. Forty-one (31.8%) of the abnormal oocytes showed only chromosome misalignment, 48 (37.2%) showed only spindle abnormalities, and 40

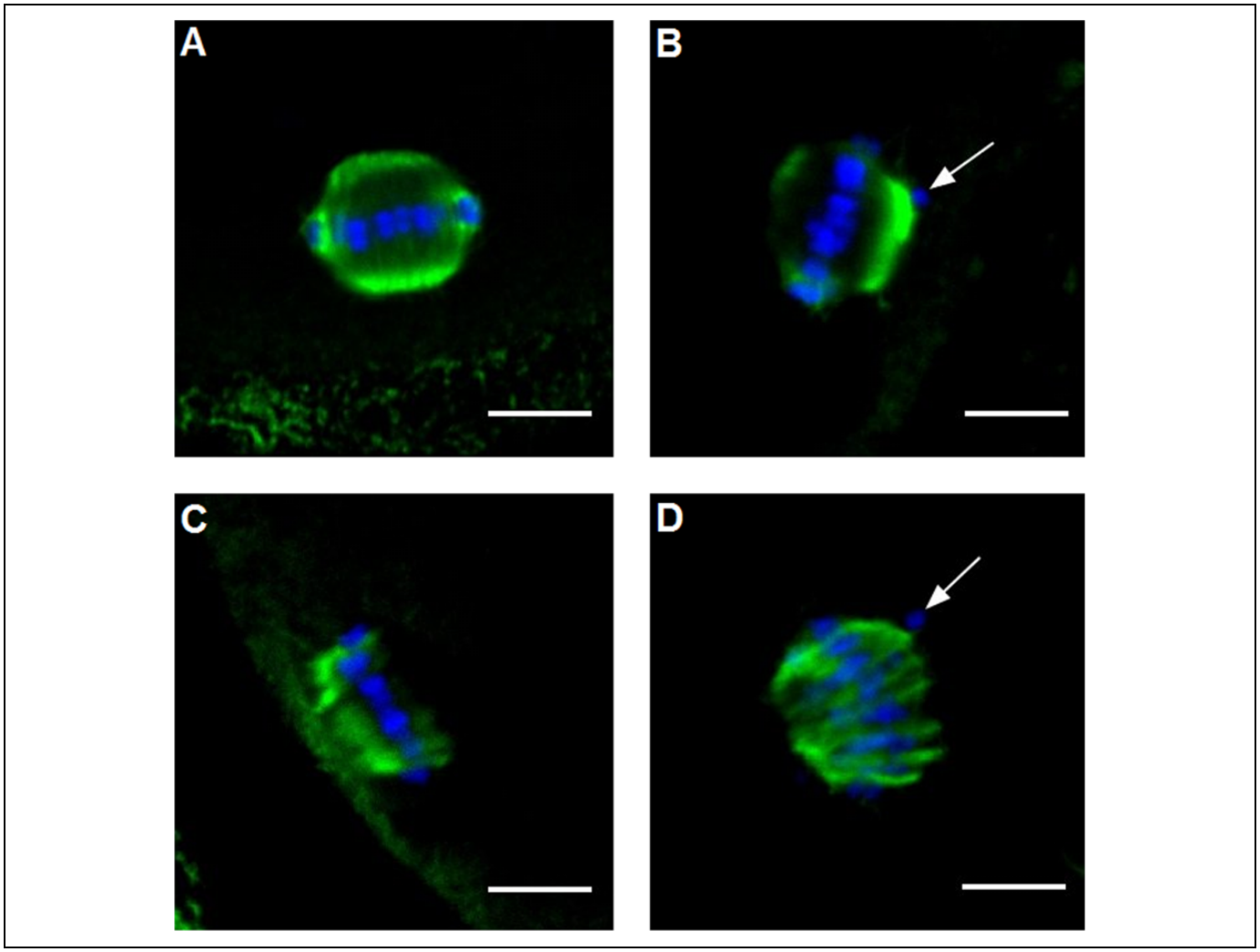

Figure 1. Confocal microscopy images of bovine oocytes matured in vitro in the presence of follicular fluid (FF) from infertile women without endometriosis (CFF) or with mild endometriosis (EFF) supplemented with N-acetyl-cysteine (NAC; C + NAC and E + NAC), with L-carnitine (LC; C + LC and E + LC), or with both antioxidants (C + 2Ao and E + 2Ao). A, Normal metaphase II (MII) oocyte of the CFF group showing properly aligned chromosomes in the central region of a barrel-shaped meiotic spindle. B, Abnormal MII oocyte of the EFF group with misaligned chromosomes and a normal barrel-shaped spindle. C, Abnormal MII oocyte of the EFF group with aligned chromosomes and an abnormal spindle (reduced longitudinal dimension). D, Abnormal MII oocyte of the EFF group with misaligned chromosomes and an abnormal spindle. Green: spindle labeled with an anti-β-tubulin antibody and a secondary antibody conjugated with fluorescein isothiocyanate; blue: chromosomes labeled with Hoechst 33342. Scale bar = 10 μm.

(31.0%) showed both chromosome misalignment and an abnormal spindle.

About meiotic abnormality types in MII oocytes, no differences were found comparing 9 groups ($P < .05$). The percentage of meiotically normal MII oocytes was similar for the No-FF group (86.36%), for all groups matured in the presence of control FF with or without antioxidant supplementation (CFF [83.52%, $P = .2817$], C + NAC [80.95%, $P = .1641$], C + LC [90.22%, $P = .5924$], C + 2Ao [81.03%, $P = .4482$]) and for the E + LC group (80.61%, $P = .0939$; Table 1).

Among the groups supplemented with FF from infertile women with ME, the EFF group was found to have a lower percentage of meiotically normal MII oocytes than the remaining 8 groups (EFF vs No-FF: $P < .0001$; EFF vs CFF: $P < .0001$; EFF vs C + NAC: $P < .0001$; EFF vs C + LC: $P < .0001$; EFF vs C + 2Ao: $P < .0001$; EFF vs E + NAC: $P = .0089$; EFF vs E + LC: $P < .0001$; EFF vs E + 2Ao: $P = .0143$). The percentage of meiotically normal MII oocytes was significantly lower in the EFF (51.4%), E + NAC (62.2%), and E + 2Ao (61.4%) groups than in the No-FF group, and all groups matured in the presence of FF from control patients. However, the percentage of meiotically normal MII oocytes in the E + LC group (80.6%) was similar to that of the No-FF group and to that of all groups matured in the presence of FF from control patients and was higher than that of the E + NAC (62.2%, $P < .0001$) and E + 2Ao (61.4%, $P < .0001$) groups. The E + NAC (61.40%) and E + 2Ao (61.40%) groups exhibited a similar percentage of meiotically normal MII oocytes ($P = .7955$), but

Table 1. Stages of Nuclear Maturation and Percentage of Normal MII Oocytes Matured in Medium Without Addition of Follicular Fluid (FF; No-FF), With the Addition of 1% FF From Infertile Patients Without Endometriosis (CFF), or With Mild Endometriosis (EFF), Supplemented With 1.5 mmol/L N-acetyl-cysteine (NAC; C + NAC and E + NAC), 0.6 mg/mL L-carnitine (LC; C + LC and E + LC), or With Both Antioxidants (C + 2Ao and E + 2Ao) and Visualized By Confocal Microscopy.[a]

	Total Number of Fixed Oocytes, n	Total Number of Viewed Oocytes, n	MI, n (%)	TI, n (%)	PA, n (%)	MII		
						Total no. of MII, n (%)	Analyzable, n (%)	Normal, n (%)
No-FF	208	200	11 (5.50)	0 (0.00)	7 (3.50)	182 (91.00)	110 (60.44)	95 (86.36)a
CFF	204	193	15 (7.77)	0 (0.00)	6 (3.11)	172 (89.12)	91 (52.91)	76 (83.52)a
C + NAC	212	200	19 (9.50)	3 (1.50)	7 (3.50)	171 (85.50)	105 (61.40)	85 (80.95)a
C + LC	205	176	14 (7.95)	2 (1.14)	2 (1.13)	158 (89.77)	92 (58.23)	83 (90.22)a
C + 2Ao	110	105	12 (11.43)	0 (0.00)	1 (0.95)	92 (87.62)	58 (63.04)	47 (81.03)a
EFF	208	189	12 (6.35)	4 (2.12)	1 (0.53)	172 (91.01)	111 (64.53)	57 (51.35)b
E + NAC	206	202	13 (6.44)	2 (0.99)	7 (3.47)	180 (89.11)	90 (50.00)	56 (62.22)c
E + LC	209	195	12 (6.15)	3 (1.54)	3 (1.54)	177 (90.77)	98 (55.37)	79 (80.61)a
E + 2Ao	120	112	12 (10.71)	0 (0.00)	4 (3.57)	96 (85.71)	57 (59.38)	35 (61.40)c

Abbreviations: MI, metaphase I; MII, metaphase II; PA, spontaneous parthenogenetic activation (extrusion of the second polar body without the occurrence of fertilization); TI, telophase I.

[a]Analyzable: oocytes with the spindle fixed in a lateral or sagittal view. Data are the results of 9 replicates. Different letters in the same column indicate a significant difference, $P < .05$, as determined by Gamma distribution and the χ^2 test.

a significantly higher percentage than that of the EFF group and a lower percentage than that of the remaining groups ($P \leq .01$; Table 1).

In the groups supplemented with FF from infertile women without endometriosis (CFF), the addition of antioxidants did not change the percentage of normal MII oocytes (CFF vs C + NAC: $P = .7526$; CFF vs C + LC: $P = .1071$, and CFF vs C + 2Ao: $P = .9038$).

Discussion

An increasing number of studies have demonstrated a possible relationship between endometriosis and OS.[31,48-50] In the present study, we investigated the use of antioxidants (LC and NAC) for the prevention of damage to the meiotic oocyte spindle induced by the FF from infertile women with ME during IVM.

When determining the percentage of MI, TI, and MII oocytes in the various groups, we demonstrated that the addition of FF from infertile women (with or without ME) did not change the rates of nuclear oocyte maturation compared to the group without FF. We expected to detect a higher percentage of MI or TI oocytes in EFF groups than for No-FF groups and CFF patients because a previous study by our group reported a greater tendency of oocytes of infertile women with endometriosis to be in TI.[11] However, this study did not separately analyze oocytes from women in the early and advanced stages of endometriosis, and the case number analyzed was very small, impairing extrapolation of the findings. Another study by our group involving an experiment similar to the present one and the same FF samples demonstrated that the addition of FF from infertile women with ME increased the percentage of MI oocytes (10.8%) compared to the group with the addition of FF from control patients (6.2%).[10] However, we wonder whether the 4.6% difference in the percentage of MI oocytes,[10] although statistically significant, is not clinically relevant. Thus, we believe that FF from patients with ME does not lead to a compromised conclusion of meiosis, and so infertile women with ME potentially exhibit the same percentage of MII oocytes, as demonstrated in the present study.

The addition of the antioxidants NAC and/or LC to the groups matured in vitro in the presence of FF also did not change the rates of nuclear oocyte maturation as demonstrated by the similar percentage of MI, TI, and MII oocytes among the various groups. In vivo, free radicals are generated as secondary products of normal cell metabolism and act as signaling molecules in various physiological and pathological processes and antioxidants are responsible for the clearance of these reactive species.[51,52] Based on a study of the literature, we carefully chose the doses of NAC and LC used in order not to hamper nuclear maturation. Our findings agree with studies using bovine and porcine models.[53-55]

Our data show that neither addition of FF nor addition of antioxidants alters types of abnormalities in MII oocytes. The meiotic spindle is an extremely vulnerable structure, and spindle abnormalities, generally, are closely related to chromosomal misalignment, as evidenced by Da Broi et al who reported that among all meiotic abnormalities, approximately, 50% represents defects in both spindle and chromosomes.[10] Recent study reported that adequate spindle assembly is dependent on guanosine triphosphatase Ran and mediated by its own chromosomes, and error in any point of this mechanism can compromise all meiosis.[56] Therefore, it is not necessary to discriminate the types of meiotic error, and we suggest that these 3 types of meiotic abnormalities be classified, in general, as oocyte abnormality.

Among the analyzable MII oocytes, the percentage of meiotic normality was similar for the No-FF group and the groups matured in the presence of FF from women without endometriosis, with or without antioxidant supplementation. We expected these findings because the control group chosen (infertility exclusively due to tubal factor without hydrosalpinx

or active pelvic inflammatory disease, and/or to male factor) would not exhibit, a priori, OS and/or worse gamete quality as the mechanisms of infertility. In this respect, when establishing the eligibility of the research participants, we carefully excluded other factors related to OS (obesity, advanced age, infectious and rheumatologic diseases, dyslipidemia, and use of medications and of vitamin supplements of any nature) and to a worse gamete quality (advanced age, evidence of compromised functional ovarian research, and obesity) to increase the internal validity of the findings. On this basis, the addition of the antioxidants NAC and/or LC to the IVM medium in the groups matured in the presence of FF from control patients, in which the presence of follicular OS was not expected, did not change the percentage of normal MII oocytes.

Otherwise, the percentage of meiotic normality was significantly lower in the oocytes submitted to IVM in the presence of FF from patients with ME than in the groups matured in the presence of FF from women without the disease and with the absence of FF. These data corroborate previous findings by our group,[10] confirming that the FF from infertile women with ME promotes meiotic oocyte anomalies. Oxidative stress damages proteins,[57,58] and the damage involves the microtubules and small molecules that are important for the organization of the cell spindle. Oxidative stress also directly damages DNA and induces chromosome misalignment.[59,60] Thus, we suggest that this may be one of the potential mechanisms of infertility related to ME. The original finding that the addition of the antioxidants NAC and LC to the IVM medium supplemented with FF from patients with ME reduces (NAC or NAC+LC) or fully prevents (LC) the meiotic oocyte damage induced by FF from patients with ME suggests that OS may be a mediator involved in this process. Although some studies have demonstrated the presence of OS in the follicular microenvironment of women with endometriosis, characterized by an increase in lipid peroxidation products and iron,[33,61] a decrease in antioxidant substances such as vitamin C and glutathione reductase and a decrease in total antioxidant capacity,[32,33] no study evaluated whether women with ME have OS in their follicular microenvironment and our findings suggests its presence.

The addition of antioxidants to the IVM medium supplemented with FF from patients with ME was beneficial in improving the percentage of normal MII oocytes. However, a considerably interesting finding was that LC was superior to NAC and completely reversed the meiotic oocyte damage provoked by FF from patients with ME. Also interesting is the fact that supplementation with NAC plus LC had an impact similar to that of NAC alone and was less effective than the impact of LC supplementation alone. An explanation for this finding is that an excessive amount of antioxidants may have been present in the IVM medium, possibly compromising in part the quality of the gamete.[53] In addition, both antioxidants, NAC and LC, are hydrophilic molecules whose antioxidant property is conferred by a reactive portion (O•) that is able to bind to free radicals, stabilizing them and preventing them from attacking other cell components.[35,37] Therefore, another possible explanation for the low effectiveness of the concomitant use of these 2 antioxidants is that a binding may occur between the reactive portions of each molecule when these substances are solubilized in aqueous medium, potentially reducing the effect of NAC and LC on the clearance of free radicals. Thus, we ask whether the concomitant use of a hydrophilic antioxidant and a hydrophobic one might potentiate the prevention of oocyte damage provoked by the FF from patients with ME, a possibility that should be investigated in subsequent studies.

Although the methodology used in the present study does not permit identification of the reasons explaining the superiority of LC over NAC, we believe that this is because LC, in addition to acting on the clearance of reactive species, is involved in fatty acid metabolism and in appropriate mitochondrial function. The increased free fatty acid levels in FF are related to a reduced morphological quality of human COCs,[62] and the lipid composition of FF is a predictor of embryonic cleavage.[63] An experiment using a murine model demonstrated that oocyte incubation with lipid-rich FF during IVM impairs oocyte development.[64] Additionally, and interestingly, FF carnitine levels seem to be related to the number of oocytes and embryos obtained, and carnitine supplementation is suggested for patients undergoing IVF.[65] We suggest that the FF from patients with ME, in addition to containing altered OS marker levels, might exhibit changes in lipid composition that might impair the oocyte, and LC might favor the energy utilization of these fatty acids by the mitochondria or prevent damage due to these changes. However, there are no studies in which fatty acid composition and FF carnitine levels from patients with ME were correlated with IVF results.

The present study made an original contribution to the elucidation of the pathogenesis of infertility related to ME. However, the study had some limitations. We used the FF from women undergoing ovarian stimulation, a fact that makes it questionable to extrapolate the results to natural cycles. However, the control group underwent the same protocol of pituitary blockade and ovarian stimulation, and their FF did not promote meiotic oocyte anomalies, and supplementation with antioxidants did not modify the percentage of meiotic normality compared to what was observed in the group with ME. We used the FF of only the first aspirated follicle of each patient because we do not know whether prolonged anesthesia and repeated ovarian punctures would promote follicular OS. However, we cannot state that FFs of different follicles would have the same impact on the oocyte meiotic spindle during IVM. Due to the rigorous criteria for the selection of FF donors, the sample size of the present study was small (11 cases and 11 controls) and therefore, new investigations using a larger patient cohort are needed to confirm these results. In addition, the data obtained from studies on animal models cannot necessarily be extrapolated to human beings, and studies on oocytes from infertile women with ME matured in vivo would be important to confirm the present results.

In conclusion, we confirmed that the FF from infertile women with ME promotes meiotic oocyte anomalies during IVM, a mechanism that might therefore be involved in the reduced fertility of these patients. We demonstrated that the

addition of NAC during IVM reduces the deleterious effects of FF on the oocyte meiotic spindle and that LC fully prevents this deleterious effect. Thus, we suggest that the deleterious effect of FF from infertile women with ME may be due not only to OS but also to a faulty energy metabolism of fatty acids and/or to the mitochondrial dysfunction detected in the oocytes and cumulus oophorus cells of these women. On this basis, the present findings open perspectives for the study of the impact of oral LC supplementation to improve the natural fertility of infertile women with ME. This would have important implications for clinical practice because access to IVF is limited even in developed countries.

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