L-carnitine supplementation reduces oocyte cytoskeleton damage and embryo apoptosis induced by incubation in peritoneal fluid from patients with endometriosis

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Objective: To investigate the protective effect of L-carnitine (LC) against deleterious substances present in the peritoneal fluid (PF) of patients with endometriosis, which may affect the oocyte cytoskeleton and embryogenesis.

Design: Experimental study.

Setting: Research embryology laboratory at an academic hospital.

Patient(s): Frozen metaphase II mouse oocytes and embryos.

Intervention(s): One hundred metaphase II mouse oocytes were divided into five groups and incubated: PF from endometriosis patients; PF from endometriosis patients + LC; PF from tubal ligation patients (patient control); LC only; and human tubal fluid (HTF) alone. A total of 180 eight-cell mouse embryos were divided into: endometriosis only; tubal ligation only; endometriosis + LC; LC alone; and HTF alone.

Main Outcome Measure(s): Protective effect of LC on oocytes and embryos.

Result(s): Incubation of the oocytes and the embryos with PF from patients with endometriosis statistically significantly damaged the oocyte microtubules and chromosomes and increased embryo apoptosis compared with controls. Incubation with LC (0.6mg/mL) statistically significantly improved microtubule and chromosome structure and decreased the level of embryo apoptosis.

Conclusion(s): We propose the use of LC as a supplement in patients with endometriosis, a novel approach that may help improve in vitro fertilization outcome in these patients. (Fertil Steril 2009;91:2079–86. ©2009 by American Society for Reproductive Medicine.)

Key Words: L-carnitine, antioxidant, endometriosis, microtubules, chromosomes, oocytes, embryo

Endometriosis is diagnosed whenever endometrial tissue is found outside the uterus. It is a common gynecologic disorder that affects approximately 14% of all women, 30% to 50% of infertile women, and 84% of women with infertility and pelvic pain (1, 2). Surgically induced endometriosis in rats results in decreased fecundity (3), and intraperitoneal injections of peritoneal fluid (PF) from women with endometriosis into mice lowers the implantation rate (4). Moderate or severe endometriosis causes infertility due to mechanical disruption of ovulation or efficient gamete transport (5). Even in its mildest forms, endometriosis may contribute to infertility (6).

The PF microenvironment has an important role in the pathogenesis and progression of endometriosis; the increased number and activation of peritoneal macrophages and increased concentrations of inflammatory cytokines such as interleukin-6 (IL-6), IL-1, and tumor necrosis factor-α (TNF-α) lead to increased vascularization and mitotic activity and stimulate the growth of ectopic endometrium (7–10).

Another pathophysiologic cause of endometriosis-associated infertility is the presence of high levels of reactive oxygen species (ROS) in the PF. Oxidative stress caused by ROS may play a role in the development and progression of endometriosis (11). Increased generation of ROS by PF macrophages, with increased lipid peroxidation in patients with endometriosis, has been demonstrated, although other researchers have reported contrary findings (12). Diminished PF antioxidants (13), elevated oxidized lipoproteins (12), lysocephatidyl choline (13), and other markers of lipid peroxidation provide further evidence of oxidative stress in the peritoneal microenvironment of patients with endometriosis (14).

The oocyte spindle is a dynamic structure composed of microtubule bundles that are polar polymers of α-tubulin and β-tubulin heterodimers. The spindle is responsible for oocyte meiotic division. During the second meiotic division,
In a study done by our group, exogenous exposure of metaphase II mouse oocytes to hydrogen peroxide (H$_2$O$_2$) caused damage in spindle structure, as evident by changes in microtubule morphology and alterations in chromosomal alignment. Significantly increased damage was seen with increased duration of incubation. Higher damage also was seen after exposure to both TNF-α alone and in combination with H$_2$O$_2$ compared with controls. Moreover, oocytes incubated with H$_2$O$_2$ and vitamin C as an antioxidant demonstrated less damage compared with those incubated with H$_2$O$_2$ alone (16).

L-carnitine (LC) is a small, water-soluble molecule that plays a very important role in fat metabolism. It is essential for the normal mitochondrial oxidation of fatty acids and excretion of acyl-CoA esters and affects adenosine triphosphate (ATP) levels (17). L-carnitine can stabilize mitochondrial membranes, increase the supply of energy to the organelle, and protect the cell from apoptotic death (18). Reduction of apoptosis through the mitochondrial pathway by the administration of LC to mouse fibroblasts in culture media has been demonstrated (18). In a recent study done by our group, we reported that supplementation of the culture media with LC (0.6 mg/mL) statistically significantly reduced the apoptosis level in mouse embryos treated with actinomycin-D (apoptosis-inducing factor). Supplementation of the culture media with LC at 0.6 mg/mL antagonized the oxidative effect of a very high concentration of H$_2$O$_2$ (500 mM). In addition, LC was able to neutralize the antiproliferative effect of TNF-α and significantly reduced the level of DNA damage in embryos (19).

Our study objective was to investigate the protective effect of LC against the toxic effects (cytokines or oxidative stress) of PF in patients with endometriosis and subsequently to examine oocyte cytoskeleton changes using metaphase II mouse oocytes or early embryo development using eight-cell mouse embryos. Antagonizing these deleterious factors may help improve oocyte and embryo quality, thereby improving in vitro fertilization (IVF) outcomes in patients with endometriosis.

MATERIALS AND METHODS
The study was approved by the Cleveland Clinic institutional review board.

Patients
The study included a cohort of 38 female patients who underwent laparoscopy at the Cleveland Clinic from March 2006 to March 2007. Patients were classified into two groups, those with endometriosis (n = 23) and a control group consisting of patients with tubal ligation or tubal ligation reversal (n = 15). The indications for laparoscopy were chronic pelvic pain, infertility or both, tubal ligation, or sterilization reversal. All patients included in the study had no significant comorbidities except for the primary indication of surgery. After obtaining informed consent, intraoperative peritoneal samples were collected. Of the 43 women who contributed their PF, five were excluded because of blood-contaminated PF. All patients included had general anesthesia using the same approach.

Peritoneal Fluid Preparation
During laparoscopy, PF was collected from the posterior cul-de-sac. Peritoneal fluid cellular constituents were removed by centrifugation at 600 × g for 5 minutes. The supernatant was then collected and stored at −70°C. All samples were collected under identical conditions.

Mouse Oocyte Preparation
Cryopreserved metaphase II mouse oocytes were obtained from Embryotech Laboratories, Inc. (Wilmington, MA). For thawing, each straw was removed from its liquid nitrogen container and placed at room temperature for 2 minutes. The straw was cut, and the oocytes were released into a Petri dish containing 500 μL of Dulbecco’s phosphate buffer saline (PBS; Irvine Scientific, Santa Ana, CA) and kept for 3 minutes. Subsequently, they were transferred into another Petri dish containing 500 μL of PBS to equilibrate for 10 minutes at room temperature. Oocytes were then incubated in 500 μL of human tubal fluid (HTF; Irvine Scientific) for 1 hour in 5% CO$_2$ at 37°C to ensure complete repolymerization of the microtubules before transfer into the PF (16).

L-Carnitine Preparation
In our recent study conducted on mouse embryos (19), we demonstrated that LC at 0.6 mg/mL was able to reduce apoptosis, act as an antioxidant, and antagonize the antiproliferative effect of TNF-α. The LC was diluted 1:1 with either PF from patients with endometriosis or with HTF to give an LC concentration of 0.6 mg/mL.

Experiment 1: Effect of Incubation of Oocytes with the Peritoneal Fluid on Spindle Structure
A total of 100 metaphase II oocytes were randomly divided into five groups: PF from endometriosis patients; PF from endometriosis patients + LC; PF from tubal ligation patients (patient control); LC only; and HTF only. Oocytes were evaluated under the microscope for maturity (presence or absence of the polar body) and incubated in HTF for 1 hour for complete repolymerization. Peritoneal fluid was diluted with HTF media (1:1).

Microtubules and chromosomal staining Microtubules were detected by modified indirect immunocytochemical techniques as reported in our earlier study. For microtubule staining, oocytes were placed in fixation solution (2% formaldehyde; 0.2% Triton X-100) in 500 mL of PBS for 30 minutes and then incubated in anti-α-tubulin monoclonal antibody (Sigma-Aldrich, St. Louis, MO) (1:300) for 60 minutes, followed by incubation in fluorescein isothiocyanate (FITC)–labeled anti-mouse antibody (Sigma-Aldrich) (1:50) for 30 minutes. For chromosome staining, oocytes were incubated in 10 μg/mL of propidium iodide (Sigma-Aldrich) for 15 minutes (16, 20).
Each staining step was followed by a minimum of three rinses in PBS. Five oocytes were loaded on a slide in a microdrop (2 μL) of Antifade (Slow Fade Light Antifade; Molecular Probes, Inc., Eugene, OR) as anti-bleach and were covered with a cover slip. Alterations in the microtubule structure were observed both by epifluorescence microscopy using the blue filter (excitation 450–490, suppression 515) for microtubules and green filter (excitation 546, suppression 580) for chromosomal alignment. Slides were stored at −20°C in the dark until they were evaluated for more details using confocal microscopy.

Confocal microscopic analysis and scoring of microtubules and chromosomes Slides were examined using a laser-scanning confocal microscope Leica TCS-SP2 (Leica Lasertechnik, GmbH, Heidelberg, Germany) equipped with an argon ion laser for the excitation of FITC for microtubules and propidium iodide for chromosome (excitation 488 nm, barrier 500–555 nm for FITC; excitation 568 nm, barrier 575–675 nm for propidium iodide). Microtubule distribution and chromosome alignment for each oocyte were examined.

Scoring of microtubules and chromosomes according to the morphologic evaluation was done by the method described earlier elsewhere by our group (16). In brief, spindle morphology was classified as normal (scores 1, 2) when a barrel-shaped structure with slightly pointed poles was formed by organized microtubules; score 3 when we found a reduction in the longitudinal dimension of the spindle; or score 4 when there was partial or total disorganization, complete absence, or remnant of dispersing spindle (16). Chromosomal configuration was regarded as normal (scores 1, 2) when chromosomes were arranged in a compact metaphase plate at the equator of the spindle. Chromosomal organization was regarded as abnormal (scores 3, 4) when chromosomes were displaced from the plane of the metaphase plate or when the chromosomes were dispersed or appeared condensed.

Experiment 2: Effect of Incubation of Mouse Embryos with Peritoneal Fluid and L-Carnitine on Apoptosis

A total of 180 eight-cell embryos were divided into endometriosis only; tubal ligation only; endometriosis and HTF alone. They were incubated in a 37°C CO2 incubator for 24 hours. Embryos were fixed in formaldehyde 3.7%. The DNA damage of the embryos was estimated by the terminal deoxynucleotidyl transferase (TdT) -mediated dUTP nick-end labeling (TUNEL) assay.

Measurement of apoptosis in embryos Individual embryos were stained with the TUNEL technique (in situ cell death detection system; Roche Diagnostic Corporation, Indianapolis, IN). After we had washed the embryos in PBS, they were fixed in 3.7% paraformaldehyde in PBS (pH 7.4) for 1 hour at room temperature. Embryos were washed at least three times in PBS containing 0.3% polyvinylpyrrolidone (PBS/PVP) and permeabilized in 0.5% Triton X-100 on ice for 2 minutes. The embryos were then washed three times in PBS/PVP and incubated in TUNEL reaction cocktail at 37°C for 1 hour in the dark. Negative controls consisted of embryos incubated without terminal transferase enzyme. The embryos were washed extensively and mounted with slight cover slip compression in Vectashield with 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) antibleaching solution (Vector Labs, Burlingame, CA). The slides were sealed with clear nail polish and stored at −20°C in the dark for analysis by confocal microscope. The TUNEL positive (apoptotic) nuclei (cell) appeared as green, and the normal chromatin appeared as blue.

Confocal microscopy for apoptosis Images were collected with a Leica TCS-SP2 laser scanning spectral confocal microscope (Leica Lasertechnik, GmbH, Heidelberg, Germany). The specimen was excited at 364 nm (ultraviolet) for DAPI and at 488 nm for visualization of TUNEL staining. Images were collected sequentially at each level of the specimen to prevent crosstalk of the fluorophores and then were collected along the z-axis of the sample with a step size of 1 to 3 mm. Each optical section of the blastocyst was analyzed for TUNEL-negative nuclei stained with DAPI and TUNEL-positive nuclei stained with DAPI and TUNEL.

The percentage of apoptotic cells in each embryo was calculated by using the projection of the three-dimensional stack of images that was created with the Leica software. The original stack of embryo images was transferred to Velocity software (Improvision, Lexington, MA) for analysis and for counting of both damaged and intact individual blastomere DNA. This software allows penetration of the scanned embryo to observe the TUNEL staining and verify whether each blastomere is independently stained. The apoptotic blastomeres in each embryo were counted, and the level of apoptosis in each group was calculated.

Differences between samples and controls were assessed using the Kruskal-Wallis test for overall group comparisons and the Wilcoxon rank sum test for pairwise comparisons. Summary statistics are presented as frequency and percent for categorical data and as median and interquartile range (25th and 75th percentile) for quantitative data. All hypothesis testing was two-tailed. P < .05 was considered statistically significant. Statistical analyses were performed using R version 2.3.1 (http://www.R-project.org).

RESULTS

The results on the effect of PF and LC on oocyte spindle and embryo apoptosis are shown in Tables 1 to 3 and Figures 1 and 2.

Experiment 1: Effect of Incubation of Oocytes with the Peritoneal Fluid on Spindle Structure

Our study showed that incubation of the oocytes in LC at a concentration of 0.6 mg/mL did not cause a statistically significant effect on the oocyte microtubules or chromosome scores, and these were similar to tubal ligation controls. Both microtubule and chromosome scores were statistically significantly decreased (>2) in oocytes incubated with PF from patients with endometriosis compared with the oocytes.
incubated in PF from patients with tubal ligation alone ($P<.001$ and $P=.006$) (see Tables 1 and 2).

Statistically significant improvement in the microtubule score was seen when 0.6 mg/mL of LC was added to the PF from endometriosis patients ($P=.009$). Oocytes incubated in PF from patients with endometriosis plus LC demonstrated a decrease in chromosomal changes ($P=.06$). Generally, there was a decrease in chromosomal changes between patients with endometriosis and other patient groups (overall $P=.032$; $.003 \leq P \leq .06$ for endometriosis vs. each of the other groups) (see Tables 1 and 2; Fig. 1).

**Experiment 2: Effect of Incubation of Mouse Embryos with Peritoneal Fluid and L-Carnitine on Apoptosis**

Our study showed that incubation of the embryos with PF from patients with endometriosis statistically significantly increased the level of apoptosis compared with the tubal ligation group control ($P<.001$). The extent of apoptosis was comparable in embryos incubated in LC (0.6 mg/mL) and tubal ligation controls. Statistically significant improvement in the level of apoptosis was seen when LC (0.6 mg/mL) was added to the PF from endometriosis patients ($P<.001$) (see Table 3; Fig. 2).

**DISCUSSION**

Reactive oxygen species are involved in the etiopathogenesis of defective embryo development (21) and retardation of embryo growth (5). An increase in ROS production leads to arrest of embryo development. Reactive oxygen species may originate in the embryo or from extraneous factors. In the in vitro fertilization (IVF) setting, strategies to reduce ROS production, such as addition of free radical scavengers and lowering the oxygen tension, are important for improving the fertility potential in assisted reproduction (22). Reactive oxygen species induce cell membrane damage, DNA damage, and apoptosis. Apoptosis results in fragmented embryos, which have limited potential to implant and hence result in poor fertility outcomes (23).

In a study evaluating the possible beneficial effects of LC on tissue injury and oxidative stress in acetic acid-induced colitis in rats, results showed that acetic acid administration significantly decreased reduced glutathione, superoxide dismutase, and catalase levels in colonic homogenate. Supplementation of LC prevented the depletion of free radical scavengers such as reduced glutathione levels and caused a statistically significant increase in superoxide dismutase levels (24).

L-carnitine was reported to down-regulate cytokines such as IL-1, IL-6, and TNF-α and/or increase clearance of these cytokines in rats implanted subcutaneously with sarcoma tumor. A statistically significant reduction in the concentrations of the cytokines (IL-1β: 423 ± 33 vs. 221 ± 60; IL-6: 222 ± 18 vs. 139 ± 38; TNF-α: 617 ± 69 vs. 280 ± 77 pg/mL; $P \leq .05$) was seen in the sarcomas groups that were untreated versus those treated with LC (25).

Recently, we demonstrated the possible role of LC in improving the outcome of IVF. We reported that incubation
of two-cell mouse embryos with 500 ng/mL of TNF-α statistically significantly decreased the blastocyst development rate (%BDR) compared with the control group (<.001). L-carnitine at 0.6 mg/mL was able to neutralize the anti-proliferative effect of TNF-α and improve the %BDR. Incubation of mouse embryos in 500 mmol/L H₂O₂ alone statistically significantly decreased the %BDR and increased the level of apoptosis (<.001 and <.01) compared with the control group. L-carnitine exhibited strong antioxidant effects as it was able to antagonize a very high concentration of H₂O₂ (up to 500 mmol/L). At 0.6 mg/mL, LC statistically significantly improved the %BDR (<.001) and decreased the level of apoptosis (<.007) compared with the group treated with H₂O₂ alone (500 mmol/L) (19).

Endometriosis is characterized by changes in the intrafollicular and PF environment and increased levels of certain cytokines such as TNF-α and ROS, which affect oocytes

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Chromosome scores after incubation of oocytes with peritoneal fluid (PF) from endometriosis, tubal ligation (control), L-carnitine (LC) alone, carnitine supplementation, and the human tubal fluid (HTF) control group.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Score 1</td>
</tr>
<tr>
<td>Endometriosis PF, group 1</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>Tubal ligation PF, (patient control), group 2</td>
<td>5 (25%)</td>
</tr>
<tr>
<td>Endometriosis PF + LC (0.6 mg/mL), group 3</td>
<td>6 (30%)</td>
</tr>
<tr>
<td>LC alone (0.6mg/mL), group 4</td>
<td>4 (20%)</td>
</tr>
<tr>
<td>HTF group, group 5</td>
<td>5 (25%)</td>
</tr>
</tbody>
</table>

Notes: Each group consisted of 20 oocytes. Overall P=.032, using Kruskal-Wallis pairwise test. P<.05 was considered statistically significant.

*P= .006 for group 1 vs. 2.
*P= .06 for group 1 vs. 3.
*P= .052 for group 1 vs. 4.
*P= .003 for group 1 vs. 5.
*P= .65 for group 2 vs. 3.
*P= .39 for group 2 vs. 4.
*P= .95 for group 2 vs. 5.
*P= .79 for group 3 vs. 4.
*P= .58 for group 3 vs. 5.
*P= .34 for group 4 vs. 5.


<table>
<thead>
<tr>
<th>TABLE 3</th>
<th>Level of apoptosis after incubation of mouse embryos with human tubal fluid (HTF), peritoneal fluid (PF) from endometriosis, tubal ligation (control), L-carnitine (LC) alone, and carnitine supplementation.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Apoptosis (%) Median (25th and 75th percentiles)</td>
</tr>
<tr>
<td>Endometriosis PF (n = 40)</td>
<td>39.1 (30.3, 46)</td>
</tr>
<tr>
<td>Tubal ligation (patient control) (n = 40)</td>
<td>6.7 (3, 10)</td>
</tr>
<tr>
<td>Endometriosis PF + LC (0.6 mg/mL) (n = 40)</td>
<td>6.2 (3.3, 10)</td>
</tr>
<tr>
<td>LC alone (0.6 mg/mL) (n = 40)</td>
<td>3.5 (0, 8.2)</td>
</tr>
<tr>
<td>HTF (n = 20)</td>
<td>3.9 (0, 6.9)</td>
</tr>
</tbody>
</table>

*P<.05 was considered statistically significant for apoptosis level using Kruskal-Wallis or Wilcoxon rank-sum test compared with the control group.

*P<.05 was considered statistically significant for differences in apoptosis level using Kruskal-Wallis or Wilcoxon rank-sum test compared with the endometriosis group.

and embryo development. In our earlier study, we investigated the effect of oxidative stress on metaphase II mouse oocyte spindle structure. Significant alterations in microtubule chromosomal alignment were reported after exposure to high concentration of H₂O₂. Exposure of the oocytes to both TNF-α alone and in combination with H₂O₂ resulted in a concentration-dependent and time-dependent increase in spindle compared with controls (16).

These results are in agreement with our present findings where we have demonstrated that incubation of oocytes with PF from patients with endometriosis statistically significantly increased the microtubule and chromosome scores compared with the oocyte group incubated in PF from patients with tubal ligation (\(P<.001\) and \(P=.006\), respectively). We attribute this to the high concentration of ROS (26) and TNF-α or other cytokines that have been reported to be higher in patients with endometriosis (27). Statistically significant improvement in the microtubule score was seen when 0.6 mg/mL of LC was added to the peritoneal fluid from endometriosis patients (\(P=.009\)). Oocytes incubated in peritoneal fluid from patients with endometriosis and LC demonstrated a decrease in chromosomal changes (\(P=.06\)). Scale of magnification bar = 10 μm.

**FIGURE 1**

Effect of incubating metaphase II mouse oocytes with peritoneal fluid from tubal ligation patients as patient control, L-carnitine (LC) alone, and peritoneal fluid from endometriosis patients with and without LC. (A) Mouse oocyte incubated with tubal ligation patients. (B) Mouse oocytes incubated in LC alone. (C) Mouse oocyte incubated in peritoneal fluid from endometriosis patients. Both microtubule and chromosome scores were statistically significantly decreased (>2) in oocytes incubated with peritoneal fluid from patients with endometriosis compared with the oocytes incubated in peritoneal fluid from patients with tubal ligation alone (\(P<.001\) and \(P=.006\), respectively). (D) Mouse oocyte incubated in peritoneal fluid from endometriosis patients and supplemented with 0.6 mg/mL of LC. Statistically significant improvement in the microtubule score was seen when 0.6 mg/mL of LC was added to the peritoneal fluid from endometriosis patients (\(P=.009\)). Oocytes incubated in peritoneal fluid from patients with endometriosis and LC demonstrated a decrease in chromosomal changes (\(P=.06\)). Scale of magnification bar = 10 μm.
endometriosis and LC demonstrated a decrease in chromosomal alignment ($P = .06$). This decrease was statistically significant for the overall endometriosis group versus all other groups ($P = .032$). The improvement in microtubule and chromosome alignment after addition of LC may be due to the strong antioxidant properties of LC. Additionally, it may be the result of the down-regulation of the cytokines that are known to be present in the PF of endometriosis patients.

The oocyte spindle is responsible for oocyte meiotic division (15). Our results may explain the significant reduction in the oocyte cleavage rate in women with endometriosis compared with controls (28). This could be explained by deleterious effects on the oocyte spindle in patients with endometriosis.

The embryotoxicity of PF from patients with and without endometriosis has been studied before, but the results have been conflicting. Although some investigators have demonstrated that the PF from individuals with endometriosis is not embryotoxic when studied in an in vitro mouse embryo model (29, 30), others have shown that embryotoxicity is increased in women with endometriosis (31). These conflicting results may be due to differences in the PF concentration, severity of the disease, incubation time, or type of culture media (32).

**FIGURE 2**

Effect of incubating eight-cell mouse embryos with peritoneal fluid from tubal ligation patients as patient control, L-carnitine (LC) alone, and peritoneal fluid from endometriosis patients with and without LC. (A) Mouse embryos incubated in peritoneal fluid from tubal ligation patients (control group). (B) Mouse embryos incubated in LC alone. (C) Mouse embryos incubated in peritoneal fluid from endometriosis patients. Incubation of the embryos with peritoneal fluid from patients with endometriosis statistically significantly increased the level of apoptosis compared with the tubal ligation group control ($P = .036$). (D) Mouse embryos incubated in peritoneal fluid from endometriosis patients and supplemented with 0.6 mg/mL of LC. Statistically significant improvement in the level of apoptosis was seen when LC (0.6 mg/mL) was added to the peritoneal fluid from endometriosis patients ($P < .001$). Scale of magnification bar = 10 μm.
Our results showed that incubation of the preimplantation mouse embryos with PF from patients with endometriosis statistically significantly increased the level of apoptosis compared with the controls \((P<.001\). Statistically significant improvement in the apoptosis level was seen after adding 0.6 mg/mL of LC to the PF from endometriosis patients \((P<.001\). We also reported earlier that PF from patients with endometriosis decreases the development of early embryogenesis in mouse embryos but does not increase the level of apoptosis \((22\). Again, this may be due to the difference in concentration of PF in these studies.

Oxygen-controlled incubators have been introduced recently in clinical embryology procedures, and these may help reduce the harmful effects of free radicals and thereby improve blastocyst rates. However, LC works with multiple mechanisms; one of them is by antagonizing ROS formation through its potent antioxidant effect. In addition, LC also can decrease the level of apoptosis in the presence of an apoptotic inducer and decrease the antiproliferative effect induced by the presence of cytokines such as TNF-α \((19\).

In conclusion, improvement in oocyte scores and embryonic developmental competence may be accomplished by addition of LC to the PF from patients with endometriosis. This effect of LC may be due to reduction in the extent of DNA damage or potent antioxidant and anti-TNF-α effects. It would be interesting to examine the association between the severity of endometriosis and the protective effect of supplementing the IVF media with LC. This may result in an improvement in the oocyte spindle structure and, subsequently, improvement in embryo quality. The use of LC as a supplement is a novel approach that can have important clinical applications in the assisted reproduction setting, and its use may improve the fertility outcomes in a category of patients in which the IVF outcome is still unsatisfactory.

Acknowledgment: The authors thank Dr. Judith Drzaiba; director of the Imagining Core Laboratory, Lerner Research Institute, Cleveland Clinic, for her valuable support. Jeff Hammel, M.S., provided statistical assistance.

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