Elevated peritoneal fluid ceramides in human endometriosis-associated infertility and their effects on mouse oocyte maturation

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Objective: To characterize the peritoneal fluid (PF) sphingolipid profile in endometriosis-associated infertility (EAI), and to assess the plausible functional role(s) of ceramides in oocyte maturation potential.
Design: Retrospective case-control study and in vitro mouse oocyte study.
Setting: University-affiliated hospital and university laboratory.
Subjects: Twenty-seven infertile patients diagnosed with endometriosis and 20 infertile patients who did not have endometriosis; BALB/c female mice.
Intervention(s): None.
Main Outcome Measure(s): PF sphingolipid concentrations. Number of metaphase II (MII) mouse oocytes.
Result(s): Liquid chromatography–tandem mass spectrometry revealed 11 significantly elevated PF sphingolipids in infertile women with severe endometriosis compared with infertile women without endometriosis (change $>50\%$, false discovery rate $\leq 10\%$). Logistic regression analysis identified three very-long-chain ceramides potentially associated with EAI. Functional studies revealed that very-long-chain ceramides may compromise or induce murine MII oocyte maturation. The oocyte maturation effects induced by the very long-chain ceramides were triggered by alterations in mitochondrial superoxide production in a concentration-dependent manner. Scavenging of mitochondrial superoxide reversed the maturation effects of $C_{24:0}$ ceramide.
Conclusion(s): EAI is associated with accumulation of PF very-long-chain ceramides. Mouse studies demonstrated how ceramides affect MII oocyte maturation, mediating through mitochondrial superoxide. These results provide an opportunity for direct functional readout of pathophysiology in EAI, and future therapies targeted at this sphingolipid metabolism may be harnessed for improved oocyte maturation. (Fertil Steril® 2018;110:767-77. ©2018 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Endometriosis, infertility, ceramides, oocytes, mitochondria

Received February 14, 2018; revised and accepted May 2, 2018.
Y.H.L. and J.X.Y. should be considered similar in author order.
Y.H.L. has nothing to disclose. J.X.Y. has nothing to disclose. J.C.A. has nothing to disclose. C.S.T. has nothing to disclose. B.S.M.C. has nothing to disclose. T.Y.T. has nothing to disclose. H.H.T. has nothing to disclose. C.N.Z.M. has nothing to disclose. J.K.C. has nothing to disclose.
Supported by a SingHealth Foundation Grant (SHF/FG560P/2014), the National Medicine Research Council (NMRC/BNIG/2033/2015 and NMRC/CG/M003/2017), and the Duke-NUS Academic Clinical Program Grant for Clinical and Translational Research in Endometriosis. J.C. received salary support from the National Medical Research Council (CSA/043/2012).
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Fertility and Sterility® Vol. 110, No. 4, September 2018 0015-0282/$36.00

https://doi.org/10.1016/j.fertnstert.2018.05.003

Infertility is a hallmark complication of endometriosis (1), and as many as one-half of infertile women have evidence of endometriosis (2). A recent genome-wide association study of 1,182 women implicated severity of endometriosis in infertility (3). Factors leading to endometriosis-associated infertility (EAI) are multifactorial, but the causal mechanistic links between endometriosis and infertility remain poorly studied. In the absence of

physical barriers, such as tubal infertility and distorted pelvic anatomy, other factors, namely, poor oocyte quality, impaired implantation due to endometrial dysfunction, and poor ovarian reserve, are linked to EAI (4–10). The peritoneal fluid (PF), which ovaries are exposed to and secondary oocytes are released into during ovulation, is an important environment for proper oocyte development (11–14). Biologic factors in the PF could interact with oocytes and fertilized eggs either in the ovaries, which are in direct contact with PF, or the ampulla, where fertilization typically takes place and which also opens into the peritoneal cavity. Evidence of the PF's detrimental effects in EAI has been shown, wherein PF from endometriotic women hindered oocyte maturation or development (15, 16). Therefore, the constitutive components of PF might reveal important information regarding the defective peritoneal environment in EAI.

Ceramides (Cers) are important second messengers in cell differentiation, proliferation, and apoptosis, and their particular ability to induce apoptosis is clinically important (17). Through an interconnected and highly regulated network of sphingolipid enzymes, Cers form the precursors to a diverse family of other sphingolipids, including sphingomyelins (SMs), ceramide-1-phosphates (C1Ps), and glucosylceramides (GlcCers) (18). The biologic roles of sphingolipids are as numerous as their numbers. For example, Cers are generally involved in apoptosis, senescence, and autophagy, whereas GlcCers are antiapoptotic. Furthermore, structural differences in the sphingolipids appear to elicit differential potencies (19). Cers have been found to be toxic to oocytes and embryos both in vivo and in vitro (20), and the inhibition of acid-sphingomyelinases, the enzyme that hydrolyzes SMs to Cers, blocks apoptosis in oocytes (21). GlcCer synthase, the enzyme that catalyzes the glycosylation of Cers to form GlcCers, is essential for oocyte and embryo membrane formation (22, 23). These studies lay the foundation of demonstrating the importance of sphingolipids in fertility and potentially EAI, but they do not clarify which sphingolipid species are associated with EAI. In the present study, we applied mass spectrometry–based sphingolipidomics to map the PF sphingolipidome and conducted functional studies in mice to identify sphingolipids that are associated with non–male factor EAI to understand the potential mechanisms of sphingolipids in affecting oocyte maturation.

MATERIALS AND METHODS

All chemicals were purchases from Sigma-Aldrich unless otherwise stated. Sphingolipid chemical standards were purchased from Avanti Polar Lipids. Liquid chromatography mass spectrometry (LC-MS)–grade solvents were purchased from Fisher-Scientific. All LC-MS consumables were purchased from Agilent Technologies unless otherwise stated.

Ethics Approval

Women provided written informed consent for collection of samples with approval from the Centralised Institutional Research Board (CIRB D-2010-167). The mouse study was conducted under approval from National University of Singapore International Animal Care and Use Committee [IACUC Research Protocol R15-1160(A)].

Patient Enrollment

Sixty-two patients from the KK Women's and Children's Hospital, Singapore, undergoing laparoscopic procedures for various indications, such as suspected endometriosis, infertility, sterilization procedures, and pelvic pain, were recruited into the study. Exclusion criteria included menstruating patients, postmenopausal patients, patients with poor ovarian reserve defined by FSH >10 IU/L, patients on any form of hormonal therapy for ≥ 3 months before laparoscopy, and other potentially confounding diseases, including diabetes, rheumatoid arthritis, inflammatory bowel disease, and systemic sclerosis. At the point of patient recruitment in this study, prediction of ovarian reserve in our hospital was through measuring FSH concentration. We have since adopted the use of antimüllerian hormone (AMH). A diagnostic laparoscopy was performed on all patients, with careful inspection of the uterus, fallopian tubes, ovaries, pouch of Douglas, and pelvic peritoneum by gynecologists subspecializing in reproductive endocrinology and infertility. PF was collected via aspiration with the use of a Veress needle under direct visualization immediately on introduction of the laparoscope to avoid contamination from blood or distension medium in women undergoing concurrent hysteroscopy, in line with Endometriosis Phenome and Biobanking Harmonisation Project standard operating procedures (24). Where there was presence of tubal infertility as assessed via hysterosalpingogram or laparoscopy and dye hydrotubation test, no indication of attempts of conceiving, or severe male-factor infertility, those patients were excluded from the study. Presence of endometriosis was systematically recorded and scored according to the revised American Fertility Society classification (rAFS) of endometriosis (25, 26). Twenty-seven infertile patients diagnosed as having endometriosis (EM+), and 20 infertile patients who did not have endometriosis or had benign gynecologic presentations, such as uterine fibroids and benign ovarian cysts, were taken as the nonendometriotic group (EM−; Supplemental Fig. 1, available online at www.fertstert.org). Fifteen subjects were excluded because they did not meet our criteria of infertility. Infertile patients were defined as those who have been trying actively to conceive for >1 year, <40 years of age, bilaterally patent fallopian tubes, and reasonable sperm parameters in their male partners (semen volume ≥ 1.5 mL, normal sperm morphology $\geq 1\%$, total sperm count $>30 \times 10^6$ (sperm density $\times$ sperm volume), motility (rapid) $\geq 32\%$) (27, 28). With reference to World Health Organization values (29), which set the lower 5th confidence interval for normal sperm morphology at $>4\%$, we assumed that using $>4\%$ sperm morphology might still be associated with fertile men. Therefore, we opted to be conservative, focusing on endometriosis-contributed factors, rather than the possibility of male-contributed factors, and used 1% as a baseline. Further details on patient characteristics can be found in Supplemental Table 1 (available online at www.fertstert.org). The phase of

the menstrual cycle (proliferative and secretory) was determined by the cycle history of patients.

Sample Preparation

Collected PF was treated with 1% protease inhibitor immediately (Complete; Roche) and spun at 1,000*g* for 10 minutes at 4°C. The supernate was transferred to a fresh 15 mL tube and stored at −80°C. Sphingolipids were extracted with the use of a modified Bligh and Dyer method. Briefly, 900 μL chloroform-methanol (1:2 v/v) was added to 100 μL PF spiked with internal standards. After 20 minutes of vortexing and incubation at 4°C, 300 μL chloroform and 300 μL double-distilled H_2O were added to the mixture. Sphingolipids were removed from the lower organic phase after centrifugation at 9,000 rpm and 4°C for 2 minutes. Subsequently, 500 μL chloroform was added, vortexed at 4°C for 20 minutes, and lipids were removed from the lower organic phase after centrifugation and combined with the previous fraction. One-half of the fraction was sent for phosphatidylcholine hydrolysis for SM analysis with the use of 10% 1 mol/L KOH, incubated at 37°C at 500 rpm for 4 hours before speed-vacuum evaporation and storage at −80°C. Quality control samples were generated by pooling equal volumes (50 μL) of all lipid extracts to assess intra-run analytical variability including retention time drift, mass accuracy, and peak intensity variation (30, 31). The sphingolipid extracts, including quality control samples, were vacuum dried and stored at −80°C. Before mass spectrometry analysis, each sphingolipid extract was reconstituted in 400 μL chloroform-methanol (1:1, v/v). Sphingolipids were analyzed within 2 weeks after extraction.

Mass Spectrometry–Based Sphingolipidomics

Positive ionization–mode liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) in multiple reaction monitoring (MRM) mode via Triple Quadrupole 6460 with Jet Stream (Agilent Technologies) was used for the relative quantification of sphingolipids as previously described with modifications (32). Briefly, C_{18} reversed-phase LC (Zorbax Eclipse 2.1 mm inner diameter × 50 mm length, 1.8 μm particle size; Agilent Technologies) was used to separate reconstituted lipids at 400 μL/min before entering the mass spectrometer. Dwell times were set at 10–50 ms per MRM transition. MS parameters were as follows: gas temperature 250°C, gas flow 10 L/min, sheath gas temperature 350°C, sheath gas flow 11 L/min, capillary voltage 5,000 V, and nozzle voltage 1,500°C. The column thermostat and autosampler temperatures were maintained at 40°C and 6°C, respectively. The mobile phase consisted of 5 mmol/L ammonium acetate in water (mobile phase A) and 5 mmol/L ammonium acetate in methanol (mobile phase B). Sphingolipids were eluted with the use of linear gradients from 60% to 100% B over 2 minutes, maintained at 100% B for 7 minutes, followed by a linear gradient to 5% B over 2 minutes, and held for another 4 minutes before returning to 60% B for 2 minutes for the next injection. Deuterated internal standards corresponding to each sphingolipid class were verified for their absence and used for relative quantification. Owing to the use of quality control samples to assess reproducibility of the LC-ESI-MS/MS analysis, and our evaluation of technical duplicate injections showing low variability (Supplemental Fig. 2, available online at www.fertstert.org), single injections per sample were made. Data acquisition and processing were performed with the use of Mass Hunter software (Agilent Technologies). Quantification of sphingolipids was performed with the use of Agilent Quantitative software (version B.05). Only sphingolipids that were >100 intensity counts (approximately three times the limit of detection of our LC-MS instrument) and were present in >50% of the samples were retained for analysis. Heatmaps (MeV version 4.9.0) were used to plot the normalized sphingolipid expression data by first centering the data to its median, and scaling it by dividing it by the standard deviation.

Mouse Work and Oocyte Harvesting

BALB/c female mice were maintained in a pathogen-free space with controlled temperature and light (24°C, 14 h light/10 h dark cycles) and ad libitum food and water. The mice and were killed by means of cervical dislocation, and their ovaries were removed and punctured with the use of a 30-gauge needle to release the cumulus-enclosed oocytes in oocyte manipulating medium (Minimum Essential Medium alpha + GlutaMAX medium [Gibco, Life Technologie]) supplemented with 25 μmol/L HEPES buffer (Gibco). The germinal vesicle (GV) oocytes were denuded mechanically by repeatedly pipetting through a 70 μm denuding micropipette (Origio; Cooper Surgical).

In Vitro Oocyte Maturation

Seven to nine-week-old adult wild-type female BALB/c mice were used for the assessment of in vitro oocyte maturation. After two washes in oocyte maturation medium (SAGE media; Cooper Surgical), denuded GV oocytes were cultured and matured in in vitro maturation (IVM) media (SAGE media supplemented with 25 mIU/mL pregnant mare's serum gonadotropin [Sigma] and 25 mIU/mL hCG [Pregnyl; MSD]). Oocytes were cultured in 30-μL drops of culture medium, at a maximum 35 oocytes/drop, layered with mineral oil, and matured for 14 hours at 37°C in a humidified atmosphere of 5% CO_2. We and others have shown that the removal of partially intact cumulus cells does not reduce oocyte maturation potential (Supplemental Fig. 3, available online at www.fertstert.org) (33, 34). To determine the effect of Cers on oocyte maturation competency, denuded murine oocytes were matured in IVM with Cers over 14 hours. $C_{24:0}$ Cer and $C_{24:1}$ Cer were dissolved in dodecane:ethanol, which was the vehicle control. Concentrations used were $C_{24:0}$ Cer 0, 50, 100, or 400 ng/mL and $C_{24:1}$ Cer at 0, 30, 60, or 240 ng/mL over 14 hours. The Cer concentrations were derived from the mass spectrometric analysis of the human PF. $C_{22:0}$ Cer was not commercially available for functional testing. After incubation, the oocytes were examined under an inverted microscope (IX70 with DP22 camera; Olympus) for the presence or absence of the GV or the extrusion of the first

polar body (PB1), which are characteristics of oocyte transition from GV to metaphase I (MI) and metaphase II (MII) stages, respectively (35). Oocytes from three female sibling mice were harvested for each independent experiment, mixed, and randomly grouped for each condition. Oocytes with PB1 that had undergone apoptosis, distinguished by the presence of fragments or remnants of the PB1, were considered to be MII oocytes (36).

For the mitochondrial reactive oxygen species (ROS) study, the denuded murine oocytes, matured in the presence of either $C_{24:0}$ or $C_{24:1}$ Cer for 14 hours, were stained live with MitoSOX red mitochondrial superoxide indicator (Molecular Probes; Invitrogen) and MitoTracker deep red FM (Molecular Probes). ImageJ software (version 2.0.0) was used to quantify the fluorescence intensity. The fluorescence intensity of the mitochondrial superoxide of each oocyte were normalized against the respective oocyte's total mitochondrial staining. Each oocyte's value was then normalized against the average ratio of the respective medium control to correct for the differences in staining intensity and imaging in each set of experiment. GV and MI oocytes were grouped together in this analysis and compared with MII oocytes. For the antioxidant study, GV oocytes were incubated in resveratrol (2 μmol/L) diluted in IVM medium for 2 hours. Subsequently, vehicle control, $C_{24:0}$ Cer, or $C_{24:1}$ Cer was added to the oocytes and incubated for a further 12 hours. Mitochondrial staining, imaging, and analysis were carried out as described above.

Statistical Analysis

Statistically significant sphingolipids were determined by applying the Benjamini-Hochberg adjustment to the two-tailed Student *t* test *P* values to control the false discovery rate (FDR) at $\leq$10%. The FDR is the proportion of incorrectly rejected hypotheses among all rejected hypotheses. Multiple logistic regression analysis incorporating forward and stepwise selection and all possible subset algorithms were applied to the subset of significant sphingolipids with the aim of identifying PF sphingolipids associating with EAI. Cumulative receiver operating characteristic (ROC) curves, and areas under the ROC curves (AUCs) were calculated and presented graphically for the predictors selected by the regression analysis. Odds ratios (ORs) and 95% confidence intervals (CIs) for the selected sphingolipid predictors are reported. *P* values $<$.05 were considered to be significant in the logistic regression analyses. In vitro oocyte maturation results were compared with the use of either unpaired two-tailed Student *t* test or Mann-Whitney test. SAS version 9.3 and Graphpad Prism were used to perform all statistical analyses.

RESULTS

Elevated Peritoneal Fluid Sphingolipids in Infertile Women with Severe Endometriosis

Supplemental Table 1 presents the patient characteristics. At the point of recruitment, the mean times for trying to conceive were 4.6 years and 2.9 years for infertile EM+ and EM− women, respectively (P=.24), with 1 year as the minimal duration for trying. Early follicular phase FSH levels were similar between EM+ (6.0 $\pm$ 1.3 IU/L) and EM− (6.1 $\pm$ 1.3 IU/L; P=.75). The mean body mass indexes (BMIs) of EM+ and EM− women were within the healthy BMI range and similar (P=.85). Both groups of patients had similar characteristics regarding menstrual phase (P=.69) and age (P=.43).

With the use of mass spectrometry, we measured sphingolipids of five classes, namely SM, Cer, C1P, monohexosylceramide, and lactosylceramide. Figure 1A shows the different PF sphingolipid profiles associated with patients' disease states. Infertile women without endometriosis (bottom row) had relatively lower sphingolipid levels among the five measured classes compared with infertile women with severe endometriosis ($EM+_{Sev}$; top row). Relative to infertile EM− women, 13 sphingolipids were significantly increased in the infertile $EM+_{Sev}$ group (rAFS III/IV; Fig. 1B). Among the sphingolipids that were significantly changed, an enrichment of Cer (62%, eight Cers) was observed in infertile $EM+_{Sev}$. The largest change was found in $C_{24:1}$ Cer (+172.6%) relative to infertile EM− women. The PF sphingolipid concentrations were similar between infertile $EM+_{Mild}$ and infertile EM− women (P>.05; FDR >10%).

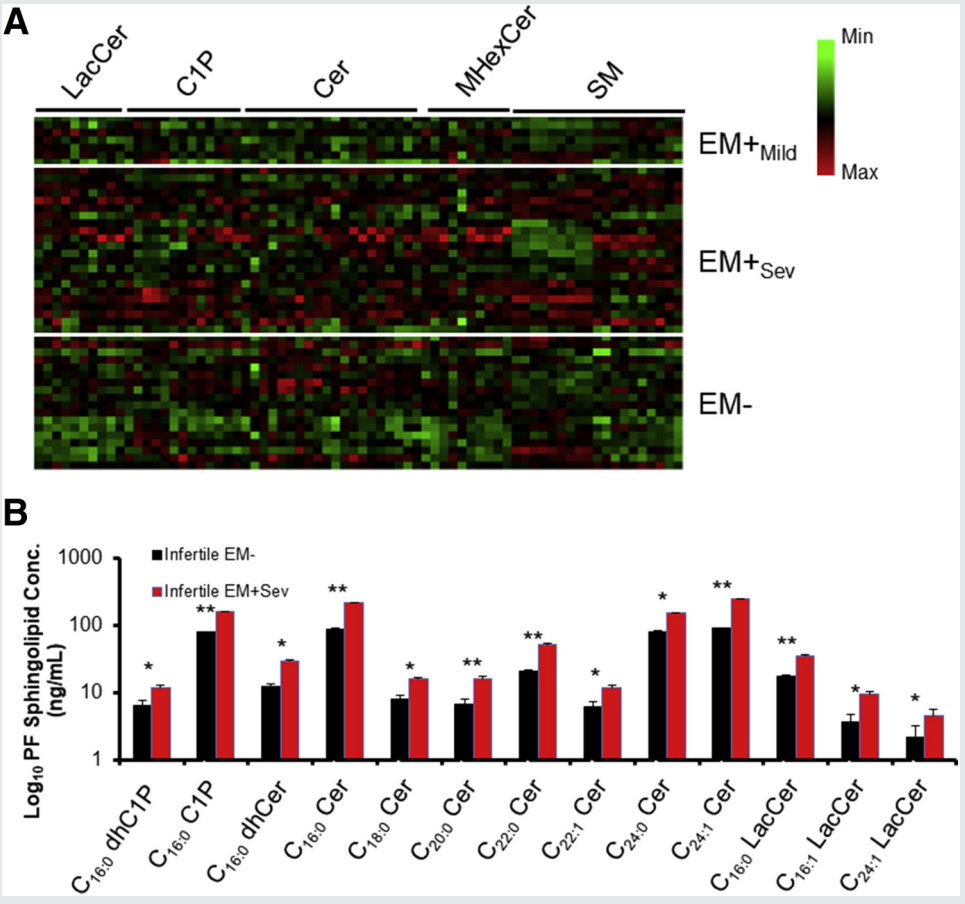
Very-Long-Chain Ceramides as Predictors of Endometriosis-Associated Infertility

Logistic regression analyses were performed on sphingolipids after adjustment for FDR $\leq$10%, with endometriosis status (EM− or $EM+_{Sev}$) as the outcome variable, to investigate their risk in association with EAI. By restricting the analysis to infertile $EM+_{Sev}$ and infertile EM− women, three ceramides, $C_{22:0}$ Cer (P=.006), $C_{24:0}$ Cer (P=.006), and $C_{24:1}$ Cer (P=.026), achieved statistical significance as predictors of severe endometriosis. Correspondingly, their individual AUCs (95% CI) were 0.85 (0.66–1.00), 0.87 (0.72–1.00), and 0.75 (0.54–0.97) respectively, with a combined AUC of 0.95 (0.83–1.00; Fig. 2A). With EM− as the reference, ORs indicated that $C_{24:1}$ Cer increases risk of EAI (OR 1.017, 95% CI 1.00–1.033), whereas $C_{22:0}$ Cer (OR 0.927, 0.86–0.995) and $C_{24:0}$ Cer (OR 0.989, 0.976–0.1001) mitigate the risk (Fig. 2B). For $C_{24:1}$ Cer, the risk of EAI was estimated to increase 1.7% per ng/mL increase in this sphingolipid level among infertile $EM+_{Sev}$ women relative to EM− women. Conversely, for $C_{22:0}$ Cer and $C_{24:0}$ Cer, the risk of EAI is reduced on average by 7.3% and 1.1%, respectively, per ng/mL increase in these sphingolipids. Additional analyses with the use of stepwise and all possible subset selection approaches identified the same three ceramides with a consistency providing assurance of a potentially real association. Across all stages of endometriosis taken (rAFS I–IV), the three very-long-chain Cers produced an AUC of 0.924 (95% CI 0.80–1.00).

Very-Long-Chain Cers Affect Oocyte Maturation Competency

Given the association of the very long Cers with EAI, we evaluated the potential functional effects of $C_{24:0}$ Cer and $C_{24:1}$ Cer on oocyte maturation. Congruent with our earlier finding that

FIGURE 1



Mapping of peritoneal fluid sphingolipidome of infertile women with and without endometriosis. **(A)** Sphingolipids were extracted from the peritoneal fluid (PF) of 27 infertile endometriotic women and 20 infertile women without endometriosis; 39 sphingolipid concentrations had their medians centered and normalized and represented as a heat map. The white lines separate women with severe endometriosis ($EM+_{Sev}$; revised American Fertility Society classification (rAFS) III and IV) from women with mild endometriosis ($EM+_{Mild}$; rAFS I and II) and nonendometriotic women (EM−). C1P = ceramide-1-phosphate; Cer = ceramide; MHexCer = monohexosylceramide; LacCer = lactosylceramide; SM = sphingomyelin. **(B)** PF sphingolipid concentrations of infertile $EM+_{Sev}$ (red; n = 27) and infertile EM− (black; n = 20) women were plotted. The 13 sphingolipids with fold change >1.5 and $P<.05$ to control the false discovery rate at ≤10% are shown. Error bars show mean ± SD. *$P<.05$; **$P<.01$ (two-tailed Student *t* test).

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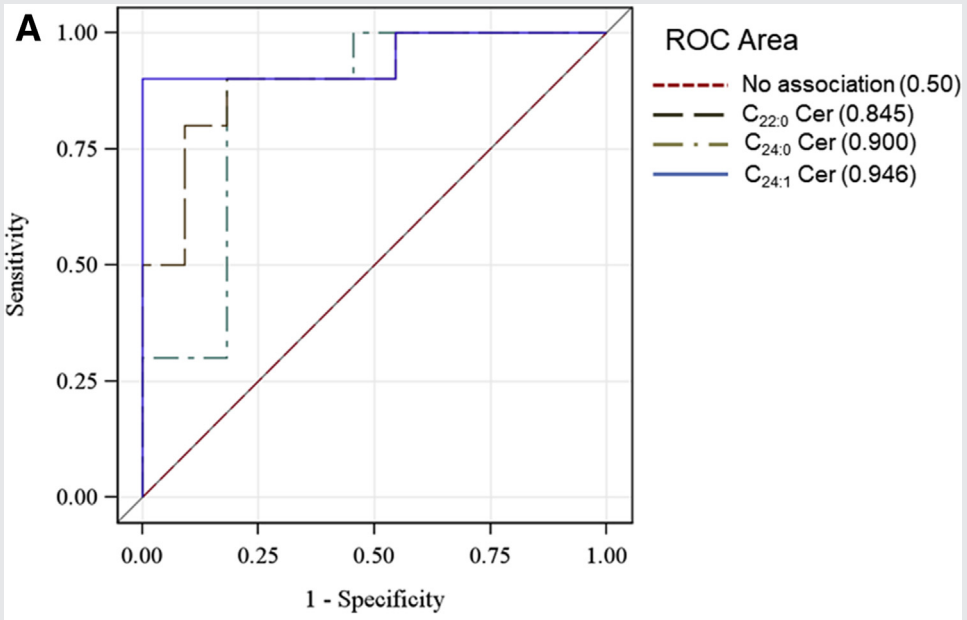
$C_{24:0}$ Cer is associated with EAI risk reduction, $C_{24:0}$ Cer at increasing concentrations of 50, 100, and 400 ng/mL significantly improved the maturation rate of GVs to MIIs compared with vehicle control (58.3%, $P<.001$; 59.9%, $P<.001$; and 79.3%, $P<.01$; respectively; Fig. 3A and B; Supplemental Table 2, available online at www.fertstert.org). In contrast, there was a reduction in the number of MII oocytes incubated with increasing concentrations of $C_{24:1}$ Cer, reaching statistical significance at 240 ng/mL $C_{24:1}$ Cer (45.5% fewer MII oocytes compared with vehicle control; $P<.01$). Correspondingly, $C_{24:1}$ Cer arrested 24.7% and 31.3% of murine oocytes at GV stage and 41.7% and 44.5% of oocytes at MI stage at 60 and 240 ng/mL, respectively (Fig. 3C and D). Notably, $C_{24:1}$ Cer did not induce apoptosis in GV oocytes up to concentrations of 30 μg/mL (Supplemental Fig. 4, available online at www.fertstert.org).

Ceramides Regulate Oocyte Maturation Through Mitochondrial Reactive Oxygen Species

Reactive oxygen species are implicated in altering oocyte maturation potential (37–41). To test the plausible mechanism of Cers in linking with ROS in eliciting pro- or antimaturation effects (42), oocytes were stained with both MitoSOX and MitoTracker dyes after exposure to the Cers in IVM to track levels and cellular distribution of mitochondrial superoxide (Supplemental Fig. 5A, available online at www.fertstert.org). After $C_{24:0}$ Cer exposure, an increase in mitochondrial superoxide production in MII oocytes was observed (50 ng/mL: 14.3%, $P<.01$; 100 ng/mL: 39.0%; $P<.0001$; 400 ng/mL: 28.7%, $P<.0001$; Fig. 4A). A similar increase in mitochondrial superoxide production was observed in GV and MI oocytes compared

FIGURE 2



B

Sphingolipid	Odds Ratio	95% Confidence Limits	
$C_{22:0}$ Cer	0.927	0.863	0.995
$C_{24:0}$ Cer	0.989	0.976	1.001
$C_{24:1}$ Cer	1.017	1.001	1.032

Peritoneal fluid sphingolipids predictive of endometriosis-associated infertility. **(A)** Receiver operating characteristic (ROC) curves showing the sequential cumulative contribution of sphingolipids predictive of endometriosis associated infertility with areas under the curves in parenthesis. **(B)** Odds ratio summary for the three best ceramide predictors. Cer = ceramide.

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with vehicle control (25.2%; $P<.01$; Supplemental Fig. 5B, available online at www.fertstert.org). Interestingly, exposure to $C_{24:1}$ Cer resulted in increased mitochondrial superoxide production at 30 and 60 ng/mL ($P<.01$) in MII oocytes; however, at higher concentrations of 240 ng/mL, superoxide production was significantly attenuated compared with control ($P<.01$; Fig. 4B). At low $C_{24:1}$ Cer concentrations, a corresponding 15.9% increase in superoxide generation was observed in GV and MI oocytes compared with vehicle control ($P<.05$; Supplemental Fig. 5C, available online at www.fertstert.org).

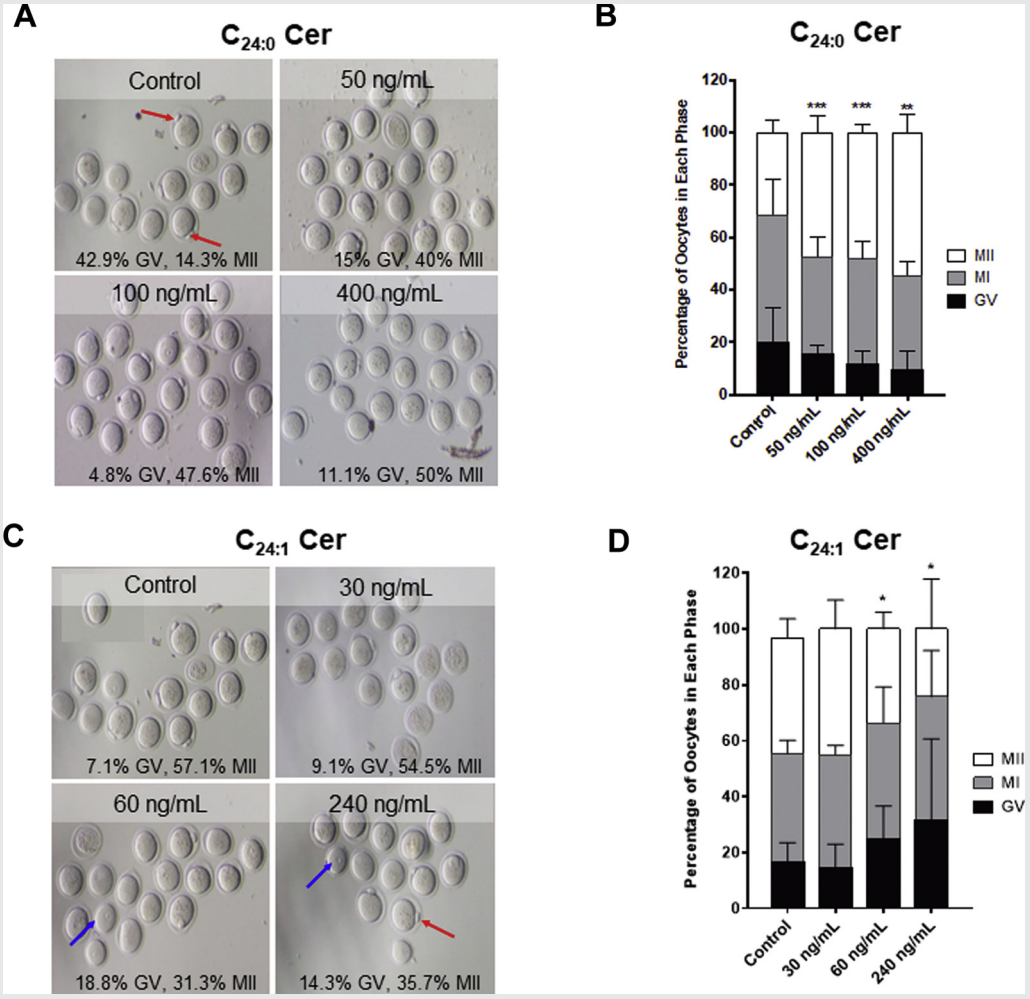
Inhibition of superoxide production with the use of the antioxidant resveratrol led to significant decrease in mitochondrial superoxide production by $C_{24:0}$ Cer ($P<.001$), reaching levels similar to that of control (Fig. 4A). The decrease in mitochondrial superoxide levels correlated with a corresponding 50% drop in the oocyte maturation potential ($P<.01$), reversing effects of $C_{24:0}$ Cer's promaturation effects (Fig. 4C), suggesting that an optimal mitochondrial superoxide level, mediated by very-long-chain Cer, is required for oocyte maturation. In $C_{24:1}$ Cer, the decline in mitochondrial superoxide was associated with a decrease in MII oocytes ($P<.01$). Taken together, a model whereby an optimal mitochondrial superoxide level for oocyte maturation mediated by very-long-chain Cers, is perturbed as EAI emerges (Fig. 4E).

DISCUSSION

Endometriosis has a complex association with infertility (1), and the underlying mechanisms are poorly understood. In this study with carefully selected infertile endometriotic women, we identified the accumulation of very-long-chain Cers in the PF that are potentially associated with EAI. Mitochondrial ROS were implicated and offered a mechanistic explanation to how very-long-chain Cers influence murine oocyte maturation competence in differing ways: $C_{24:1}$ Cer obtunded oocyte maturation, whereas $C_{24:0}$ Cer showed the opposite.

It is recognized that the PF of women with endometriosis is generally hypoxic (43), rife with elevated levels of proinflammatory cytokines and chemokines (44) and dysregulated immune cells (45) in an altered local environment for lesion implantation and growth. Owing to the juxtapositioning of

FIGURE 3



Effects of ceramides (Cers) on murine oocyte maturation. **(A, C)** Representative images of germinal vesicle (GV), metaphase I (MI), and metaphase II (MII) oocytes after 14 hours of incubation in in vitro maturation (IVM) supplemented with **(A)** $C_{24:0}$ Cer or **(C)** $C_{24:1}$ Cer. GV oocytes were characterized by the presence of the GV (*blue arrows*). MII oocytes were distinguished by the extrusion of polar bodies (*red arrows*). **(B, D)** GV, MI, and MII oocytes were expressed as a percentage of the total number of oocytes after incubation in IVM supplemented with **(B)** $C_{24:0}$ Cer or **(D)** $C_{24:1}$ Cer. Each set of data was obtained from five independent experiments. Error bars show mean ± SD. *P<.05; **P<.01; ***P<.001 (two-tailed Student t test).

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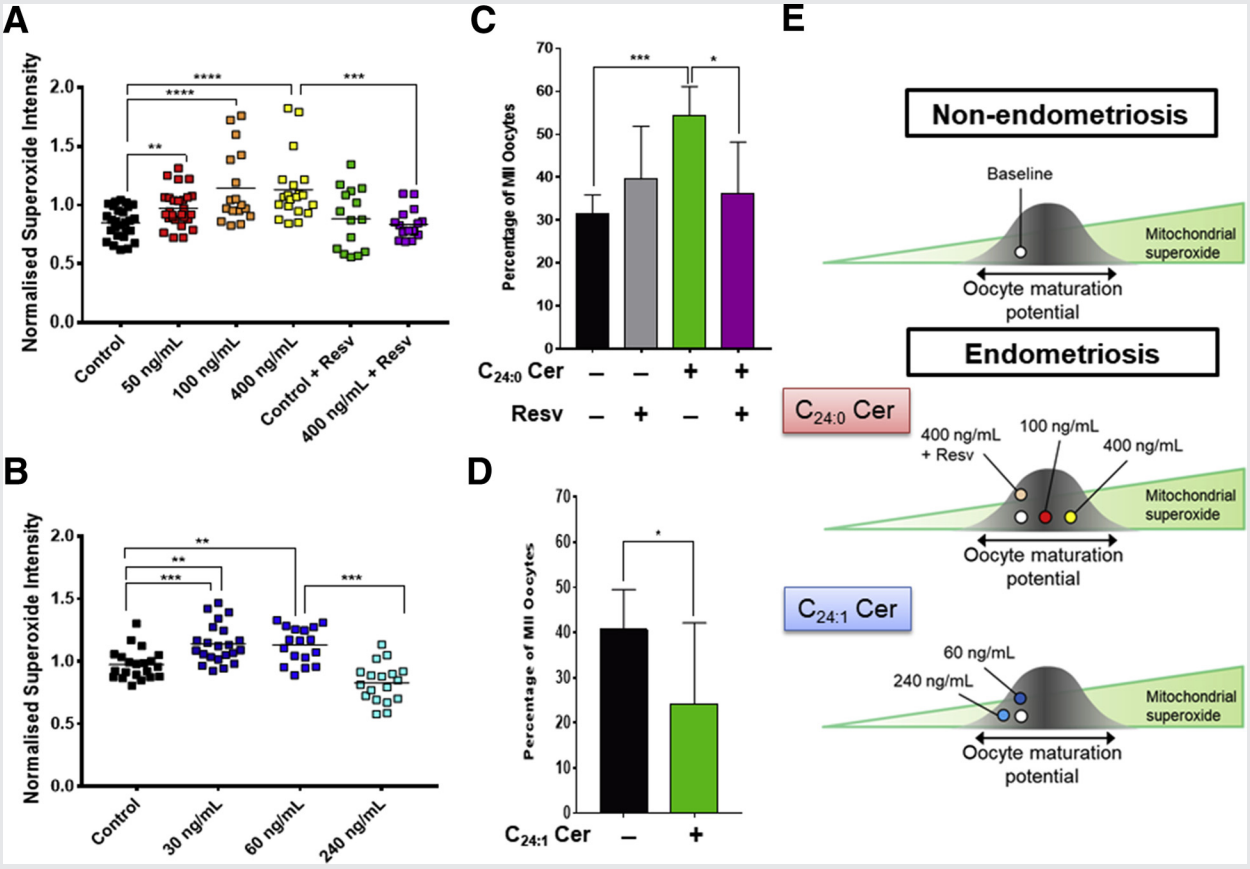
the ovaries and oocytes being released into the PF, an altered PF might have a detrimental effect on the maturation potential of oocytes, as shown here and by others (15, 16). Toxic biologic factors in the PF can affect fertility in various stages of reproduction by natural conception, e.g., gametotoxicity, or embryotoxicity in the ampulla contiguous to the peritoneal cavity, where fertilization typically takes place. Advances in mass spectrometry has provided means to profile the endometrial fluid, tissue, and serum lipidomes of patients with endometriosis and identify broadly the alterations associated with endometriosis (31, 32, 46, 47). Our sphingolipidomic study here extends the list of potentially toxic biologic factors found in the peritoneal environment of endometriotic women that could reduce a patient's ability to conceive. Among the elevated PF sphingolipids detected in this study are Cers. Experimental models of germline infertility have demonstrated that Cers accelerated oocyte apoptosis (21, 48) and perturbed normal embryo organogenesis (20). In general, Cers have pleiotropic inhibitory effects, regulating apoptosis and cell death via mechanisms involving p53 and the Bcl-2/Bax rheostat, or cell cycle arrest by activating cyclin-dependent kinase inhibitor p21 or inhibition of cyclin-dependent kinase 2 (49). However, properties of Cers may change according to their acyl chain lengths and degree of unsaturation. We have recently shown that longer-chain Cers and their glycosylated counterparts, GlcCers, have greater potency in inducing cell migration in endometrial stromal cells (19). Indeed, the three sphingolipids associated with EAI were very-long-chain Cers. Rather than a straightforward effect of Cers inducing

FIGURE 4



Effects of antioxidants on the generation of superoxide in ceramide (Cer)–treated oocytes. **(A, B)** Superoxide fluorescence intensity generated by mouse metaphase II (MII) oocytes in **(A)** $C_{24:1}$ Cer and **(B)** $C_{24:0}$ Cer exposure are represented as normalized values to the total mitochondrial fluorescence intensity. Each square represents a single oocyte, with the mean represented by the horizontal bar. **(C, D)** Histogram of percentage of MII oocytes treated with **(C)** $C_{24:0}$ Cer in the absence or presence of resveratrol (Resv), or **(D)** $C_{24:1}$ Cer. Each set of data consists of data obtained from three independent experiments. Error bars show mean. *P<.05, **P<.01, ***P<.001 (Mann-Whitney test). **(E)** Proposed model of very-long-chain Cer–mediated mitochondrial superoxide production in oocyte maturation. The *white circles* represents the untreated or baseline oocytes. The *red and yellow circles* represent 100 and 400 ng/mL $C_{24:0}$ Cer–treated oocytes, respectively. The *orange circle* represents 400 ng/mL $C_{24:0}$ Cer–treated oocytes with resveratrol pretreatment. The *dark blue and pale blue circles* represent 60 and 240 ng/mL $C_{24:1}$ Cer–treated oocytes.

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apoptosis, we found that very-long-chain Cers could inhibit or induce the maturation potential of oocytes depending on the presence of a double bond.

Cers have been shown to interfere with several mitochondrial mechanisms, including cytochrome C release, and permeabilizing the mitochondrial outer membrane, which compromises mitochondrial electron flow and triggers ROS formation (42, 50, 51). A balance between ROS and antioxidants is required for oocyte maturation (37, 52), where physiologic superoxide levels fall within an optimal concentration range for oocyte maturation ([53, 54]; Fig. 4E). It has been proposed that insufficient ROS (38, 55) or elevated ROS in endometriosis (56, 57) might adversely affect oocyte and embryo development in vivo as well as pregnancy outcomes (52). This is in agreement with the present results that $C_{24:0}$ Cer induced mitochondrial superoxide, which correlated with progression of GV oocytes to the MII stage. Furthermore, resveratrol reduced oocyte maturation potential by scavenging superoxide induced by $C_{24:0}$ Cer. In the same vein, the reduction in oocyte maturation rate on exposure to high concentrations of $C_{24:1}$ Cer was associated with reduced mitochondrial superoxide content in the MII oocytes. It is therefore tempting to speculate that the mitochondrial superoxide levels were suppressed by $C_{24:1}$ Cer below the physiologic lower limit to support proper oocyte maturation. However, with $C_{24:0}$ and $C_{24:1}$ Cers elevated in endometriosis patients, the interplay of both Cers on the oocytes is less clear. Nonetheless, our data suggest that aberrant sphingolipid metabolism in the peritoneal environment might contribute to developmentally compromised oocytes and to poorer fertility rates in women with EAI through the effects of ROS. It is noteworthy that in IVF, compromised oocyte maturation in women with endometriosis do not translate

into higher blastocyst aneuploidy rates, because similar blastocyst aneuploidy rates were reported in age-matched IVF patients with and without endometriosis (58).

In conclusion, we have identified a panel of Cers that are associated with EAI and identified plausible mechanisms underlying oocytotoxicity. The identification of functionally important oocytotoxic Cers and other sphingolipids in the PF provide an opportunity for direct functional readout of pathophysiology of circulatory compounds in a defective peritoneal environment in endometriotic women (31). Diversion of monounsaturated Cers or other sphingolipid metabolites might be protective (59), and future therapies could targeted toward this sphingolipid pathway and harnessed for improved oocyte maturation.

REFERENCES

1. de Ziegler D, Borghese B, Chapron C. Endometriosis and infertility: pathophysiology and management. Lancet 2010;376:730–8.
2. Holoch KJ, Lessey BA. Endometriosis and infertility. Clin Obstet Gynecol 2010;53:429–38.
3. Albertsen HM, Chettier R, Farrington P, Ward K. Genome-wide association study link novel loci to endometriosis. PLoS One 2013;8:e58257.
4. Schenken RS, Asch RH, Williams RF, Hodgen GD. Etiology of infertility in monkeys with endometriosis: luteinized unruptured follicles, luteal phase defects, pelvic adhesions, and spontaneous abortions. Fertil Steril 1984; 41:122–30.
5. Simón C, Gutiérrez A, Vidal A, de los Santos MJ, Tarín JJ, Remohí J, et al. Outcome of patients with endometriosis in assisted reproduction: results from in-vitro fertilization and oocyte donation. Hum Reprod 1994;9:725–9.
6. Pellicer A, Oliveira N, Ruiz A, Remohí J SC. Implantation in endometriosis: lessons learned from IVF and oocyte donation. In: Progress in endometriosis. 1994:177–183.
7. Díaz I, Navarro J, Blasco L, Simón C, Pellicer A, Remohí J. Impact of stage III–IV endometriosis on recipients of sibling oocytes: matched case-control study. Fertil Steril 2000;74:31–4.
8. Xu B, Guo N, Zhang X, Shi W, Tong X, Iqbal F, et al. Oocyte quality is decreased in women with minimal or mild endometriosis. Sci Rep 2015;5: 10779.
9. Cohen J, Ziyyat A, Naoura I, Chabbert-Buffet N, Aractingi S, Darai E, et al. Effect of induced peritoneal endometriosis on oocyte and embryo quality in a mouse model. J Assist Reprod Genet 2015;32:263–70.
10. Barnhart K, Dunsmoor-Su R, Coutifaris C. Effect of endometriosis on in vitro fertilization. Fertil Steril 2002;77:1148–55.
11. Gazeto Melo Jianini BT, Giorgi VSI, da Broi MG, de Paz CCP, Rosa e Silva JC, Ferriani RA, et al. Peritoneal fluid from infertile women with minimal/mild endometriosis compromises the meiotic spindle of metaphase II bovine oocytes. Reprod Sci 2017;24:1304–11.
12. Hunter RHF, Cicinelli E, Einer-Jensen N. Peritoneal fluid as an unrecognised vector between female reproductive tissues. Acta Obstet Gynecol Scand 2007;86:260–5.
13. Shu J, Xing L, Ding G, Luo Q, Liu X, Yan Q, et al. The effect of peritoneal fluid from patients with endometriosis on mitochondrial function and development of early mouse embryos. PLoS One 2013;8:e82334.
14. Young VJ, Brown JK, Saunders PTK, Horne AW. The role of the peritoneum in the pathogenesis of endometriosis. Hum Reprod Update 2013;0:1–12.
15. Illera MJ, Juan L, Stewart CL, Cullinan E, Ruman J, Lessey BA. Effect of peritoneal fluid from women with endometriosis on implantation in the mouse model. Fertil Steril 2000;74:41–8.
16. Mansour G, Sharma RK, Agarwal A, Falcone T. Endometriosis-induced alterations in mouse metaphase II oocyte microtubules and chromosomal alignment: a possible cause of infertility. Fertil Steril 2010;94:1894–9.
17. Young SA, Mina JG, Denny PW, Smith TK. Sphingolipid and ceramide homeostasis: potential therapeutic targets. Biochem Res Int 2012;2012: 248135.
18. Hannun YA, Obeid LM. Many ceramides. J Biol Chem 2011;286:27855–62.
19. Wimalachandra D, Yang JX, Zhu L, Tan E, Asada H, Chan JYK, et al. Long-chain glucosylceramides crosstalk with LYN mediates endometrial cell migration. Biochim Biophys Acta Mol Cell Biol Lipids 2018;1863:71–80.
20. Wang X, Rao RP, Kosakowska-Cholody T, Masood MA, Southon E, Zhang H, et al. Mitochondrial degeneration and not apoptosis is the primary cause of embryonic lethality in ceramide transfer protein mutant mice. J Cell Biol 2009;184:143–58.
21. Morita Y, Perez GI, Paris F, Miranda SR, Ehleiter D, Haimovitz-Friedman A, et al. Oocyte apoptosis is suppressed by disruption of the acid sphingomyelinase gene or by sphingosine-1-phosphate therapy. Nat Med 2000;6:1109–14.
22. Nomura KH, Murata D, Hayashi Y, Dejima K, Mizuguchi S, Kage-Nakadai E, et al. Ceramide glucosyltransferase of the nematode Caenorhabditis elegans is involved in oocyte formation and in early embryonic cell division. Glycobiology 2011;21:834–48.
23. Yamashita T, Wada R, Sasaki T, Deng C, Bierfreund U, Sandhoff K, et al. A vital role for glycosphingolipid synthesis during development and differentiation. Proc Natl Acad Sci U S A 1999;96:9142–7.
24. Rahmioglu N, Fassbender A, Vitonis AF, Tworoger SS, Hummelshoj L, d'Hooghe TM, et al, World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonization Project Working Group. World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonization Project: III. Fluid biospecimen collection, processing, and storage in endometriosis research. Fertil Steril 2014;102:1233–43.
25. American Society for Reproductive Medicine. Revised American Society for Reproductive Medicine classification of endometriosis: 1996. Fertil Steril 1997;67:817–21.
26. American Fertility Society. Revised American Fertility Society classification of endometriosis: 1985. Fertil Steril 1985;43:351–2.
27. Kersten FAM, Hermens RPGM, Braat DDM, Hoek A, Mol BWJ, Goddijn M, et al. Overtreatment in couples with unexplained infertility. Hum Reprod 2015;30:2694.
28. Dutch Society for Obstetrics and Gynecology. National network directive: subfertility [Dutch]. Available at: https://www.nvog.nl/wp-content/uploads/2018/02/Subfertiliteit-landelijke-netwerkrichtlijn-1.0-20-05-2011.pdf; 2010.
29. Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HWG, Behre HM, et al. World Health Organization reference values for human semen characteristics. Hum Reprod Update 2010;16:231–45.
30. Cui L, Fang J, Ooi EE, Lee YH. Serial metabolome changes in a prospective cohort of subjects with influenza viral infection and comparison with dengue fever. J Proteome Res 2017;16:2614–22.
31. Lee YH, Cui L, Fang J, Chern BSM, Tan HH, Chan JKY. Limited value of pro-inflammatory oxylipins and cytokines as circulating biomarkers in endometriosis—a targeted "omics" study. Sci Rep 2016;6:26117.
32. Lee YH, Tan CW, Venkatratnam A, Tan CS, Cui L, Loh SF, et al. Dysregulated sphingolipid metabolism in endometriosis. J Clin Endocrinol Metab 2014; 99:E1913–21.
33. Appeltant R, Somfai T, Nakai M, Bodó S, Maes D, Kikuchi K, et al. Interactions between oocytes and cumulus cells during invitro maturation of porcine cumulus-oocyte complexes in a chemically defined medium: effect of denuded oocytes on cumulus expansion and oocyte maturation. Theriogenology 2015;83:567–76.
34. Zhou H-X, Ma Y-Z, Liu Y-L, Chen Y, Zhou C-J, Wu S-N, et al. Assessment of mouse germinal vesicle stage oocyte quality by evaluating the cumulus layer, zona pellucida, and perivitelline space. PLoS One 2014;9:e105812.
35. Rienzi L, Balaban B, Ebner T, Mandelbaum J. The oocyte. Hum Reprod 2012; 27(Suppl 1):i2–21.
36. Schmerler S, Wessel GM. Polar bodies—more a lack of understanding than a lack of respect. Mol Reprod Dev 2011;78:3–8.
37. Kala M, Shaikh MV, Nivsarkar M. Equilibrium between antioxidants and reactive oxygen species: a requisite for oocyte development and maturation. Reprod Med Biol 2017;16:28–35.
38. Blondin P, Coenen K, Sirard MA. The impact of reactive oxygen species on bovine sperm fertilizing ability and oocyte maturation. J Androl 1997;18:454–60.
39. Xie H-L, Wang Y-B, Jiao G-Z, Kong D-L, Li Q, Li H, et al. Effects of glucose metabolism during in vitro maturation on cytoplasmic maturation of mouse oocytes. Sci Rep 2016;6:20764.

40. Han L, Wang H, Li L, Li X, Ge J, Reiter RJ, et al. Melatonin protects against maternal obesity-associated oxidative stress and meiotic defects in oocytes via the SIRT3-SOD2 dependent pathway. J Pineal Res 2017:e12431.
41. Nikseresht M, Toori MA, Rahimi HR, Fallahzadeh A, Kahshani IR, Hashemi SF, et al. Effect of antioxidants (β-mercaptoethanol and cysteamine) on assisted reproductive technology in vitro. J Clin Diagn Res 2017;11:BC10–4.
42. Yano M, Watanabe K, Yamamoto T, Ikeda K, Senokuchi T, Lu M, et al. Mitochondrial dysfunction and increased reactive oxygen species impair insulin secretion in sphingomyelin synthase 1-null mice. J Biol Chem 2011;286:3992–4002.
43. Tan CW, Lee YH, Tan HH, Lau MSK, Choolani M, Griffith L, et al. CD26/DPPIV down-regulation in endometrial stromal cell migration in endometriosis. Fertil Steril 2014;102:167–77.e9.
44. Jørgensen H, Hill AS, Beste MT, Kumar MP, Chiswick E, Fedorcsak P, et al. Peritoneal fluid cytokines related to endometriosis in patients evaluated for infertility. Fertil Steril 2017;107:1191–9.e2.
45. Ulukus M, Arici A. Immunology of endometriosis. Minerva Ginecol 2005;57:237–48.
46. Domínguez F, Ferrando M, Díaz-Gimeno P, Quintana F, Fernández G, Castells I, et al. Lipidomic profiling of endometrial fluid in women with ovarian endometriosis. Biol Reprod 2017;96:772–9.
47. Vouk K, Hevir N, Ribić-Pucelj M, Haarpaintner G, Scherb H, Osredkar J, et al. Discovery of phosphatidylcholines and sphingomyelins as biomarkers for ovarian endometriosis. Hum Reprod 2012;27:2955–65.
48. Perez GI, Robles R, Knudson CM, Flaws JA, Korsmeyer SJ, Tilly JL. Prolongation of ovarian lifespan into advanced chronological age by Bax-deficiency. Nat Genet 1999;21:200–3.
49. Taha TA, Mullen TD, Obeid LM. A house divided: ceramide, sphingosine, and sphingosine-1-phosphate in programmed cell death. Biochim Biophys Acta 2006;1758:2027–36.
50. Zigdon H, Kogot-Levin A, Park J-W, Goldschmidt R, Kelly S, Merrill AH, et al. Ablation of ceramide synthase 2 causes chronic oxidative stress due to disruption of the mitochondrial respiratory chain. J Biol Chem 2013;288:4947–56.
51. Stiban J, Perera M. Very long chain ceramides interfere with C16-ceramide–induced channel formation: a plausible mechanism for regulating the initiation of intrinsic apoptosis. Biochim Biophys Acta Biomembr 2015;1848:561–7.
52. Agarwal A, Durairajanayagam D, du Plessis SS. Utility of antioxidants during assisted reproductive techniques: an evidence based review. Reprod Biol Endocrinol 2014;12:112.
53. Das S, Chattopadhyay R, Ghosh S, Ghosh S, Goswami SK, Chakravarty BN, et al. Reactive oxygen species level in follicular fluid—embryo quality marker in IVF? Hum Reprod 2006;21:2403–7.
54. Jana SK, NB K, Chattopadhyay R, Chakravarty B, Chaudhury K. Upper control limit of reactive oxygen species in follicular fluid beyond which viable embryo formation is not favorable. Reprod Toxicol 2010;29:447–51.
55. Dunning KR, Cashman K, Russell DL, Thompson JG, Norman RJ, Robker RL. Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. Biol Reprod 2010;83:909–18.
56. Hamdan M, Jones KT, Cheong Y, Lane SIR. The sensitivity of the DNA damage checkpoint prevents oocyte maturation in endometriosis. Sci Rep 2016;6:36994.
57. Da Broi MG, Navarro PA. Oxidative stress and oocyte quality: ethiopathogenic mechanisms of minimal/mild endometriosis-related infertility. Cell Tissue Res 2016;364:1–7.
58. Juneau CR, Franasiak JM, Werner MD, Forman EJ, Molinaro T, Scott RT. Patients with endometriosis do not have a higher rate of aneuploidy. Fertil Steril 2015;104:e70.
59. Eliyahu E, Shtraizent N, Shalgi R, Schuchman EH. Construction of conditional acid ceramidase knockout mice and in vivo effects on oocyte development and fertility. Cell Physiol Biochem 2012;30:735–48.

Ceramidas elevadas del fluido peritoneal en la infertilidad asociada a endometriosis humana y sus efectos sobre la maduración de ovocitos en ratones

Objetivo: Caracterizar el perfil de esfingolípidos del líquido peritoneal (PF) en la infertilidad asociada a la endometriosis (EAI) y evaluar el/los papel (es) funcional (es) plausible (s) de las ceramidas en el potencial de maduración de los ovocitos.

Diseño: Estudio retrospectivo de casos y controles y estudio in vitro en ovocitos de ratones.

Entorno: Hospital y Laboratorio afiliados a la universidad.

Individuos: Veintisiete pacientes infértiles diagnosticados con endometriosis y 20 pacientes infértiles que no tenían endometriosis; Ratones hembras BALB / c.

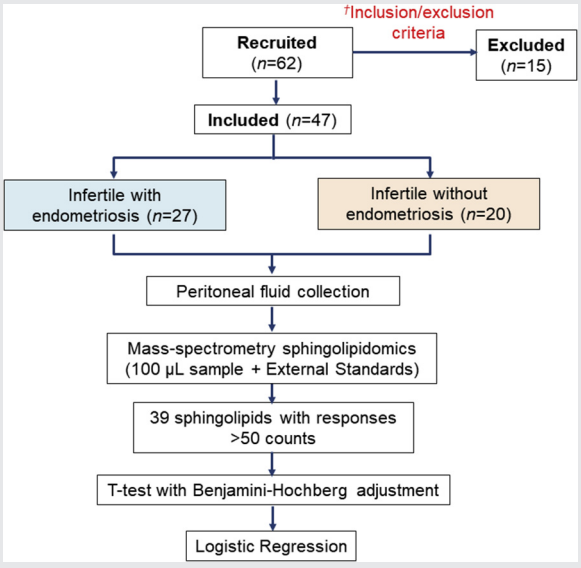
Intervención (es): ninguna.

Principales medidas de resultado: Concentraciones de esfingolípidos del PF. Número de ovocitos de ratón en metafase II (MII).

Resultado (s): La cromatografía líquida-espectrometría de masas en tándem reveló 11 esfingolípidos en el PF significativamente elevados en mujeres infértiles con endometriosis severa en comparación con mujeres infértiles sin endometriosis (cambio $>$50%, tasa de descubrimientos falsos $\geq$ 10%). El análisis de regresión logística identificó tres ceramidas de cadena muy larga potencialmente asociadas con EAI. Los estudios funcionales revelaron que las ceramidas de cadena larga pueden comprometer o inducir la maduración del ovocito MII murino. Los efectos de la maduración de los ovocitos inducidos por las ceramidas de cadena muy larga se desencadenaron por alteraciones en la producción de superóxido mitocondrial de una manera dependiente de la concentración.La eliminación del superóxido mitocondrial revirtió los efectos de maduración de la ceramida C24:0.

Conclusión (es): EAI está asociado con la acumulación de ceramidas de cadena muy larga del PF. Los estudios con ratones demostraron cómo las ceramidas afectan la maduración del ovocito MII, mediando a través del superóxido mitocondrial. Estos resultados proporcionan una oportunidad para la lectura funcional directa de la fisiopatología en EAI, y las terapias futuras dirigidas al metabolismo de estos esfingolípidos pueden aprovecharse para una mejor maduración de los ovocitos.

SUPPLEMENTAL FIGURE 1



Workflow and scheme of the study. Patients were diagnosed with the use of the revised American Fertility Society classification and further stratified by their fecundity status. Sphingolipids were extracted from peritoneal fluid and analyzed by means of mass spectrometry. Relative quantification was performed with the use of spiked external standards. Data filtering was performed and 39 sphingolipids were selected with responses >50 counts. †Exclusion criteria can be found in Materials and Methods.

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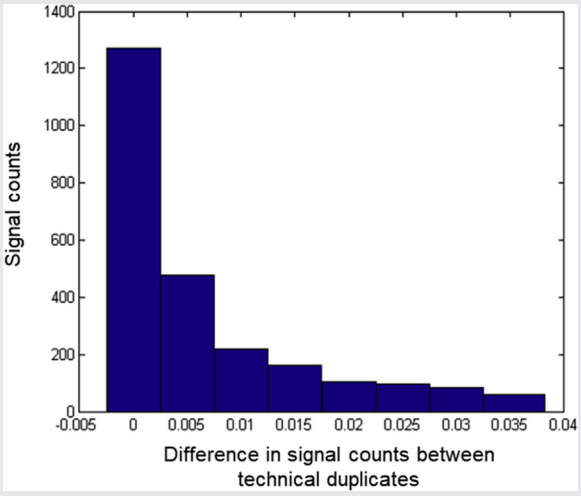
SUPPLEMENTAL FIGURE 2



Histogram of the variability from duplicate technical runs. Patient samples were run in technical duplicates, and the difference in signal counts was calculated. The differences were binned at 0.005 intervals, and most of the duplicates exhibited low variability.

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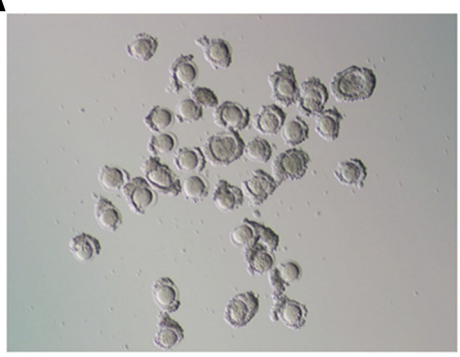
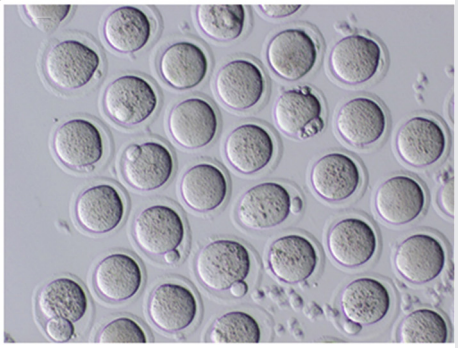
SUPPLEMENTAL FIGURE 3

A



B



C

Total no. of COCs	No. of GV oocytes after IVM	No. of MII oocytes after IVM	Maturation Rate (%)
55	3	26	47.3

In vitro maturation (IVM) of oocytes in cumulus oocyte complexes (COCs). **(A)** COCs at t = 0 h. **(B)** Denuded COCs at t = 18 h in IVM medium. Images are at ×4 and ×10 magnification, respectively. **(C)** Summary of denuded COC IVM rate. The number of germinal vesicle (GV) and metaphase II (MII) oocytes were counted at the end of the incubation period.

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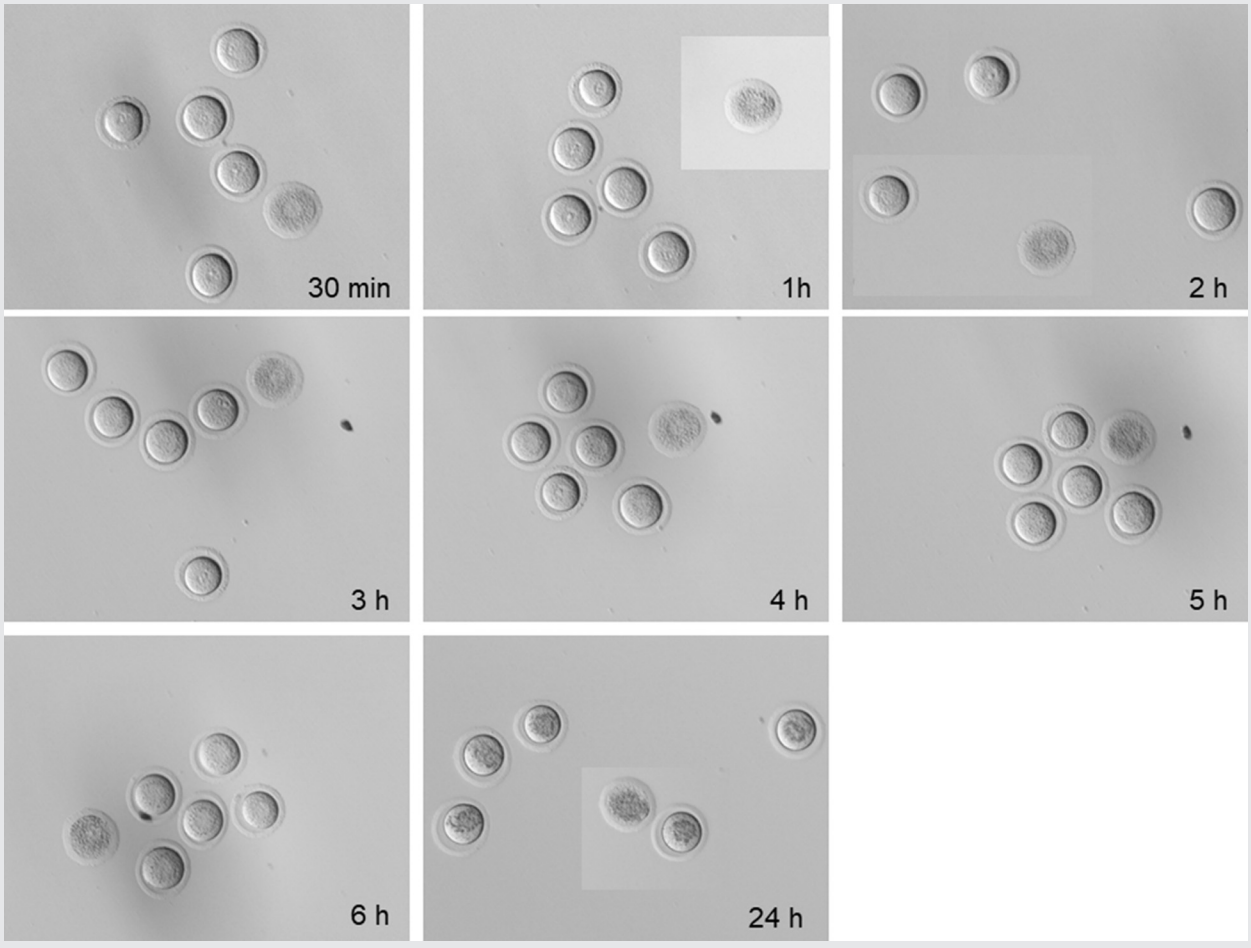
SUPPLEMENTAL FIGURE 4



$C_{24:1}$ ceramide (Cer) does not cause oocyte apoptosis. Denuded oocytes were incubated with 30 μg/mL $C_{24:1}$ Cer in MEMα media (supplemented with 10% fetal bovine serum and 25 μmol/L HEPES buffer) for up to 24 hours. Images were taken at various time points to assess for oocyte apoptosis. Where it is necessary to allow images of oocytes to be captured in the same view of vision, stitching of images was performed. Images are at $\times$10 magnification.

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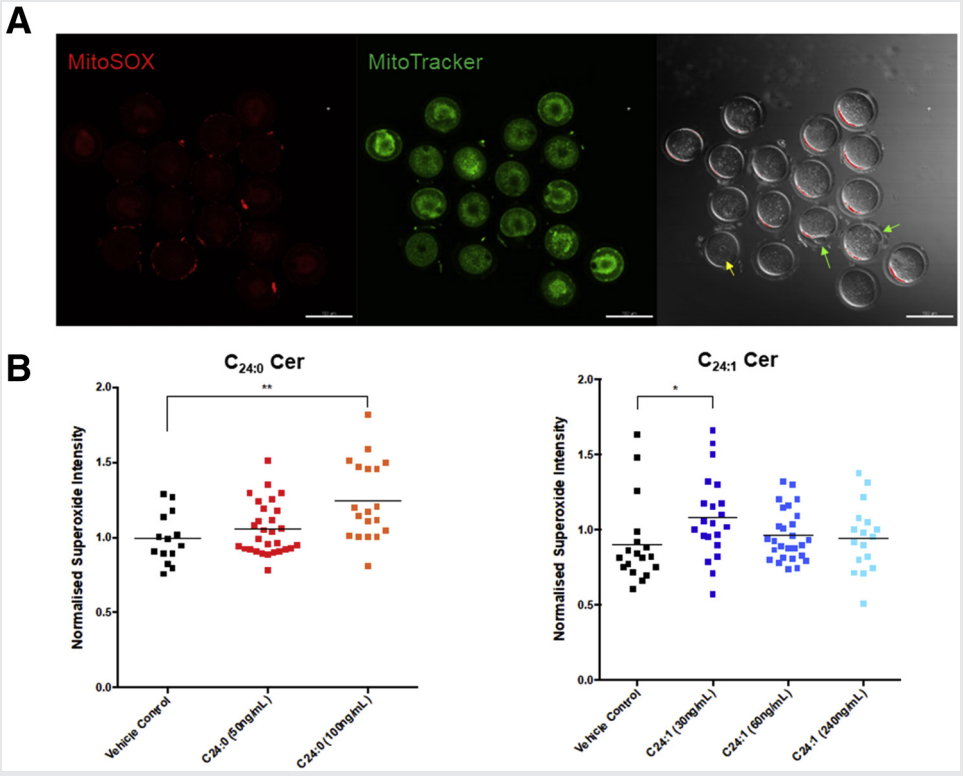
SUPPLEMENTAL FIGURE 5



Ceramides (Cers) cause mitochondrial superoxide production in germinal vesicle (GV) and metaphase I (MI) oocytes. **(A)** MitoSOX was used to track superoxide, and MitoTracker was used to track mitochondria. Overlap of both dyes indicates the generation of mitochondrial-specific superoxide. *Green arrows* indicate the presence of extruded polar body in metaphase II oocytes. *Yellow arrow* indicates the presence of germinal vesicle in GV oocytes. **(B)** Cers cause mitochondrial superoxide production in GV and MI oocytes. *$P<.05$; **$P<.01$ (two-tailed Student *t* test).

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