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

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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