# Oxidative Damage and Mitochondrial DNA Mutations with Endometriosis


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**ABSTRACT: Endometriosis, a frequently encountered disease in gynecology, is a considerable threat to the physical, psychological, and social integrity of women. Moreover, up to 50% of infertile patients have this disease. The etiology and pathogenesis of this important disease are poorly understood; it is defined as an ectopic location for endometrium-like glandular epithelium and stroma outside of the uterine cavity. It still remains an open question as to what extent the peritoneal environment influences the establishment and/or progression of endometriosis. As a result of such stress, a sterile, inflammatory reaction with the secretion of growth factors, cytokines, and chemokines is generated, which is especially deleterious to successful reproduction. Significantly higher amounts of oxidative damage were detected in endometriotic lesions than in controlled normal endometrium, including mitochondrial DNA (mtDNA) rearrangement, 8-OH-deoxyguanosine (8-OH-dG), and lipoperoxide contents. There were approximately sixfold increases in 8-OH-dG and lipoperoxides in chocolate cysts compared with normal endometrial tissues. A novel 5,335-bp deletion of mtDNA was identified in endometriotic tissue. According to these results, we propose that oxidative stress and mtDNA mutations might be anticipated in the initiation or progression of endometriosis. Only by understanding the mechanisms involved in the pathogenesis of endometriosis can we develop a basis for new diagnostic and therapeutic approaches.**


## INTRODUCTION

Endometriosis is an invasive but benign gynecological disease that is histologically characterized by the presence of endometrium-like glands and stroma outside of the uterus. It is one of the most frequently encountered diseases in gynecology, affecting 15 to 50% of women in their reproductive life span.[1] Clinical observations


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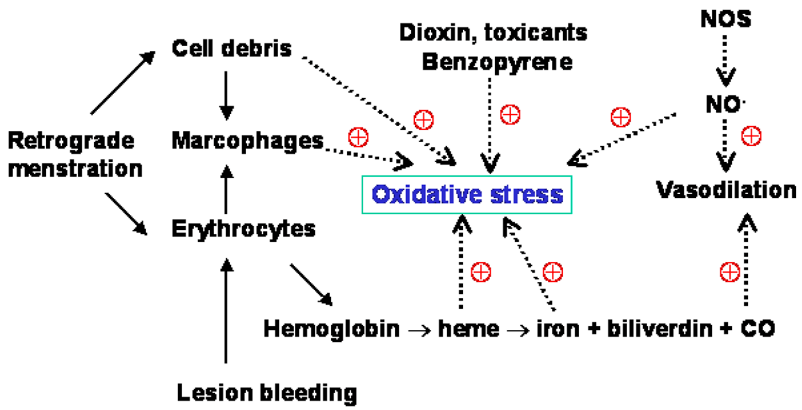


**FIGURE 1.** Hypothesis explaining oxidative stress in the peritoneal cavity of women with endometriosis.[4] Boldface type indicates factors that have specifically been studied in relation to pelvic endometriosis. CO, carbon dioxide; NO, nitric oxide; NOS, nitric oxide synthase.

and *in vitro* experiments imply that endometriotic cells are invasive and able to metastasize. The endometriotic tissue often undergoes cyclic proliferation and breakdown similar to eutopic endometrium, resulting in local inflammatory reactions. These processes cause the cyclical character of endometriosis with dysmenorrhea, dyspareunia, pelvic pain, catamenial hematuria, and other symptoms derived from the affected organ. Moreover, up to 50% of infertile patients have this disease.[2]

The pathophysiology of this disease still remains elusive. Recent studies have suggested that menstrual effluent contains factors that induce alterations in the morphology of the peritoneal mesothelium,[3] which may create adhesion sites for endometrial cells. Endometriosis is a multifactorial disease associated with a general inflammatory response in the peritoneal cavity. Oxidative stress has been proposed as a potential factor involved in the pathogenesis of the disease[4] and may be responsible for local destruction of the peritoneal mesothelium, thereby creating adhesion sites for ectopic endometrial cells. Several hypotheses have been proposed to explain why oxidative stress is induced in cases of pelvic endometriosis (FIG. 1). Oxidatively damaged erythrocytes,[5] apoptotic endometrial cells, or undigested endometrial tissue[6] may become transplanted into the peritoneal cavity and signal the recruitment and activation of mononuclear phagocytes. Women with endometriosis are prone to react to this stimulus with an inadequate macrophage scavenger receptor response. Activated macrophages in the peritoneal cavity generate oxidative stress, which consists of lipid peroxides, their degradation products, and products formed from their interaction with low-density lipoprotein (LDL), apoprotein,[7] and other proteins.[8] Moreover, autoantibodies to malondialdehyde-modified low-density lipoprotein, oxidized low-density lipoprotein, and lipid peroxide-modified serum albumin markers of oxidative stress are significantly increased in women with endometriosis.[9] As a result of such a stress, a sterile, inflammatory reaction with secretion of growth factors, cytokines, and chemokines is generated, which is deleterious especially to successful reproduction.

Production of reactive oxygen species appears to be increased in women in whom endometriosis is developing and progressing. Most reports have discussed the potential consequences of increased oxidative stress in endometriosis relative to decreased fertility. We propose that oxidative stress might be anticipated in the initiation or progression of endometriosis. In this study, we attempted to elucidate relationships among oxidative stress, mitochondrial DNA (mtDNA) mutations, and endometriosis.

## MATERIALS AND METHODS

### *Analysis of Oxidative Damage in Cellular Molecules*

*Determination of 8-OH-Deoxyguonosine (8-OH-dG)*

Tissue samples were scraped using a cell lifter in 1.5 mL TE buffer, and 75 μL of 10% SDS, 30 mL of 200 mM butylated hydroxytoluene, and 15 mL of RNase A stock solution (10 μg/mL) were added; then the lysate was incubated at 37°C for 1 h and incubated with proteinase K (100 μg/mL) at 55°C for 12 h. The lysate was extracted by a phenol/chloroform method. An aliquot of 100 μg of DNA dissolved in 100 μL of 10 mM Tris-HCl (pH 7.4)/0.1 mM DFAM was digested by incubation with 1 μL of DNase I (20 U/μL) and 11 μL of a 0.1 M $MgCl_2$ solution at 37°C for 30 min. After adjusting the pH to 5.0 by adding 4.8 μL of 1 M sodium acetate (pH 5.3) and 1.2 μL of 0.1 M $ZnSO_4$, the DNA sample was digested with 5 μL of nuclease P1 (1 U/3 μL in 20 mM sodium acetate, pH 5.3) at 65°C for 10 min. The DNA molecules were hydrolyzed to the corresponding nucleosides by incubation with 5 mL of 1 U/μL alkaline phosphatase for 30 min at 37°C. Processed DNA samples were separated on a C-18 column (particle size 5 mm, 200 × 4.6 mm; JT Baker, Phillipsburg, NJ) on an HPLC system (Jasco, Easton, MD) connected in series with an ECD detector (Bioanalytical Systems, West Lafayette, IN) and a UV detector (at 254 nm; Jasco). Elution was performed at a flow rate of 0.8 mL/min for 40 min with a mobile phase which consisted of 12.5 mM citric acid, 25 mM sodium acetate, and 10 mM acetic acid containing 6% methanol (pH 5.8).

*Determination of Lipid Peroxide*

In each analytical run, a reagent blank, 1,1,3,3-tetraethoxypropane standard working solutions, and the sample were assayed in duplicate. An aliquot of 50 μL of each sample was pipetted into a test tube containing 0.6 mL of 0.44 M phosphoric acid. After mixing, 0.2 mL of a 42 mM thiobarbituric acid solution was added to a final concentration of 7 mM and then placed in a 95°C dry bath for 1 h. The samples were then cooled and neutralized with 1 N NaOH in methanol before the HPLC analysis. An aliquot of 20 μL of supernatant obtained above was injected into a narrow-pore C18 column (4.6 × 250 mm, particle size 5 mm) using a Jasco PU-980 pump with a solvent system made of methanol and 50 mM phosphate buffer (pH 6.8; 4:6, v/v) at a flow rate of 1 mL/min. The eluent was monitored using a Jasco fluorescence detector using excitation at 525 nm and emission at 550 nm.

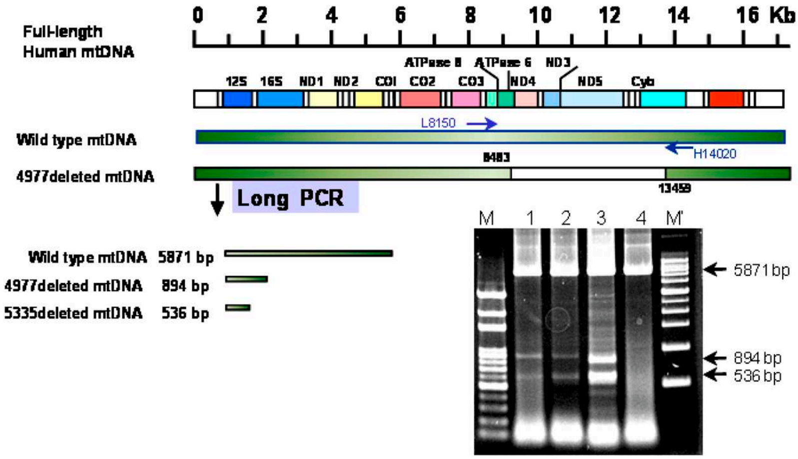


**FIGURE 2.** (**Top panel**) A scheme illustrating the strategy for the determination of multiple mtDNA deletions of the endometrium by the long-range PCR technique. Using the primer pair L8150-H14020, we generated three types of PCR products. The 5,871-bp fragment was produced from wild-type mtDNA, an 894-bp fragment was from 4,977-bp deleted mtDNA, and a 536-bp fragment was from a 5,335-bp mtDNA deletion. (**Bottom panel**) Electrophorectogram of PCR products amplified from mtDNA with specific deletions in tissues from women with or without endometriosis. Lanes 1 to 3 were examined from ovaries, myometrium, and endometrium from three individuals with endometriosis, respectively. *Lane M* is a 100-bp DNA ladder and *lane M′* is a 10-kb DNA ladder.

## *Determination of mtDNA Mutations in Human Tissue*

### *Total DNA Extraction*

All samples then were checked by microscopy for morphological changes and stored at –196°C until analysis. All tissues were minced into small pieces and incubated at 56°C for 2 h in 50 µL lysis buffer containing 2% SDS and 50 mM Tris-HCl (pH 8.3), followed by a phenol-chloroform extraction method. All DNA samples were finally preserved in 200 µL of 10 mM Tris-HCl (pH 8.3).

### *Synthesis of Oligonucleotide Primers*

Oligonucleotide primers used for amplification of the target sequences of mtDNA and genomic DNA were chemically synthesized by Protec (Taipei, Taiwan). The nucleotide sequences of used primer pairs were L8150 (5′-CCGGGGGTATACTACGGTCA-3′) and H14020 (5′-ATAGCTTTTCTAGTCAGGTT-3′). Sizes of the PCR products obtained from these primer pairs are shown in FIGURE 2.

### *Detection of mtDNA Mutations by PCR*

The desired target sequence of mtDNA was amplified from 15~20 ng of each DNA sample in a 50-µL reaction mixture containing. 200 µM of each dNTP, 0.4 µM of each primer, 1 unit of Ampli-Taq DNA polymerase (Perkin-Elmer/Cetus, Roche

**TABLE 1. Contents of 8-OH-dG and lipid peroxides examined in this study**

| Type of tissue | 8-OH-dG/dG (×10-3%) | Lipid peroxide content (pmol/μg protein) |
|---|---|---|
| Normal endometrium | 0.17 ± 0.07 ($n$ = 5) | 1.50 ± 0.25 ($n$ = 10) |
| Chocolate cyst | 1.21 ± 0.10 ($n$ = 10) | 4.48 ± 0.44 ($n$ = 10) |
| Endometriotic endometrium | 0.72 ± 0.17 ($n$ = 4) | 2.61 ± 0.66 ($n$ = 7) |
| Myometrium | 0.55 ± 0.18 ($n$ = 5) | 1.85 ± 0.11 ($n$ = 6) |
| Myoma | 0.66 ± 0.30 ($n$ = 9) | 2.80 ± 0.72 ($n$ = 12) |
| Ovary | 0.62 ± 0.05 ($n$ = 9) | 2.68 ± 0.44 ($n$ = 10) |
| Peritoneal fluid | 0.73 ($n$ = 1) | 3.34 ($n$ = 1) |

Molecular System, Branchburg, NJ), 50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl (pH 8.3). PCR was performed for 30 cycles in a DNA thermal cycler (Perkin-Elmer/Cetus) using the thermal profile of denaturation at 94°C for 40 s, annealing at 55°C for 40 s, and primer extension at 72°C for 40 s. Long PCR proceeded with the thermal profile of denaturation at 94°C for 2 min, annealing at 68°C for 1 min, and primer extension at 72°C for 2 min.

*Detection of mtDNA Mutations by PCR*

The desired target sequence of mtDNA was amplified from 500 fmol of each DNA sample in a 20-μL reaction mixture containing A-dye terminator, T-dye terminator, C-dye terminator, G-dye terminator, dITP, dATP, dCTP, dTTP, 3.2 pmol primer, 10 units of AmpliTaq DNA polymerase (Epicentric Technologies, Oldendorf, Germany), and 1 × sequencing buffer. PCR was performed for 30 cycles of denaturation at 96°C for 30 s, 50°C for 15 s, and primer extension at 60°C for 4 min. PCR products were then separated by electrophoresis on 6% polyacrylamide gels containing 8 M urea at 65 W for 4 h.

## RESULTS

### *Higher Content of Lipid Peroxides in Endometriotic Tissues*

In this study, we detected lipid peroxides (as malondialdehyde) in the endometrium, myoma, adenoma, ovary, and chocolate cyst from women with endometriosis and from normal individuals (TABLE 1). The content of lipoperoxides was 1.50 ± 0.25 ($n$ = 10), 4.48 ± 0.44 ($n$ = 10), 2.61 ± 0.66 ($n$ = 7), 1.85 ± 0.11 ($n$ = 6), 2.80 ± 0.72 ($n$ = 12), 2.68 ± 0.44 ($n$ = 10), and 3.34 ($n$ = 1) in the normal endometrium, chocolate cyst, endometriotic endometrium, myometrium, myoma, ovary, and peritoneal fluid, respectively. Higher contents of lipoperoxides were detected in tissues from eutopic and ectopic endometriosis. There was approximately 6.2-fold higher lipid peroxides in the chocolate cyst than normal endometrial tissue

### *Higher Amount of 8-OH-dG in Endometriotic Tissues*

8-OH-dG was detected using HPLC-ECD in the endometrium, myoma, adenoma, ovary, and chocolate cyst from women with endometriosis and from normal individu-

**TABLE 2. MtDNA mutation with the 4,977-bp deletion and 5,335-bp deletion were examined in each sample collected from 46 women with or without endometriosis**

| Type of tisssue | Endometriosis (endometriosis/tissue no.) | 4,977-bp mtDNA deletion | 5,335-bp mtDNA deletion |
|---|---|---|---|
| Adenomyoma | 8/8 | 2/8 | 2/8 |
| Chocolate cyst | 12/12 | 4/12 | 7/12 |
| Endometrium | 4/8 | 2/8 | 1/8 |
| Myometrium | 3/8 | 1/8 | 0/8 |
| Myoma | 11/17 | 4/17 | 4/17 |
| Ovary | 2/3 | 1/3 | 0/3 |
| Peritoneal fluid | 1/1 | 1/1 | 0/1 |

als. The content of 8-OH-dG was 0.17 ± 0.07 (×$10^{-3}$%, $n$ = 5), 1.21 ± 0.10 (×$10^{-3}$%, $n$ = 10), 0.72 ± 0.17 (×$10^{-3}$%, $n$ = 4), 0.55 ± 0.18 (×$10^{-3}$%, $n$ = 5), 0.66 ± 0.30 (×$10^{-3}$%, $n$ = 9), 0.62 ± 0.05 (×$10^{-3}$%, $n$ = 9), and 0.73 (×$10^{-3}$%, $n$ = 1) in the normal endometrium, chocolate cyst, endometriotic endometrium, myometrium, myoma, ovary, and peritoneal fluid, respectively (TABLE 1). Higher contents of 8-OH-dG were detected in tissues from eutopic and ectopic endometriosis. There was approximately 7.2-fold higher 8-OH-dG in the chocolate cyst than in the normal endometrium.

### *Accumulation of Large-scale Deletions and DNA Rearrangement of mtDNA in Endometriotic Tissues*

Accumulations of mtDNA rearrangements have been shown in aged tissues, degenerated diseases, and several types of cancer in humans. In our study, we detected mtDNA mutations in such tissues as the endometrium, myoma, adenoma, ovary, and chocolate cyst from women with endometriosis and from normal individuals (TABLE 2). Using the primer pair, L8150-H14020, we generated three types of PCR products. A 5,871-bp fragment was produced from the wild-type mtDNA, an 894-bp DNA fragment was from the 4,977-bp deleted mtDNA, and a nearly 536-bp fragment was from a 5,335-bp mtDNA deletion. A scheme illustrates the strategy for determination of multiple mtDNA deletions in various human tissues by the long-range PCR techniques (FIG. 2). In the bottom panel, lanes 1 to 3 are from ovaries, myometrium, and endometrium from three individuals with endometriosis, respectively. Lane M is a 100-bp DNA ladder, and lane M′ is a 10-kb DNA ladder in FIGURE 2.

### *A 4,977-bp Deletion and a Novel Deletion of mtDNA in Endometriotic Tissues*

We applied primer-shift PCR to ensure the existence of a 4,977-bp deletion and DNA sequencing to identify the novel 5,335-bp mtDNA deletion found in endometriotic tissue. Furthermore, we sequenced the generated PCR products. In FIGURE 3, a schematic illustration is given of the nucleotide sequence flanking the junction sites at the 5′ end of the novel 5,335-bp deletion on the heavy strand of mtDNA in the endometriotic tissue. It shows a 10-nucleotide indirect repeat (5′-CCTAT-

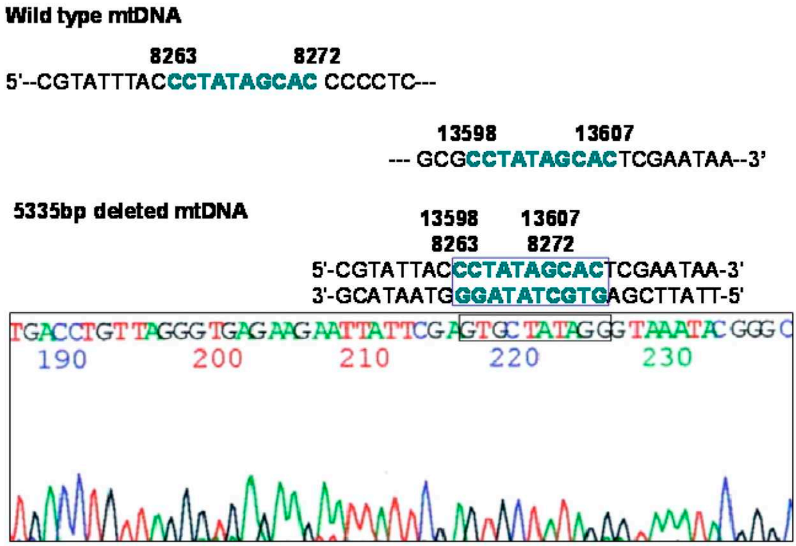


**FIGURE 3.** Schematic illustration of the nucleotide sequence flanking the junction sites at the 5′ end of the novel 5,335-bp deletion on the light strand of mtDNA in the endometrium. It reveals a three-nucleotide indirect repeat (5′-CCTATAGCAC-3′) located at the junction site at nucleotide position (np) 8263~8272 or np 13598~13607 (5′ to 3′) on the heavy strand of mtDNA.

AGCAC-3′) located at the junction sites at nucleotide position (np) 8263–8272 or np 13598–13607 (5′ to 3′) on the heavy strand of mtDNA.

## DISCUSSION

Endometriosis is a frequent disorder that commonly presents with infertility and pelvic pain and affects younger women of childbearing age. However, despite a growing number of reports on endometriosis, the pathophysiology of this disease remains poorly understood. Although the precise etiology of endometriosis is unclear, it is generally considered to involve multiple genetic, environmental, immunological, angiogenic and endocrine processes.

Recent studies have suggested that menstrual effluent contains factors that induce alterations in the morphology of the peritoneal mesothelium,[3] which may create adhesion sites for endometrial cells. Attachment of endometrial cells appears to be enhanced by induction of adhesion molecules[10] and overexpression of matrix metalloproteinases[11] and plasminogen activators,[12] which ensure local destruction of the extracellular matrix in endometriosis. After adhesion, endometrial cells proliferate and gradually invade the peritoneal tissue. Some factors induce vascularization of endometriotic implants, allowing their further development. Cytokines[13–15] and growth factors,[16] such as transforming growth factor-β, interleukin-8, interleukin-1, tumor necrosis factor, interferon-γ,[17] and vascular growth factor,[18] have been implicated as inducers of attachment, proliferation, and neovascularization.

Oxidative stress has been proposed as a potential factor involved in the pathogenesis of the disease.[8,19] This disease is characterized by the increased presence of activated macrophages, erythrocyte destruction, iron deposition,[20] and associated increases in growth-promoting activities and the production of inflammatory cytokines. In this study, significantly higher amounts of oxidative damage were detected in endometriotic lesions than in controlled normal endometrium such as the mtDNA rearrangement, 8-OH-dG, and lipoperoxide contents. In the future, we will explore the identification of the molecular pathway and factors of reactive oxygen species generation and eradication. A better understanding of the mechanisms of reactive oxygen species detoxification and further investigation of their effect on the peritoneal environment are essential to obtaining new insights into this disease and eventually developing new diagnostic and therapeutic strategies.

## ACKNOWLEDGMENTS

This work was supported by research grant NSC91-2314-B-038-013 from the National Science Council of the R.O.C.

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