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Effects of oxidants and antioxidants on proliferation of endometrial stromal cells

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Objective: To evaluate the effects of oxidative stress and antioxidants on proliferation of endometrial stromal cells.

Design: In vitro study.

Setting: Academic laboratory.

Patient(s): Women, with and without endometriosis, of reproductive age.

Intervention(s): Culture of endometrial stromal cells with antioxidants or with agents inducing oxidative stress.

Main Outcome Measure(s): Proliferation of endometrial stromal cells as determined by thymidine incorporation assay and 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) assay.

Result(s): Antioxidants induced a dose-dependent inhibition of thymidine incorporation: vitamin E succinate was inhibitory at 10–100 μM (by 43%–95%), ebselen at 10–30 μM (by 29%–77%), and *N*-acetylcysteine at 10–30 mM (by 52%–85%). In contrast, modest oxidative stress induced by hypoxanthine/xanthine oxidase (1 mM/3–30 μU/mL) stimulated proliferation by 40%–62%. H_2O_2 (1 μM) increased DNA synthesis by 56%. Comparable findings were obtained using MTT proliferation assay. Antioxidants inhibited proliferation: vitamin E succinate (100 μM) by 91%, ebselen (30 μM) by 81%, and *N*-acetylcysteine (30 mM) by 95%. Hypoxanthine/xanthine oxidase (1 mM/30 μU/mL) and H_2O_2 (1 μM) stimulated growth by 122% and 58%, respectively.

Conclusion(s): Reactive oxygen species may modulate growth of endometrial stroma. Under pathologic conditions such as endometriosis, increased oxidative stress and depletion of antioxidants may contribute to excessive growth of endometrial stromal cells. (Fertil Steril® 2004;82(Suppl 3):1019–1022. ©2004 by American Society for Reproductive Medicine.)

Key Words: Endometriosis, oxidative stress, antioxidants, endometrial stromal cells

Endometriosis is a common disorder characterized by ectopic presence of endometrial glands and stroma (1). It is a multifactorial condition associated with chronic inflammation within the peritoneal cavity. Oxidative stress is a component of this inflammatory process. An increase of autoantibodies related to oxidative stress has been documented in women with endometriosis (2). Shanti et al. found an increase in serum autoantibody titers to oxidized low-density lipoprotiens (Ox-LDL), malondialdehyde-modified LDL, and lipid peroxide-modified serum albumin (2). The same investigators have also shown that the peritoneal fluid of women with endometriosis contains higher levels of another marker of lipid peroxidation, lysophosphatidyl choline (3). Recently, Ota et al. (4) also found increased levels of several enzymes involved in the generation of reactive oxygen species (ROS) in patients with endometriosis and confirmed previous findings of increased oxidative stress in endometriosis. These observations support the concept that women with endometriosis are exposed to enhanced oxidative stress. Increased oxidative stress may be due to increased production of ROS or due to depletion of antioxidant reserve. The ROS are produced by normal oxygen metabolism, as well as by pathologic processes including inflammation (1). Cells possess a wide range of antioxidant systems protecting themselves from the toxic effects of excessive levels of ROS. These systems include, among others, vitamin E and superoxide dismutase (SOD). Increased oxidative stress in endometriosis may be induced by disruption of the

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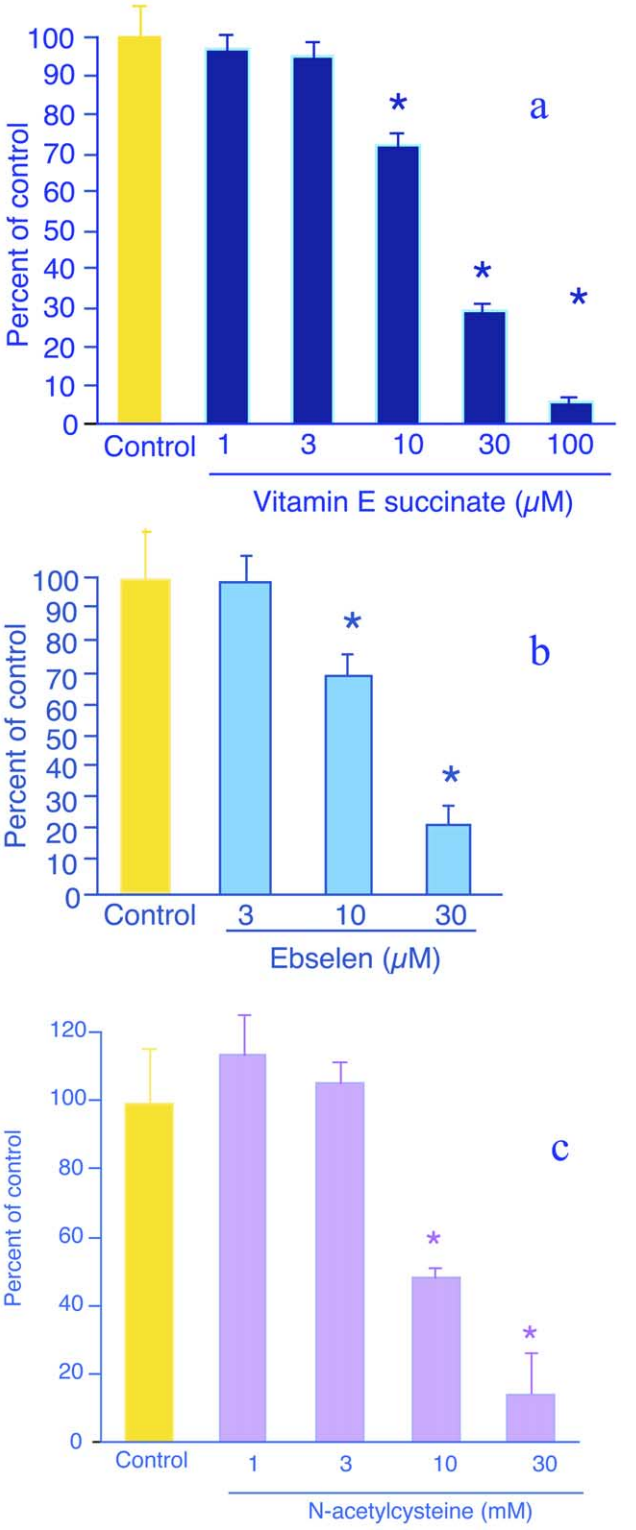
FIGURE 1

Effects of antioxidants on DNA synthesis of endometrial stromal cells cultured for 48 hours under serum-free conditions without (Control) or with treatments: (**a**) vitamin E succinate (1–100 μM), (**b**) ebselen (3–30 μM), or (**c**) *N*-acetylcysteine (1–30 mM). Each bar represents mean (±SEM) from eight replicates. *$P<.001$ significantly different from control.



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balance between ROS production and the level of antioxidants. Murphy et al. (3) showed that vitamin E levels are significantly lower in the peritoneal fluid (PF) of women with endometriosis, possibly due to a local decrease of antioxidants caused by excessive oxidative stress. Furthermore, several recent studies in women with endometriosis have shown altered expression of enzymes involved in defense against oxidative stress (4, 5). Enzymes associated with free radicals are present in the glandular epithelium of the endometrium and these levels vary dynamically throughout the menstrual cycle. In healthy women, levels of SOD and nitric oxide synthase (NOS) in the endometrium are low during the proliferative phase and increase during the early and midsecretory phase. In contrast, in women with endometriosis, levels of SOD and NOS remain constant throughout the menstrual cycle (6). Furthermore, expression of glutathion peroxidase ceases to vary during the menstural cycle in endometriosis (7).

Excessive oxidative stress may also contribute to formation of endometriosis-related adhesions. Indeed, Portz et al. (8) found that injection of antioxidative enzymes, such as SOD and catalase, into the peritoneal cavity prevented formation of intraperitoneal adhesions at endometriosis sites in rabbits.

In summary, these data strongly support the notion that endometriosis is closely related to excessive oxidative stress and antioxidant depletion. Because endometriosis is associated with inappropriate growth of endometrial cells, this study was designed to test the hypothesis that oxidative stress may induce excessive proliferation of endometrial stroma, whereas antioxidants may limit stromal proliferation.

MATERIAL AND METHODS

Endometrial samples were obtained from healthy subjects and patients with endometriosis during the follicular phase of the menstrual cycle. The study was approved by the Institutional Review Board of Yale University. Stromal cells were purified by enzymatic digestion and passing through a sieve (9). Cells were cultured in F12: Dulbecco's minimum essential medium (DMEM) with 1% antibiotic and 10% fetal bovine serum (FBS) and incubated at 37°C with humidified air and 5% CO_2 until 70%–80% confluent. Subsequently, the cells were transferred to 96-well plates with serum and phenol-free medium (at a cell density of 50,000 cells/well). The cells were incubated with vitamin E succinate (VES, 1–100 μM), ebselen (EBS, 0.3–30 μM), *N*-acetylcysteine (NAC, 0.3–30 mM), or hypoxanthine/xanthine oxidase (HX/XO, 1 mM of hypoxanthine and 1–1,000 μU/mL of xanthine

oxidase) and H_2O_2 (100 nM–10 μM) for 48 hours. Proliferation was assessed by determination of DNA synthesis using thymidine incorporation assay (10).

Results were confirmed using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) proliferation assay. This assay involves conversion of MTT to colored formazan by mitochondrial dehydrogenase of living cells, serving as a measure of cell number (11). For the purpose of this assay, the cells were transferred to fibronectin-coated 96-well plates with or without VES (100 μM), EBS (30 μM), NAC (30 mM), or HX/XO(1 mM of HX and 30 μU/mL of XO) and H_2O_2 (1 μM). Media and treatments were replaced after 48 hours of culture and the cultures were terminated at 96 hours. Cells were stained with MTT solution (5 mg/mL) for 4 hours, supernatants were then removed, isopropanol in 1 N HCl (24:1; vol:vol) was added and the optical density was measured at 570 nm (11).

Statistical analysis was performed using analysis of variance followed by pair-wise comparisons using Bonferroni correction.

RESULTS

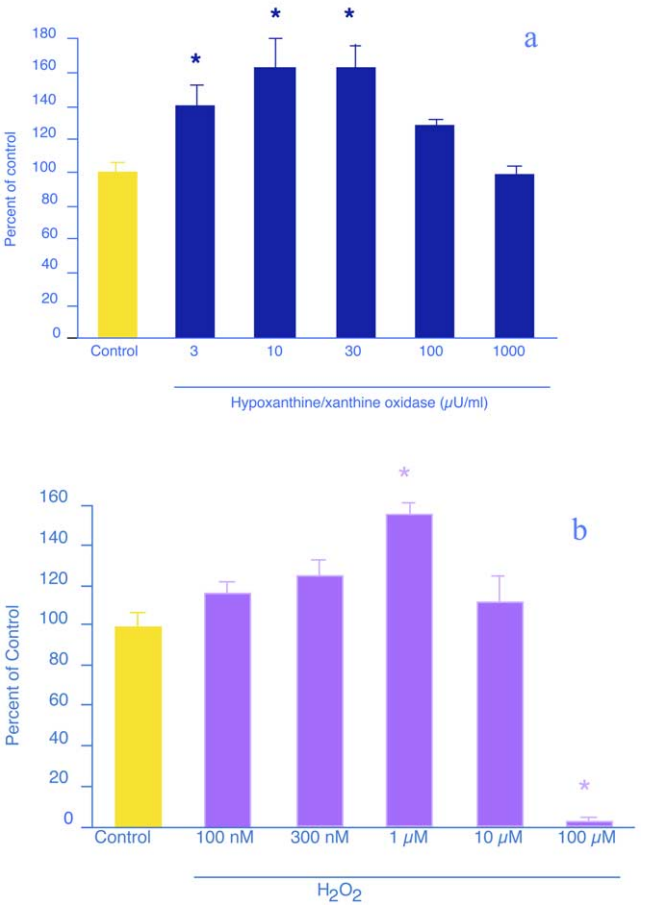
All tested antioxidants inhibited proliferation of endometrial stromal cells. Figure 1a summarizes the effects of VES on DNA synthesis, as measured by thymidine incorporation assay. The VES inhibited the DNA synthesis of endometrial stromal cells by 43%–95% ($P<.001$) in a dose-dependent fashion at concentrations ranging from 10–100 μM. Succinate alone did not show any inhibitory effect at concentrations up to 100 μM (not shown). The EBS is a seleno-organic compound with antioxidant glutathione peroxidase-like activity (12). Figure 1b demonstrates that EBS inhibited the proliferation of endometrial stromal cells at 10–30 μM concentrations by 29%–77% ($P<.001$). Similarly (Fig. 1c), NAC, a precursor of reduced glutathione (GSH) (13), inhibited endometrial stromal cell proliferation at 10–30 mM by 52%–85% ($P<.001$).

In contrast to antioxidants, moderate doses of oxidants HX/XO and H_2O_2 induced proliferation of endometrial stromal cells. Figure 2a summarizes the effects of HX/XO on DNA synthesis. The HX/XO stimulated proliferation of endometrial stromal cells at 3–30 μU/mL of XO by 40%–62% ($P<.001$). Notably, higher concentrations of XO (100–1,000 μU/mL) did not have a stimulatory effect on DNA synthesis. In a comparable fashion to HX/XO, H_2O_2 at 1 μM increased DNA synthesis by 56% ($P<.001$); a stimulatory effect was no longer observed at a concentration of 10 μM H_2O_2 (Fig. 2b). At the highest dose tested (100 μM), H_2O_2 induced profound inhibition of DNA synthesis.

The effects of oxidants and antioxidants on DNA synthesis were confirmed by MTT proliferation assay Figure 3. The VES (100 μM) inhibited MTT signal by 91% ($P<.001$), EBS (30 μM) by 81% ($P<.001$), and NAC (30 mM) by 95%

FIGURE 2

Effects of oxidative stress on DNA synthesis of endometrial stromal cells cultured for 48 hours under serum-free conditions without (Control) or with treatments: (**a**) hypoxanthine/xanthine oxidase (1 mM/3–1,000 μU/mL) or (**b**) H_2O_2 (100 nM–100 μM). Each bar represents mean (±SEM) from eight replicates. *$P<.001$ significantly different from control.



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($P<.001$). In contrast, HX/XO (30 μU/mL of XO) increased MTT signal by 120% ($P<.001$) and H_2O_2 (1 μM) by 58% ($P<.001$).

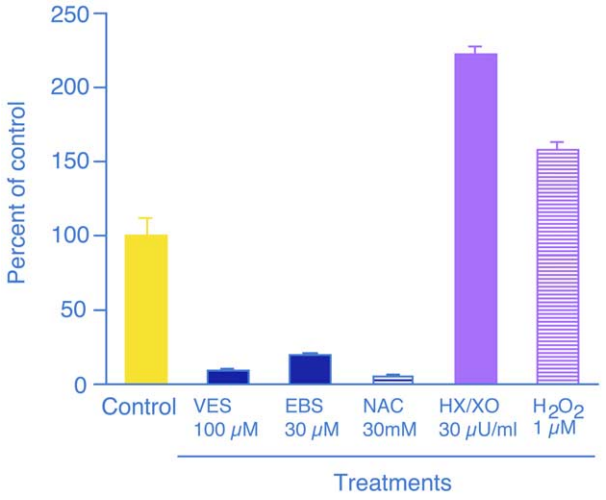
Comparable effects of antioxidants and oxidative stress were observed in cultures from women without and with endometriosis.

DISCUSSION

Previously, Murphy et al. (14) observed that RU486, a potent antiprogestagenic agent with antioxidant activity, decreases proliferation of epithelial and stromal cells. In our study, we focused more extensively on the evaluation of the effects of antioxidants and oxidants on endometrial stromal cells. The present study provides compelling ev-

FIGURE 3

The 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) proliferation assay. Effect of oxidants and antioxidants on proliferation of endometrial stromal cells. Endometrial stromal cells were cultured for 96 hours under serum-free conditions without (Control) or with vitamin E succinate (VES) (100 μM), ebselen (EBS) (30 μM), *N*-acetylcysteine (NAC) (30 mM), hypoxanthine/xanthine oxidase (HX/XO) (1 μM/30 μU/ml), and H_2O_2 (1 μM). Each bar represents mean (±SEM) from eight replicates. *$P<.001$ significantly different from control.



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idence that high levels of various antioxidants inhibit proliferation of endometrial stromal cells, whereas moderate oxidative stress has the opposite effect. We have also observed that the highest tested oxidative stress inhibits proliferation. These findings are consistent with the concept that the dose–response to oxidative stress is biphasic, whereby only moderate doses of ROS induce growth/proliferation, whereas higher doses do not, likely due to the direct cytotoxic actions and higher rate of cell death (e.g., apoptosis).

The results of this study have potential relevance to the pathophysiology of endometriosis. Inflammation and oxidative stress associated with endometriosis may explain excessive proliferation of endometrial implants, as well as the depletion of vitamin E in the PF observed in these patients (3). Ota et al. (4) demonstrated a consistently high expression of xanthine oxidase, an enzyme producing ROS in the endometrium of women with endometriosis, in contrast to cyclic variations in normal subjects. Similarly, they showed that enzymes associated with free radicals are present in the glandular epithelium of endometrium and their levels are pronounced in endometriosis. These findings suggest that the overall free radical metabolism is abnormal in endometriosis (4).

At present, the mechanisms involved in the generation of oxidative stress in endometriosis remain uncertain; nevertheless, it has been proposed that ROS may be induced by erythrocytes, apoptotic endometrial tissue, cell debris transplanted to the peritoneal cavity by menstrual reflux, macrophages, and inflammatory components (15). Thus, generated ROS may induce growth of endometriosis by promoting excessive growth of endometrial stromal cells.

In summary, our data demonstrate that oxidative stress and antioxidants modulate the growth of endometrial stromal cells. We propose that the increase of ROS and the decrease of antioxidant capacity associated with endometriosis may play an important role in inducing excessive proliferation of endometrial mesenchyme, ultimately resulting in the development/growth of endometriotic implants. Our study focused only on the effects of antioxidants and oxidative stress on endometrial stromal cells under in vitro conditions. Future studies are needed to further evaluate the effects of ROS and antioxidants on endometrial implants and on endometrial epithelial cells both in vitro and in vivo. In the long term, these studies may provide a basis for clinical use of antioxidants in the treatment of endometriosis.

References

1. Van Langendonckt A, Casanas-Roux F, Donnez J. Oxidative stress and peritoneal endometriosis. Fertil Steril 2002;77:861–70.
2. Shanti A, Santanam N, Morales AJ, Parthasarathy S, Murphy AA. Autoantibodies to markers of oxidative stress are elevated in women with endometriosis. Fertil Steril 1999;71:1115–8.
3. Murphy AA, Santanam N, Morales AJ, Parthasarathy S. Lysophosphatidyl choline, a chemotactic factor for monocytes/T-lymphocytes is elevated in endometriosis. J Clin Endocrinol Metabol 1998;83:2110–3.
4. Ota H, Igarashi S, Tanaka T. Xanthine oxidase in eutopic and ectopic endometrium in endometriosis and adenomyosis. Fertil Steril 2001;75:785–90.
5. Ota H, Igarashi S, Hatazawa J, Tanaka T. Endometriosis and free radicals. Gynecol Obstet Invest 1999;48:29–35.
6. Ota H, Igarashi S, Hatazawa J, Tanaka T. Immunohistochemical assessment of superoxide dismutase expression in the endometrium in endometriosis and adenomyosis. Fertil Steril 1999;72:129–34.
7. Ota H, Igarashi S, Kato N, Tanaka T. Aberrant expression of glutathione peroxidase in eutopic and ectopic endometrium in endometriosis and adenomyosis. Fertil Steril 2000;74:313–8.
8. Portz DM, Elkins TE, White R, Warren J, Adadevoh S, Randolph J. Oxygen free radicals and pelvic adhesion formation: I. Blocking oxygen free radical toxicity to prevent adhesion formation in an endometriosis model. Int J Fertil 1991;36:39–42.
9. Arici A, Head JR, MacDonald PC, Casey ML. Regulation of interleukin-8 gene expression in human endometrial cells in culture. Mol Cell Endocrinol 1993;94:195–204.
10. Duleba AJ, Spaczynski RZ, Olive DL, Behrman HR. Effects of insulin and insulin-like growth factors on proliferation of rat ovarian theca–interstitial cells. Biol Reprod 1997;56:891–7.
11. Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods 1989;119:203–10.
12. Muller A, Cadenas E, Graf P, Sies H. A novel biologically active seleno-organic compound—I. Glutathione peroxidase-like activity in vitro and antioxidant capacity of PZ 51 (Ebselen). Biochem Pharmacol 1984;33:3235–9.
13. De Vries N, De Flora S. *N*-acetyl-l-cysteine. J Cell Biochem Suppl 1993;17F:270–7.
14. Murphy AA, Zhou MH, Malkapuram S, Santanam N, Parthasarathy S, Sidell N. RU486-induced growth inhibition of human endometrial cells. Fertil Steril 2000;74:1014–9.
15. Santanam N, Murphy AA, Parthasarathy S. Macrophages, oxidation, and endometriosis. Ann NY Acad Sci 2002;955:183–98.