

Resveratrol and Endometrium: A Closer Look at an Active Ingredient of Red Wine Using In Vivo and In Vitro Models

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Abstract

Resveratrol is a natural phytoestrogen with antiproliferative properties present in red wine, grapes, and berries. Published reports on the effects of resveratrol in human endometrial function are limited. The objective of this study was to investigate the expression of estrogen receptor α (ESR1), Ki-67 (a proliferative marker), aryl hydrocarbon receptor (AhR), and members of the cytochrome P450 superfamily of enzymes (CYP1A1 and CYP1B1) in an in vitro and vivo assay. Alkaline phosphatase assay of estrogenicity was used to compare estrogen activity of different concentrations of resveratrol to estradiol (E2) and diethylstilbestrol (DES), using Ishikawa cell culture. Immunohistochemical expression of ESR1 and Ki67, and reverse transcriptase polymerase chain reaction of AhR, CYP1A1, and CYP1B1 were analyzed from xenograft implants of human endometrial tissue in ovariectomized immunodeficient RAG-2- γ (c) mice, after 30 days of treatment with subcutaneous pellets of E2, E2 plus progesterone (P4), or E2 plus resveratrol (6, 30, or 60 mg) for 30 days. Compared to E2, resveratrol acted as an agonist and antagonist of estrogen in low and high concentrations, respectively, when combined with E2. Xenografts of human endometrial tissues in RAG-2 mice exhibited reduced expression of ESR1 and proliferative activity (Ki67) with 60 mg of resveratrol. This study suggests that resveratrol, at high doses, has the potential benefit to reduce proliferation of human endometrium through ESR1.

Keywords

resveratrol, Ishikawa, human endometrium, endometriosis, estrogen receptor, cell proliferation

Introduction

Resveratrol (trans-3,5,40-trihydroxystilbene) is a natural phytoestrogen synthesized by plants following ultraviolet radiation. Red wine, grapes, and berries have significant concentrations of this compound. Resveratrol has been reported to have antineoplastic,¹ anti-inflammatory, and antioxidant properties.² In addition, resveratrol has been shown to inhibit growth of cancer cell lines.³⁻⁵ Researchers have investigated the antiproliferative action in endometriosis using in vivo animal and human models.⁶⁻⁸ Reduction in the size and activity of endometriotic implants has been observed with the use of resveratrol although uterine wet weight and endometrial proliferation and protein kinase B phosphorylation are increased by resveratrol treatment in the rat.⁹ Resveratrol also enhances E-cadherin and glycodeclin at sites of intercellular contact in endometrial cell lines, suggesting a possible action for resveratrol in promoting endometrial receptivity toward implantation.¹⁰

Dioxins are potentially toxic polycyclic aromatic hydrocarbons that are accumulating in our drinking water and food as a result of their production as chemical industry by-products. The

long environmental and biological half-life of dioxins coupled with their hydrophobic nature lead to accumulation in human and animal adipose tissue. Dioxins bind to and activate the aryl hydrocarbon receptor (AhR) triggering a variety of biologic responses. Dioxin has been shown to have effects on estrogen action and is implicated in the pathogenesis of endometriosis and other diseases affecting women.^{11,12}

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Endometriosis is a disease that affects 176 million women worldwide¹³ and is present in about 60% of adolescent women with chronic pelvic pain or dysmenorrhea.¹⁴ Endometriosis is defined as the presence of endometrial glands and stroma outside the uterine cavity. This condition is characterized by increased endometrial proliferation and responsiveness to estrogen and by endometrial resistance to progesterone.^{15,16} Both eutopic and ectopic endometrium in women with endometriosis overexpress estrogen receptor α (ER α ; ESR1)¹⁷ perhaps due to defective progesterone action or excessive estrogen production.¹⁸⁻²⁰ Estrogen receptor β (ER β ; ESR2) is overexpressed in endometriosis as well.^{21,22}

Resveratrol has been shown to be an AhR antagonist with possible protective activity against the effects of dioxins²³ and to have different estrogen action (agonist or antagonist) in different tissues.²⁴ Although resveratrol appears to have potential bioactivity in human endometrium, data on its effects in human endometrial function have been limited. To gain insight into the physiological aspects of resveratrol in human endometrium, this study will provide the basis for understanding pathophysiological conditions, such as endometriosis.

In this study, we investigated the effect of resveratrol on key components related to endometrial proliferation, that is, ESR1, Ki67, AhR, and its downstream target genes CYP1A1 and CYP1B1, using previously validated methods.^{25,26} These data on the effects of resveratrol on endometrial responses to estrogen will provide further insight into potential mechanisms of action of resveratrol that could help explain observed beneficial effects of this phytoestrogen.

Methods

Alkaline Phosphatase Assay

The relative estrogenicity of resveratrol was studied *in vitro*, using the alkaline phosphatase assay in a well-differentiated endometrial cell line (Ishikawa).²⁶ This cell line maintains ER α and ER β (ESR1 and ESR2), androgen receptor, and progesterone receptor and is sensitive to estrogen treatment.²⁷ Unless otherwise stated, all reagents were obtained from Sigma (St Louis, Missouri). Increased concentrations of resveratrol alone or in combination with estradiol (E2) treatment were used to study the relative estrogen activity of both substances. Ishikawa cells were cultured in medium containing phenol red-free Dulbecco Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) for 48 hours before seeding in 96-well plates (BD Bioscience, San Jose, California). The cells were washed twice with 1 \times phosphate-buffered saline (PBS), detached with trypsin-EDTA 0.25% then resuspended in culture medium. Approximately 2×10^4 cells were added to each well with 200 μ L medium and grown for 24 hours in a humid chamber at 37°C with 5% CO₂. The cells were then switched to phenol red-free DMEM/F-12 medium containing E2 (10^{-8} mol/L) and incubated up to 3 days.

Alkaline phosphatase assay was measured at 24, 48, and 72 hours. Estradiol or diethylstilbestrol (DES) at 10^{-8} mol/L, with or without resveratrol (10^{-4} - 10^{-12} mol/L), or the

antiestrogen Imperial Chemical Industries (ICI 182780— 10^{-6} mol/L, Zeneca Pharmaceuticals, Wilmington, Delaware) were incubated for 72 hours. The medium was changed daily and all experiments were performed in quadruplicates. After 72 hours of incubation, the alkaline phosphatase assay was performed. Cells were washed twice with PBS. Next, plates were frozen at -80°C for 10 minutes and then thawed to lyse the cell membranes. Cold soluble substrate of 20 μ L consisting of SigmaFast p-nitrophenyl phosphate tablets dissolved in deionized H₂O was added to each well on ice. The substrate solution produced a yellow color when alkaline phosphatase was present. After 1 to 3 hours of incubation at room temperature, the absorbance was read in a plate-reader at 405 nm (Biorad, Hercules, California). Each experiment was repeated 3 times with a minimum of 3 wells per each replicate assay.

Animal Protocols and Hormone Treatments

This study was approved by the Greenville Health System (GHS) Institutional Animal use and Care Committee protocol. The RAG-2 γ (c) knockout mice (C57BL/6J \times C57BL/10SgSnAi[-KO] γ c[KO]Rag-2— $n = 20$) were purchased from Taconic (Taconic Germantown, Philadelphia) and then housed under barrier and standard husbandry conditions. Female RAG mice were ovariectomized using sterile technique and allowed to recover for a least 1 week prior further surgery. The 20 animals were divided into 5 treatment groups ($n = 4$ in each group): (1) E2 alone, (2) E2 plus progesterone (P), (3) E2 plus 6 mg of resveratrol, (4) E2 plus 30 mg of resveratrol, and (5) E2 plus 60 mg of resveratrol. Resveratrol and the hormones were administered via implanted subcutaneous time-release hormone pellets (E2: 100 μ g/pellet; P: 7.5 mg/pellet—Innovative Research of America, Sarasota, Florida) that were placed along the dorsal paravertebral regions bilaterally as described.²⁸ The hormone pellets were designed to deliver a steady concentration daily dose of E2 and progesterone of 1.6 μ g/d for up to 60 days and 357 μ g/d for 21 days, respectively. Likewise, resveratrol pellets were designed to release a steady concentration of 6, 30, or 60 mg/d.

Human Xenografts

Human endometrial biopsies were obtained from cycling women at the time of laparoscopy using a pipelle device and the tissue was placed into Hams F12 medium for transport to the laboratory. Collection of human tissues was performed according to institutional review board-approved protocols at GHS, and all patients signed a written consent to allow the use of their tissue for research. In the laboratory, uniform samples (2 mm³) were dissected and placed into 2 separate positions in subcutaneous pockets on the abdomen of the ovariectomized, anesthetized RAG-2 mice. The incisions containing the xenograft implants were closed with 5-0 Vicryl (Ethicon, Piscataway, New Jersey) interrupted suture. At the end of 4 weeks, the mice were killed and the xenograft implants which were visible under the skin and easily identified were collected and analyzed (Figure 1).

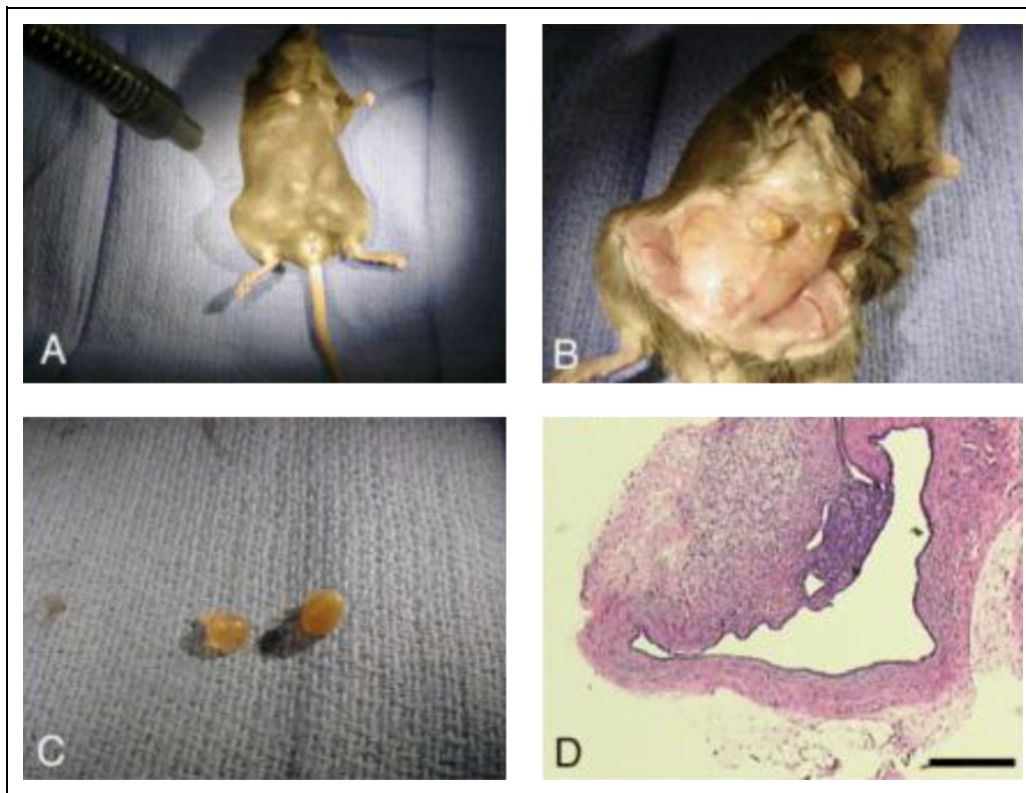


Figure 1. Composite showing the RAG2- γ (c) knockout mouse model for endometriosis. Subcutaneous implants of human endometrium were stimulated by treatment with estrogen pellets for 4 weeks. The implants are visible under the skin (A), visible endometrial xenografts (B) are harvested (C) and the histologic appearance is shown in D (scale bar = 100 μ m). In a typical experiment, 2 implants would be placed and the mice treated with E2 plus 1 of 3 doses of resveratrol prior to tissue collection after 30 days.

Immunohistochemistry

Each endometrial sample collected from the xenograft tissue was analyzed by immunocytochemistry for ER α (NCL-L-ER-6F11—dilution 1:250, Leica Biosystems, Newcastle, United Kingdom) and Ki67 (MKI67—dilution 1:75—Biogenex, San Ramon, California), a nuclear marker of cell proliferation, according to previous publication.²⁹ For this purpose, the collected xenograft tissue was fixed in 10% buffered formalin solution and then paraffin embedded. Sections of 5- μ m thick were obtained and immunostained for ESR1 and Ki67. Semiquantitative HSCORE was assigned in a blinded manner by 2 investigators (BAL and SCA) and the results were compared between groups. The use of HSCORE has been previously validated as a semiquantitative assay for immunohistochemical staining.³⁰

Reverse Transcriptase Polymerase Chain Reaction

Tissue samples were homogenized in 600 μ L of buffer RLT (Qiagen RNeasy Mini) using a Tissue Tearor (BioSpec Products, Bartlesville, Oklahoma) at 4°C. The Qiagen RNeasy Mini Kit (Valencia, California) was then used for the isolation of RNA from the lysate according to the manufacturer's instructions. RNA was quantified using the Quant-iT RiboGreen RNA

Assay Kit (Invitrogen Molecular Probes, Eugene, Oregon) and a CytoFluor Multi-Well Plate Reader Series 4000 (Perseptive Biosystems, Framingham, Massachusetts) according to the manufacturers' instructions.

The production and amplification of complementary DNA were measured in a single reaction in a DNA Opticon 2 System (MJ Research, Inc, Waltham, Massachusetts) using 2 \times SYBR Green Master Mix and Multiscribe Reverse Transcriptase (Applied Biosystems, Inc, Foster City, California). The primer sequences were: AhR, 5'-GTGCACAGCTCTGCTTCAGT-3' and 5'-CTACTCCACTTCAGCCACCA-3'; CYP1A1, 5'-CGG CCCC GGCTCTCT-3' and 5'-GTGTCGGAAGGTCTCCCAG GAT-3'; and CYP1B1, 5'-ATACAAGGCAGACGGTCCCT-3' and 5'-CTGCTCCTCCTCTTACCAG-3'. The reverse transcriptase polymerase chain reaction (RT-PCR) used the following cycles: 48 C for 30 minutes, 95°C for 10 minutes, and 40 cycles of 58°C for 1 minute and 95°C for 10 seconds. The RT-PCR data were performed using the 2^{-(Sample Ct - GAPDH Ct)} method, as described previously.³¹

The cytosolic AhR was analyzed because of its crosstalk with estrogen receptor and regulation of the expression of several isoforms of cytochrome P450, including CYP1A1 and CYP1B1. CYP1A1, and CYP1B1 are responsible for E2 hydroxylation.³² The AhR has been linked as an antiestrogenic and is a ligand to resveratrol.³³

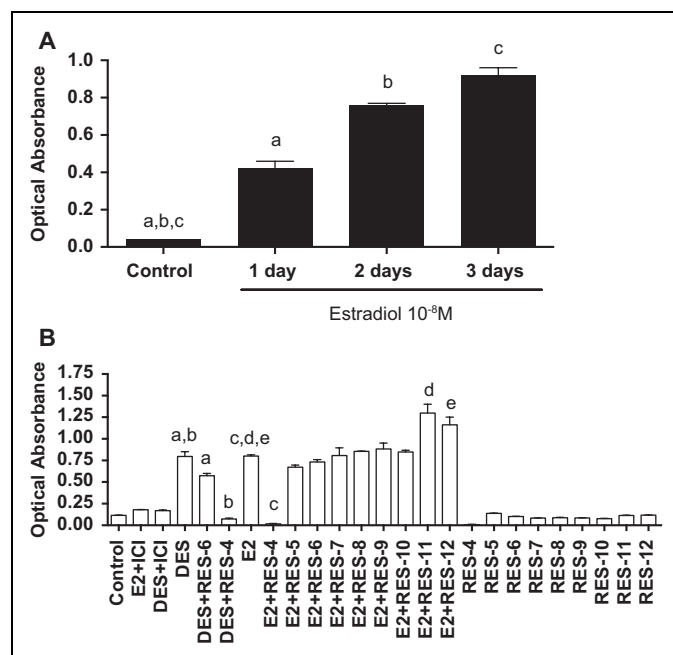


Figure 2. A, Estrogenicity in Ishikawa cells after treatment of estradiol during different time periods. Alkaline phosphatase assay of Littlefield provided a means to compare the estrogenicity. ANOVA $P < .0001$, $n = 4$ in each group. B, Estrogenicity obtained from Ishikawa cells treated for 3 days with different concentrations of resveratrol in the presence or absence of estradiol or DES at 10^{-8} mol/L, or with DES + ICI (an antiestrogen). Bars represent mean (\pm SEM); post hoc test (pairing letters), all $P < .05$. ANOVA indicates analysis of variance; DES, diethylstilbestrol; ICI, Imperial Chemical Industries; SEM, standard error of the mean.

Statistical Analysis

Comparison of results was made on Prism Software 6.0 (GraphPad, Inc, San Diego, CA) using analysis of variance with Dunn corrections for multiple comparisons. Statistical significance was assigned for $P < .05$.

Results

In Vitro Assay

Three days of incubation of Ishikawa cells with E2 at 10^{-8} mol/L resulted in the highest estrogen activity according to the alkaline phosphatase assay (Figure 2A). Based on these results, subsequent assays were done with 3 days of treatment. The comparison of different doses of resveratrol with E2 revealed that resveratrol at 10^{-4} mol/L had little estrogenic activity but combined with E2 it acted as an estrogen antagonist. In contrast, low doses of resveratrol (10^{-11} and 10^{-12} mol/L) with E2 displayed an agonistic effect (Figure 2B).

In Vivo Assay

A mouse model using human endometrial xenograft implants demonstrated that resveratrol exerted biologic activity on human endometrial tissues in vivo. Overall weight of the

recovered tissues after a month of treatment was not significantly different among treatment groups (data not shown).

Immunostaining for Ki67 and ESR1 revealed a different immunostaining expression among different forms of treatment (Figures 3 and 4). Mice treated with E2 plus P or E2 plus resveratrol showed decreased cell proliferation (Figure 3C) as well as reduced ESR1 (Figure 3A). The reduction in ESR1 was not as profound with resveratrol as with E2 plus P (Figure 4), but at the highest dose (60 mg) resveratrol exhibited a reduction in both ESR1 and Ki-67 in endometrial epithelial cells (Figure 3A and C). In stromal cells, ESR1 levels were reduced by E2 plus P, but not by resveratrol (Figure 3B), and Ki-67 expression was reduced specifically by resveratrol in human endometrial stroma in the presence of 60 mg/d of resveratrol (Figure 3D).

To further investigate the possibility that resveratrol was acting through AhR, we examined AhR itself, and 2 downstream target proteins of AhR, CYP1A1, and CYP1B1. The RT-PCR was performed on messenger RNA (mRNA) isolated from the implants recovered from the RAG mice treated with E2, E2 plus P, and E2 plus 60 mg of resveratrol. The reduction of AhR with resveratrol was not significant among any of the treatment groups (Figure 5). Likewise, mRNA expression of CYP1A1 and CYP1B1 was not significantly different compared to E2 or E2 plus progesterone (Figure 5).

Discussion

There is increasing interest in the dietary components of food and the possible benefits of specific compounds on health and disease. In the present study, we used both in vivo and in vitro techniques to investigate the role of an active component of red wine in endometrial activity. Resveratrol alone appears to have weak estrogen activity in Ishikawa cells. In the presence of estrogen, however, it acts as both an estrogen agonist and an estrogen antagonist at low and high concentrations, respectively (Figure 2B). These findings are in accordance with those published by Bowes et al whose work provided more serial dilutions with resveratrol in a cell line transfected with ER α and ER β .³⁴ The mechanism for the antagonistic effect at higher concentrations does not appear to be related to estrogen metabolism, since the effect is seen with the nonmetabolizable synthetic estrogen, DES, (Figure 1B). Increased cellular toxicity of high-dose resveratrol was not ruled out in this study but has not been shown by others.^{35,36}

We demonstrate that resveratrol slows endometrial proliferation of xenografts using the immunocompromised mouse model. Others have observed this reduction in proliferation.³⁶ Although the mechanism for this effect on blocking proliferation has been proposed to be antagonism of estrogen through AhR,³⁷ we find no difference in AhR activity in resveratrol-treated and untreated xenograft samples. We also did not find evidence for direct effects of resveratrol on ESR1 levels that paralleled proliferation data (Ki67 immunostaining). Immunohistochemical analysis of eutopic human implants in RAG-2 γ (c) knockout mice (Figure 3) suggests that the antiestrogenic, anti-proliferative activity of resveratrol may be related to reduction

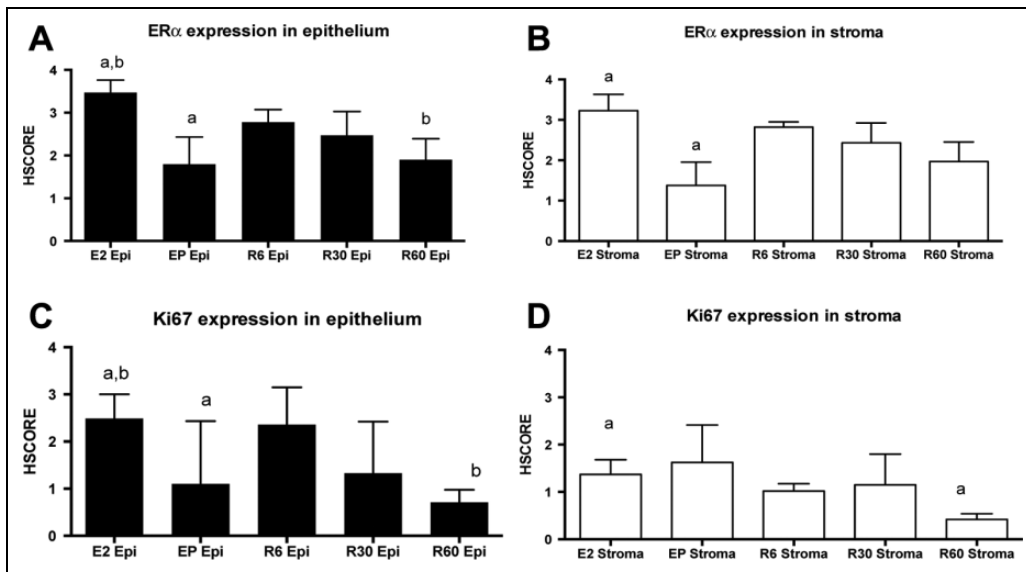


Figure 3. Immunohistochemical appearance of estrogen receptor α (ER α) and the proliferation marker Ki67 in human eutopic endometrium used as a xenografts tissue recovered from the mouse model (mean \pm SEM). A, ER α expression in epithelial endometrium is reduced in the presence of treatment with estradiol (1.6 μ g/d) and progesterone (357 μ g/d) and with estradiol (1.6 μ g + resveratrol 60 mg/d). B, ER α expression is reduced in stroma cells, compared to those treated with estradiol only. Ki-67 expression is reduced in endometrial stromal cells treated with estradiol plus progesterone (C and D). All ANOVA—Dunnett post hoc test (pairing letters)—All $P \leq .01$. Epi indicates epithelial endometrium; SEM, standard error of the mean.

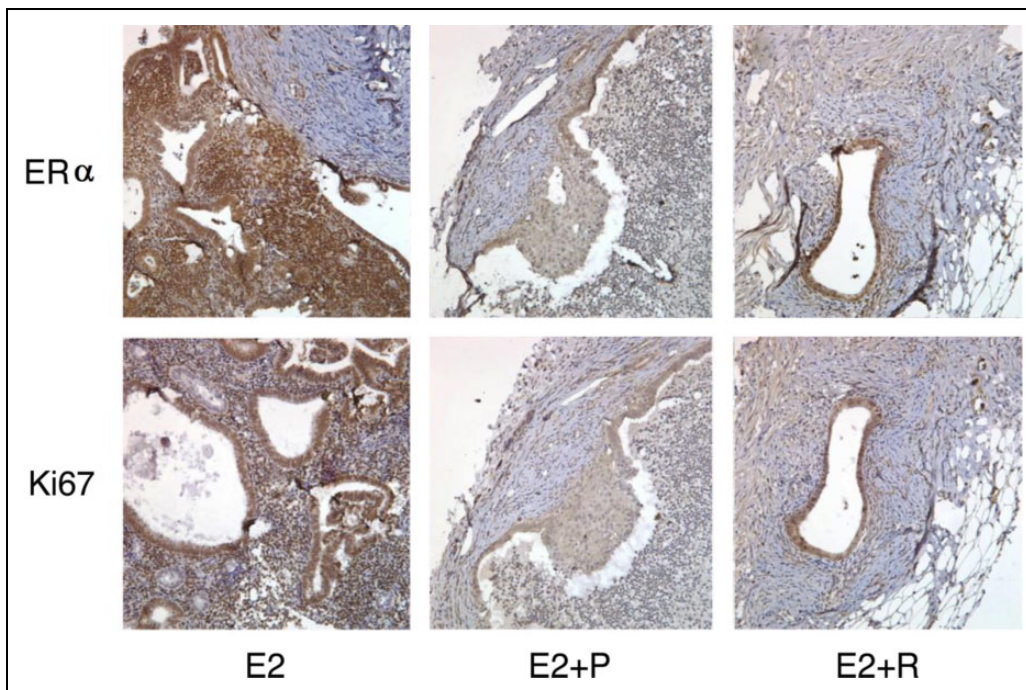


Figure 4. Photomicrograph of human eutopic endometrium used a xerograph in RAG mouse. Estrogen treated implants contained more estrogen receptors and Ki67, a marker of proliferation, than the implants from mice treated with E2 plus progesterone (E2 + P). In contrast to the E2-treated animals, mice treated with 60 mg of resveratrol (E2 + R) had reduced Ki67 and ER α immunostaining. Magnification $\times 400$. E2 indicates estradiol; P, progesterone; R, resveratrol; ER α , estrogen receptor α .

of ESR1 specifically in endometrial epithelium. These results are in accordance with those published by Kang et al.³⁶ Furthermore, resveratrol has been shown to act through

nongenomic action of alternative estrogen receptors such as GPR30³⁸ and might be acting through alternative estrogen receptors such as ESR2.²⁴

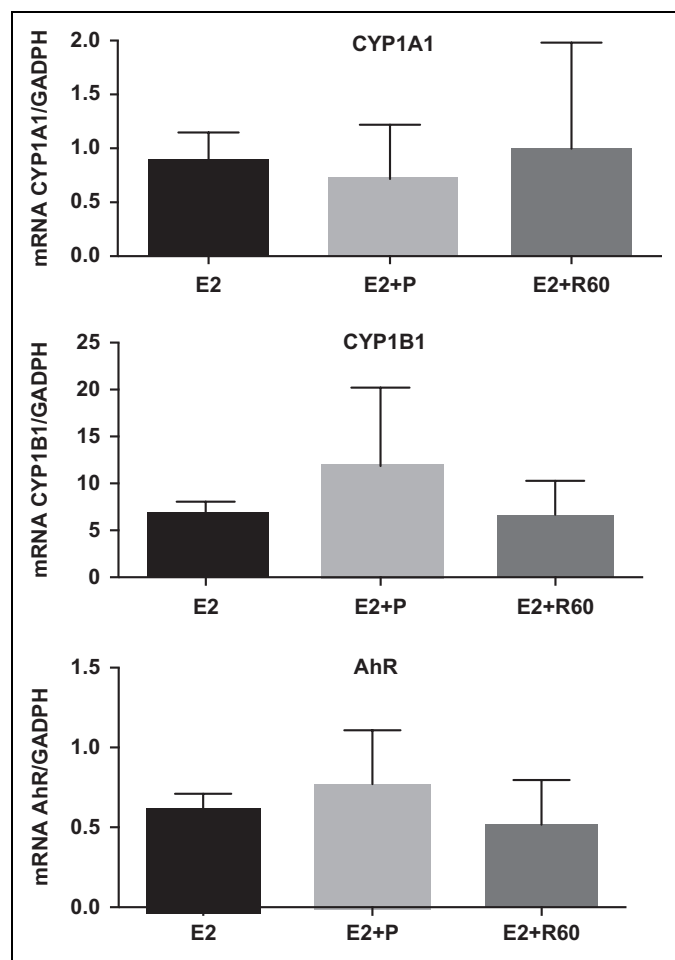


Figure 5. Comparisons are shown for AhR, Cyp1A1, and Cyp1B1 mRNA by RT-PCR (mean \pm SEM) in human xenografts endometrial tissue from RAG-2 mice treated with estradiol (E2), E2 plus progesterone (P), or E2 plus 60 mg of resveratrol. No significant difference was found among groups. AhR indicates aryl hydrocarbon receptor; mRNA, messenger RNA; RT-PCR, reverse transcriptase polymerase chain reaction; SEM, standard error of the mean.

Animal models have proven useful for the study of endometriosis. Although primate models have become the most applicable *in vivo* model to date,^{21,39} rodent models of endometriosis are more accessible and less expensive.^{40,41} Using the nude mouse model, other researchers were able to study xenografts of human endometrial tissue more effectively.⁴² The use of the RAG-2 knockout mice has been previously characterized and demonstrated to be an eloquent model for studying hormonal responses of human endometriotic tissues.²⁵ Our studies have used this particular mouse model for the study of the effect of herbal treatments on endometriosis.²⁹

Resveratrol appears to be a more potent *in vivo* antiestrogen at higher doses tested (60 mg). More recently, Chen et al reported that resveratrol inhibits the expression of CYP1A1 and CYP1B1 induced by dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin—TCDD) binding to AhR.³³ Likewise, Casper et al found that resveratrol competes with AhR in the presence of an AhR ligand, such as TCDD, and inhibits CYP1A1 activity.²³

With our sample size, we were not able to find a difference bigger than 50% among the mean expression of CYP1A1, CYP1B1, or AhR when xenograft implants were treated with E2 plus 60 mg of resveratrol (Figure 4). Possible explanations for this discrepancy might be related to the different dosages used in each study and the use of an active AhR agonist, dioxin or TCDD, in the studies by Chen et al. Indeed, the use 50 μ mol/L of resveratrol alone did not elicit the expression of CYP1A1 or CYP1B1 but 50 μ mol/L of resveratrol with 2 nmol/L of TCDD inhibits CYP1A1 and CYP1B1 expression at the mRNA and protein level.³³ Despite differences in the resveratrol doses used between these studies, the low concentration of resveratrol in combination with E2, used in our *in vitro* study, yielded a higher estrogenic activity, consistent with the results of Bhat and Pezzuto.²⁴

The findings of our study, including the reduction in endometrial ESR1, specifically in endometrial epithelium in response to resveratrol, suggest that this compound may provide a novel approach to the treatment of endometriosis. Bulun has suggested that ESR2 acts as an antiestrogen in endometriosis⁴³ and ESR2–ESR1 ratios are higher in endometriosis.⁴⁴ Resveratrol acts through both estrogen receptors⁴⁵ but has different actions in each estrogen receptor type.⁴⁶ Future studies will examine the possibility that resveratrol is acting to induce ESR2 as a mechanism for these noted effects. The dose of 60 mg/d of resveratrol used in our animal model corresponds to an unusual high dose of resveratrol for humans. However, a recent study⁷ using a 30-mg pill per day in 12 patients with endometriosis provided an important clinical ground to pursue a larger clinical trial. Oral micronized resveratrol (SRT501) can reach up to 185 μ mol/L in human plasma and it is well tolerated.⁴⁷ Together with our results, and the knowledge accumulated on the pharmacokinetics, bioavailability of oral resveratrol,⁴⁸ different formulations,^{47,49} and enhancers for bioavailability,⁵⁰ the path for larger and well-designed clinical trials is open.

In summary, using both *in vivo* and *in vitro* models we demonstrated that resveratrol acts as an antiestrogen *in vivo* and *in vitro* at high concentrations in Ishikawa cells and in human endometrium. The immunocompromised mouse model demonstrated that cellular proliferation in human endometrial xenografts declines in parallel with decreasing epithelial ER α levels. The mechanism of this reduced proliferation remains to be determined. Since overexpression or overactivity of estrogen receptor has been proposed as a central defect leading to infertility in women with endometriosis, and critical biomarkers of endometrial receptivity have been shown to be inhibited by overexpression of ESR1,¹⁷ it is possible that resveratrol may have other potential benefits for the treatment of infertility. Given the compounding effects of alcohol in reproductive-aged women and the impracticality and expense involved in achieving therapeutic doses, it seems unlikely that red wine would be a convenient source for resveratrol treatment by itself. Purified resveratrol compounds or better delivery systems for such compounds may someday prove beneficial.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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