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DDE-Induced Changes in Aromatase Activity in Endometrial Stromal Cells in Culture

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Environmental toxicants are thought to play a role in several estrogen-dependent diseases including breast cancer and endometriosis. Toxicant-induced increased aromatase activity, an enzyme complex that catalyzes the final rate-limiting step in the conversion of androgens to estrogens, has been reported in assays using placental microsomes and cancer cells in vitro. These data suggest that environmental toxicants can increase aromatase activity and thus increase local tissue estrogen levels, which could have implications for estrogen-dependent functions in target tissues. The objective of this study was therefore to quantify the effect of the stable breakdown product of DDT, 2,2-bis(*p*-chlorophenyl)ethylene (*p,p′*-DDE), a toxicant broadly detected in human adipose tissue, serum and follicular fluid, on aromatase activity in the endometrium, an estrogen-sensitive target tissue. Specifically, the effect of increasing log concentrations of *p,p′*-DDE on aromatase activity was determined in cultures of endometrial stromal cells (ESC). Relative to controls *p,p′*-DDE treatment significantly increased aromatase activity in ESC (135%). Moreover, ESC cells treated with *p,p′*-DDE were immunopositive for aromatase, whereas no aromatase staining could be demonstrated in control cultures. Our data demonstrate that *p,p′*-DDE treatment can increase aromatase activity in ESC in culture.

Key Words: Aromatase; toxicant; DDE; tissue culture; endocrine disruption.

Introduction

Although environmental toxicants with hormone-like activity can disrupt endocrine homeostasis with potentially disastrous consequences for wildlife and fish *(1)*, the consequences to human health are less clear and highly controversial.

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To date the bulk of the research attention has focused on the estrogen-like effects of xenobiotic compounds. It has been proposed, however, that the estrogenic activity of environmental toxicants is very low relative to estradiol and thus unlikely to contribute to the pathophysiology of estrogen-dependent diseases *(2,3)*. Although this view has been challenged by reports of low dose effects of estrogenic toxicants *(4,5)*, others have been unable to confirm these effects *(6,7)*. Regardless, environmental toxicants continue to be regarded as important contributing factors in the pathogenesis of estrogen-dependent diseases such as breast cancer and endometriosis *(8,9)*. We propose that, although not directly estrogenic themselves, environmental toxicants may induce estrogenic effects through alternative mechanisms and thus have important health consequences. One potential mechanism involves toxicant-induced increased available local estrogen production through increased aromatase activity.

The aromatase enzyme (CYP19) catalyzes the final rate-limiting step in the conversion of androgens or C19 steroids (androstenedione and testosterone) to estrogens, a reaction that involves removal of the C19 carbon and aromatization of the A ring of the steroid. It has recently been shown that several environmental toxicants including the Triazine herbicides and commonly used pesticides can increase aromatase activity in a human placental choriocarcinoma and adrenocortical carcinoma cell lines *(10–12)*. Toxicant-induced changes in aromatase activity have also been demonstrated in cell-free systems prepared from placental microsomes *(13)*. However, aromatase expression is regulated through several different promoter regions in a tissue-specific manner *(14)*. Therefore, it is unclear if results from cancer cell lines or cell-free systems can be generalized to cells derived from other tissues or to the whole organism. The endometrium is of interest because it is an estrogen-sensitive target tissue that is not thought to express aromatase *(15)*, although aromatase expression and activity have been documented in the endometrium of women with endometriosis *(16,17)*. Thus, changes in aromatase expression or activity in the endometrium could have important implications for gynecological diseases involving the endometrium such as endometriosis. The primary objective of this study, therefore, was to quantify environmental toxicant-induced changes in aromatase activity in cultures of endometrial stromal cells (ESC).

A broad spectrum of environmental toxicants have been detected and quantified in human serum, adipose tissue, and follicular fluid *(18–26)*. Of the many toxicants found in human reproductive fluids, the organochlorine pesticide 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT) and its stable breakdown product 2,2-bis(*p*-chlorophenyl)ethylene (*p,p′*-DDE) are of particular interest due to their persistence in the environment and their continued frequent detection in human tissues *(26)*. DDT is a pesticide that was introduced for vector control whose use in North America was banned in the early 1970s. Regardless, *p,p′*-DDE remains a global contaminant *(27)* that continues to be detected in human serum with a frequency between 50% and 99% of study subjects tested *(26,28,29)*. Furthermore, *p,p′*-DDE treatment has been reported to increase hepatic aromatase expression in male rats *(30)*. Because *p,p′*-DDE has been detected in serum, breast adipose tissue, and follicular fluid of Canadian women *(24,26,31)* and has been shown to increase hepatic aromatase expression and activity in male rats *(30)*, we hypothesized that environmentally relevant concentrations of *p,p′*-DDE will increase aromatase activity in human ESC in culture.

Results

Endometrial Stromal Cell Culture Characterization

Immunostaining by cytokeratin and vimentin demonstrated that the ESC cultures prepared as described in the Materials and Methods section consisted of approx 98% stromal cells and minimal to no epithelial cells (Fig. 1).

Aromatase Activity

The endogenous catalytic activity of aromatase in our enriched endometrial stromal cell cultures was blocked by incubation with 4-hydroxyandrostendione (data not shown). In enriched ESC cultures *p,p′*-DDE-treatment induced a significant increase in aromatase activity ($p = 0.009$, $n = 9$). The stimulatory effect of *p,p′*-DDE was significant ($p < 0.05$) at both 50 and 100 ng/mL (Fig. 2) causing a 130% and 135% increase in aromatase activity over baseline levels, respectively. *p,p′*-DDE–induced aromatase activity was similar in magnitude to the levels induced by the positive control agents prostaglandin E_2 (PGE_2, 10^{-6} *M*) and vomitoxin (250 ng/mL). In addition, treatment had no effect on cellular protein concentration suggesting that the concentrations of *p,p′*-DDE used in this study were not cytotoxic (Fig. 3).

Aromatase Expression

Immunopositive aromatase was demonstrated as a diffuse brown precipitate in the cytoplasm of some but not all ESCs treated with *p,p′*-DDE. ESCs immunopositive for aromatase were detected in *p,p′*-DDE-treated cultures whilst there was no positive staining in untreated (control) cultures (Fig. 4).

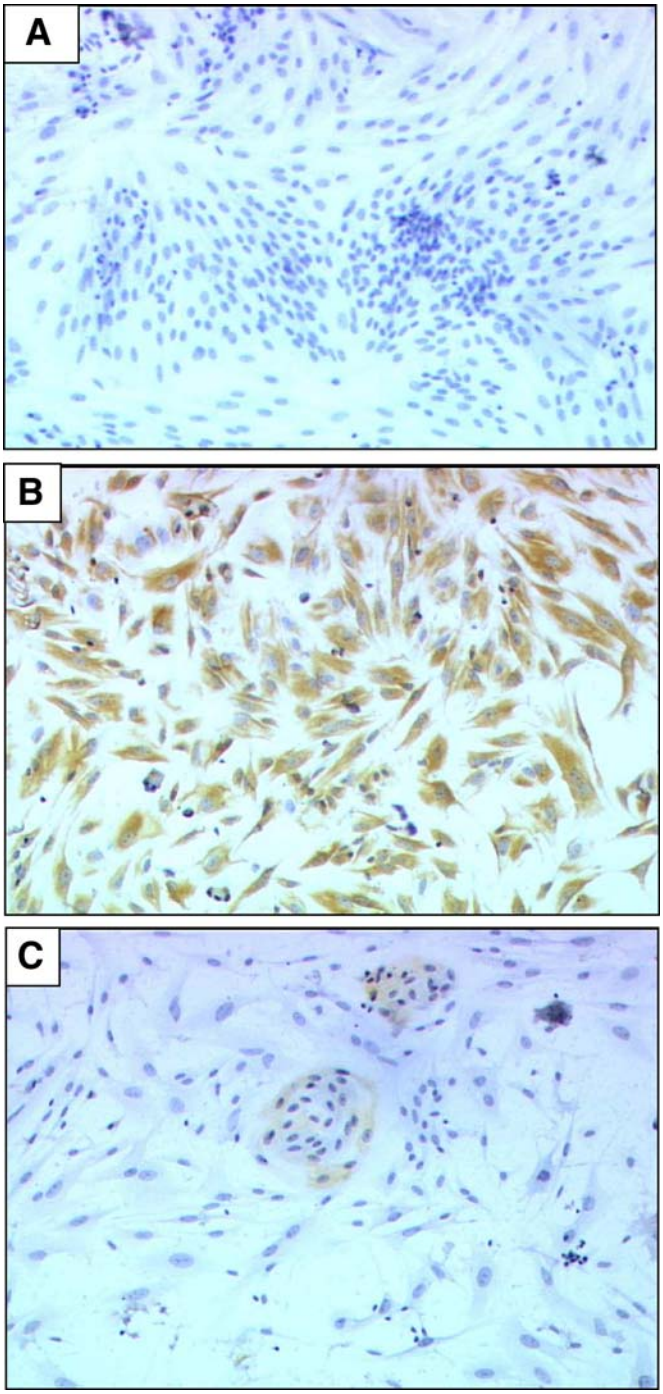

Fig. 1. Immunohistocehmical characterization of endometrial stromal cell cultures demonstrate the absence of immunopositive staining in (**A**) control (primary antibody was substituted with non-immune serum); but positive staining (**B**) for vimentin, a marker of mesenchymal cells, in endometrial stromal cells; and (**C**) no cytokeratin staining a marker for epithelial cells. Magnification bar is equal to 50 μm.

Discussion

The objective of this study was to determine the effect of environmentally relevant concentrations of *p,p′*-DDE, a persistent and ubiquitous environmental toxicant, on aromatase activity in primary cultures of estrogen-dependent ESC.

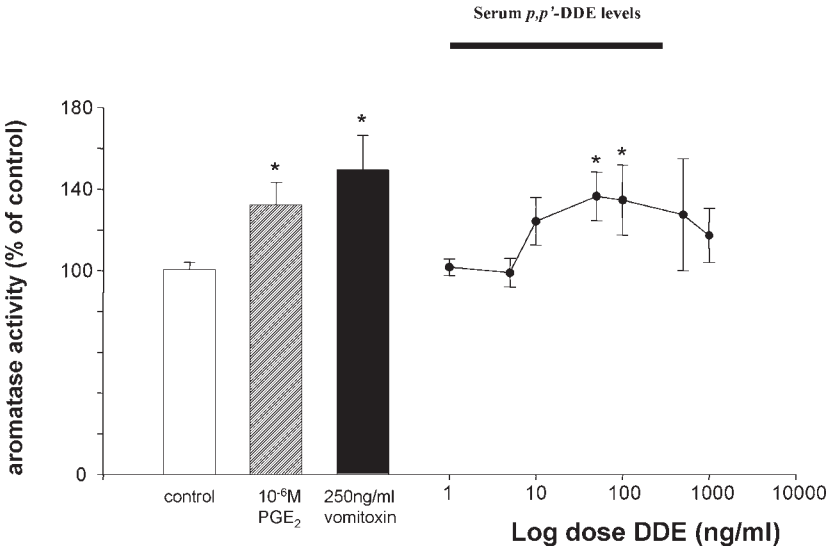

Fig. 2. Effect of treatment of enriched ESC cultures with *p,p′*-DDE compared to the positive controls PGE_2 (10^{-6} *M*) and vomitoxin (250 ng/mL). Results are shown as aromatase activity (% of control) ± SEM and means significantly different ($p < 0.05$) from controls (ANOVA followed by Bonferonni's multiple comparison vs. control) are indicated by an asterisk. The horizontal bar indicates the range of serum *p,p′*-DDE levels reported in the literature.

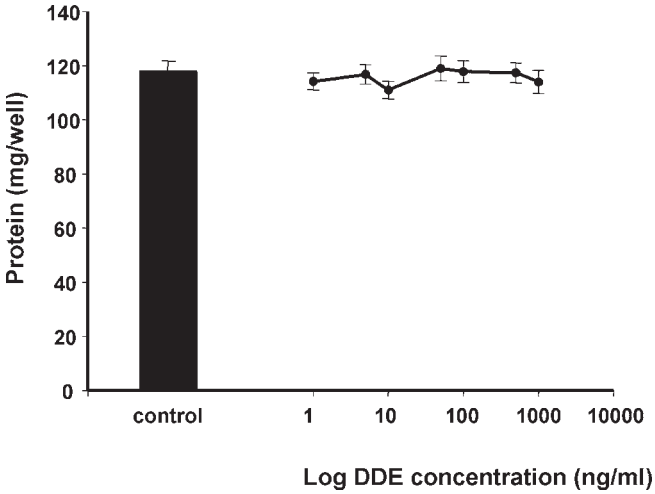

Fig. 3. Effect of *p,p′*-DDE treatment (24 h) on cellular protein levels from enriched ESC cultures. Results are shown as the mean ± SEM protein level (ng/well).

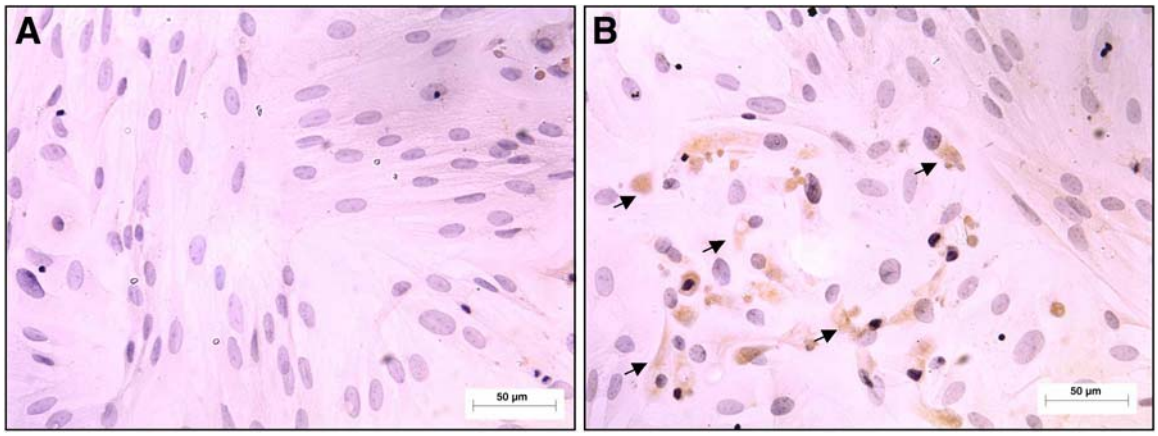

Fig. 4. Representative photomicrograph of immunopositive aromatase staining that was demonstrated as a diffuse brown precipitate in the cytoplasm of endometrial stromal cells treated in culture with *p,p′*-DDE (**A**) compared to untreated controls (**B**) which did not reveal any brown precipitate.

Our results demonstrate that *p,p′*-DDE, at concentrations representative of those measured in human serum *(26)* and follicular fluid *(24,26,31)*, can significantly increase aromatase activity in primary cultures of human ESC. While prior studies have demonstrated toxicant-induced increases in aromatase activity using transformed tumor cell lines *(10–12)*, this is the first report to document toxicant induced changes in aromatase activity using ESC in culture. Because aromatase catalyzes a terminal step in the steroidogenic pathway by converting C19 androgens into estrogens, increased aromatase activity in these tissues has the potential to increase local estrogen availability in estrogen-sensitive target tissues. Therefore, we propose that toxicant-induced changes in aromatase activity are a potentially important alternative mechanism of endocrine disruption that may also have relevance in the pathophysiology of estrogen-dependent diseases such as breast cancer and endometriosis.

In the present study we demonstrated that *p,p′*-DDE treatment can increase aromatase activity in ESC cultures, thus providing a mechanism by which an environmental toxicant can increase local tissue availability of estrogens. However, in contrast to our findings, *p,p′*-DDE treatment has been shown to either inhibit or have no effect on aromatase activity in a cancer cell line in vitro *(11,12)*. Reasons for the divergent results may stem from the use of non-transformed cells in the current study and differences in regulation of aromatase expression and activity in cells derived from different tissues. Aromatase expression is regulated via different promoter regions in a tissue-specific manner *(14)*. Hence, our results suggest that it may not be feasible to generalize results from aromatase activity assays conducted in tissues different from those of the target of concern. The aromatase enzyme is known to be expressed in steroidogenic tissues and in non-steroidogenic tissues associated with the pathophysiology of estrogen-dependent diseases. For example, aromatase expression and activity have been identified in breast cancer cell lines including MCF-7 cells *(32)*, and the expression and activity of aromatase are higher in mammary tumors compared with mammary adipose tissue of women without tumors *(33)*. Furthermore, aromatase expression has been identified in the endometrium from women with endometriosis relative to women without disease *(15–17)*. A number of investigators have also documented low-level aromatase activity in vitro in normal endometrium *(34–38)*. Therefore, we conclude that environmentally relevant concentrations of *p,p′*-DDE can increase ESC aromatase activity.

The mechanism of *p,p′*-DDE action in estrogen-dependent tissues is unknown but could involve estrogenic action of the parent compound or its metabolites, an increase in substrate availability for the aromatase enzyme, or an increase aromatase expression. It is unlikely that *p,p′*-DDE acts via an estrogenic mechanism in our culture system to induce aromatase activity, because this compound does not induce transcriptional activation of the human estrogen receptor *(39)*. Furthermore, *p,p′*-DDE is a stable compound that resists degradation and thus it is unlikely that metabolites are responsible for the observed effects. Moreover, the major metabolite of *p,p′*-DDE, methyl sulfonyl-*p,p′*-DDE was shown to be neither estrogenic nor anti-estrogenic in several distinct cell-based assay systems *(10)*. *p,p′*-DDE is a potent anti-androgen that has been shown to inhibit androgen receptor binding and androgen-dependent gene expression *(40,41)*. Although we cannot exclude the possibility that the increase in aromatase activity documented in the present study is the consequence of *p,p′*-DDE–induced displacement of androgens from the androgen receptor and thus increased availability of substrate for aromatase to act on, we propose that this is unlikely because the effects of *p,p′*-DDE diminish at concentrations that have been shown to act as a competitive antagonist with the androgen receptor *(40,41)*. In addition, we observed in the present study that *p,p′*-DDE treatment induced an increase in the number of aromatase immunopositive cells suggesting that it is an increase in aromatase expression vs increased substrate that accounts for the observed effects.

In summary, we have demonstrated that aromatase activity and expression in ESCs, which do not normally express the aromatase enzyme can be significantly increased by exposure to *p,p′*-DDE at levels that have been reported in serum, follicular fluid, and breast adipose tissue. These data suggest a mechanism by which *p,p′*-DDE, a non-estrogenic compound, can exert estrogenic effects in estrogen-sensitive target tissues and thus could have implications in the pathophysiology of estrogen-dependent diseases.

Materials and Methods

Cell Culture Techniques

Informed consent was obtained from nine fertile women aged 38–45 [mean (± SD) of 40.0 ± 2.6 yr] undergoing benign gynecologic surgery at McMaster University Medical Centre. All patients were of reproductive age and had not received endocrine therapy in the previous 6 mo before surgery and all procedures were carried out in accordance with approval of the McMaster University Research Ethics Board. Endometrial biopsy samples (1–2 g) were rinsed in HBSS containing 200 IU/mL penicillin, 0.2 mg/mL streptomycin, and 0.5 μg/mL amphotericin B (Sigma Aldrich, St Louis, MO) to remove blood and debris. Separation of the endometrial stromal cells was performed as previously described *(42)*. Briefly, following the HBSS wash, the tissue was centrifuged (5 min at 500*g*), the supernatant was removed and the tissue was minced into 1 mm^3 fragments in a sterile laminar flow hood. The tissue fragments were then digested with collagenase (2 mg/mL, Sigma-Aldrich) in dispersion medium (DMEM containing 5% FBS) (Gibco Life Technologies, Gaithersburg, MD), 200 IU/mL penicillin, 0.2 μg/mL streptomycin, and 0.5 μg/mL amphotericin B (Sigma-Aldrich) for 2.5 h at 37.5°C in a shaking

water bath. After the incubation period, the remaining tissue fragments were mechanically dispersed using a sterile glass Pasteur pipet. The homogenate was filtered through a 100 µm cell strainer, followed by filtration through a 40 µm cell strainer into a 50 mL polypropylene tube (BD Biosciences, Oakville, ON). Cells were pelleted by centrifugation (10 min at 725*g*) and resuspended in 3 mL of plating media (DMEM:F12 containing 4% FBS) (Gibco Life Technologies), 1% ITS$^+$, 100 IU/mL penicillin, 0.1 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Sigma-Aldrich). Red blood cells were removed by layering the cell suspension over 3 mL of Ficoll-Paque PLUS (Amersham Biosciences, Baie d'Urfe, PQ) in a sterile 15 mL polypropylene tube, centrifuging for 10 min (400*g*), and then removing cells at the media–Ficoll interface. The stromal cells were then pelleted by centrifugation (725*g* for 10 min) and resuspended in plating media, and the viability was assessed using the trypan blue exclusion method. Cells were seeded into 48-well Falcon tissue culture plates (BD Biosciences) at a density of 200,000 cells/well/0.5 mL. Media was changed after 48 h and the cells were treated after 72 h in culture (before reaching confluence). The purity of the ESC cultures was verified by immunohistochemical staining with cytokeratin (epithelial cell marker) and vimentin (mesenchymal cell marker). Briefly, the primary antibodies cytokeratin and vimentin (Dako Laboratories, Mississauga, ON) were used at a working dilution of 1:50. Immunopositive cells were visualized using an Envision peroxidase detection kit (Dako Laboratories, Mississauga, ON) with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, Mississauga, ON) as the chromogen and Carazzi's hematoxylin to counterstain the cells. Negative control wells were incubated in the presence of 1% bovine serum albumin in PBS.

Aromatase Activity

Cells were washed for 1 h in serum-free DMEM/F12 containing 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 µg/mL amphotericin B (Sigma-Aldrich) prior to treatment. All treatment solutions were prepared in this medium. Cells were treated for 24 h with increasing log concentrations (1–1000 ng/mL) of 2,2-*bis*(*p*-chlorophenyl)ethylene (*p,p'*-DDE) (Sigma-Aldrich). PGE_2 (10^{-6} *M*, Sigma-Aldrich) and vomitoxin (250 ng/mL, Sigma-Aldrich) were used as positive controls. At the end of the 24 h treatment period, the media was removed and replaced with 0.5 mL (2.5 µCi/mL) of [1β-^{3}H]androstenedione (Perkin-Elmer, Boston, MA) diluted in DMEM:F12 containing 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 µg/mL amphotericin B (Sigma-Aldrich) for 4 h at 37°C. Aromatase activity was determined by a tritiated water formation assay as previously described *(43)*. Briefly, aromatase activity was determined by transferring 300 µL of the incubation medium to borosilicate glass tubes, adding 300 µL of activated dextran-coated charcoal (250 mg/mL charcoal, 2.5 mg/mL T-70 dextran, Pharmacia, Uppsala, Sweden) and incubating for 2 h at 4°C. The samples were then centrifuged for 20 min at 2500*g* and the tritiated water content was determined by counting the supernatant in 5 mL of scintillation fluid (Aqueous Counting Scintillant, Amersham, Oakville, Ontario) in a liquid scintillation counter. The aromatase assay is based on the release of tritiated water and the specificity of the assay was determined by co-incubation with 4-hydroxyandrostendione, an irreversible inhibitor of the catalytic activity of aromatase *(44)*, to block the formation of tritiated water.

Aromatase Expression

Aromatase expression was examined in ESC following treatment with *p,p'*-DDE. Cells were fixed in 10% neutral buffered formalin following treatment and subsequently incubated in the presence of a mouse anti-human monoclonal aromatase antibody (clone MCA2077, Serotec, Raleigh, NC) at a dilution of 1:25. Immunostaining was identified by the avidin–biotin–peroxidase technique using the Vectastain kit (Vector Laboratories Inc., Burlingame, CA) with DAB as the chromogen (Sigma-Aldrich). Cells were counterstained with Carazzi's hemotoxylin, dehydrated and cover slipped for analysis. The primary antibody was substituted with non-immune serum for negative controls.

Statistical Methods

To control for variation in the number of cells in each well, the aromatase activity was normalized to the cell protein content in each well as determined by the Bradford method. Owing to variation in basal aromatase activity between patients, normalized aromatase activity was converted to a percentage of the control level for each culture. All data were analyzed by one-way ANOVA and tested for homogeneity of variance and normality using SigmaStat (SPSS, Chicago, IL). Where data failed the normality test and/or the equal variance test, data were analyzed using Kruskal–Wallis one-way ANOVA on ranks. Where significance was indicated ($p < 0.05$), data were compared to the control group using appropriate post-hoc comparisons ($\alpha = 0.05$).

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