

# Methoxychlor and Estradiol Induce Oxidative Stress DNA Damage in the Mouse Ovarian Surface Epithelium

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Estrogenic compounds such as 17 $\beta$ -estradiol (E<sub>2</sub>) and methoxychlor (MXC) induce oxidative stress damage in breast cells and mouse ovarian follicles, respectively. However, little is known about whether estrogenic compounds cause oxidative stress in the ovarian surface epithelium (OSE). Thus, this work tested the hypothesis that E<sub>2</sub> and MXC cause oxidative stress in the OSE. To test this hypothesis, we employed an improved mouse tissue culture assay in which OSE cells were treated with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>; positive control), MXC, or E<sub>2</sub>  $\pm$  the anti-oxidant vitamin E, or progesterone. The cells then were subjected to a novel direct immunofluorescent assay in which cells in the microtiter plate were reacted with antibodies that detect oxidative damage to DNA (8-hydroxy-2'-deoxyguanosine). The signal was identified with a tyramide Alexa Fluor fluorescent probe and quantified by microfluorimetry. Correction for cellularity was carried out for each well with a fluorescent DNA dye system (CyQuant) at a different wavelength. After 24 h, the mean Alexa Fluor CyQuant ratio was 11.3  $\pm$  0.9 for controls, 132  $\pm$  15 for H<sub>2</sub>O<sub>2</sub> treated positive control cells ( $p \leq 0.01$  from control), 105  $\pm$  6.6 for E<sub>2</sub> treated cells ( $p \leq 0.01$  from control), and 64  $\pm$  5.1 for MXC-treated cells ( $p \leq 0.01$  from control). After 72 h, the mean ratio was 121  $\pm$  10.6 for controls, 391  $\pm$  23 for H<sub>2</sub>O<sub>2</sub> treated cells ( $p \leq 0.01$  from control), 200  $\pm$  15 for E<sub>2</sub> treated cells ( $p \leq 0.03$ ), and 228  $\pm$  21 for MXC-treated cells ( $p \leq 0.01$ ). Further, vitamin E, but not progesterone, protected OSE cells from E<sub>2</sub>- and MXC-induced oxidative damage. This study demonstrates the feasibility of direct immunofluorescent quantitation of DNA adducts in cell cultures without DNA extraction. Moreover, these data indicate that E<sub>2</sub> and MXC produce oxidative DNA damage in the OSE, and that this damage is prevented by the anti-oxidant vitamin E.

**Key Words:** ovarian surface epithelium; oxidative stress damage; DNA adducts; estrogen.

An imbalance between free radical pro-oxidants and anti-oxidants has important implications for both physiological and pathological processes in the reproductive tract (Agarwal *et al.*, 2005). For example, superoxide radicals, possibly of neutro-

philic origin, are generated during ovulation and during regression of the corpus luteum in the ovary. These free radicals have important physiologic roles in regulating the ovarian follicular cycle, possibly through inhibition of steroid production (Behrman *et al.*, 2001). Nonphysiologic effects of free radicals include premature ovarian follicular atresia via apoptosis. Apoptosis is induced by many ovarian toxicants such as the xenoestrogen pesticide methoxychlor (MXC), and there is evidence that oxidative stress is central to this process (Gupta *et al.*, 2006a). Moreover, clinical observations have linked increased levels of reactive oxygen species to decreased female fertility (Agarwal *et al.*, 2006).

In distinction to the studies demonstrating that free radicals mediate apoptosis and senescence, and hence limit cell growth, is the growing body of evidence that intermediates of oxygen reduction induce DNA damage and are putative sources of neoplastic transformation. Valko *et al.* (2004) detail the sequence of DNA damage and formation of mutagenic 8-hydroxy-2'-deoxyguanosine (8-OH-dG), leading to GC-TA transversions and potential carcinogenesis. Further, Valko *et al.* (2006) note that oxidative DNA damage has been identified in various neoplasms, and suggest such damage may be some of the earliest genetic abnormality associated with malignancy. The ovarian surface epithelium (OSE) is of particular interest as a major source of ovarian cancer. Hence, the presence of 8-OH-dG as a marker of DNA mutation may serve as a sentinel event for subsequent ovarian neoplastic transformation. Because experimental evidence for ovarian carcinogenesis by exogenous agents is limited, the ability to detect abnormal DNA in the isolated OSE could prove of particular value in identifying agents with carcinogenic potential.

Previously, we have shown that MXC has a proliferative effect on the OSE, which is mediated through stimulation of cell cycle regulators and inhibition of apoptosis (Symonds *et al.*, 2005). Because MXC has been shown to induce oxidative stress in the mouse ovarian follicle (Gupta *et al.*, 2006a, b), we hypothesized it may also do so in the OSE. Using a modification of our previously described method to isolate OSE in microtiter plates (Symonds *et al.*, 2005, 2006), we exposed the cells to MXC and 17 $\beta$ -estradiol (E<sub>2</sub>) to compare their

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effects to the known free radical inducing agent hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) upon DNA oxidative damage, both in short-term (1 h) and long-term (24 and 72 h) timeframes. To measure abnormal DNA products, we developed an immunoassay directed against 8-OH-dG using an ultrasensitive tyramide-fluorescent system and fluorimetric quantitation. To counter cytotoxic effects of H<sub>2</sub>O<sub>2</sub> and possible stimulatory effects of MXC and E<sub>2</sub>, we corrected results for cellularity. Further, we evaluated the ability of the known anti-oxidant, vitamin E ( $\alpha$ -tocopherol), as well as progesterone to counteract the oxidative effect of H<sub>2</sub>O<sub>2</sub>. Finally, we assessed the effect of cotreatment of vitamin E with MXC, and with E<sub>2</sub> on 8-OH-dG products.

## MATERIALS AND METHODS

**Animals.** Female FVB mice were used in all experiments. The mice were maintained at the University of Maryland Central Animal Facility, provided food and water *ad libitum* and euthanized between postnatal days 60 and 90. Temperature was maintained at 22° ± 1°C, and animals were subjected to 12-h light:dark cycles. The University of Maryland School of Medicine Institutional Animal Use and Care Committee approved all procedures involving animal care, euthanasia, and tissue collection.

**Primary cultures of OSE cells.** Primary cells were isolated using a modification of previously reported techniques (Symonds *et al.*, 2005, 2006). In brief, ovaries were excised from mice aseptically and individually placed in 0.5 ml of Medium 199E (BioSource, Rockville, MD) with 1 mg of type IV collagenase (Sigma-Aldrich, Inc., St Louis, MO). After 30 min of incubation at 37°C, each ovary was vortexed for 2 min, the ovaries were removed, and the suspension was vortexed again for 2 min to disaggregate cell clusters. The suspension was diluted with additional Medium 199E containing 15% fetal calf serum (Atlanta Biologicals, Lawrenceville, GA) and an antimicrobial solution (10  $\mu$ l/ml) containing 10 mg/ml streptomycin, 10 mU/ml penicillin G, and 25  $\mu$ g/ml amphotericin (Sigma-Aldrich, Inc.). Aliquots containing 100–250 cells/100  $\mu$ l from a single ovary were pipetted into 96-well plates coated with type I collagen (CellCoat, Greiner Bio-One, Frickenhausen, Germany) with frequent mixing during aliquoting. Control and treatment wells were from the same suspension. Cultures were incubated at 37°C with 5% CO<sub>2</sub>, with a media change each third day, and cells were grown to confluence (usually for 7–14 days) before treatment. Use of the modifications, type IV collagenase and collagen coated plates, permitted plating a smaller number of cells and more rapid growth, with fewer growth failures and less stromal contamination than in our previous studies (Symonds *et al.*, 2005, 2006).

**Treatment of cultures.** In a first set of experiments, we evaluated the effect of E<sub>2</sub> and MXC on 8-OH-dguanine products after short-term (1 h) and long-term exposure (24 and 72 h). Controls cells were exposed to vehicle (dimethyl sulfoxide) or 15mM of H<sub>2</sub>O<sub>2</sub> (positive control). The dose of H<sub>2</sub>O<sub>2</sub> was based on pilot studies in the laboratory which indicated that this dose gave the best antibody response. Treated cells were exposed to 0.1 $\mu$ M of E<sub>2</sub> (Sigma-Aldrich, Inc.) or 3 $\mu$ M MXC (ChemService, West Chester, PA). Doses of E<sub>2</sub> and MXC were selected based on our previous studies (Symonds *et al.*, 2006). To assess the effects of vitamin E and progesterone in reversing the induction of 8-OH-dG products by H<sub>2</sub>O<sub>2</sub>, OSE cells were treated for 72 h with either 15mM of H<sub>2</sub>O<sub>2</sub>, 100mM of vitamin E ( $\alpha$ -tocopherol, Sigma-Aldrich T3251), or 100mM of vitamin E plus 15mM of H<sub>2</sub>O<sub>2</sub>. In some experiments, cells were treated with 1 $\mu$ M progesterone (Sigma-Aldrich P8783) or 1 $\mu$ M progesterone plus 15mM of H<sub>2</sub>O<sub>2</sub>. In a final experiment to determine the effect of vitamin E on E<sub>2</sub> and MXC, OSE cells were treated for 72 h either with 15mM of H<sub>2</sub>O<sub>2</sub>, 0.1 $\mu$ M of E<sub>2</sub>, 0.1 $\mu$ M of E<sub>2</sub> plus 100mM of vitamin E, 3 $\mu$ M MXC, or 3 $\mu$ M MXC plus vitamin

E. Doses of vitamin E and progesterone were based on available literature showing biologic potency at these levels (Rae *et al.*, 2004; Zhang and Omaye, 2000).

**Immunofluorescent assay for 8-OH-dG.** After treatment, cells were washed with phosphate buffered saline (PBS), air dried for 15 min, and fixed with 70% ethanol for 30 min. Plates were washed between each subsequent step three times with PBS and 100  $\mu$ l of reagent was applied at each step. After fixation, cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 10 min, followed by epitope retrieval with 0.5% dodecyl sulfate (Flukia, Sigma-Aldrich) for 10 min. Blocking was performed with 1% bovine serum albumin (BSA) for 60 min, followed by overnight incubation with 1  $\mu$ g/ml mouse monoclonal anti-8-hydroxy-2'-deoxyguanosine in 1% BSA (origin from Japanese Institute to Control Aging, Genox, Baltimore, MD). Molecular Probes Anti-mouse Tyramide Signal Amplification System with Alexa Fluor 594 (Molecular Probes, Eugene, OR) was utilized to quantify the bound anti-8-hydroxy-2'-deoxyguanosine. This procedure included incubation for 60 min with 1:100 horse-radish peroxidase in 1% blocking agent, followed by 10-min incubation with tyramide Alexa Fluor working solution constituted as per manufacturer's specifications. After washing, fluorescence was measured with a microtiter plate fluorimeter (CytoFluor 4000, PerSeptive Biosystems, Framingham, MA) with excitation at 590 nm and emission detection at 645 nm (50% gain), yielding a value in arbitrary fluorescent units. Incubation was carried out with CyQuant NF (Molecular Probes) reagent at 39°C for 60 min, followed by reading at excitation 485/emission 530 nm (30% gain). The Alexa Fluor value was then divided by the CyQuant value for each individual cell to give a ratio normalized for cellularity.

**Statistical analysis.** Data were analyzed using SPSS statistical software (SPSS, Inc., Chicago, IL). ANOVA with Tukey's *post hoc* test was used for multiple comparisons between treatment groups. Data are presented as means ± standard error of the mean ( $n = 8$  per treatment group). A  $p$  value  $\leq 0.05$  was considered significant.

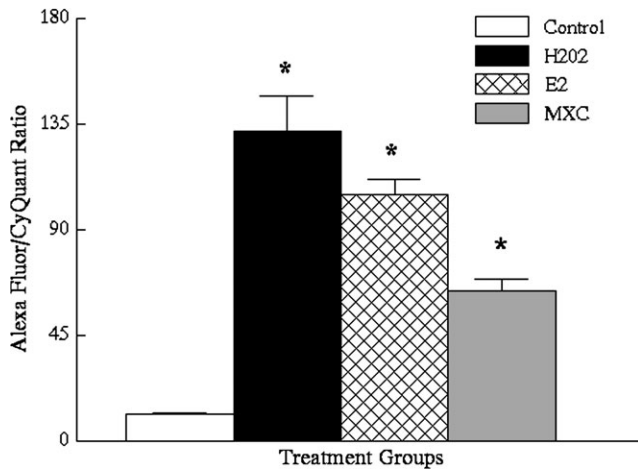
## RESULTS

The results indicate that short-term (1 h) exposure to H<sub>2</sub>O<sub>2</sub>, but not E<sub>2</sub> or MXC, significantly increased oxidative damage in the OSE. The mean adduct ratio (fluorimetric signal for anti-8-hydroxy-2'-deoxyguanosine to fluorimetric CyQuant signal) was 23.8 ± 1.7 for controls, 54.1 ± 8.2 for H<sub>2</sub>O<sub>2</sub>-treated cells ( $n = 8, p \leq 0.01$  from control), 33 ± 1.4 for E<sub>2</sub> treated cells ( $n = 8, p = 0.5$ ), and 34.9 ± 2.5 for MXC-treated cells ( $n = 8, p = 0.29$ ).

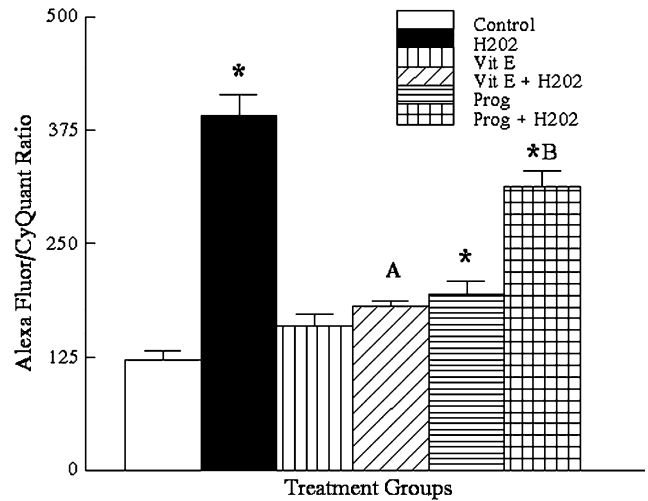
Further, the data indicate that longer-term (24 h) exposure to H<sub>2</sub>O<sub>2</sub>, E<sub>2</sub>, or MXC significantly increased oxidative damage in the OSE (Fig. 1). Specifically, the mean ratio was 11.3 ± 0.9 for vehicle controls, 132 ± 15 for H<sub>2</sub>O<sub>2</sub> treated cells ( $n = 8, p \leq 0.01$  from control), 105 ± 6.6 for E<sub>2</sub> treated cells ( $n = 8, p \leq 0.01$  from vehicle controls), and 64 ± 5.1 for MXC-treated cells ( $n = 8, p \leq 0.01$  from vehicle controls) (Fig. 1).

Similarly, the data indicate that 72-h exposure to H<sub>2</sub>O<sub>2</sub>, E<sub>2</sub>, or MXC significantly increased oxidative damage in the OSE. The mean ratio was 121 ± 10.6 for vehicle controls, 391 ± 23 for H<sub>2</sub>O<sub>2</sub> treated cells ( $n = 8, p \leq 0.01$  from vehicle controls), 200 ± 15 for E<sub>2</sub>-treated cells ( $n = 8, p \leq 0.03$  from vehicle controls), and 228 ± 21 for MXC-treated cells ( $n = 8, p \leq 0.01$  from vehicle controls) (Fig. 2).

Because H<sub>2</sub>O<sub>2</sub> caused oxidative damage in the OSE, we further evaluated whether vitamin E or progesterone could



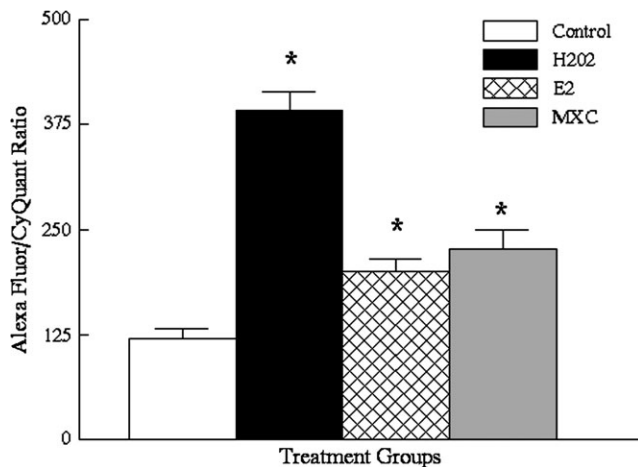
**FIG. 1.** Results of DNA adduct assay (expressed as ratio of arbitrary fluorescent units for anti-8-hydroxy-2'-deoxyguanosine/ CyQuant) for 24-h treated OSE cells. Control = vehicle-treated; H<sub>2</sub>O<sub>2</sub>-treated ( $n = 8$ ,  $p \leq 0.01$ ); E<sub>2</sub>-treated ( $n = 8$ ,  $p \leq 0.01$ ); and MXC-treated cells ( $n = 8$ ,  $p \leq 0.01$ ). Asterisks indicate significant differences from vehicle-treated controls by ANOVA.



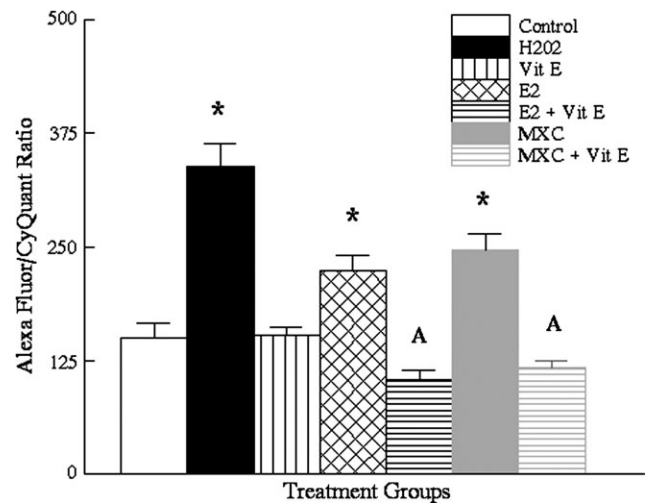
**FIG. 3.** Results of DNA adduct assay for 72-h treated OSE cells showing the effect of vitamin E and progesterone on H<sub>2</sub>O<sub>2</sub> induced oxidative stress DNA adducts (Vit E = vitamin E; prog = progesterone; other abbreviations as in Fig. 1). Asterisks indicate significant elevations from control (H<sub>2</sub>O<sub>2</sub>,  $n = 8$ ,  $p \leq 0.01$ ; prog,  $n = 8$ ,  $p \leq 0.01$ ). The letter A indicates no significant difference between vitamin E alone and vitamin E + H<sub>2</sub>O<sub>2</sub> ( $n = 8$ ,  $p = 0.88$ ). The letter B indicates a significant difference between progesterone alone and progesterone + H<sub>2</sub>O<sub>2</sub> ( $n = 8$ ,  $p \leq 0.01$ ).

protect OSE cells from H<sub>2</sub>O<sub>2</sub> induced oxidative damage (Fig. 3). The mean values were  $120 \pm 11$  for vehicle control cells,  $391 \pm 23$  for H<sub>2</sub>O<sub>2</sub> treated cells ( $n = 8$ ,  $p \leq 0.01$  compared with vehicle controls),  $159 \pm 14$  for vitamin E ( $n = 8$ ,  $p = 0.42$  compared with vehicle controls),  $181 \pm 5.5$  for vitamin E plus H<sub>2</sub>O<sub>2</sub> treated cells ( $n = 8$ ,  $p = 0.88$  compared with vitamin E treatment alone),  $194 \pm 15$  for progesterone treated cells ( $n = 8$ ,  $p \leq 0.01$  compared with vehicle controls), and  $314 \pm 17$  for progesterone plus H<sub>2</sub>O<sub>2</sub> treated cells ( $n = 8$ ,  $p \leq 0.01$  compared with progesterone alone). Thus, vitamin E, but not progesterone, prevented the production of DNA adducts by H<sub>2</sub>O<sub>2</sub> (Fig. 3).

In a second set of experiments, we evaluated the ability of vitamin E to counter the oxidative damage induced by E<sub>2</sub> and MXC (Fig. 4). The mean values were  $150 \pm 17$  for controls,  $338 \pm 25$  for H<sub>2</sub>O<sub>2</sub> treated cells ( $n = 8$ ,  $p \leq 0.01$  compared with vehicle controls),  $153 \pm 9$  for vitamin E ( $n = 8$ ,  $p = 1.00$  compared with vehicle controls),  $224 \pm 16$  for E<sub>2</sub> alone ( $n = 8$ ,  $p \leq 0.02$ , compared with vehicle controls),  $105 \pm 10$  for E<sub>2</sub> and



**FIG. 2.** Results of DNA adduct assay for 72-h treated OSE cells. Abbreviations and symbol as in Figure 1 (H<sub>2</sub>O<sub>2</sub>,  $n = 8$ ,  $p \leq 0.01$ ; E<sub>2</sub>,  $n = 8$ ,  $p \leq 0.03$ ; MXC,  $n = 8$ ,  $p \leq 0.01$ ).



**FIG. 4.** Results of DNA adduct assay for 72-h treated OSE cells showing the effect of vitamin E on E<sub>2</sub> and MXC-induced oxidative stress DNA adducts. Asterisks indicate a significant difference from vehicle-treated controls. The letter A indicates no significant difference between vitamin E alone and vitamin E + E<sub>2</sub> ( $n = 8$ ,  $p = 0.30$ ) or between vitamin E and vitamin E + MXC ( $n = 8$ ,  $p = 0.65$ ).

vitamin E treated cells ( $n = 8$ ,  $p = 0.30$  compared with vitamin E alone),  $247 \pm 17$  for MXC ( $n = 8$ ,  $p \leq 0.01$  compared with vehicle controls), and  $118 \pm 7$  (for MXC + vitamin E-treated cells ( $n = 8$ ,  $p = 0.65$  compared with vitamin E alone). Thus, treatment with vitamin E abolished the increased levels of 8-OH-dG adducts produced by E<sub>2</sub> and MXC (Fig. 4).

## DISCUSSION

The altered nucleoside, 8-OH-dG, is a major product of oxidative damage to DNA and represents a marker for such damage (Machella *et al.*, 2005). We have developed a quantitative immunoassay to measure this in cultured mouse OSE without DNA extraction based on tyramide-fluorescent localization of monoclonal antibodies to 8-OH-dG and adjustment for cellularity. Our results confirm previous work of Gupta *et al.* (2006a) that MXC induces oxidative stress damage to the ovary, and extends those findings to an additional tissue compartment, the OSE. Short-term (1 h) incubation with MXC did not significantly affect levels of 8-OH-dG in the OSE. However, after longer-term exposure (24 and 72 h), MXC produced a sixfold greater signal than untreated controls at 24 h exposure, and a twofold elevation at 72 h. Our previous studies on the effect of MXC on the OSE demonstrated proliferative effects, diminished apoptosis, and genomic expression of cell cycle regulators, Bcl-2, Bax, and cytochrome P450 (CYP450) enzymes, which occurred within a time frame of 14 days of treatment (Symonds *et al.*, 2005, 2006). Therefore, these current results suggest oxidative stress may be one of the earliest cellular alterations induced by MXC. The mechanism by which the response to MXC-induced oxidative stress relates to our previous demonstration of MXC-induced OSE proliferation and decreased apoptosis is at present unclear. However, the results appear to be consistent with the study of Young *et al.* (2004) demonstrating increased resistance to oxidative stress-induced apoptosis in human OSE which was oncogenically transformed with H-Ras<sup>V12</sup>. In the latter study, upregulation of anti-oxidant proteins in immortalized cells was the most prominent of 2200 proteins studied, suggesting that the growth characteristics of the OSE may be linked to its response to oxidative stress.

Our data indicating that E<sub>2</sub> treatment is as effective as MXC in inducing oxidative damage were unexpected because estrogen is generally considered an anti-oxidant in many tissues. For example, Lund *et al.* (1999) found that E<sub>2</sub> protected isolated ewe follicles from H<sub>2</sub>O<sub>2</sub> stress-induced apoptosis, and Miller *et al.* (2006) found that E<sub>2</sub> mitigated MXC-induced atresia. However, the effects of estrogen on oxidative stress appear complicated and may be dependent on the specific target cells. Patel and Bhat (2004) demonstrated that E<sub>2</sub> in comparison to a weak estrogen (17- $\alpha$ -ethinylestradiol) induced elevated levels of an oxidative stress marker, 8-*iso*-PGF(2 $\alpha$ ), in estrogen receptor alpha (ER- $\alpha$ ) positive

hamster kidney tumor (H301) cells. Likewise, Mobley and Brueggemeier (2004) demonstrated that E<sub>2</sub> treatment of ER $\alpha$  positive MCF-7 breast cancer cells, but not ER-negative MDA-MB-231 cancer cells, resulted in a diminished ability to metabolize peroxide, making the cells more sensitive to oxidative damage. Thus, E<sub>2</sub> appears to have a paradoxical anti-oxidant effect in some tissues (such as the follicle) and an oxidant effect on others (such as the OSE- and ER-positive breast cancer cells).

The reasons that E<sub>2</sub> acts as an anti-oxidant in the ovarian follicle and an oxidant in the OSE are unknown. It is possible that metabolic differences in ovarian follicles and the OSE lead to differences in the oxidant properties of E<sub>2</sub>. In the OSE, E<sub>2</sub> could be converted into pro-oxidant and carcinogenic metabolites. Specifically, catechol-estrogens that are produced by cytochrome P450-mediated hydroxylation of estrogens (Hiraku *et al.*, 2001; Tsuchiya *et al.*, 2005) in the position adjacent to the phenolic hydroxyl group and further oxidized to *ortho*-quinones have been implicated in these detrimental effects. From *ortho*-quinones, via a redox cycle, superoxide radical anions are generated which are ultimately responsible for oxidative damage (Bolton, 2002; Bolton *et al.*, 2000; Cavalieri *et al.*, 1997; Zhu and Conney, 1998). This hypothesis is supported by data reported by Patel and Bhat (2004), which demonstrate abolition of sensitivity to oxidative damage through inhibition of CYP450 enzymes. Previously, we demonstrated that the mouse OSE does have the appropriate CYP450 enzymes, specifically CYP 1B1, to produce catechol metabolites of E<sub>2</sub> (Symonds *et al.*, 2006), which could account for the oxidative metabolism of E<sub>2</sub> in the OSE. Although it would seem that granulosa cells have the ability to upregulate CYP 1B1 under certain circumstances (Vidal *et al.*, 2006), it is unclear that this metabolic pathway is significant for E<sub>2</sub> in the follicle. Moreover, E<sub>2</sub> may have the ability to recruit compensatory anti-oxidant enzymes more efficiently in some tissues than others. Alternatively, it is possible that the pro-oxidative properties of E<sub>2</sub> relate to differences in the distribution of ER in the ovary. The ovarian follicle predominately contains ER- $\beta$  expressed in the granulosa cells (Fitzpatrick *et al.*, 1999) and the OSE predominately contains ER- $\alpha$  (Pelletier *et al.*, 2000). It is possible that E<sub>2</sub> binding to ER- $\beta$  induces anti-oxidant pathways, whereas E<sub>2</sub> binding to ER- $\alpha$  induces oxidant pathways.

We also found it surprising that progesterone induced DNA adducts under the conditions of this study and had no protective effect, because previous studies had suggested that repair of OSE DNA damage is enhanced by exogenous progesterone administration (Murdoch, 1998). It is possible that different routes of administration (direct *in vitro* exposure in the present study) with different metabolic consequences account for these dissimilar results.

Potentially mutagenic 8-oxo-dguanine adducts have been previously identified in hen (Murdoch *et al.*, 2005), sheep, and human OSE, particularly in cells surrounding ovulation sites

(Murdoch and Martinchick, 2004). It is possible that cytokines released from inflammatory cells that accompany ovulation produce oxidative stress. Based on the results of this study, it is also possible that exposure to follicular fluid during ovulation may be contributory because follicular fluid contains levels of estrogen at least 100 times greater than serum levels (Lindgren *et al.*, 2002). Because frequency and duration of ovulation are recognized risk factors for ovarian carcinogenesis (Holschneider and Berek, 2000), oxidative induced DNA mutation at the time of ovulation could explain this association. Mutated OSE DNA could be especially prone to neoplastic transformation in epithelial inclusions (germinal inclusion cysts) formed by sequestration of surface epithelium during healing phase of the stigma. Such cells persist for long periods without apoptotic removal. As reported in this study, abnormal DNA adducts induced by MXC and E<sub>2</sub> are subject to prevention *in vitro* by the anti-oxidant vitamin E. This is in accord with the findings of Murdoch and Martinchick (2004), which indicate a protective effect of vitamin E against oxoguanine adducts in ewes. In addition, it provides an experimental basis for some epidemiological studies suggesting a protective effect of anti-oxidant intake against ovarian cancer (Fleischauer *et al.*, 2001).

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