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Transcriptional effects of Organochlorine *o,p′*-DDT and its Metabolite *p,p′*-DDE in Transfected MDA-MB 231 and MCF-7 Breast Cancer Cell Lines

Ehsan Zayerzade [1], Mohammad Kazem Koohi [2*], Azadeh Fardipour [3]

[1] Department of Biology, Faculty of Food Industry and Agriculture, Standard Research Institute, Karaj, Iran
[2] Department of Basic Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
[3] Department of Quality Control, Razi Vaccine and Serum Research Institute, Karaj, Iran

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Corresponding Author:
Mohammad Kazem Koohi
Department of Basic Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
E-mail: mkkoohi@ut.ac.ir

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Abstract

Background: The organochlorine DDT has estrogenic activity but the mechanism underlying the estrogenic activity of this pesticide remains unclear. In the present investigation, we studied the transcriptional effects of a synthetic organochlorine pesticide *o,p′*-DDT [1.1.1.-trichloro-2-(o-chlorophenyl)-2-p-chloriphenyl ethane] and its metabolite *p,p′*-DDE (2-2-bis(4/chlorophenyl)-1-1-dichloroethyl) on the bovine oxytocin and the thymidine kinase-ERE promoter by estrogen receptor α in MDA-MB 231 and MCF-7 breast cancer cell lines.

Materials and Methods: Cells were seeded for transfection into 12- well plates at a density of 100000 cells per well and were transfected with a total of 3 µg of plasmid DNA using calcium phosphate coprecipitation. *o,p′*-DDT and *p,p′*-DDE were used for stimulation of transfected MDA-MB 231 and MCF-7 breast cancer cell lines.

Results: *o,p′*-DDT showed no agonistic activity in MDA-MB 231 cells transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and estrogen receptor α. While, we found *o,p′*-DDT with an agonistic effect on both the oxytocin and the thymidine kinase-ERE promoter in MCF-7 leading to a more than two-fold stimulation of transcription at 10^{-5} M. Moreover, there was no agonistic effect with *p,p′*-DDE on transfected MCF-7 cells and MDA-MB 231 cell lines.

Conclusion: In conclusion, *o,p′*-DDT was not found to have any estrogenic activity in a classical mechanism in transfected MDA-MB 231 breast cancer cells while it showed estrogenic activity in a classical mechanism in transfected MCF-7 human breast cancer cell line.

Keywords: Organochlorinated pesticides; *o,p′*-DDT; MCF-7 cell line; Thymidine kinase-ERE promoter.

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Introduction

Environmental xenobiotics with potential estrogenic or hormonal activities are known as xenoestrogens. These compounds such as organochlorinated pesticides are ubiquitous, exhibit bioaccumulation, and act as estrogen agonists or antagonists, disrupting normal endocrine axes (1- 4). Xenoestrogens may act at the cellular and molecular levels, binding to both steroid and aryl hydrocarbon receptors exhibiting both dependent and independent receptor modulations of specific gene transcriptional elements (3, 5, 6). As a result, xenoestrogens have the potential to variably modulate cell proliferation, cell cycle progression, apoptosis and cytokine production in much the same way as 17-β-estradiol does (4, 7, 8). Organochlorinated pesticides release and persist in the environment. They accumulate in the food chain due to their lipophilicity and bioaccumulation characteristics. Human exposure occurs through inhalation of air, ingestion of food and skin contact, Thereby resulting in many public health problems.

They induce some diseases including endocrine toxicity, cancer, reproductive abnormalities, neurobehavioral disturbances and immunological toxicity via a number of mechanisms such as disruption of endocrine system, oxidative stress and epigenetic (9). Representative xenoestrogens include compounds such as DDT (o, p-dichlorodiphenyltri-chloroethane), a synthetic organochlorine pesticide, that has a weak estrogenic agonist activity (8, 10) and modulation of cell cycle and apoptosis (11, 12). Estrogen-like molecules act predominantly through estrogen mediated activation of transcription via estrogen responsive elements (ERE). Both estrogen receptor α and estrogen receptor β can interact with various cell cycle transcription factors (13, 21).

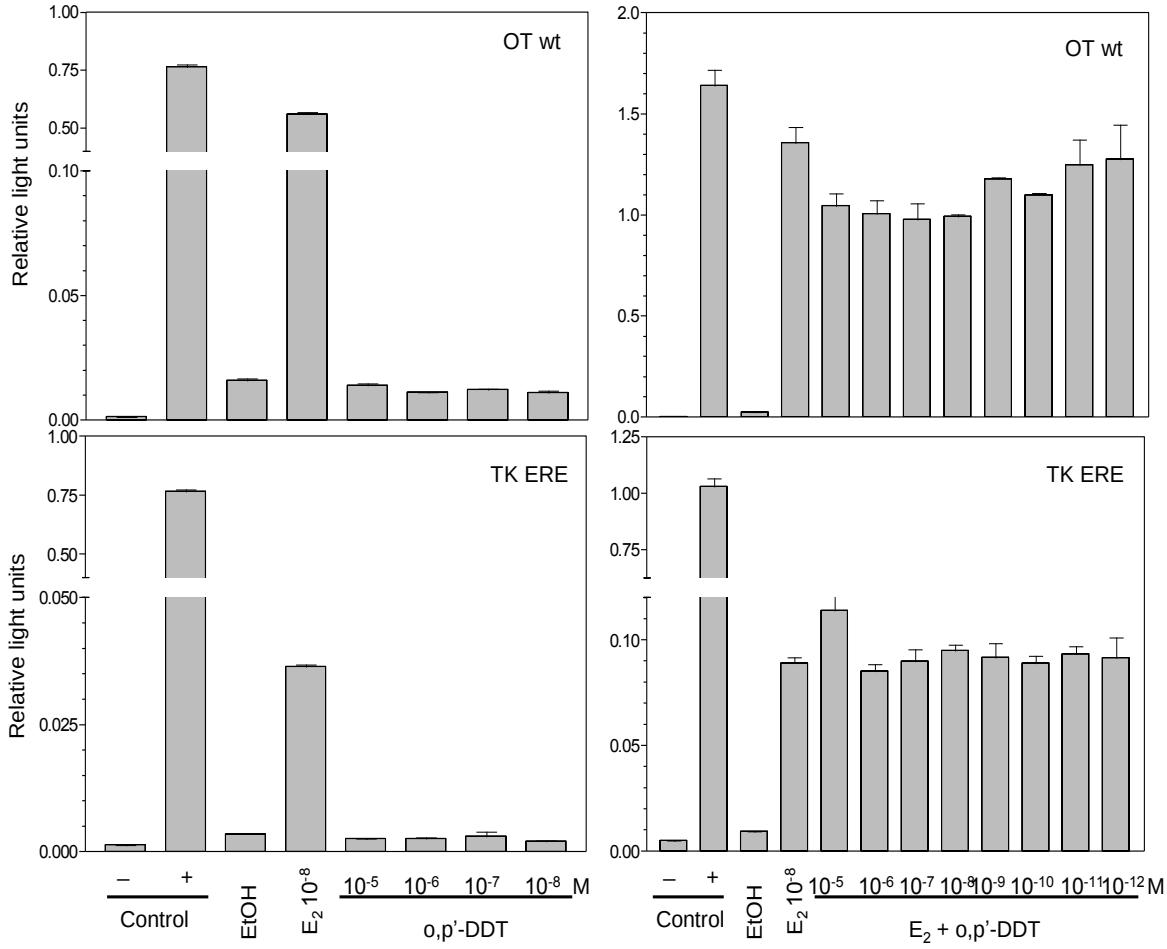

Figure 1. Transcriptional effects of, *o,p′* -DDT on the oxytocin and the thymidine kinase-ERE promoter by estrogen receptor α. MDA-MB 231 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and estrogen receptor α. Then, treated with different amounts of *o,p′*-DDT alone or simultaneously with estradiol (E2 10-8 M). There was significant difference at $p < 0.05$ according to unpaired Student t-test**.**

Kuiper et al and Lascombe et al described a stimulation of the transcriptional activity of ER α by DDT in reporter gene systems (14, 15). However, the molecular mechanisms of DDT and DDE as xenoestrogen are not well understood. In this work we studied the molecular mechanisms (classical and non-classical) of *o,p′*-DDT and *p,p′*-DDE by a detailed analysis of the components of the regulatory system controlling the oxytocin promoter. Transient transfection experiments were carried out using the breast cancer cell line MDA-MB 231 and MCF-7.

Materials and Methods

Cell Culture

MCF7 and MDA-MB 231 breast cancer cell line were seeded in flasks (Nunc, Wiesbaden, Germany) in phenol red free Dulbecco's modified Eagle's medium (DMEM) (Gibco, Karlsruhe, Germany), supplemented with 10% of steroid-free (charcoal-stripped) fetal calf serum (FCS) plus 1% L-glutamine (Sigma, Taufkirchen, Germany) and 0.5% penicillin/ streptomycin (Sigma) and incubated at 37 °C, 5% CO2. Before reaching confluence, the cells were

detached from the bottom of the flask by adding 1.5 mL 1x Viralex TM-EDTA solution (0.05% trypsin, 0.5 mM EDTA) followed by incubation at 37 °C for 2 min. Cells were then harvested by centrifugation for 2 minutes at 6500 g and resuspended in culture medium. The resuspended cells were re-plated either into another flask at 1.3 density or into 12-well plates (Nunc) at a density of 100000 cells per well for transfection.

Compounds

o,p'-DDT [1.1.1.-trichloro-2-(o-chlorophenyl)-2-p-chloriphenyl) ethane] was purchased from Chem Service, West Chester, USA. *p,p'*-DDE (2-2-bis(4/chlorophenyl)-1-1-dichloroethyl) and 17ß-estradiol were purchased from Sigma-Aldrich.

Transfection Assays

In all wells of 12-well plates (Nunc),10^5 MDA-MB 231 and MCF7 were seeded and transfected on the next day with 3 µg of plasmid DNA using calcium phosphate coprecipitation (Pro Fection Mammalian Transfection System, Promega, Mannheim, Germany). The transfected plasmid DNA included 1µg of promoter-luciferase plasmid, 1.5µg of the relevant ER expression vector and 0.5µg of a LacZ control vector driven from the CMV early promoter. Following transfection, cells were incubated for 16 h, then medium was changed, and cells were stimulated with oesterogen and phytoestrogens in 2.5 ml fresh culture medium per well .In the control group oestrogens and phytoestrogens were not added. Twenty-four hours after stimulation, cells were washed two times with phosphate-buffered saline and then 100 µl of lysis buffer (Promega) were added and the plates were incubated at room temperature for 5 min with slow shaking.

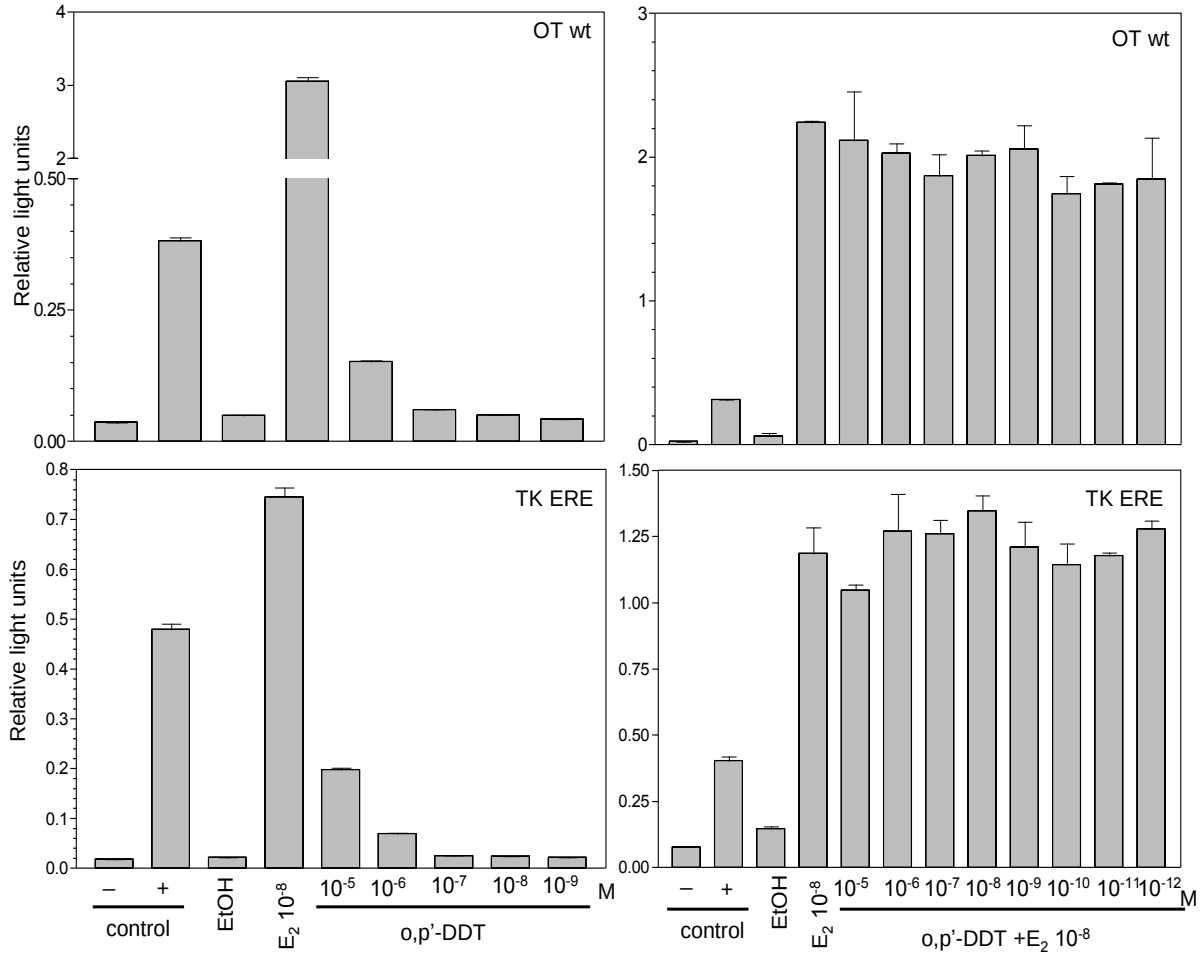

Figure 2. Transcriptional effects of *o,p'*-DDT on the oxytocin promoter and the thymidine kinase-ERE promoter by estrogen receptor α in the MCF-7 cells. MCF-7 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and estrogen receptor α. Then, treated with different amounts of *o,p'*-DDT alone or simultaneously with estradiol (E2 10^{-8} M). There was a significant difference at $p < 0.05$ according to unpaired Student t-test.

The cell lysates were then scraped off the plates with cell scrapers and transferred to Eppendorf tubes on ice. After centrifugation for 1 min at maximum speed in an Eppendorf microfuge, the supernatants were transferred to new tubes and used immediately for determination of luciferase and b-galactosidase activity. For measuring luciferase activity, the Firefly Luciferase Reporter Gene Assay System (Promega) was used according to the manufacturer's instructions. Twenty microlitres of cell lysate were added to 100 µl of Luciferase Assay Reagent and the luminescence measured for 5 s with 2 s delay time in a Berthold Sirius luminometer. β-galactosidase activity was measured using the Galacto-Light assay system (Tropix, Bedford, MA, USA) following the protocol supplied with the kit. Five microlitres of cell lysate were incubated with 200 µl of diluted substrate for 1 h at room temperature. Then 300 µl of accelerator solution were added and the luminescence was measured as described above. After determination of luciferase and β-galactosidase activities of each single cell lysate, the transcriptional activities of the promoters under investigation were normalized by dividing the luciferase values by the β-galactosidase values to control for transfection efficiency, resulting in arbitrary so-called relative light units (16).

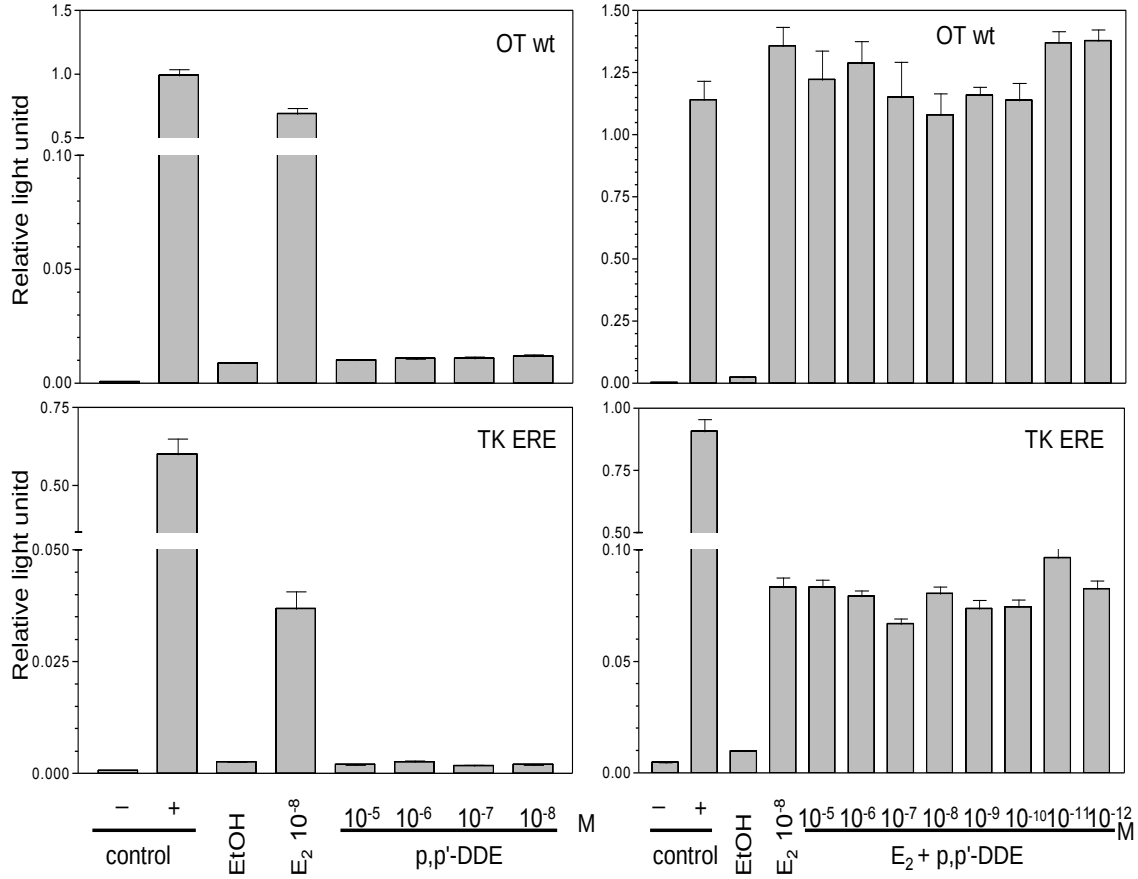

Figure 3. Transcriptional effects of *p,p'*-DDE on the oxytocin promoter and the thymidine kinase-ERE promoter by estrogen receptor α. MDA-MB 231 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and estrogen receptor α and treated with different amounts of *p,p'*-DDE alone or simultaneously with estradiol (E2 10^{-8} M). There was significant difference at $p < 0.05$ according to unpaired Student t-test.

DNA constructs

All promoter-reporter constructs were performed as previously described (16). Either the bovine oxytocin promoter (-183 to +17) was used (OXT), inserted into the pGL3-Basic vector (Promega), or the thymidine kinase promoter controlled by a single vitellogenin ERE (TKERE) was used to drive a similar luciferase reporter construct (17) As negative and positive controls, respectively, we used the pGL3-Basic plasmid, containing neither promoter nor transcriptional enhancer sequences, and the pGL3-Control vector, expressing luciferase under the control of the SV40 promoter and enhancer (both

from Promega). Erα was generated from an expression construct comprising the human ERα cDNA controlled by a CMV viral promoter (14). A version of Erα where in the DNA-binding domain (DBD) was mutated so that the receptor was no longer able to interact directly with an ERE as in Koohi et al. (16). A human ERα (hERα) expression vector was obtained as a generous gift from Dr. Katrin Stedronsky (Institute for Hormone and Fertility Research, Hamburg, Germany) and the bovine ERα (bERα) expression construct was prepared as in Walther et al (12) and driven from a CMV promoter.

Cell Toxicity Assay

First, the cells were checked for integrity following 24 h incubation with test substance by staining washed cells for 3 h using 2% neutral red, and after further washing in PBS, photometrically measuring the absorbed dye at 540 nm. Then, following exposure to the test substances, cells were replated into T75 flasks, and subjected to 7 days of culture, after which all colonies in the flasks were stained by 10% Giemsa (Merck) and cells were counted.

Statistical analysis

All experiments were performed in duplicate or triplicate dishes and each experiment was repeated at least three times. The mean ± SD was calculated for all stimulation experiments. Following correction for transfection efficiency, results were expressed as relative light units (means + S.D. for triplicate wells). Statistical significance for the differences in promoter activities was assessed by one way ANOVA followed by the Newman-Keuls test, or unpaired t-tests with Welch's correction using the GraphPad Prism 3.0 software package (GraphPad Software Inc., San Diego, CA).

Results

In the first experiment (Figure 1), neither with the oxytocin nor with the thymidine kinase-ERE promoter any agonistic effect could be detected in transfected MDA-MB 231 cells by *o,p'*-DDT.

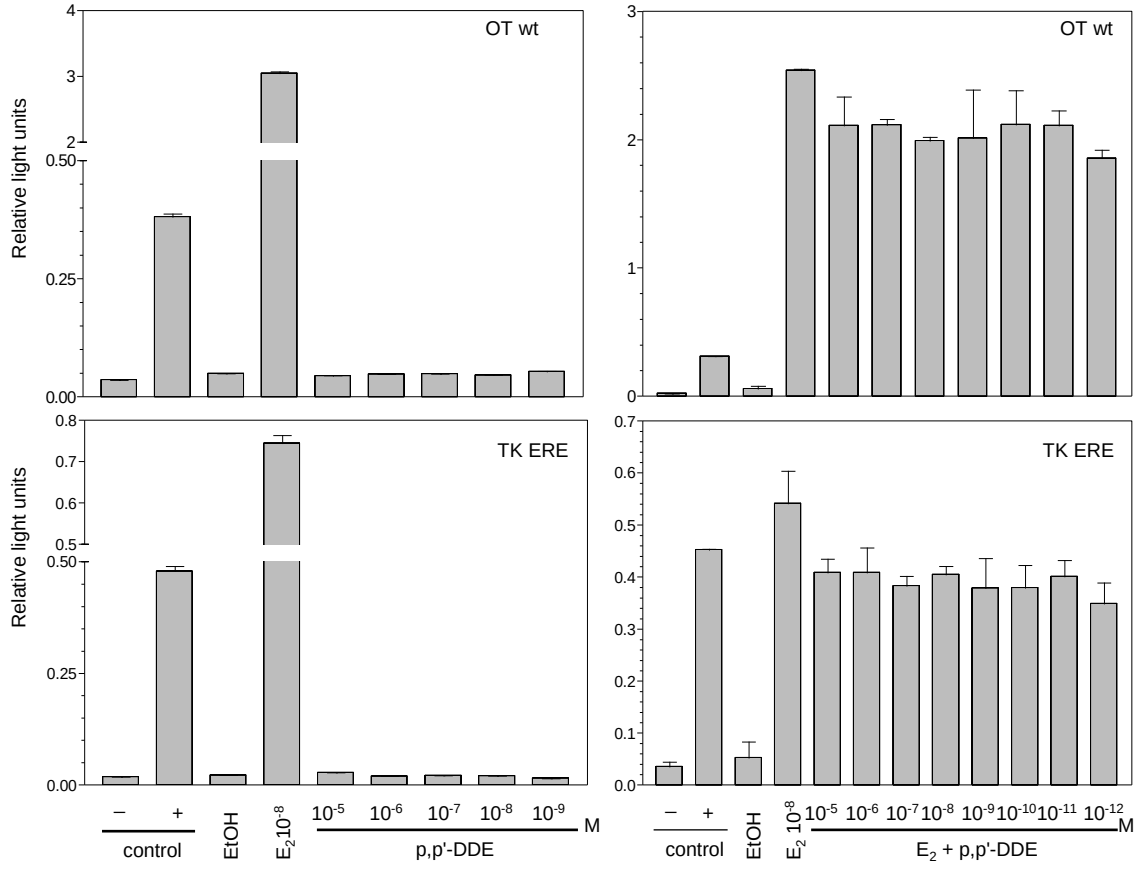

Figure 4. Transcriptional effects of *p,p'*-DDE on the oxytocin promoter and the thymidine kinase-ERE promoter by estrogen receptor α in the MCF-7 cells. MCF-7 cells were transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE (TK ERE) promoter construct and estrogen receptor α and treated with different concentration of *p,p'*-DDE alone or simultaneously with estradiol (E2 10^{-8}M). There was significant difference at $p < 0.05$ according to unpaired Student t-test.

The detection of transcriptional activity of *o,p′*-DDT in this breast cancer cell system had been expected, as *o,p′*-DDT has been shown to exert estrogenic effects in MCF-7 breast cancer cells. Therefore, the effect of *o,p′*-DDT in MCF-7 cells was measured.
The MCF-7 cells were transfected as above with the oxytocin or the thymidine kinase-ERE promoter and estrogen receptor α as shown above and cells were stimulated with increasing amounts of *o,p′*-DDT (Figure 2). The results clearly indicate that *o,p′*-DDT has an agonistic effect on both the oxytocin and the thymidine kinase-ERE promoter in this cellular context, leading to a more than two-fold stimulation of transcription at 10-5 M *o,p′*-DDT. No inhibitory or synergetic effect could be detected in this experiment on the simultaneous addition of *o,p′*-DDT and estradiol.

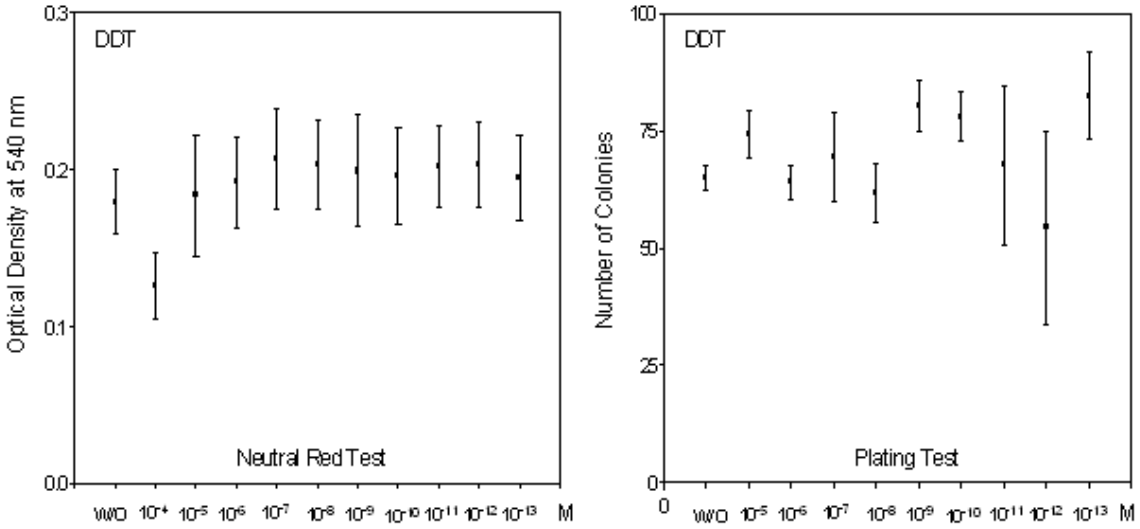

Figure 5. Cellular toxicity of *o,p′*-DDT in the MDA-MB 231 cells in the neutral red and the plating test. MDA-MB 231 cells were plated as described in Materials and Methods and treated with different concentrations of *o,p′*-DDT. (W/O: without sample)

In a further control experiment 2, 2-bis (4-chlorophenyl)-1, 1-dichloroethyl (*p,p′*-DDE) was used. MDA-MB 231 cells were transfected aforementioned with the oxytocin or thymidine kinase-ERE promoter, co-transfected with estrogen receptor α, and the transfected cells treated with increasing concentrations of *p,p′*-DDE (Figure 3). The results showed no agonistic effect with *p,p′*-DDE, neither on the oxytocin nor on the thymidine kinase-ERE promoter. In parallel transfection containing 10^{-8} M estradiol, neither an antagonistic nor a synergistic effect of *p,p′*-DDE could be detected.
In order to complete the investigation of the cell-type-specific transcriptional effect of *o,p′*-DDT and related compounds, the effects of *p,p′*-DDE were also measured in MCF-7 cells. For this experiment MCF-7 cells were transfected as described above and the transfected cells were treated with increasing amounts of *p,p′*-DDE, (Figure 4). The results indicated that *p,p′*-DDE does not have any agonistic, synergistic or antagonistic activity in MCF-7 cells. Toxic effect of *o,p′*-DDT and *p,p′*-DDE might suppress a possible agonistic effect at the high concentrations needed to obtain a stimulation of transcription by *o,p′*-DDT in MCF-7 cells. In order to check for these effects, two independent cell toxicity tests, the neutral red and the plating test, were performed. The results of both tests indicate that *o,p′*-DDT has no toxic effect on MDA-MB 231 and MCF-7 cells in the concentrations used for the transfection assays (Figure 5 and Figure 6). In a parallel experiment, *p,p′*-DDE was also tested for possible toxic effects (Figure 7). Cells were stimulated with increasing concentrations of *p,p′*-DDE and the neutral red test and the plating test were performed as described above. The results clearly showed that *o,p′*-DDT, there is not any toxic effect of *p,p′*-DDE in the concentration range used in the transfection assays. Low toxicity of *p,p′*-DDE was only detected at a concentration of 10^{-4} M, which was not used in the transfection assays.

Discussion

Exposure to persistent organochlorinated pesticides such as DDT can cause health problems including cancer, reproductive defects and behavioral abnormalities (18). Although DDT usage was banned by international organizations, but it is still used and the prevalence of DDT consumption is due to its characteristics including chemical stability, excellent

lipid solubility, and resistance to metabolism (9). As a result of the continued risk of exposure to this organochlorine, it is vital to assess possible deleterious activity of DDT, such as estrogenicity activity. Potentially, the molecular mechanism of action of DDT may serve as a model for other important organochlorines that are putative environmental estrogens. These effects might be related to their ability to disturb the functions of certain hormones, enzymes, growth factors, neurotransmitters and inducing important genes involved in metabolism of steroids and xenobiotics. Some persistent organochlorinated pesticides such as DDT show weak agonistic estrogenic activity (10).

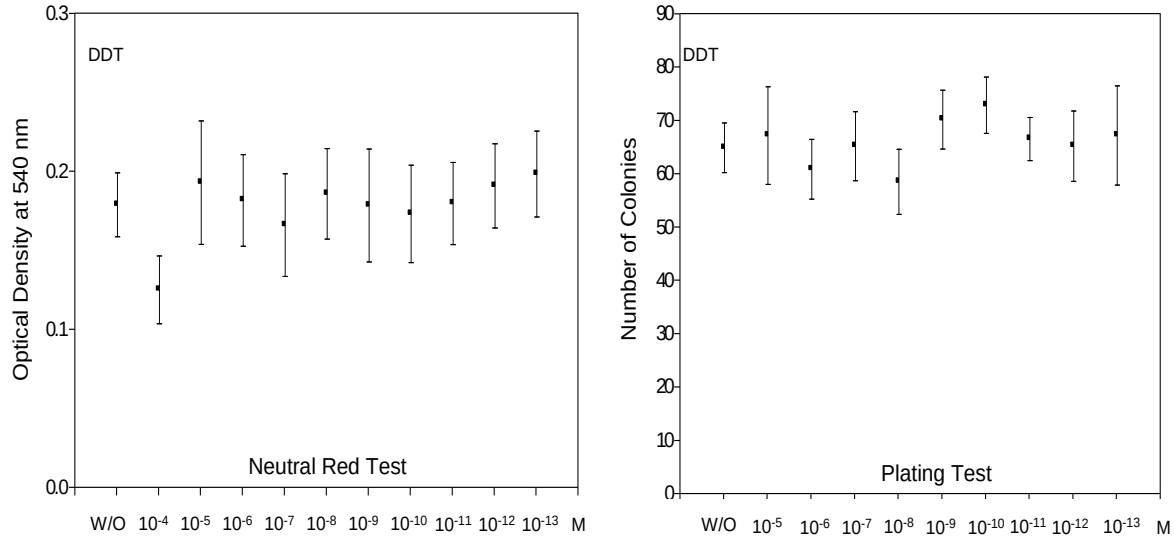

Figure 6. Cellular toxicity of *o,p'*-DDT in MCF-7 cells in the neutral red and the plating test. MCF-7 cells were plated as described in Materials and Methods and treated with different concentrations of *o,p'*-DDT. (W/O: without sample)

This Environmental xenoestrogens exert their effects through classical, genomic, or nongenomic pathways. Due to their similarity with the endogenous hormones, these compounds can bind to nuclear receptors. Their affinities for ERα and ERβ are relatively weaker compared to endogenous E2; thus, they can have agonist or antagonist activity depending on the presence of E2 (19). In the present investigation, we studied the transcriptional activity of *o,p'*-DDT and its metabolites (*p,p'*-DDE) in the MDA-MB 231 and MCF-7 transfection system.

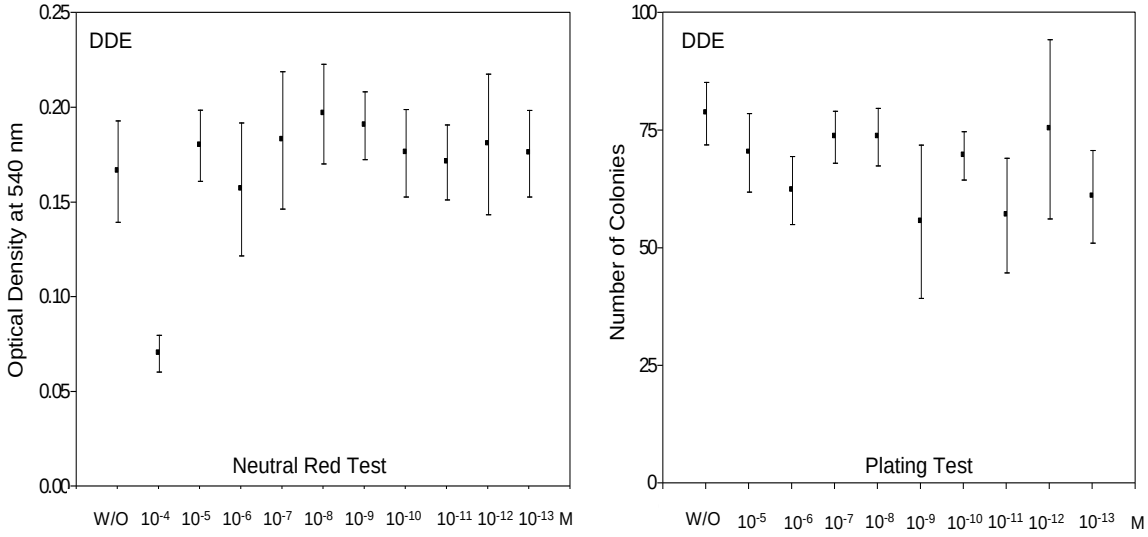

Figure 7. Cellular toxicity of *p,p'*-DDE in MDA-MB 231 cells in the neutral red and the plating test. MDA-MB 231 cells were plated as described in materials and methods and treated with different concentrations of *p,p'*-DDE.

Findings demonstrated no agonistic activity of *o,p′*-DDT in MDA-MB 231 cells transfected with the oxytocin promoter construct (OTwt) or the thymidine kinase-ERE promoter construct (TK ERE) and estrogen receptor α. While, The results obviously show that *o,p′*-DDT has an agonistic effect on both the oxytocin and the thymidine kinase-ERE promoter in MCF-7 leading to a more than two-fold stimulation of transcription at 10-5 M. In our previous studies, mechanisms of estrogenic activity of some metal ions and phytoestrogens were determined using this transfected system (20, 21). Diel et al showed DDT stimulates ER-α protein expression in MCF-7 cells (22). In addition, no inhibitory or synergetic effect could be detected in this experiment on the simultaneous addition of *o,p′*-DDT and estradiol. Some DDT isomers and metabolites affect directly as agonists and transactivate the hER at concentrations found in human tissues in MCF-7 cells. *o,p′*-DDT, *p,p′*-DDT, and the established environmental estrogen *o,p′*-DDT, were able to bind specifically to the hER with approximately 1000-fold weaker affinities for the hER than estradiol. In contrast, only *o,p′*-DDT, but not *p,p′*-DDT, bound to the rat estrogen receptor. Moreover, two yeast expression-reporter systems, constructed to test if the DDT isomers and metabolites could transcriptionally activate the hER. It was found that an *o,p′*-DDT metabolite could transactivate the hER or LexA-hER fusion protein with just a 140, to 300-fold weaker potency than estradiol (23). Bratton et al showed DDT up-regulated the expression of several genes in MCF-7 breast cancer cells that were not altered by treatment with E_2, including VEGFA (Vascular endothelial growth factor A). They proposed that this DDT-initiated, ER-independent stimulation of gene expression is due to DDT's ability to initiate crosstalk between MAPK (mitogen-activated protein kinase) signaling pathways and transcriptional coactivators (24). The results of our study show that there is no agonistic effect with *p,p′*-DDE, neither on the oxytocin nor on the thymidine kinase-ERE promoter. Also, neither an antagonistic nor a synergistic effect of *p,p′*-DDE could be detected. In addition, *p,p′*-DDE does not have any agonistic, synergistic or antagonistic activity in MCF-7 cells. In conclusion, this study indicated that *o,p′*-DDT does not act in a typical estrogen like manner in MDA-MB 231 breast cancer cells and may simulate an estrogen like action by other molecular mechanisms, which have to be investigated in future researches, while it has an agonistic effect on both the oxytocin and the thymidine kinase-ERE promoter in MCF-7 breast cancer cells.

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Conflict of interest
None declared conflicts of interest.

Authors′ contribution
Mohammad Kazem Koohi participated in the design of the study, carried out the experiments, and performed the statistical analysis and data interpretation. Ehsan Zayerzadeh was involved to carry out some experiments and interpretation of these results as well as in the preparation of the manuscript and finalized the manuscript. Azadeh Fardipour was involved in manuscript writing and data interpretation. All authors read and approved the final manuscript.

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