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Assessment of the endocrine-disrupting effects of trichloroethylene and its metabolites using *in vitro* and *in silico* approaches

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Abstract

Trichloroethylene (TCE) is a ubiquitous environmental contaminant, which may have effects on both ecosystem and human health. TCE has been reported to cause several toxic effects, but little effort has been made to assess the ecological risks of TCE or its major metabolites: trichloroethanol (TCOH), trichloroacetic acid, and oxalic acid (OA). In this study, the endocrine-disrupting potential of TCE and its metabolites were investigated using *in vitro* and *in silico* approaches. We examined alterations in the steroidogenesis pathway using the NCI-H295R cell line and utilized receptor-mediated luciferase reporter cell lines to identify effects on estrogen and androgen receptors. Molecular docking was also used to explore chemical interactions with these receptors. All test chemicals except OA significantly increased 17β-estradiol production which can be attributed to an up-regulation of 17β-hydroxysteroid dehydrogenase. Moreover, TCOH exhibited significant anti-estrogenic activity with a RIC_{20} (20% relative inhibitory concentration) of 3.7×10^{-7} M. Molecular docking simulation supported this finding with lower docking scores for TCOH, indicating that hydrogen bonds may stabilize the interaction between TCOH and the estrogen receptor binding pocket. These findings suggest that TCE contamination poses an endocrine-disrupting threat, which has implications for both ecological and human health.

Graphical Abstract

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Supporting Information

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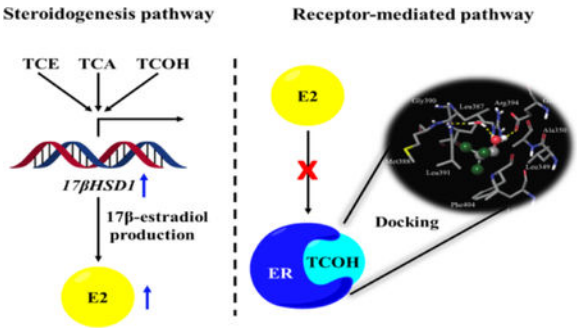

Introduction

Endocrine-disrupting chemicals (EDCs) are substances that can alter the endocrine system and cause reproductive and developmental toxicity in both humans and wildlife [1]. A variety of substances including industrial chemicals, pharmaceuticals, pesticides, and plasticizers show endocrine-disrupting properties [2, 3]. Multiple studies have identified environmental chemicals that mimic endogenous hormones through hormone receptor-mediated mechanisms [3–5]. For example, a recent study has shown that short-chain chlorinated paraffins (SCCPs) exhibit significant estrogenic and anti-estrogenic activities in luciferase reporter gene assays [6]. In addition, EDCs can modulate steroidogenesis via non-receptor mediated mechanisms [6, 7]. The widespread use of EDCs has led to environmental contamination, resulting in both ecological and public health concerns.

Trichloroethylene (TCE) is a volatile chlorinated solvent widely used in industries as a powerful vapor degreaser for manufactured metals [8, 9]. Previous studies have shown that industrial discharge containing TCE contaminates surface and ground water supplies, as well as air and sediment [10, 11]. TCE is used in several regions worldwide, including the United States [12], France [13, 14], China [15], Malaysia [16], and India [17]. In developing Asian nations, the use of TCE has been steadily increasing over the past few decades [18]. In the United States, TCE is found at more than 60 percent of Superfund and industrial waste sites proposed for inclusion on the Superfund National Priorities List, and is the most frequently reported groundwater contaminant [19]. It has been detected in water supplies at levels above the federally mandated maximum contaminant level of 5 ppb. For example, TCE levels in spring and ground water wells located in Asheville, North Carolina have been measured at 36 and 53 ppm, respectively [20]. An *et al.* reported that TCE levels measured at multiple locations around the shore of Lake Michigan ranged from 0.014 to 6.5 ppm [21]. Furthermore, trichloroacetic acid (TCA), a TCE metabolite, has been measured in various environmental media including rivers, surface water, and sediment [22]. Stidson *et al.* reported that Sitka soil collected from litter layers in Ayrshire, Scotland contained TCA concentrations as high as 2.1 ppm [23]. TCE has also been detected in human breast milk [24] and plasma [25] proving that the issue stems beyond an environmental concern.

Upon exposure, TCE is readily absorbed and rapidly distributed throughout the body due to its small molecular size and lipophilicity. TCE metabolism occurs through two pathways: glutathione conjugation and oxidation via cytochrome P450 [26]. Glutathione conjugation

produces the most genotoxic metabolites, such as *S*-(1,2-dichlorovinyl) glutathione and *S*-(1,2- dichlorovinyl)-L-cysteine [27]. However, the oxidative metabolism of TCE via cytochrome P450 is considered the major pathway and produces the primary transient metabolite chloral hydrate (Figure S1). Chloral hydrate (CH) is rapidly reduced and oxidized to form the major metabolites: TCA, trichloroethanol (TCOH), and oxalic acid (OA) [27, 28].

Exposure to TCE is associated with several adverse health outcomes, including altered immune function [29–31], cancers [32], disrupted systemic metabolism [33], and reproductive toxicity [34–37]. Kumar *et al.* reported a significant reduction in serum testosterone (T) levels in rats following exposure to 376 ppm of TCE for 12 and 24 weeks [38]. In an occupational epidemiological study of 85 workers in China, serum levels of T and SHBG were negatively correlated with years of exposure to TCE, irrespective of TCE levels measured in urine at the time of study [39]. These studies support the notion that TCE could function as an EDC. However, the endocrine-disrupting effects of TCE and its metabolites on sex hormone receptors and steroidogenic processes have yet to be thoroughly assessed. TCE, TCA, and OA were included in the Tox21 screening program with no effects being reported on the estrogen receptor (ER), but TCOH was not included in the chemical library; therefore, the endocrine-disrupting effects of this particular metabolite need to be further explored [40–42].

In the present study, we use *in vitro* and *in silico* approaches to investigate the endocrine-disrupting potential of TCE and its metabolites. First, we investigated the endocrine-disrupting effects of TCE and its major metabolites *in vitro* (TCA, TCOH, and OA) by measuring alterations on sex steroidogenesis and expression of major steroidogenic genes (*StAR*, *3βHSD2*, *17βHSD1*, *CYP11A1*, *CYP17A1*, and *CYP19A1*). Secondly, we assessed the interaction of the test chemicals with two sex hormone receptors, the ER and androgen receptor (AR), using luciferase reporter gene assays and molecular docking. Collectively, these results provide significant evidence for the endocrine-disrupting potential of TCE and its metabolites at levels detected in biological, ecological, and occupational samples.

Materials and Methods

Chemicals and reagents

Trichloroethylene (99.9 % purity) and hydrogen peroxide were obtained from Fisher Scientific (Waltham, MA, USA) while trichloroacetic acid (≥ 99.0% purity), 2,2,2-trichloroethanol (≥99% purity), forskolin (≥ 98.0% purity), mifepristone (RU486) (≥98% purity), hydroxyflutamide (OHF) (≥98% purity), and oxalic acid (≥99% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All test chemicals (Table S1) were diluted into dimethyl sulfoxide (DMSO) (≥99.7% purity) from Fisher Scientific (Waltham, MA, USA). Dihydrotestosterone (DHT) mimic, Cl-4AS-1, (>99% purity), 17β-estradiol (E2) (>99% purity), and tamoxifen (TAM) (≥99% purity) were acquired from Tocris Bioscience (Minneapolis, MN, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was obtained from Amresco (Fountain Parkway Solon, OH, USA).

Cell lines and cell culture conditions

The NCI-H295R cell line (ATCC®CRL-2128™), human adrenocortical carcinoma cells, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained according to the standardized protocol approved by the Organization for Economic Cooperation and Development (OECD) [43]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 (Gibco, Grand Island, NY, USA) medium supplemented with 1% (v/v) ITS + premix (Corning, Bedford, MA, USA), and 2.5% (v/v) Nu-Serum (Corning, Bedford, MA, USA) at 37°C in a humidified incubator with 5% CO_2.

The T47D-KBluc cell line (ATCC®CRL-2865™), a human breast cancer cell line stably transfected with luciferase reporter gene under transcriptional regulation of triplet estrogen response elements (EREs) [44], was obtained from ATCC. Cells were maintained in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Corning, Bedford, MA, USA) at 37°C in a humidified incubator with 5% CO_2. To reduce external interference from hormone mimics, cells were hormone-deprived by maintaining them in phenol red-free DMEM supplemented with 10% charcoal-dextran stripped FBS for 1 week prior to the luciferase reporter gene assay.

The MDA-kb2 cell line (ATCC®CRL-2713™), a human breast cancer cell line which is stably transfected with the murine mammalian tumor virus (MMTV) luciferase neo reporter gene construct, was obtained from ATCC. These cells highly express both endogenous AR and glucocorticoid receptor (GR), which stimulate the MMTV promoter. Chemicals that bind to either receptor are capable of activating the MMTV luciferase reporter [45]. Cells were cultured in Leibovitz's L-15 (L-15) (Gibco, Grand Island, NY, USA) supplemented with 10% FBS at 37°C in a humidified incubator with 0% CO_2. Cells were hormone-deprived by maintaining them in L-15 supplemented with 10% charcoal-dextran stripped FBS (Corning, Bedford, MA, USA) for 1 week before the luciferase reporter gene assay.

Treatment of cell cultures and assessment of cell viability

Cytotoxicity was evaluated by a quantitative colorimetric MTT assay following the corresponding procedure described in the steroidogenesis assay or the luciferase reporter gene assay. Briefly, NCI-H295R cells were seeded in 96-well microtiter plates (CellStar, Monroe, NC, USA) at a density of 4.5×10^4 cells/well. T47D-KBluc cells and MDA-kb2 cells were seeded in 96-well microtiter plates at a density of 3.0×10^4 cells/well. Due to the volatile nature of TCE and faster evaporation rate of the wells in the outer rim of the microtiter plates, treatment of cells with test chemicals or DMSO (vehicle control) was confined to the inner wells of the microtiter plate. In addition, microtiter plates were covered with low evaporation lids and wrapped in aluminum foil before being placed in an incubator that maintained relative humidity above 90%. These methods ensured that the TCE concentration remained relatively constant throughout treatment days. Mass spectrometric analysis showed that at the highest TCE dose (100 μM), there was no significant change in concentration due to evaporation (Figure S2). The concentration of DMSO used in this study did not exceed 0.1% v/v, which did not affect the cell viability in control plates. After incubation, MTT reagent (0.5 mg/mL MTT in serum-free medium) was added to each well and cells were incubated for 3 h. The plate was measured at an absorbance of 570 nm with

reference wavelength of 650 nm using microplate-scanning spectrophotometer (BioTek Instruments Inc.). No cytotoxic effects were observed for any of the test chemicals at experimental doses used (Figures S3-S5).

Steroidogenesis assay for hormone production measurement

The NCI-H295R cell line has been used as the gold standard for assessing the direct effects of endocrine disruption on steroidogenesis. Cells were maintained in DMEM/F12 culture medium containing 10 μM of forskolin for 48 h prior to chemical exposure. Cells were seeded in a 25-cm^2 flask (CellStar, Monroe, NC, USA) at a density of 3.6×10^6 cells and were exposed to the test chemicals for 48 h at 3 concentrations: 10^{-6}, 10^{-5}, and 10^{-4} M and incubated in an incubator with relative humidity above 90%. After incubation, RNA extractions were performed and supernatants were collected and stored at −80°C until further analysis. Concentrations of E2 and T were measured by competitive enzyme-linked immunoassay (ELISA) according to manufacturer's instructions (Cayman Chemical, Ann Arbor, MI, USA).

RNA isolation and real-time quantitative PCR

After a 48 h incubation period with the test chemicals, total RNA was extracted from NCI-H295R cells using RNeasy Mini Kit (Qiagen, Germantown, MD, USA). The ratio of absorbance at 260/280 greater than 2.1 qualified for cDNA synthesis. Purified RNA was used immediately for reverse transcription. Two μg of total RNA were reverse transcribed in a 20 μL reaction mixture using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. The cDNA was amplified in 10 μL of 2 × TaqMan gene expression master mix with the following TaqMan assay primers and probes from Applied Biosystems: *StAR* (Hs00986559_g1), *CYP17A1* (Hs01124136_m1), *CYP11A1* (Hs00167984_m1), *3βHSD2* (Hs00605123_m1), *17βHSD1* (Hs00166219_g1), and *CYP19A1* (Hs00903411_m1). Actin (*ACTB*) (Hs99999903_m1) was used as a reference gene. The primer and probe sets were designed and optimized according to Applied Biosystems guidelines. Quantification of the expression of target genes was based on a comparative cycle threshold (Ct) value and normalized to *ACTB*. The fold change of target genes was compared to the vehicle control using the $2^{-\Delta\Delta Ct}$ method [46].

ER-mediated luciferase reporter gene assay

T47D-KBluc cells were cultured in DMEM with 10% FBS until 1 week prior to the experiment as previously described with minor modifications [47]. Cells were seeded at a density of 3.0×10^4 cells/well in white, 96-well microtiter plates (Thermo Scientific, Grand Island, NY, USA). For assessment of antagonistic effect, cells were exposed to test chemicals for 30 min prior to co-incubation with 9.37×10^{-12} M E2. For agonistic effect, cells were exposed to test chemicals alone. After a final incubation period of 24 h, cells were lysed with 1 × passive lysis buffer (Promega, Madison, WI). Luciferase gene expression was measured using a microplate luminometer (Berthold Technologies, Centro XS3 LB 960 Instrument). The agonistic activities were expressed as 20% relative effective concentration (REC_{20}) and the antagonistic activities were expressed as 20% relative inhibitory concentration (RIC_{20}).

AR-mediated luciferase reporter gene assay

MDA-kb2 cells express both AR and GR which have homologous DNA-binding domains and act on the same MMTV promoter. To measure androgenic activities, the use of a potent glucocorticoid receptor inhibitor, RU486 was needed. L-15 contained 100 nM of RU486 to inhibit interference from GR. Cells were maintained in L-15 supplemented with 10% charcoal-dextran stripped FBS for 1 week. Then, cells were seeded at a density of 3.0×10^4 cells/well in white, 96-well microtiter plates (Thermo Scientific) and incubated at 37°C with 0% CO_2 for 24 h. Similar to the ER assay, in order to measure total antagonistic effect of the test chemicals, cells were exposed to test chemicals for 30 min prior to co-incubation with 2.5×10^{-10} M DHT. For agonistic effect, cells were exposed to test chemicals alone. The plate was read as described in the ER luciferase reporter gene assay.

Molecular docking and molecular dynamic simulation

T47D-KBluc cells express both hERα and hERβ; therefore, molecular docking against the two subtypes of ER were performed. The crystal structures of hERα and hERβ formed a complex with original reference ligands, E2 and sulfonamide, respectively. Receptor and reference ligand complexes were obtained from the Protein Data Bank (PDB). PDB codes: 3UUD (hERα) and 2YLY (hERβ) were used as a template for docking with TCE, TCA, TCOH, OA, original reference ligands, and decoy (tributyl phosphate, TBP) [5]. Considering experimental conditions and biological pH, the carboxylic acid of TCA and OA was modeled as a carboxylate with −1 charge. Modeling was mainly with Maestro (Schrödinger Release 2016–2: Maestro, Schrödinger, LLC) and tools therein. All ligand structures were first minimized using MacroModel and the proteins were prepared using the Protein Preparation Wizard. These steps added hydrogens and did a restrained minimization of the protein atoms with the OPLS_2005 force field [48]. Molecular docking was performed using Induced Fit with Glide [49]. Desmond molecular dynamics software (with OPLS_2005 force field) was used to explore the possible interactions of TCOH and the two ER subtypes [50]. Eight nanosecond (ns) trajectories were created and the root-mean-square deviation (RMSD) of the TCOH backbone was calculated.

Statistical analysis

All data are expressed as means ± S.E.M. of at least three independent experiments. Statistical comparisons between each test chemical and controls were performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison test and linear regression analysis. Differences with $p<0.05$ were considered statistically significant.

Results

Effects on the production of sex steroid hormones in NCI-H295R cells

TCE, TCA, and TCOH increased the production of E2 significantly at 10^{-5} M by 1.23-, 1.25-, and 1.26-fold, respectively (Figure 1). Similarly, at 10^{-4} M, a 1.41-, 1.41-, and 1.33-fold increase was observed for TCE, TCA, and TCOH, respectively. In contrast, OA did not affect E2 production at these concentrations. No significant fold change between the control

and test chemicals at 10^{-6} M was observed. T production was not altered by any of the chemicals at any concentration (Figure S6).

Effects on steroidogenic gene expression in NCI-H295R cells

NCI-H295R cells express all important steroidogenic genes and undertake steroid biosynthesis (Figure S7). The expression of *StAR*, *CYP11A1*, *CYP17A1*, *3βHSD2*, and *CYP19A1* showed no significant change in response to any of the test chemicals. In contrast, TCE, TCA, and TCOH selectively up-regulated the expressions of *17βHSD1* (Figure 2). At 10^{-5} M, TCE significantly increased the expression of *17βHSD1* by 1.63-fold. Additionally, at 10^{-4} M, a 1.60-, 1.61-, and 1.56-fold increase was observed for TCE, TCA, and TCOH, respectively. However, OA exposure did not affect the expression of any steroidogenic gene (Figure S8).

Agonistic and antagonistic effects of TCE and its metabolites against ER and AR

The EC_{50} value of E2 and DHT was 7.8×10^{-12} M and 6.6×10^{-10} M, respectively, as shown by the concentration-response transactivation curves (Figures S9). Moreover, TAM and OHF, known anti-estrogenic and anti-androgenic compounds, respectively, showed a significant reduction in luciferase activity from both ER and AR, establishing that the assays are performing appropriately (Figures S10). The agonistic and antagonistic effects of test chemicals against ER and AR were further investigated by measuring the transcriptional activation of ERE and MMTV in T47D-KBluc and MDA-kb2 cells, respectively. In the ER luciferase reporter gene assay, no test chemical elicited agonistic properties (Figure S11); however, TCOH exhibited a significant anti-estrogenic activity with a RIC20 value of 3.7×10^{-7} M (Figure 3 and Table 1). In contrast, no test chemical showed a significant agonistic or antagonistic activity in the AR luciferase reporter gene assay (Figures S12 and S13).

Molecular docking and molecular dynamic simulation

To further explore the potential interaction of the test chemicals against hERα and hERβ, docking results were analyzed. TCOH had the most favorable of docking scores in both ER subtypes, which suggests TCOH binds well at the pocket sites (Table S2). The pocket site of ERα is surrounded by Leu346, Leu349, Ala350, Glu353, Leu387, Met388, Gly390, Leu391, Arg394, and Phe404 (Figure 4A). The active site for ERβ mainly consists of the residues Val280, Leu298, Leu301, Ala302, Glu305, Leu339, Leu343, Arg346, and Phe356 (Figure 4B). In addition, the TCOH and ERα complex (Figure 4A) contained two hydrogen bonds which formed between TCOH and a side chain of Glu353, and between TCOH and a water molecule in the pocket site. In the TCOH and ERβ complex, a side chain of Glu305 could form a hydrogen bond with a hydroxyl group of TCOH (Figure 4B). On the contrary, we did not observe hydrogen bonds or any strong interaction in Induced Fit docking of TCE, TCA, and OA, which was consistent with the results from the ER luciferase reporter gene assay (Figures S14 and S15). Furthermore, the best Induced Fit complexes of TCOH and ER were additionally subject to molecular dynamic simulations to provide insight regarding their dynamic stability. The RMSD results for TCOH backbone atoms bound to the complexes after 8 ns of molecular dynamic simulations (Figure S16). The results reveal that the interaction reached equilibrium and RMSD fluctuations reached a plateau very close to the

initial orientation, indicating that the initial Induced Fit docking conformation is a very stable complex throughout the dynamics simulation.

Discussion

The present study assesses the endocrine-disrupting effects of TCE and its major metabolites using *in vitro* and *in silico* approaches. This is the first study to report that not only TCE, but also TCA and TCOH behave as endocrine disruptors at concentrations that can be achieved in the biological and occupational samples. TCE, TCA and TCOH increase the synthesis of E2 through a steroidogenesis pathway. Interestingly, TCOH also disrupts the hormone receptor-mediated pathway of ER. The data presented herein provide mechanisms of endocrine disruption and insights into the potential ecological and health risks of TCE and its major metabolites.

Many environmental contaminants including flame-retardants [51] and SCCPs [6] have shown endocrine-disrupting effects through the steroidogenesis pathway using NCI-H295R cells. However, the effects of TCE and its metabolites on steroidogenesis and steroidogenic gene expression have not previously been investigated. Our results showed no significant change in T production after exposure to the test chemicals, which was consistent with previous findings in humans, where the levels of TCE exposure (mean of TCE exposure was about 29.6 ppm) were not associated with serum levels of T in occupationally exposed subjects [39, 52]. However, Kumar *et al.* found a significant reduction in serum T levels following an exposure of 376 ppm of TCE in rats [38]. Discrepancies between studies may be explained by the differences in exposure levels and metabolic pathways. Interestingly, we found that TCE, TCA, and TCOH significantly increased the production of E2 in a concentration-response manner, which suggests that the estrogen synthesis pathway is a toxicological target for endocrine disruption. Our results support a previous finding that TCA at 10^{-4} M increase E2 production by 1.8 folds [53]; however, at the same concentration, we found a slightly lower fold induction of 1.41, which can be explained by the differences in experimental procedure and method used to quantify E2. Considering the relative potencies of the effects on E2 production, the rank order was as follow: TCE ≈ TCA > TCOH, indicating that TCE and TCA may be the major chemicals increasing E2 biosynthesis.

Real-time PCR analysis was used to measure steroidogenic gene expression to identify the potential endocrine-disrupting mechanism of TCE, TCA, and TCOH via a non-hormone receptor mediated pathway. Steroidogenesis is regulated by highly complex biochemical pathways, which are dependent on cytochrome P450 and hydroxyl steroid dehydrogenases. These pathways can be dysregulated by several EDCs at a multitude of entry points. In the initiation step of steroidogenesis, cholesterol is transported from the outer membrane into the inner mitochondrial membrane by the steroidogenic acute regulatory protein, or StAR (Figure S7). In the inner mitochondrial membrane, cholesterol side-chain cleavage enzyme (CYP11A1) catalyzes the biochemical conversion of cholesterol to pregnenolone. Moreover, T and E2 are synthesized by regulation of the CYP17A1, 3βHSD2, CYP19A1, and 17βHSD enzymes [54]. In particular, CYP19A1 and 17βHSD1 enzymes play a crucial role in the last step of sex hormone biosynthesis. In NCI-H295R cells, *CYP19A1* is the only gene encoding the aromatase enzyme, which irreversibly converts androgens to estrogens (estrone and E2).

Although TCE, TCA, and TCOH stimulated the production of E2, the expression of *CYP19A1* gene was not affected by our test chemicals, suggesting that endocrine-disrupting effects are not mediated through a *CYP19A1* gene-related pathway. In the last step of hormone biosynthesis, 17βHSD1 is responsible for catalyzing two independent biochemical reactions: the conversion of estrone to E2 and androstenedione to T [55]. Indeed, the results demonstrate that TCE, TCA, and TCOH selectively up-regulated the expression of *17βHSD1* gene in a concentration-dependent response, which were in agreement with the results in the E2 hormone production assay. However, the specific mechanism underlying the alteration in *17βHSD1* expression remains unknown. An effect on promoter DNA methylation may be a potential mechanism that requires further investigation [56, 57]. Despite *17βHSD1* encoding for an enzyme responsible for both T and E2 synthesis, we did not observe a significant change in T production upon exposure to all the test chemicals. This can be explained by a more favorable binding interaction of the 17βHSD1 enzyme to estrone over androstenedione [55, 58, 59]. While several studies mainly focus on the endocrine-disrupting effects of estrogenic EDCs through the aromatase pathway [7, 60, 61], our study emphasizes the importance of the *17βHSD* pathway as a potential endocrine disruption target. Further, a study showed that Wistar rats sub-chronically exposed to 376 ppm TCE had disrupted testicular 17βHSD enzymatic activity [38], supporting our findings that *17βHSD* pathway is a toxicological target for TCE toxicity.

Several studies have evaluated the anti-estrogenic activity of chlorinated chemicals including pesticides, persistence organic pollutants, and flame-retardants. The chemical RIC_{20} values against ERs have been reported within a concentration range of 10^{-9} to 10^{-6} M [5, 6, 62]. For example, SCCP C10–40.40% and C10–66.10% have a RIC_{20} value of 6.4×10^{-7} and 5.4×10^{-7} M, respectively [6]. Moreover, another study investigated the endocrine-disrupting potential of several chlorinated organophosphate flame-retardants and only tris(2-chloroethyl) phosphate elicited anti-estrogenic effect with a RIC_{20} value of 8.2×10^{-6} M [5]. In our study, we further investigated the endocrine-disrupting effects of the test chemicals through sex hormone receptors. The RIC_{20} of TCOH in the ER luciferase reporter gene assay was 3.7×10^{-7} M, suggesting that TCOH may have more potent anti-estrogenic activity than some previously reported chlorinated contaminants. Compared to Tox21, we found similar results which indicate TCE, TCA, and OA have no effect on ER and AR in cell-based assays [40, 41]. Moreover, our findings are in agreement with a previous study that showed TCOH to be capable of binding to hERβ in an E2 competition binding assay; however, the binding interaction remains unknown [2]. We used molecular docking to further explore the potential interaction between test chemicals and hERα as well as hERβ. The computational results revealed that TCOH was the only hydrogen bond donor and acceptor species within our test chemicals, which was favored in a hydrogen bond donor (water) and acceptor environment (Glu carboxylate). Therefore, it was not surprising that TCOH had the lowest value (most favorable) of docking scores in both ER subtypes as it was an ideal fit for the pocket sites. In contrast, TCA and OA showed very weak binding capacity primarily due to a charge repulsion between the carboxylate groups found in Glu, TCA, and OA. TCE does not contain a chemical group capable of hydrogen bonding. In addition, the docking scores among the test chemicals were all in accordance with the results in the ER luciferase reporter gene assay. In addition to molecular docking, quantitative structure-activity

relationship (QSAR) is also widely used to predict estrogenic properties and relative binding of chemicals [63,64]. Applying QSAR with the CERAPP model [64,65], TCE and its metabolites were predicted as non-binders against the ER (data not shown), thus QSAR does not seem to accurately predict the ER binding and activation capability of TCOH.

SCCP residues with C10 and C11 were found in soft-shell clams at level of 623 ppb and 1.01 ppm, respectively [66]. These values are higher than lowest-observed effect levels (LOELs) from the *in vitro* assay, implying EDCs can potentially cause adverse outcomes in wildlife [6]. Considering the potential ecological impacts of TCE and its metabolites we compared the LOELs in our *in vitro* assays to internal levels detected in the biota. Alarmingly, TCE possesses the ability to transport from aquatic environment to organisms and present in tissues of marine organisms up to 0.5 ppm [67, 68], which was close to TCE's LOEL (1.3 ppm = 1×10^{-5} M). In addition, TCA has been found in tissues of soil-dwelling biota as high as 0.4 ppm which was close to TCA's LOEL value (1.6 ppm = 1×10^{-5} M) [69]. We note that both TCE's and TCA's LOEL values were similar to chemical residues detected in organisms, indicating the potential to pose adverse effects and significant risks to the environment and wildlife including aquatic and soil-dwelling biota via direct contact. While, there is limited information on TCOH levels in biota, we should not ignore the ecological risks of TCOH due to its ability to interfere with endocrine function at low concentrations (LOEL = 55.3 ppb).

In addition to the ecological risks, health risks associated with TCE and its metabolites should be evaluated in different settings and among vulnerable populations. Previous studies have demonstrated the anti-estrogenic effects of EDCs in association with potential health risks on reproductive and endocrine systems [70–72]. Early life exposure to EDCs is of particular concern as several chemicals have been shown to be present in breast milk [24, 73–75]. Considering lipophilicity characteristics, TCE, like other EDCs of this nature, can be found in breast milk from exposed mothers. More importantly, mothers who live near industrial sites have greater amounts of TCE present in their breast milk, with reports as high as 6 ppb (4.5×10^{-8} M) [24]. Though the concentration of TCE detected in human samples are lower than the TCE's LOEL value (1.3 ppm) in this study, breast milk could still be a hazardous exposure for the susceptible infants of lactating mothers. Young children may be an especially vulnerable population to the effects of both TCE and its anti-estrogenic metabolite TCOH due to the lack of complete defense mechanisms [76, 77]. In occupational settings, where levels of TCE exposure are typically higher than the general population, Varshney *et al.* reported plasma concentrations of TCA and TCOH detected in TCE exposed workers as high as 2.49 ppm and 0.94 ppm, respectively [17]. These plasma concentrations of TCA and TCOH are higher than our LOELs and suggest TCE and its metabolites may be endocrine-disrupting occupational health hazards. The biological effects of TCOH need to be carefully interpreted due to TCOH's endocrine disrupting effects on both pathways. At low concentrations, the endocrine-disrupting effect of TCOH through ER appears to be the predominant pathway as it inhibits ER. However, at high concentrations, there can be two competing effects as a result of ER inhibition and E2 production.

In summary, this study aimed to assess the endocrine-disrupting property of TCE and its major metabolites at biologically relevant concentrations. The results showed that TCE,

TCA, and TCOH elicited endocrine disruption via the steroidogenesis pathway by increasing E2 production through the *17βHSD1*-related pathway. In addition, TCOH, possesses endocrine-disrupting abilities through the hormone receptor-mediated pathway. TCOH displayed anti-estrogenic activity by forming hydrogen-bonding interactions at the ER binding pocket. Our study suggests that TCE and its major metabolites may pose a biological and ecological risk, through at least two mechanisms of endocrine disruption.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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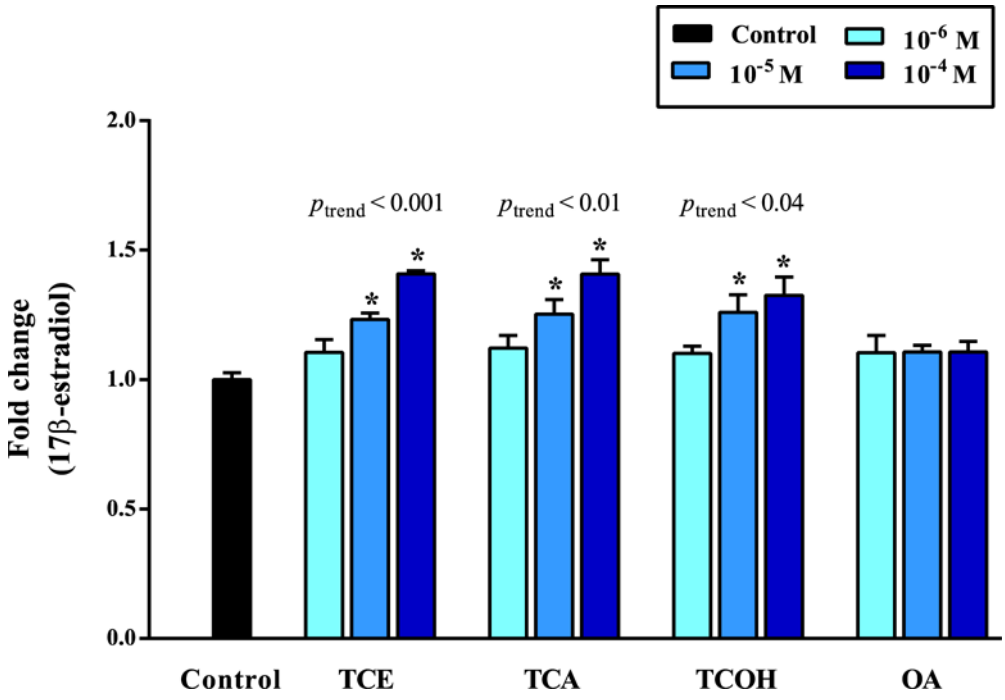

Figure 1.

Effects of TCE and its metabolites on E2 production in NCI-H295R cells after a 48 h exposure. Values are expressed as the mean fold change ± S.E.M. of duplicate measurements in three independent experiments. * $p<0.05$ indicates a significant difference between exposure groups and the corresponding control group (0.1% v/v DMSO). The p trend is determined based on linear regression analysis.

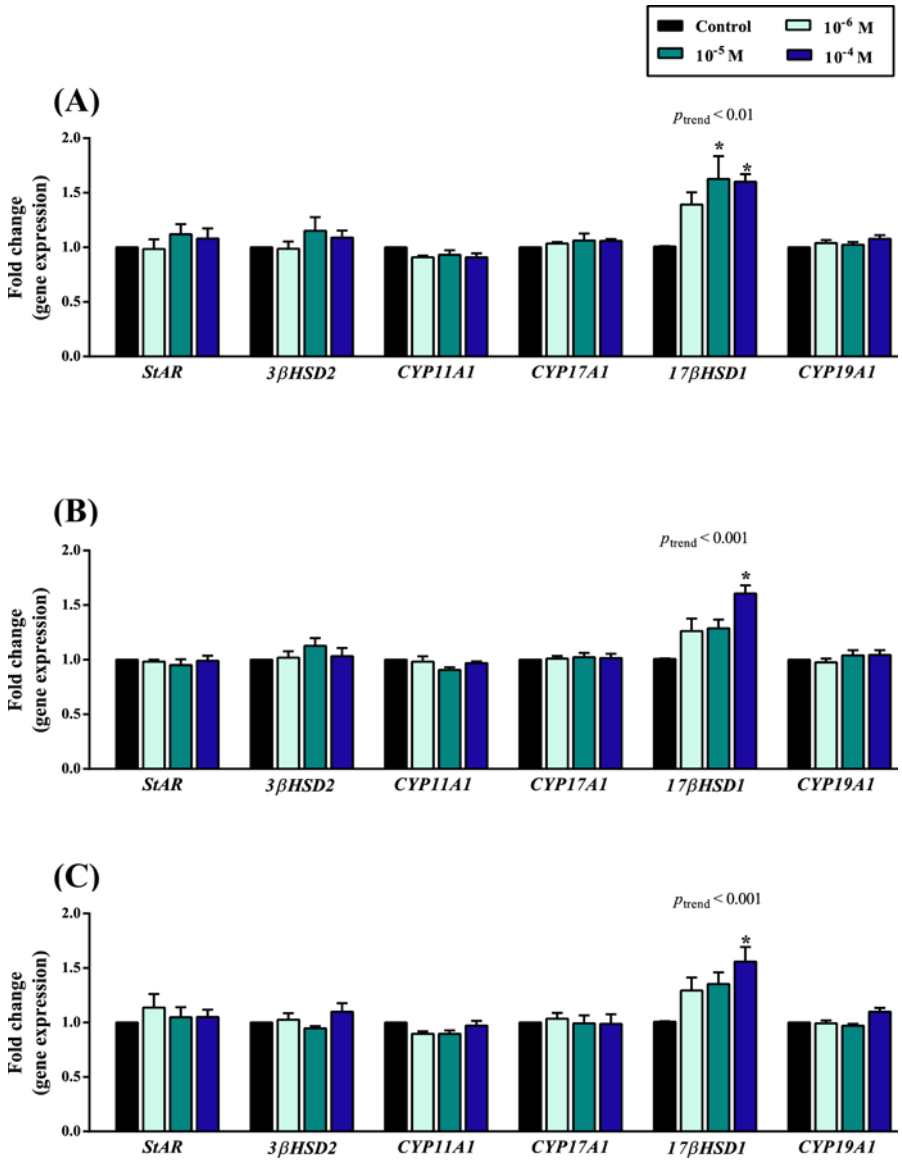

Figure 2.

Effects of TCE and its metabolites on the expression of genes related to steroidogenesis pathways in NCI-H295R cells. Cells were exposed to TCE (A), TCA (B), and TCOH (C) at various concentrations for 48 h. Values are expressed as the mean fold change ± S.E.M. of duplicate measurements in at least three independent experiments. * $p<0.05$ indicates a significant difference between exposure groups and the corresponding control group (0.1% v/v DMSO). The p trend is determined based on linear regression analysis.

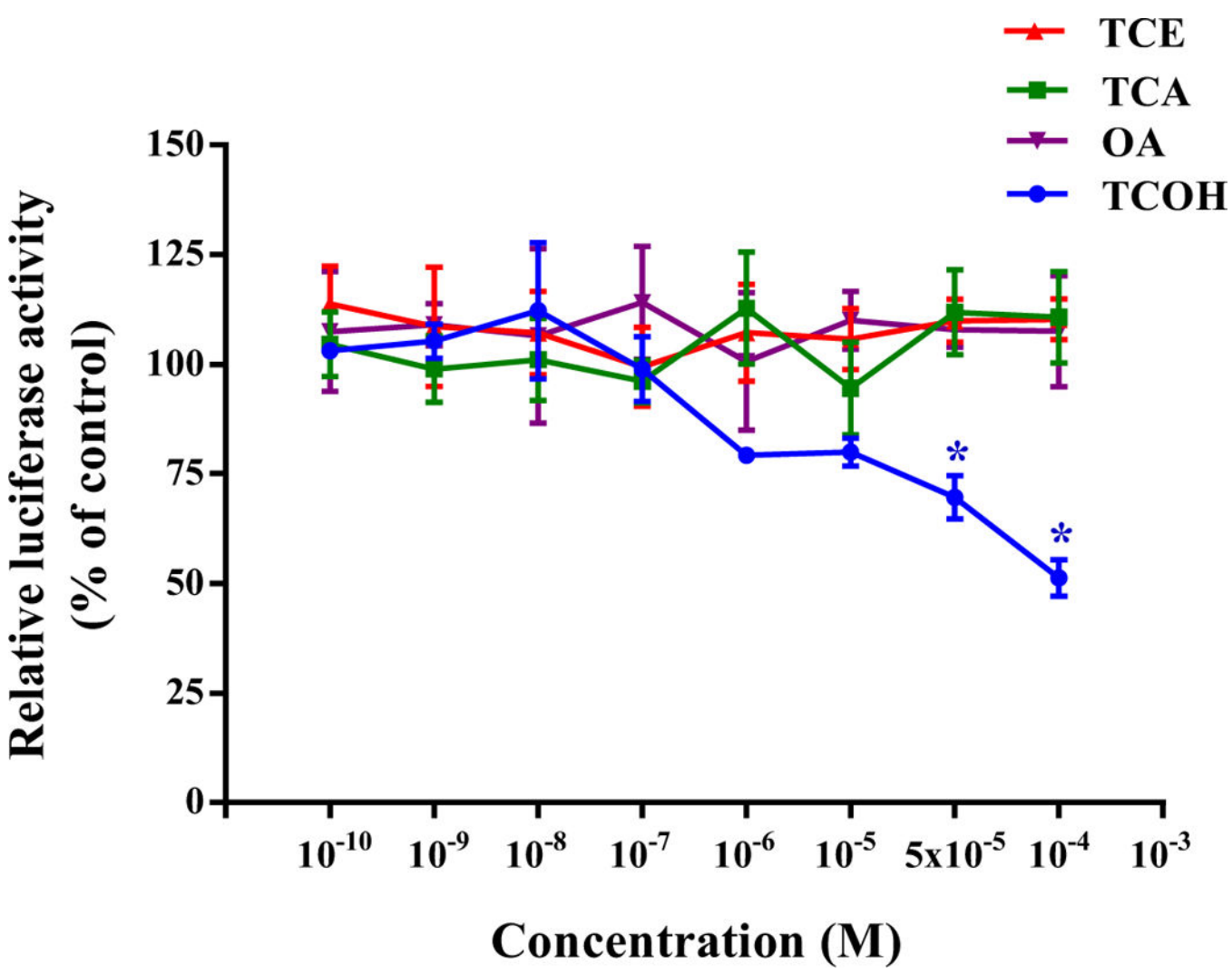

Figure 3.

Anti-estrogenic effects of TCE, TCA, TCOH, and OA in in the ER-mediated luciferase reporter gene assay. T47D-KBluc cells were stably transfected with triple ERE. Cell were exposed to test chemicals for 30 min prior to co-incubation with 9.37×10^{-12} M of E2. Values are expressed as the mean percentage of control ± S.E.M. of triplicate measurements in three independent measurements. * $p<0.05$ indicates a significant difference between exposure groups and the corresponding control group (9.37×10^{-12} M E2).

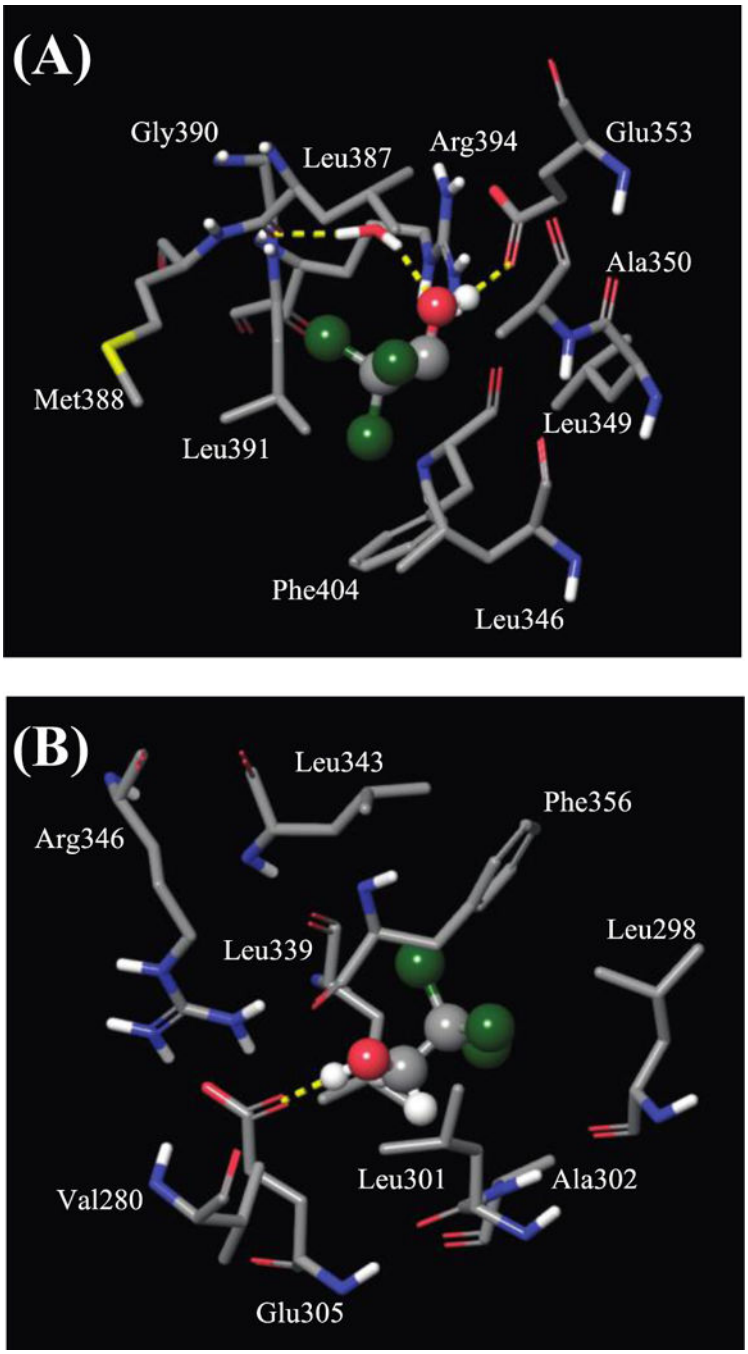

Figure 4.

Proposed binding mode between TCOH and ERα (A) and ERβ (B). Oxygen, nitrogen, sulfur, carbon, hydrogen, and chlorine atoms are displayed in red, blue, yellow, grey, white, and green, respectively, and hydrogen bonds are represented with dashed lines (yellow).

Table 1.

Agonistic and antagonistic activities of TCE and its metabolites in ER-mediated luciferase reporter gene assay.

Chemicals	REC_{20} (M)	RLA[a] (%)	RIC_{20} (M)	RLA[b] (%)	LOEL (ppb)
17β-estradiol (E2)	5.4×10^{-12}	100	-	100	-
TCE	NE	-	-	-	-
TCA	NE	-	-	-	-
TCOH	NE	-	3.7×10^{-7}	51.2	55.3
OA	NE	-	-	-	-

NE: no effect.

REC_{20}: 20% relative effective concentration. The concentration of the test chemicals showing 20% of the agonistic activity of 2.5×10^{-11} M E2 via ER.

RIC_{20}: 20% relative inhibitory concentration. The concentration of the test chemicals showing 20% of the antagonistic activity of 9.37×10^{-12} M E2 via ER.

[a]RLA: relative luciferase activity. Percentage response of maximum activity of the test chemicals with 100% activity defined as the activity obtained from E2 at 2.5×10^{-11} M.

[b]RLA: relative luciferase activity. Percentage response of maximum inhibition of the test chemicals with 100% activity defined as the activity obtained from E2 at 9.37×10^{-12} M.

LOEL: The lowest observed effect level, which is the lowest concentration causing the 20% inhibition of luciferase activity.