Effects of the organochlorine *p,p'*-DDT on MCF-7 cells: investigating metabolic and immune modulatory transcriptomic changes

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Abstract

The organochlorine pesticide dichloro-diphenyl-trichloroethane (DDT) is persistent in the environment and leads to adverse human health effects. High levels in breast milk pose a threat to both breast tissue and nursing infants. The objectives of this study were to investigate DDT-induced transcriptomic alterations in enzymes and transporters involved in xenobiotic metabolism, immune responses, oxidative stress markers, and cell growth in a human breast cancer cell line. MCF-7 cells were exposed to both environmentally-relevant and previously-tested concentrations of *p,p'*-DDT in a short-term experiment. Significant up-regulation of metabolizing enzymes and transporters (*ACHE*, *GSTO1*, *NQO1* and *ABCC2*) and oxidative stress markers (*CXCL8*, *HMOX-1*, *NFE2L2* and *TNF*) was clearly observed. Conversely, *UGT1A6*, *AHR* and cell growth genes (*FGF2* and *VEGFA*) were severely down-regulated. Identification of these genes helps to identify mechanisms of *p,p'*-DDT action within cells and may be considered as useful biomarkers for exposure to DDT contamination.

Keywords: *p,p'*-DDT, MCF-7 cells, transcriptomics, biomarkers

Abbreviations: ABCC2, ATP binding cassette subfamily C member 2; ACHE, acetylcholinesterase; AHR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; CXCL8, C-X-C motif chemokine ligand 8; CYP1A1, cytochrome P450 family 1 subfamily A member 1; CYP3A5, cytochrome P450 family 3 subfamily A member 5; DDD, dichloro-diphenyl-dichloroethane; DDE, dichloro-diphenyl-dichloroethylene; DDT, dichloro-diphenyl-trichloroethane; ESR1, estrogen receptor 1; FGF2, fibroblast

growth factor 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSTO1, glutathione S-transferase omega 1; HMOX-1, heme oxygenase 1; NFE2L2, nuclear factor erythroid 2 like 2; NQO1, NAD(P)H quinone dehydrogenase 1; NR1I2, nuclear receptor subfamily 1 group I member 2; PXR, pregnane X receptor; RXRα, retinoid X receptor α; SULT1A1, sulfotransferase family 1A member 1; TNF, tumor necrosis factor; UGT1A6, UDP glucuronosyltransferase family 1 member A6; VEGFA, vascular endothelial growth factor A

1. Introduction

Over several decades, the organochlorine pesticide dichloro-diphenyl-trichloroethane (DDT) was used for agricultural and disease vector control purposes. DDT and its metabolites (most commonly dichloro-diphenyl-dichloroethylene (DDE) and dichloro-diphenyl-dichloroethane (DDD)) are persistent in the environment, bioaccumulate, and show toxicity in many species. Use of DDT is now strictly regulated, with the main use to control insect vectors of disease (Weiss, 2011).

Residues of DDT and its metabolites (collectively known as DDTs) in foods have been described in many countries (Thompson et al., 2017). Although levels in foods are declining, contamination levels in people are still a concern. In particular, high levels of lipophilic DDTs in human breast milk are a potential risk for infant health. Mean levels of DDTs in breast milk from malaria-endemic villages in South Africa using DDT regularly were 9.5-18 mg/kg milk fat (25-50 μM), sufficiently high to exceed the provisional tolerable daily intake (PTDI) for infants and the maximum residue limit (MRL) set by FAO and the WHO (Bouwman et al., 2012; JMPR, 2010). The postnatal period is considered a critical phase of development, and exposure at this time may have significant impact on infants (Desaulniers et al., 2005). Concurrently, high levels of DDTs in lipid-rich breast milk may result in toxic effects within breast tissue. Studies have linked exposure to DDTs with an increased risk of breast cancer in people (Cohn et al., 2015). However, the underlying metabolic changes for this risk are poorly understood. DDT is classed by the International Agency for Research on Cancer (IARC) as a group 2A carcinogen, a “probable cause of cancer in humans”.

Exposure of human breast cells to high levels of DDTs contamination from the environment *in vivo* means that *in vitro* exposure studies using human breast cell lines, such as MCF-7, are useful to investigate molecular changes at the cellular level. Use of the MCF-7 breast cancer cell line allows investigation of agents that may encourage progression of cancer in a cell line that maintains many characteristics of mammary epithelium. Also, estrogens simulate growth of many human breast cancers, and MCF-7 cells are one of the few breast cancer cell lines expressing the estrogen receptor α, a target for therapy. DDT is a known endocrine disruptor chemical, with some estrogen-like properties (Zhong et al., 2013).

Xenobiotic exposure has been linked to various biochemical changes, including acetylcholinesterase (*ACHE*) which has been used as a biomarker for neurotoxic substances in vertebrate and invertebrate species (Binelli et al., 2006; Vieira et al., 2016). Exposure to DDT induced cytochrome P450 family 1 subfamily A member 1 (*CYP1A1*) expression in rat livers and ovaries (Chanyshev et al., 2014). Cytochrome P450 family 3 subfamily A member 5 (*CYP3A5*) is another enzyme involved in phase I metabolism of xenobiotics, and is associated with DNA damage in workers exposed to organophosphate pesticides (Singh et al., 2011). Glutathione S-transferase omega 1 (GSTO1) plays a role in cellular stress responses but also is a significant phase II metabolizing enzyme (Ramkumar et al., 2016). Although not previously linked to DDTs, overexpression has additionally been shown in invasive breast cancer cell lines (Adam et al., 2002). The phase II enzyme UDP glucuronosyltransferase family 1 member A6 (UGT1A6) has been

linked to hepatic tumor promotion in a rat model when exposed to the pyrethroid-like insecticide etofenprox (Hojo et al., 2012). NAD(P)H quinone dehydrogenase 1 (*NQO1*), sulfotransferase family 1A member 1 (*SULT1A1*) and ATP binding cassette subfamily C member 2 (*ABCC2*, also known as *MRP2*) have been linked to metabolism of other xenobiotics (Hockley et al., 2006; Pascussi et al., 2008; Saengtienchai et al., 2014). The nuclear factor, erythroid 2 like 2 (*NFE2L2*, also known as *Nrf2*) gene is involved in regulation of the *ABCC2* gene, and is inhibited by *p,p'*-DDE (Jin et al., 2014; Vollrath et al., 2006). C-X-C motif chemokine ligand 8 (*CXCL8*, also known as *IL8*), tumor necrosis factor (*TNF*), and AHR inflammatory changes have been linked to persistent organic pollutants (Buoso et al., 2017; Kim et al., 2012). AHR also plays a major role in regulation of xenobiotic metabolizing enzymes such as CYP1A1 and NQO1. Heme oxygenase (*HMOX-1*) is a biomarker for oxidative stress, and its expression has been linked to environmental contamination by xenobiotics such as PCBs in fish and *in vivo* exposure of mice to *p,p'*-DDE (Morales-Prieto et al., 2017; Schlenk et al., 2002). *p,p'*-DDT is known to induce adipocyte differentiation, and vascular endothelial growth factor A (*VEGFA*) and fibroblast growth factor 2 (*FGF2*) were included in the genes of interest due to their involvement in adipose tissue angiogenesis (Cao, 2010; Moreno-Aliaga and Matsumura, 2002). Various regulatory elements have been implicated in effects of endocrine disruptor pesticides, including aryl hydrocarbon receptor (*AHR*), nuclear receptor subfamily 1 group I member 2 (*NR1I2*, also known as *PXR*) and estrogen receptors (including estrogen receptor 1, *ESR1*) (Chanyshev et al., 2014; Mnif et al., 2011).

Previous studies on the MCF-7 human breast cancer cell line have investigated various effects of DDTs such as cell viability and proliferation, invasiveness and glucose metabolism (He et al., 2015; Norberto et al., 2017; Pestana et al., 2015). However, genes related to xenobiotic metabolism, cellular stress, immunity and cell growth have not yet been investigated. In some studies, alterations in gene expression are detected even after short-term exposure. Therefore, the objective of this study was to assess a selection of such genes to elucidate some of the mechanisms by which *p,p'*-DDT, the main component of technical grade DDT, affects cells after a brief period of exposure. Therefore, the objective of this study was to assess a selection of such genes to elucidate some of the mechanisms by which *p,p'*-DDT, the main component of technical grade DDT, exposure affects cells.

2. **Materials and methods**

2.1 Chemicals and reagents

We obtained the MCF-7/GFP cell line from Cell Biolabs, Inc. (distributed by Funakoshi Co. Ltd., Tokyo, Japan). For culture, Dulbecco's modified Eagle's medium (DMEM)-high glucose with L-Glutamine and Phenol Red (Wako, Tokyo, Japan), penicillin-streptomycin solution (Wako, Tokyo, Japan) and fetal bovine serum (FBS, Biowest, France) were purchased. Treatments were the solvent dimethyl sulfoxide (DMSO, Sigma Chemical Co., St. Louis, MO, USA) and dichloro-diphenyl-trichloroethane (*p,p'*-DDT, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). The Cell Counting Kit-8 (CCK-8,

Dojindo, Kumamoto, Japan) was purchased for cellular viability assessment. TRI reagent (Sigma, St. Louis, MO, USA), chloroform (Kanto Chemical Co., Inc., Tokyo, Japan), Nucleospin® RNA (Machery-Nagel, Germany), ReverTraAce® qPCR RT Master Mix with gDNA remover (Toyobo Co., Osaka, Japan), Fast SYBR Green master mix (Applied Biosystems, Life Technologies Japan Ltd., Tokyo, Japan) and primer sets (Invitrogen, Carlsbad, CA, USA) were purchased for RNA isolation, cDNA synthesis and qPCR analysis.

2.2 Cell culture conditions

MCF-7 cells were grown in DMEM, supplemented with 10% FBS and 5% antibiotic, this contains 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin. They were maintained in a fully humidified atmosphere with 5% CO_2 at 37°C. Culture medium was changed every 2–3 days and subcultured when confluent (every 5–7 days).

2.3 Cell treatments

Once cell confluency was reached, treatments were added to the medium. DMSO was used as the carrier for *p,p'*-DDT, prepared by mixing powdered *p,p'*-DDT ultrasonically with DMSO. The final concentration of DMSO was the same for all treatment wells (0.1%). It notes worthy to report that the physiological levels of DDT in human blood ranged from 0.01 to 0.27 µM (Beard et al., 2003; Eskenazi et al., 2009) and in breast milk fat from 25-to 50 µM (Bouwman et al., 2012). Concentrations used for *p,p'*-DDT ranged from 0 to 100 µM. Such concentrations agree with that used in previous studies (Ballard

and Morrow, 2013; Bratton et al., 2012; Desaulniers et al., 2005; Ennaceur and Driss, 2013; Gregoraszczuk et al., 2008; He et al., 2015). Cellular RNA was collected 24 h after addition of the treatment, and stored at -80°C until further analysis.

2.4 Cell viability

Cellular viability was determined by CCK-8 assay as per the manufacturer's instructions. Cell suspension (100 µl) was inoculated in a 96-well plate (collagen-coated microplate, Iwaki, Japan), and once confluency was attained, treatments were added. Previous studies have reported no effect on MCF-7 cell viability at 0.1% DMSO concentration (Machado et al., 2016). Medium with DMSO only served as the solvent carrier negative control. Cells were pre-incubated with the treatments for 24 h before performing the assay to determine cell number. Absorbance was measured at 450 nm using a Thermo Scientific Multiskan® GO microplate spectrophotometer (Thermo Scientific, Japan). The assay was repeated three times. Cultures were also visually assessed by light microscopy (Olympus CK40, Tokyo, Japan).

2.5 RNA isolation and quantitative RT-PCR

For treatment prior to RNA isolation, cells were inoculated into 6-well collagen-coated microplates (Iwaki, Japan). Total RNA was isolated using a modified protocol for NucleoSpin® RNA (Macherey-Nagel GMbH & Co. KG, Düren, Germany). The initial steps were replaced by TRI reagent (Sigma-Aldrich) used according to the manufacturer's instructions to lyse cells, and chloroform (Kanto Chemical Co., Inc., Tokyo, Japan) for

phase separation. At this point, the aqueous phase was mixed with 70% ethanol and transferred to NucleoSpin® columns for DNA binding, desalting, DNA digestion, membrane washing, and RNA elution according to the NucleoSpin® protocol. For cDNA synthesis, ReverTraAce® qPCR RT Master Mix with gDNA remover (Toyobo Co. Ltd., Osaka, Japan) was used as described in the manufacturer's instructions. cDNA samples were stored at -20°C pending further analysis.

The mRNA expression levels were determined using real-time reverse transcriptase-PCR (RT-PCR), carried out using the Step One Plus Real-Time PCR system (Applied Biosystems, Foster, CA). The PCR mixture contained 600 ng of cDNA, Fast SYBR® Master mix, 10 μM of each primer, with RNase-free water added to a final volume of 10 μL. The reaction cycle comprised a holding stage for 20 s at 95°C, followed by 40 denaturation cycles of 3 s at 95°C and 30 s at 60°C (*GAPDH, ACHE, CYP1A1, CYP3A5, GSTO1, NQO1, UGT1A6, ABCC2, CXCL8, HMOX-1, NFE2L2, TNF, FGF2, VEGFA, AHR, ESR1,* and *NR1I2*) or 62°C (*GAPDH* and *SULT1A1*), and 15 s extension at 95°C. Single amplicon amplification was confirmed using melting curve analysis, and absence of primer dimers and genomic DNA amplification by agarose gel electrophoresis. GAPDH was used for normalization by the comparative Ct method, and each experiment repeated at least three times. Genes of interest were selected from a range of genes shown to be involved in exposure to xenobiotics, including various stages of xenobiotic metabolism (phase I, II, III), oxidative stress, inflammation and growth factors. Primer sets used are shown in Table 1.

2.6 Statistical analysis

Microsoft Excel® 2014 and JMP® Pro 13 (SAS Institute Inc., Cary, NC, USA) were used for data analysis. One way analysis of variance followed by Dunnett's test were performed to evaluate statistical significance between exposure groups and the control, with a *p*-value of <0.05 considered significant.

3. Results

3.1 Cellular viability

After 24 h exposure to either the carrier compound, DMSO, or the carrier with *p,p'*-DDT, cellular viability was determined by CCK-8 assay. As shown in Figure 1, there were no statistically significant effects observed on cell viability between wells containing the carrier (DMSO) and carrier with *p,p'*-DDT concentrations up to 50 μM. However, there was a significant reduction in cellular viability at 100 μM ($p < 0.0001$), and on microscopic evaluation some non-adherent cells were seen in wells with this concentration. Comparisons of gene expression were conducted using solvent carrier samples as controls.

3.2 Gene expression

Results of gene expression analysis are shown in Figures 2–5. First, the enzymes involved in metabolism were analysed (Figure 2). No significant differences were seen between negative carrier control and *p,p'*-DDT treatments for the phase I enzyme *CYP3A5*, nor the phase II enzyme *SULT1A1*. *ACHE* was up-regulated 3-fold at 50 μM ($p = 0.03$) and

16-fold at 100 μM ($p < 0.0001$). For the phase I enzyme *CYP1A1*, gene expression was down-regulated 5-fold at 5 μM *p,p'*-DDT ($p = 0.04$). Of the other phase II enzymes assessed, *NQO1* was up-regulated 2–fold at 10 μM and 20 μM ($p < 0.0001$ for both), while *GSTO1* was up-regulated 2-fold at 20 μM and 4-fold at 50 μM and 100 μM ($p < 0.0001$ for both). Conversely, expression of *UGT1A6* was down-regulated significantly ($p < 0.0001$) for all exposure groups, with a minimum of 17-fold change at 10 μM. The phase III metabolising enzyme, *ABCC2*, was up-regulated 14-fold at 100 μM ($p = 0.01$).

Oxidative stress and inflammatory markers were then analysed (Figure 3). *CXCL8* expression was up-regulated 71-fold at 100 μM ($p < 0.0001$), and *NFE2L2* up-regulated 10-fold at 50 μM ($p = 0.001$). Both *HMOX-1* and *TNF* showed dose-dependent up-regulation from 10 μM to 100 μM treatments ($p < 0.0001$ at 20 μM and above). Gene expression for *HMOX-1* ranged from 3-fold up-regulation at 10 μM ($p = 0.02$) to 16-fold at 100 μM (with $p < 0.0001$ from 20 μM upwards). Up-regulation for *TNF* was from 4-fold at 10 μM ($p = 0.008$) to 24-fold at 100 μM (with $p < 0.0001$ from 20 μM upwards).

Next, cell growth genes were analysed (Figure 4). *FGF2* was down-regulated at all exposure concentrations, to a low of 398-fold compared to control at 20 μM ($p = 0.0003$ at 50 μM, $p < 0.0001$ for all other concentrations). *VEGFA* was down-regulated between 5 μM ($p = 0.006$) and 20 μM ($p = 0.04$), with a low of 3-fold at 10 μM ($p = 0.005$).

Finally, regulatory elements were analysed (Figure 5). No significant differences were seen between negative carrier control and *p,p'*-DDT treatments for either *ESR1* or *NR1I2*. *AHR* expression was down-regulated between 5 μM ($p = 0.003$) and 50 μM ($p = 0.006$), with a low of 2–fold at 10 μM ($p = 0.002$).

4. **Discussion**

4.1 Cellular viability

Concentrations up to 50 μM *p,p'*-DDT did not affect cell viability. However, the difference at 100 μM shows significant adverse cellular changes. Data for the highest exposure were considered with this in mind. Exposure of the neuronal PC12 cell line to *p,p'*-DDE induced apoptosis via TNF signalling at concentrations ≥ 20 μM (Wang et al., 2014). Wang et al.'s study also demonstrated dose-dependent neuronal apoptosis in zebrafish embryos exposed to *p,p'*-DDE. Cell cytotoxicity appears to be dependent on the cell line, exposure concentration, and congener (Nuñez et al., 2002). Even within the same cell line, there may be discrepancies in viability results, perhaps due to differences in culture conditions or cell passage number. Reduction in cellular viability could be due to oxidative stress identified in some of the genes assessed in this study, for example *TNF* or *CXCL8*, which may lead to cell death.

4.2 Gene expression

Although previous studies suggested that significant changes may be expected in expression of the genes selected, this was not so in all cases.

4.2.1 Enzymes and transporters involved in metabolism

Environmental studies frequently use inhibition of ACHE activity as a biomarker for xenobiotic, including DDTs, contamination (Jung et al., 2012; Vázquez-Boucard et al., 2014). Activity of ACHE in the brain appears to be an important biomarker but even serum levels are less reliable for toxicity assessment (Walker et al., 2012). Responses to drugs or traumatic insults may induce *ACHE* transcription (Soreq and Seidman, 2001). Long term exposure to organophosphorus pesticides was linked to amplification of the *BCHE* gene which encodes butyrylcholinesterase (BuChE), an enzyme related to ACHE (Prody et al., 1989). Although a non-significant inhibition of *ACHE* was observed at low exposure concentrations, this *in vitro* study has identified a substantial promotion of expression in MCF-7 cells at higher *p,p'*-DDT concentrations. Thus, ACHE may play a more important role at high levels of contamination.

No significance was detected in *NR1I2* expression. A systematic study conducted by Chaturvedi and others concluded that different mouse cell types responded discordantly to xenobiotic exposure, with the DDT treatment resulting in up-regulation of the NR1I2 receptor in liver but unchanged in testis (Chaturvedi et al., 2010). Species-specific regulation of the *NR1I2* gene has also been demonstrated in mouse and rat liver experiments after *o,p'*-DDT exposure (Kiyosawa et al., 2008a). The nuclear receptor

NR1I2 pathway (originating at the constitutive androstane receptor/pregnane X receptor (CAR/PXR) ligand on the nucleus) is involved in *CYP3A5* expression. It is therefore unsurprising that this gene was not significantly affected by treatment.

Expression of the phase II enzyme *SULT1A1* and phase III enzyme *ABCC2* are also under control via the CAR/PXR ligand, but via the nuclear receptor subfamily 1 group I member 3 (NR1I3) pathway. Phase I enzyme *CYP3A5* expression is also influenced via this pathway. Of these three enzymes, only *ABCC2* expression showed a significant dose-dependent up-regulation at the highest treatment. This gene is also induced by NFE2L2 and NR1I2, and it may be useful to assess comparative expression of these with NR1I3 to further clarify the mechanism involved. The ABCC2 protein plays a major role in elimination of endo- and xenobiotics, and regulation by *p,p'*-DDT is likely to impact detoxification in the body (Arana and Tocchetti, 2016). For the MCF-7 cell line, elimination of *p,p'*-DDT at phase III may be more important than earlier stages of metabolism. Further examination of other phase I and phase II enzymes should be conducted to elucidate this.

Expression of the phase I enzyme *CYP1A1* was significantly downregulated only at the 5 μM concentration. In the MCF-7 cells, *AHR* expression was also down-regulated after exposure to *p,p'*-DDT. AHR is known to induce expression of *CYP1A1*. This finding concurs with a study using placental cells, which found suppression of both AHR protein and CYP1A1 activity after exposure to *p,p'*-DDT, *o,p'*-DDT and *o,p'*-DDE (Wójtowicz et al., 2011) Another regulator of *CYP1A1* expression is ESR1; expression of *ESR1* was

not significantly affected by *p,p'*-DDT in this study. Although some pesticides including DDTs have previously been linked to estrogenic effects, and CYP1A1 has been implicated in some cell line exposures, a study exposing MCF-7 cells with *p,p'*-DDE and another exposing peripheral blood mononuclear cells with DDE did not result in induction of the *CYP1A1* gene via this estrogenic mechanism (Gaspar-Ramírez et al., 2015; Liu et al., 2014; McDougal et al., 1997). Also, expression of *TNF* was significantly up-regulated in a dose-dependent manner. This protein is known to inhibit expression of *CYP1A1*. NFE2L2, which was up-regulated at 50 μM, also inhibits *CYP1A1* expression. The balance between ESR1, NFE2L2 and TNF effects is likely to be important for *CYP1A1* expression.

After oxidation of xenobiotics (phase I metabolism), some resultant electrophiles undergo glutathione conjugation catalysed by glutathione transferases (GSTs). GSOT1 has been linked to arsenic metabolism and associated atherosclerosis (Hsieh et al., 2011). GST activity in rat testes was decreased at high dose (100 mg/kg intraperitoneally daily for 10 days) *in vivo* exposure to *p,p'*-DDT (Marouani et al., 2017). Induction of *GSTO1* gene expression is regulated by retinoid X receptor α (RXRα) and PXR pathways (Dai et al., 2005). These pathways should be investigated to clarify species and/or tissue differences in GST expression and activity in response to *p,p'*-DDT exposure.

NQO1 is a phase II enzyme, known to detoxify xenobiotics and provide cytoprotection to exposed tissues. Exposure to the carcinogen benzo(a)pyrene upregulated expression of NQO1 in both MCF-7 and HepG2 human cell lines (Hockley et al., 2007). Expression of

the *NQO1* phase II enzyme was significantly up-regulated in MCF-7 at 10 μM and 20 μM *p,p'*-DDT concentrations. Reactive oxygen species activate NFE2L2 (up-regulated in this study) via the MAPK pathway, in turn inducing *NQO1*. AHR may induce *NFE2L2*, but down-regulation of *AHR* seen in this study suggests this pathway is not involved during *p,p'*-DDT exposure. TNF is known to induce expression of *NQO1*, and expression of *TNF* was significantly up-regulated in the study. The balance between NFE2L2, TNF and AHR effects is likely to be important for *NQO1* expression.

The phase II enzyme UGT1A6 is an antioxidant, induced by NFE2L2 and AHR. In this study, decreased expression of *UGT1A6* therefore likely is linked to the down-regulation of *AHR*. Conversely, an *in vivo* exposure study of deer mice (*Peromyscus maniculatus*) with *p,p'*-DDE resulted in increased conjugation, as denoted by measurement of a general UGT substrate (Dickerson et al., 1999). This difference may be attributed to the inter-species and/or tissue differences. Thus, confirmation of function should therefore be established in the MCF-7 cell line before final determination of the role of this enzyme in DDT metabolism. UGTs are also known to play a significant role in first pass metabolism of many medical drugs, and therefore any functional alterations after DDT exposure may potentially increase susceptibility to toxic effects of drugs (Fisher et al., 2001).

4.2.2 Oxidative stress and inflammatory markers

A highly significant ($p < 0.0001$) increase in gene expression of *CXCL8* was seen at the 100 μM exposure concentration. This inflammatory cytokine and indicator of oxidative stress may be related to the reduced cell viability seen at this concentration, and this mechanism should be further investigated. A study with exposure of THP-1 cells (a human monocytic cell line) to *p,p'*-DDT or *p,p'*-DDE resulted in a significant down-regulation of *CXCL8* expression, suggesting cell-specific effects on this gene (Buoso et al., 2017). CXCL8 is thought to be involved in pathogenesis of bronchiolitis, and prenatal DDE exposure has been linked to children's respiratory health. However, although regression modelling suggested the chemokine interleukin 10 (*IL10*) plays a role in such respiratory pathology, the modelling did not confirm a link with *CXCL8* (Gascon et al., 2014).

Expression of the anti-oxidant enzyme *HMOX-1* was up-regulated in a dose-dependent manner when exposed to *p,p'*-DDT. Inducers of this gene include heavy metals, endotoxin, and inflammatory cytokines (Choi and Alam, 1996). NFE2L2 has been shown to up-regulate expression of this gene (Salazar et al., 2006). In this study, a significant increase was also noted in *NFE2L2* at 50 μM concentration. Conversely, a study using HepG2 cells showed ROS-mediated down-regulation of *NFE2L2* following exposure to *p,p'*-DDE (Jin et al., 2014). It would be of interest to repeat exposure of MCF-7 cells with *p,p'*-DDE at similar exposure concentrations to compare effects. This indicator of oxidative stress is an important intermediary in several xenobiotic metabolism pathways and therefore a useful biomarker.

TNF is an important pro-inflammatory cytokine, regulating immune response to pathogens. Massawe et al. demonstrated an increase in TNF secretion after DDT exposure at 2.5 μM to various human immune cells, thought to occur via the MAPK pathway (Massawe et al., 2017). Conversely, Burow et al. proposed that suppression of TNF-induced apoptosis by *o,p'*-DDT occurred via an estrogen receptor pathway (Burow et al., 1999). However, a study on macrophages showed that although DDT alone induced *TNF*, suppression of *TNF* was seen with DDT in the presence of lipopolysaccharide (found on the outer membrane of Gram negative bacteria and a strong immune stimulant) (Dutta et al., 2008). TNFα is a pro-inflammatory cytokine, and altered production will result in an imbalance in the immune system. An association was shown clinically between maternal *p,p'*-DDE and *o,p'*-DDD, *TNF* expression, and preterm birth of infants (Tyagi et al., 2016). A correlation has also been demonstrated between chlorinated pesticides, including DDT, in mothers' milk and depressed TNF secretion in infants (Schaalan et al., 2012). These data suggest immunotoxicity relating to organochlorine exposure may occur in infants during development.

4.2.3 Cell growth

Interestingly, expression of both cell growth factors—*FGF2* and *VEGFA*—were significantly down-regulated with exposure to *p,p'*-DDT. With *VEGFA*, the suppression is most obvious at lower exposure doses. These lower doses are closer to the environmental exposures seen with breast milk contamination. An 18 h exposure experiment with MCF-7 cells showed up-regulation of *VEGFA* expression after exposure to 10 μM *o,p'*-DDT, suggesting a difference in effects between DDT congeners (Bratton

et al., 2012). AHR induces *FGF2* and *VEGFA* expression, and thus the down-regulation of these genes seen in this study may be explained by concomitant *AHR* down-regulation (Lahoti et al., 2013). Downregulation of *VEGF* expression has been linked to suppression of tumor-mediated angiogenesis (Yen et al., 2017). However, elevated VEGF levels have also been linked to a better survival rate in breast cancer (Gasparini, 2000). This is the first report of *FGF2* expression effects in association with *p,p'*-DDT exposure, and may indicate another mechanism by which *p,p'*-DDT exerts effects on cells, particularly adipose tissue. FGF2 has been shown to mediate cell proliferation in hepatocellular carcinoma via inhibition of apoptotic cell death, thus a reduction may be protective in some cases of cancer (Pan et al., 2013). Another metabolite of DDT, *o,p'*-DDD (also known as mitotane) is used to inhibit cell proliferation in the treatment of adrenal adrenocortical carcinoma (Waszut et al., 2017).

4.2.4 Regulatory elements

There was a significant down-regulation of *AHR* gene expression at exposure doses between 5–50 μM. This differs from results in a study using MCF-7 cells with 1 μM *p,p'*-DDT exposure, which showed enhanced proliferation of cells associated with up-regulation of *AHR* expression after 12 h (Ociepa-Zawal et al., 2007). Peripheral blood mononuclear cells with 28 nM DDE exposure also showed up-regulation of *AHR* expression, which was abolished by TNF (Gaspar-Ramírez et al., 2015). It can be concluded that differing the exposure chemical, dose and period have very different effects on cells.

Although there appears to be a trend for increasing expression of *ESR1* with increasing concentration of *p,p'*-DDT, no statistical significance was detected between exposure groups. Previous work on mice brains has linked prenatal *p,p'*-DDT exposure to a depressive-like effect associated with a decrease in estrogen receptors including ESR1 (Kajta et al., 2017). An *in vivo* exposure study using *o,p'*-DDT in immature rats did not elicit estrogen receptor-mediated responses (Kiyosawa et al., 2008b).

At the concentrations used, no statistically significant effects were observed in *NR1I2* expression after exposure of MCF-7 cells with *p,p'*-DDT. Exposure of other cell lines to other DDT congeners is generally reported to result in induction of *NR1I2*: for example in HepG2 cells exposed to 10 μM *o,p'*-DDT, liver samples from rats which received technical grade DDT, and liver from salmon receiving DDE (Kiyosawa et al., 2008b; Medina-Díaz et al., 2007; Mortensen and Arukwe, 2006). However, Kiyosawa et al. reported species differences in PXR and CAR activation in mice and rats (Kiyosawa et al., 2008a).

Many experiments have been conducted using DDTs to assess the molecular effects. Differences in cell line, tissue, species, DDT congener used and exposure time vary greatly between studies. Genes were selected in this study to give an overview of several metabolic processes that may be affected by xenobiotic exposure. Also, expression in the target cell line was considered (for example CYP1A1 is highly expressed in breast cells while CYP1A2 is not). This study has demonstrated a number of modulatory effects by *p,p'*-DDT on the transcriptome, and future studies can follow these to further elucidate

pathways involved. Detailed assessment at the proteomic and metabolomic levels would enlighten our understanding of these mechanisms and their importance in toxicity relating to DDTs. Therefore, future approaches are still required to investigate the posttranslational effects of DDTs of XMEs and other regulatory factors. It would be useful to investigate these areas of metabolism in more depth, for example selecting oxidative stress markers such as superoxide dismutase (SOD), or inflammatory mediators such as Type 1 interferon or other interleukins. We still have much to learn about the toxic effects of DDTs, especially at levels and mixtures present in the environment.

This acute exposure study used levels of *p,p'*-DDT that are higher (10–100 μM) than those previously reported in breast milk in Tunisian women (estimated to be 0.0395 μM using an average milk fat concentration of 3.2%) (Ballard and Morrow, 2013; Ennaceur and Driss, 2013). However, high-level exposure acutely may mimic the chronic exposure experienced by people living in countries using DDT regularly. It would therefore be useful to assess genes identified in the study with expression in these populations.

5. **Conclusions**

This experimental study has identified several genes to be significantly up-regulated (*ACHE*, *GSTO1*, *NQO1*, *ABCC2*, *CXCL8*, *HMOX-1*, *NFE2L2*, and *TNF*) and down-regulated (*CYP1A1*, *UGT1A6*, *FGF2*, *VEGFA*, and *AHR*) after acute exposure of MCF-7 cells to the *p,p'*-DDT congener. Effects are dose-dependent in some genes. These genes are involved in an array of metabolic processes, including inflammation, oxidative stress,

and growth of fibroblast and vascular endothelial cells. These may be useful biomarkers for exposure to DDT contamination, and may also help identify the mechanisms of toxicity of DDT and its metabolites in breast cells and in nursing infants.

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Table 1: Primers sets used for qRT-PCR analysis in this study.

Symbol	Description	Function	Sequence		Accession number	Product length (bp)
			Forward	Reverse		
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	(Housekeeping gene)	ACCCAGAAGACTGTGGATGG	CAGTGAGCTTCCCGTTCAG	NM_001289746.1	139
ACHE	Acetylcholinesterase	Metabolism	CATCAACGCGGGAGACTT	GAGACTCGTTGTCTTTGCTGAA	NM_001302621.1	113
CYP1A1	Cytochrome P450 family 1 subfamily A member 1	Phase I metabolism	CTATCTGGGCTGTGGGCAA	CTGGCTCAAGCACAACTTGG	NM_001319217.1	138
CYP3A5	Cytochrome P450 family 3 subfamily A member 5	Phase I metabolism	TGACCCAAAGTACTGGACAG	TGAAGAAGTCCTTGCGTGTC	NM_001291830.1	240
GSTO1	Glutathione S-transferase omega 1	Phase II metabolism	AGGACGCGTCTAGTCCTGAA	TTCCCTGGGTATGCTTCATC	NM_004832.2	191
NQO1	NAD(P)H quinone dehydrogenase 1	Phase II metabolism	GGATTGGACCGAGCTGGAA	AATTGCAGTGAAGATGAAGGCAAC	NM_001286137.1	140
UGT1A6	UDP glucuronosyltransferase family 1 member A6	Phase II metabolism	CATGATTGTTATTGGCCTGTAC	TCTGTGAAAAGAGCATCAAACT	NM_001072.3	105
SULT1A1	Sulfotransferase family 1A member 1	Phase II metabolism	AAAGCCCCAGGGATTCCCTCA	GGAAACTGCCACATCCTTTGCGT	NM_177530.2	162
ABCC2	ATP binding cassette subfamily C member 2	Phase III metabolism	CTTCGGAAATCCAAGATCCTGG	TAGAATTTTGTGCTGTTCACATT	NM_000392.4	284
CXCL8	C-X-C motif chemokine ligand 8	Inflammation, oxidative stress	ACTTTCAGAGACAGCAGAGCACACA	CCTTCACACAGAGCTGCAGAAATC	NM_001354840.1	151
HMOX-1	Heme oxygenase 1	Oxidative stress	ATGGCCTCCCTGTACCACATC	TGTTGCGCTCAATCTCCTCCT	NM_002133.2	55
NFE2L2	Nuclear factor, erythroid 2 like 2	Oxidative stress	CTTGGCCTCAGTGATTCTGAAGTG	CCTGAGATGGTGACAAGGGTTCTA	NM_001313904.1	124
TNF	Tumor necrosis factor	Inflammation	CAGCCTCTTCTCCTTCCTGAT	GCCAGAGGGCTGATTAGAGA	ENST00000376122.3	123
FGF2	Fibroblast growth factor 2	Growth factor, fibroblast cells	GGCTTCTTCCTGCGCATCCA	GCTCTTAGCAGACATTGGAAGA	NM_002006.4	354
VEGFA	Vascular endothelial growth factor A	Growth factor, especially vascular endothelial cells	ACATTTACACGTCTGCGGATCT	AGGGAAAGGGGCAAAAACG	NM_001025367.2	104
AHR	Aryl hydrocarbon receptor	Regulatory element	ATCACCTACGCCAGTCGCAAG	AGGCTAGCCAAACGGTCCAAC	NM_001621.4	137
ESR1	Estrogen receptor 1	Regulatory element	ATTGGTCTCGTCTGGCGCTCC	CCCTGCAGATTCATCATGCGG	NM_001328100	161
NR1I2	Nuclear receptor subfamily 1 group I member 2	Regulatory element	CATGAGGGGGGTAGCAAAGC	TGCAGGGGATCTCCCTCTTC	NM_022002.2	248

Effects of the organochlorine *p,p'*-DDT on MCF-7 cells: investigating metabolic and immune modulatory transcriptomic changes

Captions to figures and tables

Table 1: Primers sets used for qRT-PCR analysis in this study.

Figure 1: Comparison of *p,p'*-DDT on MCF-7 cell viability, as evaluated by the CCK-8 assay after 24 h exposure. DMSO concentration in all wells was 0.1%. Data are mean ± SE of percent viability compared to DMSO. Range of *p,p'*-DDT concentrations was 5–100 μM. Column carrying *** mark is significantly different at $p < 0.0001$ compared to DMSO wells (Dunnett's test).

Figure 2: Effect of *p,p'*-DDT on metabolizing enzymes in MCF-7 cells after 24 h exposure. A) Acetylcholinesterase (*ACHE*), B) cytochrome P450 family 1 subfamily A member 1 (*CYP1A1*), C) cytochrome P450 family 3 subfamily A member 5 (*CYP3A5*), D) glutathione S-transferase omega 1 (*GSTO1*), E) NAD(P)H quinone dehydrogenase 1 (*NQO1*), F) UDP glucuronosyltransferase family 1 member A6 (*UGT1A6*), G) sulfotransferase family 1A member 1 (*SULT1A1*), and H) ATP binding cassette subfamily C member 2 (*ABCC2*). Values are expressed as mean ± SE of mRNA expression relative to the control, DMSO (ΔΔCt method, $n \geq 3$). Columns carrying a * mark are significantly different at $p < 0.05$, and *** at $p < 0.001$ (Dunnett's test).

Figure 3: Effect of *p,p'*-DDT on oxidative stress and inflammatory markers in MCF-7 cells after 24 h exposure. A) C-X-C motif chemokine ligand 8 (*CXCL8*), B) heme oxygenase 1 (*HMOX-1*), C) nuclear factor, erythroid 2 like 2 (*NFE2L2*), and D) tumor necrosis factor (*TNF*). Values are expressed as mean ± SE of mRNA expression relative

to the control, DMSO (ΔΔCt method, $n \geq 3$). Columns carrying a * mark are significantly different at $p < 0.05$, ** at $0.001 < p < 0.01$, and *** at $p < 0.001$ (Dunnett's test).

Figure 4: Effect of *p,p'*-DDT on cell growth factors in MCF-7 cells after 24 h exposure. A) Fibroblast growth factor 2 (*FGF2*), and B) vascular endothelial growth factor A (*VEGFA*). Values are expressed as mean ± SE of mRNA expression relative to the control, DMSO (ΔΔCt method, $n \geq 3$). Columns carrying a * mark are significantly different at $p < 0.05$, ** at $0.001 < p < 0.01$, and *** at $p < 0.001$ (Dunnett's test).

Figure 5: Effect of *p,p'*-DDT on regulatory elements in MCF-7 cells after 24 h exposure. A) aryl hydrocarbon receptor (*AHR*), B) estrogen receptor 1 (*ESR1*), and C) nuclear receptor subfamily 1 group I member 2 (*NR1I2*). Values are expressed as mean ± SE of mRNA expression relative to the control, DMSO (ΔΔCt method, $n \geq 3$). Columns carrying a ** mark are significantly different at $0.001 < p < 0.01$ (Dunnett's test).

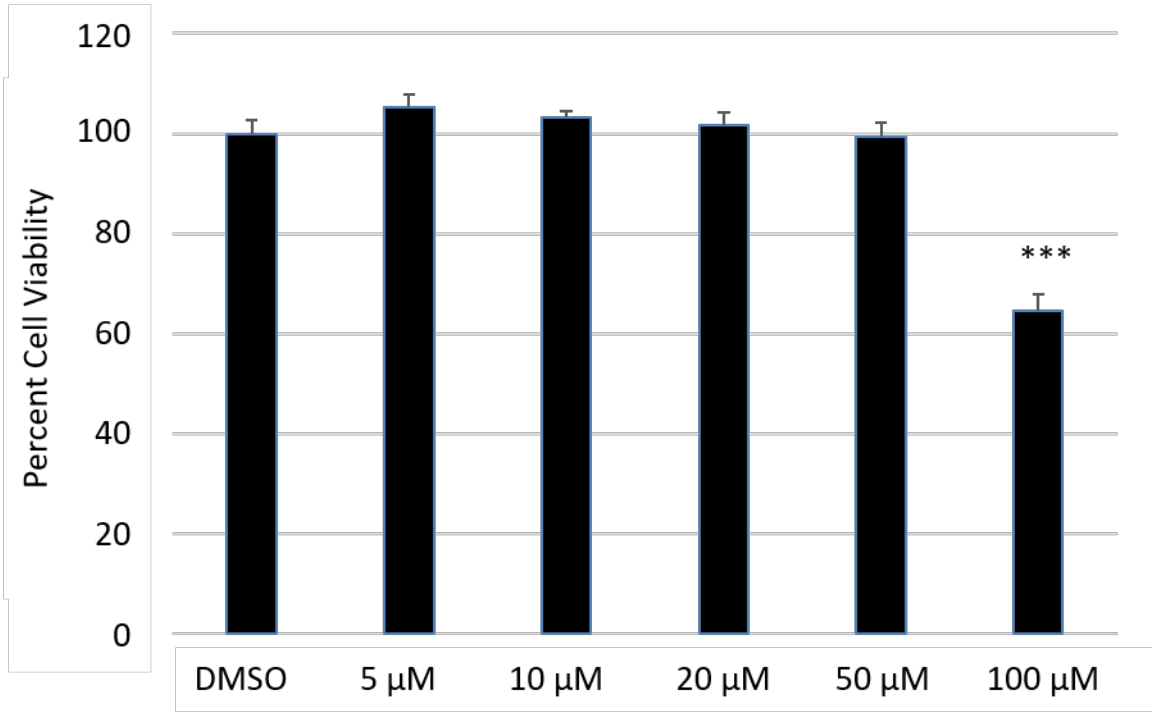

Figure 1
Comparison of *p,p'*-DDT on MCF-7 cell viability, as evaluated by the CCK-8 assay after 24 h exposure.

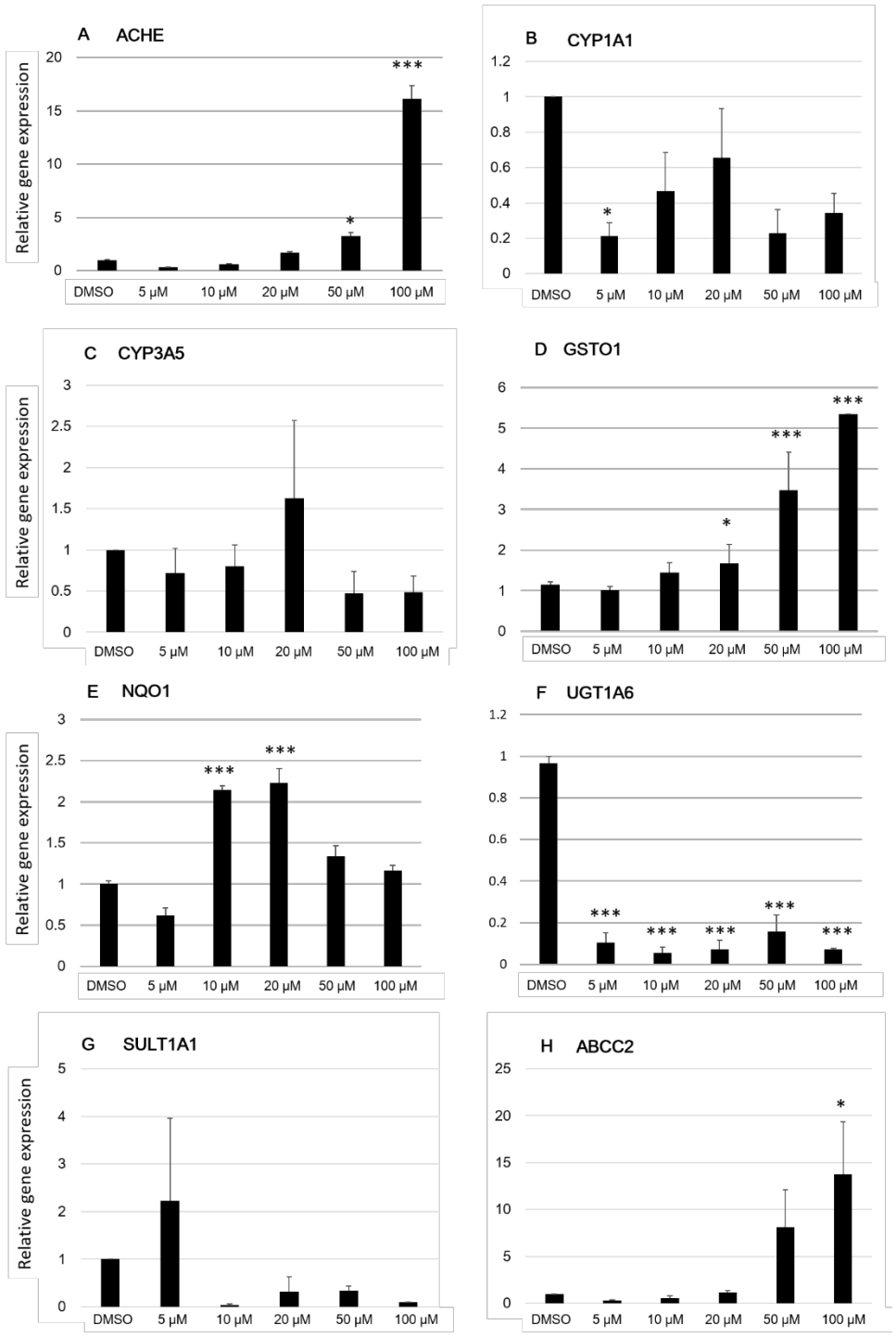

Figure 2
Effect of *p,p'*-DDT on metabolizing enzymes in MCF-7 cells after 24 h exposure.

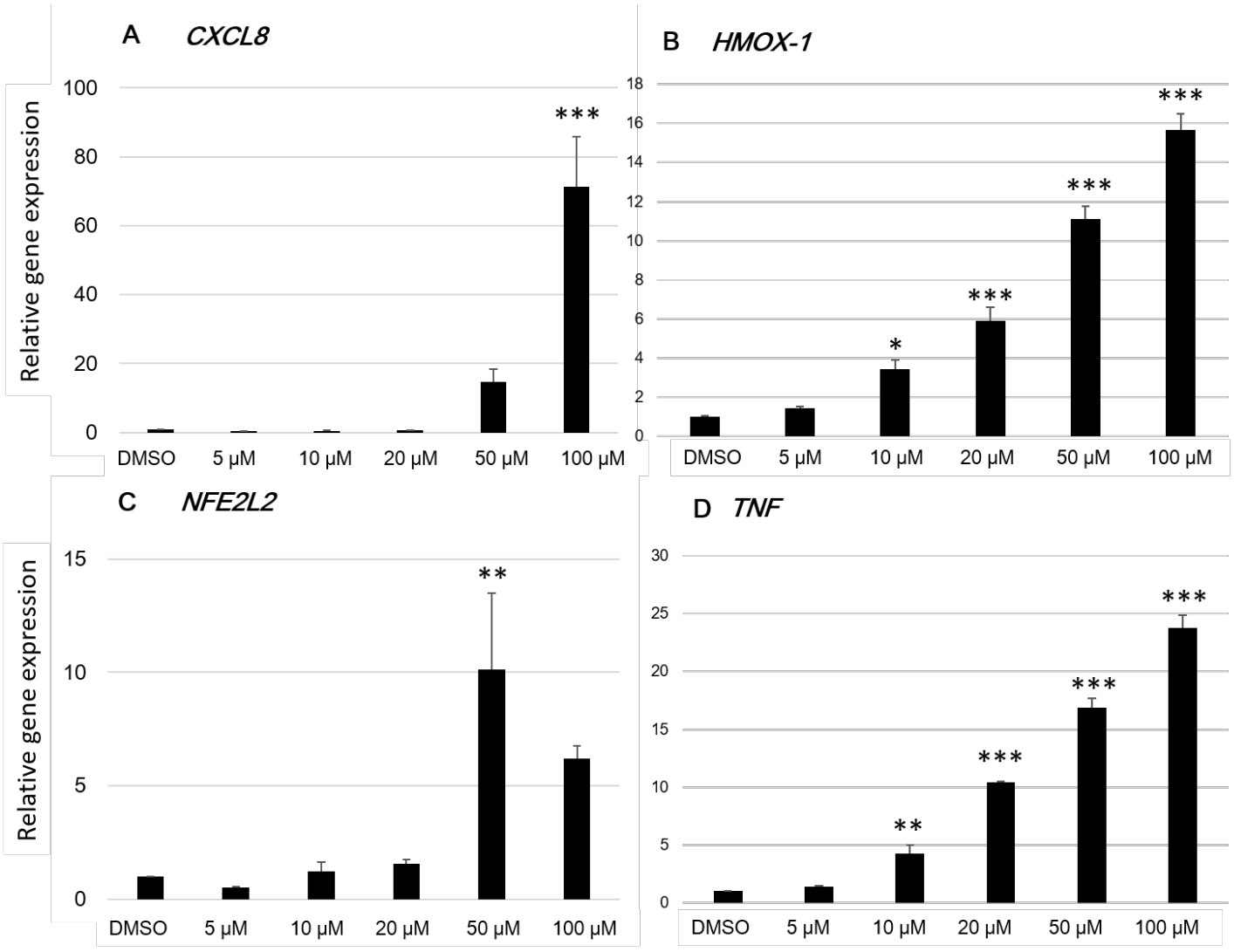

Figure 3
Effect of *p,p'*-DDT on oxidative stress and inflammatory markers in MCF-7 cells after 24 h exposure.

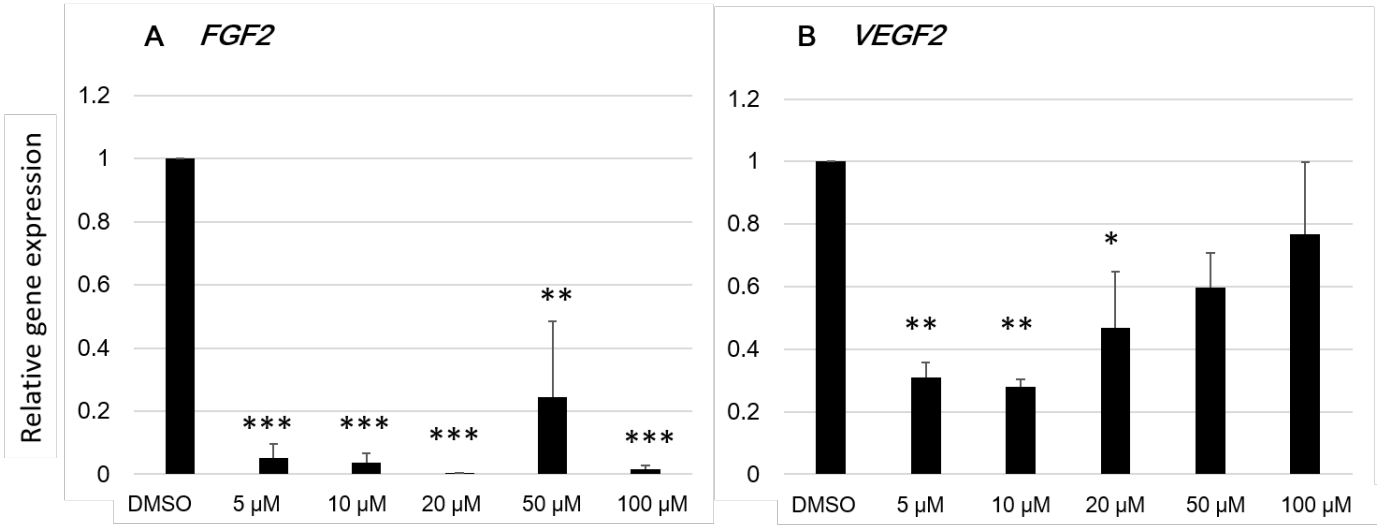

Figure 4
Effect of *p,p'*-DDT on cell growth factors in MCF-7 cells after 24 h exposure.

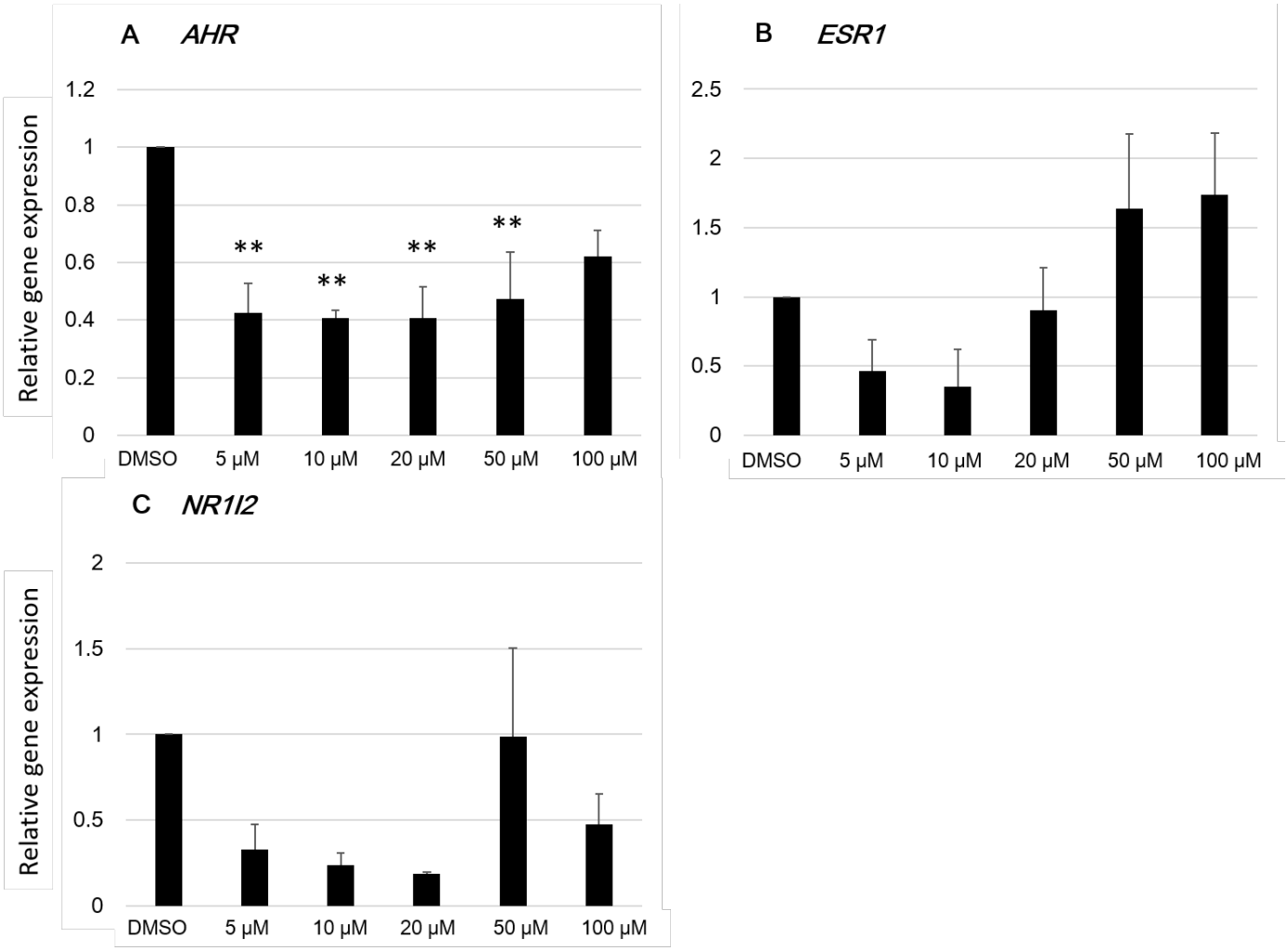

Figure 5
Effect of *p,p'*-DDT on regulatory elements in MCF-7 cells after 24 h exposure.