Phthalates and Phthalate Alternatives: Effects on Proliferative and Estrogenic Target Genes in Ishikawa Cells

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BRIEF. Effects of phthalates and phthalate alternatives in Ishikawa cells were compared against vehicle and 17β-estradiol treatments to observe changes in expression of estrogenic and proliferative genes.

ABSTRACT: Phthalates are used as plasticizers in many of the products found in medical, household, and industrial applications. Much research has not been completed on the effects of these phthalates as potential endocrine disrupting chemicals (EDCs). As these chemicals are ingested, the mechanism by which they affect the reproductive system is largely unknown. The purpose of this study was to observe how 2 phthalates, Di-n-butyl phthalate (DBP) and Diisononyl phthalate (DINP), and 2 phthalate alternatives, Dioctyl terephthalate (DOTP) and BHT (butylated hydroxytoluene)affect uterine cells in comparison to a vehicle treatment and 17β-Estradiol treatment. Changes in expression of mRNA were observed using reverse transcription polymerase chain reaction. Results from this study show that based on trends of change in the genes CD1, C-myc, ERα, PR, and HOXA10, each of the four chemical treatments changed proliferation in Ishikawa cells. Our results have opened possible classifications for mechanisms that the chemical treatments may follow as potential EDCs.

INTRODUCTION.

Endocrine disruption occurs when a synthetic chemical, endocrine disruptor, is absorbed into the body and disrupts the body's normal functions by mimicking or blocking hormones [1]. Endocrine disrupting chemicals lead to unnecessary intracellular responses by attaching to receptors and activating the cell's normal response to an excessive extent or at the wrong time [2]. The disruptor could have an antagonistic effect by simply bonding to the cellular receptor, preventing the natural hormone from bonding. Disruptors can also bind to transport proteins which can affect metabolic rates, which affect desynthesis of the natural hormone [2]. Investigating EDCs is important in improving public health because presence of EDCs within the body can lead to reproductive diseases such as endometriosis, infertility, diabetes, metabolic syndrome, breast or prostate cancer, early puberty, and obesity [3]. One common hormone that is mimicked by toxic chemicals is estrogen. Estrogen is produced in all vertebrates and it is important that estrogen is released at the right time and stage in the organism to maintain homeostasis [2]. Most endocrine disruptors are man-made pollutants that mimic or block the function of estrogen. These compounds can bind to the estrogen receptor and can cause transcription to occur, which may happen even when homeostasis is not disrupted [4]. Known endocrine disruptors such as Bisphenol-A and dichlorodiphenyltrichloroethane (DDT) have been extensively researched and are known to mimic estrogen in both male and female reproductive systems, as shown in both in *vivo* and in *vitro* models [5,6]. Although some phthalates are classified as endocrine disrupting chemicals, many of the ones used often in industrial production have not been researched extensively, and therefore have not been classified as endocrine disrupting chemicals yet [5]. Although the phthalates Di-n-butyl phthalate (DBP) and diisononyl phthalate (DINP) have been banned in production, phthalate alternatives such as Di-octyl terephthalate (DOTP) and butylated hydroxytoluene (BHT) have replaced traditional phthalates [7]. The effects of these chemicals on the female reproductive system have not been researched to a great extent. Instead of only focusing on the ability of the four potential endocrine disrupting chemicals (DBP, DINP, DOTP, and BHT) to interfere with or promote estrogenic activity, the effect of each treatment on proliferative genes was also observed in this study. Genes known to indicate change in proliferation and differentiation include HOXA10, Cyclin D1, and C-myc, while genes such as Estrogen Receptor (ER) and Progesterone Receptor (PR) were used to determine estrogenic properties [8,9,10,11]. We hypothesized that the ability of each phthalate to affect expression of each target gene will vary based on mechanism of action. Currently, many studies have not been completed observing the effects of phthalate and phthalate alternatives on changes of these target genes in Ishikawa cells or even leiomyoma or myometrium cells [5]. DBP has been shown to have a possibility of decreasing androgenic function, and therefore may act as an androgen inhibitor, but the effects on estrogen related genes are not clear [6]. The purpose of this study was to observe and compare the effects of two phthalates, DBP and DINP, and two phthalate alternatives, DOTP and BHT, in comparison to vehicle and 17β-estradiol (E2) treatments on target genes affecting proliferation and estrogenic function in Ishikawa cells.

MATERIALS AND METHODS.

Cell Culture and Treatment.

Ishikawa cells, a well-differentiated line of endometrial adenocarcinoma human cells which express both estrogen and progesterone receptors, were used because they express not only Progesterone Receptor (PR) and Estrogen Receptor Alpha (ERα), but also express HOXA10, Cyclin D1 (CD1), and C-Myc [8,11,12]. Ishikawa cells were used because they are more sensitive to chemical treatment than normal myometrium cells and are smaller in size, allowing for culturing of more cells. However, because Ishikawa cells share the same hormone receptors as healthy uterine cells and tissue and both types of cells are found in the uterus, the results can still be applied to studies on healthy uterine tissue and cells [12]. The chemicals, DBP, DINP, DOTP, and BHT were obtained from Sigma-Aldrich Chemical Company. These chemicals were diluted in ethanol to prepare a stock solution of each chemical at 10^{-2} M concentration. All cells were cultured (all below passage 10) in DMEM/$F12_{1:1}$ (GIBCO/BRL, Grand Island, NY) which contained 10% Fetal Bovine Serum (FBS). Cells were grown to confluence in two 150 mm cell culture plates and were maintained in a 37°C 5% CO_2, humid environment within the incubator. These cells were trypsinized and seeded into three 6-well plates. After cells reached 70-80% confluence, cells were starved in phenol red-free DMEM/F12 for 24 hours. For the first experiment the starved cells were treated with DBP, DINP, DOTP, or BHT, at 10^{-5} concentration (primarily diluted to 10^{-2} concentration in ethanol and further diluted in starvation media) for 24 hours and one set of cells was treated with 17β-Estradiol only (E_2; Sigma-Aldrich) at 10^{-7} Molarity concentration for 24 hours. The control set of cells (VEH) was treated with the same starvation media which contained 10^{-5} concentration ethanol only. Each experiment was performed in triplicate.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

Cells were directly lysed using RLT lysis buffer and RNA was extracted using the RNeasy Mini Kits (QIAGEN Gmbh, Hilden, Germany). RNA was quantified and 1 μg RNA was reverse transcribed into 20 μL of complimentary DNA using the qScript cDNA SuperMix (Quanta BioSciences, Inc., Gaithersburg, MD). Using a 386-well PCR plate, 10 μL of reactant were prepared in each well. The reactants for each PCR reaction consisted of 3 μL of RNAse-free H_2O, 5 microliters of SYBR green DNA-binding dye (Applied Biosystems, Foster City, CA), 0.5 μL of reverse primer (2 micromolar), 0.5 μL of forward primer (2 μm), and 1 μL of cDNA. The ABI Prism 7900HT Detection System (Applied Biosystems) was used to determine relative amounts of each transcript and gene expression using the setting comparative CT wherein PCR amplification

is parallel between all samples. Cycle Threshold (CT) is the number of cycles required for the fluorescent signal (after PCR reaction) to exceed the threshold (background reactions). CT count is inversely proportional to the amount of nucleic acid of the target gene in the sample. Cycling conditions started at 50° C for 2 min, followed by 95°C for 10 min, then 40 cycles of 95°C for 15 sec and 60°C for 1 min. PCR Amplification process was completed in triplicate for the following genes: GAPDH, Cyclin D1, C-myc, HOXA10, ER, and PR. Fold change was calculated by comparing CT values to those of GAPDH (unaffected by treatment), the housekeeping gene. The following primers were used:

GAPDH
Forward: 5'- GAAGGTGAAGGTCGGAGTC-3'
Reverse: 5'- GAAGATGGTGATGGGATTTC-3'

ERα
Forward: 5'- CACCAACCAGTGCACCATTG-3'
Reverse: 5'- AAGGTTGGCAGCTCTCATGTC-3'

C-myc
Forward: 5'-TCGGAAGGACTATCCTGCTG-3'
Reverse: 5'-GTGTGTTCGCCTCTTGACATT-3'
Cyclin D1: Ordered from Qiagen (Hs_CCND1_1_SG QuantiTect primer assay; Catalog Number: QT00495285)

HOXA10
Forward: 5'-AGGTGGACGCTGCGGCTAATCTCTA-3'
Reverse: 5'-GCCCCTTCCGAGAGCAGCAAAG-3'

PR
Forward: 5'-TGGAAGAAATGACTGCATCG-3'
Reverse: 5'-TAGGGCTTGGCTTTCATTTG-3'

Data Analysis Equation.

Fold Change= $2^{(-(\text{Normalized CT count value for treatment-normalized CT count value for vehicle}))}$
Equation 1: CT count value was normalized to GAPDH by subtracting coordinating GAPDH value from CT count of target gene.

Statistical Analysis.

In order to determine statistical significance of results, multivariate analysis using ANOVA statistics was performed followed by Tukey's post - hoc test for significance set at two-tailed $p<0.05$ using kaleidagraph program (synergy Software, Reading, PA).

RESULTS.

The objective of completing reverse-transcription polymerase chain reaction was to determine change in expression of the 5 target genes marking proliferation, differentiation, and estrogen-mediated action. The housekeeping gene was approximately equal in Cycle threshold count for each treatment in the experiment [Figure 2]. Figure 1 shows the average fold change of each gene in response to treatment. The order of treatment shown in the graph is as follows: E2, VEH, DBP, DINP, DOTP, and BHT.

HOXA10 is a transcription factor associated with uterine development. This gene affects cell proliferation and differentiation [8]. Differences in HOXA10 expression may change cell proliferation [8]. All of the compounds slightly increased expression of HOXA10 by approximately 20% in comparison to vehicle treatment [Figure 1]. The E2 treatment did not noticeably increase HOXA10 expression [Figure 1]. Typically, HOXA10 expression in Ishikawa cells should be significantly upregulated by approximately 60% with the presence of E2 [8] however the results in our study do not support that claim.

C-myc is a regulatory transcription factor which affects cell proliferation, cell growth, and differentiation. In comparison to the vehicle, all treatments increased expression of C-myc. Treatment with BHT increased expression of C-myc by approximately 40%, while other compounds DBP, DINP, and DOTP increased expression of C-myc by approximately 25% [Figure 1]. Treatment with E2 only slightly increased expression, closer to 5% increase.

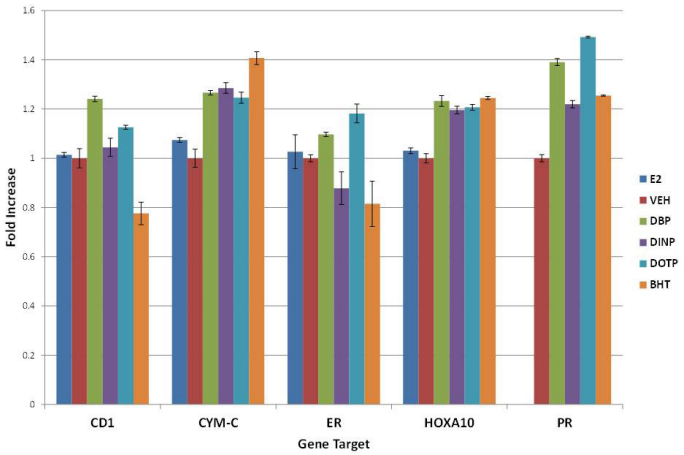

Figure 1. Fold changes calculated using Equation1 are shown for each target gene. The most statistically significant difference in comparison to vehicle occurred in CD1 expression when cells are treated with BHT ($p=0.09$); DBP and BHT showed the most statistically significant differences, compared to each other particularly in expression of CD1 and Cym-C ($p=0.09,0.08$, respectively). Treatment with BHT significantly decreased CD1 expression. Phthalate and Phthalate alternatives: DBP, di-n-butyl phthalate; DINP, diisononyl phthalate; DOTP, dioctylterephthalate; BHT, butylated hydroxytoluene; E2, 17β-Estradiol; Veh, Control.

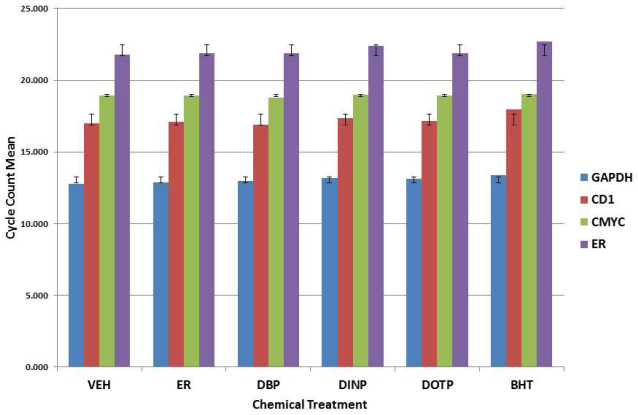

Figure 2. Cycle counts were calculated to average fold change using Equation 1. A standard deviation below 1 was observed for each group. Based on these results from the Comparative CT analysis, fold magnification or decrease was calculated using equation 1. The normalizing gene (GAPDH) did not vary much between treatments ($p>0.4$).

Progesterone receptor (PR) is a major target gene of estrogen action and plays an important role of differentiation within the uterus. Treatment with E2 heavily increased expression of PR by 32-fold compared to the Vehicle, as expected since Estradiol is a known PR-mediator [8]. DOTP, DBP, BHT, and DINP also increased expression of PR (by 50%, 40%, 25%, 20%, respectively) compared to the Vehicle [Figure 1].

Estrogen receptor is a nuclear receptor that is responsive to estrogenic compounds [13]. Expression of the Estrogen Receptor in E2 treated cells was slightly greater than that of the vehicle group. Compared to the Vehicle, DBP and DOTP slightly increased ER expression (by 10% and 20%, respectively), while DINP and BHT treatments decreased the expression of ER (by 10% and 20%, respectively). Expression of CD1 was approximately the same in both Vehicle treated cells and E2 treated cells.

Cyclin D1 (CD1) works primarily during the G1 phase of the cell cycle and is shown to be a marker of cell proliferation [10]. There was an increase in expression of CD1 in cells treated with DBP, DINP, and DOTP (by 25%, 5%, and 15%, respectively) while cells treated with BHT showed a decrease in expression of CD1 by approximately 25% compared with the Vehicle group [Figure 1].

Overall, although there was not much statistically significant change [p>0.1], trends observed may provide insight into the potential pathways and mechanisms of action of the four target chemicals. In a similar primarily quantitative PCR based study, although only around 1.5-2 fold change in many of the genes was seen by treating the cells with compound, when cells were treated with compound and estradiol together, magnified change was seen in the same trend [8]. Therefore, the trends noticed in this study are still valuable in investigating mechanisms of these chemicals.

DISCUSSION.

Previously, many studies have not been conducted observing the effects of DBP, DINP, DOTP, and BHT on expression of HOXA10, C-myc, CD1, PR, and ERα. However, based on previous studies, it is known that Estradiol (E2) is a major regulator of growth and differentiation within endometrial cells and increases expression of PR and ER [13]. We hypothesized that the ability of each phthalate or phthalate alternative to affect expression of each target gene will vary. Specifically, based on studies conducted on the male reproductive system, DBP may act as an estrogenic compound and therefore it is hypothesized that DBP may also increase proliferation, since within the female reproductive system proliferation is increased in the presence of estrogenic compounds [6]. Although estrogenic compounds are known to increase proliferation within endometrial cells, increase in proliferation does not necessarily indicate presence of an estrogenic compound. However, these compounds may be classified as potential endocrine disruptors which are characterized as substances that have properties that may lead to endocrine disruption [3]. In this study, for all compounds, except for BHT, as c-myc is upregulated, CD1 is also upregulated, which is expected because as C-myc is increased in expression, the expression of cyclins is also upregulated. Since both genes are upregulated, these compounds likely contribute to increase in cell proliferation, since these genes control cell growth and proliferation.

PR and ERα are activated by estrogenic action [13]. In DBP and DOTP the correlation between increase in expression of both PR and ER indicate that these compounds may contribute to estrogenic activity, and may be potential endocrine disruptors. However, in DINP and BHT, while expression of PR was increased, expression of ER was decreased in comparison to the vehicle group. In a previous study, it was shown that progesterone downregulated nuclear estrogen receptor while upregulating cytosol progesterone receptor [13]. Based on the similar behavior that DINP and BHT exhibit, DINP and BHT may follow a mechanism similar to progesterone. However, both of these observations must be verified through further future experimentation. As shown in other studies, treating cells with just the target compound will lead to a less prominent change in expression of target genes [8]. However, in combination with E2, these treatments show magnified results of the changes already seen.

Although this study showed results for the endocrine disruptive effects of DBP, DINP, DOTP, and BHT only on Ishikawa cells, the claims and observations reported can be applied to healthy myometrium cells and even leiomyoma cells. Before deciding to test only Ishikawa cells, the effects of these chemicals on normal myometrium cells and leiomyoma cells was observed, and slightly similar trends were observed, however it was difficult to confirm positive correlation since these cells did not respond as strongly to the treatment, partly because of the larger cell size and experimental limitations such as less mRNA collection and number of cells cultured.

In addition, although the changes in this study may seem subtle, we argue that in combination with other natural hormones in an *in vivo* environment, the actual effect of phthalate or phthalate alternative exposure is likely to be more impactful [8]. Based on similar studies, it is observed that fold change increases with the addition of natural hormones such as estradiol in addition to the chemical.

It is important to remember that our results do not declare DBP, DINP, DOTP, and BHT as endocrine disruptors, but as potential endocrine disruptors because we must perform other quantitative procedures in order secure knowledge of these chemical mechanisms. However, our study found that the treatment groups changed proliferation of Ishikawa cells. Also, we think that DBP and DOTP may follow mechanisms similar to estradiol while DINP and BHT may follow mechanisms more similar to progesterone, however this hypothesis can only be validated with further experimentation. Therefore, it is important to control the use of these substances in industrial, medicinal, and household products because of the effects of these chemicals on the female reproductive system.

FUTURE DIRECTIONS/CONCLUSIONS.

In order to better understand the further effects of cell proliferation that several of these compounds induce, future investigations will consist of testing for change in expression of genes associated with oncogenesis and apoptosis. In addition, protein analysis will be completed in order to observe changes in trend with the results observed in this study due to change in transcription and translation. Previously, we employed similar testing using myometrium and leiomyoma cells, however tests repeated in Ishikawa cells produced magnified results, likely because of the smaller cell size (allows collection of more RNA) and the sensitive nature of these cells. It would also be advantageous to complete similar experimentation within an *in vivo* environment or with longer chemical exposure. In addition, in order to further observe mechanisms of action of these chemicals and verify the previously discussed hypotheses based on observations in the current study, these chemicals will be compared in effect to Progesterone and estradiol using gene targets such as KLF11 and SGK1 along with others.

In conclusion the results of this study have opened up options to categorize the mechanisms of four popularly used phthalates/phthalate alternatives. In addition, it is has also shown that observing changes in expression of PR and ER with chemical treatment and documenting these trends in comparison to natural hormones may help us better understand the potential effects and diseases that these chemicals can cause when ingested in excess.

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