In Vitro Exposure of Human Luteinized Mural Granulosa Cells to Dibutyl Phthalate Affects Global Gene Expression

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The authors certify that all research involving human subjects was done under full compliance with all government policies and the Helsinki Declaration.

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ABSTRACT

Exposure to dibutyl phthalate (DBP) is ubiquitous among women of reproductive age. Previous studies in animal models and in human cells in vitro have shown that exposure to DBP disrupts ovarian function. Here, we examined the effect of DBP on global gene expression in mural granulosa cells (MGCs) in vitro. Primary cultures of MGC obtained from 48 patients undergoing IVF were treated with increasing concentrations of DBP (0, 0.01, 0.1, 1, 10, or 100 μg/ml) for 48 h. Microarray analysis was used to identify genes exhibiting expression changes following DBP exposure. When compared with untreated cells, exposure to 100 μg/ml DBP resulted in significant differences in expression of 346 annotated genes (> 2-fold; q value < .05). Of them, 151 were upregulated and 195 downregulated. The main functional annotations affected by DBP were associated with cell cycle, mitosis, Rho GTPases, PLK1, Aurora B signaling pathways, and E2F-mediated regulation of DNA replication. No significant differences in gene expression were observed for the lower concentrations of DBP (0.01, 0.1, 1, and 10 μg/ml) compared with controls for both the microarray analysis and genes validated by quantitative real-time (qRT)-PCR. This study provides important molecular inputs on the effect of short-term DBP exposure on human MGCs in vitro. Our results indicate that acute treatment with high concentrations of DBP alters gene expression pathways mainly associated with the cell cycle.

Key words: dibutyl phthalate; granulosa cells; microarray transcriptomic analysis; cell cycle; endocrine disruptors.

Phthalates are man-made chemicals commonly found in a variety of daily-use products, including personal care items, packaging, inflatable toys, medical devices, dietary supplements, polyvinyl chloride plastics, and building materials (Hauser and Calafat, 2005; Kelley et al., 2012). Because phthalates are not covalently bound to plastic polymers, they can leach into the environment during product use or disposal. Consequently, human exposure to phthalates is ubiquitous via inhalation, ingestion, or direct contact (Parlett et al., 2013; Wittassek et al., 2011), and phthalate metabolites are detectable in body fluids including blood, breast milk, urine, and follicular fluid (Berman et al., 2009; Hines et al., 2009; Krotz et al., 2012; Latini et al., 2003; Parlett et al., 2013; Silva et al., 2004; Specht et al., 2014). Among the most common phthalates, dibutyl phthalate (DBP) is found in cosmetics, deodorant, shampoo, conditioner, lotion, fragrances, soap, nail polish, and nail polish remover (Hannon and Flaws, 2015;
Parlett, et al., 2013). These products are used regularly among women of reproductive age. Accordingly, the National Health and Nutrition Examination Survey reported mean urinary levels of the DBP metabolites: mono-n-butyl phthalate (MnBP) of 20.2–22.2 ng/ml, while those of mono-isobutyl phthalate were 2.7–3.6 ng/ml (CDC 2015; Marsee, et al., 2006; Silva, et al., 2004). DBP metabolites were also detected in the follicular fluid, at mean levels of 1–2 ng/ml (Du, et al., 2016; Krotz, et al., 2012).

DBP and other phthalates are classified as endocrine disrupting chemicals (EDCs) due to their ability to alter the normal function of endocrine system (Marie, et al., 2015; Swan, 2008). EDCs, including phthalates, have varying effects on reproductive function in mammals. In mice, prenatal exposure to an environmentally relevant phthalate mixture impaired aspects of female reproduction (Zhou, et al., 2017). Treatment of mice with 0.01, 0.1, and 1000 mg/kg/day DBP in vivo decreased serum estradiol at all concentrations tested and exposure to 0.1 mg/kg/day of DBP also decreased antral follicle numbers (Sen, et al., 2015). In vitro exposure of mouse antral follicles to DBP at a concentration of 1000 μg/ml, significantly suppressed follicular growth, altered cell cycle, and increased apoptosis (Craig, et al., 2013). Exposure of mice antral follicles at ≥ 10 μg/ml resulted in growth inhibition followed by cytotoxicity at ≥ 500 μg/ml in another study (Rasmussen, et al., 2017). In rats, in vitro culture of granulosa cells with 100 μg/ml DBP did not induce cell death but it reduced the follicle-stimulating hormone (FSH)-induced production of estradiol and progesterone and aromatase expression, along with an attenuation of the cell proliferation that is otherwise induced by FSH and activin (Wang, et al., 2016). We recently showed, using quantitative real-time PCR (qRT-PCR), that short exposure to a high concentration of DBP (100 μg/ml) impairs mRNA expression of genes associated with steroidogenesis as well as a subset of luteinizing hormone (LH)-dependent genes in primary cultures of human mural granulosa cells (MGCs) (Adir, et al., 2017). These findings suggest that DBP may disrupt normal reproductive function in mammals.

We hypothesized that DBP exposure may also impair the global gene expression profile of MGC around the critical stage of ovulation. Given the pivotal roles of granulosa cells and gene expression dynamics during follicle and oocyte development, and at ovulation (Khan, et al., 2016), it is now essential to extend the evaluation of DBP effects beyond the analyses of a few target genes (as solely done to date). We applied microarray transcriptome profiling in primary cultures of MGC treated with a range of DBP concentrations (0, 0.01, 0.1, 1, 10, or 100 μg/ml), with the lowest concentration equivalent to the concentrations of DBP metabolites previously detected in human urine (Hauser, et al., 2016; Silva, et al., 2004).

MATERIALS AND METHODS

The study was approved by the Institutional Review Board committee (approval number: SMC-8707-11). All patients provided written informed consent.

Cell Collection

MGC, which are routinely aspirated and discarded, were collected during oocyte retrieval in women undergoing in vitro fertilization (IVF). To reduce a possible bias due to interpatient variability and to increase the number of cells available for the experiments, cells from 2 to 3 patients (depending on the number of retrievals that day) were pooled before culture for the microarray-based gene expression experiments or for some of the qRT-PCR validations and cell viability testing. Cell preparation and culture were described previously (Mansur, et al., 2017).

Cell Culture

Cells were cultured in 6- or 24-well plates (250,000 and 100,000 live cells/well for microarray and for the qRT-PCR, respectively) for 48 h at 37°C in Dulbecco’s modified Eagle’s medium (DMEM/F12) (GIBCO, UK) with 1% glutamax (GIBCO, USA), 10% fetal bovine serum (GIBCO, Brazil), and 1% penicillin/streptomycin (GIBCO, UK) in a humidified atmosphere of 5% CO2 and 20% O2. After 48 h, medium was replaced by fresh medium containing, 1μM 4-Androstene-3, 17-dione (Sigma-Aldrich, Missouri), serving as an androgenic substrate for estradiol production, 1 IU/ml FSH (Merck-Serono, Switzerland) (final concentration of 73.3 ng/ml), and DBP (Sigma Aldrich, France): 0, 0.01, 0.1, 1, or 10 μg/ml DBP dissolved in Dimethyl sulfoxide (DMSO) 99% (Amresco, Canada). Fresh working solutions were prepared from a thawed DBP stock aliquot for each experiment with the DMSO concentration held constant at 0.1% DMSO, regardless of the DBP concentration being tested. After 48 h, cells were lysed with 350 or 300 μl of lysis buffer for either the microarray or qRT-PCR experiments and stored at –80°C until subsequent RNA isolation was performed.

Cell Viability

As dying cells may influence the interpretation of the gene expression data, culture wells were washed twice (with PBS) before RNA preparation to eliminate dead cells. In a separate set of experiments, we tested granulosa cell viability following DBP treatment (with the same protocol described earlier) through 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulphophenyl)-2H-Tetrazolium-5-Carboxanilide inner salt (XTT), using cell proliferation kit (Biological industries, Israel) according to the manufacturer’s instructions. Following granulosa cell culture, 50 μl of XTT was added to each well and plates were incubated at 37°C for additional 2 h. XTT absorbance was measured at 450 nm against a reference wavelength at 650 nm using a microplate reader (Infinite F500, Tecan, Switzerland).

RNA Isolation for Microarray Analysis

For the microarray analysis, RNA was extracted from 6 biological replicates of MGC cultured with indicated concentrations of DBP using the EZ-10 DNAaway RNA Mini Preps Kit (Bio-basic, Canada) according to the manufacturer’s instructions. RNA concentrations were measured using Nano Drop 2000C spectrophotometer (Thermo Scientific, Waltham, MA, USA). To analyze the quality and integrity of RNA samples, we used gel agarose (Lonza Inc, NJ, USA) in a gel electrophoresis system (Mini Gel Migration trough, Cosmo Bio, California, USA) according to the procedure of Dingman et al. (Peacock and Dingman, 1968). One hundred nanogram of RNA were used from each sample.

Microarray Analysis

Expression profiling was performed using the HumanHT-12 whole-genome gene expression array and direct hybridization assay (Illumina, Inc, San Diego, California). To prepare the array hybridization target, 150 ng total RNA were converted to cDNA, then in vitro transcription was used to generate biotin-labeled cRNA using the Ambion Illumina Total Prep RNA Amplification Kit (Thermo Fisher, Waltham, MA, USA) according to the manufacturer’s instructions. The labeled probes were hybridized...
overnight at 58°C to the Illumina HumanHT-12 Bead Chips, which targets over 47,000 transcripts. Following washing and staining with Cy3-streptavidin conjugate, the BeadChips were imaged using the Illumina iScan System to measure fluorescence intensity at each probe. beads were processed and analyzed with Illumina’s GenomeStudio gene expression module to determine gene expression signal levels.

Validation of Microarray Results by qRT-PCR

A total of 10 culture experiments were conducted. RNA extraction was performed for all biological replicates; depending on amount of RNA available, qRT-PCR for the 5 gene targets could be conducted 6–10 times.

RNA was extracted from MGC using the Quick-RNA Microprep Kit according to the manufacturer’s instructions (Quick-RNA Microprep Kit, ZYMO Research, California, USA). RNA concentrations were measured using Nano Drop 2000C spectrophotometer (Thermo Scientific). Twenty-five nanograms of RNA were used for reverse transcription with a high-capacity cDNA RT kit (Applied Biosystems, California, USA). We used fluorescent SYBR Green PCR mix (Applied Biosystems, Carlsbad, CA, USA) to quantify PCR products.

Selection criteria for the validation of candidate genes were a high fold-change (> 2-fold; q value < .05) after exposure to DBP at the highest concentration of 100 μg/ml; hybridization intensity; and biological relevance to the most enriched functional annotations. To exclude any possible effect of DMSO, a second control without DMSO was included for the qRT-PCR validation experiments. Specific primers for the genes of interest (baculoviral repeat-containing 5; CDC20 - cell-division cycle protein 20; BUB1B - budding uninhibited by benzimidazoles 1 homolog beta; CCNB1 - Cyclin B1; TRIB3 - tribbles pseudokinase 3).

Table 1. Primers for Genes Selected for Validation Using qRT-PCR

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Forward</th>
<th>Reverse</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actb</td>
<td>5'-CTGCTCGGTTGCGCTTTT-3'</td>
<td>5'-TTCTCTGTCCAGTTTCAAAATTCA-3'</td>
<td>NM_001101</td>
</tr>
<tr>
<td>BIRC5</td>
<td>5'-TGCTCCGCTTGCGCTTTT-3'</td>
<td>5'-TCGTCCGGTTGCGCTTT-3'</td>
<td>NM_001168</td>
</tr>
<tr>
<td>CDC20</td>
<td>5'-CGGAAGACGCTGCGTTACAT-3'</td>
<td>5'-CCCAAAGTTCAGTTAAGTTATTC-3'</td>
<td>NM_002270</td>
</tr>
<tr>
<td>BUB1B</td>
<td>5'-CTGCAAAATGCCCTGAGCTCT-3'</td>
<td>5'-CCCAAATGTCTCCGAGAAGT-3'</td>
<td>NM_001121</td>
</tr>
<tr>
<td>CCNB1</td>
<td>5'-TGAAATGGCAACACACTCTCAACAA-3'</td>
<td>5'-CCCGATGTACATGACTATCCTACAG-3'</td>
<td>NM_019663</td>
</tr>
<tr>
<td>TRIB3</td>
<td>5'-CTGGCTCTCCTGCCAGATG-3'</td>
<td>5'-CTCCAGCCCCCTTCTCCTC-3'</td>
<td>NM_021158.4</td>
</tr>
</tbody>
</table>

Actb - beta actin; BIRC5 - baculoviral inhibitor of apoptosis repeat-containing 5; CDC20 - cell-division cycle protein 20; BUB1B - budding uninhibited by benzimidazoles 1 homolog beta; CCNB1 - Cyclin B1; TRIB3 - tribbles pseudokinase 3.

Statistical Analysis

Bioinformatics. Microarray data were processed via the Limma (Ritchie et al., 2015) R package from the Bioconductor project. First, NormExp background correction (Ritchie et al., 2007; Silver et al., 2009) followed by quantile normalization using control probes was done using the “neqc” command. Next, probes that were not expressed were filtered out. Filtering was done by retaining probes expressed in at least 6 arrays according to a detection P value of 5%. Unsupervised hierarchical clustering using Euclidian distance and complete linkage method showed that one sample clustered separately and distantly from all other samples. This observation was validated by multidimensional scaling analysis. To avoid masking true statistical significance, this outlier was removed. Differential expression analyses were conducted using linear models provided in the Limma package (Ritchie et al., 2015). We used the false discovery rate (FDR) method to adjust for multiple testing (Benjamini and Hochberg, 1995). Genes were considered as differentially expressed if their FDR was below 5%. Differentially expressed genes were analyzed for enriched GO terms and pathways using GeneAnalytics (Ben-Ari Fuchs et al., 2016).

qRT-PCR and cell viability data analysis. For the qRT-PCR and cell viability assessments, data were analyzed using SPSS statistical software version 23.0 (SPSS Inc Chicago, Illinois). Comparisons between the different DBP concentrations were made using 1-way ANOVA with repeated measures. No statistical difference was found between 2 control groups (with/without DMSO), and cell culture qRT-PCR data as well as cell viability data were normalized to the non-DMSO group. All comparisons were made between treatment groups and non-DMSO group. Adjustments were performed to the alpha level for multiple comparisons between groups by using Bonferroni correction (5 comparisons). P value < .05 was considered statistically significant.

RESULTS

Patients

We collected MGC from 48 women undergoing IVF. Depending on the number of available cells, samples were pooled from 2 to 3 patients and different samples were used for the different parts of the experiment. We used cells from 5 patients for mural
samples into subgroup clusters (Supplementary Figure 1). Using these 2653 genes segregated the higher concentration of the groups under study (analysis identified 2653 annotated genes that varied across any which clustered distinctly from the other samples. Linear model revealed clear separation of the 100 $\mu$g/ml-treated group, along with hierarchic clustering dendrograms for samples and genes.

Functional Annotations of Differentially Expressed Genes

Genes affected by exposure to 100 $\mu$g/ml DBP were assessed with GeneAnalytics, Gene Ontology (GO) and pathway analysis (Ben-Ari Fuchs et al., 2016). The most over represented canonical signaling pathways were related to “cell cycle and mitotic regulation,” “signaling by Rho GTPases,” “PLK1 signaling,” “Aurora B signaling,” “CDK-mediated phosphorylation,” “E2F-mediated regulation of DNA replication,” “Kinesins,” and “oocyte meiosis” (Figure 1). Figure 2 presents a gene expression heatmap of the transcripts associated with the cell cycle that were differentially expressed when exposed to 100 $\mu$g/ml DBP, along with hierarchical clustering dendrograms for samples and genes.

String analysis (Poswar Fde et al., 2015) of these genes revealed one main interaction network, which involves genes related to cell cycle, mitosis, and oocyte maturation, together with 7 other small-interacting clusters.

Validation of Microarray Results by qRT-PCR

From the results obtained in the functional annotations analyses, we chose to focus on genes involved in cell cycle progression, mitosis, Rho GTPases, PLK1, CDK-mediated phosphorylation, and Aurora B pathways, as well as E2F-mediated regulation of DNA replication. Candidate genes with significant fold changes and known central functions in these pathways (CDC20, BIRC5, CCNB1, BUB1B, and TRIB3) were selected for validation. Based on 6–10 replicates (depending on the gene under analysis), expression changes (decreases and increases) observed by microarray analysis were indeed confirmed by qRT-PCR (Figure 3).

DISCUSSION

In vitro exposure of primary cultures of human MGC to DBP resulted in significant differences in gene expression only at a high concentration of 100 $\mu$g/ml. The main functional pathways affected by DBP were associated with cell cycle, mitosis, Rho

**Figure 1.** Functional annotations of differentially expressed genes after exposure to 100 $\mu$g/ml dibutyl phthalate (DBP).
GTPases, PLK1, Aurora B signaling, and E2F-mediated regulation of DNA replication. The effect of DBP on several genes that play crucial roles in these pathways was validated using qRT-PCR.

The cell cycle comprises 4 consecutive phases: (1) gap 1 (G1), i.e., synthesis of mRNA and proteins; (2) synthesis phase (S), i.e., duplication of the chromosomes and DNA synthesis; (3) gap 2 (G2), resumption of cell growth and preparation for division; and (4) mitosis (M) phase, i.e., segregation of the chromosomes, assembly of the spindle, and cell division (Morgan, 2016; Murray, 1993; Rhind and Russell, 2012). Mitotic progression is regulated by several kinases including CDK1, Aurora A, and B (David et al., 2012; Ma and Poon, 2011; Nigg, 2001). Rho GTPases exert crucial functions during cell cycle progression and mitosis (David et al., 2012; Villalonga and Ridley, 2006). They are involved in the onset of mitosis by regulation of the expression of genes associated in G1/S transition (Villalonga and Ridley, 2006). Later, they are essential for the regulation of spindle assembly and attachment of microtubules to kinetochores (Bakal et al., 2005; Yasuda et al., 2004) and play key roles in the regulation of the dynamics of the actin cytoskeleton (and therefore in sustaining cell shape) and in chromosome alignment (David et al., 2012). Moreover, Rho GTPases are required for the cleavage furrow to separate daughter cells later in mitosis (Kamijo et al., 2006; Madaule et al., 1998; Miller and Bement, 2009; Narumiya and Yasuda, 2006; Niiya et al., 2006; Yuce et al., 2005). Similarly, E2F factors play an important role during the G1/S transition in mammalian cell cycle (Gaubatz et al., 2000). E2F transcriptional targets include cyclins, CDKs, checkpoints regulators, DNA repair, and replication proteins (Gaubatz et al., 2000). Aurora B also plays a crucial role in the cell cycle, serving as a mitotic checkpoint kinase that ensures appropriate chromosome segregation and normal mitosis progression (Gully et al., 2012). Another kinase, PLK1, localizes at centrosomes and spindle poles and is important for mitotic progression (Asteriti et al., 2015). It is crucial for the control of mitotic entry, recovery from DNA damage checkpoint-mediated G2 arrest and spindle formation. In addition, it is activated by E2F factors (Tategu et al., 2008). Using qRT-PCR, we confirmed the decrease in expression of BIRC5, BUB1B, CCNB1, and CDC20 following exposure to DBP at a concentration of 100 μg/ml. BIRC5 is a member of the inhibitor of apoptosis proteins and its mRNA expression is elevated in granulosa cells particularly during the G2/M phase of mitosis (Johnson et al., 2002). BUB1B, CDC20, and CCNB1 play key roles in the spindle assembly checkpoint in human (Holt et al., 2013; Kapanidou et al., 2017; Shi et al., 2016; Vleugel et al., 2015). The processes of follicle selection, growth, and atresia depend on a dynamic control and balance of granulosa cell proliferation, differentiation, and apoptosis. Therefore, alterations in the expression of molecular regulators of mitosis and cell death (as reported herein) may impair follicle development and periovulatory events, in turn perhaps leading to ovarian malfunc tion and infertility. Our findings thus delineate cell cycle molecular targets that merit future evaluations when considering the impact of DBP on reproduction.
In a mouse model, Craig et al. (2013) used a follicle culture system to evaluate the effects of DBP on cell cycle gene expression, with exposures of antral follicles from adult mice to DBP at concentrations similar to our study (1, 10, and 100 μg/ml) as well as a higher concentration (1000 μg/ml). Exposure to DBP impaired follicle growth at very high concentration of 1000 μg/ml. In addition, flow cytometry revealed that DBP exposure (at this highest concentration) disrupts the cell cycle, resulting in cycle arrest at G1 (Craig et al., 2013). These results are in line with this study in which exposure to 100 μg/ml of DBP impaired the transition from G1 to S phase (Figure 1). Taken together, evidence is thus accumulating for a vulnerability of the cell cycle (and its control mechanisms) in granulosa cells exposed to DBP. Now that the cell cycle is identified as a target pathway, future studies will thus need to focus on the functional evaluation of cell proliferation upon DBP exposure in human MGC.

The microarray and qRT-PCR data both showed an increase in TRIB3 due to exposure to 100 μg/ml DBP. TRIB3 has been implicated in cell division and migration, tissue homeostasis, inflammation, and lipid metabolism (Basatvat et al., 2015; Lohan and Keeshan, 2013). High expression of TRIB3 has been detected in cumulus cells from mature bovine and murine oocytes, with TRIB3 modulating peroxisome proliferator-activated receptor gamma (PPARγ) and lipid metabolism (Brisard et al., 2014). Studies using human and rat liver cell lines showed that DBP activates PPARs and induces oxidative stress (Lapinskas et al., 2005). Interestingly, another EDC Bisphenol A (BPA) provoked an increase in TRIB3 mRNA expression in human MGC (Mansur et al., 2017), thus making TRIB3 a particularly relevant target for further investigation into the effects of EDCs.

To understand the potential adverse health effects of the most abundantly used phthalates, di-(2-ethylhexyl) phthalate (DEHP)/mono-(2-ethylhexyl) phthalate (MEHP), butyl benzyl phthalate (BBP), monobutyl phthalate (MBP) and DBP, the curated interactions between these 5 phthalates and genes/proteins were studied using data obtained from the Comparative Toxicogenomics Database (Singh and Li, 2011). Of the 249 phthalate-interacting genes/proteins that were analyzed for their GeneOntology, pathways, networks, and human diseases, 34 genes/proteins had 3 and more interactions. DBP/BBP/MBP was found to have 11 interactions with estrogen receptor 1 (ESR1) and 9 with androgen receptor. Further, ESR1 and endothelial growth factors (VEGFs) genes/proteins had 6 interactions with DBP/BBP/MBP in humans but none in rodents. In a recent study (in primary cultures of MGC from another cohort of women), we investigated the effects of DBP on LH-dependent gene expression. Interestingly, we found that mRNA expression of VEGF was significantly higher in human MGC exposed to 100 μg/ml DBP (Adir et al., 2017). We also showed that DBP exposure altered the expression of LH-dependent genes, including steroidogenesis targets (Adir et al., 2017). Importantly, the same genes were also altered in the current microarray analysis; in both studies, the change in the mRNA expression of these genes was significant only for the 100 μg/ml DBP concentration. Using a global approach, our current findings thus corroborate, and expand on, our previously published changes in select target genes.

It is relevant to note that the parent compound DBP has not yet been evaluated in follicular fluid; but, because one of its metabolites, MnBP, has been reported in human follicular fluid (Du et al., 2016), we also tested the effects of MnBP on the expression of the same genes that we used for validation. The effects on gene expressions were similar with the only significant effects after exposure to 100 μg/ml MBP (unpublished data).
In our study, DBP was not cytotoxic for MGCs up to the concentration of 100 μg/ml tested. A recent study compared the effects of DBP and its metabolite MnBP on in vitro growth and viability of mouse ovarian antral follicles. Cells were treated for 24–72 h with increasing doses of these chemicals (0.001 and up to 1000 and 100 μg/ml for DBP and MnBP, respectively). Exposure to DBP at ≥10 μg/ml resulted in inhibition of antral follicular growth and in cytotoxicity at ≥500 μg/ml, while MnBP did not cause toxicity at the doses tested (Rasmussen et al., 2017).

Similar to our study, the effects of DBP were pronounced only at high doses, although in contrast with our findings in human cultures, MnBP in mice did not cause follicular toxicity after exposure to high doses, although in contrast with our findings in human cultures, MnBP in mice did not cause follicular toxicity after exposure to <100 μg/ml. The dissimilarity between the 2 studies for MnBP effects can be attributed to the different outcomes assessed as well as to the variance in species. Importantly, future studies are needed to evaluate levels of DBP (as well as its metabolites) within the follicle, along with a testing of any potential ability of human granulosa cells to metabolize DBP.

Our study has a few limitations. First, using an in vitro model, the exposure of MGCs to DBP was relatively acute (48 h). The effects of DBP were significant only in high concentration, therefore generalization might be limited. Second, the effects of phthalates were tested at the level of mRNAs, and it is possible therefore generalization might be limited. Second, the effects of DBP were significant only in high concentration, although in contrast with our findings in human cultures, MnBP in mice did not cause follicular toxicity after exposure to high doses, although in contrast with our findings in human cultures, MnBP in mice did not cause follicular toxicity after exposure to <100 μg/ml. The dissimilarity between the 2 studies for MnBP effects can be attributed to the different outcomes assessed as well as to the variance in species. Importantly, future studies are needed to evaluate levels of DBP (as well as its metabolites) within the follicle, along with a testing of any potential ability of human granulosa cells to metabolize DBP.

In conclusion, our study provides important molecular data. (2016). GeneAnalytics: An integrative gene set analysis tool for next generation sequencing, RNAseq and microarray data. OMICS 20, 139–151.


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This study also has important strengths. To the best of our knowledge, it is the first study assessing changes in gene expression following DBP exposure in human granulosa cells. Moreover, using cells from both fertile and infertile women may help generalize our results.

In conclusion, our study provides important molecular inputs on the effect of short DBP exposure in human MGCs in vitro, with the identification of pertinent pathways that relate to the cell cycle.

SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

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