Published in final edited form as:

Toxicol Appl Pharmacol. 2009 September 15; 239(3): 325–336. doi:10.1016/j.taap.2009.06.014.

Improving *in vitro* Sertoli cell/Gonocyte co-culture model for assessing male reproductive toxicity: lessons learned from comparisons of cytotoxicity versus genomic responses to phthalates

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Abstract

Gonocytes exist in the neonatal testis and represent a transient population of male germ-line stem cells. It has been shown that stem cell self-renewal and progeny production is probably controlled by the neighboring differentiated cells and extracellular matrix (ECM) *in vivo* known as niches. Recently, we developed an *in vitro* three-dimensional (3D) Sertoli cell/Gonocyte coculture (SGC) model with ECM overlay, which creates an *in vivo*-like niche and supports germ-line stem cell functioning within a 3D environment. In this study, we applied morphological and cytotoxicity evaluations, as well as microarray-based gene expression to examine the effects of different phthalate esters (PE) on this model. Known *in vivo* male developmentally toxic PEs (DTPE) and developmentally nontoxic PEs (DNTPE) were evaluated. We observed that DTPE induced significantly greater dose-dependent morphological changes, a decrease in cell viability and an increase in cytotoxicity compared to those treated with DNTPE. Moreover, gene expression was more greatly altered by DTPE than by DNTPE and non-supervised cluster analysis allowed the discrimination of DTPE from the DNTPE. Our systems-based GO-Quant analysis showed significant alterations in gene pathways involved in cell cycle, phosphate transport and apoptosis regulation with DTPE but not with DNTPE treatment. Disruptions of steroidogenesis related-gene expression such as Star, Cyp19a1, Hsd17b8, and Nr4a3 were observed in the DTPE group, but not in the DNTPE group. In summary, our observation on cell viability, cytotoxicity, and microarray-based gene expression analysis induced by PEs demonstrate that our *in vitro* 3D-SGC system mimicked *in vivo* responses for PEs and suggests that the 3D-SGC system might be useful in identifying developmental reproductive toxicants.

Keywords

Sertoli cell/Gonocyte co-culture; male reproductive toxicity; microarray; phthalate esters

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Introduction

Reproductive toxicity is one of the most complicated, time-consuming, and expensive endpoints to assess experimentally. At the moment there is no validated battery of alternative tests that would cover the different aspects of the reproductive cycle (ECVAM, 2002). The establishment of *in vitro* models for the evaluation of testis development will provide important alternative approaches to *in vivo* systems and allow for new tools for the assessment of reproductive and developmental toxicants. Such models can provide important information regarding specific mechanisms of toxicant action in the testis and this information could lead to improvements in data interpretation within *in vivo* models. By improving the efficiency of data interpretation, animal usage within *in vivo* models can be reduced and refined. To date, various *in vitro* systems for evaluating testicular changes during normal development have been reported in the literature including Sertoli cell/germ cell co-cultures (Hadley *et al.*, 1985; Gray, 1986), Sertoli cell-enriched cultures (Chapin *et al.*, 1988), germ cell-enriched cultures (Lejeune *et al.*, 1998), Leydig cell cultures (Yang *et al.*, 2003), and Leydig-Sertoli cell co-cultures (Bilinska, 1989). Sertoli cell/germ cell co-cultures (SGC) have been used to examine the interactions and effects of various hormones and growth factors on spermatogonial survival and proliferation *in vitro* (Mather *et al.*, 1990). SGC, isolated from fetal rat testes, have also been developed and their interactions have been studied *in vitro* (Orth and Jester, 1995). However, limited applications in toxicological studies have been reported, specifically due to the lack of reproducibility of the cell isolation procedure and the poor ability of these *in vitro* culture systems to replicate the complex biochemical, molecular and functional interactions observed in the testis *in vivo* (Gregotti *et al.*, 1992; Li *et al.*, 1998).

Gonocytes exist in the neonatal testis and represent a transient population of male germ-line stem cells. It has been shown that stem cell self-renewal and progeny production is probably controlled by the neighboring differentiated cells and extracellular matrix (ECM), *in vivo* known as niches. Improvements have focused on the employment of an extracellular matrix coating (Matrigel™) in tissue culture-treated dishes (2 dimensional substratum) to enhance Sertoli cell attachment. Although such ECM pre-coated dishes have been used with relative success in the culture of SGC (Hadley *et al.*, 1985; Li *et al.*, 1998; Orth *et al.*, 2000), the associated mechanism of improvement in cell survival and proliferation remains unclear. Recently, we developed an *in vitro* three dimensional Sertoli cell/gonocyte co-culture model (3D-SGC) with overlay of ECM and demonstrated that this culture system creates *in vivo*-like niches, supporting germ-line stem cell functioning within a 3D environment (Yu *et al.*, 2005; Yu *et al.*, 2008a). Validation of this culture system has been conducted on the morphology, stress and survival signaling pathways as well as c-kit protein expression (Yu *et al.*, 2005). The presence of an ECM overlay at optimized concentrations of 200 µg/ml resulted in suppression of the stress-signaling pathway associated with the survival of spermatogonia. Moreover, up-regulation of the expression of c-Kit proteins confirmed functional integrity of this system. These results suggest that the ECM overlay enables a physiologically more stable 3D-SGC system by forming a testicular-like multilayered architectural structure that mimics *in vivo* characteristics of seminiferous tubules (Faustman *et al.*, 2003; Yu *et al.*, 2005).

In a subsequent study, we further employed this *in vitro* 3D-SGC system to examine the effects of cadmium, a ubiquitous environmental pollutant that has been reported to cause male reproductive toxicity both in humans and animals. We investigated the time- and dose-dependent effect of cadmium on morphological alterations, cell viability, the activation of stress signaling proteins, and the disruption of the ubiquitin proteasome system (UPS) as well as the cell cycle regulatory protein p53 (Yu *et al.*, 2008a). These results suggest that UPS dysfunction plays a key role in the underlying mechanism of cadmium-induced testicular toxicity and confirms that our established 3D-SGC can be used to investigate mechanistic pathways of testicular toxicants. The purpose of this study is to further examine whether our

established *in vitro* 3D-SGC model can be effectively used for screening a series of related testicular developmental toxicants. In this study we have chosen several phthalate esters (PE) including both known *in vivo* male developmentally toxic PE (DTPE) as well as developmentally non-toxic PE (DNTPE) (Liu *et al.*, 2005). PEs are used as plasticizers, solvents and emulsifiers, and have a widespread use in consumer products, including toys, health and beauty supplies and medical equipments. Reproductive male toxicity of some PEs including phthalate diester congeners and their monoester metabolites has long been recognized in both fetal and prepubertal male rodents (Sharpe *et al.*, 1995; Mylchreest *et al.*, 1998; Gray *et al.*, 2000; Parks *et al.*, 2000; Barlow *et al.*, 2004; Boekelheide, 2004; Liu *et al.*, 2005) and has been extensively discussed by an expert panel of the NTP-CERHR (National Toxicology Program, Center for the Evaluation of Risks to Human Reproduction) (McKee *et al.*, 2004; Kavlock *et al.*, 2006). The reproductive tract abnormalities described after PE exposure *in utero* include underdeveloped or absent reproductive organs, hypospadias, cryptorchidism, decreased anogenital distance, retained nipples, and decreased sperm production (Sharpe *et al.*, 1995; Mylchreest *et al.*, 1998; Gray *et al.*, 2000; Parks *et al.*, 2000; Barlow *et al.*, 2004; Liu *et al.*, 2005). The large number of published *in vivo* studies available, allow us to make comparisons between published *in vivo* data and our *in vitro* model. We hypothesized that 3D-SGC model can be useful in identifying male developmentally toxic PEs. To test this hypothesis, we examined cell viability, cytotoxicity and microarray-based gene expression of seven PEs including both DTPE and DNTPE. The DTPE group was represented by dibutyl phthalate (DBP), diethylhexyl phthalate (DEHP), dipentyl phthalate (DPP) or benzyl butyl phthalate (BBP), whereas DNTPE included diethyl phthalate (DEP), dimethyl phthalate (DMP) and dioctyl tere-phthalate (DOTP).

MATERIALS AND METHODS

Sertoli cell-gonocyte co-culture (3D-SGC) and treatments

The 3D-SGC co-culture was set-up as previously described (Faustman *et al.*, 2003; Yu *et al.*, 2005). Briefly, male pups were obtained by mating Sprague–Dawley rats (Charles River Laboratories, Wilmington, USA). Testes were dissected from 5-day-old rats, and a cell suspension containing primarily Sertoli cells and Type A spermatogonia was isolated with multiple digestion steps. Cells were re-suspended in hormone- and serum-free Eagle's Minimal Essential Medium (Life Technologies Inc., Gaithersburg, MD) containing 0.1 mM non-essential aminoacids, 1 mM sodium pyruvate, 3 mM sodium lactate, 1% ITS+™ premix (a culture supplement containing insulin, transferrin, selenium, linoleic acid, and bovine serum albumin; BD Biosciences, Bedford, MA, U.S.A.). Cells were plated in 35-mm tissue culture-treated dishes at 2.4×10^6 density in serum-free medium. Immediately after seeding, an ice-cold extracellular matrix medium (5 µg/ml, BD Science) was applied to these dishes at a final concentration of 200 µg/ml, and dishes were gently shaken 5 times. Serial dilutions were prepared from a stock solution of PEs in distilled water, and added directly to the culture medium 48 h after the addition of ECM overlay. The final concentrations tested in the culture medium were 0, 50, 100, 200 and 400 µM. Dibutyl phthalate (DBP, Sigma # D2270, 99% purity), diethylhexyl phthalate (DEHP, Sigma # 4–8557, 99% purity), dipentyl phthalate (DPP, Sigma # 80154, 99% purity), or benzyl butyl phthalate (BBP, Sigma # 44–2503, 99% purity), diethyl phthalate (DEP, Sigma # 524972, 99.5% purity), dimethyl phthalate (DMP, Sigma # 525081, 99% purity), and dioctyl tere-phthalate (DOTP, Sigma # 525189, 96% purity) were obtained from Sigma-Aldrich (MO, USA).

Morphology, Cell viability and Cytotoxicity

All cultures were viewed with a Nikon inverted microscope equipped with phase-contrast optics (Nikon, Tokyo, Japan) at intervals during the culture to assess their general appearance. Resultant images were captured, digitized using a Coolsnap camera (Roper Scientific, Inc,

Duluth, GA) and processed with Adobe Photoshop. A three-color fluorescence assay [calcein acetoxymethyl ester (AM), propidium iodide (PI), Hoechst 33342] was applied to show the morphologic changes after 24 h treatments as previously described (Yu *et al.*, 2005; Yu *et al.*, 2008a). PI (1 mg/ml), Hoechst 33342 (1 mg/ml) and calcein AM (Sigma, 1 mg/ml) were added directly to co-cultures and incubated at 37°C for 5 min. Live cells have intracellular esterases that convert the non-fluorescent, cell permeable calcein AM to the intensely green fluorescent, calcein. PI, a live cell-impermeable dye, selectively stains nuclei of apoptotic cells with increased membrane permeability, while Hoechst 33342 selectively stains nuclei with blue fluorescence. The nuclei of live cells are evenly stained, whereas dead cells are intensely and irregularly stained. This three-color assay was conducted in 3 dishes from each treatment group and the morphological changes were analyzed qualitatively.

Cytotoxicity was evaluated through measurement of released lactate dehydrogenase (LDH) and cell viability was measured through neutral red (NR) uptake assay. To measure the release of lactate dehydrogenase (LDH) in the culture media after treatment with PE for 4, 8 and 24 h, CytoTox 96® non-radioactive cytotoxicity assay (Promega Corporation, WI, USA) was used in three plates per treatment group, per time point, and repeated in three different experiments. LDH, a stable cytosolic enzyme, is released upon cell lysis. The results of the release of LDH were expressed as corrected absorbance value by the mean of three maximum LDH release controls at each time point. The NR assay was used to determine viability of the cultured cells after 24 h treatment as previously reported (Borenfreund and Puerner, 1985). Briefly, cells were treated with different concentrations of both DTPE and DNTPE groups (0, 50, 100, 200 and 400 µM). The media with treatment was removed and fresh media containing 50 µg/ml neutral red was added to the dish. After incubation for 3 h at 37°C and 5% CO₂, the cells were washed with PBS and neutral red was eluted with a 1% acetic acid / 50% ethanol solution. Finally, 200 µl of the resulted neutral red solution was added to 96 well plates and measured at 490 nm. The results of quantitative analysis of cell viability were expressed as the percentage of the mean of the control group. For this assay, 3 independent experiments were conducted and each experiment includes 3 dishes per treatment group.

Microarray-based gene expression analysis

For the microarray-based gene expression analysis, SGC were treated with PEs at 100 µM for 24 h. This dose was chosen based on minimal impact on neutral red based cell viability assay, in which no significant decrease in cell viability of the all PEs treatment data was found. Total RNA was isolated using Trizol Reagent (Gibco-BRL, Gaithersburg, MD), according to the manufacturer's instructions. Subsequently, the total RNA was purified using an RNeasy kit (Qiagen, Valencia, CA, USA). RNA labeling, gene chip hybridizations, and gene chip scans were conducted by the University of Washington's Center for Ecogenetic and Environmental Health (CEEH), Functional Genomics Laboratory. Each total RNA sample was evaluated for quality on an Agilent 2100 bioanalyzer, and then converted into biotin-labeled cRNA using the Affymetrix eukaryotic target labeling protocol (Harrington *et al.*, 2000). Briefly, 5 µg of total RNA was reversely transcribed to double-stranded cDNA via a round of transcription with Superscript II and then a round with T4 DNA polymerase. The resulting cDNA was converted to biotinylated cRNA in the presence of T7 DNA polymerase and biotin-labeled nucleotides. This resulting cRNA was fragmented and used for hybridization to an Affymetrix Rat Genome 230 2.0 Array including 31,000 probe sets according to standard Affymetrix protocols. Three arrays per PE or control group were conducted.

Image processing and expression analysis were performed using Affymetrix GeneChip Operating Software (GCOS). Each GeneChip (GC) image underwent GCOS absolute expression analysis. The quality of the hybridizations and overall chip performance were determined by visual inspection of both the raw scanned image and the extracted quality control

metrics. The resultant cell intensity files (CEL) were input into BRB ArrayTools v3.0 (Biometric Research Branch, National Cancer Institute, NIH) for further statistical analysis (Wright and Simon, 2003). Data normalization was performed using GC-RMA (GeneChip-Robust Multiarray Averaging) method within the BRB ArrayTools, which uses the GC content of the mismatch probes for a better background adjustment of the perfect match probes (Wu and Irizarry, 2004).

Statistical Analysis

The results of quantitative analysis of cell viability and cytotoxicity are the mean $\pm$ standard error of the mean. Statistical significance among the treatments was determined using two-way analysis of variance (ANOVA, treatments and concentrations). Statistical significance of each treatment was determined using one-way analysis of variance (ANOVA), followed by the comparison with the control using the Dunnett's method. A $p \leq 0.05$ denoted the presence of a statistically significant difference. The percent of change of cell viability and cytotoxicity was calculated by dividing the value of individual treatment with the average of three controls at each time point. Furthermore, a non-supervised two dimensional hierarchical clustering analysis was conducted on each sample in three different experiments using average linkage and elucidation dissimilarity to examine whether the cytotoxicity or cell viability endpoints can be used to discriminate the PEs into different groups such as DTPE or DNTPE (Eisen *et al.*, 1998; De Hoon *et al.*, 2004). The complete output of cluster analysis is shown with the percentage change indicated colorimetrically.

For the microarray data comparison analysis, we applied randomized variance t/F tests, which was reported to be a powerful alternative to the standard t/F tests when there are few samples ($n = 2$ to 5) in each class (Wright and Simon, 2003). The significantly changed genes among treatment were selected based on a multivariate permutations test under the following conditions: nominal significance level of each univariate test was $p \leq 0.001$ and confidence level of false discovery rate assessment was 90%. The maximum allowed number of false-positive genes and the minimum allowed proportion of false-positive genes were 10 and 0.1, respectively. Significant genes were selected and ratios were derived from dividing each normalized gene value with the average value of the controls which were transformed to log2 ratio. These log2 transformed ratios were then input to MeV (MultiExperiment Viewer) software, and further non-supervised two dimensional hierarchical clustering analysis of each group was conducted using average linkage and correlation similarity (Saeed *et al.*, 2003).

To establish the association between the treatment and the affected gene ontology (GO) terms, we applied the MAPPfinder to identify enriched biological themes, particularly GO terms at $p \leq 0.001$ (Dahlquist *et al.*, 2002). Z-score and permutation p-value were used to rank these terms for biological significance (Dahlquist *et al.*, 2002). Furthermore, a systems-based GO-Quant quantitative functional analysis developed by our lab was conducted (Yu *et al.*, 2006; Robinson *et al.*, 2009). The output of the MAPPfinder calculation was input into the GO-Quant program and GO-Quant Index (the average ratio of each GO item) was calculated, which allowed for quantitative comparisons across treatments (Yu *et al.*, 2006). Furthermore, since the disruptions of steroidogenesis related-gene expression have been well recognized in PEPE induced testicular developmental toxicity, a set of seventy-six genes that have been annotated in rat in the Gene Ontology database was identified and statistical analysis of 84 probes linked to these 76 genes across treatments using F test was conducted (Harris *et al.*, 2004). Nominal significance level of each univariate test and permutation p-values for significant genes was set at 0.001, and the permutation test was computed based on 10000 random permutations.

Results

In the control group, the SGC formed a 3D structure 24 h after plating as shown in a three-color staining (Fig. 1A). Compared to the control (Fig. 1A), no obvious morphological changes or visual increases in PI-positive cells (dead cells) were observed in cultures treated with DNTPEs including DEP, DMP and DOTP at 200 μ M for 24 h (Fig. 1 B–D). Obvious morphological changes such as decrease of cytoplasm calcein green staining and increase of number of PI positive cells (dead cells) were observed in those cultures treated with DTPEs including DBP, DPP, DEHP and BBP (Fig. 1E–H).

There are significant differences in cell viability among the treatments as measured by a neutral red uptake assay of SGC and analyzed by two-way ANOVA analysis (interaction of concentrations and treatments, $p \leq 0.0001$). One-way ANOVA analysis complemented with Dunnett's test revealed dose-dependent decreases in cell viability following treatment ($\dagger p \leq 0.05$) with DTPE including DBP, DEHP, BBP and DPP, but were not observed following exposure to DNTPE including DOTP, DMP, DEP (Fig. 2A). Significant decreases in cell viability were observed at 200 and 400 μ M for DPP and BBP, and 400 μ M for DBP and DEHP (Dunnett's test with the control, $* p \leq 0.05$). In order to examine whether the cell viability endpoint was able to discriminate PEs into DTPEs and DTNPEs groups, non-supervised two dimensional hierarchical cluster analysis of the concentration of phthalates (0 to 400 μ M) and cell viability at 24h and these comparisons are shown in Fig 2B. Although there is no clear cutoff between the DTPEs and DTNPEs, Fig 2B visually illustrates the degree of PEs affecting cell viability, high impact PEs at the top and low at the bottom. Cluster 1 on the top includes DPP, BBP, DEHP (DTPE group), and cluster 3 at the bottom includes DMP, DOTP, DEP (DTNPE group). Cluster 2 in the middle includes mostly the DTPE group (DEHP, DBP), but also two samples of DEP, a DTNPE compound.

Significant dose-dependent increases in cytotoxicity as measured by the cellular release of LDH were observed in the DTPE group including DPP (Fig 3A), DBP (Fig. 3B), DEHP (Fig. 3C) and BBP (Fig. 3D) at 4, 8 and 24 h (one-way ANOVA, $\dagger p \leq 0.05$). The comparison of each dose with the controls by Dunnett's test revealed significant increases of LDH releases measured after 4, 8 and 24 h treatments were observed for all PEs in the DTPE group ($* p \leq 0.05$ vs control). However, the magnitude of the LDH release caused by the DTPE was different. The amount of LDH release caused by DPP and DBP was similar and significant changes were first observed at concentrations of 50 μ M for DPP, and 200 μ M for DBP for all three time points examined. Significant increases of LDH release were observed in the treatment with DEHP and BBP, but to a much lesser extent in comparison to the treatment with DPP and DBP. A sharp increase in LDH release at 24 h was observed in the DEHP treatment in a non-progressive concentration-dependent manner. Minimal increases of LDH were also observed after treatment with BBP (4, 8 and 24 h) as well as DEP (24 h). No significant changes in LDH release assay were observed at any time-points or doses for DOTP or DMP as compared to control. Similar to the cell viability, hierarchical cluster analysis was used to compare the concentration of phthalates (0 to 400 μ M) and cytotoxicity at 24 h. As shown in Figure 3H, the percentage of LDH released by these PEs is visually presented with three distinct clusters (1, 2 and 3). Cluster 1 on the bottom, which includes DBP and DPP, has the highest impact on the LDH release. Cluster 3 at the top includes DBP, DEHP and BBP phthalates and Cluster 2 in the middle includes all the DNTPE (DOTP, DEP, and DMP), as well as 4 sets of BPP samples.

We conducted a microarray-based gene expression comparison study to further compare whether the gene expression alteration by different PEs is more sensitive (at low doses) than conventional toxic endpoints, such as cytotoxicity, in order to discriminate the DTPE from DTNPE in our established 3D-SGC model. Based on the neutral red-based cell viability assay,

we chose a less toxic dose of 100 μ M of PEs, where no significant decrease was observed in any PEs treatment, in order to evaluate microarray-based gene expression analysis as a tool for looking at more subtle effects. As shown in Fig. 4, treatment with DTPE in the 3D-SGC induced a significantly greater number of genes changed than cells treated with DNTPE at the same concentration (100 μ M) for 24 h. The number of significantly changed genes (two group comparison with the control, $p \leq 0.001$) in the DTPE group was 741 (365 increased and 376 decreased), 1344 (790 increased and 554 decrease), 1348 (706 increased and 646 decreased) and 1291 (616 increased and 675 decreased) for BBP, DBP, DPP, and DEHP, respectively. In the DNTPE group, the number of significantly changed genes (two-group comparison with the control, $p \leq 0.001$) was only 69 (27 increased and 42 decreased), 20 (9 increased and 11 decreased), and 191 (60 increased and decreased 131) for DEP, DMP and DOTP, respectively.

One way-ANOVA analysis across treatments identified 2640 genes at $p \leq 0.001$. From these genes, the ones whose the geometric mean intensities varied ≥ 2 -fold over the control (in either direction) in at least one treatment (1670 genes) were included in a hierarchical clustering analysis using average linkage and Euclidean dissimilarity (Fig. 5). Similar gene expression patterns within the DNTPE or DTPE group were observed in a non-supervised cluster analysis for the genes changed across the treatments (Fig. 5). Based on their gene expression level, each treatment (biological sample) formed a specific cluster. The DEP, DMP and DOTP, part of the DNTPE group, formed a cluster. The control formed a distinct cluster, and BBP, DEHP, DBP and DPP, part of the DTPE group, formed another separate cluster.

Fig. 6 shows the relative changes in each Gene Ontology category (GOID) in the biological process (A) and molecular function (B). These have been identified by the Gene Ontology analysis with MAPPfinder and further quantitatively analyzed by GO-Quant. GO-Quant automatically links functional gene category analysis result from MAPPfinder with the original gene expression data and calculates the average magnitude in change for those significant genes within each GO term, i.e., the GO-Quant index. As shown in Fig. 6A, significant down-regulation in DNA-dependent DNA replication (GOID 6261), cell cycle (GOID 7049), cell division (GOID 51301), phosphate transport (GOID 6817) and anion transport (GOID 6820), in addition to positive regulation of apoptosis (GOID 43065), were observed in the DTPE group but not in the DNTPE group (Fig. 6A). In the molecular function category (Fig 6B), significant down-regulation in the extracellular matrix constituent (GOID 5201); copper ion binding (GOID 5507) and up-regulation in G-protein-coupled receptor binding (GOID 1664); chemokine activity (GOID 8009 and 42379) and glutathione transferase activity (GOID 4364) were observed only in the DTPE group, but not within the DNTPE group.

Fig. 7 shows the effects of PEs treatments on the genes involved in steroidogenesis. Forty-two probe sets out of a total of 84 gene probes identified as being probes involved in steroidogenesis (Harris *et al.*, 2004) were found to be differentially expressed across PEs treatment. Genes significantly up-regulated at least 2 fold in at least one treatment such as Star, Akrb8, Cyp19a1, and Hsd17b8, and Atp1a1 were shown in Fig. 7A. Genes significantly down-regulated at least 2 fold in at least one treatment such as Nr4a3, Igfbp7, Hsd11b2, Abcg1, Srd5a1, Bmp6, and Nsdhl were shown in Fig. 7B.

Discussion

Gonocytes in the neonatal testis represent a transient population of male germ-line stem cells that bridge the developmental gap between primordial germ cells in the embryo and spermatogonial stem cells in the pre-pubertal testis. To date, there has been limited application of *in vitro* systems for the evaluation of reproductive developmental toxicants because of the lack of reproducibility of the cell isolation procedure and compromised stem cell survival. However, modifications to *in vitro* models of gonocyte cultures developed in our laboratory

have significantly improved cell culture stability and quality. In this way, the 3D-SGC model, which captures both the 3-D organization and multicellular complexity of testis, may provide a powerful tool for screening the effects of potential testicular toxicants on these cells. In fact, we have already reported that the model positively demonstrates the reproductive toxicity of cadmium as well as possible mechanisms underlying its effect (Yu *et al.*, 2008a).

Continuing our efforts to validate our 3D-SGC culture system in screening testicular developmental toxicants, the present study investigates whether our SGC model is able to discriminate PEs that are developmental reproductive toxicants (DTPE) from those that do not have this property (DNTPE). We chose these PEs because their reproductive male toxicities have been well-documented in *in vivo* animal studies, allowing us to make comparisons between published *in vivo* data and our *in vitro* model (Mylchreest *et al.*, 1998; Gray *et al.*, 2000; Parks *et al.*, 2000; Barlow *et al.*, 2004; Liu *et al.*, 2005; Howdeshell *et al.*, 2008a).

In our comparison of the endpoints of cell viability between the DNTPE and DTPE treated cells, our results show DTPE-treated SGCs including DBP, DEHP, BBP, and DPP presented concentration-dependent decreased cell viability, but not in DNTPE-treated cells (Fig. 2A). Although the two-dimensional hierarchical clustering analysis of cell viability with the PEs illustrated general relative degree of these PEs in reducing cell viability, high impact PEs at the top and low at the bottom (Fig. 2B), and separated these PEs into three distinct clusters, it is still hard to clearly discriminate DTPE including DEHP and DBP from DNTPE including DEP in the middle cluster (B). In agreement with the decreases in cell viability in DTPE-treated SGCs, significant increases in the release of LDH in DPP, DBP, DEHP and BBP were observed (Fig. 3A). DPP increased LDH release in all the doses and time-points evaluated. However, the order of magnitude of the other PEs was not exactly the same as the one observed in the neutral red assay. In the LDH assay, DBP was the second most effective PE to affect LDH release, followed by DEHP, BBP and DEP, respectively. DMP and DOTP did not alter LDH release (Fig. 3B). Similar to our observations of the cell viability assay, hierarchical clustering analysis can separate DTPEs (including DPP, DBP, DEHP) from DNTPEs, however, it cannot clearly discriminate BBP from the DNTPE group using only these endpoints. Few studies have investigated multiple PEs at the same time and it is difficult to discuss potency and efficacy when comparing different studies because of the differences in experimental protocols, endpoints investigated, species and strain used, and time and duration of dosing, in addition to the dose itself. In an early comparative study of PEs (including DEHP, BBP, DINP, DEP, DMP, or DOTP) Gray *et al.* reported that oral administration of BBP, DEHP, and DINP to the dam at 750 mg/kg/d from gestational day (GD) 14 to postnatal day (PND) 3 induced significant increases in the rate of malformations among male rats at rates of 84%, 82% and 7.7%, respectively (Gray *et al.*, 2000). DEHP, BBP, and DINP all altered sexual differentiation, whereas DOTP, DEP, and DMP were ineffective at this dose. In another study, Liu *et al.* compared the effect of seven PEs (DPP, DBP, DEHP, BBP, DMP, DOTP and DEP) on anogenital distance (AGD) and gene expression alteration of male rat fetuses after gestational exposure (GD 12 to 19) to 500 mg/kg/d of different PEs (Liu *et al.*, 2005). As compared to the control, all of the DTPEs evaluated, (including DPP, DBP, DEHP, and BBP) reduced the AGD, with DPP being the most effective. Recently, Howdeshell *et al.* compared the dose-response of six individual PEs (BBP, DBP, DEHP, DPP, DEP and diisobutyl phthalate [DiBP]) on testicular testosterone production following the exposure of rats on GD 8–18 (Howdeshell *et al.*, 2008b). DPP was found to be three times more potent in reducing fetal testicular testosterone production as compared to BBP, DBP, DEHP and DiBP. DEP had no effect on fetal testosterone production.

These studies reveal complexity of reproductive toxicity signaling following PE exposure *in vivo*, however, are also reflecting the types of complex profiles seen with many reproductive toxicants. In our study we focused on assessment on the evaluation of several relative simple

endpoints, cytotoxicity and changes in gene expressions to see how effectiveness of such in vitro assessment might be in tracking array of these in vivo signals. In the current study we demonstrated that gene expression alteration (gene expression pattern) by different PEs was greater than conventional toxic endpoints such as cell viability and cytotoxicity in vitro. The gene expression changes were able to separate DTPE from DNTPE in our established 3D-SGC model. At a concentration of 10 μ M of PE treatment in 3D-SGC, we did not observed obvious morphological changes and significant decrease in cell viability. However at this concentration, we observed a greater number of significantly altered genes in the DTPEs, their genes changed in the DNTPEs. The number of genes altered by each DTPE, was similar among DPP, DBP, and DEHP while BBP was less effective (Fig. 4). Compared to our cytotoxicity evaluation endpoints, the global gene expression analysis revealed a much more distinct pattern between DTPE and DNTPE responses. A non-supervised two-dimensional cluster analysis for the genes changed across the treatments showed the presence of distinct clusters, corresponding to the control, the DNTPE group (including DEP, DMP and DOTP) and the DTPE group (including BBP, DEHP, DBP and DPP). Microarray technology has become a powerful tool for exploring the expression levels of thousands of genes, and even complete genomes, after exposure to toxicants. Alterations in gene expression have also been interrelated to conventional toxicological endpoints (Hamadeh *et al.*, 2002; Moggs, 2005; Yu *et al.*, 2006; Yu *et al.*, 2008b). Interestingly, the gene expression pattern that resulted from the cluster analysis of our *in vitro* model results is consistent with the pattern reported in an *in vivo* study that also applied microarray technology to evaluate testicular gene expression after maternal exposure (GD 12–19) to 7 different PE (DEP, DMP, DOTP BBP, DBP, DPP and DEHP) (Liu *et al.*, 2005). Similarly to our results, a cluster analysis conducted with the 391 genes whose expressions were found to be significantly (ANOVA) differentially altered, showed that PEs belonging to the DNTPE group (including DEP, DMP, DOTP) clustered together with the control group and were separated from the PEs belonging to the DTPE group (BBP, DBP, DPP and DEHP). The gene expression pattern among the compounds belonging to the DTPE group were indistinguishable (Liu *et al.*, 2005).

The functional interpretation of the microarray datasets were assessed through gene ontology (GO) and pathway mapping using MAPPfinder, followed by quantitative analyses by the GO-Quant approach (Yu *et al.*, 2006). GO-Quant facilitates quantitative interpretation of dose- or time-dependent genomic data because it extracts quantitative gene expression values and calculates the average intensity or ratio for those significantly altered by the functional gene category based on MAPPFinder results (Yu *et al.*, 2006). In the present study, we applied this systems-based approach to compare functional alterations across treatments. We found that there are more significant functional alterations in the DTPE group than within the DNTPE group. Changes induced by DTPEs in biological processes included down-regulation in DNA-dependent DNA replication; cell cycle; cell division; phosphate transport and anion transport; as well as positive regulation of apoptosis. There are a number of genes involved in the regulation of apoptosis with this GO category. The molecular function category included significant changes in down-regulation in the extracellular matrix constituent and copper ion binding GO categories in addition to significant up-regulation in G-protein-coupled receptor binding gene pathways. Chemokine activity and glutathione transferase activity gene expression changes were also observed only in the DTPE group. This GO-Quant based quantitative comparative analysis further supports that our improved 3D-SGC is useful in identifying the DTPEs based on their cellular and molecular responses.

Consistent with previous reported findings PEs (Barlow *et al.*, 2003; Lehmann *et al.*, 2004; Thompson *et al.*, 2005; Borch *et al.*, 2006; Lahousse *et al.*, 2006; Clark and Cochrum, 2007; Ryu *et al.*, 2007), disruptions of steroidogenesis related-gene expression were observed in the DTPE group, but not in the DNTPE group (Fig.7). Alterations in gene and protein expression and testosterone synthesis have been well recognized and used as sensitive indicators of

testicular response to PEs (Barlow *et al.*, 2003; Lehmann *et al.*, 2004; Thompson *et al.*, 2005; Borch *et al.*, 2006; Lahousse *et al.*, 2006; Clark and Cochrum, 2007; Ryu *et al.*, 2007). Utero treatment with DBP at 500 mg/kg/day on GD 12–19 down-regulated *Scarb1*, *Star*, *P450scc*, *3beta-HSD*, *P450c17*, and *c-kit* (Barlow *et al.*, 2003). In another in utero exposure study with daily utero exposure DBP treatment in rats from gestation day (GD) 12 to 19, decreases in expression of genes involved in cholesterol transport and steroidogenesis such as *Star*, *Scarb1* and *Ins13* were observed with a reduction in testosterone production in the fetal testis (Lehmann *et al.*, 2004). However, in our *in vitro* SGC model, we observed significant up-regulations of *Star*, *Akr1b8*, *Cyp19a1*, *Hsd17b8* and *Atp1a1*. The differences in the gene expression of *Star*, *Akr1b8*, *Cyp19a1*, *Hsd17b8* and *Atp1a1* might be due to temporal difference of treatment along with the testis development. The majority of *in vivo* utero exposure studies occurred repeatedly from gestational day 12 to 19 along with testis development (Barlow *et al.*, 2003; Lehmann *et al.*, 2004; Thompson *et al.*, 2005; Borch *et al.*, 2006; Lahousse *et al.*, 2006; Clark and Cochrum, 2007; Ryu *et al.*, 2007). Testicular genes involved in cholesterol metabolism (*Dhcr7*) and steroidogenesis (*Cyp17a1* and *StAR*) were examined following postnatal exposure through a single dose of 10, 100 and 1000 mg/kg of mono-(2-ethylhexyl) phthalate (MEHP) to 28-day old rats (Lahousse *et al.*, 2006). Decreased expression of these genes at 1000 mg/kg MEHP were observed 6 h following exposure, but increased expression was observed in the lower MEHP dose levels (10 and 100 mg/kg) 12 h following exposure. In a recent study, Culty et al examined the effects of utero exposure from GD 14 to the day of parturition (Postnatal Day, PN 0) to a wide range of DEHP doses (234 and 1250 mg/kg/day) on fetal, neonatal, and adult testosterone production in rats (Culty *et al.*, 2008). Adult serum testosterone levels were reduced significantly compared to those of controls at all DEHP doses. However, significant increases were seen in the gene expression of *Cyp11a1*, *Cyp17a1*, *Star*, and *Tspo* transcripts after birth (PN 60), which are different at the early time points observed (Culty *et al.*, 2008). In another male adult rat study with DBP at levels of 250, 500, and 750 mg/kg/day for 30 days, significant increases in the mRNA levels of steroidogenesis related genes (*scarb1*, *Star*, *P450scc*, *CYP17*) were also observed in the high dose DBP-treated rats (Ryu *et al.*, 2007). Further dose and time-dependent experimentation is needed to examine the dynamic gene expression alternation as well as protein levels that occur in our *in vitro* conditions. In the future, a detailed comparison of these altered signaling pathways with the *in vivo* study will further validate our 3D-SGC model for the screening of male reproductive toxicant effects.

Although the current study presented comparable results to our *in vitro* 3D-SGC model with *in vivo* results reported in the PE literature (Liu *et al.*, 2005), limitations of this *in vitro* model still exist. The necessity of bioactivation for some toxicants to induce their toxic effect is a limitation for most *in vitro* models, including the 3D-SGC. It is reported after *in vivo* exposure to diester phthalates, the parent compounds are hydrolyzed in the small intestines to monoester phthalates, which have been considered to be putative toxicants (Lampen *et al.*, 2003; Janer *et al.*, 2008; Svehnikov and Soder, 2008). However, diester phthalates have also been shown to have direct impacts on other *in vitro* systems for different system endpoints (Picard *et al.*, 2001; Seek Rhee *et al.*, 2002; Lu *et al.*, 2004; Hong *et al.*, 2005; Bredhult *et al.*, 2007). Whether these *in vitro* effects are caused by the direct effect of the parent compounds or by metabolites formed in the cultures after hydrolysis of the parent compound is a question that still needs to be addressed. Another limitation of *in vitro* models in screening for testicular toxicants is the presence, *in vivo*, of the blood/testis barrier, which can limit putative toxicants from reaching their target cells. For phthalates specifically, this would not have been a problem because they are able to cross the blood/testis barrier (Ono *et al.*, 2004).

In summary, using the 3D-SGC model, we demonstrated that DTPE induced significantly greater dose-dependent morphological changes, a decrease in cell viability and an increase in cytotoxicity compared to those treated with DNTPE. Moreover, microarray based-gene

expression was more altered by DTPE than by DNTPE and non-supervised cluster analysis allowed for the discrimination of DTPE from DNTPE. Consistent disruptions of steroidogenesis related gene expression such as *Star*, *Cyp19a1*, *Hsd17b8*, and *Nr4a3* were observed in DTPE group, but not in the DNTPE group. Our observation on cell viability, cytotoxicity, and microarray-based gene expression analysis induced by PEs demonstrate that our *in vitro* 3D-SGC system mimicked *in vivo* responses for phthalates and suggests that the 3D-SGC system might be useful in identifying the effects of developmental reproductive toxicants.

Acknowledgements

This work was supported in part by the USEPA-NIEHS UW Center for Child Environmental Health Risks Research (EPA R826886 and NIEHS 1P01ES09601), the NIEHS grant R01-ES1063, UW NIEHS Center for Ecogenetics and Environmental Health (5 P30 ES07033), Colgate-Palmolive Grants for Alternative Research, Society of Toxicology and the Johns Hopkins Center for Alternatives to Animal Testing (CAAT). Estefania G Moreira is a post-doctoral fellow from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil.

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Fig. 1.

Typical high-magnification fluorescence-based photographs of live/dead cells assessed at 24 h after treatment with phthalates esters (PE). Propidium iodide, Hoechst 33342 and calcein AM were added directly to co-cultures and incubated at 37°C for 5 min at 24 h after the treatment with PEs. The stained co-cultures were viewed by epi-fluorescence with appropriate band pass filters for PI, Hoechst, and calcein. The panels refer to the control (A) and variable phthalates (B–H) at concentration of 200 μ M /ml at 24 h (dead cell in red). Obvious morphological changes and increases of the number of PI-stained nucleus (dead cells) were observed in those cultures treated with previously known as *in vivo* male developmentally toxic PE including dibutyl phthalate (DBP), dipentyl phthalate (DPP) diethylhexyl phthalate

(DEHP), and benzyl butyl phthalate (BBP). No obvious visual morphologic changes no increases in dead cells were observed in the control, or in the cultures treated with previously known as developmentally nontoxic PE including phthalates diethyl phthalate (DEP), dimethyl phthalate (DMP), and dioctyl tere-phthalate (DOTP).

Fig. 2.

Cell viability in response to phthalates esters (PE) treatment. Sertoli cells and gonocytes were co-cultured for 48 h. Cells were treated with 0 to 400 μM for 24 h. Cell viability assessments (A) were conducted using the neutral red dye uptake assay which assesses lysosomal accumulation of this dye. Data is presented as mean $\pm$ SE of 3 samples. Significant dose-dependent decreases of cell viability with one way ANOVA analysis were observed in the groups of PE, which were previously known as *in vivo* male developmentally toxic PE (DTPE, ($\dagger p \leq 0.05$)) including dibutyl phthalate (DBP), dipentyl phthalate (DPP) diethylhexyl phthalate (DEHP), and benzyl butyl phthalate (BBP, but there was no difference in cell viability compared to control in the group of PE which were previously known as developmentally nontoxic PE (DNTPE) including phthalates diethyl phthalate (DEP), dimethyl phthalate (DMP), dioctyl tere-phthalate (DOTP). Significant decreases in cell viability were observed at

200 and 400 μM for DPP and BBP, and 400 μM for DBP and DEHP (comparison with the control using the Dunnett's test, * $p \leq 0.05$). Non-supervised two dimensional hierarchical clustering analysis of cell viability (fold change over the mean of the control, B) was conducted on samples using average linkage and Euclidean dissimilarity. The fold changes in cell viability in SGC are visually illustrated - high at the top and low at the bottom. Grey represents data missing. Cluster 1 on the top only includes DPP, BBP, DEHP (DTPE group), and cluster 3 at the bottom only includes DMP, DOTP, DEP (DTNPE group). Cluster 2 in the middle includes DEHP, DBP and DEP.

Fig. 3.

Dose-dependent increases in cytotoxicity as determined by LDH release in response to phthalate ester (PE) treatment within Sertoli cell-gonocyte. Cells were treated with previously known as *in vivo* male developmentally toxic PE (DTPE) such as dibutyl phthalate (DBP, A), dipentyl phthalate (DPP, B), diethylhexyl phthalate (DEHP, C), or benzyl butyl phthalate (BBP, D) and previously known as developmentally nontoxic PEs (DNTPE) such as diethyl phthalate (DEP, E), dimethyl phthalate (DMP, F), dioctyl tere-phthalate (DOTP, G) at concentrations from 0 to 400 μM for up 24 h. Cytotoxicity was measured using CytoTox 96® non-radioactive cytotoxicity assay at 4, 8 and 24 h after treatment. The results of the release of LDH are presented as the mean of corrected absorbance values corrected by the maximum

LDH release of 3 independent experiments at each time-point $\pm$ SE. Significant dose-dependent increases in cytotoxicity were observed in the DTPE group at 4, 8 and 24 h (one-way ANOVA, $\dagger p \leq 0.05$). * $p \leq 0.05$ shows the significance of the comparison of the each dose with the control by the Dunnet's test. Non-supervised two dimensional hierarchical clustering analysis of cytotoxicity, the percentage over the mean of the controls was conducted (H). Three distinct clusters (1, 2 and 3) were identified based on the dose-dependent cytotoxicity measurement (H). Cluster 1 on the bottom, which includes DBP and DPP, has the highest impact on the LDH release. Cluster 3 at the top includes DBP, DEHP and BBP and Cluster 2 in the middle includes all the DNTPE (DOTP, DEP, and DMP), as well as 4 sets of BPP samples.

Fig. 4.

Evaluation of genes expression changes following treatment with phthalates: the number of significantly up-regulated or down-regulated genes are shown in comparison with control expression at 100 μ M PE concentrations 24 hours post treatment in Sertoli cell-gonocytes co-culture. Sertoli cell-gonocytes were treated with phthalates at 100 μ M for 24 h. Total RNA was isolated and microarray analysis was conducted using Affymetrix Rat Genome 230 2.0 Array (three arrays per phthalate or control condition). The significant changed genes following treatment were selected based on a multivariate permutations test (1000 random permutations) at the following conditions: nominal significance level of each univariate test was $p \leq 0.001$ and confidence level of false discovery rate assessment was 90 %. Maximum allowed number of false-positive genes and maximum allowed proportion of false-positive genes were 10 and 0.1, respectively.

Fig. 5. Hierarchical clustering analysis of significant gene expression changes after exposure to phthalates. Sertoli cell-gonocytes were treated with phthalates at 100 μ M for 24 h. Total RNA was isolated and microarray analysis was conducted using Affymetrix Rat Genome 230 2.0 Array (three arrays per phthalate or control). A total of 2640 genes were identified as significantly changed across treatments. These genes were further filtered using the geometric mean intensities that varied ≥ 2 -fold over control (in either directions) in at least one treatment for further evaluation. For these selected genes (1670 genes), the gene expression ratio derived from dividing each normalized gene value with the average value of the control as transformed to log2 ratio. These log2 transformed ratio were input to the Multi Expression View (MEV)

(Saeed et al. 2003) and a non-supervised two dimensional hierarchical clustering analysis was conducted on the output genes using average linkage and Euclidean dissimilarity (Eisen et al. 1998).

Fig. 6.

GO-Quant based quantitative Gene Ontology analysis on the effects of phthalate treatments at 100 μ M for 24 h on gene expression pathways in Sertoli cell-gonocytes co-culture. Sertoli cell-gonocytes were treated with phthalates at 100 μ M for 24 h. Total RNA was isolated and microarray analysis was conducted using Affymetrix Rat Genome 230 2.0 Array (three arrays per phthalate or control). The functional interpretation of the microarray datasets were assessed through gene ontology (GO) and pathway mapping using MAPPfinder was followed by quantitative analyses by GO-Quant approach (Yu et al., 2006). Briefly, MAPPfinder was used to link identified significant genes at $p \leq 0.01$ with GO functional gene categories, and assign overall z-score to each category and identify the significant changes of GOID, then apply GO-

Quant to quantify each GOID (Yu *et al.*, 2006). Relative changes (GO-Quant index) in biological process (A) and molecular function (B) were shown.

Fig. 7.

Effects of phthalate treatments on the genes involving in the steroidogenesis in Sertoli cell-gonocytes. Sertoli cell-gonocytes were treated with phthalates at 100 μ M for 24 h. Total RNA was isolated and microarray analysis was conducted using Affymetrix Rat Genome 230 2.0 Array (three arrays per phthalate or control). A set of seventy-six genes that has been annotated in rat in Gene Ontology database was identified and statistical analysis of 84 probes linked to these 76 genes across treatments using F test was conducted. Nominal significance level of each univariate test and permutation p-values for significant genes was set at 0.001. Forty-two probe sets were found differentially expressed across phthalates treatment. Significantly up-regulated genes (at least 2 fold up-regulated in at least one treatment) were shown in Fig. 7A,

and significantly down-regulated genes (at least 2 fold down-regulated in at least one treatment) were shown in Fig. 7B.