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Comparison of toxicogenomic responses to phthalate ester exposure in an organotypic testis co-culture model and responses observed *in vivo*

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ABSTRACT

We have developed a three-dimensional testicular co-culture system (3D-TCS) which mimics *in vivo* testes. In this study, transcriptomic responses to phthalate esters (PE's) were compared in the 3D-TCS with responses in rat testes *in vivo*. Microarray data from the 3D-TCS and from *in vivo* testes were used to compare changes in gene expression patterns after exposure to developmentally toxic (DTPE) or developmentally non-toxic (DNTPE) phthalate esters. DTPE treatments clustered separately from DNTPE treatments based on principle components analysis both *in vitro* and *in vivo*. Pathway analysis using GO-Elite software showed that terms relating to steroid metabolism or reproductive development were enriched both *in vitro* and *in vivo* after DTPE exposure. Processes such as cell cycle, cell proliferation and apoptosis were enriched for DTPE treatments *in vitro*, but not *in vivo*. Based on these analyses we concluded that transcriptomic responses in the 3D-TCS reflect key aspects of *in vivo* phthalate toxicity.

1. Introduction

Current models of male reproductive toxicity are expensive, time consuming and require a large number of animals. Rovida and Hartung [1] estimated a cost of ~€6.9 billion and ~49 million animals would be required to implement all reproductive toxicity testing proposed under Europe's REACH program (Registration, Evaluation, Authorisation and Restriction of Chemical substances). New *in vitro* methods may aid in reducing these burdens [1,2]. However, the use of alternative models of reproductive development has been limited by difficulties in modeling the complex cellular interactions and multiple endpoints which are involved in these processes [3]. Due to these difficulties, there is currently no reliable and reproducible *in vitro* model for testicular toxicity screening [4]. With the aim of addressing some of these issues, we have developed an *in vitro* model of testes development [5]. This cellular co-culture system (3D-TCS) contains several testes cell types (Sertoli, germ and Leydig cells) grown in a three dimensional conformation facilitated by an extracellular matrix (ECM) overlay. Earlier experiments have demonstrated that addition of ECM in this co-culture model results in a more physiologically stable system and that cells form a testicular-like architectural structure representative of *in vivo* characteristics of seminiferous tubules [5].

Phthalate esters (PE's) represent a class of well-known male reproductive toxicants. Male reproductive effects of exposure to certain phthalate esters (PE's) include endpoints such as underdeveloped reproductive organs, hypospadias, cryptorchidism and decreased anogenital distance [6–8]. Previously, we demonstrated that our 3D-TCS model was able to distinguish between developmentally toxic PEs (DTPE) and developmentally non-toxic PEs (DNTPE) based on observed differences in microarray-based gene expression profiles, with significant changes occurring in the

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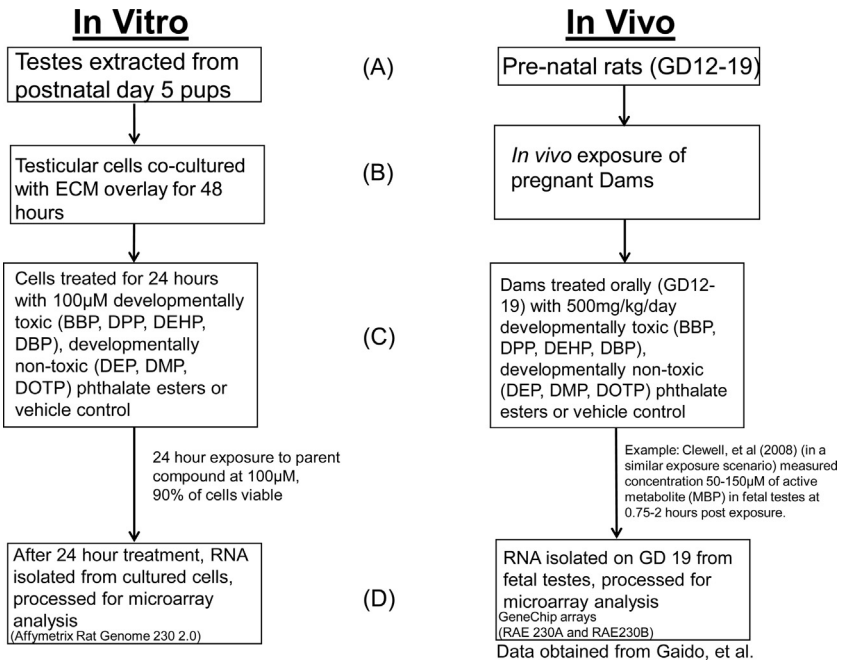

Fig. 1. Experimental design for the *in vitro* three-dimensional testicular cell co-culture system (3D-TCS) and *in vivo* prenatal rats exposed to phthalate esters.

steroid biosynthetic pathway. In contrast, a general cytotoxicity assay (neutral red uptake assay) was not able to distinguish between the toxic and non-toxic phthalates at the same dose. These results suggested that the 3D-TCS system provides a sensitive tool for identifying male reproductive toxicants through alterations in important biological pathways with functional relevance to male reproductive development [9].

Previously, Liu et al. exposed pregnant rats to the same group of DTPE and DNTPE's. This was followed by microarray-based gene expression profiling in the testes of male fetuses, which identified a number of cellular pathways disrupted by DTPE exposure. These pathways included lipid and cholesterol homeostasis, insulin signaling, oxidative stress and steroidogenesis. In addition to cellular pathway changes, DTPE exposure was associated with the phenotypic outcome of significantly decreased anogenital distance [10]. The purpose of the current study was to compare transcriptomic responses induced by PEs in the 3D-TCS model with those observed from the Liu et al. *in vivo* exposure using analysis of microarray gene expression profiles and pathway based analysis.

Our comparative analyses will allow us to determine the extent to which DTPE induced gene expression changes in the 3D-TCS reflect those occurring under an *in vivo* exposure scenario and aid in the evaluation of a proposed adverse outcome pathway (AOP) for phthalate reproductive toxicity [11,12]. As shown in Fig. 1, there were significant differences in the experimental design between the *in vivo* and *in vitro* datasets used in this study, for example the *in vivo* data was a repeated multiple dose exposure while the *in vitro* study was a 24 h acute exposure. Bearing these differences in mind, we sought to determine whether the transcriptomic response in the 3D-TCS revealed key aspects of the mode of action (MOA) for phthalate reproductive toxicity. In order to accomplish this, we examined overall changes at the pathway level with a particular focus on the steroid biosynthesis pathway because disruption of steroidogenic gene expression has been phenotypically anchored to a number of male reproductive outcomes and is an important MOA for phthalate toxicity [6,7,13]. These comparisons have allowed us to link effects on gene expression observed *in vitro* with those observed *in vivo* and provide critical information in supporting *in vitro* models for evaluating mechanisms of male reproductive toxicity.

2. Materials and methods

2.1. Three dimensional co-culture model for testes (3D-TCS), treatments and gene expression profiling

Fig. 1 shows the experimental designs for both the *in vitro* and *in vivo* data used in this study. The method for the three dimensional co-culture model for testes (3D-TCS) and PE treatments was described previously [9]. Briefly, testes were dissected from 5-day-old male pups obtained from mated Sprague-Dawley rats (Charles River Laboratories, Wilmington, USA). A single cell suspension containing primarily Sertoli, germ and Leydig cells was then plated followed by an overlay of extracellular matrix medium (Matrigel™). Benzyl butyl phthalate (BBP, Sigma #44-2503, 99% purity), dibutyl phthalate (DBP, Sigma #D2270, 99% purity), diethylhexyl phthalate (DEHP, Sigma #4-8557, 99% purity), diethyl phthalate (DEP, Sigma #524972, 99.5% purity), dimethyl phthalate (DMP, Sigma #525081, 99% purity), dioctyl tere-phthalate (DOTP, Sigma #525189, 96% purity) or dipentyl phthalate (DPP, Sigma #80154, 99% purity) were obtained from Sigma–Aldrich (MO, USA). Phthalate solutions (or vehicle control, DMSO, Sigma–Aldrich, MO,USA) with a final concentration of 100 μM were then added directly to the culture medium 48 h after the addition of ECM overlay. This dose was selected based on earlier experimental data showing changes in gene expression with a minimum effect on cell viability (90% of the cells were still viable). Total RNA was extracted from cells and used as starting material for probing of Affymetrix Rat Genome 230 2.0 microarrays (3 replicate arrays for each PE treatment and control).

2.2. Gene expression profiling following in utero exposure to phthalate esters

In vivo exposure to phthalate esters was reported previously by Liu et al. [10]. Briefly, time-mated Sprague-Dawley outbred CD rats were obtained from Charles River Laboratories, Inc. (Raleigh, NC) on Gestation Day 0 (GD 0). Dams were treated by gavage daily from GD 12 to GD 19 with corn oil vehicle or BBP, DBP, DEHP, DEP, DMP, DOTP or DPP (Aldrich Chemical Company, Milwaukee, WI) in corn