Boekelheide Laboratory-Material and Methods

Description of the test articles used, study material evaluations (diet, drinking water, cage and bedding leachates), general study design, animal treatments, and animal allocations to the 2 year toxicology study conducted at NCTR and the grantee studies can be found in Heindel et al. (2015).

Data from the current study were reported in Dere *et al.* (2018) Effects of continuous bisphenol A exposure from early gestation on 90 day old rat testes function and sperm molecular profiles: A CLARITY-BPA consortium study. Toxicol App Pharmacol 347:1-9.

Sample Collection

The current study used samples from 6 dose groups (n=10 per group; 1 pup per litter); vehicle and 2.5, 25, 250, 2500 and 25000 µg/kg/d BPA. Additionally, a 250000 µg/kg/d BPA and vehicle group (n=20 per group; 2 pups per litter) were included (herein designated as "high-dose study"). Animals were necropsied ±10 days of postnatal day (PND) 90, and the body and reproductive organ weights were recorded. Left testes were fixed in modified Davidson's and transferred to 10% neutral-buffered formalin after 24 hours for histological examination, and a portion of each animal's right testis was detunicated and snap frozen in liquid nitrogen for the automated determination of homogenization resistant spermatid head (HRSH) counts (Pacheco *et al.*, 2012). The epididymides were weighed and sperm from the caudal regions of the epididymides were used to isolate RNA and DNA.

Histological Endpoints

Two cross sections from the center of the fixed testes were embedded in glycol methacrylate (Technovit 7100; Heraeus Kulzer GmBH, Wehrheim, Germany) for histological examination of stage-specific retained spermatid heads (RSH) or embedded in paraffin for detection of apoptosis by TUNEL staining. The Aperio ScanScope (Aperio Technologies, Vista, CA) was used to create digital images of the microscope slides, and all histological endpoints were analyzed using ImageScope software.

Enumeration of RSH was performed using two cross sections (3 µm) of testes from six randomly selected rats per treatment group stained with periodic acid-Schiff's reagent followed by hematoxylin counter stain (PASH). Each section was evaluated for seminiferous tubules in spermatogenesis stages IX-XI, each of which was required to be nearly round (major:minor axis of less than 1.5:1) (Bryant *et al.*, 2008). RSH were identified as condensed spermatid heads in the basal epithelium in stages IX-XI, and the counts were averaged together on an individual rat

basis. The counts were log-transformed to ensure normally distributed errors prior to statistical analysis.

For the evaluation of apoptosis, paraffin sections (5 µm) of testes from the subset of rats utilized above were stained using the ApopTag Peroxidase In Situ Apoptosis Detection Kit (EMD Millipore, Burlington, MA) following the manufacturer's protocol and were counterstained with methyl green. Apoptotic cells were counted in a minimum of 50 seminiferous tubules, systematically selected using a grid, having a major:minor axis of less than 1.5:1. The percentage of seminiferous tubules containing TUNEL positive cells was assessed.

Sperm Isolation and RNA Extraction

Sperm from the cauda epididymides were isolated as previously described (Pacheco *et al.*, 2012; Dere *et al.*, 2016). Briefly, the cauda epididymides were punctured repeatedly with 26 and 30 gauge needles, placed into micro-centrifuge tubes containing phosphate buffered saline (PBS, Life Technologies, Grand Island, NY), and incubated in a water bath at 37 °C for 10 minutes to allow sperm release. Epididymal tissue and debris were pelleted by centrifugation for 3 minutes at 300 x *g*, and the supernatant was removed and centrifuged for 5 minutes at 2000 x *g* to pellet the sperm. Somatic cell contaminants were removed by resuspending the sperm in a somatic cell lysis buffer (0.15 M ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA) (Thermo Fischer Scientific Inc., Pittsburgh, PA) and incubating for 30 seconds prior to centrifugation at 16,100 x *g* for 1 minute, leaving the sperm intact. The pelleted sperm were subsequently washed with PBS and centrifuged again at 16,100 x *g* for 1 minute and RNA was extracted using the mirVana miRNA Isolation Kit (Applied Biosystems, Austin, TX). The isolated RNA was further purified and concentrated using Qiagen's RNase-free DNase and RNeasy MinElute Cleanup kits (Qiagen Sciences, Germantown, MD), following the manufacturer's protocol. RNA yields and concentrations were measured on a NanoDrop 1000 spectrophotometer (Thermo Scientific).

Sperm mRNA Transcript Analysis

RNA samples from all the BPA treatment groups (n=10 for the CLARITY-BPA consortium doses, and n=20 and n=18 from the vehicle and 250000 µg BPA/kg/d from the high-dose study, respectively) were used to perform whole-genome microarray profiling of sperm mRNA using Affymetrix Rat Gene 1.0 ST GeneChips (Affymetrix, Santa Clara, CA). Microarray data were processed in the R software environment (R Core Team) and Bioconductor (Gentleman *et al.*, 2004) using the RMA algorithm in the *oligo* package (Carvalho and Irizarry, 2010) for probe-level summarization and analyzed using the *limma* package (Ritchie *et al.*, 2015). Gene annotation information was joined to the transcriptomic data using the ragene10sttranscriptcluster.db package. Significantly altered transcripts were defined as having a q-value < 0.05 with a $|\text{fold change}| > 1.5$. This analysis used the litter as the unit of

replication for the consortium dose groups and the individual pups as the unit for the high-dose study.

A Monte Carlo method implemented in R that was previously used to analyze the robustness of –*omic* signatures (Dere *et al.*, 2016) was used to repeatedly identify significantly altered sperm mRNA transcripts from five randomly selected BPA and vehicle treated samples from the CLARITY-BPA consortium dose groups using 100 iterations, and significantly altered mRNA transcripts were identified for each iteration. The analysis of the high-dose study dose groups was conducted with 1000 iterations, where five randomly selected litters from the 250000 µg BPA/kg/d and concurrent vehicle control groups were chosen, and a pup from each of those selected litters was randomly picked for the analysis. This selection process ensured that pups from the same litter were not chosen, thereby maintaining the litter as the unit of replication.

RRBS Library Construction

Reduced representation bisulfite sequencing (RRBS) libraries were constructed using previously published protocols from sperm samples with sufficient amounts of DNA (Gu *et al.*, 2011; Boyle *et al.*, 2012). The number of samples for each dose group with sufficient amounts of DNA library construction are provided in Figure 2. A modified guanidine thiocyanate DNA extraction method was utilized to extract highly compacted DNA from sperm (Griffin, 2013), and the quality and quantity were assessed using a NanoDrop 1000 (Thermo Scientific). Briefly, for each sample, 500 ng of DNA were digested with MspI (New England Biolabs, Ipswich, MA) and then purified using QIAquick Nucleotide Removal Kit (Qiagen, Germantown, MD). Subsequently, end-repair A-tailing was performed using Klenow fragment (3'-5' exonuclease; New England Biolabs), and TruSeq methylated indexed adaptors (Illumina, San Diego, CA) were ligated with T4 DNA ligase (New England Biolabs). Fragments were size selected using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN) and then followed with two rounds of bisulfite conversion to ensure complete conversion of the methylated cytosines using the EpiTect kit (Qiagen). Following bisulfite treatment, the DNA was purified as directed and amplified using Pfu Turbo Cx Hotstart DNA polymerase (Agilent Technologies, Santa Clara, CA). Library quantification was performed using the Quant-iT high sensitivity DNA assay kit (Invitrogen, Grand Island, NY) and the Bioanalyzer DNA 1000 kit (Agilent Technologies). Single-end 50 bp sequence runs were performed for each constructed multiplexed library on an Illumina HiSeq 2500 (Illumina). The data from the sequence runs were processed using trim galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), and then aligned to the rat reference genome RGSC Rnor_6.0 using Bismark (Krueger and Andrews, 2011).

RRBS Methylation Analysis

Differentially methylated CpGs and 100 bp tiles were identified using the methylKit package (Akalin *et al.*, 2012) for R (https://www.R-project.org) as previously described (Dere *et al.*,

2016). Logistic regression analysis was used to compare the methylation means of the BPA and vehicle treated groups to calculate methylation levels and p-values, and the p-values were corrected to genomic-wide false discovery rate (FDR)-based q-values by using the SLIM method (Wang *et al.*, 2011). The analysis was performed both on an individual CpG and 100 bp stepwise tiling window level. The methylation level of a tile represents the average of all the individual CpGs within the tile, and averaged across replicates. Differentially methylated CpGs or 100 bp tiles elicited by BPA treatment were defined as having >10% methylation difference relative to vehicle controls and a q-value < 0.05. This analysis used the litter as the unit of replication for the consortium dose groups and the individual pups as the unit for the high-dose study.
The Monte Carlo method was applied to repeatedly identify differentially methylated CpGs and tiles from randomly selected BPA and vehicle treated samples from both the CLARITY-BPA consortium dose groups, and the 250000 µg/kg/d high-dose group. The random selection process was conducted as described in the sperm mRNA transcript analysis methodology to ensure that the litter was treated as the unit of replication. Additionally, Monte Carlo analysis was performed with the two vehicle groups, using the litter as the unit of replication. For this analysis, five randomly selected samples per group were selected for 100 or 1000 iterations, and differentially methylated regions were identified for each iteration.

Data Decoding

All the blinded raw data for the CLARITY-BPA consortium dose groups were submitted to the NTP Chemical Effects in Biological Systems (CEBS) database. Data were then independently verified to account for all expected data sets and data points, and "locked" such that data could not be altered. The researchers at Brown University were then provided with the exposure code for data analysis. Unblinded data for the high-dose study dose groups were also uploaded into the CEBS database.

Statistical Analysis

Body and organ weights, and quantitative RSH, HRSH and TUNEL data from the CLARITY-BPA consortium dose groups, and the sperm mRNA transcript data from all dose groups were analyzed using one-way ANOVA followed by the Dunnett's multiple correction test to identify significant changes relative to vehicle control. For the high-dose study groups, there were two male pups per dam and the litter was used as the unit of replication for comparing body and organ weights, and quantitative RSH, HRSH and TUNEL data. These data were analyzed using a one-tailed unpaired *t*-test relative to the high-dose group's concurrent vehicle control. The statistical analyses of the data, with the exception of the microarray and RRBS data, were performed using Prism 6 by GraphPad (GraphPad Software, La Jolla, CA). Dose-response modeling of the sperm DNA methylation data was performed using the *drc* package for R (Ritz *et al*., 2015).

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