vom Saal Laboratory - Material and Methods (1 Year)

Animal Husbandry and Dosing

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).

NCTR-CD-SD male rats were randomly assigned to one of eight treatment groups (0.3% CMC vehicle control, 2.5, 25, 250, 2500, 25000ug/kg BPA, 0.05 or 0.5ug/kg EE) and dosed via oral gavage, daily, for one year. The 48 one year old males (6 per treatment group) used in this study were from loads 2, 3, 4 and 5, from animal set number 13.

Tissue Collection and Embedding

The urogenital tracts (UGT) from 1 year old males were fixed in 10% neutral-buffered formalin for 24 hours and then shipped to the vom Saal Lab in 70% ethanol. A one centimeter portion of prostatic urethra was collected from the UGTs and placed into RNAse-free ddH2O overnight until agar- and paraffin-embedding the next day. The one centimeter prostatic urethra was embedded vertically, cranial side down, to make a tissue array. The final four cassettes of agar-embedded urethras were processed using a standard overnight protocol and paraffin-embedded in the University of Wisconsin Carbone Cancer Center Histology Core.

Immunohistochemistry

Formalin-fixed, paraffin-embedded 1 year old rat prostatic urethras were serially sectioned in RNAse-free conditions, 5µm thick. Every tenth slide was then stained with Hematoxylin and Eosin for 3D reconstruction contouring. Immunohistochemistry was completed using 2 different antibodies, Androgen Receptor (Santa Cruz, sc-816, 1:100) and Estrogen Receptor alpha (ProteinTech, 21244-1-AP, 1:400), as well as Picrosirius Red (PSR) to stain collagen fibers focusing on the the slides nearest to the middle of the prostatic urethra we reconstructed.

3D Reconstruction

Photographs were acquired with a 2x objective lens (N.A.=0.06) on an 80i microscope using the DS-Fi2 camera and NIS Elements software (Nikon Instruments, Melville, NY). All images were then imported into BioVis3D software (Montevideo, Uruguay) defining the distance between each image as 100µm. BioVis3D was used to trace the urethral lumen on every image in order to render it in 3D. To ensure the 3D reconstruction of the urethral lumen had the same start and end points between samples, a set of criteria was defined. The cranial endpoint occurred when the lateral ducts branched outside of the rhabdosphincter, while the caudal endpoint occurred when the urethra was enclosed and the appearance of dual ducts below the ejaculatory ducts occurred.

Picrosirius Red (PSR) quantification

After the slides around the middle of the prostatic urethra were stained with PSR, brightfield and birefringent images were collected using a 2x objective (N.A.=0.06) on an 80i microscope using the DS-Fi2 camera through circular polarized lenses and NIS Elements software (Nikon Instruments, Melville, NY). Two regions of interest (ROI) were determined, including the prostatic urethra ROI, which included all the prostatic tissue within the rhabdosphincter and the luminal urethra ROI, which was an ellipse shape drawn from the top of the urethral lumen and out to the inside edge of the rhabdosphincter. Collagen bundles were isolated and colored pixels quantified using ImageJ macros corresponding to thickness (Red=very thick, orange=thick, yellow=medium, green=thin).

Data Decoding and Statistical Analysis

The raw, blinded data files were uploaded to the NTP Chemical Effects in Biological Systems (CEBS) database and "locked" down to prevent further editing while being independently verified. Once all data was submitted from involved researchers, the data was decoded, so final statistical analyses could be performed. Collagen deposition via PSR staining and 3D urethral morphometric measurements were statistically analyzed using t-tests or linear one-way analysis of variance (ANOVA) followed by LSmeans test comparing controls to treatment groups; separate analyses were conducted for BPA and EE2 data. In addition, R Statistic software was used to compare the goodness of fit of just the BPA data to a linear or non-monotonic function by addition of a quadratic term. In all analyses $*P < 0.05$ was considered statistically significant and $P < 0.1$ as trending.

References:

Heindel JJ, Newbold RR, Bucher JR, Camacho L, Delclos KB, Lewis SM, Vanlandingham M, Churchwell MI, Twaddle NC, McLellen M, Chidambaram M, Bryant M, Woodling K, Gamboa da Costa G, Ferguson SA, Flaws J, Howard PC, Walker NJ, Zoeller RT, Fostel J, Favaro C, Schug TT. NIEHS/FDA CLARITY-BPA research program update. Reprod Toxicol. 2015 Dec; 58:33-44.