vom Saal Laboratory - Material and Methods (Postnatal Day 1)

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 female rats were stratified by body weight and 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). Starting at gestational day 6 and continuing until parturition, dams were administered daily with 5ml/kg treatment doses via oral gavage. Mating pairs were assigned randomly and occurred in five "loads", spaced 4 weeks apart. One day old pups used in this study were from loads 2, 3, 4 and 5, from animal set number 2. Forty eight animals (six per treatment group) were used. These were selected from the 10 animals per treatment group available, in order to keep the body weight range as narrow as possible (original body weight range: 3.7-9.6 grams; used body weight range: 5.2-8.1 grams at PND1).

Tissue Collection and Embedding

Dams and pups remained undisturbed on the day of birth (=PND0) until terminal pup weights were taken and euthanized by decapitation on PND1. The hind end from the males were fixed in 10% neutral-buffered formalin for 24 hours and then shipped to the vom Saal Lab in 70% ethanol. The entire male lower urogenital tract, including the urogenital sinus (UGS), bladder, and associated accessory sex glands were collected and placed into RNAse-free ddH2O overnight until agar and paraffin-embedding the next day. Before agar-embedding, the top 1/3 of the bladder was trimmed off in order to arrange 20 urethras per block, dorsal side down. The final three cassettes of agar-embedded UGTs were processed using a standard overnight protocol and paraffin-embedded in the University of Wisconsin Carbone Cancer Center Histology Core.

Immunohistochemistry and 3D Reconstruction

Formalin-fixed, paraffin-embedded PND1 rat UGTs were serially sectioned in RNAse-free conditions, 5µm thick. Every third slide was then stained for basal epithelial cell nuclear marker, p63 ΔN (BioLegends, 619002, 1:500). Photographs were acquired with a 4x objective lens (N.A.=0.13) 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 15µm. BioVis3D was used to trace the urethra, seminal vesicles (SV), vas deferans (VD), ventral prostate (VP), lateral prostate (LP), dorsal prostate (DP) and anterior prostate (AP) on every image in order to render it in 3D.

Immunohistochemistry was completed using 10 different antibodies focusing on the urethral epithelial area on the slides nearest to the laterally widest part of the urethra. Antibodies used and dilutions are summarized in the table below.

Antibody Name	Alt. Name	Company	Cat#	Dilution
AR	Androgen Receptor	Santa Cruz	SC-816	1:100
CYP19A1	Aromatase	Abcam	ab18995	1:125
CYP11A1	Cytochrome P450	ProteinTech	13363-1-AP	1:100
BMP4	Bone Morphogenetic Protein 4	ProteinTech	12492-1-AP	1:100
DKK2	Dickkopf	ProteinTech	21051-ap-1	1:50
ESR1	Estrogen Receptor 1	ProteinTech	21244-1-AP	1:400
IGF-1	Insulin-like Growth Factor-1	Santa Cruz	SC-9013	1:400
SF1	NR5A1	ProteinTech	18658-1-ap	1:100
Thbs2	Thombospondin 2	Bioss	bs-7524R	1:100
SFRP4	Secreted Frizzled-Related Protein 4	ProteinTech	15328-1-ap	1:800

3D Reconstruction Criteria and Morphometric Definitions

To ensure consistent traces were made for each UGT 3D reconstruction, a set of criteria was defined as follows: The cranial trace followed the verumontanum p63 positivity whenever possible. However, if this was not possible, a straight line across from the points where the VP last branch from the urethral epithelium or where the epithelium thickens at the bladder neck was used instead. The first dorsal and last ventral trace were made when p63 positivity first appeared dorsally and last appeared ventrally or, in some cases, when the last VP bud tip was present in the epithelium before reaching the ventral mesenchymal pad (VMP). The caudal trace was a fixed distance of 600µm down from the top of the colliculus.

Looking down from the cranial aspect of the 3D UGT, the widest slice was chosen to take the following morphometrics: The urethral area and perimeter are calculated by BioVis3D, along with the luminal area and perimeter. The urothelium area is found by subtracting the luminal area from the urethral area. To calculate the urothelium thickness, two distances were measured on the left and right lateral sides of the urothelium at the widest point of the urethra and averaged. The utricle distance is measured from the lowest caudal point of the colliculus to the lowest caudal point of the urethra. Lastly, the lateral width was measured across the widest, lateral part of the p63 positive uroepithelium. The same morphometrics were taken on the halfway slice, which was chosen at the point halfway between the AP duct's first urethral attachment and the bladder's first urethral attachment. Further morphometrics were taken on the entire 3D reconstruction, including dorsal to ventral length, caudal to cranial length as well as volume and surface area of the cranial urethra, prostatic urethra and colliculus. Lastly, the colliculus angle was measured by drawing a point from where the AP first branch to where the colliculus angles from the urethra and then down to the most caudal point of the colliculus.

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. A number of morphometric measurements were statistically analyzed using 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.