Flaws 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).

The methods below were published as part of our manuscript: Patel, S., Brehm, E., Gao, L., Rattan, S., Ziv-Gal, A., Flaws, J.A. (2017) Effects of bisphenol A exposure on ovarian follicle numbers and sex steroid hormone levels: Results from a CLARITY-BPA study. *Endocrinology* 158:1727-1738.

Sample Collection

At necropsy, the individual weight of the ovaries was recorded, the right ovary was flash-frozen in liquid nitrogen, and the left ovary was fixed in Dietrich's solution. Serum was prepared from terminal blood and frozen. The ovary and serum samples were shipped from NCTR to Flaws laboratory. The samples were collected from animal set 1 [postnatal day (PND) 1], 4 (PND 21), 6 (PND 90), 8 (6 months), and 10 (1 year) from all BPA and EE_2 dose groups, including stop and continuous dosing arms.

Assessment of Ovarian Follicle Numbers

The fixed ovaries from NCTR were transferred to 70% ethanol, embedded in paraffin wax, and serial sectioned (8 µm) using a microtome. The serial sections were mounted on a glass slide and stained with hematoxylin and eosin. Follicle numbers were counted in every tenth section using previously defined criteria (Benedict et al., 2000; Borgeest et al., 2004; Hannon et al., 2015). Briefly, primordial follicles contained an oocyte surrounded by a single layer of squamous granulosa cells, primary follicles contained an oocyte surrounded by a single layer of cuboidal granulosa cells, pre-antral follicles contained an oocyte surrounded by at least two layers of cuboidal granulosa cells and theca cells, and antral follicles contained an oocyte surrounded by multiple layers of cuboidal granulosa cells with a fluid filled antral space and theca cells. All primordial and primary follicles were counted in each section regardless of nuclear material in the oocyte, whereas only pre-antral and antral follicles with nuclear material in the oocyte were counted to avoid double counting the larger follicle types that can span multiple sections. Follicles transitioning between stages were counted as follicles within the more immature stage of the two stages. Follicles were considered unhealthy if they showed numerous apoptotic granulosa cells or oocyte abnormalities. All other follicles were considered healthy. Data were collected without knowledge of treatment group.

Measurement of Estradiol and Progesterone

The serum estradiol and progesterone samples were obtained from NCTR. Hormone levels were quantified by enzyme-linked immunosorbent assays (ELISAs) at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (estradiol: Calbiotech, Spring Valley CA; progesterone: IBL, Minneapolis, MN). The Core used assays that were validated according to the Endocrine Society's Sex Steroid Assay Reporting Task Force (https://med.virginia.edu/research-in-reproduction/wp-content/uploads/sites/311/2015/12/Steroid-Method-Valid-Proc.pdf). The assays were read on an EL800 Universal Microplate Reader (Bio-Tek Instruments, Inc.). The Core laboratory performed all analyses without knowledge of treatment group. The intra-assay and inter-assay coefficient of variations for estradiol were 6.1% and 8.9%, respectively. The intra-assay and inter-assay coefficient of variations for progesterone were 3.6% and 9.0, respectively.

Measurement of Gene Expression

Gene expression was analyzed as described in Berger et al. (2016). RNA from snap frozen ovaries was extracted using a miRNeasy Micro Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's protocol. The samples were treated with DNase (Qiagen, Inc., Valencia, CA) during the process. RNA (100 ng) was reversed transcribed to cDNA and subjected to quantitative real time PCR (qPCR) using the CFX96 Real-Time PCR Detection System (Bio-Rad Inc.) and accompanying software (CFX Manager Software). The initial incubation temperature was 95 °C for 5 min. This was followed by 36 cycles at 95 °C for 10 s, at 60 °C for 10 s, and at 72 °C for 10 s. Melting from 65 °C to 95 °C was followed by final extension at 72 °C for 2 min. Standard curves, melting temperature graphs, and threshold cycle (Ct) values were generated for each run. All samples were run in duplicate. The expression data from each sample were normalized to the corresponding values of beta-actin (*Actb*).

Statistical Analysis

Data analysis was conducted using SPSS statistical software (SPSS, Inc., Chicago, IL). In the case of data collected from cycling females (PND 90, 6 months, and 1 year), only the data collected from females in estrus were used in hormone analysis. Therefore, the n is less than 10 for some endpoints. Data were expressed as means and standard error of the means (SEM). Multiple comparisons between normally distributed experimental groups were made using one-way analysis of variance (ANOVA). In cases when the ANOVA was significant at $p \leq 0.05$, Dunnett's 2-sided t tests were used to determine which treatment groups were significantly different from the control. Non-normally distributed data were analyzed using a Kruskall-Wallis test. BPA and EE_2 were compared to the vehicle in separate analyses. Statistical significance was assigned at $p \leq 0.05$.

References:

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