Belcher 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 manuscript: Gear R, Kendziorski JA, Belcher SM. Effects of Bisphenol A on Incidence and Severity of Cardiac Lesions in the NCTR-Sprague-Dawley Rat: A CLARITY-BPA Study. Toxicol Lett. 2017 Jun 5;275:123-135.

Tissue Collection, Preparation, and Staining

At each of the three time points analyzed (PND 21, PND 90, or 6 months), animals were weighed, sacrificed, and hearts were harvested with heart weights recorded at NCTR. Each tissue specimen and all corresponding experimental endpoints (*e.g.*, body and heart weight measurements) were assigned a coded identification and all tissue preparation and subsequent histopathologic analysis and scoring was done blinded to exposure group, exposure duration, or sex. Hearts were fixed for 24 h in 10% neutral buffered formalin (NBF), post-fixed in fresh 10% NBF for an additional 24 h, and then transferred to 70% ethanol for shipping. Hearts were shipped to the Belcher laboratory with these coded identification numbers to ensure that the investigators were completely blinded to all experimental variables except age. Upon arrival, specimens were washed in 70% ethanol, prepared by automated tissue processing for 40–45 min each in seven changes of graded alcohols followed by embedding with three changes in paraffin at 58 °C with applied vacuum (Tissue-Tek VIP 3000; Sakura Torrence, CA). Hearts were then cut into concentric 1 mm transverse sections, and embedded into paraffin blocks (Histocenter 3; Thermo-Shandon Kalamazoo, MI). Serial 5 µm microtome sections were cut from these blocks at 4 °C, placed on positively charged slides for staining, and analyzed as described previously (Belcher et al., 2015; Patisaul et al., 2013). Heart sections were stained with hematoxylin and eosin (H & E; Richard Allan, Kalamazoo, MI) using a standard protocol to examine tissue structure, morphology and pathology. Left ventricular (LV) free wall area was measured and the average LV free wall thickness was calculated from a single section at the level of the papillary muscle for each study animal. Serial transverse sections at the level of the papillary muscle were also stained with Picrosirius Red (Polysciences; Warrington, PA) to visualize total collagen (red) with bright field illumination (Junqueira et al., 1979, 1978; Dayan et al., 1989; Kendziorski and Belcher, 2015). For picrosirius red staining, tissue sections were deparaffinized, rehydrated, and stained for 8 min with Weigert's hematoxylin (American MasterTech; Lodi, CA). Stained sections were rinsed with tap water for 5 min, incubated for 2 min in 0.2% phosphomolybdic acid hydrate, and rinsed in deionized water for 30 s. Slides were then stained for 1 h in picrosirius red F3 B solution (1.3% 2,4,6-trinitrophenol, 0.4% Direct Red 80), transferred to 0.1 N hydrochloric acid solution for 2 min, washed in 70% ethanol for 45 s, dehydrated, and then coverslipped. Stained sections were examined on Nikon Eclipse 55i and 80i microscopes equipped with DS-Fi1 CCD cameras controlled by Digital Sight Software (Nikon; Melville, NY). Digital images of each section were collected using a 1 x and 10 x objectives, with additional higher magnification images collected using 20 x and 40 x objectives. Acquired images of picrosirius red sections were captured in RGB file format and then converted to HSI file format using Image Pro v4.5 (Media Cybernetics Silver Springs, MD). Images were confirmed as not

containing saturated pixels, and threshholded to background staining intensity. The total LV area and total LV collagen staining were calculated with collagen staining reported as a percent of LV area. Visual inspection of each slide was performed to qualitatively confirm the accuracy of the computed levels of staining. From the H & E stained sections, 10× digital images were acquired to examine gross and microscopic tissue structure and to measure LV free wall thickness. The average free LV wall thickness, LV diameter, and total LV area were measured as described previously with Image-Pro v4.5 (Belcher et al., 2015; Patisaul et al., 2013). For wall thickness measurements, five evenly spaced digital lines spanning the width of the left ventricular free wall were superimposed onto the digital image from a single stained section at the level of the papillary muscle. The average LV wall thickness was calculated as the mean length of those five lines relative to a stage micrometer of known length. For morphometric data collection and analysis, all samples including controls were comprehensively masked and were analyzed by a single observer blind to exposure dose, exposure duration (stop vs. continuous dosing arm) and sex (Gibson-Corley et al., 2013). All digital results were confirmed by direct microscopic observation.

Evaluation of Cardiac Pathology

Pathology of PND 21, PND 90, and 6 month deidentified specimens was evaluated by examination of H & E stained sections at final magnifications of 100–200 x. Cardiomyopathy, late stage cardiomyopathy (focal fibrosis), diffuse degeneration, and inflammatory infiltration phenotypes were each scored according to the Standardized System of Nomenclature and Diagnostic Criteria (SSNDC) Guide (Ruben et al., 2000). No threshold for morphological changes was applied to the analysis and any lesion consistent with each pathology was scored as positive. Cardiomyopathy and LV pathology were assessed using a standardized four point severity scale employed by Jokinen et al. (Jokinen et al., 2005, 2011) with 1= <10%, 2 =11–40%, 3 = 41–80%, 4= >81% of LV area involvement. Specimens containing visible hemosiderin were also noted. In consultation with a board-certified veterinary pathologist (Diplomat of the American College of Veterinary Pathologists), all pathology was assessed by the same investigator. An independent blinded pathology review was performed by a second investigator with any differences in lesion grading reviewed and resolved by consensus of the research team.

Statistical Analysis

Detailed review and analysis of all the research plans included in the CLARITY-BPA hypothesis driven studies followed NIEHS and NTP recommendation for evaluation of 10 animals per sex per group. Our previous analysis had found that n =10 was a sufficient sample size with enough statistical power to detect BPA exposure induced changes in the CD-1 mouse heart for endpoints assessed here (Belcher et al., 2015). Confound from litter effects were avoided by limiting analysis at each time point to one animal of a given sex from each litter, with each sex being considered separately. The statistical unit used was the litter for all analyses. A minimal level of statistical significance for differences in values among or between groups was considered $p < 0.05$. All statistical analyses for differences in values compared to control were made independently for BPA and EE_2 exposures and followed guidelines for low dose endocrine disrupting chemicals (Haseman et al., 2001). Percentage data were arcsine transformed (arcsine of the square root of the value) prior to statistical analysis. Data analysis were performed using Dunnett's multiple comparison tests, one-way or two-way analysis of variance as

indicated, and for pathology severity scores, a rank order ANOVA Kruskal-Wallis H test with Dunn's multiple comparisons tests were used. All data were analyzed using Excel (Microsoft; Redmond, WA) and GraphPad Prism® v6 software (GraphPad; La Jolla, CA).

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