

Greenberg Laboratory – Material and Methods

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). Studies were performed on rat studies for 12 month continuous and stop dosage studies. During analysis of samples, researchers were blinded to sample history. Pancreatic histological studies were performed only on female samples. While for other studies by Dr. Greenberg and his laboratory studies on both male and female samples were performed.

Insulin-Glucose Determinations. Serum insulin levels were assayed for insulin using the Rat/Mouse Insulin ELISA kit (Millipore Corporation, Cat # EZRMI-13K). The raw measurements were generated using EPOCH Plate Reader (GEN5 software), read at 450nm, corrected from 590nm. The serum was assayed for glucose using the Beckman Coulter AU400 enzymatic assay system. The measured values of Insulin, originally in ng/mL, were converted to $\mu\text{U/mL}$. The HOMA-IR index was calculated using the following equation: $(\text{Glucose} \times \text{Insulin})/405$ [1].

Liver Histology:

Liver samples were fixed in 10% NBF for a day and then transferred to PBS. They were kept cold for up to a year before further processing. 301 50 mL conical tubes each containing many sections of liver in cold PBS were received by our lab. One randomly-selected section of liver per tube was trimmed and cossetted. Tissues were routinely processed, embedded in paraffin, sectioned at 5 microns, and stained with H&E. Slides were scored by a board certified veterinary pathologist (me) who was masked to any information regarding treatment groups, sex, age, etc. The scoring system used for steatosis was that of Y. Wang et al, Increased apoptosis in high-fat diet-induced nonalcoholic steatohepatitis in rats is associated with c-Jun NH2-terminal kinase activation and elevated proapoptotic Bax,[2]. Briefly, "steatosis was graded 0-4 based on the average percent of fat-accumulated hepatocytes per field based on the average percent of fat-accumulated hepatocytes per field at ~ 200 magnification under H&E staining (grading: 0 = <5%, 1 = 5-25%, 2 = 26-50%, 3 = 51-75%, 4 = >75%). Please note that using this scoring system, a grade of "0" does not necessarily indicate a complete lack of fatty droplets, as samples could have up to 5 percent of hepatocytes containing fat droplets and still receive a grade of "0." Samples were scored for macro and micro steatosis.

RT-PCR of Liver Gene Expression:

RNA was extracted from frozen livers samples using RNAeasy Mini Kit (250), Qiagen, Cat #74106. RNA was quantified and checked for purity using the Nanodrop spectrophotometer (Nanodrop 1000, Wilmington, DE). cDNA was generated from 1 mg of RNA and real-time quantitative PCR was performed using SYBR Green (Applied Biosystems 7300, Carlsbad, CA). Fold changes were calculated using as 2eDDCT, with cyclophilin B used as the endogenous control [3].

Pancreatic Histological studies:

Female pancreas was isolated and weighed and formalin fixed. Pancreatic tissue from each animal was then embedded in paraffin, and multiple 5 micrometer consecutive sections were

mounted on microscope slides. **Immunohistochemistry:** The unstained slides were then stained for either insulin or glucagon to determine the presence of beta cells and alpha cells respectively, and a separate image for each stain was captured [3]. Serial longitudinal pancreatic sections (5 µm) were deparaffinized in Histo-Clear (National Diagnostics Inc., Manville, N.J.) and rehydrated ethanol (100% and 95%) followed by deionized water. Heat induced epitope retrieval in 10mM sodium citrate buffer, pH 6.0, was used to unmask antigens. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide for 10 minutes followed by incubation in a 5% normal goat serum (Sigma-Aldrich) diluted in 1X TBS containing 0.1% Tween-20, pH 7.6 (1X TBST) for one hour at room temperature to avoid non-specific binding of primary antisera. Following blocking, tissue was incubated in primary antisera—glucagon (1:100; Cell Signaling, Danvers, MA, in 0.5% NGS diluted in 1X TBST) and insulin (1:400; Cell Signaling, Danvers, MA, diluted in SignalStain® Antibody Diluent #8112)—overnight at 4°C. After several 1X TBST washes tissue was incubated in biotinylated goat anti rabbit secondary (1:200; Vector Labs, Burlingame, CA) for 30 minutes followed by additional 1X TBST washes and incubation in avidin-biotin-peroxidase complex according to manufacturer's instructions (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA, PK-6100) for 30 minutes. Slides were developed using VECTOR NovaRED Peroxidase (HRP) Substrate Kit (Vector, Labs SK4800), rinsed several times with deionized water, and cover slipped using Fluoromount Aqueous Mounting Media (Sigma-Aldrich). **Data Analysis:** A CRi Panoramic Scan Whole Slide Scanner (Perkin Elmer, Waltham, MA) was used to capture images. The proportion of insulin stained beta cells and glucagon stained alpha cells relative to total pancreatic area were quantified using the freehand polygonal tracing feature in Case Viewer (3DHistech Ltd, Budapest, Hungary). The ratio of alpha (or beta) cells was calculated by dividing its area by the total pancreatic area. Mass of alpha (or beta) cells was obtained by multiplying the ratio of alpha (or beta) cell by pancreas weight.

Statistical Data Analysis: Rats were grouped by sex (Male, Female), Treatment Type (EE2, BPA), Dose (vehicle control in addition to dosage ranging between 0.05 and 25000 µg/kg body weight/day) and Experimental Arm (Continuous Treatment, Stop Dose). All groups were pre-processed by removing outliers (as defined by being more than 2 standard deviations away from the mean). Means and standard errors of the mean are reported. All comparisons were carried out in R, using the Welch two-sample t-test with unequal variances. Bonferroni correction for multiple testing was applied.

1. Matthews, D.R., et al., *Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man*. Diabetologia, 1985. **28**(7): p. 412-9.
2. Wang, Y., et al., *Increased apoptosis in high-fat diet-induced nonalcoholic steatohepatitis in rats is associated with c-Jun NH2-terminal kinase activation and elevated proapoptotic Bax*. J Nutr, 2008. **138**(10): p. 1866-71.
3. Bowman, T.A., et al., *Acyl CoA synthetase 5 (ACSL5) ablation in mice increases energy expenditure and insulin sensitivity and delays fat absorption*. Mol Metab, 2016. **5**(3): p. 210-20.