

Science Supplemental Online Material

**Epigenetic Transgenerational Actions of Endocrine Disruptors and Male Fertility**

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**MATERIALS & METHODS**

*A. In Vivo Procedures*

Gestating outbred Sprague-Dawley and inbred Fisher mother rats from timed pregnant colonies housed at the Washington State University Vivarium were given intraperitoneal injections of vinclozolin (100 mg/kg per day) and methoxychlor (200 mg/kg/day) from embryonic day 8-15 (E8-E15) of gestation ( $F_0$  generation) as previously described (SI). Sperm positive vaginal smear date being embryonic day 0. Gestating control mothers received vehicle alone (i.e. sesame oil or DMSO). At least 6 lines (individual  $F_0$  injected females) were generated for controls and treated groups for these analyses. Male rats from control and treated groups were collected at P60-P180 for analyses.  $F_1$  treated males were bred to  $F_1$  treated females to generate the  $F_2$  treated generation and  $F_2$  treated males were bred to  $F_2$  treated females to generate the  $F_3$  generation and the  $F_3$  were bred in the same manner to generate the  $F_4$  generation. Rats for the control groups were bred in the same manner for all the generations. No inbreeding or sibling crosses were generated. The outcross group (VOC) was generated by breeding the  $F_2$  treated males with wild-type females (total of 6 litters) and reverse outcross group (RVOC) was generated by breeding  $F_2$  treated females with wild-type males (total of 3 litters). Control ( $n = 4$ ) and treated ( $n = 4$ ) male offspring from the  $F_1$  generation from Fisher strain rats were collected at P6 for DNA methylation analysis, see below. All procedures have been approved by the Washington State University Animal Use and Care Committee. The numbers of animals used for replicates in the experiments (i.e.,  $n$  value) for vinclozolin treatment are as follows:  $F_1$  (control 11, treated 12);  $F_2$  (control 19, treated 30);  $F_3$  (control 19, treated 26);  $F_4$  (control 15, treated 21); outcrosses (VOC 18, RVOC 6, wild-type controls 8). The methoxychlor treatment animal number was for  $F_1$  (control 6, treated 9) and  $F_2$  (control 16, treated 22).

### *B. Sperm Motility and Concentration Analysis*

Animals were sacrificed and cauda epididymal sperm motility was determined using cauda epididymal sperm. Briefly, the epididymis was dissected free of connective tissue and a small cut made to the cauda. The tissue was placed in 5 ml of culture medium containing 0.1% BSA for 10 min at 37°C. An aliquot was placed on a pre-warmed slide and gently cover-slipped. The specimen was immediately examined using phase contrast microscopy with 100x magnification. All the motile sperm (i.e rapid progressive, slow progressive, and non-progressive) were counted according to *WHO category (S2)*. Percent ratio of the motile sperm to the total number of sperm including immotile sperm was calculated. Epididymal sperm count was determined using the same epididymis sample according to a previously described method with some modifications (*S3*).

### *C. Histology*

Tissues were fixed in Bouin's (Sigma, St. Louis, MO), embedded in paraffin, sectioned and then sections stained with hematoxylin and eosin according to standard procedures. The Center for Reproductive Biology, Histology Core Laboratory assisted with these procedures. The animal numbers are  $n = 50$  for vinclozolin treatment and  $n = 42$  for controls.

### *D. Detection of Cell Apoptosis*

To detect apoptotic cells in testis sections, the Fluorescein In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN) was utilized (*S1*). This system measures the fragmented DNA from apoptotic cells by enzymatically incorporating fluorescein-12-dUTP at the 3'-OH DNA ends using the enzyme terminal deoxynucleotidyl transferase which forms a polymeric tail using the principle of the TUNEL assay. Fluorescent apoptotic cells were imaged on a confocal microscope and number of apoptotic cells per testis cross section determined. A minimum of  $n = 8$  for vinclozolin and  $n = 6$  for controls for each generation was used. All cross sections used for TUNEL analysis had normal testis morphology.

### *E. DNA Methylation Assays*

The methylation status of DNA isolated from control and endocrine disruptor treated P6 testis or epididymal sperm was determined using a combination of methylation sensitive restriction enzymes and a PCR approach previously described (*S4, S5*). Briefly, isolated

genomic DNA was incubated and digested with Rsa I with either methylation-sensitive (Hpa II) or insensitive (Msp I) restriction enzymes, followed by PCR with 10 primer sets designed to amplify methylation sites. PCR products were electrophoretically separated and visualized by SYBR green staining (Molecular Probes, Eugene, OR) and effects of endocrine disruptors determined by the presence or absence of specific bands. The PCR products of interest were isolated, cloned and sequenced to determine chromosomal location using BLAST Genbank analysis (S6, S7). P6 testis analysis was repeated using 4 animals from different litters.

#### *F. Bisulfite Sequencing*

Genomic DNA was isolated from F<sub>1</sub>-F<sub>4</sub> sperm samples using the DNeasy Tissue Kit (Qiagen, Valencia, CA) and digested with Rsa I. Genomic DNA was then treated with sodium bisulfite following methods previously established (S8). Briefly, 5 µg of digested DNA was denatured in NaOH then treated with 4.0 M sodium metabisulfite pH 5.0 for 16 hours at 55°C. Following desalting, DNA was desulfonated with NaOH, neutralized and precipitated with ammonium acetate and ethanol. DNA samples were resuspended in 30 µL of 1mM Tris buffer, pH 8.0. Sequence specific primers were generated to amplify the region of interest. PCR products were cloned into pGEMT Easy vector (Promega Corporation) and sequenced. Approximately 35 clones from each PCR product were sequenced to determine the methylation state of the CpG sites. Confirming altered methylation states involved comparisons of the sequences following the bisulfite treatment. The primers for the lysophospholipase PCR bisulfite analysis were 5'GGT ATA TAT AGA GGA AGG TAG GTA GG3' and 5'TAA AAA CCT CCA AAA AAC AAA CAC T3'.

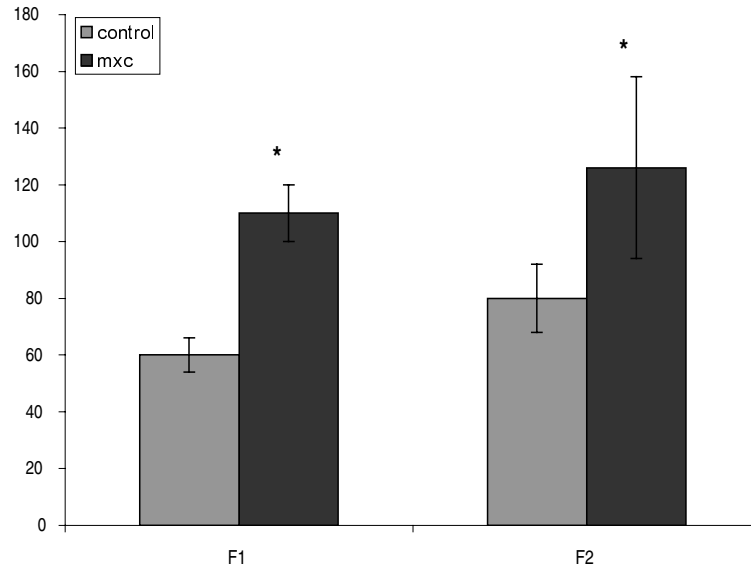
#### *G. Statistical Analysis*

The data from apoptotic cell numbers, sperm motility and sperm count were analyzed using a SAS program. The values were expressed as the mean ± SEM. Statistical analysis was performed and the difference between the means of treatments and respective controls was determined using two-way Analysis of Variance (ANOVA). Analysis performed with JMP v3.1.6, SAS Institute Inc, Cary, NC. In vivo experiments were repeated with 6-30 individuals for each data point. A statistically significant difference was confirmed at  $P < 0.05$ .

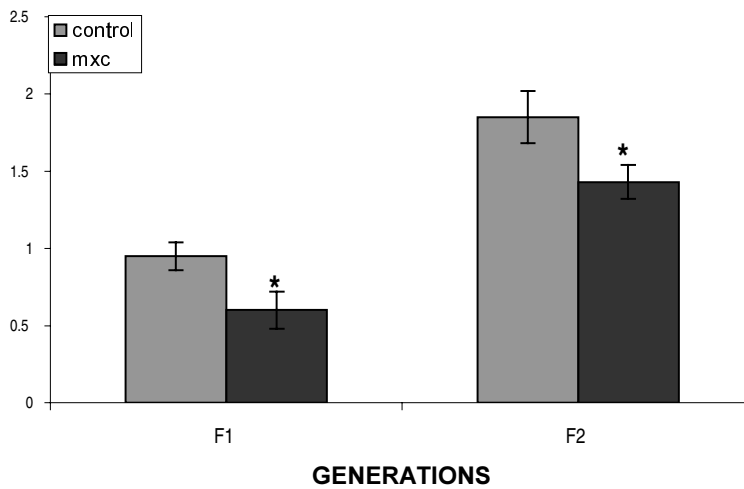
### References and Notes

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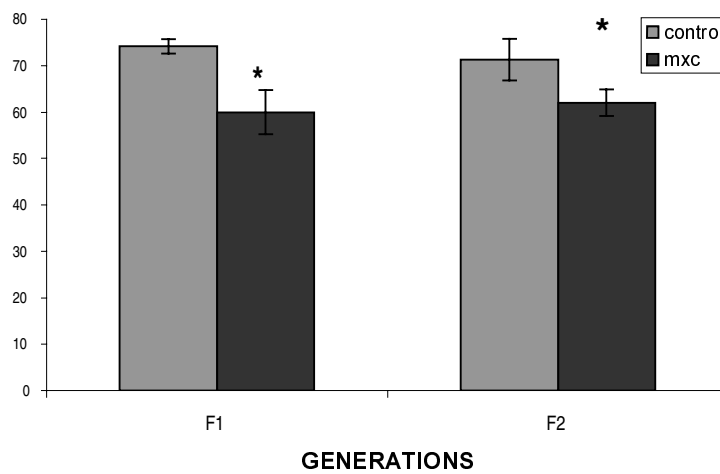
**A.**



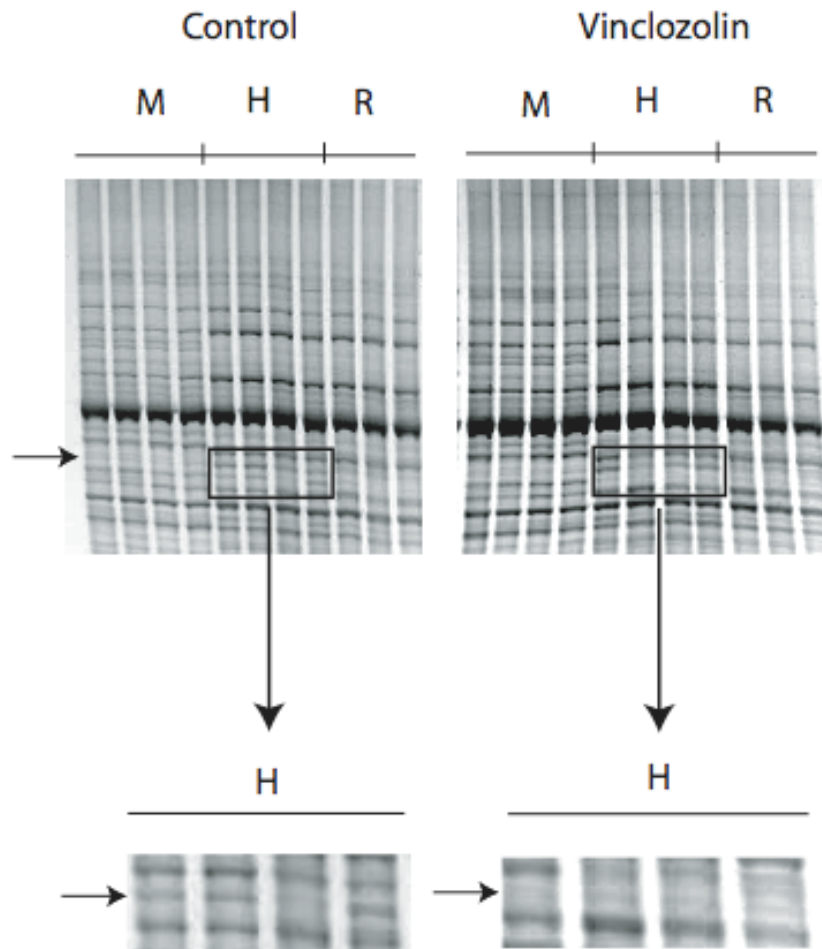
**B.**



**C.**



**Fig. S1.** Transgenerational phenotype after methoxychlor treatment. **(A)** Spermatogenic cell apoptosis. **(B)** epididymal sperm counts. **(C)** Epididymal sperm motility in P60-P120 control and treated Fisher rats in F<sub>1</sub> and F<sub>2</sub> generations. Statistically significantly differences between control and treated animals within a generation are indicated by (\*) for  $P < 0.05$ . The number of animals for each bar ranged between 6 and 12.



**Supplemental Figure S2.** DNA methylation analysis from control and treated P6 testis. Representative gel image of the PCR based methylation sensitive H-HpaII+RsaI (H) and insensitive MspI +RsaI (M), and RsaI (R) as control. restriction enzyme digest analysis with representative band (arrow) (200bp) affected by in vivo exposure to vinclozolin shown. Postnatal day 6 (P6) testis from control (control) and vinclozolin (treated) treatment presented. Each lane represents the analysis of a different animal P6 testis (n=4).

GGCACATACAGAGGAAGGTAGGCAGG**CG**AGGGACCCAGCAGGTTCTTGACA  
GCTTCTCCC**CG**TGCCCTCCCCACTCCCAGGCTGGCATCAAGGG**CG**GACA  
G**CG**AAGGCCTG**CG**GG**CG**TGGTGGCAGGCTGGGGCTGACCTAGGGACAGC  
G**CG**ACTATGATGGACACTGTGCCCTGCAAGTAGTGAGTGCCTGCCTCCTGGA  
GGTCCTCA

Supplemental Figure S3. DNA sequence from the lysophospholipase (LPL) gene with altered methylation pattern. The LPL genomic sequence (accession #NW\_047762.1) from base pairs 32270685 to 32270889. Methylated cytosine residues (Bold CG) present in both control and vinclozolin generation animal sperm are marked with a closed circle (•) and those altered in vinclozolin F1-F4 generation sperm samples are marked with an open circle (°).