

Supplemental Material

Supplemental Material includes Supplemental Methods, Supplemental Figure S1, and Supplemental Tables S1 and S2.

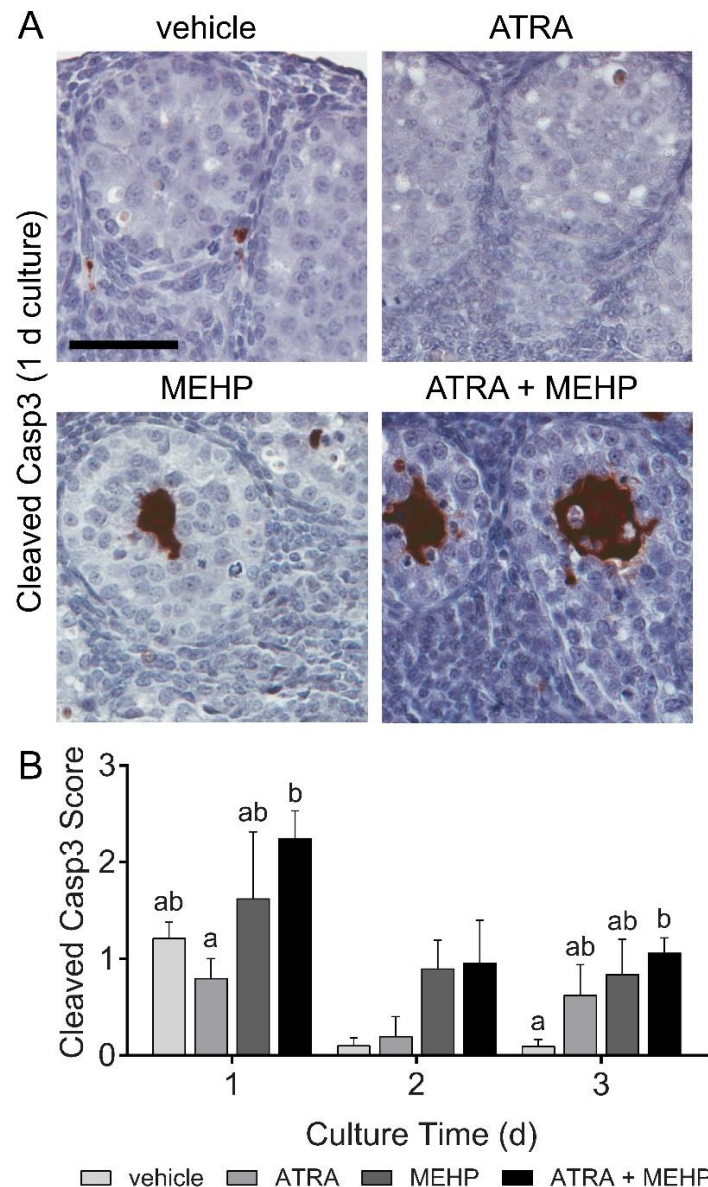
Spade DJ, Hall SJ, Wortzel JD, Reyes G, Boekelheide K. All-trans retinoic acid disrupts development in *ex vivo* cultured fetal rat testes. II: Modulation of mono-(2-ethylhexyl) phthalate toxicity.

Supplemental Methods

To determine the mechanism and rate of germ cell death in ATRA and MEHP-treated fetal rat testis cultures, a three-day time-course experiment was performed, in which testes were cultured on 600 μ L of cell culture media consisting of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 supplemented with 80 μ g/mL gentamicin (Gibco/ThermoFisher Scientific). One or both testes from each fetus was placed on a Millicell membrane and cultured with either 10^{-4} M MEHP (Takeda), 10^{-6} M ATRA (Sigma Aldrich), a combination of 10^{-4} M MEHP and 10^{-6} M ATRA, or 1:4000 dimethyl sulfoxide (DMSO) vehicle. Testes were collected after 1, 2, or 3 d, fixed in Bouin's solution for 2 h, then transferred to 70% ethanol and stored at 4°C until processing for histology.

Samples were processed and embedded in paraffin for histology as described in the Materials and Methods and trimmed to their approximate center prior to cutting. Immunohistochemical staining for cleaved caspase-3 was performed on slides from days 1-3 of the time-course testis cultures. Antigen retrieval was performed by heating slides in 10 mM citrate buffer, pH 6.0, in a vegetable steamer for 20 min, followed by 20 min at room temperature. Staining was performed using the SignalStain Apoptosis (Cleaved Caspase-3) IHC Detection Kit (Cell Signaling Technology, Danvers, MA, USA), per manufacturer's instructions, and sections were counterstained with hematoxylin. The provided isotype control was used as negative control for each sample. Slides were scanned at 40x magnification using an Aperio ScanScope CS (Aperio Digital Pathology/Leica Biosystems, Buffalo Grove, IL). Digital slide images were used for scoring of d 1-3 cleaved caspase-3 IHC, using the scoring scale reported in Supplemental Table S1.

Testes from fetuses in the same litter were assigned to different treatment groups. If more than 4 male fetuses were obtained from the same litter, littermate testes assigned to the same treatment group (technical replicates) were averaged and entered in the dataset as a single value. Cleaved caspase-3 scores were analyzed in Prism software (GraphPad) by two-way ANOVA, followed by Tukey's multiple comparison test.



Supplemental Figure S1. Time course of testicular germ cell apoptosis in culture.

A. Representative cleaved Casp3 from cultures exposed to vehicle, 10^{-6} M ATRA, 10^{-4} M MEHP, and 10^{-6} M ATRA + 10^{-4} M MEHP for 1 d. Following 1 d culture, all treatment groups showed at least infrequent germ cell labeling. Co-exposed samples had frequent large clusters of Casp3-positive cells in the center of seminiferous cords. Scale bar = 50 μ m. **B.** Cleaved Casp3 scores indicate that the rate of apoptosis in vehicle-treated cultures decreased throughout the 3 d culture period, while Casp3 remained elevated in treated samples. ATRA/MEHP co-exposure

resulted in a significant increase in Casp3 score on d 3, while the co-exposure score was significantly higher than ATRA alone on d 1. Within each day, bars not connected by the same letter are significantly different by two-way ANOVA followed by Tukey's multiple comparison test. No significant differences were detected on d 2. Time ($p=0.0003$) and treatment ($p=0.0033$), but not the interaction term, were significant in the overall ANOVA.

Supplemental Table S1. Cleaved caspase-3 IHC scoring scale

Score	Scoring Criteria
0	no or little signal
1	infrequent single germ cells stained
2	frequent single germ cells or small germ cell clusters stained
3	large germ cell clusters stained

Supplemental Table S2. Primers used for real-time PCR

Target	UniGene ID	RefSeq Accession	Band Size (bp)	Reference Position
B2m	Rn.1868	NM_012512.2	128	132
Ddx4	Rn.198577	NM_001077647.1	155	2124
Fstl3	Rn.48835	NM_053629.3	156	522
Ldha	Rn.107896	NM_017025.1	67	852
Nr0b1	Rn.10596	NM_053317.1	161	1333
Pou5f1	Rn.161748	NM_001009178.2	137	737
Rbp1	Rn.902	NM_012733.4	158	329
Star	Rn.11399	NM_031558.3	90	839
Wnt4	Rn.34782	NM_053402.2	176	791