Co-Exposure to Mono(2-ethylhexyl) Phthalate and Elevated Temperature Inhibits Mouse Antral Follicle Growth and Steroidogenesis *In Vitro*

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Di(2-ethylhexyl) phthalate (DEHP) is a plasticizer found in common consumer goods, such as household items, food packaging, and medical devices. DEHP is rapidly metabolized upon ingestion to mono(2-ethylhexyl) phthalate (MEHP), which is bioactive and known to exert endocrine-disrupting toxicities. Due to the extensive use of DEHP in consumer goods, humans are ubiquitously exposed and are vulnerable to endocrine and reproductive dysfunction by MEHP. Studies have shown that MEHP exposure disrupts the essential ovarian processes of antral follicle growth and steroidogenesis. Simultaneously, humans are ubiquitously exposed to non-chemical stressors, such as rising global temperatures, and studies have shown that exposure to elevated temperatures can impair female reproductive health. Given the progression of climate change and the unavoidable exposure to both increasing heat and DEHP, this study used an *in vitro* follicle culture system to test the hypothesis that exposure to high temperature (HT) will exacerbate the negative effects of MEHP exposure on antral follicle growth and steroidogenesis. Antral follicles were isolated from 4-5-week-old CD-1 mice and cultured for 96 hours in media containing follicle-stimulating hormone at a control temperature (CT; 37°C) or high temperature (HT; 42°C; 8hr/day). Follicles were treated with vehicle control (dimethyl sulfoxide; DMSO) or MEHP (0.2, 2, and 20µg/ml) in both temperature groups for the 96-hour culture period. Separate incubators were used for the CT and HT treatment groups, in which all HT treatments occurred for 8 hours per 24-hour period to model daytime, occupational exposure to high temperatures. Follicular growth was measured every 24 hours via inverted microscopy. Follicles and media were collected at 96 hours for steroidogenic gene expression analysis and sex steroid hormone measurements, respectively (n=3-7 with 5-10 follicles/group/replicate, $p \leq 0.05$). Antral follicle growth was decreased by HT+MEHP 2µg/ml and HT+MEHP 20µg/ml (72hr, 96hr) treatment compared to the CT+DMSO control group. Further, antral follicle growth was decreased by HT+DMSO (72hr, 96hr) and HT+MEHP 20µg/ml (96hr) treatment compared to their CT treatment equivalents. Estradiol levels were decreased by CT+MEHP 0.2µg/ml treatment and in HT groups (DMSO, MEHP 0.2, 2, and 20µg/ml) compared to CT+DMSO and in HT groups (DMSO, MEHP 0.2, 20µg/ml) compared to their CT treatment equivalents. Testosterone levels were increased by CT+MEHP 20µg/ml treatment compared to CT+DMSO and decreased by HT+MEHP 20µg/ml treatment compared to its CT treatment equivalent. The mRNA levels of *Hsd3b1* were decreased in CT groups (MEHP 0.2, 2, and 20µg/ml) and by HT+MEHP 20µg/ml treatment compared to CT+DMSO. The mRNA levels of *Cyp17a1* and *Hsd17b1* were decreased by HT+MEHP 20µg/ml treatment compared to CT+DMSO and by HT+DMSO and HT+MEHP 0.2µg/ml treatment compared to their CT equivalents. The mRNA levels of *Cyp19a1* were decreased in CT groups (MEHP 2, 20µg/ml) and HT groups (DMSO, MEHP 0.2, 2, and 20µg/ml) compared to CT+DMSO and decreased in HT groups (DMSO, MEHP 0.2, 2, and 20µg/ml) compared to their CT treatment equivalents. These findings suggest that exposures to HT and MEHP inhibit antral follicle growth and disrupt steroidogenesis. The observed decreases in steroidogenic mRNA levels correlate to the decreases in estradiol levels, with HT exposure

exacerbating the MEHP 2µg/ml and 20µg/ml-mediated decreases in *Cyp19a1* levels. Thus, combined exposures to these ubiquitous chemical and non-chemical stressors may potentiate ovarian dysfunction. Supported by R01ES033767 and pilot funding from UL1TR001998 and P30ES026529.