Title: A Study on Human Developing Neurons and Male Bias: Effects of Different Levels of Testosterone and Endocrine Disrupting Chemicals.

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Background: Endocrine Disrupting Chemicals (EDCs) are exogenous substance that may impact normal synthesis, secretion, transport, binding, action or elimination of natural hormones responsible for the maintenance of homeostasis, reproduction, development and differentiations of developing fetal brains. We normally encounter myriad of chemicals in our daily lives (i.e. cosmetics, perfumes, freshener, moisturizers, fragrances, plastic product, sunscreen etc.) that can be harmful to the developing fetal brain neurons. These chemicals have potential of getting stored in adipose tissue and alter the hormonal and homeostatic system, and thus affect the metabolism, sexual development, growth, stress response, insulin production, gender behavior, reproduction and fetal development. Autism Spectrum Disorder (ASD) is developmental disabilities that can cause significant social, communication and behavioral challenges. The male-to-female ratio is nearly 5:1. Autism is generally believed to be a genetic disease and investigators have identified over 1,000 genes linked to ASD. We have proposed that natural testosterone along with EDCs (i.e., Benzyl Benzoate, Benzyl Salicylate, Diethyl phthalate, Eugenol, Musk Ketone, Octinoxate), and other androgenic and estrogenic present in our environment can interfere in normal neurodevelopment, especially in a developing fetus if exposed to EDCs or abnormally concentration of testosterone during the prenatal periods (especially during week 8-24 week of gestation).

Methods: In this study we exposed neuroblastoma cell lines (NBCs) of male and female origins (CRL 2267 and CRL 2266, respectively), representing human developing fetal brain neurons, to six most commonly encountered chemicals, found in regularly used household products to three different concentration of chemicals; (i.e., 62.5µg/ml (high), 31.25µg/ml (medium) and 15.625µg/ml (low) and to testosterone 50 nM (high), 30 nM (medium), 25 nM (low) for 48-72 h and evaluated at morphological, molecular and immunological levels to determine the impacts of EDCs on developing human fetal brain neurons. We carried out morphological analyses by determining the degree of central chromatolysis, neurite formation (i.e., neurite length and thinning, degeneration and syncytia formation) in exposed NBCs and compared to unexposed controls. We also analyzed the expressions levels of oxytocin (OXY) and Arginine-vasopressin receptors (AVPR) in controls *versus* EDC and testosterone exposed NBCs. Finally, we utilized gene expression analysis by qRT-PCR for *RORA*, *Aromatase* (*CYP19A*), oxytoxin and arginine-vasopressin, Estrogen *and Androgen receptors* expressions in cells exposed to EDCs and different concentrations of testosterone *versus* controls.

Results: The NBCs exposed to EDCs and testosterone at various concentrations exhibited significant neuromodifications. The morphologic analyses showed significant differences in axonal length and axonal degeneration in EDCs exposed NBCs from both genders. Furthermore, there was significant downregulation in oxytocin receptor expression in cell lines that were exposed to EDCs at all level of concentrations as compared to controls in both male and female cell lines.

The molecular analyses by qRT-PCR showed dysregulation of *RORA, Aromatase, Estrogen, Androgen receptor* expressions in both the neuronal cell lines exposed to 6-EDCs.

Among three different concentration, high concentration dysregulated those genes in male than female. Those genes were downregulated in higher percentage in male than female.

Conclusion: We concluded that exposures to EDCs as well as different levels of testosterone concentrations, induce significant neuromodifications as evaluated by neurohistologic, immunologic and at molecular levels, suggesting that these chemicals may play an important role in neurodevelopment of a fetal brain if exposed during early stages of gestation and may result in neurodevelopmental disorders including ASD. Also, testosterone might be one of the reasons for male bias prevalent in Autism.

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