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Research Article

Gender-Dependent Effects of Endocrine Disrupting Chemicals on Retinoic Acid-Related Orphan Receptor Alpha [*RORA*] and Aromatase Genes on Human Progenitor Neurons

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Abstract

Normally we encounter a myriad of chemicals in our daily lives. Endocrine disrupting chemicals [EDCs] are ubiquitous in our environment and upon bodily entry many be stored in adipose tissue and, in pregnant woman, can reach the developing fetal brain, disrupting normal fetal brain development. EDC-induced aberrant levels of sex hormones can alter sexual dimorphism [i.e. degree of feminization or masculinization; sex differences in brain and behavior] and may contribute to the differential susceptibility of males and females to autism. Fetal development is guarded by the placenta which expresses high levels of aromatase, an enzyme that converts testosterone [T] to estrogen [E], and it appears that this process prevents the transfer of maternal T to the vulnerable developing fetus. Many investigators have shown a clear association between exposure to high levels of T during the prenatal and

early postnatal periods, and development of autism spectrum disorder [ASD]. Retinoic acid-related orphan receptor-alpha [RORA] is a transcription factor that regulates aromatase. We analyzed the effects of six commonly encountered EDCs on human progenitor neurons from both genders and evaluated the differential expression of both aromatase and RORA by real-time PCR using an *in vitro* approach. We also evaluated the effects of the EDCs on neurite formation. Male and female human neuroblastoma cell lines were used to evaluate the effects of EDCs on neurite formation and the expression of RORA and CYP19A1. The morphologic analyses showed significant neuromodifications in neurite formation and a significant differential downregulation of RORA in male, but not in female neurons. Dysregulation in CYP19A1 was not gender-associated but was dependent on the dose of EDCs. Our findings confirm previous reports of differential downregulation of RORA in male neurons while expanding the gene-environment connection where estrogenic or androgenic EDCs can profoundly dysregulate RORA and aromatase gene expression, leading to a cascade of a highly integrated gene-network, associated with ASD.

Keywords
Aromatase; autism; androgenic; endocrine disrupting chemicals; estrogenic; human progenitor neurons; male bias; neuromodifications; neurite formation; retinoic acid-related orphan receptor alpha [rora]

1. Introduction

EDCs are xenobiotics which adversely affect the human fetal neurodevelopment [1-3]. The developing human fetus is highly vulnerable to disturbances in sex hormones and endocrine disruptors during the prenatal period, and especially during early developmental stages, where they can impart permanent damage into adulthood and across generations [4-6]. The molecular and cellular mechanisms that can affect human neurodevelopment, and expressions of *AROMATASE* [*CYP19A1*] and retinoic acid-related orphan receptor alpha [*RORA*] genes, on human progenitor neurons and the differential effects of EDCs has not been thoroughly explored [1, 7, 8]. Since testosterone [T] is regulated by the interaction of *RORA* and aromatase, these two genes have been leading candidates in the study of sex bias in ASD. *RORA* has been shown to regulate aromatase transcriptionally and aromatase converts T to estradiol [E]. Deficiency of *RORA* results in aromatase deficiency, causing increases in T [1, 4]. The role of T in autism spectrum disorder [ASD] has been extensively explored by Baron-Cohen's group [4, 9, 10], who have forwarded the *extreme male brain* [EMB] hypothesis [10]. Here we analyzed the effects of six EDCs on differential expressions of aromatase and *RORA* genes as well as the disturbance in neurite formations using an *in vitro* model [11, 12]. Our data clearly show a profound differential dysregulation of RORA expression in males *vs* females as well as significant changes in aromatase gene expression. In addition, we show significant disturbance in neurite formations from both gender cell lines when exposed to EDCs. To the best of our knowledge this is the first study that has explored dysregulation in *RORA,* and *AROMATASE* gene expressions exposed to EDCs. We hypothesize that exposure to certain EDCs may have direct impact in the development of ASD.

2. Methods and Materials

2.1 Materials

All the EDCs; Benzyl Benzoate [BB], Benzyl Salicylate [S], Diethyl Phthalate [DEP], Eugenol [EU], Musk Ketone [MK] and Octinoxate [ON] were purchased from Sigma [Saint Louis, MO, USA]. The cell culture supplies: Eagle's Minimum Essential Media [EMEM], Fetal Bovine Serum [FBS], Penicillin/Streptomycin solutions were purchased from Fisher Scientific [Hanover Park, IL, USA]. Neuroblastoma cells [CRL 2266 and CRL-2267] cell lines were purchased from ATCC [Manassas, VA, USA].

2.2 Methods

2.2.1 Cell Culture

CRL-2267, of male origin, and CRL-2266, of female origin, were cultured with Eagle's Minimum Essential media [EMEM] supplemented with 10% heat inactivated Fetal Bovine Serum [FBS] and 1% L-Glutamine-penicillin-streptomycin solution [complete media] [Sigma, St. Louis, MO] at 37 °C, 5% CO_2. The stock cell cultures were grown in 25 mL or 75 mL flasks [Thermo-Scientific, Nunc, Rochester, NY].

In order to determine the lowest non-cytotoxic concentrations [LNC] of each of the EDCs, cells were seeded in 96-well flat surface plates [Nunc] at $1x10^4$ cells per well. The cells were incubated for 6 hours at 37 °C, 5% CO_2, allowing them to attach on the surface and then were exposed to each of the EDC [11, 12]. The LNC were determined by subjecting each of the chemicals to 1:10 serial dilutions of each of the EDCs at 1 mg/mL in 96-well plates. Sub-cytotoxic concentrations were determined by Eosin-Y vital stain dye. The specific experimental methods have been previously described [11]. The LNC that showed no cytotoxicity was further diluted 1:10 which was used as the starting concentration for each of the EDCs and designated as High [H]. Further 1:2 and 1:4 dilutions were used and designated Medium [M] and Low [L], respectively. For example, if LNC was 310 mg/mL for an EDC, we used the 1:10 dilution of the sub-cytotoxic concentration i.e., 31.25 µg/mL as H, and 15.625 µg/mL and 7.812 µg/mL [designated medium and low, respectively]. The cells were cultured in 8-well chambers, each of the 2-chambers representing control [C] where no EDC was added and others for H, M and L, respectively for 3-5 days, for each of the six EDCs.

2.2.2 Hematoxylin and Eosin [H & E] Staining

For morphologic studies, each cell line was grown in 8-well chamber slides with ~$1x10^5$ cells in 100 mL of media for 5-days. For seeding the NB cell lines, the stock cell cultures, grown in the flasks, were gently washed once with 1x sterile phosphate buffered saline [PBS] [Fisher Scientific, Fair Lawn, NJ], followed by trypsinization, until single cell suspension and inactivation of trypsin with 1mL FBS. The cells were counted using a hemocytometer chamber and adjusted to $1x10^6$ cells/mL. The 8-well chamber slides were labeled, and 100 µL of cells added to each well together with 5 µL of each of the three dilutions of EDCs. After 5-days culture, the media was removed from the slides and the cells were fixed by adding 500 µL of 2% PFA to each well and allowed to set overnight at room temperature [12]. The caskets of the glass slides were removed by the device provided by the

manufacturer and the slides were washed gently three times using 1× PBS. These slides were stained with freshly prepared H&E [Leica Biosystems Richmond, Inc., Richmond, IL], washed in distilled water for a minute, mounted with mounting buffer containing 50% PBS and 50% glycerol, observed under 10X and 40X magnification, and analyzed for neurite formation: central chromatolysis, axonal length, thickness, thinning, and degeneration. In addition, syncytia formation, other morphologic and cytotoxic changes were recorded for comparison to controls. The experiments were repeated at least 6-times and the observations were recorded using a digital camera [Olympus BX51].

2.2.3 Qualitative Real-Time PCR [RT-qPCR]

RORA/CYP19A1-F were quantified using one-step RT-PCR. Viral RNA was extracted from culture pellets using GeneElute Mammalian Total RNA Miniprep kit [Sigma, St. Louis, MO] according to the manufacturer's protocol. Two sets of primer pairs were designed for each of the target. RORA-F 5'-CAATGCCACCTACTCCTGTCC-3'', RORA-R 5'-GCCAGGCATTTCTGCAGC-3' and CYP19A1-F, 5'-GACACATCATGCTGGACACC-3', CYP19A1-R 5'-CAAGTCCTTGACGGATCGTT-3'. Glyceraldehyde 3-phosphate dehydrogenase [GAPDH] was used as internal control to normalize the expression levels of *RORA* and *CYP19A1* [11]. The following primer pair was used to detect internal control GAPDH-F 5'-AGGTCGGTGTGAACGGATTTG-3', and GAPDH-R 5' GGGGTCGTTGATGGCAACA-3'. The reactions of RT-qPCR were carried out using an iScript One-step RT-PCR kit with SYBR Green [Bio-Rad, Hercules, CA] and quantification was performed using an ABI 7500 real-time cycler [Applied Biosystems, Foster City, CA]. The thermal cycling profile of this assay consisted of a 15 min cDNA synthesis step at 50 ºC, 5 min of iScript reverse transcriptase inactivation at 95ºC, followed by 40 cycles of PCR at 95 ºC for 10 sec and a step of a single fluorescence emission data collection at 55 °C for 30 sec. Each experiment was repeated three times. Results were analyzed using HID Real-Time PCR Analysis Software v1.2 [Thermo Scientific, Waltham, MA]. The relative degrees of gene expressions in the two cell lines were calculated using cycle threshold values [C_t]. The copy number calculations were carried by the standard curve utilizing GAPDH for internal control as described previously [13].

2.2.4 Statistical Analysis

All results are expressed as the mean ± standard deviation [SD]. The differences between EDC-exposure groups and the negative control were tested using one-way analysis of variance with specific mean comparisons. Differences were considered significant at a p value<0.05. The images from H&E staining were analyzed by counting the total number of cells and identifying the neurite formations as well as cellular death, such as central chromatolysis, axon elongation [length], axon degeneration, axonal thinning, and syncytia formation, comparing control *vs.* experimental variations [with added chemicals at different concentrations]. The data were entered into an Excel spreadsheet for statistical analysis by calculation of the degree of significance [P values]. Frequency data were analyzed with the chi-square statistical test or pair-student t-test. Statistical analyses were carried out by utilizing Stata for Windows; software version 11 The copy number of RORA and CYP19A1 were calculated by using a standard curve utilizing GAPDH as the internal control or house-keeping gene [13].

3. Results

3.1 Study of Neurite Formation

We analyzed several aspects of morphological changes in developing human neurons after they were exposed to various concentrations of EDCs. The morphologic changes included significant modulation in neurite formation such as axonal length, axonal elongation, thinning, degeneration, and central chromatolysis on neural cells, before and after the cells were exposed to different EDCs at three different concentrations and compared to controls.

All six chemicals induced significant changes in neurite formations. Only representative figures are shown in Figures 1 and supplemental Tables 1. Briefly, Figure 1A-B shows the morphologic patterns of the two progenitor neuronal cell lines we utilized: one of female and another of male origin. Figure 1 A-B shows how the cell lines morphology appears in control cell lines. We used these images as the baselines and the fundamental references to compare with the cell lines exposed to various concentrations of EDCs. Therefore, as displayed in Figure 1 C-H exhibits the representative images of female and male neuronal cell lines [CRL-2266 and CRL 2267, respectively] exposed to low, medium and high concentrations of DEP. As clearly apparent from the images that exposure to DEP induced profound neurite dysregulation, including axonal thinning, elongation, and increased chromatolysis. In many cases, we observed emergence of multiple axonal connections, suggesting increased neurite network formation. We observed similar neurite dysregulation in neural cell lines exposed to various concentrations of Ketone Musk [Figure1 I-N], Benzyl Benzoate [Figure1 O-T], Benzyl Salicylate [Figure 1 U-Z], Eugenol [Figure 1 a-f] and Octinoxate [Figure 1 g-l]. Of note, both, estrogenic EDCs: OT, EU, BB and BS and androgenic EDCs: MK and DEP, induced similar degrees of neurite dysregulation. As shown in STable 1 [supplementary data] despite profound morphological neuromodulations observed, differences [$p<0.05$] were not noted between either estrogenic or androgenic EDCs with respect to their neuromodulating effects. Similarly, we were unable to decipher differential changes between the male and female cell lines. We believe this was due to the many variations in neurite formations which were difficult to calculate using technology currently available. However, the degree of axonal degenerations in all the EDC-exposed neurons were significant [$p<0.05$ or below]. And axonal elongation was significant in neurons exposed to all estrogenic EDC and in one of the two androgenic EDC-i.e. DEP in male neurons. Therefore, in the case of DEP, female neurons exhibited significant neurite dysregulation, but the male exhibited much reduced axonal length changes. However, the exposure to KM in both genders imparted a significant degree of neurite deformation. Of note, the numbers of surviving neurons in 8-well chamber cell cultures, were remarkably lower when compared to controls after 3-5 days incubation, especially in high dose EDCs. This might account for variations in the degrees of significance noted in Table S1.

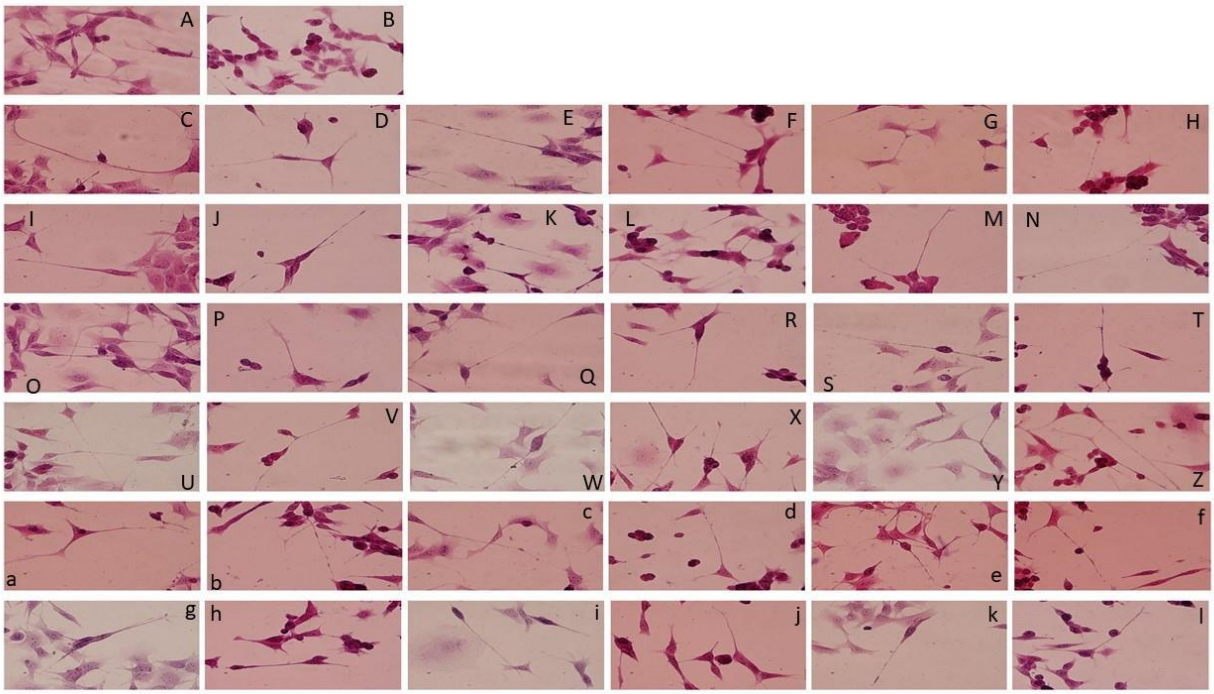
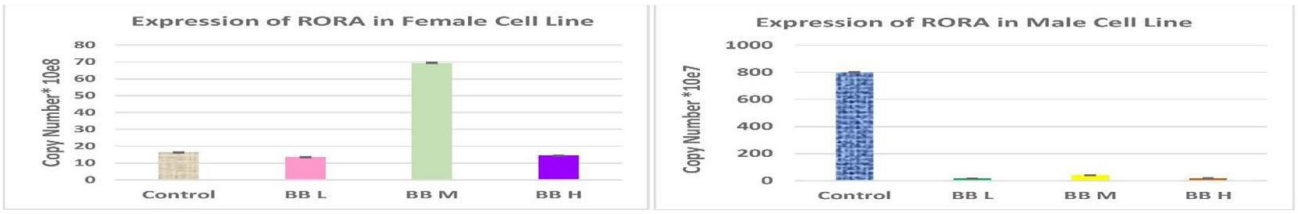


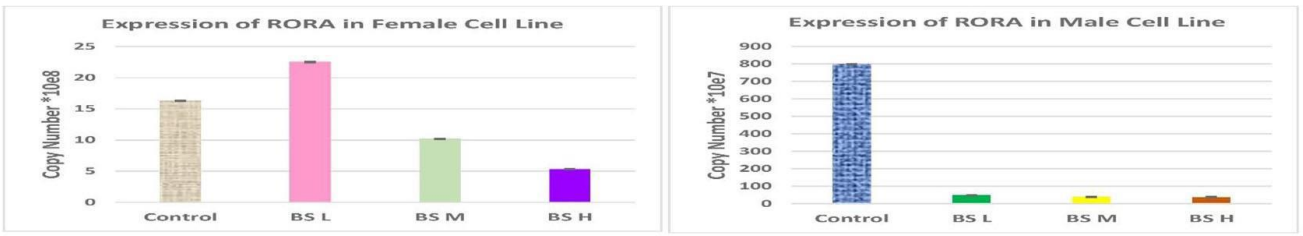
Figure 1 Effects of various EDCs on Neurite formation.

3.2 Differential Expression of RORA: A Gender Dependent Downregulation of RORA in Male Progenitor Neurons

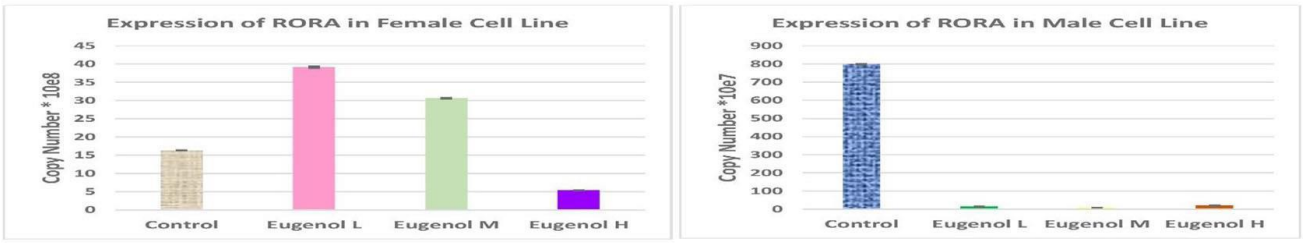
The qPCR analyses of *RORA* expression in progenitor neurons exposed to three different concentrations of EDCs resulted in significant downregulation of *RORA* in male progenitor neurons. As illustrated in Figure 2, both, estrogenic EDCs: OT, EU, BB and BS, and androgenic EDCs: KM and DEP induced highly significant downregulation of *RORA* in the male neurons when compared to controls [unexposed to any of the EDCs] [Figure 2]. The degree of expression of *RORA* in female neurons exposed to estrogenic EDCs varied significantly depending on the concentration employed. For example, in female neurons exposed to BB, *RORA* expression was significantly upregulated as in neurons exposed to the medium dose of BS. In female neurons exposed to BS and EU there were dose-depended on effects with higher doses exhibiting lower degrees of upregulation compared to controls. This may reflect the cytotoxicity observed in neurons after 3-5 days of culture, and at certain concentrations there was an upregulation of *RORA*. In case of two androgenic EDCs i.e., DEP and MK, we observed a downregulation of RORA, but at lower magnitude to that observed in male neurons [Figure 2].



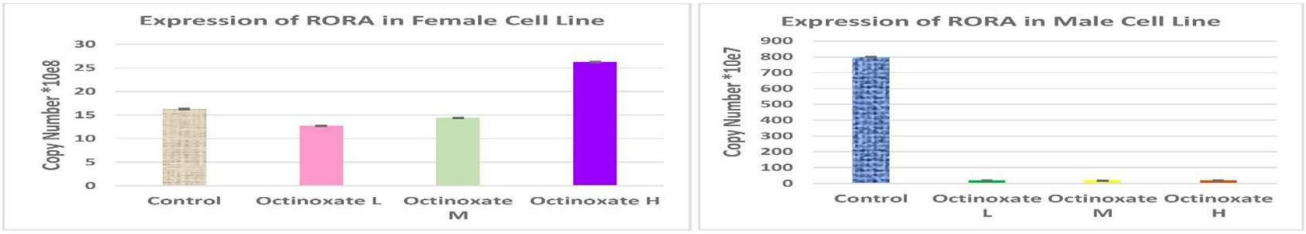
Expression of RORA on Benzyl Benzoate Treated Human Progenitor Neurons.



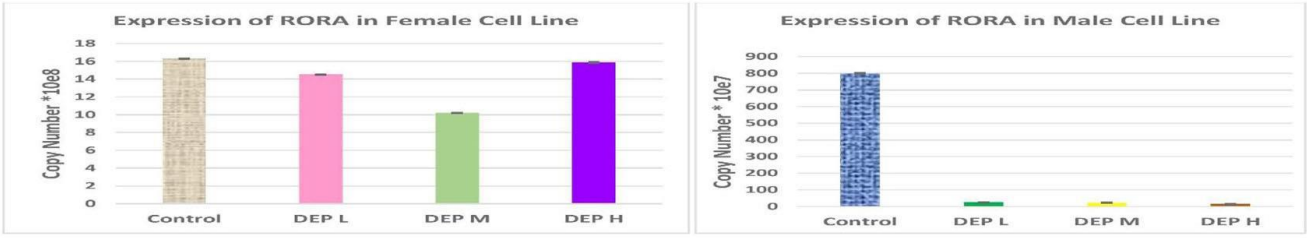
Expression of RORA on Benzyl Salicylate Treated Human Progenitor Neurons.



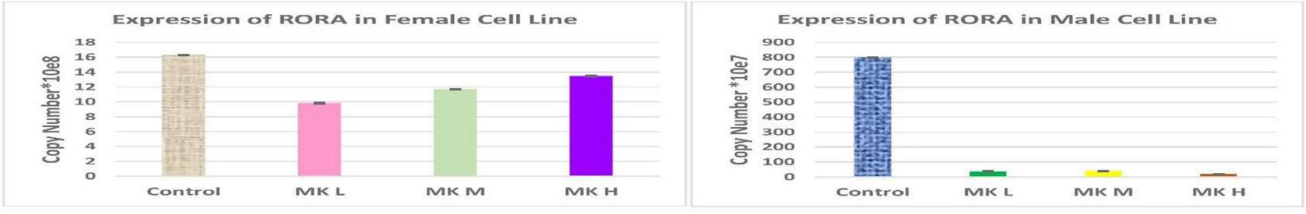
Expression of RORA on Eugenol Treated Human Progenitor Neurons.



Expression of RORA on Octinoxate Treated Human Progenitor Neurons.



Expression of RORA on DEP Treated Human Progenitor Neurons.



Expression of RORA on Musk Ketone Treated Human Progenitor Neurons.

Figure 2 Differential downregulation of *RORA* in male progenitor neurons vs female neurons exposed to six different EDCs.

3.3 Expression of AROMATASE [CYP19A1]: A Gender Dependent Downregulation of RORA in Male Progenitor Neurons

We measured the expression levels of *CYP19A1* [*AROMATASE*] in both female and male progenitor neurons exposed to three different concentrations of both estrogenic and androgenic EDCs and did not observe a gender-dependent differential expression. Therefore, in case of exposure to BB, the expression of *CYP19A1* was significantly upregulated at the medium dose of BB in both female and male progenitor neurons. Exposure to BS resulted in down regulation of *CYP19A1* in neurons from both genders. On the other hand, exposure to EU resulted in significant upregulation of *CYP19A1* in the neurons of female origin for all three concentrations, whereas CYP19A1 exposure resulted in down regulation in male neurons at low and medium doses but upregulation at high concentrations of EU. Exposure to OT resulted in significant upregulation of CYP19A1 at the high concentration in female neurons whereas in male neurons down regulation was recorded at all three concentrations of OT. The expression to the two androgenic EDCs we tested, induced CYP19A1 upregulations in neurons from both genders at high concentration but down-regulation at low and medium concentrations. Exposure to MK resulted in significant upregulation of CYP19A1 at high doses in female and low doses in male neurons. It appears our observations did not show a clear pattern of gene dysregulation for CYP19A1, irrespective of neuron exposure to either estrogenic or androgenic EDCs at either of the three concentrations utilized.

4. Discussion

EDCs are exogenous substances that may impact normal synthesis, secretion, transport, binding actions, or elimination of natural hormones responsible for the maintenance of homeostasis, reproduction, development, and differentiations of fetal brains during the prenatal as well as postnatal periods [2, 5, 7, 8, 10]. We normally encounter a myriad of chemicals in our daily lives in the form of fragrances, perfumes, air fresheners, moisturizers, cosmetics, laundry detergents, household cleaners, sanitizers, plastic products, sunscreens and numerous aromatic foods and drinks, etc. Many of these consumer products contain EDCs [7, 8]. Of note, there are numerous fragrances in the form of perfumes, colognes, cosmetics and even fruit drinks that contain untested chemicals, many with EDC properties [11, 12, 14-17]. EDCs are ubiquitous in our environment and upon bodily entry are stored in adipose tissue [18-20]. A pregnant woman recycles stored fat to provide energy for the developing fetus and hence may expose fetal tissues, and especially the developing fetal nervous system, to the stored EDCs, potentially modifying neurodevelopment in an adverse fashion [18, 21]. The same EDCs may likewise alter the hormonal and homeostatic system, and thus affect fetal metabolism, sexual development, growth, stress responses, insulin production, gender behavior, reproduction, fetal brain differentiation and development, and perhaps gender selection [2-7]. Most EDCs are synthetic chemicals that are generally industrial by-products that are released into the environment, polluting rivers and other aquatic habitats, and may thereby enter our drinking-water and food supplies [16]. However, some naturally occurring EDCs can also be found in plants or fungi. Exposure to EDCs is not limited to drinking-water and food, but EDCs can enter via breathing contaminated air, or through skin contact [15]. The range of EDCs is very broad and includes substances like plasticizers [*e.g.*, bisphenols, phthalates found in cosmetics, nail polish, hair spray and as solvents and fixatives in perfumes], preservatives [*e.g.*,

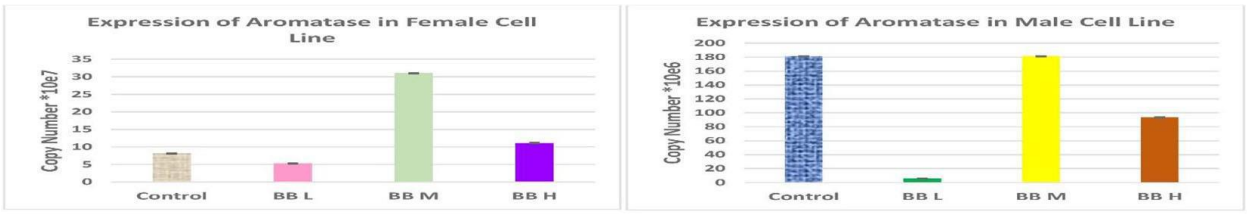
parabens, used in perfumes and sunscreens], surfactants [*e.g.*, alkylphenols, perfluoroalkyls which are used in non-stick utensils], flame retardants [*e.g.*, halogenated bisphenols], and ultraviolet filters [*e.g.*, benzophenones], and the byproducts of various industrial processes [*e.g.*, dioxins] [1-3]. Many are banned, but DEP is the only phthalate still commonly used in cosmetics [5, 22, 23]. Many natural compounds, such as the phytoestrogens, genistein and daidzein, or the mycoestrogen zearalenone are considered as EDCs [24].

Of note, the expression of *CYP19A1* is essential for the functional differentiation of the syncytiotrophoblast cells. E_2 ensures high production of progesterone in the placenta to maintain pregnancy [Chen et al 1986]. Placental *CYP19A1* is essential for the differentiation of the female external genitalia since it prevents the female fetus from the androgenic effect of fetal androgens. Placental *CYP19A1* prevents excessive androgen accumulation in the maternal circulation.

Many environmental chemicals in contaminated foods act as anti-estrogens via the inhibiting *CYP19A1* enzymatic activity, lowering E_2 levels and causing estrogen deficiency. Many food components and environmental chemicals, including phthalates, pesticides, biocides, and fungicides target *CYP19A1* with various modes of action [25].

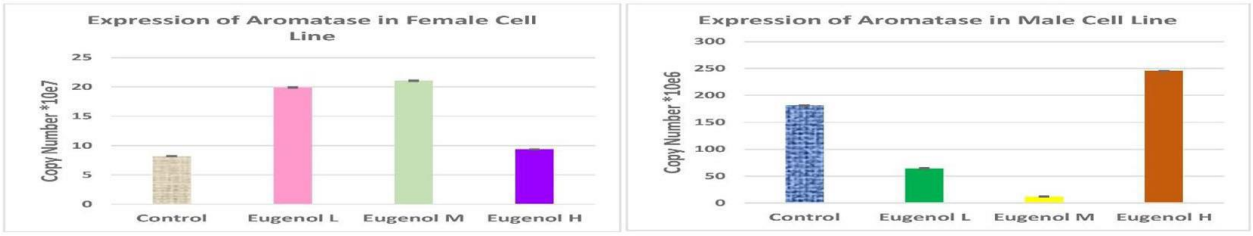
EDCs can disrupt normal homeostasis of endocrine systems of organisms by a wide variety of mechanisms [1-8, 10]. For example, they may mimic natural hormones, can antagonize hormonal action, interfere in their anabolism or catabolism, transport or intracellular signaling pathways [1, 2, 6]. Herein, we examined the effects of six EDCs i.e., estrogenic EDCs: EU, BB, BS and OT, and two androgenic EDCs: MK and DEP on neurite formation from a male and a female developing neuronal cell lines and ascertain significant neuromodifications at manifest as disturbance in neurite formation including in axonal length, axonal degeneration, thinning and central [Figure 1]. The neuromodifications in neurite formation was generally dose related: the higher the dose, the more statistically significant were the morphological abnormalities. We believe that in cases, where the dose-dependent severity of effects was not observed it was due to toxicity of the EDC that significantly reduced the number of surviving neurons. Moreover, we analyzed the effects the same compounds on *RORA* regulation in human developing neurons and show a significant down regulation of *RORA* in male neurons as compared to female neurons when exposed to four estrogenic EDCs. The effects of the EDCs were generally dose dependent on *CYP19A1* expression and varied between each gender.

We used three different concentrations of each of the EDCs [i.e. 31.25 µg/mL as H, and 15.625 µg/mL and 7.812 µg/mL] and although lacking the data to verify concentrations of all six EDCs found in wastewater contaminations, much larger amount of DEP, phthalates, musk ketone, EU, BB, BS and OT are used in cosmetics, sunscreens, perfumes and in daily household items, including laundry detergents, cleaning liquids and other items than employed here [7, 8, 12, 25, 26]. Many pesticides and herbicides also use several of the EDCs we tested at much higher concentrations [27, 28]. Xiao et al [29] have recently measured several EDCs concentration found in river waters in certain parts of China as environmental contaminants. They showed that the concentrations ranged from mM to nM levels. For example, the concentration of estrogenic compounds ranged from approximately 1–10 ng/L. Similarly, a recent study indicated that the concentration of DEHP was 78 μg/L in Bohai Sea [30], whereas MEHP was found at much higher concentrations. DEHP has been reported to have carcinogenic, mutagenic, and hepatotoxic effects. A significant amount of EDCs, pesticides and herbicides have been reported in the FARs providence of Iran and in the sludge found near Bander-e-Imam petrochemical complex [31, 32].

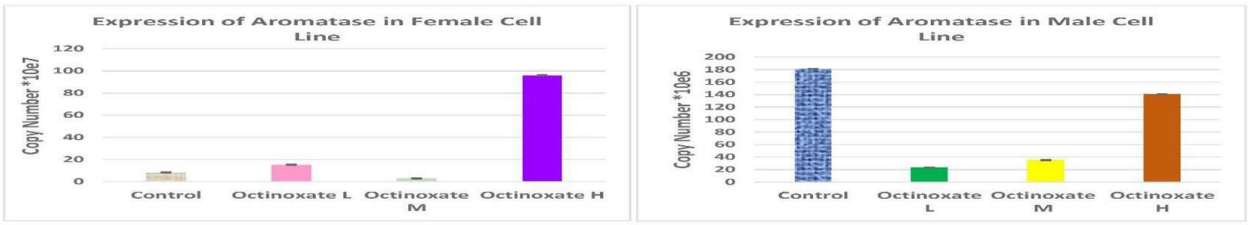


Expression of Aromatase on Benzyl Benzoate treated Human Progenitor Neurons.

Expression of Aromatase on Benzyl Salicylate Treated Human Progenitor Neurons.

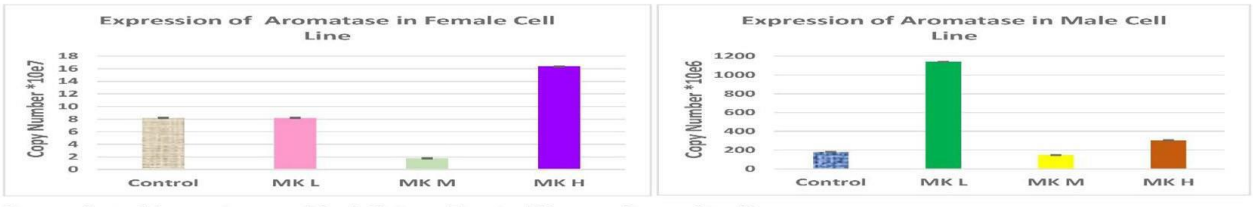


Expression of Aromatase on Eugenol Treated Human Progenitor Neurons.



Expression of Aromatase on Octinoxate Treated Human Progenitor Neurons.

Expression of Aromatase on DEP Treated Human Progenitor Neurons.



Expression of Aromatase on Musk Ketone Treated Human Progenitor Neurons.

Figure 3 Expression of AROMATASE in male and female progenitor neurons.

Gene expression analyses by qPCR showed gender-dependent downregulation of *RORA.* Therefore, human developing neurons of male origin exhibited a significant downregulation of RORA when exposed to estrogenic EDCs. Similar downregulations were not observed in female neurons. It should be noted that several EDCs are found in nature, especially estrogenic chemicals

and evolution has developed a fine defense mechanism to protect a developing fetus from the adverse effects of EDCs [23]. Prenatal exposure to sex hormones has significant lifelong as well as transgenerational effects on neurodevelopment [33, 34]. Fetal exposure to aberrant levels of sex hormones can alter sexual behavior, the degree of feminization or masculinization, and may contribute to the differential susceptibility of males and females to several neurodevelopmental disorders, including autism, schizophrenia, and Alzheimer's disease [7-9]. The placenta regulates the *in utero* environment and especially the endocrine hormones that can have long-term adverse effects if a fetus is exposed to abnormal levels of sex hormones [9, 10]. As shown in Figure 4, the placenta expresses high levels of aromatase, which converts testosterone to estrogen, which prevents the transfer of maternal testosterone to the fetus [35]. *RORA* regulates aromatase, thus perhaps serving as a gate-keeper gene to maintain hormonal homeostasis. *RORA*, which is a transcription factor, regulates aromatase and exposure to an unusual amount of synthetic androgenic chemicals that aromatase may not be able to convert, could result in neurodevelopmental disorders including autism spectrum disorder [ASD]. RORA is considered by many to be an ASD candidate gene [1].

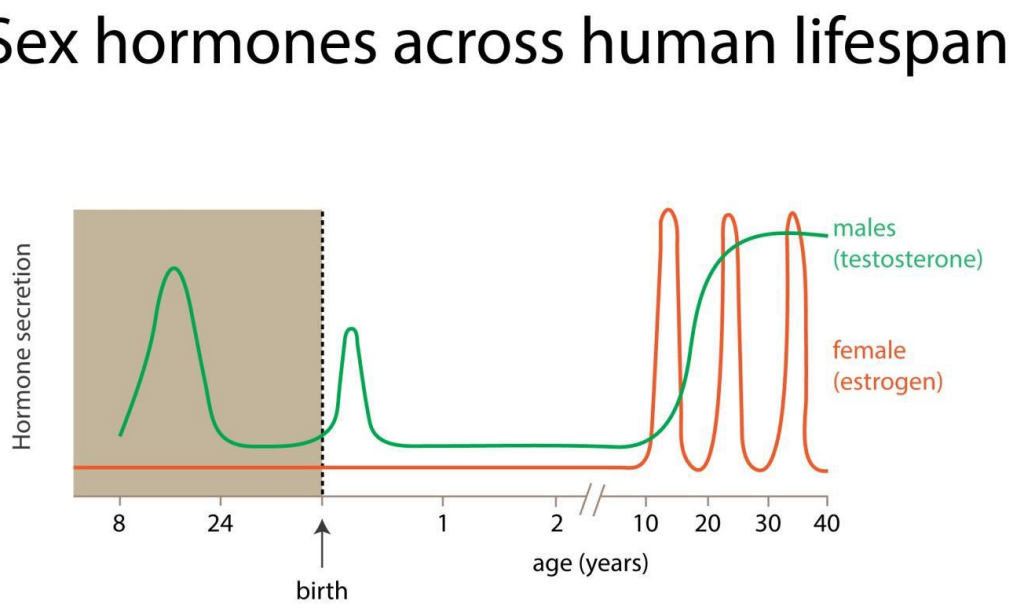


Figure 4 Normal range of sex hormones in male and female during the lifetime [8].

There is a large body of work on phthalate neurotoxicity. Phthalates are some of the most common environmental EDCs and are used as plasticizers in PVC plastics. For example, Heudorf et al [36] have reviewed the adverse effects of phthalates. As the phthalate plasticizers are not chemically bound to PVC, they can leach, migrate or evaporate into indoor air and atmosphere, foodstuff, other materials, etc. Consumer products containing phthalates can result in human exposure through direct contact and use, indirectly through leaching into other products, or general environmental contamination. Humans are exposed through ingestion, inhalation, and dermal exposure during their whole lifetime, including intrauterine development. This paper presents an overview on current risk assessments done by expert panels as well as on exposure assessment data, based on ambient and on current human biomonitoring results. Some phthalates are reproductive

and developmental toxicants in animals and suspected endocrine disruptors in humans. Exposure assessment via modelling ambient data give hints that the exposure of children to phthalates exceeds that in adults. Current human biomonitoring data prove that the tolerable intake of children is exceeded to a considerable degree, in some instances up to 20-fold. Very high exposures to phthalates can occur via medical treatment, i.e. via use of medical devices containing DEHP or medicaments containing DBP phthalate in their coating. Because of their chemical properties exposure to phthalates does not result in bioaccumulation. However, health concern is raised regarding the developmental and/or reproductive toxicity of phthalates, even in environmental concentrations. Gray and Billock [37] have reviewed the adverse effects of EDCs, especially phthalates can have gender-specific effects on boys but not girls with regards to ASD [Swan et al [38] exposure and evidence that developmental neurotoxicity could account for some of the gender and birth order effects in autism spectrum disorders. Most of their focus was on brominated flame retardants which have multiple pathways to neurotoxicity, and can modulate both thyroid and androgen receptors, but they also found some evidence for specifically estrogenic chemicals like phthalates with roles in autism. Similarly, Miodovnik et al. [39] found that high prenatal phthalate resulted in children with worse Social Responsiveness Scale scores [i.e., ASD]. They analyzed third trimester urines of 404 women enrolled in the Mount Sinai Children's Environmental Health Study between 1998-2002 for phthalate metabolites and BPA. Mother-child pairs follow-up assessment of the child was between the ages of 7 to 9 years. At this visit, mothers completed the Social Responsiveness Scale (SRS), a quantitative scale for measuring the severity of social impairment related to Autistic Spectrum Disorders (ASD) in the general population. They showed that the low molecular weight phthalate (LMW) metabolite concentrations were associated with greater social deficits. Among the subscales, LMWP were also associated with poorer Social Cognition. However, they did not find any significant association with BPA was found. Prenatal phthalate exposure was associated with childhood social impairment in a multiethnic urban population. These results extend our previous finding of atypical neonatal and early childhood behaviors in relation to prenatal phthalate exposure. Larsson et al. [40] carried out a large, long-term study and followed 4,779 children who lived in homes with phthalate-treated vinyl flooring. From a total of 4,779 eligible children, 72 (60 boys, 12 girls) were identified with parentally reported ASD, diagnosed by medical professionals, in accordance with the Swedish system for monitoring children's health. An analysis of the associations between indoor environmental variables in 2000 as well as other background factors and the ASD diagnosis indicated five statistically significant variables: (1) maternal smoking; (2) male sex; (3) economic problems in the family; (4) condensation on windows, a proxy for low ventilation rate in the home; (5) PVC flooring, especially in the parents' bedroom. In addition, airway symptoms of wheezing and physician-diagnosed asthma in the baseline investigation (2000) were associated with ASD five years later. Results from the second phase study indicated PVC flooring to be one important source of airborne phthalates indoor, and that asthma and allergy prevalence are associated with phthalate concentrations in settled dust in the children's bedroom. Chen et al [41] have utilized Zebra Fish embryo as a model to explore the toxicity of phthalates and found that the typical toxicity symptoms caused by phthalates were death, tail curvature, necrosis, cardio edema, and no touch response. Using an estrogen-responsive *ChgH-EGFP* transgenic medaka (*Oryzias melastigma*) eleuthero embryos based 24 h test, they demonstrated estrogenic activity of various phthalates, and the mixture of the six phthalates exhibited enhanced-estrogenic activity. These

findings indicate that the widespread use of phthalates may cause potential health risks to human beings.

The present study clearly shows that the exposure to synthetic estrogenic EDCs imparts a profound neurodevelopmental effect both at the morphologic and molecular levels. The finding that exposures to estrogenic EDCs significantly downregulates RORA expression in male progenitor neurons confirms the reports of Hu *et al* [36] and Sarachana *et al* [1, 37].

Sarachana et al [36] were the first to describe in detail the differential expression of *RORA* and its regulatory effect on the *AROMATASE* [36]. They showed that male and female hormones differentially regulate the expression of a novel autism candidate gene, in a neuronal cell line, SH-SY5Y. In addition, they showed that RORA transcriptionally regulated aromatase and that aromatase protein was significantly reduced in the frontal cortex of autistic subjects relative to sex- and age-matched controls and was strongly correlated with *RORA* protein levels in the brains of ASD patients *vs* controls. Their results suggested that *RORA* has the potential to be under both negative and positive feedback regulation by male and female hormones, respectively, via aromatase, and forwarded the notion that male ASD may be related to *RORA*/aromatase regulation [36]. Hu *et al*., [37] showed that *RORA* protein levels were higher in the brains of typically developing females compared to typically developing males, presumably providing females with a buffer against *RORA* deficiency.

Our qPCR analyses confirmed that there was a profound downregulation of RORA in the male progenitor neurons exposed to four estrogenic EDCs but not in the female neurons. Hu et al., have suggested that RORA deficiency may be related to EDCs as potential risk factors for autism [37]. RORA dysregulation induced by EDCs may increase the risk for ASD in a sexually dimorphic manner based on differential regulation in male *vs* female hormones. The hormone sensitivity of RORA to EDCs further suggests that EDCs, many of which mimic sex hormones or their antagonists, may also interfere with the normal expression and function of this critical transcription factor, thus increasing risk for ASD.

Dysregulation of *RORA* is likely to impair androgen-dependent neurological development and functions. A downregulation of the RORA gene can result in dysregulated feed-back loops with sex hormones, as well as the many autism-associated genes regulated by RORA. Hu et al [37] suggested that, at least in a subgroup of autism, males and females may be differentially affected by dysregulated RORA expression. In their rodent model they showed that disruption of *RORA* appears to have a higher level of adverse effect in male than female mice.

Here we show that there is a gene-environment interplay between the expression of *RORA* and exposure certain EDCs. Our study reveals a potential etiological role of *RORA* downregulation and prenatal hormonal effects and points to gene-environment correlation in which biological sex plays a moderating role. Baron-Cohen's group has shown increased prenatal steroidogenic activity being an early 'environmental' risk for later autism diagnosis in males [7, 8]. Since intentional exposure of pregnant mothers to EDCs would not be an ethical form of research, we, instead, have utilized human progenitor neurons to evaluate the male gender-bias in ASD. Exposure to EDCs during early fetal development has been linked to seriously harmful outcomes in newborns. EDCs can modify the actions of estrogenic and androgenic hormones, thus disrupting the homeostasis of sex hormones and modifying neurodevelopment [1-6]. Further, EDCs can alter nuclear receptor signaling, and can act through membrane receptors, as well as interfere with enzymatic pathways that are involved in steroid biosynthesis and/or

metabolism, and through numerous other mechanisms that converge upon endocrine and reproductive systems [38].

In conclusion, our study further extends the studies of Hu and Sarachana [1, 36, 37] and confirms the potential role of environmental EDCs on neurodevelopment of both genders and sheds some light on male gender bias that has been reported previously [1, 36, 37]. We hypothesize that male gender bias in ASD may partially be due to exposure to various EDCs during the early gestation period [9, 10]. Also, a case study report by Long et al., [6] on amniotic fluid of pregnant women concluded that prenatal exposure to EDCs results in high risk of ASD. The morphologic analyses of profound disturbance in neurite formation exhibits the results of exposure to EDCs vividly and may impact pruning mechanisms and disturbance in normal development of neuronal network during gestation [39].

Of note, it is well documented that many of the EDCs are lipophilic and can accumulate in the fatty tissues. These EDCs can remain for decades [18-20]. During pregnancy fatty acids serve as one of the major sources of reserve energy that supports a healthy outcome. It is safe to assume that older women have higher EDC levels accumulated in fatty tissues than younger females, perhaps providing one explanation for why older women give birth to higher numbers of ASD children [40, 41].

Baron-Cohen's group has shown increased prenatal androgen levels can be risk factor with later ASD diagnosis for males [9, 10]. This finding strengthens the gene-environment interplay and potential role of androgenic EDCs but also estrogenic EDCs that may increase the androgenic levels directly or indirectly through receptor redistribution, displacement or *via* cell signaling pathways. Our study points to male-specific risks by environmental factors, and specifically EDCs.

There are over 4,000 EDCs found in the environment [2, 3]. There are infinite differences between the *in vivo* human brain development and the *in vitro* state as presented herein. Nevertheless, despite this limitation, we believe that a clear picture of susceptibility of neurons is demonstrated and a potential mechanism of male gender bias mediated by certain EDCs resulting in *RORA* deficiency. In the present study we have not explored the receptors mediated signaling or the molecular mechanisms that may be involved in the pathogenesis of autism; investigations that will further explore and define these interrelationships with EDCs-*RORA*-aromatase network.

5. Conclusion

From our studies we conclude that environmental factors contribute significantly to neurodevelopment of both male and female developing fetal brain neurons. All six EDCs we examined induced significant downregulation in *RORA* in male developing neurons but not in female neurons, perhaps explaining male-gender bias of ASD. In addition to *RORA*, we determined that almost all EDCs downregulated *CYP19A1*, leading towards dysregulation in neurodevelopment. Our morphologic analyses show severe disruption in neurite formation and neural network. These EDCs induce significant adverse effects in developing neurons even at low concentrations.

Abbreviations

Autism spectrum disorder [ASD], Aromatase [CYP19A1], Benzyl Benzoate [BB], Benzyl Salicylate [S], Diethy Phthalate [DEP], Eagle's Minimum Essential Media [EMEM], Endocrine Disrupting

Chemicals [EDCs], Eugenol [EU], Fetal Bovine Serum [FBS], Musk Ketone [MK], Neuroblastoma cells [NBC], Octinoxate [ON], Phosphate buffer saline [PBS], Quantitative Real Time PCR [qRT-PCR].

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Additional Materials

The following additional materials are uploaded at the page of this paper.

1. Table S1A: Neuromodifications induced by Benzyl Benzoate in female 2266.
2. Table S2A: Neuromodifications induced by Benzyl Benzoate (BB) in male 2267 cell lines.
3. Table S1B: Neuromodifications induced by Benzyl Salicylate (BS) in female cell line.
4. Table S2B: Neuromodifications induced by Benzyl Salicylate (BS) in male cell line.
5. Table S1C: Neuromodifications induced by Eugenol in female cell line.
6. Table S2C: Neuromodifications induced by Eugenol in male cell line.
7. Table S1D: Neuromodifications induced by Octinoxate in female cell line.
8. Table S2D: Neuromodifications induced by Octinoxate in male cell line.
9. Table S1E: Neuromodifications induced by DEP in female cell line.
10. Table S2E: Neuromodifications induced by DEP in male cell line.
11. Table S1F: Neuromodifications induced by Musk Ketone in female cell line.
12. Table S2F: Neuromodifications induced by Musk Ketone in male cell line.

Author Contributions

OB initially conceived the idea, designed, and planned the study. The experiments were further refined by NS. OB and PP significantly contributed to performing the experiments. All authors significantly contributed to preparation of the manuscript. EM contributed to preparation and refinement of the manuscript.

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Competing Interests

All authors declare no conflict of interest.

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