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Outcomes of developmental exposure to total particulate matter from cigarette smoke in zebrafish (*Danio rerio*)

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ABSTRACT

The effects of prenatal exposure to cigarette smoke remain a subject of major interest, especially as it relates to neural development and adverse behavioral outcomes. Several studies have investigated the developmental toxicity of cigarette smoke components in a zebrafish model, showing that developmental exposure to total particulate matter (TPM; particulate phase of cigarette smoke) leads to adverse physiological aberrations and locomotor hyperactivity. Thus, the current study examines whether developmental TPM exposure of zebrafish embryos/larvae (F0) leads to physiological and behavioral alterations, and whether adverse effects are observed in adult fish and the next generation (F1; i.e. F0 offspring). We also examine whether behavioral effects are associated with changes in neural development, stress response, neurotransmitters, and bioenergetics. We demonstrate that TPM exposure during F0 development increased the incidence of deformities in F0 larvae, but F1 larvae did not exhibit any deformities. TPM exposure also resulted in swimming hyperactivity in F0 larvae and several behavioral changes were noted in F0 fish when they grew into adulthood. These behavioral changes were generally not associated with changes in markers of neural development in larvae, stress response in F0 adults, and concentration of neurotransmitters (acetylcholine, dopamine, and serotonin) in F0 adult brain. There were also no changes in F0 or F1 embryonic oxygen consumption rate (OCR; marker of bioenergetics and mitochondrial health); however, the OCR in the brain of F0 males was reduced with TPM. We conclude that developmental exposure to TPM affects larval physiology and induces hyperactive swimming behavior, but these effects do not persist in F1 larvae. Moreover, developmental TPM exposure leads to long-lasting sex-specific behavioral outcomes in the F0 adult fish.

1. Introduction

The average reported prevalence of maternal smoking any time during pregnancy in the USA in 2014 was estimated at 8.4%, ranging from 1.8% in California to 27.1% in West Virginia (Curtin and Mathews, 2016). The effects of maternal smoking on fetal development remain an area of major interest, since prenatal exposure to cigarette smoke is associated with low birth weight and increased risk of stillbirth (Knopik, 2009), neonatal malformations, cardiovascular defects (Hacksaw et al., 2011), and neurobehavioral effects (Rogers, 2009).

Moreover, epidemiological studies have documented that children, who were exposed to cigarette smoke prenatally were more likely to display delinquent and aggressive behavior, as well as locomotor hyperactivity and increased impulsiveness (Cornelius et al., 2011). Despite the abundant literature on neurobehavioral effects due to prenatal exposure to cigarette smoke, there is still very little known about how exposure to substances in cigarette smoke during early development leads to long-lasting effects that are detected from infancy to adulthood (Morris et al., 2011).

Nicotine, the psychoactive compound in cigarette smoke, has long been thought to play a major role in neurobehavioral effects, and several studies have documented its adverse effects on mammalian brain development, which manifest in cognitive and behavioral deficits (e.g. Levin and Slotkin, 1998; Levitt, 1998; Slikker et al., 2005; Slotkin et al.,

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2006). However, recent studies demonstrate that developmental exposure to other components of cigarette smoke also produces neurobehavioral effects. For example, polycyclic aromatic hydrocarbons (PAHs) are abundant in cigarette smoke and have been shown to cross the placenta and fetal blood brain barrier (Brown et al., 2007). Although the neurotoxic properties of PAHs are still not very well understood (Schroeder, 2011), prenatal exposure to PAHs has been shown to induce neurobehavioral effects, including anxiety, depression, and attention problems (Perera et al., 2012). The neurotoxic properties of PAHs are associated with increased DNA adducts in cord and maternal blood, as well as their metabolism and generation of reactive oxygen species (Schroeder, 2011; Perera et al., 2012; Bollinger et al., 2015).

Undoubtedly, additional research with refined methods/analyses is necessary to better understand the effects of maternal smoking on offspring behavior. While murine model systems have been extensively used in understanding the effects reported in humans, inclusion of less complex high-throughput systems, such as zebrafish, could further enhance these efforts. Our previous work has demonstrated that exposure to total particulate matter (TPM; the particulate phase of cigarette smoke), which contains nicotine, PAHs, and hundreds of other chemicals, disrupts early development of zebrafish, including higher incidence of deformities, reduced cranial angiogenesis, and larval hyperactivity, and that these effects are not attributed to nicotine, but are partially mediated through the activation of aryl hydrocarbon receptor (AHR) pathway and downregulation of Wnt signaling pathway (Massarsky et al., 2015, 2016, 2018b). Notably, the behavioral effects in larvae were not attributed to nicotine, since locomotor hyperactivity was not observed in nicotine-exposed larvae (Massarsky et al., 2015). We have also shown that components of cigarette smoke (such as PAHs) accumulate in the brain of developing zebrafish, potentially contributing to disrupted vascular development and altered larval behavior (Massarsky et al., 2018b).

Considering the significant effects of TPM exposure on zebrafish development, the current study examines whether developmental exposure of zebrafish embryos/larvae can result in long-lasting effects in adults and their offspring. To this end, we examined the effects of developmental exposure to TPM on behavior of F0 larvae and whether adverse effects could be observed in adults (F0) and/or offspring (F1 embryos/larvae). We also assessed several factors that might be underlying these behavioral changes, including early neural development in larvae, stress response in adults, and the concentrations of neurotransmitters in adult brain. Differences in bioenergetics were also examined, since cigarette smoke contains many compounds (e.g. PAHs) that can disrupt mitochondrial function (Fetterman et al., 2017), and mitochondrial dysfunction is associated with neuronal degeneration (Schon and Manfredi, 2003). Ultimately, this study aims to improve our understanding of the effects of developmental exposure to cigarette smoke components and potential long-lasting outcomes in adults and their offspring.

2. Materials and methods

2.1. Chemicals

TPM was prepared at Labstat International Inc., (Kitchener, ON, Canada) by mechanical smoking of 3R4F reference cigarettes (University of Kentucky) following the ISO standard 3308:2012 (35 mL puff volume, 60 s interval between puffs, and 2 s puff duration) (Johnson et al., 2009). TPM was collected onto Cambridge glass fiber filter pads. These filter pads typically retain at least 99.9% of TPM, and the particle size of TPM is reported to be ≥ 0.3 µm in diameter (Johnson et al., 2009). The collected TPM was then dissolved in DMSO at a concentration of 20 mg TPM/mL to generate the stock solution and stored at −80 °C until used. The partial analysis of the TPM stock solution was performed at Labstat International as reported previously (Massarsky et al., 2015). All other reagents were purchased from Sigma Aldrich unless otherwise specified.

2.2. Fish husbandry and zebrafish embryo collection

Adult EkkWill zebrafish (EkkWill Waterlife Resources, Ruskin, FL, USA) were maintained in holding tanks on a 14:10 h light-dark cycle at 28 °C in circulating AHAB system (Aquatic Habitats, Apopka, FL, USA) in 60 mg/L salt water (Instant Ocean, Foster & Smith, Rhinelander, WI, USA). Tg(*Neurog1*:GFP) *Mitfa* mutant zebrafish (a generous gift from Dr. Linney, Duke University) were maintained in a separate circulating AHAB system under similar conditions; these transgenic zebrafish were used to assess the neuronal development (see Section 2.6.1.2 for more details). Fish were fed brine shrimp in the morning and Zeigler's Adult Zebrafish Complete Diet (Aquatic Habitats) in the afternoon. Breeding tanks were set at 5 PM and breeding was initiated at 9 AM the following morning. Embryos were collected within half an hour of spawning. Transgenic zebrafish were maintained in the same manner as the wild-type zebrafish. All procedures were approved by the Duke University Institutional Animal Care & Use Committee (A279-08-10).

2.3. Experimental setup

The exposures were setup such that 15 embryos were randomly assigned into 6 cm diameter glass Petri dishes, containing a total volume of 15 mL 30% Danieau's (in mM: 58 NaCl, 0.7 KCl, 0.4 $MgSO_4$, 0.6 $Ca(NO_3)_2$, 5 HEPES) supplemented with 0.00003% methylene blue (a fungicide), and separated into treatment groups. There were 4 replicate Petri dishes per treatment group per cohort to ensure sufficient quantity of larvae for various endpoints. Separate experiments were performed with 6 replicate Petri dishes per treatment group per cohort to ensure sufficient quantity of larvae for DNA isolation (see details in next paragraph), which requires larger pools of larvae to have sufficient DNA quantity. The embryos were exposed to three concentrations of TPM, corresponding to 0.1, 0.2, and 0.3 µg/mL equi-nicotine units (abbreviated as $TPM_{0.1}$, $TPM_{0.2}$, and $TPM_{0.3}$, respectively). These concentrations translate into 1.4, 2.8, and 4.2 µg TPM/mL and are similar to the range of concentrations used in previous studies (Ellis et al., 2014; Massarsky et al., 2015, 2016, 2018b). Control embryos received an equivalent volume of DMSO (0.1%). The exposures started at 6 h post-fertilization (hpf) and lasted up to 96 hpf.

During exposure, the viability, hatching success, deformities, and bioenergetics were assessed (see sections below for details) in wild-type embryos/larvae. At the end of the exposure the larvae were (i) imaged in methylcellulose (15 larvae/treatment group/cohort); (ii) collected and stored at −80 °C until RNA analysis (15 larvae/treatment group/cohort); (iii) transferred to fresh Danieau's and larval activity was assessed at 144 hpf (30 larvae/treatment group/cohort); (iv) transferred into 3 L holding tanks (45 larvae/treatment group/cohort) and raised to adulthood (see Section 2.2 for details on fish husbandry), in order to assess tissue bioenergetics, adult behavior, and to generate the F1 generation. Samples of F0 and F1 larvae (90 larvae/treatment group/cohort) were also collected and stored at −80 °C for DNA isolation and methylation analysis; a manuscript detailing the methylation changes is currently in preparation. Additionally, some of the F0 adult fish were preserved, sectioned, and stained for histopathological analysis of various organs; a manuscript detailing the histopathology is currently in preparation.

Tg(*Neurog1*:GFP) zebrafish were exposed exactly as outlined above. The embryos/larvae were visualized to assess neural development (see Section 2.6.1.2 for details).

2.4. General physiology

2.4.1. Larvae

Several parameters were assessed in F0 embryos/larvae during the exposure. Viability was assessed throughout the exposure and dead