Gestational exposure to particulate air pollution exacerbates the growth phenotypes induced by preconception paternal alcohol use: a multiplex model of exposure

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Abstract

It is now clear that parental histories of drug use, toxicant exposure, and social stress all have a significant influence on the health and development of the next generation. However, the ability of epigenetic parental life memories to interact with subsequent gestational exposures and cumulatively modify the developmental trajectory of the offspring remains an unexplored perspective in toxicology. Studies from our laboratory have identified male-specific postnatal growth restriction in a mouse model of chronic, preconception paternal alcohol exposure. The goal of the current study was to determine if paternal alcohol use, before conception, could modify the susceptibility of the offspring to a completely separate exposure encountered by the mother during pregnancy. In independent experiments, we previously identified altered developmental programming and increased markers of severe asthma induced by gestational exposure to particulate air pollution. In this study, male mice were exposed to either the control or alcohol preconception treatments, then mated to naive females, which we subsequently exposed to an ultrafine mixture of particulate matter via inhalation. Individually, neither preconception paternal drinking nor gestational exposures to particulate air pollution impacted the postnatal growth of female offspring. However, when both exposures were combined, females displayed a 30% reduction in weight gain. Unexpectedly, this exposure paradigm resulted in a dramatic postnatal increase in litter loss due to maternal cannibalism, which prevented additional measures of offspring health. These preliminary studies provide evidence of a complex interplay between preconception life history and intrauterine environmental factors in the control of postnatal growth.

Key words: developmental programming; epigenetic programming; preconception exposure; early life exposure; fetal growth restriction; particulate air pollution; multiplex model of exposure

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Introduction

Rather than being attributable to any one single risk factor, emerging evidence now indicates that most instances of birth defects and disease are the consequence of complex interactions between genetic, epigenetic, and environmental or lifestyle factors [1]. Although we recognize the importance of each of these factors individually, understanding how and when these separate elements interact with each other remains a core question in the field of developmental toxicology. For example, maternal exposures to cigarette smoke or traffic-related air pollution are both known to interact with a stressful home environment and increase the risk of asthma in the offspring [2]. Similarly, gestational exposures to alcohol increase both the sensitivity of the offspring to carcinogens, as well as the propensity to develop cancer as an adult [3]. Therefore, understanding how separate life events interact and their capacity synergize is crucial to our efforts to decipher the developmental basis of birth defects and disease.

Today, it is well-accepted that in addition to maternal intrauterine exposures, environmental stressors encountered by both parents before conception also exert a significant influence on child health and development [4, 5]. However, despite clear evidence that parental histories of drug use, social stress, and environmental exposures all impact fetal development, the capacity of preconception exposures to interact with and exacerbate outcomes associated with subsequent intrauterine encounters remain unknown. This is especially true of preconception male exposures, which are seldom considered relevant in a clinical setting [5].

In the USA, male alcohol use far exceeds that of women, with 70% of men reporting consistent patterns of consumption and 40% drinking at a binge level [6, 7]. Although maternal exposure to alcohol during pregnancy has well-established effects on fetal development, preconception paternal exposures have only recently been recognized as potent modulators of offspring health [8]. Using an established mouse model, our laboratory recently described sex-specific patterns of both fetal and postnatal growth restriction in the offspring of males chronically exposed to alcohol [9–12]. Collectively, our studies reveal that preconception alcohol use by the father exerts a powerful impact on the sperm-inherited developmental program. Given that the life expectancy of patients with fetal alcohol syndrome is 34 years, which is 58% lower than the general population [13], it is crucial to understand the long-term impact paternal alcohol use has on offspring health and the predisposition to develop disease.

The goal of the current study was to determine if chronic paternal alcohol use prior to conception could modify the susceptibility of his offspring to a completely separate environmental exposure encountered by the mother during pregnancy. Our group recently developed a mouse model of intrauterine exposure to ultraviolet particles, which is an exposure paradigm highly relevant to pregnant women living in polluted urban environments [14]. Here, we hypothesized that epigenetic defects induced by preconception male alcohol use would increase the susceptibility of the fetus to intrauterine exposure to particulate matter (PM) and unmask an enhanced predisposition to develop disease. As a large number of environmental exposures are associated with delays in perinatal growth, which in turn, is a major determinant of child mortality and morbidity, as well as disease in adult life [15, 16], we focused on this initial study on offspring growth.

Materials and Methods

Animal Work

Experiments were conducted under AUPs 2015-0279 and 2017-0308 and approved by the Texas A&M University IACUC.

Preconception Male Alcohol Exposure

Individually caged, postnatal day 90, C57BL/6j adult male mice were fed a standard diet (catalog#2019, Teklad Diets, Madison, WI, USA) and maintained in the Texas Institute of Genomic Medicine on a 12-h light/dark cycle. Males were provided limited access to ethanol during a 4-h window, beginning 1 h after the initiation of the dark cycle, using methods previously described [9–12]. During this 4-h window, we provided experimental males access to a solution of 10% (w/v) ethanol (catalog# E7023; Millipore-Sigma, St. Louis, MO, USA) and 0.066% (w/v) Sweet’N Low (Cumberland Packing Corp, Brooklyn NY, USA), while control males received a solution of 0.066% (w/v) Sweet’N Low alone. The addition of Sweet’N Low is necessary to encourage male mice to develop consistent drinking habits.

After established, we maintained males on this protocol for a period of 70 days (approximately two spermatogenic cycles), which ensured that sperm formed prior to the initiation of treatment could not confound the results. After 70 days, we placed two naturally cycling females into a new cage along with each exposed male. During these matings, we did not provide males access to the alcohol/control preconception treatments. The next morning, we confirmed matings by the presence of a vaginal plug, at which point, the males were returned to their original cages and the dams were employed in the prenatal exposure paradigms described below. Males were allowed a 72-h rest period, during which the preconception exposure continued. We repeated this procedure until each male had produced a minimum of 3 litters.

Gestational Exposures to PM

We maintained pregnant dams on a Breeder diet (catalog # 5058; LabDiet, St. Louis, MO, USA). We purchased Diesel Particulate Matter Standard Reference Material (SRM 2975) from the National Institute of Standards and Technology (Gaithersburg, MD, USA). After confirmed mating, we randomly placed females into whole-body exposure chambers, which consisted of a 12” x 8” x 32” stainless steel box with separated inner compartments and a 1/4” clear cast acrylic lid. Air was continuously pumped through the chamber by stainless steel aerosol distribution lines attached to the lid and out return lines on the bottom. PM was generated utilizing a commercial constant output atomizer (TSI 3076, TSI, Inc., Shoreview, MN, USA). Particles were conditioned, including adjustment of particle concentration in a dilutor, water vapor removed using a multi-tube Nafion drier, organic vapor removed using a denuder filled with Spectrum XB-17 reactive adsorbent, and particle charge neutralized using a Po-210 bipolar diffusion charger. The concentration and size distribution of the PM pumped into the exposure chambers was constantly monitored with a Scanning...
Mobility Particle Sizer (SMPS) system and adjusted by changing the corresponding PM dilution ratios at the source. The SMPS operated with a sheath flow of 6.5 L/min and a sample flow of 1 L/min. The mass concentration of accumulation mode PM was maintained near 100 μg/m³, corresponding to a total number concentration of about 10⁵ particles/cm³ for 150 nm geometric mean diameter particles. Using this system, we exposed pregnant dams to either ultrafine particles (PM) or filtered air (FA) for 6 h/day. Exposures continued until gestational day 18.5 (GD18), in which point dams were removed to individual housing and allowed to deliver spontaneously.

Data Handling, Experimental Replicates, and Statistical Analysis

For all experiments, measures were input into the statistical analysis program GraphPad (RRID: SCR_002798; GraphPad Software, Inc., La Jolla, CA, USA) and statistical significance set at alpha = 0.05. All datasets were first verified for normality using the Brown–Forsythe test. In this study, the effect of two independent variables (preconception treatment vs. postnatal treatment) was assessed using a two-way analysis of variance test (ANOVA), and differences among the means evaluated using Sidak’s post-hoc test of contrast.

Results

To determine if paternal exposure history could modify offspring susceptibility to an environmental pollutant encountered by the mother during pregnancy, we combined our established mouse model of preconception male alcohol-exposure [9–12] with our recently developed mouse model examining the developmental consequences of intrauterine exposure to particulate matter [14]. In these experiments, we used a 2 × 2 factorial design to compare the postnatal growth of the offspring of control males mated to dams exposed to filtered air (Control-FA), control males mated to dams exposed to PM (Control-PM), alcohol-exposed males mated to dams exposed to filtered air (EtOH-FA), and alcohol-exposed males mated to dams exposed to PM (EtOH-PM) (Fig. 1).

Alterations in Fetal Growth Induced by Paternal Alcohol Exposure

We exposed five males to each of the alcohol and control experimental treatments described in our previously published study [12] and, after 70-day exposure, mated them to naive females. To first verify an effect of paternal drinking on offspring growth, we terminated the pregnancies from three control and three alcohol-exposed males on gestational day 16.5 and measured

Figure 1: Experimental design. Schematic representation of the experimental model employed to assay interactions between preconception paternal drinking and intrauterine exposures to particulate air pollution. Males were exposed to either the control or alcohol preconception treatments for 70 days, which is roughly equivalent to two spermatogenic cycles. Exposed males were then mated to naive dams, which were then placed into exposure chambers for 6 h each day, from gestational day 0.5 to 18.5, then returned to their original cages and allowed to spontaneously deliver. Postnatal growth was monitored for 30 days.

Figure 2: Preconception paternal alcohol exposure induces heritable fetal growth restriction in the offspring. (A) Comparisons of average weights of the male and female offspring sired by three control and three alcohol-exposed males. (B) Placental weights compared between offspring sired by control and ethanol-exposed males (control male n = 9, alcohol male n = 12, control female n = 8, and alcohol female n = 9). Differences in fetal and placental weights were assayed using a two-way ANOVA followed by Sidak’s post-hoc test of contrast. Errors bars represent the SEM. *P < 0.05 and **P < 0.01.
fetal growth. Consistent with our previous data [12], the offspring of alcohol-exposed sires displayed a reduction in fetal weight (Fig. 2A), which was accompanied by a respective 18% and 12% increase in placental weight in the male and female offspring of alcohol-exposed sires (Fig. 2B).

Combined Exposures Induce Sex-Specific Alterations in Postnatal Growth

To model prenatal exposures to particulate air pollution, we exposed pregnant C57Bl/6 dams to either filtered air or aerosolized PM for 6 h/day, using inhalation chambers. PM composition included black carbon, nitrate, and sulfate, which is an exposure paradigm highly relevant to human populations living in urban environments [17]. During the prenatal exposures, daily PM mass loads averaged 99.52 mg/m³ (Fig. 3A). At GD18, we removed pregnant dams from the exposure chambers and allowed them to spontaneously deliver. There were no differences in litter size between treatment groups (Fig. 3B). At postnatal day 12, we did not observe any significant differences in the weight of either male or female offspring between treatment groups. However, beginning on postnatal day 19 and continuing through Day 28, male and female offspring within the EtOH-PM treatment group...
displayed an average ~20% and ~30% respective reduction in body weight, compared to the Control-FA, Control-PM, and EtOH-FA treatment groups (Fig. 3C and E). We then calculated the growth rate between postnatal days 12 and 28, and compared treatment groups. Compared to the corresponding controls, male offspring within the EtOH-FA group displayed a 22% reduction in growth rate, while males in the combined EtOH-PM treatment group displayed a 45% reduction in growth rate (Fig. 3D). No statistically significant differences in growth rate were observed between EtOH-FA and EtOH-PM male offspring. In contrast to male offspring, there were no differences in the growth rates of the female offspring between the Control-FA and EtOH-FA treatment groups. However, similar to the male offspring, females in the EtOH-PM group displayed a 45% reduction in their growth rate as compared to both the Control-PM and EtOH-FA females (Fig. 3F). These observations indicate that although male offspring of alcohol-exposed sires display growth restriction under both postnatal treatments, only the female offspring exposed to the combined EtOH-PM treatment were affected.

**Combined Exposures Reduced Postnatal Survival and Increased Cannibalism**

In this study, a significant number of dams in the Control-PM, EtOH-FA, and EtOH-PM treatment groups began to cannibalize their litters. This behavior consistently eliminated the whole litter, and predominantly occurred on or before postnatal day 5 but was also observed on postnatal days 12, 19, and 26 in PM-exposed litters. Importantly, the mice were not handled from GD18.5 through to postnatal day 5 when most of the cannibalism was identified. During these experiments, we utilized a well-established breeder diet, to which dams had unrestricted access and subjected the mice to minimal handling stress. We did not observe cannibalism in any of our previous postnatal studies examining the offspring of alcohol-exposed sires [10, 11], or in the Control-FA treatment group, indicating the strong possibility that these outcomes were associated with the treatments. After 30 days of postnatal growth, we terminated the experiments due to the low number of surviving offspring (Fig. 4).

**Discussion**

In our previous studies, both the male and female offspring of alcohol-exposed sires displayed fetal growth restriction, while after birth, only the male offspring continued to exhibit reductions in growth [9–12]. Using an inhalation model of preconception paternal alcohol exposure, Rompala et al. [18] also observed similar patterns of male-specific growth restriction. Collectively, these observations indicate that females are protected from the long-term deficits in growth induced by paternal drinking. In this study, we sought to determine if alterations in epigenetic programming caused by paternal alcohol exposure could be exacerbated when combined with a second hit. As alcohol-induced birth defects and environmentally induced asthma both exhibit a higher prevalence among socially disadvantaged populations [19, 20], we chose to combine our paternal model of exposure with one examining altered programming induced by in utero exposure to ultrafine PM [14]. The major finding of this study is the significant synergistic effect the combined exposures have on the growth of female offspring. Individually, neither preconception paternal alcohol nor gestational exposures to particulate air pollution impact the postnatal growth of female offspring [10, 14]. However, when combined, we observed a significant reduction in female weight gain. These preliminary experiments indicate that prenatal exposures to particulate air pollution interact with male exposure history to sensitize female offspring to growth defects induced by paternal drinking.

Although interactions between parental life histories and intrauterine environmental encounters are hypothesized to influence offspring phenotypes, multiplex models of exposure with the potential to identify additive or synergistic interactions between these life stages have yet to be reported. The objective of this study was to develop a proof of principle multiplex model that would test the ability of paternal exposure history to synergistically interact with an unrelated intrauterine encounter and influence both the prevalence and severity of environmentally induced pediatric disease. Our data indicate that our two completely separate mouse models of exposure do interact and influence postnatal growth. Although compelling, our studies are preliminary. Given the previously unobserved cannibalistic behavior of the dams, we acknowledge that the reduced growth of exposed offspring may be partially due to a lack of maternal care, and not directly to exposure. Further, due to the postnatal loss of litters in the Control-PM, EtOH-FA, and EtOH-PM treatment groups due to cannibalism, we were unable to use average litter weights as the statistical unit or extend our observations to more functional measures of offspring health. Despite these limitations, we propose that combinatorial interactions between parental life histories and gestational exposures represent a significant influence on the incidence of congenital defects and disease. Future studies are necessary to determine the mechanisms by which the memories of exposures persist and how they interact.

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