Uterine Artery Flow and Offspring Growth in Long-Evans Rats following Maternal Exposure to Ozone during Implantation


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BACKGROUND: Epidemiological studies suggest that increased ozone exposure during gestation may compromise fetal growth. In particular, the implantation stage of pregnancy is considered a key window of susceptibility for this outcome.

OBJECTIVES: The main goals of this study were to investigate the effects of short-term ozone inhalation during implantation on fetal growth outcomes and to explore the potential for alterations in uterine arterial flow as a contributing mechanism.

METHODS: Pregnant Long-Evans rats were exposed to filtered air, 0.4 ppm ozone, or 0.8 ppm ozone for 4 h/d during implantation, on gestation days (GD) 5 and 6. Tail cuff blood pressure and uterine artery Doppler ultrasound were measured on GD 15, 19, and 21. To assess whether perimplantation ozone exposure resulted in sustained pulmonary or systemic health effects, bronchoalveolar lavage fluid, serum metabolic and inflammatory end points, and kidney histopathology were evaluated in dams at GD 21. Growth parameters assessed in GD 21 offspring included fetal weight, length, and body composition.

RESULTS: Measures of maternal uterine arterial flow, including resistance index and mean velocity, indicated that resistance increased between GD 15 and GD 21 in 0.8 ppm dams but decreased in controls, although absolute values were similar in both groups on GD 21. Ozone-exposed dams also had lower serum glucose and higher free fatty acid concentrations than controls on GD 21. On GD 21, both male and female offspring had lower body weight than controls, and pooled subsets of 3 male and 3 female fetuses from litters exposed to 0.8 ppm ozone had lower lean mass and fat mass than pooled control offspring.

CONCLUSIONS: Findings from our experimental model suggest that the offspring of dams exposed to ozone during implantation had reduced growth compared with controls, possibly as a consequence of ozone-induced vascular dysfunction. https://doi.org/10.1289/EHP2019

Introduction

Intrauterine growth restriction (IUGR) is an adverse pregnancy outcome defined by Peleg et al. (1998) as a reduction in fetal weight (below the 10th percentile for gestational age) with an abdominal circumference below the 2.5th percentile, although inconsistencies in the definition of IUGR exist. IUGR affects 5.8–11.2% of all pregnancies in the United States, varying by ethnicity (Kramer 2003), and 23.8% of pregnancies in developing countries (de Onis et al. 1998). Growth-compromised offspring have increased risk of perinatal death (Bernstein et al. 2000) and of later-in-life adverse health outcomes including neurodevelopmental delay (Murray et al. 2015) and cardiometabolic diseases (Cianfarani et al. 2012). The pathogenesis of IUGR is complex and incompletely understood (Sankaran and Kyle 2009), and effective prevention recommendations and treatment options are currently limited.

Known risk factors for IUGR include, but are not limited to, poor maternal nutrition, infection, gestational hypertension (preeclampsia), and diabetes (Albu et al. 2014). With regard to air pollutants, studies have found relationships between prenatal ozone exposure and reduced birth weight (Zheng et al. 2016). More specifically, Tu et al. (2016) reported that ambient ozone was inversely associated with term birth weight among 105,818 singleton births in the state of Georgia. Similarly, a study in 3,545,177 singleton births in California also demonstrated an inverse relationship with ozone exposure and birth weight (Morello-Frosch et al. 2010). In contrast, others have not found associations between ozone exposure during pregnancy and fetal weight. For example, Liu et al. (2007) failed to find a positive association between IUGR and gestational ozone exposure in Canadian singleton births between 1986 and 2000; Gouveia et al. (2004) also did not find a relationship between ozone exposure and low birth weight in 213,973 births in São Paulo, Brazil. Inconsistent findings among observational studies may be related to differences in seasonal temperature or to variability in sensitivity at different stages of gestation. Arroyo et al. (2016) reported a positive relationship between second-trimester ambient temperature and low birth weight, as well as a similar relationship between first-trimester ozone exposure and birth weight in all singleton births in Madrid, Spain between 2001 and 2009. Additionally, Geer et al. (2012) reported that estimated ozone exposure during the first and second trimesters was associated with lower term birth weight among >1,500,000 births in Texas between 1998 and 2004. Further, among California participants in the Children’s Health Study, positive associations between ozone and IUGR were reported for exposures during both the second and third trimesters (Salam et al. 2005). Thus, the influence of the period during gestation in which exposure to ozone takes place requires further study.

Adverse pregnancy outcomes such as IUGR may initiate from suboptimal implantation in the uterine wall [reviewed by Young et al. (2010)]. It is hypothesized that disrupted stem cell invasion may lead to poor spiral and uterine artery remodeling with subsequent impaired placental development. In turn, the stress from...
diminished uterine arterial blood flow and insufficient placental nutrition may result in maternal systemic endothelial dysfunction and inflammation, hypertension, and kidney injury related to glomerular damage. Poor arterial remodeling also impairs fetal blood flow, which may promote disrupted placental development and decreased fetal weight gain.

Given this evidence, we hypothesized that ozone exposure exclusively during the period of implantation receptivity in the rat would compromise fetal growth and maternal health in late gestation. Anticipating that impairments in fetal growth may be related to diminished uterine arterial flow, we used Doppler ultrasonography periodically throughout gestation to assess changes in uterine artery blood flow. The duration of implantation receptivity in rats and humans is similar (2 d). Thus, investigating the effects of ozone exposure during the time of implantation may provide a plausible mechanism that links gestational ozone exposure and IUGR.

**Methods**

**Rats and Experimental Design**

Timed-pregnant Long-Evans female rats (*n* = 10/group) from Charles River Laboratories were transferred to the U.S. Environmental Protection Agency (EPA) animal facility on gestation day (GD) 1 (plug-positive day). All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the National Health and Environmental Effects Research Laboratory, an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility. All animals were treated humanely and with regard for alleviation of suffering. A modified, phytoestrogen-free American Institute of Nutrition growth and lactation diet (D15092401; Research Diets) was provided *ad libitum* to dams after arrival. Food intake and body weight were measured daily. The primary caretaker was blinded to the exposure groups throughout the study.

Rats were randomly assigned to one of three groups: filtered air, 0.4 ppm ozone, or 0.8 ppm ozone. These concentrations were selected based on evidence that there is a four- to five-fold difference in relative lung ozone dosimetry between resting rats and humans exercising heavily on an intermittent basis, as determined by heavy (13O-labeled) reaction product concentrations in the lung (Hatch et al. 1994). Thus, the 0.4 and 0.8 ppm concentrations in rats correspond approximately to 0.08 and 0.16 ppm exposure concentrations, respectively, in humans. The approved 2015 U.S. national ambient air quality standard (NAAQS) for ozone is 0.070 ppm, with an 8-h averaging time (U.S. EPA 2015).

On GD 5 and 6, rats were exposed for 4 h (0700–1100 hours) in Rochester-style “Hinners” chambers that were controlled for temperature (22.5 ± 0.14°C) and humidity (50.0 ± 0.65%). Ozone was generated in a silent arc discharge generator (OREC Model 400; Teledyne Instruments). To quantify the extent of ozone-induced ventilatory dysfunction (which has not been previously reported in pregnant Long-Evans rats), conscious, unrestrained rats were placed in whole-body plethysmographs immediately following exposure. Estimates of tidal volume, breathing frequency, minute volume, peak flow rates, and enhanced pause [Penh; an index of airflow limitation and a surrogate measure for bronchoconstriction (Hamelmann et al. 1997)] were obtained using emka iox2 software (emka TECHNOLOGIES).

On GD 15, 19, and 21, blood pressure was measured using tail cuff photoplethysmography. Rats were acclimated to a Plexiglass restraint tube for 5 min at 22.2–23.3°C and then for 5 min in a prewarmed 32°C chamber. Tail blood pressure was determined from 3–5 readings of each rat on an IITC Life Science Inc. system and was analyzed using Model 51 NIBP software (v.1.32; IITC Life Science Inc.).

**Uterine Artery Blood Flow**

Because blood flow through the uterine artery does not increase until GD 14 (Osol and Mandala 2009), we assessed uterine arterial blood flow after that time point. On GD 15, 19, and 21, pulsed-wave Doppler ultrasonography (Vevo 2100 Imaging System; FUJIFILM VisualSonics Inc.) was performed to evaluate blood flow in the left uterine artery, similar to procedures that have been described previously (Qu et al. 2014). In brief, rats were anesthetized with 3% isoflurane and were maintained at 1.5% with a targeted heart rate range of 400–450 beats per minute. Dams were placed in dorsal recumbency on a heated monitoring platform (FUJIFILM VisualSonics Inc.), which allowed monitoring of heart rate and electrocardiogram (ECG) traces, respiration rate, and body temperature. Fur was clipped from the abdomen, and a depilatory agent was used to remove any remaining fur. Pulsed-wave Doppler ultrasonography was performed using a 12.5-MHz MS201 transducer (FUJIFILM VisualSonics Inc.). All measurements and analyses were performed using Vevo LAB software (v.1.7, FUJIFILM VisualSonics Inc.) by researchers who were blinded to the experimental groups. Data were derived by analyzing six arterial pulsatile blood flow waveforms from each dam at each time point. Peak systolic velocity (PSV) was measured at the point of peak velocity, and end diastolic velocity (EDV) was marked at the next ECG R-wave after PSV was measured (Figure 1). Markers of resistance were calculated using the following formulas: resistance index = (PSV – EDV)/PSV; pulsatility index = (PSV – EDV)/mean blood velocity (mV); and systole/diastole (S/D) ratio = (PSV/EDV).

**Necropsy and Tissue Collection**

On GD 21, dams were euthanized with a lethal intraperitoneal dose of pentobarbital (>400 mg/kg) following a 5 h fast; this dose also euthanized the fetuses in utero. Whole blood was obtained from the abdominal aorta and was collected into serum separator tubes and ethylenediaminetetraacetic acid (EDTA) tubes for plasma collection. Following centrifugation for 10 min at 2,465 × g and 4°C, aliquots were prepared from the resulting supernatant and were stored at −80°C until use. Bronchoalveolar lavage fluid (BALF) was collected and analyzed from dams as previously described (Wallenborn et al. 2009). Briefly, the following markers of pulmonary injury were assessed: total protein (μg/mL BALF), albumin (μg/mL BALF), lactate dehydrogenase (LDH) activity [U/L (units per liter) BALF], N-acetyl glucosaminidase (NAG) activity (U/L BALF), and γ-glutamyl transferase (GGT) activity (U/L BALF). The following markers of pulmonary inflammation were assessed: alveolar macrophages, eosinophils, lymphocytes, and neutrophils. The right kidney was collected, divided midsagittally, placed in Bodian’s fixative (2% formaldehyde, 5% acetic acid, 72% ethanol, and 21% water), and stored at 4°C for histopathology. The gravid uterus was weighed and assessed for implantation sites. Fetuses were counted and assessed for crown-rump length, weight, and sex. Ultimately, one dam from the air group and one dam from the 0.4 ppm ozone–exposed group were removed from the study at the time of necropsy owing to insufficient litter size (*n* < 5 fetuses). Excessively small litters reduce maternal nutritional requirements (Mattingly and Mcclure 1982) and promote larger fetal size because of reduced competition for nutrients within the litter (Romero et al. 1992).
Status in the mother (Mihu et al. 2015). Hence, in a multiplexed assay including interferon-
kit provided a broad spectrum of proinflammatory cytokines in a multiplexed assay including interferon-γ (IFN-γ), interleukin-1β (IL1β), interleukin 4 (IL4), interleukin 5 (IL5), interleukin 6 (IL6), interleukin 10 (IL10), interleukin 13 (IL13), keratinocyte chemoattractant/human growth-regulated oncogene (KC-GRO), and tumor necrosis factor-α (TNFα). Assay was performed according to the manufacturer’s protocols.

Inflammatory Measurements
Preeclampsia is associated with increased serum inflammatory status in the mother (Mihu et al. 2015). Hence, inflammation markers were measured in maternal serum using a Meso Scale Proinflammatory Panel 2 kit for rats on a Meso Scale QuickPlex SQ 120 system (Meso Scale Diagnostics, LLC). This predesigned kit provided a broad spectrum of proinflammatory cytokines in a multiplexed assay including interferon-γ (IFN-γ), interleukin-1β (IL1β), interleukin 4 (IL4), interleukin 5 (IL5), interleukin 6 (IL6), interleukin 10 (IL10), interleukin 13 (IL13), keratinocyte chemoattractant/human growth-regulated oncogene (KC-GRO), and tumor necrosis factor-α (TNFα). Assay was performed according to the manufacturer’s protocols.

Histopathology
After fixation, right kidneys were embedded in paraffin wax and sectioned at 5 μm. Kidney sections were deparaffinized and stained with hematoxylin and eosin (H&E) (BBC Biochemical) using standard procedures and were evaluated via light microscopy by a certified study pathologist (C.E.W.). According to best practices for nonclinical safety studies (Crissman et al. 2004), primary histopathological review was performed unblinded to experimental treatment, and findings that might have been treatment-related or might otherwise have affected interpretation were re-reviewed in a blinded manner to confirm differences from controls, severity grade, or both. Histopathological diagnoses were based on standard criteria and nomenclature (Frazier et al. 2012). Severity of lesions was qualitatively graded using a 0–4 scale (0 = absent, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe) based on increasing extent, complexity of change, or a combination of the two.

Fetal Body Composition Measurements
At necropsy, three arbitrarily selected fetuses from each sex were grouped for each litter and were stored at 4°C. If there was an insufficient number of fetuses, a measurement could not be obtained. Hence, an n = 7/litter group was analyzed for air and for 0.8 ppm ozone, whereas an n = 8/litter group was analyzed for 0.4 ppm ozone. After warming to room temperature (22.2–23.3°C), body composition (grams of fat and lean body mass) was measured using magnetic resonance on the n = 3 representative group for each litter (measured at the same time) using an EchoMRI™-100 system (EchoMRI™) at the University of North Carolina at Chapel Hill Nutrition Obesity Research Center.

Statistics
For fetal growth metrics, all analyses were performed separately by sex, and litters were pooled by sex. Data were analyzed using a two-tailed Fisher’s least significant difference (LSD) test for all planned multiple comparisons (filtered air vs. 0.4 ppm vs. 0.8 ppm ozone for all data) within a one-way analysis of variance (ANOVA) (GraphPad Prism v.6; GraphPad Software, Inc.). Fisher’s LSD test does not correct for multiple comparisons; however, it can be used in study designs with three groups without increasing Type I error rate (Liu and Tu 2008). Upon determining that fetal weight was reduced in fetuses from ozone-exposed pregnancies, a priori body composition measurements were performed. Therefore, a one-tailed Fisher’s LSD test within a one-way ANOVA was used to assess differences in fetal body composition. Finally, because renal pathology findings were only assessed in the positive direction (i.e., increasing pathological changes), histopathology incidence data were analyzed using a one-sided Fisher’s exact test. For all measurements, significance was set at p < 0.05.
Results

Effects of Ozone Exposure during Implantation on Dams

Food intake and body weight were monitored throughout gestation as markers of growth and overall maternal health. Because the size of the litter affects the nutrient demands of the pregnant rodent (Mattingly and McClure 1982), the two dams having litters with <5 fetuses (one from the air group and one from the 0.4 ppm ozone group) were removed from all analyses in the study. Exposure to 0.8 ppm ozone reduced 48 h body weight gain and food intake in pregnant dams over the exposure period (difference between GD 5 and GD 7) (Figure 2A, B). There were no significant differences between treatment groups in total weight gain or in food intake during pregnancy (Table 1).

Whole-body plethysmography assessments performed immediately after ozone exposure on GD 5 and 6 and assessments of controls were performed at the same time of day. Penh, an index of airway resistance, was significantly higher in the 0.8 ppm ozone group than in controls on both days and in the 0.4 ppm ozone group on GD 5 (Figure 2C), whereas minute volume was significantly lower in the 0.8 ppm ozone group compared with controls on GD 6 (Figure 2D). However, both Penh and minute volumes were comparable among controls and both exposure groups on GD 15, 19, and 21. Therefore, exposure to ozone did not appear to have lasting effects on respiratory parameters after GD 15.

Table 1. Litter characteristics according to exposure group [mean ± standard error of the mean (SE)].

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Air</th>
<th>0.4 ppm</th>
<th>0.8 ppm</th>
<th>ANOVA p-value (totals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of litters maintained to GD 21</td>
<td>9</td>
<td>9</td>
<td>10</td>
<td>0.35</td>
</tr>
<tr>
<td>Gestational weight gain (g)</td>
<td>152.4±5.83</td>
<td>147.6±5.91</td>
<td>139.6±6.81</td>
<td>0.35</td>
</tr>
<tr>
<td>Gestational food intake (g)</td>
<td>521.6±15.8</td>
<td>492.9±18.4</td>
<td>503.2±16.8</td>
<td>0.50</td>
</tr>
<tr>
<td>Implantation sites per litter</td>
<td>13.5±0.64</td>
<td>14.1±0.59</td>
<td>13.2±0.57</td>
<td>0.57</td>
</tr>
<tr>
<td>Resorptions per litter</td>
<td>1.4±0.31</td>
<td>2.1±0.77</td>
<td>1.5±0.50</td>
<td>0.62</td>
</tr>
<tr>
<td>Full term fetuses</td>
<td>12.1±0.50</td>
<td>12.0±0.37</td>
<td>11.7±0.72</td>
<td>0.87</td>
</tr>
<tr>
<td>Percent males per litter</td>
<td>47.0±3.95%</td>
<td>50.6±4.55%</td>
<td>50.4±5.24%</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Note: ANOVA, analysis of variance; GD, gestational day.
*Owing to low litter size (<5 fetuses), one rat each was removed from the air and the 0.4 ppm ozone group data sets.
*Data were analyzed by two-tailed Fisher’s least significant difference (LSD) posttest within a one-way ANOVA.
Effects of Peri-implantation Exposure to Ozone on Maternal Health in Late Gestation

We used Doppler ultrasonography to assess the effects of ozone exposure during implantation on maternal uterine artery blood flow at various points during gestation. In a normal pregnancy, resistance to blood flow decreases in the uterine artery, which facilitates perfusion of the fetoplacental unit (Mose 2014). In air-exposed dams, mean uterine arterial resistance [represented by the resistance index (RI)] decreased 3.3% (±2.8%) from GD 15 and 21, and mV increased 4.6% (±8.6%) (Figure 3B, E; see also Table S1). The mean RI for 0.8 ppm ozone–exposed dams was slightly lower than that of controls on GD 15 (0.59 ± 0.01 vs. 0.61 ± 0.01, respectively) but was comparable to that of controls on GD 21 (both 0.60), resulting in a 2.2% increase (±2.1%, p < 0.10) from GD 15 and GD 21, in contrast to a 3.3% decrease (±2.8%) in the controls over the same time period. Patterns were similar for the systolic/diastolic (S/D) ratio and the pulsatility index (PI) (Figure 3C, D; see also Table S1), which are additional indicators of resistance. The average mV was significantly higher in 0.8 ppm ozone–exposed dams than in controls on GD 15, and although it decreased from GD 15 to GD 21 (23% ± 9.2%), there was no significant difference in the average mV between 0.8 ppm dams and controls on GD 21 (Figure 3E; see also Table S1).

Examination of a panel of nine proinflammatory cytokines in the serum of dams on GD 21 revealed no significant differences among the control and treatment groups (see Table S3). In addition, there were no significant differences among groups in BALF cell counts (alveolar macrophages or other leukocytes) or in protein concentrations in BALF collected on GD 21 (see Table S4). BALF concentrations of GGT and NAG were significantly higher in 0.4 ppm ozone–exposed dams than in controls, but concentrations in BALF from 0.8 ppm dams were not significantly different from those in controls.

Effects of Peri-implantation Exposure to Ozone on Developmental End Points

All dams successfully maintained their pregnancies to GD 21. After excluding one dam from the control group and one from the 0.4 ppm ozone group that each had <5 fetuses per litter, there were no significant differences among the treatment groups with regard to implantation sites or resorptions on GD 21, or with regard to the sex distribution of the fetuses (Table 1).

There were no significant differences among treatment groups in the average length of male and female fetuses from each litter on GD 21 (Figure 4A and B, respectively). However, average weights of fetuses in each litter were lower in the litters exposed to 0.4 ppm and 0.8 ppm ozone on GD 5 and 6 compared with controls (significant for males in both exposed groups and for females in the 0.8 ppm group). Reduced fetal weight with normal fetal length is consistent with asymmetrical IUGR, which is purportedly related to reduced fetal nutrition and oxygen supply in the second half of pregnancy (Moh et al. 2012); this is in contrast with symmetrical IUGR, in which both weight and length are...
reduced owing to poor cellular development in the first trimester, the second trimester, or in both (Brodsky and Christou 2004).

Because asymmetrical IUGR is associated with reduced adipose tissue deposition due to decreased nutritional supply in the second half of pregnancy (Lapillonne et al. 1997), we next investigated the potential influence of ozone-induced growth restriction on the body composition of the offspring. Average litter values for lean mass and fat mass (based on pooled data for 3 randomly selected male and female fetuses from each litter) were lower in the ozone-exposed litters (n = 8 for 0.4 ppm, n = 7 for 0.8 ppm) than in controls (n = 7), with significant differences from controls (based on the one-tailed Fisher’s LSD posttest) in the 0.8 ppm groups for both outcomes in both male and female fetuses (Figure 4C, D). The ratio of fat mass to lean body mass [fat mass (g)/lean body mass (g) of the pooled group for each litter, separated by sex] was then assessed because asymmetrical IUGR is related to reduced fat development in utero (Lapillonne et al. 1997), and the ratio provides an estimate of relative adiposity. Although the fat-to-lean mass ratio was not statistically different (based on the one-tailed Fisher’s LSD posttest) in female offspring from ozone-exposed pregnancies, male offspring from 0.8 ppm ozone–exposed dams had a modest, nonstatistically significant reduction in fat mass relative to lean mass (p = 0.06; Figure 4E, F).

Mean serum FFAs on GD 21 were significantly higher in dams exposed to either concentration of ozone than in air controls (Figure 5A), whereas mean blood glucose concentrations were significantly lower in ozone-exposed dams (Figure 5B). There were no significant differences in other maternal serum parameters on GD 21, including insulin and triglycerides, among the treatment groups (see Table S5).

Discussion

In this study, the offspring of Long-Evans rats with acute ozone exposure exclusively during the period of implantation

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Figure 4. Body size measurements in male and female offspring on gestational day (GD) 21 according to treatment group and sex. (A) and (B) Distribution of litter average values for length (cm) and weight (g) in males and females, respectively, from each litter (n = 9, 9, and 10 litters for air, 0.4 ppm ozone, and 0.8 ppm ozone groups, respectively). (C) and (D) Distribution of litter average values for fat mass and lean mass (relative to controls) in males and females, respectively (n = 3 fetuses pooled per litter for each group and sex; n = 7, 8, and 7 litters for air, 0.4 ppm ozone, and 0.8 ppm ozone groups, respectively). (E) and (F) Distribution of litter average values for fat mass to lean mass ratio in males and females, respectively (calculated by the obtained fat and lean mass for the n = 3 fetuses pooled per litter for each group and sex; n = 7, 8, and 7 litters for air, 0.4 ppm ozone, and 0.8 ppm ozone groups, respectively). The data were analyzed by one-way analysis of variance (ANOVA) within a two-tailed Fisher’s least significant difference (LSD) posttest (for length and weight) or a one-tailed Fisher’s LSD posttest (for fat mass, lean mass, and fat-to-lean mass ratios). Boxes extend from the 25th to the 75th percentile, horizontal bars represent the median, whiskers indicate the 10th and 90th percentiles, and outliers are represented as points. Average values for individual litters (by sex) are overlaid on each plot. # p < 0.10, * p < 0.05, ** p < 0.01 versus air controls.
were evaluating. The prenatal environment, including both fetal development and placental adaptations, differs between males and females (Buckberry et al. 2014; Reinius and Jazin 2009). We did not formally compare differences in outcomes between male and female offspring in this study, but potential sex-specific health implications of prenatal exposures to air pollutants on the risk of adverse pregnancy outcomes present an important subject for future research.

The short duration of ozone exposure was chosen to target the timing of the receptivity window for implantation in the rat, which occurs at approximately GD 5 to GD 6.75 (Witschi 1962). The suspected origin of some adverse pregnancy outcomes, including IUGR, is impaired implantation of the blastocyst into the uterine wall (Young et al. 2010). According to accepted theory, blastocysts that do not implant at the correct depth and length can affect the ability of stem cells to form the placenta and engage in arterial remodeling. Our results suggest that implantation may be a critical time window for the effects of ambient ozone on IUGR, although we cannot rule out potential effects of exposure during other time periods. Our findings also support the value of our experimental model for the study of environmental pollutant effects on gestational outcomes in general. Epidemiological studies have reported associations between ambient air pollution exposures during early pregnancy and a variety of outcomes; for example, fetal growth parameters measured at 13–26 wk of gestation (Hansen et al. 2008), preterm birth and preeclampsia (Ohsson et al. 2013), and placenta previa (Michikawa et al. 2016).

Average maternal serum FFA concentrations on GD 21 were higher in the exposed groups than in the controls in our study, consistent with findings from a case–control study reporting that serum FFAs at the time of cesarean section were higher in 24 women with IUGR pregnancies than in 30 women delivering term births by cesarean section and in 25 women at 35 wk gestation (Ortega-Senovilla et al. 2009). The higher FFAs and lowered blood glucose in exposed dams compared with controls might be related to a prolonged stress response resulting in increased lipolytic capacity as an adaptive mechanism to raise FFA availability and glucose uptake in the fetus, as has been observed in studies of pregnant ewes (Hay 2006). In the present study, we did not investigate the role of placental insufficiency in reducing fetal growth. In principle, disrupted implantation would also impair placental development. Thus, it is plausible that in this model, poor placental vascular development further compounded impaired uterine blood flow, resulting in reduced oxygen and nutrients available for development. In addition to assessing altered uterine blood flow, studies are in progress to determine if ozone exposure caused placental insufficiency and dysfunction in our model.

A previous study of mice exposed to urban air pollutants during the entirety of gestation reported that the maternal blood surface area and diameter in the placenta was reduced in exposed mice compared with controls (Veras et al. 2008). A recent study of diesel exhaust exposure throughout gestation in rabbits also reported reduced placental blood flow as measured by 3D Doppler ultrasound (Valentino et al. 2016). Relatedly, a recent prospective cohort study of 366 pregnant women reported that ambient ozone exposure during the second trimester was associated with higher umbilical artery PI (greater vascular resistance), whereas exposure during the third trimester was associated with lower PI values (Carvalho et al. 2016). Together, these studies suggest the potential for gestational exposure to air pollutants to disrupt placental function, thus contributing to impaired fetal weight gain.

In otherwise healthy Long-Evans rats, ozone exposure exclusively during implantation was not associated with significant

![Figure 5. Serum free fatty acids (A) and blood glucose (B) on gestational day (GD 21) dams exposed to air (n = 9), 0.4 ppm ozone (n = 9), or 0.8 ppm ozone (n = 10) on GD 5 and GD 6. The data were analyzed by two-tailed Fisher’s least significant difference (LSD) posttest within a one-way analysis of variance (ANOVA). Boxes extend from the 25th to the 75th percentile, horizontal bars represent the median, whiskers indicate the 10th and 90th percentiles, and outliers are represented as points. Values for individual dams are overlaid on each plot. * p < 0.05, ** p < 0.01 versus air controls.](image-url)
differences in renal pathology or markers of systemic inflammation in the dams at the end of gestation. Epidemiological findings have suggested that maternal ozone exposure may induce preeclampsia (Pedersen et al. 2014), a potentially fatal pregnancy disorder. Although we did not observe any signs of preeclampsia development in ozone-exposed dams (e.g., altered blood pressure, renal changes), it is important to note that the increased uterine arterial resistance observed in rats exposed to ozone during implantation is similar to that occurring in preeclamptic pregnancies and is indicative of impaired arterial remodeling. Uterine and spiral arterial remodeling are hypothesized to contribute to the etiology of preeclampsia (Young et al. 2010).

Many of the cardiometabolic and pulmonary effects of acute ozone exposure in rodent models are believed to occur in part because of impaired respiration (Jang et al. 2003), induction of cardiac arrhythmia (Farraj et al. 2012), glucose intolerance (Bass et al. 2013), and activation of the stress response (Kodavanti et al. 2013), and activation of the stress response (Kodavanti et al. 2013). The cardiometabolic and pulmonary effects induced by acute ozone exposure further complicate the identification of mechanisms for the observed reduction in fetal weight; however, the findings presented here suggest an important interplay between the quality of implantation and alterations in arterial remodeling across gestation.

Conclusion

To our knowledge, we are the first to report experimental evidence that ozone exposure during early pregnancy may cause growth restriction in offspring. Our findings suggest that exposure during the period of implantation receptivity, which has a similar (approximately 2-d) duration in rats and humans, may cause diminished uterine artery flow later in gestation. Because 40% of U.S. children reside in areas that do not meet the NAAQS for ozone (McCarthy and Lattanzio 2015), many (approximately 2-d) duration in rats and humans, may cause diminished uterine artery flow later in gestation. Because 40% of U.S. children reside in areas that do not meet the NAAQS for ozone (McCarthy and Lattanzio 2015), many of the cardiometabolic and pulmonary effects induced by acute ozone exposure further complicate the identification of mechanisms for the observed reduction in fetal weight; however, the findings presented here suggest an important interplay between the quality of implantation and alterations in arterial remodeling across gestation.

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