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Aspirin pre-treatment modulates ozone-induced fetal growth restriction and alterations in uterine blood flow in rats

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Abstract

Prenatal exposure to ozone has been linked to low birth weight in people and fetal growth restriction in rats. Clinical recommendations suggest use of low dose aspirin to lower risk of preeclampsia and intrauterine growth restriction in high-risk pregnancies, yet its utility in mitigating the postnatal effects of gestational ozone exposure is unknown. The present study investigated the possibility of low dose aspirin to mitigate the effects of ozone exposure during pregnancy. Exposure to ozone impaired uterine arterial flow and induced growth restriction in fetuses of both sexes. Aspirin treatment induced marginal improvements in ozone-induced uterine blood flow impairment. However, this resulted in a protection of fetal weight in dams given aspirin only in early pregnancy. Aspirin administration for the entirety of gestation increased placental weight and reduced antioxidant status, suggesting that prolonged exposure to low dose aspirin may induce placental inefficiency in our model of growth restriction.

Keywords

aspirin; ozone; intrauterine growth restriction; uterine arterial flow; placenta; antioxidants

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Introduction

In the U.S., approximately 40 million people live in areas with ozone levels that exceed the National Ambient Air Quality Standard (NAAQS) established by the U.S. Environmental Protection Agency (EPA) [1]. Exposure to ozone, an oxidant gaseous air pollutant, results in a variety of health effects in both humans and animals. Acute effects include increased pulmonary vascular leakage [2], disruption of glucose homeostasis [3], and cardiac autonomic dysfunction [4]. Epidemiological studies further indicate that ozone exposure during gestation is associated with various adverse pregnancy outcomes including preeclampsia [5], intrauterine growth restriction (IUGR) [6], and gestational diabetes [7]. We recently reported that gestational exposure to ozone in rats resulted in reduced fetal growth and increased uterine artery resistance to blood flow without evidence of preeclampsia in the dam [8]. While the cardiopulmonary and metabolic effects have been thoroughly investigated in nonpregnant models, the mechanisms by which ozone exposure may impact pregnancy outcomes are poorly understood.

Aspirin is a nonsteroidal anti-inflammatory drug widely used both as an analgesic and as a prophylactic in the prevention of cardiovascular complications [9]. In fact, daily low-dose aspirin is currently recommended for use in the prevention of preeclampsia in individuals considered to be at higher risk for pregnancy complications [10]. Further, the protective effects of low-dose aspirin have also been associated with improving the risk of IUGR [11]. To date, however, randomized control trials report conflicting evidence on the beneficial impact of aspirin use for the prevention of preeclampsia and/or IUGR, and the studies indicating positive associations raise additional questions about the optimal dosage [11] and timing of aspirin administration [12], as well as underlying maternal conditions [13,14]. Most recently, the Aspirin for Evidence-Based Preeclampsia Prevention (ASPRE) trial found that in well-identified high-risk pregnancies, incidence of preeclampsia was reduced in the aspirin-treated group [15]. However, fetal adverse outcomes such as IUGR did not appear to differ between groups. Nevertheless, based on the collective evidence to date, early, first trimester initiation of aspirin may reduce the incidence of preeclampsia and possibly IUGR in certain high-risk pregnancies [11,16].

To our knowledge, the ability of aspirin to mitigate IUGR in a rodent model has not been thoroughly examined. However, a study by Ates *et al.* (2007) reported that low-dose aspirin treatment from estrus into GD4 increased the thickness of the uterine endometrial lining, which may improve the quality of implantation and protect against adverse pregnancy outcomes related to poor implantation [17]. Therefore, in the present study, we hypothesized that prophylactic, aspirin administration (0.6 mg/day; equivalent to 2 mg/kg in a 250 g rodent) in Long-Evans rats could lessen the degree of ozone-mediated fetal growth restriction. Herein, we investigated the influence of aspirin administration for two durations; the first spanning GD1 through implantation (GD7), and the second covering the majority of gestation (GD 1 – 20). Ozone-induced effects on maternal weight gain, uterine arterial flow, and fetal growth characteristics were evaluated at GD21. In addition, we evaluated differences in placental mitochondrial, nutrient, and antioxidant status related to peri-implantation ozone exposure with or without aspirin administration.

Materials and Methods

Animal Model and Experimental Timeline

GD1 (plug-positive) Long-Evans rats were purchased from Charles River Laboratories (Raleigh, NC). The Long-Evans strain was selected for this work as they are known to produce larger litter sizes [18] and have more attentive maternal behavior [19] compared to available data reported in other strains. Dams were 11 weeks of age at breeding. This age was chosen because breeding of less mature rats (e.g., 8-weeks) is associated with greater estrous cycle irregularity [20], less pregnancy success [21], and smaller fetuses [22]. Lastly, water and a phytoestrogen-free, growth and lactation diet (Research Diets, D15092401, Table 1), which removes the influence of contaminating phytoestrogens that are known to have uterotrophic [23], behavioral [24], and metabolic effects in rodents [25], were provided *ad libitum* throughout the study. Food intake and body weight were monitored daily.

To determine if low-dose aspirin administration could mitigate the ozone-induced effects on fetal growth, dams were randomized into air (group 1) or ozone (groups 2, 3 and 4) exposure groups. Groups 1 and 2 were controls and the remaining groups were further randomized into one of two aspirin treatment groups: aspirin administration between GD 1-7 (7-day; group 3) or aspirin administration between GD 1-20 (20-day; group 4). Aspirin was delivered in a banana-flavored Supreme Mini-Treat (Bio-Serv, Flemington, NJ) at 0.6 mg/day (2 mg/kg in a 250 g rat) to approximate the low-dose recommendation in humans. We chose not to deliver aspirin by oral gavage as this procedure comes with the risk of aspiration, induces some degree of an acute stress response [26], increases blood pressure and heart rate [27], and may affect neurodevelopment in the offspring [28]. To ensure that each dam consumed the entire treat at approximately the same time each day, the treat was coated in a small amount of peanut butter, which ensured immediate consumption in front of the investigators. A placebo mini-treat was provided for groups 1 and 2 to maintain behavioral consistency between all groups. The study design is summarized in Fig. 1; no air + aspirin groups were investigated in the current study as low dose aspirin use is currently recommended in high-risk pregnancies with no known adverse effects [10]. The Institutional Animal Care and Use Committee of the U.S. Environmental Protection Agency approved all procedures prior to the start of the study (Protocol #18-08-003).

Ozone Exposure

As described recently [8], dams underwent whole body inhalation exposures to either filtered air or ozone (0.8 ppm $\times$ 4 hours, between 0700 - 1100) on GD5 and again on GD6. Briefly, ozone was generated from a silent arc discharge generator (OREC; Phoenix, AZ) and dispersed into Rochester style “Hinners” chambers. Ozone concentrations were measured continuously throughout the exposures using photometric analyzers (API Model 400, Teledyne Instruments; San Diego, CA) and temperature was maintained at approximately 23.3°C and 46% relative humidity. In our previous study [8], the higher exposure concentration (0.8 ppm $\times$ 4 hours) appeared to result in significantly reduced fetal weight at GD21 in both male and female fetuses, whereas the lower concentration (0.4 ppm $\times$ 4 hours) primarily affected male offspring. To better assess whether aspirin administration would ameliorate this outcome in both sexes, 0.8 ppm $\times$ 4 hours was selected for the current

study. Lastly, while the 2015 NAAQS for ozone is 0.070 [29], ¹⁸O-labeled ozone experiments have revealed that an approximately five-fold difference in dosimetry exists between the intermittent exercising human and the resting rat [2]. Using this adjustment, a 0.8 ppm exposure concentration in the rat provides similar lung dosimetry to that of 0.16 ppm ozone in an exercising human.

Doppler Ultrasonography

On GD15 and GD21, blood flow through the uterine artery was measured using pulsed-wave Doppler ultrasonography (Vevo® 2100 Imaging System, VisualSonics Inc., Toronto, Ontario) as described previously [8]. Briefly, anesthesia induction was achieved using 3% isoflurane and anesthesia was maintained using 1.5% isoflurane in order to achieve a heart rate of 400 ± 25 beats per minute. Following removal of abdominal fur, pulsed-wave Doppler of the uterine artery was obtained using a 12.5 MHz MS201 transducer (VisualSonics, Inc.). Indicators of arterial resistance including resistance index (RI), pulsatile index (PI), and systolic/diastolic (S/D) ratio were calculated from six pulsatile blood flow waveforms in a blinded manner using the Vevo Lab software (v.1.7, VisualSonics, Inc.).

Necropsy

On GD21 (~1.5 days prior to parturition), dams and their fetuses were euthanized with an intraperitoneal dose of pentobarbital (>400 mg/kg). Whole blood from the dams was collected from the abdominal aorta in EDTA blood collection tubes or serum separator tubes. Complete blood counts (CBC) were performed on a Beckman-Coulter AcT blood analyzer (Beckman-Coulter, Inc., Fullerton, CA) on EDTA blood. Remaining samples were centrifuged at 3500 rpm for 10 minutes at 4°C; serum was stored at -80°C for later use. The gravid uterus was dissected and then weighed. All fetuses were subsequently removed to obtain measurements on weight, length, and sex. The decidual layer of the placenta was removed, and following weighing, the labyrinth zone was snap-frozen and stored at -80°C until further processing. Upon dissection, corpora lutea of both the left and right ovaries were enumerated using a dissecting microscope. Corpora lutea numbers and fetal numbers are positively correlated in rodents [30]. Hence, in a normal rat pregnancy there should be little discrepancy between the number of corpora lutea and implantation sites at the end of pregnancy. Under this assumption, dams with a high divergence between their corpora lutea count and the number of implantation sites ($n = 6$) were removed from the study due to excessive pre-implantation loss and an underlying pregnancy complication unrelated to ozone-exposure; there was no effect of either ozone or aspirin treatment on this assessment. Additional acceptance criteria for inclusion in the study included adequate daily treat consumption and appropriate water and food consumption during the exposure/implantation period as this could indicate a malfunctioning ligit, which would influence the health of the dam during this critical time (see details in Table 2).

Fetal Body Composition Assessment

Body composition was measured by magnetic resonance on the Bruker LF90 II 'minispec' body composition analyzer (Bruker Optics, Inc., Billerica, MA). In order to meet the minimum weight requirement for this system, female and male fetuses from each dam were

separated and randomly pooled into sex-specific groups of three for body composition assessments. If an n=3 fetuses for a specific sex within the litter was not available, data was not obtained for the litter (n=7-10/group/sex).

Platelet Factor-4 Assay

Whole blood was collected through the tail vein at GD7 and at necropsy on GD21 from the abdominal aorta. To obtain serum, blood was centrifuged at 4°C for 10 minutes at 1,000 rpm. Serum samples were stored at -80°C until assayed for the marker of platelet activation, platelet factor 4 (PF-4). Non-hemolyzed GD7 and GD21 samples (n=7-12/group for GD7 and n=5-9/group for GD21) were evaluated using the PF-4 ELISA by Cloud-Clone Corp. (Houston, TX) as per the manufacturer's instructions. As both aspirin groups did not differ at the GD7 measurement, these groups were combined for data analysis. Absorbance was measured on a SpectraMax i3 microplate reader (Molecular Devices, Sunnyvale, CA).

Relative Placental Mitochondrial Quantification

Placental DNA was isolated using the Quick-DNA™ Miniprep Plus Kit (Zymo Research, Irvine, CA) and quantified on the Qubit (ThermoFisher Scientific, Waltham, MA) from a subset of placentas (n=8/group/sex/dam; selected across litters). Primers were designed for a nuclear gene (β -Actin) and a mitochondrial gene (cytochrome c oxidase subunit-1, COX1) using the publically accessible NCBI database. Forward and reverse primers were purchased from Integrated DNA Technologies, Inc. (Coraville, IA): β -Actin f- GATCGTGAGGAACACTCAGAAG, r- CACCCTAGGCGGAAAGTTAAG; COX1 f- TGAGCAGGAATAGTAGGGACAG, r- GGGCTGTGACGATGACATTATAG. Using 15 ng of DNA, the relative difference of mitochondrial DNA was measured by qPCR on a QuantStudio™ 7 system (ThermoFisher Scientific, Waltham, MA) using Sybr Green PCR Master Mix (ThermoFisher Scientific, Waltham, MA).

Placental Metabolic Assessment

A suspension of placental tissue was prepared from frozen samples by homogenizing ~50 mg of tissue in ice-cold RIPA buffer containing protease inhibitors for a subset of samples (n=7-8/group/sex/dam; selected across litters). Homogenates were centrifuged at 10,000 $\times$ g for 10 minutes at 4°C. The supernatant, including the lipid layer, were collected for assessment on the Konelab Arena 30 system (Thermo LabSystems, Espoo, Finland). The following kits were adapted for use on the system: cholesterol and triglycerides (TECO Diagnostics, Anaheim, CA), free fatty acids (Cell Biolabs, Inc, San Diego, CA), glutathione peroxidase activity (GPx; adapted from Jaskot, *et al.* [31]), superoxide dismutase (SOD) activity and total antioxidant status (TAS; Randox Laboratories, Ltd., United Kingdom), and total protein (Coomassie Plus Protein Assay Kit, Pierce Scientific, Rockford, IL). All data were normalized to sample weight (e.g. analyte/mg tissue).

Statistical Analysis

This experimental approach was used to test for differences between each group receiving aspirin (group 3, 7-days or group 4, 20-days) compared to the air (group 1) and ozone (group 2). Statistical differences were calculated using a One-Way ANOVA within a Fisher

LSD post-test. To reduce the influence of litters with extreme values, data was trimmed at the 90th and 10th percentiles for average fetal weight within each group. Significance was set at $p < 0.05$. For data obtained prior to GD8 (food intake and body weight gain during the exposure and PF-4), the groups 3 and 4 data were combined. All statistical analyses and graphs were prepared using GraphPad Prism (v.6.07; La Jolla, CA).

Results

Gestational Weight Gain and Food Intake

Following the first day of ozone exposure (GD5), as expected, ozone-exposed, placebo-treated dams (group 2) failed to gain body weight in a 24-hour period. Aspirin administration from GD 1-7 in ozone-exposed rats blocked the effect of ozone on body weight ($p < 0.01$ vs. group 2 rats; Fig. 2A). No group differences in body weight change were observed on GD6, the second day of the exposure. Across the two-day period of air or ozone exposure, all ozone-exposed rats (groups 2, 3, and 4) had reduced food intake compared to group 1, air-exposed rats ($p < 0.05$; Fig. 2B). Thus, while aspirin administration partially protected against reduced weight gain acutely following the initial exposure, this was not due to increased food intake. In all groups, daily body weight gain (Fig. 2C) and grams of food consumed (Fig. 2D) increased as gestation advanced. However, neither extrauterine weight gain (difference between GD21 and GD5 body weight) nor total food intake differed between groups through the entirety of gestation (Table 2).

Uterine Arterial Blood Flow

No between-group differences of uterine arterial resistance were observed on GD15 or GD21 (Fig. 3A). However, as previously demonstrated [8], air-exposed (group 1) rats showed a significant reduction of the RI, PI, and S/D ratio in the uterine artery from GD15 to GD21 ($p < 0.01$; Fig. 3A–C). Also, like our previous results, this reduction in RI across gestation day was not evident in the group 2, ozone-exposed (placebo) dams. Hence, the data suggest that ozone-exposed rats had poorer arterial adaption relative to air-exposed rats. In ozone-exposed rats receiving aspirin for 7-days (group 3), there was a tendency for reduced uterine resistance at GD 21 compared to GD 15.

In Fig. 3D, differences in arterial resistance indices (e.g. RI, PI, and S/D ratio) are presented as the percent change between GD15 and GD21. Accordingly, group 2 rats showed a negligible change in resistance over that period compared to group 1 across all indices ($p < 0.05$). By comparison, both the group 3 (7-day) and group 4 (20-day) aspirin dams had intermediate values compared to groups 1 and 2, but were not statistically different than either group 1 or 2.

Effects of Gestational Aspirin Treatment on Ozone-Induced Changes in Maternal Health

We assessed serum PF-4 at GD7 and GD21 to estimate changes in activated platelets related to pregnancy and daily aspirin administration. No between-group differences in PF-4 levels were found at GD7 (Table 3). By the end of gestation (GD21), PF-4 levels in all groups were markedly higher than at GD7 (nearly a 300-fold increase), consistent with pregnancy-induced increases in PF-4 [32,33]. Ozone-exposed rats provided with aspirin for 20-days

(group 4) had increased PF-4 levels compared to ozone-exposed, placebo-treated group 2 dams ($p<0.01$). There were no differences observed between group 1 (air) and ozone-exposed rats provided aspirin for 7-days (group 3).

Notably, one rat from group 4 was euthanized on GD16 due to serosanguinous vaginal discharge. Blood collected at necropsy indicated severe anemia (hematocrit of 12%). Histopathologic findings included arteritis of the mesometrial arteries with focal necrosis and hemorrhage, but without evidence of ulceration within the gastrointestinal tract. The serum PF-4 level (4.75 ng/mL) was 300-fold lower than rats at GD21, however there were no PF-4 values from any other rats from this study for comparison at GD16. Findings were consistent with non-immune mediated platelet consumption or destruction, and suggest a likely cause of vaginal discharge being blood loss associated with poor platelet function at or near the implantation sites.

In light of the aforementioned dam, at necropsy we closely examined CBCs and found no between group differences in any parameter measured (Table 3). Lastly, we observed negligible impacts of ozone or aspirin treatment on most of pregnancy outcomes including post-implantation loss, number of fetuses/litter, and the number of litters maintained to GD21 (Table 4). However, the percentage of males was decreased in group 3 compared to group 2 ozone-controls ($p<0.05$), which was attributed to reduced number of males/litter and not an increase in the number of female offspring.

Fetal Growth Characteristics

On GD21, male fetuses from ozone-exposed, placebo-treated (group 2) dams weighed significantly less than air-control (group 1) male fetuses, but were of similar length ($p<0.05$; Fig. 4A). Notably, the average weight of the males in group 3 (ozone-exposed, 7-day aspirin) was not significantly different from group 1, but was increased relative to group 2 males ($p<0.05$). There were no significant differences in male fetal weight between group 4 males (20-day aspirin) compared to groups 1 or 2. Body composition, assessed by magnetic resonance, further revealed that fat mass was reduced in group 2 male fetuses ($p<0.05$; Fig. 4B); whereas, this trend was not evident in male fetuses from aspirin groups 3 or 4. These findings remained consistent in terms of the calculated relative adiposity (fat-to-lean mass ratio); with male fetuses from group 2 having reduced relative adiposity ($p<0.05$). These results are consistent with our previous findings [8]. Conversely, group 4 males had elevated adiposity relative to ozone-controls (group 2). There were no differences in body composition in male fetuses in group 3 (7-day aspirin) compared to groups 1 and 2.

Similar results were observed in the female fetuses. Specifically, females from group 2 had reduced weight at GD21 with no effect on body length ($p<0.05$; Fig. 4C); whereas, group 3 females did not weigh significantly less than air controls (group 1), suggesting a small effect of aspirin on ozone-induced changes in fetal weight ($p=0.07$ vs. group 2, ozone-exposed). By comparison, group 4 female fetuses weighed marginally less than group 1 ($p=0.06$), and their average fetal weight was not different than group 2 controls. Unlike with the male fetuses, there were no differences between groups 1 or 2 female fetuses in either lean or fat mass (Fig. 4D). However, female fetuses from group 2 and group 4 (20-day aspirin) had increased relative adiposity compared to air controls ($p<0.05$). By contrast, there were no

between group differences in body composition in group 3 females relative to group 1 air controls.

Placental indices

The placental weight of fetuses from group 1, air-exposed dams were not different from that of group 2, ozone-exposed dams in either sex. However, placental weight from the male fetuses in group 4 (20-day aspirin) were significantly greater compared to group 2 ($p<0.05$; Fig. 5A). A similar, but insignificant trend was observed in females in group 4 ($p=0.12$).

Placental nutrient status was further analyzed according to sex and all results were normalized to the wet-weight of the placenta (Table 5). Notable differences observed in male placentas from group 2 dams included a reduction in placental free fatty acids and triglycerides ($p<0.05$ vs. controls); whereas, there were negligible differences in glucose, cholesterol, and protein content. Placentas from males of ozone-exposed group 3 (7-day aspirin) showed changes in free fatty acids and triglycerides, similar to group 2 ($p<0.05$). Notably, group 3 had significantly higher protein content ($p<0.001$), while group 4 had mildly reduced triglycerides and cholesterol ($p<0.05$) compared to group 1. In the female placenta, ozone exposure (group 2) resulted in increased glucose content compared to group 1 ($p<0.05$; Table 5). This effect was not observed in either aspirin groups 3 or 4. As in the males, free fatty acids were significantly reduced in the female placentas of group 2 compared to group 1. Free fatty acids were also lower in group 4 ($p<0.05$ vs. group 1), but not in group 3 compared to group 1. Female placentas from both aspirin groups 3 or 4 had lower placental triglyceride content relative to group 2 ($p<0.05$).

Finally, because poor placental adaption and IUGR may be related to mitochondrial dysfunction, relative mitochondrial DNA levels were measured. Both aspirin durations, 7-days (group 3) and 20-days (group 4), resulted in a reduction in mitochondrial density in both male and female placentas compared to groups 1 and 2 ($p<0.05$; Fig. 5B). We then assessed antioxidant markers in the placental homogenates. In males, total antioxidant status (TAS) was reduced in group 4 compared to group 1 ($p<0.05$; Fig. 5C), which trended toward statistical significance compared to group 2 ($p=0.06$). In female placentas from group 2 dams, glutathione peroxidase (GPx) activity and TAS were increased compared to group 1 ($p<0.05$; Fig. 5D). However, the 20-day aspirin duration (group 4) resulted in a significant downregulation of GPx and SOD activity, in addition to TAS, compared to group 2 ($p<0.05$).

Discussion

Given that ozone exposure during implantation impairs uterine artery blood flow and promotes fetal growth restriction, as we have previously shown [8], and that low-dose aspirin has been proposed to reduce the risk of preeclampsia and IUGR [10], the goal of this study was to examine if a comparably low dose of aspirin could improve pregnancy outcomes induced by ozone inhalation in rats. We demonstrate for the first time that once daily, oral “low-dose” aspirin (~2 mg/kg) administration between GD 1 – 7, but not throughout gestation, was sufficient to prevent growth restriction at GD21 in male and female fetuses exposed to ozone during implantation. Despite public health recommendations that encourage the use of aspirin to prevent preeclampsia and IUGR,

evidence from clinical trials shows that aspirin is most effective when its use is initiated within the first trimester (<16 weeks) [11]. The results presented corroborate these findings, as administration of aspirin only during early pregnancy was more effective than aspirin treatment throughout gestation in quelling some of the adverse effects of ozone.

While aspirin administration throughout the entirety of gestation in the current study may have had some negative consequences, early treatment of aspirin from GD 1 – 7 successfully protected against ozone-induced growth restriction. Ates *et al.* (2007) reported that daily aspirin treatment during estrus into embryonic day 4 resulted in a thicker uterine endometrial layer [17]. Endometrial thickness is important in pregnancy success [34] and numerous clinical trials in women undergoing *in vitro* fertilization report improved pregnancy rates with low-dose aspirin treatment [35]. Such direct links between endometrial thickness and disease etiology are not as clear in preeclamptic and IUGR pregnancies. Although the mechanisms by which aspirin might improve pregnancy outcomes and increase endometrial thickening are unclear, it has been shown that aspirin may improve arterial remodeling by inhibiting vasoconstriction [36], thereby promoting normal placentation and thus, fetal development. The partial reversal of ozone-induced increases in uterine artery resistance and the prevention of growth restriction from ozone-exposed dams in the 7-day aspirin group (i.e. through implantation only) supports this hypothesis and suggests that aspirin may have improved implantation, by preventing, in part, the cascade of events that impair arterial remodeling and placental development.

Despite clear differences between the two durations of aspirin administration in alleviating ozone-induced fetal growth outcomes, both durations appeared to reduce placental mitochondrial density to a similar extent. While alteration (increases or decreases) in mitochondrial density is not indicative of specific disease syndromes, it does suggest a potential disturbance in mitochondria health and function. Several studies have reported positive associations between mitochondrial DNA concentrations in the placenta and severity of growth restriction in the fetus [37,38]. Likewise, prenatal exposure to air pollutants have been positively associated with placental mitochondrial levels [39]. Furthermore, other studies of IUGR in air pollution research have found reduced mitochondrial DNA content in the placenta, suggesting a reduction in mitochondrial density [40,41]. This is in contrast to the findings here where we observed an effect of aspirin on mitochondria, since placental tissue from placebo-treated pregnant dams exposed to ozone did not undergo a change in nuclear to mitochondrial DNA ratio.

To obtain a better understanding of the relevance of such changes, we investigated additional markers of mitochondrial health such as antioxidant capacity and nutrient status in the placenta. We built upon our previous work [8] and report here a sexually dimorphic response in the placentas from dams that were exposed to ozone. In response to ozone exposure during implantation, the female placenta appeared to upregulate antioxidant status. However, this adaptation was not observed in the male placentas. Elevated oxidative damage and reduced antioxidant activity has been reported in male placentas of pregnancies from women exposed to betamethasone during gestation [42]. Furthermore, cord blood samples and placentas from female fetuses have increased levels of the antioxidant, selenium, compared to males [43]. Likewise, female placentas have elevated expression of immune-regulating

genes and suppressed cell proliferation genes compared to male placentas [44]. Along with our data, these findings point to potentially more active protective mechanisms in female placentas that better maintain appropriate fetal development during stressful events.

Placental antioxidant status appears to play an important role in both placental adaptations to stress as well as in the maintenance of nutrient transfer capacity [45]. Hence, as expected, female placentas from ozone-exposed, placebo pregnancies (group 2) also demonstrated increases in a number of nutrient markers in the current study. While female offspring from ozone pregnancies were still growth restricted, these fetuses had the same amount of fat mass and relative adiposity compared to air controls, similar to our previous findings [8]. Thus, this may suggest that the female neonate in our model is able to sustain adequate lipogenesis in the developing fat depots, which in part, is related to placental adaptation (i.e. nutrient absorption, utilization and availability). The placental alterations observed in our current work may reflect critical sex differences during developmental programming, differences which repeatedly suggest increased male sensitivity and require continued study [44]. Furthermore, the reduction in placental antioxidants in the female placentas of group 4 (20-day aspirin) could relate to impairment in adaptation, accounting for, in part, the reduced placental cholesterol and triglycerides and lack of mitigation of the impact of ozone exposure on fetal weight.

Within the current study, it is difficult to ascertain what differences occurred between groups 3 and 4 that resulted in the divergence of fetal weight at GD21. While both groups had comparable reductions in uterine artery resistance, the effect of continued aspirin administration on fetal outcomes may be attributable to the increased placental weight and decreased antioxidant production within the placentas in group 4. Herein, we did not incorporate an aspirin-only control group as no toxicological impacts were anticipated at the 2 mg/kg dose and its use would not be recommended in healthy pregnancies. It is also further important to note that the U.S. Preventative Services Task Force concluded that low-dose aspirin use in pregnancy has little risk to maternal health and developmental effects in the offspring [10], and prophylactic use is currently recommended in high-risk pregnancies. Hence, the results observed in group 4 on placental weight and antioxidant status was a surprise. It remains unknown if the antioxidant reductions observed with prolonged aspirin treatment and ozone exposure were due to aspirin itself or an interaction between the two. While the effects of low-dose aspirin treatment on placental function remains poorly understood, aspirin administration may serve to prevent lipid peroxide production and release from the placenta [46], which could reduce the need for antioxidant upregulation. As others have demonstrated [47,48], gestational antioxidant treatment protects against growth restriction in maternal undernutrition models. Therefore, it is reasonable to hypothesize that the aspirin-induced decreases in placental antioxidants is a likely mechanism behind the reduced fetal weight in group 4. Nonetheless, the effects of low-dose aspirin on the placental antioxidant response requires further study.

Importantly, administration of aspirin throughout gestation (e.g. group 4, 20-days) resulted in an elevated PF-4 level compared to ozone control rats. Limited data suggest that increases in PF-4 occurs in individuals with preeclampsia [49,50]. Furthermore, excessive platelet activation is associated with placental insufficiency (failure to provide adequate nutrients to

the fetus), preeclampsia and poor fetal growth [51]. In our current investigation, dams from group 4 (20-day aspirin) also had increased placental weight, indicating placental insufficiency. Interestingly, placental insufficiency has been positively correlated with fetal PF-4 levels, although no relationship was found in the maternal serum [52]. The link between placental PF-4, platelet activation, and IUGR requires further study. However, it is likely that the prolonged aspirin administration in some manner served to impair placental adaptation and/or resulted in excessive platelet activation that may have impaired placental delivery of nutrients to the fetus.

In summary, our findings identify significant sexual dimorphism in the placental reaction to maternal ozone exposure and highlights a critical window during gestation where aspirin treatment may be effective against ozone-induced growth restriction. However, continued aspirin administration throughout pregnancy reverses some of the beneficial placental antioxidant adaptations, resulting in only marginal effects on fetal weight, while also eliciting placental insufficiency. The efficacy of early aspirin administration in mitigating the effects of ozone may relate to its effects on arterial remodeling, which is a key event in early pregnancy, and suggesting that the timing of treatment is critical.

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Figure 1. Study Design

Pregnant Long-Evans rats were exposed to either air or 0.8 ppm on gestation days (GD) 5 and 6. Air-exposed rats (group 1) and ozone-only exposed rats (group 2) were provided a daily placebo treat (dotted lines) between GD 1 – 20. Ozone-exposed dams provided a daily treat with 0.6 mg aspirin (filled line) were further divided into two groups based off of days of administration: group 3 (GD 1-7; 7-day) and group 4 (GD 1-20; 20-day). To maintain consistency, group 3 was given the placebo treat starting on GD8. Differences in uterine arterial flow, fetal growth characteristics, and placental mitochondria, nutrient and antioxidant status were measured between dams given aspirin and rats exposed to either air (group 1) or ozone (group 2) at GD21.

Figure. 2. Effect of ozone exposure with and without aspirin administration on gestational food intake and body weight gain.

A) Body weight change and B) food intake immediately following exposure on GD5, GD6, and GD 5 and 6 combined (note that rats receiving aspirin combined into a single group). C) Daily body weight gain and D) food intake (GD 1-21; note that rats receiving aspirin are depicted separately by treatment length). Group sizes: n=9 (air), n=10 (ozone), n=10 (ozone + 7-day), and n=8 (ozone + 20-day). Data presented as mean $\pm$ SE. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

Abbreviations: air (group 1), ozone (group 2), 7-day aspirin group (7-day; group 3), 20-day aspirin group (20-day; group 4), gestation day (GD).

Figure 3. Uterine arterial resistance indices at GD 15 and 21.

Results of Doppler ultrasonography of the uterine artery. Group differences in A) resistance index, B) pulsatile index, and C) S/D ratio at GD 15 and 21. D) Percent change of arterial resistance indices from GD 15 to GD 21. Group sizes: n=9 (air), n=10 (ozone), n=10 (ozone + 7-day), and n=8 (ozone + 20-day). Data presented as mean $\pm$ SE. **p<0.01, *p<0.05.

Abbreviations: air (group 1), ozone (group 2), 7-day aspirin group (7-day; group 3), 20-day aspirin group (20-day; group 4), gestation day (GD), resistance index (RI), pulsatile index (PI), S/D (systole/diastole) ratio.

Figure 4. Effects of ozone with or without aspirin on fetal growth outcomes by sex.

The effects of ozone and aspirin on male (n=8-10/group) A) fetal size and B) body composition, and female C) fetal size and D) body composition. For panels A and C, group sizes: n=9 (air), n=10 (ozone), n=10 (ozone + 7-day), and n=8 (ozone + 20-day). For body composition measurements an n=3 fetuses were pooled for each litter for each group, separated by sex. Group sizes for panels B and D for males were n=9 (air), n=10 (ozone), n=10 (ozone + 7-day), and n=7 (ozone + 20-day); females were n=9 (air), n=9 (ozone), n=10 (ozone + 7-day), n=8 (ozone + 20-day). Data presented as mean $\pm$ SE. *p<0.05.

Abbreviations: air (group 1), ozone (group 2), 7-day aspirin group (7-day; group 3), 20-day aspirin group (20-day; group 4).

Figure 5. Measurements of placental mitochondria and antioxidant capacity.

Effects of peri-implantation ozone exposure and aspirin treatment on (A) placental weight; group sizes: n=9 (air), n=10 (ozone), n=10 (ozone + 7-day), and n=8 (ozone + 20-day). (B) Relative mitochondrial number was assessed by qPCR in the placenta. Antioxidant indexes were measured in placental homogenates normalized to sample weight from (C) males and (D) females. Group sizes for all groups were n=8, except females from the ozone + 20-day (n=7). Data presented as mean $\pm$ SE. ****p<0.0001, ***p<0.001, *p<0.05.

Abbreviations: air (group 1), ozone (group 2), 7-day aspirin group (7-day; group 3), 20-day aspirin group (20-day; group 4), mitochondrial DNA (mtDNA), nuclear DNA (nucDNA), glutathione peroxidase (GPx), superoxide dismutase (SOD), total antioxidant status (TAS).

Table 1.

Nutritional composition of the D15092401 diet.

Nutrient	Gram%	Kcal%
<i>Protein</i>	20	20
<i>Carbohydrate</i>	64	64
<i>Fat</i>	7	16
<i>Total</i>		100
<i>Kcal/gram</i>	4.0	
Ingredient	Gram	Kcal
<i>Casein</i>	200	800
<i>L-Cystine</i>	3	12
<i>Corn Starch</i>	391.5	1566
<i>Maltodextrin 10</i>	75	300
<i>Sucrose</i>	172.8	691
<i>Cellulose, BW100</i>	50	0
<i>Soybean Oil</i>	52	468
<i>Lard</i>	20	180
<i>Mineral Mix, S10026</i>	10	0
<i>DiCalcium Phosphate</i>	13	0
<i>Calcium Carbonate</i>	5.5	0
<i>Potassium Citrate, 1 H₂O</i>	16.5	0
<i>Vitamin Mix V10001</i>	10	40
<i>Choline Bitartrate</i>	2	0
<i>FD&C Yellow Dye #5</i>	0	0
<i>FD&C Red Dye #40</i>	0.05	0
<i>FD&C Blue Dye #1</i>	0	0
<i>Total</i>	1021.35	4057

Table 2.

Effect of ozone exposure without or without aspirin administration on maternal weight gain and food intake.

Characteristics ^{a,b}	Air (Group 1)	Ozone (Group 2)	Ozone + 7-day (Group 3)	Ozone + 20-day ^c (Group 4)
<i>Litters maintained to GD 21</i>	9	10	10	8
<i>Extrauterine weight gain (g)</i>	143.5 ± 5.88	140.1 ± 6.35	143.6 ± 7.84	146.9 ± 6.27
<i>Gestational food intake (g)</i>	431.3 ± 17.1	434.8 ± 13.2	429.5 ± 16.2	437.1 ± 20.6

Values presented as means ± SE. Extrauterine weight gain was measured as the difference in weight between GD21 and GD5. Abbreviations: 7-day aspirin group (7-day), 20-day aspirin group (20-day), gestation day (GD).

^aLitters with high corpus luteum to implantation site counts (n = 6) were removed from the study due to pre-implantation loss; n=1 removed from air, 7-day, and 20-day aspirin groups; n=2 removed from ozone.

^bTo remove the influence of extreme values that could falsely impact the group mean, litters with average fetal weight above the 90th percentile and below the 10th percentile were trimmed.

^cA single rat was euthanized prior to gestational day 21 due to serosanguinous vaginal discharge.

Table 3.

Effect of ozone exposure with or without aspirin administration on PF-4 and GD21 CBC indices.

Blood values		Air (Group 1)	Ozone (Group 2)	Ozone + 7-day (Group 3)	Ozone + 20-day (Group 4)
PF-4	GD 7	16.6 ± 5.64	14.9 ± 4.75	8.22 ± 2.15 ^a	
(ng/mL)	GD 21	315 ± 12.2	294 ± 12.7	318 ± 34.7	369 ± 29.0 ^τ
CBCs					
	Platelets (10³/uL)	910 ± 49.6	893 ± 36.1	921 ± 30.6	936 ± 44.3
	White blood cells (10³/uL)	4.43 ± 0.15	4.13 ± 0.24	4.07 ± 0.30	4.58 ± 0.32
	Red blood cells (10⁶/uL)	5.51 ± 0.06	5.63 ± 0.11	5.54 ± 0.06	5.60 ± 0.09
	Hemoglobin (g/dL)	11.7 ± 0.30	11.9 ± 0.24	11.8 ± 0.13	12.0 ± 0.20
	Hematocrit (%)	32.1 ± 0.48	32.5 ± 0.49	32.1 ± 0.18	32.5 ± 0.38
	MCV (fL)	58.2 ± 0.58	57.7 ± 0.52	57.9 ± 0.64	58.0 ± 0.43
	MCH (pg)	21.2 ± 0.38	21.1 ± 0.32	21.4 ± 0.34	21.4 ± 0.26
	MCH concentration (g/dL)	36.4 ± 0.52	36.5 ± 0.37	36.9 ± 0.42	36.9 ± 0.39

^aGroups 3 and 4 data were combined as there were no differences in treatments at this time

Due to effects of hemolysis on PF-4, only non-hemolyzed samples were measured. At GD7, group sizes were n=7 (air), n=9 (ozone), and n=12 (aspirin); at GD21 group sizes were n=5 (air), n=9 (ozone), n=5 (ozone + 7-day), and n=6 (ozone + 20-day). Group sizes for CBC measurements: n=9 (air), n=10 (ozone), n=10 (ozone + 7-day), and n=8 (ozone + 20-day). Data presented as mean ± SE.

^τp<0.01 vs. ozone (group 2).

Abbreviations: complete blood count (CBC), 7-day aspirin group (7-day), 20-day aspirin group (20-day), gestation day (GD), platelet factor-4 (PF-4), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH).

Table 4.

Effect of ozone exposure with or without aspirin administration on pregnancy outcomes at GD21.

Reproductive Endpoints at GD21	Air (Group 1)	Ozone (Group 2)	Ozone + 7-day (Group 3)	Ozone + 20-day (Group 3)
<i>Implantation sites per litter</i>	12.9 ± 0.73	12.9 ± 0.55	12.1 ± 0.59	12.1 ± 0.79
<i>Resorptions per litter</i>	0.33 ± 0.24	0.70 ± 0.50	0.40 ± 0.16	0.63 ± 0.26
<i>Fetuses per litter</i>	12.6 ± 0.75	12.2 ± 0.70	11.7 ± 0.60	11.5 ± 0.85
<i>Percent males per litter</i>	52.3 ± 3.21	59.1 ± 2.66	50.0 ± 3.07 ^τ	53.7 ± 7.41

Values presented as means ± SE. Group sizes: n=9 (air), n=10 (ozone), n=10 (ozone + 7-day), and n=8 (ozone + 20-day).

^τp<0.05 vs. ozone (group 2).

Abbreviations: 7-day aspirin group (7-day), 20-day aspirin group (20-day).

Table 5.

Effect of ozone exposure with and without aspirin administration on placental nutrient status.

	Sex	Air (Group 1)	Ozone (Group 2)	Ozone + 7-day (Group 3)	Ozone + 20-day (Group 3)
<i>Glucose (ng/mg)</i>	Male	109 ± 7.08	106 ± 6.10	96.7 ± 6.54	101 ± 5.42
<i>FFA (μM/mg)</i>	Male	2.37 ± 0.18	1.62 ± 0.07 ^{***}	1.82 ± 0.03 ^{**}	1.89 ± 0.23
<i>TAG (ng/mg)</i>	Male	12.7 ± 0.42	11.1 ± 0.32 [*]	11.1 ± 0.55 [*]	10.7 ± 0.33 ^{**}
<i>Cholesterol (ng/mg)</i>	Male	13.1 ± 0.45	12.3 ± 0.42	12.5 ± 0.20	11.7 ± 0.33 [*]
<i>Protein (μg/mg)</i>	Male	54.2 ± 2.19	58.3 ± 2.86	72.1 ± 4.22 ^{***} τ	57.2 ± 3.82
<i>Glucose (ng/mg)</i>	Female	111 ± 5.62	141 ± 7.29 [*]	115 ± 9.17 ^{τ}	100 ± 7.63 ^{τ}
<i>FFA (μM/mg)</i>	Female	2.22 ± 0.30	1.43 ± 0.19 [*]	1.82 ± 0.24	1.45 ± 0.14 [*]
<i>TAG (ng/mg)</i>	Female	13.5 ± 0.45	14.7 ± 1.06	11.9 ± 0.39 ^{τ}	10.9 ± 0.58 ^{τ}
<i>Cholesterol (ng/mg)</i>	Female	12.5 ± 0.14	12.9 ± 0.50	12.5 ± 0.42	12.14 ± 0.24
<i>Protein (μg/mg)</i>	Female	48.8 ± 2.88	53.3 ± 5.67	57.3 ± 3.01	59.9 ± 3.18

Values presented as means ± SE following adjustment for placental homogenate and presented as concentration / mg of tissue.

^{*}
p<0.05^{**}
p<0.01^{***}
p<0.001 vs. air controls; ^{τ}
p<0.05 vs. ozone controls.

Group sizes in males were n=8/group in all non-lipid endpoints; n=1 was removed from lipid-related data in the ozone + 20-day group due to a system read error; group sizes in females: n=7 (air), n=7 (ozone), n=8 (ozone + 7-day), and n=7 (ozone + 20-day).

Abbreviations: 7-day aspirin group (7-day), 20-day aspirin group (20-day), free fatty acids (FFA), triglyceride (TAG).