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MECHANISMS AND TREATMENT OF HALOGEN INHALATION-INDUCED PULMONARY AND SYSTEMIC INJURIES IN PREGNANT MICE

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Abstract

Inhalation of oxidant gases has been implicated in adverse outcomes in pregnancy, but animal models to address mechanisms and studies to identify potential pregnancy-specific therapies are lacking. Herein we show that inhalation of bromine at 600 parts per million for 30 minutes by pregnant mice on the 15th day of embryonic development results in significantly lower survival after 96 hours than an identical level of exposure in non-pregnant mice. On the 19th embryonic day, bromine-exposed pregnant mice have increased systemic blood pressure, abnormal placental development, severe fetal growth restriction, systemic inflammation, increased levels of circulating anti-angiogenic short fms-like tyrosine kinase one, and evidence of pulmonary and cardiac injury. Treatment with tadalafil, an inhibitor of type 5 phosphodiesterase, by oral gavage one hour post-exposure and then once daily thereafter, attenuated systemic blood pressures, decreased inflammation, ameliorated pulmonary and cardiac injury, and improved maternal survival (from 36% to 80%) and fetal growth. These pathological changes resemble those seen in preeclampsia. Non-pregnant mice did not exhibit any of these pathological changes, and were not affected by tadalafil. These findings suggest that pregnant women exposed to bromine may require particular attention and monitoring for signs of preeclampsia-like symptoms.

Keywords

Hemodynamics; Vascular Biology; Pregnancy; Heart Failure; Preeclampsia; Lung Edema

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INTRODUCTION

Recent epidemiological evidence indicates that inhalation of noxious gases such as NO_2, N_2O_3, SO_2, and O_3 during pregnancy is associated with fetal growth restriction (FGR) and/or the development of preeclampsia-type symptoms [1, 2]. Additional observational studies have further corroborated this association between inhaled toxicants and preeclampsia-like symptoms. For instance, maternal cigarette smoking impairs fetal growth via excess placenta-derived secreted frizzled-related protein 1 (FRP1) [3]. Additionally, in a state-wide birth cohort, maternal exposure during pregnancy to fine particulate matter (PM2.5) and ozone was positively associated with small-for-gestational age and low birth weight births [4]. Finally, formaldehyde inhalation has been strongly linked to fetal growth restriction and adverse outcomes in pregnancy [5].

Exposure to halogens (e.g. chlorine (Cl_2) or bromine (Br_2)) causes significant risk to human populations resulting in both pulmonary and systemic injury followed by death from respiratory and cardiac failure; reviewed in [6]. Over 56 million tons of halogens are produced each year worldwide for use in the manufacture of flame-retardants, medicinal compounds, gasoline additives, dyes, and water disinfectants. Accidental exposures during transit of halogens has occurred in Geneva Switzerland (1988, Br_2), Graniteville, SC (2005, Cl_2) and Chelyabinsk Russia (2011, Br_2), resulting in significant short and long term morbidity and mortality [7].

In addition to major industrial accidents and/or intentional release of halogens, a common source of halogen inhalation toxicity are swimming pool sanitation accidents [8]. Furthermore, pre-existing viral infections increase the severity of lung injury secondary to halogen exposure [9]. Accordingly, there is an extensive body of literature available regarding halogen inhalation lung injury both in humans and in animal models [10]. Yet, there is a paucity of systematic studies addressing mechanisms of lung and systemic injury caused by halogen gas injury in pregnancy. We observed that exposure of pregnant mice to concentrations of Br_2, likely to be encountered in the vicinity of industrial accidents, resulted in increased mortality as compared to non-pregnant mice; furthermore, surviving pregnant mice developed preeclampsia-like symptoms and their fetuses failed to thrive.

Preeclampsia is a life-threatening complication of pregnancy characterized by maternal hypertension and is considered severe when accompanied by pulmonary or systemic pathologies [11]. Preeclampsia is commonly associated with fetal growth restriction (FGR). Circulating factors, in part released by an injured placenta such as soluble fms-like tyrosine kinase-1 (sFLT-1), soluble endoglin (sENG) and inflammatory cytokines, have been mechanistically linked to features of preeclampsia such as increased blood pressure, decreased trophoblast maturation and proliferation and retarded fetal growth [12, 13]. sFLT-1 and sENG, in particular, are thought to promote endothelial dysfunction, by inhibiting vascular endothelial growth factor (VEGF) [14]. Since inhibitors of type 5 phosphodiesterase ($PDE5_i$) are efficient in treating endothelial dysfunction, they have been used successfully in animal models of preeclampsia and are in clinical trials for the treatment of preeclampsia [15, 16].

We hypothesized that pregnancy sensitizes mice to Br_2 inhalation injury through a potential amplifying effect on endothelial dysfunction by placenta-derived inflammatory and anti-angiogenic mediators. We further hypothesized that treatment with the $PDE5_i$, tadalafil, can counteract pregnancy-specific Br_2 toxicity via a beneficial effect on blood pressure and perfusion. In a series of experiments we found that exposure of pregnant mice at gestational day 14.5 (E14.5) to Br_2 in concentrations likely to be encountered in industrial accidents [17] (600 parts per million (ppm) for 30 min) caused a significantly higher rate of mortality compared to non-pregnant female and male mice. We show that this is associated with severe FGR, cardiac and pulmonary injuries, and development of preeclampsia-like features. Furthermore, maternal mortality and morbidity, as well as fetal growth restriction, were alleviated by maternal post-exposure administration of tadalafil.

METHODS

(Please see Online Supplement for detailed description of methods, procedures and reagents used).

Animals

Pathogen-free, age-matched male (20–25 g), non-pregnant female (20–25 g), and pregnant female C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) and housed in the University of Alabama at Birmingham Animal Facility under standard conditions with food and water access *ad libitum*. All procedures were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC #20343).

Exposure of mice to Br_2

Mice were exposed to Br_2 for 30 or 45 min at 600 ppm Br_2 as described previously [10] at E14.5. After exposure, mice were returned to room air. Control mice were exposed to air for 30 or 45 min under identical conditions.

Tadalafil administration

Stock solutions of tadalafil (Santa Cruz Biotechnology, Santa Cruz, CA, SC-208412), were administered to pregnant and non-pregnant mice via oral gavage at 2 mg/kg body weight at one h post-exposure and every 24 h thereafter. Control mice received equal volumes of sterile saline by gavage.

Statistics

Survival between treatment groups under different experimental conditions and at different time points was evaluated using the Pearson chi-squared test, and Fisher's exact test. The overall survival rates were estimated according to the Kaplan-Meier method and compared using the log-rank test. To eliminate unequal variances, data was log transformed. No outliers were identified via ROUT. One-way (for pregnant-only variables such as placental analyses) or two-way (for analyses that included pregnant and non-pregnant data) ANOVA (for three or more groups) was used to compare continuous variables. Analysis was conducted using Graphpad Prism ver. 7 software (La Jolla, CA). A p-value < 0.05 was

considered statistically significant in the two-tailed statistical tests that were used. All data is represented as mean ± standard error of the mean (SEM).

RESULTS

Br_2 exposure causes higher mortality and loss of body weight in pregnant mice compared to non-pregnant mice

We exposed male and non-pregnant female mice and pregnant mice to Br_2 (Figure 1A) at E14.5 to an exposure level that causes minimal lethality in non-pregnant mice (600ppm for 30 min). All mice exposed to Br_2 lost weight similarly by E16.5 (Figure 1B). However, non-pregnant mice began to gain weight after E16.5. In contrast, pregnant mice continued to lose weight until either death or euthanasia at E18.5.

Male and non-pregnant female mice exposed to Br_2 at 600 ppm for 30 min had a mean survival of 80% within five days post-exposure. However, pregnant females exposed to Br_2 had significantly lower mean survival (36%) than non-pregnant mice. Treatment of Br_2-exposed mice with tadalafil post-exposure increased survival to 80%. Additionally, tadalafil had no effect on mortality in non-pregnant mice exposed to similar or even greater doses of Br_2 (Figure S1: please see http://hyper.ajajournals.org). These data suggest a pregnancy-specific mechanism of injury. Subsequent studies were performed to identify potential mechanisms involved.

Br_2 inhalation injury causes severe fetal growth restriction, and treatment with tadalafil partially rescues fetal growth

Pregnant mice were exposed to Br_2 at 600 ppm for 30 min or air at E14.5 and then returned to room air with or without tadalafil at 2 mg/kg by gavage daily, starting one h post-Br_2 exposure. Fetal length, measured at E14.5, E16.5, and E18.5 *in utero* with echosonography (Figure 1C), was decreased at both E16.5 and E18.5 as compared with air controls (Figure 1D). Tadalafil treatment returned fetal lengths to air control levels by E18.5 (Figures 1D and 1E). Also, fetuses of pregnant mice exposed to 600ppm Br_2 for 30 min weighed less as compared to fetuses of air breathing mice (Figure 1E); administration of tadalafil increased fetal weight. Fetuses of Br_2-exposed mice showed no signs of viability (breathing, movement, or skin turning pink) after delivery via cesarean section on E18.5, as opposed to the fetuses of air-exposed mice which all demonstrated these signs. Even though fetuses of mice exposed to Br_2 exhibited no signs of viability when delivered at E18.5 by cesarean section, prior ultrasonography at E18.5 detected heartbeats in all but one fetus. With such a marked decrease in fetal growth following Br_2 exposure, we next investigated whether this was due to injury to the placenta.

Br_2 inhalation injury hinders placenta development and induces inflammation and production of the anti-angiogenic molecule sFLT-1

Fetal growth is dependent on normally developing placenta, and abnormal placenta development or placenta injury can negatively affect maternal physiology by sustaining an inflammatory state and producing anti-angiogenic molecules. As shown in Figures 2A–C, there were significant reductions of the placental junctional zones in mice exposed to Br_2

(despite similar placental weights) and a return to baseline values in tadalafil treated mice. Visualization of glycogen-containing cells by PAS staining and detection of CDX2 by immunohistochemistry corroborated the findings of altered placenta development by detecting a reduced area occupied by glycogen-containing and CDX2-positive cells.

Placental TNFα mRNA was significantly reduced in Br_2-exposed mice that received tadalafil (Figure 2D). This indicated a less inflamed state in placentas of Br_2-exposed mice with PDE5 inhibition. sFLT-1:FLT-1 mRNA ratio increased in placentas of mothers exposed to Br_2 (Figure 2E). Tadalafil therapy post-Br_2 decreased the sFLT-1:FLT-1 mRNA ratio to air control levels. These changes in sFLT-1 mRNA levels were reflected by a five-fold increase of circulating sFLT-1 levels in maternal plasma of Br_2-exposed mice, which was normalized by tadalafil therapy (Figure 2F).

Pregnant mice exhibit signs of severe lung injury 96 h post-Br_2 exposure, which are absent in non-pregnant mice

Br_2 inhalation in non-pregnant animals is known to cause a temporary increase in alveolar epithelial permeability. This leads to increased protein content in the bronchoalveolar lavage (BAL) fluid, which begins to resolve after three days [10]. Our data show that non-pregnant mice exposed to 600 ppm for 30 min have lung wet/dry weight ratios and BAL fluid protein values similar to those of the corresponding air controls three days post-exposure. Pregnant mice, however, had increased lung wet/dry weight ratios and BAL fluid protein at E18.5. Treatment with tadalafil significantly reduced wet/dry lung weight ratios and BAL fluid protein (Figures 3A and 3B).

P_aO_2 was unaffected by Br_2 inhalation (Figure 3C), however, there was a significant increase of P_aCO_2 in pregnant mice (Figure 3D) accompanied by a severe drop in pH (to 7.05 Figure 3E). These findings are consistent with alveolar hypoventilation. Similar findings have been reported in sheep exposed to 100% oxygen at 1ATA for three-four days [18]. Tadalafil administration had no significant effect on P_aCO_2, but significantly increased arterial pH to 7.18, presumably due to an increase of bicarbonate. S_aO_2 was significantly decreased in Br_2-exposed pregnant mice and was restored by tadalafil administration (Figure 3F).

Br_2 inhalation causes systemic hypertension in pregnant mice

A life threatening complication of pregnancy is severe hypertension, the etiology of which is driven by placental inflammation and injury as well as production of anti-angiogenic mediators such as sFLT-1. Both systemic systolic and diastolic blood pressures of non-pregnant mice exposed to Br_2 were unchanged as compared to non-pregnant air controls. Systolic pressures in pregnant mice exposed to Br_2 were not significantly increased (Figure 4A). However, there was a 40% increase of systemic diastolic blood pressures in pregnant mice at E18.5. Additionally, these values returned to baseline in tadalafil treated mice (Figure 4B). Right ventricle pressures were unchanged in Br_2-exposed pregnant mice (Figure 4C and S2: please see http://hyper.ahajournals.org).

Br_2 inhalation causes decreased cyclic guanosine monophosphate (cGMP) levels in pregnant mice

We hypothesized that changes in lung and aortic cGMP levels may account for the systemic hypertension seen in pregnant mice exposed to Br_2. cGMP levels in pregnant mice were decreased significantly as compared to air controls in the lung, placenta, and aorta (Figure 4D–F). Treatment with tadalafil increases cGMP values in the lung and placenta.

Br_2 increases circulating inflammatory cytokines

At E18.5, circulating levels of tumor necrosis factor alpha (TNFα) and keratinocyte chemoattractant/ growth related oncogene (KC/GRO) (the murine functional equivalent of human interleukin-8) were increased only in the pregnant, Br_2-exposed mice. Interleukin-6 (IL-6) was increased in both non-pregnant and pregnant mice exposed to Br_2. These cytokines have been shown to be associated with the onset of preeclampsia [12, 19–24]. Tadalafil therapy subsequently reduced these levels to those similar to air controls (Figure 5A–C).

Br_2 inhalation decreases cardiac function in pregnant mice that is reversed by tadalafil

At E18.5, cardiac output calculated from echo-Doppler analysis (Figure 6A) was diminished in pregnant mice exposed to Br_2 as compared to air-exposed control pregnant mice (Figure 6B). Administration of tadalafil restored cardiac output. Ejection fraction (Figure 6C), fractional shortening, and heart rate were unaffected in any of the experimental groups, but stroke volume was reduced in Br_2-exposed pregnant mice. Left ventricle (LV) end diastolic volumes were decreased in Br_2-exposed pregnant mice (Figure 6E), explaining the decrease of LV cardiac output despite the preserved ejection fraction. These findings are consistent with the presence of heart failure with preserved ejection fraction, historically referred to as diastolic heart failure.

DISCUSSION

We show for the first time that exposure of pregnant mice to the oxidant and electrophile gas Br_2, in concentrations likely to be encountered in the vicinity of industrial accidents, results in significant mortality and morbidity as compared to male and non-pregnant female mice exposed to identical concentrations of Br_2. Pregnant mice experienced severe injury to their placentas resulting in the release of inflammatory cytokines and the anti-angiogenic molecule sFLT-1 in the plasma, fetal growth restriction, as well as increased systemic diastolic pressures and non-cardiogenic pulmonary edema. These symptoms are reminiscent of those seen in pregnant women with preeclampsia.

One factor that may account for this preeclampsia-like condition is the observed decrease of cGMP in lungs, aorta, and placenta. This may be caused by increased breakdown of cGMP by PDE5 or decreased production due to the systemic inactivation of eNOS as seen in mice and rats following exposure to Cl_2 [25]. Phosphodiesterase 5 inhibitors ($PDE5_i$) have shown therapeutic benefit in animal models of preeclampsia, [26, 27] and the most widely used $PDE5_i$, sildenafil, is in human clinical trials to treat preeclampsia [15, 16]. These protective effects are most likely due to the inhibition of cGMP breakdown by PDE5 and prevention of

hypertension. We chose to administer the $PDE5_i$ tadalafil due to its long half-life (17.5 h) as well as its specificity. For example, tadalafil is more than 700x more potent for PDE5 than for PDE6 and at least 9,000x more potent for PDE5 than PDE1-4 and PDE7-10 [28].

However, the protective effects of restoring cGMP may be due to a variety of additional mechanisms. cGMP has also been shown to maintain endothelial barrier function [29] and increase sodium-dependent alveolar fluid clearance via regulation of epithelial Na^+ [30] and K^+ channels [31]. This may explain the restoration of blood gas permeability and resolution of pulmonary edema seen in pregnant mice that received tadalafil post-Br_2 exposure. In the placenta, which lacks significant smooth muscle, cGMP-dependent mechanisms have been shown to participate in maintaining trophoblast survival and invasiveness [32]. Therefore, it is plausible that some of the beneficial effects of tadalafil therapy were mediated by direct effect on the trophoblasts. Furthermore, within the limits of this study, we were unable to exclude any off-target effects (e.g. inhibition of other PDEs) of tadalafil.

Exposure to Br_2 represents a clear danger to public health. World production of Br_2 exceeds 300,000 tons per year; it is used in the manufacturing of medicinal compounds, flame-retardants, agricultural chemicals, gasoline additives, dyes, photographic chemicals, bleaching agents, and water disinfectants. It is produced in central facilities and transported by trains or trucks. A major accident recently occurred during railway Br_2 transport in the city of Chelyabinsk, Russia (pop.1.1 million), resulting in 42 people being hospitalized and over 200 individuals seeking medical attention [33]. A study conducted on human volunteers demonstrated that a small exposure to 0.9 ppm bromine (Br_2) for five minutes results in cough, headache, and irritation of the eyes, nose, and upper respiratory tract [34]. Accidental exposure to higher doses of Br_2 and its ensuing respiratory complications can lead to severe morbidity and mortality, yet there is a lack of human and animal data available on Br_2 toxicity, especially during pregnancy. This is particularly pertinent, since, according to the US census bureau, four percent of women in the United States are pregnant at any given time. Interestingly, two epidemiological studies in western Australia and the southern United States reported associates among levels of halogenated intermediates in drinking water (used as disinfectants) and premature births [35] and birth defects [36].

The exact mechanism by which Br_2 inhalation induces placental injury remains unclear. Br_2 and HOBr are strong oxidants, likely to react with targets on the plasma membranes of lung epithelial cells (such as plasmalogens), forming secondary, longer living intermediates, such as bromopalmitaldehyde (2-BrPALD) and 2-bromostearaldehyde (2-BrSALD) which may be oxidized to 2-bromopalmitic acid (2-BrPA) and 2-bromostearic acid (2-BrSA) capable of transducing injury to distal sites [37]. 2-BrPA is a potent inhibitor of mitochondrial fatty acid oxidation and of protein palmitoylation [38, 39]. In addition, in eosinophils, the catalytic action of eosinophil peroxidase on hydrogen peroxide and bromide generates 2-BrFALD [39]. Normal physiological concentrations of bromide are in the range of 20–150 μM and will most likely rise in the mM range following inhalation of Br_2 gas. Thus, production of 2-BrFALD by inhaled Br_2 and inflammatory cell myeloperoxidase is likely to occur. Recently Ford *et al.* showed that the formation of 2-chloropalmitaldehyde (2-ClPALD), 2-chlorostearaldehyde (2-ClSALD), and their oxidized products, free and esterified 2-chloropalmitic acid (2-ClPA) and 2-chlorostearic acid could be detected in the lungs and

plasma of mice and rats for up to 72 h post-exposure to 400 ppm Cl_2 for 30 min [40]. Furthermore, intranasal administration of 2-ClPA or 2-ClPALD resulted in increased distal lung permeability and inflammation and systemic endothelial dysfunction characterized by loss of eNOS-dependent vasodilation. Thus reactive intermediates formed during halogen inhalation are capable of causing distal organ injury and may be responsible both for placental and cardiac injury. In another study, Zaky *et al.* demonstrated that perfusion of isolated hearts with chloramines (another long term intermediate formed during Cl_2 inhalation) resulted in severe cardiac dysfunction [41].

Placental injury can be induced by a variety of agents that induce endothelial dysfunction, including antagonists of nitric oxide synthesis [42], genetic ablation of endothelial nitric oxide synthase (eNOS) in gene-targeted mice [43], exogenous administration of the vascular endothelial growth factor (VEGF) antagonist short variant of FMS-like tyrosine kinase 1 (sFLT-1) [44], or the short form of endoglin [45]. In these models, placental injury or decreased uterine/placental perfusion leading to placental ischemia, causes placental inflammation and the production of anti-angiogenic molecules which, in turn, aggravate systemic endothelial dysfunction and worsen vasoconstriction that further aggravates placenta injury [46]. In Br_2-exposed pregnant mice we were able to clearly demonstrate placental injury by evaluating placenta development based on the size of the junctional zone and by detecting inflammation and increased production of short-fms-like tyrosine kinase (sFLT-1) by quantifying their respective mRNA levels. We also detected increased circulating sFLT-1 and decreased vascular and tissue cGMP levels that is considered to be a diagnostic sign of decreased VEGF signaling. Acting as a decoy receptor for VEGF, sFLT-1 inhibits maternal VEGF signal transduction resulting in endothelial dysfunction, vasoconstriction and hypertension.

Similarly, inflammatory cytokines may play an important role in injuring the placenta. TNFα and IL-6 have been well established as circulating biomarkers in human preeclampsia and have been shown to be causative in pathogenesis in experimental animal models of preeclampsia [20, 47, 48]. Recently IL-8 has been shown to be associated with preeclampsia in humans [49]. Indeed, our analysis of plasma cytokines determined that pregnant mice exposed to Br_2 exhibited significant elevation of all three of these pro-inflammatory mediators whereas there were no significant differences in cytokine or chemokine levels (except IL-6) at this time point in non-pregnant mice. These data indicate the presence of systemic inflammation in pregnant mice post-Br_2, which may contribute to the observed injuries to cardiac and pulmonary systems.

An additional intriguing factor in our studies is the development of severe respiratory acidosis in the pregnant mice, which is caused by alveolar hypoventilation. Respiratory acidosis contributed to pulmonary hypertension, cardiac dysfunction and placenta hypoperfusion and injury [50, 51]. Interestingly, tadalafil administration did not decrease P_aCO_2; however, it had a significant therapeutic effect by normalizing the arterial pH.

In human clinical studies, left ventricular diastolic dysfunction has been observed in preeclampsia [52]. In cases when preeclampsia was accompanied with pulmonary edema, diastolic dysfunction was highly prevalent [53]. We observed decreased cardiac output in

pregnant mice exposed to Br_2, and this was accompanied with a decreased filling of the left ventricle under diastole but also accompanied with preserved ejection fraction. In addition to pulmonary edema, we also observed increased BAL fluid protein, indicating that both barriers at the air-blood interface have been compromised.

CONCLUSIONS

Our data shows conclusively that pregnant C57BL/6 mice exposed to Br_2 at E14.5 exhibit increased mortality as compared to non-pregnant mice and display signs of placental damage accompanying symptoms reminiscent of preeclampsia. Potential mechanisms involved are shown in Figure S3 (please see http://hyper.ahajournals.org). Tadalafil, the orally available, long-acting $PDE5_i$, administered following Br_2 exposure, increases survival exclusively in pregnant mice and eliminates signs of placental damage. The key mechanistic driver of pathology, similar to models of preeclampsia, appears to be a positive feedback loop between endothelial dysfunction and the placenta via the anti-angiogenic sFLT-1. The resulting increased vascular resistance is likely combined with additional cGMP-mediated mechanisms in non-vascular tissues such as the damage to alveolar epithelium and trophoblasts in the placenta. Damage to the placenta as well as eNOS is likely mediated by long acting mediators formed by the interaction of inhaled Br_2 with plasmalogens, as recently reported in Cl_2-exposed mice, as well inflammatory cytokines and chemokines. This is the first demonstration that a brief exposure of pregnant mice to an oxidant and electrophile gas (Br_2) results in extensive injury to the placenta, initiating a cycle of events leading to significant morbidity and mortality.

PERSPECTIVES

In summary, we have determined exposure to Br_2 causes preeclampsia-like signs and increased mortality in pregnant mice. These signs include, hypertension, placental injury, and fetal growth restriction that are accompanied by increased pulmonary and cardiac injury. We demonstrate that Br_2 decreased cGMP in multiple tissues including the vasculature. Administering the $PDE5_i$ tadalafil restores cGMP levels and reduces signs of injury seen in pregnant mice exposed to Br_2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Sadis Matalon and Tamas Jilling were responsible for the main conceptual design of these studies, They jointly directed the project and finalized the manuscript.

James A. Lambert and Matthew A. Carlisle performed the majority of experiments, contributed to experimental design and drafted the manuscript.

Wayne E. Bradley and Tamas Jilling catheterized mice and performed and analyzed echosonography.

Louis Dell'Italia directed the design and interpretation of cardiac function studies.

Saurabh Aggarwal, Namasivayam Ambalavanan, David Ford, and Rakesh Patel contributed to experimental design, critical interpretation of the data and editing of the manuscript.

Adam Lam, Stephen Doran, Changchun Ren contributed to the execution of the experimental plan and the drafting of the manuscript.

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NOVELTY AND SIGNIFICANCE

1) WHAT IS NEW?

We demonstrate that exposure of pregnant mice to Br_2 damages the placenta, causes fetal growth restriction, systemic hypertension, cardiac injury and lung injury. Furthermore, we show tadalafil, administered post-exposure, alleviates all pregnancy-specific injury, and increases survival in pregnant mice from 36% to 80%.

2) WHAT IS RELEVANT?

Four % of American women are pregnant at any given time. Currently, there are no studies assessing injury to pregnant animals exposed to halogens, such as Br_2.

3) SUMMARY

We demonstrate that following Br_2 exposure, pregnant mice display signs reminiscent of preeclampsia with fetal growth restriction. Additionally, we documented increased cardiac and pulmonary injuries in pregnant mice exposed to Br_2. Post-exposure administration of tadalafil in pregnant mice, decreases mortality and morbidity.

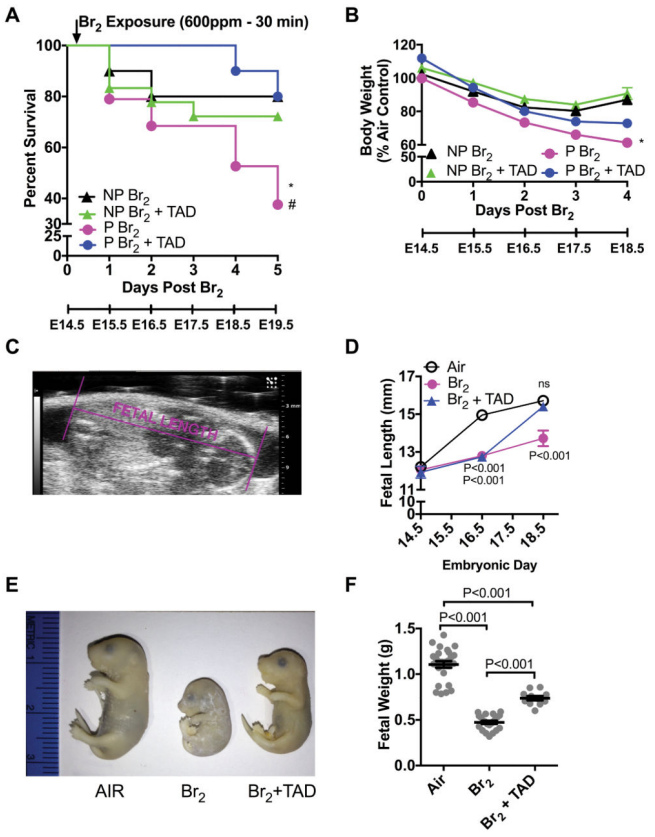

Figure 1. Pregnant mice exhibit increased body weight loss and mortality and fetal growth restriction

Non-pregnant (NP) and pregnant (P) (E14.5) mice were exposed to air or to Br_2 at 600 ppm for 30 min and returned to room air; they received tadalafil (TAD; 2 mg/kg BW in 0.1 ml of sterile saline) or vehicle via oral gavage at 1 h post-exposure and every 24 h thereafter. Body weights and survival times were recorded daily. **A)** Kaplan-Meyer curves of pregnant and non-pregnant mice with tadalafil or vehicle, post Br_2 exposure. Non-pregnant mice exposed to Br_2 and returned to room air lived longer than similarly exposed pregnant mice (* = $P<0.05$). Tadalafil improved survival times of pregnant mice post Br_2 (# = $P<0.05$) but not of non-pregnant mice; n=10–19; Log-Rank Test. **B)** Body weights normalized to weights of air-exposed mice. Pregnant mice exposed to Br_2 and returned to room air exhibit more severe weight loss compared to similarly exposed non-pregnant mice (* = $P<0.05$). Tadalafil administration mitigated weight loss in pregnant mice at four days post-exposure, but has no effect in non-pregnant mice; n=6–8; ANOVA. **C)** Representative ultrasound of a fetus showing how fetal length was measured. **D)** Summary data of fetal length measurements at E14.5, E16.5, & E18.5. Exposure to Br_2 resulted in decreased fetal lengths which were restored to their air control values at E18.5 in the tadalafil group; n= 6–10 pups (2 pups per litter) for each condition; ANOVA; p values as compared to the corresponding air controls for the indicated gestational age. **E)** Representative photograph of paraformaldehyde-fixed fetuses at E18.5 for the indicated conditions. Fetuses of Br_2-exposed pregnant mice exhibit severe fetal growth restriction, and tadalafil improves fetal growth. **F)** Fetal weights were recorded after extraction of fetuses at E18.5. Fetal weights of fetuses from Br_2-exposed

pregnant mice weighed considerably less and were partially rescued by tadalafil (TAD); n=pups (11–25) (2 pups per litter); ANOVA. All data are means±S.E.M.

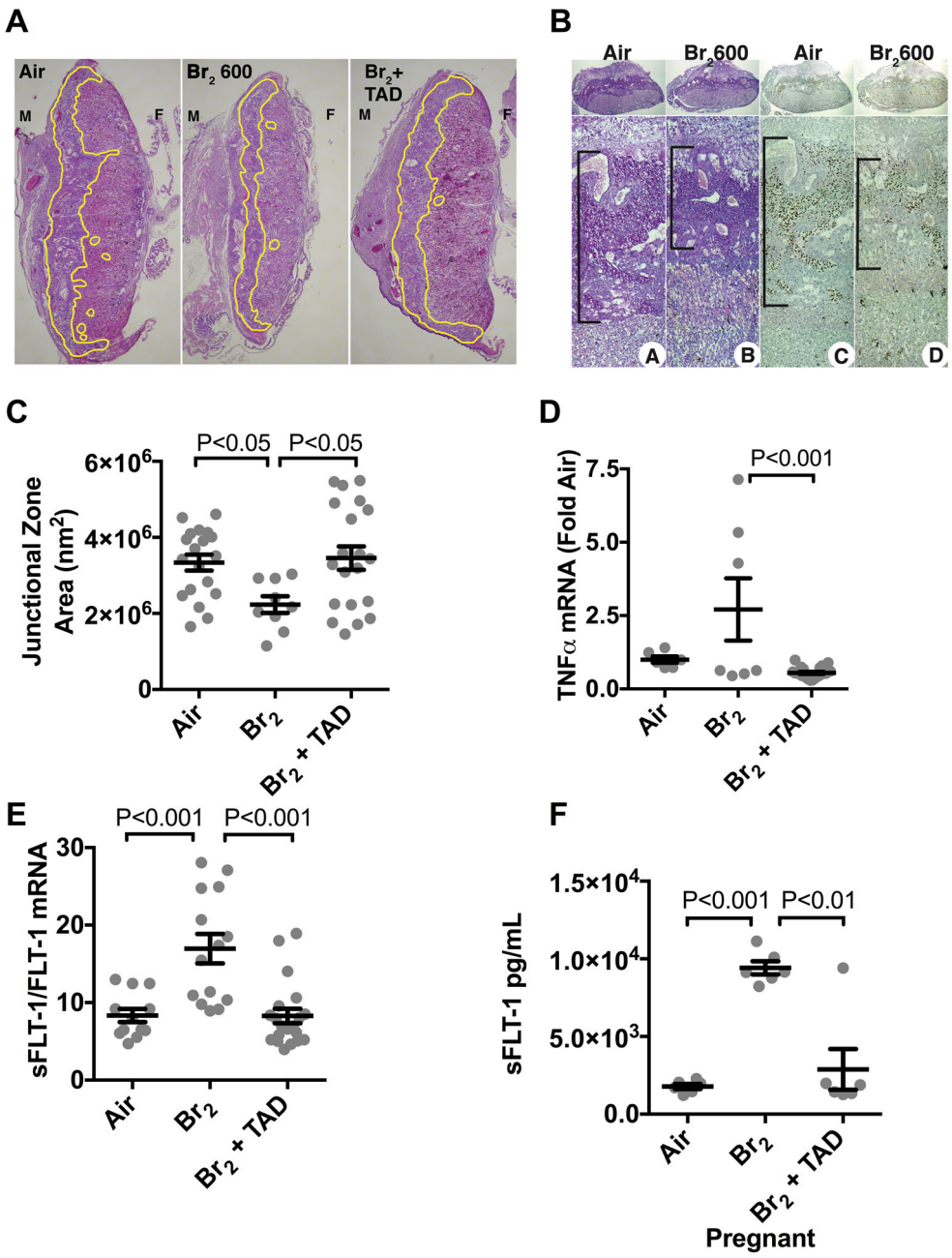

Figure 2. Exposure of pregnant mice to Br_2 damages their placentas

Pregnant (E14.5) mice were exposed to air or 600ppm Br_2 for 30 minutes, returned to room air and received tadalafil (TAD) or vehicle. **A**–**B)** Representative H&E stained (A) placenta sections at E18.5 with the junctional zone demarcated with yellow highlighting (A) as well as (B) PAS staining (left) and CDX2 staining (right) of Br_2-exposed pregnant mice revealed a reduced junctional zone (B: black bars) at E18.5; tadalafil administration restored junctional zones to normal size. **C)** Junctional zone areas at E18.5 for the indicated groups; n=9–20; ANOVA. **D**–**E)** TNFα mRNA (n=6–23) was reduced in Br_2-exposed pregnant mice treated with tadalafil. sFLT-1/FLT-1 mRNA (=12–18) was increased in placentas of Br_2-

exposed pregnant mice at E18.5 (ANOVA), and was reduced to air control values by tadalafil. **F)** sFLT-1 in plasma at E18.5 increased in pregnant mice exposed to Br_2 and was reduced with tadalafil; ANOVA. Only one placenta per pregnant mother was used. All data are means±S.E.M.

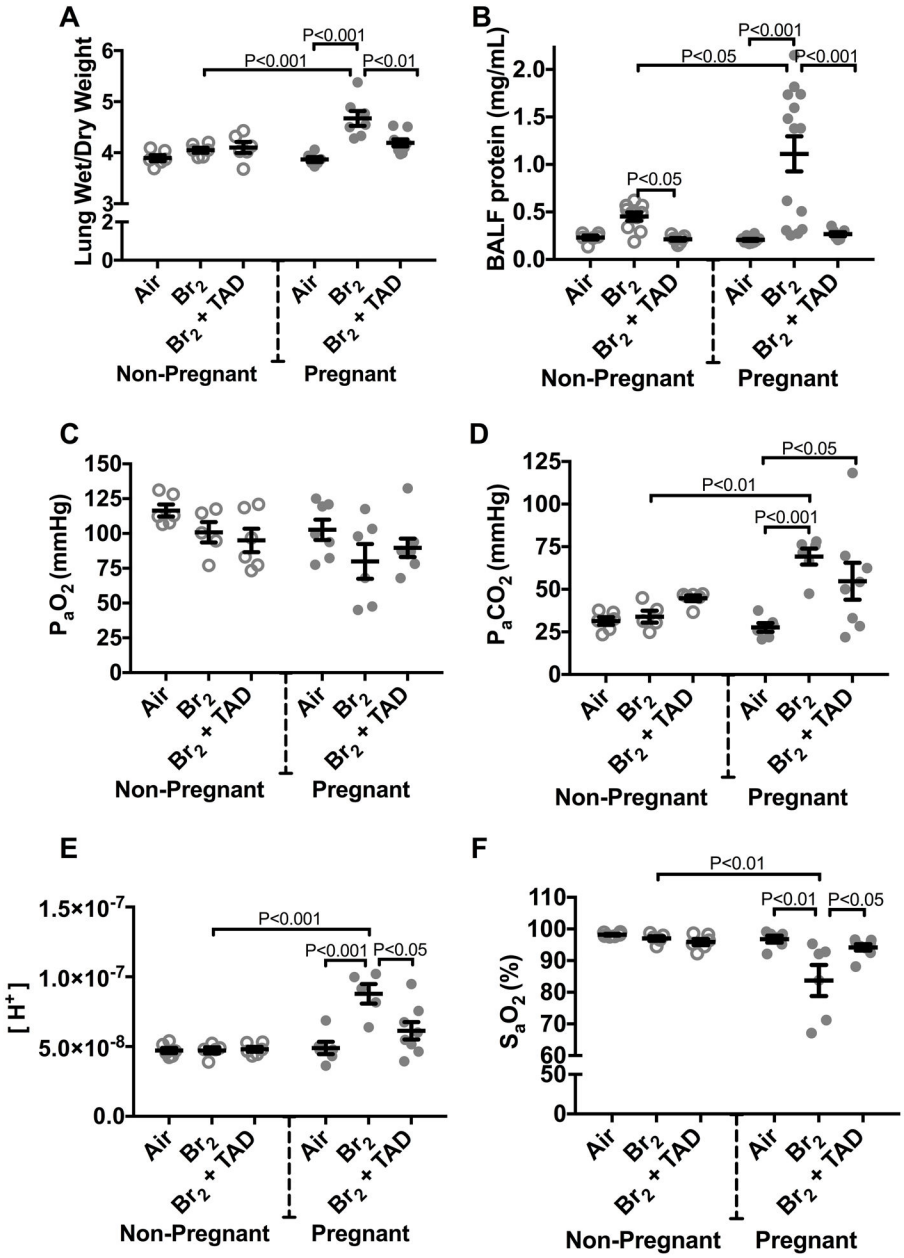

Figure 3. Pregnant mice exhibit pulmonary injury 96 h post-exposure

Non-pregnant and pregnant (E14.5) mice were exposed to air or 600ppm Br_2 for 30 minutes, returned to room air, then administered tadalafil (TAD) or vehicle. **A)** Lung wet/dry weight ratios at E18.5 are increased in pregnant mice exposed to Br_2 and are reduced by tadalafil. **B)** Protein concentrations in the bronchoalveolar lavage fluid (BALF) of pregnant mice were increased at E18.5 and returned to the air control values following administration of tadalafil (TAD). **C–F)** P_aO_2 was unchanged, P_aCO_2 increased, [H^+] increased and S_aO_2 was decreased in pregnant mice exposed to Br_2 at E18.5. Administration of Tadalafil returned [H^+] and S_aO_2 to their air control values but did not improve P_aCO_2; All data n=6–14 mice; ANOVA; means±S.E.M.

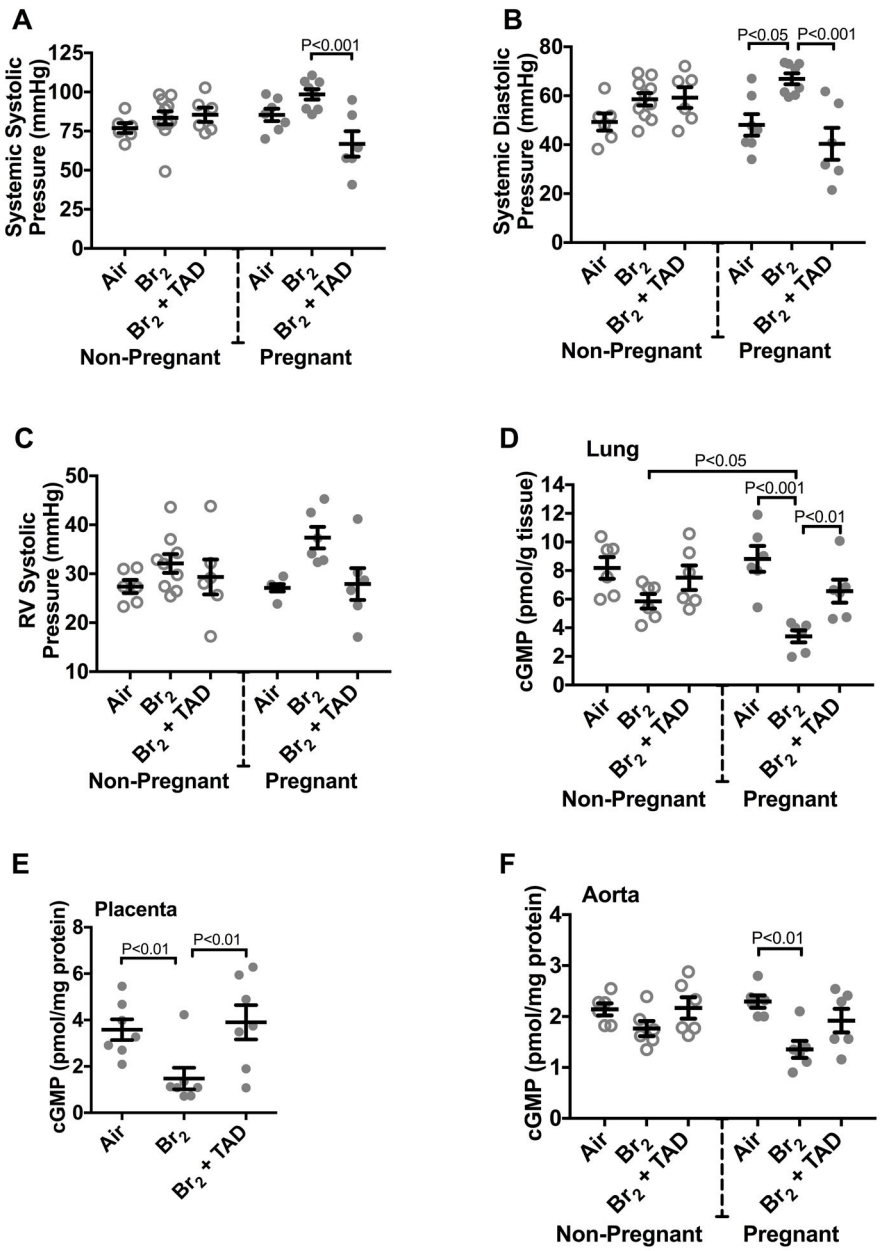

Figure 4. Exposure to Br_2 increases the systemic blood pressure of pregnant mice and decreases cGMP in the lungs, placentas and aortas

Non-pregnant and pregnant (E14.5) mice were exposed to air or 600ppm Br_2 for 30 minutes, returned to room air, then administered tadalafil (TAD) or vehicle. At E18.5, a pressure-transducer catheter was inserted into either the aortic arch via the carotid or into the right ventricle through the jugular vein in anesthetized mice. **A–B)** Br_2 has no significant effect on systemic systolic or diastolic blood pressures in non-pregnant mice (NP). However, systemic diastolic pressures were increased in pregnant mice (P). Both systemic systolic and diastolic pressures were decreased to their corresponding air controls following tadalafil administration; n=6–11; ANOVA. **C)** Right ventricular (RV) systolic pressures remained unchanged in pregnant mice exposed to Br_2. **D–F)** Lung tissue, placenta, and aorta cGMP

levels of Br_2-exposed pregnant mice decreased significantly. Lung and placenta cGMP levels were restored following tadalafil administration; n=6–7 per group; ANOVA. All data are means±S.E.M.

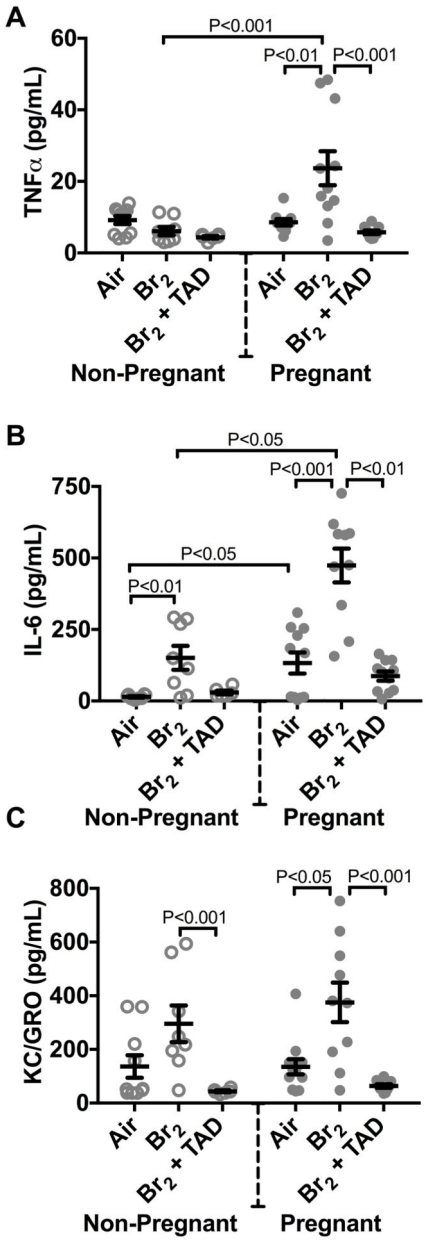

Figure 5. Pregnant mice exposed to Br_2 demonstrated systemic inflammation at E18.5

Non-pregnant and pregnant (E14.5) mice were exposed to air or 600ppm Br_2 for 30 minutes, returned to room air, then administered tadalafil (TAD) or vehicle. **A–C)** Plasma TNFα, IL-6, and KC/GRO increased in pregnant mice exposed to Br_2 but only IL-6 increased in non-pregnant mice post-Br_2. All three cytokines in pregnant mice exposed to Br_2 returned to air control values following tadalafil administration; n=6–12 per group; ANOVA. All data are means±S.E.M.

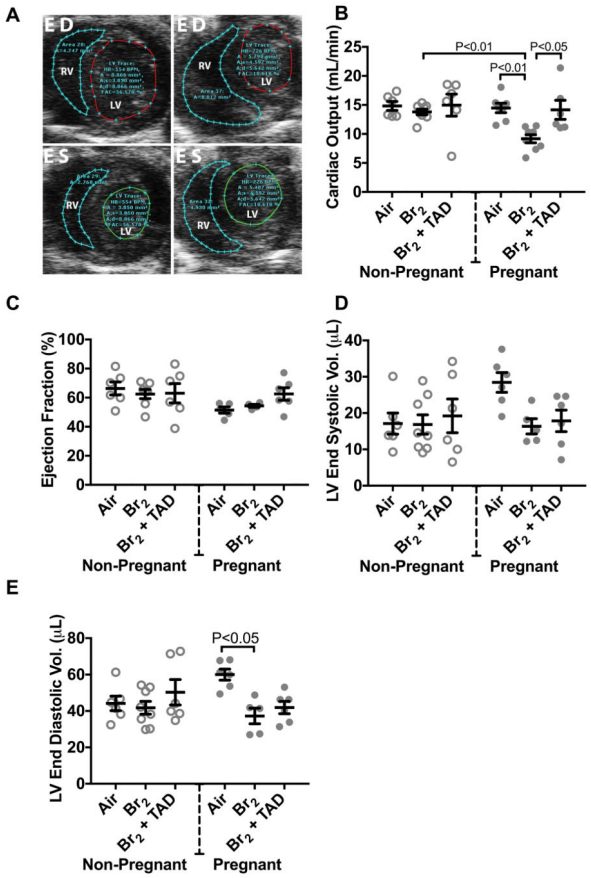

Figure 6. Br_2-exposed pregnant mice exhibit diminished cardiac function at E18.5

Non-pregnant and pregnant (E14.5) mice were exposed to air or 600ppm Br_2 for 30 minutes, returned to room air, then administered tadalafil (TAD) or vehicle. **A)** Representative echosonography LV and RV traces at E18.5 of pregnant mice exposed to air or Br_2 with demarcation of ventricular sizes. HR = Heart rate, A = Area, A;s = Area systole, A;d = Area diastole, FAC = Fractional area change. **B)** Cardiac output (LV) determined by echosonography was decreased in Br_2-exposed pregnant mice, which increased following administration of tadalafil; ANOVA: n=6–8. **C)** Ejection fraction (LV) was unchanged in all groups; ANOVA. **D)** LV end-systolic volume was similar in all groups. **E)** However, LV end-diastolic volume was diminished in Br_2-exposed pregnant mice; n=6–8; ANOVA. All data are means±S.E.M.