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Sex-specific Differences in Hyperoxic Lung Injury in Mice: Implications for Acute and Chronic Lung Disease in Humans

Krithika Lingappan*, Weiwu Jiang*, Lihua Wang*, Xanthi I. Couroucli*, Roberto Barrios**, and Bhagavatula Moorthy*

*Department of Pediatrics, Section of Neonatology, Texas Children's Hospital, Baylor College of Medicine, Houston, Texas, USA. Address: 1102 Bates Avenue, MC: FC530.01, Houston, Texas 77030

** Department of Pathology and Genomic Medicine, The Methodist Hospital Physician Organization. Address: 6565 Fannin Street, Suite M227, Houston, Texas 77030

Abstract

Sex-specific differences in pulmonary morbidity in humans are well documented. Hyperoxia contributes to lung injury in experimental animals and humans. The mechanisms responsible for sex differences in the susceptibility towards hyperoxic lung injury remain largely unknown. In this investigation, we tested the hypothesis that mice will display sex-specific differences in hyperoxic lung injury. Eight week-old male and female mice (C57BL/6J) were exposed to 72 h of hyperoxia (FiO₂>0.95). After exposure to hyperoxia, lung injury, levels of 8-iso-prostaglandin F₂ alpha (8-iso-PGF 2α) (LC-MS/MS), apoptosis (TUNEL) and inflammatory markers (suspension bead array) were determined. CytochromeP450 (CYP)1A expression in the lung was assessed using immunohistochemistry and western blotting. After exposure to hyperoxia, males showed greater lung injury, neutrophil infiltration and apoptosis, compared to air-breathing controls than females. Pulmonary 8-iso-PGF 2α levels were higher in males than females after hyperoxia exposure. Sexually dimorphic increases in levels of IL-6 (F>M) and VEGF (M>F) in the lungs were also observed. CYP1A1 expression in the lung was higher in female mice compared to males under hyperoxic conditions. Overall, our results support the hypothesis that male mice are more susceptible than females to hyperoxic lung injury and that differences in inflammatory and oxidative stress markers contribute to these sex-specific dimorphic effects. In conclusion, this paper describes the establishment of an animal model that shows sex differences in hyperoxic lung injury in a temporal manner and thus has important implications for lung diseases mediated by hyperoxia in humans.

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Corresponding author: Krithika Lingappan, Assistant Professor, Department of Pediatrics, Section of Neonatology, Texas Children's Hospital, Baylor College of Medicine, 1102 Bates Avenue, MC: FC530.01, Houston, Texas 77030. Phone: +1-832-824-3208. Fax: +1-832-825-3204. lingappa@bcm.edu.

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Keywords

Hyperoxia; Sex- differences; Lung injury; Oxidative stress; Inflammation; Cytochrome P4501A1

Introduction

Sex-specific differences exist in various forms of organ injury in adults and children. Acute respiratory distress syndrome (ARDS) is a devastating clinical disorder in critically ill patients with a high mortality. Mortality in ARDS was higher in males compared to females (Moss and Mannino, 2002; Agarwal et al., 2006). Neonatal outcomes for males are worse than females for many diseases, including bronchopulmonary dysplasia (BPD). The incidence of BPD is lower among preterm girls after adjusting for other confounders (Stevenson et al., 2000). Male sex is considered an independent predictor for the development of BPD (Kraybill et al., 1989). The lung function in boys both in the neonatal period and at 1 year of age was noted to be worse when compared to girls (Stocks et al., 1997; Thomas et al., 2006). The reasons behind this are not known though better antioxidant defense mechanism in girls is thought to contribute to this advantage (Vento et al., 2009a; Hamon et al., 2011).

Oxygen toxicity is thought to play a role in both acute lung injury and BPD. Exposure to high concentrations of oxygen (hyperoxia) leads to pathological changes similar to ARDS in mammalian species (Clark and Lambertsen, 1971; Freeman and Crapo, 1981; Bryan and Jenkinson, 1988; Matute-Bello et al., 2008; Budinger et al., 2011) and prolonged exposure of newborn mice to hyperoxia, leads to lung pathology similar to human BPD (Warner et al., 1998). In critically ill patients, hyperoxia may exacerbate or even cause acute lung injury (ALI). In the acute phase, after exposure to hyperoxia, lung epithelial injury and neutrophilic infiltration is observed. Exposure to hyperoxia postnatally is thought to contribute to the development of BPD in neonates (Vento et al., 2009b). Hyperoxia leads to the production of reactive oxygen species (ROS) and these molecules lead to lung injury via oxidation of cellular macromolecules including DNA, protein and lipid (Freeman and Crapo, 1981).

The cytochrome P450 (CYP) enzymes belong to a super family of heme proteins, involved in the metabolism of exogenous and endogenous chemicals (Guengerich, 1990). Induction of the CYP family of proteins has been implicated in the potentiation of hyperoxic lung injury (Hazinski et al., 1995), but on the other hand, we have demonstrated the protective effect of CYP1A enzymes (Jiang et al., 2004; Sinha et al., 2005; Couroucli et al., 2011).

The impact of sex and sex hormones on lung physiology and disease has been extensively studied in animal models. Gender also contributes to differential lung development, and has a major role in the causation of disease conditions from the neonatal (respiratory distress syndrome, BPD) to the adult period (asthma, lung cancer, interstitial lung disease) (Carey et al., 2007). This is probably due to modulation by sex hormones that may contribute to the disease pathogenesis or serve as protective factors, depending on the disease involved. With respect to acute lung injury due to hyperoxia it has been showed that castration prolonged tolerance of young male rats to pulmonary oxygen toxicity (Neriishi and Frank, 1984). In other acute lung injury models, testosterone was found to increase (Card et al., 2006) and

estrogen to ameliorate inflammation and injury (Speyer et al., 2005). However, there are no studies on the temporal effects of exposure to hyperoxia on lung injury in a sex-specific model in adult mice. The sex based dimorphic response in lung injury due to hyperoxia and the differences in inflammatory and oxidative stress markers have also not been studied. In this investigation, we tested the hypothesis that males are more susceptible to hyperoxic lung injury than females and that sex-specific differences in inflammatory and oxidative stress markers contribute to the gender differences under hyperoxic conditions.

Material and methods

Animals

This study was conducted in accordance with the federal guidelines for the human care and use of laboratory animals, and was approved by the Institutional Animal Care and Use Committee (IACUC) of Baylor College of Medicine. Breeding pairs of mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Eight-week old male (C57BL/6J) mice were maintained at Texas Children's Hospital animal facility and used for the study. They were fed standard mice food and water *ad libitum*. Animals were maintained in a 12-h day/night cycle. Briefly, we used a total of 20 animals per sex in the study. The mice were maintained in either room air (21% oxygen) or exposed to hyperoxia (95–100% oxygen) environment using pure O₂ at 5 l/min for 72 h in a sealed Plexiglas chamber, as reported previously (Gonder et al., 1985). After sealing the chamber, the oxygen concentration in the Plexiglas chamber was measured frequently by an analyzer (Getronics, Kenilworth, New Jersey). Purified tap water and food (Purina Rodent Lab Chow 5001 from Purina Mills, Inc., Richmond, IN) were available *ad libitum*. After 72 h of hyperoxia exposure, the animals were anesthetized with 200 mg/kg of sodium pentobarbital (i.p.) and euthanized by exsanguination while under deep pentobarbital anesthesia. The lung tissues were harvested for further analysis.

Chemicals

Tris, sucrose, bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). All Real Time RT-PCR reagents were from Applied Biosystems (Foster City, CA). 8-iso-PGF₂α and 8-iso-PGF₂α-d₄ were purchased from Cayman Chemical Co. (Ann Arbor, MI).

Lung wet weight and Body weight

The mice were weighed immediately after being anesthetized, and the lungs were weighed after the sacrifice and harvesting.

Preparation of tissues for histology, histopathology and immunohistochemistry

Tracheotomy was performed on the anesthetized mice and the lung tissue was fixed by intratracheal instillation of 10% zinc formalin at constant pressure of 25 cm of H₂O (Couroucli et al., 2002). Samples were left in solution for 24 h in formaldehyde, and then transferred to 70% ethanol for long-term storage. Routine histology was performed on lung tissues from individual animals following staining of the paraffin sections with hematoxylin and eosin. 5 microns deparaffinized lung sections were immunostained with CD5

monoclonal CYP1A1/2 antibody (generous gift from Dr. Paul E. Thomas; dilution 1:50) for CYP1A1/2 and rat anti-mouse neutrophil antibody (Serotec, Raleigh, NC; MCA771G, dilution 1:200) for neutrophils, followed by staining with biotinylated secondary antibodies (Vector Laboratories Burlingame, CA). To analyze the degree of pulmonary neutrophil infiltration, the positively stained cells were counted in 20 non-adjacent areas per mouse under 40x magnification. A pulmonary pathologist, who was blinded to the treatment of mice with various regimens, evaluated the histopathology and immunohistochemistry slides. Assessment of lung injury in the histopathological lung sections was performed as follows: 20 random high-power fields (400x total magnification) were independently scored in a blinded fashion taking care that 50% of each field was occupied by lung alveoli. Five histological findings: neutrophils in the alveolar space, neutrophils in the interstitial space, hyaline membranes, proteinaceous debris filling the airspaces and alveolar septal thickening were graded using a three tiered schema as described in the official American thoracic society workshop report on measurement of acute lung injury in experimental animals. (Matute-Bello et al., 2011) resulting in a lung injury score between zero and one.

TUNEL Analysis

Lung tissue was analyzed for terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) using the Millipore Apoptag Peroxidase In Situ apoptosis detection kit (Millipore, S7100). Tissue sections were deparaffinized and rehydrated in an ethanol series. The tissue sections were then pretreated with proteinase K for 15 minutes at room temp. Endogenous peroxide was quenched using 3% hydrogen peroxide in PBS for 5 minutes at room temp. Nicked DNA ends were labeled by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method following the protocol provided by the manufacturer. Color was developed in peroxidase substrate, counter stained with methyl green. The sections were then dehydrated and permanently mounted. Twenty consecutive hpf under 40x magnification were observed for the number of TUNEL positive cells. Negative controls (performed simultaneously but without TdT) were examined at the same time.

Lung F₂-Isoprostane Analyses

Mouse tissues (lung) were prepared for LC-MS/MS analysis by homogenizing the tissues in dPBS (Dulbecco's Phosphate Buffered Saline) or methanol using MP Biomedical lysing matrix D in an MP FastPrep 24 instrument (MP Biomedical, Solon, OH). The tissues were homogenized at a tissue/volume (w/v) concentration of 50 mg tissue per ml of methanol. Complete homogenization required 40 seconds duration at a power setting of 6. Lung tissues were suspended in PBS. Following homogenization, the lung tissue homogenates were vortex mixed and 200 μ L of the homogenates were removed and placed into a 15 mL glass tube, 22 μ L of 1 μ g/mL deuterated 8-isoprostane F₂ α (8-iso-PGF₂ α) was added as an internal standard and the solution was then extracted twice with 3 mL of methyl tertiary butyl ether. The extracts were combined and evaporated to dryness with nitrogen gas. The dried extract was reconstituted to 100 μ L volume with 50:50 methanol:0.2% acetic acid in water and transferred to an LC-MS/MS vial for analysis.

The detection and quantification of 8-iso-PGF2 α was done using a Waters QuattroUltima mass spectrometer (Waters, Milford, MA) coupled to an Agilent 1100 binary HPLC system (Agilent, Santa Clara, CA). A Phenomenex Luna phenyl-hexyl 150x2.1 mm, 3 μ particle size analytical column (Phenomenex, Torrance, CA) was used to chromatographically resolve 8-iso-PGF2 α from background. Chromatography was done using a linear methanol: 0.2% formic acid in water gradient. The initial conditions were 50% methanol to 80% methanol at four minutes, then increasing to 100% at 5 minutes and held at 100% until 7 minutes. The column temperature was maintained at 60°C with a flow rate of 400 μ L per minute. The injection volume was 25 μ L with 8-iso-PGF2 α eluting at 3.9 $\pm$ 0.1 minutes. The compound was detected and quantified by selected reaction monitoring using a mass transition of m/z 353.25>353.25 in electrospray negative ionization. The lower limit of quantification was 12.5 pg on column with a signal to noise >10. Quantification was done using a quantification curve prepared using control mouse lung tissues at 50 mg/tissue (wt. per vol.) spiked with concentrations of 8-iso-PGF2 α and its deuterated internal standard which were extracted using the same sample preparation protocol as experimental animal tissues.

Preparation of Lung Homogenates for Luminex Suspension Bead Array

The snap frozen lung samples were weighed and added to 1ml of lysis buffer (PBS+ cocktail of protease inhibitor). The tissues were ground and the samples were incubated on a rocker at 4°C for 15 minutes followed by centrifugation at 10,000 G for 15 minutes. The supernatants were removed and protein concentrations were determined using the BCA (bicinchoninic acid) method. The samples were subsequently diluted to 2mg/ml and 50 μ g of lung sample (1:1 dilution) was subjected to the Luminex assay.

Luminex Suspension Bead Array

The proteomic platform for the detection of inflammatory markers in the lungs was the luminex suspension bead array (Luminex, Austin, TX, USA). The mouse cytokine/chemokine MCYTOMAG-70K-06 kit (Cat# MCYTOMAG-70K-06 Lot#2058538,) was used according to the user manual to evaluate the levels of IL-1 β , IL-6, TNF- α , VEGF, MCP-1 and MIP- α in lung samples. Each sample was assayed in duplicate. Medium florescent intensity was generated for each well was calculated based on standard curve data using 5-parameter logistic-fitting method in Bio-Plex manager software 6.0. Lysis buffer mentioned above was used as matrix for accurate concentration determination in the lung lysate samples. The plate was analyzed by Bio-Plex 200 system and data analyzed by Bio-Plex manager software 6.0 (Bio-Rad, Hercules, CA). An 80-120% Observed/Expected concentration criterion was used. Fifty beads per analyte were collected and a timeout of 60s was set while analyzing the palette average values of the replicates were used in the final analysis.

Western Blotting

Lung whole protein (20 μ g of protein) was prepared and subjected to SDS polyacrylamide gel electrophoresis in 10% acrylamide gels. The separated proteins on the gels were transferred to polyvinylidene difluoride membranes, followed by western blotting. For the western blot analysis, a monoclonal antibody to CYP1A1, which cross-reacts with CYP1A2

was used as a primary antibody. The primary antibody was detected by incubation with the horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody. For loading controls, the membranes were stripped and incubated with antibodies against β -actin, followed by electrochemical detection of bands.

Data Analyses

The comparison between male and female mice was done using two-way ANOVA, followed by Bonferroni post-hoc tests, and $p < 0.05$ was considered significant. The statistical analysis was performed using Graphpad Prism version 5 (Graphpad Software, San Diego, CA)

Results

Lung Injury

a) Lung wet weight and body weight—Lung wet weights were determined to evaluate the severity of lung edema. Differences existed in body weights between male and female animals at baseline (room air) conditions ($p < 0.001$ between male and female mice body weight at room air, 24 and 48 h of hyperoxia exposure and $p < 0.01$ at the 72 h time point). After exposure to hyperoxia (Fig. 1A), at 48 h ($p < 0.05$) and at 72 h ($p < 0.001$), male mice showed significant weight loss whereas female mice did not. The progressive worsening in pulmonary edema as shown by increase in lung weight with increasing duration of hyperoxia exposure can be seen in Figure 1B ($p < 0.001$ for the comparison between lung weight after 72 h of hyperoxia exposure to room air breathing controls). Also, after exposure to hyperoxia (Fig. 1B), males showed greater increase in lung wet weight compared to corresponding room air animals than females at 72 h after hyperoxia ($p < 0.05$).

b) Lung histopathology and lung injury score—Lungs of male and female mice were evaluated for lung injury by histology. Figure 2A shows lung sections at 10X magnification and Figure 2B at 20X magnification at room air and after 24–72 h of hyperoxia exposure. At 24 h, the animals did not show any appreciable injury. After 48 h, there were signs of alveolar edema. At 72 h, the lungs showed greater perivascular, bronchiolar edema, and alveolar hemorrhage in male mice when compared to corresponding room air animals than females. Figure 3 shows the objective assessment of lung injury according to the official American thoracic society workshop report on the measurement of acute lung injury in experimental animals. Increase in lung injury score in animals after exposure to hyperoxia for 72 h can be seen in both male ($p < 0.001$) and female ($p < 0.01$) animals. Also, the lung injury score was higher in female mice compared to male mice ($p < 0.001$) at the 72 h time point.

c) Neutrophil infiltration—We performed immunohistochemistry using anti-neutrophil antibodies to quantify neutrophil infiltration in the lungs upon hyperoxia exposure. Neutrophil recruitment in the lungs can be seen in the lung sections upon exposure to hyperoxia (Figure 3A). Upon quantification, at 24 h male mice showed greater neutrophil infiltration in the lungs when compared to corresponding room air animals than females ($p < 0.05$). At 48 h, there was a decrease in the lung neutrophils, and this was more significant

in males ($p<0.001$). After 72 h, males showed a trend towards higher neutrophil infiltration in the lungs when compared to corresponding room air animals than females (Figure 3B)..

Pulmonary apoptosis after hyperoxia

TUNEL assay was used for the determination of apoptotic cells in the lung after exposure to hyperoxia. TUNEL staining was increased in the lungs of male mice exposed to 72 h of hyperoxia when compared to corresponding room air controls than females. (Figure 5A). Quantitative analyses showed a significantly greater number of TUNEL positive cells in the lungs in males after hyperoxia exposure than females (Figure 5B; $p<0.003$).

Pulmonary F₂-Isoprostane levels

To test differences in the levels of ROS-mediated lipid peroxidation products such as F₂-isoprostanes, we determined levels of 8-iso-PGF₂ α by LC-MS/MS in the lungs of male and female mice in room air and after exposure to hyperoxia. The representative chromatograms of the standard and lung samples are shown in Figure 6A, 6B and 6C. Even in room air, animals showed appreciable levels of F₂-isoprostanes in their lungs. At 24 h, there was no difference between male and female mice. 8-iso-PGF₂ α levels were higher in males after 48 h of hyperoxia exposure compared to similarly exposed females. ($p<0.05$) (Figure 6D). At 72 h, the levels were lower compared to other time points.

Lung cytokine levels

Lung IL-6 levels increased after exposure to hyperoxia (48 h) in both male ($p<0.05$) and female mice ($p<0.0001$), but the interaction of sex was statistically significant with greater increase in IL-6 levels seen in female mice ($p<0.05$) (Figure 7A). Lung VEGF (Figure 7B) levels also increased after exposure to hyperoxia (48 h) in both male and female mice ($p<0.01$). Lung VEGF levels was higher in males both at room air and after exposure to hyperoxia ($p<0.05$). Lung IL-1 β (Fig.7C) levels were higher in females ($p<0.001$) at room air compared to males. After exposure to hyperoxia for 48 h, the levels decreased in female mice compared to room air breathing controls than males ($p<0.001$). There was no sex-specific difference noticed in MIP-1 α (Fig.7D) levels but the levels decreased in both male ($p<0.001$) and female ($p<0.05$) mice after 48 hours of hyperoxia exposure. No change was observed in lung MCP-1 (Fig.7E) levels. TNF- α levels were too low in the lung homogenates and hence not reported.

CYP1A1 immunohistochemistry and western blot

To determine the expression of CYP1A1 protein in specific regions of the lung, we performed immunohistochemistry on fixed lung sections of male and female mice using monoclonal antibodies against CYP1A1. Immunohistochemistry showed that after 72 h of hyperoxia exposure, enhanced CYP1A1 staining was noted both in the bronchial and alveolar epithelial cells in the lung sections of female mice compared to room air breathing controls than male mice (Figure 8A). Western blot analysis of lung protein (Fig 8B) at room air and after 48 h of hyperoxia showed higher CYP1A levels in female mice.

Discussion

In this study, we demonstrated the sex-based dimorphic response in lung injury due to hyperoxia and the possible correlation with the differences in inflammatory markers and oxidative stress response in male and female mice. Male mice were more susceptible to hyperoxic lung injury compared to corresponding room air animals than female mice, as evidenced by greater pulmonary edema (lung wet weight) after hyperoxia exposure. Histological analyses of the lung tissues confirmed the above, with male mice showing greater perivascular and bronchiolar edema, and alveolar hemorrhage. Lung injury scores were higher in male mice after 72 h of hyperoxia exposure compared to corresponding room air animals than female mice. Neutrophil infiltration in the lungs was greater in male lungs probably due to greater injury in the male animals or increased neutrophil recruitment to the lungs may have led to increased inflammation and injury in male animals. Apoptosis in the lungs after exposure to hyperoxia was also greater in males as assessed by the TUNEL assay. F₂-isoprostanes, a marker of lipid peroxidation and oxidative stress were elevated in male lungs after 48 h of hyperoxia exposure compared to similarly exposed females. Interestingly, the inflammatory response showed increased IL-6 in the lungs in females while the VEGF levels were greater in males. Immunohistochemistry and western blot analysis for CYP1A1 revealed greater expression of pulmonary CYP1A1 in female mice compared to males after hyperoxia exposure. We hypothesize that the interaction among the above measured parameters mechanistically contribute to the sex-specific differences in hyperoxic lung injury. We postulate that differences in the levels of ROS (e.g., F₂-isoprostanes), CYP1A1 expression, levels of cytokines and other inflammatory markers, and extent of pulmonary neutrophil recruitment contribute to the noted sex differences in hyperoxic lung injury.

Epidemiological data point to the effect of sex in the incidence, susceptibility, and severity of lung diseases. This could be due to underlying hormonal, physiological, and developmental differences between males and females. Gender affects inflammation and prevalence and severity of various lung diseases including pulmonary fibrosis, asthma and lung cancer (Carey et al., 2007). In ALI models with LPS-induced lung inflammation, males showed more inflammation than females, and it was observed that testosterone given to female animals and gonadectomy in males decreased inflammation (Card et al., 2006). Another study in a similar animal model showed the protective effect of estrogen (Speyer et al., 2005). Similar sex-specific dimorphic outcomes were observed in models of shock-induced lung injury (Ananthakrishnan et al., 2005). Similar to the studies above we observed more lung injury and neutrophil infiltration in male mice compared to corresponding room air animals than females after exposure to hyperoxia. This was in agreement with previous studies (Neriishi and Frank, 1984). Hormones like estradiol and testosterone are a major contributor to sex-specific outcomes, there may be other mechanisms underlying these differences.

We observed greater extent of apoptosis in male mice after hyperoxia exposure compared to corresponding room air animals than females (Figure 4). Sex-specific differences in apoptosis have been shown in neuronal ischemia, heart failure and renal ischemia (Hutchens et al., 2008; Lang and McCullough, 2008; Dunlay and Roger, 2012). Divergent cell death

pathway activation could be one of the reasons leading to this phenomenon (Lang and McCullough, 2008).

Oxidative stress has been implicated in the pathogenesis of many disease processes. Lipids are major target of ROS and they undergo peroxidation under conditions of oxidative stress. Measurement of F₂-isoprostanes formed non-enzymatically by peroxidation of arachidonic acid esterified in membrane phospholipids is considered one of the most reliable approaches for assessing oxidative stress status *in vivo* (Fessel et al., 2002). At room air, both male and female mice had detectable levels of F₂-isoprostanes in the lungs (Figure 6D), and after 24 h of hyperoxia, there was no difference between males and females. At 48 h, levels of F₂-isoprostanes in the lungs of female animals were less compared to males, which may suggest that female metabolize the isoprostanes generated during hyperoxia to non-toxic intermediates. The levels at 72 hours were lower. This could be attributed to the short half life of F₂-isoprostanes and the advanced stages of injury and cell death at this time point. Lipid peroxidation products generated due to ongoing cellular injury are generated at earlier time points and subsequently metabolized or excreted.

IL-6 type cytokines have been shown to confer significant protection against hyperoxic lung injury. This might be mediated thro PI3K/Akt-mediated Bax phosphorylation (Kolliputi and Waxman, 2009). In another study, IL-6 markedly diminished by peroxic lung injury, cell death and DNA fragmentation and this ws associated with induction in bcl-2 (Ward et al., 2000). In our experiments, female mice showed higher IL-6 levels in the lungs compared to male mice.

Reactive oxygen intermediates generated under hyperoxic conditions increase the expression of VEGF *in vitro* and *in vivo* (Kuroki et al., 1996). VEGF overexpression increases pulmonary permeability and leads to the development of pulmonary edema (Kaner et al., 2000). We found increased VEGF levels in the lungs after hyperoxia exposure in males compared to females. VEGF levels were elevated in males with COPD when compared to females (de Torres et al., 2011). The role of VEGF in ALI and recovery from ALI remains controversial. Lung IL-1 β in females and MIP-1 α levels in both male and female mice decreased at the 48h time point. This also corresponds to the decrease in neutrophil infiltration in the lungs at this time point as shown in Fig.4B. IL-1 β and MIP-1 α are known to contribute to neutrophil accumulation and lung injury (Patton et al., 1995; Shanley et al., 1995).

The protective effect of CYP1A system against hyperoxic lung injury has been well documented. Induction of the CYP1A enzymes by 3-methylcholanthrene and beta-naphthoflavone (Mansour et al., 1988; Sinha et al., 2005; Moorthy, 2008; Couroucli et al., 2011) attenuates, while treatment with the CYP1A inhibitor 1-aminobenzotriazole (Moorthy et al., 2000) exacerbates lung injury in hyperoxic conditions. In the present investigation, immunohistochemistry for CYP1A1 in the lungs showed more enhanced staining in female lungs compared to males after exposure to 72 h of hyperoxia. Although the antibodies we used cross-react with CYP1A2, the fact that CYP1A2 is liver-specific and absent in lung indicated that our immunohistochemical studies revealed the expression of CYP1A1. These results support the hypothesis that the higher expression of CYP1A1 in the lungs of female

mice under hyperoxic conditions may have in part contributed to the lesser susceptibility of females to hyperoxic lung injury than males. The mechanism by which increased CYP1A1 expression contributes to lesser lung injury is not yet clear, but it is possible that detoxification of lipid hydroperoxides such as F₂-isoprostanes by CYP1A1 may have contributed to the lesser injury seen in female mice (Moorthy et al., 2000; Couroucli et al., 2011)

In this study, we did not time our experiments with the estrous cycle in female mice, which could have led to some of the variability in our findings. However, consistent results across multiple experiments support the interpretation of our findings. Other mechanisms have been proposed explaining the resistance of female mice to hyperoxia induced oxidative stress such as upregulated expression of heme oxygenase-1 and CYP enzymes such as Cyp2a5 (Ma ak-Šafranko et al., 2011). Antioxidant enzymes like superoxide dismutase may also have contributed towards the observed gender-based differences (Park and Rho, 2002). Enomoto et al showed that male pups have lower superoxide dismutase (SOD) content and fail to upregulate SOD when exposed to hyperoxia (Enomoto et al., 2012).

Our findings show clear sex-specific differences in hyperoxic lung injury, in that males are more susceptible than females, and the fact that similar effects are seen in clinical settings such as ARDS makes our studies in animal models to be highly relevant to the study of acute lung injury in humans. . In conclusion, this paper describes the establishment of an animal model that shows sex differences in hyperoxic lung injury in a temporal manner that are similar to that observed in human premature infants and adults, and therefore, the studies have important implications for lung diseases mediated by hyperoxia in humans. In addition, the increased expression of CYP1A in the lungs of female mice point towards the sex-specific protective effect of this enzyme. In summary, our findings show clear gender differences in hyperoxic lung injury, in that males are more susceptible than females, and the fact that similar effects are seen in clinical settings such as ALI/ARDS in humans makes our studies in animal models to be highly relevant to these diseases.

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Abbreviations

BPD	Bronchopulmonary dysplasia
ALI	Acute Lung Injury
ROS	Reactive oxygen species
ARDS	Acute respiratory distress syndrome
8-iso-PGF2α	8-iso-prostaglandin F ₂ .alpha

CYP

CytochromeP450

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Highlights

1. Male mice were more susceptible to hyperoxic lung injury than females.
2. Sex differences in inflammatory markers were observed.
3. CYP1A expression was higher in females after hyperoxia exposure.
4. Male mice were more susceptible to hyperoxic lung injury than females.
5. Sex differences in inflammatory markers were observed.
6. CYP1A expression was higher in females after hyperoxia exposure.

Figure 1.

Male mice have greater pulmonary edema and body weight loss compared to females after exposure to hyperoxia. 1A: Effect of hyperoxia (24, 48 and 72 h) on body weight. 1B: Effect of hyperoxia (24, 48 and 72 h) on lung wet weight. Values are means $\pm$ SEM from at least 5 individual animals. Significant differences are indicated by *, $p < 0.05$ and ***, $p < 0.001$.

Figure 2.

Male mice show more lung injury on histopathological analysis compared to female mice after exposure to hyperoxia at 72 h. Representative hematoxylin and eosin stained lung sections obtained from male and female mice (n=5 mice per group) exposed to hyperoxia (24, 48 and 72 h). Figure 2A: at 10x magnification and Figure 2B at 20x magnification. Males show more perivascular, bronchiolar edema and alveolar hemorrhage (as shown by arrows) at 72 h when compared to female mice.

Figure 3.

Animals show increase in lung injury scores after hyperoxia exposure and male mice have higher lung injury scores on histopathological analysis compared to female mice after exposure to hyperoxia at 72 h. Lung injury scores in 20 random high-power fields (400x total magnification, n=3 mice per group) in hematoxylin and eosin stained lung sections obtained from male and female mice at room air and after exposure to hyperoxia (24, 48 and 72 h). Significant differences are indicated by **, $p < 0.01$ and ***, $p < 0.001$.

Figure 4.

Male mice had greater neutrophil infiltration in the lungs compared to females. **Fig 4A** shows the lung sections from male and female mice immunostained with rat anti-mouse neutrophil antibody at room air and after 24, 48 and 72 h of hyperoxia exposure. **Fig 4B** shows the representative quantitative analysis of neutrophil infiltration in the lungs at room air and after exposure to 24, 48 and 72 h of hyperoxia ($\text{FiO}_2 > 95\%$). Neutrophil count per high power field (40x magnification) was done as described under materials and methods. Data represent means $\pm$ SEM from at least 3 individual animals in each group. Significant differences are indicated by *, $p < 0.05$ and *** $p < 0.001$.

Figure 5.

Increased terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) staining in the lungs of male mice exposed to 72 h of hyperoxia when compared to females. **5A:** Representative sample of TUNEL staining form lung of male and female mice at room air and 72 hours after hyperoxia exposure. **5B:** Significant increase in TUNEL staining in lung of male mice exposed to hyperoxia compared with lung of female mice ($p < 0.003$ indicated by **), $n = 3$ for each group. Twenty consecutive hpf under 40x magnification were observed for the number of TUNEL positive cells. Negative controls (performed simultaneously but without TdT) were examined at the same time.

Figure 6.

Male mice show greater lipid peroxidation in the lungs compared to females after hyperoxia exposure. **Fig 6A, 6B and 6C** shows the representative chromatograms of the standard and lung samples. **Fig 6D** shows the results of the LC-MS/MS analysis for 8-iso-PGF2α in lungs at room air and after 24, 48 and 72h of hyperoxia exposure. Values are means ± SEM from at least 3 individual animals. Significant differences between the male and female mice are indicated by *, $p < 0.05$.

Figure 7.

Effect of hyperoxia on cytokine expression in the lungs of male and female mice. The suspension bead array analysis shows cytokine concentration in lungs at room air levels and after 48 h of hyperoxia exposure. 7A: IL-6. 7B: VEGF, 7C: IL-1β 7D: MIP-1α 7E: MCP-1. Significant differences are indicated by *, $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Figure 8A.

CYP1A1 immunohistochemistry: Female mice have more enhanced immunostaining for CYP1A in the lungs after hyperoxia exposure compared to room air breathing controls. Lung sections immunostained for CYP1A at room air and after 72 h of hyperoxia exposure in male and female mice. Positively stained areas in bronchial epithelial cells are shown by arrows. **Figure 8B:** Whole lung protein (20 μ g) was subjected to Western blotting using monoclonal antibodies raised CYP1A1, as described in “Materials and methods.” Under each sample lane is the corresponding β -actin blot to assess for protein loading. The positive control (labeled “PC”) was 0.5 μ g of liver microsomes from mice treated with 3-methylcholanthrene.