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Quercetin attenuates the hyperoxic lung injury in neonatal mice: Implications for Bronchopulmonary dysplasia (BPD)

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ABSTRACT

Quercetin (QU) is one of the most common flavonoids that are present in a wide variety of fruits, vegetables, and beverages. This compound possesses potent anti-inflammatory and anti-oxidant properties. Supplemental oxygen is routinely administered to premature infants with pulmonary insufficiency. However, hyperoxia is one of the major risk factors for the development of bronchopulmonary dysplasia (BPD), which is also termed chronic lung disease in premature infants. Currently, no preventive approaches have been reported against BPD. The treatment of BPD is notably limited to oxygen administration, ventilatory support, and steroids. Since QU has been shown to be effective in reducing inflammation and oxidative stress in various disease models, we hypothesized that the postnatal QU treatment of newborn mice will protect against hyperoxic lung injury by the upregulation of the phase I (CYP1A/B) and/or phase II, NADPH quinone reductase enzymes. Newborn C57BL/6J mice within 24 h of birth with the nursing dams were exposed to either 21% O_2 (air) and/or 85% O_2 (hyperoxia) for 7 days. The mice were treated, intraperitoneally (*i.p.*) once every other day with quercetin, at a concentration of 20 mg/kg, or saline alone from postnatal day (PND) 2–6. The mice were sacrificed on day 7, and lung and liver tissues were collected. The expression levels of CYP1A1, CYP1B1, NQO1 proteins and mRNA as well as the levels of MDA-protein adducts were analyzed in lung and liver tissues. The findings indicated that QU attenuated hyperoxia-mediated lung injury by reducing inflammation and improving alveolarization with decreased number of neutrophil and macrophage infiltration. The attenuation of this lung injury correlated with the up-regulation of CYP1A1/CYP1B1/NQO1 mRNA, proteins and the down regulation of NF-kB levels and MDA-protein adducts in lung and liver tissues. The present study demonstrated the potential therapeutic value of quercetin in the prevention and/or treatment of BPD.

1. Introduction

Flavonoids are part of the human diet, and they have been extensively studied for their beneficial effects on various diseases. It has been estimated that more than 8000 flavonoids exist in plants, fruits, vegetables, cocoa, wine and tea, whereas only 20 of them are available in the market that possess pharmacological activities at human physiological levels (Bjorklund et al., 2017; Sharma et al., 2018). The protective effects of flavonoids on various diseases, notably cancer, have been well documented and it has been shown that these compounds interact with the cytochrome P450 CYP1 family of enzymes, which are overexpressed in various tumors (Androutsopoulos et al., 2009; Androutsopoulos et al., 2010; Androutsopoulos et al., 2013; Androutsopoulos and Tsatsakis, 2014; Hashemzaei et al., 2017; Margina et al., 2015; Spanidis et al., 2016; Spyrou et al., 2014; Surichan et al., 2012; Wilsher et al., 2017). Quercetin (QU, 3,5,7,3^1,4^1-pentahydroxyflavone), is one of the most common flavonoids found in a wide variety of fruits, vegetables, and beverages (Boots et al., 2008). QU and its metabolites have been reported to have potent anti-inflammatory (Basu et al., 2018) anti-oxidant (Goutzourelas et al., 2015; Hayashi et al., 2012; Herraiz and Galisteo, 2017), neuro protective (Pandey et al., 2012) and anti-tumor (Lee and Lee, 2008) properties. The

Abbreviations: AhR, Aryl hydrocarbon receptor; BNF, beta-naphthoflavone; BPD, Bronchopulmonary dysplasia; CYP1A, Cytochrome P450 1A; LW/BW, Lung weight /Body weight; MLI, Mean Linear Intercept; NF-kB, Nuclear Factor -kappa B; NQO1, Nicotinamide adenine dinucleotide phosphate quinone Oxidoreductase 1; QU, Quercetin; RAC, radial alveolar count; vWF, von Willebrand Factor

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protective effect of QU in heart and lung injury has also been reported (Griffiths et al., 2016; Hertog et al., 1993; Schafer et al., 2017; Takashima et al., 2014). Although QU has already been administered to human adults as a natural anti-inflammatory remedy (Moon et al., 2008), there is little information and research on the effects of QU in term and preterm newborn diseases. Bronchopulmonary dysplasia (BPD) is the most common complication in premature infants with a gestational age of < 30 weeks (Baud et al., 2016; Cui et al., 2017; Davidson and Berkelhamer, 2017; Jobe, 2016) with long term consequences. BPD is a chronic lung disease of premature infants that is characterized by inhibition of postnatal lung development due to damage caused by various factors, namely mechanical ventilation, prolonged usage of oxygen, episodes of infections and other causes of inflammation (Yee et al., 2013). Approximately 50% of preterm infants have BPD, which is characterized by lack of normal pulmonary function. Approximately 50% of preterm infants develop BPD, which is characterized by lack of normal pulmonary function that can persist until adulthood (Sureshbabu et al., 2016). Unfortunately, neither a definite treatment nor a preventive strategy for BPD have been developed (Couroucli et al., 2016). The main treatment includes supportive measures, such as oxygen administration, ventilator support, and steroids that can lead to increased incidence of cerebral palsy and other complications (Lim et al., 2015). Hence, the identification of preventive strategies and active compounds for BPD, which could be a lifelong disease, is imperative (Jain and Bancalari., 2017).

Although the adverse effects of hyperoxia have been well studied, supplemental oxygen administration is still a lifesaving therapeutic measure in premature infants with pulmonary insufficiency. Over the past several years, a limited number of studies have shown beneficial effects with regard to pharmacological interventions for the amelioration of hyperoxic lung injury in animal models and preterm infants, but the incidence of BPD remains the same (Cui et al., 2017; Jagarapu et al., 2015; Vaidya et al., 2017). Studies from our laboratory have shown that the induction of the enzymes of the Cytochrome P450 1A (P4501A) family plays protective role in hyperoxic lung injury of the newborn rodents, although this protection may have important clinical implications for the prevention of BPD (Lingappan et al., 2018; Maturu et al., 2017). QU is known to inhibit and/or enhance CYP1 enzyme activity, depending on the experimental conditions, the bioavailability of the compound and the access to the catalytic site of the enzymes (Androutsopoulos et al., 2010; Wilsher et al., 2017). It has further been shown that QU upregulates the phase II metabolizing/detoxifying enzymes via activation of the nuclear factor (erythroid derived 2)-like 2 (Nrf2) antioxidant response element (Nrf2-ARE) signaling pathway (Saw et al., 2014).

Although the role of QU has been reported recently in various disease models, no experimental studies have investigated the use of QU in the prevention and/or treatment of hyperoxia-mediated lung injury in newborns. In general, BPD is characterized by alveolar simplification, reduced vascular growth and increased inflammation due to accumulation of inflammatory cells (Vaidya et al., 2017). It has been shown that reactive oxygen species (ROS) that are formed under hyperoxic conditions contribute to the formation of protein, lipid and nucleic acid oxidative adducts, resulting in structural and functional alterations in the newborn mouse lung similar to those found in human patients with BPD (Berger and Bhandari, 2014).

Oxidative stress caused by hyperoxia has been implicated in the development of lung injury and inhibition of pulmonary and vascular tissue development, although the exact role and mechanism of action of antioxidant compounds with regard to the protection against this injury and the inhibition of inflammation remain unclear. Therefore in the present study, we hypothesized that postnatal intraperitoneal (i.p.) administration of QU in newborn C57BL/6J mice could attenuate hyperoxic lung injury due to its anti-inflammatory and antioxidant properties by a mechanism that involves the upregulation of the phase I (CYP1A/B) and/or phase II NADPH quinone reductase (NQO1) enzymes.

In the current study, we report that QU ameliorates hyperoxia mediated lung injury by reducing inflammation, alveolar simplification, vaso-obliteration and oxidative stress, and by upregulating the expression levels of the CYP1A1, CYP1B1, and NQO1 enzymes. The aim of this study was to explore the potential of QU to prevent BPD, which is a lifelong disease with major health and financial consequences.

2. Materials and methods

2.1. Animal care

The animal studies and the protocol were approved by the Ethics committee of the Baylor College of Medicine. All experiments were conducted in accordance with the standards established by the United States Animal Welfare Acts set forth in the National Institutes of Health (NIH) guidelines and the Policy and Procedures by the Institutional Animal Care and use Committee of Baylor College of Medicine. The C57BL/6J mice were obtained from the Charles River laboratories (Wilmington,DE). The mice were bred and handled in accordance with the NIH guidelines. All animals were housed at the Feigin Center animal facility and were permitted access to food (Purina rodent Lab Chow no: 5001 from Purina Mills, Inc., Richmond, IN) and water *ad libitum* at a temperature range of 20–23 °C under a 12:12-h light-dark cycle.

2.2. Animal experimentation: oxygen exposure and quercetin treatment

Newborn mice were randomly assigned and equally distributed with the nursing mothers. The mice were placed in plexi-glass chambers and exposed to either 21% O_2 (room air) and/or 85% O_2 (hyperoxia) for 7 d as described previously (Park et al., 2007). Newborn mice were treated, intraperitoneally (*i.p.*) once every other day with Quercetin (QU) (20 mg/kg) dissolved in saline, from postnatal day 2 to postnatal day 6 (PND). The dose of QU was selected based on the pilot experiments performed with the minimal mortality in room air and/or hyperoxia exposed newborn mice. The nursing mothers were transferred between room air and hyperoxia-exposed litters every 24 h in order to prevent oxygen toxicity and to eliminate maternal effects between the groups. The excess CO_2 was absorbed using anhydrous Soda Lime (J.T Baker, Cat# 3447-05) from the chambers. The oxygen concentration (85%) was always maintained with a humidified circuit at a flow rate of 5 L/min through a blender (Nelin et al., 1996). Neonatal mice were humanely sacrificed with intraperitoneal sodium pentobarbital injection on PND 7 with the recommended dosage (200 mg/kg).

2.3. Lung perfusion and collection of tissues

The lung tissues were inflated (3-4 mice) through the trachea with buffered zinc formalin (10%) and fixed overnight in the same solution. The fixed tissues were dehydrated, cleared, and paraffin-embedded. From each group (without formalin fixation), lung and body weights from 4 to 6 newborn mice were measured for Lung weight/Body weight (LW/BW) ratio calculations as an index of lung injury. The lung and liver tissues were immediately frozen and stored at −80 °C for subsequent molecular analyses.

2.4. Histopathology and immunohistochemistry analysis of lungs

Embedded lung tissues were cut into 4-μm sections for subsequent histological analyses of lung injury and immunohistochemical analysis. The sections were stained with Hematoxylin and Eosin (H&E) as described previously (Couroucli et al., 2016a; Couroucli et al., 2006b; Maturu et al., 2017). The immunohistochemical analysis of the inflammatory markers was performed with rat anti-mouse ly-6b.2 antibody (AbD Serotec-Cat # MCA771G) and rat anti-mouse F4/80 antibody (Bio legend-Cat #123102) at 1:200 dilution as described

previously (Maturu et al., 2017). At least 20 random high power fields (at ×20 magnification) were used for the quantitation of neutrophils and macrophages (Couroucli et al., 2011; Ramsay et al., 1998).

2.5. Lung vascular density analysis by immunohistochemistry

Pulmonary vascular density was determined with the average number of von Willebrand Factor (vWF) stained vessels by the immunohistochemical analysis using a rabbit anti-mouse Anti-von Willebrand Factor polyclonal antibody (Abcam-Cat# ab6994) at 1:500 dilution. The vWF is an endothelial specific marker that stains vessels (< 100 μm diameter) in high power field (×20 magnification). A total of 10 random images on each lung section were used for quantification.

2.6. Pulmonary morphometry and alveolar development analysis

Quantitative analyses of the radial alveolar count (RAC) and the Mean Linear Intercept length (MLI) were performed on H&E sections as previously described (Cooney and Thurlbeck, 1982; Knudsen et al., 2010; Maturu et al., 2017). These two parameters were used to evaluate the hyperoxia induced lung histological damage and to assess the modulating efficacy of QU in hyperoxia-induced lung injury in newborn mice. Representative and proportional lung samples from each group with 15 random fields were assessed by histological slides using an ×20 magnification. The analysis included quantification of the degree of histological damage.

2.7. Protein expression analysis by immunoblotting

Total proteins were collected from the lung and liver tissue samples of the newborn pups. The proteins were equally loaded (10 μg of protein) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 10% acrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes The membranes were incubated with monoclonal antibodies for CYP1A1, which cross-reacts with CYP1A2 (Couroucli et al., 2000; Jiang et al., 2010; Moorthy et al., 2000; Thakur et al., 2014). The detection of NQO1 and NF-kB proteins was conducted by a standard western blotting protocol with specific antibodies [NQO1-Santacruz Biotechnology, SC-393876(F8); NF-kB-p-65 Santa Cruz Biotechnology, SC-8008(F6)]. For loading controls, the PVDF membranes were incubated with antibodies against β-actin. The protein bands were visualized with the HyGlo chemiluminescence HRP antibody detection reagent kit (Denville Scientifics, Holliston, MA. Cat# E-2500) on a Chemidoc touch imaging system (Bio-Rad Laboratories Inc, Hercules, CA, USA). Subsequently, the density values of the individual protein bands were quantified using the Image Lab software. The relative protein expression levels of CYP1A1, NQO1 and NF-kB were normalized to β-actin.

2.8. Analysis of oxidative stress

Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds such as Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), natural biproducts of lipid peroxidation. The modified proteins by MDA and 4-HNE were analyzed by the OxiSelect™ MDA Immunoblot Kit (Cell Biolabs, Inc, San Diego, California; STA-331, dilution 1:1000) and an anti-β-actin-HRP (C4) (Santa Cruz Biotechnologies, Santa Cruz, California; sc-47778, dilution 1:2000) antibodies to assess oxidative stress. The experiments were conducted as described by manufacturer's recommendations.

2.9. RNA isolation and real Time-PCR analysis

Total RNA was isolated from the snap frozen lung and liver tissues using Direct-Zol RNA mini prep kit (Zymo research, Cat#R2052) and was reverse transcribed to produced cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, Cat#170-8841). The cDNA was then amplified using QuantiTect SYBR Green reagent (Qiagen-Cat#204143). Real-Time PCR was conducted using ABI PRISM 7700 Sequence Detection System with β-actin as an internal control. The RT-PCR reaction cycling conditions included an initial denaturation step at 95 °C for 10 min, followed by 40 PCR cycles of amplification that consisted of a denaturation step at 95 °C for 15 s and an annealing and extension steps at 60 °C for 1 min. The relative quantification of mRNA expression for CYP1A1, 1A2, 1B1, NQO1 was conducted using the 2-ΔΔCt method as described previously (Anwar-Mohamed et al., 2012; Maturu et al., 2017; van Ede et al., 2014). The sequences of the primers for these genes are provided upon request.

2.10. Statistical analyses

Data were expressed as means ± SE and analyzed by two-way ANOVA (the effect of QU treatment and hyperoxia) followed by Student-test. A p value of < 0.05 was considered significant following a multiple comparison analysis (Tukey) with GraphPad version 5 software.

3. Results

3.1. Attenuation of alveolar simplification and lung injury in newborn mice following postnatal administration of QU

To determine the role of QU in the attenuation of lung injury, we analyzed the histological examination of newborn mice lungs treated with saline or QU postnatally, followed by exposure to room air or O_2. As shown in Fig. 1, lungs from saline and hyperoxia-exposed newborn animals displayed impairment of alveolarization as demonstrated by edema, perivascular inflammation and simplified alveoli (Fig. 1C). Treatment of hyperoxia-exposed newborns with QU improved the alveolar structure and significantly attenuated hyperoxic lung injury to a similar level to that noted for room air controls. Upon morphometric analysis, a significant decrease in the radial alveolar count (RAC) was noted in saline treated newborns exposed to hyperoxia. However, the treatment of the mice with QU during hyperoxia significantly increased RAC (Fig. 1F). Similarly, the mean linear intercept (MLI) was significantly increased in the saline-hyperoxia newborn lung tissues, whereas this parameter was restored in the lung tissues of the newborn mice that were exposed to both QU and hyperoxia (Fig. 1G). As indicated by the histological analysis, the restoration of the alveolar number and size following QU treatment was significant. The extent of lung injury was further examined by the Lung weight/Body weight (LW/BW) ratios, which is an index of increased cellularity and pulmonary edema. The LW/BW ratios of the newborn mice from the saline-hyperoxia treated group indicated a significant increase compared with the room air controls suggesting an increase in the lung injury of these animals. However, the treatment of the newborn mice with QU reduced the LW/BW ratios significantly following hyperoxia exposure. These ratios were not significantly different than those noted in the room air control subjects. The results indicated that QU attenuated the hyperoxia-induced lung injury.

3.2. Reduced lung inflammation following postnatal treatment of QU during hyperoxia

The saline-hyperoxia treated newborn mice showed an increased recruitment of neutrophils and macrophages in their lungs as determined by immunohistochemical analysis (Fig. 2). This was significantly reduced following exposure to both QU and hyperoxia and the number of reduced neutrophils (Fig. 2D and E) and macrophages (Fig. 2I and J) were comparable to that noted in the ling tissues of the saline + room air newborn mice.

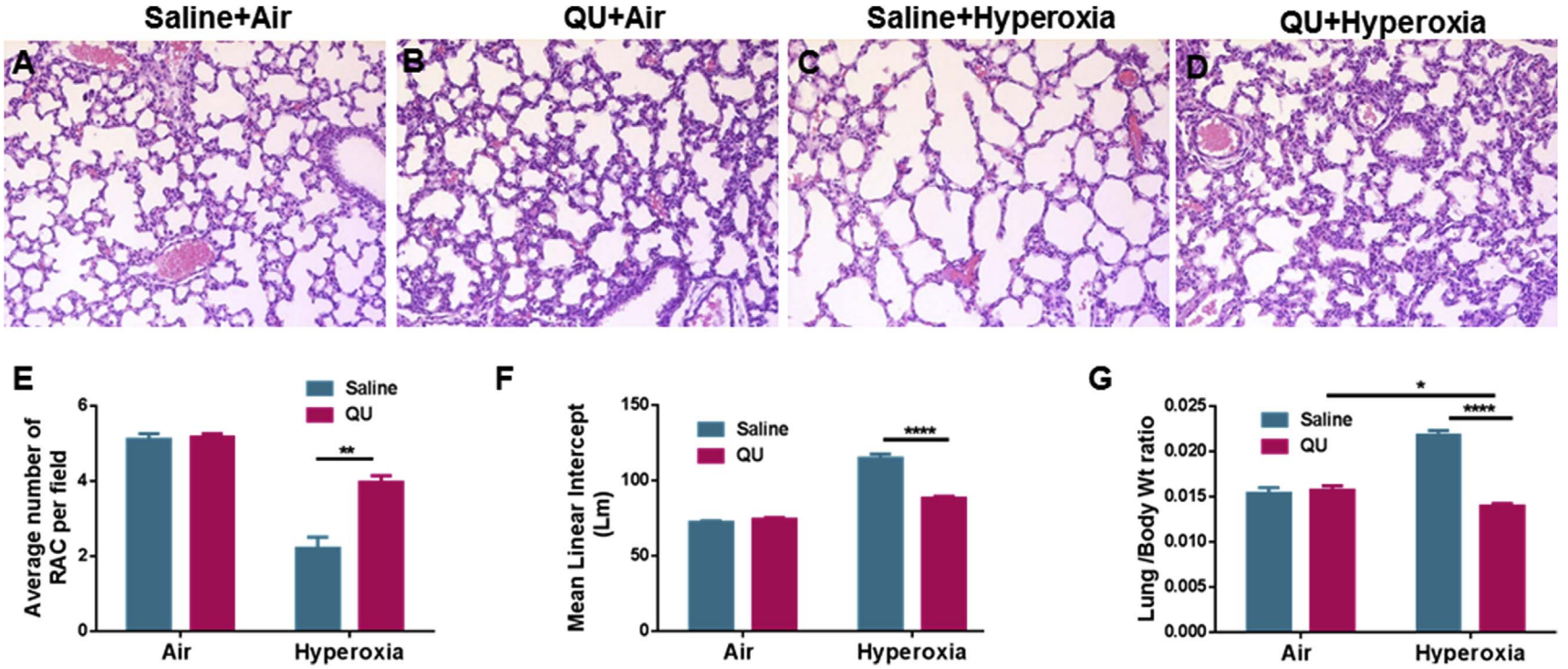

Fig. 1. Attenuation of alveolar simplification and lung injury in newborn mice following postnatal administration of QU: Newborn mice were exposed to room air or hyperoxia and treated intraperitoneally (*i.p.*) once every other day with Quercetin (QU) (20 mg/kg) or saline, from postnatal day (PND) 2–6 as described in the methods. **A-D).** Histological images of newborn lung tissues were stained with Hematoxylin and Eosin (H&E). Saline + Hyperoxia group showed increased lung injury with perivascular edema and increased alveolar simplification. QU treatment (QU + Hyperoxia) resulted in significant attenuation and improved alveolarization. The images were obtained at 200× magnification. The effects of Hyperoxia and postnatal QU on **(E)** Radial Alveolar Count (RAC), Mean Linear Intercept (MLI) or Lm **(F)** and Lung/Body Wt ratios **(G),** which are indexes of lung injury, were determined in newborn mice. The values are means ± S.E.M. from 3 to 5 individual newborn lung mice in air or hyperoxia groups with saline and/or QU treatment. *, statistically significant differences between QU and saline treated mice at $P \leq 0.05$ by two-way ANOVA.

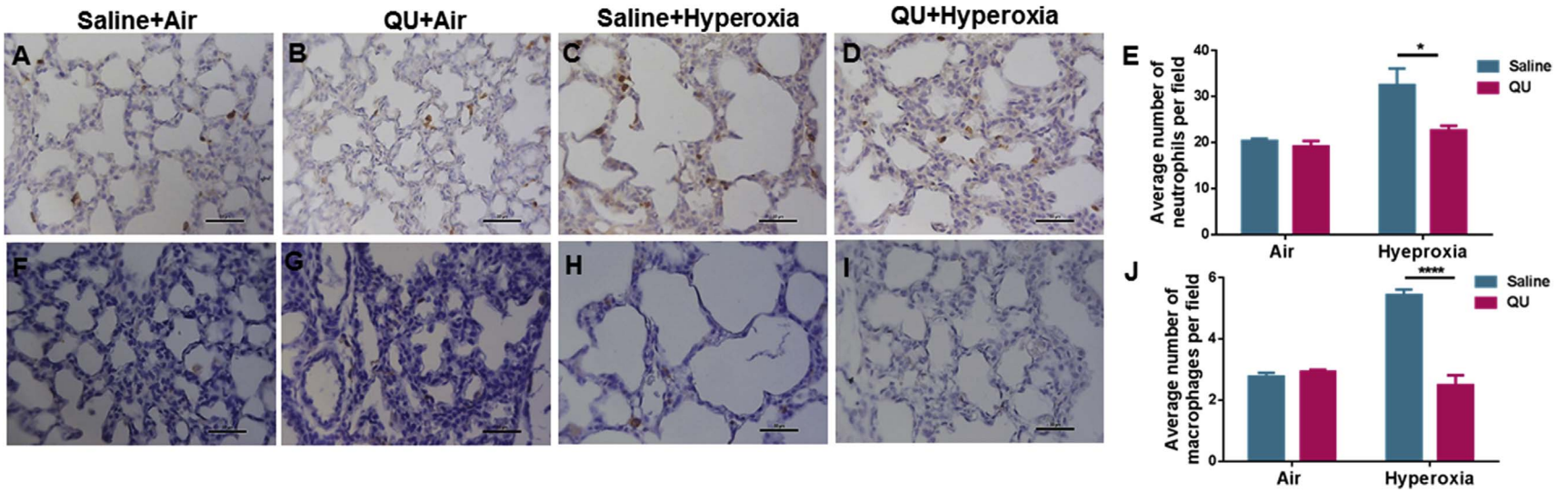

Fig. 2. Reduced lung inflammation following postnatal treatment of QU during hyperoxia. The immune cell infiltration in newborn lungs was assessed by immunohistochemistry with anti-neutrophil and macrophage specific antibodies as described in the methods section. Representative images of immunostained lung sections (×400 magnification) obtained from anti-neutrophils specific antibody **(A, B, C and D)** and anti-macrophage specific antibody **(F, G, H and I)** are shown. Furthermore, neutrophil **(E)** and macrophage **(J)** infiltrations were quantified after collecting 20 representative high-power field images at × 200 magnification from all different groups.*, statistically significant differences between QU and vehicle-treated mice at $P \leq 0.05$ by two-way ANOVA.

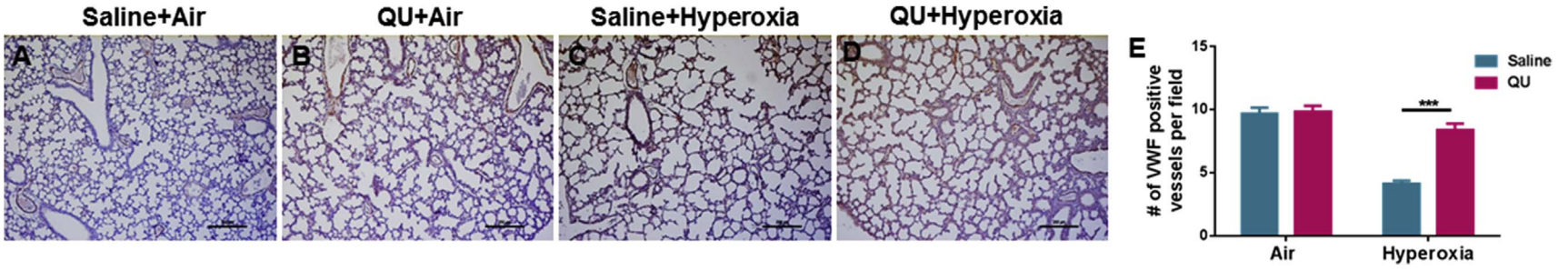

Fig. 3. Postnatal QU treatment improved pulmonary vascular development during hyperoxia. Representative immunohistochemical expression of von Willebrand factor (vWF) in newborn lungs from **(A, B, C and D)** indicated that QU treatment restored the endothelial cells in moderate size vessels, with modestly increased vascular count **(D)**. A total of 10–15 representative high-power field images were collected at × 200 magnification for each group and quantified **(E).** *, statistically significant differences between QU and vehicle-treated mice at $P \leq 0.05$ by two-way ANOVA.

3.3. Postnatal QU treatment improved pulmonary vascular development during hyperoxia

To determine the effect of QU on the lung vascularization in the different treatment groups, we performed immunohistochemistry of VWF in lung tissues and quantified the VWF positive vessels (Fig. 3). The VWF positive vessels were significantly decreased in saline-hyperoxia (Fig. 3C and E) exposed lungs compared with the room air samples. Administration of QU to hyperoxia exposed newborn lungs modestly increased vascular count (Fig. 3D and E) to similar levels of those noted in the room air control mice.

3.4. QU treatment up-regulated pulmonary CYP1A1, CYP1B1 and NQO1 mRNAs following postnatal treatment of newborn mice

To investigate the mechanism of action of QU during hyperoxia, we evaluated the pulmonary mRNA levels of CYP1A1 and NQO1. No significant increase in the pulmonary CYP1A1 mRNA levels was noted in mice treated with postnatal QU in room air compared with the saline controls. Interestingly, the QU treatment during hyperoxia induced CYP1A1 mRNA significantly compared with the saline treated hyperoxia controls (Fig. 4A). Similarly, the CYP1B1 (Fig. 4B), and NQO1 (Fig. 4C) mRNA induction pattern was similar between these two groups and the CYP1A1 mRNA levels did not change considerably during room air treatment although a substantial increase was observed following QU and hyperoxia treatment.

3.5. Induction of pulmonary CYP1A1 protein in newborn mice following postnatal QU and hyperoxia treatment

To elucidate the role of QU in the induction of pulmonary CYP1A1 protein, we determined the lung CYP1A1 apoprotein expression under room air and hyperoxic conditions by western blot analysis. Pulmonary CYP1A1 (Fig. 5A and B) apoprotein expression was significantly increased in newborn mice that were exposed to 7 d of hyperoxia in the presence of QU treatment compared with to room air (Saline + Air) and/or QU + Air and/or Saline + Hyperoxia mice.

3.6. Upregulation of pulmonary NQO1 protein following postnatal treatment of QU and hyperoxia

QU treatment further induced NQO1 protein in the room air and Hyperoxia groups. However, there was a prominent increase in NQO1 protein in the QU + Hyperoxia group compared with the saline + hyperoxia and the remaining groups (Fig. 5C and D).

3.7. Postnatal QU treatment reduced the pulmonary inflammation by down regulating the expression of NF-kb protein

Given that QU treatment reduced inflammation as determined by morphometric and LW/BW analysis, we analyzed the expression of NF-kb protein in order to add insight in the potential anti-inflammatory action of QU. A significant reduction was noted in the pulmonary NF-kb protein levels in the QU + Hyperoxia newborn mice compared with the saline + hyperoxia group (Fig. 5E and F).

3.8. QU treatment reduced hyperoxia-induced lung MDA protein adduct formation

Hyperoxia-induced oxidative stress has been documented to contribute to the formation of MDA adducts. Consequently, western blotting analysis was performed using an anti-MDA antibody in order to determine the effects of QU on hyperoxia-induced lung oxidative stress. As expected, hyperoxia increased lung MDA protein adduct (Fig. 6A and B) formation in the regions between 40 and 80 kDa. Similarly, QU reduced significantly hyperoxia-induced increase of MDA protein adducts

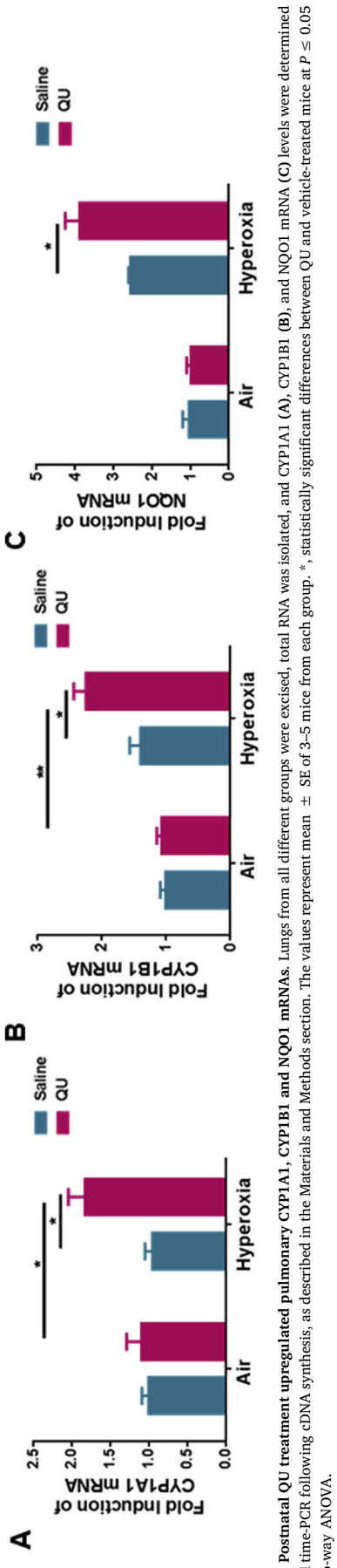

Fig. 4. Postnatal QU treatment upregulated pulmonary CYP1A1, CYP1B1 and NQO1 mRNAs. Lungs from all different groups were excised, total RNA was isolated, and CYP1A1 (**A**), CYP1B1 (**B**), and NQO1 mRNA (**C**) levels were determined by real time-PCR following cDNA synthesis, as described in the Materials and Methods section. The values represent mean ± SE of 3–5 mice from each group. *, statistically significant differences between QU and vehicle-treated mice at $P \leq 0.05$ by two-way ANOVA.

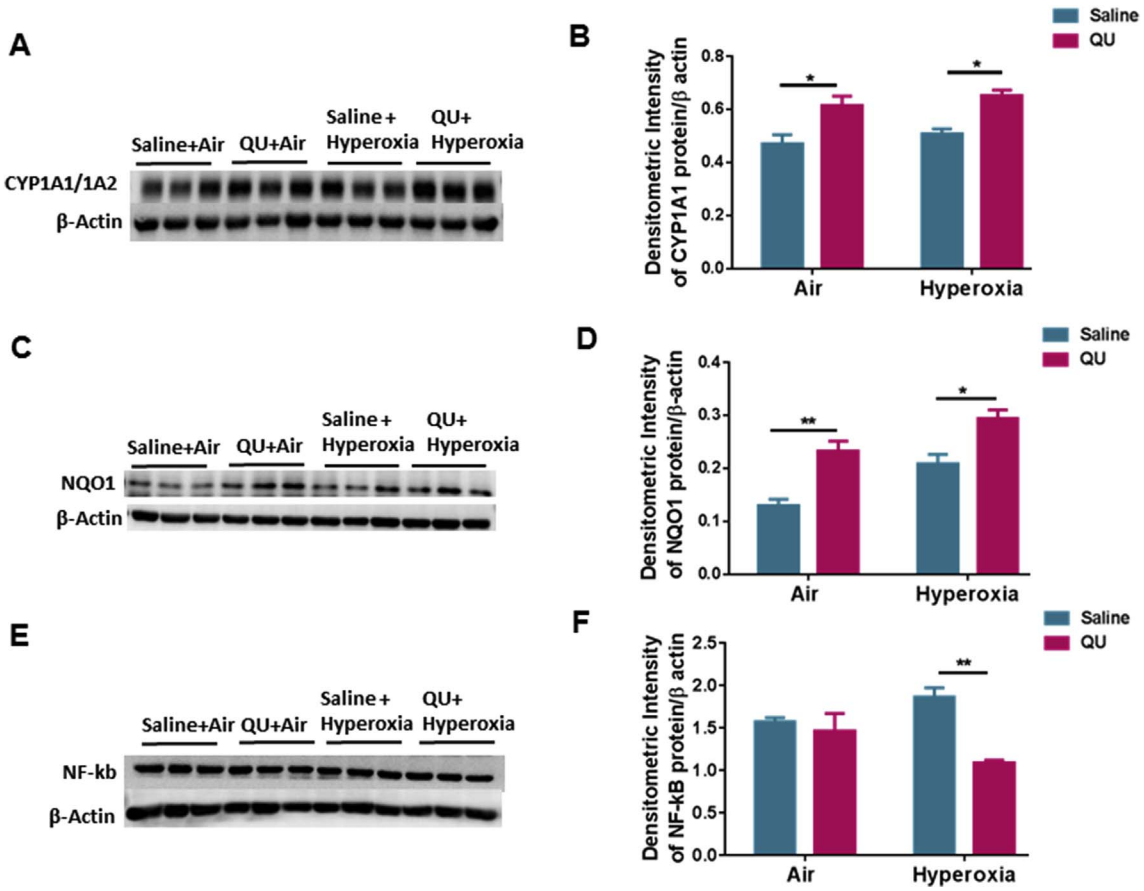

Fig. 5. Induced pulmonary CYP1A1/1A2, NQO1 proteins, and downregulated NF-kB protein following postnatal QU and hyperoxia treatment. The lung homogenates prepared from different treatment groups of newborn mice were subjected to immunoblot analysis. Representative immunoblots showing the expression of CYP1A1/1A2 in lungs **(A &B)**, NQO1 **(B &C)** and NF-kb **(C&D)** were shown with β-actin as a loading control. The densitometric intensities of these proteins normalized to β-actin were quantified and shown separately. Values represent means ± SE of 3 mice from each group. *, statistically significant differences between QU and vehicle-treated mice at $P \leq 0.05$ by two-way ANOVA.

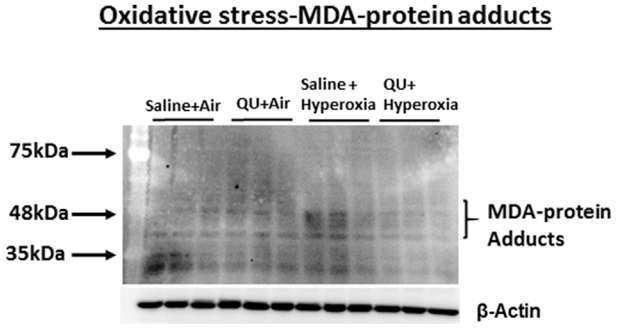

Fig. 6. QU treatment reduced hyperoxia-induced lung MDA protein adduct formation. The hyperoxia induced MDA-protein adducts were analyzed by immunoblotting by an anti-MDA antibody in lung homogenates and representative immuno blots were shown with β-actin as a loading control. The hyperoxia-increased lung MDA protein adduct formation between the regions of 40–80 kDa. QU treatment reduced hyperoxia-induced lung MDA protein adduct expression.

in the lung tissues of QU treated mice (Fig. 6) compared with saline-treated animals.

3.9. Postnatal treatment of newborns with QU induced hepatic CYP1A1/2 and NQO1mRNAs

Exposure to hyperoxia along with QU treatment of newborn mice led to the marked induction of hepatic CYP1A1 (Fig. 7A) mRNA levels compared with saline treated animals that were kept under room air and/or hyperoxia conditions. A similar increase in CYP1A2 (Fig. 7B) mRNA was noted in the QU treated hyperoxia group when compared to the other groups. No induction of CYP1A1 expression was noted in newborn mice that were exposed to room air (Fig. 7A). In order to determine the effect of postnatal QU treatment on the induction of gene expression of the phase II enzyme, NQO1, we determined the mRNA levels of NQO1 in the liver tissues of mice treated with saline and/or QU under room air or hyperoxic conditions. Postnatal treatment with QU induced NQO1 expression in newborn liver tissues compared with vehicle treated animals under room air conditions (Fig. 7C). Under hyperoxic conditions, the animals indicated a similar induction of NQO1mRNA in the QU treated group compared with the saline treated controls (Fig. 7 C).

3.10. Treatment with QU induced hepatic CYP1A1/1A2 apoprotein in newborn mice exposed to hyperoxia

To confirm that treatment of newborn mice with QU treatment further induced liver CYP1A1/1A2 apoprotein expression under room air and hyperoxia exposed conditions, we analyzed the expression of these enzymes by immuno blot analysis followed by the densitometric quantification of the band intensities. The hepatic CYP1A1/1A2 (Fig. 8A and B) apoprotein expression was significantly increased in newborn mice exposed to 7 days of hyperoxia in the presence of QU compared to the room air (Saline + Air) the QU + Air and/or the Saline + Hyperoxia groups.

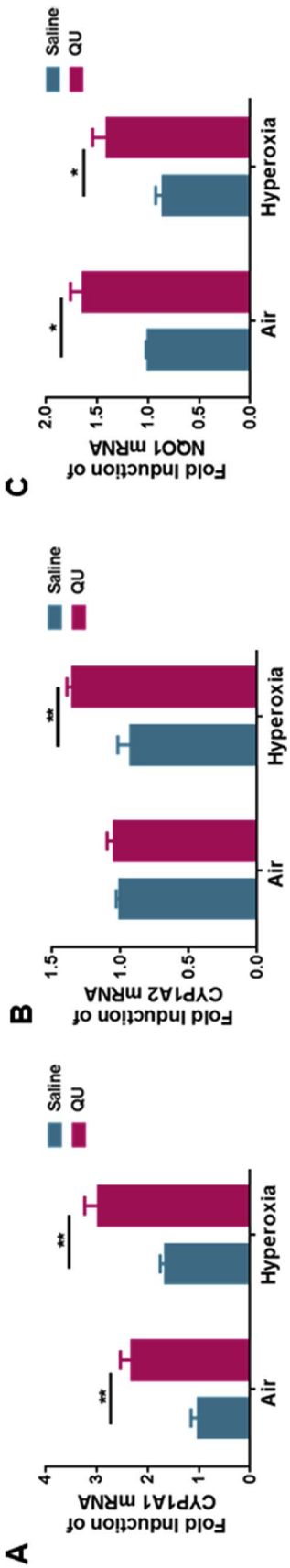

Fig. 7. Postnatal treatment of newborns with QU induced hepatic CYP1A1/2 and NQO1 mRNAs. Real-time (Q-PCR) analysis data showing the liver mRNA levels of CYP1A1 **(A)**, CYP1A2 **(B)** and NQO1 **(C)** following cDNA synthesis of different genotypes. Following the postnatal treatment of newborn mice with hyperoxia and QU, liver tissue homogenates were prepared as described. The values represent means ± SE of at least 3 mice from each group. *, Statistically significant differences between QU and saline treated newborn mice at $P \leq 0.05$ by two-way ANOVA.

3.11. QU treatment enhanced hepatic NQO1 and inhibited the NF-kB proteins

To determine the effect of QU treatment in the induction of the antioxidant enzymes in hyperoxia, we performed western blot analysis and a significant induction of NQO1 protein in QU treated newborn mice was noted following hyperoxia exposure compared to the room air (Saline + Air) the QU + Air and/or the Saline + Hyperoxia mice (Fig. 8C and D). It is noteworthy that QU + Air newborn liver tissues exhibited a significant down regulation of NF-kB protein content although a similar non-significant decrease in NF-kB protein was noted (not significant) in the QU + Hyperoxia group compared with the Saline + Hyperoxia group (Fig. 8E and F).

4. Discussion

QU is one of the most potent and abundantly available antioxidant among 8000 flavonoids (Choi et al., 2012). The diversified role of QU in various diseases has been reported (Basu et al., 2018; Hayashi et al., 2012; Lee and Lee, 2008; Pandey et al., 2012). Although the protective role of flavonoids in the management of adult chronic obstructive pulmonary disease (COPD) has been studied (Biswas et al., 2013; Mitani et al., 2017), no investigations on the role of quercetin in the chronic lung disease of newborns have been conducted. In the present study, we tested the hypothesis that postnatal administration of QU (20 mg/kg) in newborn C57BL/6J mice during hyperoxia will prevent abnormal alveolarization and vaso-obliteration by upregulating phase I (CYP1) and phase II enzymes (NQO1), as well as by reducing oxidative stress. We demonstrated for the first time that QU attenuated hyperoxia induced alveolar simplification, and lung inflammation, while it promoted the progression of angiogenesis in the newborn mice.

The pathophysiology of BPD is characterized by alveolar simplification and vascular abnormalities with small and medium vessel obliteration, increased inflammation and pulmonary edema. Oxidative stress leads to destruction of the alveoli with increased alveolar size and decreased number, as well as vaso-obliteration of the alveolar capillaries. It has been reported that quercetin has beneficial effects on lung inflammation modulating epithelium derived cytokines and epithelial apoptosis. The present study demonstrated that QU treatment during hyperoxia increased the number of alveoli (RAC) as well as reduced the alveolar wall size (MLI) and pulmonary edema (LW/BW ratios). Taken together, these results suggest that QU can ameliorate the pathological perturbations of the lung development. BPD is a multifactorial disease. However in the newborn mouse model of hyperoxia-mediated oxidative stress and inflammation, the dysregulation of alveolar developmental correlated with the pathological findings of human BPD.

In the current study, the postnatal QU treatment improved pulmonary vascular development during hyperoxia, as demonstrated by the increased vWF staining. This suggests that QU protects the pulmonary endothelium during lung development with an increase in the angiogenesis and/or vascularization processes. It has been shown that QU can exert vasodilatory and anti-inflammatory effects by modulating the epithelium derived cytokines and the induction of epithelial apoptosis. This is fundamental in BPD where abnormal angiogenesis may lead to pulmonary hypertension in human preterm neonates which may further cause complications in their adult life. Additionally, the pulmonary vascular and endothelial development are interdependent, notably under hyperoxic conditions.

The hyperoxia-mediated oxidative stress or ROS in the lung is due to over production of free radicals, and the lung is the most susceptible organ in order to sustain oxidative damage (Bargagli et al., 2009). It has been proposed that the antioxidant network can provide sufficient protection against ROS damage (Poljsak et al., 2013). In newborn infants, the endogenous antioxidant network may not be sufficiently developed in order to provide such protection. The modulatory role of QU against hyperoxia-induced lung injury and oxidative stress has not been

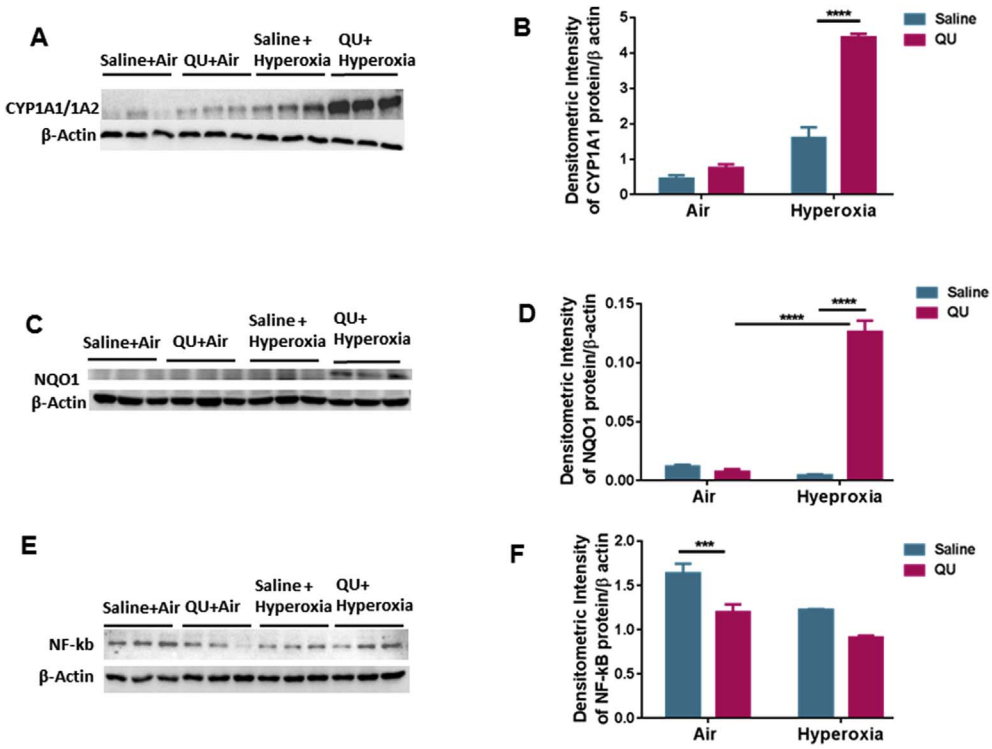

Fig. 8. Postnatal treatment with QU increased hepatic CYP1A1/1A2, NQO1 proteins and decreased NF-kB in newborn mice exposed to hyperoxia. Protein expression in liver tissue samples from each group was analyzed by western blot analysis. Representative western blots and their respective quantitative data showing the expression of CYP1A1/1A2 in **(A &B)**, NQO1 **(B&C)** and NF-kb **(C&D)** in liver samples were shown. β-actin was used to verify equivalent loading. Values are the means ± SE (n = 3). *P < 0.05 is considered significant, when compared between QU and vehicle-treated mice by two-way ANOVA.

well studied in animal models of BPD, although QU has been reported to have anti-inflammatory (Basu et al., 2018) anti-oxidant (Hayashi et al., 2012; Herraiz and Galisteo, 2017; Jomova et al., 2017; Nascimento et al., 2017) neuro protective (Pandey et al., 2012) and anti-tumor (Lee and Lee, 2008) properties in different disease models. In the present study, we evaluated the molecular mechanism of action of QU in the attenuation of hyperoxia-induced lung injury using a neonatal mouse model of BPD.

The anti-inflammatory effects of QU may be due to its interplay between oxidative stress and inflammation. The ROS induced hyperoxia-mediated inflammation was caused by activation of the transcription factors, namely nuclear factor NF-kB. The NF-kB expression in the Saline + hyperoxia group was highly induced and reverted to normal following QU administration, which indicated the anti-inflammatory role of QU. The significant reduction in inflammatory immune cell infiltration such as neutrophils and macrophages in the lungs following QU treatment and hyperoxia exposure suggest the anti-inflammatory role of QU. The radical scavenging activity of QU is offering protection against oxidative stress and may also mitigate inflammation. It has been reported that NF-kB will lead to the induction of radical producing enzymes (Boots et al., 2008), and that the anti-inflammatory effects of QU may be due to the decrease in NF-kB activation, leading to attenuation of ROS formation and oxidative stress (Poljsak et al., 2013).

The present study further indicated an induction of oxidative stress in lung tissues with an increased MDA-protein adduct formation and a reduced protein expression of the anti-oxidative enzyme NQO1 in the saline + hyperoxia group. However, following administration of QU, the levels of MDA–protein adducts (a marker of lipid peroxidation) were diminished, with concurrent enhancement of NQO1 expression in the QU + hyperoxia group compared with the saline + hyperoxia group. This is suggestive of the anti-oxidant role of QU that possibly occurs by a similar mechanism as described in other studies (Mira et al., 2002; Yasui et al., 2015) that includes quercetin effects against oxidative stress in various tissues including fetal brain (Dogan et al., 2018). With the exception of the free radical scavenging activity, the potent action of QU against oxidative stress may occur due to the chelation of iron and calcium and the inhibition of lipid-peroxidation (Mira et al., 2002). In addition, other biochemical mechanisms and the activation of NQO1 and other anti-oxidant enzymes via the nuclear factor erythroid 2 like 2-antioxidant response elements (Nrf2-ARE) signaling pathway (Saw et al., 2014; Spyrou et al., 2014) have been reported. It is established that the antioxidant role of QU may be directly involved in the redox regulation of proteins and transcription factors, which are inhibited by elevated ROS.

In the present study, we observed that QU treatment during hyperoxia exposure induced Phase I enzymes, such as CYP1A1 and CYP1B1 both in lung and liver tissues. Interestingly, the induction of mRNA and protein levels of CYP1A/1B1 following QU treatment correlated with less hyperoxic lung injury indicating the protective effect of QU against hyperoxic lung damage. To the best of our knowledge, this is the first study to report that QU treatment can induce the expression of phase I enzymes. In addition, this induction correlated with reduced lung damage in a newborn mouse model of BPD. Based on these results, the upregulation of the CYP1A1/2/1B1 genes correlated with considerably lesser hyperoxic lung injury in newborn mice, which is suggestive of a protective role. It has been reported that QU and other flavonoids induced CYP1A1 in Caco-2 cells (Sergent et al., 2009) *in* vitro. QU was proposed as an effective CYP1A1 inducer that can be provided by dietary fruit and vegetable ingestion. Similarly, the induction of CYP1A1 and CYP1B1 in human breast epithelial cells by QU was also reported (Mense et al., 2008).

QU has been studied as an AhR ligand in breast cancer MCF-7 cells (Ciolino et al., 1999), and in rat hepatocytes with weak AhR agonistic activity (Ashida, 2000). In the human hepatoma HepG2 cells (Walle and Walle, 2002), QU was shown to be effective as a CYP1A1 and CYP1B1 inducer. Furthermore, several reports indicated the anti-oxidant properties of QU with agonistic (Sergent et al., 2009) and antagonistic (Choi et al., 2012) roles in inducing phase I enzymes via the AhR pathway in different disease conditions. These studies indicated that the AhR agonistic activities of QU are cell dependent. We have recently reported that postnatal administration of beta-naphthoflavone (BNF) induces Phase I and Phase II enzymes such as CYP1A1/CY1B1/CYP1A2/NQO1 in lung and liver tissues (Maturu et al., 2017). We further reported the detoxification of F2-isoprostanes that were

produced by lipid peroxidation during hyperoxia, by the enzyme CYP1A2 (Wang et al., 2015). Interestingly, in the present study, the expression of CY1A2 was upregulated in the liver tissues by QU under hyperoxic conditions, which correlated with reduced lung injury. Therefore, the hepatic CYP1A2 could have a protective role against hyperoxic lung injury in newborn mice. Indeed we have previously shown that newborn CYP1A2 null mice can exert severe hyperoxic lung injury, which is ameliorated by the administration of BNF (Lingappan et al., 2018). These results indicated that QU administration in the mouse model of BPD exerts anti-oxidant and anti-inflammatory roles with the induction of phase I enzymes, such as CYP1A1/CYP1B1 in the lung and CYP1A2 in the liver, as well as the phase II enzyme NQO1.

In summary, the present study provided novel findings that QU regulates the resolution of hyperoxia-induced lung anti-oxidant in a newborn mouse model of BPD, by modulating the expression of xenobiotic enzymes such as CYP1A1/2/1B1 and the anti-oxidant enzymes such as NQO1, and by reducing the pulmonary MDA protein adducts. Additionally, enhancement of this protective response of QU was achieved by down regulation of NF-kb, which resulted in decreased inflammatory immune cell infiltration, such as neutrophils and macrophages in lung tissues, and improved the lung architecture with regard to the alveolarization and vascularization following exposure to hyperoxia. The present study invokes the possibility that QU treatment may be an effective preventative measure of BPD, and further animal and clinical studies are warranted in order to prevent this devastating disease at the beginning of human life.

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