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## Caffeine Ameliorates Hyperoxia-Induced Lung Injury by Protecting GCH1 Function in Neonatal Rat Pups

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### Abstract

**Background**—Bronchopulmonary dysplasia (BPD) is a major morbidity in premature infants, and impaired angiogenesis is considered a major contributor to BPD. Early caffeine treatment decreases the incidence of BPD; the mechanism remains incompletely understood.

**Methods**—Sprague-Dawley rat pups exposed to normoxia or hyperoxia since birth were treated daily with either 20 mg/kg caffeine or normal saline by intraperitoneal injection from day 2 of life. Lungs were obtained for studies at day 10 and 21.

**Results**—Hyperoxia impaired somatic growth and lung growth in the rat pups. The impaired lung growth during hyperoxia was associated with decreased levels of cyclic AMP (cAMP) and tetrahydrobiopterin (BH4) in the lungs. Early caffeine treatment increased cAMP levels in the lungs of hyperoxia-exposed pups. Caffeine also increased the levels of phosphorylated endothelial nitric oxide synthase (eNOS) at serine<sup>1177</sup>, total and serine<sup>51</sup> phosphorylated GTP-cyclohydrolase-1 (GCH1), and BH4 levels, with improved alveolar structure and angiogenesis in hyperoxia-exposed lungs. Reduced GCH1 levels in hyperoxia were due, in part, to increased degradation by the ubiquitin-proteasome system.

**Conclusion**—Our data support the notion that early caffeine treatment can protect immature lungs from hyperoxia-induced damage by improving eNOS activity through increased BH4 bioavailability.

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**Category of study:** Translational.

## Introduction

Preterm infants are born with immature lungs and frequently require oxygen support and mechanical ventilation. They are at high risk for lung injury, which can progress to chronic lung disease or bronchopulmonary dysplasia (BPD). BPD represents an arrest of lung development, initiated by inflammation and oxidative stress (1, 2). Impaired angiogenesis is considered a key contributor to BPD (3).

Both supplemental oxygen (4) and mechanical ventilation (5) increase reactive oxygen species (ROS) formation and deplete nitric oxide (NO) availability in the lungs. Decreased formation of NO by endothelial nitric oxide synthase (eNOS) in pulmonary vascular endothelial cells, contributes to impaired angiogenesis (6). Thus, maintaining adequate endogenous eNOS activity may prevent preterm infants from developing BPD.

Adequate eNOS coupling requires the interaction of eNOS with multiple co-factors. Tetrahydrobiopterin (BH4) is an essential cofactor for all members of the nitric oxide synthase (NOS) family (7–8). BH4 deficiency uncouples eNOS, which leads to superoxide formation instead of NO formation, when NOS is activated. Uncoupled eNOS has been shown to be the major source of oxidative stress in a rat model of ventilator-induced lung injury, which was improved by BH4 supplementation (9).

Rat pups are born at the sacular stage of lung development, similar to premature infants at 24–28 weeks' gestation and reach the alveolar stage between postnatal day 4 (P4) and 14 (P14) (10). The histological changes that develop after exposure to hyperoxia in rat pups mimic BPD in premature human infants (11). Caffeine, a nonselective phosphodiesterase (PDE) inhibitor, is commonly used to treat apnea of prematurity (12–13). The benefit of early caffeine treatment in preventing BPD had not been recognized until a randomized clinical trial identified this unexpected association in the secondary analysis of data (14). A recent retrospective study by Canadian researchers further supported the efficacy of caffeine in preventing BPD (15). However, the mechanisms involved in the lung protective effect of caffeine remain unclear.

It has been previously shown that cyclic AMP (cAMP) increases GTP-cyclohydrolase-1 (GCH1) protein levels in endothelial cells (16) and phosphorylation enhances GCH1 activity (17). GCH1 is the rate-limiting enzyme for BH4 synthesis in endothelial cells. We previously reported that BH4 supplementation in the presence of oxidative stress recouples eNOS and improves in vitro angiogenesis (18). We hypothesized that hyperoxia decreases GCH1 levels and activity in developing lungs while early caffeine treatment protects developing lungs from hyperoxia-induced injury through increased cAMP levels and improved GCH1 function, which leads to eNOS recoupling. Our results show that hyperoxia decreases cAMP and BH4 levels in the lungs, whereas early caffeine treatment protects developing lungs from hyperoxic injury by increasing BH4 levels. Our findings provide the biological basis for the role of early caffeine treatment in the prevention of BPD in premature infants.

## Methods

### Antibodies and Chemicals

Rabbit anti-phospho-eNOS serine<sup>1177</sup> (p-eNOS<sup>1177</sup>) antibody was from Cell Signaling (Beverly, MA); mouse anti-GCH1 antibody was from Abnova (Taipei, Taiwan); rabbit anti-DHFR antibody was from GeneTex (Irvine, TX); mouse anti- $\beta$ -actin antibody was from Sigma-Aldrich (St. Louis, MO); rabbit anti-VEGF antibody was from Santa Cruz (Dallas, TX); mouse anti-ubiquitin antibody was from Thermo Fisher Scientific (Waltham, MA); rabbit anti-rat endothelial cell antigen (RECA)-1 antibody was from Abcam (Cambridge, MA); horse raddish peroxidase tagged secondary antibodies for western blot were from Bio-Rad (Hercules, CA); Biotinylated horse anti-rabbit IgG and horse anti-mouse IgG were from Vector Laboratories (Burlingame, CA). Rabbit anti-phospho-GCH1 serine<sup>51</sup> was custom-made by 21<sup>st</sup> Century Biochemicals by using a keyhole limpet hemocyanin conjugated short peptide based on rat GCH1 sequence.

Caffeine/pentoxifylline ELISA kit was from Neogen (Lexington, KY); direct cAMP enzyme immunoassay kit was from Arbor Assays (Ann Arbor, MI); EZ DNA Methylation Kit was from Zymo Research (Irvine, CA); PyroMark Gold Q96 Reagents for performing pyrosequencing reactions were from QIAGEN (Germantown, MD); PureLink™ genomic DNA mini kit was from Invitrogen (Carlsbad, CA); Van Giesons and Resorcin-Fuchsin solutions were from Electron Microscopy Sciences (Hatfield, PA); bicinchoninic acid (BCA) protein assay kit was from Thermo Scientific (Rockford, IL); Vectastain ABC kit and Bloxall blocking solution were from Vector Laboratories (Burlingame, CA); protein block solution, antibody diluent and liquid diaminobenzidine (DAB)+substrate chromogen system were from Dako (Carpinteria, CA); In situ cell death detection kit was from Roche (Indianapolis, IN); all other chemicals were from Sigma (St Louis, MO).

### Animal care (Figure 1)

Animal use was approved by Institutional Animal Care and Use Committee (IACUC) of Medical College of Wisconsin (AUA#2268). Rats were cared under 12-hour day/night cycle with unlimited access to chow and water. For each experiment (n=5), four litters of rat pups were exposed simultaneously, within 12 hours of birth, to either hyperoxia (>90% oxygen chamber) or normoxia (room air) until day 10 of life (P10). Oxygen concentrations were monitored continuously with an oxygen sensor (Drägerwerk AG, Lübeck, Germany). Within each assigned group, pups received either daily intraperitoneal injections of 20 mg/kg caffeine base or the same volume of normal saline as control from P1 to P21. Nursing dams were rotated between normoxia and hyperoxia litter every day to reduce O<sub>2</sub> toxicity. All remaining pups were recovered in room air from P11 to P21. Lungs from at least 6 pups per group were obtained at P10 and P21 for histology and downstream assays.

### Body weight

Rat pups were checked and weighed at 9 AM every day by an electronic scale to the closest gram.

### Determination of serum caffeine levels

Blood was collected at euthanasia. To obtain serum, the whole blood samples were centrifuged at  $2,000 \times g$  for 10 minutes at  $4^{\circ}\text{C}$  after they were clotted. Serum caffeine levels were determined at P10 and P21 by Caffeine/Pentoxifylline ELISA test kit according to the manufacturer's protocol. Standards were freshly prepared prior to the assay.

### Measurement cAMP levels

Aliquots of lung homogenates were assayed for levels of cAMP by direct cAMP enzyme immunoassay kit and expressed as pmol/mg-protein.

### Western blotting

Lung tissue was homogenized in MOPS buffer (20 mM 3-N-morpholino-propanesulfonic acid, 2 mM EGTA, 5 mM EDTA, 30 mM NaF, 10 mM  $\beta$ -glycerophosphate, 10 mM Na pyrophosphate, 2 mM Na orthovanadate, 1 mM PMSF, 0.5% NP-40, 1% protease inhibitor cocktail, and 1% phosphatase inhibitor cocktails 2 and 3, pH 7.0) using Bullet Blender (Next Advance, Inc., Averill Park, NY) to obtain tissue homogenate. For Western blotting analysis, 30  $\mu\text{g}$  of lysate protein was resolved by SDS-PAGE, transferred to nitrocellulose membranes (0.2  $\mu\text{m}$ ), and then probed with VEGF, eNOS, p-eNOS<sup>1177</sup>, GCH1, p-GCH1<sup>51</sup> and DHFR antibodies overnight at  $4^{\circ}\text{C}$ . Signals were generated after incubation with horseradish peroxidase conjugated goat anti-rabbit (1:10,000) or anti-mouse (1:10,000) IgG using SuperSignal West Pico (Pierce, Waltham, MA), or Femto mixed with Pico for some low abundant proteins, and recorded on CL-Xposure films. Integrated optical density (IOD) was processed by ImageJ, and the  $\beta$ -actin signal was used as loading control.

### Quantification of BH4

BH4 was assayed by high-performance liquid chromatography (HPLC) method using an electrochemical detector (ESA Biosciences CoulArray System, Chelmsford, MA) as previously described (19). Multichannel coulometric detection was set between 0–600 mV. Intracellular concentrations were calculated using authentic BH4 standards. BH4 levels were then normalized to protein concentrations.

### Histology

For histology studies, the trachea was cannulated by an Instech Solomon (20G) stainless steel feeding tube (Plymouth Meeting, PA), and the lungs were inflated with 10% neutral buffered formalin at 2.4 kPa for 3 h. Lungs were removed and then fixed additionally in 10% neutral buffered formalin for 72 h.

Five  $\mu\text{m}$ -thick sections from the paraffin block of four groups of P10 and P21 old pups' lungs were cut and stained with hematoxylin and eosin for tissue morphology. Six random non-overlapping fields per pup in the distal lung sections were used for the morphometric examinations. Sections were photographed using a digital camera. Photographs were analyzed using Image J. RAC was obtained as described by Emery and Mithal (20). AWT was calculated using  $\text{AWT} = \text{VD}_T (\text{Tissue volume density}) \times L_m (\text{cord length})$  (21). For measurement of secondary septa, elastin was stained with resorcin-fuchsin and Van Gieson's

solution. To quantify capillary density, we measured the rat endothelial cell antigen-1 (RECA-1) immunostained area compared with total parenchymal cell area, using Colour Deconvolution (22).

### GCH1 promoter methylation

DNA extracted from ~5 mg of lung tissue was purified using PureLink™ Genomic DNA Mini Kit. Then, 500 ng extracted genomic DNA was bisulfate-converted using the EZ DNA Methylation Kit. The pyrosequencing system (PyroMark Q96 MD, Qiagen, Germantown, MD) was used to quantify methylated CpGs. Primer sequences for the GCH1 promoter region were designed by PyroMark Q96 MD.

*Forward primer:* 5'-GGTTTTTTAGTTGGAGGTTTGTTTTAGATT-3'

*Reverse primer:* 5'-biotin-ACCCATTAATACACCTTACACC-3'

*Sequencing primer:* 5'-AATTTTTGTTTAGGTGATAGTTATA-3'

### Immunoprecipitation

Immunoprecipitation was performed to detect ubiquitinated GCH1. Lung tissue homogenates were obtained as we described. 1mg of each lysate protein was incubated overnight at 4°C with anti-GCH1. Immunocomplexes were adsorbed to 100µl of protein A/G plus agarose beads (Thermo Fisher Scientific, Waltham, MA). Samples were denatured with SDS loading buffer, and proteins were separated by SDS-PAGE. Western blotting was performed with anti-ubiquitin and anti-GCH1 antibodies. Ratio of ubiquitin/total GCH-1 was assessed.

### Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, CA). Values are expressed as mean±SD. Significant differences among groups were determined by ANOVA with Student-Newman-Keuls post-hoc test. A p-value <0.05 was considered statistically significant.

## Results

### Caffeine decreases mortality and improves weight gain in hyperoxia-exposed rat pups

A total of 20 pregnant dams (4 dams per experiment) were used in this study. The mortality rate increased in rat pups exposed to hyperoxia compared to the pups exposed to normoxia; however, daily caffeine treatment decreased the mortality rate in the hyperoxia group (Figure 2A). Pups' weight (Figure 2B) was significantly decreased by hyperoxia as early as P4. Daily caffeine therapy improved postnatal weight gain. Conversely, caffeine reduced the weight gain after P20 in the normoxia group.

### Serum caffeine levels

Serum caffeine levels were undetectable in the saline-treated pups. The levels for the caffeine-treated pups were 19.3±5.3 µg/ml (n=12) at P10 and 18.4±4.9 µg/ml (n=12) at P21

without difference between groups (hyperoxia+caffeine vs. normoxia+caffeine,  $p=0.8$  by ANOVA).

### Effects of hyperoxia and caffeine on lung structure

There were no differences in the radial alveolar count (RAC), alveolar wall thickness (AWT) (Figure 3A), secondary septa formation (Figure 3B), and capillary count (Figure 3C) between caffeine treated and saline treated normoxia groups. Hyperoxia exposure impaired lung growth at P10 with decreased RAC (19%), increased AWT (60%), and a marked decrease in number of secondary septa (40%) and capillaries (60%). Caffeine treatment improved alveolar development during hyperoxia by increasing RAC (17%), decreasing AWT (30%), increasing the number of secondary septa (16%), and increasing capillary density (100%) compared to the saline-treated group.

Continuation of the caffeine treatment during recovery in room air maintained the beneficial effect on lung structure in the hyperoxia group (Figure 2). At P21 the lung structure remained impaired in the hyperoxia+saline group with decreased RAC (37%), increased wall thickness (60%), decreased number of secondary septa (28%) and capillaries (54%). Lung structure remained significantly improved by caffeine in the hyperoxia group at P21.

### Effects of caffeine on cAMP level

Exposure to hyperoxia decreased the cAMP levels in the lungs compared to normoxia controls. Caffeine treatment restored the cAMP levels in the hyperoxia-exposed lungs at P10. At P21, cAMP levels of the hyperoxia group were lower than those of the normoxia group, with no difference between caffeine and saline treated hyperoxic pups (Figure 4).

### Effects of caffeine on BH4 metabolism and angiogenic factors

To investigate the changes in angiogenesis by hyperoxia, we evaluated the proteins that are known to modulate angiogenesis, including vascular endothelial cell growth factor (VEGF) and eNOS and proteins that are known to affect BH4 levels, including GCH1 and dihydrofolate reductase (DHFR). Hyperoxia decreased VEGF and phospho-eNOS serine<sup>1177</sup> (p-eNOS<sup>1177</sup>) levels at P10 and caffeine partially abrogated this effect (Figure 5A). Recovery in room air restored the VEGF expression in hyperoxia group to normoxic levels at P21, but p-eNOS<sup>1177</sup> levels remained lower in hyperoxia group compared to normoxia; caffeine significantly increased the p-eNOS levels in the hyperoxia group at P21 compared to saline treatment (Figure 5A).

Hyperoxia also decreased GCH1 and phospho-serine<sup>51</sup>-GCH1 (p-GCH1<sup>51</sup>) (17) levels in the lungs at P10; caffeine treatment significantly increased both GCH-1 and p-GCH1<sup>51</sup> in the hyperoxia group. The GCH-1 levels show a persistent decrease at P21 after hyperoxia; caffeine increased the GCH-1 levels at P21 in the hyperoxia group (Figure 5B). The p-GCH1<sup>51</sup> levels, however, recovered to normoxic levels by P21, with no additional changes with caffeine treatment (Figure 5B). DHFR, the enzyme for salvage pathway BH4 formation, levels were unchanged by either hyperoxia or caffeine treatment. Changes in BH4 levels paralleled the changes in GCH-1 expression; BH4 levels decreased in hyperoxia alone at P10 and P21 and were increased at both time points by caffeine (Figure 5C).



## Methylation of the GCH1 promoter and ubiquitination of GCH1

Previous studies demonstrated that hyperoxia-induced lung injury is associated with alterations in the methylation of promoter regions of certain genes (23, 24). The GCH1 promoter area contains 12 potential methylation sites. We did not observe difference in methylation levels of GCH1 promoter among the 4 groups (Figure 6A). The methylation percent was very low in all groups, suggesting that GCH1 gene has high transcriptional potential in the postnatal developing lungs.

Immunoprecipitation analysis demonstrated an increase in GCH1 ubiquitination by hyperoxia in lungs at both P10 and P21 (Figure 6B), when compared to normoxia lungs. Caffeine decreased GCH1 ubiquitination at both P10 and P21.

## Discussion

BPD is a common cause of long-term morbidity, with more than 10,000 new cases annually in the USA, increasing the health care resource utilization for premature babies. Impaired angiogenesis is believed to be a key contributor to decreased alveolar growth in BPD (3, 25) and may further increase the risk of pulmonary hypertension. Our study investigated the mechanism by which caffeine improves lung growth during hyperoxia. We observed that caffeine helps maintain lung growth under hyperoxia, in part, by ameliorating impaired angiogenesis through improving GCH1 activity.

Premature infants commonly receive oxygen and mechanical ventilation right after birth for their respiratory support. Both oxygen and mechanical ventilation increase oxidative stress in the immature lungs (26–28). Increased oxidative stress depletes BH4 in vascular endothelial cells (10). BH4 promotes eNOS association with heat-shock-protein-90 (HSP90) and eNOS dimerization, both of which are required steps for adequate eNOS coupling (17). BH4 is also an intracellular antioxidant (29) which may facilitate NO bioavailability and stabilize soluble guanylate cyclase (30). Studies in animal models have demonstrated that NO is both the activator and mediator of VEGF and its effects on angiogenesis and alveolar growth in the developing lungs is well described (31, 32). We previously reported that increased oxidative stress depletes BH4 in pulmonary artery endothelial cells, which contributes to the impaired angiogenesis in developing lungs (33).

In this study, we showed that DHFR, which regenerates BH4 through the salvage pathway (34), did not change with either hyperoxia or caffeine treatment. On the other hand, we observed that GCH1 and p-GCH1<sup>51</sup> expression in the pups' lungs were significantly reduced by hyperoxia exposure, and restored by caffeine treatment; indicating that the changes in BH4 levels were due mainly to changes in GCH1 levels and the post-translational modifications.

The mechanism(s) by which hyperoxia decreases GCH1 levels remain unclear. Our data indicate that neither hyperoxia nor caffeine treatment alter the methylation level of the GCH1 gene promoter area. Some previous studies identified that oxidative stress stimulates ubiquitin-proteasomal protein degradation (35, 36). We investigated whether ubiquitin-proteasomal degradation explains the changes we observed in GCH1 protein levels in

hyperoxia-exposed rat lungs. Our results suggested that hyperoxia increased GCH1 ubiquitination in the lungs; this effect was reversed by caffeine treatment. The increase in cAMP levels we observed with caffeine treatment may provide protection to GCH1 by reducing both ubiquitin-dependent degradation and reduction in ROS formation (37).

While we show early caffeine treatment ameliorates hyperoxia-induced lung injury, our results are different from those reported by Londhe et al (38). Londhe showed caffeine aggravates the impaired growth of hyperoxia-exposed lungs with increased apoptosis in alveolar cells. These contradicting observations deserve further investigation, but there are at least two distinct differences between the two studies. Our study used a higher dose of caffeine (20 mg/kg/d) than reported by Londhe, et al (10 mg/kg/d). However, the measured serum caffeine levels in our study were within the clinically suggested therapeutic range (5–20 µg/ml) (39). Whether a higher caffeine dose confers the observed benefits deserves future investigation. The studies by Londhe used inbred mouse pups while we used outbred Sprague-Dawley rat pups and it is possible to have an inter-strain difference in response. Future studies in different models of lung injury are necessary to validate the potential beneficial effects of caffeine.

We observed that caffeine treatment provides benefits only in hyperoxia-exposed pups. Hyperoxia decreased the weight gain in our study even with switching the nursing dams which may be secondary to our >90% oxygen environment but caffeine improved weight gain despite exposure to hyperoxia. In fact, caffeine decreased postnatal weight gain in the normoxia group. However, most extremely premature infants require supplemental oxygen to provide adequate oxygenation early in life, so the early caffeine treatment in this susceptible group with poor weight gain would be less concerning.

In conclusion, our study provided plausible biological mechanisms by which early caffeine treatment protects developing lungs from hyperoxia-induced injury. By maintaining adequate GCH1 activity caffeine can preserve eNOS function during hyperoxia exposure.

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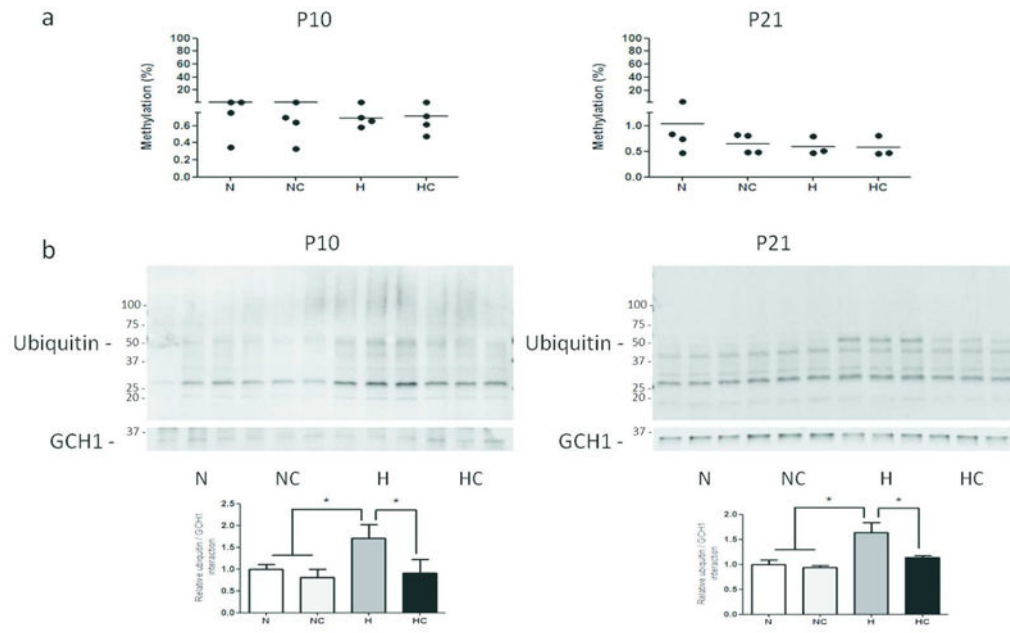
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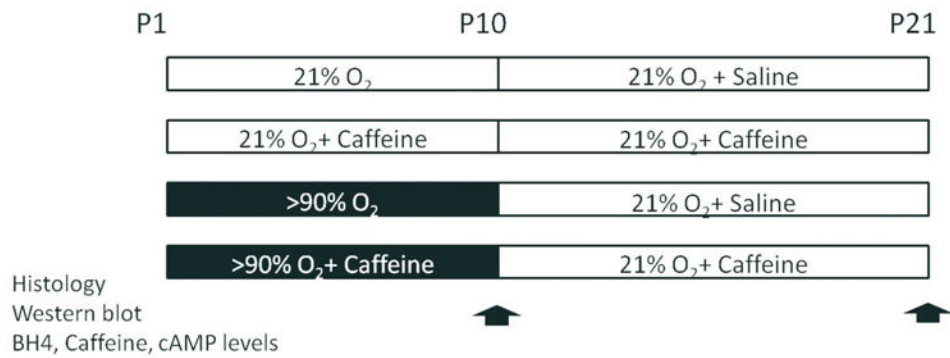
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Caffeine maintains adequate GCH1 level in lungs under hyperoxia.

**Figure 1.**

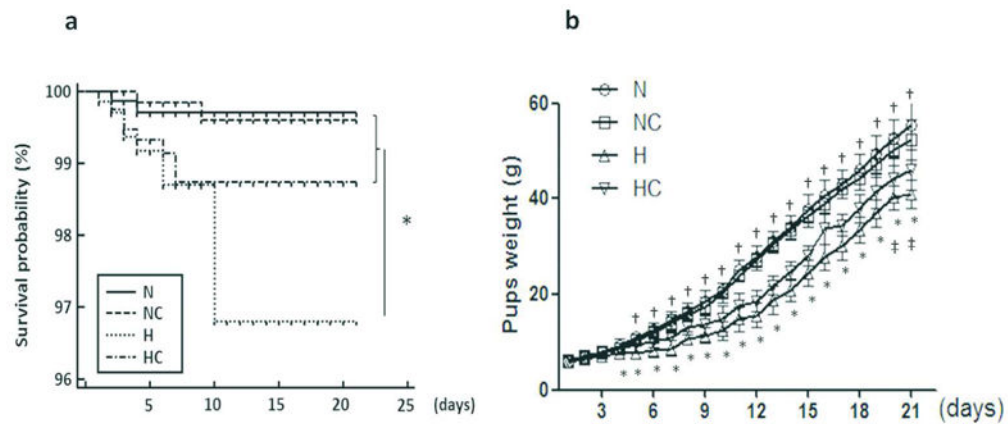
Study protocol for exposure of pups to hyperoxia and caffeine. P1, P10, and P21 are postnatal days 1, 10, and 21, respectively.



Study protocol for exposure of pups to hyperoxia and caffeine.

**Figure 2.**

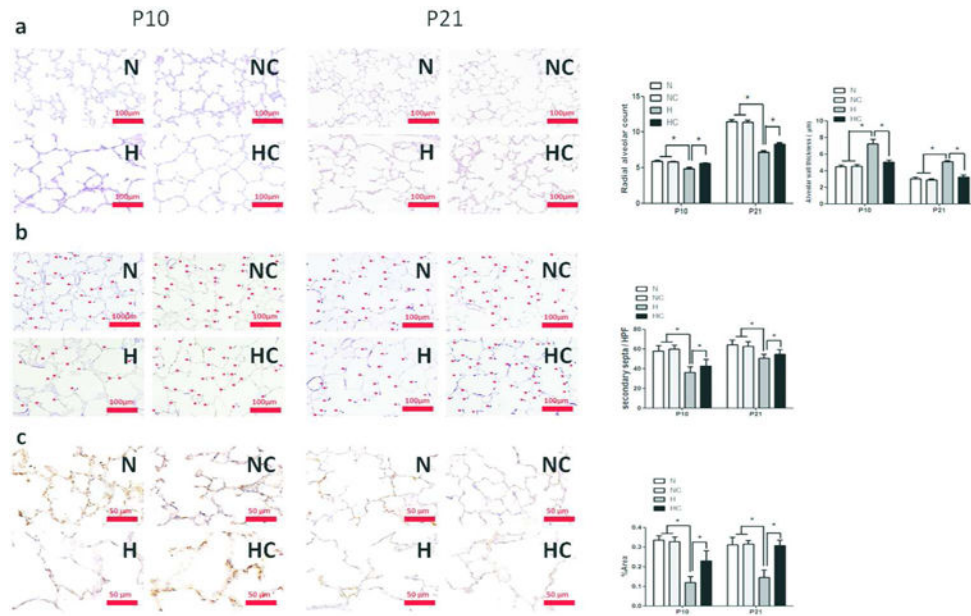
Hyperoxia decreased *the survival* and weight gain in pups and early caffeine treatment ameliorated the growth inhibiting effect of hyperoxia. (a) The survival rate decreased in pups exposed to hyperoxia and daily caffeine treatment improved the survival rate ( $*p < 0.05$ ,  $n = 50/\text{group}$ ). Although there was a significant difference, more than 95% of all rat pups survived the experiments. (b) Weight gain was significantly reduced by hyperoxia from the fourth day (P4) after birth. Pups in hyperoxia group were average 26% lighter than the ones from normoxia group at P21. Caffeine attenuated the weight loss effect in hyperoxia group but caused weight loss in the normoxia group. Normoxia (N, O), normoxic pups treated with 20 mg/kg caffeine per day (NC, ), age-matched hyperoxia controls (H, ), and hyperoxia pups treated with 20mg/kg caffeine per day (HC, ▽). Significant restriction of the pups' weight gain by hyperoxia was seen since P4 ( $*p < 0.05$ ,  $n = 50/\text{group}$ ). From P5, caffeine treated pups' weight was significantly higher compared to saline treated pups in hyperoxia ( $†p < 0.05$ ,  $n = 34/\text{group}$ ). Body weights of the caffeine-treated normoxia group were lower than the saline-treated group at P21 ( $‡p < 0.01$ ,  $n = 17/\text{group}$ ). N=normoxia, NC=normoxia+caffeine, H=hyperoxia and HC= hyperoxia+caffeine.



Hyperoxia decreased the survival and weight gain in pups and early caffeine treatment ameliorated the growth inhibiting effect of hyperoxia.

**Figure 3.**

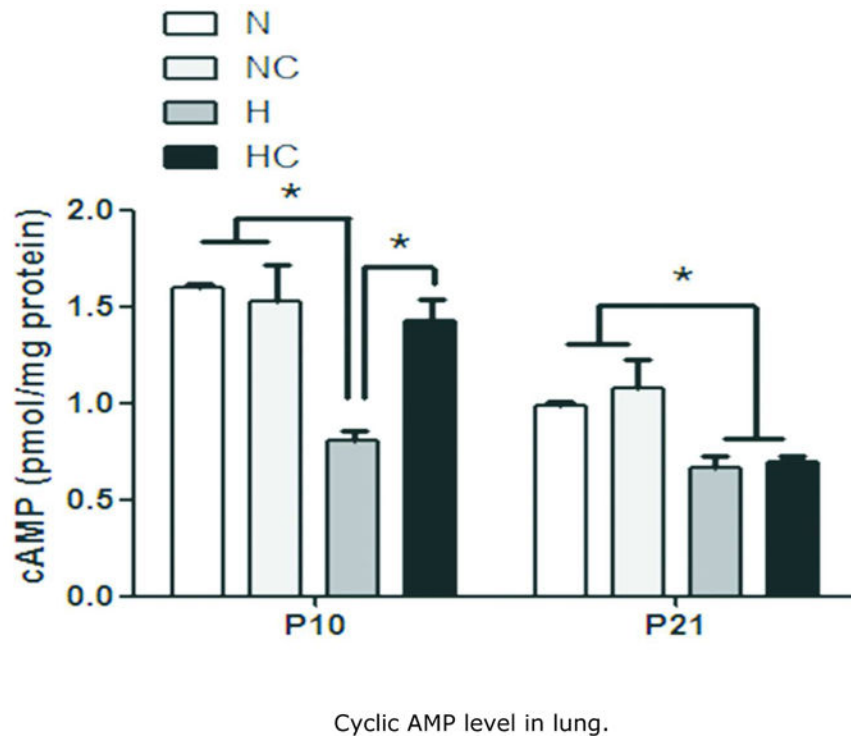
Caffeine ameliorates the effects of hyperoxia on lung growth. Lung sections were stained by hematoxylin and eosin for histology at P10 and P21 (a). The radial alveolar count (RAC) was decreased by hyperoxia. Caffeine increased the RAC in the hyperoxia group. Alveolar wall thickness (AWT) was increased in hyperoxia. Caffeine decreased the AWT in the hyperoxia group (A). Weigert's resorcin-fuchsin stain for elastin (purple dots) showed decreased secondary septa formation in hyperoxia at P10 and P21 (b). The area of RECA-1-immunostained endothelial cells as a proportion of total lung parenchymal cell area was measured by ImageJ. Ten random fields were chosen from each slide for 7~8 animals at each time point (c). Caffeine treated pups maintained their blood vessel counts in hyperoxia. \* $p < 0.01$ , n (N)=8, n (NC)=8, n (H)=7, n (HC)=7.



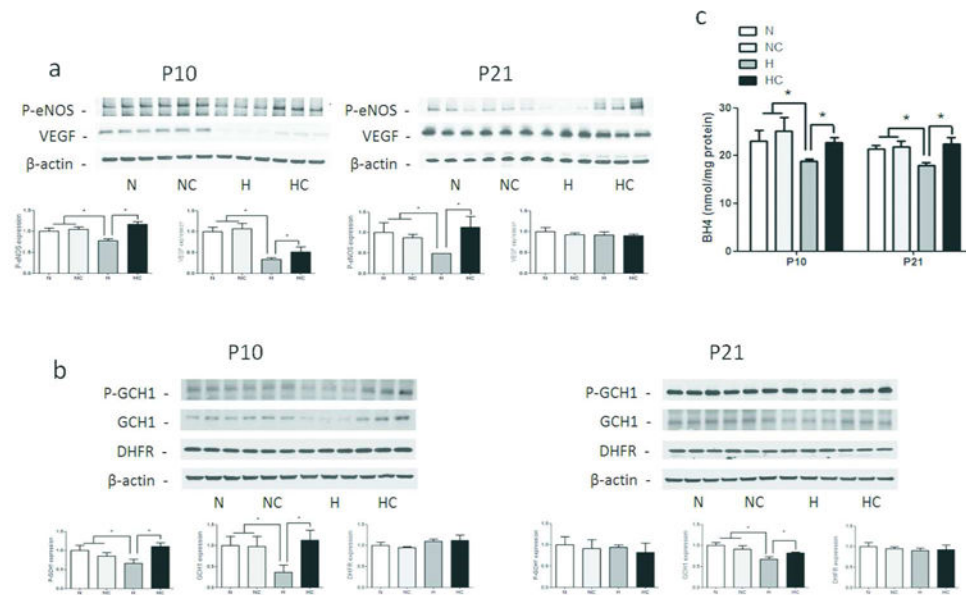
Caffeine ameliorates the effects of hyperoxia on lung growth.

**Figure 4.** Levels of cyclic AMP (cAMP) decreased at P10 and P21 in hyperoxia; caffeine restored cAMP to normoxia levels at P10. Caffeine did not increase cAMP levels at P21 in the hyperoxia group. \* $p < 0.05$ ,  $n = 6$ /group.





**Figure 5.** Effects of hyperoxia and caffeine on the levels of angiogenesis proteins and BH4 in the rat lungs. (a) Hyperoxia decreased vascular endothelial cell growth factor (VEGF) levels at P10, but not at P21; caffeine restored VEGF levels in the hyperoxia group at P10. Hyperoxia decreased endothelial nitric oxide synthase serine<sup>1177</sup> phosphorylation (p-eNOS<sup>1177</sup>) at both P10 and P21 and caffeine increased p-eNOS levels at both time points. (b) Hyperoxia decreased phospho-GTP cyclohydrolase-1 serine<sup>51</sup> (p-GCH1<sup>51</sup>) and GTP cyclohydrolase-1 (GCH1) at P10, and caffeine increased their levels. There was no change in p-GCH1 at P21 while GCH1 levels were lower in the hyperoxia group and this was increased by caffeine. There was no change in dihydrofolate reductase (DHFR) levels in any group at either P10 or P21. (c) The changes in tetrahydrobiopterin (BH4) levels paralleled the changes in GCH1 level, with hyperoxia group showing decreases at both P10 and P21. Caffeine increased BH4 levels during hyperoxia at P10 and P21. \* $p < 0.05$ ,  $n = 6$ /group.



Effects of hyperoxia and caffeine on the levels of angiogenesis proteins and BH4 in the rat lungs.

**Figure 6.**

(a) GCH1 promoter methylation levels were not affected by either hyperoxia or caffeine at both P10 and P21. (b) Hyperoxia and caffeine effects on GCH1 levels are mainly at the post-translational level. Hyperoxia increased GCH1 ubiquitination and caffeine decreased this effect at both P10 and P21. \* $p < 0.05$ ,  $n = 4$  for P10 all 4 groups;  $n = 4$  for N and NC at P21 but  $n = 3$  for H and HC at P21.