Neonatal exposure to mild hyperoxia causes persistent increases in oxidative stress and immune cells in the lungs of mice without altering lung structure.

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Abstract

Preterm infants often require supplemental oxygen due to lung immaturity, but hyperoxia can contribute to an increased risk of respiratory illness later in life. Our aim was to compare the effects of mild and moderate levels of neonatal hyperoxia on markers of pulmonary oxidative stress and inflammation, and on lung architecture; both immediate and persistent effects were assessed.

Neonatal mice (C57BL6/J) were raised in either room air (21% O₂), mild (40% O₂), or moderate (65% O₂) hyperoxia from birth until postnatal day 7 (P7d). The mice were killed at either P7d (immediate effects), or lived in air until adulthood (P56d, persistent effects). We enumerated macrophages in lung tissue at P7d and immune cells in bronchoalveolar lavage fluid (BALF) at P56d. At P7d and P56d, we assessed pulmonary oxidative stress (heme oxygenase-1 (HO-1) and nitrotyrosine staining) and lung architecture. The data were interrogated for sex differences. At P7d, HO-1 gene expression was greater in the 65% O₂ group than in the 21% O₂ group. At P56d, the area of nitrotyrosine staining and number of immune cells were greater in the 40% O₂ and 65% O₂ groups relative to the 21% O₂ group. Exposure to 65% O₂, but not 40% O₂, led to larger alveoli and lower tissue fraction in the short-term and to persistently fewer bronchiolar-alveolar attachments. Exposure to 40% O₂ or 65% O₂ causes persistent increases in pulmonary oxidative stress and immune cells, suggesting chronic inflammation within the adult lung. Unlike 65% O₂, 40% O₂ does not affect lung architecture.

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45 **Key words**

46 Supplemental oxygen, newborn, heme oxygenase, nitrotyrosine,

47 macrophages
Introduction

Preterm birth, defined as birth before 37 completed weeks of gestation, accounts for approximately 11% of all live births worldwide (12, 17). Preterm birth causes infants to be born while the lungs are immature and unable to maintain adequate gas-exchange. Consequently, preterm infants, especially those born very preterm (before 32 weeks of gestation), often require respiratory support including assisted ventilation and supplemental oxygen therapy using hyperoxic gas (58).

In recent decades there have been significant improvements in neonatal respiratory care, including the use of antenatal corticosteroids (53), exogenous surfactant therapy (36) and the introduction of gentler ventilation techniques (30) with lower concentrations of inspired oxygen (64). Despite these improvements, the risk of very preterm infants developing the chronic lung disease bronchopulmonary dysplasia (BPD) remains high (34). BPD is characterized by altered lung development, including enlarged and simplified alveoli, variable interstitial fibrosis and airway smooth muscle (ASM) hyperplasia (18, 35). It is now apparent that persistent changes within the lungs of preterm infants increase the risk of respiratory illness and insufficiency up to adulthood. In later life, individuals born very preterm have an increased risk of impaired lung function and reduced exercise capacity (23, 38, 59), asthma (28), chronic obstructive pulmonary disease (COPD) (14), and respiratory infections that require re-hospitalization (15, 52).

Although BPD is a multi-factorial disease, a major factor in its etiology is the
extended use of supplemental oxygen (65). Numerous large-scale epidemiological studies have shown that use of supplemental oxygen is associated with BPD (2, 31, 37, 57, 61). Prolonged inhalation of hyperoxic gas can lead to the accumulation of reactive oxygen species (ROS) within the developing lung, and ultimately a state of oxidative stress. Preterm infants are likely to be particularly vulnerable to oxidative stress as they have an immature antioxidant system and therefore have a limited ability to mount an antioxidant defense in response to supplemental oxygen (21). Very preterm infants resuscitated with 90% O₂, but not those resuscitated with 30% O₂, exhibited an increase in oxidative stress at both days 1 and 7 after birth, which correlated with the development of BPD (67). Of concern is that this increase in oxidative stress may persist into later life, as a recent study reported high oxidative stress levels in the lungs of adolescents born before 32 weeks of gestation (27).

It is now understood that the accumulation of ROS has the potential to activate transcription factors that are involved in both the recruitment and activation of inflammatory cells and mediators (51, 54). Experimental studies have shown that neonatal hyperoxia upregulates pro-inflammatory cytokines, which results in an influx of inflammatory cells into the lungs (3, 10, 70). Furthermore, preterm infants who develop BPD exhibit increased blood concentrations of the pro-inflammatory cytokines interleukin (IL)-1β, -6, -8 and -10, tumor necrosis factor-α and macrophage chemoattractant protein-1 (1, 13), and increased numbers of macrophages and neutrophils in bronchoalveolar lavage fluid (BALF) during infancy (29, 39, 60); however the
role of inflammation in the pathogenesis of BPD remains unclear. It is possible
that persistent oxidative stress and inflammation may not only play a role in
altering lung development, but may increase the risk of later respiratory
illness. Current understanding of how neonatal hyperoxia leads to oxidative
stress, inflammation, and lung injury resembling BPD, is largely derived from
animal models using high oxygen concentrations (80-100% O₂) (9, 44, 45, 49,
70, 72, 73). However, little is currently known about the effects of neonatal
exposure to lower oxygen concentrations, which are more reflective of those
used in current clinical practice, on oxidative stress, inflammation and lung
development (16, 67). We hypothesized that a lower level of neonatal
hyperoxia would not lead to persistent effects on the lung. Therefore, our
objective was to compare the effects of mild (40% O₂) and moderate (65%
O₂) levels of neonatal hyperoxia on markers of pulmonary oxidative stress,
immune cell infiltration and lung architecture. A concentration of 40% O₂ was
chosen as preterm infants are currently maintained on 30 – 50% O₂ (20, 41,
64, 68), and 65% O₂ was chosen for comparison as it is established that
neonatal exposure to 65% O₂ results in altered lung structure (46, 47). We
used an established model of murine exposure to neonatal hyperoxia (46, 47),
with some animals being studied immediately after the 7 days of neonatal
hyperoxia and others at adulthood, in order to assess both the immediate and
persistent effects.

Methods

**Experimental groups:** Female mice (C57BL6/J) were time-mated and
housed individually in clear plastic chambers (approximate volume 7.5 litres,
Tecniplast, Italy). Offspring were born at term and litters randomly allocated into three groups: mild hyperoxia (40% O2), moderate hyperoxia (65% O2) and controls that breathed only room air (21% O2). Hyperoxic groups were exposed to hyperoxic gas from 0.5 days before expected delivery to 7 days after delivery (postnatal day 7, P7d), which coincides with the late saccular and early alveolar stages of lung development. Concentrations of O2 and CO2 within the chambers were monitored (Servoflex MiniMP 5200, Servomex, USA). Food and water were available ad libitum. Within each of the three treatment groups, dams and litters were allocated into short-term and long-term survival studies. All experimental procedures were approved by the Monash University Animal Ethics Committee.

Short-term survival study (P7d): Newborn mice were exposed to 21% O2 (total n=23; 12M, 11F), 40% O2 (total n=28; 13M, 15F), or 65% O2 (total n=25; 12M, 13F). At P7d, the mice were killed by cervical dislocation and underwent necropsy for collection of both lungs.

Long-term survival study (P56d): Newborn mice were exposed to either 21% O2 (total n=23; 12M, 11F), 40% O2 (total n=28; 14M, 14F) or 65% O2 (total n=25; 12M, 13F) until P7d; all mice were then exposed to room air until adulthood (P56d). At P56d, they were killed by cervical dislocation and underwent necropsy for collection of BALF and both lungs.

Tissue collection and processing: Fresh lung tissue from eight offspring (4M and 4F) in each group was snap-frozen in liquid N2 for molecular
analysis. The lungs of remaining animals were infused \textit{in situ} via the trachea with 4\% paraformaldehyde in 0.1M phosphate buffered saline (pH 7.4) at 25cmH$_2$O, processed and wax-embedded. Sagittal sections (5μm) through both lungs were used for histological and immunohistochemical analyses, and sections through the right lung were used for immunofluorescent analysis. Researchers were blinded to the treatment group and sex of each animal.

\textbf{Pulmonary heme-oxygenase-1 gene expression:} Heme-oxygenase-1 (HO-1) mRNA expression was assessed as the HO-1 protein is known to be induced by oxidative stress (26, 55, 62). Total RNA extraction and cDNA synthesis was performed on frozen lung tissue at P7d and P56d using TRIZol reagent (Invitrogen™, Life Technologies, CA, USA) and M-MLV Reverse Transcriptase RNase H Minus, Point Mutant (Promega, WI, USA), respectively. Oligonucleotide primer pairs for \textit{HO-1} (GenBank accession number NM_010442.2; PrimePCR, Bio-Rad, CA, USA) were used for qPCR. \textit{β-actin} (GenBank accession number NM_007393.3; Bioneer, Korea) and \textit{18S rRNA} (GenBank accession number NC_000072.6; GeneWorks, Australia) were used as the housekeeping genes at P7d and P56d, respectively. Efficiency of primers was tested and samples were assayed in triplicate. In each run, a melt curve was conducted and a negative control sample was assayed. qPCR was performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, CA, USA) on a CFX384 C1000 Touch Thermal Cycler (Bio-Rad, CA, USA). Differential gene expression was determined using the comparative delta-delta $C_T$ (cycle threshold) method (50). HO-1 mRNA levels were expressed as a fold-change relative to the mean mRNA
levels of the 21% O$_2$ group.

Light microscopy analysis: Histologically and immunohistochemically stained tissue sections were examined using a light microscope (Nikon Eclipse E400, Nikon, Japan). Color images (x400 magnification) were captured using a digital camera (SPOT Insight 4meg Fire Wire Color Mosaic 14.2 Diagnostic Instruments, MI, USA) and analyzed using image analysis software (Image-Pro Plus, version 6.2, Media Cybernetics, MD, USA).

Oxidative stress in lung parenchyma: Immunohistochemical staining for nitrotyrosine was used as a marker of oxidative stress in the lung parenchyma (44). Nitrotyrosine is produced by tyrosine nitration after the formation of peroxynitrite by nitric oxide and the superoxide free radical (66). Nitrotyrosine was detected with a primary antibody (1:300 rabbit anti-nitrotyrosine, Merck Millipore, Germany) and secondary antibody conjugated with horseradish peroxidase (goat anti-rabbit, Dako, Denmark). Antibody binding was identified using 3,3'-Diaminobenzidine (DAB) (Dako, Denmark); sections were counterstained with Harris hematoxylin. Nitrotyrosine staining was assessed using three and five fields of view of each lung from the P7d and P56d groups, respectively; this was the maximum number of fields of view that could be consistently obtained at each time-point that were free of any major blood vessels or airways. The area of positive staining for nitrotyrosine (brown staining; located in the extracellular matrix and intracellularly) was expressed as a percentage of the total area of lung tissue (blue hematoxylin-stained tissue and brown staining) in each field of view;
data were averaged to produce a single value for each animal and these
averages were used to calculate the mean for each group.

**Pulmonary macrophages at P7d:** An immunofluorescent antibody against
galectin-3 was used to identify macrophages in the lung parenchyma at P7d
(74). Galectin-3 was identified using a primary antibody (1:1000 rat anti-
galectin-3, eBioscience, CA, USA) and a fluorescent secondary antibody
(1:1000 goat anti-rat antibody conjugated with Alexa Fluor 594-red,
Invitrogen™, Life Technologies, CA, USA). Sections were co-stained with
4′,6-Diamindino-2-Phenylinole (DAPI) (1:10000, Invitrogen™, Life
Technologies, CA, USA) to identify nuclei (blue).

Stained sections were examined by fluorescent microscopy (Zeiss Imager A1,
Zeiss, Germany) and images (x200 magnification) captured with a digital
camera (AxioCam MRc5, Zeiss, Germany) linked to image analysis software
(AxioVision, version 4.8, Zeiss, Germany). In the right lung, three fields of
view of lung parenchyma, free of large bronchioles and blood vessels, were
analyzed. The number of macrophages (identified by red staining) was
expressed as a percentage of the total number of cells (identified by nuclei
stained blue by DAPI) in each field of view of the lung parenchyma; the values
were averaged for each animal and these averages were used to calculate
the mean for each group.

**Immune cells in BALF at P56d:** BALF was collected via cannulation of the
trachea and lung lavage with 2 x 1ml of saline. The total number of immune
cells was counted using a hemocytometer and expressed per ml of BALF. Cells were also prepared by cytocentrifugation (Shandon Cytospin, Thermo Fisher Scientific, MA, USA) and stained with May-Grünwald-Geimsa (Sigma-Aldrich, MO, USA) for differential cell counting of macrophages, lymphocytes, neutrophils and eosinophils; leukocytes were enumerated based on strict morphological criteria (33). A total of 250 cells were counted per animal by light microscopy at x400 magnification.

**Lung architecture:** Fifteen fields of view that were free of large conducting airways and blood vessels were randomly selected for analysis from each left and right lung. Sections stained with Masson’s Trichrome were used to determine lung tissue fraction and mean linear intercept (MLI) by superimposing test grids over each field of view (71).

**Bronchiolar wall structure:** Wall structure was analyzed in six bronchioles (three from each lung) from each offspring at P7d and ten bronchioles (five from each lung) from each offspring at P56d; this was the maximum number of bronchioles that could be consistently obtained at each time-point that appeared in near-circular cross-section and had an intact bronchiolar wall that lacked cartilage. The bronchioles analyzed had basement membrane perimeters ($P_{BM}$) ranging from 200-1000μm. Bronchiolar measurements were standardized by dividing them by $P_{BM}$. Masson’s Trichrome-stained sections were used to determine bronchiolar epithelial area (48) and number of bronchiolar-alveolar attachments ($N_{BA}$) (48, 56). We used Gordon and Sweet’s Reticular Fibre stain to assess the area of types I and III collagen...
fibres, which were stained black; sections were counterstained with nuclear fast red (48). To assess the area of ASM (48), immunohistochemistry was performed using an antibody against α-smooth muscle actin (α-SMA; 1:200 mouse anti-α-SMA, Sigma-Aldrich, MO, USA). The secondary antibody conjugated with biotin (1:200 goat anti-mouse, Sigma-Aldrich, MO, USA) was detected using an ABC kit (Vector Laboratories Inc, CA, USA). Antibody binding was identified using DAB (Dako, Denmark), which resulted in detection of α-SMA by brown staining; sections were counterstained with Harris hematoxylin.

**Statistical analysis:** Data are presented as mean ± SEM. A normality test (Shapiro-Wilk) was performed and data transformed if required. Comparisons between groups were made using a two-way ANOVA with treatment and sex as factors. Effects of sex were examined, as sex is known to affect lung development (7). To identify differences between individual treatment groups, a least significant difference post-hoc test was conducted. Statistical significance was set at p<0.05.

**Results**

**Oxidative stress:** At P7d, the relative gene expression of HO-1 was significantly greater in the 65% O₂ group than in both the 21% O₂ (p<0.001) and 40% O₂ (p<0.001) groups. The relative gene expression of HO-1 tended to be greater in the 40% O₂ group than the 21% O₂ group but this did not reach statistical significance (p=0.07). Values were: 21% O₂, 1.0±0.1; 40% O₂, 1.3±0.1; 65% O₂, 1.9±0.1 (Figure 1A). At P56d, the relative gene
expression of HO-1 was not significantly different between the three treatment
groups (p=0.851, 21% O2, 1.0±0.2; 40% O2, 1.0±0.2; 65% O2, 1.1±0.2, Figure
1B).

At P7d, the proportion of lung tissue stained for nitrotyrosine was not
significantly different (p=0.336) between the three treatment groups (21% O2,
0.5±0.1 %; 40% O2, 0.6±0.1 %; 65% O2, 0.7±0.1 %). However at P56d, the
nitrotyrosine-stained area was significantly greater in the 40% O2 (p=0.047)
and 65% O2 (p=0.008) groups than in the 21% O2 group (21% O2, 0.8±0.6 %;
40% O2, 2.5±0.5 %; 65% O2, 3.3±0.6 %, Figure 2).

*Pulmonary macrophages:* At P7d, the proportion of cells in lung tissue that
were macrophages was significantly lower in the 40% O2 group compared to
the 21% O2 (p=0.004) and 65% O2 (p=0.001) groups; however, the
macrophage proportion in the 65% O2 group was not significantly different to
that in the 21% O2 group (21% O2, 1.1±0.1 %; 40% O2, 0.5±0.1 %; 65% O2,
1.1±0.1 %, Figure 3).

*Immune cells in BALF:* At P56d, the number of immune cells in BALF,
relative to the 21% O2 group, was greater (by 117%, p=0.011) in the 40% O2
group, and was further increased (by 150%, p=0.001) in the 65% O2 group.
Values were: 21% O2, 11115±1847 cells/ml; 40% O2, 18005±1565 cells/ml;
65% O2, 23797±1660 cells/ml (Figure 4A).
Enumeration of different populations of immune cells showed that there were no significant differences between treatment groups in the proportions of macrophages, lymphocytes or neutrophils (Figure 4B-E). The majority of immune cells in BALF were macrophages (>98%), and no eosinophils were observed in any of the treatment groups.

**Lung architecture:** At P7d, immediately following hyperoxia, the lung tissue fraction was significantly lower in the 65% O$_2$ group compared to the 21% O$_2$ (p=0.001) and 40% O$_2$ (p<0.001) groups (21% O$_2$, 39.5±1.1 %; 40% O$_2$, 40.4±1.0 %; 65% O$_2$, 34.0±1.0 %, Figure 5A, C-E). The MLI was significantly greater in the 65% O$_2$ group than in the 21% O$_2$ (p=0.009) and 40% O$_2$ (p=0.001) groups, indicating larger air spaces (21% O$_2$, 106.9±1.8 μm; 40% O$_2$, 106.8±1.6 μm; 65% O$_2$, 113.3±1.7 μm, Figure 5B, C-E). At P7d, the number of bronchiolar-alveolar attachments (N$_{ba}$) was significantly lower in the 65% O$_2$ group than in the 21% O$_2$ (p=0.004) and 40% O$_2$ (p<0.001) groups (21% O$_2$, 26.6±0.7 N$_{ba}$/mm; 40% O$_2$, 27.9±0.6 N$_{ba}$/mm; 65% O$_2$, 23.8±0.7 N$_{ba}$/mm, Figure 6A).

At P56d, the lung tissue fraction was not significantly different between the three treatment groups (p=0.535, 21% O$_2$, 33.7±0.8 %; 40% O$_2$, 32.7±0.7 %; 65% O$_2$, 32.7±0.7 %). The MLI had a tendency to be greater in the 65% O$_2$ group than in the 21% O$_2$ and 40% O$_2$ groups (p=0.075). Values were: 21% O$_2$, 82.0±1.9 μm; 40% O$_2$, 82.8±1.6 μm; 65% O$_2$, 87.5±1.7 μm). The number of bronchiolar-alveolar attachments remained significantly lower in the 65% O$_2$ group than in the 21% O$_2$ (p=0.004) and 40% O$_2$ (p<0.001) groups (21%
O$_2$, 31.3±0.7 N$_{ba}$/mm; 40% O$_2$, 32.3±0.6 N$_{ba}$/mm; 65% O$_2$, 28.3±0.7 N$_{ba}$/mm, Figure 6B).

**Bronchiolar wall structure:** At P7d and P56d, the bronchiolar epithelial area, and the areas of collagen and α-SMA in the outer bronchiolar wall, relative to P$_{BM}$, were not significantly different between the treatment groups (Table 1).

**Sex effects:** No significant sex differences were observed in any of the analyses at either P7d or P56d.

**Discussion**

In this study we investigated the immediate and persistent effects of neonatal exposure to both 40% O$_2$ and 65% O$_2$ on pulmonary oxidative stress and pulmonary immune cells, and related these to lung pathology. Importantly, we found that neonatal exposure to either mild (40% O$_2$) or moderate (65% O$_2$) levels of hyperoxia led to increased oxidative stress immediately after exposure and this persisted into adulthood; moreover, we found increased numbers of pulmonary immune cells in adulthood. Although mild hyperoxia led to persistent changes in oxidative stress and immune cells, it did not cause structural changes within the developing lung. Our findings could help explain why individuals who were born very preterm and received prolonged oxygen therapy are more susceptible to respiratory infections, COPD and asthma later in life (8, 14, 24).

Our study was designed to determine the effects of a clinically relevant
oxygen concentration (40% O₂) on lung development, and to compare the
effects with 65% O₂, which has previously been shown to induce structural
and functional changes within the lung (46, 47). With improvements in the
respiratory care of very preterm infants, supplemental oxygen concentrations
have declined substantially in recent years (40, 64). In contrast, very few
experimental studies have investigated the effects of prolonged inhalation of
gas containing less than 80% O₂ on lung development (5, 46, 47, 69, 72). Our
study design using mild and moderate degrees of hyperoxia has allowed us to
demonstrate dose-dependent responses to hyperoxia within the developing
lung.

**Oxidative stress:** A major finding of our study was an increase in both the
relative gene expression of \( \text{HO-1} \) at P7d in the 65% O₂ group and protein
expression of nitrotyrosine at P56d in both hyperoxia groups. To the best of
our knowledge, this is the first demonstration that exposure to a currently
clinically relevant level of hyperoxia (40% O₂) results in oxidative stress that
persists into adulthood. This increase in oxidative stress was not
accompanied by structural alterations in the lung, suggesting that structural
alterations caused by higher oxygen concentrations may not be mediated
together entirely by oxidative stress. Indeed, previous studies have attributed the
hyperoxia-induced disruption to alveolarization to alterations in the expression
of growth factors, in particular the downregulation of vascular endothelial
growth factor (42, 63).

The increase in oxidative stress that we observed following neonatal
hyperoxia in mice is consistent with findings in humans. It has been shown that preterm infants who suffered from respiratory distress and required supplemental oxygen exhibited an eight-fold increase in HO-1 expression within the blood 1-5 days after birth compared to term controls (25). Furthermore, this increase in oxidative stress persists; adolescent children born very preterm had greater expression of an oxidative stress marker (8-isoprostane) in exhaled breath condensate than term-matched controls, regardless of whether they developed BPD or not (27). Therefore our finding that exposure to 40% O₂ leads to persistent oxidative stress but not lung pathology correlates with human data showing that preterm infants who do not develop BPD in infancy may display increased oxidative stress in the lungs later in life (27).

Pulmonary macrophages: Exposure to 40% O₂ led to a transient decrease in the percentage of macrophages in the lung parenchyma at P7d. This was an unexpected finding as a previous study found an influx of inflammatory cells into the lung immediately following hyperoxia (70); however in that study the cells were predominantly localized to the perivascular and peribronchiolar regions (70), whereas we studied only gas-exchanging tissue. This discrepancy could also be due to the use of different mouse strains and oxygen concentrations.

Immune cells in BALF: Neonatal exposure to both 40% and 65% O₂ led to a dose-dependent, persistent increase in the number of pulmonary immune cells in adult mice. Further characterization of the immune cells showed no
significant differences between the proportion of macrophages, lymphocytes and neutrophils between the treatment groups, with the majority (>98%) of cells being macrophages. This appears to be the first demonstration that neonatal exposure to 40% O₂ results in an increase in pulmonary immune cells that persists into adult life. Previous studies have shown that neonatal exposure to hyperoxia (>50% O₂) led to an increase in the number of pulmonary immune cells in the BALF; however this was observed immediately after exposure (4, 11, 22, 70).

Our findings suggest that neonatal exposure to 40% and 65% O₂ leads to a chronic inflammatory state within the lung which could be mediated by the prolonged state of oxidative stress. This chronic inflammation may be a result of a positive feedback cycle occurring between the production of ROS by inflammatory cells, which in turn recruits more inflammatory cells to the lung (54). The presence of such a process may help explain why very preterm infants are more susceptible to respiratory infections and diseases, such as COPD, that are characterized by an increase in macrophages within the lung (6).

**Lung architecture:** Our study showed that neonatal exposure to 40% O₂ during the saccular and early alveolar stages of lung development did not lead to any immediate or persistent structural alterations in the lung parenchyma. These findings concur with those of a previous study showing that exposure of C57BL6/J mice to 40% O₂ during the saccular stage of lung development (from P0d-P4d) did not lead to alterations, at P56d, in MLI or mean chord
length, which are estimates of “alveolar” size (72). Together, these studies indicate that prolonged inhalation of 40% O₂, unlike higher concentrations, does not cause alveolar simplification or adversely affect lung architecture. In contrast, a recent study in which C57BL6/J mice were exposed to 40% O₂ from P0d-P7d showed a decrease in alveolar number and radial alveolar count at P21d (69). Although these results seem to contradict both the present study and the study by Yee et al (72), the difference could be due to the lungs being examined at a different time-point after hyperoxia and differences in the methods used to measure the degree of alveolarization.

We have confirmed that neonatal exposure to 65% O₂ for 7 days leads to a decrease in alveolarization immediately after the exposure period (46). Structural alterations continued into adulthood, with a persistent decrease in the number of bronchiolar-alveolar attachments and a tendency for the MLI to be increased. Our findings in adulthood are consistent with previous studies showing that exposure to either 60% or 65% O₂ leads to persistent alterations in alveolar structure, together with a decrease in the number of bronchiolar-alveolar attachments (46, 47, 72). We, and others (44, 70, 72), have previously observed that structural changes induced by neonatal hyperoxia are associated with altered lung function in adult mice (46). A decrease in the number of bronchiolar-alveolar attachments is considered to favor small airway narrowing and is found in asthma victims (43) and smokers, in whom it was associated with poor lung function (56). A reduced number of attachments has been related to increased risk of asthma (8), COPD (14) and poor lung function (24) in individuals born very preterm.
**Bronchiolar wall structure:** We found no significant differences following neonatal exposure to 40% O₂ and 65% O₂ on the epithelial, collagen or ASM areas in the bronchiolar walls at P7d and P56d. Previous studies on the effects of hyperoxia on airway wall structure have produced conflicting results. Our laboratory previously reported that exposure of neonatal mice to 65% O₂ from P0d-P7d led to epithelial thickening (in males only), a ~25% decrease in bronchiolar collagen and no changes in ASM at P56d (46). The apparent discrepancy between studies could be due to the mouse line being re-derived. Furthermore, other studies have demonstrated epithelial thickening after exposure to >95% O₂ (19, 32), increased collagen deposition at P21d following exposure to 40% and 70% O₂ from P0d-P7d (69) and an increase in ASM at P21d following exposure to 40% O₂ from P0d-P7d; in contrast, exposure to 70% O₂ from P0d-P7d did not alter the area of ASM (69). Moreover, other studies have reported an increase in ASM following exposure to 50% O₂ for 15 days in neonatal rats (22). Further studies are required to elucidate why discrepancies exist between the present and previous studies concerning the effects of hyperoxia on bronchiolar wall morphometry.

**Conclusions**

Neonatal exposure to both 40% and 65% O₂ during the saccular and early alveolar stages of lung development leads to an immediate and persistent increase in oxidative stress and a persistent increase in the number of pulmonary immune cells. In contrast, only exposure to 65% O₂ affects lung architecture. Together, our findings are consistent with the concept of a
persistent positive feedback cycle between prolonged oxidative stress
induced by hyperoxia and subsequent chronic inflammatory response. Our
findings may help explain why preterm infants, particularly those who do not
develop BPD, are at increased risk of respiratory infections, asthma, COPD
and poorer lung function later in life.

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Authors’ Contributions
S.B, M.O.R., R.H and F.S are responsible for the conception and design of
the experiments, S.B, M.O.R and F.S performed the animal and laboratory
experiments, S.B analyzed the data, S.B, R.H and F.S interpreted the results,
S.B drafted the manuscript and prepared the figures, S.B, M.O.R, R.H and F.S edited the manuscript, and S.B, M.O.R, R.H and F.S approved the final manuscript.
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Figure legends

Figure 1: Relative gene expression of HO-1 at P7d and P56d. At P7d, the relative gene expression of HO-1 was significantly increased in the 65% O₂ group and had a tendency to be increased in the 40% O₂ group compared to the 21% O₂ group (A). At P56d, no significant differences were observed between the treatment groups (B). An asterisk (*) indicates a significant difference (p<0.01) in relation to the 21% O₂ and 40% O₂ groups.

Figure 2: Percentage of nitrotyrosine staining in the lung parenchyma at P56d. At P56d, the percentage of nitrotyrosine staining was significantly increased in the 40% O₂ and 65% O₂ groups in comparison to the 21% O₂ group (A). An asterisk (*) indicates a significant difference (p<0.05) in relation to the 21% O₂ group. Immunohistochemical images (B-D) showing lung tissue stained with an antibody against nitrotyrosine (brown staining) are representative of the parenchymal fields of view analyzed at P56d (B; 21% O₂, C; 40% O₂, D; 65% O₂). Scale bar=50μm.

Figure 3: Percentage of macrophages in the lung parenchyma at P7d. At P7d, the proportion of cells stained positive for galectin-3 (macrophages) was significantly reduced in the 40% O₂ group in comparison to the 21% O₂ group (A). Immunofluorescent images (B; 21% O₂, C; 40% O₂, D; 65% O₂) showing lung tissue stained with an antibody against galectin-3 (red) are representative of the parenchymal fields of view analyzed at P7d. An asterisk (*) indicates a significant difference (p<0.01) in relation to the 21% O₂ and 65% O₂ groups. Scale bar=30μm.
Figure 4: Number and type of immune cells present in BALF at P56d. At P56d, the number of immune cells per mL of BALF was significantly increased in the 40% O₂ group in relation to the 21% O₂ group and this was further increased in the 65% O₂ group (A). The proportion of macrophages, lymphocytes and neutrophils was not significantly different between treatment groups (B). An asterisk (*) indicates a significant difference (p<0.05) in relation to the 21% O₂ group. A caret (^) indicates a significant difference (p<0.05) between the 40% O₂ and 65% O₂ groups. Histological images (C-E) showing immune cells stained with May-Grünwald-Geimsa are representative of the fields of view analyzed at P56d (C; 21% O₂, D; 40% O₂, E; 65% O₂). Scale bar=50μm.

Figure 5: Lung parenchyma structure at P7d. At P7d, the percentage of tissue (A) and mean linear intercept (B) were significantly altered immediately following exposure to 65% O₂ compared to the 21% O₂ and 40% O₂ groups. An asterisk (*) indicates a significant difference (p<0.01) in relation to the 21% O₂ and 40% O₂ groups. Histological images (C-E) showing lung tissue stained with Masson’s Trichrome are representative of the fields of view analyzed at P7d (C; 21% O₂, D; 40% O₂, E; 65% O₂). Scale bar=50μm.

Figure 6: Bronchiolar-alveolar attachments at P7d and P56d. The number of bronchiolar-alveolar attachments was significantly lower in the 65% O₂ group in comparison to the 21% O₂ and 40% O₂ groups at both P7d (A) and at P56d (B). An asterisk (*) indicates a significant difference (p<0.01) in
relation to the 21% O₂ and 40% O₂ groups.
HO-1 mRNA levels
(Fold-change relative to 21% O₂)

A

B

HO-1 mRNA levels
(Fold-change relative to 21% O₂)
Area nitrotyrosine/tissue area (%) for different oxygen concentrations:

- 21% O₂
- 40% O₂
- 65% O₂

Significant differences are indicated with an asterisk (*) in the graph.
Galectin-3 positive cells / total cells (%)

- 21% O₂
- 40% O₂
- 65% O₂

B C D
A

Number of bronchiolar-alveolar attachments (N_{BA}/mm)

- 21% O_2
- 40% O_2
- 65% O_2

B

Number of bronchiolar-alveolar attachments (N_{BA}/mm)

- 21% O_2
- 40% O_2
- 65% O_2

* Statistically significant difference
Table 1: Neonatal exposure to 40% O₂ and 65% O₂ did not significantly alter the epithelial, collagen or α-SMA area of the bronchioles at P7d or P56d.

<table>
<thead>
<tr>
<th></th>
<th>P7d</th>
<th>P56d</th>
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<tbody>
<tr>
<td></td>
<td>21% O₂</td>
<td>40% O₂</td>
</tr>
<tr>
<td>Epithelial Area (μm²/μm)</td>
<td>7.1±0.2</td>
<td>7.0±0.1</td>
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<tr>
<td>Collagen Area (μm²/μm)</td>
<td>3.6±0.3</td>
<td>3.4±0.2</td>
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<tr>
<td>α-SMA Area (μm²/μm)</td>
<td>0.9±0.2</td>
<td>0.7±0.1</td>
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