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Lung Development Alterations in Newborn Mice after Recovery from Exposure to Sublethal Hyperoxia

Esther Rieger-Fackeldey,*† Min S. Park,*‡ Brandon L. Schanbacher,* Mandar S. Joshi,* Louis G. Chicoine,* Leif D. Nelin,* John A. Bauer,* Stephen E. Welty,* and Charles V. Smith*§

From the Section of Neonatology, Department of Pediatrics, Center for Perinatal Research, Nationwide Children's Hospital Research Institute, Columbus, Ohio; the Neonatology and Pediatric Critical Care,† Department of General Pediatrics, University Children's Hospital of Muenster, Muenster, Germany; the Department of Pediatrics,‡ Yonsei University College of Medicine, Seoul, Republic of Korea; and the Center for Developmental Therapeutics,§ Seattle Children's Hospital Research Institute, Seattle, Washington*

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Address correspondence to Stephen E. Welty, M.D., 6621 Fannin St, WT6-104, Houston, TX 77030. E-mail: welty@bcm.edu.

Exposure of newborn mice to hyperoxia arrests lung development, with resultant pathological characteristics similar to bronchopulmonary dysplasia in infants born prematurely. We tested the hypothesis that aberrations in lung development caused by 14 days of sublethal hyperoxia would be reversed during 14 days of recovery to room air (RA) when the concentration of oxygen exposure was weaned gradually. Newborn FVB mice were exposed to 85% oxygen or RA for 14 days. Weaning from hyperoxia was by either transfer directly into RA or a decrease in the concentration of oxygen by 10% per days. At 28 days, pups were euthanized, and the lungs were inflation fixed and assessed. At postnatal day 28, lungs of mice weaned abruptly from hyperoxia had fewer (6 ± 0.6 versus 10 ± 0.7; $P < 0.001$) alveoli per high-powered field and larger alveoli (4050 ± 207 versus 2305 ± 182 μm^2) than animals weaned gradually; both hyperoxia-exposed groups were different from lungs obtained from air-breathing controls (20 ± 0.5 alveoli per high-powered field; $P < 0.001$). The results are consistent with the absence of catch-up alveolarization in this model and indicate that the long-term consequences of early exposures to hyperoxia merit closer examination. The effects of abrupt weaning to RA observed further suggest that weaning should be considered in experimental models of newborn exposure to hyperoxia. *(Am J Pathol 2014, 184: 1010—1016; http://dx.doi.org/10.1016/j.ajpath.2013.12.021)*

Bronchopulmonary dysplasia (BPD) is a chronic lung disease originally reported in prematurely born infants exposed to mechanical ventilation and supplemental oxygen, to provide respiratory support to infants with hyaline membrane disease.[1] The development of hyaline membrane disease and its supportive treatments have been associated with lung inflammation and lung injury.[2] In subsequent decades, refinements in clinical care have provided great improvements in avoiding the acute lung injury and suboptimal tissue repair that were associated with BPD originally, but the incidence of BPD and chronic respiratory morbidity in prematurely born infants has not declined similarly, in large part because of increased survival of infants born at extremely premature gestations.[3] Limiting acute lung injury is critical, and efforts have been focused on minimizing atelectatrauma and volutrauma associated with respiratory support from mechanical ventilation, more judiciously using supplemental oxygen, and limiting pulmonary inflammatory responses. However, BPD in prematurely born infants also develops in infants supported with minimal or no mechanical ventilation and low supplemental oxygen concentrations. Therefore, acute lung injury may not be the only factor in development of BPD.

At the level of lung tissue, BPD in the modern era is characterized largely by poorly developed lungs as a result of interrupted alveolarization,[4] and animal models have been developed that show arrested alveolarization with minimal lung inflammation and acute injury.[5,6]

The lungs of term newborn mice are comparable structurally with lungs of human infants born at 26 to 28 weeks'

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Current address of S.E.W., Department of Pediatrics, Baylor College of Medicine, Houston, TX.

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gestation, but are functionally sufficient for support of extrauterine life.[7] The stages of structural growth and maturation of lungs appear to be similar in mice and humans, and newborn mice, therefore, offer useful models for studies of lung development. Mechanisms proposed for dysregulation of lung development in preterm infants include lung inflammation, extracellular matrix remodeling, protease-antiprotease imbalance, mesenchymal cell apoptosis and proliferation, and epigenetic changes affecting cell cycle regulation.[8–10] Although many preterm infants are supported with supplemental oxygen, even room air (RA) can constitute an oxidant stress to those born that are extremely premature.[11]

Studies have shown diminished alveolarization in animal models of BPD, such as the 125 days premature baboon model,[12] with some preliminary studies of possible mechanisms.[13,14] However, studies in newborn mice provide the unique opportunity to study a pure oxidant stress in lungs that are in an immature stage of embryology and are surfactant sufficient, which may mimic lung maldevelopment and the development of BPD in premature infants receiving low concentrations of supplemental oxygen and nasal continuous positive airway pressure. Warner et al[15] reported that exposure of newborn FVB/N mice to 85% oxygen for 14 days inhibited lung alveolarization and development, providing a lung alveolar phenotype similar to that observed in infants with BPD of more recent years.[15] They also observed 40% pup lethality, inhibition of body growth, and marked inflammation in the lungs of animals exposed to hyperoxia. With the same strain and exposure protocol, we similarly observed arrested lung development, but, in contrast to observations made by Park et al,[5] we did not observe associated pup death, stunted growth, or marked lung inflammation. In agreement with the working hypothesis of oxidant-mediated alterations in signal transduction mechanisms, we observed temporal and spatial alterations in expressions of fibroblast growth factor 7 and fibroblast growth factor receptors 3 and 4. By using more sensitive methods for assessing lung inflammation, Rogers et al[6] observed small, but measurable, increases in neutrophil contents of lungs of newborn C3H/HeN mice exposed to 85% oxygen.

Previously, we had examined whether exposure of FVB/N newborns to supplemental oxygen at concentrations that do not cause mortality, retardation of animal growth, or acute tissue necrosis, in the absence of insults arising from endotracheal intubation, mechanical ventilation, atelectatrauma, or acute tissue necrosis, and the inflammatory responses initiated by these insults, would alter lung development. Other than the inescapable fact that mice born at term and premature infants differ in several ways, the intent of the present line of research is to attempt to define the potentially limiting factors in lung development in infants in the absence of adverse effects of supportive care. The results of our earlier studies[5] supported this working hypothesis, but a critical question is whether the deficits in alveolarization and other differences in lung development observed at postnatal day 14 (P14) would persist or be reversed by lung growth and maturation after weaning the animals exposed to hyperoxia to RA. Differences observed at P14 in animals exposed to hyperoxia that are mitigated after weaning to RA and further maturation would be of less concern than the effects that persist. In fact, we did report persistent effects of hyperoxia in a follow-up report, but in those studies, the dams of the study mice were all exposed to interventions at embryonic day 16, and the most pronounced effects of hyperoxia were in mice whose mothers were exposed to subacute doses of lipopolysaccharide; the persistent effects on alveolarization of hyperoxia alone were not studied in this report.[16] However, recently, Husain et al[17] have reported persistent effects in infants exposed to hyperoxia in the newborn period.

In contrast to the studies of hyperoxia exposure and recovery in which the transfer to RA for recovery is abrupt,[15,16] in supportive respiratory care for premature infants, supplemental oxygen support is weaned gradually, rather than abruptly. However, weaning protocols for studies in experimental animals have received no attention, and transfers from hyperoxia to RA typically have been abrupt.

We tested the hypotheses that the effects on lung structure in newborn FVB mice exposed to 14 days of sublethal hyperoxia [fraction of inspired oxygen (F_{IO_2}) = 0.85] do not recover by compensatory growth, and that gradual weaning to RA (decreasing F_{IO_2} by 0.1 per day, until 0.21 was reached) mitigates the detrimental effects of hyperoxia exposure and recovery on lung structure (morphometry) and function (static lung compliance).

Materials and Methods

Animals

Time-dated pregnant FVB/N mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and Charles River (Wilmington, MA) and arrived at the vivarium at least 5 days before term to become acclimatized before delivery. Dams were maintained on standard laboratory food and water ad libitum and kept on a 12:12-hour light-dark cycle. All animal study protocols were approved by our Institutional Animal Care and Use Committee.

Gas Exposures and Study Design

Animals were randomized to one of the three 28-day treatment groups, which included the following: i) RA control for 28 days, ii) 85% oxygen for 14 days, with abrupt weaning to RA, or iii) 85% oxygen for 14 days, with graded weaning to RA over 6 days. After these treatments, each animal was euthanized at 28 days for tissue studies.

Newborn FVB/N mice from pairs of time-dated pregnant dams delivered naturally within 6 hours of each other were pooled and randomly redistributed back to the two dams in separate cages within 12 hours of birth. One group of pups was placed in 85% oxygen (hyperoxia exposed), and the other group was maintained in RA for 14 days. Nursing dams were rotated between the hyperoxia-exposed and RA

litters every 24 hours, to avoid oxygen toxicity in the dams and eliminate maternal effects between groups. Exposures were conducted in Plexiglass chambers into which oxygen was delivered continuously through an oxygen blender, to achieve constant levels of 85% oxygen. Oxygen levels were monitored with an oxygen analyzer (Hudson RCI, Chicago, IL). The flow rate was maintained at 5 L/minute if one cage was placed in the Plexiglass chamber or at 10 L/minute if two cages were placed in the chamber. Soda lime was used to remove CO_2. After 14 days, hyperoxia-exposed animals were either removed and placed in RA (abrupt weaning) or weaned gradually by a reduction of oxygen over 6 days (75% × 1 day, 65% × 1 day, 55% × 1 day, 45% × 1 day, 35% × 1 day, and 21% × 9 days). Dam rotation was continued until day 21, when all pups were weaned from their mothers. The ambient temperature was kept constant at 23°C, and humidity was maintained between 40% and 60%. Animals were monitored every 12 hours for evidence of morbidity or mortality. Body weights were recorded at pooling and 7, 14, 21, and 28 days later. In most animals, tissue samples were prepared as described in *Tissue Preparation*. In a subset of animals, assessments of static lung compliance were obtained by standard pressure manometry.

Tissue Preparation

On day 28, animals were euthanized by i.p. injection of 200 mg/kg of sodium pentobarbital and exsanguinated by carotid artery transection. In all animals, tracheas were cannulated with 25-gauge Silastic catheters (Harvard Apparatus, Holliston, MA), and 10% neutral-buffered formalin was instilled at 25 cm H_2O pressure over 5 minutes of equilibration. We defined a pressure decrease during the inflation fixation to <20 cm of H_2O to be considered a leakage, and tissue specimen was excluded from morphometric analyses; however, no leaks were detected, and no animals were excluded. After 5 minutes, tracheas were tied, and lungs were removed and fixed overnight in 10% neutral-buffered formalin. The next day, fixed lungs were washed in PBS five times and stored at 4°C. Intact left and right lungs were serially dehydrated in increasing concentrations of ethanol, before being embedded in paraffin. Left lungs were cut transversely (perpendicular to the longitudinal or craniocaudal axis of the animal) at the level of entry of the left main bronchus into the lung parenchyma, to obtain 2-mm thick slices, whereas the right lungs were cut coronally through all four lobes (along the longitudinal axis and perpendicular to the dorsal-ventral axis) at the level of entry of the right main bronchus. The cut slices of lung tissues were oriented identically and embedded into paraffin blocks.

Morphometric Analysis, Image Capture, and Digital Image Analysis

Lungs from 44 paired experimental and control animals were examined. The left lung lobes embedded in paraffin were cut into sections (5 μm thick), deparaffinized, and stained with H&E. Five photomicrographs per lung section were captured at the same anatomical sites at ×64 magnification (Olympus BX-40 microscope; Olympus Optical, Melville, NY) and a digital camera (Diagnostic Instruments, Ontario, NY), under identical lighting conditions and optical settings. Images were analyzed using research-based digital image analysis software (Image Pro Plus version 4.0; Media Cybernetics, Silver Spring, MD) and a custom written macro for automated investigations of alveolar morphological characteristics. Complete alveoli within the image were selected by color segmentation, and any incomplete alveoli (those touching the image edge) were excluded from analysis. The macro subroutine then measured the number of complete alveoli in the image, as well as the area and the perimeter of each alveolus. By using this approach, we obtained morphometric parameters in an objective manner, with high throughput and low variability. Preliminary studies showed that intra-animal and interobserver variabilities in these measurements were routinely <10%. The following parameters were determined for each alveolar space: number of complete alveoli per field of view (at ×64 magnification); alveolar size (area in microns^2); alveolar perimeter (in microns): perimeter/area ratio (1/μm); theoretical surface area/volume ratio (a three-dimensional estimation from measured perimeter/area ratios, based on assuming cross sections of spherical alveoli).

Statistical Analysis

Data are expressed as means ± SEM and were analyzed by one- or two-way analysis of variance, with hyperoxia-exposed/RA and gradual/abrupt weaning as independent variables. Student-Newman-Keuls tests and modified *t*-tests were applied as post hoc tests. Lung compliance and body weight were analyzed by unpaired Student's *t*-tests. Differences were attributed at $P < 0.05$.

Results

At P28, body weights of mice raised in RA were not different from the weights of mice kept in 85% oxygen for 14 days, then in RA (17.8 ± 3.6 versus 16.0 ± 3.5; $n = 22$ per group, respectively).

Histological sections of lungs of mice raised in RA, then euthanized for study at P28 (Figure 1A), reflected the expected continuation of alveolarization and lung maturation, whereas mice exposed to 85% O_2 from P0 through P14, then weaned to RA, showed much lower extents of alveolar development, both in animals weaned abruptly and gradually (Figure 1, B and C, respectively), with the lungs of the animals weaned abruptly to room air showing the greatest deficits in alveolarization.

Quantitative morphometric assessments of lungs indicated that numbers of alveoli per high-powered field (HPF)

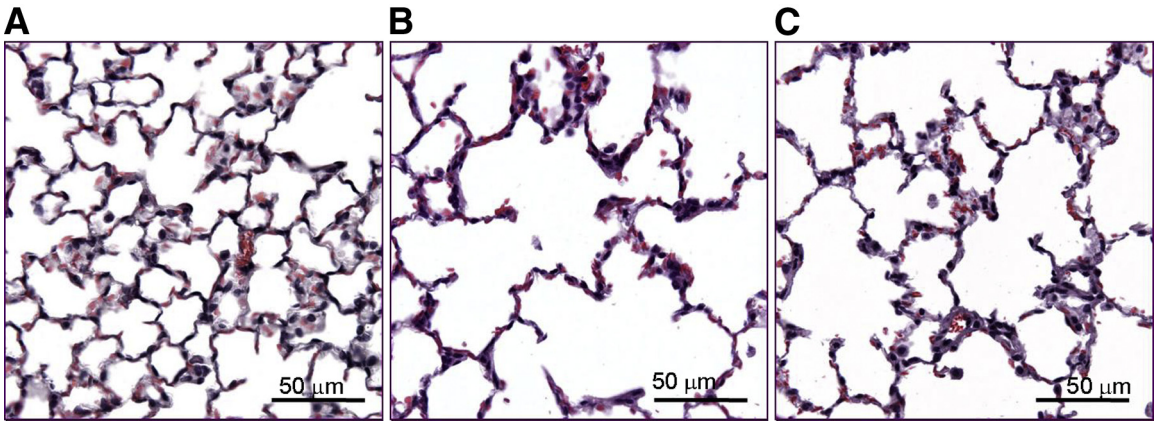

Figure 1 Effects on lung structure of newborn mice after 14 days of exposure to hyperoxia, followed by abrupt or gradual weaning to room air (RA). Lung sections are from neonatal mouse pups, at 28 days of age, raised in RA or exposed to 85% O_2 for P0 to P14 days and weaned gradually (10% reduction per day of F_{IO_2} over 6 days) or weaned abruptly from hyperoxia versus RA for a further 14 days. **A:** RA control at 28 days of age shows formation of many small alveoli. **B** and **C:** Lungs of 28-day-old mice exposed to 85% O_2 from P0 through P14 show retarded alveolar formation with larger lung saccules. **B:** Lungs of 28-day-old mice exposed to 85% O_2 from P0 through P14, then weaned to RA abruptly. **C:** Lungs of 28-day-old mice exposed to 85% O_2 from P0 through P14, then weaned gradually to RA, exhibit visibly better alveolarization than mice weaned abruptly, shown in **B**. H&E stain was used. Original magnification, ×64.

were lower in animals exposed to hyperoxia, then weaned to RA, than in RA control animals, even after 14 days of recovery. The deficits in alveolar numbers were more pronounced in animals weaned abruptly than in mice weaned gradually (Figure 2A). Alveolar cross-sectional areas were larger in hyperoxia-exposed animals than normoxic controls and lungs of mice weaned abruptly than in mice weaned gradually (Figure 2B). Per HPF, total alveolar areas of lungs of mice weaned abruptly were less than were areas in lungs of their corresponding controls, but the data did not indicate differences between weaning groups (Figure 2C). Average alveolar perimeters were larger in lungs of mice exposed to hyperoxia than in mice raised entirely in normoxia, and hyperoxia-exposed mice weaned abruptly showed larger average alveolar perimeters than did mice weaned gradually (Figure 3A). Per HPF, total perimeters, the sums of all individual alveolar perimeters, were smaller in hyperoxia-exposed mice than in normoxic animals. Total alveolar perimeters of abruptly weaned mice were lower than gradually weaned animals (Figure 3B). Perimeter/area ratios, reflecting total surface areas for gas exchange per unit volumes of lungs, were much lower in lungs of mice exposed to hyperoxia than in normoxic animals. Mice weaned abruptly showed lower ratios than animals weaned gradually (Figure 3C). Mice exposed to hyperoxia during P0 to P14 and weaned abruptly to RA exhibited greater static lung compliance on P43 than RA control animals (Figure 4).

No differences between the two normoxia control groups are indicated for any of the structural parameters presented in Figures 2 and 3. Although the pups in these groups were maintained consistently in RA, the dams nursing and caring for the pups were exposed differently. Possible differential effects, such as alterations in amount or composition of milk produced or extended hyperoxia in the rotating dams of the

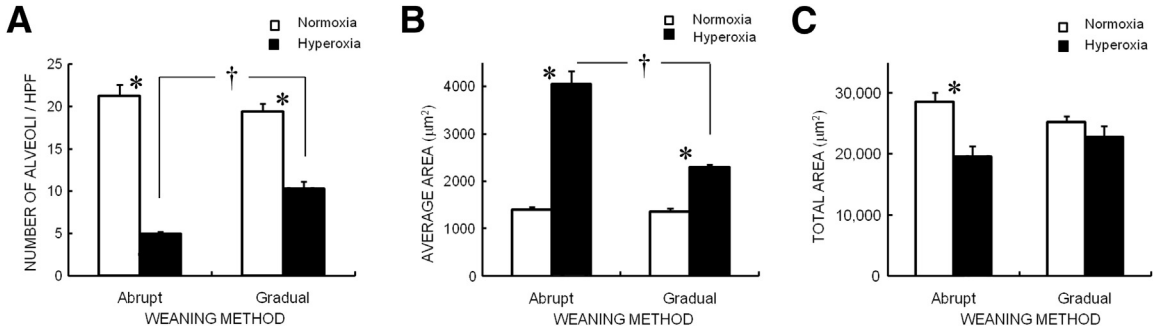

Figure 2 Lung morphometric assessments reflecting defects in alveolar development at P28 after exposure of newborn mice to 85% O_2 for 14 days and weaning to room air (RA). After 14 days of 85% O_2, weaning from hyperoxia was conducted, as indicated, abruptly or gradually (75% × 1 day, 65% × 1 day, 55% × 1 day, 45% × 1 day, 35% × 1 day, and 21% × 9 days, respectively). At P28, all measured morphological variables were different between hyperoxia-exposed and RA animals. Hyperoxic exposure resulted in fewer (**A**) and larger (**B**) alveoli than exposure to RA. **C:** Total alveolar areas were lower in hyperoxia-exposed animals at P28 than RA animals. Animals weaned abruptly from hyperoxia had fewer alveoli (**A**) and more alveolar areas (**B**) than animals weaned gradually. $^*P < 0.05$ for hyperoxia exposure compared with RA exposure; $^\dagger P < 0.05$ for abrupt weaning to RA compared with gradual weaning to RA after 14 days of exposure to hyperoxia.

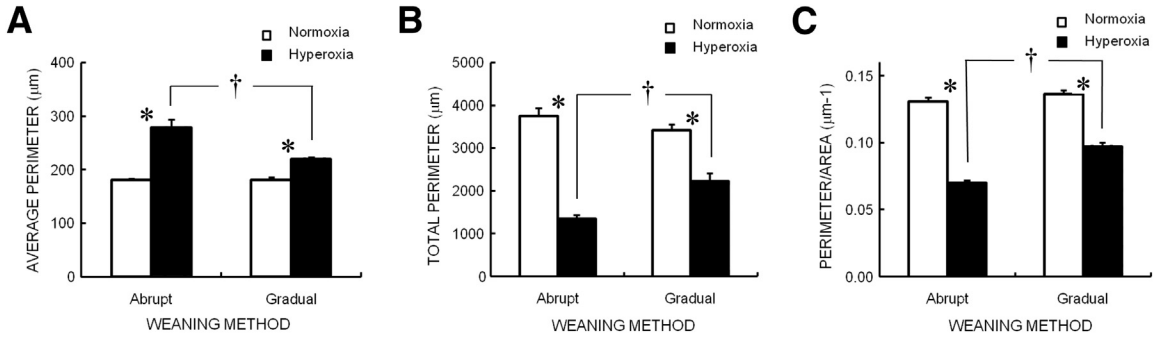

Figure 3 Lung morphometric assessments reflecting surface area for air exchange at P28 after exposure of newborn mice to 85% O_2 for 14 days and weaning to room air (RA). After 14 days of exposure to 85% O_2, mice were weaned to RA, as indicated, abruptly or gradually (75% × 1 day, 65% × 1 day, 55% × 1 day, 45% × 1 day, 35% × 1 day, and 21% × 9 days, respectively). At P28, all measured morphological variables were different between hyperoxia-exposed and RA animals. Hyperoxia exposure resulted in larger alveolar perimeters (**A**) and lower perimeter/area ratios (**C**) than in mice maintained in RA. **B:** Total perimeters were lower in hyperoxia-exposed animals at P28 than in RA animals, in conjunction with the fewer alveoli per field. Animals weaned abruptly from hyperoxia had higher alveolar perimeters and lower perimeter/area ratios than animals weaned gradually. Low perimeter/area ratios reflect lower lung volume for air exchange in hyperoxic animals than in animals exposed to RA and mitigation of the effect through gradual weaning. $^*P < 0.05$ for hyperoxia exposure compared with RA exposure; $^\dagger P < 0.05$ for abrupt weaning to RA compared with gradual weaning to RA after 14 days of exposure to hyperoxia.

gradual weaning groups, are not suggested by the data. The effects of exposures of the neonatal animals to hyperoxia and weaning mode are larger and clearly distinguishable.

Discussion

Infants that develop BPD have long-term functional and structural defects. Furthermore, the incidence of BPD in premature infants has not changed, despite changes in neonatal care that limit exposures to supplemental oxygen and invasive mechanical ventilation. Models of arrested alveolarization have been described, exposure of newborn mice to hyperoxia has demonstrated arrested alveolarization, and some have even found persistence in the effects after exposure to hyperoxia. The present study showing persistence in the initial hyperoxia-exposed arrested lung development confirms the findings reported recently. The finding that gradual weaning from hyperoxia partially mitigated the effects of hyperoxia on arrested lung development is important, and studies of the mechanisms responsible for the differences between the two modes of weaning are needed.

In human BPD, the prevailing information in the field of persistent functional and structural defects suggests that the defects observed in the initial hospital course persist into childhood.[17] Interestingly, persistent effects have not been reported from animal models of alveolar loss, but most of the experimental models of loss and regeneration have been conducted with dietary restriction and refeeding of adult animals,[18,19] which may not reflect the course in humans affected during embryological development. Restoration of alveoli lost in response to caloric restriction of adult animals is facile. In these two studies,[18,19] the authors observed that adult animals subjected to caloric restriction would undergo extensive loss of alveoli, resulting in lungs similar in appearance to the lungs we observe arising from exposure of newborn mice to sublethal hyperoxia. Furthermore, refeeding the animals gave essentially complete recovery from the disrupted alveolarization of the lungs. However, alveoli not formed during normal lung development, as in the present studies, appear to present a different problem.[20] Lung alveolarization in rhesus monkeys continues throughout linear growth, into young adulthood.[21] The potential significance to human health of disrupted lung alveolarization during the neonatal period, infancy, and perhaps childhood is underlined by the age-dependent decline in lung structure and function that could become clinically relevant at earlier ages in individuals who were compromised during development.[22] Exposure to hyperoxia during key developmental

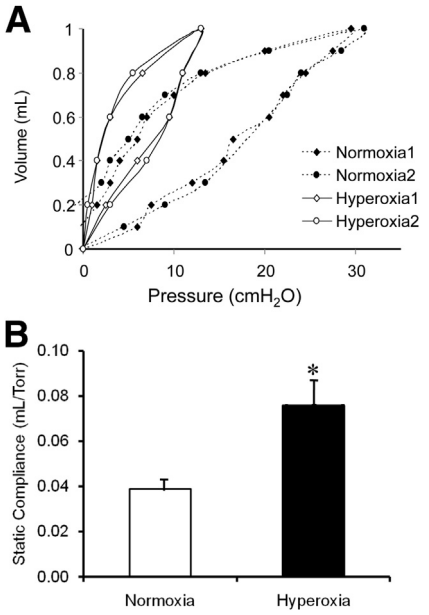

Figure 4 Lung compliance at day 43 (typical examples given in **A** and **B**) after exposure for 14 days to either RA or 85% oxygen, followed by a recovery period in RA. **B:** Values are means ± SEM ($n = 3$ animals per group). $^*P = 0.039$ when compared with controls by unpaired Student's *t*-test.

periods resulted in pulmonary vascular disease, increased lung compliance, and shortened life spans in aging mice.[23] Similar temporal profiles in humans would extend the period of critical susceptibility to life-span, limiting effects on lung development through phases in which many individuals are exposed to tobacco smoke, environmental or primary, and other environmental hazards and put infants born prematurely at increased risk for pulmonary and cardiac disease in their later stages of life.

Few systematic studies of infants' lung structures or alveolarization in infants recovering from BPD have been reported. One small autopsy study in patients recovered from BPD and dying as a result of circumstances unrelated to respiratory failure[17] and another small study assessing chest computed tomographic scans in young adults recovering from BPD[24] demonstrated persistent morphometric changes and computed tomographic scan changes, respectively, supporting the relevance of the persistent defects in experimental models as mimicking this important human problem.

The fact that gradual weaning from hyperoxia resulted in attenuation of the persistent effects of hyperoxia on lung structure is encouraging, because the gradual weaning protocol more closely mimics typical supportive treatment of patients. The longer period of exposure to hyperoxia needed for the gradual weaning protocol did not result in greater extents of altered lung development, as was the concern. Rodent models of BPD studied might, therefore, overestimate changes in lung development and function in preterm infants, because abrupt weaning has been the experimental design applied in most studies reported thus far.

Exposures of newborn animals to hyperoxia have been reported to decrease[12,25–29] or increase[30,31] lung compliance. Although the experimental designs in these reports encompass other differences, the distinguishing feature in the studies appears to be that lung compliance measurements are lower than in controls at the end of exposure, whereas with sufficient time of recovery in RA, lung compliances are greater in animals exposed to hyperoxia than in control animals. Dauger et al[30] studied lung compliance after 9.5 months in RA, Yee et al[23,31] allowed 8 weeks, and, in some animals, 67 weeks was allowed. Yee et al[23,31] found alveolar simplification, a loss of type II epithelial cells, an increase in type I cells, and increased elastin at 8 weeks of age. In an apparent contradiction to this working hypothesis, Auten et al[27] observed lower compliance in lungs of animals exposed to hyperoxia, then weaned to RA for 6 days. In addition to the shorter time allowed for recovery in RA, the studies by Auten et al[27] used 95% O_2 for P1 to P8, which is likely to have induced acute lung damage and necrosis, as further indicated by the extensive inflammatory responses they observed. The increased compliance found in the present report, in conjunction with the persistent defects in alveolarization, could be explained by the lack of tethering effects because of fewer alveoli. In a large animal model of mechanical ventilation and hyperoxia, Pierce et al[32] noted quantitative and qualitative differences in elastin expression; if similar changes were observed in the present model, altered elastin expression could explain, at least in part, the findings observed herein.

The findings of altered morphological characteristics and increased static compliance could be interpreted such that the increased compliances in hyperoxia-exposed animals led to higher lung volumes on fixation and resultant artifactual increases in alveolar sizes. In fact, in systematic lung volume studies, Knudsen et al[33] showed that measurements of airspace size were affected by lung volumes such that the mean chord length, a measure of airspace size, was proportional to lung inflation in mice that had emphysematous lungs. Furthermore, the results from Figure 4 indicate that lung volumes at fixation pressure may be higher in hyperoxia-exposed animals than in animals exposed to RA, so that the differences in morphometry could be related to these volumes and not persistent defects in alveolarization, or a combination of those two possibilities. In the absence of postfixation lung volume measurements, we cannot exclude the possibility that inflation artifact contributes to the difference between groups. However, it does not negate the importance of the findings that gradual weaning appeared to have better long-term studies than in the animals weaned abruptly.

The apparent retardation of lung alveolarization observed in the present studies was not accompanied by potentially confounding effects of animal death, inhibition of somatic growth, lung volutrauma from mechanical ventilation, or substantial lung necrosis or inflammatory responses. The results of the studies with this animal model, therefore, suggest that the effects observed are likely to involve alterations in mechanisms that regulate lung development, such as oxidant shifts in steady-state conditions that mediate signal transduction mechanisms, rather than secondary consequences of suboptimal tissue repair. However, most tissues under almost all conditions probably will exhibit some levels of processes that reasonably can be termed inflammation, and determinations of the levels of effects that are associated with incremental differences in low levels of inflammation will be difficult and unlikely to be sufficiently informative to merit investigation.

de Visser et al[34] found that the lungs of neonatal rat pups exposed to 100% oxygen for 9 days, followed by 42 days of recovery in RA, did not exhibit improvements in alveolarization or medial wall thickness. Interestingly, in mice exposed to 100% oxygen between P1 and P4, the alveolar simplification seen at 8 weeks was no longer evident at 67 weeks of age, although the mice exhibited greater lung compliances than control mice not exposed to hyperoxia.[31] If RA constitutes an oxidant stress for prematurely born infants,[11] avoiding deficits in lung development may require adequate oxygenation of micropreemies with subambient oxygen ($F_{IO_2} < 0.21$) or mitigation of the effects on the critical signal transduction mechanisms of the oxidant stresses produced by RA. If the metabolic demands for O_2 of premature infants are greater than can be supported by subambient oxygen, as may well be the case, modulation of

oxidant determinants of signal transduction mechanisms that regulate pulmonary development will require investigation and more detailed understanding than is available.

References

1. Northway WH Jr, Rosan RC, Porter DY: Pulmonary disease following respirator therapy of hyaline-membrane disease: bronchopulmonary dysplasia. N Engl J Med 1967, 276:357–368
2. Carlton DP, Albertine KH, Cho SC, Lont M, Bland RD: Role of neutrophils in lung vascular injury and edema after premature birth in lambs. J Appl Physiol 1997, 83:1307–1317
3. Jobe AH, Bancalari E: Bronchopulmonary dysplasia. Am J Respir Crit Care Med 2001, 163:1723–1729
4. Ehrenkranz RA, Walsh MC, Vohr BR, Jobe AH, Wright LL, Fanaroff AA, Wrage LA, Poole K: Validation of the National Institutes of Health consensus definition of bronchopulmonary dysplasia. Pediatrics 2005, 116:1353–1360
5. Park MS, Rieger-Fackeldey E, Schanbacher BL, Cook AC, Bauer JA, Rogers LK, Hansen TN, Welty SE, Smith CV: Altered expressions of fibroblast growth factor receptors and alveolarization in neonatal mice exposed to 85% oxygen. Pediatr Res 2007, 62:652–657
6. Rogers LK, Tipple TE, Nelin LD, Welty SE: Differential responses in the lungs of newborn mouse pups exposed to 85% or >95% oxygen. Pediatr Res 2009, 65:33–38
7. Warburton D, Schwarz M, Tefft D, Flores-Delgado G, Anderson KD, Cardoso WV: The molecular basis of lung morphogenesis. Mech Dev 2000, 92:55–81
8. Zhu L, Li H, Tang J, Zhu J, Zhang Y: Hyperoxia arrests lung development through suppression of histone deacetylases in neonatal rats. Pediatr Pulmonol 2012, 47:264–274
9. Hilgendorff A, Parai K, Ertsey R, Jain N, Navarro EF, Peterson JL, Tamosiuniene R, Nicolls MR, Starcher BC, Rabinovitch M, Bland RD: Inhibiting lung elastase activity enables lung growth in mechanically ventilated newborn mice. Am J Respir Crit Care Med 2011, 184:537–546
10. Londhe VA, Sundar IK, Lopez B, Maisonet TM, Yu Y, Aghai ZH, Rahman I: Hyperoxia impairs alveolar formation and induces senescence through decreased histone deacetylase activity and up-regulation of p21 in neonatal mouse lung. Pediatr Res 2011, 69:371–377
11. Smith CV, Hansen TN, Martin NE, McMicken HW, Elliott SJ: Oxidant stress responses in premature infants during exposure to hyperoxia. Pediatr Res 1993, 34:360–365
12. Coalson JJ, Winter VT, Siler-Khodr T, Yoder BA: Neonatal chronic lung disease in extremely immature baboons. Am J Respir Crit Care Med 1999, 160:1333–1346
13. Maniscalco WM, Watkins RH, Pryhuber GS, Bhatt A, Shea C, Huyck H: Angiogenic factors and alveolar vasculature: development and alterations by injury in very premature baboons. Am J Physiol Lung Cell Mol Physiol 2002, 282:L811–L823
14. Subramaniam M, Bausch C, Twomey A, Andrefffa S, Yoder BA, Chang LY, Crapo JD, Pierce RA, Cuttitta F, Sunday ME: Bombesin-like peptides modulate alveolarization and angiogenesis in bronchopulmonary dysplasia. Am J Respir Crit Care Med 2007, 176:902–912
15. Warner BB, Stuart LA, Papes RA, Wispe JR: Functional and pathological effects of prolonged hyperoxia in neonatal mice. Am J Physiol 1998, 275:L110–L117
16. Velten M, Britt RD Jr, Heyob KM, Welty SE, Eiberger B, Tipple TE, Rogers LK: Prenatal inflammation exacerbates hyperoxia-induced functional and structural changes in adult mice. Am J Physiol Regul Integr Comp Physiol 2012, 303:R279–R290
17. Husain AN, Siddiqui NH, Stocker JT: Pathology of arrested acinar development in postsurfactant bronchopulmonary dysplasia. Hum Pathol 1998, 29:710–717
18. Massaro D, Alexander E, Reiland K, Hoffman EP, Massaro GD, Clerch LB: Rapid onset of gene expression in lung, supportive of formation of alveolar septa, induced by refeeding mice after calorie restriction. Am J Physiol Lung Cell Mol Physiol 2007, 292:L1313–L1326
19. Massaro D, Massaro GD, Baras A, Hoffman EP, Clerch LB: Calorie-related rapid onset of alveolar loss, regeneration, and changes in mouse lung gene expression. Am J Physiol Lung Cell Mol Physiol 2004, 286: L896–L906
20. Massaro D, Massaro GD: Critical period for alveologenesis and early determinants of adult pulmonary disease. Am J Physiol Lung Cell Mol Physiol 2004, 287:L715–L717
21. Hyde DM, Blozis SA, Avdalovic MV, Putney LF, Dettorre R, Quesenberry NJ, Singh P, Tyler NK: Alveoli increase in number but not size from birth to adulthood in rhesus monkeys. Am J Physiol Lung Cell Mol Physiol 2007, 293:L570–L579
22. Massaro D, Massaro GD: Developmental alveologenesis: longer, differential regulation and perhaps more danger. Am J Physiol Lung Cell Mol Physiol 2007, 293:L568–L569
23. Yee M, Vitiello PF, Roper JM, Staversky RJ, Wright TW, McGrath-Morrow SA, Maniscalco WM, Finkelstein JN, O'Reilly MA: Type II epithelial cells are critical target for hyperoxia-mediated impairment of postnatal lung development. Am J Physiol Lung Cell Mol Physiol 2006, 291:L1101–L1111
24. Wong PM, Lees AN, Louw J, Lee FY, French N, Gain K, Murray CP, Wilson A, Chambers DC: Emphysema in young adult survivors of moderate-to-severe bronchopulmonary dysplasia. Eur Respir J 2008, 32:321–328
25. Auten RL, Mason SN, Whorton MH, Lampe WR, Foster WM, Goldberg RN, Li B, Stamler JS, Auten KM: Inhaled ethyl nitrite prevents hyperoxia-impaired postnatal alveolar development in newborn rats. Am J Respir Crit Care Med 2007, 176:291–299
26. Yi M, Jankov RP, Belcastro R, Humes D, Copland I, Shek S, Sweezey NB, Post M, Albertine KH, Auten RL, Tanswell AK: Opposing effects of 60% oxygen and neutrophil influx on alveologenesis in the neonatal rat. Am J Respir Crit Care Med 2004, 170:1188–1196
27. Auten RL Jr, Mason SN, Tanaka DT, Welty-Wolf K, Whorton MH: Anti-neutrophil chemokine preserves alveolar development in hyperoxia-exposed newborn rats. Am J Physiol Lung Cell Mol Physiol 2001, 281:L336–L344
28. Chetty A, Cao GJ, Severgnini M, Simon A, Warburton R, Nielsen HC: Role of matrix metalloprotease-9 (MMP- 9) in hyperoxic injury in developing lung. Am J Physiol Lung Cell Mol Physiol 2008, 295: L584–L592
29. Alejandre-Alcazar MA, Kwapiszewska G, Reiss I, Amarie OV, Marsh LM, Sevilla-Perez J, Wygrecka M, Eul B, Kobrich S, Hesse M, Schermuly RT, Seeger W, Eickelberg O, Morty RE: Hyperoxia modulates TGF-beta/BMP signaling in a mouse model of bronchopulmonary dysplasia. Am J Physiol Lung Cell Mol Physiol 2007, 292: L537–L549
30. Dauger S, Ferkdadji L, Saumon G, Vardon G, Peuchmaur M, Gaultier C, Gallego J: Neonatal exposure to 65% oxygen durably impairs lung architecture and breathing pattern in adult mice. Chest 2003, 123:530–538
31. Yee M, Chess PR, McGrath-Morrow SA, Wang Z, Gelein R, Zhou R, Dean DA, Notter RH, O'Reilly MA: Neonatal oxygen adversely affects lung function in adult mice without altering surfactant composition or activity. Am J Physiol Lung Cell Mol Physiol 2009, 297: L641–L649
32. Pierce RA, Albertine KH, Starcher BC, Bohnsack JF, Carlton DP, Bland RD: Chronic lung injury in preterm lambs: disordered pulmonary elastin deposition. Am J Physiol 1997, 272:L452–L460
33. Knudsen L, Weibel ER, Gundersen HJ, Weinstein FV, Ochs M: Assessment of air space size characteristics by intercept (chord) measurement: an accurate and efficient stereological approach. J Appl Physiol 2010, 108:412–421
34. de Visser YP, Walther FJ, Laghmani el H, Steendijk P, Middeldorp M, van der Laarse A, Wagenaar GT: Phosphdiesterase-4-inhibition attenuates persistent heart and lung injury by neonatal hyperoxia in rats. Am J Physiol Lung Cell Mol Physiol 2012, 302:L56–L67