

# Bronchopulmonary Dysplasia in a Rat Model Induced by Intra-amniotic Inflammation and Postnatal Hyperoxia: Morphometric Aspects

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**ABSTRACT:** Antenatal inflammation is a known risk factor of bronchopulmonary dysplasia. The authors hypothesized that lipopolysaccharide (LPS) administration amplifies hyperoxia-induced lung injury in neonatal rats. LPS (0.5 or 1.0  $\mu\text{g}$ ) or normal saline was injected into the amniotic sacs of pregnant rats at 20 d gestation (term 22.5 d). After birth, rats were exposed to 85% oxygen or room air for 1 or 2 wk. Morphometric analysis of lungs was performed on 14 d. One week of hyperoxia without LPS administration resulted in modest lung injury. LPS at 0.5  $\mu\text{g}$  alone did not alter lung morphology, but amplified the effect of 1 wk of hyperoxia resulting in marked inhibition of alveolarization (airspace were enlarged and alveolar surface areas further reduced). LPS at 1.0  $\mu\text{g}$  independently induced modest lung injury and also amplified the effect of 1 wk of hyperoxia. However, this sensitizing effect of LPS was not observed in rats subjected to 2 wks of hyperoxia, which in itself caused extensive lung injury (possibly masking the effect of LPS). The authors concluded that intra-amniotic LPS sensitizes neonatal rat lungs, and thus, amplifies the hyperoxia-induced inhibition of alveolarization. (*Pediatr Res* 65: 323–327, 2009)

The pathophysiology of bronchopulmonary dysplasia (BPD) has evolved from its classical form following the introduction of antenatal glucocorticoid, surfactant replacement therapy, and gentler ventilatory support (1). However, BPD remains a major chronic pulmonary complication in small preterm infants. In previous studies, antenatal inflammation has been shown to increase the risk of BPD (2–5), although ironically antenatal inflammation may improve pulmonary function during the immediate neonatal period (6). Furthermore, in infants who subsequently develop BPD, we have reported increased levels of inflammatory biochemical factors in serum and tracheal aspirate at birth, thus implicating inflammation in the development of BPD (7,8). In addition, prolonged hyperoxic exposure has been found to lead to BPD in clinical and animal studies, but much remains to be determined concerning the role of antenatal inflammation in the development of BPD. Recently, Van Marter *et al.* (4) reported that mechanically ventilated preterm infants with chorioam-

nionitis or postnatally acquired sepsis showed a several fold increase in the prevalence of BPD compared with mechanically ventilated preterm infants without these complications. Ikegami and Jobe (9) suggested that endotoxin had a sensitizing effect after observing that fetal sheep exposed to intra-amniotic endotoxin showed further increased proinflammatory responses to mechanical ventilation. Jobe (10) subsequently suggested antenatal inflammation has a sensitizing effect and that it increases the incidence and severity of BPD when neonates are exposed to oxygen and mechanical ventilation. A number of animal models of BPD have been devised (9,11,12), but to date this concept that antenatal inflammation increases susceptibility to lung injury by hyperoxia has not been demonstrated in any of these models.

Given the above background, we hypothesized that intra-amniotic lipopolysaccharide (LPS) would amplify hyperoxia-induced lung injury in neonatal rats, which is known to markedly inhibit alveolarization, a histological hallmark of human BPD. The objectives of this study were to establish a rat model of BPD induced by intra-amniotic LPS and postnatal hyperoxia exposure, and to examine the sensitizing role of intra-amniotic LPS in the pathogenesis of BPD.

## MATERIALS AND METHODS

**Experimental animals.** The animal experiment was performed at the Clinical Research Institute in Seoul National University Bundang Hospital, Korea, and the experimental protocol was approved by its Animal Care and Use Committee. The experimental animals were rat pups, and these were divided into eight groups according to whether LPS had been administered antenatally and whether animals were exposed to room air or hyperoxia for 1 or 2 wks, as shown in Fig. 1 and below.

Control group: no LPS administration and 2 wks in room air ( $n = 23$ ),  
L(0.5) group: 0.5  $\mu\text{g}$  LPS and 2 wks in room air ( $n = 20$ ),  
L(1.0) group: 1.0  $\mu\text{g}$  LPS and 2 wks in room air ( $n = 29$ ),  
O<sub>2</sub>(1) group: no LPS and 1-wk exposure to 85% oxygen ( $n = 20$ ),  
O<sub>2</sub>(2) group: no LPS and 2-wk exposure to 85% oxygen ( $n = 13$ ),  
L(0.5) + O<sub>2</sub>(1) group: 0.5  $\mu\text{g}$  LPS and 1-wk exposure to 85% oxygen ( $n = 11$ ),  
L(1.0) + O<sub>2</sub>(1) group: 1.0  $\mu\text{g}$  LPS and 1-wk exposure to 85% oxygen ( $n = 23$ ),  
and  
L(0.5) + O<sub>2</sub>(2) group: 0.5  $\mu\text{g}$  LPS and 2-wk exposure to 85% oxygen ( $n = 17$ ).

Timed-pregnancy Sprague-Dawley rats (term, 22.5 d) weighing 300–356 g were used throughout. Rats were housed in individual cages with free access

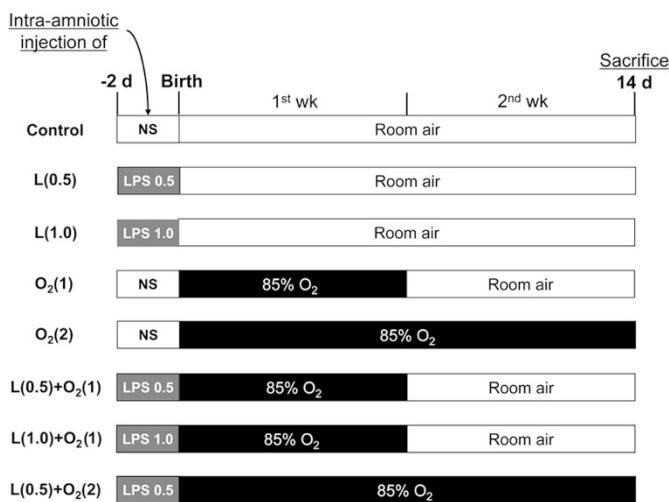
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**Abbreviations:** BPD, bronchopulmonary dysplasia; **Lm**, mean cord length; **LPS**, lipopolysaccharide; **S<sub>A</sub>**, alveolar surface area; **W<sub>T</sub>**, alveolar wall thickness



**Figure 1.** Schematic outline of the experimental protocol. NS: normal saline; L(0.5), L(1.0): LPS administered at 0.5 or 1.0  $\mu\text{g}$  at gestation day 20, respectively;  $\text{O}_2(1)$ ,  $\text{O}_2(2)$ : exposure to 85% oxygen for 1 or 2 wk, respectively.

to food and water. Lighting was provided from 6 am to 6 pm. One to three pregnant rats were used per study group. Litter sizes ranged from 8 to 13, including stillborns. The male to female ratio in the study groups ranged from 0.8 to 1.2 and this was not different between groups.

**Intra-amniotic LPS administration.** On gestation day 20, rats were anesthetized by isoflurane inhalation. Following a midline abdominal section, 0.5 or 1.0  $\mu\text{g}$  LPS (*Escherichia coli* 0111:B4, Chemicon International, Tamecula, CA) solubilized in 0.05 mL of normal saline was injected into the amniotic sacs of animals in the LPS groups [L(0.5), L(1.0), L(0.5) +  $\text{O}_2(1)$ , L(1.0) +  $\text{O}_2(1)$ , and L(0.5) +  $\text{O}_2(2)$  groups]. The same volume of normal saline without LPS was injected into the amniotic sacs of animals in the nonLPS groups [control,  $\text{O}_2(1)$ , and  $\text{O}_2(2)$  groups]. Pups were delivered spontaneously 2–2.5 d after these injections. Ten to 12 h after birth, pups were weighed and given to foster rats (5–8 pups/foster rat).

**Exposure to hyperoxia.** Rat pups in the hyperoxia groups were kept with a foster rat in cage within 40 L Plexiglas hyperoxic chambers containing 85% oxygen for one [ $\text{O}_2(1)$ , L(0.5) +  $\text{O}_2(1)$ , and L(1.0) +  $\text{O}_2(1)$  groups] or 2 wks [ $\text{O}_2(2)$  and L(0.5) +  $\text{O}_2(2)$  groups]. Rat pups in the first week hyperoxia groups were kept in room air for the second week, and room air groups [control, L(0.5), and L(1.0) groups] were kept in room air for the entire 2-wk period. Foster rats in hyperoxic chamber were rotated daily to avoid oxygen toxicity; oxygen concentrations in the chamber were monitored daily using an oxygen sensor (Extech 407510, Extech Instruments Corp., Waltham, MA). Body weights were measured on days 1, 7, and 14 after birth. Breeding conditions, which can affect nursing and feeding, were similar for the eight study groups. Foster rats only reared pups allocated to a single group.

**Tissue preparation.** On 14th day of life, all surviving rats were anesthetized i.p. with ketamine (50 mg/kg, Yuhan Corp., Seoul) and xylazine (50 mg/kg, Bayer AG, Leverkusen, Germany). Lungs were exposed by thoracotomy, and after exsanguination by transecting aorta and inferior vena cava, right ventricle was punctured and lungs were perfused with 3 mL of PBS at 25  $\text{cmH}_2\text{O}$ . After removing lungs, tracheas were cannulated and buffered formaldehyde (4% paraformaldehyde solubilized in PBS, pH 7.4) was instilled at 25  $\text{cmH}_2\text{O}$  for 5 min. They were then closed with a suture and lungs were fixed in buffered formaldehyde for 24 h at 4°C. Paraffin sections (4  $\mu\text{m}$ ) cut from left upper lobes were mounted onto Super Frost Plus slides (VWR Scientific, West Chester, PA), and slides were then deparaffinized and stained with hematoxylin and eosin (H&E).

**Lung morphometry.** Six random nonoverlapping fields per pup in two distal lung sections were used for the morphometric examinations. Sections were photographed using a digital camera (Axioskop MRc5, Carl Zeiss, Oberkochen, Germany) attached to an Axioskop 40 microscope (Carl Zeiss, Oberkochen, Germany) at  $\times 200$  and saved as JPEG files. Photographs were analyzed using the morphometric methods designed by Weibel (13). All measurements were made by a single observer unaware of group identities. Tissue volume density ( $\text{VD}_T$ ) was determined using a  $10 \times 10$  grid (grid element side length  $\sim 29 \mu\text{m}$ ). Mean cord length ( $L_m$ ) is an estimate of the distance from one airspace wall to another airspace wall and was determined by counting intersections of airspace walls including alveoli, alveolar sacs, and alveolar ducts with an array of 84 lines, each  $\sim 24 \mu\text{m}$  long. Alveolar

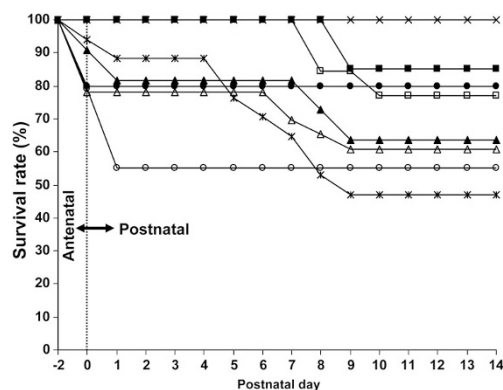
surface area ( $S_A$ ) was calculated using  $S_A = 4 \times \text{VD}_T \times \text{lung volume}/L_m$ . Lung volume was determined by measuring the displacement of water by the lungs after fixation. No correction was made for perfusion or tissue shrinkage. Alveolar wall thickness ( $W_T$ ) was calculated using  $W_T = \text{VD}_T \times L_m$  (14).

**Statistical analysis.** Group live birth rates and postnatal and overall (perinatal) survival rates were compared by Kaplan-Meier survival analysis. Postnatal changes in body weights, mean cord lengths, alveolar surface areas, and alveolar wall thicknesses were expressed as means  $\pm$  SD and compared by one-way ANOVA. The Least Significant Difference test was used for *post hoc* analysis;  $p$  values of  $< 0.05$  were considered to be statistically significant.

## RESULTS

### Survival rates and postnatal changes in body weights.

Group survival patterns varied (Fig. 2). No fetal death occurred in the untreated (LPS or hyperoxia) control group and all rat pups survived to the end of experiment. Animals treated with LPS but not exposed to hyperoxia showed high fetal and day 1 death rates, particularly those treated with 1.0  $\mu\text{g}$  LPS, but thereafter survived without further loss. However, no fetal death occurred among animals not treated with LPS, but deaths occurred after 7 d of hyperoxia. Animals in the LPS and hyperoxia treated groups had both fetal deaths similar to LPS only treated animals and postnatal deaths similar to hyperoxia alone treated animals. Overall survival rate in the L(0.5) +  $\text{O}_2(1)$  group was not significantly different from that of the  $\text{O}_2(1)$  group (63.6% versus 85.0%,  $p = 0.115$ ), but the overall survival rate of the L(1.0) +  $\text{O}_2(1)$  group was significantly lower than that of the  $\text{O}_2(1)$  group (60.9% versus 85.0%,  $p = 0.037$ ). The L(0.5) +  $\text{O}_2(2)$  group had the lowest overall survival rate and this was significantly lower than that of the  $\text{O}_2(2)$  group (47.1% versus 76.9%,  $p = 0.046$ ). The postnatal changes in group body weights of surviving rats are



**Figure 2.** Survival rates in the experimental groups. The combined live birth rates in the LPS-treated groups [L(0.5), L(1.0), L(0.5) +  $\text{O}_2(1)$ , L(1.0) +  $\text{O}_2(1)$ , and L(0.5) +  $\text{O}_2(2)$  groups] were significantly lower than in the combined LPS-untreated groups [control,  $\text{O}_2(1)$ , and  $\text{O}_2(2)$  groups] (83.0% versus 100.0%,  $p = 0.001$ ). The combined postnatal survival rates in the hyperoxia-exposed groups [ $\text{O}_2(1)$ ,  $\text{O}_2(2)$ , L(0.5) +  $\text{O}_2(1)$ , L(1.0) +  $\text{O}_2(1)$ , and L(0.5) +  $\text{O}_2(2)$  groups] were significantly lower than that of the combined hyperoxia-unexposed groups [control, L(0.5), and L(1.0) groups] (72.7% versus 88.7%,  $p = 0.032$ ). Overall survival rate in the L(0.5) +  $\text{O}_2(1)$  group was not significantly different from that of the  $\text{O}_2(1)$  group (63.6% versus 85.0%,  $p = 0.115$ ). However, overall survival rate in the L(1.0) +  $\text{O}_2(1)$  group was significantly lower than that of the  $\text{O}_2(1)$  group (60.9% versus 85.0%,  $p = 0.037$ ). Overall survival rate was poorest in the L(0.5) +  $\text{O}_2(2)$  group and this was significantly lower than in the  $\text{O}_2(2)$  group (47.1% versus 76.9%,  $p = 0.046$ ). Control [ $\times$ ]; L(0.5) [ $\bullet$ ]; L(1.0) [ $\circ$ ];  $\text{O}_2(1)$  [ $\blacksquare$ ];  $\text{O}_2(2)$  [ $\square$ ]; L(0.5) +  $\text{O}_2(1)$  [ $\blacktriangle$ ]; L(1.0) +  $\text{O}_2(1)$  [ $\triangle$ ]; L(0.5) +  $\text{O}_2(2)$  [ $\ast$ ]. See "Materials and Methods" for full group definitions.

presented in Fig. 3. No significant differences were found between the eight groups on day 1. The body weights at day 7 of the O<sub>2</sub>(2) and the L(1.0) + O<sub>2</sub>(1) groups were significantly lower than those of control group ( $p < 0.05$ ). The L(0.5) + O<sub>2</sub>(1) and L(1.0) + O<sub>2</sub>(1) groups had significantly lighter day 7 body weights than the O<sub>2</sub>(1) group ( $p < 0.05$ ). However, on day 14, no significant body weight differences were observed between the eight study groups ( $p = 0.059$ ).

### Light microscopy

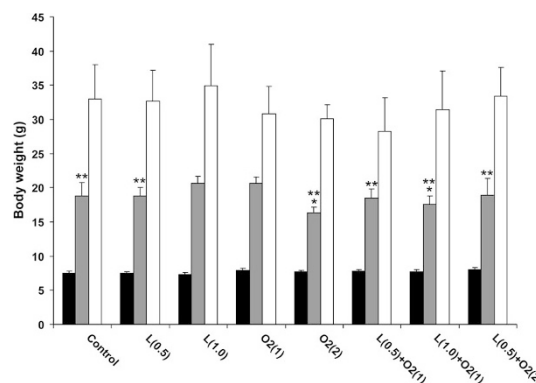
Rats in the control and L(0.5) groups showed normal alveolarization, whereas rats in the L(1.0) and O<sub>2</sub>(1) groups showed modest inhibition of alveolarization. Marked inhibition of alveolarization was observed in the L(0.5) + O<sub>2</sub>(1) and L(1.0) + O<sub>2</sub>(1) groups. Most extensive inhibition was observed in the L(0.5) + O<sub>2</sub>(2) and O<sub>2</sub>(2) groups, and at these levels the inhibition was so severe that the exacerbating effect of LPS could not be detected (Fig. 4).

**The mean cord length ( $L_m$ ).** No significant difference was observed between the  $L_m$  values of the L(0.5) group and untreated control group (Fig. 5A1), but animals in the L(1.0), O<sub>2</sub>(1), and O<sub>2</sub>(2) groups had significantly larger  $L_m$  values than control and L(0.5) groups [L(1.0),  $65.5 \pm 6.0 \mu\text{m}$ ; O<sub>2</sub>(1),  $68.8 \pm 10.4 \mu\text{m}$ ; O<sub>2</sub>(2),  $98.9 \pm 17.4 \mu\text{m}$  versus control,  $58.9 \pm 5.6 \mu\text{m}$ ; L(0.5),  $58.6 \pm 6.1 \mu\text{m}$ ,  $p < 0.05$ ]. Increases in  $L_m$  values in the L(1.0) and O<sub>2</sub>(1) groups were similar. However,  $L_m$  increased more in the O<sub>2</sub>(2) group than in the L(1.0) and O<sub>2</sub>(1) groups (Fig. 5A1).

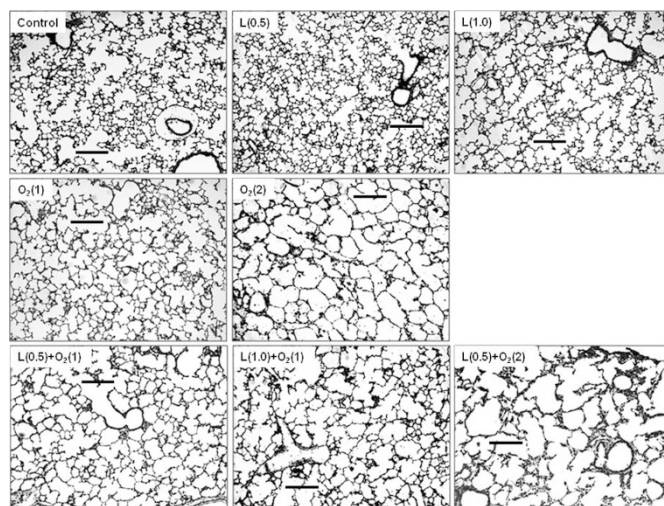
Intra-amniotic LPS appeared to sensitize animals to hyperoxia in terms of average alveolar size. The L(0.5) group showed no increase in  $L_m$ , but the L(0.5) + O<sub>2</sub>(1) group amplified the effect of 1 wk of hyperoxia on  $L_m$  [L(0.5) + O<sub>2</sub>(1),  $83.5 \pm 7.7 \mu\text{m}$  versus O<sub>2</sub>(1),  $68.8 \pm 10.4 \mu\text{m}$ ,  $p < 0.05$ ] (Fig. 5A2). Moreover, this sensitizing effect of LPS was not dose dependent. Greatest  $L_m$  increase was observed in the animals exposed to hyperoxia for 2 wks regardless of LPS treatment [O<sub>2</sub>(2),  $98.9 \pm 17.4 \mu\text{m}$ ; L(0.5) + O<sub>2</sub>(2),  $103.8 \pm 14.6 \mu\text{m}$  versus L(0.5) + O<sub>2</sub>(1),  $83.5 \pm 7.7 \mu\text{m}$ ; L(1.0) + O<sub>2</sub>(1),  $84.2 \pm 13.0 \mu\text{m}$ ,  $p < 0.05$ ] (Fig. 5A2). Prior treatment with LPS resulted in slightly higher  $L_m$  values in animals exposed to hyperoxia for 2 wks, but this difference was not statistically significant.

**The alveolar surface area ( $S_A$ ).** Mean  $S_A$  values were similar for the L(0.5) and control groups, but the L(1.0), O<sub>2</sub>(1), and O<sub>2</sub>(2) groups had lower  $S_A$  values than the control group [L(1.0),  $307.4 \pm 54.8 \mu\text{m}^2/\text{lung}$ ; O<sub>2</sub>(1),  $342.3 \pm 119.6 \mu\text{m}^2/\text{lung}$ ; O<sub>2</sub>(2),  $213.1 \pm 72.6 \mu\text{m}^2/\text{lung}$  versus control,  $403.8 \pm 58.7 \mu\text{m}^2/\text{lung}$ ; L(0.5),  $447.6 \pm 101.2 \mu\text{m}^2/\text{lung}$ ,  $p < 0.05$ ] (Fig. 5B1).  $S_A$  was lowest in the O<sub>2</sub>(2) group, and this was significantly lower than in the L(1.0) and O<sub>2</sub>(1) groups.

The sensitizing effect of LPS was noted on  $S_A$  values (Fig. 5B2). High dose LPS markedly amplified the effect of 1 wk of hyperoxia on  $S_A$  [L(1.0) + O<sub>2</sub>(1),  $242.9 \pm 77.8 \mu\text{m}^2/\text{lung}$  versus O<sub>2</sub>(1),  $342.3 \pm 119.6 \mu\text{m}^2/\text{lung}$ ,  $p < 0.05$ ] (Fig. 5B2). Two weeks of hyperoxia had the most potent effect. In the L(0.5) + O<sub>2</sub>(2) group LPS treatment further reduced  $S_A$ , but this



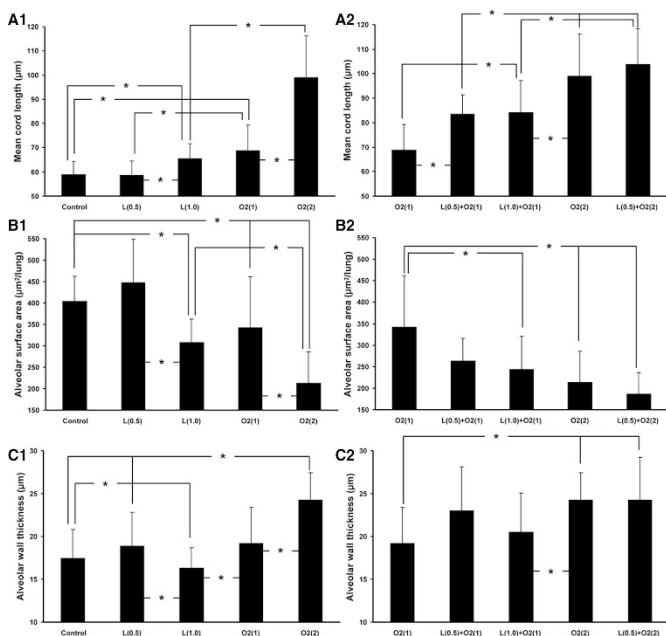
**Figure 3.** Postnatal changes in group body weights. Black, gray, and white bars indicate body weights on days 1, 7, and 14, respectively. Data are based on survival to day 14. No significant differences were found between the eight groups on day 1. On day 7, body weights in the O<sub>2</sub>(2) and L(1.0) + O<sub>2</sub>(1) groups were significantly lower than those of control group ( $p < 0.05$ ). The L(0.5) + O<sub>2</sub>(1) and L(1.0) + O<sub>2</sub>(1) groups had significantly lighter mean body weights on day 7 than the O<sub>2</sub>(1) group ( $p < 0.05$ ). However, no significant difference in body weight was present between individual groups on day 14 ( $p = 0.059$ ). See "Materials and Methods" for full group definitions. \*Significantly lower than control group ( $p < 0.05$ ). \*\*Significantly lower than the O<sub>2</sub>(1) group ( $p < 0.05$ ).



**Figure 4.** Representative photomicrographs of rat lungs on day 14. The control and L(0.5) groups showed normal alveolarization, the L(1.0) and O<sub>2</sub>(1) groups modest alveolarization inhibition, and the L(0.5) + O<sub>2</sub>(1) and L(1.0) + O<sub>2</sub>(1) groups marked inhibition of alveolarization. Alveolarization was most inhibited in the O<sub>2</sub>(2) and L(0.5) + O<sub>2</sub>(2) groups. H&E stained. Magnification  $\times 100$ . Scale bars indicate  $200 \mu\text{m}$ .

reduction was not statistically significant [O<sub>2</sub>(2),  $213.1 \pm 72.6 \mu\text{m}^2/\text{lung}$ ; L(0.5) + O<sub>2</sub>(2),  $186.1 \pm 49.8 \mu\text{m}^2/\text{lung}$ ] (Fig. 5B2).

**Alveolar wall thickness ( $W_T$ ).** LPS ( $0.5 \mu\text{g}$ ) or exposure to hyperoxia of 1 wk did not alter  $W_T$  (Fig. 5C1). However, high dose ( $1.0 \mu\text{g}$ ) LPS reduced  $W_T$ , while exposure to hyperoxia for 2 wks markedly increased  $W_T$  [L(1.0),  $16.3 \pm 2.3 \mu\text{m}$ ; O<sub>2</sub>(2),  $24.3 \pm 3.2 \mu\text{m}$  versus control,  $17.5 \pm 3.3 \mu\text{m}$ ,  $p < 0.05$ ]. When pups were exposed to low or high dose LPS and hyperoxia for 1 wk, mean  $W_T$  values were similar to those of the O<sub>2</sub>(1) group, and no LPS sensitization was observed [L(0.5) + O<sub>2</sub>(1),  $23.0 \pm 5.2 \mu\text{m}$ ; L(1.0) + O<sub>2</sub>(1),  $20.5 \pm 4.6 \mu\text{m}$ ; O<sub>2</sub>(1),  $19.2 \pm 4.2 \mu\text{m}$ ]. However, exposure to LPS and hyperoxia for 2 wks significantly increased  $W_T$ , but no sensi-



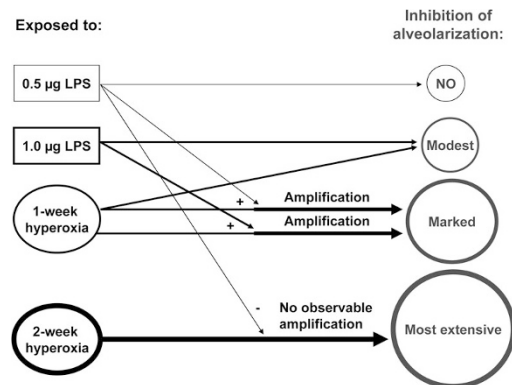
**Figure 5.** Group morphometric data. In panel A1, mean cord length ( $L_m$ ), average alveolar size, was significantly longer in the L(1.0), O<sub>2</sub>(1), and O<sub>2</sub>(2) groups than in the control group, but this was not observed in the L(0.5) group.  $L_m$  was more increased by hyperoxia than LPS. Panel A2 shows the synergism between LPS and hyperoxia with respect to alveolar size.  $L_m$  was significantly greater in the L(0.5) + O<sub>2</sub>(1) and L(1.0) + O<sub>2</sub>(1) groups than in the O<sub>2</sub>(1) group.  $L_m$  values were not significantly different between the L(0.5) + O<sub>2</sub>(1) and L(1.0) + O<sub>2</sub>(1) groups or between the O<sub>2</sub>(2) and L(0.5) + O<sub>2</sub>(2) groups. Panel B1 shows that alveolar surface area ( $S_A$ ) was significantly smaller in the L(1.0), O<sub>2</sub>(1), and O<sub>2</sub>(2) groups than in the control group, but that this was not the case for the L(0.5) group. Panel B2 shows the synergistic interaction between LPS and hyperoxia on  $S_A$ . The trend was evident, but statistical significance was only found between the O<sub>2</sub>(1) and L(1.0) + O<sub>2</sub>(1) groups. Panel C1 shows that alveolar wall thickness ( $W_T$ ) in the O<sub>2</sub>(2) group was significantly greater than that of the control group, and that alveolar wall thickness in the L(1.0) group was significantly thinner than that of the control group. Longer exposure to hyperoxia increased  $W_T$ , and conversely, larger dose of LPS decreased  $W_T$ . Panel C2 shows that LPS did not amplify the effect of short-term or prolonged hyperoxia on  $W_T$ . \* $p < 0.05$ .

tization by LPS was observed [O<sub>2</sub>(2),  $24.3 \pm 3.2 \mu\text{m}$ ; L(0.5) + O<sub>2</sub>(2),  $24.3 \pm 5.0 \mu\text{m}$  versus O<sub>2</sub>(1),  $19.2 \pm 4.2 \mu\text{m}$ ,  $p < 0.05$ ] (Fig. 5C2).

## DISCUSSION

In 1967, Northway *et al.* (15) described the pathologic characteristics of BPD as necrotizing bronchiolitis, alveolar cell hyperplasia, and bronchiolar squamous metaplasia leading to alveolar septal fibrosis. However, in the postglucocorticoid, postsurfactant era, the classic diagnostic features of BPD are infrequently seen and BPD is now characterized by an arrest of alveolarization, fewer and larger alveoli and a smaller alveolar surface area (1).

In the present study, we devised a rat model of BPD induced by intra-amniotic LPS administration and hyperoxia. The sensitizing effect of antenatal LPS administration on lung injury is schematically shown in Fig. 6. LPS at  $0.5 \mu\text{g}$  did not inhibit alveolarization alone, whereas LPS at  $1.0 \mu\text{g}$  modestly inhibited alveolarization to a similar extent to that shown by hyperoxia for 1 wk. However, compared with hyperoxia for 1



**Figure 6.** Schematic presentation of experimental results showing the independent effect of antenatal LPS treatment and synergism between LPS and hyperoxia on alveolarization.

wk, treatment with low or high dose LPS plus hyperoxia for 1 wk resulted in a markedly increase in the inhibition of alveolarization, which demonstrates the sensitizing effect of LPS. However, this sensitization was not dose-dependently related to LPS, which suggests that LPS doses of  $<0.5 \mu\text{g}$  would be equally effective.

In the described rat model, the most extensive inhibition of alveolarization observed occurred after 2 wk of hyperoxia, and this was unaffected by LPS presumably because of the overwhelming effect of 2 wk of hyperoxia. Our morphometric results are on pups killed at the end of the experimental period (day 14). The highest death rate was observed in pups treated with LPS and exposed to hyperoxia for 2 wk, and these pups probably died of severe lung injury, biasing our results. Nonetheless, it is obvious that the sensitizing effect of LPS stands out more conspicuously under the short-term hyperoxia than prolonged hyperoxia. This pattern of lung injury is more consistent with BPD, as it is now encountered as most preterm infants are exposed to much milder degrees of hyperoxia and gentler ventilatory support.

Chorioamnionitis has shown to be associated with an increased BPD prevalence (2,4,5), which suggests that antenatal infection and/or inflammation amplify postnatal lung injury. In preterm lambs and baboons, the intra-amniotic administration of endotoxin or *Ureaplasma urealyticum* was found to decrease alveolarization or alter developmental signaling in the immature lung (11,12,16). However, these studies did not demonstrate the sensitizing effect of endotoxin on the inhibitory effect of hyperoxia or mechanical ventilation. Ikegami and Jobe (9) suggested sensitization by endotoxin after finding that fetal sheep exposed to intra-amniotic endotoxin 30 d before preterm delivery had larger proinflammatory responses to 6 h of mechanical ventilation than nonexposed controls, but they failed to identify other detectable pathologic effects on fetal lungs. However, in this study, the short postnatal experimental period of 6 h might have been inadequate in terms of observing the full sensitizing effect of endotoxin. To our knowledge, the present study is the first to demonstrate that endotoxin promotes the postnatal hyperoxia-induced inhibition of alveolarization.

At  $1.0 \mu\text{g}$ , LPS increased mean cord lengths and reduced alveolar surface areas, which indicates a significant inhibition of alveolarization, which is a histological hallmark of modern

BPD. Moreover, in the present study, LPS induced alveolar wall thinning, which contrasts with septal thickening in classical BPD. This result was also reported by Willet *et al.* (11) in preterm lambs that had been treated antenatally with endotoxin or betamethasone. In particular, these lambs showed inhibited alveolarization, reduced alveolar numbers, increased average alveolar volumes, and alveolar wall thinning. This phenomenon has also been reported in cases of human antenatal infection and/or inflammation (2,4–6). These conditions have been reported to reduce the risk of RDS and improve surfactant production and gas exchange (2,6), but paradoxically to increase the prevalence of BPD (2,4,5).

In cases of antenatal infection and/or inflammation, lung injury is believed to be mediated by inflammatory cytokines (3,7,8,10). Preterm fetal sheep exposed to intra-amniotic endotoxin and preterm baboons antenatally colonized with *Ureaplasma urealyticum* had inflammatory cells in the airspaces and lung parenchyma, and elevated levels of proinflammatory cytokines in bronchoalveolar lavage fluid and lung tissues (11,17–20). In the present study, the presence of fetal inflammatory response was not investigated either by determining inflammatory mediators levels or histologically. However, in lung tissues at 7 and 14 d inflammatory changes, such as inflammatory cell infiltration and alveolar destruction, were not observed (data not shown), which concurs with the findings of a study conducted by Ueda *et al.* (12). In their antenatal endotoxin rat model, serial morphometric examinations up to the 60th day of life showed no signs of inflammation. We believe that our rat pup model of BPD could be used to investigate biochemical aspects of the effect of LPS on alveolarization and its sensitizing effect on hyperoxia-induced inhibition of alveolarization.

Factors related to mechanical ventilation, such as stretch, were not considered during the present study. It is known that mechanical ventilation contributes to preterm lung injury independently or in combination with other injuries (*e.g.* antenatal inflammation and hyperoxia) (9,21). Moreover, in the present study, we used term newborn rat pups and not preterm pups. Therefore, our rat pup model is unlikely to fully simulate human preterm infants or the pathophysiology of BPD. However, it is well known that hyperoxia inhibits alveolarization *per se* in newborn rats during the first two wks of life in a dose-dependent manner (22). In terms of lung development, term newborn rats correspond to human at gestation wks 26–28 (23). Term newborn rats are born in the sacular phase and alveolarization takes place during the first 2 wks of life, and thus, the term newborn rat is a useful animal model, at least, for investigations on alveolarization.

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## REFERENCES

- Husain AN, Siddiqui NH, Stocker JT 1998 Pathology of arrested acinar development in postsurfactant bronchopulmonary dysplasia. *Hum Pathol* 29:710–717.
- Watterberg KL, Demers LM, Scott SM, Murphy S 1996 Chorioamnionitis and early lung inflammation in infants in whom bronchopulmonary dysplasia develops. *Pediatrics* 97:210–215.
- Yoon BH, Romero R, Jun JK, Park KH, Park JD, Ghezzi F, Kim BI 1997 Amniotic fluid cytokines (interleukin-6, tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8) and the risk for the development of bronchopulmonary dysplasia. *Am J Obstet Gynecol* 177:825–830.
- Van Marter LJ, Dammann O, Allred EN, Leviton A, Pagano M, Moore M, Martin C 2002 Chorioamnionitis, mechanical ventilation, and postnatal sepsis as modulators of chronic lung disease in preterm infants. *J Pediatr* 140:171–176.
- Alexander JM, Gilstrap LC, Cox SM, McIntire DM, Leveno KJ 1998 Clinical chorioamnionitis and the prognosis for very low birth weight infants. *Obstet Gynecol* 91:725–729.
- Van Marter LJ, Allred EN, Pagano M, Sanocka U, Parad R, Moore M, Susser M, Paneth N, Leviton A 2000 Do clinical markers of barotraumas and oxygen toxicity explain interhospital variation in rates of chronic lung disease? *Pediatrics* 105:1194–1201.
- Kim BI, Lee HE, Choi CW, Jo HS, Choi EH, Koh YY, Choi JH 2004 Increase in cord blood soluble E-selectin and tracheal aspirate neutrophils at birth and the development of new bronchopulmonary dysplasia. *J Perinat Med* 32:282–287.
- Choi CW, Kim BI, Kim HS, Park JD, Choi JH, Son DW 2006 Increase of interleukin-6 in tracheal aspirate at birth: a predictor of subsequent bronchopulmonary dysplasia in preterm infants. *Acta Paediatr* 95:38–43.
- Ikegami M, Jobe AH 2002 Postnatal lung inflammation increased by ventilation or preterm lambs exposed antenatally to *E. coli* endotoxin. *Pediatr Res* 52:356–362.
- Jobe AH 2003 Antenatal factors and the development of bronchopulmonary dysplasia. *Semin Neonatol* 8:9–17.
- Willet KE, Jobe AH, Ikegami M, Newnham J, Brennan S, Sly PD 2000 Antenatal endotoxin and glucocorticoid effects on lung morphometry in preterm lambs. *Pediatr Res* 48:782–788.
- Ueda K, Cho K, Matsuda T, Okajima S, Uchida M, Kobayashi Y, Minakami H, Kobayashi K 2006 A rat model for arrest of alveolarization induced by antenatal endotoxin administration. *Pediatr Res* 59:396–400.
- Weibel ER 1963 Morphometry of the Human Lung. Berlin: Springer-Verlag.
- Snyder JM, Jenkins-Moore M, Jackson SK, Goss KL, Dai HH, Bangsund PJ, Giguere V, McGowan SE 2005 Alveolarization in retinoic acid receptor-beta-deficient mice. *Pediatr Res* 57:384–391.
- Northway WH Jr., Rosan RC, Porter DY 1967 Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. *N Engl J Med* 276:357–368.
- Viscardi RM, Atamas SP, Luzina IG, Hasday JD, He JR, Sime PJ, Coalson JJ, Yoder BA 2006 Antenatal *Ureaplasma urealyticum* respiratory tract infection stimulates proinflammatory, profibrotic responses in the preterm baboon lung. *Pediatr Res* 60:141–146.
- Kramer BW, Moss TJ, Willet KE, Newnham JP, Sly PD, Kallapur SG, Ikegami M, Jobe AH 2001 Dose and time response after intraamniotic endotoxin in preterm lambs. *Am J Respir Crit Care Med* 164:982–988.
- Kramer BW, Kramer S, Ikegami M, Jobe AH 2002 Injury, inflammation, and remodeling in fetal sheep lung after intra-amniotic endotoxin. *Am J Physiol Lung Cell Mol Physiol* 283:L452–L459.
- Kallapur SG, Willet KE, Jobe AH, Ikegami M, Bachurski CJ 2001 Intra-amniotic endotoxin: chorioamnionitis precedes lung maturation in preterm lambs. *Am J Physiol Lung Cell Mol Physiol* 280:L527–L536.
- Yoder BA, Coalson JJ, Winter VT, Siler-Khodr T, Duffy LB, Cassell GH 2003 Effects of antenatal colonization with *Ureaplasma urealyticum* on pulmonary disease in immature baboon. *Pediatr Res* 54:797–807.
- Ehlert CA, Truog WE, Thibeault DW, Garg U, Norberg M, Rezaiekhailigh M, Mabry S, Ekekezie II 2006 Hyperoxia and tidal volume: independent and combined effects on neonatal pulmonary inflammation. *Biol Neonate* 90:89–97.
- Bucher JR, Roberts RJ 1981 The development of the newborn rat lung in hyperoxia: a dose-response study of lung growth, maturation, and changes in antioxidant enzyme activities. *Pediatr Res* 15:999–1008.
- Massaro D, Teich N, Maxwell S, Massaro GD, Whitney P 1985 Postnatal development of alveoli. Regulation and evidence for a critical period in rats. *J Clin Invest* 76:1297–1305.