Functional assessment of hyperoxia-induced lung injury after preterm birth in the rabbit

Jute Richter,1 Jaan Toelen,1 Jeroen Vanoirbeek,2 Aiko Kakigano,1 Philip DeKoninck,1 Eric Verbeken,3 and Jan Deprest1

1Research Unit Fetus Placenta Neonate, Faculty of Medicine, Department of Development and Regeneration, KU Leuven, Leuven, Belgium; 2Laboratory of Occupational and Environmental Toxicology, Department of Public Health and Primary Care, KU Leuven, Leuven, Belgium; and 3Department of Pathology, University Hospitals of Leuven, KU Leuven, Leuven, Belgium

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Richter J, Toelen J, Vanoirbeek J, Kakigano A, DeKoninck P, Verbeken E, Deprest J. Functional assessment of hyperoxia-induced lung injury after preterm birth in the rabbit. Am J Physiol Lung Cell Mol Physiol 306: L277–L283, 2014. First published December 27, 2013; doi:10.1152/ajplung.00315.2013.—The objective of this study was to document early neonatal (7 days) pulmonary outcome in the rabbit model for preterm birth and hyperoxia-induced lung injury. Preterm pups were delivered at 28 days (term = 31 days; early saccular phase of lung development) by cesarean section, housed in an incubator, and gavage fed for 7 days. Pups were divided into the following groups: 1) normoxia (21% O2; normoxia group) and 2) hypoxia (>$95%$ O2; hyperoxia group). Controls were pups born at term who were housed in normoxic conditions (control group). Outcome measures were survival, pulmonary function tests using the whole body plethysmograph and forced oscillation technique, and lung morphometry. There was a significant difference in survival of preterm pups whether they were exposed to normoxia (83.3%) or hyperoxia (55.9%). Hyperoxic exposure was associated with increased tissue damping and elasticity and decreased static compliance compared with normoxic controls ($P < 0.01$). Morphometry revealed an increased linear intercept and increased mean wall transection length, which translates to larger alveoli with septal thickening in hyperoxia compared with normoxia ($P < 0.01$). In conclusion, the current experimental hyperoxic conditions to which preterm pups are exposed induce the typical clinical features of bronchopulmonary dysplasia. This model will be used to study novel preventive or therapeutic interventions.

bronchopulmonary dysplasia
HYPEROXIA-INDUCED LUNG INJURY AFTER PRETERM BIRTH IN THE RABBIT

MODEL DESCRIBED BY MASCARETTI ET AL. (21) AND MATALOUN ET AL. (21, 22) LED US TO SELECT RABBITS AS THE BEST CANIDATE MODEL TO STUDY THE EFFECTS OF HYPEROXIA AFTER PRETERM BIRTH. OUR AIM WAS TO CREATE A MODEL WITH A FULL METHODOLOGICAL DESCRIPTION, INCLUDING HISTOLOGICAL AND IMMUNOHISTOCHEMICAL OUTCOMES, AS WELL AS FUNCTIONAL LUNG TESTS. THIS MODEL CAN BE USED TO INVESTIGATE NOVEL PERINATAL INTERVENTIONS TO PREVENT OR TREAT BPD.

MATERIALS AND METHODS

Animals and definition of experimental groups. Time-mated pregnant does (hybrid of New Zealand White and Dendermonde) were obtained from the animalium of the group Biomedical Sciences at the KU Leuven. Animals were treated according to current guidelines of animal well-being, and the experiments were approved by the Ethics committee for Animal Experimentation of the Faculty of Medicine. Does were housed in separate cages before delivery, with free access to water and chow and a light-dark cycle of 12 h. Four does were allowed to deliver naturally at term (day 31 postconception), and their pups remained with their mother in normoxic conditions and were used as an external control group (control group). The other does underwent a cesarean section, resulting in preterm delivery at 28 days of gestation (early saccular lung developmental phase). The pups were divided into two different groups: 1) the normoxia group where pups were housed in 21% oxygen for 7 days and 2) the hyperoxia group where pups were nursed in hyperoxia (≥95% oxygen) for 7 days. Oxygen concentration inside the incubator was monitored daily using an oxygen analyzer (MiniOX 1; Ohio Medical).

For cesarean section the doe was premedicated with intramuscular ketamine (35 mg/kg), ketamine 1000 CEVA; CEVA Santé Animal, Brussels, Belgium) and xylazine (6 mg/kg, Vexylan; CEVA Santé Animal). Subsequently, spinal anaesthesia was administered with 2% lidocaine hydrochloride (Lisonil; B Braun) at the level of L7–S1. The doe was placed in the supine position, and the abdomen was opened through a low midline abdominal incision. The bicornuate uterus was exposed, and all pups were extracted through hysterotomy. Following delivery the doe was killed with a mixture of 200 mg embutramide, 50 mg mebezonium, and 5 mg tetracain hydrochloride (iv bolus of 1 ml T61; Intervet Belgium, Mechelen, Belgium).

At delivery, the pups were dried, stimulated, weighed, and placed in an incubator (Dräger Incubator 7310; Dräger, Lübeck, Germany) at 32°C. Within the first 30 min of life, 0.025 ml glucose 10%/g body wt was administered through a 3.5-Fr orogastric tube. Thereafter they were fed two times daily via an orogastric tube placed just before the feeding. The feeding consisted of a mixture of soybean oil (Intralipid 30%, 270 kcal/100 ml; Fresenius Kabi), pure casein (100% Casein, 360 kcal/100 mg; Optimum Nutrition), pediatric milk formula (Nutrition HA nr 2, 453 kcal/100 mg; Nutricia), and water. This mixture gives a nutritional value of 16 g lipids, 5.3 g proteins, 2.7 g carbohydrates (176 kcal in total)/100 ml and was chosen in accordance to prior studies using a preterm rabbit model (21, 22). The amount of feeding steadily increased from 80 to 100 to 150 finally 200 mg·kg⁻¹·day⁻¹ on days 0, 1, 2, and 3 (until 7), respectively. On day 2, vitamin K was administered intramuscularly (0.002 mg/kg, Kon- akion pediatrique; Roche, Basel, Switzerland), and from that point pups were given a daily intramuscular injection of benzylpenicillin (20,000 E·kg⁻¹·day⁻¹), penicilline; Kela, Sint-Niklaas, Belgium) and amikacin (20 mg·kg⁻¹·day⁻¹, Amukin; Bristol-Myers-Squibb) (21, 22). The pups remained in the incubator except for the feeding moments for which they were removed from the incubator for a maximum of 10 min/feed.

At day 7 pups were randomly divided for either death to allow lung morphometry or performing pulmonary function testing with the whole body plethysmograph or the FlexiVent (with a minimum of 5 pups/group).

Lung function tests. A functional assessment was performed with an unrestrained whole body plethysmograph (EMKA Technologies, Paris, France), as described in rodents (14, 15). Three groups were compared at day 7: 1) animals born term (day 31) and held in normoxia (21% O₂), 2) animals born preterm (day 28) and housed in normoxia, and finally 3) animals born preterm (day 28) and housed in hyperoxia (>95% O₂). The pups were first kept in the plethysmograph for 9 min to stabilize. Thereafter, over a period of 10 min, the pups were monitored, and the following different ventilatory parameters were determined every 30 s: time of inspiration (Ti), time of expiration, peak inspiratory flow, peak expiratory flow, tidal volume, expiration volume, relaxation time, minute volume (MV), frequency of breathing (Freq), end-inspiratory pause (EIP), end-expiratory pause, and enhanced pause.

Invasive lung function testing was performed using a forced oscillation technique with the Flexivent system (FlexiVent; SCIREQ, Montreal, Canada) (15) after anaesthetizing the pups with ketamine (35 mg/kg) and xylazine (6 mg/kg). These were placed in the supine position, a tracheostomy was performed, and an 18-gauge metal needle was inserted in the trachea and connected to the FlexiVent device with a ventilation frequency of 120 breaths/min. The following parameters were assessed: airway resistance (Rn), tissue damping (resistance, G), and tissue elasticity using Primewave-8 forced oscillation and the total lung capacity and static compliance (Cst) using the pressure-volume perturbation. All measurements were performed until three consistent measurements were obtained, with a coefficient of determination of >0.95 as the limit to accept the measurement. The average of three measurements was calculated and used in the results. All measurements were performed within a time frame of 10–15 min, after which the pups were killed with 0.1 ml T61 administered intraperitoneally.

Histological examination of the lungs. The pups allocated to morphometry were first premedicated with an intramuscular injection of ketamine (35 mg/kg) and xylazine (6 mg/kg) and subsequently killed with 0.1 ml T61 administered intraperitoneally. A thoracotomy was performed, and the lungs and trachea were removed “en bloc” after which the left bronchus was ligated and the left lung was removed and snap-frozen for other research purposes. A 20-G catheter was inserted in the trachea whereafter the right lung was fixed with 4% paraformaldehyde by immersion and under a constant hydrostatic pressure of 25 cmH₂O for 24 h before embedding. Paraffin sections were stained with hematoxylin and eosin (H&E). A pulmonary pathologist performed a qualitative blinded assessment of the lung slides. Morphometric measurements consisted of 1) the linear intercept (Lₐ₀), which is a measure of alveolar size, and 2) the mean wall transection length (Lₐₜₜ), a measure of the interalveolar septal thickness. Both param-

Fig. 1. Survival curves. Survival curves of all animals born preterm and held in normoxia or hyperoxia, and of term controls. Survival at 7 days of life: normoxia group 83.3% (n = 36), hyperoxia group 55.9% (n = 34), and term controls 84.6% (n = 26).
Table 1. Lung function with the whole body plethysmograph

<table>
<thead>
<tr>
<th>Unit</th>
<th>Normoxia</th>
<th>Hyperoxia</th>
<th>Term Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_i$ ms</td>
<td>152.20 ± 30.59</td>
<td>231.80 ± 38.8*§</td>
<td>151.90 ± 22.94</td>
</tr>
<tr>
<td>$T_e$ ms</td>
<td>184.60 ± 37.98</td>
<td>373.80 ± 112.3§</td>
<td>243.30 ± 78.77</td>
</tr>
<tr>
<td>PIF ml/s</td>
<td>3.14 ± 0.74</td>
<td>1.87 ± 0.88*</td>
<td>3.90 ± 0.91</td>
</tr>
<tr>
<td>PEF ml/s</td>
<td>2.83 ± 0.54</td>
<td>1.92 ± 0.94</td>
<td>2.78 ± 0.63</td>
</tr>
<tr>
<td>$V_r$ ml</td>
<td>0.26 ± 0.09</td>
<td>0.19 ± 0.09*</td>
<td>0.34 ± 0.08</td>
</tr>
<tr>
<td>EV ml</td>
<td>0.26 ± 0.09</td>
<td>0.20 ± 0.09*</td>
<td>0.33 ± 0.08</td>
</tr>
<tr>
<td>RT ms</td>
<td>113.80 ± 19.12</td>
<td>195.50 ± 50.52§</td>
<td>150.40 ± 35.91</td>
</tr>
<tr>
<td>MV ml</td>
<td>51.00 ± 13.44</td>
<td>24.00 ± 13.96*§</td>
<td>60.31 ± 15.30</td>
</tr>
<tr>
<td>Freq beats/min</td>
<td>238.40 ± 14.87</td>
<td>120.10 ± 34.74*§</td>
<td>198.60 ± 31.37</td>
</tr>
<tr>
<td>EIP ms</td>
<td>5.81 ± 1.75</td>
<td>17.78 ± 8.62*§</td>
<td>4.44 ± 0.62</td>
</tr>
<tr>
<td>EEP ms</td>
<td>20.25 ± 3.74</td>
<td>46.28 ± 22.82§</td>
<td>31.19 ± 15.68</td>
</tr>
<tr>
<td>PenH</td>
<td>1.01 ± 0.70</td>
<td>0.93 ± 0.49*</td>
<td>0.49 ± 0.04</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD. $T_i$, time of inspiration; $T_e$, time of expiration; PIF, peak inspiratory flow; PEF, peak expiratory flow; $V_r$, tidal volume; EV, expiration volume; RT, relaxation time; MV, minute volume; Freq, frequency of breathing; EIP, end-inspiratory pressure; EEP, end-expiratory pressure; PenH, enhanced pause. Results of the whole body plethysmograph. Normoxia group ($n = 8$), hyperoxia-group ($n = 9$), and term controls ($n = 8$). *$P < 0.05$ compared with the term controls and §$P < 0.05$ compared with the normoxia group.

RESULTS

Survival rates. The average birth weight of term pups was 47.8 g compared with 31.8 g ($P < 0.001$) for preterm pups (normoxia group: 31.9 g; hyperoxia group: 31.8 g). Weight gain of the term pups housed with their mother was significantly higher (69.6 g at day 7, increase of 45.6%) compared with the preterm pups [normoxia: 37.5 g (+17.5%) and hyperoxia 35.7 g (+12.3%)]. Figure 1 shows a survival analysis of the pups (Fig. 1). Out of 36 pups kept in normoxia, six pups died (3 immediately after birth, 2 at day 1, and 1 at day 5), which results in a 7-day survival rate of 83.3%. In the hyperoxia group this was 55.9% ($P = 0.029$ compared with normoxia controls). Out of 34 pups, three died immediately after birth, two at day 3, one at day 5, four at day 6, and five more at day 7. In the term control group ($n = 26$), three pups died within hours after birth and one more at day 3, which results in a survival of 84.6%.

The deaths occurring immediately after delivery were most likely the result of postnatal adaptation problems and acute respiratory failure. Mortality occurring after day 4 was because of severe respiratory insufficiency, or to skin and generalized infections or intestinal (obstruction) problems.
Lung function tests. Performing the unrestrained whole body plethysmography has the important disadvantage of a very quick desaturation of the pups that were kept in hyperoxia (within a few minutes). Several pups died during the plethysmography and were excluded from further analysis. When comparing the group with hyperoxia vs. the preterm and term controls, statistical differences were found in $T_i$, MV, EIP, and Freq (Table 1). There were no differences between the term or preterm pups held in normoxia.

In the next cohort, the forced oscillation technique, which is a more precise method to obtain lung function testing, was used. Again there was no detectable difference between the normoxic preterm and control pups.

The $R_n$, $G$, and $H$ were assessed by forced oscillation (Primewave-8). $R_n$ did not differ between the preterm groups (Fig. 2A) (normoxia 0.09 ± 0.01, hyperoxia 0.12 ± 0.04 cmH$_2$O s$^{-1}$ ml$^{-1}$). Both $G$ and elasticity were significantly increased in the hyperoxia group (G 7.16 ± 2.1 cmH$_2$O/ml and H 47.39 ± 25.4 cmH$_2$O/ml), compared with the normoxia group (G 2.16 ± 0.1 and H 5.93 ± 0.5 cmH$_2$O/ml) (Fig. 2B and C) ($P = 0.0037$ and $P = 0.0031$ respectively). With the use of maximal pressure-volume loops, the maximal vital total lung capacity was significantly higher in the normoxia group (2.59 ± 0.3 ml) compared with the hyperoxia group (0.55 ± 0.3 ml, $P = 0.0012$) (Fig. 2D). The results of the static compliance measurements (Fig. 2E) demonstrated a significantly decreased compliance in the hyperoxia group (0.025 ± 0.01 ml/cmH$_2$O) compared with normoxia pups (0.134 ± 0.02 ml/cmH$_2$O, $P = 0.0060$).

Histology and morphometry of the lungs. H&E-stained, formalin-fixed, paraffin-embedded lung tissues were first analyzed by a pulmonary pathologist who was blinded to the study conditions of the different cohorts. A clear discrimination could be made between the hyperoxic and normoxic conditions. Hyperoxic conditions showed evidence of extensive and homogenous lung injury with interstitial and alveolar edema, and acute inflammatory cell infiltration compared with normoxic conditions (Fig. 3, A and B). The edema did not appear to be the result of an endothelial pathology since there was an absence of septal necrosis and microthrombosis. The inflammatory cells were located mainly in the peribronchial alveolar parenchyma. The developmental changes induced by hyperoxia were characterized by a simplification of the acinar structure, with a decreased number of alveoli and greatly enlarged terminal airways. These findings were corroborated by morphometric findings, and are suggestive of a developmental arrest of the pulmonary tissue (4).

Morphometry revealed no significant differences between term controls and preterm normoxia pups ($P = 0.3$ and $P = 0.7$ for $L_m$ and $L_{mw}$, respectively). Yet $L_m$ and $L_{mw}$ in the hyperoxia group (81.8 ± 6.5 and 29.5 ± 2.9 μm, respectively) were significantly higher than in normoxia preterm controls ($L_m$ 67.40 ± 3.5 and $L_{mw}$ 16.46 ± 1.5 μm, $P = 0.0003$ and $P = 0.0017$, respectively) (Fig. 3, C and D). The number of MIB-1-positive cells was significantly higher following hyperoxia (30.5 ± 3.9 pos cells/HPF compared with 5.2 ± 5.5 pos cells/HPF in normoxia, $P = 0.0048$) (Fig. 4, A-C). Sirius Red staining indicated a marked increase in collagen content (red

![Fig. 3. Hematoxylin and eosin (H&E) staining and morphometry (linear intercept ($L_m$) − mean wall transection length ($L_{mw}$)) of the right lung. H&E staining of a sagittal section of the lung. A: normoxia group; B: hyperoxia group. Magnification ×10 and ×40. There is a clear difference between the groups held in hyperoxia and the normoxic animals with larger alveoli and thickened septa. C and D: morphometric analysis of the $L_m$ (C) and $L_{mw}$ (D). n = 5–6 Animals, *$P < 0.01$.](http://ajplung.physiology.org/ by 10.220.33.3 on April 5, 2017)
DISCUSSION

BPD remains one of the most important clinical problems of preterm born survivors. In humans the window of opportunity for prenatal intervention is situated in the early saccular phase. The smallest species in which in utero manipulation like intra-amniotic or direct intravenous or intratracheal injections during the saccular or initial alveolar phase of development can be modeled is the rabbit. The use of preterm born rabbits has been previously described to study neonatal nutrition (5), the antioxidant system (31), intraventricular hemorrhage (3), the effect of betamethasone (29), or hyperoxia exposure (21, 22). The latter two studies have provided very important information about the housing and feeding of premature rabbits as well as insights in the lung pathology after hyperoxic exposure. Further advantages of those two studies were the survival up to 7 and 11 days, but outcome measures were solely lung morphology, hence functional assessment was lacking. In our hands premature birth combined with artificial feeding alone did not result in detectable changes in histology nor lung function or mortality. This suggests that postnatal care with artificial feeding and without the presence of a maternal animal has no significant effect on lung development. At this stage the presence of a hyperoxic environment is necessary to induce the toxicity and pulmonary changes. We also tried a more obvious prematurity insult by birth before day 27 of gestation. This resulted indeed in death resulting from respiratory failure within minutes (data not shown). This is possibly because of the extreme immaturity of the developing lung at that time (late canalicular-early saccular stage), because the rabbit rushes through the last lung developmental stages in only a few days. At that stage the fetal lung is not capable to perform adequate gas exchange with unsupported breathing movements.

For the functional analysis of the lungs, we used whole body plethysmography and the forced oscillation technique. We previously used the FlexiVent system (8, 11) in neonatal rabbits before the onset of breathing. To our knowledge, assessment of functional lung parameters in spontaneously breathing and preterm born rabbit pups has not been described before. This allowed the detection of significant changes in the pups kept in hyperoxia, which is in line with previous observations in rodents (14, 15, 24, 25).

The morphometry of the lung sections revealed a significant increase in alveolar size as well as thickened septa. These results parallel those observed in other hyperoxic lung injury animal models (2, 6, 24, 27, 32) as well as what is clinically observed (16). These changes represent a lung developmental arrest, typically characterized by a simplification with enlarged alveoli and thickened septa (6, 24, 27, 32).

Immunohistochemical analysis of the lungs revealed a more intense staining with Picro Sirius Red in the hyperoxia group corresponding to increased collagen content and thickened septa. This corroborates data from previously published models (21, 22). The MIB-1 staining (which detects the proliferation marker Ki67) demonstrated a significant increase of proliferating cells in pups exposed to hyperoxia. This could be
because of increased proliferation of pulmonary cells or secondary immune cells. We have been unsuccessful to do double staining for either lung or immune markers to discriminate which cell type is responsible for the increased proliferation. This is mainly because of the fact that few antibodies are available for this kind of staining procedures in this particular species. All tested antibodies resulted in significant background staining (data not shown). A morphological assessment of the Ki67-positive cells suggests however that both lung and immune cells are proliferating. This suggests a regenerative effort of the pulmonary tissue in the context of oxygen-induced toxicity.

The advantage of the used model is that it can be considered as a large animal where the lung can be assessed both functionally as well as by morphometry. Because BPD is a chronic disorder, survival up to 7 days may be more informative than short-term (24–48 h) studies. Nevertheless, there are several limitations to our study. First, we only performed assessment at a single time point (7 days of life). Second, the continuous high dose of oxygen for 7 days might be too toxic to demonstrate any positive effects of different therapies, and in the near future more studies will be performed to decrease the oxygen level after several days, mimicking more closely the current neonatal course in humans. Furthermore, the design, and in particular the use of the FlexiVent, does not permit a longitudinal analysis of the same pup at different time points. These limitations can be overcome by the use of a double-chamber plethysmography, which is more sensitive than whole body plethysmography.

In conclusion, we comprehensively documented the effect of continuous exposure to hyperoxia (>95% O2) for 7 days in preterm born rabbit pups. Hyperoxic conditions induced functional and histological features compatible with the clinical features of BPD. This animal model will enable us to study perinatal interventions to modulate the pathophysiology of hyperoxia-induced lung injury.

DISCLOSURES
No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES