Injury and repair in the very immature lung following brief mechanical ventilation

Nadine Brew,1 Stuart B. Hooper,2,3* Beth J. Allison,2 Megan J. Wallace,2,3 and Richard Harding1*

Departments of Anatomy and Developmental Biology and Obstetrics and Gynaecology, Monash University, Victoria; and Monash Institute of Medical Research, Clayton, Victoria, Australia

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Brew N, Hooper SB, Allison BJ, Wallace MJ, Harding R. Injury and repair in the very immature lung following brief mechanical ventilation. Am J Physiol Lung Cell Mol Physiol 301: L917–L926, 2011. — Mechanical ventilation (MV) of very premature infants contributes to lung injury and bronchopulmonary dysplasia (BPD), the effects of which can be long-lasting. Little is currently known about the ability of the very immature lung to recover from ventilator-induced lung injury. Our objective was to determine the ability of the injured very immature lung to repair in the absence of continued ventilation and to identify potential mechanisms. At 125 days gestational age (days GA, 0.85 of term), fetal sheep were partially exposed by hysterotomy under anesthesia and aseptic conditions; they were intubated and ventilated for 2 h with an injurious MV protocol and then returned to the uterus to continue development. Necropsy was performed at either 1 day (short-term group, 126 days GA, n = 6) or 15 days (long-term group, 140 days GA, n = 5) after MV; controls were unventilated (n = 7–8). At 1 day after MV, lungs displayed signs of injury, including hemorrhage, disorganized elastin and collagen deposition in the distal airspaces, altered morphology, significantly reduced second-order septal crest density, and decreased airspace. Bronchioles had thickened epithelium with evidence of injury and sloughing. Relative mRNA levels of early response genes (connective tissue growth factor, cysteine-rich 61, and early growth response-1) and proinflammatory cytokines [ interleukins (IL)-1β, IL-6, IL-8, tumor necrosis factor-α, and transforming growth factor-β] were not different between groups 1 day after MV. At 15 days after MV, lung structure was normal with no evidence of injury. We conclude that 2 h of MV induces severe injury in the very immature lung and that these lungs have the capacity to repair spontaneously in the absence of further ventilation.

ventilator induced lung injury; bronchopulmonary dysplasia; repair; inflammation; preterm birth

VERY PRETERM INFANTS OFTEN require mechanical ventilation (MV) due to respiratory insufficiency. However, respiratory support including MV can injure the immature lung and contribute to the development of bronchopulmonary dysplasia (BPD) (10). Structural changes in the lung that are associated with MV and BPD can persist, resulting in deficits in lung function in children (17, 19) and young adults (18). Consistent with this, exercise capacity is reduced in children (23, 32) and adolescents (31, 33) who were born very preterm and developed BPD. The structural changes seen in the lungs of infants with BPD include hypercellularity, fewer and larger alveoli, altered vascular growth, and disorganized elastin and collagen deposition (12). In addition, alterations to the small conducting airways have been reported in BPD-affected infants, including increases in airway smooth muscle and the number of goblet cells (47).

The causes of BPD remain unclear. While MV is known to be a significant contributor to BPD, other factors are thought to be involved, including infection, use of supplemental oxygen, and impaired nutrition (27). Indeed it has been difficult to identify the role of MV alone, or any other single factor contributing to BPD, because other potentially confounding treatments are required for survival of infants or animal models. To understand the effects of MV alone on the very immature lung, we have developed a novel technique for ventilating the lungs of very immature fetal sheep in utero. Using this technique, we reported BPD-like changes in the lung parenchyma (3) and small airways (38) after only 6 or 12 h of MV; the effects persisted for 7 days in the absence of further interventions. These studies showed that, following MV, the immature lungs were injured and structurally abnormal for up to 7 days. However, it was not apparent from these studies whether the injury persists beyond 7 days or whether the lung has the capacity to repair over time.

To determine if MV-induced injury in the very immature lung persists or resolves in the absence of further ventilation, we have exposed very immature fetal sheep to a brief period of MV and have examined the lungs 15 days later. We hypothesized that the immature lung has the capacity to repair, restoring normal lung structure, following MV-induced injury. If our hypothesis is correct, it would suggest that continued ventilation of an already injured lung is a major contributor to persisting BPD-like changes in lung structure. We ventilated the very immature lungs of fetal sheep at 125 days gestational age (GA) for 2 h. At this age, the fetal sheep lung is at the late saccular-early alveolar stage of development (2). Very preterm infants (<30 wk gestation) are at similar stage of lung development as used in our model, and usually require MV. Our objectives were 1) to characterize the effects of a short period of MV (2 h) on the very immature lung, 2) to determine if MV-induced injury persists for up to 15 days, and 3) to investigate genes that could be involved in the lung injury. We have assessed injury to the lung parenchyma and bronchioles.

METHODS

Fetal preparation. All experimental procedures were approved by the Monash University Animal Ethics Committee. Under general anesthesia (1.5% halothane in NO2-O2, 70:30), aseptic surgery was performed on pregnant ewes at 125 days after mating (term is ~147 days). After the head and crest of the fetus were exposed, auffed (3 mm diameter) endotracheal tube was inserted in the trachea via the mouth. A polyvinyl catheter was implanted in a carotid artery to allow monitoring of fetal arterial blood gases and electrolytes during MV and after surgical recovery. Amniotic fluid loss was minimized by clamping the cut edges of the uterus to the fetal skin.
**MV protocol.** Before MV, fetal lung liquid was drained by gravity from the endotracheal tube and stored aseptically. The endotracheal tube was then connected to a neonatal ventilator (Dräger Babylog 8000+), and the fetus was ventilated for 2 h in ‘volume guaranteed’ mode targeting 5 ml/kg; we ventilated with unhumidified air (FiO₂ = 0.21, balance N₂, ~22°C), a peak inspiratory pressure of 40 cmH₂O, a positive end-expiratory pressure of 0 cmH₂O, and a frequency of 50 min⁻¹. This ventilation strategy was specifically chosen to induce lung injury (3). Throughout this time, the fetus was perfused via the umbilical-placental circulation. Fetal body weight was estimated from head length and fetal growth charts (11). Airway pressure and tidal volume were recorded continuously using a data acquisition system (Powerlab; ADI). Arterial blood (1 ml) was sampled for blood gas and electrolyte analysis immediately before MV commenced and at 1 and 2 h after commencing MV. After 2 h, MV ceased and the drained lung liquid was returned to the fetal lungs, the endotracheal tube was removed, and the fetus was returned to the uterus. The uterus and then the abdominal wall of the ewe were sutured closed, and catheters were exteriorized through the ewe’s right flank. Control fetuses either underwent sham surgery or were the twin of a sham or ventilated fetus. After surgery, ewes were allowed to recover and were housed in individual cages; fetal blood gas and electrolyte status were measured on alternate days. To reduce the risk of preterm labor, all ewes were administered progesterone (150 mg im; Sigma-Aldrich) at 1 day before surgery and for 10 days after surgery.

**Treatment groups.** We used two treatment groups, each with its age-matched control group, to assess the short- and long-term effects of MV at 125 days GA (0.85 of term). The short-term survival group was used to determine the early effects of MV; in this group, fetuses were killed 2 h after commencing MV (MV +1 day, n = 6). The control fetuses for this group (C + 1 day, n = 8) underwent identical surgery but did not receive MV. This control group consisted of sham-operated fetuses (n = 3) as well as the unexposed twins of sham-operated (n = 2) and ventilated (MV) fetuses (n = 3).

A long-term survival group was used to determine the prolonged effects of MV and the capacity for repair; in this group (MV + 15 days, n = 5), fetuses underwent necropsy 15 days after the period of MV, at 140 days GA. The control fetuses for this treatment group (C + 15 days, n = 7) were also killed at 140 days GA. This control group consisted of sham-operated (n = 2) and the unexposed twins of sham-operated (n = 1) and MV fetuses (n = 4). Lung morphology was not different between the sham control and the unexposed control fetuses for both groups.

**Lung tissue collection at necropsy.** At necropsy (126 or 140 days GA), the fetal lungs were removed and weighed; the left bronchus was ligated, and portions of the left lung were snap-frozen at −70°C. The right lung was fixed at 20 cmH₂O with 4% paraformaldehyde infused via the trachea, postfixed in Zamboni’s fixative, and processed for light microscopy; 20 cmH₂O expands the fetal lungs to a luminal volume similar to that observed in vivo (37). Lung volume was estimated using Cavalieri’s method of point counting (15). The percent water content of lung tissue was determined by drying tissue at 70°C. The percent tissue and blast content were determined using Ki-67 (1:100, M7240; DakoCytoMation) antibodies and sections were counterstained with hematoxylin to identify nuclei. The number of Ki-67-labeled cells, expressed as the proportion of total cells, was used to determine the lung cell proliferation rate.

**Assessment of bronchiolar wall injury.** Bronchioles with detached or absent epithelium were classified as injured (38). Briefly, the degree of injury was quantified by expressing the number of injured airways as a percentage of total airways (intact and injured) for each animal. The extent of epithelial loss or detachment in injured bronchioles was then classified as mild (<45° epithelium detached or absent, i.e., the injury affected <1/8 of the bronchiolar epithelial perimeter), moderate (45°–180° epithelium detached or absent, i.e., affecting 1/8 to 1/2 of bronchiolar epithelial perimeter), or extreme (>180° epithelium detached or absent, i.e., affecting >1/2 of bronchiolar epithelial perimeter) as previously described (35).

The presence or absence of cellular or acellular debris within the lumen of all bronchioles was expressed as a percentage of the total number of bronchioles examined. Bronchioles with epithelial injury, but not those with luminal debris and an intact epithelium, were excluded from further morphometric analysis.

**Morphometric analysis of intact bronchioles.** Light microscopy was used to measure the luminal area, the perimeter of the basement membrane, the area of epithelium, and the number of mucus-containing cells in the bronchial epithelium. For each parameter, 15 randomly chosen bronchioles from three sections from each animal were analyzed. PBM was used as an index of bronchiolar size, and epithelial area was expressed in relation to PBM. PBM is considered an appropriate indicator of bronchiolar dimension, since it is not significantly affected by alterations to lung volume or bronchoconstriction (5).

The epithelial area was calculated as the difference between the area enclosed by the epithelial basement membrane and the area of the lumen. Staining with PAS, a marker of mucus glycoconjugates secreted by goblet cells, was used to identify mucus-containing cells in the bronchial epithelium. The number of mucus-containing cells was expressed as a percentage of the total number of epithelial cells

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**Histological staining.** Sections were stained with either hematoxylin and eosin for general morphometry, Hart’s resorcin-fuscin stain to identify elastin (14, 28), Gordon and Sweet’s reticular stain to identify collagen, or periodic acid Schiff (PAS) stain to identify mucus-containing cells. Immunohistochemical staining. Proliferating cells and myofibroblast content were determined using Ki-67 (1:100, M7240; DakoCytoMation) and α-SMA antibodies (1:500, M0851; DakoCytoMation), respectively. Sections were incubated for 60 min using an immunohistochemistry kit [EnVision + Dual Link System-HRP (DAB+)] (Dako Cytomation), and sections were counterstained with hematoxylin to identify nuclei. The number of Ki-67-labeled cells, expressed as the proportion of total cells, was used to determine the lung cell proliferation rate.

**Analysis of lung injury.** The right lung was cut into 5-mm slices; three 2-cm² sections were randomly selected from slices of the cranial, middle, and caudal lobes and embedded in paraffin. One block was randomly chosen from each of the three lobes and sectioned at 5 μm. Sections were incubated at 60°C (2 h), deparaffinized, rehydrated, and washed in PBS. Sections of tissue analyzed were chosen randomly and included both injured and noninjured regions of the lung.

**Histological staining.** Sections were stained with either hematoxylin and eosin for general morphometry, Hart’s resorcin-fuscin stain to identify elastin (14, 28), Gordon and Sweet’s reticular stain to identify collagen, or periodic acid Schiff (PAS) stain to identify mucus-containing cells. Immunohistochemical staining. Proliferating cells and myofibroblast content were determined using Ki-67 (1:100, M7240; DakoCytoMation) and α-SMA antibodies (1:500, M0851; DakoCytoMation), respectively. Sections were incubated for 60 min using an immunohistochemistry kit [EnVision + Dual Link System-HRP (DAB+)] (Dako Cytomation), and sections were counterstained with hematoxylin to identify nuclei. The number of Ki-67-labeled cells, expressed as the proportion of total cells, was used to determine the lung cell proliferation rate.
counted. All analyses were performed on coded slides by a single observer (Brew) blinded to the experimental groups.

Quantitative real-time polymerase chain reaction. To provide information on lung injury, we measured mRNA levels of early response genes and cytokines that are highly expressed following MV that reflect the severity of lung injury and that are thought to play an important role in lung injury manifestation (24, 49). Connective tissue growth factor (CTGF), early growth response 1 (EGR-1), cysteine rich 61 (CYR-61), interleukins-1β (IL-1β), -6 (IL-6), and -8 (IL-8), tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β) mRNA levels in lung tissue were measured by quantitative real-time polymerase chain reaction (qRT-PCR) using ovine-specific primers (49). The “housekeeping” gene 18S was used to account for minor differences in sample preparation between animals. Total RNA was extracted and DNase-treated, and 1 μg of RNA was reverse-transcribed into cDNA (Superscript III cDNA synthesis kit; Invitrogen). qRT-PCR was performed using an Applied Biosystems 7900HT real-time PCR machine using reactions that contained cDNA template, forward and reverse primer, SYBR green (Platinum SYBR Green qPCR Supermix-UDG; Invitrogen Life Technologies), and nuclelease-free water. The thermal profile used to amplify the PCR products included an initial 2-min incubation at 95°C followed by 35–40 cycles of denaturation at 95°C for 3 s, annealing at 60°C for 20 s, and elongation at 72°C for 20 s. Fluorescence was recorded after each 72°C step. Dissociation curves were performed to ensure that a single PCR product had been amplified for each primer pair. A control sample containing no template was included in each run, and each sample was measured in triplicate. A threshold value (CT value) for each sample was determined. Minor differences in the amount of cDNA template added to each reaction were accounted for by subtracting the CT value for 18S from the CT value for the gene of interest and expressing it relative to the mean mRNA levels of the gene of interest in control fetuses.

Statistical analysis of data. Data are expressed as means ± SE. For all morphological analyses, comparisons were made using a nested ANOVA with the field of view, lung lobe, and treatment as factors. qRT-PCR data were compared by t-test. Differences with P values <0.05 were considered statistically significant.

RESULTS

Blood gas and electrolyte status. During the period of ventilation, there were no significant differences between control and MV fetuses in pH, PaCO₂, PaO₂, SaO₂, hematocrit, or concentrations of hemoglobin, glucose, and lactate in arterial blood. Following recovery from surgery, the fetal blood gas parameters were stable and indicated that all fetuses remained healthy until necropsy.

Necropsy data. Fetal body weights, relative wet and dry lung weights, and fixed lung volumes were not different between control and MV fetuses, both at 1 and 15 days after MV (Table 1). In the MV + 1 day group, there was a trend toward a lower lung volume following MV compared with controls (P = 0.09, Table 1).

DNA and protein concentration in lung tissue. The DNA concentration of lung tissue in MV fetuses was not different from controls, both at 1 and 15 days after MV (Table 1). Similarly, the total protein concentration of lung tissue was not significantly different between MV and control lungs, at either 1 or 15 days after MV. There was, however, a significant

Table 1. Fetal body weights, lung weights, volumes, DNA and protein concentration for MV and control fetuses 1 and 15 days after MV

<table>
<thead>
<tr>
<th></th>
<th>C + 1 Day</th>
<th>MV + 1 Day</th>
<th>C + 15 Days</th>
<th>MV + 15 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>6</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Body wt, kg</td>
<td>2.6 ± 0.2</td>
<td>2.8 ± 0.2</td>
<td>4.9 ± 0.3</td>
<td>4.1 ± 0.3</td>
</tr>
<tr>
<td>Wet lung wt, g/body wt, kg</td>
<td>37 ± 2.2</td>
<td>36 ± 2.1</td>
<td>40 ± 8.1</td>
<td>44 ± 8.9</td>
</tr>
<tr>
<td>Dry lung wt, g/body wt, kg</td>
<td>4.09 ± 0.3</td>
<td>4.1 ± 0.2</td>
<td>3.59 ± 0.2</td>
<td>3.47 ± 0.3</td>
</tr>
<tr>
<td>Left lung volume, cm³/body wt, kg</td>
<td>49 ± 3.1</td>
<td>41 ± 3.0</td>
<td>58 ± 4.4</td>
<td>59 ± 4.9</td>
</tr>
<tr>
<td>DNA concentration, mg/g tissue</td>
<td>4.1 ± 0.18</td>
<td>4.03 ± 0.3</td>
<td>2.6 ± 0.19</td>
<td>2.3 ± 0.21</td>
</tr>
<tr>
<td>Protein concentration, mg/g tissue</td>
<td>28.4 ± 1.4</td>
<td>31.6 ± 1.1</td>
<td>23.2 ± 3.5</td>
<td>18.7 ± 3.1</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of fetuses. C, control; MV, mechanical ventilation.

Fig. 1. Examples of lung injury in mechanical ventilation (MV) + 1 day lungs stained with hemotoxylin and eosin. These lungs showed heterogeneous injury, hypercellularity, and enlarged connective tissue septa (arrow) (A); bronchioles that displayed epithelial injury and contained debris within the lumen (arrow, B); and airspaces that contained red blood cells (arrow) indicative of hemorrhage (C). Scale bar = 100 μm for A and 50 μm for B and C.
decrease in pulmonary DNA and protein concentration of both MV and control groups between 1 and 15 days after the MV period (see Table 1).

General lung morphometry. At 1 day after MV, lung tissue showed heterogeneous injury. Injured regions of MV lungs displayed hypercellularity, enlarged alveoli, and regions of atelectasis as well as areas containing red blood cells, indicative of localized hemorrhage (Fig. 1C). Noninjured regions of the MV lungs appeared structurally normal and similar to age-matched control lungs. In fetuses examined 15 days after MV, lung structure showed normal morphology with no detectable regions of injury (Fig. 2H).

Percentage of lung occupied by tissue. At 1 day after MV, the percentage of lung occupied by tissue (48.3 ± 3.0%) was significantly greater than in controls (36.8 ± 2.4%, \( P < 0.001 \)). Fifteen days following MV, ventilated lungs had significantly less tissue area (29.6 ± 2.3%) than control lungs (34.7 ± 2.2%, \( P = 0.02 \), Fig. 2).

Elastin deposition and abundance. In control fetuses at 1 and 15 days after MV, elastin staining in the distal lung parenchyma was mostly localized in bundles at the tips of secondary septal crests and to a lesser extent in the alveolar walls (Fig. 3, D and F). In fetuses examined 1 day after MV, elastin deposition appeared abnormal; deposition was predominantly thin, ribbon-like fibers in primary septal walls rather than at the tips of secondary septal crests (Fig. 3E). However, at 15 days after MV, elastin deposition was similar to that of controls (Fig. 3G).

![Fig. 2. Lung morphology and percentage tissue space in MV and control (C) fetal lungs. Light micrographs stained with hematoxylin and eosin depicting lung morphology in C 1 day (A and E), MV + 1 day (B and F), C + 15 days (C and G), and MV + 15 days (D and H) lung tissue. The percentage tissue space of the lung was greater in MV + 1 day (B and F) than in matched control fetuses (A and E); the interstitium was thicker with an increase in tissue cellularity. The low-power image of the MV + 1 day fetus (F) shows heterogeneous airspace expansion and hemorrhage (arrow). Fifteen days after MV, fetuses (D and H) had less tissue space than control lungs (C and G); lungs in both groups displayed uniform expansion and thinning of the airspace wall. Values that do not share a common letter are significantly different from each other \( P = <0.05 \), scale bar = 10 \( \mu \text{m} \) (A–D) and 200 \( \mu \text{m} \) (E–H)].
The relative abundance of elastin in lung tissue in MV + 1 day fetuses (3.9 ± 0.2%) was significantly less than in age-matched controls (5.2 ± 0.5%, P = 0.01, Fig. 3A); however, when quantified 15 days after MV (5.6 ± 0.7%), there was a significant increase compared with age-matched controls (4.8 ± 0.4%, P = 0.02, Fig. 3A).

Secondary septal crest density. At 1 day after MV, secondary septal crests appeared stunted and thicker than in controls, as well as occupying a significantly smaller proportion of the tissue (5.5 ± 0.7% in MV vs. 9.2 ± 0.5% in controls, P < 0.001). Fifteen days after MV, secondary septal crest density and morphology were not different between MV and control lungs (10.7 ± 0.8% in MV vs. 10.3 ± 0.6% in controls, Fig. 3C). With increasing age, sepal crest density in control fetuses underwent a small but significant increase (~16%, P = 0.01, Fig. 3C). In comparison, MV fetuses underwent a very large increase in septal crest density over the same time period (~95%, P < 0.001).

Collagen deposition and abundance. In control fetuses, collagen appeared as discrete dense fibers within the alveolar wall (Fig. 3, H and J). At 1 day after MV, the collagen deposition appeared disorganized, and fibers were present in thinner, highly folded ribbons, diffusely located throughout the perialveolar tissue (Fig. 3I). Fifteen days after MV, collagen deposition in both control and ventilated fetuses appeared similar to that in controls after 1 day (Fig. 3, J and K). The proportion of collagen staining in the distal lung was lower in MV fetuses both 1 day (24.8 ± 1.0%) and 15 days (26.9 ± 1.1%) after MV, relative to their age-matched controls (C + 1 day 26.9 ± 1.4%, P = 0.007; C + 15 days 32.0 ± 1.4%, P = <0.001, Fig. 3B).

Alveolar myofibroblasts. At 1 day after MV, the relative abundance of α-SMA, a marker of myofibroblasts, was not different from that in control fetuses (22.9 ± 0.9% in MV vs. 22.2 ± 1.6% in controls, Fig. 4A). Fifteen days following MV, there was significantly less α-SMA staining in ventilated lungs.
Luminal debris was uncommon in control fetuses and accounted for much less of the luminal space; most often, it consisted of a few red blood cells within the lumen. At 1 day after MV, bronchiolar debris in MV lungs was twice as prevalent (39.3 ± 4.7%) as in controls (19.1 ± 2.4%, \( P < 0.05 \), Fig. 5D). At 15 days after MV, the proportion of bronchioles that contained debris was not significantly different between MV (23.2 ± 4.9%) and control (13.4 ± 3.2%) fetuses (Fig. 5D).

**Bronchiolar epithelial injury.** In ventilated fetuses, epithelial injury was present in 41.1 ± 7.6% of bronchioles 1 day after MV (Table 2 and Fig. 5E). Sixty-five percent of this injury was categorised as severe, i.e., \( >180^\circ \) of the epithelium was affected (Table 2). At 1 day after MV, fetuses had a significantly higher proportion of injured bronchioles (41.1 ± 7.6%, Table 2) than their controls (8.2 ± 2.2%). Fifteen days after MV, the proportion of bronchioles that were injured was 8.8%, which was not different from that in controls (5.6%), and none of the MV or control fetuses had bronchioles that were classified as severely injured.

**Mucin-containing cells in the bronchiolar epithelium.** At 1 day after MV, the proportion of epithelial mucin-containing cells in bronchioles (5.1 ± 0.4%) was less than in control fetuses (7.6 ± 1.7%, Fig. 5C). Fifteen days after MV, there was no difference in the proportion of mucin-containing cells between MV (12.2 ± 3.2%) and control (14.1 ± 4.0%) fetuses (Fig. 5C).
Lung inflammatory and injury gene expression. There was no difference in the relative mRNA levels of CTGF, CYR-61, EGR-1, IL-1\beta, IL-6, IL-8, TNF-α, and TGF-β, between MV and control lungs at 1 day after MV (Table 3).

**DISCUSSION**

This study demonstrates that the very immature ovine lung, at a similar stage of lung development to that of very preterm infants, has the capacity to repair itself following a brief episode of injurious MV. We have previously shown that MV of the extremely immature ovine lung (equivalent to \(~25\) wk of human gestation) for 6–12 h causes structural changes to the parenchyma and bronchioles consistent with the structural changes observed in BPD, which persist for up to 7 days after MV (3, 38). In the present study, we ventilated lungs at a later developmental stage, the late saccular to early alveolar stage of lung development (2), when MV is often required for respiratory support. With just 2 h of injurious MV using normoxic gas, we produced considerable structural injury evident 1 day after the injurious MV. The resolution of lung injury, assessed of the extremely immature ovine lung (equivalent to \(~25\) wk of human gestation) for 6–12 h causes structural changes to the parenchyma and bronchioles consistent with the structural changes observed in BPD, which persist for up to 7 days after MV (3, 38). In the present study, we ventilated lungs at a later developmental stage, the late saccular to early alveolar stage of lung development (2), when MV is often required for respiratory support. With just 2 h of injurious MV using normoxic gas, we produced considerable structural injury evident 1 day after the injurious MV. The resolution of lung injury, assessed

**Table 2. Bronchiole epithelial injury severity in MV and control fetuses**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>No. of Airways</th>
<th>No. Intact</th>
<th>No. Injured</th>
<th>Injured, %</th>
<th>No. Mild</th>
<th>No. Moderate</th>
<th>No. Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>C +1 Day</td>
<td>8</td>
<td>305</td>
<td>280</td>
<td>25</td>
<td>8.2</td>
<td>20 (80)</td>
<td>0 (0)</td>
<td>5 (20)</td>
</tr>
<tr>
<td>MV +1 Day</td>
<td>6</td>
<td>251</td>
<td>146</td>
<td>105*</td>
<td>41.1*</td>
<td>21 (20)</td>
<td>16 (15)</td>
<td>68 (65)</td>
</tr>
<tr>
<td>C +15 Days</td>
<td>5</td>
<td>270</td>
<td>255</td>
<td>15</td>
<td>5.9</td>
<td>15 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>MV +15 Days</td>
<td>7</td>
<td>215</td>
<td>196</td>
<td>19</td>
<td>9.7</td>
<td>16 (84)</td>
<td>3 (16)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Percents are in parentheses. Mild injury: <45° bronchiole epithelium detached or absent; moderate: 45°–180° bronchiole epithelium detached or absent; extreme: \(>180\)° bronchiole epithelium detached or absent. Injured data represent total no. of mild, moderate, and severely injured bronchioles. (*\(P < 0.05\))
15 days after MV (just before term), occurred in the absence of continuing MV and without any further treatment or intervention.

We modified an established model of VILI (3) to assess the ability of the very immature lung to repair in the absence of continued ventilation. Following a brief period of injurious MV, fetuses were replaced in the uterus to continue normal development in the absence of other potentially injurious procedures. Our previous studies (3, 38) indicate that evidence of injury and altered development persist for 7 days after MV; therefore, in the present study, we extended the recovery period to 15 days. We found that our MV protocol caused extensive lung injury, similar to that observed by Allison et al. (3) after 12 h of MV, and that, after 15 days, the repair of lung tissue and bronchioles was virtually complete, with few detectable differences from controls.

Using the fetus to study the effects of MV on the immature lung has many advantages. Fetal support by the placenta enables us to ventilate the lungs with standardized settings at a very immature stage of lung development, while normal blood gases are maintained. This avoids the high mortality rates that would occur in an ex utero model (and in humans) at this very early stage of lung development. Fetal MV also enables us to assess how MV, in the absence of other contributing factors such as supplemental oxygen, impaired nutrition, and infection, injures the very immature lung and to assess its capacity for repair as well as the processes involved. This is important, since little is known about the ability of the immature lung to recover from injury.

At 1 day after MV, lung parenchyma and bronchioles displayed structural abnormalities similar to those seen in preterm infants with BPD. Changes in lung parenchyma included increased tissue area, edema, atelectasis, reduced secondary septa density (26), and disorganized collagen and elastin structure. In the bronchioles, we found epithelial thickening (13, 38, 47), loss or detachment of the epithelium (34, 44), as well as the presence of luminal debris. As in BPD, the injury was heterogeneous, indicative of nonuniform inflation, which is typical of stiff, immature, surfactant-deficient lungs (43). The increase in percent tissue space is consistent with previous BPD models, including those using baboons (13) and fetal sheep (3, 40).

In an attempt to understand the basis for the increase in lung tissue area at 1 day after MV, we measured cell proliferation rates but found that they were not different from values in controls. This suggests that cell proliferation rates may have increased earlier in response to MV, as previously reported (3), and had returned to control levels by 1 day. Indeed, the Ki-67 antigen, used as a maker of cell proliferation, has a relatively short half-life of only 90 min (22). Other factors that may have contributed to the increased percentage of tissue space include atelectasis and tissue edema.

It is apparent that the lung parenchyma and bronchioles underwent considerable repair during the recovery period, since the lung tissue and bronchioles showed no evidence of injury at 15 days after MV. Specifically, at 15 days after MV, the tissue space was 39% lower than at 1 day after MV, whereas the percentage of tissue space was reduced by only 5% over the same period in controls. Although resolution of edema, atelectasis, and alveolar injury likely contributed to this reduction, the MV lungs displayed less tissue than age-matched controls after 15 days. The thinner septal walls and reduced bronchiolar epithelial thickness present following 15 days of recovery suggest that the maturation of lung structure was enhanced in fetuses exposed to MV.

Despite considerable lung injury, molecular markers of injury, in particular the expression of inflammatory cytokines and early response genes, were not elevated 1 day after MV. Expression of these genes increases very rapidly in the immature lung following MV before declining. For example, preterm lambs that received brief MV had 10- to 120-fold increased mRNA expression levels of IL-1β, IL-6, IL-8 (24, 41, 49), and early response genes (49). Early response genes (49) and IL-1β, IL-6, and IL-8 (36, 49) mRNA expression increase significantly and then decline during 2 h of MV, and EGR1, IL-1β, and IL-6 mRNA levels normalize by 6 and 1 day after MV (24).

In our study, it is likely that the expression of early response and inflammatory genes was elevated during and soon after the period of MV but were normalized by 1 day, suggesting that, by 1 day, the initial injury response has ceased. It is likely that structural repair takes up to 7–15 days to occur, commencing with normalization of injury gene mRNA levels within hours, followed by ongoing structural repair in the absence of continued MV. Further investigation of these mechanisms is required.

Secondary septal crests are definitive features of alveoli, and their reduced numbers and stunted appearance have been described in preterm infants with BPD (7) as well as in lambs following MV (40). The mechanism responsible for the reduction in secondary septal crest density within 1 day of MV is unknown, but it is possible that they are mechanically disrupted by overinflation and regress back into the primary septa (3, 4). Such a process could account for the altered elastin deposition, which was predominantly seen in the saccular-alveolar wall rather than at the tips of secondary septa. Edema, hypercellularity, and/or atelectasis may also contribute to reduced secondary septa density, in addition to the destruction of secondary septa.
Fifteen days after MV, secondary septal crest density was not different from that of control lungs, indicating that, in the absence of further intervention, the developing lung is capable of repair; importantly, alveolarization and septation are not permanently impaired. Indeed, over the 15-day recovery period, secondary septal crest density increased by 95% in MV-exposed fetuses, whereas it increased by only 16% over this time in controls. Similarly, Allison et al. (3) found that secondary septal crest density was restored 7 days after in utero ventilation at 110 days GA in fetal sheep. This important finding, in conjunction with the decreased proportion of tissue space, means that MV-exposed lungs are likely to have a similar surface area for gas exchange and blood-gas diffusion distance as control lungs.

Disorganization of extracellular matrix (ECM) proteins is another feature of BPD (9). Pulmonary ECM is exposed to external mechanical load during MV, which could contribute to rupture of ECM proteins (39). This has previously been demonstrated in experimental (6, 20) and clinical (16) studies of BPD.

Infants with BPD have increased elastin synthesis (46) in their lungs, and this feature is replicated in animal models of BPD (40). However, elastin synthesis was not elevated in the present study, although its deposition was abnormal, which could have contributed to impaired septation or may be a consequence of it. It is possible that, in our study, the ventilation period was too brief to have stimulated elastogenesis; this could arise because the injury-induced increase in the activation of genes that regulate increased production and assembly of elastin are time-dependent (8).

The relative abundances of collagen and elastin in the lung were reduced 1 day after MV, and the collagen fibers appeared highly folded and more dispersed throughout the tissue compared with controls, potentially altering the mechanical properties of lung tissue. Similarly, MV in preterm baboons reduces collagen expression and increases the expression of matrix metalloproteinase 9, which is known to break down type I collagen (45). However, it is also possible that the decreased relative collagen and elastin abundances we observed were due to the increase in tissue space, rather than loss of ECM. Indeed, pulmonary elastin and collagen fiber structure after 15 days of recovery from MV was no longer different from that of controls.

Myofibroblasts synthesise both collagen and elastin and play an important role in both lung development and lung remodeling following injury (42). The abundance of myofibroblasts is increased in the lungs of infants with BPD (29, 48) and in animal models of BPD (40). Increased numbers of myofibroblasts are thought to primarily result from increased myofibroblast differentiation, which is also associated with increased ECM deposition within the lung (51). However, we found that 1 day after MV, the relative abundance of myofibroblasts in lung tissue was not different from that of controls, potentially due to a similar increase in tissue space. In accordance with this finding, the relative abundance of elastin and collagen was also not increased.

It is likely that myofibroblast stimulation following MV in the immature lung is influenced by multiple factors, including the stage of lung development and ventilatory parameters, such as $F_{O_2}$ and MV duration (4). Although increased myofibroblast expression occurs in infants with BPD as well as in some animal models (40), other studies have observed no change in myofibroblast content following MV of the immature lung (3, 25), as seen in the present study.

Further insults imposed on the immature lung, such as inflammation or hyperoxia, in addition to MV, may delay or inhibit the repair process described in this study, possibly leading to the development of BPD. In addition, the fetal lung may respond differently to injury compared with the postnatal lung by having greater repair capacity following injurious stimuli. Lung repair and plasticity have previously been reported in other models of injury (30, 35), and it is known that the development of BPD is exacerbated by multiple insults or challenges. The mechanisms involved in normal and abnormal repair processes during lung development are complex and require further investigation.

Our study confirms that brief MV of the very immature lung causes considerable structural injury in the parenchyma and bronchioles at 1 day. Importantly, we show, for the first time, that the immature lung is capable of virtually total repair within 15 days in the absence of further treatments. Our model of tissue repair following MV-induced injury to both the lung parenchyma and bronchioles will enable us to identify repair processes in the immature lung. This understanding could provide valuable data for the development of therapeutic strategies for preterm infants at risk of developing BPD.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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