Effects of vagal denervation on cardiorespiratory and behavioral responses in the newborn lamb

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Received 24 January 2001; accepted in final form 21 June 2001

Lalani, Salim, John E. Remmers, Francis H. Green, Ashfaq Buhkari, Gordon T. Ford, and Shabih U. Hasan. Effects of vagal denervation on cardiorespiratory and behavioral responses in the newborn lamb. J Appl Physiol 91: 2301–2313, 2001.—Recently, Wong et al. (Wong KA, Bano A, Rigaux A, Wang B, Bharadwaj B, Schurch S, Green F, Remmers JE, and Hasan SU, J Appl Physiol 85: 849–859, 1998) demonstrated that fetal lambs that have undergone vagal denervation prenatally do not establish adequate alveolar ventilation shortly after birth. In their study, however, vagal denervation was performed prenatally and the deleterious effects of vagal denervation on breathing patterns and gas exchange could have resulted from the prenatal actions of the neurotomy. To quantify the relative roles of pre- vs. postnatal vagal denervation on control of breathing, we studied 14 newborn lambs; 6 were sham operated, and 8 were vagally denervated below the origin of the recurrent laryngeal nerve. Postoperatively, all denervated animals became hypoxemic and seven of eight succumbed to respiratory failure. In vagally denervated lambs, expiratory time increased, whereas respiratory rate, minute ventilation, and lung compliance decreased compared with the sham-operated animals. In the early postoperative period, the frequency of augmented breaths was lower but gradually increased over time in the denervated vs. sham-operated group. The dynamic functional residual capacity was significantly higher than the passive functional residual capacity among the sham-operated group compared with the denervated group. No significant differences were observed in the prevalence of various sleep states and in the amount of total phospholipids or large- and small-aggregate surfactants between the two groups. We provide new evidence indicating that intrauterine actions of denervation are not required to explain the effects of vagal denervation on postnatal survival. Our data suggest that vagal input is critical in the maintenance of normal breathing patterns, end-expiratory lung volume, and gas exchange during the early neonatal period.

augmented breaths; fetus; functional residual capacity; gas exchange; hypoxemia; pulmonary mechanics; pulmonary surfactant; sighs; sleep states

AS OPPOSED TO EPISODIC FETAL breathing movements, breathing becomes continuous at birth. The mechanisms of the establishment of continuous breathing remain unknown. Evidence suggests that vagal innervation plays an important role in the maintenance of normal postnatal breathing patterns and alveolar ventilation (7, 12–14, 50). In these studies, however, vagal denervation was performed in the cervical region, which could have resulted in the compromise of upper airway function, including vocal cord paralysis. Furthermore, the animals in these studies were either anesthetized, tracheotomized, and/or studied after the immediate newborn period (7, 14, 32, 48, 50).

To avoid the limitations associated with the previous studies, Wong et al. (57) performed intrathoracic vagal denervation prenatally to investigate the role of vagal innervation on the establishment of breathing and gas exchange at birth. In this study, sham-operated animals established effective gas exchange, whereas vagally denervated animals developed profound respiratory failure. However, prenatal vagal denervation might have had confounding effects on a number of physiological adaptations occurring during transition from fetal to neonatal life, including increases in pulmonary blood flow, surfactant secretion, and lung liquid absorption (18, 26).

Although the study by Wong et al. (57) provided unequivocal evidence that vagal innervation is critical for the establishment of continuous breathing and adequate gas exchange at birth, the mechanisms for the postnatal respiratory failure remain unclear. Two possibilities exist: 1) the intrauterine vagal denervation may have impaired fetal lung development (1), or 2) after birth, vagal denervation may have compromised breathing, most likely by eliminating the afferent feedback from the lung. Because Wong et al. performed vagal denervation 10–14 days before birth, both types of effects could have contributed. On the other hand, evidence suggests that vagal innervation plays an important role in the regulation of breathing during the early postnatal period. Consequently, elucidating the contribution of the vagal afferents in establishing and maintaining breathing and pulmonary gas exchange immediately after birth represents an area of research of fundamental significance to neonatal respiratory control.
To exclude the confounding effects of prenatal vagal denervation on breathing patterns and gas exchange, we performed bilateral intrathoracic vagal denervation during the early postnatal period. We hypothesized that vagal innervation is critical for pulmonary gas exchange and maintenance of normal breathing patterns in the early neonatal period.

**METHODS**

**Surgical procedures.** All procedures were performed in accordance with the Canadian Council on Animal Care, and the study protocol was approved by the animal care committee of the University of Calgary. Fourteen newborn lambs underwent either intrathoracic vagal denervation (n = 8) or sham surgery (n = 6) within 24 h of birth. Surgery was performed under general anesthesia using 4% halothane in oxygen for induction and 1.5% halothane for maintenance. With the use of sterile techniques, a 2-cm incision lateral to the trachea and immediately below the thyroid cartilage was made in the neck to expose the jugular vein and carotid artery. Polyvinyl catheters (1 mm ID, 2 mm OD; Portex, Hythe, Kent, UK) were inserted 7 cm into the jugular vein and carotid artery and secured in place. The arterial catheter was used to draw blood samples for arterial pH and blood-gas tension measurements and to record arterial blood pressure and heart rate, whereas the venous catheter was used to administer antibiotics and fluids intra- and postoperatively. During surgery, rectal temperature (39°C) and arterial pH (7.35–7.45) and blood-gas tensions (arterial P\(_{CO_2}\) (P\(_{CO_2}\)) = 35–45 Torr and arterial P\(_{O_2}\) (P\(_{O_2}\)) = 90–110 Torr) were maintained by adjusting the heating pad temperature and ventilator settings, respectively.

After implantation of vascular catheters, the lamb was placed on its left side and a 2-cm incision was made at the fourth intercostal space. The vagus and phrenic nerves were identified, and a 2-cm section of the right vagus nerve was removed below the origin of the recurrent laryngeal nerve. To minimize accumulation of intrapleural air, an 8-Fr chest tube (Argyle) was inserted through a small incision made at the sixth intercostal space, and a purse-string suture was placed around the incision site. The procedure was then repeated on the left side. Through a 2-cm incision made parallel to the ribs at the 10th intercostal space, three diaphragmatic electrodes were implanted into the right costal diaphragm. Although three diaphragmatic electrodes are implanted, only two electrodes (bipolar) are used to obtain the optimum diaphragmatic signal. All the incisions were closed in layers using size 0 silk and placed under water seal at −12 cm H\(_2\)O.

After vagal denervation was performed, lambs were placed in prone position to implant electrodes to record electromyograms (EMG\(_{DKN}\), EMG\(_{NK}\), and EMG\(_{dia}\)). The surgical details for electrode placement have been given elsewhere (24). Briefly, the two ECoG electrodes were placed on the dural surface, 2 cm caudal to the coronal suture line and 3 cm apart through two holes drilled in the skull. To record EMG\(_{NK}\), a 3-cm incision was made in the dorsal neck region, and pair of electrodes were sewn into one of the right nuchal muscles. Two 0.5-cm incisions were made along the superior and inferior orbital ridges and EOG electrodes were implanted as described previously (23). ECoG, EOG, and EMG\(_{NK}\) were used to define various sleep states as described previously (23). All electrode wires (AS 633 Cooner, Chatsworth, CA) were subcutaneously tunneled, secured, and soldered to a Lemo connector (Lemo, Ecublens, Switzerland) for diaphragmatic and sleep-state recordings.

If the animals were unable to establish effective pulmonary gas exchange and spontaneous breathing after surgery, supplemental oxygen and/or manual positive pressure ventilation was administered. Effective pulmonary gas exchange was defined as arterial pH >7.30, P\(_{CO_2}\) <50 Torr, and P\(_{O_2}\) >50 Torr (41). Establishment of spontaneous breathing was defined as a respiratory rate of at least 15 breaths/min.

Augmented breaths were measured using a catheter filled with saline placed in the midesophagus. In the current study, two definitions of augmented breaths were used: 1) a biphasic spontaneous inspiration having at least a twofold increase in esophageal pressure (30, 53) and 2) a biphasic response with at least 50% increase in esophageal pressure and diaphragmatic EMG (EMG\(_{dia}\)) compared with the preceding 10 breaths. We used both definitions, since a twofold increase in tidal volume has been used previously but on a purely arbitrary basis. In contrast, an increased and biphasic nature of esophageal pressure reflects respiratory center output and is more relevant to our current study especially in view of the changes in pulmonary compliance. The esophageal pressure was continuously recorded in both sham-operated and vagally denervated animals.

The animals were considered awake and alert when they were able to open their eyes and lift their heads. To help maintain normal body temperature (39.0°C), we used a neonatal transport incubator during transfer from the operating room to the laboratory and a heat lamp during observations in the laboratory.

**Experimental design.** The experimental design is given in Fig. 1. Postoperation sleep states, EMG\(_{dia}\), arterial blood pressure, heart rate, esophageal pressure, and rectal temperature levels were continuously recorded. The ECoG, EOG, EMG\(_{NK}\), and EMG\(_{dia}\) signals were amplified and filtered appropriately with frequency ranges of 0.5–10 Hz, 5–40 Hz, 50 Hz to 1 kHz, and 50 Hz to 1 kHz, respectively. Arterial blood pressure and heart rates were recorded using a pressure transducer (Statham P23 ID; Gould Instrument Division, Cleveland, OH). Arterial blood samples were drawn every 60–120 min or more frequently if clinically indicated for measurement of arterial pH, and blood-gas tensions were corrected for body temperature (IL 1312 blood-gas manager). No arterial blood samples were withdrawn while the lambs were receiving supplemental oxygen. Esophageal pressure was obtained by placing an 8-Fr feeding tube in the midesophagus and then recorded using a pressure transducer (Statham P23 ID; Gould). All bioelectric signals were displayed on an eight-channel chart recorder (Gould Brush 2800s), digitized, and stored on a videocassette using an eight-channel Neurocorder (DR-886; Neurodata Instruments, New York, NY).

Dextrose (10%) in normal saline was continuously infused intravenously at 90–120 ml kg\(^{-1}\) day\(^{-1}\) to prevent hypoglycemia and dehydration. Two doses of 25 mg/kg of cefazolin sodium in saline (Ancef, Smith Kline Beecham Pharma, Oakville, ON) and 2.5 mg/kg of gentamicin sulfate (Garamycin injectable, Schering Canada, Pointe-Claire, PQ) were administered intravenously every 8 h.

Pulmonary function tests were performed using a 4.5- or 5-Fr endotracheal tube 1 h before surgery, during recovery, and 6 and 24 h after surgery or earlier if respiratory failure occurred. The animals were only intubated during the pulmonary function tests, each lasting ~10–15 min. The rationale for intubation was to avoid air leak and to maintain the accuracy of the data during lung compliance and resistance measurements. The animals were extubated immediately after surgery.
after each pulmonary function test was completed. With the exception of pulmonary function tests, all data were collected while the animals were breathing spontaneously with intact upper airway. Neither assisted ventilation nor continuous positive airway pressure was used once the animals were extubated. The recovery period was defined as the time when the animal was breathing spontaneously. A Fleisch pneumotachograph (size 00), a Hans-Rudolph flow occluder (Hans Rudolph, Kansas City, MO), and Validyne pressure transducers (DP45-32-A-3.5-S-4-D and DP45-14-A-3.5-S-4-D, Validyne Engineering, Northridge, CA) were used to measure tidal volume, respiratory rate, minute ventilation, inspiratory and expiratory times, and static and dynamic compliance and resistance. Data were stored on a PC (Dell 233 MHz) and then analyzed with the use of the Anadat, Labdat, and Auto programs (version 5.2, RHT-Info Dat, Montreal, PQ).

For calculation of compliance, resistance, and the time constant, the pressure signal during occlusion and the flow signal during exhalation were used. The data were recorded for 4 s with a sampling rate of 250 samples per second for each channel. The high sampling rate was necessary to record the rapid changes in the flow signal in small volumes, especially in noncompliant lungs. Static respiratory system compliance and resistance were measured by occluding airflow at end inspiration from 10 breaths in each subject for each period of pulmonary function testing (29). Dynamic lung compliance and pulmonary resistance were calculated using the computerized method of multiple linear regression (29). To obtain information regarding lung volume and expiratory braking, flow-volume graphs were constructed after end-inspiratory occlusions for 300 ms. After occlusion, the flow signal was integrated to obtain volume and a flow-volume curve was constructed. The slope of the curve was established by using regression analysis with a regression coefficient greater than 0.99. The regression line (slope) was then extrapolated to obtain the volume that represents the complete emptying of the lung to the normal functional residual capacity (FRC), whereas the y-intercept (flow) of the regression line provides the peak flow (see Fig. 6). The static lung compliance was calculated as the relation of occlusion pressure to the extrapolated volume and the total lung resistance as the peak flow to the occlusion pressure.

The animals were euthanized using Euthanyl (MTC Pharmaceuticals, Cambridge, ON) either 24 h postoperatively or earlier if severe respiratory failure occurred (pH < 7.0). At autopsy, sectioning of both vagi and the integrity of the phrenic nerves were confirmed in all animals. The right middle lobe was tied using size 4 silk to avoid leakage of bronchoalveolar lavage as described below. A 1-cm² section of the right middle lobe was removed and fixed with 1% osmium tetroxide in fluorocarbon and postfixed in 2.5% freshly prepared gluteraldehyde. The lung tissues were embedded in epon, sectioned and stained with uranyl acetate/lead citrate, and examined using a Hitachi 7000 transmission electron microscope for presence or absence of type II cells and lamellar bodies in a blinded fashion.

Bronchoalveolar lavage was performed on both lungs except the right middle lobe while still intact. Saline (100 ml/kg) was infused using the gravitational method at 20 cmH₂O, intratracheally in four aliquots as described previously, and gently withdrawn using a 60-ml syringe. The gravitational method was used to minimize precipitous lung distension to avoid lung rupture and an inadvertent surfactant release (43). The lavagate was centrifuged for 8 min at 150 g to remove cells and debris. The supernatant was then centrifuged for 20 min at 40,000 g (Ti 60 rotor, Beckman) to separate the lavagate into large and aggregates of surfactant. Large aggregates were resuspended in 15 ml of saline and analyzed for total phospholipids using Bartlett’s method (3). Surface tension‐lowering properties were assessed using the captive bubble technique as described by Schürch et al. (49).

After the lavage, the lungs were fixed using formalin at 25 cmH₂O. Tissues were processed through paraffin, and standard 5-μm sections were stained with hematoxylin and eosin for light microscopic examination.

Statistical analysis. The effects of intrathoracic vagal denervation and sham surgery on arterial pH, blood-gas tensions, breathing patterns, pulmonary mechanics, heart rate, arterial blood pressure, and surface tension of the bronchoalveolar lavage were analyzed with the use of ANOVA for repeated measures. If a significant difference was observed, Tukey’s test was performed to determine where the differences were across time but within a given group. Differences in sleep state, postoperative course, and surfactant aggre-
gates were analyzed using Student’s t-test. Regression analysis using the generalized estimating equation approach, a method analogous to ANOVA, was utilized to determine the frequency of augmented breaths and the effects of time within and between the groups. Furthermore, a generalized additive model was used to explore the possible nonlinearity of the slopes of the frequency of augmented breaths. The incidence of manual positive pressure ventilation and oxygen requirement during the postoperative course was analyzed using the Fisher’s exact test. All values are given as means ± SD, and statistical significance was considered as $P < 0.05$.

### RESULTS

**Postoperative course.** The vagally denervated animals were significantly slower to initiate spontaneous breathing after surgery and required manual ventilation and supplemental oxygen to establish effective pulmonary gas exchange compared with the sham-operated group ($P < 0.05$; Table 1). Similarly, sham-operated animals, compared with the denervated animals, were significantly faster in achieving an awake and alert state (0.5 vs. 6 h, respectively; $P < 0.05$). Furthermore, all denervated animals required additional heating via a heat lamp as rectal temperature decreased to 36.6°C by 30 min postoperatively (Table 1). Six of the eight denervated animals developed respiratory failure ~20 h postoperatively, as evidenced by prolonged apneic periods and gasping. In contrast, all sham-operated lambs maintained normal breathing patterns throughout the study course.

**Arterial pH and blood-gas tensions.** Arterial pH and blood-gas tensions were measured while the animals were breathing room air and are given in Table 2. Postoperatively, there were no significant differences in PaCO$_2$ and arterial pH between sham-operated and denervated lambs until 16 and 20 h, respectively. PaO$_2$ was significantly lower in the denervated than in sham-operated lambs immediately after surgery and remained lower until the end of the study compared with the sham-operated lambs ($P < 0.05$).

**Pulmonary mechanics and breathing patterns.** Pulmonary function variables including respiratory rate, inspiratory and expiratory times, tidal volume, minute ventilation, augmented breaths, respiratory system and lung compliance, and respiratory system and pulmonary resistance are given in Figs. 2–5. The respiratory rate and inspiratory and expiratory times were similar in both groups before surgery. However, respiratory rate was significantly reduced in the denervated group after the immediate postoperative period compared with the sham-operated group ($P < 0.05$; Fig. 2A). Vagally denervated animals exhibited a significant increase in inspiratory time compared with sham-operated animals during the recovery period; however, there were no significant differences thereafter (Fig. 2B). In contrast, the expiratory time was consistently twofold higher in the denervated group compared with the sham-operated animals ($P < 0.05$; Fig. 2C).

The tidal volume was similar between the sham-operated and the vagally denervated animals both before and after surgery (Fig. 3A). In contrast, minute ventilation significantly decreased in the denervated group compared with the sham-operated lambs at 6 and 24 h postoperatively ($P < 0.05$; Fig. 3B).

The frequency of augmented breaths is presented in Fig. 4 in five 4-hour postoperative time bins (2–5, 6–9, 10–13, 14–17, and 18–21 h). The first hour could not be included because various data acquisition apparatuses were still being connected to record physiological variables from both groups and vagally denervated animals were still requiring assisted ventilation. Similarly, only two vagally denervated animals were alive more than 21 h after surgery, and a statistical analysis of the data from these two animals would not be appropriate.

### Table 1. Postoperative course

<table>
<thead>
<tr>
<th></th>
<th>Onset of Spontaneous Breathing, min</th>
<th>Manual Positive Pressure Ventilation, no. of animals</th>
<th>Oxygen Requirement, no. of animals</th>
<th>Awake and Alert, min</th>
<th>Duration of Supplemental Heating, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>$10.5 ± 11.8$</td>
<td>$0$</td>
<td>$1$</td>
<td>$33.5 ± 24.5$</td>
<td>$93 ± 66.8$</td>
</tr>
<tr>
<td>Denervated</td>
<td>$70 ± 33.1$</td>
<td>$5$</td>
<td>$7$</td>
<td>$356.3 ± 534.9$</td>
<td>$481.9 ± 420.3$</td>
</tr>
<tr>
<td>$P$</td>
<td>$&lt;0.05$</td>
<td>$&lt;0.05$</td>
<td>$&lt;0.05$</td>
<td>$&lt;0.05$</td>
<td>$&lt;0.05$</td>
</tr>
</tbody>
</table>

Values are means ± SD; $n = 6$ sham-operated animals (Sham) and $n = 8$ vagally denervated animals (Denervated).

### Table 2. Arterial pH and blood-gas tensions at various postoperative time bins (hours)

<table>
<thead>
<tr>
<th></th>
<th>0–0.5</th>
<th>0.5–1</th>
<th>1–1.5</th>
<th>1.5–2</th>
<th>2–3</th>
<th>3–4</th>
<th>4–6</th>
<th>6–8</th>
<th>8–12</th>
<th>12–16</th>
<th>16–20</th>
<th>20–24</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH$_a$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>7.35 ± 0.02</td>
<td>7.37 ± 0.01</td>
<td>7.42 ± 0.01</td>
<td>7.34 ± 0.08</td>
<td>7.34 ± 0.04</td>
<td>7.38 ± 0.02</td>
<td>7.31 ± 0.05</td>
<td>7.33 ± 0.07</td>
<td>7.34 ± 0.05</td>
<td>7.36 ± 0.08</td>
<td>7.33 ± 0.05</td>
<td>7.34 ± 0.07</td>
</tr>
<tr>
<td>Denervated</td>
<td>7.26 ± 0.20</td>
<td>7.18 ± 0.20</td>
<td>7.35 ± 0.07</td>
<td>7.23 ± 0.22</td>
<td>7.26 ± 0.14</td>
<td>7.32 ± 0.07</td>
<td>7.21 ± 0.05</td>
<td>7.29 ± 0.11</td>
<td>7.32 ± 0.09</td>
<td>7.30 ± 0.04</td>
<td>7.34 ± 0.04</td>
<td>7.14 ± 0.02*</td>
</tr>
<tr>
<td>PaCO$_2$, Torr</td>
<td>44 ± 2</td>
<td>45 ± 2</td>
<td>41 ± 0.4</td>
<td>44 ± 2</td>
<td>46 ± 0.6</td>
<td>42 ± 0.4</td>
<td>43 ± 7</td>
<td>43 ± 9</td>
<td>40 ± 5</td>
<td>41 ± 4</td>
<td>40 ± 6</td>
<td>46 ± 5</td>
</tr>
<tr>
<td>Sham</td>
<td>50 ± 9</td>
<td>58 ± 10</td>
<td>40 ± 11</td>
<td>51 ± 8</td>
<td>50 ± 7</td>
<td>42 ± 5</td>
<td>40 ± 3</td>
<td>45 ± 7</td>
<td>46 ± 7</td>
<td>46 ± 6</td>
<td>50 ± 7*</td>
<td>62 ± 15*</td>
</tr>
<tr>
<td>Denervated</td>
<td>42 ± 2</td>
<td>51 ± 3</td>
<td>67 ± 2</td>
<td>57 ± 23</td>
<td>67 ± 15</td>
<td>73 ± 3</td>
<td>72 ± 20</td>
<td>64 ± 16</td>
<td>75 ± 10</td>
<td>72 ± 10</td>
<td>79 ± 13</td>
<td>77 ± 13</td>
</tr>
<tr>
<td>PaO$_2$, Torr</td>
<td>27 ± 4*</td>
<td>41 ± 11</td>
<td>50 ± 7</td>
<td>35 ± 12</td>
<td>38 ± 17*</td>
<td>39 ± 8*</td>
<td>48 ± 8*</td>
<td>38 ± 13*</td>
<td>39 ± 14*</td>
<td>44 ± 16*</td>
<td>42 ± 13*</td>
<td>29 ± 14*</td>
</tr>
</tbody>
</table>

Values are means ± SD. pH$_a$: arterial pH; PaCO$_2$: arterial PCO$_2$; PaO$_2$: arterial PO$_2$. *$P < 0.05$ sham vs. denervated group.
The generalized additive model showed no evidence of nonlinearity in the sham-operated group. However, the relationship with time was more complex in the denervated group, showing evidence of nonlinearity ($P = 0.0013$). The frequency of augmented breaths showed a negative correlation ($P = 0.001$) over time in the sham-operated group, whereas the reverse was true for the denervated group ($P = 0.001$). The degree of linearity and negative correlation in the sham-operated group and the nonlinearity and positive correlation in the denervated group was true for both definitions of the augmented breaths ($P = 0.001$). Furthermore, there was a significant difference in the frequency of augmented breaths over time between the sham-operated and denervated groups ($P < 0.001$; Fig. 4).

Respiratory system and pulmonary resistance using static and dynamic methods, respectively, exhibited no significant differences between sham-operated and vagally denervated animals at any time periods after surgery (Fig. 5, C and D). Lung and respiratory system compliances were significantly lower in vagally denervated animals compared with the sham-operated ani-

Fig. 2. Respiratory rate, inspiratory time, and expiratory time. A: respiratory rate (per minute) significantly decreased in vagally denervated animals by 6 and 24 h postoperatively compared with the sham-operated group. B: no significant differences were observed in inspiratory time except during the recovery period in the denervated animals. C: expiratory time was significantly higher in the denervated lambs during recovery and 6 and 24 h postoperatively compared with the sham-operated group.

The generalized additive model showed no evidence of nonlinearity in the sham-operated group. However, the relationship with time was more complex in the denervated group, showing evidence of nonlinearity ($P = 0.0013$). The frequency of augmented breaths showed a negative correlation ($P = 0.001$) over time in the sham-operated group, whereas the reverse was true for the denervated group ($P = 0.001$). The degree of linearity and negative correlation in the sham-operated group and the nonlinearity and positive correlation in the denervated group was true for both definitions of the augmented breaths ($P = 0.001$). Furthermore, there was a significant difference in the frequency of augmented breaths over time between the sham-operated and denervated groups ($P < 0.001$; Fig. 4).

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Fig. 3. Pulmonary function variables: Vt/kg (A) and MV/kg (B). A: no differences were observed in Vt/kg between sham-operated and vagally denervated animals. B: MV/kg (ml·min⁻¹·kg⁻¹) was significantly lower in vagally denervated animals at 6 and 24 h postoperatively compared with sham-operated animals.

Fig. 4. Augmented breaths. A: frequency of augmented breaths, defined as an inspiration on inspiration and at least 50% increase in esophageal pressure and EMGdia compared with the preceding 10 breaths. The frequency of the augmented breaths decreased over time in the sham-operated group (8.41 – 0.30 × time; $P = 0.001$). In contrast, a significant increase in the slope of the augmented breaths [(8.41 – 5.13) + (0.54 – 0.30) × time] was observed in the denervated group ($P = 0.001$; A). B: frequency of augmented breaths, defined as an inspiration on inspiration and a twofold increase in esophageal pressure compared with the preceding 10 breaths. The sham-operated group showed a decrease in the frequency of the augmented breaths over time (5.97 – 0.20 × time; $P = 0.001$). In contrast, a significant increase in the slope of the augmented breaths [(5.97 – 4.22) + (0.32 – 0.20) × time; $P = 0.001$] was observed in the denervated group. The differences in the direction of the slopes using both definitions (A and B) was also significantly different between the 2 groups over the duration of the study ($P < 0.001$).
mals at 6 and 24 h after surgery (P < 0.05; Fig. 5, A and B).

Representative flow-volume graphs from the sham-operated and vagally denervated animals are shown in Figs. 6 and 7. The sham-operated animal maintained the dynamic FRC to be ~50 ml above the passive FRC (Fig. 6A). In contrast, the dynamic FRC was equivalent to the passive FRC in the denervated animal (Fig. 6B). Overall, the sham-operated animals interrupted their expiration before reaching the “zero” flow, whereas the denervated animals emptied the lungs right to the passive FRC (P < 0.02; Fig. 7).

Sleep states. The three sleep states [non-rapid eye movement (NREM), rapid eye movement (REM), and awake] are given as a percentage of total recorded time in sham-operated and vagally denervated animals in Table 3. No significant differences existed in the distribution of NREM and REM sleep or arousal states between the two groups.

Cardiovascular variables: systolic, diastolic, and mean blood pressures and heart rate. No significant differences were observed between sham-operated and vagally denervated animals in systolic, diastolic, or mean blood pressures and heart rates (Figs. 8 and 9).

Large- and small-aggregate surfactants, surface tension, and light and electron microscopy. Phospholipid content of large- and small-aggregate surfactants were similar in both the sham-operated and the denervated groups. The phospholipid content in large-aggregate surfactant was 39 ± 21 and 35 ± 6 mg/kg in sham-operated and denervated lambs, respectively. The phospholipid levels in small-aggregate surfactant were 61 ± 36 mg/kg in the sham-operated and 58 ± 18 mg/kg in the denervated animals (P > 0.05).

Measurement of the ability of large-aggregate surfactant to reduce surface tension at an air-liquid interface using the captive bubble technique showed no significant differences between the two groups (Fig. 10). Transmission electron microscopy showed no differences in alveolar type II cell morphology, lamellar body content/structure, or tubular myelin formation in the extracellular space (Fig. 11). In addition, light microscopy showed no differences in lung architecture between the two groups (Fig. 12). Specifically, there was no evidence of inflammation or interstitial edema in the two groups. Alveolar edema could not be assessed because the lungs had been lavaged.

DISCUSSION

General. This is the first study to investigate the effects of intrathoracic vagal denervation in unanesthetized and spontaneously breathing animals during early postnatal life. We have shown that vagal denervation leads to hypoxemia and decreased pulmonary compliance. Furthermore, vagal denervation during
this time leads to changes in breathing patterns, including prolongation of the duration of expiration and reductions in respiratory rate and minute ventilation. In the early postoperative period, the frequency of augmented breaths was lower in the denervated group but gradually increased and then plateaued over the course of the study. In contrast, the sham-operated group showed a negative correlation in the frequency of augmented breaths over time. Finally, we find no aberrations in the surfactant system, as evidenced by the absence of changes in biochemical and physical properties of the bronchoalveolar lavage and presence of normal type II cells. The absence of differences between sham-operated and denervated lambs in light and electron microscopy examinations and surface tension suggests that pulmonary edema is not the likely cause of hypoxemia and decreased compliance in vagally denervated animals. Thus our results indicate that postnatal vagal denervation does not directly influence the status of the lung. Rather, its primary effects relate to alteration of neural control of ventilation.

![Expiratory flow-volume curve](image)

**Fig. 6.** Expiratory flow-volume curve. Flow-volume curves of a sham-operated (A) and a vagally denervated (B) animal with identical body weights indicate that the sham-operated animal interrupted expiration (the “knee”) before reaching passive functional residual capacity (FRC). The dynamic FRC is 50 ml above the passive FRC, as calculated by subtracting the real expired volume (67 ml) from the extrapolated volume (117 ml) in the sham-operated animal (A). In contrast, the vagally denervated animal exhibits no difference between the dynamic and passive FRC, indicating that expiration continues until passive FRC (B).

![Comparison of the difference between dynamic and passive FRC](image)

**Fig. 7.** Comparison of the difference between dynamic and passive FRC ($\Delta$FRC) in sham-operated and vagally denervated animals indicates that sham-operated animals maintained a significantly higher $\Delta$FRC compared with the denervated group (*$P = 0.02$).

### Table 3. Distribution of various sleep states as a percentage of total time in sham-operated and vagally denervated animals

<table>
<thead>
<tr>
<th></th>
<th>NREM (%)</th>
<th>REM (%)</th>
<th>Awake (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>34 ± 5</td>
<td>8 ± 5</td>
<td>58 ± 7</td>
</tr>
<tr>
<td>Denervated</td>
<td>35 ± 10</td>
<td>8 ± 6</td>
<td>57 ± 10</td>
</tr>
</tbody>
</table>

Values are means ± SD. NREM, non-rapid eye movement sleep; REM, rapid eye movement sleep.

![Systolic, mean, and diastolic blood pressure](image)

**Fig. 8.** Systolic (A), mean (B), and diastolic (C) blood pressure. Arterial blood pressure data were obtained at various time bins postoperatively up to 24 h. There were no significant differences in systolic, mean, or diastolic blood pressures between sham-operated and vagally denervated animals.
increased PaCO2 and decreased PaO2 and pH near the
Respiratory failure gradually ensued, as evidenced by
alveolar-arterial oxygen difference, and hypoxemia.
problem characterized by a normal PaCO2, increased
nervated animals exhibited an intrinsic gas-exchange
mocapneic throughout the study.
and/or excitatory input provided to the inspiratory
activity by the unmyelinated vagal fibers (8).
and functions to prevent pulmonary atelectasis and to
increase lung compliance and contributes to intrapul-
mocapneic throughout the study.
Postoperative course. In the immediate postoperative
period, denervated animals required manual ventila-
tion, supplemental oxygen, an external heating source
and took longer times to be extubated compared with
the sham-operated animals. This difference is most
likely due to lack of afferent vagal feedback influencing
the breathing pattern, ventilation, lung volume (14),
and/or excitatory input provided to the inspiratory
activity by the unmyelinated vagal fibers (8).
Pulmonary gas exchange. In our current study, de-
nervated animals consistently displayed arterial hy-
poxemia during the postoperative period. We observed
no significant differences in either arterial pH or PaCO2
until the end of the study, when vagally denervated
lambs developed respiratory acidosis and hypercapnea
compared with the sham-operated animals. These ef-
facts cannot be ascribed to surgical instrumentation
because the sham-operated animals underwent similar
surgical manipulation but remained normoxic and nor-
mocapneic throughout the study.
During the early postoperative period, vagally de-
nervated animals exhibited an intrinsic gas-exchange
problem characterized by a normal PaCO2, increased
alveolar-arterial oxygen difference, and hypoxemia.
Respiratory failure gradually ensued, as evidenced by
increased PaCO2 and decreased PaO2 and pH near the
end of the study. Although hypoxemia may arise from
a number of different pathological mechanisms, the
three possible mechanisms relevant to this study are
low ventilation-to-perfusion ratio, right-to-left in-
trapulmonary shunt, or diffusion impairment. We
speculate that the hypoxemia may arise from either
pulmonary atelectasis or perhaps pulmonary edema,
both of which would both result in a low ventilation-
to-perfusion ratio and, possibly, right-to-left intrapul-
monary shunt. Occurrence of these mechanisms is sup-
ported by the observed changes in the pulmonary
mechanics, namely, the reduction in both lung and
respiratory system compliance.
Augmented breaths or sighs, defined as a biphasic
spontaneous inspiratory effort, i.e., an “inspiration on
inspiration,” occurs in almost all mammalian species
and functions to prevent pulmonary atelectasis and to
increase lung compliance and contributes to intrapul-
monary gas diffusion (9, 11, 34, 35, 53). Their fre-
cuency is, however, species dependent, occurring as
frequency as 45 cycles per hour in adult mice vs. 3
cycles per hour in adult humans (34). Furthermore, in
human infants, Cross et al. (9) and Thach and Taeusch
(53) have shown that augmented breaths tend to occur
more frequently on postnatal day 1 compared with
days 2–5.
Mead and Collier (35) and Williams et al. (56) pro-
vided evidence that, in the absence of large inflations,
both lightly anesthetized spontaneously breathing and
artificially ventilated animals experience a decreased
pulmonary compliance. The decrease in transpulmo-
nary pressure was directly proportional to the decrease
in pulmonary compliance, and as few as four inflations
restored the pulmonary compliance to the control val-
ues (56). Furthermore, postmortem examination did
not show gross atelectasis among animals that re-
ceived intermittent inflations (56). Studies by Larrabee
and Knowlton (28) and Knowlton and Larrabee (27)
provided indirect evidence that slowly adapting and
rapidly adapting vagal fibers mediate the inspiratory
inhibitory and excitatory responses to lung inflation,
respectively. Similarly, Glogowska et al. (17) showed
that vagal afferents mediated both spontaneous and
cyanide-induced augmented breaths. However, Bart-
lett (2) showed that both vagal denervation and/or
chemodenervation eliminate spontaneous deep breaths.
It is important to note that hypoxia (10% O2) caused a
severalfold increase in the incidence of sighs compared
with normoxic conditions, suggesting a complex inter-
action between afferent chemoreceptor and vagally me-
diated inspiratory drives (2).
In our current study, during the early postoperative
period, augmented breaths were lower in the dener-
vated group and, along with decreased respiratory rate
and low dynamic FRC, could result in atelectasis and
poor pulmonary compliance (35, 56), which gradually
worsened over time. Provision of supplemental oxygen
might have also exacerbated the pulmonary atelecta-
thesis. Although the frequency of augmented breaths in-
creased in the denervated group after the initial few
hours after surgery, late resurgence of such breaths

Fig. 9. Heart rate analysis of sham-operated and vagally denervated
animals at various time bins postoperatively shows no significant
differences between the 2 groups at any of the time bins postopera-
tively.

Fig. 10. Analysis of surface tension. No differences were observed
between the 2 study groups in the ability of large-aggregate surfac-
tants to reduce surface tension (mN/m) at an air-liquid interface over
a period of 300 s.
might not have been able to reverse the atelectasis, resulting in persistent hypoxemia and low pulmonary compliance. In contrast, depending on the definition of augmented breaths used, sham-operated animals did not manifest a decrease in frequency until 14–18 h postoperatively despite normoxemia. A remarkable and persistent increase in the frequency of augmented breaths in the denervated group, 5 h after surgery, is likely due to hypoxemia, as shown by Bartlett (2) and Glogowska et al. (17). Although evidence from the previous studies performed in anesthetized animals suggests that the decreased or absent augmented breaths due to lack of vagal input are not restored by hypoxemia (2, 17), our data show this not to be the case.

Several possible explanations for the persistence of augmented breaths in our denervated animals can be entertained. The animals in our studies were less than 24 h old and would be expected to have higher number of sighs (9) compared with the older neonates or adult subjects (45, 53). Persistence of sighs in the denervated group after vagal denervation may reflect the net effect of loss of the sigh-promoting action of vagal afferents and the addition of such an effect by hypoxia. After the immediate postoperative period, we did not correct hypoxemia with supplemental oxygen, which might be the underlying stimulatory mechanism as shown previously (2, 17). Furthermore, vagal denervation was performed in the intrathoracic region, which would leave aortic chemoreception and laryngeal afferent input intact, possibly playing a role in the production of augmented breaths. Finally, the relatively noninvasive method of recording the augmented breaths using the esophageal pressure, which correlates well with tidal volume measurements, provided us an opportunity to record breathing patterns over a prolonged period and might have mitigated the effects of acute and short-term studies (9). Further experiments are required to clarify the relative roles of anesthesia, hypoxemia, postnatal age, and the site of vagal denervation on the frequency and depth of augmented breaths.

Toward the end of the study (~16 h postoperatively), vagally denervated lambs exhibited an increase in \(P_{\text{aCO}_2}\) and a decrease in \(pH\) relative to sham-operated animals, which did not increase minute ventilation. This time period was also associated with a lowered respiratory rate and high expiratory times associated with periods of apnea in vagally denervated animals. Similar results were reported by Schwieri (50) who showed that vagal denervation in the newborn leads to decreased \(pH\) and increased \(P_{\text{aCO}_2}\), without any effective stimulation of respiration. Another possible factor that could contribute to the observed apnea is diaphragmatic muscle fatigue as a result of increased anaerobic respiration toward the end of the study (20).

Breathing patterns/pulmonary mechanics. We observed a significant reduction in respiratory rate and an increase in respiratory time, followed by irregular breathing and apneas, in vagally denervated compared with the sham-operated animals. Similar results were described in a study by Schwieri (50), in which cervical vagal denervation in newborns reduced breathing rate that became periodic or gasplike and was followed by apneic episodes (50). Increases in expiratory and inspiratory times have been shown by a number of investigators who have performed either cervical vagal denervation or vagal cooling in rabbits, cats, dogs, and newborn rats (10, 14, 46, 54).

In addition, we observed that minute ventilation was significantly lower in vagally denervated lambs by 6 h postoperatively compared with sham-operated animals. Decreased minute ventilation has also been observed in cervically vagally denervated rabbits, lambs, and rats and may be responsible for the resulting hypoxemia in our vagally denervated lambs (14, 33, 38).

Previous studies have shown up to a threefold increase in tidal volume of vagally denervated rabbits and rats (newborn) compared with control animals (14, 55). However, tidal volume did not change in the denervated lambs compared with the sham-operated animals over the course of the study. The failure of tidal
volume to increase may have two explanations. First, the small increase in inspiratory time would limit the inspiratory volume. Because previous studies showed large increases in inspiratory time, this could lead to an increase in tidal volume. Second, the low lung compliance in vagally denervated animals presumably offsets an increase in the driving pressure to produce a tidal volume comparable to sham-operated animals. These changes in pulmonary function data and mechanics support the hypothesis that vagal-afferent input provides the positive feedback in newborns required for the maintenance of breathing patterns, ventilation, and pulmonary mechanics.

No changes were observed in either static or dynamic respiratory system resistance between the sham-operated and denervated animals. Similar results in adult rabbits were reported by Mortola et al. (38). In addition, our experiments showed that both respiratory system compliance and lung compliance were lower in vagally denervated animals compared with the sham-

Fig. 12. Light micrographs of vagally denervated (A) and sham-operated (B) animals. Light microscopy of lung parenchyma and airways of sham-operated and vagally denervated animals showed no evidence of perivascular pulmonary edema, vascular congestion, or inflammation in either group.
operated group. This may be due to the development of alveolar derecruitment (atelectasis), connective tissue abnormalities, disturbances in surfactant function, or pulmonary edema (6, 19, 25).

Atelectasis would reduce lung volume history over time, depending on the breathing patterns exhibited by the subject. In our study, vagally denervated animals exhibited increased expiratory times without an elevation in inspiratory times. Together with the loss of lower airway afferent input from slowly adapting and rapidly adapting pulmonary vagal receptors to the upper airway, this pattern of breathing could result in substantial derecruitment of alveoli, which would change lung volume history over time and explain the lower lung compliance values obtained from vagally denervated animals compared with the sham-operated animals.

In our present study, we have shown that dynamic FRC was maintained above the passive FRC in the sham-operated animals. In contrast, dynamic end-expiratory lung volume was similar to the passive FRC in the denervated group as determined by expiratory flow-volume curves. In newborns, evidence suggests that end-expiratory lung volume is dynamically maintained above the passive FRC (5, 36). This strategy is essential for a number of reasons, including the presence of an oxygen reserve, to minimize the energetic losses during lung expansion, and to limit the cyclic oscillations in alveolar and blood gas (5). Establishment of a dynamic FRC above the passive FRC in newborns is accomplished via expiratory braking, which serve to prolong the expiratory time constant, resulting in airway pressures more positive than during normal respiration (36, 47, 52). During expiratory braking, lung volume is above the end-expiratory volume and expiratory flow approaches the zero baseline (47).

Studies have shown that expiratory braking may be attained via two separate mechanisms. One mechanism involves the upper airway and larynx, which increases the resistance of airflow during expiration by contraction of the thyroarytenoid muscles, resulting in adduction of the vocal cords (5). In newborns, Fisher et al. (15) showed that this mechanism of expiratory braking lengthens expiratory time by up to 75% during the early newborn period. The second mechanism of expiratory braking involves retardation of the expiratory flow by postinspiratory contraction of the diaphragm and other inspiratory muscles (47). Tonic activity of the diaphragm and intercostal muscles was documented at end expiration in newborns, and lung volume decreased when this activity disappeared during apneic periods (31, 40). In our present study, the animals were endotracheally intubated during pulmonary function tests, eliminating laryngeal braking. Thus braking mechanisms involving the diaphragm and intercostals were responsible for the differences in FRC of the sham-operated and vagally denervated animals. In the lamb and puppy, it has been observed that deflation of the lung whether by opening a tracheal window, thus bypassing the upper airway, or by exposing the airway to an end-expiratory subatmospheric pressure causes an increase in thyroarytenoid activity, resulting in the adduction of the vocal cords. This response reflects an attempt by the animal to maintain an elevated lung volume (42). In addition, when the upper airway is bypassed, postinspiratory diaphragmatic activity will increase in an attempt to maintain lung volume (21). We speculate that the differences in expiratory braking between the two groups would have been greater had the larynx not been intubated.

Pulmonary edema in vagally denervated animals has previously been described by Berry et al. (4). Furthermore, pulmonary edema could lead to dysfunction of the surfactant system, which, in turn, could be responsible for the observed changes in arterial pH and blood-gas tensions. However, in our current study, vagally denervated animals showed no differences in phospholipid content of large- or small-aggregate surfactants compared with sham-operated animals, indicating that total phospholipid levels remained intact in vagally denervated animals. In addition, electron micrographs of tissue samples taken from the right middle lobes of vagally denervated and sham-operated animals depict the presence of tubular myelin formation and secretion of surfactant from lamellar bodies, indicating that secretion of surfactant phospholipids and assembly of tubular myelin from phospholipids and surfactant-associated proteins was preserved in vagally denervated animals. To quantify surfactant function, we measured surface tension-lowering properties of the bronchoalveolar lavage using the captive bubble technique of Schürch et al. (49) and observed no difference in large-aggregate surface tension-lowering properties between the sham-operated and vagally denervated lambs, indicating that the surfactant produced was fully capable of reducing surface tension and promoting stability at the alveolar interface (49).

**Thermoregulation.** Although the primary strategy adopted by adults to increase alveolar ventilation is hyperpnea, many newborn species respond to hypoxemia predominantly with hypometabolism as opposed to hyperpnea (37, 51). Hypoxic reduction in metabolic rate is also supported by the observation that denervated animals were unable to maintain normal body temperatures over the postoperative period and had to be warmed using a heat lamp. Hypoxia decreases body temperature in a number of animal species (16, 39, 44, 57) via a decrease in thermogenesis, although metabolic and ventilatory responses vary depending on species and postnatal age and weight (16).

**Sleep states.** We observed no significant differences in sleep states between the vagally denervated and sham-operated lambs over the 24-h study course; thus the observed decreases in breathing frequency, arterial gas tensions, and pH cannot be explained on the basis of deficiency or excess of one particular sleep state. The prevalence of various sleep states in our denervated and sham-operated animals may not represent the normal (physiological) distribution due to the surgical procedures. However, the lack of difference in the dis-
tribution of sleep states between the two groups suggests that the respiratory failure was not sleep dependent.

*Cardiovascular variables.* Vagal innervation influences heart rate and contractility via a parasympathetic mechanism. To avoid the cardiac effects of vagal denervation, we minimized trauma to the vagal fibers, leading to the cardiac plexus, and observed no significant differences between sham-operated and denervated animals in heart rate, systolic pressure, or diastolic pressure. Absence of changes in postnatal pulmonary blood flow with antenatal intrathoracic vagal denervation has also been previously established by Hasan et al. (22).

In summary, we report that vagally denervated neonatal lambs developed hypoxemia and respiratory failure as well as lower minute ventilation and breathing rate compared with the sham-operated group. Furthermore, in the denervated animals, respiratory system and lung compliance were reduced without any pulmonary histological changes or aberrations in the surfactant system. The results suggest that vagal innervation is necessary for the maintenance of normal breathing patterns and pulmonary gas exchange during the early neonatal period. Because the FRC in the neonates is determined dynamically, it is likely that the deleterious effects of intrathoracic vagal denervation on pulmonary mechanics and gas exchange during the early newborn period are due to a progressive atelectasis secondary to decreased respiratory frequency and reduced end-expiratory lung volume. Furthermore, a deficiency in the frequency of augmented breaths during the early postoperative period in the denervated animals likely exacerbated the progressive atelectasis.

We acknowledge the statistical consultation provided by Dr. Gordon Fick, the technical assistance of Ather Bano, Helena Frndova, Anita Rigaux, Stanley Cheng, and the secretarial support of Marion Taftgart.

Financial support was provided by the Medical Research Council of Canada, the Alberta Heritage Foundation for Medical Research, and in part by the Alberta Children’s Hospital Foundation.

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