**Phrenic Nerve Block and Respiratory Effort in Pigs and Critically Ill Patients with Acute Lung Injury**

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ONLINE DATA SUPPLEMENT

**Methods**

These studies were approved by two different ethics committees: one for animals experiments and one for clinical studies. Both protocols were funded by the Faculty of Medicine of the University of São Paulo.

**Animal Protocol: Pigs**

After the approval by the Ethics Committee for Animal Studies (No. 967/2017, Faculdade de Medicina da Universidade de São Paulo), six female Landrace pigs weighing on average 36.9 kg were continuously monitored using ECG, and pulse oximetry. Animals were fasted for more than 12h with free access to water before the protocols. All protocols started at 9 AM. Protocols were performed between October/2018 and April/2019. Pigs were initially anesthetized with intramuscular ketamine and midazolam, followed by intravenous fentanyl and pancuronium, a neuromuscular blocking agent. A jugular internal vein catheter and a femoral arterial line were placed to sample blood gases, administer vasoactive drugs, and monitor blood pressure. Before starting the protocol, pigs were submitted to a short recruitment maneuver with PEEP of 20 cmH2O and pressure support of 15 cmH2O followed by controlled ventilation on PEEP of 10 cmH2O, respiratory rate of 24 and pressure support of 10 cmH2O for 10 minutes. An arterial blood gas was then collected, and the animal was included in the protocol if they had low shunt levels identified by the sum of PaO2 and PaCO2 > 400 mmHg (1). An esophageal balloon (Nutrivent, Sidam, Mirandola, Italy) was inserted to allow continuous measurement of esophageal pressure (ΔPeso); correct placement was verified using the occlusion technique (2). We measured electrical activity of the diaphragm using a dedicated Neurally Adjusted Ventilatory Assist catheter (Maquet, Sweden). SERVO-i® has a unique function that guides the user for the correct positioning of the catheter. Electrical impedance tomography data were recorded using Enlight monitor (Timpel, São Paulo, Brazil) with 32 electrodes imbedded in a customized silicon belt, placed on the perimeter defining a cross-sectional plane of the thorax at the level of the 6th intercostal space (parasternal line).

To improve reliability of the measurements, several steps were included during monitoring for each tool involved. Before insertion of both esophageal catheters, the distances from the nostrils to the ear lobe, and from the ear lobe to the xiphoid appendix were measured. Neurally Adjusted Ventilatory Assist catheter and the esophageal pressure catheter were placed and tested according to manufacturer instructions. Electrical activity of the diaphragm was analyzed by two or more different people involved in the protocol and if its waveform were not in synchronized with the pig’s respiratory effort or if there were no waveform, the catheter would be further inserted or pulled out. Of note, to improve measures of pleural pressure using the esophageal catheter, we inflated the esophageal catheter balloon to its optimal volume, identified in a balloon pressure-volume curve (3). The esophageal catheter placement was confirmed with the Baydur maneuver. The electrical impedance tomography electrodes were always placed between the fourth and fifth intercostal spaces, to minimize any interference from the diaphragm displacement. After data acquisition, all files were synchronized. Finally, respiratory variables reported were calculated as an average of 20-30 seconds of data collected, hence reducing any bias. An average from a shorter period could have biased data from coughing or sighing; an average from a longer period would likely not provide further information.

The experimental protocol consisted of two phases: (1) lung injury and pressure support titration, and (2) phrenic nerve blockade. In phase 1, we induced severe ARDS by lung lavage with *tween* (an astringent solution) followed by injurious mechanical ventilation. Once a PaO2/FIO2 ratio ≤150 mmHg with a positive end-expiratory pressure (PEEP) of 10 cmH2O was reached, deep sedation was achieved by a continuous infusion of intravenous propofol, ketamine, and remifentanil. Then anesthesia was titrated down until spontaneous inspiratory effort reached a ΔPeso ≥ 10 cmH2O during an airway occlusion procedure. PEEP was maintained at 10 cmH2O during the entire protocol.

Following lung injury and sedation titration, succinylcholine 1mg/kg and propofol 1-2 mg/kg were administered and pigs were briefly ventilated on pressure-controlled ventilation. We then calculated respiratory system compliance (Cdyn), and titrated inspiratory pressure that was kept unchanged after the effect of succinylcholine and propofol had weaned and the pig was back to assisted ventilation. This way, regardless of the pig’s inspiratory effort, we would guarantee VT > 4ml/kg after bilateral phrenic nerve block.

In the current protocol, we followed a predefined sequence, initiating with the left branch of the phrenic nerve. Ten minutes after the left phrenic nerve blockade, the right phrenic nerve blockade was performed following the same steps. We continuously monitored the decrease of electrical activity of the diaphragm and ΔPeso. Of note, in the first four pigs, we also assessed VT and ventilation distribution online on electrical impedance tomography on continuous positive airway pressure during baseline, after left phrenic nerve block, and after right phrenic nerve block (**figures 1A, 1B, and 1C**).

The phrenic nerve emerges from the swine’s cervical plexus between the third and fourth cervical vertebrae. The nerve has a caudal direction, posterior to cervical musculature and vessels, with an intimate relationship with the vagosympathetic trunk. The anatomical landmarks are left subclavian vein, superior vena cava, common carotid artery, internal jugular vein, cutaneous colli muscle, sternocephalic muscle, and longus capitis muscle.

In phase 2, we performed bilateral phrenic nerve block using an ultrasound and a neurostimulator to identify the cervical plexus by the motor response on the ipsilateral arm (Plexygon, Vygon, Italy). The identification was followed by the injection of 20ml of lidocaine 2%.

The following variables were analyzed: electrical activity of the diaphragm, ΔPeso, VT in ml per kg, peak transpulmonary pressure, and driving pressure. VT in ml per kg was calculated as VT/Predicted Body Weight. Predicted body weight was calculated according to previous studies (4). Peak transpulmonary pressure was calculated as pressure support - ΔPeso (**figure 2**). Driving pressure was calculated as VT/Cdyn as no inspiratory pauses were performed. For ventilation distribution analysis, the electrical impedance tomography image was divided in right and left ventilation (**figure 3A**) and into four regions of interest, each covering 25% of the ventrodorsal diameter (**figure 3B**) and the percentage of ventilation in the most dependent region was calculated. Finally, percentage of left lung ventilation was analyzed. Data acquisition in pigs occurred in five time points: before phrenic nerve blockade (Baseline), 4 minutes after left phrenic block (LPB-4 min), 10 minutes after the left phrenic block (LPB-10 min), 4 minutes after the right phrenic block (RPB-4 min), and 10 minutes after right phrenic block (RPB- 10min).

In this experimental study, our primary goal was to evaluate whether this technique was feasible and efficient at reducing VT or peak transpulmonary pressure. Our secondary objectives were to quantify the decrease in electrical activity of the diaphragm, and ΔPeso, and driving pressure. We also aimed to evaluate pigs during continuous positive airway pressure to estimate unassisted VT. Finally, we analyzed ventilation distribution using electrical impedance tomography and assessed pendelluft, a phenomenon that occurs when the pressure generated by diaphragmatic contraction is not uniformly transmitted to the lungs of subjects with ARDS, eventually leading to deflation of the ventral portion of the lung and overdistention of the dorsal portion of the lung during early inflation (5). The gas subject to pendelluft does not contribute to gas exchange and may result in wasted work of breathing and carbon dioxide retention and the only available way to measure pendelluft is with an electrical impedance tomography. Our hypothesis was that bilateral phrenic nerve blockade would reduce or abolish pendelluft.

Of note, to assess pendelluft in anesthetized pig model, we performed a stepwise analysis of flow, pressure, VT, and plethysmogram variation. First, electrical impedance tomography image was divided in regions of interest, each covering 25% of the ventrodorsal diameter (figure 2). Second, we screened flow, pressure, VT, and plethysmogram variation waveforms to identify a respiratory cycle with pendelluft. Third, we analyzed VT in the selected respiratory cycle. Fourth, we calculated VT/plethysmogram variation to quantify the amount of volume, in milliliters, per unit measured in the plethysmogram for every region of interest (ROI). Finally, we calculated the volume that was displaced from the ventral to the dorsal portion of the lungs during early inflation before initiation of the respiratory cycle. The data in the results are expressed as percentage of tidal volume that was displaced from the most independent region of the lung to the most dependent region of the lung before the ventilator was triggered.

The study was over once both sides were blocked. The pigs were then sedated with midazolam, fentanyl and pancuronium and were killed with high dose of potassium chloride.

**Human Protocol**

Between May 2019 and September 2020, patients admitted for ARDS and under assisted spontaneous breathing were screened for inclusion in the study after obtaining Institutional Review Board approval (CAAE number: 02029118.2.0000.0068) and informed consent from legal guardians. Data collection was carried out in the respiratory intensive care unit at Heart Institute (Instituto do Coração) of the Hospital das Clínicas da Faculdade de Medicina da USP. This trial was registered at clinicaltrials.gov (NCT03978845).

Patients eligible to the study protocol had to be older than 18 years old, on invasive pressure support, with a PaO2/FIO2 ratio <300 mmHg, capable of triggering the ventilator, and having a driving pressure greater than 15 cmH2O or with a VT greater than 10 ml/Kg of predicted body weight. Of note, to assess whether a patient had a driving pressure greater than 15 cmH2O, a short inspiratory pause during pressure support ventilation was performed (6, 7). First, we evaluated if a reliable plateau pressure could be obtained. Then, we calculated the driving pressure by subtracting plateau pressure minus PEEP (6). If the driving pressure was higher than 15 cmH2O and/or if the VT was > 10 ml/kg, this patient reached inclusion criteria. Exclusion criteria were: use of long-acting neuromuscular blocking agents for less than 3 hours, pain or Richmond Agitation-Sedation Scale > 0, arterial pH < 7.25, hemodynamic instability or need to increase doses of vasopressor drugs in the past 2h, intracranial hypertension, presence of chest or abdominal fistula, neuromuscular disease, spinal cord trauma, massive ascites, burns in the thoracic region, patients with tetanus or pregnant women.

To improve reliability of the measurements, several steps were included during monitoring for each tool involved. Before insertion of both catheters, the distances from the nostrils to the ear lobe, and from the ear lobe to the xiphoid appendix were measured. Both Neurally Adjusted Ventilatory Assist catheter and the esophageal pressure were placed and tested according to manufacturer instructions. Electrical activity of the diaphragm was analyzed by two or more different people involved in the protocol: if its waveform were not in synchronized with the patient’s respiratory effort, or if there were no electrical activity of the diaphragm waveform, the catheter would be further inserted or pulled out. The esophageal catheter placement was confirmed with the Baydur maneuver. The electrical impedance tomography electrodes were always placed between the fourth and fifth intercostal spaces, to minimize any interference from the diaphragm displacement. After data acquisition, all files were synchronized. Finally, respiratory variables reported were calculated as an average of 20-30 seconds of data collected, hence reducing any bias. An average from a shorter period could have biased data from coughing or sighing; an average from a longer period would likely not provide further information.

The study protocol started with patient monitoring using standard monitoring. Two patients were monitored with bispectral index to assess sedation depth. Concomitant analysis of the Richmond Analgesia-Sedation Scale was also performed. An electrical impedance tomography electrode strap (Timpel Enlight 1800 Model, Timpel, Brazil) was installed in the thoracic region between the 4th and 5th intercostal space. Two esophageal catheters- Nutrivent and Neurally Adjusted Ventilatory Assist catheter- were positioned and calibrated to measure esophageal pressure and electrical activity of the diaphragm, respectively. Flow, pressures (peak, plateau, and PEEP) and VT were obtained from the SERVO-i® ventilator connected to a laptop for continuous capture of these signals using Servo Tracker® and from the electrical impedance tomography. In 3 patients, the esophageal pressure was monitored using an Optivent (Mirandola, Italy); in the other 6 patients, esophageal pressure was monitored using a Pneumodrive (São Paulo, Brazil). Correct placement of the esophageal catheter was verified using the occlusion technique (2). Full recovery was defined as the time after the bilateral phrenic nerve blockade when electrical activity of the diaphragm, ΔPeso and VT after blockade were within ±20% of their baseline values. Heart rate, blood pressure, and peripheral oxygenation were continuously monitored. After inclusion of the patient in the protocol, sedation was not changed, and vasoactive drugs were titrated according to standard care in the intensive care unit.

The clinical protocol consisted of 3 phases: (1) monitoring and inspiratory pressure titration, (2) bilateral phrenic nerve block, and (3) recovery phase. During phase 1, succinylcholine 1 mg/kg and propofol 1.5-2.5 mg/kg were administered and patients were briefly ventilated on pressure-controlled ventilation. We then calculated respiratory system compliance (Cdyn), and titrated inspiratory pressure that was kept unchanged after the effect of succinylcholine and propofol had weaned and the patient was back to pressure support ventilation. From baseline until the end of the study, patients were on pressure support ventilation to guarantee VT > 4mL/kg. This inspiratory pressure was kept unchanged during the entire protocol.

During phase 2, a trained anesthesiologist performed phrenic nerve block using an ultrasound (Mindray M6 Ultrasound System, Shenzhen, China) and a peripheral nerve stimulator (Stimuplex® HNS 12B Braun, Melsungen, Germany). Of note, an anesthesiologist who has performed 15 peripheral blocks is able to distinguish anatomical landmarks and has a success rate of close to 90% (8). The combination of ultrasound and neurostimulator reduces intravascular or intraneural injection, direct neural damage by the needle and vascular bruise during needle manipulation (9). Specifically, the ultrasound minimizes the risk of complications and allows observing the dispersion of the local anesthetic, while the neurostimulator avoids intraneural administration of local anesthetics. Intraneural administration of local anesthetics is associated with neural damage (9). Of note, when the patient has motor response at low current output (<0.2 mA), one cannot ensure that the needle is not intraneural. In our study, we did not administer lidocaine if the phrenic nerve could not be visualized and/or if the patient had a motor response at a current output lower than 0.50 mA, thus minimizing the risk of intraneural injection (10). Finally, we used lidocaine due to its pharmacokinetics and pharmacodynamics properties: shorter half-life, better cardiovascular stability, low latency, and faster block than other local anesthetics (11).

Cervical ultrasound evaluation began at the level of the 6th cervical vertebra, identifying its characteristic bone anatomical landmark- the Chassaignac tubercle. We visualized the main cervical vessels (internal jugular vein and carotid artery), and important muscles references to perform the block (anterior scalene muscle, middle scalene muscle, and sternocleidomastoid muscle). The following step was to identify the cervical nerve roots of the 4th cervical to the 6th cervical vertebrae, assessed anteriorly to the 6th cervical vertebra. To recognize the origin of the phrenic nerve, we performed a caudal scan, paying close attention to the formation of a branch - mainly of the 5th cervical vertebrae root - a hypoechoic structure with medial direction and anterior to the anterior scalene muscle (**figure 4**). Finally, the phrenic nerve block was performed with a special needle (Needle for anesthesia – Stimuplex BBraun A100, Brazil) connected to the peripheral nerve stimulator (set with the electrical current generated greater than 0.48mA to avoid intraneural injection). The motor response of phrenic nerve stimulation was an ipsilateral diaphragm muscle contraction, similar to hiccups. Then, we administered 10-15 ml of lidocaine 2% on each side.

The study protocol was conducted under a predefined sequence: protocol initiated on the left nerve followed by blockade of the right branch of the phrenic nerve. Ten minutes after the left phrenic nerve blockade, the right phrenic nerve blockade was performed following the same steps. We assessed phrenic nerve block with three tools: electrical activity of the diaphragm signal and ΔPeso swings on pressure support; and real-time ventilation distribution on continuous positive airway pressure. Based on the findings observed in pigs, we defined thresholds to classify whether the phrenic nerve block was successful and designed the following real-time stepwise approach. To assess left phrenic nerve block, the percentage of right lung ventilation on continuous positive airway pressure was briefly evaluated and the block was considered successful if the percentage of right lung ventilation was greater than 75. In addition, on pressure support ventilation, we expected a decrease in 40% of electrical activity of the diaphragm and ΔPeso when LPB-10 min was compared to baseline. To assess right phrenic nerve block, a similar approach was performed. On continuous positive airway pressure, we briefly assessed percentage of right lung ventilation and expected it to be lower than 65. On pressure support ventilation, we expected a decrease of 80% in electrical activity of the diaphragm and ΔPeso when RPB-10 min was compared to baseline. We evaluated: (1) the percentage of patients blocked, (2) the amount of time spent from scanning to administration of lidocaine and (3) the duration until recovery.

The following variables were analyzed: electrical activity of the diaphragm, ΔPeso, VT in ml per kg, peak transpulmonary pressure, driving pressure, respiratory rate (RR), and ventilation distribution. VT in ml per kg was calculated as VT/Predicted Body Weight. Predicted body weight was calculated according to previous studies (4). Peak transpulmonary pressure was calculated as pressure support - ΔPeso (**figure 2**). Driving pressure was calculated as VT/Cdyn. For ventilation distribution analysis, the electrical impedance tomography image was divided in right and left lungs (**figure 3A**) and into four regions of interest, each covering 25% of the ventrodorsal diameter (**figure 3B**) and the percentage of ventilation in the most dependent region was calculated. Finally, percentage of left lung ventilation was analyzed. Data acquisition in humans occurred in seven time points: before phrenic nerve blockade (Baseline), 4 minutes after left phrenic block (LPB-4 min), 10 minutes after the left phrenic block (LPB-10 min), 4 minutes after the right phrenic block (RPB-4 min), 10 minutes after right phrenic block (RPB- 10min), 1h after right phrenic block, and in the following day (“Final” time-point).

From baseline until the end of the study, patients were ventilated on support pressure mode. The study was over once ΔPeso and electrical activity of the diaphragm after blockade were within ±20% of their baseline values.

In the human protocol, our primary goal was to evaluate the reduction in VT or peak transpulmonary pressure. Our secondary objectives were to assess the decrease in electrical activity of the diaphragm, and ΔPeso, driving pressure and RR. We also analyzed ventilation distribution using electrical impedance tomography and assessed whether bilateral phrenic nerve block could reduce or abolish pendelluft. Furthermore, we aimed to evaluate the time to wean from the block, feasibility, safety, and efficacy of this technique. Finally, we passively monitored for severe tachycardia, defined as a heart rate higher than 140 beats per minute, and prolonged phrenic nerve palsy, defined as elevation of the hemidiaphragmatic dome in imaging exams after the end of protocol.

**Statistical Analysis**

No statistical power calculation was used to guide sample size. The unadjusted results were reported as median and interquartile range [25th–75th]. We fit linear mixed models for each outcome, accounting for the repeated measurements on each animal or individual with a random intercept. Models were adjusted for time (Baseline, LPB-4 min, LPB-10 min, RPB-4 min, RPB-10 min, one hour after right phrenic block, and in the following day), PEEP (continuous variable), and pressure support (continuous variable) at each time for humans, and for time (Baseline, LPB-4 min, LPB-10 min, RPB-4 min, RPB-10 min) and pressure support (continuous variable) at each time point for pigs. Data from pigs and humans were analyzed separately. No data imputation was performed for missing data. All outcome variables were log transformed to obtain a normal distribution of residuals. P values were corrected for multiple comparisons using the Bonferroni method. A two-sided corrected p-value<0.05 was considered statistically significant. In humans, we performed 21 comparisons (pairwise combination of 7 levels). Therefore, the target P value was 0.05/21 = 0.002. We presented instead the “corrected P-value”, i.e., the unadjusted P-value x 21. The same method was applied for pigs, where we performed 10 comparisons (pairwise combination of 5 levels). For the analysis of variables (heart rate and blood pressure) that were collected in two time points, we performed a Wilcoxon signed rank test. All figures were plotted, and statistical analyses were performed in software R 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria) and Rstudio (Rstudio Team, Boston, MA, USA).

**Results**

Six pigs and nine patients were successfully included in this study. **Figure 5** shows lost, missing, or corrupted data.

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**Figures**

**Figure 1** – Online evaluation of phrenic nerve block.

To evaluate the phrenic nerve block, we briefly ventilated the pigs on continuous positive airway pressure before phrenic nerve block , after left phrenic block, and after bilateral phrenic nerve block. On the left side of the figure, there is a ventilation distribution map. In the middle of the picture, there is a variation of the plethysmograph of the right and left lungs. The blue line corresponds to the right lung, and the red line corresponds to the left lung. The green mark is the initiation of a respiratory cycle. On the right side, there is the tidal volume in mL per lung. (A) Both lungs are inflated during inspiration, and there was a ventilation distribution of 59.7% to the right lung and 40.3% to the left lung. The tidal volume of the right lung was 108 mL, and the left lung was 72 mL (B) The ventilation distribution of 83.7% to the right lung and 16.3% to the left lung suggested that the left phrenic nerve was blocked. Also, the decrease in plethysmogram variation in the left lung, while there is an increase in plethysmogram variation in the right lung, suggests pendelluft. Pendelluft and asymmetric distribution of ventilation were considered markers of an effective phrenic nerve block. Finally, the tidal volume of the right lung was 113 mL, and the left lung was 23 mL (C) When both phrenic nerves were blocked, the ventilation distribution was similar to baseline, with 63.7% to the right lung and 36.3% to the left lung. The tidal volume was 65 ml in the right lung and 37 mL in the left lung. All graphs have the same x-axis scale.

**Figure 2** – Representative imaging of transpulmonary pressure calculation in pigs and humans. A. Airway pressure (in cmH2O) with PEEP and pressure support. B. Esophageal pressure (in cmH2O) with ΔPeso. Transpulmonary pressure was calculated as pressure support - ΔPeso. *Abbreviations: PEEP: positive end-expiratory pressure. ΔPeso: esophageal pressure swing.*

**Figure 3** – Representative imaging of electrical impedance tomography regional ventilation analysis in pigs and humans. A. Electrical impedance tomography imaging was divided in left and right lungs and the percentage on ventilation to the left lung was calculated. B. Electrical impedance tomography imaging was divided in into four regions of interest, each covering 25% of the ventrodorsal diameter and the percentage of ventilation in the most dependent region of the lung (region of interest 4) was calculated.

**Figure 4** - The ultrasound-guided cervical approach to phrenic nerve block in humans.

The inset picture shows a patient in dorsal decubitus with the probe placed on the lateral portion of the neck. (A) First, we visualize the Chassaignac Tubercle and the root of the 5th cervical vertebrae. Then, we performed a slight translational movement, dislocating the probe inferiorly. (B) The electrical stimulation of the phrenic nerve in the root of the 5th cervical vertebrae promoted diaphragmatic contractions. The needle was inserted, and lidocaine was administered in the location indicated by the arrowheads, perineurally to the phrenic nerve.

**Figure 5** – Data collection during animal and clinical protocols.

Six pigs and nine patients were included in this study. All six pigs were successfully bilaterally paralyzed and there were no missing or corrupted data. All patients were successfully paralyzed, and none was excluded. In one patient, we recorded esophageal pressure, but offline analysis was not possible because the data was corrupted. In another patient, despite good positioning of the Neurally Adjusted Ventilatory Assist catheter, we did not record waveforms due to software malfunctioning. Furthermore, one hour after bilateral phrenic nerve block, electrical activity of the diaphragm of only 6 patients was included. Finally, one patient was not assessed during in the “Final”.