ORIGINAL BASIC SCIENCE REPORT

OPEN

Diaphragm Neurostimulation Mitigates Ventilation-Associated Brain Injury in a Preclinical Acute Respiratory Distress Syndrome Model

Thiago G. Bassi, MD, PhD[1]

Elizabeth C. Rohrs, BSc, RRT, PhD[2,3]

Mr. Karl C. Fernandez, BSc, RRT[2,3]

Ms. Marlena Ornowska, BSc[3]

Ms. Michelle Nicholas, BHSc, RN, MSc[2,3]

Ms. Jessica Wittmann, BSc[2]

Mr. Matt Gani, BSc, BE[1]

Mr. Doug Evans, MS, MBA[1]

Steven C. Reynolds, MD[2,3]

CONTEXT: In a porcine healthy lung model, temporary transvenous diaphragm neurostimulation (TTDN) for 50 hours mitigated hippocampal apoptosis and inflammation associated with mechanical ventilation (MV).

HYPOTHESIS: Explore whether TTDN in combination with MV for 12 hours mitigates hippocampal apoptosis and inflammation in an acute respiratory distress syndrome (ARDS) preclinical model.

METHODS AND MODELS: Compare hippocampal apoptosis, inflammatory markers, and serum markers of neurologic injury between never ventilated subjects and three groups of mechanically ventilated subjects with injured lungs: MV only (LI-MV), MV plus TTDN every other breath, and MV plus TTDN every breath. MV settings in volume control were tidal volume 8 mL/kg and positive end-expiratory pressure 5 cm H_2O. Lung injury, equivalent to moderate ARDS, was achieved by infusing oleic acid into the pulmonary artery.

RESULTS: Hippocampal apoptosis, microglia, and reactive-astrocyte percentages were similar between the TTDN-every-breath and never ventilated groups. The LI-MV group had a higher percentage of these measures than all other groups ($p < 0.05$). Transpulmonary driving pressure at study end was lower in the TTDN-every-breath group than in the LI-MV group; systemic inflammation and lung injury scores were not significantly different. The TTDN-every-breath group had considerably lower serum concentration of homovanillic acid (cerebral dopamine production surrogate) at study end than the LI-MV group ($p < 0.05$). Heart rate variability declined in the LI-MV group and increased in both TTDN groups ($p < 0.05$).

INTERPRETATIONS AND CONCLUSIONS: In a moderate-ARDS porcine model, MV is associated with hippocampal apoptosis and inflammation, and TTDN mitigates that hippocampal apoptosis and inflammation.

KEY WORDS: acute respiratory distress syndrome; diaphragm neurostimulation; mechanical ventilators; neuroinflammation; ventilation-induced

Acute respiratory distress syndrome (ARDS) is common in mechanically ventilated patients admitted to ICUs, with one-third of patients presenting with moderate ARDS (1, 2). Clinically and preclinically, ARDS has been associated with hippocampal apoptosis and inflammation (3–6). The association between ARDS and hippocampal inflammation is concerning because the standard of care for patients with ARDS includes mechanical ventilation (MV) (1). MV has recently been implicated in triggering hippocampal apoptosis and inflammation, also called ventilation-associated brain injury (VABI) and may therefore potentiate further hippocampal apoptosis and neuroinflammation in ARDS patients (7, 8).

DOI: 10.1097/CCE.0000000000000820

KEY POINTS

Question: What specific question does the article address?

Does phrenic-nerve stimulation, in combination with mechanical ventilation (MV) for 12 hours, mitigate hippocampal apoptosis and inflammation in an acute respiratory distress syndrome (ARDS) preclinical model?

Findings: What is the key result?

The group receiving MV alone had higher percentages of hippocampal apoptosis and inflammation than all other groups. Hippocampal apoptosis and inflammation were similar when comparing the group receiving phrenic-nerve stimulation on every breath with the never ventilated control group.

Meaning: What are the key conclusion and implications based on the primary finding(s)?

In a moderate-ARDS porcine model, MV is associated with hippocampal apoptosis and inflammation, and phrenic-nerve stimulation in combination with MV mitigates that hippocampal apoptosis and inflammation.

It has been proposed that systemic inflammation might trigger neuroinflammation and apoptosis in ARDS/MV subjects (9). While some preclinical studies that induced lung injury via either volutrauma or barotrauma have shown brain injury associated with systemic inflammation, other preclinical studies that used healthy subjects undergoing lung-protective MV have also demonstrated that MV itself is associated with brain injury and that systemic inflammation played a secondary role (7, 8, 10).

Our group has previously reported that a hybrid ventilation strategy employing temporary transvenous diaphragm neurostimulation (TTDN) as an adjunct to MV resulted in neuroprotection after 50 hours of MV (10–12). This novel ventilatory strategy uses a central line catheter embedded with electrodes inserted in the left subclavian vein to stimulate bilaterally the phrenic nerve in synchrony with MV (10). In a deeply sedated porcine model with healthy lungs ventilated for 50 hours, our group reported that the degree of neuroprotection was higher with greater exposure to TTDN (10). This was the first preclinical study that investigated a hybrid ventilatory intervention to mitigate cellular apoptosis and neuroinflammation in an ICU model during deep sedation with MV (10). In preclinical studies, greater hippocampal apoptosis and neuroinflammation have been associated with worse cognitive scores (3, 4, 13). Clinically, a post-mortem study also showed that patients who had delirium prior to death had greater hippocampus inflammation compared with patients who did not have delirium prior to death (5). Although our group demonstrated that a therapy could mitigate hippocampal inflammation and cellular apoptosis associated with MV, the neuroprotection observed was in a healthy lung preclinical model, thereby limiting its translational generalizability. To extend our previous findings of VABI mitigation, we hypothesize that TTDN will also result in neuroprotection in a moderate-ARDS porcine model.

METHODOLOGY

Animals

Female juvenile Yorkshire pigs (4–5 mo old) were procured, housed, maintained, and studied following the local Animal Care Committee guidelines, after University of British Columbia. Ethics Committee and Animal Care Committee approvals (ethics certificate number A20-0245 and approval date January 5, 2022). Yorkshire pigs were chosen based on their anatomical similarities with humans. The choice of only using females was due to the animal facility used for the experiments only having female animal providers and due to easier housing and husbandry (**Supplemental Digital Content**, http://links.lww.com/CCX/B103).

Experimental Protocol

Subjects were assigned to four groups: mechanically ventilated with injured lungs (LI-MV); mechanically ventilated with injured lungs plus TTDN every other breath (LI-MV + TTDN50%); mechanically ventilated with injured lungs plus TTDN every breath (LI-MV + TTDN100%); and never ventilated (NV). All mechanically ventilated subjects were ventilated for 12 hours post-injury. All subjects from all groups received a central line catheter in the left subclavian vein. Subjects from the NV group were immediately euthanized to provide histological baseline data.

Temperature, mean arterial pressure, heart rate, glucose levels, and arterial blood gases were monitored during the

experiment to ensure values stayed within normal ranges (**Table S1**, http://links.lww.com/CCX/B103).

Mechanical Ventilation

Mechanically ventilated subjects were ventilated in volume control mode (Evita XL, Dräger, Draeger, Germany) using lung-protective settings (5 cm H_2O positive end-expiratory pressure, 8 mL/kg tidal volume, due to greater dead space in pigs) (14). Respiratory rate and F_{IO_2} were adjusted to achieve normal arterial blood gases.

Esophageal pressure, plateau pressure, driving pressure, and transpulmonary plateau pressure during the first and the last hour of the experiments were compared (FluxMed GrT, MBMed). Esophageal pressure was measured throughout the breath. Plateau, driving pressure, and transpulmonary plateau pressure were all measured during an end-inspiratory pause.

Transpulmonary driving pressure was calculated as (end-inspiratory transpulmonary plateau pressure) minus (end-expiratory plateau pressure). Real-time ventilator pressure-time product was obtained by respiratory monitoring using a respiratory monitor (FluxMed GrT, MBMed).

P_{aO_2}/F_{IO_2} Ratio

Arterial blood gas samples were used to calculate P_{aO_2}/F_{IO_2} ratio. Mechanically ventilated subjects had samples taken at baseline, every 4 hours, or as appropriate. NV subjects had one sample taken before euthanasia.

Lung Injury

To induce ARDS in each mechanically ventilated subject, 0.1–0.4 mL of oleic acid (cis-9-octadecenoic acid) mixed with the animal's fresh blood was injected into the pulmonary artery via pulmonary artery flotation catheter (via distal port) until a P_{aO_2}/F_{IO_2} ratio less than 200 mm Hg was achieved. Our study targeted the induction of moderate ARDS, which was confirmed by P_{aO_2}/F_{IO_2} ratio between 100 mm Hg and 200 mm Hg (14).

Diaphragm Contractions and Diaphragm Contribution With TTDN

In subjects receiving TTDN, a central venous catheter embedded with electrodes (LIVE Catheter; Lungpacer Medical, Exton, PA; **Fig. S1**, http://links.lww.com/CCX/B103) was used to stimulate the phrenic nerves bilaterally for diaphragm contractions in synchrony with the ventilator's inspiratory phase. Neurostimulated breaths targeted a ventilator pressure-time-product reduction of 15–20%, as previously described (**Figs. S2** and **S3**, http://links.lww.com/CCX/B103). A respiratory monitor (FluxMed GrT, MBMed) was used to monitor real-time ventilator pressure-time product to ensure standardized diaphragm contribution during the experiments (10, 15). Neurostimulation intensity was adjusted during the experiment to keep ventilator pressure-time-product within the targeted range.

Serum Samples

All subjects had blood samples taken to determine the serum concentration of biomarkers at the end of the experiment (**Table S2**, http://links.lww.com/CCX/B103). Brain injury biomarkers were S100β, neuron-specific enolase (NSE), glial fibrillary acidic protein (GFAP), and ubiquitin carboxy-terminal hydrolase L1 (UCHL1). Systemic inflammatory biomarkers were interferon-gamma (IFN-γ), granulocyte/monocyte colony-stimulating factor (GM-CSF), tumor necrosis factor-alpha (TNFα), interleukin (IL)-1α, IL-1β, IL-6, IL-8, and IL-10. Biomarkers for tryptophan metabolism were tryptophan, kynurenine, and kynurenic acid (KYNA). The biomarker for cerebral dopamine metabolism was homovanillic acid (HVA). An independent laboratory (Eve Technologies Corporation, Calgary, Canada), blinded to study group allocation, analyzed the samples.

Hippocampal Sampling and Preparation

An independent laboratory (Wax-it Histology Services, Vancouver, Canada), blinded to sample group, performed immunochemistry preparation and processing for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling, GFAP, and ionizing calcium-binding adaptor molecule-1 markers (**Figs. S4** and **S5**, http://links.lww.com/CCX/B103).

Cell Counting

Hippocampal tissue was classified (positive-stained cells, negative-stained cells, and extracellular matrix) and cells were counted by machine-learning software (ImageJ, Fiji, New Zeland). The percentage of positive-stained cells was calculated by the formula: ([positive-stained cells] divided by [positive-stained cells plus negative-stained cells]).

Lung Histology

Post-euthanasia, lungs were harvested, and an independent laboratory (Wax-it Histology Services), blinded to study group allocation, stained the lung slides with hematoxylin and eosin (**Fig. S7**, http://links.lww.com/CCX/B103). Samples were then scored for lung injury by an examiner who was blinded to the study groups, following a method adapted from Matute-Bello, as used in our previous experiments (**Table S3**, http://links.lww.com/CCX/B103) (16).

Heart Rate Variability Analysis

The root mean square of the SD (RMSSD) of R-R intervals, a surrogate measure of parasympathetic activity, was calculated for the first 4 hours after experiment initiation, and the last 4 hours before subject euthanasia (**Fig. S6**, http://links.lww.com/CCX/B103).

Statistical Analysis

Nonparametric tests were used, and included either the Kruskal-Wallis test and Dunn multiple comparison test, or the Wilcoxon signed-rank test or Spearman correlation test, as appropriate. Data are expressed as median and interquartile range unless otherwise stated. *p* values of less than or equal to 0.05 are considered statistically significant. Statistical analyses used Prism 8.4.2 software (GraphPad, San Diego, CA).

A power calculation was performed based on data from González-López et al (17) (ratios of cleaved to complete poly [adenosine triphosphate-ribose] polymerase 1, an apoptotic marker, in the control group [mean 1.0, SD ± 0.2] and in the mechanically ventilated group [mean 6.0, SD ± 0.8]) using $\alpha = 0.05$ and $\beta = 0.90$. The calculation indicated that one subject per arm would be sufficient to achieve statistical power. As this would pose a risk of bias due to the small sample size required and one subject per arm would not show any variance in the data, the number of samples was increased to six per arm, thereby increasing statistical power.

RESULTS

The study protocol, detailed description of the methods used, additional information about the power calculation, ventilatory settings, summary of the total drug used during the experiments, and arterial and venous blood work results can be found in the **Tables S5-S17** (http://links.lww.com/CCX/B103).

Twenty-four female subjects were studied, with median weights of 64 kg (62–65 kg) in the LI-MV group ($n = 6$), 67 kg (64–84 kg) in the LI-MV + TTDN50% group ($n = 6$), 69 kg (67–73 kg) in the LI-MV + TTDN100% group ($n = 6$), and 54 kg (52–56 kg) in the NV group ($n = 6$; $p = 0.0011$). Differences in weights were not statistically significant between the mechanically ventilated groups (**Table S4**, http://links.lww.com/CCX/B103). No statistically significant differences were observed in the body length-to-weight ratio (the equivalent of the body mass index for animals) between the groups, including the NV group. Temperature, mean arterial pressure, heart rate, glucose levels, and $Paco_2$ were within normal ranges for all subjects from all groups. Mean arterial pressures and central venous pressures are shown in **Table 1**. Differences in mean arterial pressures at study end were statistically significant between LI-MV and LI-MV + TTDN100% groups ($p = 0.0124$) (Table 1). All subjects receiving TTDN had bilateral phrenic-nerve capture. Fluid balance (input minus output) in each ventilated subject was within the target range of 0.1–2.0 mL/kg/hr with no statistically significant difference between the mechanically ventilated groups (Table S7, http://links.lww.com/CCX/B103) (18).

The median number of episodes of spontaneous breathing activity (defined as subject-triggered breaths) was 6–10 per total study period and not significantly different between groups.

Differences in total administered doses of propofol, midazolam, fentanyl, ketamine, phenylephrine, norepinephrine bitartrate, and oleic acid were not statistically significant between the groups, when normalized to weight (Table S14, http://links.lww.com/CCX/B103).

Hippocampal Apoptosis and Neuroinflammation

Differences in apoptotic cell percentages were statistically significant between the groups ($p = 0.0002$) (**Table S18**, http://links.lww.com/CCX/B103; and **Fig. 1*A***).

Percentages of microglia and reactive astrocytes were significantly different between the groups ($p = 0.0002$ and $p = 0.0085$, respectively) (Table S18, http://links.lww.com/CCX/B103; and **Fig. 1**, ***B*** and ***C***).

Serum Concentrations of Systemic Inflammatory Biomarkers

Systemic inflammatory markers IL-1α, IL-1β, IL-6, IL-8, IL-10, and TNFα levels were not significantly different between the groups (Table S15, http://links.lww.com/CCX/

TABLE 1.

Lung Physiology Data, Mean Arterial Pressures, and Central Venous Pressures

Time	Measurement	Median (IQR)			*p* (Kruskal-Wallis Test)	*p* (Dunn Multiple Comparison Test)	
		LI-MV Group ($n = 6$)	LI-MV + TTDN50% Group ($n = 6$)	LI-MV + TTDN100% Group ($n = 6$)			
Baseline	Esophageal pressure (cm H_2O)	13 (10–15)	11 (8–11)	11 (9–14)	NS	LI-MV vs LI-MV + TTDN50%	–
						LI-MV vs LI-MV + TTDN100%	–
						LI-MV + TTDN50% vs LI-MV + TTDN100%	–
	Plateau pressure (cm H_2O)	18 (16–19)	17 (15–19)	16 (13–16)	NS	LI-MV vs LI-MV + TTDN50%	–
						LI-MV vs LI-MV + TTDN100%	–
						LI-MV + TTDN50% vs LI-MV + TTDN100%	–
	Transpulmonary driving pressure (cm H_2O)	8 (6–9)	8 (6–9)	5 (5–6)	0.0404	LI-MV vs LI-MV + TTDN50%	NS
						LI-MV vs LI-MV + TTDN100%	NS
						LI-MV + TTDN50% vs LI-MV + TTDN100%	NS
	Transpulmonary plateau pressure (cm H_2O)	6 (4–6)	6 (5–6)	5 (4–5)	NS	LI-MV vs LI-MV + TTDN50%	–
						LI-MV vs LI-MV + TTDN100%	–
						LI-MV + TTDN50% vs LI-MV + TTDN100%	–
	Mean arterial pressure (mm Hg)	66 (61–69)	64 (56–76)	70 (61–82)	NS	LI-MV vs LI-MV + TTDN50%	–
						LI-MV vs LI-MV + TTDN100%	–
						LI-MV + TTDN50% vs LI-MV + TTDN100%	–
	Central venous pressure (cm H_2O)	7 (6–9)	9 (8–10)	7 (5–8)	NS	LI-MV vs LI-MV + TTDN50%	–
						LI-MV vs LI-MV + TTDN100%	–
						LI-MV + TTDN50% vs LI-MV + TTDN100%	–
Study end	Esophageal pressure (cm H_2O)	11 (10–12)	9 (8–12)	10 (7–13)	NS	LI-MV vs LI-MV + TTDN50%	–
						LI-MV vs LI-MV + TTDN100%	–
						LI-MV + TTDN50% vs LI-MV + TTDN100%	–
	Plateau pressure (cm H_2O)	24 (23–27)	21 (19–24)	19 (17–21)	0.0015	LI-MV vs LI-MV + TTDN50%	NS
						LI-MV vs LI-MV + TTDN100%	0.0380
						LI-MV + TTDN50% vs LI-MV + TTDN100%	NS
	Transpulmonary driving pressure (cm H_2O)	15 (13–17)	11 (10–14)	9 (6–14)	0.0055	LI-MV vs LI-MV + TTDN50%	NS
						LI-MV vs LI-MV + TTDN100%	0.0008
						LI-MV + TTDN50% vs LI-MV + TTDN100%	NS
	Transpulmonary plateau pressure (cm H_2O)	13 (12–16)	12 (9–14)	8 (5–12)	NS	LI-MV vs LI-MV + TTDN50%	–
						LI-MV vs LI-MV + TTDN100%	–
						LI-MV + TTDN50% vs LI-MV + TTDN100%	–
	Mean arterial pressure (mm Hg)	73 (65–74)	73 (66–82)	85 (82–90)	0.0057	LI-MV vs LI-MV + TTDN50%	NS
						LI-MV vs LI-MV + TTDN100%	0.0124
						LI-MV + TTDN50% vs LI-MV + TTDN100%	NS
	Central venous pressure (cm H_2O)	7 (6–9)	8 (5–9)	5 (3–6)	0.0402	LI-MV vs LI-MV + TTDN50%	NS
						LI-MV vs LI-MV + TTDN100%	NS
						LI-MV + TTDN50% vs LI-MV + TTDN100%	NS

IQR = interquartile range, LI-MV = mechanically ventilated with injured lungs, NS = not significant, TTDN = temporary transvenous diaphragm neurostimulation.
Dashes indicate not applied.

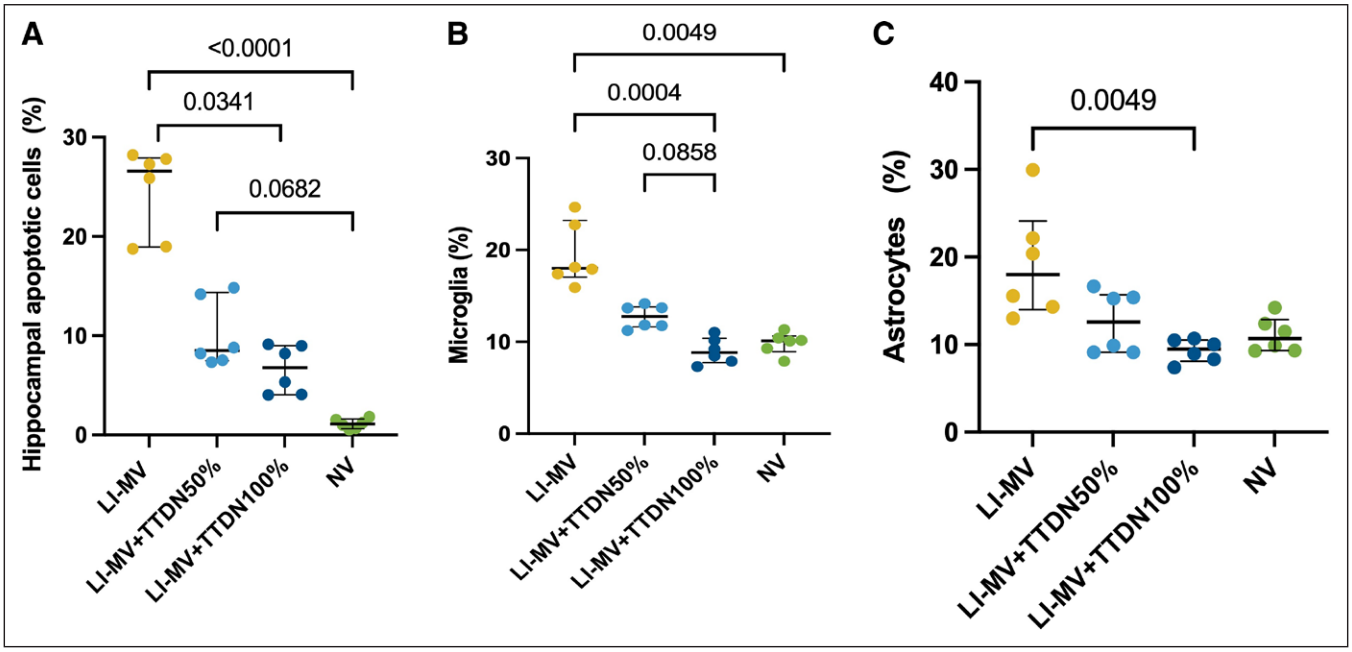

Figure 1. *Dot plot* of hippocampal apoptosis, microglia and astrocytes percentages. **A**, *Dot plot* of hippocampal apoptotic cell percentages (%) for all groups ($n = 6$ per group). Hippocampal apoptotic cell percentages found were 26.5 (18.9–27.9) for the mechanically ventilated with injured lungs (LI-MV) group, 8.5 (7.4–14.3) for the LI-MV + temporary transvenous diaphragm neurostimulation (TTDN) 50% group, 6.7 (4.0–9.0) for the LI-MV + TTDN100% group, and 0.9 (0.5–1.6) for the never ventilated (NV) group. Post hoc analysis using Dunn multiple comparison test showed statistically significant differences between the LI-MV and NV groups (26.5 vs 0.9; $p < 0.0001$), and between the LI-MV and LI-MV + TTDN100% groups (26.5 vs 6.7; $p = 0.0341$). Also, there was a tendency to statistical significance in the difference between the LI-MV + TTDN50% and NV groups (8.5 vs 0.9; $p = 0.0682$). **B**, *Dot plot* of microglia percentages (%) for all groups ($n = 6$ per group). Microglia percentages found were 18.0 (17.0–23.2) for the LI-MV group, 12.7 (11.6–13.8) for the LI-MV + TTDN50% group, 8.8 (7.7–10.3) for the LI-MV + TTDN100% group, and 10.1 (8.9–10.6) for the NV group. Post hoc analysis using Dunn multiple comparison test showed statistically significant differences between the LI-MV and NV groups (18.0 vs 10.1; $p = 0.0049$), and between the LI-MV and LI-MV + TTDN100% groups (18.0 vs 8.8; $p = 0.0004$). Also, there was a tendency to statistical significance in the difference between the LI-MV + TTDN50% and the LI-MV + TTDN100% groups (12.7 vs 8.8; $p = 0.0858$). **C**, *Dot plot* of astrocyte percentages (%) for all groups ($n = 6$ per group). Astrocyte percentages found were 17.9 (13.9–24.1) for the LI-MV group, 12.5 (9.1–15.7) for the LI-MV + TTDN50% group, 9.4 (8.0–10.5) for the LI-MV + TTDN100% group, and 10.6 (9.3–12.8) for the NV group. Post hoc analysis using Dunn multiple comparison test showed a statistically significant difference between the LI-MV and LI-MV + TTDN100% groups (17.9 vs 9.4; $p = 0.0049$).

B103). However, IFN-γ and GM-CSF serum concentrations were significantly different between the groups. The LI-MV + TTDN50% group showed significantly lower IFN-γ serum concentration at study end compared with the LI-MV + TTDN100% group ($p = 0.0133$). The LI-MV + TTDN100% group showed significantly higher GM-CSF serum concentration at study end compared with the LI-MV group ($p = 0.0475$).

Tryptophan Metabolism and Cerebral Dopamine Production

Tryptophan, kynurenine, and KYNA serum concentrations and kynurenine/tryptophan and KYNA/kynurenine ratios were not significantly different between the groups. HVA serum concentrations were significantly higher in the LI-MV group (8 ng/mL) when compared with the LI-MV + TTDN100% group (3 ng/mL; $p = 0.0020$) (**Fig. S9**, http://links.lww.com/CCX/B103).

Serum Concentrations of Markers for Neuronal Injury

GFAP, UCHL1, S100β, and NSE serum concentrations were not significantly different between the mechanically ventilated groups (Table S18, http://links.lww.com/CCX/B103).

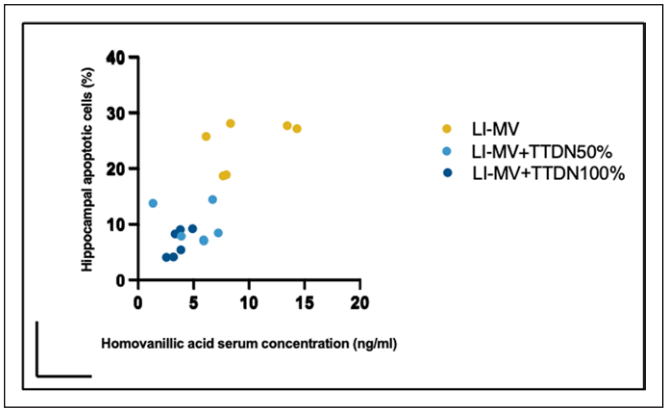

Figure 2. Hippocampal apoptotic cell percentages plotted against homovanillic acid serum concentrations (ng/mL). Spearman test showed a strong, moderate, positive correlation between hippocampal apoptotic cell percentages and serum concentrations of homovanillic acid ($r = 0.75$; 95% CI, 0.43–0.90; $p = 0.0003$). LI-MV = mechanically ventilated with injured lungs, NV = never ventilated, TTDN = temporary transvenous diaphragm neurostimulation.

Correlation Between Hippocampal Apoptosis Percentage and Homovanillic Acid Serum Concentration

A post hoc analysis using the Spearman correlation test showed strong, linear, and positive correlation between hippocampal apoptosis percentage and HVA serum concentration, with $r = 0.75$ (95% CI, 0.43–0.90; $p = 0.0003$) (**Fig. 2**).

Lung Physiology and Lung Injury Score

Esophageal pressures, plateau pressures, driving pressures, and transpulmonary plateau pressures are shown in Table 1 and **Figure S8** (http://links.lww.com/CCX/B103) Analysis of lung injury scores showed no statistically significant difference between the mechanically ventilated groups, but it showed a considerable difference between the LI-MV and NV groups ($p = 0.0132$) and between the LI-MV + TTDN50% and NV groups ($p = 0.0003$) (**Table 2**). Pao_2/Fio_2 ratios at the study end showed a significant difference between the LI-MV and LI-MV + TTDN100% groups ($p = 0.0386$) (Table 2).

Heart Rate Variability

Analysis of the RMSSD changes from study start to study end showed that the LI-MV group had a 9% decline in RMSSD, the LI-MV + TTDN50% group had a 10% increase in RMSSD, and the LI-MV + TTDN100% group had a 34% increase in RMSSD (**Fig. 3**). Change in RMSSD showed a statistically significant difference between the LI-MV and the LI-MV + TTDN100% groups (–9% vs +34%; $p = 0.0026$) (Fig. 3).

DISCUSSION

Our study found that lung-protective MV for 12 hours in pigs with moderate ARDS is associated with hippocampal apoptosis and inflammation. The magnitudes of these results are similar to our previous findings in a porcine model with healthy lungs ventilated for 50 hours, in which we showed that lung-protective MV also resulted in hippocampal apoptosis and neuroinflammation (8). When compared with our previous findings in pigs with healthy lungs, ventilated for 50 hours, moderate ARDS likely accelerated hippocampal apoptosis and inflammation.

In addition to the brain injury associated with the combination of moderate ARDS and MV, our study showed that TTDN on every breath mitigated hippocampal injury associated with the combination of moderate ARDS and MV. The LI-MV + TTDN100% group showed lower apoptotic cell, microglia, and astrocyte percentages than the LI-MV group and statistically similar percentages compared with the NV group. Furthermore, the percentage of pro-inflammatory microglia was considerably lower in the LI-MV + TTDN100% group than in the LI-MV group. Although this study has not analyzed clinical outcomes secondary to hippocampal apoptosis and neuroinflammation after ARDS/MV, in preclinical studies, hippocampal apoptosis and inflammation have been associated with cognitive dysfunction (3, 4, 13). Previously, our group had also shown mitigation of apoptotic cell, microglia, and astrocyte percentages provided by TTDN in a healthy lung porcine model ventilated for 50 hours (10). Thus, diaphragm neurostimulation on every breath provides neuroprotection both in pigs with healthy lungs, ventilated for 50 hours, and those with induced moderate ARDS, ventilated for 12 hours. These are the first steps that will assist in the design of clinical studies to investigate whether the neuroprotection observed in this model will be seen in a human clinical scenario.

Interestingly, although the LI-MV group showed the highest percentage of hippocampal apoptosis, the serum concentration of biomarkers for brain injury (GFAP, UCHL1, S100β, and NSE) were similar between

TABLE 2.
Gas Exchange Measures and Lung Injury Scores

Gas Exchange Measures and Lung Injury Scores	Median (IQR)				*p* (Kruskal-Wallis Test)	*p* (Dunn Multiple Comparison Test)	
	LI-MV Group ($n = 6$)	LI-MV + TTDN50% Group ($n = 6$)	LI-MV + TTDN100% Group ($n = 6$)	NV Group ($n = 6$)			
Pao_2/Fio_2 ratio, study start (mm Hg)	511 (467–543)	513 (406–536)	520 (481–531)	568 (546–571)	0.8543	LI-MV vs LI-MV + TTDN50%	–
						LI-MV vs LI-MV + TTDN100%	–
						LI-MV vs NV	–
						LI-MV + TTDN50% vs LI-MV + TTDN100%	–
						LI-MV + TTDN50% vs NV	–
						LI-MV + TTDN100% vs NV	–
Pao_2/Fio_2 ratio, lung injury achieved (mm Hg)	190 (156–197)	180 (166–192)	191 (186–194)	NA	0.6794	LI-MV vs LI-MV + TTDN50%	–
						LI-MV vs LI-MV + TTDN100%	–
						LI-MV vs NV	–
						LI-MV + TTDN50% vs LI-MV + TTDN100%	–
						LI-MV + TTDN50% vs NV	–
						LI-MV + TTDN100% vs NV	–
Pao_2/Fio_2 ratio, study end (mm Hg)	271 (250–389)	363 (267–430)	431 (382–490)	NA	0.0360	LI-MV vs LI-MV + TTDN50%	NS
						LI-MV vs LI-MV + TTDN100%	0.0386
						LI-MV vs NV	NA
						LI-MV + TTDN50% vs LI-MV + TTDN100%	NS
						LI-MV + TTDN50% vs NV	NA
						LI-MV + TTDN100% vs NV	NA
Lung injury score	0.46 (0.41–0.49)	0.56 (0.48–0.62)	0.39 (0.37–0.39)	0.19 (0.17–0.21)	0.0003	LI-MV vs LI-MV + TTDN50%	NS
						LI-MV vs LI-MV + TTDN100%	NS
						LI-MV vs NV	0.0132
						LI-MV + TTDN50% vs LI-MV + TTDN100%	NS
						LI-MV + TTDN50% vs NV	0.0003
						LI-MV + TTDN100% vs NV	NS

IQR = interquartile range, LI-MV = mechanically ventilated with injured lungs, NA = not available, NS = not significant, NV = never ventilated, TTDN = temporary transvenous diaphragm neurostimulation.
Dashes indicate not applied.

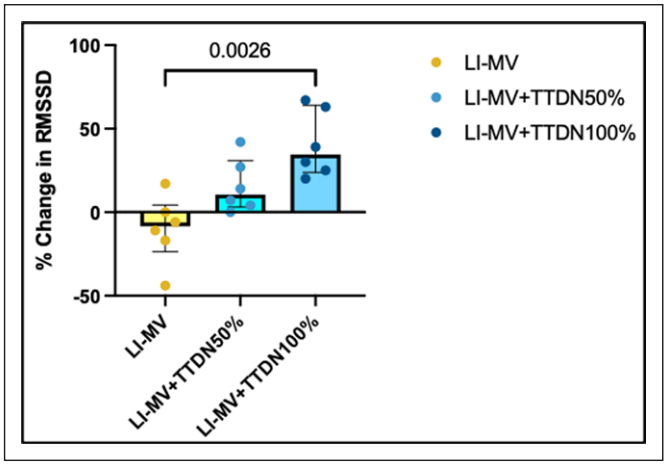

Figure 3. *Dot plot* showing percentage changes in root mean square of SD (RMSSD) of R-R intervals from study start to study end ($n = 6$ per group). The mechanically ventilated with injured lungs (LI-MV) group showed a decline in RMSSD from study start to study end (–9%). The LI-MV + temporary transvenous diaphragm neurostimulation (TTDN) 50% group showed an increase in RMSSD from study start to study end (+10%). The LI-MV + TTDN100% group showed an increase in RMSSD from study start to study end (+34%). Kruskal-Wallis test showed a statistically significant difference between the groups in the RMSSD changes from study start to study end. Post hoc analysis using Dunn multiple comparison test indicated a statistically significant difference between the LI-MV group and the LI-MV + TTDN100% group (–9% vs +34%; $p = 0.0026$). NV = never ventilated.

all mechanically ventilated groups, indicating that the hippocampal apoptosis and inflammation observed did not result in significant changes in serum concentration of biomarkers for brain injury. In our previous study, we have shown significantly higher GFAP and UCHL1 serum concentrations at study end in the MV group compared with the MV + TTDN100% group (10). It is possible that either the relatively short duration of our experiment (12 hr) or the power of our experiment was not sufficient to capture any statistically significant difference in serum concentrations between the groups.

Only the serum concentrations of IFN-γ and GM-CSF were significantly different between the groups. The group receiving TTDN every breath showed a significantly higher serum concentration of IFN-γ when compared with the LI-MV + TTDN50% group and also a significantly higher serum concentration of GM-CSF when compared with the LI-MV group. IFN-γ and GM-CSF are proteins that regulate the activation and inactivation of immunological cells (19, 20). GM-CSF also plays an important role in surfactant homeostasis, assisting in keeping alveoli open (20). One of the mechanisms by which IFN-γ controls and limits inflammation is by shifting tryptophan metabolism toward the kynurenine pathway, reducing the serum concentration of free tryptophan in the bloodstream. It has been shown that the activation of the kynurenine pathway is linked to neuroinflammation and delirium (19). Our study has not found any statistically significant differences in serum concentrations of tryptophan and its metabolites between groups. A recently published preclinical study showed that stimulation of different branches of the subdiaphragmatic vagus nerve (the celiac, gastric, and hepatic branches) resulted in considerable increases in IFN-γ and GM-CSF serum concentrations (21). In particular, the stimulation of the celiac and the hepatic branches of the subdiaphragmatic vagus nerve induced a greater release of IFN-γ compared with the gastric branch, resulting in increased serum concentrations of IFN-γ and GM-CSF after electrical stimulation, via activation of the neuroimmunological reflex (21). It has previously been established that the subdiaphragmatic phrenic nerve is also connected directly to the celiac ganglion (22). It can therefore be hypothesized that the greater serum concentrations of IFN-γ and GM-CSF observed in the TTDN-every-breath group might have been the result of direct stimulation of the celiac ganglion via phrenic-nerve stimulation.

The significantly lower HVA serum concentration observed at the end of the study in the TTDN-every-breath group, compared with the LI-MV group, provides interesting insights into the potential mechanism for the protective effect of TTDN. HVA is a product of dopamine metabolism (23). The serum concentration of HVA is correlated with cerebral dopamine production in the CNS (23). Previously, it has been hypothesized that injurious MV leads to the activation of the pulmonary transient receptor potential cation channel subfamily V member 4 receptors (mechanoreceptors), which, in turn, activate the pulmonary afferent purinergic receptors (vagal afferent receptors) increasing dopamine release in the hippocampus and triggering the intrinsic apoptotic cascade (17, 24). The LI-MV group had a considerably higher serum concentration of HVA at study end, compared with the LI-MV + TTDN100% group. Further, our data showed a strong,

linear, and positive correlation between hippocampal cellular apoptosis and serum concentration of HVA across all mechanically ventilated groups. Higher serum concentration of HVA in the LI-MV group at study end, and the correlation between serum concentration of HVA and hippocampal apoptosis across all the mechanically ventilated groups, support the hypothesis that a cerebral hyperdopaminergic state triggers the apoptotic cascade.

In addition to the analysis of HVA serum concentration, our study investigated parasympathetic system activity. The vagus nerve signal is the main component of the parasympathetic system (25). Parasympathetic activity was quantified by determining the RMSSD of R-R intervals (26). Our results showed different behaviors of RMSSD between the mechanically ventilated groups. The LI-MV group had a statistically significant decrease in RMSSD from the beginning to the end of the experiments; conversely, both TTDN groups had statistically significant increases in RMSSD from the beginning to the end of the experiments. This means that the LI-MV group displayed decreased parasympathetic activity, while the TTDN groups displayed increased parasympathetic activity between the start and end of the experiment. Clinically, increased heart rate variability has been associated with lower morbidity and mortality (26). The transpulmonary driving pressure and plateau pressure at study end were considerably lower in the LI-MV + TTDN100% group, compared with the LI-MV group. Lower transpulmonary driving pressure is extremely relevant for pulmonary stretch-receptor activity, since transpulmonary driving pressure is the pressure generated by pulmonary tissue expansion during the respiratory cycle (15). It can be hypothesized that diaphragm neurostimulation might have affected parasympathetic activity by restoring a more physiologic pulmonary stretch during MV, thereby modulating pulmonary afferent stretch-receptor signaling to the brain via the vagus nerves in a way that mitigates hippocampal cellular apoptosis and neuroinflammation.

Our results showed that the LI-MV group had a significant decline in the RMSSD and also a greater level of hippocampal apoptosis, which contradicts previous results from other groups that used vagal blocking to prevent hippocampal apoptosis associated with volutrauma (17, 24). In preclinical experiments, systemic inflammation and neuroinflammation were induced by different methods, vagus stimulation was shown to result in a reduction of hippocampal apoptosis and neuroinflammation compared with the control group, and conversely, vagotomy resulted in increased hippocampal apoptosis and inflammation (25, 27–30). Our results align with the hypothesis that increased parasympathetic system activity could provide neuroprotection. Although our study has provided interesting data showing mitigation of hippocampal apoptosis and neuroinflammation by TTDN, it was not designed to investigate the mechanism of protection observed with diaphragm neurostimulation. The contradictory results found in our study compared with the results reported by other groups could be due to differences in many factors including but not limited to, study design, animal model, animal species used for the studies, level of sedation, duration of MV, MV settings, and the short-term anti-inflammatory effects of surgical vagotomy and vagal blockage.

It could also be argued that the brain injury observed might have been a consequence of the lung injury induced by oleic acid (ARDS), systemic inflammation, physical immobility, sedative drugs, and vasoactive drugs. Our data showed that the lung injury scores, the duration of physical immobility, the consumption of sedatives, the total amount of oleic acid used, the serum concentration of pro-inflammatory proteins, and vasoactive drug consumption were not statistically different between the mechanically ventilated groups, and therefore these factors are unlikely to have resulted in the differences in neural injury observed.

Additionally, during our studies, mean arterial pressures remained between the minimum (50 mm Hg) and maximum (150 mm Hg) thresholds that would disrupt cerebral vascular autoregulation (31–33). Therefore, changes in mean arterial pressure during the experiments cannot account for either brain injury or neuroprotection.

Our study had limitations. While 12 hours of MV after achieving moderate ARDS might be considered long-term for a preclinical study, it is short-term for a clinical study, limiting the generalizability of our findings. In addition, we did not analyze dopamine gene expression in the hippocampus, although HVA is an accepted surrogate for dopamine cerebral production (23). Most importantly, we have not investigated the clinical effects of the hippocampal injury associated

with ARDS with MV. Our experiment might have been underpowered to detect statistically significant differences between the groups' biomarkers for brain injury and systemic inflammation. Also, we cannot rule out the possibility that dyssynchrony or reverse triggering might have affected our results, although our data showed that the number of episodes of spontaneous breathing effort was minimal in all groups during the experiments. Another limitation is that we did not strictly follow the low-positive end-expiratory pressure (PEEP) ARDS ladder. During our experiments, only a few animals qualified for an increase of PEEP from 5 to 8 cm H_2O. Based on our previous experience, pigs are relatively intolerant to increases in PEEP. Also, due to the potential confounding effects of increasing PEEP in our experiment, maintaining a PEEP of 5 cm H_2O for all ventilated groups allowed comparison of the results from this study with those of our previous study. TTDN has significant impacts on pulmonary parameters that are beyond the specific scope of this article. A discussion on the pulmonary impacts of TTDN in this experiment has recently been published elsewhere (34). Finally, the association between MV, ARDS and cognitive impairment in critically ill patients is well documented; however, multiple factors alongside MV are involved in the development of cognitive impairment. Although the hybrid ventilatory strategy used in this study mitigated hippocampal apoptosis, and astrocyte and microglia percentages in deeply sedated animals in ICU conditions, clinical studies are needed to investigate ventilator-associated brain injury and its clinical outcomes.

CONCLUSIONS

Our study showed that porcine subjects with moderate ARDS, undergoing lung-protective MV for 12 hours, exhibited hippocampal apoptosis and inflammation, independent of systemic inflammation and lung injury. TTDN during the inspiratory phase of every breath resulted in neuroprotection, with lower hippocampal cellular apoptosis, and microglia and astrocyte percentages, compared with the other mechanically ventilated groups. Furthermore, the levels of hippocampal apoptosis and inflammation in the group receiving TTDN every breath were not different from NV subjects. Further, the group receiving TTDN every breath also showed considerably lower serum concentrations of HVA at study end, compared with the group receiving MV only. Future studies should investigate the mechanism of neuroprotective effect resulting from TTDN every breath. Furthermore, future translational studies should investigate whether TTDN could also mitigate neural injury in ARDS patients.

ACKNOWLEDGMENTS

We thank Suzette Willems, Kate Orchard, Stephanie Smith, Jessica Rabang, Pamela Zurek, Carli Peters, Samar Hejazi, and Brett Hannigan.

1 Lungpacer Medical Inc., Vancouver, BC, Canada.

2 Fraser Health Authority, Royal Columbian Hospital, New Westminster, BC, Canada.

3 Simon Fraser University, Burnaby, BC, Canada.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website (http://journals.lww.com/ccejournal).

Dr. Bassi and Evans were responsible for hypothesis generation. Drs. Bassi, Rohrs, and Reynolds were responsible for the conception of this study. Dr. Bassi, Dr. Rohrs, Gani, and Dr. Reynolds contributed to study design and data interpretation. Dr. Bassi, Dr. Rohrs, Gani, and Dr. Reynolds were responsible for writing the article. Dr. Bassi, Dr. Rohrs, Fernandez, Ornowska, Nicholas, and Wittmann performed data acquisition. Dr. Bassi, Dr. Rohrs, Ornowska, and Nicholas conducted data analysis. All authors have agreed with the final version of the article before submission.

Supported, in part, by grant from Lungpacer Medical, Royal Columbian Hospital Foundation, TB Vets Foundation, BC Lung Foundation, and Mathematics of Information Technology and Complex Systems.

The authors have disclosed that they do not have any potential conflicts of interest.

For information regarding this article, E-mail: tbassi@lungpacer.com

REFERENCES

1. Fan E, del Sorbo L, Goligher EC, et al; American Thoracic Society, European Society of Intensive Care Medicine, and Society of Critical Care Medicine: An official American Thoracic Society/European Society of Intensive Care Medicine/Society of Critical Care Medicine clinical practice guideline: Mechanical ventilation in adult patients with acute respiratory distress syndrome. *Am J Respir Crit Care Med* 2017; 195:1253–1263
2. Rezoagli E, Fumagalli R, Bellani G: Definition and epidemiology of acute respiratory distress syndrome. *Ann Transl Med* 2017; 5:282–282

3. Chen T, Chen C, Zhang Z, et al: Toll-like receptor 4 knockout ameliorates neuroinflammation due to lung-brain interaction in mechanically ventilated mice. *Brain Behav Immun* 2016; 56:42–55
4. Chen C, Zhang Z, Chen T, et al: Prolonged mechanical ventilation-induced neuroinflammation affects postoperative memory dysfunction in surgical mice. *Crit Care* 2015; 19:1–12
5. van Munster BC, Aronica E, Zwinderman AH, et al: Neuroinflammation in delirium: A postmortem case-control study. *Rejuvenation Res* 2011; 14:615–622
6. Kamuf J, Garcia-Bardon A, Ziebart A, et al: Lung injury does not aggravate mechanical ventilation-induced early cerebral inflammation or apoptosis in an animal model. *PLoS One* 2018; 13:e0202131
7. Bassi T, Rohrs E, Reynolds S: Systematic review on brain injury after mechanical ventilation. *Crit Care* 2021; 25:99
8. Bassi T, Rohrs E, Fernandez K, et al: Brain injury after 50 h of lung-protective mechanical ventilation in a preclinical model. *Sci Rep* 2021; 11:5105
9. Barton SK, Tolcos M, Miller SL, et al: Ventilation-induced brain injury in preterm neonates: A review of potential therapies. *Neonatology* 2016; 110:155–162
10. Bassi T, Rohrs E, Fernandez K, et al: Transvenous diaphragm neurostimulation mitigates ventilation-associated brain injury. *Am J Respir Crit Care Med* 2021; 204:1391–1402
11. Bassi TG, Rohrs EC, Fernandez KC, et al: Reply to Salimi et al. *Am J Respir Crit Care Med* 2022; 205:590–592
12. Bassi T, Rohrs E, Fernandez K, et al: Increased hippocampal apoptotic index after 50 hours of gold-standard mechanical ventilation in a pre-clinical pig model. *Am J Respir Crit Care Med* 2020; 201:A5961–A5961
13. Bickenbach J, Biener I, Czaplik M, et al: Neurological outcome after experimental lung injury. *Respir Physiol Neurobiol* 2011; 179:174–180
14. Grasso S, Stripoli T, de Michele M, et al: ARDSnet ventilatory protocol and alveolar hyperinflation: Role of positive end-expiratory pressure. *Am J Respir Crit Care Med* 2007; 176:761–767
15. Rohrs EC, Bassi TG, Fernandez KC, et al: Diaphragm neurostimulation during mechanical ventilation reduces atelectasis and transpulmonary plateau pressure, preserving lung homogeneity and PaO2/FiO2. *J Appl Physiol (1985)* 2021; 131:290–301
16. Matute-Bello G, Downey G, Moore BB, et al. An official American Thoracic Society workshop report: Features and measurements of experimental acute lung injury in animals. *Am J Respir Cell Mol Biol* 2011; 44:725–738
17. González-López A, López-Alonso I, Aguirre A, et al: Mechanical ventilation triggers hippocampal apoptosis by vagal and dopaminergic pathways. *Am J Respir Crit Care Med* 2013; 188:693–702
18. Voldby AW, Brandstrup B: Fluid therapy in the perioperative setting-a clinical review. *J Intensive Care* 2016; 4:27
19. Lee SH, Kwon JY, Kim SY, et al: Interferon-gamma regulates inflammatory cell death by targeting necroptosis in experimental autoimmune arthritis. *Sci Rep* 2017; 7:10133
20. Paine R, Standiford TJ, Dechert RE, et al: A randomized trial of recombinant human granulocyte-macrophage colony stimulating factor for patients with acute lung injury. *Crit Care Med* 2012; 40:90–97
21. Somann JP, Wasilczuk KM, Neihouser K, et al: Characterization of plasma cytokine response to intraperitoneally administered LPS & subdiaphragmatic branch vagus nerve stimulation in rat model. *PLoS One* 2019; 14:e0214317
22. Verlinden TJM, van Dijk P, Herrler A, et al: The human phrenic nerve serves as a morphological conduit for autonomic nerves and innervates the caval body of the diaphragm. *Sci Rep* 2018; 8:11697
23. Sternberg D, Heninger G, Roth R: Plasma homovanillic acid as an index of brain dopamine metabolism. *Life Sci* 1983; 32:2447–2452
24. González-López A, López-Alonso I, Pickerodt PA, et al: Lung purinoceptor activation triggers ventilator-induced brain injury. *Crit Care Med* 2019; 47:e911–e918
25. Meneses G, Bautista M, Florentino A, et al: Electric stimulation of the vagus nerve reduced mouse neuroinflammation induced by lipopolysaccharide. *J Inflamm (Lond)*. 2016; 13:33
26. Task Force of the European Society of Cardiology the North American Society of Pacing Electrophysiology: Guidelines heart rate variability. *Eur Heart J* 1996; 17:354–381
27. Huffman WJ, Subramaniyan S, Rodriguiz RM, et al: Modulation of neuroinflammation and memory dysfunction using percutaneous vagus nerve stimulation in mice. *Brain Stimul* 2019; 12:19–29
28. Kaczmarczyk R, Tejera D, Simon BJ, et al: Microglia modulation through external vagus nerve stimulation in a murine model of Alzheimer's disease. *J Neurochem* 2018; 146:76–85
29. Hiraki T, Baker W, Greenberg JH: Effect of vagus nerve stimulation during transient focal cerebral ischemia on chronic outcome in rats. *J Neurosci Res* 2012; 90:887–894
30. Kox M, Vaneker M, van der Hoeven JG, et al: Effects of vagus nerve stimulation and vagotomy on systemic and pulmonary inflammation in a two-hit model in rats. *PLoS One* 2012; 7:e34431
31. Lee KFH, Wood MD, Maslove DM, et al: Dysfunctional cerebral autoregulation is associated with delirium in critically ill adults. *J Cereb Blood Flow Metab* 2019; 39:2512–2520
32. Schramm P, Klein KU, Falkenberg L, et al: Impaired cerebrovascular autoregulation in patients with severe sepsis and sepsis-associated delirium. *Crit Care* 2012; 16:R181
33. Toth P, Szarka N, Farkas E, et al: Traumatic brain injury-induced autoregulatory dysfunction and spreading depression-related neurovascular uncoupling: Pathomechanisms, perspectives, and therapeutic implications. *Am J Physiol Heart Circ Physiol* 2016; 311:H1118–H1131
34. Rohrs EC, Bassi TG, Nicholas M, et al: Negative-pressure-assisted ventilation lowers driving pressure and mechanical power in an ARDS model. *J Appl Physiol (1985)* 2022; 133:1237–1249