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Original Article

Vagotomy Improves Hypoxic Pulmonary Vasoconstriction in Rats Subjected to Brain Ischemia-Reperfusion Injury

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Abstract

Background: Pulmonary dysfunction is one of the critical complications of a stroke. However, it remains unclear whether the mechanism is caused by either neurogenic or inflammatory reactions. The present study aimed to determine the effect of cerebral ischemia-reperfusion injury and the role of the vagus nerve on hypoxic pulmonary vasoconstriction (HPV) in rats.

Methods: This study was performed at Shiraz University of Medical Sciences, Shiraz, Iran, 2018. Male Sprague Dawley rats (n=56) were divided into four groups, namely the sham, vagotomy (Vag), 1 hour of ischemia followed by 23 hours of reperfusion without vagotomy (I/R) and with vagotomy (I/R+Vag). Neurological deficit scores and total infarct volumes of brains were measured in the I/R and I/R+Vag groups. Pulmonary artery pressure and lung weight were continuously registered during ventilation with normoxic and hypoxic gases in the isolated lungs. The blood gas parameters and the lung malondialdehyde (MDA) level of each group were also evaluated. ANOVA, with Tukey's *post hoc* test and *t* test, was used to compare the variables in the experimental groups.

Results: The infarct volume of the brains in the I/R and I/R+Vag groups were similar. HPV in the I/R group was lower than those in the sham and Vag groups, while vagotomy reversed this response in the I/R+Vag group (P=0.004). In the I/R group, PO_2 and pH were lower, and PCO_2 was higher than those in the sham and Vag groups. The lung MDA level in the I/R group was higher than that in the Vag group (P=0.019).

Conclusion: Brain ischemia-reperfusion injury decreased HPV independent of increased MDA in the lung, whereas vagotomy improved HPV by repairing the blood-gas barrier and oxygen sensing.

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Keywords ● Brain ischemia ● Hypoxia ● Pulmonary artery ● Vagus nerve

What's Known

- Stroke may be associated with distant organ dysfunctions including the lung.
- The mechanism of lung injury induced by brain ischemia has not been fully understood. To date, no study has reported the role of the vagus nerve on hypoxic pulmonary vasoconstriction in brain ischemia-reperfusion injury.

What's New

- Hypoxic pulmonary vasoconstriction was disrupted after 1 hour of brain ischemia followed by 23 hours of reperfusion regardless of increased lung malondialdehyde level, as an indicator of reactive oxygen production.
- Vagotomy recovered hypoxic pulmonary vasoconstriction, partly related to the repair of gas exchange through the blood-gas barrier and oxygen sensing.

Introduction

Stroke is the second leading cause of death in the world. In addition to brain damage, it may increase the risk of distant organ dysfunctions including the lung. However, the mechanism of lung injury induced by brain damage has not been entirely clarified. It has been proposed that brain ischemia produces pro-inflammatory and inflammatory mediators passing through the

systemic circulation to the lung.[1, 2] As a result, it leads to the accumulation of neutrophils and generation of reactive oxygen (ROS) and nitrogen (NOS) species in the lung, which ultimately leads to pulmonary edema.[3, 4]

In addition to its autonomic functions, the vagus nerve may also play a role in the inflammatory reactions of the organ tissues. However, the effect of the vagus nerve in this regard is controversial. It has been shown that the vagus nerve has an anti-inflammatory effect. Vagal stimulation decreases serum tumor necrosis factor-alpha (TNF-α) 10 minutes before or after the administration of lipopolysaccharide (LPS).[5] It has been reported that α7 nicotinic cholinergic receptor (α7nAchR) plays an important role in mediating the systemic immunosuppression observed after 1 hour of the middle cerebral artery occlusion (MCAO) and 24 hours of reperfusion.[6] On the other hand, the inflammatory effect of the vagus nerve has been demonstrated in some studies. The cervical vagotomy reduces inflammation induced by capsaicin in the lower airways.[7] Also, serum TNF-α significantly decreases with bilateral cervical vagotomy in LPS-injected animals.[8] Furthermore, mechanical ventilation with moderate tidal volume potentiates the pulmonary inflammation caused by the injection of LPS, whereas vagal stimulation has no effect.[9]

There are a few experimental models indicating lung injury induced by brain damage. It has been shown that brain injury worsened mechanical lung injury in an isolated rabbit lung model.[10] Also, MCAO for 30 minutes and reperfusion for 48 hours increased brain and lung water contents which attenuated after the administration of hypertonic saline.[11] Nevertheless, no study has reported the effect of brain ischemia-reperfusion injury on the gas exchange through the respiratory membrane.

Hypoxic pulmonary vasoconstriction (HPV) is a normal response of pulmonary vessels to alveolar hypoxia. These vessels, unlike the systemic circulation, constrict the vessels and shift the blood from the low oxygen pressure alveoli to the well-ventilated ones. As a result, the ventilation/perfusion ratio will be optimized. The precise mechanism of HPV pathogenesis has not been determined so far. Many studies have reported the role of ROS in HPV. However, it is not clear whether it is the increase or the decrease in ROS that induces HPV.[12-15] Furthermore, little attention has been paid to the effect of brain ischemia on HPV. Moreover, the effect of vagotomy on HPV in brain ischemia remains to be investigated.

The present study aimed to investigate the pulmonary vascular response to alveolar hypoxia in an isolated rat lung after 1 hour of MCAO followed by 23 hours of reperfusion. The infarct size of ischemic brains and neurological scores were also determined. Furthermore, we measured blood gas parameters and lung tissue malondialdehyde (MDA), as an indicator of lipid peroxidation and ROS production. All the above parameters were evaluated in the vagotomized rats accordingly.

Materials and Methods

Male Sprague-Dawley rats weighing 220-250 g were purchased from the Laboratory Animal Breeding Center of Shiraz University of Medical Sciences, Shiraz, Iran, in 2018. The rats were housed in standard cages under controlled laboratory temperature, humidity and 12:12 hour light:dark cycles. They had free access to water and standard food a few days before starting the experiments.[16] Animals (n=56) were divided into four groups, namely sham (n=14), vagotomy (Vag, n=14), cerebral ischemia without vagotomy (I/R, n=14), and cerebral ischemia with vagotomy (I/R+Vag, n=14). Each group was divided into two subgroups (n=7 in each subgroup) to measure: (i) the pulmonary artery pressure (PAP) and lung weight in the first subgroup, and (ii) arterial blood gas parameters and MDA in the second subgroup.

All experimental procedures were approved by the Center for Comparative and Experimental Medicine and the Ethical Committee of Animal Care at Shiraz University of Medical Sciences (Shiraz, Iran), and performed according to the provisions of the declaration of Helsinki (code number: IR.SUMS.REC.1395.S26).

Brain Ischemia-Reperfusion Injury

Focal cerebral ischemia was induced using the MCAO method.[17] Briefly, the animals were anesthetized with intraperitoneal injection of ketamine (60 mg/kg), Alfasan, Netherland, and Xylazine (10 mg/kg), Alfasan, Netherland. The right common carotid artery and its branches were exposed by a midline neck incision. A silicone-coated monofilament suture (Doccol, 4043PK5Re, USA) inserted into the external carotid artery and advanced into the internal carotid artery such that the middle cerebral artery was occluded for 1 hour. The monofilament was then removed, followed by 23 hours of reperfusion. The skin was sutured using a 5-0 nylon thread (SUPA, Iran). After surgery, the animals were placed in separate and clean cages and returned to the animal house.

Vagotomy

In the vagotomized groups, immediately after

1 hour of the first surgery, the right cervical vagus nerve was separated from the right common carotid artery. Then, a 0.5-cm segment of the nerve distal to the origin of the superior laryngeal nerve was transected.[18] In the sham and I/R groups, the surgical intervention was similar to those used in the denervated groups, but the vagus nerve was not transected.

Neurological Deficit Score and Infarct Volume

After 23 hours of reperfusion, the neurological deficit score (NDS) was used to grade the outcomes in all four groups: 1, no observable neurological deficit; 2, failure to extend right forepaw; 3, circling to the right; 4, falling to the right; 5, inability to walk spontaneously and a depressed level of consciousness. Then, the animals were anesthetized with an IP injection of sodium thiopental (60 mg/kg), Vuab Pharma A.s, Czech, tracheostomized, and decapitated. The skull was opened by means of a bone fracture and the brain was removed in its entirety with caution in all experimental groups. Each brain was sectioned coronally into 2 mm slices (rat brain matrix, USA), stained with three phenyl tetrazolium chloride (TTC, Sigma, Germany), and transferred to 10% buffered formalin. The next day, the slices were photographed individually using a digital camera (Olympus, Japan). The cortical and striatal infarct areas of the slices were measured using an image analyzer software (NIH Image Analyzer). Then, all calculations were performed using Microsoft Excel 2010. The area of the infarct size was multiplied by the thickness of each slice to calculate the infarct volume. Finally, all infarct volumes of the slices were added to measure the total cortical and striatal infarct volumes of brains.[6, 17]

Preparation of Isolated Lung

Preparation of the isolated lung in the first subgroup was performed in accordance with the method described in previous studies.[19, 20] Briefly, at the same time as the brain preparation, the lungs were tracheostomized and ventilated (Harvard, USA) with room air (tidal volume: 1.2 ml/100 g BW and respiratory rate: 50 breaths/minute) with the help of an assistant to prevent any lung damage. The chest was opened and the right ventricle sectioned, the pulmonary artery was cannulated and perfused (Infusion pump, SN-1500 H, China) with a perfusate solution (4 °C and 2 mL/min). The lung was carefully separated from the chest and the left atrium cannulated while the heart and lung were suspended over a stand. Then, the ventilated-perfused lung was carefully transferred to a humidified chamber of the isolated lung system. Meanwhile, the ventilated gas was replaced with normoxic gas to maintain the pH of the lung within the normal range. Also, the temperature of the whole system increased up to 37 °C and the perfusate flow rate was gradually increased to a maximum value of 10 mL/min. The lungs were subsequently rinsed to remove residual blood and the perfusion circuit was closed for circulation. The left atrial pressure was adjusted to about 1.5-2 cmH_2O, and positive expiratory pressure (PEEP) of 1 cmH_2O was employed. The PAP and lung weight were recorded by pressure and force transducers connected to the PowerLab system, respectively (ADInstruments, Australia).

Composition of Ventilatory Gas and Perfusate

In the present study, two different gas mixtures were used; normoxic gas (15.5% O_2, 5.5% CO_2 balanced with N_2) and hypoxic gas (1.0% O_2 and 5.5% CO_2 balanced with N_2). The perfusate contained 120.0 mM NaCl, 1.1 mM K_2HPO_4, 1.3 mM $MgCl_2$, 4.3 mM KCl, 2.4 mM $CaCl_2$, 13.3 mM glucose, 1 g dextran/100 mL (mw 70,000), and 24 mM $NaHCO_3$.[20]

Study Protocol

After 1-hour surgery followed by a 23-hour recovery, the rats in each group (sham, ischemia with or without vagotomy) were anesthetized, the brains removed, and sectioned. Then, the animals were divided into two subgroups. The first subgroup was used for the isolated lung measurements in which the PAP and lung weight were continuously recorded throughout the experiments. Lungs were first ventilated with normoxic gas for during 30 minutes of the steady-state period, followed by the ventilation with hypoxic gas for 10 minutes in the presence of phenylephrine 30 µM phenylephrine (Sigma, Germany).[20] The mean values of the PAP changes (ΔPAP) during minutes 5-10 of hypoxic maneuver was considered as the response of pulmonary vasculature to hypoxia. In the second subgroup, arterial blood was taken from the femoral artery to analyze the blood gas parameters (Medica, Easy blood gas, USA). Finally, the animals were sacrificed under deep anesthesia. The chest was opened and the lower parts of the right and left lung sectioned and stored at -70 °C for the MDA measurement.

MDA Assay

The lung tissue (50 mg) was homogenized in 450 µL of 1.15% KCl for 3 minutes. Then, 250 µL of sodium dodecyl sulfate (SDS) was mixed with the homogenized solution and

centrifuged at 12,000 rpm for 20 minutes at 4 °C. The supernatant was used for the MDA measurement by thiobarbituric acid reactive substances assay (TBARS). Briefly, samples and standards (1,1,3,3-tetra ethoxy propane, Sigma, Germany) were mixed with 0.25 N HCl (Sigma, Germany), 20% trichloroacetic acid (Sigma, Germany), and 0.8% thiobarbituric acid (Sigma, Germany) incubated at 90 °C for 60 minutes and centrifuged at 12,000 rpm for 3 minutes at 4 °C. Finally, the absorbance was read at 532 nm using a microplate reader (Biotek, USA).[19, 21]

Statistical Analysis

The data were analyzed using SPSS software (version 23.0). Analysis of variance (ANOVA) with Tukey's *post hoc* test was used to compare the experimental groups. The student *t* test was used to compare between the groups. Data were expressed as mean±SE. P<0.05 with 95% confidence intervals was considered statistically significant.

Results

There was no significant difference in the mean values of neurological deficit score (NDS) or total infarct volumes of the brains in the I/R and I/R+Vag groups (figure 1 and 2). Since the atmospheric pressure in the city (Shiraz, Iran) was about 630 mmHg, the alveolar oxygen pressure was set to about 74 mmHg under normal conditions. Therefore, an arterial oxygen pressure (PaO_2) of about 5-10 mmHg less than that in the alveoli was considered normal. PaO_2 in the I/R group was lower than those in the sham and Vag groups (P=0.001) and vagotomy prevented this reduction in the I/R+Vag group (P=0.001). The arterial carbon dioxide pressure ($PaCO_2$) in the I/R group was higher than those in the sham (P=0.006) and Vag (P=0.003) groups, and vagotomy decreased $PaCO_2$ in the I/R+Vag group compared with the I/R group (P<0.001). The pH level in the I/R group was lower than those in the sham (P=0.036) and Vag (P=0.019) groups, whereas in the I/R+Vag group, it was recovered compared with the I/R group (P=0.001). There was no alteration between concentrations of HCO_3^- and base excess in the experimental groups (table 1).

There was no difference between the increase of ΔPAP in the sham and Vag groups during hypoxic maneuvers. However, the increase of PAP in the I/R group was insignificantly lower than those in the other groups. Also, the reduction of lung weight (ΔLW) in the I/R group was less than that in the Vag

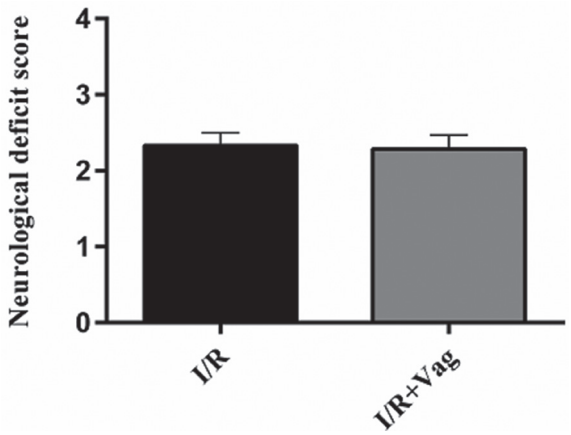

Figure 1: The figure shows identical neurological deficit scores in the I/R and I/R+Vag groups (n=7). Data are expressed as mean±SE.

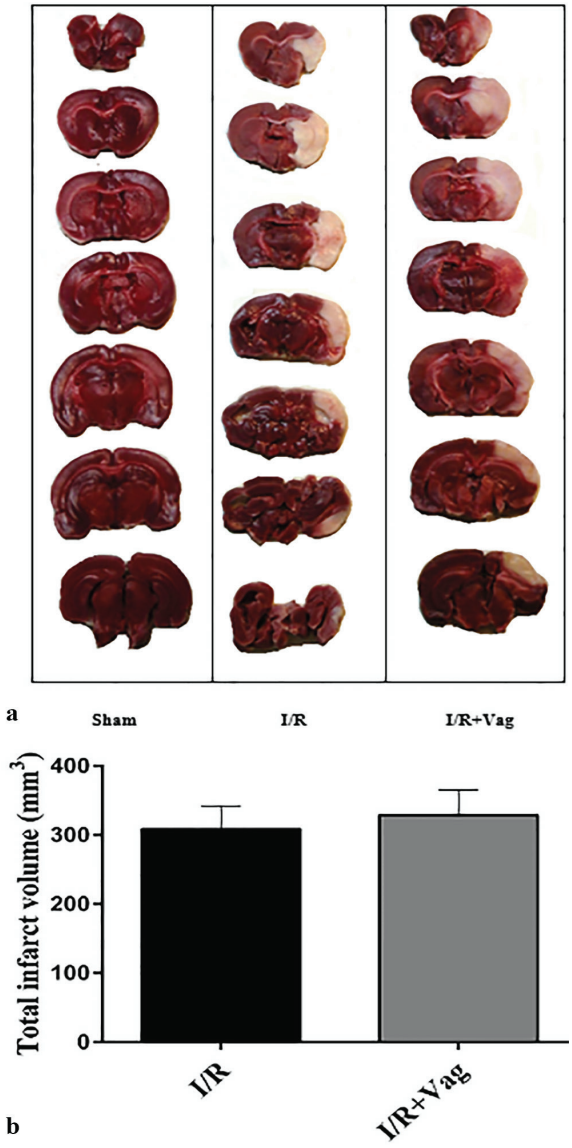

Figure 2: TTC-stained brain sections indicated a normal tissue in the sham group (red color) and ischemia tissues (white color) in the I/R and I/R+Vag groups (a). There was no alteration in the total infarct volume of the I/R and I/R+Vag groups (b). Data are expressed as mean±SE and n=7 in each group.

Table 1: Blood gas parameters in the second subgroup (n=7) after 24 hours

	Sham	Vag	I/R	I/R+Vag
pH	7.34±0.01	7.35±0.01	7.26±0.03*#	7.39±0.02†
PCO_2 (mmHg)	45.98±1.98	44.24±1.50	56.66±2.49*#	40.60±1.28††
PO_2 (mmHg)	64±2.61	62.20±4.16	41.80±1.70**##	61±0.3$
HCO_3^- (mmol/L)	24.48±0.77	24.42±1.04	25.50±0.66	24.72±0.52
BEb	-1.83±0.65	-2.58±0.23	-2.74±1.13	-0.38±0.68

Data are expressed as mean±SE. PCO_2: Carbone dioxide pressure; PO_2: Oxygen pressure; HCO_3^-: Sodium bicarbonate concentration; BEb: Blood base excess; *P<0.05 and **P<0.01 vs. the sham group; #P<0.05 and ##P<0.01 vs. the Vag group, †P<0.05 and ††P<0.01 vs. the I/R group

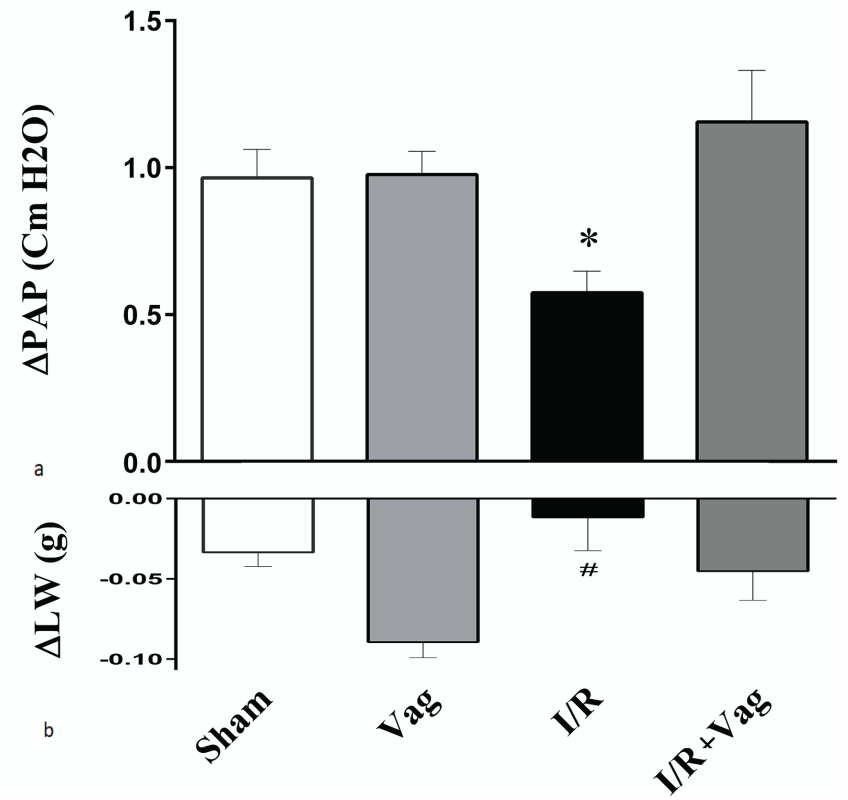

Figure 3: The mean values of ΔPAP (a) and lung weight (ΔLW) (b) during hypoxic gas ventilation in the I/R group were lower than that in the sham, Vag and I/R+Vag groups. Data are expressed as mean±SE and n=7 in each group. HOX: Hypoxia, NOX: Normoxia, Phe: Phenylephrine. *P<0.05 vs. the sham group.

group (P=0.003). Vagotomy in the I/R+Vag group returned the normal hypoxia response so that ΔPAP was significantly higher than that in the I/R group (P=0.004); figure 3. There was no significant variation between the mean values of MDA in the sham and Vag groups. MDA in the I/R group was higher than those in the other groups and significant compared with the Vag group (P=0.019). Note that vagotomy returned MDA to the normal values in the I/R+Vag group (figure 4).

Discussion

In the present study, brain ischemia-reperfusion injury impaired gas exchange through the blood-gas barrier and decreased HPV in the isolated lung in spite of increased MDA in the lung tissue. Vagotomy prevented respiratory acidosis and returned the pulmonary vascular response to hypoxia in brain ischemia-reperfusion injury.

No alteration in the infarct volume of the ischemic brain and NDS was detected in the I/R and I/R+Vag groups, whereas the hemodynamic and chemical parameters of the lung differed among these groups. This may suggest different roles for the afferent and efferent pathways of the vagus nerve and therefore, different effects of vagotomy on inflammatory reactions in the brain and lung. Furthermore, the inflammatory or anti-inflammatory effect of the vagus nerve is controversial. Activation of the afferent vagus nerve may trigger the hypothalamic-pituitary axis, leading to the release of glucocorticoids and consequently inhibiting the production of local cytokines.[8, 22] Also, acetylcholine (ACh)

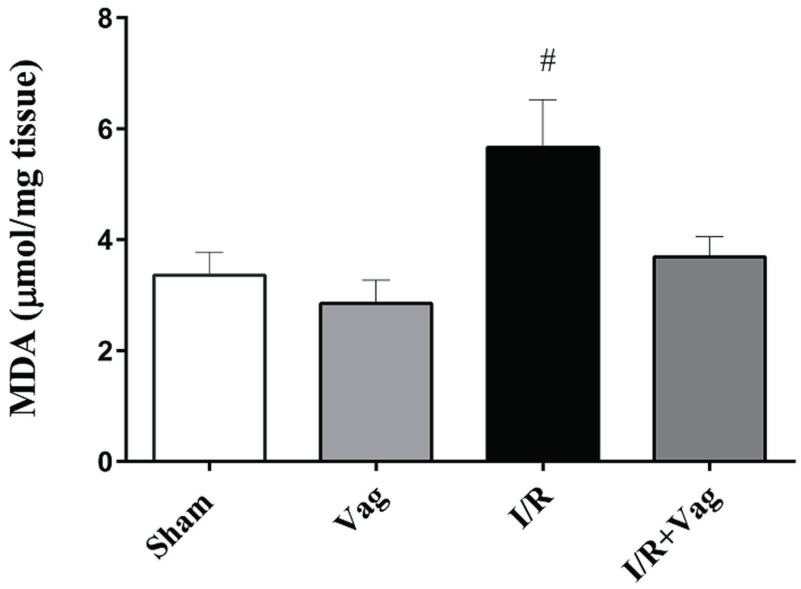

Figure 4: Lung MDA in the I/R group was higher than that in the sham, Vag, and I/R+Vag groups (n=7 in each group). Data are expressed as mean±SE. #P<0.05 vs. the Vag group.

release from the efferent fibers of the vagus nerve inhibited NF-κB and cytokines production through the activation of α7nAchR and the signaling pathways of cAMP, CREB, and c-fos genes. In addition, ACh inhibited NF-κB by the activation of the JAK-STAT pathway.[23-25] In LPS-stimulated human macrophage cultures, ACh attenuated the release of inflammatory cytokines but not of the anti-inflammatory cytokine interleukin 10 (IL-10). Furthermore, to reduce the local cytokines, the systemic inflammatory reactions can be affected by the vagus nerve activity as the electrical stimulation of peripheral vagus nerve attenuated serum TNF-α during endotoxaemia.[5]

In contrary to the aforementioned studies, the vagus nerve may cause the inflammatory reactions. Perhaps the timing of vagus nerve stimulation is critical for the inflammatory or anti-inflammatory response, since vagal stimulation at 4 hours after the induction of polymicrobial sepsis by cecal ligation and puncture (CLP) does not reduce the lung injury or pulmonary and systemic inflammatory markers.[26] Nevertheless, the acetylcholinesterase inhibitor physostigmine attenuated inflammation and septic shock when administered prior to and not after the induction of CLP.[27] Francis and colleagues also indicated that vagotomy 30 minutes before the injection of LPS decreased plasma TNF-α. However, vagotomy increased TNF-α at 3 days prior to LPS injection in rats subjected to myocardial infarction.[28] Fuentes and colleagues stated that vagotomy decreased 50% of serum TNF-α and interleukin 6 (IL-6) at 24 hours prior to LPS injection.[8] Zielinski and colleagues reported that the expression of TNF-α in the brain of mice infected with LPS reduced 2 weeks after vagotomy.[29]

In the present study, vagotomy was performed immediately after 1 hour of ischemia and at the onset of 23 hours of reperfusion period. The blood gas analyses showed an uncompensated respiratory acidosis due to increased PCO_2, decreased PO_2, and decreased pH with no significant change in the arterial blood HCO_3 in the I/R group, while vagotomy prevented any of these alterations in the I/R+Vag group. This result was in line with the hypothesis of the inflammatory effect of the vagus nerve, as vagotomy prevented the damage in the blood-gas barrier following brain ischemia. On the other hand, the parasympathetic nerve is responsible for airway constriction in the lung. Therefore, the activation of the vagus nerve may lead to hypoventilation and impairment of gas exchange, whereas vagotomy has the opposite effect. As a result, it may be suggested that the constrictor effect of the vagus nerve in the lung is predominant over its anti-inflammatory reaction. Vagotomy may also improve the blood gas parameters by bronchodilation. However, due to the preventive effect of vagotomy on lung MDA in the I/R+Vag group, it is probable that vagotomy corrected the gas exchange through the repair of the respiratory membrane.

Alveolar hypoxia alleviated the pulmonary vascular response in the I/R group, whereas vagotomy improved HPV in the I/R+Vag group. Some studies have shown that lung injury in animal models impaired HPV linked to impairment of the respiratory membrane and oxygen sensing.[30, 31] Since the abnormal blood

gas parameters in the I/R group were corrected in the I/R+Vag group it can be concluded that the disruption of HPV in the I/R group may be related to the impairment of the alveolar-capillary barrier, and vagotomy could return normal oxygen sensing and HPV by preventing lung tissue damage in the I/R+Vag group.

Although the MDA level increased in the I/R group, HPV in this group was lower than those in the sham and Vag groups. On the other hand, the MDA level in the I/R+Vag group was less than that in the I/R group, while the response to the hypoxia was similar to that in the sham group. These results indicated that lung injury induced by brain ischemia had a negative effect on hypoxia response. The role of ROS in HPV is still controversial. Many studies have reported the increase of ROS in HPV, whereas some investigations demonstrated the opposite results.[15] However, due to the damage to the lung parenchymal tissue, the decrease or increase in MDA could not be considered as a marker for HPV response in our study.

The main limitation of the study was the high mortality rate of animals in the MCAO method. Furthermore, data collection in the isolated lung system was difficult and must be performed with caution. Therefore, we measured blood gas parameters and lung MDA in separated subgroups to prevent any interference of the experimental protocols with the results.

Conclusion

The findings of the present study indicated that HPV was disrupted in cerebral ischemia-reperfusion injury despite increased MDA in the lung tissue. Vagotomy recovered this response probably by repairing the gas exchange through the blood-gas barrier. However, the effects of efferent and afferent vagal stimulation on lung injury induced by brain ischemia and HPV needs to be clarified.

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Conflict of Interest: None declared.

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