

Neuroprotection by hypercapnia in brain ischemic injury followed by persistent hypoxia: an in vivo and in vitro experimental study

Wanchao Yang

Second Affiliated Hospital of Harbin Medical University

Qi Wang

Second Affiliated Hospital of Harbin Medical University

Xiaohong Wu

Third Affiliated Hospital of Harbin Medical University

Tingting Li

Second Affiliated Hospital of Harbin Medical University

Qiang Wan

Second Affiliated Hospital of Harbin Medical University

Wen-Zhi Li (wenzhili9@126.com)

Second Affiliated Hospital of Harbin Medical University https://orcid.org/0000-0003-4196-524X

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Abstract

Backgrounds: Therapeutic hypercapnia was shown to have a potential neuroprotective role in our previous studies in a rat model of ischemia followed by hypoxia; however, it is unknown how hypercapnia affects blood-brain barrier (BBB) function under hypoxic conditions in cerebral ischemia. We aimed to observe the BBB permeability changes in response to cerebral ischemia followed by acute hypoxia or hypercapnic hypoxia using *in vivo* and *in vitro* models.

Methods: Adult rats underwent unilateral common carotid artery ligation, <u>at 60 min of ligation, they were</u> <u>exposed to</u> systemic hypoxia with ventilation of 15% oxygen (O_2) combined with 8% carbon dioxide (CO_2) for 180 min. Cerebral blood flow, BBB integrity, infarct volume and behavior were assessed in this study. *In vitro*, rat brain microvascular endothelial cells (BMECs) were isolated and cultured under O_2 (1% or 21%) with or without 15% CO_2 for 6 h. Cell viability and transendothelial electrical resistance <u>(TEER)</u> were measured. The ZO-1 and occludin protein levels were explored in BMECs by Western blotting.

Results: Arterial blood O_2 (Pa O_2) tensions averaged 56.1 mmHg during simple hypoxia, and arterial blood O_2 tensions (Pa O_2) were maintained at normal values or 60–80 mmHg. Hypercapnia treatment significantly reduced brain infarct volume and pathophysiological changes in hypoxic ischemia rats. Furthermore, in the *in vitro* experiment, hypercapnia significantly improved the growth condition of BMECs, reduced endothelial cell permeability and attenuated the loss of ZO-1 and occludin protein in BMECs induced by hypoxia.

Conclusions: Hypercapnia exerts beneficial effects on the BBB permeability in the rat model of hypoxicischemic injury and recovers the neurologic status especially within one week, possibly by preventing the loss of tight junction proteins.

Backgrounds

Acute ischemic stroke is a major cause of mortality and long-term neurologic morbidity in the adult population.[1] Hypoxia is one of the most important mechanisms in the development of stroke pathological processes.[2, 3] Cerebrovascular endothelial cells play a central role in the formation of the blood-brain barrier (BBB) which has the function to limit the transport of potentially harmful substances from blood to brain and provides a steady state environment for proper neural function. Hypoxia and ischemia may affect the integrity of the BBB.[4] Several lines of evidence have shown that hypoxic-ischemic (HI) insults trigger a cascade of biochemical, cellular, and pathological events that result in cell injury and death in the brain.[5–7] Hypoxic factors were reported to be important pathogenic factors in the alteration of tight junction (TJ) proteins and induction of vascular leakage in the brain.[8] The disruption of BBB can be major part of the pathology of cerebral ischemia through altered TJ permeability.[9, 10] Exposure of brain-derived endothelial cells to hypoxia was reported to result in the depressed localization of occludin and ZO-1 to the plasma membrane, although their protein levels were

barely affected.[11, 12] However, some studies have demonstrated that TJ proteins of cerebral microvessels are down-regulated in response to hypoxia/aglycemia.[13, 14]

Hypercapnia has been shown to have a significant therapeutic effect on brain injury,[15–17] and arterial blood CO_2 tension (PaCO_2) at 60–100 mmHg had a protective impact on the brain during ischemic injury. [18–20] In our previous study, hypercapnia exerted beneficial effects under mild to moderate hypoxemia and augmented the detrimental effects on the brain during severe hypoxemia in a rat model of hypoxia-ischemia.[7] However, BBB dysfunction did not further deteriorate after combined exposure to moderate hypoxia and hypercapnia in this animal model. Moreover, little is known about how the hypercapnia affects molecular and functional changes, such as hypoxia-induced disruption of the TJs, in BBB dysfunction.

In the present study, the effect of moderate hypercapnia on ischemic outcomes was determined in a rat model of transient cerebral hypoxic ischemia by assessing the following parameters: infarct volumes, BBB permeability and neurological function. Furthermore, a primary culture model for brain microvascular endothelial cells (BMECs) was established to explore the effect of hypercapnia on permeability and TJ protein expression after hypoxic exposure for 6 h. This study provided experimental evidence to help elucidate the effects of hypercapnia on hypoxic-ischemic brain injury.

Methods

Animal care

In accordance with the ARRIVE (Animal Research: Reporting *In Vivo* Experiments) guidelines for animal research, adult male Wister rats weighing 250–300 g were purchased from the Laboratory Animal Center of Harbin Medical University (Harbin, China). The experimental protocol and animal care were approved by the Ethics Committee of Animal Care and Guidelines in Harbin Medical University for the care and use of laboratory animals.

Experimental protocol

Eighty-five rats were divided randomly into three groups: control group (normoxia, FiO₂ 21%, PaCO₂ 35–45 mmHg), hypoxia-ischemia (HI) group (FiO₂ 15%, PaO₂ 50–59 mmHg, PaCO₂ 35–45 mmHg), and hypercapnia group (PaCO₂ 60–80 mmHg + HI group). The rats in the control group (n = 25) were anesthetized with an intraperitoneal injection of 30 mg/kg pentobarbital sodium (Abbott, North Chicago, IL) and underwent left common carotid artery (CCA) was exposed and ligated. At 1 h of ligation, rats were mechanically ventilated with air via tracheostomy (Harvard ventilator 683, USA) for 3 h. 0.5% ropivacaine was injected for local infiltration when the brain skin was made incision and when performed tracheostomy. In the HI group (n = 30), under the same anesthetic conditions and surgical procedures as rats in the control group, the CCA was ligated, and at 1 h of ligation, followed by ventilation and

inhalation of a gas mixture of 15% O_2 for 3 h. In the hypercapnia group (n = 30), rats received the same HI protocol and the same fraction of inspired oxygen as described for the HI group with an additional 8% CO₂, but hypercapnia was applied at the beginning of ventilation. The body temperature of rats was kept constant at 37 °C using a rectal thermometer and a servo-controlled heating pad. The skull was fixed in a stereotaxic frame (model 51600; Stoelting Co., Wood Dale, IL) for preparation of the cranial window over the left parietal hemisphere for laser Doppler flowmetry assessment. We first made a midline incision of the rat brain skin, then carefully stripped the subcutaneous tissue as well as skull periosteum of the left parietal lobe to make the skull surface smooth and clean. Then we used a 20 ml syringe needle to drill a craniotomy (a diameter of 1.5 mm) to perfectly place a laser doppler flow probe (PeriFlux 5000, Perimed, Sweden) onto the surface of dura and was fixed in place by dental acrylate to record regional cortical blood flow every 30 min. In this process, the probe was inserted to 1–2 mm, avoiding to puncture the dura. When the probe was pulled out, the craniotomy was sealed by dental acrylate. Inhaled and exhaled CO₂ and O₂ were monitored continuously using a gas monitor (MindaryBeneView T8, Mindray Medical International Limited, P. R. China). Systemic mean arterial blood pressure (mmHg) was measured in the left ligated proximal CCA. A cannula was inserted to the left femoral vein for blood gas determination and peripheral drug administration. After 3 h of ventilation, the catheters were removed, a thin layer of erythromycin ointment on each layer of tissue before the closure of skin as prophylactic antibiotics and the animals were extubated and placed in an oxygenated Plexiglass box until the animals were awake. Rats were given free access to food and water immediately after recovering from anesthesia. Rats that died during systemic hypoxia with or without hypercaphic ventilation were excluded from measuring infarct size and BBB permeability. The neurological severity scores (mNSS) were calculated for the HI and HP groups and used as an important parameter to assess the beneficial effect of hypercapnia.

Assessment of BBB permeability

Five rats from each group were randomly selected to assess the BBB permeability. The quantitative evaluation of BBB disruption during HI and hypercapnia was achieved by measuring fluorescence in the defined brain areas as previously described ^[21]. Before the end of ventilation with hypoxic and hypercapnic gas for 10 min, rats were injected (0.5 mL) with fluorescent dextran (10 kDa in saline, 2 mg/mL) via a unilateral cannula implanted into the left CCA. The rats were then transcardially perfused with 0.9% saline to remove the intravascular dextran. The brain was rapidly removed, and the left and right hippocampus and left and right cortex were dissected. Brain tissues were homogenized in 50% wt/vol trichloroacetic acid (Sigma, St. Louis, MO, USA). After centrifugation (10,000 g), the supernatant was collected. The fluorescence intensity (ng/mL) in 96-well plates was measured by a microplate fluorescence reader (TECAN Infinite M200, Männedorf, Switzerland) and analyzed at an excitation wavelength of 495 nm and an emission wavelength of 520 nm. The differences in the fluorescence intensity between the control and HI groups were calculated as tracer leakage, and the data are presented as percent changes from the control (normoxia) tissues.

Infarct volume measurement

Infarction volumes were measured by analysis of the 2,3,5-triphenyltetrazolium chloride (TTC) staining in brain sections taken 24 h after HI using standard protocols ^[22]. Briefly, five rats from the HI and HP groups were randomly selected for analysis. Brain tissues were immediately collected and cut into 2 mm coronary slices using a stainless steel mold (68709, RWD Life Science Co., Ltd., China). These slices were placed flat in 2% TTC (Sigma, St. Louis, MO, USA) for 30 min. The sections were immersed in 10% formalin at 37 °C for 12 h. Non-infarct tissues were stained red, and the infarct regions were white after TTC staining. The slices were photographed, and the area of ischemic injury as well as the hemisphere area was measured by Image-Pro Plus software (Version 6.0, Media Cybernetics, Bethesda, MD, USA). The infarct volume was calculated as follows: $(a1 + a2 + a3...+a6) \times t$, where a1 to a6 is the infarct area of each section, and t is the serial section thickness. Edema area was defined as the ipsilateral hemisphere area minus the contralateral hemisphere area. For elimination of the effects of edema, the percentage of ischemic area was assessed according to the following formula: percentage of infarction=[measured infarct area-edema area]/[(ipsilateral hemisphere area + contralateral hemisphere area].

Anti-Endothelial Cell antibody (RECA-1) expression in the brain

After hypoxia for 3 h with or without hypercapnic treatment, four rats from each group were randomly selected. Rats were transcardially perfused with ice-cold phosphate buffered saline (PBS) (pH 7.2) under anesthesia. The brain was removed, fixed with 4% paraformaldehyde and sliced into coronal sections (4 µm thick). The brain sections were incubated in the primary antibody anti-RECA-1 (mouse monoclonal antibodies, 1:20 dilution, ab9774; Abcam, Cambridge, MA, USA) overnight at 4 °C. After washes with PBS, the sections were then incubated with a goat anti-mouse fluorescein-conjugated secondary antibody (1:1000 dilution, 1031-01; Southern Biotech, Birmingham, USA) for 20 min at 37 °C. The sections were incubated with DAPI (1:1000) in 1% bovine serum albumin for 10 min, mounted on albumin-coated slides, and sealed. Images were captured under a laser scanning confocal microscope (Fluoview 1000; Olympus, Tokyo, Japan).

Evaluation of neurological function

The neurological function test was performed in a double-blinded manner by two unbiased investigators. The neurological function was evaluated at 2 h before and 3 ,7,14d after hypoxia with or without hypercapnic exposure using the mNSS score as described in a previous modified assay. [23] The mNSS was a composite of motor, sensory, reflex, and balance tests. The neurological function evaluation was graded on a scale of 0-18 (0 = normal score; 18 = maximal deficit score). In the injury severity scores, 1 score point was awarded for the inability to perform the test or for the lack of a tested reflex; thus, the higher the score is, the more severe is the injury.

Primary cell culture

Brain microvascular endothelial cells (BMECs) were extracted from 3-week-old rats as a described in a previous study. [24] Briefly, BMECs were cultured in DMEM/F12 with puromycin at 37 °C with a humidified atmosphere of 5% $CO_2/95\%$ air for 2 d. On the 3rd day, the puromycin was removed from the medium. When the cells reached 80% confluence (5th – 6th day), the endothelial cells were passed using a trypsin/EDTA mixture (Sigma). The cells were transferred to 12-well Transwell inserts coated with collagen type IV and fibronectin to form the BBB model *in vitro*. The endothelial monolayer showed positive expression of the endothelial marker von Willebrand factor (supplementary Fig. 1) after immunostaining. BMECs were incubated at 37 °C for 6 h. The cells were divided into three groups (each group, n = 3): the normoxia (21% O_2), hypoxia (1% $O_2 + 5\%$ $CO_2 + 94\%$ N_2) and hypercapnia groups (1% $O_2 + 15\%$ $CO_2 + 84\%$ N_2).

Assessment of gas analysis and cell viability

Cell culture media (before hypoxia, after hypoxia with or without hypercapnia) were removed, and gas values (pH, PCO₂, PO₂) were measured using a blood gas analyzer (Bayer Rapid Lab 855, Leverkusen, Germany). The viability of the endothelial cells in response to oxidative stress was assessed by measuring the lactate dehydrogenase (LDH) activity in the cell culture medium after each experiment. For this purpose a Lactate Dehydrogenase Activity Assay Kit (Sigma,MAK066) has been used. For further analysis only cultures with no increased LDH activity has been used.

Measurement of transendothelial electrical resistance (TEER)

Since resistance is inversely proportional to permeability, TEER (in $\Omega \times cm^2$) was measured using an EVOM₂ membrane potentiometer (World Precision Instruments, Inc., U.S.). After the samples were exposed to hypoxia with or without hypercapnia for 2, 4 or 6 h, the TEER values were measured in BMECs. The resistance of blank inserts was subtracted as background resistance from the total resistance of each treatment. [25]

Western blotting

The treated or control cells were lysed for 30 min on ice. The total protein was measured by a BCA kit (Beijing Kangwei Century, China). The electrophoresis was performed and the membrane was hybridized as described in previous studies. [26, 27] The primary antibodies were used as follows: rabbit polyclonal anti-ZO-1, anti-occludin, and mouse anti- β -actin antibodies (Zhongshan Golden Bridge, China). Anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies were purchased from Zhongshan

Golden Bridge. Band intensity was normalized to protein loading band density and expressed as a percentage of normoxic values.

Statistical analysis

Statistical analysis was performed using the Statistical Package of Social Sciences (SPSS) 13.0 (Beijing Stats Data Mining Co., Ltd.). The data are expressed as the mean \pm standard deviation (S.D.). One-way analysis of variance (ANOVA) was used to assess the differences followed by a post hoc test to evaluate the differences in variables among groups. The changes in variables with time were analyzed using repeated measures ANOVA followed by a post hoc test. All *P* values are 2-tailed, and *P*<0.05 was considered significant.

Results

Physiological data

No significant differences in body weight and rectal temperature were found among the three groups (date not shown). Three rats (two from the HI and one from the HP group) that died after delivery of low oxygen were excluded from this study because of progressive hypotension.

Changes in cortex cerebral blood flow induced by hypoxia and hypercapnia

Cortex CBF was measured in each group for 10 min as a baseline. After the initial hypoxia/hypercapnia, CBF in each group was measured every 30 min, and the trend of CBF was calculated using the percentage of the baseline value. When ischemia was induced, the CBF in the left cortex was decreased to approximately 30% of the baseline value. No changes of CBF were observed in the control group during mechanical ventilation. CBF in the rats was decreased to approximately 50% of the baseline values in the HI group after ventilation with 15% O_2 for 3 h; however, CBF was significantly increased to 75% of the baseline values of the HP group after ventilation combined with 8% CO₂ for 3 h (Fig. 1).

Effect of hypercapnia on infarct volume after HI

Cerebral infarction volume was assessed after hypoxic ischemia for 24 h using TTC staining. TTC staining in the brain sections of the HI group showed dramatic lesions as pale regions in the areas that were supplied by the middle cerebral artery. Hypercapnia treatment significantly attenuated the brain damage compared to that of the hypoxic ischemia alone group. The infarct volume in the rats of the hypercapnia with HI group was smaller than that in the HI rats ($12.2 \pm 3.3\%$ vs. $22.4 \pm 3.6\%$, *P* < 0.05, Fig. 2).

Hypercapnia inhibited the increasing BBB permeability of hypoxia induced by ischemia and affected the RECA-1 expression in the cortex

RECA-1 expression was observed and quantified by fluorescence intensity in the rat brain sections of the control group (Fig. 3A). The fluorescence intensity of RECA-1 expression in the rat left cortex was significantly lower in the HI group than the control and HP groups. Obviously incomplete endothelial cell layers and disjunction were observed in the HI group. An increased permeability to 10 kDa fluorescent dextran was observed in both the left and right brains of rats in the HI group compared with the rats in the control group (P < 0.05; Fig. 3B). However, there was a significantly decreased BBB permeability in both the left and right brains of compared with the HI group (P < 0.05; Fig. 3B). However, there was a significantly decreased BBB permeability in both the left and right hippocampus and cortex in the HP group compared with the HI group (P < 0.05; Fig. 3B). These results suggested that hypercapnia could reverse the damage to the brain microvascular endothelial barrier function caused by HI.

Hypercapnia improved the recovery of neurological function in HI rats

The mNSS scores in rats was significantly increased after 3 day in the HI and HP groups compared to the control group (P < 0.05) (Fig. 4), the mNSS scores in the Group HP rats were significantly reduced at day 7 after hypoxia ischemia compared with the Group HI rats (p < 0.05), however, there were no differences in the mNSS between the hypoxia with or without hypercapnia groups at day 14 (p > 0.05) (Fig. 4).

Blood gas analysis in the culture medium treated by hypoxia and hypercapnia

After hypoxia, pH values in the cultural medium were decreased to 6.91 ± 0.02 and PO₂ to 50.2 ± 3.11 mmHg at 6 h as shown by blood gas analysis. These values were significantly lower than those in the normoxic group (pH value was 7.27 ± 0.03 and PO₂ was 152.6 ± 3.6 mmHg) (*P*< 0.05). PCO₂ gradually increased and was maintained at 75–80 mmHg after hypoxia for 6 h in the hypercapnia group (Fig. 5).

Hypercapnia increased the cell viability of BMECs exposed to hypoxia

To exclude that the changes observed after hypoxia with or without hypercapnia arises from nonspecific cell injury, LDH release was determined in order to asses cell viability and membrane integrity. Hypoxia alone (6 h) caused a significant increase in the LDH activity of the culture medium (36 ± 8 and $67 \pm$

11 mU/mL), however, when BMECs exposed to hypoxia with hypercapnia (6 h), the change of the LDH activity of the culture medium (33 ± 7 and 45 ± 8 mU/mL) were not significant which indicating that the endothelial cells maintained their membrane integrity during the hypercapnic exposure.

Hypercapnia increased the TEER of BMECs exposed to hypoxia

TEER values in BMECs remained stable $(54 \pm 6 \ \Omega \cdot cm^2)$ during normoxic control conditions. After hypoxic treatment for 6 h, TEER values were dramatically decreased to $147 \pm 14 \ \Omega \cdot cm^2$. There were significant differences between hypoxia and normoxia (*P*<0.01). However, hypercapnia caused a significant increase in the TEER values (88 ± 9 $\Omega \cdot cm^2$) in BMECs after hypoxia for 6 h (Fig. 6A).

Hypercapnia inhibited the expression of ZO-1 and occludin protein in BMECs induced by hypoxia

After hypoxia for 4 h and 6 h, ZO-1 protein expression was significantly decreased in BMECs compared with the cells in normoxia (Fig. 6B). In the hypercapnia group, ZO-1 protein expression was significantly increased after hypoxia for 2 h, reached a maximum at 4 h, and was then markedly reduced to approximately 60% compared to that of cells in the normoxic group at 6 h. The protein expression was significantly higher than that in the hypoxia group (P< 0.01). In addition, occludin protein expression in the hypoxia for 4 h or 6 h (Fig. 6C). In the hypercapnia group, occludin protein expression was also significantly decreased compared to that of the normoxia group after hypoxia for 4 h or 6 h (Fig. 6C). In the hypercapnia group, occludin protein expression was also significantly decreased compared to that of the normoxia group after hypoxia for 4 h (P< 0.05). Occludin protein expression in the hypercapnia group was significantly higher than that of the normoxia group of the normoxia for 4 h (P< 0.05). Occludin protein expression in the hypercapnia group was significantly higher than that of the normoxia group of the hypoxia for 4 h (P< 0.05). Occludin protein expression in the hypercapnia group was significantly higher than that in the hypoxia group (P< 0.05).

Discussion

Our data demonstrated that hypercapnia improved cerebral blood flow and ameliorated BBB permeability disturbances induced by ischemic injury followed by hypoxic, and hypercapnia promote the recovery of neurological function in this rat model within 7 days. Furthermore, hypercapnia significantly increased the expression levels of ZO-1 and occludin protein in BMECs under hypoxic conditions.

The principal pathogenetic mechanism underlying most of the neuropathology attributed to HI is impairment of the cortex CBF.[28] In the present study, the ipsilateral cortical CBF fell to approximately 30% or 40% of the baseline value after left CCA ligation. Following exposure to hypoxia ($15\% O_2$, $PaO_2 > 50 \text{ mmHg}$) at 60 min of ischemia, the CBF was decreased over time; however, this decreased CBF was fully recovered when the treatment was combined with moderate hypercapnia. Cerebral vascular reactivity was shown to be strongly influenced by CO_2 , [29] Cyclo-oxygenase-1(COX-1)-derived PGE2 and

EP1 receptors, which may play a role in the hypercapnic regulation of the cerebral circulation.[30] Hypercapnia may maximally dilate the cerebral vessels during mild or moderate hypoxia ($PaO_2 > 50 \text{ mmHg}$); thus, it can improve O_2 delivery to the cerebral hemisphere even when the CCA has been previously ligated, which in turn promotes cerebral glucose utilization and oxidative metabolism for optimal maintenance of tissue high energy phosphate reserves. [15]

Edema volume has a deleterious impact on the morbidity and mortality after stroke by increasing ICP and impairing cerebral perfusion and oxygenation during reperfusion.[31-33] BBB disruption permits the extravasation of albumin and other high molecular weight compounds, resulting in edema formation and increased ICP.[34, 35] In this study, acute hypoxia rapidly increased cortical and hippocampal vascular permeability to 10 kDa dextran. While mild hypoxemia was associated with restoration of vascular integrity by hypercapnia, based on the immunofluorescence results of RECA-1, brain microvascular endothelial barrier function in the ischemic cerebral region was protected by hypercapnia in rats during acute hypoxia. However, in this study, a significant functional recovery within 7d after hypercapnia treatment was observed but not seen the obvious protective effect at 14d compared to that of the rat model of CCA that only underwent hypoxic ventilation. This result was not the same as a previous study [18] showing that mild and moderate hypercapnia were associated with better neurologic deficit scores (at 72 h) compared with normocapnia in a rat model of global cerebral ischemia. It may be due to the use of different animal cerebral ischemic models. In this study, the left CCA was permanently ligated followed by hypoxic condition, which causes ipsilateral infarction together with hypoxic stress. Hypoxia is frequently associated with a range of clinical conditions, such as chronic obstructive pulmonary disease, neuromuscular disease and sleep apnea syndrome. This model simulates these patients, when underwent ischemic disease, would suffer double stress from both stroke and hypoxia and this was much severer than that of cerebral ischemic followed by reperfusion. We believe hypercapnia though did not significantly improve the mNSS scores after one week, it still exerts beneficial effects to this severe and complicated physiopathological process as shown in our results.

This BBB model *in vitro* could approximately mimic *in vivo* oxygen and CO_2 changes in the cerebral ischemia rats under moderate hypoxia (PaO₂ between 50–60 mmHg) with high CO_2 ventilation. Since endothelial cells are the initial cells within the vascular wall exposed to changes in the ambient O_2 and CO_2 concentrations in the blood, the effects of hypoxia with or without hypercapnia on endothelial BBB function were explored in primary BMECs. To assess the membrane integrity of BMECs after hypoxia/hypercapnia, we have measured the LDH activity in the culture medium. When BMECs were exposed to only hypoxic stress for 6 h, our results show that BMECs exhibit a significant increase in LDH activity indicating that the experimental conditions used by us do affect cell viability. Although there is a general agreement that BMECs are sensitive to oxidative stress, the effect on their viability is still controversial. Mertsch et al. demonstrated a significant membrane damage even after 120 min hypoxia/30 min reoxygenation.[36] On the other hand, Plateel et al. reported that LDH activity in the culture medium of BMECs did not increase even after prolonged hypoxia.[37] This discrepancy may arise from the differences in the experimental conditions. Furthermore, our findings also showed that hypoxic

stress causes a rapid and intense decrease of the TEER which is a sign of increased paracellular permeability. However, the results presented here clearly indicate that the integrity and viability of the cells are little affected by exposure to hypercapnia (15%) for 6 h. This finding was similar to those previously described by Tsuji, in which both hypercapnic acidosis and buffered hypercapnia improved the rate of wound healing due to stimulation of cell proliferation under the influence of hypoxia in human umbilical venous endothelial cells. [38]

Here we show that hypoxic stress leads to a downregulation of ZO-1 and occluding, this led us to assume that junctional proteins which are responsible for the maintenance of the paracellular barrier may represent a potential target of hypoxic stress. Changes in the expression of junctional proteins in response to hypoxia/reoxygenation have been demonstrated in several recent studies as well. Fischer et al. have shown that hypoxia causes a decrease in ZO-1 levels accompanied by an increased phosphorylation and redistribution of this protein. ZO-1 is directly linked to the COOH terminus of occludin [39] and is important for localization of occludin to the TJs [40]. In this study, although the translocalization of the ZO-1 and occludin proteins was not determined, the expression levels of ZO-1 and occludin protein were explored and determined to be associated with increased paracellular permeability along with hypoxic stress for 6 h in BMECs. Conversely, hypercapnia also significantly inhibited the loss of ZO-1 and occludin induced by hypoxia and may protect against the disruption of BBB permeability during hypoxia. Interestingly, in our previous study in a rat model of lateral FPI, we have confirmed that only the neuroprotective isozyme PKCE (but not PKCa and PKCBI) was significantly elevated after hypercapnia, and also found hypercapnia significantly increased the mRNA expression of PKCs at 48 h post-FPI.[17] This was accompanied by augmented expression of TJ proteins ZO-1, occludin, and claudin-5 as well as decreased brain edema. These results suggest that hypercaphia exerts neuroprotective effects via upregulation of PKCc expression.[17]

An important aspect of this study is the attempt to translate our findings from the ligation of a CCA hypoxia model *in vivo* to the BBB model *in vitro*. HI primarily affects infants, and mild hypercapnia was shown to be protective in an immature rat model of cerebral hypoxia and ischemia.[15, 29] To the best of our knowledge, few studies have been performed on adult rats that were subjected to cerebral HI with or without CO_2 added to the hypoxic gas mixture to which the animals were exposed. In our previous study, our findings suggested that the addition of hypercapnia aggravated the injury after CCA ligation along with severe hypoxemia (PaO₂ < 50 mmHg), but hypercapnia produced protective effects against HI-induced brain damage in rats treated with mild to moderate systemic hypoxia (15% and 18% O_2 , PaO₂ > 50 mmHg).[7] It is possible that the severe injury exceeded the short-term therapeutic potential of exposure to hypercapnia gas. The large volume of ischemic tissue may have overwhelmed any possible benefit of hypercapnia gas exposure. However, in our clinical study and under intravenous anesthesia, the induction of therapeutic hypercapnia by continual inhalation of carbon dioxide during one-lung ventilation (OLV) could improve respiratory function and mitigate the OLV-related lung and systemic inflammation in patients undergoing a lobectomy.[41] Based on the studies presented above, the differential effects of hypercapnia after cerebral ischemia on functional and structural changes for weeks

and even months after the insult should be examined, and further experimental analyses are needed to justify clinical use.

Conclusions

In summary, we found significant recovery of neurologic status by hypercapnia treatment in the rat model of hypoxic-ischemic injury within one week, our findings demonstrated that hypercapnic potentiation increased neuroprotective efficiency during moderate ($PaO_2 > 50 \text{ mmHg}$) hypoxia-ischemic injury including decreasing infarct volumes and improving BBB permeability. The principal mechanisms of these effects may include maintenance of BBB integrity by increasing the expression of TJ proteins in BMECs.

Abbreviations

TH therapeutic hypercapnia CO_2 carbon dioxide PaCO₂ arterial blood CO₂ tension TBI traumatic brain injury BBB blood-brain barrier ΤJ tight junction FPI fluid percussion injury CCA common carotid artery HI hypoxic-ischemic MAP mean arterial pressure HR heart rate ICP Intracranial pressure CBF cerebral blood flow

TTC 2,3,5-triphenyltetrazolium chloride BMECs brain microvascular endothelial cells RECA Anti-Endothelial Cell antibody TEER transendothelial electrical resistance PBS phosphate buffered saline LDH lactate dehydrogenase OLV one-lung ventilation

Declarations

Ethics approval and consent to participate

The experimental protocols were approved by the Institutional Animal Care Committee of Harbin Medical University, and all procedures were conducted in strict accordance with the guidelines for the care and use of laboratory animals of Harbin Medical University as well as the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines for animal research.

Consent for publication

Not applicable

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

WCY and WZL designed the study. QW and QW performed the animal modes. XHW performed the cell experiments. TTL collected and analyzed the data. WCY drafted and wrote the manuscript. WZL revised the manuscript critically for intellectual content. All authors gave intellectual input to the study and approved the final version of the manuscript.

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Figures

Figure 1

The values of cortex cerebral blood flow (CBF) in the ipsilateral hemisphere during hypoxia-ischemia (HI) insulted with 15% oxygen or hypercapnia (HP) ventilated with an additional 8% carbon dioxide. A and B show the differences in CBF between HI and HPThe CBF values are expressed as a percentage of the preligation value (100%) in each animal (n=10). *P<0.05, when compared to the baseline; # P<0.05, when compared to the control group.



Figure 2

Hypercapnia reduced infarct volume in rats after hypoxia-ischemia for 24 h. (A) Representative photographs of brain sections stained with 2% TTC in the HI and HIP groups. (B) Measurement of infarct volume showed a significant decrease in the infarct areas of the HP group compared to the HI group (n=5). * P<0.05, when compared to the HI group.

Α



Figure 3

Vascular endothelial cells in the cortex (A) and changes in BBB permeability (B) determined by immunofluorescence. (A) The RECA-1 expression in cerebral cross-sections of rat brain. RECA-1 (green) and DAPI (blue) were stained in cerebral cross-sections by double immunostaining. The pictures were captured at ×60 with a fluorescence microscope (n=4, scale bar=100 μ m). Arrows indicate the incomplete and lack of endothelial barrier. (B) The magnitude of blood-brain barrier disruption was quantified by

measuring the extent of leakage of 10 kDa dextran. The values are expressed as the mean \pm S.D. as a percentage of the values in the control group (n=5). * P<0.05, when compared to the control group; # P<0.05, when compared to the HI group.



Figure 4

Neurological severity scores were evaluated with or without hypercapnia exposure 2 h before and 1, 3, or 7 d after hypoxia. There were no differences between the HI and HP groups (P<0.05) (n=6).



Figure 5

Effects of hypoxia with or without hypercapnia on blood gas analysis and cell proliferation assayed by CCK8 for BMEC. *P<0.05 indicate differences versus Normoxia group. ΦP<0.05 indicate differences versus Hypoxia group.





Effects of hypoxia with or without hypercapnia on TEER in BMECs. TEER was measured in BMECs exposed to hypoxia with or without hypercapnia (A). The TEER values are expressed as the mean \pm S.D. (Ω ·cm2). The expression of ZO-1 (B) and occludin (C) protein in BMECs by Western blotting, (n=3).*P<0.05, when compared to the normoxia group. Φ P<0.05, when compared to the hypoxia+15% CO2 group.

Supplementary Files

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