Effectiveness of carbon dioxide per-conditioning before recanalization in hyperacute ischemic stroke rat model

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Effectiveness of carbon dioxide per-conditioning before recanalization in hyperacute ischemic stroke rat model

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I am currently positioned in front of a fresh beginning line before I graduate. The knowledge I gained while earning my doctorate would be an excellent starting point for me to become a humble scientist. I'll never forget their help and compassion, once more.

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ABSTRACT

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Background: This study investigated whether carbon dioxide (CO_2) per-conditioning reduced oxidative stress, blood-brain barrier breakdown, and neurologic deficit in a rat model of middle cerebral artery occlusion (MCAO).

Methods: MCAO rat models received intermittent inhalation of mixed gas (20% CO_2, 20% O_2, 60% N_2) or room air. After the surgery, arterial blood gas analysis and behavior test were conducted, and animals were euthanized for calculating infarct size, western blot analysis, and immunohistochemistry.

Results: Our results showed that CO_2 per-conditioning reduced infarct size and neurological deficit. Number of 8-OHdG positive cell and MMP-9/PDGFRβ double positive cell expressions were significantly decreased after CO_2 per-conditioning. The expression of tight junction protein and PDGFRβ were significantly elevated after CO_2 per-conditioning.

Conclusion: CO_2 per-conditioning not only protects the ischemic penumbra from ischemia-reperfusion injury and reduces neurologic deficit, but also maintains the integrity of blood-brain barrier and neurovascular unit, and reduces oxidative stress in a hyperacute stroke rat model. CO_2 per-conditioning is promising and requires further assessment for clinical application.

Key words: carbon dioxide, per-conditioning, ischemic rat model, pericyte, oxidative stress, blood-brain barrier, ischemia-reperfusion injury

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Effectiveness of carbon dioxide per-conditioning before recanalization in hyperacute ischemic stroke rat model

1. Introduction

Acute stroke is the second leading cause of morbidity and mortality worldwide.[1] Ischemic stroke accounts for 85% of all strokes followed by hemorrhagic stroke.[2,3] A significant proportion of patients with a history of acute ischemic stroke experience long-term disability, resulting in substantial healthcare costs. Nevertheless, clinicians have limited treatment options for hyperacute ischemic stroke, which mainly consist of intravenous thrombolytic therapy and endovascular treatment (EVT). Up until now EVT is considered the most effective treatment for hyperacute ischemic stroke with large vessel occlusion.[4] Due to developments in mechanical devices and the technical skills associated with EVT, the recanalization rate has dramatically increased to approximately 80%.[5] However, the rate of favorable outcomes remains at approximately 50%, despite proper recanalization with timely treatment.[6]

During the post-reperfusion state, the rapid restoration of blood flow leads to an elevation in tissue oxygenation levels, triggering a surge in the generation of reactive oxygen species (ROS).[7] It makes ischemia-reperfusion injury that affects peri-lesional penumbra.[8] Ischemia-reperfusion injury has multiple mechanisms to make cell dysfunction like overproduction of ROS, consumption of antioxidants, inflammation, blood-brain barrier (BBB) disruption, apoptosis, and increasing excitotoxicity.[9,10] As a result, cerebral damage expands to encompass the penumbra and adjacent brain parenchyma.[3]

Therefore, how to avoid or reduce ischemia-reperfusion injury is the prominent point of treatment, which is called neuroprotection.[3,11] Until recently, a number of neuroprotective agents were developed and subjected to clinical trials; however, despite their success in preclinical studies, none

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of these agents have proven successful in clinical trials.[12-14]

Most of the neuroprotective trials are aimed for pre-conditioning mechanism. It is not relevant to clinical setting because acute ischemic stroke is unpredictable event, therefore, pre-conditioning trials may have limitations in the real life.[15] Rather than pre-conditioning, per-conditioning may be more suitable because this intervention is carried out between onset of ischemia and recanalization. Clinically, per-conditioning corresponds to a treatment just before EVT. In addition, a recent clinical trial demonstrated the successful implementation of per-conditioning in conjunction with EVT.[16]

Carbon dioxide (CO_2) is harmless gas and has powerful vasodilative effects on the cerebral vessels.[17] CO_2 is maintained within narrow range (35–45 mmHg) in physiologic state and has an intense diffusion capacity with an ability to cross the BBB. Several studies showed that arterial carbon dioxide tension ($PaCO_2$) is strong determinant of cerebral blood flow. Furthermore, CO_2 has anti-oxidant, anti-epileptic, and anti-inflammatory effects.[15,18-23]

This study aims to show that intermittent CO_2 inhalation during the per-conditioning phase can improve outcomes in hyperacute ischemic stroke animal models by reducing oxidative stress and blood-brain barrier disruption, and saves the neurovascular unit, especially pericytes.

2. MATERIALS AND METHODS

2.1. Ethics statement and animals

All experiments were carried out in accordance with Institutional Animal Care and Use Committee of Yonsei University Health System, and the protocol was approved by the Institutional Animal Care and Use Committee of Yonsei University Health System (approval number: 2020-0331). All efforts were made to minimize suffer and the number of rats. In total, 42 male Wistar rat (8-week-old, 270–320g) were obtained (ORIENT BIO, Seongnam, South Korea). Experimental animals could freely access to food and water. The animal room was adjusted on a 12-h light/dark cycle with constant temperature of 22°C ± 2°C.

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2.2. Experimental design and protocol

Before the operation, rotarod training had been performed for three days. A heating lamp were used to keep the rectal body temperature at 36.6–37.4°C. Wistar rats were deeply anesthetized with 4.5% isoflurane and maintained with 1.5% isoflurane using a mask during surgery. We made middle cerebral artery infarction models. In the process of induction of middle cerebral artery infarction, first, the animal was placed in a supine position, midline neck incision was made, and the soft tissues over the trachea were gently retracted. Second, the left common carotid artery (CCA) was separated from the vagus nerve and then ligated. Following that, the left external carotid artery (ECA) was ligated consecutively. Third, both the occipital artery and pterygopalatine artery were ligated together to obstruct the blood flow. Fourth, the microvascular clip was temporarily positioned in the left internal carotid artery (ICA) close to the CCA junction and the tied section of ECA was incised using microscissors to insert the monofilament (4035556PK10; Doccol Co., Albuquerque, NM, USA). Finally, the filament was cautiously advanced up to 20 mm into the ICA to occlude the origin of the middle cerebral artery (MCA). MCA was occluded for 90 min. After 30 min from induction of middle cerebral artery occlusion (MCAO), the rat began inhaling a mixed gas (20% CO_2, 20% O_2, 60% N_2; flow rate = 1L/min) with 3 cycles of inhalation for 5 min followed by 10 min recovery session (normoxic atmosphere). At the end of 90 min transient MCAO (tMCAO), the rats were anesthetized, and the microfilament was removed for recanalization of the MCA. Sham animals underwent the surgical procedure without any occlusion, while the animals with permanent MCAO (pMCAO) did not undergo the microfilament removal procedure (Figure 1).

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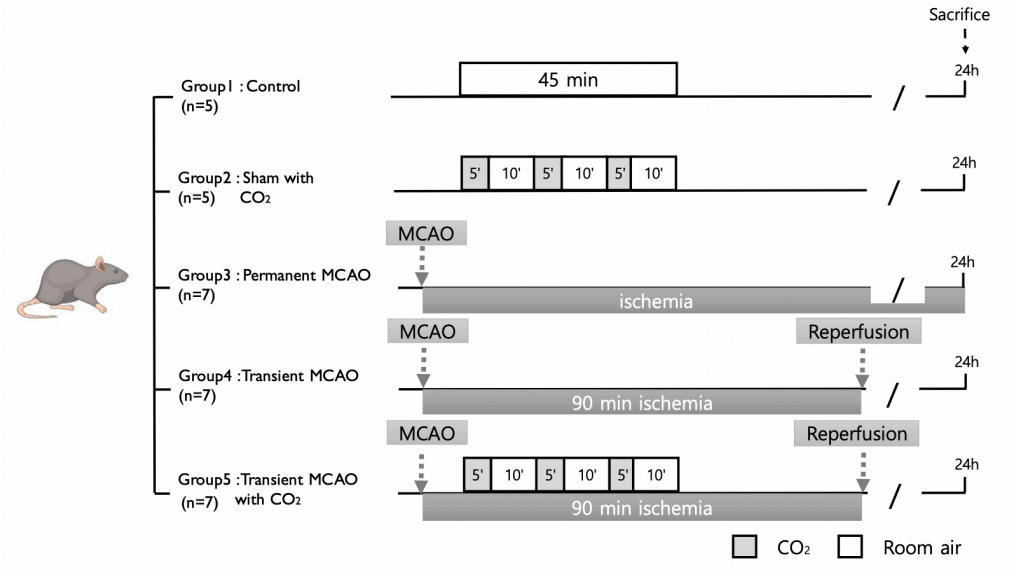

Figure 1. Experimental design.

After the operation, the rats were placed on a cage. The physiological variables including pH, pO_2, pCO_2, HCO_3 were recorded by arterial blood gas analysis before and after the CO_2 inhalation.

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2.3. Sample size and experimental group

The total sample size for the study was determined using MedCalc statistical software version 22.017 (MedCalc Software by Ostend, Belgium), targeting an alpha level of 0.05 and a power of 0.8. The calculation was based on the mean difference in infarct size observed between the groups in a preliminary study. The comparison of two means' method indicated that a minimum of 25 animals were needed for the study. To account for an anticipated mortality rate and technical errors of 40%, the study included 42 rats in total. Forty-two rats practiced rotarod training, but 11 rats did not successfully complete the training. 31 rats were randomly allocated into five groups (Figure 2).

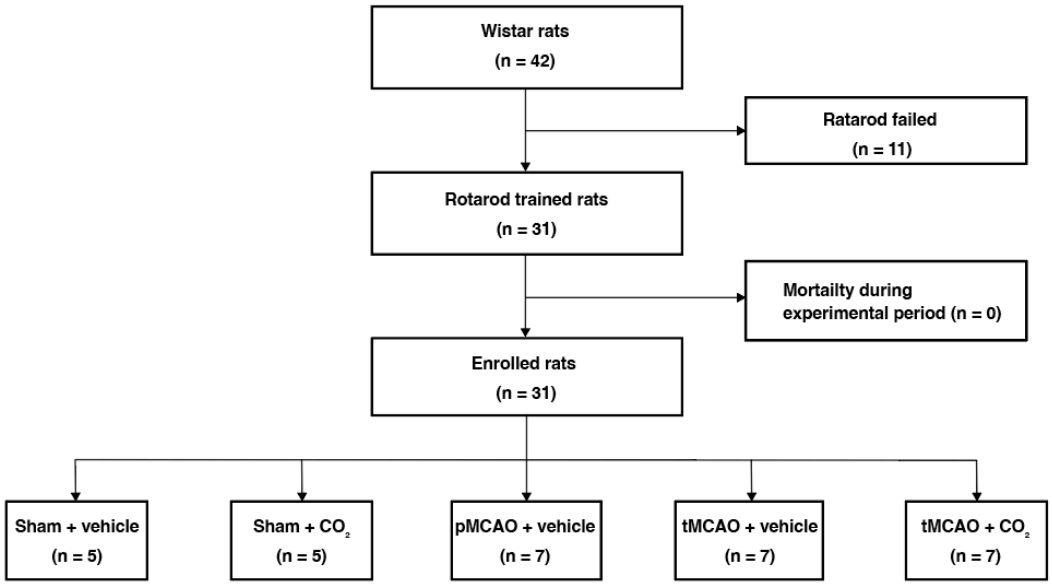

Figure 2. Study groups.

sham-operated rat with room air inhalation (Sham + vehicle group, n=5), sham-operated rat with intermittent CO_2 inhalation (sham + CO_2 group, n=5), pMCAO-operated rat with room air inhalation (pMCAO + vehicle group, n=7), tMCAO-operated rat with room air inhalation (tMCAO + vehicle group, n=7), and tMCAO-operated rat with intermittent CO_2 inhalation (tMCAO + CO_2 group, n=7).

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2.4. Assessment of neurologic function

Neurologic evaluation was performed at 24 h after the initiation of the surgical procedure. We carried out various scoring methods for appropriate assessment – Garcia test, Longa test, rotarod motor test, and Modified Neurological Severity Score (mNSS). The neurologic deficits of the rats were examined by a blinded observer. To evaluate neurologic function, blinded investigator measured Longa test, Garcia test, rotarod motor test and, mNSS for motor, sensory and reflex of experimental animals. Baseline assessment of rotarod motor test was conducted. The Garcia test consists of a 6-factor scale with each factor having a maximum score of 3, with total scores ranging between 3 and 18.[24] The Longa test is a 5-point scale with a minimum score of 0 for no neurological deficit and a maximum score of 4 for no spontaneous walking and depressed.[25] The rotarod motor test, animals are placed on the rotarod cylinder, and duration the animals endured on the rotarod is measured.[26,27] The last is mNSS, which included motor, sensory, reflex and balance tests. Neurological function is graded on a scale of 0 to 18.[26,28] The higher the score, the more severe is the injury.[26]

2.5. Arterial blood gas analysis

The artery on the ventral aspect of the experimental animal's tail was used for the collection of arterial blood. For baseline blood gas results, we collected arterial blood of whole rats before the operation, and in the groups with CO_2 inhalation, additional arterial blood was obtained immediately after the 3 cycles of inhalation. Arterial blood gases were analyzed using OPTI CCA blood gas and electrolyte analyzer (OPTI Medical System, Roswell, GA, USA).

2.6. Infarct size measurement

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At 24 h after surgery, experimental animals were euthanized with intraperitoneal injection of trichloroacetaldehyde hydrate (0.4 g/kg) and followed by cardiac perfusion with 4% paraformaldehyde and normal saline. The brains were removed, dissected at 2 mm intervals, totally generating 7 sections and 4 of total sections were stained with 2% 2-3-5-triphenyl tetrazolium chloride (TTC) solution (T8877; Sigma-Aldrich, Darmstadt, Germany) and fixed in formalin. The infarct size of each section was measured by computer image analyzing software (Scion Image ®, Frederick, MD, USA). Infarct size was normalized to the contralateral area and expressed as a percentage. Average values from the 4 sections were calculated.

2.7. Immunofluorescence analysis

Immunofluorescence analysis was performed between 8-hydroxy-2-deoxyguanosine (8-OHdG), platelet derived growth factor receptor beta (PDGFRβ) and matrix metalloproteinase 9 (MMP-9), zonula occludens-1 (ZO-1), or PDGFRβ to analyze the oxidative damage of deoxyribonucleic acid (DNA), MMP-9 positive pericytes, tight junction expression, and pericytes expression. Staining was carried out using 4-μm thick paraffin sections. A two-day immunofluorescence staining for paraffin protocol was performed. After being deparaffinized and rehydrated, the sections were steamed with epitope retrieval solution (IW-1100; IHC-TEK, Austin, TX, USA) using steamer (IHC World, Woodstock, MD, USA) for 40 min. After 20 min of cooling, the sections were rinsed by phosphate-buffered saline (PBS) and permeabilization was performed with 1xPBS-T (PBS with 0.3% triton X-100) for 10 min at room temperature. Then the sections were blocked with blocking solution (1% bovine serum albumin + 10% horse serum + 1xPBS-T) for 1 hr at room temperature. Subsequently, the sections were incubated in primary antibodies against PDGFRβ (anti-rabbit monoclonal antibodies, 1:200 dilution, ab32570; Abcam, Cambridge, UK), ZO-1 (anti-rabbit polyclonal antibodies, 1:200 dilution, 61-7300; Thermo Fisher Scientific, Waltham, MA, USA), MMP-9 (anti-goat polyclonal antibodies, 1:100 dilution, sc-6841; Santa Cruz biotechnology, Dallas, TX, USA), and 8-OHdG (anti-mouse monoclonal antibodies, 1:200 dilution, ab48508; Abcam, Cambridge, UK)

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overnight at 4°C. Second day, the sections were incubated with enzyme-conjugated secondary Ab (Alexa fluor 488-labeled goat anti-rabbit IgG antibody (1:200 dilution, ab150077; Abcam, Cambridge, UK), Alexa fluor 647-labeled goat anti-mouse IgG antibody (1:200 dilution, ab150115; Abcam, Cambridge, UK), and Alexa fluor 647-labeled donkey anti-goat IgG antibody (1:200 dilution, ab150131; Abcam, Cambridge, UK) for 1 hr at room temperature in the dark to avoid photobleaching. After washing by PBS for 3 times, the sections were mounted with antifade mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Newark, CA , USA). The sections were scanned with a confocal microscope (LSM 710, Zeiss Instruments, Oberkochen, Germany). The investigator chose cortical or striatal penumbra region of interest (ROI) per section and observed the expression of 8-OHdG, PDGFRβ/MMP-9, ZO-1 and PDGFRβ. Quantification of the positively stained cells in the ROI and fluorescence intensity was performed with image analysis software (ImageJ, National Institutes of Health, Bethesda, MD, USA).

2.8. Western blot analysis

Proteins from the rat brain samples were extracted in homogenization buffer (50 mM Tris, 120 mM NaCl, pH 7.4) containing protease inhibitors (complete Mini, GIBCO, UK), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride (PVDF) membrane (cat. #1620177, Bio-Rad Laboratories, Hercules, CA, USA). The blocked membranes were incubated with the primary antibody overnight at 4°C. The primary antibodies were against PDGFRβ (anti-rabbit monoclonal antibodies, 1:1000 dilution, ab32570; Abcam, Cambridge, UK) and ZO-1(anti-rabbit polyclonal antibodies, 1:500 dilution, 61-7300; Thermo Fisher Scientific, Waltham, MA, USA). After the membranes were rinsed, the membranes were incubated with enzyme conjugated secondary antibodies for 1 hour at room temperature. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (1:10,000 dilution, ab205718; Abcam, Cambridge, UK) were used as secondary antibodies. Electrochemiluminescent detection was performed by LAS4000 (GE healthcare life sciences, NY, USA). Blot bands were quantified

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with ImageJ software (v1.4, NIH, Bethesda, MD, USA). β-actin (sc-47778; Santa Cruz biotechnology, Dallas, TX, USA) was blotted on the membrane as a loading control. The quantified values were presented as a percentage of sham group (100%).

2.9. Statistical analysis

All the data in this study were presented as the median and interquartile range (IQR). Kruskal-Wallis H test was performed to compare the baseline results of whole groups, and Wilcoxon signed rank test was performed to compare pairwise results of arterial blood gas analyses of groups with CO_2 inhalation. Kruskal-Wallis H test followed by the post-hoc Dunn's multiple comparison was performed in score of behavior test and cell count of immunohistostaining. The differences were considered statistically significant at a value of $p < 0.05$, and all p-values are two-tailed. Statistical analyses were performed using R version 4.2.1 (R Foundation for Statistical Computing, Vienna, Austria).

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3. RESULTS

3.1. Physiologic variables before and after CO_2 per-conditioning

No rat experienced mortality throughout the duration of the experiment (0/31, 0%). Body weight and rectal temperature were not statistically different among the experimental groups (data not shown). pH, pCO_2, pO_2, and HCO_3 levels of baseline arterial blood gas analyses did not show significant difference among the groups (For pH, $p = 0.8$, for pCO_2, $p > 0.9$, for pO_2, $p = 0.8$, and for HCO_3, $p > 0.9$). After the CO_2 inhalation, pH of sham + CO_2 and tMCAO + CO_2 groups were reduced ($p = 0.062$, $p = 0.022$, respectively, Table 1). The opposite patterns were shown in pCO_2 level of both groups.

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Table 1 ABGA results of experimental animals with pre and post CO_2 inhalation

Experimental group	Before CO_2 inhalation[1]	After CO_2 inhalation[1]	p-value[2]
pH			
Sham (median [IQR])	7.48 [7.45, 7.50]		
Sham + CO_2	7.46 [7.45, 7.48]	7.40 [7.39, 7.42]	0.062
pMCAO	7.47 [7.45, 7.48]		
tMCAO	7.46 [7.45, 7.48]		
tMCAO + CO_2	7.47 [7.47, 7.51]	7.31 [7.29, 7.44]	0.022
pCO_2 (mmHg)			
Sham	34.0 [27.0, 35.0]		
Sham + CO_2	30.0 [29.0, 31.0]	38.0 [31.0, 41.0]	0.062
pMCAO	31.0 [23.0, 39.0]		
tMCAO	31.0 [28.0, 35.5]		
tMCAO + CO_2	27.0 [24.0, 33.5]	40.0 [34.0, 45.5]	0.016
pO_2 (mmHg)			
Sham	134.0 [113.0, 143.0]		
Sham + CO_2	135.0 [124.0, 144.0]	106.0 [64.0, 109.0]	0.062
pMCAO	143.0 [116.0, 148.5]		
tMCAO	135.0 [127.0, 148.5]		
tMCAO + CO_2	146.0 [122.0, 153.0]	97.0 [84.5, 109.5]	0.016
HCO_3 (mEq/L)			
Sham	24.2 [17.4, 25.0]		
Sham + CO_2	21.7 [21.5, 22.1]	19.4 [18.6, 26.1]	0.81
pMCAO	22.2 [16.7, 26.4]		
tMCAO	21.0 [20.4, 24.0]		
tMCAO + CO_2	19.2 [18.7, 23.8]	21.1 [20.4, 21.7]	0.94

[1]Data were shown as median [IQR].

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[2]Wilcox signed rank test was performed for paired data.

Abbreviations: CO_2 = carbon dioxide; HCO_3 = bicarbonate; IQR = interquartile range; PCO_2 = partial pressure of carbon dioxide; pH = potential of hydrogen; pMCAO = permanent middle cerebral artery occlusion; tMCAO = transient middle cerebral artery occlusion.

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3.2. CO_2 per-conditioning attenuates infarct size after tMCAO

To evaluate the potential protective effects of intermittent CO_2 inhalation on the outcomes of rat models with MCAO, we assessed infarct size and conducted four different behavior tests to assess functional outcomes. In the TTC staining analyses, the tMCAO + CO_2 group showed a significantly smaller infarct size compared to both the tMCAO + vehicle and the pMCAO + vehicle group. (For tMCAO + vehicle, $p < 0.0001$; for pMCAO + vehicle, $p < 0.0001$, respectively, Figure 3). Between the tMCAO + vehicle and the pMCAO + vehicle groups, significant infarct size difference was found ($p < 0.0001$, Figure 3, Table 2).

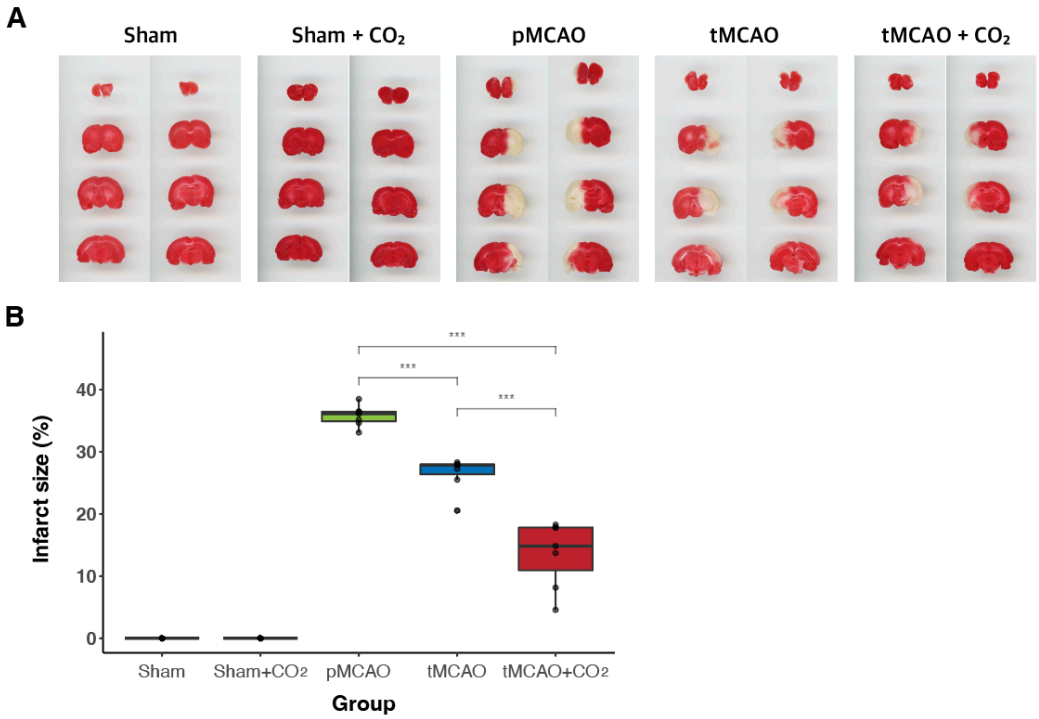

Figure 3. Infarct size measurement.

(A) Representative Images of TTC stain. (B) Quantitative analysis of infarct size according to the groups (***$p < 0.001$).

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Abbreviations: CO_2 = carbon dioxide; pMCAO = permanent middle cerebral artery occlusion; tMCAO = transient middle cerebral artery occlusion; TTC = 2-3-5-triphenyl tetrazolium chloride.

Table 2 Infarct size measurement

Experimental group	Infarct size (%)[1]
Sham, median [IQR]	0
Sham + CO_2	0
pMCAO	36.1 [34.9, 36.5]
tMCAO	27.9 [26.4, 28.0]
tMCAO + CO_2	14.8 [11.0, 17.8]

[1]Data were shown as median [IQR].

Abbreviations: CO_2 = carbon dioxide; IQR = interquartile range; pMCAO = permanent middle cerebral artery occlusion; tMCAO = transient middle cerebral artery occlusion.

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3.3. CO_2 per-conditioning alleviates neurologic deficit after tMCAO

Regarding behavior tests, the tMCAO + CO_2 group exhibit lower mNSS scores compared to both the tMCAO + vehicle group and the pMCAO + vehicle group (Figure 4C). In addition, Garcia and rotarod test score of the tMCAO + CO_2 group were significantly higher than those of the other MCAO groups. (Figure 4A, 4D) In the Longa test, the tMCAO + CO_2 groups showed a lower median score compared to the other MCAO groups; however, this difference was not statistically significant (Figure 4B).

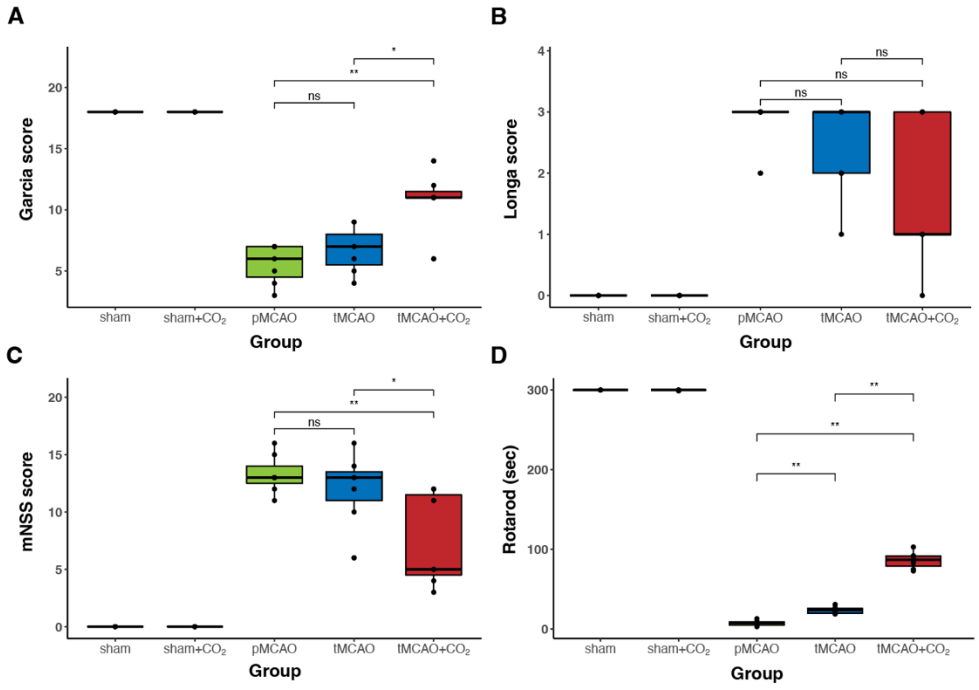

Figure 4. Behavior test score of Garcia, Longa, mNSS, and rotarod test.

Abbreviations: CO_2 = carbon dioxide; pMCAO = permanent middle cerebral artery occlusion; tMCAO = transient middle cerebral artery occlusion.

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3.4. CO_2 per-conditioning downregulates peri-lesional oxidative stress after tMCAO

As shown in Figure 5, the number of 8-OHdG positive cells was significantly reduced in the tMCAO + CO_2 group compared to both the tMCAO + vehicle and pMCAO + vehicle groups (p = 0.0026, p = 0.00058, respectively). Furthermore, the pMCAO + vehicle group exhibited a significant increase in the number of 8-OHdG positive cells compared to the tMCAO + vehicle group (p = 0.00058). The sham + vehicle and sham + CO_2 groups showed a minimal presence of 8-OHdG positive cells.

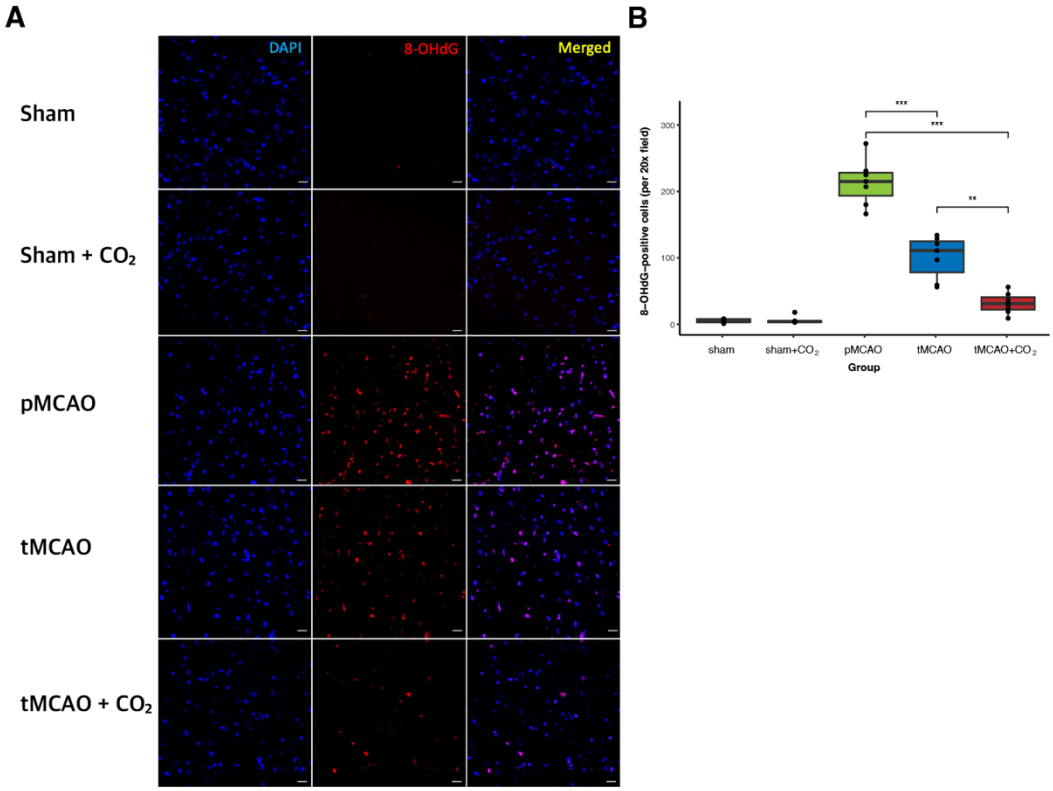

Figure 5. CO_2 per-conditioning decreased the number of 8-OHdG positive cells.

(A) Representative Images of immunofluorescence staining illustrated the 8-OHdG positive cells. scale bar = 20 μm. (B) Quantitative analysis of 8-OHdG expression in immunofluorescence staining (**$p < 0.01$, ***$p < 0.001$). 8-OHdG expressions in all rat models presented similar pattern.

Abbreviations: 8-OHdG = 8-hydroxy-2-deoxyguanosine; CO_2 = carbon dioxide; DAPI = 4′,6-diamidino-2-phenylindole; pMCAO = permanent middle cerebral artery occlusion; tMCAO = transient middle cerebral artery occlusion.

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3.5. CO_2 per-conditioning alleviates BBB failure after tMCAO

In the peri-infarct areas, the tMCAO + CO_2 group showed a significant reduction in the number of MMP-9/PDGFRβ double positive cells compared to the other MCAO groups ($p = 0.0014$, $p = 0.011$, respectively, Figure 6). Compared to the tMCAO + vehicle group, the pMCAO + vehicle group revealed a higher expression of MMP-9/PDGFRβ double positive cells ($p = 0.016$, Figure 6).

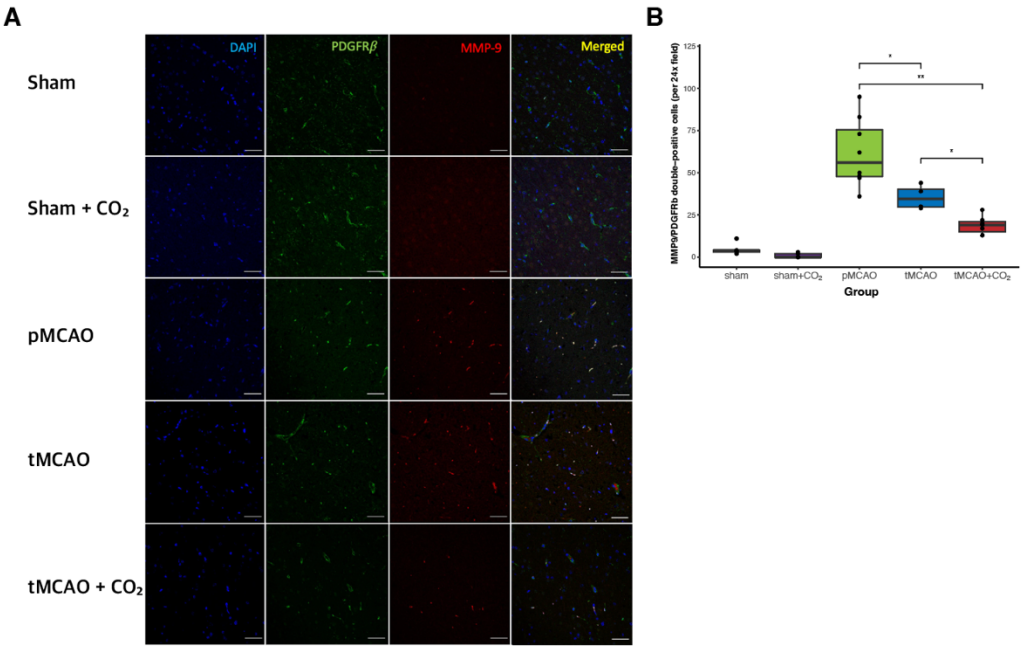

Figure 6. CO_2 per-conditioning decreased the number of MMP-9/PDGFRβ double positive cells.

(A) Representative Images of immunofluorescence staining illustrated the PDGFRβ, MMP-9, and MMP-9/PDGFRβ double positive cells. scale bar = 20 μm. (B) Quantitative analysis of MMP-9/PDGFRβ expression in immunofluorescence staining (*$p < 0.05$, **$p < 0.01$). MMP-9 and PDGFRβ expressions in all rat models presented similar pattern.

Abbreviations: CO_2 = carbon dioxide; DAPI = 4′,6-diamidino-2-phenylindole; MMP-9 = matrix metallopeptidase 9; PDGFRβ = platelet derived growth factor receptor beta; pMCAO = permanent middle cerebral artery occlusion; tMCAO = transient middle cerebral artery occlusion.

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3.6. CO_2 per-conditioning upregulates tight junction protein expressions after tMCAO

The tMCAO + CO_2 group showed reserved number of ZO-1 positive cells compared with the other MCAO groups ($p = 0.0019$, $p = 0.047$, respectively, Figure 7). Compared to the tMCAO + vehicle group, the pMCAO + vehicle group revealed a lower expression of ZO-1 positive cells ($p = 0.0019$, Figure 7). Western blot analysis showed that the tMCAO + CO_2 group had higher protein levels of ZO-1 than the other MCAO groups (Figure 7).

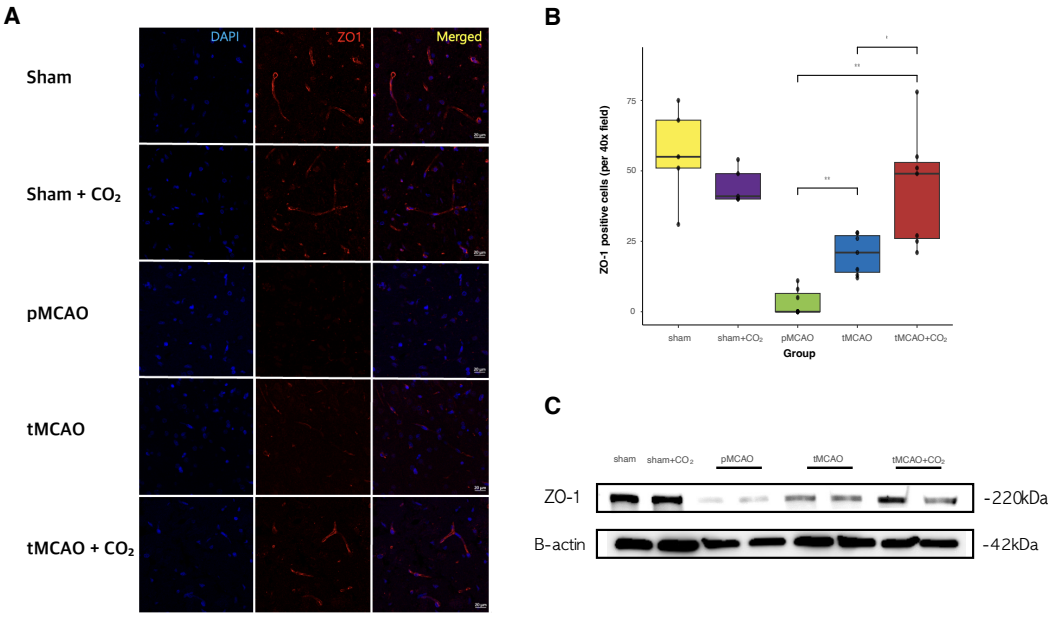

Figure 7. CO_2 per-conditioning increased the number of ZO-1 positive cells.

(A) Representative Images of immunofluorescence staining illustrated the ZO-1 positive cells. scale bar = 20 μm. (B) Quantitative analysis of ZO-1 expression in immunofluorescence staining (*p < 0.05, **p < 0.01). (C) Western blot analysis of ZO-1 expression. ZO-1 expressions in all rat models presented similar pattern.

Abbreviations: CO_2 = carbon dioxide; DAPI = 4′,6-diamidino-2-phenylindole; ZO-1 = zonula occludens-1; pMCAO = permanent middle cerebral artery occlusion; tMCAO = transient middle cerebral artery occlusion.

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3.7. CO_2 per-conditioning upregulates PDGFRβ expressions after tMCAO

The tMCAO + CO_2 group showed reserved number of PDGFRβ positive cells compared with the other MCAO groups ($p = 0.0021$, $p = 0.0025$, respectively, Figure 8). Western blot analysis showed that the tMCAO + CO_2 group had higher protein levels of PDGFRβ than the other MCAO groups (Figure 8).

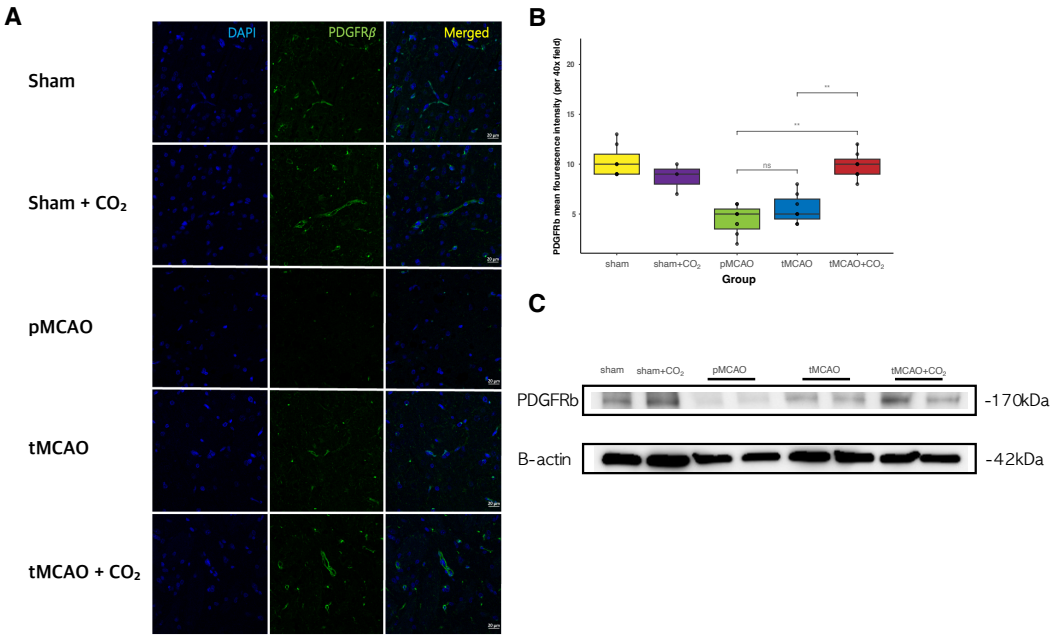

Figure 8. CO_2 per-conditioning increased the number of PDGFRβ positive cells.

(A) Representative Images of immunofluorescence staining illustrated the PDGFRβ positive cells. scale bar = 20 μm. (B) Quantitative analysis of PDGFRβ expression in immunofluorescence staining (**$p < 0.01$). (C) Western blot analysis of PDGFRβ expression. PDGFRβ expressions in all rat models presented similar pattern.

Abbreviations: CO_2 = carbon dioxide; DAPI = 4′,6-diamidino-2-phenylindole; PDGFRβ = platelet derived growth factor receptor beta; pMCAO = permanent middle cerebral artery occlusion; tMCAO = transient middle cerebral artery occlusion.

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4. DISCUSSION

In the present study, we evaluated neuroprotective effect of intermittent CO_2 per-conditioning in tMCAO rat model. Taking advantage of experimental models, our data illustrated that CO_2 per-conditioning significantly decreased infarct size and neurologic deficit by reducing the oxidative stress, BBB breakdown, and death of pericytes after tMCAO. In addition, there were a link between structural lesion and functional deficit in our models.

Several studies have shown that mild acidosis via hypercapnia decreases infarct size and has a neuroprotective effect on cerebral ischemia in mouse or rat models.[22,29-31] Previous studies have used pre-or post-conditioning methods, however, none of them performed hypercapnia intervention during ischemic period, which is per-conditioning. In this study, we focused on per-conditioning, that can be used in conjunction with the treatment of patients with hyperacute ischemic stroke. Additionally, CO_2 is fast acting vasodilatory gas and can cross BBB owing to its fat-soluble characteristics.[32,33] Due to these specific features, CO_2 may exert a neuroprotective effect on ischemic state during per-conditioning. We suggest per-conditioning and induced mild hypercapnia will work synergistically with each other. Our study showed that intermittent CO_2 inhalation as a per-conditioning method is effective for neuroprotection. Future studies that compare this method with pre-conditioning or post-conditioning will further clarify the efficacy of per-conditioning.

The vasodilatory effect of hypercapnia is known to be modulated by vascular smooth muscle relaxation, which is caused by decreased endothelial intracellular Ca^{2+} level.[34,35] Relaxation of cerebral artery smooth muscles are induced as $PaCO_2$ increases, which mediated through accompanying changes in pH and/or directly by CO_2.[22,30,36] Cerebral blood flow (CBF) decreases by 2-3% for each mmHg decrease of $PaCO_2$ from its normal resting value owing to the vasoconstrictive effects of hypocapnia, conversely, CBF increases by 3-4% for each unit increase in $PaCO_2$, reaching when $PaCO_2$ is elevated by 10-20 mmHg above normal resting state.[37] In this study, we intermittently inhaled 20% CO_2, which is the most efficient CO_2 concentration identified in pilot and other studies.[22,38] Based on ABGA results of CO_2 inhalation groups, the $PaCO_2$ values in our

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study did not rise more than 20 mmHg from the baseline $PaCO_2$ value, indicating the absence of excessive hypercapnia. Since $PaCO_2$ is a major determinant of CBF regulation, especially in acute hypoxia, mild hypercapnia in cerebral ischemia models may have a significant impact on reversing the penumbra by alleviating the decrease in CBF.[37] In contrast, excessive increase in CO_2 concentration resulted in poor outcomes through increased intracranial pressure rather than neuroprotective effects.[29] In clinical field, recent multi-national, multi-center, randomized trial showed that mild hypercapnia is unlikely to cause clinically relevant elevations in intracranial pressure.[39] In this regard, it is critical to maintain adequate mild hypercapnia during ischemia for safety aspect. In clinical field, one randomized controlled trial demonstrated beneficial effect of targeted therapeutic mild hypercapnia after cardiac arrest.[40] In this clinical trial, the median difference of $PaCO_2$ between the intervention and control groups was 8 mmHg, and this result was similar to our ABGA results. Our findings could help to design future studies on the clinical efficacy of mild hypercapnia for patients with ischemic stroke.

In our study, we revealed that oxidative stress and BBB breakdown after cerebral ischemia can be attenuated by CO_2 per-conditioning. Oxidative stress which is caused by excessive production of ROS due to imbalance between pro-oxidants and anti-oxidants is known as a key deleterious factor in brain ischemia. During the reperfusion phase, while thrombolysis therapy removed the blood clot, blood reperfusion can have detrimental effects on brain cells due to the excessive production of ROS.[41] Initiation of reperfusion produces large amounts of superoxide by reducing NAD^+ to NADH in mitochondrial respiratory chain, also ROS occurs in the cytoplasm through the reaction of NADPH-oxidase.[42] One study showed that CO_2 can interact with reactive oxygen species, scavenge the peroxynitrite which is produced from superoxide, and prevent oxidative stress.[43] This anti-oxidative effect of CO_2 may have contributed to the reduction of oxidative stress induced by the ischemia-reperfusion state, as shown by the attenuation of 8-OHdG in our experimental results.

MMP-9 is well known mediator of BBB breakdown caused by degradation of basement membrane proteins in ischemia-reperfusion injury.[44,45] In addition, high MMP-9 levels were associated with size of infarction and unsuccessful recanalization.[46,47] Our result showed that mild hypercapnia attenuated MMP-9 expression and upregulated ZO-1. These findings suggested that mild

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hypercapnia can decrease BBB breakdown by attenuating MMP-9 expression and increasing ZO-1 expression.

In the present study, we found that mild hypercapnia attenuated death of pericytes in ischemic state. Pericytes, one of neurovascular unit components, are perivascular mural cells on blood microvessels. Pericytes can stabilize the capillary wall, maintain the BBB, prevent neuroinflammation, and regulate the capillary diameter and blood flow.[48-50] In the ischemic state, contraction of pericytes triggers capillary constriction, and then erythrocytes are trapped in microvessels.[51,52] After recanalization of occluded vessels, pericytes remain contracted despite successful recanalization.[51] It is a main cause of no-reflow phenomenon. As such, pericytes play an important role in the microcirculation environment. One study showed that increasing $PaCO_2$ induces relaxation of pericytes, whereas decreasing $PaCO_2$ causes contraction of pericytes.[21] This study implies that CO_2 can affect alteration of pericytes, in this regard, the mechanism of beneficial roles of CO_2 may be mediated by pericytes. In addition, because pericytes contraction also can be induced by ROS, the anti-oxidant effect of hypercapnia may relieves pericytes contraction, which triggers capillary constriction.[51]

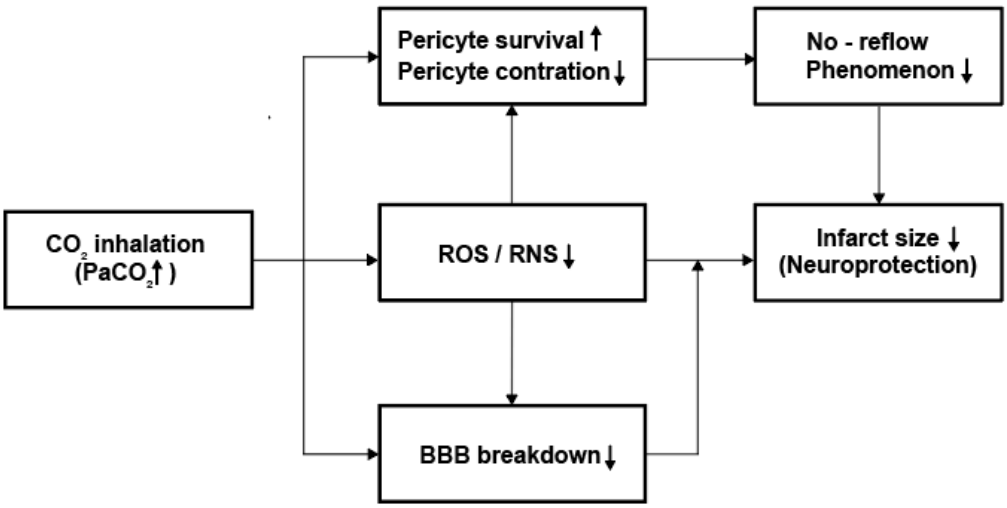

Figure 9. Schematic flow of effectiveness of CO_2 per-conditioning in acute ischemia.

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These various effects of CO_2 inhalation during transient cerebral ischemia may alleviate size of ischemic stroke and neurologic deterioration (Figure 11). Due to its minimal reactivity and low risk in daily life, CO_2 can be used outside of hospital. As there is no significant clinical risk, the early initiation of mild hypercapnia can be considered even before patients with acute neurologic deficits arrive at the hospital. If the stability of mild hypercapnia is confirmed for conditions such as cerebral hemorrhage and infectious brain disease in the future, conducting clinical trials may lead to promising outcomes.

There are several limitations in this study. First, the relative limited observational period was followed (24 hr). In clinical fields, the modified Rankin Scale, which measures degree of disability or dependence after a stroke is evaluated at some weeks or even months after the stroke insult. If the rats equivalent of a human year is nearly 2 weeks, then the evaluations need to be prolonged to a week, or longer. Future studies need to clarify structural and functional changes of insulted experimental groups until weeks and even months. Second, stroke has sexually dimorphism in clinical field. Because women own a protective female sex hormone, they have a lower risk of stroke incidence relative to men.[53] The results from experimental stroke model may have difference in sex. From all these considerations, sex difference should be considered in further study to determine the effect of intermittent CO_2 inhalation in ischemic stroke models. Third, we did not measure cerebral blood flow in the present study. However, another study showed hypercapnia preserved cerebral blood flow in the ischemic state.[54] In addition, one study indicated hypercapnia has been reported to cause vasodilation and increase cerebral blood flow.[55] Finally, we did not measure concurrent $PaCO_2$ level during intervention and surgery. The follow-up ABGA results after CO_2 inhalation showed a lower $PaCO_2$ level than anticipated. This occurred because, despite conducting the ABGA procedure immediately after three cycle of CO_2 inhalation, there was at least 10-min gap from the last CO_2 inhalation due to the recovery phase.

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5. CONCLUSION

Mild hypercapnia not only protects the ischemic penumbra from ischemia-reperfusion injury and reduces neurologic deficit, but also maintains the integrity of BBB and neurovascular unit, and reduces oxidative stress in a hyperacute stroke rat model. Therapeutic hypercapnia is promising and requires further assessment for clinical application.

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Abstract in Korean

급성 뇌경색 동물 모델에서 재관류 치료에 동반한 이산화탄소 흡입 치료의 효과

연구 배경: 중대뇌동맥 폐쇄 (MCAO) 설치류 모델에서 재관류 전 이산화탄소 흡입이 산화 스트레스, 뇌혈관장벽의 손상, 신경학적 손상을 감소시키는지를 조사하였다.

방법: Sham, Sham + CO_2, 일시적 중대뇌동맥 폐쇄(tMCAO), 영구적 중대뇌동맥 폐쇄(pMCAO), 그리고 tMCAO + CO_2군으로 나눠서 실험을 진행하였다. , Sham + CO_2, tMCAO + CO_2군은 혼합 가스(20% CO_2, 20% O_2, 60% N_2)를 흡입하였다. MCAO 수술 후 각 실험군마다 동맥혈가스분석 및 행동 검사를 시행하였으며 24 시간 이후 뇌경색 크기 확인을 위한 조직 염색, 웨스턴 블롯, 면역형광염색을 시행하였다.

결과: 이산화탄소 흡입 치료를 한 tMCAO + CO_2군은 tMCAO군에 비해 뇌경색 크기가 작았고(tMCAO+CO_2군, 14.8 [11.0, 17.8] vs. tMCAO군, 27.9 [26.4, 28.0], $p < 0.0001$) 신경학적 점수도 tMCAO + CO_2군이 tMCAO군 또는 pMCAO군 보다 더 우수하였다. tMCAO + CO_2군에서 tMCAO군 또는 pMCAO군에 비해 8-OHdG와 MMP-9 의 발현이 유의미하게 감소하였다. tMCAO + CO_2군에서 tMCAO군 또는 pMCAO군에 비해 ZO-1과 PDGFRβ 의 발현은 유의미하게 증가하였다.

결론: 경도의 고탄산혈증은 허혈 재관류 손상으로부터 허혈성 반음영을 보호하며,

`

신경학적 결손을 줄일 뿐 아니라, 뇌혈관장벽과 신경혈관단위의 보전 및 산화 스트레스 줄인다. 치료적 고탄산혈증은 유망하며 임상 적용을 위한 추가 평가가 필요하다.

핵심되는 말: 이산화탄소, 허혈성 뇌졸중, 뇌경색 동물 모델, 산화 스트레스, 허혈 재관류 손상